The neurochemical basis of locust behavioural phase change: a pharmacological, behavioural and molecular approach

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

By
Jonathan M. Smith, MSc
Department of Neuroscience,
Psychology and Behaviour
University of Leicester
2017

The neurochemical basis of locust behavioural phase change: a pharmacological, behavioural and molecular approach

Jonathan M. Smith, MSc

Locusts undergo dramatic changes in behaviour, morphology and physiology in response to changing population densities, leading to the formation of devastating swarms. Of these forms of phenotypic plasticity, known collectively as phase polyphenism, behavioural plasticity is the most rapid. Shy, cryptic *solitarious* phase locusts can change their behaviour within 4 h of exposure to conspecifics to resemble that of locusts in the active, swarming *gregarious* phase ("gregarise"). In this thesis, I use a multidisciplinary approach to investigate the neurochemical mechanisms underpinning behavioural phase polyphenism in the locust.

In Chapters 3 and 4, my investigations using an established behavioural assay, High-Performance Liquid Chromatography and pharmacology found no evidence supporting published claims that the neuromodulator 5-hydroxytryptamine in the thoracic ganglia is required for gregarisation. My work indicates a need for further re-investigation of the roles of monoamines in locust phase polyphenism.

In Chapter 5, I ran locusts through repeated behavioural assays to reproduce an unexpected phase-specific behavioural change observed in Chapter 4, a potentially novel phase-specific familiarity response to repeated behavioural observations. Solitarious locusts increased their exploratory activity after repeated observations at 2 h intervals, but this effect was not present for longer intervals nor in a different paradigm. I suggest methodological refinements to examine the origins of this potential phase-specific familiarity response.

In Chapter 6, I used an assembled transcriptome to identify locust orthologues of four immediate-early genes widely used to create neuronal "activity maps" in other species, in an attempt to apply this to *S. gregaria* during gregarisation. Quantitative Polymerase Chain Reaction analyses found no activity-related changes in transcription of these genes in solitarious locusts crowded for 30 min compared with uncrowded controls. My work suggests no involvement of these genes in the early stages of gregarisation. This paves the way for optimisation of this novel tool in the locust.

Acknowledgements

First thanks go to my supervisors Drs Tom Matheson and Swidbert Ott for their kind support and mentoring over the four years of this PhD. Thank you to both for all of your time and energy that you have spent helping this project progress. Second thanks to all current and past members of the Leicester Locust Labs who have given me valuable mentoring, critique, encouragement, pub trips, and some unforgettable experiences of this project. I am specifically grateful to Dr Rien de Keyser, for his amazeballs mentoring, assistance and suffering getting the HPLC to work, Dr Georgy Fenton for her awesomevilles mentoring, and assistance with data collection, Dr Ben Warren for his mentoring and help in learning molecular techniques and writing skills, Chanida Fung for her incredible support in her time here, Dr Jon Shand for his technical expertise and for welcoming me to Leicester, Anthony Vencatasamy for his stellar assistance with HPLC data collection, and Ben Cooper for his expertise and good taste in video games. Many thanks to everyone who kept the locust colonies thriving in my time at the lab, including Chanida, Anthony, Laura, Carl, Rubén and Jon.

Massive thanks go to Ceinwen Tilley, Dr Will Norton, Ben Hunt, Jon and Ben Warren for their assistance in the molecular techniques in Chapter 6, without whom I would never have known how to begin.

I would like to thank all my family for their caring support. Thanks Em for being my best mate and sibling partner in crime. Thanks Dad, Mum, Sarah, Serena, Dan and Dean for being amazing. Thanks also to my nephews Isaac and Austin, at whose dancing video I can never stop laughing.

Thanks to all my departmental mates for the amazing times, especially Boris Berkhout who has fed me many times when I needed it. Thanks also to my Spanish buddies: Héctor, Rosana, Nuria, Rubén and Judit, who have all kept me sane in my last year, and kept me practising their lingo.

I'd also like to thank the more abstract influences in my life. A shout out goes to my favourite authors Joe Abercrombie, R. A. Salvatore, Brandon Sanderson, and the late J. R. R. Tolkien, Terry Pratchett and Philip K. Dick, whose stories shaped my early years and still keep life exciting. Furthermore, a huge thanks to musicians whose work helped me to relax in the harder times, particularly Miracle of Sound, Oscar Peterson, Ludovico Einaudi, Jeremy Soule, Two Steps From Hell, and more.

Finally, thanks to the BBSRC for their funding, and the MIBTP programme for giving me this opportunity.

Collaborations and contributions

As I worked in a highly collaborative environment and project, this section describes the contributions from colleagues towards much of the data in this thesis.

Chapter 3:

All HPLC and behavioural work in the Leicester strain was carried out by me.

Mauritanian behavioural and HPLC data collection was carried out by Dr Rien de Keyser and Chanida Fung, with assistance from myself. I analysed the Mauritanian strain data with the Leicester strain.

Chapter 4:

All behavioural experiments except for the reserpine experiments (injections of DMSO control, and reserpine in gregarious and solitarious locusts) were carried out by me. Anthony Vencatasamy carried out HPLC data collection on solitarious reserpine experimental animals and I analysed the data. Otherwise, all HPLC procedures were carried out by me with assistance from Anthony Vencatasamy, Chanida Fung and Rien de Keyser.

All behavioural assays in the reserpine experiments were carried out by undergraduate project student Jadesola Dinakin, and the analysis was performed by me.

Chapter 5:

The Y-maze experiments, and assays of some experimental animals in the 'Empty' groups in the repeated measures experiments were carried out by undergraduate project student Samantha Purton. I then carried out data analysis on the Y-maze behavioural data, with the assistance of Swidbert Ott.

All of the rest of the behavioural assays in the chapter were carried out by me.

Chapter 6:

The cloning steps of the chapter were carried out with assistance and training from Ceinwen Tilley. All other practical work and analyses were carried out by me.

Contents

The neurochemical basis of locust behavioural phase change: a pharmacological, behavioural and molecular approach i

Acknowledgements ii

Collaborations and contributions iii

Contents iv

List of Tables vii

List of Figures xi

List of Abbreviations xiv

1 General Introduction 1

1.1 Plagues and food security 1

1.2 Behind the swarming: phenotypic plasticity 2

1.3 Phase polyphenism in the locust 3

1.4 S. gregaria as a model for phenotypic plasticity 5

1.5 Behavioural quantification in phase change 9

1.6 The neurochemical basis of locust behavioural phase change 11

1.7 Learning, memory and behavioural phase change 14

1.8 Neuronal circuitry in phase change 16

1.9 Thesis rationale 17

1.10 Overview of the chapters 19

2 General Methods 22

2.1 Animal husbandry 22

2.2 Phase-changing treatments 27

2.3 Behavioural assay 27

2.4 HPLC 36

2.5 Pharmacology 38

2.6 Statistical analysis 39

2.7 Appendix 41

3 Does the established role of 5-HT in locust behavioural gregarisation stand up to scrutiny? 44

3.1 Summary *44*

3.2 Introduction *45*

3.3 Methods *49*

3.4 Results *53*

3.5 Discussion *69*

4 Pharmacological reinvestigations into the role of monoamines in behavioural phase change in the Desert Locust 78

4.1 Summary *78*

4.2 Introduction *79*

4.3 Methods *88*

4.4 Results *99*

4.5 Discussion *123*

5 Phase-specific responses to repeated stimulus exposure in the Desert Locust 131

5.1 Summary *131*

5.2 Introduction *132*

5.3 Methods *135*

5.4 Results *144*

5.5 Discussion *157*

6 Identification of activity-related immediate-early genes in the Desert Locust 162

6.1 Summary *162*

6.1 Introduction *163*

6.2 Methods *168*

6.3 Results *180*

6.4 Discussion *192*

7 General Discussion 200

7.1 Is the P_{greg} model a suitable indicator of behavioural phase state? *200*

7.2 What mechanisms control behavioural phase state in S. gregaria? *201*

7.3 What is the future of the Desert Locust phase change model? *202*

7.4 Is behavioural phase change a viable target mechanism of swarm control?..... 204

7.5 Can the locust phase change model be translated across species?..... 205

8 Bibliography..... 207

List of Tables

Chapter 2:

Table 2.1:	List of behavioural variables generated by automated tracking script *tandy*.	32
Table 2.2:	Variables in logistic regression model for Leicester colony.	33
Table 2.3:	Variables in logistic regression model for Mauritanian colony.	35
Table 2.4:	Terminology assigned to statistical evidence levels.	40
Table 2.5:	Statistical treatments of the effect of generation on gregariousness in the Leicester colony.	43

Chapter 3:

Table 3.1:	Sample sizes for the maternal lineage experiment.	51
Table 3.2:	Statistical tests of the effect of tickling treatments on gregariousness.	55
Table 3.3:	Statistical tests of the effect of 2 h crowding on gregariousness.	56
Table 3.4:	Statistical tests of the effect of 4 h crowding on gregariousness.	57
Table 3.5:	Statistical tests of the effect of all phase-changing paradigms on logit values gregariousness and ganglionic 5-HT amounts.	58
Table 3.6:	Statistical tests of the effect of crowding and maternal lineage on gregariousness and ganglionic 5-HT amounts.	61
Table 3.7:	Statistical tests of the effect of crowding on gregariousness in two different strains.	63
Table 3.8:	Statistical tests of the effect of crowding on ganglionic 5-HT in two different strains.	67
Table 3.9:	Statistical tests of the covariance between ganglionic 5-HT and behavioural gregariousness in two different strains.	68

Chapter 4:

Table 4.1:	Statistical tests of the effect of fluphenazine injections on phase maintenance in gregarious nymphs.	101
Table 4.2:	Statistical tests of the effect of fluphenazine injections on phase maintenance in solitarious nymphs.	103
Table 4.3:	Statistical tests of the effect of AMTP injections on crowd-induced gregarisation.	105
Table 4.4:	Statistical tests of the effect of AMTP injections on regregarisation.	106
Table 4.5:	Statistical tests of the effect of AMTP injections on tickle-induced gregarisation.	109
Table 4.6:	Statistical tests of the effect of AMTP injections on tickle-induced gregarisation and ganglionic 5-HT.	111
Table 4.7:	Statistical tests of the effect of 5-HT injections on gregarisation.	115
Table 4.8:	Statistical tests of the effect of DMSO injections on gregariousness in gregarious nymphs.	116
Table 4.9:	Statistical tests of the effect of reserpine on gregariousness, ganglionic 5-HT and ganglionic DA.	118
Table 4.10:	Statistical tests of the effect of reserpine on gregarisation, ganglionic 5-HT and ganglionic DA.	120

Chapter 5:

Table 5.1:	Treatments investigating an effect of repeated behavioural arena runs, handling and injection procedures on behavioural gregariousness.	136
Table 5.2:	Statistical tests of the effect of animal handling, injections and behavioural assays on gregariousness in gregarious nymphs.	146
Table 5.3:	Statistical tests of the effect of animal handling, injections and behavioural assays on gregariousness in solitarious nymphs.	148

Table 5.4:	Statistical tests of the effect of repeated behavioural assays and the crowd stimulus exploratory behaviour in solitarious nymphs with 2 h intervals.	150
Table 5.5:	Statistical tests of the effect of repeated behavioural assays and a crowd stimulus on exploratory behaviour in solitarious nymphs with 24 h intervals.	151
Table 5.6:	Statistical tests of the effect of repeated behavioural assays and a crowd stimulus on exploratory behaviour in solitarious nymphs with 48 h intervals.	152
Table 5.7:	Statistical tests of the effect of repeated behavioural assays and a crowd stimulus on exploratory behaviour in gregarious nymphs with 24 h intervals.	153
Table 5.8:	Statistical tests of the effect of phase and the crowd stimulus on exploratory behaviour.	154
Table 5.9:	Logistic regression model fit for fixed effects on side choice in the Y-maze setup.	156

Chapter 6:

Table 6.1:	cDNA synthesis steps in thermocycler in chronological order.	171
Table 6.2:	Specifications for primer design.	173
Table 6.3:	PCR reaction thermocycler steps in chronological order.	174
Table 6.4:	qPCR reaction thermocycler steps in chronological order.	178
Table 6.5:	IEG queries and the longest matching contigs within the existing transcriptome.	181
Table 6.6:	Open Reading Frame (ORF) protein sequences and lengths for identified IEGs.	182
Table 6.7:	Primer design details for each IEG identified.	187
Table 6.8:	Statistical analysis of the effect of crowding on IEG expression.	188
Table 6.9:	Raw C_t values of amplification in HKGs and IEG targets, arranged in order of transcript abundance.	189
Table 6.10:	Analysis of *EGR*-associated transcripts.	189

Table 6.11: Analysis of *FOS*-associated transcripts. 190
Table 6.12: Analysis of *Hr38*-associated transcripts. 190
Table 6.13: Analysis of *JRA*-associated transcripts. 191

List of Figures

Chapter 2:

Figure 2.1:	Locust holding cages.	25
Figure 2.2:	The behavioural assay method.	30
Figure 2.3:	Output of logistic regression models in the Leicester and Mauritanian colonies.	34
Figure 2.4:	Only weak evidence for an effect of generation in solitarious behavioural gregariousness.	43

Chapter 3:

Figure 3.1:	No evidence for an increase in ganglionic amounts of 5-HT in solitarious locusts given phase-changing stimuli.	54
Figure 3.2:	Behavioural gregariousness does not correlate with ganglionic 5-HT amounts in a large pooled dataset.	58
Figure 3.3:	Maternal lineage does not influence the gregarisation response in solitarious locusts.	60
Figure 3.4:	No evidence for a 5-HT increase during crowd-induced gregarisation in two separate strains of locust.	64

Chapter 4:

Figure 4.1:	Schematic timeline of methods for fluphenazine experiments.	90
Figure 4.2:	Schematic timeline of methods for experiments AMTP1 and AMTP2.	92
Figure 4.3:	Schematic timeline of methods for experiments AMTP3, AMTP4 and AMTP5.	94
Figure 4.4:	Schematic timeline of methods for 5-HT injection experiment.	95
Figure 4.5:	Schematic timeline the reserpine experiments.	97
Figure 4.6:	Inconclusive effect of DA receptor block on behavioural phase maintenance and phase change.	100

Figure 4.7:	No evidence for AMTP effect on behavioural phase change.	104
Figure 4.8:	No evidence for AMTP effect on tickle-induced behavioural phase change.	108
Figure 4.9:	Very little evidence for AMTP effects on tickle-induced behavioural gregarisation or CNS 5-HT concentrations.	110
Figure 4.10:	No evidence for a correlation between CNS 5-HT concentrations and gregariousness with AMTP treatment.	113
Figure 4.11:	Weak evidence for a solitarising effect of central 5-HT injections on behavioural phase state.	114
Figure 4.12:	No evidence for an effect of DMSO on behavioural gregariousness.	116
Figure 4.13:	Very little evidence for reserpine effects on gregariousness or ganglionic 5-HT concentrations.	117
Figure 4.14:	Very little evidence for ganglionic monoamine depletion effects on crowd-induced behavioural gregarisation or ganglionic 5-HT concentrations.	119

Chapter 5:

Figure 5.1:	Schematic timeline of methods in Experiment 1.	137
Figure 5.2:	Schematic timeline of methods in Experiment 2.	139
Figure 5.3:	Y-maze and odour release designs.	141
Figure 5.4:	Schematic timeline of methods in Experiment 2.	143
Figure 5.5:	Weak evidence for an effect of repeated arena runs, handling and injections on behavioural gregariousness.	145
Figure 5.6:	Evidence for an effect of repeated arena runs and the presence of a crowd stimulus in the arena on exploratory behaviour.	149
Figure 5.7:	No evidence for an effect of odour pre-exposure on odour preference in solitarious or gregarious adult locusts.	155

Chapter 6:

Figure 6.1:	EGR protein.	181
Figure 6.2:	FOS protein.	183
Figure 6.3:	Hr38 protein.	184
Figure 6.4:	JRA protein.	185
Figure 6.5:	Confirmation of primer specificity for IEG targets.	186
Figure 6.6:	No evidence for changes in the CNS expression of four IEGs over 30 min crowding.	188

List of Abbreviations

5-HT	5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
AA	Amino Acid
AANAT	Arylalkylamine N-acetyltransferase
ACN	Acetonitrile
ACS	American Chemical Society
AMTP	α-methyltryptophan
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
CaMKII	Calcium-calmodulin dependent Kinase II
cAMP	cyclic Adenosine Monophosphate
CNLA	Centre National de Lutte Antiacridienne
CNS	Central Nervous System
C_t	Threshold cycle
Ct_{jk}	Mean C_t value per animal sample per gene
Ct_{ref}	Reference C_t for each gene
DA	Dopamine
DCMD	Descending Contralateral Movement Detector
DHBA	3,4-dihydroxybenzylamine hydrobromide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EFa48D	Elongation factor 1α
EGR	Early Growth Response gene
FETi	Fast Extensor Tibiae motorneuron
FractalDim	Fractal dimension
FSCV	Fast Scan Cyclic Voltammetry
GPCR	G-Protein-Coupled Receptor
GUI	Graphical User Interface
HKG	Housekeeping gene
HPLC	High Performance Liquid Chromatography

Hr38	Hormone receptor-like 38 gene
IQR	Interquartile range
JRA	Jun-related Antigen
L-DOPA	L-3,4-dihydroxyphenylalanine
Longwalkbouts	Long walk bouts
NADA	N-acetyldopamine
NAS	N-acetylserotonin
NF_k	Normalising Factor for each animal sample
NMS	N-methyl-serotonin
NRQ	Normalised Relative Quantity
Nurr1	Nuclear receptor subfamily 4 group A member 2
OA	Octopamine
PCA	Perchloric acid
PCR	Polymerase Chain Reaction
P_{greg}	Probability of belonging to the gregarious phase
PKA	cAMP-dependent protein kinase A
PKC	Protein Kinase C
PKG	Protein Kinase G
qPCR	quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
RP49	Ribosomal Protein 49
RPM	Revolutions per minute
RT-qPCR	Real-Time quantitative Polymerase Chain Reaction
StimTF	Stimulus time fraction
TH	Tryptophan Hydroxylase
vat1	Vesicular Amine Transporter 1
ZnF_C4	C4 Zinc Finger in nuclear hormone receptors

1 General Introduction

Locusts are notorious pests that aggregate into swarms of billions of individuals, causing economic damage to agriculture worldwide (Cullen et al., 2017). Plagues of these insects have been described in historical texts such as official Chinese records, the Old Testament and the Quran, demonstrating their dramatic impact on ancient agricultural society (Pener and Simpson, 2009). Despite advances in swarm prevention strategies and tools, locust plagues continue to cause concern in the modern world, with the most problematic and widespread locust species including the Desert Locust (*Schistocerca gregaria,* Forskål), particularly in North Africa and the Middle East; the Migratory Locust (*Locusta migratoria,* Linnaeus), found across Asia, Australasia and Central and South Africa, and the Australian Plague Locust *Chortoicetes terminifera* (Cullen et al., 2017).

1.1 Plagues and food security

While locust pests are not directly dangerous to human health, plagues can damage food security on a vast scale. Outbreaks of *S. gregaria* alone are estimated to threaten agriculture across 20% of the total land surface of the world, and the livelihoods of 10% of the world's population (Steedman, 1988). For example, a large-scale upsurge in outbreaks of *S. gregaria* in 2003-5 across the Sahel region of Africa resulted in approximately 80% losses in cereal production in surveyed regions, and affected approximately eight million people (Brader et al., 2006). These crop losses exacerbated pre-existing issues with poverty and malnutrition within vulnerable countries with limited resources, with additional impacts on household debt, education and tensions between pastoralists and farmers (Brader et al., 2006).

Current strategies for swarm control and management consists of organophosphate, carbamate, and pyrethroid insecticide treatment sprayed across breeding grounds (Peveling, 2001), though the use of preventative, slow-acting fungal biopesticides such as *Metarhizium acridum* is on the rise, particularly in China (Cullen et al., 2017).

However, the difficulties of these strategies are numerous, such as the limited economic capacity of affected countries and regions to orchestrate regular structured prevention efforts in the absence of large plagues, the transnational span of the affected areas complicating coordinated strategies, political instability or lack of security in many regions, and the fact that the breeding areas are often distant from the vulnerable crop regions due to the migratory nature of locusts (Cullen et al., 2017, Peveling, 2001).

One major drawback of the use of broad-spectrum synthetic pesticides is their collateral effect on the ecosystem of the treated areas. Adverse effects of the pesticide treatments can be observed in other arthropods (Peveling et al., 1999), as well as vertebrate predators such as birds (Goldstein et al., 1999). These can also have unpredictable indirect effects on predator populations in the ecosystem depending on the temporal and spatial distribution of the pesticide treatments (reviewed in Peveling, 2001). While biopesticides such as *M. acridum* are more specific, they are more dependent on optimum environmental conditions in the field, and require more study to optimise their costs and efficacy for countries with fewer financial resources (Lecoq, 2007). Thus, investigating the biological basis of locust swarming may inform strategies for improving locust control efforts.

1.2 Behind the swarming: phenotypic plasticity

The inducible expression of a swarming phenotype in locusts is an example of phenotypic plasticity, a phenomenon in which a single genotype can express multiple phenotypes (West-Eberhard, 2003). This universal phenomenon can be expressed at morphological, biochemical, physiological and behavioural levels across the animal kingdom and is crucial for an organism's survival in fluctuating habitats (West-Eberhard, 2003). Well-studied fields of physiology such as acclimation, immune system adaption and learning and memory are forms of this phenomenon (Fusco and Minelli, 2010). The range of phenotypes possible for an organism is defined as a reaction norm (Woltereck, 1909), and this plasticity varies across species and environmental conditions (Pigliucci et al., 2006).

The type of phenotypic plasticity of interest in this thesis is polyphenism, a specific form of phenotypic plasticity in which different discrete phenotypes are expressed depending on environmental factors (Fusco and Minelli, 2010; Mayr, 1970). For example, many species express temperature-dependent sex determination (Janzen and Phillips, 2006). Phytoplankton can develop morphological and physiological anti-predator defences such as spines in response to predator chemical cues (Donk et al., 2011). Larvae of some amphibian species express omnivorous or carnivorous phenotypes determined by resource availability in the habitat (Pfennig et al., 2006), and the butterfly species *Araschnia levana* exhibits different colouration based on season (Nijhout, 2003).

A particularly striking example of polyphenism is caste determination in eusocial insects such as ants, termites and bees (Evans and Wheeler, 2000; Hartfelder et al., 2006; Miura, 2005; Nijhout and Wheeler, 1982; Roisin, 2000; Schwarz et al., 2007). Sterile females in many bee species can be induced to express reproductive phenotypes depending on nutritional and pheromonal cues (Schwarz et al, 2007). Termite species express multiple caste phenotypes determined postembryonically by physical environmental factors and social interactions. Notably, expression of the seasonal reproductive winged termite alate caste and the wingless soldier caste is strongly influenced by the actions of Juvenile Hormone (see Miura, 2005). Studies in eusocial insects have thus shed light on how physiological mechanisms link environmental stimuli with the ensuing phenotypic expression.

1.3 Phase polyphenism in the locust

Some of the most dramatic examples of polyphenism occur in the grasshopper family (Orthoptera: Acrididae), in the 19 species currently considered as locusts (Cullen et al., 2017; Pener and Simpson, 2009). Locusts are grasshoppers that have the capacity to reversibly transform between two behavioural and morphological phenotypes, or 'phases', the non-swarming *solitarious* phase and the swarming *gregarious* phase, depending solely on the population density. This capacity for so-called density-dependent phase polyphenism, or phase change, is present across all developmental stages of the locust, including eggs and adults. Furthermore, phase polyphenism has a continuous range of phenotypes in between the two extremes. For example, crowding

solitarious phase locusts for just a few hours induces an intermediate *transiens* phase, in which the behavioural phenotype has changed to become more gregarious-like while morphological and physiological characters remain solitarious-like. Changes to the physical phenotypes can take 1-2 generations of altered population density to fully manifest (Pener and Simpson, 2009).

The 19 locust species exhibiting phase polyphenism are spread across separate subfamilies in the Acrididae, suggesting that the phenomenon has evolved multiple times (Cullen et al., 2017; Pener and Simpson, 2009). The reaction norm of phase change varies across locust species, with the most striking phase differences found in *S. gregaria* and *L. migratoria*, both showing density-dependent differences in morphology, colouration and behaviour. In fact, until the phase polyphenism theory was suggested, the solitarious and gregarious phases were considered as two separate species in both *L. migratoria* (*L. danica* and *L. migratoria*; Uvarov, 1921), and *S. gregaria* (*S. flaviventris* and *S. gregaria*; Uvarov and Zolotarevsky, 1929). Other locust species show less extreme forms of polyphenism. The Australian Plague Locust (*C. terminifera*), for example, displays only behavioural density-dependent polyphenism (Gray et al., 2009). Some grasshopper species such as *Melanoplus sanguinipes*, which are not considered to be true locusts can nevertheless aggregate to form nymphal bands (Cullen et al., 2017; Song, 2010) and show density-dependent differences in colouration (Song and Wenzel, 2008). *S. americana*, which is thought to have diverged from the swarming *S. gregaria* in North America (Lovejoy et al., 2006), has retained a degree of phase polyphenism in both behaviour and colouration (Gotham and Song, 2013). This diversity in reaction norms suggests that the boundary between 'locust' and 'grasshopper' species is less defined than the labels suggest. Furthermore, the multiple evolutions of phase polyphenism imply diversity in the biological mechanisms underlying phase change across locust species.

In addition to locust control applications, the study of phase polyphenism in locusts provides a controllable model system to elucidate biological mechanisms underlying polyphenism, which will have important applications in ecological and translational disciplines. To understand the biological mechanisms linking population density with the resulting phase in locusts, the phenomenon must be broken down into its component parts. The first component is the precise sensory stimuli from conspecifics, or lack

thereof, that determine the phase of an individual locust. The second component is the biological mechanisms processing the sensory stimuli into both short-term and long-term phenotypic changes, which may include neuronal, biochemical, molecular or epigenetic processes (Pener and Simpson, 2009). With their large reaction norm, the model organisms *S. gregaria* and *L. migratoria* have provided useful tools for addressing questions about the mechanisms underlying phenotypic plasticity (Cullen et al., 2017).

1.4 *S. gregaria* as a model for phenotypic plasticity

S. gregaria is a well-studied model organism for the biological underpinnings of environmentally-induced phenotypic plasticity due to its ease of rearing in captivity, relatively large size and its large reaction norm in response to just population density. Furthermore, considerable work has been carried out characterising the anatomy and circuitry of its nervous system, which can help with investigations into behavioural plasticity in phase polyphenism (Burrows, 1996).

1.4.1 Life cycle

S. gregaria undergoes hemimetabolous development, which involves moulting through 5 or 6 nymphal instar stages before the adult stage (Symmons and Cressman, 2001; Uvarov, 1966). Female locusts lay egg pods of 80-100 eggs in moist sand at a depth of 5-10 cm. They then cover the egg pods with a foam plug. These eggs develop in the substrate for ~ 14 days, depending on the humidity and temperature. Freshly-hatched vermiform larvae wriggle to the surface of the substratum and immediately moult into 1st instar nymphs, which have the appearance of miniature adult locusts without wings (Symmons and Cressman, 2001; Uvarov, 1966). Some nymphs undergo an extra moult and this is a more common occurrence in solitarious females than other individuals (Injeyan and Tobe, 1981; Ott and Rogers, 2010). Each larval instar stage lasts for 6-11 days depending on the phase and environmental conditions. The final instar nymphal moult takes place after a total ~ 21 days of development, and this stage undergoes the imaginal moult into the winged adult stage after ~ 28 days total development, though this can be delayed by low humidity or temperatures (Symmons and Cressman, 2001; Uvarov, 1966).

1.4.2 Phase differences

The phase characteristics of *S. gregaria* differ over the stages of its life cycle and reflect distinct anti-predation survival strategies (Burrows et al., 2011; Pener and Simpson, 2009; Uvarov, 1966). Locusts in the solitarious phase utilise cryptic and crepuscular strategies in order to reduce predation risk. Nymphs of this phase express camouflage colouration ranging from bright green in higher humidity to beige in lower humidity conditions, whereas adults are light brown in colour. Solitarious locusts typically hide on host vegetation during the day, fly at night, exhibit low activity, adopt a slow, crawling gait and avoid conspecifics. In contrast, gregarious locusts use a strategy of aposematic colouration and swarming behaviours to reduce predation risk. Nymphs exhibit vivid black and yellow patterning, and mature adult males are a conspicuous yellow colour. Gregarious locusts are highly active during the day, adopt a fast, upright walking gait and are attracted to conspecifics. Solitarious females are larger than gregarious females, but the opposite is true in males (Pener and Simpson, 2009). Solitarious locusts also have proportionally larger antennae, eyes, hind leg femurs, and wings than gregarious locusts (Burrows et al., 2011; Rogers et al., 2010; Uvarov, 1966). Gregarious, but not solitarious, locusts prefer foods containing toxic alkaloids, hypothesised to be an anti-predation strategy when combined with their bright aposematic colouration and lack of cryptic behaviours (Despland and Simpson, 2005). In the field, gregarious nymphs can form marching bands, whose migrations are driven, in part, by cannibalism (Bazazi et al., 2008). Densities of gregarious nymphs within a swarm can reach 20-150 million animals per km^3, and the density of a flying adult swarm can reach 10 individuals per m^3 (Symmons and Cressman, 2001; Uvarov, 1977, 1966).

The lifestyle of each phase is reflected in its internal anatomy and physiology. As group living imposes a complex sensory environment with the threat of intraspecific competition and cannibalism, gregarious phase locusts express more close-proximity touch receptors than do solitarious locusts (Burrows et al., 2011; Rogers et al., 2003). They also have 30% more total brain volume in proportion to their bodies than solitarious locusts, suggesting a compensatory increase in higher order sensory integration in response to the more complex environment. In contrast, solitarious locusts have larger primary visual and olfactory neuropils, suggesting a higher emphasis on sensory sensitivity (Ott and Rogers, 2010). Another physiological correlate to the ecological

strategies is seen in the Descending Contralateral Movement Detector (DCMD) neuron, an intersegmental visual interneuron that fires spikes in response to looming stimuli (Rowell, 1971). It synapses with the Fast Extensor Tibiae motor neuron (FETi) and flight motor neurons, which contribute respectively to jumping and flight avoidance behaviours. Thus, the DCMD is useful for swarming adults avoiding collisions in mid-air, as well as predation (Rind and Simmons, 1997, 1999; Santer et al., 2006). The DCMD fires more spikes in response to looming stimuli in gregarious locusts and also shows less pronounced habituation responses than in solitarious locusts (Matheson et al., 2004), suggesting that it is adapted for the avoidance of numerous looming stimuli in a flying swarm.

1.4.3 Phase-changing stimuli

Fluctuating spatial distribution of vegetation forms the ecological basis for swarming phenotype expression in *S. gregaria*. Clustered vegetation forces together solitarious locusts more than widely distributed vegetation (Collett et al., 1998; Despland et al., 2000). Once this is achieved, the consequent crowding with conspecifics rapidly induces behavioural gregarisation in nymphs and adults. In the lab, *S. gregaria* gregarise strongly within 4 h of crowding with conspecifics (Bouaïchi et al., 1995; Roessingh and Simpson, 1994). This rapid behavioural gregarisation is a *transiens* stage, as re-isolation reverses the change equally quickly (Roessingh and Simpson, 1994). Only sustained crowding stimuli consolidate the behavioural changes and induce the more gradual morphological differences over 1-2 generations of epigenetic accumulation (Pener and Simpson, 2009). Once gregarisation has taken place, there is a phase transmission effect from gregarious females to their offspring, due to the actions of a 'gregarising factor' secreted into the egg pod foam (Islam, 1997; McCaffery et al., 1998; Miller et al., 2008; Simpson and Miller, 2007). Such transgenerational transmission and hatchling emergence within a swarm setting contribute towards a positive feedback effect of swarm formation in the field (Symmons and Cressman, 2001; Uvarov, 1966).

Solitarisation takes place if individuals are separated from the group (Pener and Simpson, 2009), if the nutritional value of the food increases, the distribution of food becomes wider (Despland and Simpson, 2000), or at night when locusts are quiescent with no visual stimuli from conspecifics (Roessingh and Simpson, 1994). In *S. gregaria*,

solitarisation is reported to be a slower process, with 96 h of isolation failing to fully solitarise the behaviour of 5th instar gregarious locusts (Roessingh and Simpson, 1994). However, gregarious adult locusts in another study exhibited a loss of attraction behaviours after just 1 h isolation (Alessi et al., 2014). As the studies were carried out in different developmental stages in different arena paradigms, however, comparisons between them are difficult.

Gregarisation in the wild involves clustering the normally shy solitarious animals with conspecifics onto sparse vegetation, where they are exposed to the sight, smell and tactile 'jostling' stimuli associated with this crowding (Pener and Simpson, 2009). Advances have been made in pinpointing the precise sensory stimuli evoking behavioural gregarisation. Early studies indicated a role of tactile stimulation (Ellis, 1959; Hägele and Simpson, 2000). While 4 h exposure to odour stimuli derived from conspecifics alone was not sufficient, and visual stimuli produced only partial gregarisation, the combination of visual and olfactory stimuli gregarised solitarious locusts (Roessingh et al., 1998). Tactile stimulation using bombardment with small paper balls was by itself sufficient to gregarise solitarious locusts (Roessingh et al., 1998). Chemosensory stimulation of the femur was not sufficient to gregarise the animal (Rogers et al., 2003). Further work using a fine paintbrush to stroke ("tickle") solitarious 5th instar nymphs revealed that tactile stimulation of the hind leg femur for 2 h gregarised them more strongly than tickling other regions of the cuticle (Simpson et al., 2001). This gregarising effect of stroking the hind leg femur required an additional proprioceptive input from receptors at the thoraco-coxal joint signalling leg movements: restraining the hind leg prevented tickling stimulation from producing gregarisation. Furthermore, electrical stimulation of metathoracic nerve 5, the nerve innervating the entire hind leg, had the same gregarising effect (Rogers et al., 2003). These findings indicate that the neuronal pathways processing both tactile and proprioceptive stimuli are required for touch-evoked behavioural gregarisation.

In other locust species alternative sensory pathways are important for behavioural gregarisation. For example, stroking the antennae of *C. terminifera* was a stronger gregarising stimulus than stroking the hind leg femur. In this species, neither olfactory stimuli, nor visual stimuli, nor a combination of the two evoked behavioural gregarisation

(Cullen et al., 2010). No comparative analysis of visual, olfactory and tactile stimuli on behavioural gregarisation has been carried out in *L. migratoria*, and it would be difficult since the gregarisation time course of *L. migratoria* is longer than that of *S. gregaria*; 96 h crowding is only sufficient to partially gregarise solitarious nymphs (Ma et al., 2011).

1.5 Behavioural quantification in phase change

As behaviour is the first overt phenotype to change in locust phase change, behavioural analyses have shed light on the mechanisms of the early stages of phase change. Early studies used assays aimed at understanding the effects of crowd stimuli on aggregation behaviours (Pener and Simpson, 2009). For example, a ring-shaped arena was used to assess marching behaviours in solitarious nymphs of *L. migratoria* (Ellis, 1953), and the role of cannibalism in driving nymphal marching (Bazazi et al., 2008). A circular arena was used for measurements of grouping behaviours (Heifetz et al., 1997). Other assays have been used to assess aggregation behaviour, but only with focus on measurements of distances between individuals. While these were highly useful for the understanding of the acquisition of aggregation, marching and intraspecific interactions in groups (Ellis, 1959, 1963a, 1963b, 1964; Pener and Simpson, 2009), they were less informative for understanding individual behaviour.

The development of an assay of individual locust behaviours was instrumental in assessing individual behavioural phase state (Roessingh et al., 1993). This arena assay used a rectangular arena with a crowd stimulus chamber at one end and an empty control stimulus chamber at the other end. The test locust was placed into the arena through an entry hole in the floor midway between the empty and crowd chambers. An experimenter could then record the locust's trajectory over ~10 min, as well as measuring more subtle behaviours such as grooming. Observations of many known solitarious and gregarious locusts provided baseline data against which could be compared the behaviours of animals of unknown phase state. These comparisons were made formally using logistic regression methods, which were used to predict an individual's behavioural phase state in terms of the probability of belonging to the gregarious model population (expressed as P_{greg}, or its reciprocal P_{sol} in some studies). Typical behaviours used to form the logistic regression model in this assay include time spent near to the crowd stimulus, time spent at rest, time spent grooming and the ground covered in the arena. For example,

solitarious locusts show less activity, less exploration, less time near to the crowd stimulus, and more time jumping inside the arena than gregarious locusts (Roessingh et al., 1993). This arena and method of analysis has facilitated characterisations of behavioural phase change across *S. gregaria* (Anstey, 2012; Bouaïchi et al., 1995; Roessingh et al., 1998; Rogers et al., 2003; Simpson et al., 2001), *L. migratoria* (Guo et al., 2015, 2013; Hoste et al., 2002; Ma et al., 2011) and a range of other locust species (Anstey, 2012; Cullen et al., 2010; Gray et al., 2009; Sword, 2003). This 'Roessingh arena' has mostly been used with nymphs to reduce complications of flight and of sexual attraction to conspecifics. Further advances of the assay technique were made using automated object tracking software to increase throughput, but the technology is currently not advanced enough to detect more subtle behaviours such as grooming that were included in the earlier manually-scored versions (Cullen et al., 2012, 2010; Gray et al., 2009; Stettin, 2014). This technique has received criticism for being unduly influenced by changes in a single underlying behaviour such as activity (Tanaka and Nishide, 2013), but a detailed meta-analysis argued that the technique was robust and sensitive to changes in all behaviours comprising the logistical regression model (Rogers et al., 2014).

Specific aspects of phase-related behaviours have been investigated using other methods. Pure attraction and repulsion assays have been used to investigate mechanisms of behavioural phase change in *S. gregaria* (Alessi et al., 2014; Tanaka and Nishide, 2013) and *L. migratoria* (Guo et al., 2011; Ma et al., 2015). A Y-maze has been used to identify odour preferences and appetitive or aversive conditioning in *S. gregaria* (Simões et al., 2016). This was based on an innate preference of locusts for vanilla over lemon odour. Geva et al., (2010) recorded the behaviours of crowds of solitarious and gregarious locusts placed together into a circular arena. Interestingly, contrary to the low activity observed individually in the Roessingh arena (Roessingh et al., 1993), solitarious locusts showed higher activity than gregarious locusts when entering this crowded chamber, and their activity steadily decreased over time in the crowd. This was suggested to be an escape response driven by aversion to conspecifics, which decreases over time as they habituate to the crowd stimulus (Geva et al., 2010). In this thesis, which focusses on the behaviours of individual animals, a Roessingh arena was used to characterise the phase state of most experimental animals. Some specific experiments used a Y-maze.

1.6 The neurochemical basis of locust behavioural phase change

Neurochemicals form the basis of neurotransmission and neuronal plasticity across the animal kingdom (Kandel et al., 2012). For this reason, they are very likely to play roles in the initiation of behavioural phase change. Neurochemicals fall into 3 broad categories based on their functions: neurotransmitters, neuromodulators and neurohormones. Neurotransmitters (e.g. glutamate, acetylcholine) are released across specialised synaptic junctions. They generally act on postsynaptic ligand-gated ion channels to change the membrane potential of the postsynaptic cell, and initiate or inhibit action potentials. Neuromodulators (e.g. dopamine, DA) are typically released by a neuron and act through second messengers to exert long-term effects on the function of a somewhat distant target cell population. Neurohormones (e.g. neuroparsin) are typically released into the blood or haemolymph to affect more peripheral targets, however, the distinction between these and neuromodulators is not always clear-cut. Some molecules defy these broad classifications, such as octopamine (OA), which can satisfy all three definitions (see Orchard et al., 1993), and nitric oxide, which is a neurotransmitter capable of diffusion through cell membranes into neighbouring cells (Burrows, 1996; Garthwaite et al., 1988).

Unlike neurotransmitters, the often broad and long-lasting effects of neuromodulators and neurohormones make them prime candidates for modulating behaviour and behavioural plasticity in vertebrates and invertebrates. There is extensive evidence for the role of neuromodulators and neurohormones in behaviour in vertebrates such as the neurohormones oxytocin and vasopressin in social behaviours (Keverne and Curley, 2004; Veenema and Neumann, 2008), a variety of neuromodulators in mammal aggression (Nelson and Chiavegatto, 2001) and more (see Levin, 2006). This concept of the 'orchestration of behaviour' by neuromodulators is also well-supported in invertebrates such as molluscs and arthropods (Hunt, 2007; Sombati and Hoyle, 1984; Lockard et al., 2017; Malagoli and Ottaviani, 2017; Libersat and Pflueger, 2004; Verlinden et al, 2010).

1.6.1 Neurohormones and neuromodulators in behavioural phase change

A prominent group of neuromodulators with widespread effects on behaviour are the biogenic amines (Libersat and Pflueger, 2004; Verlinden et al., 2010; Vleugels et al.,

2015; Pener and Simpson, 2009). Biogenic amines that have received the most attention in the context of behavioural phase change are the monoamines DA, OA and 5-hydroxytryptamine (5-HT, serotonin) due to their roles in well-defined behaviours (Kamhi and Traniello, 2013). For example, 5-HT is implicated in stress-coping behaviours, boldness and social dominance in fish, as well as regulation of aggression and feeding behaviour in mice (Mosienko et al., 2015; Voigt and Fink, 2015). DA is linked with behaviours such as social pair bonding (Johnson and Young, 2015), reward-seeking behaviour (Schultz, 2016) and maternal behaviour (Bridges, 2015) in vertebrates.

Invertebrates such as the Desert Locust make useful models for studying the monoamine orchestration of behaviour due to the smaller assemblies of neurons involved in coordinating behaviours and comparative ease with which invasive techniques can be applied (Libersat and Pflueger, 2004). Hoyle and Barker (1975) found that key neurons modulating the contraction of metathoracic extensor tibia motor fibres, the Dorsal Unpaired Median (DUM) neurons, are octopaminergic. A later study by Sombati and Hoyle (1984) found that experimental application of OA to regions of the thoracic CNS- evoked specific behaviours such as tibial extension and flight in locusts. Injections of 5-HT into the lobster initiate flexion of appendages whereas injections of OA have the opposite effect (Livingston et al., 1980). Furthermore, 5-HT has been linked with social behaviours in invertebrates such as the cricket and the lobster (Kravitz, 2000; Steddon et al., 2000; Stevenson et al., 2000). OA and DA are thought to be involved in appetitive and aversive conditioning in arthropods (Barron et al., 2010). Furthermore, DA and OA appear to mediate aggressive behaviours in the cricket, in opposition to the action of 5-HT (Stevenson et al., 2000).

Amounts of monoamines show transient changes in the CNS of *S. gregaria* during behavioural phase change, suggesting a role in this process (Rogers et al., 2004). The role of 5-HT in behavioural gregarisation of *S. gregaria* was identified by the finding that amounts of 5-HT underwent a dramatic and transient 9-fold increase in the thoracic ganglia at the same time as behavioural gregarisation, that ganglionic 5-HT correlated with behavioural gregariousness and that pharmacological manipulation of 5-HT affected behavioural gregarisation (Anstey et al., 2009; Rogers et al., 2004). Furthermore,

increases in DA amounts in the CNS over 24 h isolation, as well as pharmacological studies, have implicated DA in behavioural solitarisation in *S. gregaria* (Alessi et al., 2014; Rogers et al., 2004; Stettin, 2014). Interestingly, this role is suggested to be reversed in *L. migratoria*, though this is suggested to be dependent on the specific receptor activated (Guo et al., 2015, 2013; Ma et al., 2011). Though the role of OA in behavioural phase change has not been thoroughly investigated in *S. gregaria*, both it and its precursor molecule tyramine have been implicated in conspecific attraction and repulsion in *L. migratoria* (Ma et al., 2015). The role of 5-HT and other monoamines in behavioural phase change is covered in more detail in the introductions of Chapters 3 (Section 3.2) and 4 (Section 4.2).

Although some neurohormones are implicated in the expression of morphological and physiological phase characteristics, the consensus is currently that they are not involved in the expression of behavioural phase. A peptide neurohormone produced in the locust brain, [His7]-corazonin, induces expression of gregarious-like colouration in solitarious nymphs, but it does not affect behaviour (Hoste et al., 2002). Juvenile Hormone has also been suggested to mediate morphological and colouration aspects of the solitarious phase, although there is no evidence for a role in expression of solitarious behaviour (Pener and Simpson, 2009).

1.6.2 Quantification and manipulation of neurochemicals in the locust

The use of High-Performance Liquid Chromatography (HPLC) has revealed overall changes in the amounts of monoamines over different stages of behavioural phase change in the locust CNS (Anstey, 2012; Ma et al., 2011, 2015; Rogers et al., 2004). While HPLC provides a precise estimate of the total content in the CNS, it is less informative regarding the specific actions of the monoamines; for example, the localisation of action or the synaptic release dynamics. Immunocytochemical methods have revealed populations of serotonergic neurons in the thoracic ganglia that increase their 5-HT content in response to gregarising stimuli (Rogers and Ott, 2015). Unpublished work using the electrochemical technique Fast Scan Cyclic Voltammetry (FSCV) suggests increases in extracellular 5-HT release in the thoracic ganglia during hind leg tickling (Fenton, G., unpublished, 2017), but it is not known if this is related to phase change. To measure 5-HT and DA levels in individual locusts following experimental treatments and

behavioural assays, this thesis uses extremely sensitive HPLC with electrochemical detection. This makes it possible to seek correlations between behaviour and neurochemical content during phase change.

Investigations into the role of monoamines in behavioural phase change in *S. gregaria* have predominantly employed pharmacological techniques (Alessi et al., 2014; Anstey, 2012; Anstey et al., 2009; Stettin, 2014), although RNA interference against 5-HT, DA and OA receptors has been carried out in *L. migratoria* (Guo et al., 2015, 2013; Ma et al., 2011). While providing valuable insights into the neurochemical basis of phase change, administration of pharmacological compounds is often non-specific and invasive, and comes with the caveat that it is difficult to confirm actions of the drug. Improvement in this field will certainly involve genetic approaches. In comparison with *Drosophila melanogaster*, locusts have relatively few genetic tools available for investigations into the neurochemical basis of behavioural phase change. Progress has been made with the publication of the genome of *L. migratoria* (Wang et al., 2014), and of a modern genetic modification technique (CRISPR/CAS9) in *L. migratoria* (Li et al., 2016). No genome has yet been published for *S. gregaria*, although efforts are in progress (Camacho et al., 2015). Published transcriptomes have revealed important differences between long-term phases in S. *gregaria* and *L. migratoria*, but no transcriptome has been published regarding short-term gregarisation in either species (Badisco et al., 2011; Chen et al., 2010; Ma et al., 2006). Thus, a wider range of molecular tools will be useful to investigate the neurochemical basis of behavioural phase change.

1.7 Learning, memory and behavioural phase change

It has been suggested that behavioural gregarisation includes habituation to crowded conditions, and can be used as a model for learning and memory (Ellis, 1959; Geva et al., 2010). Learning is a behavioural change in response to an experience; and memory is the representation of the experience stored in the organism (Kandel, 2001). If one considers gregarising stimuli as an ‘experience’ and behavioural gregarisation as the ‘learning’ response, then the two forms of phenotypic plasticity may be very similar. Two principal forms of memory are short-term memory, a labile memory formed through short-term changes in synaptic transmission, and long-term memory, a more stable memory that requires gene transcription and protein synthesis within neurons for

consolidation (reviewed in Kandel, 2014). In a reflection of short-term and long-term memory processes, the separate processes of acquisition and consolidation of gregarious behaviour are dependent on the intensity and duration of the stimulus; gregarious behaviour acquired over a brief 4 h crowding stimulus is quickly lost upon re-isolation, whereas the behaviour of long-term gregarious locusts takes much longer to change (Roessingh and Simpson, 1994). Stettin (2014) found that the gregarisation rate of 5th instar nymphs that had been reared in a crowd until the 4th instar stage and then isolated for 2 stadia was higher than that of long-term isolated nymphs, suggesting a 'recall' effect of the crowding stimulus. These lines of evidence suggest parallels between behavioural phase change and learning and memory.

Given the phenomenological similarities between learning and memory and behavioural phase change, it is plausible that they share a mechanistic basis. The molecular basis of learning and memory is well understood (Kandel et al., 2014), and it is notable that major signalling mechanisms implicated in locust behavioural phase change such as 5-HT (Anstey et al., 2009; Guo et al., 2013; Ma et al., 2011; Rogers et al., 2004; Rogers and Ott, 2015), DA (Alessi et al., 2014; Guo et al., 2015; Ma et al., 2011) and the downstream effector molecule cyclic adenosine monophosphate (cAMP)-dependent Protein Kinase A (PKA; Ott et al., 2012) are also crucial to learning and memory (Kandel et al., 2014). Like short-term memory but not long-term memory formation (Fulton et al., 2008), short-term behavioural gregarisation is insensitive to cold-induced anaesthesia and does not require gene transcription or translation (Stettin, 2014). No evidence for a role in behavioural gregarisation was found for three other molecules involved in learning and memory: Protein Kinase G (PKG) (Ott et al., 2012), Protein Kinase C (PKC) and Calcium-Calmodulin Dependent Kinase II (CaMKII) (Stettin, 2014). However, as there are many forms of learning and memory with diverse biological underpinnings (Kandel, 2001), further work is required to understand where behavioural gregarisation falls in this spectrum.

The possibility that behavioural phase change may selectively modify 'classical' forms of learning and memory such as appetitive and aversive conditioning, has exciting implications for the organism as a model of learning and memory (see Byrne et al., 2014). For example, gregarious locusts lack the aversive conditioning responses to a noxious

food stimulus that is shown in solitarious locusts. Crowding solitarious locusts abolished the formation of new aversive responses, but aversive memories formed prior to crowding were maintained. Furthermore, appetitive conditioning responses were unchanged across phases (Simões et al., 2016, 2013). It will thus be useful to investigate this possible interaction in other behavioural learning paradigms such as operant conditioning, and contextual, social, or object familiarity.

1.8 Neuronal circuitry in phase change

Relatively little is known about the neuronal circuits mediating behavioural phase change in the locust. The observations that behavioural gregarisation can be elicited by tactile and proprioceptive stimulation of the hind leg alone (Roessingh et al., 1998; Rogers et al., 2003; Simpson et al., 2001), or by combined visual and olfactory cues (Roessingh and Simpson, 1994), imply that there are both cephalic and thoracic pathways effecting behavioural gregarisation in *S. gregaria*. There are CNS region-specific changes of 5-HT, DA and OA during solitarisation and gregarisation, suggesting that the brain, optic lobes and thoracic ganglia each process particular aspects of behavioural phase change (Rogers et al., 2004). Direct application of 5-HT to the thoracic ganglia is sufficient to increase an locust's behavioural gregariousness (Anstey et al., 2009), suggesting that this region of the nerve cord contains key targets for this neuromodulator. Inferences concerning the likely sensory input elements of the thoracic pathway have been made by the electrical stimulation of sensory nerves such as the metathoracic nerves 5A (containing thoraco-coxal joint proprioceptive afferents) and 5B (containing tactile and proprioceptive afferents from the more distal parts of the leg; Anstey et al., 2009; Rogers et al., 2003). All of these afferents form connections with diverse populations of interneurons within the thoracic ganglia (Burrows and Newland, 1993, 1994, Newland and Burrows, 1994, 1997). Beyond these brief glimpses, however, the neuronal circuitry connecting the gregarising sensory stimuli with behavioural responses over the time frame of behavioural phase change remains unclear.

Another consideration in this question is that the concept of a discrete 'gregarisation circuit' within the CNS may be too simplistic. Although behavioural phase change is evoked by population density alone, a suite of different behaviours undergo phase transition in a short time frame, such as conspecific attraction, activity and exploration

(Roessingh et al., 1993; Uvarov, 1966; Roessingh and Simpson, 1994). As these changes in different behaviours are initiated and controlled by diverse neuronal networks (Burrows, 1996), it may be more appropriate to approach behavioural gregarisation as a CNS-wide 'reconfiguration' process that involves the modification of many networks. To tackle this challenge, new tools are required. One such tool that has been used extensively in mammals and invertebrates is the visualisation of immediate-early gene (IEG) transcription. IEGs are genes whose transcription is upregulated rapidly and transiently when a neuron has been activated. The imaging of IEG transcription has allowed spatial analyses of neuronal activity during learning, behaviour and sensory stimulation in rodents and invertebrates (Chen et al., 2016; Fujita et al., 2013; Guzowski et al., 2005; Lutz and Robinson, 2013; McNeill and Robinson, 2015). This technique is brought to bear on behavioural phase change for the first time in this thesis, and is introduced in detail in Chapter 6.

1.9 Thesis rationale

This thesis began with the main aim of examining mechanisms underpinning consolidation of gregarisation and mechanisms underpinning solitarisation, to build on the established role of 5-HT in short-term behavioural gregarisation in *S. gregaria*. Multiple lines of evidence indicated that 5-HT was sufficient and necessary to induce behavioural gregarisation over 2-4 h (Anstey et al., 2009; Rogers et al., 2004; Rogers and Ott, 2015), but there was apparently no role for 5-HT in behavioural solitarisation (Stettin, 2014). Although it was presumed that monoamines were not involved in the consolidation of phase behaviours beyond the first 24 h of behavioural gregarisation (Anstey et al., 2009; Ott et al., 2012; Rogers et al., 2004), no studies had been carried out investigating the role of 5-HT in maintenance of behavioural phase. Another important question was whether 5-HT is involved in the re-gregarisation of transiently-isolated long-term gregarious locusts.

Using the same behavioural and HPLC methods as Anstey et al. (2009), experiments in Chapter 4 failed to confirm an effect of the 5-HT-depleting compound α-methyltryptophan (AMTP) on crowding or tickle-induced behavioural gregarisation, nor on CNS 5-HT amounts. This created the necessity to re-investigate key findings in the original papers, most importantly the 9-fold increase in ganglionic 5-HT amounts

reported in the first 4 h of gregarisation, and correlations of 5-HT content with behavioural gregariousness (Anstey et al., 2009; Rogers et al., 2004). Once again, these published findings could not be replicated (Chapter 3), so the 5-HT hypothesis was investigated across family lines and strains of *S. gregaria*. This work provided no evidence for a role of 5-HT in behavioural gregarisation.

These conclusions led to investigations into the role of other monoamines such as DA in behavioural phase change in *S. gregaria*. With a lack of effect of the DA receptor antagonist fluphenazine (Alessi et al., 2014) on behavioural phase change, it was decided to resort to a non-specific monoamine depletion protocol using the drug reserpine (Chapter 4). This drug strongly depleted 5-HT and DA, and blocked the effect of crowding on behavioural gregarisation. However, it also raised the longstanding issue of how to distinguish true solitarious behaviours from solitarious-like behaviours of movement-impaired individuals in the Roessingh arena assay (Roessingh et al., 1993).

During the experiments of Chapter 4, unexpected behavioural changes in a control group of animals also raised a further issue regarding a possible effect of repeated behavioural assays and handling on behaviour. In essence, did *S. gregaria* show a familiarity response to contextual stimuli of the behavioural arena, and was the nature of this response dependent on the phase? This question was investigated in detail in Chapter 5, which found strong effects of repeated arena exposure in solitarious locusts at short time intervals (2 h). This effect was weak, but apparent in gregarious locusts, and was absent across longer time intervals, as well as in a behavioural Y-maze paradigm established in previous work (Simões et al., 2016). Thus, it remained largely unclear what had caused the unusual behaviour of the control group in Chapter 4.

In contrast to the whole-tissue and whole-animal scale experiments of the first chapters, the work in Chapter 6 investigated the potential for generating neuronal 'activity maps' of the locust CNS during behavioural phase change using immediate-early gene transcription. Thus, Chapter 6 identified the *S. gregaria* orthologues of 4 commonly used IEG targets and investigated their expression in solitarious locusts using quantitative Polymerase Chain Reaction (qPCR) after 30 min crowding. Their long-term expression was also compared in long-term phases using a transcriptome generated in previous work

(Shand, 2015). Though no evidence was found for upregulation of the 4 IEGs, this chapter laid important groundwork for further use of this technique in the future.

1.10 Overview of the chapters

Chapter 2: General Methods

This general methods chapter describes in detail the key methods used throughout the thesis, including animal husbandry, crowding, tickling and isolation protocols, drug injections, HPLC procedures and quantification of behaviour. It also outlines the statistical approaches and terminology employed in this thesis in an effort to minimise the use of misleading phrasing.

Chapter 3: Does the established role of 5-HT in locust behavioural gregarisation stand up to scrutiny?

This chapter re-investigates the role of 5-HT in behavioural gregarisation in relation to previous literature. It examines the correlation between 5-HT and behavioural gregariousness using 3 different paradigms:

1. Across 2 h hind leg tickling, 2 h crowding and 4 h crowding in the Leicester colony of *S. gregaria*.
2. Across 4 h crowding in different maternal lineages of animals in the Leicester colony.
3. Across 1, 2, 4 h crowding in the Leicester colony and a recently derived strain from eggs collected in Mauritania.

Chapter 4: Pharmacological reinvestigations into the role of monoamines in behavioural phase change in the Desert Locust

This chapter re-examines and expands upon previous reports of the importance of monoamines in behavioural phase change. This encompasses investigations into the role of DA in behavioural phase change and maintenance, the role of 5-HT in behavioural gregarisation and the role of monoamines in behavioural gregariousness in gregarious and crowded solitarious locusts. The different experiments investigate:

1. The effect of blocking DA receptors with the antagonist fluphenazine on the behavioural phase state and behavioural phase change in gregarious and solitarious locusts.
2. The effect of injections of the 5-HT-depleting compound AMTP on 5-HT concentrations in the CNS and behavioural gregariousness.
3. The effect of injections of a drug vehicle solution dimethyl sulfoxide (DMSO) on behavioural gregariousness in gregarious locusts.
4. The effect of injections of the nonspecific monoamine-depleting compound reserpine on DA and 5-HT concentrations and behavioural phase gregariousness in gregarious, solitarious and 4 h crowded solitarious locusts.

The fluphenazine experiment in both solitarious and gregarious locusts provided surprising trends for changes in behavioural gregariousness over 96 h with repeated assays, handling and injections in the control groups. This unexpected change was investigated in more detail in the following chapter.

Chapter 5: Phase-specific responses to repeated stimulus exposure in the Desert Locust
In this chapter, I aim to understand the effects of repeated behavioural assays, handling and injections on behavioural gregariousness and exploration in the arena. When weak evidence was found for increases in behavioural gregariousness with repeated assays in solitarious locusts, I then aimed to understand if this behavioural change over time was as strong in an arena lacking a gregarising crowd stimulus as in an arena with this stimulus. Finally, I aimed to investigate if behavioural changes over repeated arena runs were detectable in a behavioural binary (Y-maze) paradigm. The main investigations were:

1. Whether the changes observed in the fluphenazine experiment are reproducible in solitarious and gregarious locusts, and whether they are due to repeated arena assay runs, handling or injections.
2. If changes in behavioural exploration over repeated runs take place at different time intervals, and if a crowd stimulus is required in the behavioural arena for this behavioural change.
3. Whether familiarisation to an odour in solitarious and gregarious locusts changes their odour preferences in a Y-maze setup.

Chapter 6: Identification of activity-related immediate-early genes in the Desert Locust

I aim to understand which, if any, of four target IEGs are upregulated during behavioural gregarisation in the CNS in *S. gregaria*. I first characterise the nucleotide and protein sequences of these 4 IEGs in *S. gregaria*. I then study the changes in expression of these 4 IEGs over long-term phase change, and within 30 min of behavioural gregarisation of solitarious locusts. The main components of this chapter are to:

1. Use an existing transcriptome to obtain nucleotide and protein sequences of *S. gregaria* homologues of the 4 IEGs EGR, Fos, Hr38 and JRA.
2. Use a Polymerase Chain Reaction (PCR) technique to validate primers based on the sequences obtained in step 1.
3. Use qPCR to understand the effect of 30 min crowding on mRNA expression of these 4 IEG targets in solitarious locusts.
4. Use existing quantification of the transcriptome to analyse changes in IEG transcripts between male and female locusts in the gregarious and solitarious phases.

Chapter 7: General Discussion

This chapter interprets the results and discusses how they advance the study of phase change.

2 General Methods

2.1 Animal husbandry

2.1.1 Gregarious colony

Gregarious phase locusts *S. gregaria* (Forskål) were reared over many generations in large cages (50 cm L × 50 cm W × 50 cm D; 100–300 adult locusts per cage, mixed males and females; Figure 2.1A). Locusts were kept under a 12:12 h light-dark cycle, with temperatures of 35–38°C (light period) and 25°C (dark period), and relative humidity at 20–25%. The holding rooms were lit with high-frequency daylight fluorescent bulbs in the light period. Lights were turned on at 8am GMT (9am BST) and were turned off at 8pm GMT (9pm BST). Holding cages were constructed with a solid metal floor, perforated metal side walls and top, and a clear acrylic rear wall. Slots at the front held a sliding clear acrylic front wall for feeding and cleaning access. All locusts were fed *ad libitum* with trays of fresh wheat seedlings and dishes of bran flakes administered three times per week. Faeces and dead locusts were cleaned from the floor of the cages with a vacuum cleaner three times per week. Rectangular self-supporting shelves of metal mesh were added inside each cage containing 4th instar juveniles and older to allow the addition of larger amounts of wheat, as well as to provide an extra platform from which animals could hang while moulting. Cages containing more than 300 4th instar, 5th instar or adult locusts were thinned out to minimise overcrowding. Locusts were sucked through a 5 cm diameter vacuum cleaner hose into a small cage under vacuum, and then frozen. Cages containing breeding adults were fitted with glass tubes (12 cm L × 3 cm D) filled with loose compost for egg deposition. Breeder cages were maintained for 1–3 weeks before culling. Empty cages were washed thoroughly in a Lancer washer with 10% bleach (78638, Eastern Shires Purchasing Organisation, UK). Every 3 d, egg tubes containing egg pods from multiple females were removed, placed in clear plastic tubs and incubated at 30°C for ~ 10 d until hatching. Gregarious hatchlings were placed in a clean gregarious cage containing a tray of wheat seedlings, a dish of bran and a beaker of wet cotton wool to allow the animals to drink. Each month, several locusts were dissected and their Malpighian tubules screened for infections of the gut parasite *Malamoeba*. If the screening gave a positive result, wheat seedlings and bran were spray-coated with 6% sulfamethazine, 6% sulfacetamide and 12% sulfathiozole for several weeks until the infection was cleared. In practice the colony was treated for

Malamoeba approximately every 6 months; animals fed treated wheat were not used for experiments. Locusts reared in the gregarious colony all showed full gregarious colouration, indicating that the conditions were sufficient to hold them in the gregarious phase. Gregarious locusts used in all experiments were selected from the 5th instar within 2–5 days after the 4th moult, prior to the separation of their wing buds, as 5th instar nymphs nearing the end of the instar stage (8–9 d) become less active due to the moulting process.

2.1.2 Solitarious colony

First generation male and female solitarious locust breeders were isolated from the gregarious colony at hatching and housed individually in a different holding facility under the same temperature and light conditions as for gregarious locusts. The ventilation in the colony was maintained at 20 air changes per hour. The solitarious locust facility was purpose-built to ensure that animals could be raised in complete isolation from the sight, smell and touch of other locusts. Hatchlings were separated into individual holding tubs (15 cm H × 5 cm D) consisting of a lower clear plastic pot containing water with a modified screw-on lid (Figure 2.1B, lower compartment), which formed the floor of an upper holding chamber (8 cm H) consisting of a modified clear plastic pot (Figure 2.1B, upper compartment). In the centre of the lid/floor was cut a 15 mm hole through which a bundle of fresh wheat leaves and an absorbent string wick (to allow animals to drink) was pushed. The leaves and wick thus dipped into the water below and passed into the upper holding chamber. Each week, the lower chamber was replaced with a new one containing fresh water and wheat leaves, with bran flakes scattered on the holding chamber floor. Opaque white tape was applied to the lateral sides of the holding chamber to prevent sight exposure to conspecifics in the neighbouring holding tubs. To ensure total isolation from the smell of conspecifics, each holding chamber was provided with an independent clean air supply drawn from the outside of the building, heated to 36°C (day) or 25°C (night) and humidified as necessary. This clean conditioned air was pumped into the holding chambers at ~ 950 air changes per hour, at a slight positive pressure compared with the holding room air pressure. Upon reaching the 4th instar stage, solitarious juveniles were transferred to metal holding cages (10 cm L × 10 cm W × 20 cm H; Figure 2.1C) with a clear acrylic lid and clear acrylic sliding door on the front. These ‘adult cages’ contained a metal clip to hold a glass vial of wheat seedlings, a removable wire mesh floor suspended 10 cm above the base of the cage to allow easy

cleaning of faecal matter from the lower compartment, and a strip of wire mesh as a perch to facilitate moulting. They were provided with individually ducted clean air at ~ 45 air changes per hour. All locusts were fed *ad libitum* with vials of fresh wheat seedlings in water and dishes of bran flakes provided twice per week. Faecal deposits were vacuum cleaned from the cages twice per week.

Once sexually mature, selected male and female solitarious breeders were paired in clear plastic tubs (25 cm L × 15 cm W × 15 cm D) and left for 3–4 h to allow mating to take place. Mating was deemed successful if the male had mounted the female and commenced copulation by the end of the waiting period. The breeders were then returned to their separate holding cages. The removable wire mesh floors of the cages holding female breeders were modified with a hole to allow the female to lay in a clear plastic egg tube containing moist sand (sand + 10% water mix) or compost. Egg tubes were checked for egg pods twice per week, and any egg tubes containing an egg pod were removed and incubated individually at 30°C until hatching. The offspring of these breeders, second generation solitarious hatchlings, were set up in the same conditions and holding tubs. All dirty holding tubs and metal cages were washed thoroughly using a dishwasher (washing tablets: EDISHTAB25, Classic, Ecover, Belgium). The same *Malamoeba* checks and treatment procedures as for gregarious animals were implemented in the solitarious colony. Locusts reared in the solitarious colony showed full solitarious colouration (bright green, green with dappled black or light brown/beige in nymphs), indicating that the conditions were sufficient to hold them in the solitarious phase. Second generation solitarious locusts used in all experiments were selected from the 5th instar within 2–6 days after the 4th moult, prior to the dorsal separation of their wing buds. Subject to availability, some experiments also used third or fourth generation solitarious locusts, which showed weak evidence for a behavioural distinction from second generation solitarious animals in the Leicester locust colony (See Appendix 2.7.1). Unless otherwise specified, solitarious hatchlings were set up in sibling groups of 10–20, from different solitarious breeding pairs. Animals from several such sibling groups were pooled to achieve the required sample sizes in any given experiment. Unless stated otherwise, control and experimental treatments were always run simultaneously so that siblings were approximately evenly distributed across control and experimental treatments.

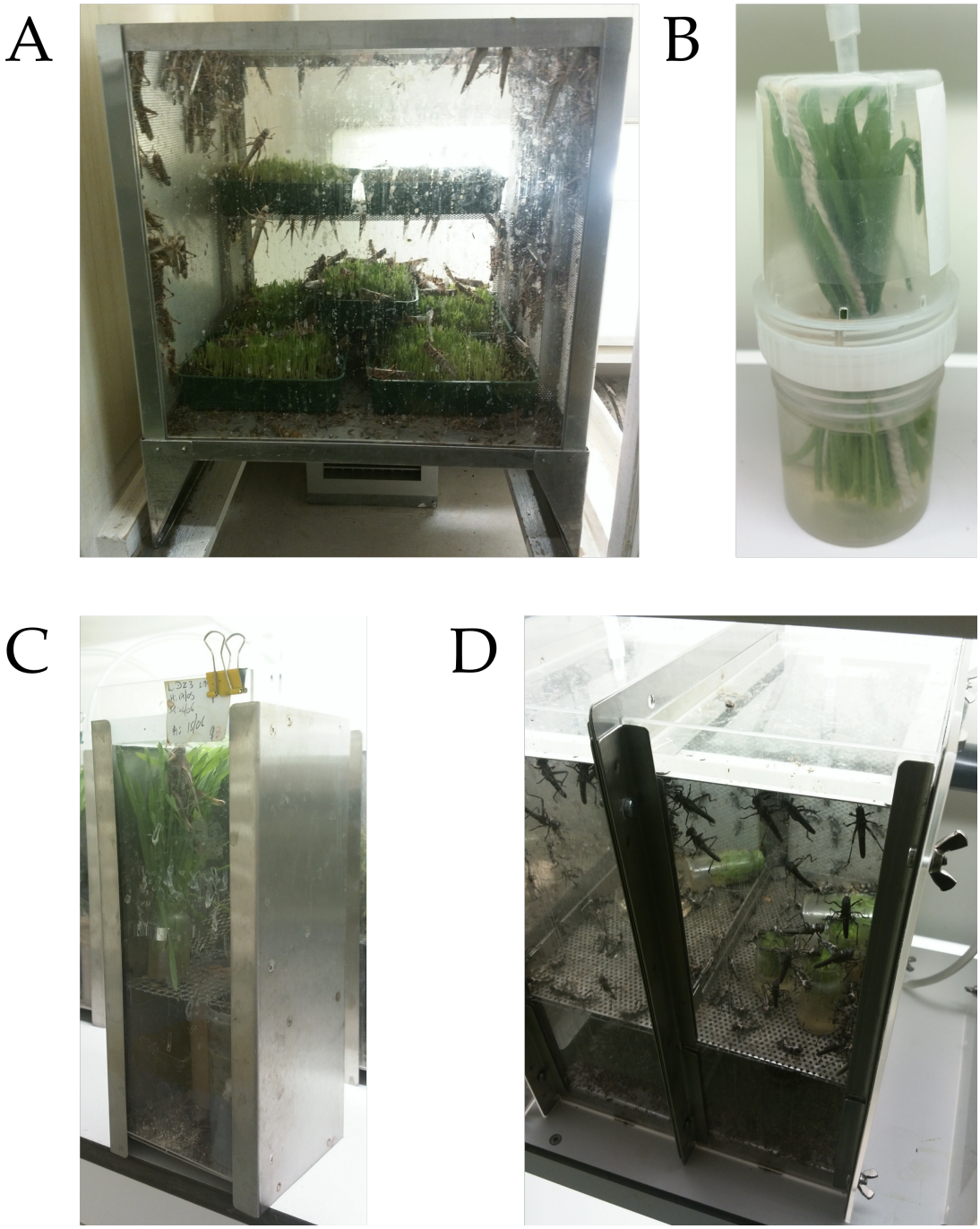


Figure 2.1: Locust holding cages. (A) Gregarious holding cage containing 100-300 adult locusts. (B) Solitarious hatchling holding cage. (C) Solitarious adult holding cage. (D) Modular holding cage containing ~ 100 3rd instar locusts. Shown here are two modules connected together.

2.1.3 Establishing a field strain from Mauritania

To generate a colony from a field strain from Mauritania, collaborators Mohamed Abdallahi Ould Babah, Sidi Ould Ely and colleagues sent eggs collected from field populations in October 2015 (Centre National de Lutte Antiacridienne (CNLA), Nouakchott, Mauritania). The parents of the first batches received were copulating solitarious adults collected in a farming area close to their Akjoujt station (Inchiri region; 19°53'58"N, 14°17'49"W). These adults were collected and reared in 2 m × 2 m × 2 m holding cages in Akjoujt. Their eggs were collected in rocky and sandy soil collected at the same site, and shipped to the University of Leicester prior to hatching. On arrival, the locust eggs were incubated at 30°C until hatching. The colony was topped up in spring 2016 with eggs of gregarious adults, the offspring of solitarious adult populations collected in Sbaiya (18°53'14"N, 13°45'24"W) in November 2015. Gregarious mothers were kept in crowded conditions in CNLA for 3 months during which time there was some evidence for solitarisation of some individuals (S. O. Ely, personal communication).

Fresh hatchlings of the field strain were maintained in modular cages (22 cm L × 11 cm W × 31 cm D per module, ~ 30–40 hatchlings per module; Figure 2.1D) consisting of a metal frame with clear acrylic walls, roof and sliding door. The modular cages had a removable wire mesh floor to allow easy cleaning of faecal matter and a separate clean air supply at 8 air changes per hour primarily to control humidity, as described for Leicester solitarious cages. The hatchlings were provided wheat seedlings and bran *ad libitum*, with a beaker of moist cotton wool to allow the hoppers to drink. The Mauritanian locusts were initially kept in the solitarious locust facility under identical conditions to those described in Section 2.1.2. Once the first-generation Mauritanian locust population was stable, it was moved into large gregarious cages and maintained as per the Leicester gregarious colony (Section 2.1.1) but in a separate quarantined holding facility. Mauritanian solitarious breeders were given the same rearing conditions as Leicester solitarious animals and reared in the same holding facility. The two colonies were quarantined by changing gloves and vacuum cleaner attachments. Mauritanian solitarious experimental animals were set up and reared in the same conditions as the Leicester strain.

2.2 Phase-changing treatments

Forced crowding and repeated hind leg stimulation procedures were adapted from Anstey et al. (2009). For solitarious 5th instar locusts undergoing forced crowding, test animals were labelled bilaterally on the thorax and wingbuds with a permanent marker pen to permit individual identification. They were then placed in clear plastic tubs (25 cm L × 15 cm W × 15 cm D) with a crowd of gregarious conspecifics (~ 30 individuals, mixed male and female, wheat seedlings and bran *ad libitum* unless otherwise specified) in the solitarious colony holding room (Section 2.1.2).

Before hind leg tactile stimulation (tickling), test animals were bilaterally labelled on the thorax and wingbuds with a permanent marker pen to permit individual identification. Tickling was performed by placing solitarious nymphs individually into opaque plastic beakers (8 cm H × 5 cm D) with a wire mesh lid, through which a thin paintbrush was inserted to stroke the left lateral hind leg femur for 5 s per minute for 2 h in Chapter 3 and 4 h in Chapter 4. Control (untickled) animals were either left in their holding cages or placed in the opaque plastic tubs and visually presented with the paintbrush only (sham group; see Chapter 3), without tactile stimulation for 5 s per minute for 2 h. The same paintbrush was used for all animals in each session and rinsed after each session. The opaque plastic beakers were washed between sessions in a dishwasher.

Isolation of gregarious 5th instar locusts was carried out by removing them from the crowd cages and housing them in individual cages in the solitarious colony for 24–48 h (details in Chapter 4), under identical conditions to solitarious holding conditions.

2.3 Behavioural assay

2.3.1 Arena setup

Behavioural assays were carried out in the solitarious holding facility (20 air changes per hour) using an established behavioural arena method developed and automated in previous studies (Roessingh et al., 1993; Stettin, 2014). This procedure used a white acrylic arena with a clear acrylic lid (66.7 cm L × 30.7 cm W × 10.0 cm D). The arena consisted of a central chamber (40.5 cm L × 28.7 cm W × 10.0 cm D; Figure 2.2B and C) – to contain the test locust – and removable stimulus chambers on each end (28.3 cm

L × 13.0 cm W × 10.0 cm D). The central chamber had an entry hole (2 cm diameter) located at centre of the chamber floor with a screw attachment glued to the underside to allow the entry of the test locust from below; and the floor was lined with removable A3 white paper to allow better grip for the test locust and to reduce odour contamination between sessions. To reduce climbing behaviours in the arena, thin Teflon sheets (3 mm) were glued to the long walls of the inner chambers of the arena. The stimulus chambers were made of white acrylic for the floor and three of the walls, while the removable roof was transparent acrylic. The side of the stimulus chamber facing towards the central chamber was made of perforated transparent plastic to allow the passage of visual and olfactory stimuli to the central chamber. Movement of the crowd stimulus animals in the stimulus chambers was limited to the rear 2/3 of the chamber with a coarse wire mesh to prevent them appearing in the tracking footage. One stimulus chamber held ~ 30 gregarious locusts (5^{th} instar, mixed male and female) as a crowd stimulus while the other was kept empty as a control chamber. The position of the stimulus chambers was switched for each session to minimise any potential side bias. The arena was lit obliquely from above using bright LED lamps (Planetsaver, 932030521BC, DFx Technology Ltd., Witney, UK) placed on both lateral sides of the top of the stimulus chambers. The arena was held above the desk top with a metal frame to permit access to the animal entry mechanism from below, and was surrounded with white cotton sheets to shield the test animal from observers. A digital camera (Guppy F-036B, Allied Vision Technologies GmbH, Germany) connected via a firewire cable to a desktop PC running Ubuntu version 12.04 was mounted above the arena to record the test animal from directly above.

2.3.2 Behavioural procedure

To carry out the assay, the test locust was placed into a 10 mL plastic syringe tube (9.8 cm L × 2.3 cm D), which was modified with a screw lid attachment and screw-on cap, and covered with opaque tape (Figure 2.2A). The test locust was left inside this capped opaque tube with the plunger minimally inserted for 6 min to recover from handling stress. The tube was then opened by removing the screw-on cap and it was then screwed into the receptacle on the underside of the arena floor. The plunger of the syringe was gently raised to encourage the test locust upwards into the arena's central chamber through the central hole in the floor. The locust's behaviour was then recorded by the digital camera, using the open-source Linux Graphical User Interface (GUI) software

Coriander (version 2.0.1, 2008, http://sourceforge.net/projects/coriander/) on Ubuntu version 12.04. Video recordings were captured at 30 frames per second and saved directly onto a removable hard drive in raw format video files (4.3 GB). Recording duration was 466 s (due to the 4.3 GB file size limitation of the FAT-32 system format of the hard drive). Immediately after behavioural observations, test animals were either culled at -10°C or were snap frozen in liquid nitrogen for bioaminergic analysis (see Section 2.4).

Raw format behavioural video files were converted to lossless FFV1-encoded AVI files (for archiving) and lossy MPEG-encoded MP4 files (for tracking) using the open source software FFmpeg (version 0.8.17-4:0.8.17-0, Bellard, F., https://www.ffmpeg.org/) on a separate desktop computer running Ubuntu 12.04. Automated behavioural tracking was then run on MP4 video files with the open-source tracking software Swistrack (Version 4.1, EFPL, Lausanne, Switzerland; Lochmatter et al., 2008; Stettin, 2014) on the same computer, outputting the tracked locust's x-y coordinate and body orientation for each video frame. These data were then processed using an Octave script named *tandy* to quantify behavioural variables from the animal's trajectory (Stettin, 2014). Behavioural variables returned by *tandy* that were based only on the locust's position in the arena were: the time spent on the walls of the arena, time spent on the $1/6^{th}$ of the arena floor next to the crowd stimulus side, and time spent on the opposite $1/6^{th}$ of the arena floor from the crowd stimulus side. The other variables quantified involved the activity of the locust, or a combination of activity and position. Activity-related behaviours were defined using threshold locust speeds, with the locust being defined as 'at rest' at speeds below 0.21 $cm \cdot s^{-1}$ to discount 'jittering' of pixels during tracking. These behaviours can be found in Table 2.1. To distinguish true walking and climbing from short movements with the same threshold speeds, long bouts of walking or climbing were also measured. These were defined as walks or climbs made above the speed threshold for 18 out of a 20 frame time window (Table 2.1).

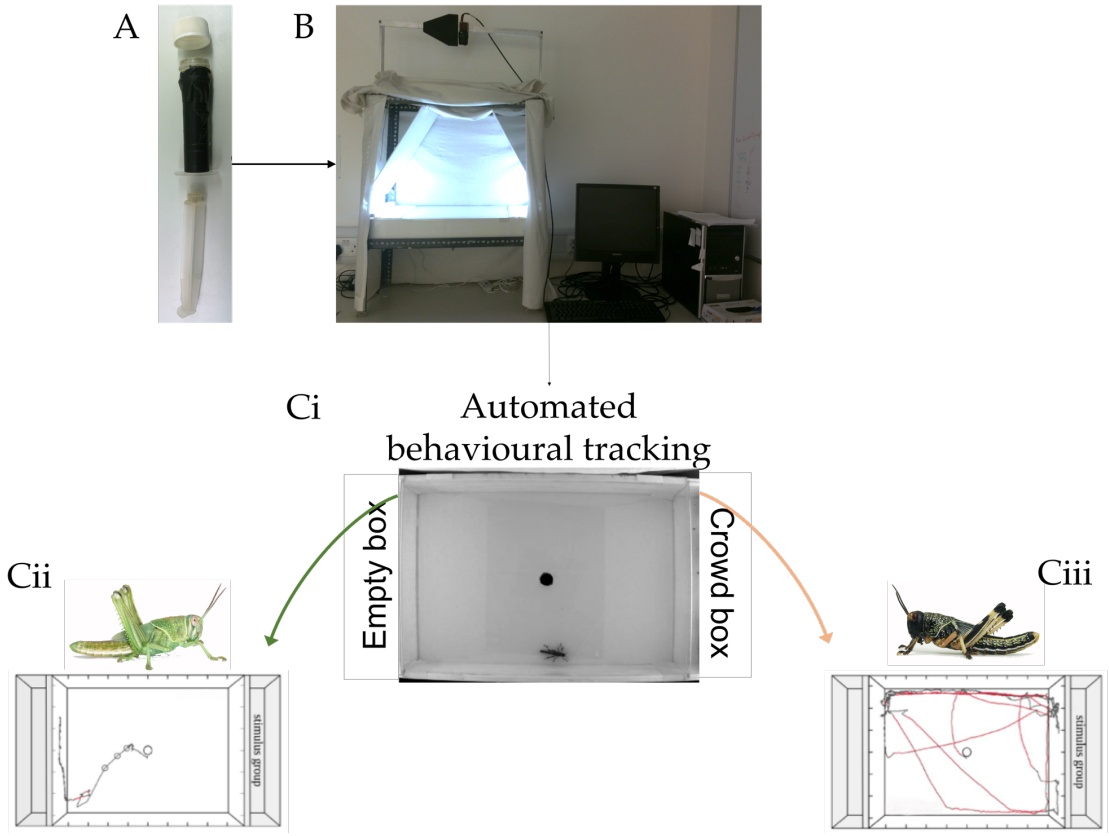


Figure 2.2: The behavioural assay method. (A) A test locust is placed in an opaque holding syringe (shown dismantled) for 6 min before introduction upwards onto the floor of the behavioural arena. (B) Behavioural arena (left) with a camera gantry for top-down recording of arena floor. Camera is connected to desktop PC (right). (Ci) Image of the behavioural arena floor (top-down view), with the main chamber partitioned from the empty and crowd stimulus boxes, shown here schematically, which were alternated each session. (Cii) Green arrow points to a schematic (adapted from Stettin, 2014) showing the tracked trajectory of a typical solitarious locust, with low activity and little time near to the crowd stimulus. (Ciii) Orange arrow points to similar schematic for a typical gregarious locust, with higher activity levels (longer trajectory) and more time near to the crowd stimulus. Adapted from Stettin, 2014.

2.3.3 Logistic regression model

To form predictions of behavioural phase state throughout this thesis, a binomial logistic regression model was fit to a model population of long-term solitarious and gregarious locusts (Roessingh et al., 1993). All analyses were performed in RStudio v1.0.143. The model was fitted using the *glm* function in R for generalised linear models with a logistic-link function. The phase of the locusts was set as the dependent variable and had a binary outcome: 0 for solitarious, 1 for gregarious. The model was formulated on a previous model selected due to its relative parsimony and incorporation of phase-related

behaviours based on both activity and positional variables (Stettin, 2014). The formula for the logistic regression model is:

$$Phase \sim StimTF + LongWalkBouts + FractalDim + Jumps, \quad \text{Eqn 2.1}$$

where *StimTF* is stimulus time fraction, *LongWalkBouts* is the number of long walk bouts made, *FractalDim* is fractal dimension, and *Jumps* is number of jumps.

The same variables were fitted to the two model populations analysed in this thesis: the Leicester colony and the Mauritanian colony. The model output has two alternative metrics: a probability of belonging to the gregarious phase (P_{greg}; $0 < P_{greg} < 1$), or a logit (log-odds = $\ln(P_{greg} / (1 - P_{greg}))$; $-\infty < \text{logit} < \infty$) value. The next sections outline the sample sizes and model characteristics with each population.

Table 2.1: List of behavioural variables generated by automated tracking script *tandy*.

Behavioural variable	Type	Description
Rest time fraction	Activity	Fraction of total time spent below the walking threshold of 1.30 $cm \cdot s^{-1}$
Walk time fraction	Activity	Fraction of total time spent moving above the walking threshold on the arena floor
Walk speed	Activity	Mean speed of the animal when walking on the floor of the arena ($cm \cdot s^{-1}$)
Walk bouts	Activity	Amount of times the animal started walking
Walk bout length	Activity	Mean duration of the walking bouts (s)
Mean bout speed	Activity	Mean walking speed of the bouts ($cm \cdot s^{-1}$)
Long walk bouts (Longwalkbouts)	Activity	Number of walk bouts above the speed threshold for 18 out of a 20 frame time window
Ground covered	Activity	The number of 1 cm^2 squares visited by the animal in the arena
Fractal dimension (FractalDim)	Activity	The Minkowski–Bouligand or box-counting dimension of the animal's trajectory; a measure of path complexity.
Turns	Activity	Number of body rotations made above the turn threshold of 0.9 $rad \cdot s^{-1}$
Jumps	Activity	Number of movements made above the jump speed threshold of 30 $cm \cdot s^{-1}$
Laterality	Activity	Mean ratio of sideways over forwards velocity components
Stimulus time fraction (StimTF)	Positional	Fraction of total time in $1/6^{th}$ of arena closest to the crowd side
Opposite time fraction	Positional	Fraction of total time in $1/6^{th}$ of arena furthest from the crowd side
Wall time fraction	Positional	Fraction of total time spent on the walls of the arena (both long and side walls)
On wall bouts	Positional	Number of times the animal approached the walls
Wall bout length	Positional	Mean duration of the wall bouts (s)
Climb time fraction	Positional and activity	Fraction of total time spent moving on the walls above the climbing threshold speed of 0.45 $cm \cdot s^{-1}$
Climb bouts	Positional and activity	Number of times the animal started moving on the walls above the threshold speed.
Climb bout length	Positional and activity	Mean duration of the climbing bouts (s)
Long climb bouts	Positional and activity	Number of climb bouts above the speed threshold for 18 out of a 20 frame time window
Long climb time fraction	Positional and activity	Fraction of total time spent making long climb bouts
Long climb length	Positional and activity	Mean duration of the long climb bouts (s)

2.3.3.1 Leicester colony

The model population consisted of 296 long-term 5th instar solitarious and 224 long-term 5th instar gregarious locusts, collected over > 2 years. It was therefore slightly unbalanced in favour of solitarious individuals. The solitarious population consisted of 84 first, 151 second, 42 third and 19 fourth generation animals. Using the threshold of $P_{greg} \geq 0.5$ for a correct gregarious classification and $P_{greg} < 0.5$ for a correct solitarious classification, the model correctly classified 88.5% of the solitarious model population and 84.8% of the gregarious model population (Figure 2.3Ai). The logit values of the models indicate a clear separation of the two phases in this model, with the interquartile ranges (IQR) showing no overlap (Figure 2.3Aii). The behavioural variables in the model all showed strong predictive *p*-values in this model (Table 2.2). The Area Under the Curve (AUC) was analysed to estimate the relationship between specificity (1 – false positive rate) and sensitivity (1 – false negative rate) across all threshold values of 'correct classification'. In this test, an AUC of 1 indicates perfect prediction and a value of 0.5 indicates the model predictions are no different from random guessing (Hosmer and Lemeshow, 2000). The model AUC was 0.939, indicating a very strong separation of the two phases in the model populations.

Table 2.2: Variables in logistic regression model for Leicester colony using the formula in *Eqn* 2.1.

Effect	Estimate	Standard Error	z-value	*p*-value
Intercept	-8.733	0.914	-9.550	< 0.0001
StimTF	1.896	0.495	3.833	0.000127
Longwalkbouts	0.155	0.0490	3.154	0.00161
FractalDim	7.89	0.893	8.835	< 0.0001
Jumps	-0.679	0.216	-3.139	0.00170

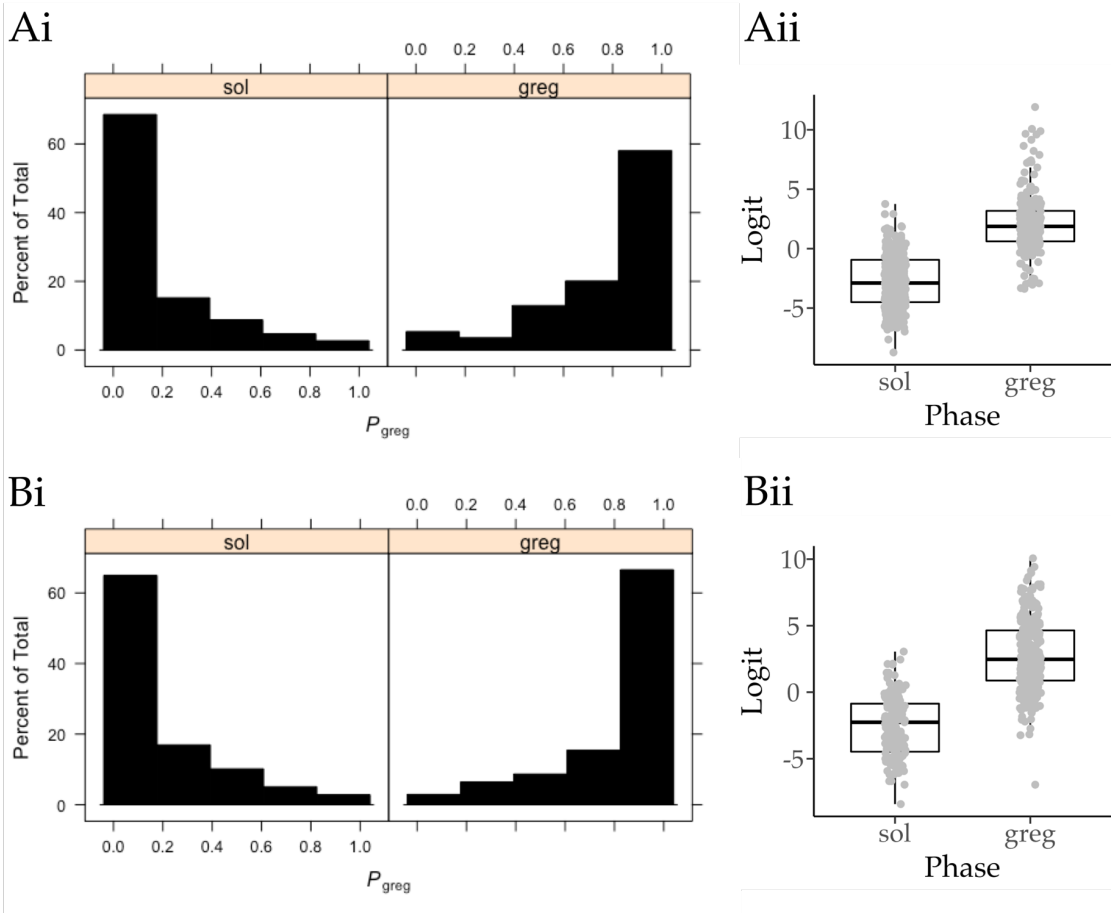


Figure 2.3: Output of logistic regression models for predicting probability of gregariousness (P_{greg}) values in the Leicester and Mauritanian colonies. (Ai) Distribution of P_{greg} values within long-term phase groups of the Leicester colony populations (sol = solitarious, $N = 296$; greg = gregarious, $N = 224$). (Aii) Comparisons of logit-transformed P_{greg} values between long-term phase groups within the Leicester populations. Each grey point represents one locust. (Bi) as (Ai) except for the Mauritanian population (sol $N = 177$; greg $N = 311$). (Bii) As Aii except for the Mauritanian population.

2.3.3.2 Mauritanian colony

For consistency of comparisons, the logistic regression model developed for the Leicester population was fit to solitarious and gregarious Mauritanian populations. The model population consisted of 177 solitarious 5th instar locusts and 311 gregarious 5th instar locusts collected over > 2 years. Of the 177 solitarious locusts, 105 were 1st generation solitarious locusts and 72 were in the 2nd generation of isolation. This model population was highly unbalanced in favour of gregarious phase locusts, requiring a correction for the model intercept $\hat{\beta}_0$ (King and Zeng, 2001):

$$\hat{\beta}_0 - \ln\left(\frac{1-\tau}{\tau} \times \frac{\bar{y}}{1-\bar{y}}\right) \qquad \text{Eqn 2.2}$$

where τ is the true probability of a locust being in the gregarious phase (assumed as 0.5), and $\bar{y}$ is the probability of a locust being in the gregarious phase in the model population (i.e., 311 / (177 + 311)).

This corrected intercept value was then subtracted from all logit values predicted from the model (i.e., for all experimental animals), and the corrected P_{greg} values were back-calculated from the corrected logits. The accuracy of the model was 85.6% in gregarious locusts and 88.1% in solitarious locusts based on a cut-off of $P_{greg} \geq 0.5$ (Figure 2.3Bi). The logit values derived from the corrected model indicated a clear separation of the two phases for the Mauritanian animals, with no overlap in the IQR (Figure 2.3Bii). The *Longwalkbouts* and *FractalDim* variables in the model provided strong predictive value in this population, but *StimTF* and *Jumps* did not (Table 2.3). The AUC value was 0.945, indicating a very strong separation of the two phases in the model population.

Table 2.3: Variables in the logistic regression model for long term solitarious and gregarious Mauritanian animals using the formula in Eqn 2.2.

Effect	**Estimate**	**Standard Error**	**z-value**	***p*-value**
Intercept	-7.847	1.080	-7.268	< 0.0001
Stimulus time fraction	0.876	0.574	1.526	0.127
Longwalkbouts	0.0991	0.0312	3.181	0.00147
Fractal dimension	6.563	1.007	6.516	< 0.0001
Jumps	-0.00268	0.287	-0.009	0.992

2.4 HPLC

Monoamine concentrations were quantified using HPLC with electrochemical detection in order to make comparisons with Anstey et al. (2009) and Rogers et al. (2004). Chemicals used in this section were obtained from Sigma-Aldrich Company Ltd. (UK), unless otherwise stated.

2.4.1 Sample preparation

Unless otherwise stated, nymphs in the 5th instar stage were snap-frozen in liquid nitrogen immediately after their final behavioural assay in order to minimise monoamine degradation. Frozen samples were then stored at -80 °C for 2 weeks to 18 months before processing further. The prothoracic, mesothoracic and metathoracic ganglia were dissected out on a custom-made Peltier-cooled platform that maintained tissue at approximately -10°C, and were pooled together into one empty chilled 1.5 mL microcentrifuge tube kept on dry ice. The midbrain and optic lobes were also collected in separate tubes. These samples were then homogenised in 50 µL of 150 mM perchloric acid (PCA, 311421) with 0.5 mM ethylenediaminetetraacetic acid (EDTA, E9884, American Chemical Society (ACS) grade), as well as 15 µM 3,4-dihydroxybenzylamine hydrobromide (DHBA, 858781) and 19 µM N-methyl-serotonin (NMS; M1514) as internal standards, using a motorised pestle (Z359971-1EA, Kontes, SigmaAldrich, UK) for 10 s. Each microcentrifuge tube was spun in a refrigerated centrifuge (5415 R, Eppendorf, UK) at 13,200 RPM for 10 min at 4 °C. The supernatant was collected using a 100 µL pipette and placed into 0.22 µm centrifuge filters (M9160, Ultrafree, Durapore, UK) and spun at 13,200 rpm for a further 30 min before being transferred to a HPLC autosampler tube (27423, Supelco) kept at 4°C, and run through the HPLC monoamine detection system.

2.4.2 Monoamine quantification

2.4.2.1 Equipment and maintenance

The HPLC system (Dionex Ultimate 3000, Unity Lab Services, Thermo Fisher Scientific Inc., UK) consisted of a biocompatible rapid separation pump module (LPG-3400RS), a temperature-controlled autosampler module (SR3000), column oven (LPE3000RS) and electrochemical detector module (ECD, ECD3000RS). The system was connected to a desktop computer running Windows 7, and controlled automatically by module

processors, and by the user from the desktop using Chromeleon software (version 7.2 SR4, Thermo Fisher Scientific Inc., UK). The ECD was fitted with two electrochemical cells: one coulometric guard cell against contaminants (6020RS, 6070.2100, Unity Lab Services) and one analytical amperometric cell (6070.3200, Unity Lab Services). The column oven was fitted with an Acclaim PolarAdvantage II analytical column (068990, Unity Lab Services) and a guard column (069692, Acclaim PolarAdvantage II) held at 35°C. The pressure of the system was held at 260–280 bar and the autosampler kept samples at a constant 4°C until analysis (up to 4 h). The rear seals on the pump were automatically washed hourly with degassed 10% methanol (M/4049/PB17, Fisher, HPLC grade) in HPLC-grade water (W/0108/17, Fisher, UK). When not running samples, the system was maintained by running 10% acetonitrile (ACN, A/0626/PB17, Fisher, HPLC grade) in HPLC grade water through the system into the waste. During sample runs, the system ran mobile phase through to waste. This mobile phase was a solution of HPLC grade water with 90 mM sodium dihydrogen phosphate (10049-21-5, ACS grade), 50 mM citric acid (C0759, reagent grade), 3.2 mM 1-octanesulfonic acid (O8380, reagent grade), 0.50 mM EDTA and 9% ACN, pH 2.90, degassed and filtered using nylon filter paper (0.45 µm pore size, 58060, Supelco, UK). Mobile phase solutions were replaced weekly. Needles were automatically washed with a solution of 10% ACN in HPLC water before and after every run of samples and standards. Columns were cleaned by reverse flushing in 90% methanol and 0.1% formic acid (A117-50, Fisher, UK) every 3–4 weeks, and replaced when standard peaks in the chromatography became distorted.

2.4.2.2 Sample runs

Autosampler tubes containing homogenised samples were placed into the autosampler temperature-controlled rack. Autosampler tubes containing a single mixture of the external standards 5-HT (H9623), DA (H8502), N-acetyldopamine (NADA; Tractus Chemicals, UK), N-acetylserotonin (NAS, A1824), and the internal standards DHBA and NMS, were added in every session to form calibration curves based on three standards concentrations: 10, 50, and 100 $ng \cdot mL^{-1}$. Before each session, the amperometric cell and coulometric guard cells were switched on at potential differences of 350 mV and 500 mV respectively and the mobile phase was run through the system at a flow rate of 0.3 $mL \cdot min^{-1}$. The cells were then left for at least 30 min to stabilise. The samples were then

loaded into the autosampler and an automatic injection protocol was launched using Chromeleon. The autosampler automatically injected 10 µL sample or standards into the sample loop, with each electrochemical detection run lasting for 10–15 min. Samples were processed in sequences of 6–8 sample runs flanked by runs of the three external standards. Initial optimisation of the entire system led to extremely high repeatability of measurement, so in subsequent experiments each sample was run only once. The baseline of the chromatogram was calculated and chromatogram peak areas were analysed using the automated peak integration function in Chromeleon. Concentrations of 5-HT in each 10 µL injection were automatically quantified in the peak integration tool in Chromeleon, based on the calibration curve generated from external standard concentrations and corrected for run variation based on relative area of the internal standard peak NMS. The value in pmol/sample was derived using:

$$Amount\ (pmol/sample) = \frac{\left(Amount\left(\frac{ng}{\mu L}\right) \times 50\ \mu L\right)}{molecular\ mass\left(\frac{g}{mol}\right) \times 10^{-9}} \times 10^{-3} \qquad \text{Eqn 2.3}$$

2.5 Pharmacology

All chemicals were purchased from Sigma Aldrich, USA, unless otherwise stated. In Chapter 4, the compounds 5-HT, AMTP (M8377) or fluphenazine (F4765) were dissolved in locust saline (140 mM NaCl (S/3160/60, Fisher), 10 mM KCl (BPE366, Fisher), 4 mM $CaCl_2$ (C1016, Fluka), 4 mM $NaHCO_3$ (13433), 6 mM NaH_2PO_4 (P/4520/53, Fisher; pH 6.5; details in Chapter 4). Reserpine (R0875) was dissolved in 100% DMSO (D8419). In Chapter 5, locusts were injected with saline only.

Before each injection procedure, 5th instar locusts were marked with a black non-toxic permanent pen on their wingbuds for individual identification. Gregarious 5th instar locusts were also marked with a spot of blue or red acrylic paint, coded to the treatment, on their pronotum in order to simplify individual identification.

All locusts were restrained on a block of plasticine to minimise tissue damage. A Hamilton microsyringe containing the drug or control solution was then inserted ventrally between the first and second abdominal segments and pushed into the thoracic cavity. The solution was then injected and the needle gently withdrawn. Although 10–30

μL of haemolymph was lost from approximately 60% of the animals injected, no animals showed locomotion impairments immediately after injections of any compound, indicating minimal internal tissue damage from the injection procedure. After injections, locusts were returned to their holding cages, underwent phase-changing treatments (Section 2.2) or went through a behavioural assay depending on the experimental design (see chapters for details).

2.6 Statistical analysis

2.6.1 Statistical treatments

All experimental data were analysed in the open source software RStudio (Version 1.0.143, RStudio Inc., http://www.rstudio.com). Unless stated otherwise, all data were presented in boxplots generated in R (see Figure 2.4 for an example). The boxes describe the median and the first and third quartiles. The whiskers signify the first quartile – 1.5 × IQR and the third quartile + 1.5 × IQR. Individual samples are displayed as points on the boxplots and, where applicable, connecting lines indicate observations belonging to the same individuals in repeated measures experiments.

For all statistical analyses of behavioural gregariousness in single observation trials, linear models (*lm* function in R) were fitted to the derived logit values from the behavioural model (Section 2.3.3). In experiments with behavioural assays run at repeated time points for each individual locust, linear mixed models (*lme* function in R package 'nlme') were fitted, using the animal identity (*Animal.ID*) as a random effect. Fixed effects included time point, drug treatment and phase-changing treatment. Unless otherwise stated, Analysis Of Variance (ANOVA) tests were used to analyse the overall fixed effects. If sample sizes were imbalanced, interaction models were first applied to the data to investigate any interactions present. If the ANOVA provided less than weak evidence for an interaction effect (see Section 2.6.2), then an additive model with the same factors was fit instead. Post-hoc comparisons between factor levels within the linear models were performed using the *t* statistics associated with the regression coefficients (R *summary* function). Logit comparisons between pairs of groups were carried out using a Student's *t*-test. Any behavioural analyses carried out differently from this are described in their specific chapter.

Ganglionic 5-HT levels were fitted with similar linear models to behavioural gregariousness. To quantify covariance between behavioural gregariousness and 5-HT concentrations in the thoracic ganglia, brain or optic lobes, 5-HT concentrations were included as a fixed effect in a linear model fit for logit values and analysed with an Analysis of Covariance (ANCOVA). A Spearman's Rank test was performed for all correlation analyses between 5-HT concentrations and logit values.

2.6.2 Statistical reporting

The outcomes of statistical tests are described throughout this thesis using the approach and terminology recommended by Colquhoun (2014). Much debate has taken place regarding the use the word 'significant' in publications. Counterintuitively, the 'significance level threshold' for *p*-values of 0.05, suggested nearly a century ago (Fisher, 1925), has a false discovery rate of at least 26% (Colquhoun, 2014). Thus, the loaded phrase 'significant' is misleadingly based on an arbitrary and often misunderstood cutoff, particularly considering that a *p*-value of 0.049 could be referred to as 'significant' whereas a virtually identical *p*-value of 0.051 may often be considered 'non-significant'. Therefore, I decided to adapt a more nuanced terminology for statistical reporting, as shown in Table 2.4 (Colquhoun, 2014, 2015). This provides a clearer representation of the statistical evidence of the experiment, without the reliance on the term 'significance', and reflects more appropriately the false discovery rate. Stronger evidence is indicated on figures and in tables by asterisks (*; Table 2.4).

Table 2.4: Terminology assigned to statistical evidence levels in this thesis based on recommendations from Colquhoun (2014, 2015).

***p*-value threshold**	**Terminology**	**Symbol**
$p \geq 0.1$	No evidence for a real effect	N/A
$0.05 \leq p < 0.1$	Very little evidence for a real effect	N/A
$0.01 \leq p < 0.05$	Weak evidence for a real effect	*
$0.001 \leq p < 0.01$	Moderate evidence for a real effect	**
$p < 0.001$	Strong evidence for real effect	***

2.7 Appendix

2.7.1 Model population generational differences

The behavioural gregariousness of solitarious locusts measured in 5th instars has previously been suggested to decrease over three generations (Roessingh et al., 1993), although Stettin (2014) found no evidence for the effect of parental crowding conditions on their solitarious 5th instar offspring's behavioural gregariousness. As an inter-generational effect could present a confounding effect in the behavioural arena, it is important to understand the effect of generation in the animals used in this thesis. Therefore, an inter-generational comparison was carried out in the Leicester and Mauritanian model populations using the P_{greg} and logit values predicted from the fitted logistic regression models outlined in Section 2.3.3.

2.7.1.1 Leicester colony

The Leicester model population used 4 generations of solitarious locusts subject to availability. All of the solitarious groups showed median P_{greg} values of close to 0 except for the 4th generation (Figure 2.4A), thus indicating that there was no trend for increasing solitarious behaviour over generations. There was an unexpectedly high median P_{greg} value ([1st quartile, 3rd quartile]) of 0.20 [0.03, 0.57] in the 4th generation solitarious group, but this is likely to be a random outcome due to the very small sample size. The long-term gregarious population showed a high median P_{greg} value as expected.

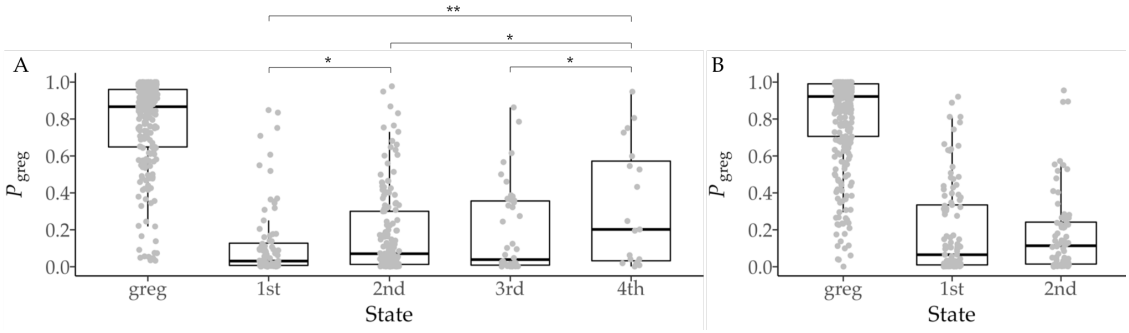


Figure 2.4: Only weak evidence for an effect of generation in solitarious behavioural gregariousness. (A) Leicester colony comparison of P_{greg} values across long-term gregarious (greg; $N = 224$), and 1st ($N = 84$), 2nd ($N = 151$), 3rd ($N = 42$) and 4th generation ($N = 19$) solitarious 5th instar locusts. (B) As (A) except in the Mauritanian colony with long-term gregarious ($N = 311$), 1st ($N = 105$) and 2nd generation ($N = 72$) 5th instar locusts. Each grey point represents one locust. Asterisks show evidence for generational differences using regression coefficients in a linear model (Table 2.4).

The effect of the solitarious generation was analysed using a fitted linear model of generations 1-4 (Table 2.5). Long-term gregarious locusts were excluded from this analysis due to the focus on the effect of solitarious generations. A linear regression model provided weak evidence for an effect of generation on solitarious 5th instar behaviour in the Leicester colony ($p = 0.0126$; Table 2.5). Further analysis of the regression coefficients provided weak evidence that the 2nd generation behaved more gregariously than the 1st generation ($p = 0.0311$; Table 2.5), but not the 3rd generation ($p = 0.592$; Table 2.5). There was no evidence that the 3rd generation behaved more gregariously than the 1st generation ($p = 0.287$; Table 2.5). There was weak evidence that the 4th generation behaved more gregariously than the other generations, however, this was possibly due to a low sample size in the dataset (Table 2.5). Furthermore, although the 2nd generation seemed to show more gregarious behaviour than the 1st generation, the P_{greg} median increase was only 0.04, thus the biological significance of this increase, if real, is questionable. Previous work reported a *decrease* of gregariousness with successive generations of isolation (Roessingh et al., 1993). This analysis overall provided only weak evidence for a minimally confounding effect of solitarious generations in this thesis.

Table 2.5: Table of statistical treatments of the effect of solitarious locust generation on P_{greg} values (behavioural gregariousness) in the Leicester colony. The linear model used to fit the generation factor to behavioural gregariousness over time points was Logit ~ Generation. Df = degrees of freedom. The long-term gregarious locusts were not included in this analysis as the difference between long-term solitarious generations was the focus.

Test	Comparison	Statistic	Df between groups	Df within groups	*p*-value
Linear model	Generation fixed effect (ANOVA)	$F = 2.673$	3	292	0.0126
Regression coefficient	2nd vs 1st generation	$t = 2.167$	3	N/A	0.0311
Regression coefficient	3rd vs 1st generation	$t = 1.066$	3	N/A	0.287
Regression coefficient	4th vs 1st generation	$t = 3.100$	3	N/A	0.00213
Regression coefficient	3rd vs 2nd generation	$t = -0.536$	3	N/A	0.592
Regression coefficient	4th vs 2nd generation	$t = 2.023$	3	N/A	0.0439
Regression coefficient	4th vs 3rd generation	$t = 2.120$	3	N/A	0.0349

2.7.1.2 Mauritanian colony

Gregarious Mauritanian 5th instar locusts in this analysis showed a high median P_{greg} value (Figure 2.4B). Both 1st and 2nd generation Mauritanian solitarious 5th instar locusts showed low median P_{greg} values. As with the Leicester colony, the gregarious group were excluded from the inter-generation comparisons. Although the 2nd generation had higher median P_{greg} value than the 1st generation (an increase of 0.07; Figure 2.4B), a Student's *t*-test provided no evidence for a difference between the behavioural gregariousness of the first and second generation solitarious Mauritanian strain animals (*t*-test, $t = -0.564$, $p = 0.574$, df = 160). This provides no evidence that the generation of the Mauritanian solitarious locusts provides a confounding effect on behavioural gregariousness.

3 Does the established role of 5-HT in locust behavioural gregarisation stand up to scrutiny?

3.1 Summary

The neurotransmitter 5-HT has previously been implicated in behavioural gregarisation of the desert locust, *S. gregaria*, but data presented here cast significant doubts on this claim. Crowding juvenile solitarious phase locusts for 4 h transforms their behavioural phenotype towards that of gregarious locusts, which tend to aggregate (Roessingh and Simpson, 1994). During this rapid transformation over 4 h, the amount of 5-HT in the thoracic ganglia has been reported to increase 9-fold (Rogers et al., 2004), and to correlate with a measure of behavioural gregariousness (Anstey et al., 2009; Rogers et al., 2004). This chapter re-investigates the role of 5-HT in behavioural gregarisation over different phase-changing paradigms, maternal origins, and animal strains. Solitarious 5th instar nymphs were reared at the University of Leicester and exposed to either tickling of the hind leg femur with a paintbrush for 2 h, or placed in a crowd of age-matched conspecifics for 2 or 4 h to induce gregarisation. They were then assessed in a behavioural arena (Roessingh et al., 1993) and assigned a P_{greg} value. After the behavioural assay, ganglionic 5-HT content was quantified using HPLC. Whilst crowded solitarious nymphs showed robust behavioural gregarisation within 4 h, there was no evidence for a link between ganglionic 5-HT content, which did not increase upon crowding, and behavioural gregariousness, across multiple family groups or strains. These findings suggest that the role of 5-HT in behavioural gregarisation, if present, must be more complex or labile than previously suggested.

3.2 Introduction

3.2.1 The role of 5-HT in behavioural gregarisation

This chapter aims to re-examine the previously established role of 5-HT in behavioural gregarisation of *S. gregaria*. The indolamine 5-HT is a neuromodulator universally found across the animal kingdom and is involved in synaptic plasticity, behaviour, learning and memory (Vleugels et al., 2015). Given its role in such a large range of behaviours across species, it would be no surprise for 5-HT to play an important role in behavioural gregarisation. This section outlines published evidence that 5-HT is key to behavioural gregarisation in *S. gregaria*.

Rogers et al. (2004) report that long-term solitarious adults of *S. gregaria* have twice as much 5-HT in the brain and optic lobes as gregarious adults. There was no such difference in 5th instar nymphs. A role for 5-HT in behavioural gregarisation was initially suggested by Rogers et al., (2004) who reported that 5-HT increased 9-fold in the thoracic ganglia after 4 h crowding of 5th instar solitarious locusts, the same time that behaviour changed to a more gregarious phenotype. While other neurochemicals also showed changes in amounts over the gregarisation process, ganglionic 5-HT was the only candidate to undergo a dramatic, transient increase at the same time as behavioural gregarisation. Amounts of 5-HT in the midbrain and optic lobes also increased, but did so more slowly and increased only 4-fold. Rogers et al. (2004) therefore suggested that a surge in 5-HT in the thoracic ganglia might underpin the process of gregarisation. The increased thoracic 5-HT levels fell to long-term gregarious levels by 24 h crowding, suggesting a transient action of 5-HT with downstream cellular effects.

A subsequent study by some of the same authors reported that 5-HT is necessary and sufficient to induce gregarisation in *S. gregaria*. Ganglionic 5-HT amounts were reported to correlate with behavioural gregariousness over 0 h, 1 h and 2 h crowding of 5th instar solitarious locusts, as well as over other phase-changing paradigms (Anstey et al., 2009). Furthermore, injections of the 5-HT-synthesis inhibitor AMTP into the thoracic cavity, and direct injections of 5-HT receptor antagonists into the mesothoracic and metathoracic ganglia, blocked behavioural gregarisation over 1-2 h (discussed in Chapter 4). Direct applications of 5-HT to enzymatically desheathed thoracic ganglia, and injections of

5-HT receptor agonists, were sufficient to increase gregarious behaviour in solitarious locusts. Boosting the production of endogenous 5-HT using 5-hydroxytryptophan (5-HTP) enhanced the behavioural gregarisation response to 30 min of crowding. Further work (Anstey, 2012) suggested that injections of 5-HT into the thoracic haemocoel were also sufficient to increase gregarious behaviour in solitarious locusts, but in another study by a different group, such injections failed to increase behavioural attraction to other locusts, a key phase-related behaviour (Tanaka and Nishide, 2013). This was confirmed in a later meta-analysis of the body of work of Anstey et al. (Rogers et al., 2014), which reported that injections of 5-HT into the haemocoel were not effective in increasing gregarious behaviour, whereas 5-HT was able to increase behavioural gregariousness upon direct application to the desheathed thoracic ganglia.

A role for 5-HT in gregarisation was supported by a later semi-quantitative immunostaining study which reported that 5-HT immunoreactive (serotonergic) neurons in the 5th instar solitarious CNS increase their 5-HT immunofluorescence following 1 h exposure to gregarising stimuli in heterogeneous and cell-specific ways (Rogers and Ott, 2015). Circumstantial evidence for the role of 5-HT can also be inferred from the finding that the intracellular effector molecule PKA, one of the downstream targets of 5-HT receptor 5-HT$_7$, is important for the acquisition of gregarious behaviour after 2 h crowding (Ott et al., 2012). These various lines of evidence thus strongly indicate a role of 5-HT in behavioural gregarisation in *S. gregaria*.

Whereas 5-HT has been strongly implicated in the behavioural gregarisation process in *S. gregaria*, this does not appear to apply for all species of locust. In *L. migratoria*, amounts of 5-HT in the brain showed no changes over 32 h crowding, and did not differ between the long-term phases (Guo et al., 2013; Ma et al., 2011). In these studies, however, even 64 h crowding was only sufficient to achieve partial gregarisation of these locusts (Guo et al., 2011, 2013; Ma et al., 2011). Pharmacological experiments nevertheless indicated a role for 5-HT in both behavioural gregarisation and solitarisation in *L. migratoria*, as injections of 5-HT and 5-HT receptor agonists into the thoracic haemocoel promoted gregarious behaviour (Ma et al., 2011). However, another study found that solitarious *L. migratoria* administered with a 5-HT$_2$ receptor antagonist exhibited more gregarious behaviour, whilst gregarious locusts injected with the same

compound appeared less gregarious in behaviour (Guo et al., 2013). An increase in 5-HT_2 transcription during gregarisation led the authors to suggest a receptor-specific role of 5-HT in behavioural phase transition in *L. migratoria* (Guo et al., 2013). Overall, 5-HT was suggested to principally drive the behavioural solitarisation process in *L. migratoria*, whereas DA was suggested to be the main mediator for gregarisation. In another swarming species, *Chortoicetes terminifera,* there is no link between 5-HT (or DA) and behavioural gregariousness during tickling-induced gregarisation (Anstey, 2012).

These widely different observations may reflect real diversity in the behavioural phase change mechanism across species within the Acrididae, or they may reflect lab strain differences, or they may be artifacts due to low sample sizes and technical differences between the procedures used in different labs. Thus, a vital issue in locust phase change research is to determine whether results obtained from one animal strain in one lab can be generalised to the species as a whole. This chapter explores this issue of intraspecific (*within* species) differences in the phase change mechanism.

3.2.2 Intraspecific variation in behavioural phase change

3.2.2.1 By population

Different species of locust may use different mechanisms to mediate behavioural phase change. However, it is possible that different populations or strains *within* a species also vary (i.e. intraspecific variation). Different field populations of *L. migratoria* from different historical backgrounds of outbreaks show differences in the reaction norms for morphological (Chapuis et al., 2008; Heifetz et al., 1994) and behavioural (Chapuis et al., 2008) phase differences. Different populations of the non-swarming American species *S. americana* also show differences in behavioural reaction norms (Sword, 2003). The southern African subspecies of the Desert Locust, *S. gregaria flaviventris* (Burmeister), shows little capacity for phase change compared to the North African *S. gregaria gregaria* (Forskål) (Chapuis et al., 2017). This principle of intraspecific variation in phase change capacity likely applies to different lab strains of *S. gregaria* as well, since lab strains generally have high inbreeding rates and low genetic diversity (Berthier et al., 2010). Genetic drift between different lab strains could affect the mechanisms underlying phase change, and reduce the replicability of phase change studies across labs. Work investigating this possibility so far is lacking.

3.2.2.2 By maternal factors

The parents of an individual insect exert genetic effects on its phenotype, as does the parental environmental and interactions thereof (Rossiter, 1998). In the locust, therefore, the maternal breeder can influence the phase reaction norm and neurochemical phenotypes of her offspring through 4 main avenues. First, through pure heritable genetic differences in phase-relevant genes; second, through pure heritable epigenetic differences such as in DNA methylation, influenced by environmental factors (Bird, 2002; Mousseau and Fox, 1998); third, from maternal genetic variation in this epigenetic transmission; fourth, through epigenetic variation in the epigenetic transmission, for example, with increasing age and successive clutches of the breeder. Eusocial insects are particularly well studied regarding these four sources of variation in behavioural plasticity (Yan et al., 2014). In locusts, the maternal transmission of phase state through epigenetic factors has been closely studied in the locust, particularly with attention to a 'gregarising factor' in the foam sealing egg pods (Islam, 1997; McCaffery et al., 1998; Miller et al., 2008; Simpson and Miller, 2007). However, the effect of maternal genetic variation on phase reaction norm has received no attention. This may be important in lab studies since experimental animals may often come from cohorts with different parents. If care is not taken in experimental design to split animals from mating lines across experimental treatments, potential differences between mating lines may be misconstrued as the consequences of experimental manipulations.

3.2.3 Experimental aims:

1. Re-examine the link between ganglionic amounts of 5-HT and behavioural gregariousness in solitarious 5th instar nymphs from the established lab colony at the University of Leicester.
2. Compare the link between ganglionic amounts of 5-HT and behavioural gregariousness in solitarious 5th instar nymphs from different maternal lineages.
3. Compare the link between ganglionic amounts of 5-HT and behavioural gregariousness in solitarious 5th instar nymphs from the Leicester lab strain and a recent wild-derived strain.

3.3 Methods

Chapter 2 describes the general methods in detail. What follows is a brief recap and explanation of experiment-specific methodology.

3.3.1 Animals

All experiments utilised solitarious and gregarious 5th instar locusts reared in the University of Leicester. Mauritanian colony locusts were used after ~ 1 y of laboratory rearing when the colony was stable and disease-free (Section 2.1.3).

3.3.2 Experimental designs

3.3.2.1 5-HT link with gregarisation after 2 h tactile stimulation of the hind leg

To understand the role of 5-HT in tickling-induced gregarisation, second and third generation solitarious 5th instar nymphs were gregarised with either hind leg tickling (Section 2.2) for 2 h ('tickle 2h' group; $N = 22$), a non-contact paintbrush presentation treatment for 5 s per min for 2 h ('sham' group; $N = 22$) or left in their holding cage for 2 h ('control' group; $N = 13$). They were then run through the behavioural arena assay (Section 2.3) and then left isolated for 30 min in a holding cage to recover. They were then snap-frozen and their ganglionic 5-HT amounts quantified using HPLC (Section 2.4) within 2 months of storing the undissected samples at -80 °C (control $N = 12$; sham $N = 17$; tickle 2 h $N = 18$).

3.3.2.2 5-HT link with gregarisation over 2 h and 4 h crowding

Second and third generation solitarious 5th instar locusts in the Leicester colony were exposed to crowd-induced gregarisation to understand the role of 5-HT in rapid crowd-induced behavioural gregarisation. Test locusts in the 2 h crowding experiment were either crowded for 2 h ('crowd 2h' group; $N = 24$) or left for 2 h in their holding cage ('control' group; $N = 22$). Test locusts in the 4 h crowding experiment were treated with either crowding for 4 h ('crowd 4h' group; $N = 21$) or left for 4 h in their holding cage ('control' group; $N = 20$). They were then run through the behavioural arena assay (Section 2.3) and then left isolated for 30 min in a holding tub to recover. They were then snap-frozen and their ganglionic 5-HT amounts were then quantified using HPLC (Section 2.4) within 2 months after sample collection. The final sample sizes for the

HPLC groups were slightly lower than for the behavioural groups (control $N = 19$; crowd 4h $N = 20$) due to tissue loss during processing.

3.3.2.3 5-HT links with gregarisation over 4 h crowding for different maternal lineages

To understand if the maternal lineage has an effect on ganglionic 5-HT and behavioural phase change, second and third generation solitarious nymphs were exposed to crowd-induced gregarisation. Test offspring locusts were collected from 11 monogamously paired parental breeders over a period < 3 months per breeding pair (Table 3.1). Testing hatchlings from all 11 breeding pairs (at 5th instar) took place over 6 months. Test locusts in the 4 h crowding experiment were treated with either crowding for 4 h ('crowd 4h' group; $N = 131$) or left for 4 h in their holding cage ('control' group; $N = 119$). They were then run through the behavioural arena assay (Section 2.3), immediately snap-frozen after this and their tissue samples stored at -80 °C. Due to a mixture of dissection errors, technical issues with the HPLC equipment and time constraints, a proportion of samples, including one sibling group, was excluded from the 5-HT analysis, with the final sample sizes being 97 (crowd 4h) and 96 (control; Table 3.1). Their ganglionic 5-HT content were then quantified using HPLC (Section 2.4) within 5 months after sample collection. A linear mixed model was fit to analyse the effect of treatment on the logit values. The first model fit an interaction of the treatment fixed effect with the *mother* random effect to identify any differences in gregarisation rate between mothers (*Logit* ~ *Treatment*|*Mother*). If no evidence for an interaction was found, an additive model was employed to identify maternal effects on behavioural gregariousness (*Logit* ~ *Treatment* + (1|*Mother*)). This approach was repeated for the ganglionic 5-HT. To identify covariance of ganglionic 5-HT with behavioural gregariousness, the *Amt_5HT* continuous variable was added to the model in either an interaction or additive model (interaction model: *Logit* ~ *Treatment* + (1|*Mother*) + *Treatment*|*Amt_5HT*; additive model: *Logit* ~ *Treatment* + (1|*Mother*) + *Amt_5HT*). The *lmer* function was employed in RStudio to analyse the *mother* random effect. Comparisons of ganglionic 5-HT between two maternal groups were carried out using a non-parametric Mann-Whitney U test.

Table 3.1: Table of sample sizes for the maternal lineage experiment in the uncrowded controls (Control columns) and 4 h crowded treatment group (Crowd columns). Due to technical issues, only a proportion of behaviourally tested animals (Behav columns) reached the HPLC stage (HPLC columns). Three pairs of mothers were siblings (L.1CV2 & L.1CV6, L.1CX1 & L.1CX4, L.2CS39 & L.2CS41). Dates are shown for when the offspring were run through the behavioural arena and snap-frozen.

Mother	Control Behaviour	Control HPLC	Crowd Behaviour	Crowd HPLC	Experiment date range
L.1CV2	6	6	6	7	Aug-Oct 2016
L.1CV6	7	7	6	6	Aug-Sept 2016
L.1CW3	9	9	14	11	Aug-Oct 2016
L.1CX1	12	0	18	0	Nov-Dec 2016
L.1CX4	7	5	8	6	Oct-Nov 2016
L.1DA1	7	3	9	6	Oct-Dec 2016
L.2CP26	8	8	7	6	Jul-Aug 2016
L.2CQ12	17	16	13	11	Aug-Oct 2016
L.2CS39	19	18	16	15	Jul-Oct 2016
L.2CS41	9	9	12	11	Aug-Sept 2016
L.2CU35	18	17	21	18	Jul-Aug 2016
Total	119	98	131	97	

3.3.2.4 5-HT link with gregarisation in lab and wild strains

To understand the effect of animal strain on 5-HT and behavioural gregariousness, second generation solitarious nymphs from the wild-derived Mauritanian strain (Chapter 2) were exposed to crowd-induced gregarisation. Test locusts were either uncrowded (N = 19), crowded with age-matched Mauritanian strain gregarious locusts for 1 h (N = 21), 4 h (N = 22) or 24 h (N = 22). They were then run through the behavioural arena assay and then immediately snap-frozen and their tissue samples stored at -80 °C. Their ganglionic 5-HT amounts were then quantified using HPLC within 2 months after sample collection.

The logits and 5-HT amounts of this strain were plotted alongside all behavioural and HPLC data pooled from the previous crowding experiments from Leicester strain locusts (Sections 3.3.2.2 and 3.3.2.3). Note, however, that because the Leicester and Mauritanian logistic regression models were calibrated separately to each of the populations (Section 2.3), the absolute values of the logits and P_{greg} values are not directly comparable between

strains. Specifically, they correctly reflect the individual's phase state relative to that strain's long term solitarious and long term gregarious phenotypes, but they do not provide a precise measure of 'absolute gregariousness' across strains. In contrast, ganglionic 5-HT content is directly comparable. However, as the tissue mass/volume was not used as a correction in this study, a confounding effect of strain size cannot be ruled out.

For each pooled strain, linear models were fitted in RStudio to analyse the effects of treatment on behavioural gregariousness (*Logit ~ Treatment*) and ganglionic 5-HT (*Amt_5HT ~ Treatment*). Linear models analysing the effect of treatment on ganglionic 5-HT were the same as for logit. A further strain comparison in ganglionic 5-HT was carried out between the controls and 4 h crowding time points in the strains (*Amt_5HT ~ Treatment + Strain)*, where treatment includes only controls and crowd 4h treatment groups. Correlation analyses were carried out on all pooled time points in the Leicester animals (0, 2 and 4 h crowding, $N = 116$, 24 and 116 respectively) but only up to 4 h crowding in the Mauritanian animals (0, 1 and 4 h crowding) for consistency.

3.4 Results

3.4.1 No changes in 5-HT levels after 2 or 4 h of crowding or tickling

3.4.1.1 Tickling 2 h

Leicester locusts in the sham group ($N = 22$) unexpectedly behaved more gregariously than those tickled for 2 h ($N = 22$) and the control group kept in their holding cage for 2 h ($N = 13$; Figure 3.1Ai). A linear model provided strong evidence for a real treatment effect ($p = 0.0009$; Table 3.2). Analysis of the regression coefficients provided strong evidence that the sham group behaved more gregariously than the control ($p = 0.0005$; Table 3.2) and tickle 2h groups ($p = 0.0043$; Table 3.2), and no evidence that the tickle 2h group behaved more gregariously than the control group ($p = 0.260$; Table 3.2). There was no evidence for a treatment effect on ganglionic 5-HT amounts (control $N = 12$; sham $N = 17$; tickle 2h $N = 18$, $p = 0.7707$; Table 3.2; Figure 3.1Aii). An ANCOVA provided no evidence for an interaction effect of ganglionic 5-HT amounts and treatment groups on behavioural gregariousness ($p = 0.1804$; Table 3.2), nor for covariance between 5-HT and logit ($p = 0.1734$; Table 3.2; Figure 3.1Aiii). There was also no evidence for a correlation between ganglionic 5-HT amounts and logit values (Spearman's Rank, $p = 0.250$; Table 3.2).

3.4.1.2 Crowding 2 h

There was strong evidence that locusts exposed to the 2 h crowding treatment behaved more gregariously than control locusts left in their holding cage for 2 h (control $N = 22$; crowd 2h $N = 24$; Student's t-test $p = 0.0004$; Table 3.3; Figure 3.1Bi). However, there was no evidence for a ganglionic 5-HT increase in the crowded group (Student's t-test, $p = 0.3416$; Table 3.3; Figure 3.1Bii).

An ANCOVA provided no evidence for an interaction effect of ganglionic 5-HT amounts and treatment groups on behavioural gregariousness ($p = 0.6687$; Table 3.3; Figure 3.1Biii), nor for covariance between 5-HT and logit values ($p = 0.1520$; Table 3.3). A Spearman's Rank test provided no evidence for a correlation between ganglionic 5-HT amounts and logit values ($p = 0.9854$; Table 3.3).

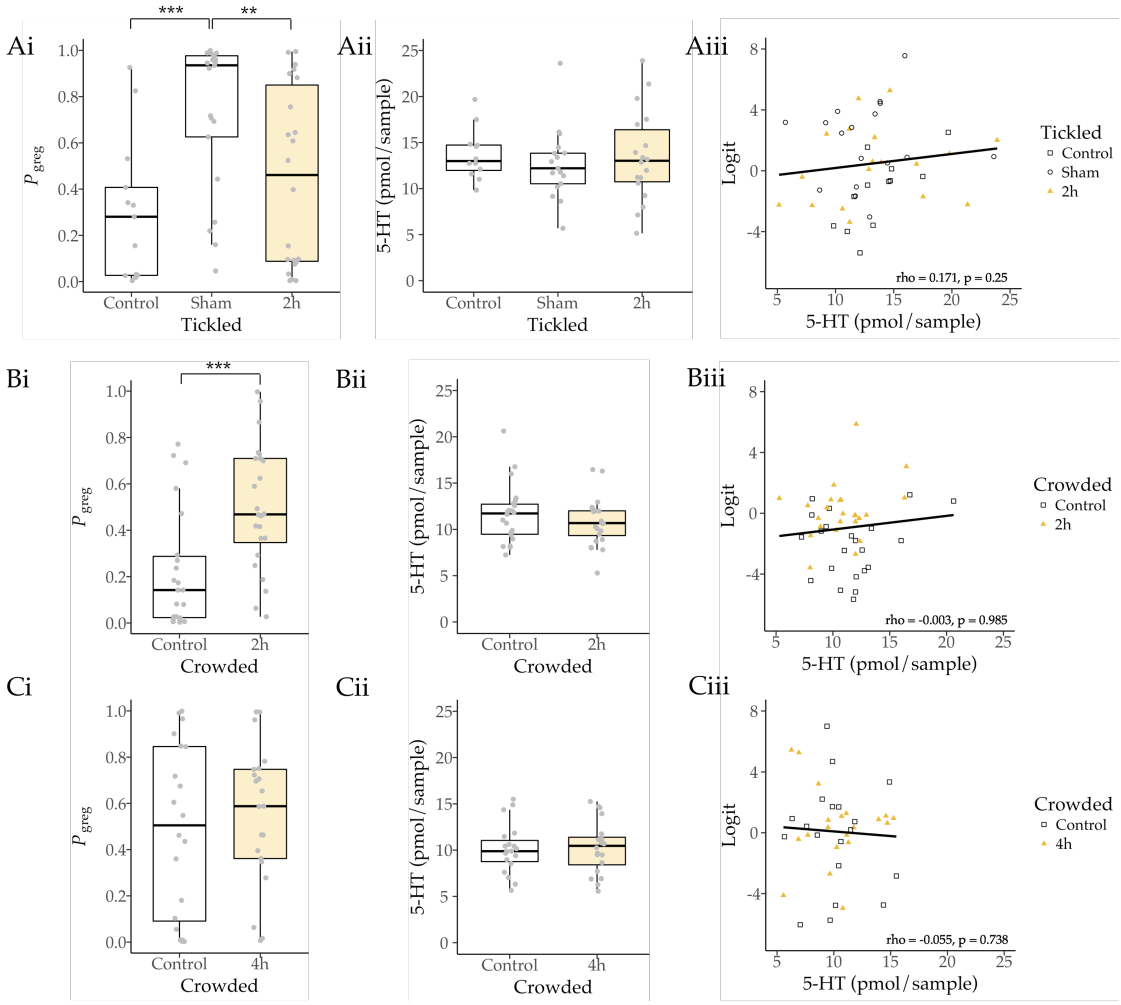


Figure 3.1: No evidence for an increase in ganglionic amounts of 5-HT in solitarious locusts given phase-changing stimuli. (Ai) Comparison of P_{greg} values in solitarious nymphs undergoing hind leg tickling for 2 h (2h group, orange, $N = 22$), nymphs exposed to a paintbrush without physical contact for 2 h (sham group, $N = 22$) and locusts kept in their holding cages for 2 h (control group, $N = 13$). Points indicate individual observations. (Aii) As (i) except comparing ganglionic 5-HT amounts between the control ($N = 12$), sham ($N = 17$) and 2h group ($N = 18$). (Aiii) Relationship between ganglionic 5-HT amount and behavioural gregariousness (logit-transformed P_{greg} values). Line of best fit to pooled data. Animals undergoing gregarising treatments plotted as orange triangles. *N*-values as (Aii). Also displayed are the ρ and p statistics from a Spearman's Rank correlation test between 5-HT and logit. (Bi) As (Ai) except in nymphs undergoing 2 h crowding treatment (2h group; $N = 22$) or uncrowded controls (control group; $N = 24$). (Bii) As (Aii) except with groupings from (Bi). *N*-values as in (Bi). (Biii) As (Aiii) except with 2h crowding and control group. (Ci) As (Bi) except in locusts undergoing 4 h crowding (4h group; $N = 21$) and controls (control group; $N = 20$). (Cii) As (Bii) except in 4 h crowding group ($N = 19$) vs controls ($N = 20$). (Ciii) As (Biii) except with 4 h crowding vs controls. *N*-values as in (Cii). Asterisks show evidence for differences between groups using regression coefficient analyses in a linear model (Ai) and a t-test (Bi; Table 2.4).

Table 3.2: Statistical tests of the effect of tickling treatments on logit values (behavioural gregariousness). Df = degrees of freedom. Linear model analyses = type 2 ANOVA and ANCOVA.

Test	Comparison	Statistic	Df between groups	Df within groups	*p*-value
		Logit ~ Treatment			
Linear model	*Treatment* fixed effect	$F = 8.00$	2	53	0.0009
Regression coefficient	Sham vs control	$t = 3.71$	2	N/A	0.0005
Regression coefficient	Tickle 2h vs control	$t = 1.14$	2	N/A	0.2602
Regression coefficient	Tickle 2h vs sham	$t = -2.99$	2	N/A	0.0043
		Amt_5HT ~ Treatment			
Linear model	*Treatment* fixed effect	$F = 0.26$	2	44	0.7707
		Logit ~ Treatment + Amt_5HT + Treatment:Amt_5HT			
Linear model	*Treatment* fixed effect	$F = 6.57$	2	41	0.0034
Linear model	*Amt_5HT* fixed effect	$F = 1.92$	1	41	0.1734
Linear model	Interaction fixed effect	$F = 1.79$	1	41	0.1804
		Logit ~ Treatment + Amt_5HT			
Linear model	*Treatment* fixed effect (ANOVA)	$F = 6.57$	2	42	0.0034
Linear model	*Amt_5HT* fixed effect (ANCOVA)	$F = 1.92$	1	42	0.1734
Spearman's Rank	*Amt_5HT* vs *logit*	$\rho = 0.17$	N/A	N/A	0.250

Table 3.3: Statistical tests of the effect of 2 h crowding on logit values (behavioural gregariousness) and ganglionic 5-HT amounts. Df = degrees of freedom. Linear model analyses = type 2 ANOVA and ANCOVA.

Test	Comparison	Statistic	Df between groups	Df within groups	*p*-value
		Logit ~ Treatment			
Student's t-test	Crowd 2h vs Control	t = 3.82	1	40	0.0004
		Amt_5HT ~ Treatment			
Student's t-test	Crowd 2h vs Control	t = -0.96	1	40	0.3416
		Logit ~ Treatment + Amt_5HT + Treatment:Amt_5HT			
Linear model	*Treatment* fixed effect	F = 16.13	1	42	0.0002
Linear model	*Amt_5HT* fixed effect	F = 2.09	1	42	0.1561
Linear model	Interaction fixed effect	F = 0.19	1	42	0.6687
		Logit ~ Treatment + Amt_5HT			
Linear model	*Treatment* fixed effect	F = 16.44	1	43	0.0002
Linear model	*Amt_5HT* fixed effect	F = 2.12	1	43	0.1520
Spearman's Rank	*Amt_5HT* vs *logit*	ρ = -0.003	N/A	N/A	0.9854

3.4.1.3 Crowding 4 h

There was no evidence that locusts crowded for 4 h behaved more gregariously than control locusts (control $N = 20$; 4 h crowded $N = 21$; Student's t-test, $p = 0.5161$; Table 3.4; Figure 3.1Ci). Note, however, that the control group in this experiment showed unusually large variation in behavioural gregariousness (Figure 3.1Ci, *cf.* Ai, Bi). There was also no evidence that locusts crowded for 4 h had higher ganglionic 5-HT amounts (control $N = 19$; 4 h crowded $N = 20$; Student's t-test, $p = 0.8540$; Table 3.4; Figure 3.1Cii).

An ANCOVA analysis provided no evidence for an interaction effect of ganglionic 5-HT amounts and treatment groups on behavioural gregariousness ($p = 0.9636$; Table 3.4), nor for covariance between 5-HT and logit values ($p = 0.7266$; Table 3.4). A Spearman's Rank test provided no evidence for a correlation between ganglionic 5-HT amounts and logit values ($p = 0.8832$; Table 3.4).

Table 3.4: Statistical tests of the effect of 4 h crowding on logit values (behavioural gregariousness) and ganglionic 5-HT amounts. Df = degrees of freedom. Linear model analyses = type 2 ANOVA and ANCOVA.

Test	Comparison	Statistic	Df between groups	Df within groups	*p*-value
		Logit ~ Treatment			
Student's t-test	*Crowd 4h vs Control*	$t = 0.66$	1	37	0.5161
		Amt_5HT ~ Treatment			
Student's t-test	*Crowd 4h vs Control*	$t = 0.19$	1	34	0.8540
		Logit ~ Treatment + Amt_5HT + Treatment:Amt_5HT			
Linear model	*Treatment* fixed effect	$F = 0.37$	1	35	0.5492
Linear model	*Amt_5HT* fixed effect	$F = 0.12$	1	35	0.7303
Linear model	Interaction fixed effect	$F = 0.002$	1	35	0.9636
		Logit ~ Treatment + Amt_5HT + Treatment:Amt_5HT			
Linear model	*Treatment* fixed effect	$F = 0.38$	1	36	0.5435
Linear model	*Amt_5HT* fixed effect	$F = 0.12$	1	36	0.7266
Spearman's Rank	*Amt_5HT* vs *logit*	$\rho = -0.02$	N/A	N/A	0.8832

3.4.1.4 Correlation between 5-HT and behavioural gregariousness across phase-changing paradigms

To investigate a correlation or covariance between behavioural gregariousness and ganglionic 5-HT amounts across all phase-changing paradigms, the data from all 3 previous sections were pooled. There was no evidence for an interaction effect of ganglionic 5-HT amounts and treatment groups on behavioural gregariousness (ANCOVA, $p = 0.4091$; Table 3.5; Figure 3.2), nor for covariance between 5-HT and logit values (ANCOVA, $p = 0.3018$; Table 3.5). As expected, there was moderate evidence for an effect of treatment on behavioural gregariousness (ANCOVA, $p = 0.001$; Table 3.5). A Spearman's Rank test provided no evidence for a correlation between ganglionic 5-HT amounts and logit values ($p = 0.257$, Table 3.5).

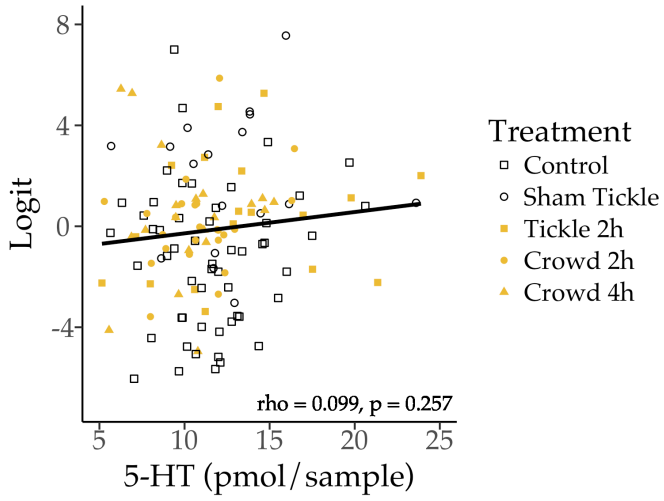


Figure 3.2: Behavioural gregariousness does not correlate with ganglionic 5-HT amounts in a large pooled dataset. Ganglionic 5-HT amounts are plotted against the animal's behavioural gregariousness (logit-transformed P_{greg} values). The data include all solitarious controls ($N = 56$), and solitarious nymphs administered with a non-contact paintbrush treatment for 2 h (Sham, $N = 17$), tickled for 2 h ($N = 18$), crowded for 2 h ($N = 22$) or crowded for 4 h ($N = 19$). Line of best fit to pooled data. All animals subjected to gregarising treatments plotted as orange symbols. Also displayed are the ρ and p statistics from a Spearman's Rank correlation test between 5-HT and logit.

Table 3.5: Statistical tests of the effect of all phase-changing paradigms on logit values (behavioural gregariousness) and ganglionic 5-HT amounts. Df = degrees of freedom. Linear model analyses = type 2 ANOVA and ANCOVA.

Test	**Comparison**	**Statistic**	**Df between groups**	**Df within groups**	***p*-value**
Logit ~ Treatment + Amt_5HT + Treatment:Amt_5HT					
Linear model	*Treatment* fixed effect (ANOVA)	$F = 4.40$	5	120	0.0010
Linear model	*Amt_5HT* fixed effect (ANCOVA)	$F = 1.08$	1	120	0.3017
Linear model	Interaction fixed effect (ANCOVA)	$F = 1.02$	5	120	0.4091
Logit ~ Treatment + Amt_5HT					
Linear model	*Treatment* fixed effect (ANOVA)	$F = 4.40$	5	125	0.0010
Linear model	*Amt_5HT* fixed effect (ANCOVA)	$F = 1.08$	1	125	0.3018
Spearman's Rank	*Amt_5HT* vs *logit*	$\rho = 0.099$	N/A	N/A	0.257

3.4.2 The effect of maternal lineage on gregarious behaviour and ganglionic 5-HT amounts

In light of the unexpectedly high apparent gregariousness of animals in the sham tickling experiment (Figure 3.1Ai) and the unexpectedly large variance of control animals in the 4h crowding experiment (Figure 3.1Ci), the following experiments sought to determine if this could be explained by maternal lineage. To investigate the effect of maternal lineage on ganglionic 5-HT and behavioural gregarisation, 11 solitarious adult mothers were selected for monogamous breeding and their 5th instar solitarious offspring crowded for 4 h or left uncrowded. There was strong evidence that locusts crowded for 4 h (crowd 4h group $N = 131$) behaved more gregariously than locusts left in their holding cage for 4 h (control group $N = 119$; linear mixed model $p < 0.0001$; Table 3.6; Figure 3.3A). A linear mixed model provided no evidence for an effect of maternal lineage on the rate of gregarisation (*Treatment|Mother* interaction random effect, $p = 0.2631$; Table 3.6), nor on behavioural gregariousness across both treatments (*Mother* random effect, $p = 1.0000$; Table 3.6).

There was no evidence for an effect of 4 h crowding on ganglionic 5-HT amounts (control $N = 98$; crowd 4h $N = 97$; linear mixed model, $p = 0.7659$; Table 3.6; Figure 3.3B). The sibling group (i.e. all offspring from a single mother, regardless of subsequent treatment) with the highest median ganglionic 5-HT amount (L.1CW3, median 5-HT [Q1, Q3] = 10.79 [9.76, 12.47] pmol/sample, $N = 20$) had 26.9% more ganglionic 5-HT than the group with the lowest (L.2CP26, median 5-HT = 8.50 [7.61, 11.54] pmol/sample, $N = 14$). A Mann-Whitney-U Test provided weak evidence for a real difference between the two most extreme sibling groups ($W = 198$, $p = 0.0431$).

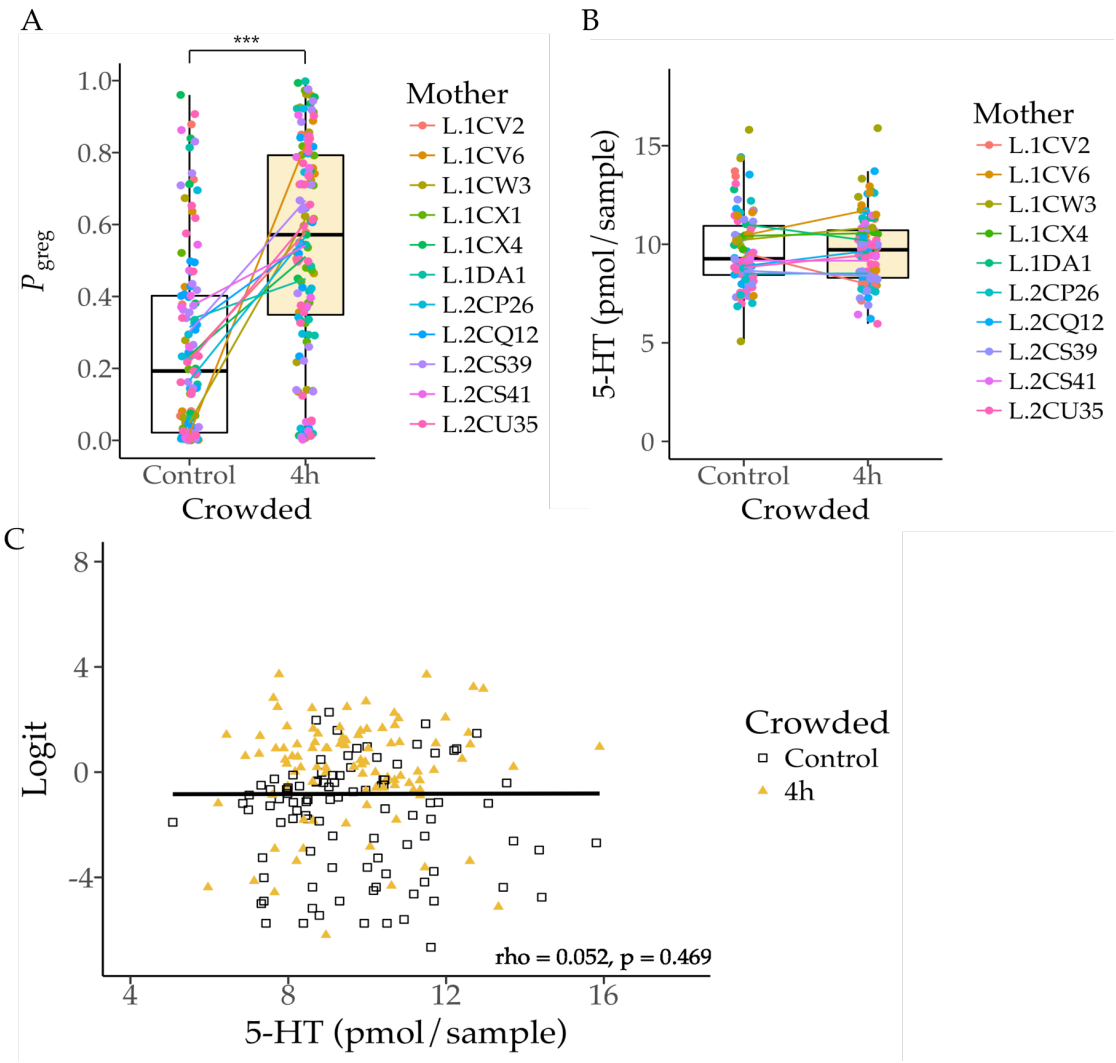


Figure 3.3: Maternal lineage does not influence the gregarisation response in solitarious locusts. (A) Comparisons of the behavioural phase state (P_{greg}) between solitarious locusts crowded for 4 h (4h group, $N = 131$) and controls left in their cage for 4 h (Control group, $N = 119$). Individual data points are coloured by their maternal origin; coloured lines connect the medians of uncrowded and crowded members of the same sibling groups. Sample sizes of each maternal lineage grouping are shown in Table 3.1. (B) As (A) except comparing the ganglionic 5-HT (pmol/sample) between the crowded 4h locusts ($N = 98$) and control groups ($N = 97$). (C) Individual animal points plotted of ganglionic 5-HT amounts against their behavioural gregariousness (logit-transformed P_{greg} values). Line of best fit to pooled data. All animals subjected to gregarising treatments plotted as orange triangles. Also displayed are the ρ and p statistics from a Spearman's Rank correlation test between 5-HT and logit. Asterisks show evidence for differences between groups using regression coefficient analyses in a linear model (Table 2.4).

Table 3.6: Statistical tests of the effect of 4 h crowding and maternal lineage on logit values (behavioural gregariousness) and ganglionic 5-HT amounts. Df = degrees of freedom. Linear model analysis: type 2 ANOVA and ANCOVA.

Test	Comparison	Statistic	Df between groups	Df within groups	*p*-value
Logit ~ Treatment + (1|Mother) + (Treatment|Mother)					
Linear mixed model	*Treatment* fixed effect	$\chi^2 =$ 40.83	1	250	< 0.0001
Linear mixed model	*(1|Mother)* random effect	$\chi^2 =$ 0.00	1	250	1.000
Linear mixed model	*(Treatment|Mother)* random effect	$\chi^2 =$ 2.671	1	250	0.2631
Regression coefficient	Crowd 4h vs control	$t =$ 7.857	1	N/A	< 0.0001
Amt_5HT ~ Treatment + (1|Mother) + (Treatment|Mother)					
Linear mixed model	*Treatment* fixed effect	$\chi^2 =$ 0.089	2	185	0.7659
Linear mixed model	*(1|Mother)* random effect	$\chi^2 =$ 10.88	1	185	0.0001
Linear mixed model	*(Treatment|Mother)* random effect	$\chi^2 =$ 0.00	1	185	1.0000
Logit ~ Treatment + Amt_5HT + Treatment:Amt_5HT					
Linear model	*Treatment* fixed effect	$F =$ 40.21	1	189	< 0.0001
Linear model	*Amt_5HT* fixed effect	$F =$ 0.047	1	189	0.8292
Linear model	Interaction fixed effect	$F =$ 0.64	1	189	0.4249
Logit ~ Treatment + Amt_5HT					
Linear model	*Treatment* fixed effect	$F =$ 40.29	1	190	< 0.0001
Linear model	*Amt_5HT* fixed effect	$F =$ 0.047	1	190	0.8290
Spearman's Rank	*Amt_5HT* vs *logit*	$\rho =$ 0.052	N/A	N/A	0.469

An ANCOVA provided no evidence for an interaction effect between ganglionic 5-HT amounts and treatment groups on behavioural gregariousness (p = 0.4249; Table 3.6; Figure 3.3C) nor for covariance between behavioural gregariousness and ganglionic 5-HT (p = 0.8290; Table 3.6). A Spearman's Rank test provided no evidence for a correlation between gregariousness and ganglionic 5-HT amounts (p = 0.469; Table 3.6; Figure 3.3C).

3.4.3 Ganglionic 5-HT and behavioural gregariousness in two strains of *S. gregaria*

3.4.3.1 Gregariousness of two strains assessed at different crowding time points

Leicester colony crowding data were pooled from previous crowding experiments (Sections 3.4.1.2, 3.4.1.3 and 3.4.2) to form three experimental groups: controls (N = 140), crowded 2 h (N = 24) and crowded 4 h (N = 118). Comparable Mauritanian colony data were obtained by crowding solitarious nymphs for 1 h (N = 22), 4 h (N = 25), 24 h (N = 22), or leaving them uncrowded as controls (N = 23). Data for the two strains were modelled separately, using either Leicester or Mauritanian long-term phase animals as model populations. This means that the P_{greg} values reported in what follows, while providing a correct estimate of relative gregariousness within each strain, do not give an accurate measure of 'absolute gregariousness' that could be formally contrasted across strains.

There was very strong evidence for an effect of crowding on behavioural gregariousness in the Leicester strain (linear model, $p < 0.0001$; Table 3.7; Figure 3.4A). Regression coefficient analysis provided moderate evidence that 2 h crowding increased behavioural gregariousness compared with controls (p = 0.0051; Table 3.7) and strong evidence for the same effect with 4 h crowding compared with controls ($p < 0.0001$; Table 3.7). There was no evidence that 4 h crowding increased behavioural gregariousness more than did 2 h in the Leicester strain (p = 0.9456; Table 3.7).

There was also very strong evidence for an effect of crowding treatments on behavioural gregariousness in the Mauritanian strain (linear model, $p < 0.0001$; Table 3.7; Figure 3.4A). There was strong evidence that all crowding periods strongly gregarised solitarious nymphs compared with controls (Regression coefficient analyses of time points vs 0 h; Table 3.7). Regression coefficient analyses provided no evidence for a

difference in gregarisation between 1 h and 4 h crowding (p = 0.9693; Table 3.7) but moderate evidence that 24 h crowding further gregarised nymphs compared with 1 h and 4 h crowding time points (Table 3.7).

Table 3.7: Statistical tests of the effect of 1, 2, 4 or 24 h crowding on logit values (behavioural gregariousness) in two different strains, analysed separately. Df = degrees of freedom. Linear model analysis: type 2 ANOVA.

Test	**Comparison**	**Statistic**	**Df between groups**	**Df within groups**	***p*-value**
Leicester strain: Logit ~ Treatment					
Linear model	*Treatment* fixed effect	F = 22.00	2	279	< 0.0001
Regression coefficient	Crowd 2h vs control	t = 3.52	2	N/A	0.0051
Regression coefficient	Crowd 4h vs control	t = 6.34	2	N/A	< 0.0001
Regression coefficient	Crowd 4h vs crowd 2h	t = 0.07	2	N/A	0.9456
Mauritanian strain: Logit ~ Treatment					
Linear model	*Treatment* fixed effect	F = 12.45	3	88	< 0.0001
Regression coefficient	Crowd 1h vs control	t = 3.84	3	N/A	0.0002
Regression coefficient	Crowd 4h vs control	t = 3.92	3	N/A	0.0002
Regression coefficient	Crowd 24h vs control	t = 5.98	3	N/A	< 0.0001
Regression coefficient	Crowd 4h vs crowd 1h	t = -0.04	3	N/A	0.9693
Regression coefficient	Crowd 24h vs crowd 1h	t = 2.12	3	N/A	0.0372
Regression coefficient	Crowd 24h vs crowd 4h	t = 2.31	3	N/A	0.0241

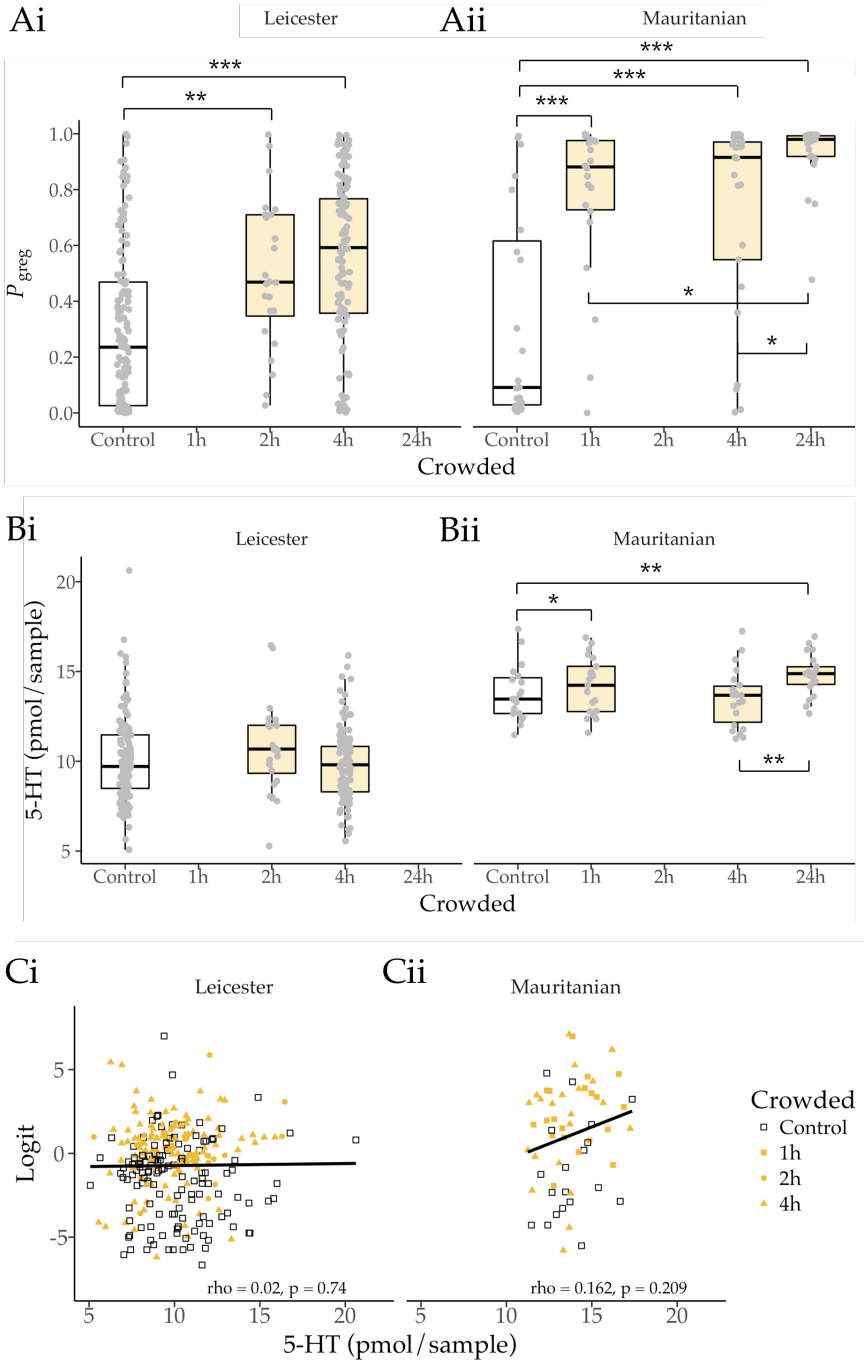


Figure 3.4: No evidence for a large 5-HT increase during crowd-induced gregarisation in two separate strains of locust. (A) (i) Comparisons of the behavioural phase state (P_{greg}) between pooled Leicester strain solitarious nymphs crowded for 2 h (N = 24), 4 h (N = 118) or left uncrowded (control, N = 140). Points indicate individual observations. (ii) As left but with Mauritanian strain locusts crowded for 1 h (N = 22), 4 h (N = 25), 24 h (N = 22) or left uncrowded (control, N = 23). (B) (i and ii) As (Ai) except with ganglionic 5-HT (pmol/sample). (C) Ganglionic 5-HT amounts are plotted against the animal's behavioural gregariousness (logit-transformed P_{greg} values) in the Leicester strain (i) and Mauritanian strain (ii). Line of best fit to pooled data. All animals subjected to gregarising treatments plotted as orange symbols. Also displayed are the ρ and p statistics from a Spearman's Rank correlation test between 5-HT and logit. Asterisks show evidence for a crowding effect using regression coefficient analyses in a linear model (Table 2.4).

3.4.3.2 Ganglionic 5-HT of two strains with crowding

There was no evidence for a crowding treatment effect on ganglionic 5-HT amounts in the Leicester strain (linear model, $p = 0.1144$; Table 3.8; Figure 3.4B). There was, however, moderate evidence for a crowding treatment effect on ganglionic 5-HT amounts in the Mauritanian strain (linear model, $p = 0.0068$; Table 3.8; Figure 3.4B). Analysis of regression coefficients provided weak evidence that crowding for 1 h increases ganglionic 5-HT by 5.7% ($p = 0.0439$; Table 3.8; Figure 3.4B), and moderate evidence that ganglionic 5-HT in solitarious nymphs crowded for 24 h is 10.5% higher than in controls ($p = 0.0081$; Table 3.8; Figure 3.4B). The regression coefficients revealed no evidence for a difference between the crowd 4h group and controls ($p = 0.5350$; Table 3.8), or between the crowd 4h group and the crowd 1h group ($p = 0.1451$; Table 3.8), but moderate evidence that crowd 24h group animals have 8.7% more ganglionic 5-HT than the crowd 4h group ($p = 0.0081$; Table 3.8). There was no evidence for a difference between the crowd 1h and crowd 24h groups ($p = 0.2324$; Table 3.8).

To understand strain differences in ganglionic 5-HT amounts and changes after 4 h crowding, the data from the controls and the crowd 4h group of each strain were pooled and analysed using the variables *Treatment* and *Strain*. These were analysed in an interaction (*Treatment*|*Strain*) and additive model (Table 3.8). The linear model provided no evidence for a *Treatment*|*Strain* interaction effect on ganglionic 5-HT ($p = 0.3583$; Table 3.8) but strong evidence for a strain effect ($p < 0.0001$; Table 3.8). There was no evidence for a treatment effect ($p = 0.4876$; Table 3.8). Analysis of the regression coefficients in the model provided strong evidence that Mauritanian control locusts have 30% more ganglionic 5-HT than Leicester controls ($p < 0.0001$; Table 3.8).

3.4.3.3 Correlation between ganglionic 5-HT and behavioural gregariousness for two strains

To understand the link between ganglionic 5-HT and behavioural gregariousness across all control and crowding groups in either strain, linear models for covariance with treatment variables were fitted to the Leicester and Mauritanian strains separately. To target the first four hours of behavioural gregarisation, the 24 h crowding time point was removed from the analysis. There was no evidence for an interaction effect between ganglionic 5-HT amounts and treatment groups on behavioural gregariousness in the

Leicester strain (p = 0.5644; Table 3.9; Figure 3.4C), nor for covariance between behavioural gregariousness and ganglionic 5-HT (p = 0.6221; Table 3.9). A Spearman's Rank test provided no evidence for a correlation between gregariousness and ganglionic 5-HT amounts in the Leicester strain (p = 0.740; Table 3.9; Figure 3.4C).

An ANCOVA in the Mauritanian strain provided no evidence for an interaction effect between ganglionic 5-HT amounts and treatment groups on behavioural gregariousness in the Mauritanian strain (p = 0.4569; Table 3.9; Figure 3.4C), nor for a covariance between behavioural gregariousness and ganglionic 5-HT (p = 0.3154; Table 3.9). There was no evidence for a correlation between gregariousness and ganglionic 5-HT amounts in the Mauritanian strain (Spearman's Rank test; p = 0.209; Table 3.9; Figure 3.4C).

Table 3.8: Statistical tests of the effect of 1, 2, 4 or 24 h crowding on ganglionic 5-HT in two different strains. Df = degrees of freedom. Linear model analysis = type 2 ANOVA.

Test	Comparison	Statistic	Df between groups	Df within groups	*p*-value
Leicester strain: Amt_5HT ~ Treatment					
Linear model	*Treatment* fixed effect	$F = 2.19$	2	275	0.1144
Mauritanian strain: Amt_5HT ~ Treatment					
Linear model	*Treatment* fixed effect	$F = 4.33$	3	88	0.0068
Regression coefficient	Crowd 1h vs control	$t = 2.04$	3	N/A	0.0439
Regression coefficient	Crowd 4h vs control	$t = 0.62$	3	N/A	0.5350
Regression coefficient	Crowd 24h vs control	$t = 3.26$	3	N/A	0.0016
Regression coefficient	Crowd 4h vs crowd 1h	$t = -1.47$	3	N/A	0.1451
Regression coefficient	Crowd 24h vs crowd 1h	$t = 1.20$	3	N/A	0.2324
Regression coefficient	Crowd 24h vs crowd 4h	$t = 2.71$	3	N/A	0.0081
Both strains at 0 h and 4 h: Amt_5HT ~ Treatment + Strain + Treatment:Strain					
Linear model	*Treatment* fixed effect	$F = 0.48$	1	298	0.4877
Linear model	*Strain* fixed effect	$F = 73.57$	1	298	< 0.0001
Linear model	*Treatment|Strain* interaction effect	$F = 0.85$	1	298	0.3583
Both strains at 0 h and 4 h: Amt_5HT ~ Treatment + Strain					
Linear model	*Treatment* fixed effect	$F = 0.48$	1	299	0.4876
Linear model	*Strain* fixed effect	$F = 73.61$	1	299	< 0.0001
Regression coefficient	Mauritanian vs Leicester	$t = 8.58$	1	N/A	< 0.0001

Table 3.9: Statistical tests of the covariance between ganglionic 5-HT and behavioural gregariousness across crowding treatments in two different strains. Df = degrees of freedom. Linear model analysis = type 2 ANOVA and ANCOVA.

Test	Comparison	Statistic	Df between groups	Df within groups	*p*-value
Leicester strain: Logit ~ Treatment + Amt_5HT + Treatment:Amt_5HT					
Linear model	*Treatment* fixed effect	$F = 21.79$	2	272	< 0.0001
Linear model	*Amt_5HT* fixed effect	$F = 0.24$	1	272	0.6227
Linear model	Interaction fixed effect	$F = 0.57$	2	272	0.5644
Leicester strain: Logit ~ Treatment + Amt_5HT					
Linear model	*Treatment* fixed effect	$F = 21.85$	2	274	< 0.0001
Linear model	*Amt_5HT* fixed effect	$F = 0.24$	1	274	0.6221
Leicester strain: Spearman's Rank	*Amt_5HT* vs *logit*	$\rho = 0.02$	N/A	N/A	0.740
Mauritanian strain: Logit ~ Treatment + Amt_5HT + Treatment:Amt_5HT					
Linear model	*Treatment* fixed effect	$F = 5.14$	2	56	0.0089
Linear model	*Amt_5HT* fixed effect	$F = 1.02$	1	56	0.3173
Linear model	Interaction fixed effect	$F = 0.79$	2	56	0.4569
Mauritanian strain: Logit ~ Treatment + Amt_5HT					
Linear model	*Treatment* fixed effect	$F = 5.18$	2	58	0.0085
Linear model	*Amt_5HT* fixed effect	$F = 1.03$	1	58	0.3154
Mauritanian strain: Spearman's Rank	*Amt_5HT* vs *logit*	$\rho = 0.162$	N/A	N/A	0.209

3.5 Discussion

3.5.1 The link between ganglionic 5-HT and behavioural gregariousness in different phase-changing paradigms

5-HT shows no large increases in any phase-changing paradigms compared with untreated controls, nor any correlation with behavioural gregariousness (Figure 3.1A-C). This was carried out using phase-changing paradigms that have previously been reported to both induce gregarisation and increase ganglionic 5-HT amounts 9-fold (Anstey, 2012; Anstey et al., 2009; Rogers et al., 2004, 2014). The sample sizes used in the present work are at least twice as large as those reported in the earlier work, and up to 9 times as large for the overall correlations. The data reported here are therefore not in agreement with the previous work and cast doubt on the extent of the role of 5-HT in behavioural phase change in *S. gregaria*.

3.5.1.1 Tickling for 2 h

There was weak evidence that 2 h tickling of the hind leg femur with a paintbrush behaviourally gregarised solitarious 5th instars compared with controls in this experiment. The large IQR of the tickled group (Figure 3.1A) indicates variation in the efficacy of the treatment, which could have been due to inconsistent placement of the paintbrush onto the most sensitive regions of the hind leg (Rogers et al., 2003). Furthermore, there was an unexpectedly strong gregarious-like behaviour in the sham group, which made closer comparisons difficult. Although the 2 h tickled group's median P_{greg} (0.46) is lower than that of previous work (greater than 0.75; Anstey, 2012; Anstey et al., 2009; Rogers et al., 2014), the increase over controls still indicates that the animals gregarised. It is difficult to explain the apparently strong gregarisation of the sham group, since visual stimuli alone are reported not to gregarise *S. gregaria* (Roessingh et al., 1998).

The single previous study linking gregariousness with HPLC-quantified ganglionic 5-HT in locusts tickled for 2 h published neither 5-HT quantification of a sham group nor a parallel solitarious control group, resulting in a single tickled group of 25 animals (Anstey et al., 2009). This makes it unclear what group was used as a baseline in their

analysis, if any, which would have been essential to control for day-to-day variation. A separate semi-quantitative study demonstrated changes in 5-HT immunostaining in the thoracic ganglia after 1 h tickling, but only in pre-selected animals with the desired behavioural gregariousness ($P_{greg} > 0.9$ or solitarious controls with a $P_{greg} < 0.05$), complicating the interpretation (Rogers and Ott, 2015). Earlier behavioural gregarisation studies applied tactile stimulation for 4 h, rather than 2 h (Roessingh et al., 1998; Rogers et al., 2003; Simpson et al., 2001), however, data in Chapter 4 of this thesis indicate that 4 h tickling is no more efficacious than 2 h in the Leicester strain. Regardless of whether the 2 h tickling treatment reported in the present work was as efficacious as that in previous work, the large dataset might still have been expected to confirm the reported correlation between gregariousness and ganglionic 5-HT levels, but as discussed below, this was also not the case.

The unexpectedly gregarious-like behaviour of the sham tickle group (Figure 3.1Ai) confounds this experiment. This appears to be a robust effect in a sample size of 21 animals spanning a number of individual hatchling cohorts that were appropriately split across the three treatment groups. The sham group was included to control for the potentially stress-inducing factors of handling and placing in individual tubs, and presenting animals with a moving object. Similar shams have been used as an effective control in some, but not all previous work (Anstey, 2012; Simpson et al., 2001). In the present work the same paintbrush was used for all animals including those that were tickled, so cross-contamination via olfactory sources cannot be ruled out. This may resemble findings that the combined sight and smell of a crowd of conspecific locusts can partially gregarise solitarious animals, though that was only tested over 4 h (Roessingh et al., 1998). Prolonged presentation of non-conspecific moving object stimuli to *S. gregaria* nymphs evokes gregarious-like colouration over stadia, implying a gradual gregarising effect of these stimuli (Tanaka and Nishide, 2012) but the effect of such stimuli on short-term behaviour has not been examined. It is thus difficult to postulate a plausible mechanistic basis for the apparent increase in gregariousness observed in the non-contact group given what is currently known. If the effect is real, a further explanation would be required to explain why the tickled group, receiving both tactile stimulation and presentation of the brush (plus possible odour), did not gregarise as strongly. Regardless of the origin of the unexpected gregarious behaviour, it was not

accompanied by a corresponding increase in ganglionic 5-HT (Figure 3.1Aii and Figure 3.1Aiii).

3.5.1.2 Crowding 2 h and 4 h

Crowding for 2 h was sufficient to increase the gregariousness of solitarious nymphs compared to uncrowded controls (Figure 3.1Bi), but the behaviour of the uncrowded control group for the 4 h crowding experiment was unusually variable, yielding a high median P_{greg} value of 0.51 (Figure 3.1Ci), compared with 0.23 in the pooled Leicester control data in the strain. This unusual control group thus complicates the comparison. Despite the unusual control data, the median P_{greg} values of both crowded groups are typical for the values obtained from the maternal lineage experiment and other crowding treatments in Chapter 3. The following discussion therefore makes the tentative assumption that the control group of the 4 h experiment behaved in an unusually gregarious way for unknown reasons, rather than that the crowding treatment was ineffective.

The data from the crowding experiment, like those from the tickling experiment, also provide no evidence for the reported change in ganglionic 5-HT in response to crowding (Anstey et al., 2009; Rogers et al., 2004; Rogers and Ott, 2015) despite using very similar methods to the previous work. It is worth noting, however, that previous work also has conflicting findings regarding 5-HT quantification before and after crowding. First, the amount of 5-HT measured in the ganglia differed considerably between studies: Rogers et al. (2004) reported ganglionic levels of approximately 10 pmol/sample 5-HT in control 5th instar solitarious locusts, whereas Anstey et al. (2009) reported only 5 pmol/sample. Second, the reported increases of 5-HT differ markedly in magnitude. In comparison to the dramatic 9-fold increase in ganglionic 5-HT amounts reported after 4 h crowding by Rogers et al. (2004), Anstey et al. (2009) reported more modest increases of approximately 2-fold. Though semi-quantitative, an immunostaining study in the thoracic ganglia reported that 5-HT immunofluorescence increased by no more than approximately 50% in individual serotonergic neurons (Rogers and Ott, 2015). Overall, these previous studies concur that 5-HT levels increase after crowding for 4 h, but large discrepancies in the estimated effect sizes between them cast doubts on the reliability of these reported increases. The results of this chapter carried out using a highly optimised

HPLC system in-house and with larger sample sizes than previous work failed completely to replicate the reported effect. It is also difficult to reconcile the small percentage changes in 5-HT content of just a small number of serotonergic neurons (Rogers and Ott, 2015) with 9-fold changes in total 5-HT content reported by Rogers et al. (2004), casting significant doubt on that figure obtained from a small sample size of 9-12 animals.

In the present experiments, ganglionic 5-HT levels of control locusts in the tactile stimulation experiment are approximately 3 pmol/sample (30%) higher than those in crowding experiments, and the amounts show a slight downward trend over the experiments (Figure 3.1A), which were performed in sequence. Regardless of this small discrepancy between experiments, there remains no evidence for changes in ganglionic 5-HT amounts over time points of tickling or crowding. Though an attempt was made to experimentally increase thoracic 5-HT amounts through central 5-HT injections in Chapter 4 (Section 4.4.3), a HPLC-supported positive control experiment will be useful to test if increased 5-HT action in the thoracic ganglia has an effect on behavioural gregariousness.

3.5.1.3 Correlation analyses across phase change paradigms

The correlation and covariation analyses of these experiments revealed no evidence for any link between ganglionic 5-HT and gregariousness over several phase-changing paradigms. This is evident both when analysing experiments separately and when pooling together the data from tactile stimulation and the two crowding experiments. This is in conflict with Anstey et al. (2009) and Anstey (2012), in which there was apparently robust evidence for correlations between gregariousness and ganglionic 5-HT over multiple phase changing paradigms. As has been mentioned above, however, the crowding-induced gregarisation correlation over 0, 1, 2 and 4 h crowding of that study used sample sizes of only 10 animals per group (a total of 29 points in Anstey et al. (2009) and 39 points in Anstey (2012), increasing the likelihood of a false positive result. In the other phase-changing paradigms, the sample sizes were larger (12-25 points), but each treatment was reported in the absence of a control group, showing only correlations within the treatment group. The other studies reporting ganglionic 5-HT increases after gregarising stimuli included no behavioural component for correlation analyses (Rogers

et al., 2004; Rogers and Ott, 2015). The data in this chapter add to the complexity of the situation by finding no link between 5-HT and behavioural gregariousness, with much larger sample sizes and more control groups than reported before.

3.5.2 Effect of maternal line on the link between ganglionic 5-HT and behavioural gregariousness

Overall, while the experimental animals showed robust behavioural gregarisation after 4 h crowding as expected, there was no evidence that maternal origin can explain variation in behavioural gregariousness across sibling groups, nor that it explains variations in behavioural gregarisation or in ganglionic 5-HT changes over 4 h crowding (Figure 3.3A). There was, however, strong evidence that sibling groups had different baseline levels of ganglionic 5-HT.

3.5.2.1 No evidence for maternal effects on behavioural gregarisation

To my knowledge, this is the first study to investigate the effect of maternal genetic variation on behavioural gregariousness, behavioural gregarisation and ganglionic 5-HT amounts. This focus on genetic variation of the mothers differs from work carried out in the maternal effects on behavioural phase previously, which targeted the epigenetic effects of mother crowding experience on the offspring (Simpson and Miller, 2007). The lack of evidence for a maternal role on any of the variables is surprising, since the effect of parental genotypes on their offspring is considered a confounding effect in assessments of genetic and phenotypic variation in insect populations (Rossiter, 1998). The lack of an observed effect in this study suggests that, even if present, this variation was too subtle to detect in this experiment, particularly since the test animals are derived from an inbred lab strain with low genetic diversity (Berthier et al., 2010).

3.5.2.2 Moderate evidence for maternal effects on ganglionic 5-HT amounts, but not on changes in 5-HT over 4 h crowding

This experiment is the first that has investigated the effect of maternal line on ganglionic 5-HT amounts in offspring and changes thereof during crowd-induced gregarisation in *S. gregaria*. There was strong evidence that sibling groups had different ganglionic 5-HT amounts (Figure 3.3B). For example, the sibling group with the most 5-HT had 26.9% more 5-HT than the lowest group. This finding requires a cautious interpretation,

however, since ganglionic 5-HT amounts in this chapter have shown some variation across experiments in Section 3.5.1.2, likely due to differences in sample preparation. This is supported by a post-hoc analysis using a linear model to seek an effect of experimenter dissection skill. There was strong evidence for a role of sample preparation date in the amount of 5-HT in this experiment (Model: *Amt_5HT ~ HPLC.Date*: $F_{8,184}$ = 10.979, $p < 0.0001$). Notwithstanding this effect, however, the key comparison was maternal effects across treatment groups, and this was always carried out on data from the same day.

The extensive dataset created from this experiment independently supports the earlier data in this chapter, finding no evidence that 5-HT increases over 4 h crowding, even though the animals gregarised robustly. With all sibling groups combined, this experiment provides a highly robust sample size of ~ 100 animals per treatment group in comparison to the 9-12 animals per treatment group used in each experiment in the earlier work of Rogers et al. (2004) and Anstey et al. (2009). One further factor that may explain the difference in results could be that the University of Leicester and the University of Oxford lab strains differ. This is not unlikely, since genetic drift and loss of diversity have been observed over generations of establishing locust lab strains (Berthier et al., 2010; discussed further in Section 3.5.3).

3.5.3 The link between ganglionic 5-HT and behavioural gregariousness across locust strains

These experiments found no evidence for a role of 5-HT in behavioural gregarisation at 4 h crowding in either the Leicester or the Mauritanian strain. While there was weak evidence that 5-HT levels in the Mauritanian strain's thoracic ganglia were higher at 1 h crowding than controls, this increase was only 5.7% higher than controls, far from the up to 9-fold increases reported in Anstey et al. (2009) and Rogers et al. (2004). The same previous studies used an inbred lab strain maintained in the University of Oxford for many generations, whereas initial work carried out in this chapter used an inbred lab strain reared in the University of Leicester over many generations. Thus, there was the possibility that the University of Leicester strain had lost a 5-HT-based phase change mechanism demonstrated in the Oxford strain, whilst still exhibiting robust behavioural phase change. An alternative would be that the Oxford strain expressed a novel 5-HT

mechanism not found in other strains. As the Oxford strain of locusts no longer exists, the approach taken here was to obtain animals from the field, and examine the role of 5-HT in behavioural gregarisation in this wild-derived strain.

3.5.3.1 Moderate evidence for strain effects on behavioural gregarisation

Both the Mauritanian and Leicester strains showed robust behavioural gregarisation after 4 h crowding as expected (Figure 3.4A). As they were distinct strains with different logistical regression calibrations, comparability of the reaction norm in behavioural gregarisation was not possible with the P_{greg} metric. However, it was evident that the Mauritanian strain had fully behaviourally gregarised after 1 h crowding (median P_{greg} of 0.88), with no additional increases at 4 h. This scale of increase is not observed in the Leicester strain, which typically gregarise to a median P_{greg} of ~ 0.6 over 2-4 h of tickling or crowding. This suggests that there are population-specific differences in behavioural plasticity, and shows similarities to previous work on intraspecific variation. Chapuis et al. (2008) compared the phase transition characteristics of two separate populations of *L. migratoria* and found that first instar hatchlings from a historically outbreaking population of *L. migratoria* showed a larger behavioural reaction norm than those from a population without a history of outbreaks. Different geographical populations of the non-swarming species *S. americana* express different behavioural reaction norms during crowding (Sword, 2003). Separate populations of *L. migratoria* were also found to have strong differences in morphological, but not behavioural, reaction norms (Heifetz et al., 1994). If the difference between Mauritanian and Leicester strain reaction norms over 4 h crowding is a true effect, then this may be the first study to demonstrate a population effect on reaction norm in short-term behavioural gregarisation, as previous work has only studied effects in long-term phase characteristics.

3.5.3.2 Very weak evidence for strain effects on ganglionic 5-HT levels

Contrary to the reports of 9-fold (Rogers et al., 2004) or 2-fold (Anstey et al., 2009) changes in ganglionic 5-HT amounts over 4 h crowding, this experiment once more found no evidence for an increase in ganglionic 5-HT amounts over the same time frame (Figure 3.4B). Although there was moderate evidence for a crowding treatment effect on 5-HT content in the Mauritanian strain, the increase in median 5-HT over 1 h crowding was only 6% compared with controls. This effect size in a field strain fails to resemble

the 200-900% increases observed previously in Anstey et al. (2009) and Rogers et al. (2004). It thus appears extremely unlikely that the Leicester lab strain has lost the capacity for a 5-HT-related behavioural phase change mechanism in comparison to wild-type locusts. This strengthens the conclusions of Sections 3.5.1, 3.5.2 and 3.5.3, which similarly found no evidence for increases in ganglionic 5-HT amounts during gregarisation. This is also apparent in the complete lack of covariance and correlation between behavioural gregariousness and 5-HT in either strain. Thus, this experiment strengthens the earlier sections of this chapter, finding no evidence for a link between 5-HT and behavioural gregariousness across gregarisation. A perhaps unlikely explanation for the results seen in the previous studies is that the Oxford strain may have developed a novel 5-HT response to crowding, though this has no supporting evidence.

3.5.4 Do methodological differences explain the conflict?

An alternative explanation for the observed results in both strains may arise from a systematic difference in methodology between the work in this thesis and in the previous studies. For example, the Rogers et al. (2004) study did not employ a behavioural assay. Instead, after the crowding treatment, they placed each animal into a holding pot for 1 h to recover and then plunged the pot into the liquid nitrogen. As Anstey et al. (2009) did employ the behavioural assay step, they snap-froze animals immediately after behavioural assay. My methods were developed over time, thus the initial tickling and crowding 2 h and 4 h experiments left the animal for 30 min in a holding pot to recover after the behavioural assay before snap freezing, whereas the later Maternal lineage experiment and Mauritanian experiments snap-froze the animals immediately after the behavioural assay. The fact that these changes yielded no differences in the experimental outcome likely discounts these differences as a factor. Similarly, inclusion of the behavioural assay prior to snap-freezing is unlikely to be a factor since, while the Anstey et al. (2009) showed a smaller 5-HT increase over 4 h gregarisation than Rogers et al. (2004), they still found a 2-fold increase, much larger than that seen in my work.

Other differences in methodology between this thesis and the previous studies (Anstey et al., 2009; Rogers et al., 2004) may affect the rate of 5-HT degradation in the samples. For example, the use of a Peltier coil cooling system allows a more stable, automated apparatus at a similar temperature to the ice-cooled dissection blocks from the previous

papers, which should reduce degradation. Furthermore, many of the samples were stored at -80 °C for longer than the 2-4 weeks reported in previous work due to technical issues. Most samples in this chapter were stored for up to 4 months before processing, which means that the 5-HT may have degraded more than that in the previous papers. However, the fact that all treatment groups were processed with the same method in a balanced structure means that any treatment effects should still be present even with degradation. Similarly, the use of different internal standards in this thesis (NMS rather than DHBA in Rogers et al. (2004) and no reported internal standards in Anstey et al. (2004)) should affect all treatment groups equally, and is a more rigorous method. As discussed in Section 4.3.4, the use of NMS in this work was based on unpublished work in our lab indicating that DHBA degrades at a different rate to 5-HT. The different degradation rate of DHBA to 5-HT thus reduces the reliability of the internal standardisation. It is, however, unlikely to explain the large group differences observed in previous work. Furthermore, the agreement between the quantified 5-HT amounts of this thesis and those of Rogers et al. (2004) also cast doubt on the DHBA creating significant spurious standarisations. The differences in methodology are therefore unlikely to explain the conflict between this thesis and previous studies.

3.5.5 Overall lack of evidence for a role of ganglionic 5-HT in behavioural gregarisation

The various experiments of this chapter all come to the same conclusion: while *S. gregaria* nymphs readily behaviourally gregarise within 4 h of gregarising stimuli, their ganglionic 5-HT amounts do not show the same 9-fold or 2-fold increases indicated by previous literature (Anstey et al., 2009; Rogers et al., 2004). This applies across two phase-changing paradigms, multiple sibling groups and two independent strains of *S. gregaria*.

Previous work also used pharmacological techniques to manipulate 5-HT action in *S. gregaria* and these approaches supported the hypothesis of a link between 5-HT and behavioural gregariousness (Anstey et al., 2009). Chapter 4 of this thesis re-examines those experiments in more detail.

4 Pharmacological reinvestigations into the role of monoamines in behavioural phase change in the Desert Locust

4.1 Summary

The monoamines 5-HT, DA and OA have been suggested to have important roles in behavioural gregarisation and solitarisation in the swarming locust species *S. gregaria* and *L. migratoria*. However, the roles of these monoamines in the maintenance of established phase behaviour, and re-gregarisation after initial isolation are unknown. This chapter aimed to shed light on these processes by means of pharmacological tools used in previous work. The effects of the DA D1/D2 receptor antagonist fluphenazine on phase maintenance and phase transition were examined in long-term gregarious or solitarious 5th instar nymphs by injecting them every day and comparing their probability of belonging to the gregarious phase (P_{greg}) with that of saline-injected controls in an established behavioural arena assay (Roessingh et al., 1993). Unexpected confounding changes in behavioural gregariousness in both phases suggested a phase-specific response to repeated arena assays, and were followed up in Chapter 5. To investigate the roles of 5-HT in behavioural gregarisation and regregarisation, solitarious and gregarious nymphs were injected with the 5-HT synthesis inhibitor AMTP and treated with phase-changing stimuli (isolation for solitarisation, crowding and hind leg tickling for gregarisation). However, AMTP was shown via HPLC to reduce CNS 5-HT by just 27%, suggesting that better pharmacological tools are required for 5-HT manipulation. To understand the effects of non-specific monoamine depletion on expression of gregarious behaviour, solitarious and gregarious nymphs were injected with the vesicular amine transporter inhibitor reserpine and were observed in the behavioural arena. Solitarious locusts were observed before and after 4 h crowding. Total monoamine depletion observed in HPLC corresponded with decreases in behavioural gregariousness in both long-term gregarious and crowded solitarious locusts, suggesting that monoamine activity is required equally for the expression of gregarious behaviour induced by long-term crowding and 4 h crowding.

4.2 Introduction

4.2.1 Behavioural phase change in the locust

One major target of investigation into behavioural phase change in locusts is the role of biogenic amines. Common neuromodulators or neurohormones across the animal kingdom are derived from aromatic amino acids and have a variety of roles in insect behaviour and plasticity in the CNS (Blenau and Baumann, 2001; Libersat and Pflueger, 2004; Vleugels et al., 2015). These compounds are involved in short-term cellular signalling processes underlying plasticity such as regulating the concentrations of intracellular second messengers, such as cyclic adenosine monophosphate (cAMP) and calcium ions, downstream kinases influencing protein functions, and long-term functions such as regulating gene transcription (Verlinden et al., 2014). Of these biogenic amines, particular interest has been devoted to the monoamines, such as the indolamine 5-HT, the catecholamine DA and the phenolamines tyramine and OA and their roles in behavioural phase change in the locust. This chapter expands on work carried out in Chapter 3 on the role of 5-HT in behavioural gregarisation to include investigations of the roles of these monoamines in various aspects of behavioural phase change such as gregarisation, solitarisation, regregarisation and phase maintenance.

4.2.2 5-hydroxytryptamine (5-HT)

5-HT is a monoamine neurotransmitter found across the animal kingdom. It is synthesised by hydroxylation of the amino acid tryptophan to 5-HTP by the enzyme tryptophan hydroxylase (TH), and a subsequent decarboxylation of 5-HTP to 5-HT by the enzyme aromatic amino acid decarboxylase (Vleugels et al., 2015). The release of 5-HT across synapses via exocytosis is controlled by the enzyme vesicular amine transporter 1 (*vat1*). Six types of 5-HT receptors have been identified in insects: 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{7} and 5-HT_{8} (Cullen et al., 2017; Vleugels et al., 2015). These are all G protein-coupled receptors which have the same cell signalling effects as their vertebrate counterparts, with 5-HT_{1A} and 5-HT_{1B} receptors decreasing the intracellular concentration of cAMP, 5-HT_{2A} and 5-HT_{2B} receptors increasing the intracellular concentration of calcium ions, 5-HT_{7} receptors increasing intracellular cAMP concentrations, and 5-HT_{8} increasing intracellular calcium ion concentrations (Nichols and Nichols, 2008; Vleugels et al., 2015). 5-HT_{8} has been identified in some species of insects, but not others, nor in vertebrate species (Qi et al., 2014). Unlike

vertebrates, the monoamine breakdown enzyme monoamine oxidase is not active in insects, thus one main route for 5-HT inactivation in insects is through acetylation to NAS via the enzyme arylalkylamine *N*-acetyltransferase (AANAT) (Sloley, 2004; Smith, 1990). It plays a role in a wide range of behaviours in insects such as olfactory learning (Menzel et al., 1999; Sitaraman et al., 2012), place memory (Sitaraman et al., 2008) and aggression (Asahina, 2017; Stevenson et al., 2000). Of the monoamine candidates for the modulation of behavioural phase change in the locust, it has received the most attention in recent work in *S. gregaria* (see also Chapter 3).

4.2.2.1 5-HT in *S. gregaria*

As discussed in Chapter 3, 5-HT has been reported to be sufficient and necessary for behavioural gregarisation of solitarious locusts (Rogers et al., 2004; Anstey et al., 2009). Chapter 3 found no supporting evidence for these findings across multiple phase-changing paradigms, maternal lineages or strains. However, pharmacological manipulation of 5-HT in Anstey et al. (2009) supported the role of 5-HT in behavioural gregarisation. Injections of the competitive TH inhibitor AMTP into the thoracic haemocoel reportedly reduced behavioural gregarisation of 5th instar solitarious locusts in response to hind leg tactile stimulation (tickling; Anstey et al., 2009). Critically, however, this effect was not found for crowd-induced gregarisation (Anstey, 2012). Direct application of the 5-HT receptor antagonists ketanserin and methiothepin into the thoracic ganglia blocked behavioural gregarisation induced by tickling and sight and smell stimuli (Anstey et al., 2009). Conversely, direct application of 5-HT onto enzymatically desheathed thoracic ganglia increased gregarious behaviour in solitarious locusts, as did application of 5-HT receptor agonists α-methylserotonin and 5-carboxamidotryptamine. Boosting the production of endogenous 5-HT using injections of 5-HTP enhanced the behavioural gregarisation response to 30 min of crowding but did not increase behavioural gregariousness of uncrowded solitarious locusts (Anstey et al., 2009). Anstey (2012) also suggested that central injections of 5-HT into the thoracic haemocoel were sufficient to increase gregarious behaviour in solitarious locusts, however 5-HT injections into the haemocoel did not increase behavioural attraction to other locusts (Tanaka and Nishide, 2013), a key phase-related behaviour (Roessingh et al., 1993; Uvarov, 1977). A meta-analysis (Rogers et al., 2014) of the work of Anstey, Rogers and colleagues, revealed that central injections of 5-HT were not effective in increasing gregarious behaviour, whereas applications to the desheathed thoracic ganglia

(bypassing the blood brain barrier) were effective. More circumstantial evidence for a role of 5-HT in behavioural gregarisation was provided by a study identifying serotonergic neurons in *S. gregaria* 5th instar nymphs that increase 5-HT immunoreactivity after 1 h gregarising stimuli (Rogers and Ott, 2015).

Less work has investigated the potential role of 5-HT in behavioural solitarisation in *S. gregaria*. One reason for this is that the process of solitarisation in gregarious 5th instar *S. gregaria* is slower than gregarisation in the lab, requiring more than 96 h isolation (Roessingh and Simpson, 1994). Rogers et al. (2004) found that CNS concentrations of 5-HT increased after 24 h isolation, with 8-fold, 3-fold and 2-fold increases observed in the optic lobes, midbrain and thoracic ganglia respectively. A recent study, however, found a more rapid solitarisation process, with loss of attraction to conspecifics within just 1 h isolation of gregarious adults, though this was in an assay of attraction and repulsion behaviours only, and in adults instead of 5th instar nymphs (Alessi et al., 2014). It also found 2-3 fold increases in 5-HT amounts in the metathoracic ganglion over 1 h isolation, and this increase was still present at 72 h isolation. Stettin (2014) found no effect of AMTP on behavioural solitarisation in gregarious 5th instars, however, there was no confirmation of 5-HT depletion using HPLC. There is therefore evidence for a response of 5-HT to behavioural solitarisation, but the exact role has yet to be elucidated.

4.2.2.2 5-HT in *L. migratoria*

Recent studies suggest two seemingly opposing roles of 5-HT in behavioural phase change in *L. migratoria*. While concentrations of 5-HT in the brains of 4th instar solitarious showed no differences over 32 h of crowding (Guo et al., 2013), it is important to note that full behavioural gregarisation is not achieved in this species even after 64 h crowding (Guo et al., 2011; Ma et al., 2011, 2015). Pharmacological experiments provided support for a role of serotonin in behavioural gregarisation, and this was proposed to be through 5-HT_2 activation only, rather than all 5-HT signalling (Guo et al., 2013; Ma et al., 2011). Equally, Guo et al. (2013) found evidence for a role of 5-HT in behavioural solitarisation. Injections of 5-HT into the head cavities of 4th instar gregarious nymphs potentiated the solitarising effect of 30 min isolation in these animals, and 5-HT receptor antagonists reduced the solitarising effect of isolation (Guo et al., 2013).

4.2.3 DA

The monoamine DA is a monoamine derived from tyrosine. The synthesis pathway of DA in insects involves the hydroxylation of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase (encoded by the *pale* gene) (Walter et al., 1996). L-DOPA is then decarboxylated to DA by the enzyme DOPA decarboxylase. The release of DA across synapses via exocytosis is controlled by *vat1* (Wittkopp et al., 2003) and is inactivated via acetylation to NADA (Sloley, 2004). Invertebrate DA receptors are all G-Protein-Coupled Receptors (GPCRs) corresponding to vertebrate D1 and D2 receptors. These range from the Dop1 and Dop2 receptors, which increase intracellular cAMP concentration, to Dop3 receptors which decrease cAMP like vertebrate D2 receptors (Cullen et al., 2017). DA plays an important role in social behaviours in invertebrates. For example, DA signalling is implicated in pheromonal attraction in the honey bee (Vergoz et al., 2009), social defeat recovery in the cricket (Rillich and Stevenson, 2014) and complex social processing in ants (Penick et al., 2014; Shimoji et al., 2017). It is thus possible that a similar role exists for DA in behavioural phase change in the locust.

4.2.3.1 DA in *S. gregaria*

Work in *S. gregaria* has focused more on investigations for a role of 5-HT in behavioural gregarisation than DA. This due to the finding that, while DA increased in the midbrain and optic lobes during crowding, the changes were smaller and more delayed than those of 5-HT (Rogers et al., 2004). Long-term solitarious 5th instar locusts had twice as much DA in their brain than long-term gregarious nymphs. At 4 h crowding, DA amounts in the brain increased from 2 to approximately 2.5 times more than long-term gregarious concentrations, DA amounts in the thoracic ganglia were reduced from 1.5 times to 0.75 times more than long-term gregarious concentrations, and amounts in the optic lobes decreased to 0. By 24 h crowding, brain DA amounts were 3 times higher than long-term gregarious concentrations, and DA amounts in the thoracic ganglia and optic lobes increased to approximately 2 times more than long-term gregarious nymphs (Rogers et al., 2004). There were no correlations of ganglionic DA concentrations with behavioural gregariousness across different paradigms of short-term behavioural gregarisation, however, there was a dramatic increase in ganglionic DA concentrations with gregarising electrical stimulation of metathoracic nerve 5 (Anstey, 2012).

There is evidence for a role of DA in behavioural solitarisation in *S. gregaria*. After 24 h isolation of 5th instar gregarious locusts, Rogers et al. (2004) found that amounts of DA transiently increased over 3-fold in the midbrain, over 4-fold in the optic lobes and over 5-fold in the thoracic ganglia, suggesting a role of DA across the CNS in behavioural solitarisation. This effect size was not confirmed by Alessi et al. (2014), who found less than 50% increases in DA concentrations over 1, 4, 24 and 72 h isolation, though comparability is limited due to the use of just the metathoracic ganglion from isolated adults in this study (Alessi et al., 2014). Isolating long-term gregarious adult locusts for 72 h and injecting them with DA decreased their likelihood to approach a conspecific stimulus after both 1 h re-crowding or 1 h isolation compared with saline controls (Alessi et al., 2014). Conversely, injections of fluphenazine, an antagonist of D1 and D2 receptors, increased their propensity to approach other locusts after 1 h isolation compared with saline controls, but did not increase approaches after 1 h recrowding compared with saline controls (Alessi et al., 2014).

It is also interesting to note that 5th instar nymphs isolated for > 1 generation showed 1.5-3-fold higher DA concentrations in the midbrain than long-term gregarious locusts (Rogers et al., 2004). This raised the possibility that this higher brain DA concentration in solitarious locusts acts as a mechanism to maintain solitarious behaviour during isolation, and that disruption of this mechanism leads to gregarious-like behaviour. Further work will be able to explore this mechanism and shed light on the little-studied maintenance of phase behaviours.

4.2.3.2 DA in *L. migratoria*

Investigations in the swarming locust species *L. migratoria* suggested a modulatory role of DA activity in behavioural phase transition, dependent on the receptor subtypes activated (Guo et al., 2015; Ma et al., 2011). The brains of 4th instar gregarious nymphs of *L. migratoria* contained 30% more DA than solitarious nymphs (Guo et al., 2015; Ma et al., 2011), but the two studies differed by more than 25-fold in their estimates of total content. As noted before, full gregarisation of L. migratoria takes more than 64 h crowding (Guo et al., 2011; Ma et al., 2011, 2015). Guo et al. (2015) found that crowding solitarious 4th instar nymphs for 4 h induced a transient 30% increase in brain DA concentrations up to the levels of gregarious nymphs, accompanied by a transient 4-fold increase in brain expression of the DA receptor D1 at 4 h and key genes involved in the

synthesis of DA. Injections of the D1 agonist SKF38393 into the brain of 4th instar solitarious nymphs promoted gregarious behaviour (Guo et al., 2015), suggesting that D1 activation plays a trigger role in the slow behavioural gregarisation of *L. migratoria*.

DA also plays a role in solitarisation of *L. migratoria*. Isolation of gregarious 4th instar nymphs induces more than a 50% reduction in brain DA concentrations by 1 h – corresponding with a rapid behavioural solitarisation – and these concentrations remain lower than the solitarious levels by 32 h isolation (Guo et al., 2015). This accompanied a 3-fold increase in D2 expression after 1 h isolation which remained stable over 32 h (Guo et al., 2015). Blocking the actions of D2 with injections of the D2 antagonist S(-)-sulpiride and D2 double-stranded ribonucleic acid (dsRNA) reduced behavioural solitarisation responses of 4th instar gregarious nymphs to 1 h isolation (Guo et al., 2015). These experiments suggest that D2 receptor activation specifically has a role in the loss of gregarious behaviour in *L. migratoria* in response to isolation.

4.2.4 OA and Tyramine

Tyramine and OA are highly abundant monoamines in the CNS of invertebrates (Verlinden et al., 2010). They are thought to have the functional roles of noradrenaline and adrenaline in vertebrates respectively (Davenport and Evans, 1984; Lange, 2009; Verlinden et al., 2010), though it is found in trace amounts in vertebrate systems. The OA synthesis pathway begins with tyrosine as does that for DA. The enzyme tyrosine decarboxylase converts tyrosine to the precursor molecule tyramine, which also has its own neurotransmitter functions in invertebrates (Cole et al., 2005; Lange, 2009; Livingstone and Tempel, 1983), and which is converted to OA by the enzyme tyramine ß-hydroxylase (Monastirioti et al., 1996). The GPCR receptors Octα1R, OctβR and Octα2R bind preferentially for OA over tyramine. Octα1R increases intracellular cAMP and calcium concentrations, while OctβR increases cAMP concentrations and the Octα2R receptor decreases cAMP and increase calcium ion concentrations (Verlinden et al., 2010). Furthermore, the receptors TyrRI – which decreases intracellular cAMP – and TyrRII – which increases intracellular calcium ion concentrations – preferentially bind tyramine over OA (Verlinden et al., 2010). OA and tyramine are inactivated via acetylation in invertebrates (Sloley, 2004; Verlinden et al., 2010). Given its varied role in broad modulation of physiological processes across invertebrates (Pflüger and Stevenson, 2005), it is a potential mechanism for behavioural phase change in the locust.

4.2.4.1 OA and tyramine in *S. gregaria*

The role of OA and tyramine in behavioural phase change in *S. gregaria* has received little attention. There are no differences seen in OA concentrations between long-term gregarious and solitarious 5th instar locusts, although tyramine concentrations across the CNS are higher in gregarious locusts than solitarious (Rogers et al., 2004). After 4 h crowding, OA concentrations decreased to almost undetectable levels in the CNS and subsequently displayed transient dramatic increases in concentration at 24 h crowding (8-fold in the thoracic ganglia and 14-fold in the optic lobes), whereas tyramine concentrations showed little change (Rogers et al., 2004). After 24 h isolation of long-term gregarious 5th instar locusts, concentrations of tyramine were reduced by 75% in the optic lobes, 60% in the brain and under 50% in the thoracic ganglia whereas OA concentrations showed little change over the CNS except for an approximately 30-40% reduction in the thoracic ganglia (Rogers et al., 2004). A separate study in the metathoracic ganglion of gregarious adults reported a similar pattern, where OA concentrations were reduced by approximately 30% after 1 h isolation and remained at this level over 72 h isolation (Alessi et al., 2014). More work is required to understand the role of OA in this process in *S. gregaria* more fully.

4.2.4.2 OA and tyramine in *L. migratoria*

L. migratoria has received more attention regarding the role of OA and tyramine in behavioural phase change than *S. gregaria*. Studies have suggested that long-term solitarious animals show higher concentrations of OA than gregarious animals (Fuzeau-Braesch and David, 1978; Ma et al., 2015). Injections of OA into the thoracic haemocoel modestly increased behavioural gregariousness of 4th instar solitarious locusts and increased their attraction to conspecifics in a Y-maze assay, whilst silencing expression of OARα prevent crowding-induced increases in gregarious-like behaviour (Ma et al., 2015). This suggests that OA signalling through activation of OARα plays a role in behavioural gregarisation in *L. migratoria*. In contrast, there was no evidence in this study that brain concentrations of tyramine and the expression of tyramine receptor TAR were linked to behavioural gregarisation.

In the same study suggesting a role of OA signalling in behavioural gregarisation of solitarious 4th instar *L. migratoria*, there was evidence for a role in tyramine signalling in behavioural solitarisation of gregarious animals (Ma et al., 2015). During isolation of

long-term gregarious 4th instar locusts, brain concentrations of tyramine correlated with the percentage of animals choosing the clean air arm of the Y-maze. Furthermore, injections of tyramine into the thoracic haemocoel reduced behavioural gregariousness in long-term gregarious locusts, while silencing the expression of TAR reduced behavioural solitarisation during isolation, indicating a role of tyramine signalling in the acquisition of solitarious behaviour.

4.2.5 Downstream cellular mechanisms for behavioural plasticity

All of the GPCRs for the monoamine neurotransmitters control intracellular concentrations of the second messengers cAMP or calcium. Downstream effectors of phase change in the locust have received less attention. Protein Kinase A (PKA), a cell signalling protein activated downstream of cAMP via adenylyl cyclase, is implicated in synaptic plasticity and learning and memory (Glanzman, 2010; Kandel, 2001). It is also reported to be necessary for behavioural gregarisation in *S. gregaria* (Ott et al., 2012), whereas another cell signalling protein implicated in synaptic plasticity, PKG (Kleppisch and Feil, 2009), is not (Ott et al., 2012). Stettin (2014) found no evidence for roles of PKC and CamKII, other synaptic proteins important in plasticity and learning and memory (Kandel, 2001). Interestingly, the same work indicated that behavioural gregarisation during 2 h crowding does not depend on protein transcription or translation (Stettin, 2014). Other modifications to cell functions such as epigenetic and transcriptional changes may consolidate the behavioural changes. Crowding for 24 h induces a change in overall DNA methylation patterns in solitarious *S. gregaria*, suggesting a role for this process in longer-term behavioural phase change (Mallon et al., 2016). Transcriptional changes have been observed over the phase change process in *L. migratoria* (Chen et al., 2010), though the causative role of these changes has not been investigated. This evidence suggests that rapid behavioural gregarisation observed in *S. gregaria* is a labile process induced by monoamine signalling via PKA. It likely involves rapid synaptic plasticity, but is only consolidated only with long-lasting changes in neuronal circuitry in the CNS.

4.2.6 Experimental aims

1) Investigate the effect of DA receptor antagonist fluphenazine on behavioural phase maintenance, solitarisation and gregarisation in 5th instar locusts.
2) Re-examine the effect of the 5-HT synthesis inhibitor AMTP on behavioural phase transition across time points of crowd-induced gregarisation and regregarisation in the desert locust.
3) Re-examine the effect of AMTP on behavioural phase transition across time points of tickle-induced gregarisation in the desert locust, and on HPLC-quantified concentrations of 5-HT across the CNS.
4) Re-examine the effect of 5-HT injections on behavioural phase transition across time points of crowd-induced gregarisation in the desert locust.
5) Investigate the effect of drug vehicle solution DMSO on behavioural gregariousness in 5th instar gregarious locusts compared with control saline.
6) Investigate the effect of vesicular monoamine transporter inhibitor reserpine on HPLC-quantified ganglionic concentrations of DA and 5-HT and behavioural gregariousness in 5th instar gregarious locusts.
7) Investigate the effect of reserpine on HPLC-quantified ganglionic concentrations of DA and 5-HT and behavioural gregarisation in 5th instar solitarious locusts.

4.3 Methods

Chapter 2 describes the core methods in detail. What follows is a brief recap and explanation of experiment-specific methodology.

4.3.1 Animal husbandry

All locusts used in this chapter were reared in the long-term gregarious and solitarious lab colonies explained in Chapter 2. All experiments were performed on 5th instar nymphs at 2-6 d into the instar stage.

4.3.2 Phase-changing treatments

4.3.2.1 Solitarious

Forced crowding and repeated hind leg stimulation procedures in solitarious locusts were the same as those used in Chapter 2, unless otherwise stated.

4.3.2.2 Gregarious

Experimental gregarious 4th instar nymphs selected for assays in the 5th instar stage undergoing crowding were injected with their assigned compound and labelled with a treatment-coded blob of non-toxic acrylic paint before placement into a segregated crowd tub with others of the same treatment and conspecific crowd stimulus animals. They were then held inside the crowding tub, which was itself placed into the larger gregarious colony cages for 48 h. Experimental gregarious 5th instar locusts kept in crowded conditions were injected with their assigned compound, bilaterally labelled on the wingbuds using a permanent marker pen and with a treatment-coded blob of non-toxic acrylic paint. They were then either kept in crowd tubs or in isolation depending on the protocol. Gregarious locusts undergoing isolation were removed from the gregarious colony and housed individually in the solitarious colony, isolated from the touch, sight and smell of conspecifics. They were given wheat and bran flakes *ad libitum* during their isolation period (see Chapter 2 for solitarious holding conditions).

4.3.3 Behavioural assay

Detailed methods for the behavioural assay can be found in Chapter 2.

4.3.4 HPLC

Detailed methods for 5-HT quantification can be found in Chapter 2. HPLC methods for the reserpine experiment are as in Chapter 2; experiment 'AMTP5' was carried out prior

to final adjustments with the HPLC protocol so differs in preparation steps. The dissected midbrains, optic lobes and thoracic ganglia of experimental animals undergoing HPLC in experiment AMTP5 (see Section 4.3.7.2.3 for details) were homogenised and mixed with 0.1 M PCA along with the internal standard DHBA, without NMS. Whereas HPLC quantification for experiments carried out subsequent to experiment AMTP5 involved multiple external standard concentrations, AMTP5 used just one concentration. To make this experiment more comparable with others in this thesis, a manual calibration of the raw peak areas of the external 5-HT standards (x) was carried out, by fitting them with a coefficient curve of $f(x) = 0.1721x$, with the coefficient derived from the mean of 10 experiments carried out with multiple external standards. An internal standard NMS was used only in subsequent HPLC experiments (Chapter 2 and reserpine experiments of this chapter), so the raw peak areas in experiment AMTP5 were left uncorrected (unpublished work from our lab shows that DHBA, although generally considered to be a reliable standard, does not degrade at the same rate as 5-HT, making it unreliable as an internal standard for 5-HT).

4.3.5 Pharmacology

The compounds 5-HT, AMTP and fluphenazine were mixed in locust saline (concentration details in Section 4.3.7; saline and injection details in Chapter 2). Reserpine was mixed in 100% DMSO (details in Section 4.3.7). A Hamilton microsyringe containing the drug or vehicle solution was then inserted ventrally between the first and second abdominal segments and pushed into the thoracic haemocoel. The drug was then injected and the needle was gently withdrawn.

4.3.6 Statistical analysis

See Section 2.6.1 for the core statistical treatments.

4.3.7 Experimental designs

4.3.7.1 Fluphenazine

The aim of two experiments using fluphenazine was to understand the effect of blocking DA receptors on behavioural phase maintenance and phase transition in *S. gregaria*. Long-term solitarious and gregarious locusts were run through an initial behavioural assay 2 d after moulting to the 5th instar stage (Figure 4.1). Locusts in the treatment group

were then immediately injected with 5 µL 500 µM fluphenazine dissolved in saline (Alessi et al., 2014), with the control group receiving injections of saline. Injections were given every day after this. For each phase, behavioural assays were carried out every 2 d in the absence of phase-changing stimuli until 96 h, when phase-changing treatments were applied. The following subsections explain the designs of each experiment in more detail.

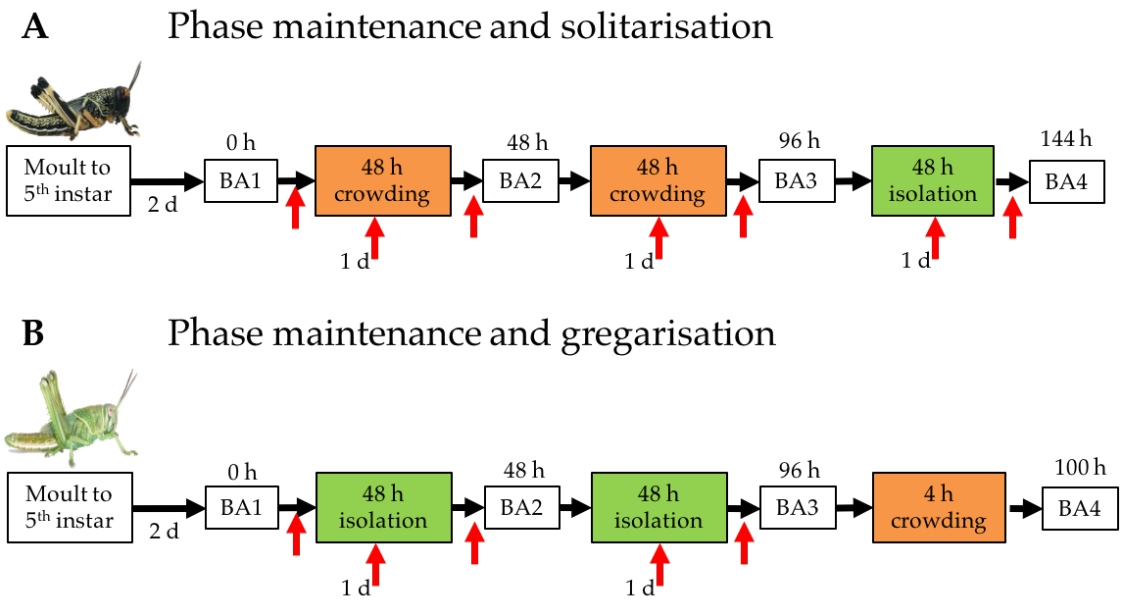


Figure 4.1: Schematic timeline of methods for fluphenazine experiments. (A) Phase maintenance and solitarisation: Gregarious locusts were run through the behavioural assay (BA) 2 d after moulting to the 5th instar stage. They were then immediately injected with either 5 µL 500 µM of the DA receptor antagonist fluphenazine dissolved in saline, or control saline (red arrows, 1 d label confirms that the injection took place 1 d into the crowding/isolation period), and this injection was then repeated daily for 6 d. During this 6 d period, they were run through the same behavioural assay every 2 d (48 h crowding), with injections of fluphenazine immediately prior to placing in the holding tube. At 96 h, gregarious locusts were exposed to 48 isolation, followed by a final behavioural assay (BA4) and culled at -10°C. (B) Phase maintenance and crowd-induced gregarisation: As (A) except with solitarious 5th instar locusts undergoing isolation for 96 h followed by 4 h crowding.

4.3.7.1.1 Phase maintenance and solitarisation

The aim of this experiment was to understand the effect of blocking DA receptors on behavioural phase maintenance and solitarisation of long-term gregarious 5th instar nymphs. 2 d after moulting to the 5th instar stage, gregarious locusts were run through the behavioural assay (0 h time point). They were then injected with fluphenazine (Flu group; $N = 18$) or a saline control (Saline group; $N = 18$) immediately after the assay and

returned to their holding cages within the crowding tub for easier location in future assays (Figure 4.1A). They were then injected each day for 6 d. At 48 h and 96 h, they were run through the behavioural assay again to measure the effect of the drug on baseline behavioural gregariousness. After the 96 h assay, they were then isolated for 2 d and run through a final behavioural assay to measure the effect of fluphenazine injections on behavioural solitarisation (144 h). They were then culled at -10°C.

4.3.7.1.2 Phase maintenance and gregarisation

The aim of this experiment was to understand the effect of blocking DA receptors on behavioural phase maintenance and gregarisation of long-term solitarious 5th instar nymphs. 2 d after moulting to the 5th instar stage, the locusts were run through the behavioural assay (0h time point). They were then injected with fluphenazine (Flu group; $N = 21$) or a saline control (Saline group; $N = 21$) immediately after the assay and returned to a holding tub (Figure 4.1B). They were then injected each day for 6 d. At 48 h and 96 h, they were run through the behavioural arena to measure the effect of the drug on baseline behavioural gregariousness. After the 96 h assay, they were then crowded for 4 h and run through a final behavioural assay (100 h) to measure the effect of fluphenazine injections on behavioural gregarisation. They were then culled at -10°C.

4.3.7.2 AMTP

To understand the effect of 5-HT depletion on behavioural phase transition, the 5-HT-specific synthesis-blocking compound AMTP was used in an attempt to pharmacologically manipulate 5-HT concentrations in the CNS. The protocol was adopted from Anstey et al. (2009), where it was reported to result in an 80% depletion in 5-HT concentrations in the thoracic ganglia of the desert locust. In all experiments using AMTP (experiments AMTP1-5), the treatment group was injected with 40 µL 100 mM AMTP dissolved into locust saline and the control group received injections of locust saline only. For AMTP1-4 (Sections 4.3.7.2.1 & 4.3.7.2.2) the injection period was extended from 5 d prior to the experimental procedures (Anstey et al., 2009) to 7 d prior to the behavioural assay in order to maximise 5-HT depletion. The injections of AMTP or control saline were thus commenced 7 d prior to behavioural assay in solitarious 4th instar locusts. Injections continued every 2 d until 3 d before the first experimental procedure, when injections were administered every day (3, 2, 1 d before). The final injection was delivered to the test locusts 1 h before the first experimental procedures.

AMTP5 (Section 4.3.7.2.3) – a direct replication of Anstey et al. (2009) – used the same injection procedure as AMTP1-4 except that it commenced after the moult to the 5th instar stage (5 d prior to experimental procedures as in Anstey et al. (2009)).

4.3.7.2.1 Crowd-induced gregarisation and regregarisation (AMTP1-2)

Experiments AMTP1 and 2 aimed to understand the effect of 5-HT depletion on crowd-induced gregarisation and re-gregarisation respectively, using a repeated measures approach to maximise the data obtained from each experimental animal and to provide a robust time course of behavioural phase change (Figure 4.2). The following subsections explain the designs of each experiment in more detail.

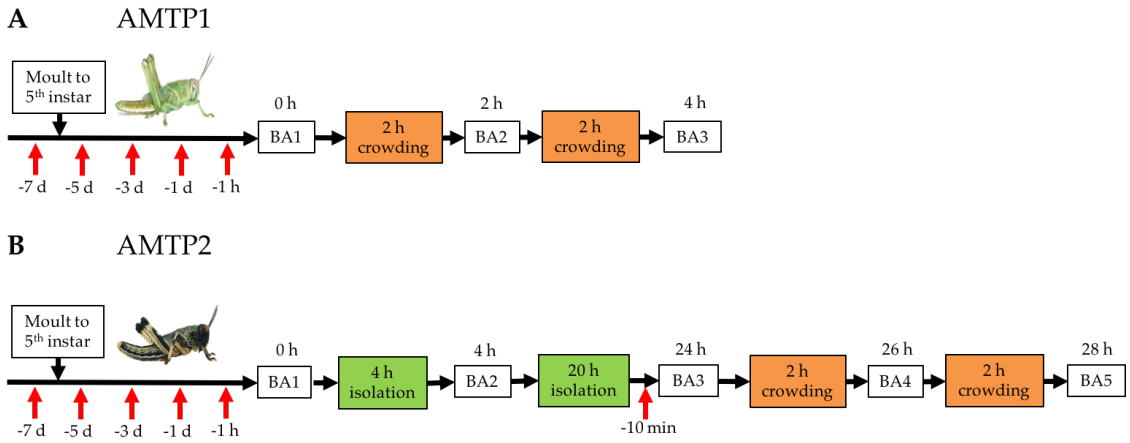


Figure 4.2: Schematic timeline of methods for experiments AMTP1 and AMTP2. (A) AMTP1: Solitarious locusts were injected with 40 µL of 5-HT-depleting compound AMTP (100 mM) dissolved in saline, or control saline (red arrows) every two days starting from 7 d prior to their first behavioural assay (BA). They were then crowded for 2 h, re-assayed and then crowded again. They were then culled at -10°C. (B) AMTP2: Gregarious locusts were given the same injection regime and were isolated for 24 h, with one behavioural assay after 4 h isolation. They were injected again prior to undergoing re-crowding for 4 h and then culled the following day.

4.3.7.2.1.1 Gregarisation (AMTP1)

Injections of AMTP or control saline (AMTP group: $N = 31$; Saline group: $N = 33$) were commenced 7 d prior to behavioural assay in solitarious 5th instar locusts (Figure 4.2A). Starting 1 h after the final injection, the behavioural gregariousness of the solitarious nymphs was quantified using the behavioural assay (BA1; 0 h time point). Immediately after this, both treatment groups were exposed to 2 h crowding, and run through the behavioural assay once more (BA2; 2 h time point), before returning to the crowd tub for another 2 h. After the final behavioural assay (BA3; 4 h time point), test locusts were

culled at -10°C. HPLC confirmation of the drug effect was not carried out in these first experiments because the effect had been described by Anstey (2012) and Anstey et al. (2009).

4.3.7.2.1.2 Regregarisation (AMTP2)

The repeated measures approach was used to test the effect of 5-HT depletion on regregarisation of isolated gregarious locusts (Figure 4.2B). After saline- and AMTP-injected (Saline group: N = 23; AMTP group: N = 23) crowded gregarious nymphs completed their injection regimen, their behavioural gregariousness was quantified using the behavioural assay (BA1; 0 h time point). The treatment groups were then exposed to 4 h isolation before being run through the behavioural assay (BA2; 4 h time point). They were returned to isolation for another 20 h. After this, they were injected with another dose of AMTP or saline and run through the behavioural assay once more 1 h later (BA3; 24 h time point). After this behavioural assay, the animals were re-crowded for 2 h, then run back through the same behavioural assay (BA4; 26 h time point). After returning to the crowd for another 2 h, they underwent a final behavioural assay (BA5; 28 h time point) and were then culled at -10°C. HPLC confirmation of the drug effect was not carried out as described in experiment AMTP1.

4.3.7.2.2 Tickling-induced gregarisation (AMTP3-4)

Experiments AMTP3 and 4 aimed to understand the effect of 5-HT depletion on tickle-induced gregarisation using the same repeated measures approach as AMTP1. AMTP3 followed the same protocol as experiment AMTP1, except that hind leg tickling rather than crowding (Chapter 2; AMTP group: N = 16; Saline group: N =18) was used to induce behavioural gregarisation (Figure 4.3A). Experiment AMTP4 used the same methods as AMTP3 (AMTP group N = 20, Saline group N = 20), except was carried out two months later with the extra step of collecting HPLC samples after the final behavioural assay in order to determine the actions of AMTP injections on 5-HT content (Figure 4.3A). Due to unforeseen technical difficulties with installation of the HPLC equipment, HPLC quantification of the samples was not carried out for this thesis. Instead, the behavioural data from each cohort were pooled for analysis.

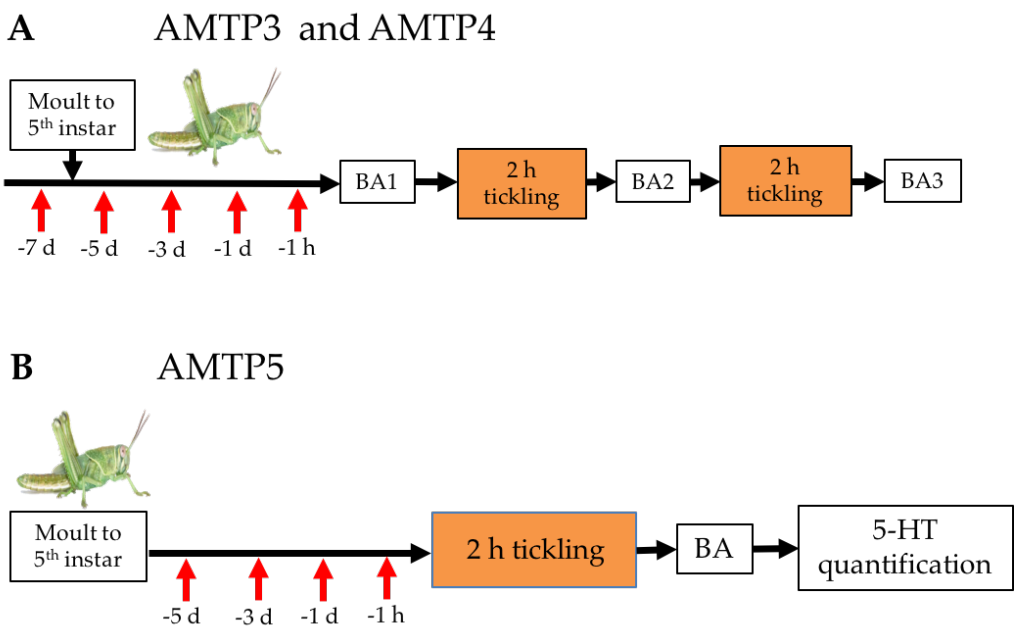


Figure 4.3: Schematic timeline of methods for experiments AMTP3, AMTP4 and AMTP5. (A) AMTP3: Solitarious locusts were injected with 40 µL of 5-HT-depleting compound AMTP (100 mM) dissolved in saline, or control saline (red arrows) every two days starting from 7 d prior to their first behavioural assay (BA) before undergoing hind leg tickling with a fine paintbrush. They were then tickled for 2 h before another behavioural assay, before this was repeated again. They were then culled at -10°C. AMTP4: As AMTP3, but locusts were snap-frozen in liquid nitrogen immediately after the final behavioural assay (BA3). (B) AMTP5: as AMTP4 but animals were administered with AMTP 5 d prior to experimental procedures. Furthermore, animals were exposed to tickling for 2 h without prior behavioural observations, and behaviourally assayed before HPLC quantification of 5-HT.

4.3.7.2.3 Tickling-induced gregarisation with one observation and HPLC (AMTP5)

Experiment AMTP5 was a direct attempt to replicate previously reported effects of AMTP injections on tickle-induced gregarisation (Anstey, 2012; Anstey et al., 2009) – since such effects were absent in experiments AMTP1-4. Solitarious nymphs were injected with AMTP or saline control (AMTP group: $N = 24$; Saline group: $N = 31$) 5 d, 3 d, 2 d, 1 d and 1 h prior to tickling for 2 h (Figure 4.3B) and their behavioural gregariousness was quantified using the behavioural assay. The samples were then snap-frozen and processed for HPLC. Due to sample loss (incomplete retrieval of tissue from frozen locusts), the sample sizes for HPLC quantification are lower than for than the behavioural assay (AMTP group: $N = 17$; Saline group: $N = 22$).

4.3.7.3 5-HT injections

This experiment aimed to understand the behavioural and neurochemical effects of injecting 5-HT into solitarious 5th instar nymphs. Due to potentially confounding issues encountered in repeated measures work prior to this (see Section 4.4.1), all groups in this study underwent only one behavioural observation. There were four experiment groups in this experiment: 5-HT uncrowded (0 h 5-HT: $N = 21$), 5-HT crowded (2 h 5-HT: $N = 21$), saline uncrowded (0 h Saline: $N = 21$) and saline crowded (2 h Saline: $N = 20$). Two days after moulting to the 5th instar stage, 2 h before the behavioural assay, animals in the control saline uncrowded and crowded groups were then injected with 40 µL saline and the 5-HT treatment group animals were injected with 40 µL 10 mM 5-HT dissolved in saline (Anstey, 2012). The crowded treatment groups were then placed into forced crowding conditions, whereas the uncrowded control groups were returned to their holding cages for 2 h (Figure 4.4). After 1 h and 1 h 50 min (1 h, and 10 min before behavioural assay respectively), the same injections were repeated. After the final injection, the locusts were run through the behavioural assay and snap-frozen in liquid nitrogen. Due to time constraints, HPLC quantification was not carried out for this experiment (Figure 4.4).

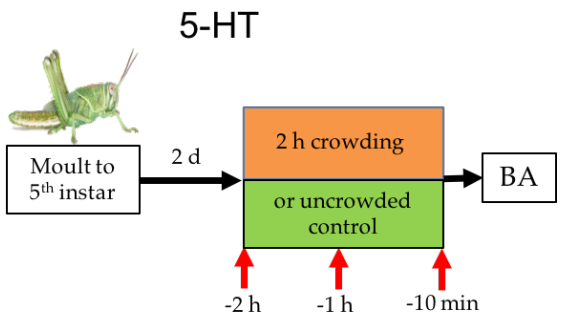


Figure 4.4: Schematic timeline of methods for 5-HT injection experiment. From 2-4 d into the 5th instar stage, solitarious locusts were injected with 40 µL of 10 mM 5-HT dissolved in saline, or control saline (red arrows) and underwent either 2 h crowding with conspecifics or remained in their isolated holding cage for 2 h. They were then run through a behavioural assay (BA) and immediately snap-frozen in liquid nitrogen.

4.3.7.4 Reserpine

The aim of these experiments was to establish a monoamine-depletion protocol in *S. gregaria* to reassess the roles of monoamines in behavioural phase change, given the limited efficacy of AMTP for 5-HT revealed in earlier experiments (Section 4.3.7.2). For

all experiments in this section, reserpine was dissolved into 100% DMSO vehicle (Ma et al., 2011; Stevenson et al., 2000; Vieira et al., 2007). The first experiment of this section measured the behavioural effects of injecting 2.5 µL vehicle 100% DMSO (Figure 4.5A) compared with 2.5 µL control saline in 5th instar gregarious locusts. The second experiment of this section measured the behavioural and neurochemical effects of injecting 2 separate doses of reserpine (2.5 µL 50 µg per g bodyweight of locust or 2.5 µL 100 µg per g bodyweight of locust (µg/g; Figure 4.5B) compared with 2.5 µL DMSO in 5th instar gregarious locusts. The third experiment of this section measured the effect of 2.5 µL 50 µg/g reserpine on behavioural gregarisation and monoamine concentrations in solitarious 5th instar locusts (Figure 4.5C). The following subsections explain the experimental designs of each experiment in more detail.

4.3.7.4.1 DMSO control vs saline control

The aim of this experiment was to measure the behavioural effect of DMSO injections on long-term gregarious 5th instar nymphs over time (Figure 4.5A). 2 d after moulting to the 5th instar stage, locusts were injected with 2.5 µL 100% DMSO (DMSO group: N = 19) or control saline (Saline group: N = 22). They were then returned to their crowding tub, which was placed inside a gregarious colony for 24 h. They were then run through a behavioural assay (24 h time point). After this assay, this step was repeated for another 24 h (48 h time point), before culling the locusts at -10 °C.

4.3.7.4.2 Reserpine in long-term gregarious locusts

The aim of this experiment was to measure the behavioural effect of two doses of reserpine injections on long-term gregarious 5th instar nymphs over time compared with DMSO (Figure 4.5B). Two days after moulting to the 5th instar stage, the locusts were injected with either 2.5 µL 50 µg/g, 2.5 µL 100 µg/g reserpine, or 100% DMSO control reserpine (50 µg/g group: N = 28; 100 µg/g group: N = 27; DMSO group: N = 28). They were then returned to their crowding tub, which was placed inside a gregarious colony for 24 h. They were then run through the behavioural assay to quantify their behavioural gregariousness. The locusts were then snap-frozen in liquid nitrogen, their thoracic ganglia were dissected out and processed for HPLC quantification of DA and 5-HT. Due

to incomplete dissections, some samples were discarded and the final sample sizes were 18 (100 µg/g), 16 (50 µg/g) and 15 (DMSO).

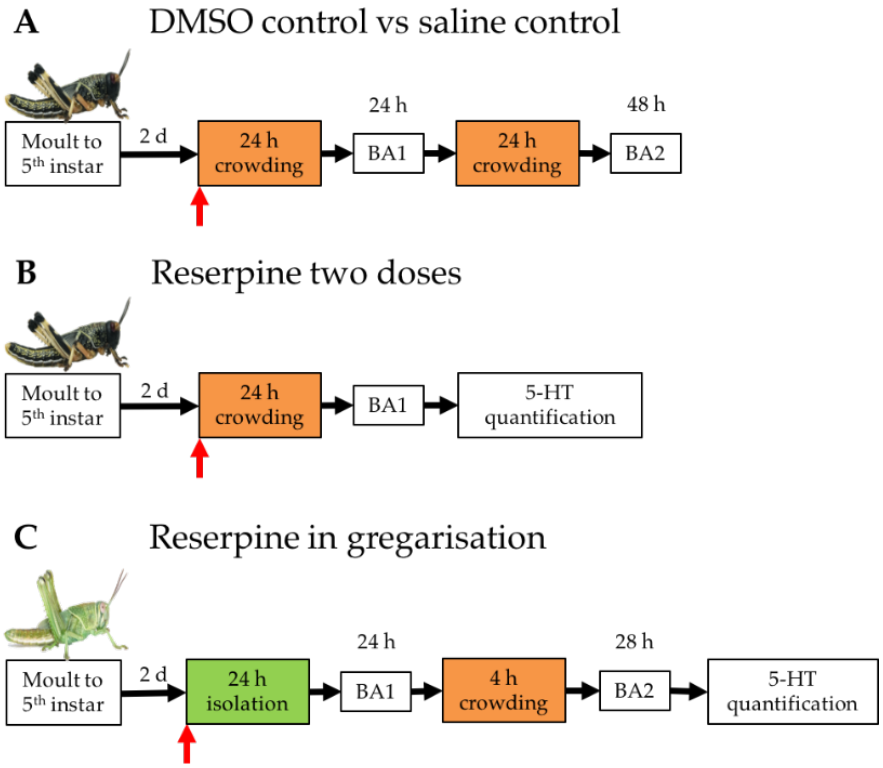


Figure 4.5: Schematic timeline of the reserpine experiments. (A) DMSO control vs. saline control: 2 d after moulting to the 5th instar stage, gregarious locusts were injected with 2.5 µL DMSO or control saline (red arrow). They were then returned to their crowding tub for 24 h. They were then run through a behavioural assay (BA) and then returned to the crowd tub for another 24 h. They were then run through a behavioural assay and culled at -10 °C. (B) Reserpine two doses: as (A) except the locusts were injected with either 2.5 µL DMSO, 2.5 µL 50 µg/g reserpine dissolved in DMSO or 100 µg/g reserpine dissolved in DMSO and assayed only once, 24 h after drug injection. They were then snap-frozen in liquid nitrogen and their brain and thoracic ganglia processed for 5-HT quantification using HPLC. (C) As (B) except with solitarious locusts that were injected, then left in their isolated holding cage for 24 h. They were then run through a behavioural assay, crowded for 4 h, then run though a second behavioural assay before 5-HT quantification.

4.3.7.4.3 Reserpine in behavioural gregarisation

The aim of this experiment was to measure the effect of 2.5 µL 50 µg/g reserpine on behavioural gregarisation and monoamine content in the thoracic ganglia of solitarious 5th instar locusts (Figure 4.5C). 2 d after moulting to the 5th instar stage, locusts were injected with either 2.5 µL 50 µg/g reserpine or 100% DMSO control (50 µg/g group: N = 21; DMSO group: N = 21). They were then returned to their holding cage for 24 h.

They were then run through a behavioural assay (24 h time point) to quantify their behavioural gregariousness and then crowded with conspecifics for 4 h. They were then run through a final behavioural assay (28 h). The locusts were then snap-frozen in liquid nitrogen, their thoracic ganglia were dissected out and processed for HPLC quantification of DA and 5-HT. Due to time restraints, only half of the behavioural cohort were processed for HPLC analysis, and the final sample sizes were 10 (50 μg/g group) and 11 (DMSO group).

4.4 Results

4.4.1 Confounding effects observed when injecting with fluphenazine

4.4.1.1 Phase maintenance and solitarisation

To understand the effect of blocking DA receptors on behavioural phase maintenance and solitarisation, long-term gregarious nymphs were injected with fluphenazine or saline daily, and run through the behavioural assay at 0 h, 48 h and 96 h, before being isolated for 48 h and run through a final behavioural assay (Figure 4.1A). Both treatment groups showed gregarious behaviour at the 0 h time point before injections (Figure 4.6A). However, both groups showed an unexpected decrease in P_{greg} over time, reaching a minimum at the end of 96 h without phase-changing stimuli, and with the Flu group decreasing less than the Saline group (0 h to 96 h: Saline: median P_{greg} decrease of 0.49; Flu: median P_{greg} decrease of 0.23; Figure 4.6A). Subsequent isolation for 48 h did not change the median P_{greg} value of the Saline group but sharply decreased the median P_{greg} value of the Flu group by 0.36 (Figure 4.6A green boxes).

All statistical details are in Table 4.1. A linear mixed model provided very strong evidence for an effect of time point on behavioural gregariousness ($p < 0.0001$), however, there was only weak evidence for a treatment effect on behavioural gregariousness ($p = 0.0790$). The model provided no evidence for an interaction between the treatment and the crowding time point fixed effects ($p = 0.1349$). Analysis of regression coefficients provided moderate evidence for a reduction effect in behavioural gregariousness after 48 h (48 h vs 0 h: $p = 0.0057$; Figure 4.6A) and weak evidence for a further reduction effect after another 48 h (96 h vs 48 h time point: $p = 0.0368$). Although there was very strong evidence for a solitarising effect of 48 h isolation compared with the starting time point in the experiment (144 h vs 0 h time point: $p < 0.0001$), there was no evidence for an effect of 48 h isolation compared with the end of the 96 h period across the groups (144 h vs 96 h time point: $p = 0.2055$). Further analysis of regression coefficients revealed very little evidence for a treatment effect in any time point (Flu vs Saline: $p = 0.0790$).

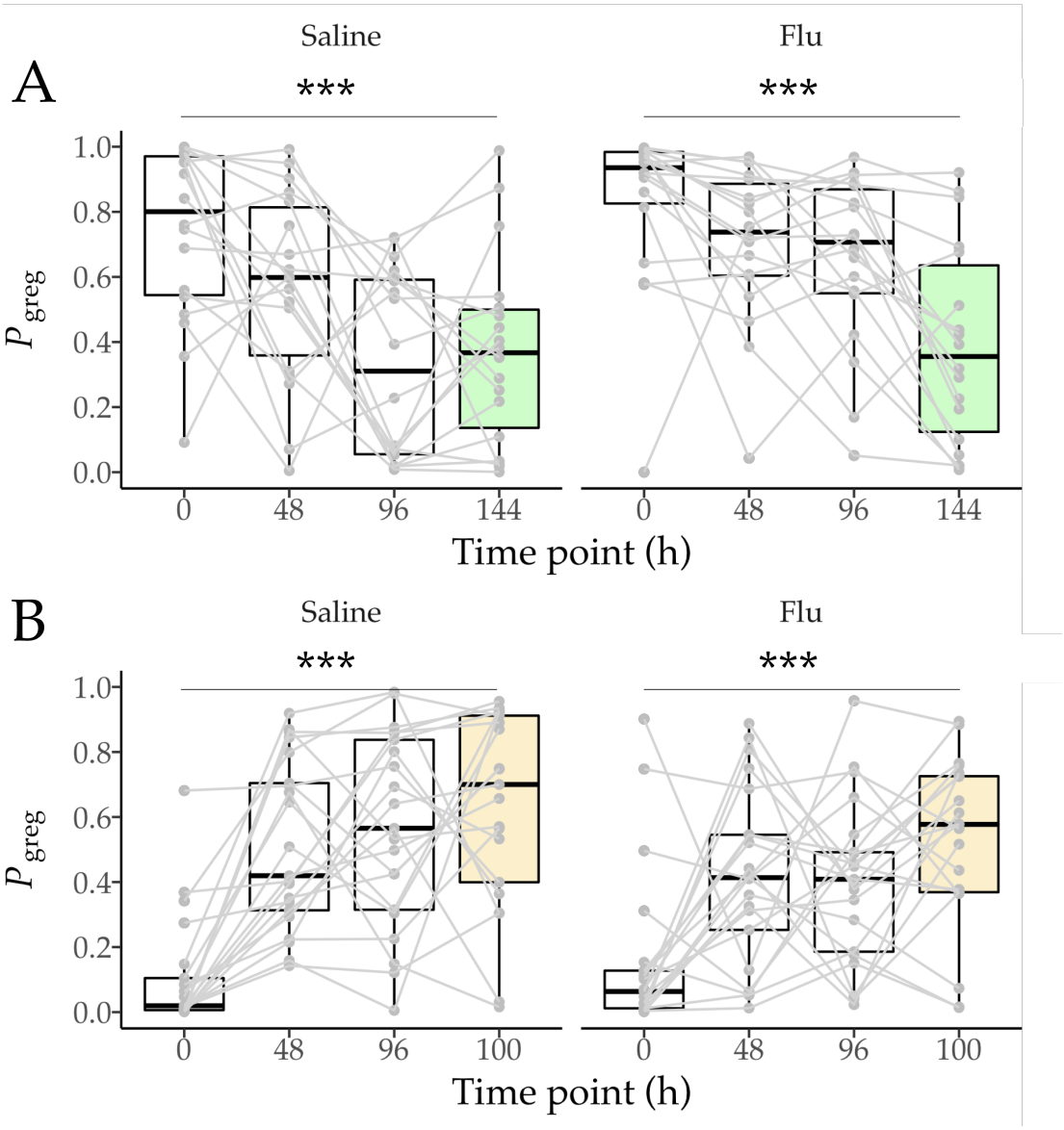


Figure 4.6: Inconclusive effect of DA receptor block on behavioural phase maintenance and phase change. (A) Comparison of P_{greg} values over 96h of phase maintenance (0 h, 48 h, 96 h, white boxes) between gregarious 5th instar locusts injected with fluphenazine or saline control ($N = 18$ for each group). The gregarious animals finally underwent 48 h isolation (144 h time point, green boxes). (B) As (A) except with 5th instar solitarious locusts observed during 96 h continued isolation and ending with 4 h crowding (orange boxes). Both groups $N = 21$. Each point represents one observation, and lines connect observations over time. Asterisks show evidence for the time point effect of an additive linear mixed effects model (Table 2.4).

Table 4.1: Statistical tests of the effect of fluphenazine injections and time point on logit values (behavioural gregariousness) in gregarious nymphs. Df = degrees of freedom. Linear model analysis: type 1 ANOVA.

Test	Comparison	Statistic	Df between groups	Df within groups	*p*-value
Logit ~ Time point + Treatment + Time point:Treatment + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	$F = 15.12$	3	102	< 0.0001
Linear mixed model	*Treatment* fixed effect	$F = 3.28$	1	34	0.0790
Linear mixed model	*Time point:Treatment* interaction	$F = 1.90$	3	102	0.1349
Logit ~ Time point + Treatment + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	$F = 14.74$	3	105	< 0.0001
Linear mixed model	*Treatment* fixed effect	$F = 3.28$	3	34	0.0790
Regression coefficient	48 h vs 0 h	$t = -2.82$	3	N/A	0.0057
Regression coefficient	96 h vs 0 h	$t = 1.14$	3	N/A	0.2600
Regression coefficient	144 h vs 0 h	$t = -6.21$	3	N/A	< 0.0001
Regression coefficient	96 h vs 48 h	$t = -2.99$	3	N/A	0.0368
Regression coefficient	144 h vs 96 h	$t = -1.274$	3	N/A	0.2055
Regression coefficient	Flu vs Saline	$t = 1.81$	3	N/A	0.0790

4.4.1.2 Phase maintenance and gregarisation

To understand the effect of blocking DA receptors on behavioural phase maintenance and gregarisation, long-term solitarious nymphs were injected with fluphenazine or saline daily, and run through the behavioural assay at 0 h, 48 h and 96 h, before being crowded for 4 h and run through a final behavioural assay (Figure 4.1B). Both treatment groups showed solitarious behaviour at the 0 h time point before injections (Figure 4.6B). Over time injected and assayed, the Saline group unexpectedly increased over 48 and 96 h without phase-changing stimuli (0 h to 96 h: median P_{greg} increase of 0.55; Figure 4.6B). The fluphenazine-injected group showed the same increase by 48 h, but it did not increase further by 96 h (0 h to 96 h: median P_{greg} increase of 0.35; Figure 4.6B). With already high median P_{greg} values, crowding for 4 h (96 h to 100 h) increased the median

P_{greg} value of the control group by just 0.13, and increased the median P_{greg} value of the Flu group by just 0.17 (Figure 4.6B orange boxes).

All statistical details are in Table 4.2. A linear mixed model provided very strong evidence for an effect of time point on behavioural gregariousness ($p < 0.0001$), however, there was no evidence for a treatment effect on behavioural gregariousness ($p = 0.3247$). There was also very little evidence for an interaction between the treatment and the crowding time point fixed effects ($p = 0.0823$). Across both treatment groups, analysis of regression coefficients provided very strong evidence for an increase in behavioural gregariousness after 48 h (48 h vs 0 h: $p < 0.0001$; Figure 4.6B) and no evidence for a further reduction effect after another 48 h (96 h vs 48 h: $p = 0.7637$). Although there was very strong evidence for a gregarising effect of 4 h crowding compared with the starting time point in the experiment (100 h vs 0 h: $p < 0.0001$), there was no evidence for a gregarising effect of 4 h crowding compared with the end of the 96 h period across the groups (100 h vs 96 h: $p = 0.2671$). Further analysis of regression coefficients revealed very little evidence for a treatment effect at any time point (Flu vs Saline: $p = 0.3209$).

4.4.2 AMTP had limited effects on behavioural phase change and maintenance

4.4.2.1 AMTP1: no evidence for an AMTP effect on crowd-induced gregarisation

To determine the effect of AMTP on crowd-induced gregarisation in solitarious nymphs, AMTP and Saline groups received pretreatment before crowding for 4 h (Figure 4.2A; Figure 4.7A). Crowding for 2 h increased P_{greg} by 0.43 in the saline control group and by 0.4 in the AMTP group. Crowding for a further 2 h (4 h time point) had no further effect on P_{greg}.

Table 4.2: Statistical tests of the effect of fluphenazine injections and time point on logit values (behavioural gregariousness) in solitarious nymphs. Df = degrees of freedom. Linear model analysis: type 1 ANOVA.

Test	Comparison	Statistic	Df between groups	Df within groups	*p*-value
Logit ~ Time point + Treatment + Time point:Treatment + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	$F = 19.61$	3	119	< 0.0001
Linear mixed model	*Treatment* fixed effect	$F = 0.98$	1	119	0.3247
Linear mixed model	*Time point:Treatment* interaction	$F = 2.29$	3	119	0.0823
Logit ~ Time point + Treatment + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	$F = 19.67$	3	122	< 0.0001
Linear mixed model	*Treatment* fixed effect	$F = 0.99$	3	122	0.3209
Regression coefficient	48 h vs 0 h	$t = 8.19$	3	N/A	< 0.0001
Regression coefficient	96 h vs 0 h	$t = 8.49$	3	N/A	<0.0001
Regression coefficient	100 h vs 0 h	$t = 9.60$	3	N/A	< 0.0001
Regression coefficient	96 h vs 48 h	$t = -0.30$	3	N/A	0.7637
Regression coefficient	100 h vs 96 h	$t = 1.115$	3	N/A	0.2671
Regression coefficient	Flu vs Saline	$t = -0.100$	1	N/A	0.3209

All statistical details are in Table 4.3. As expected, a linear mixed model provided very strong evidence for a crowding effect on gregariousness ($p < 0.0001$), however, there was no evidence for a treatment effect ($p = 0.2491$). There was also no evidence for an interaction between the treatment and the crowding time point effects (linear mixed model $p = 0.9228$). Analysis of regression coefficients provided strong evidence for a gregarising effect of 2 h crowding compared with the 0 h time point in both groups (0 h vs 2 h: $p < 0.0001$; Figure 4.7A) but no evidence for further gregarisation after another 2 h crowding (4 h vs 2 h: $p = 0.8952$; Figure 4.7A). Further analysis of regression coefficients revealed no evidence for a treatment effect in any time point (AMTP vs Saline: $p = 0.2535$; Figure 4.7A).

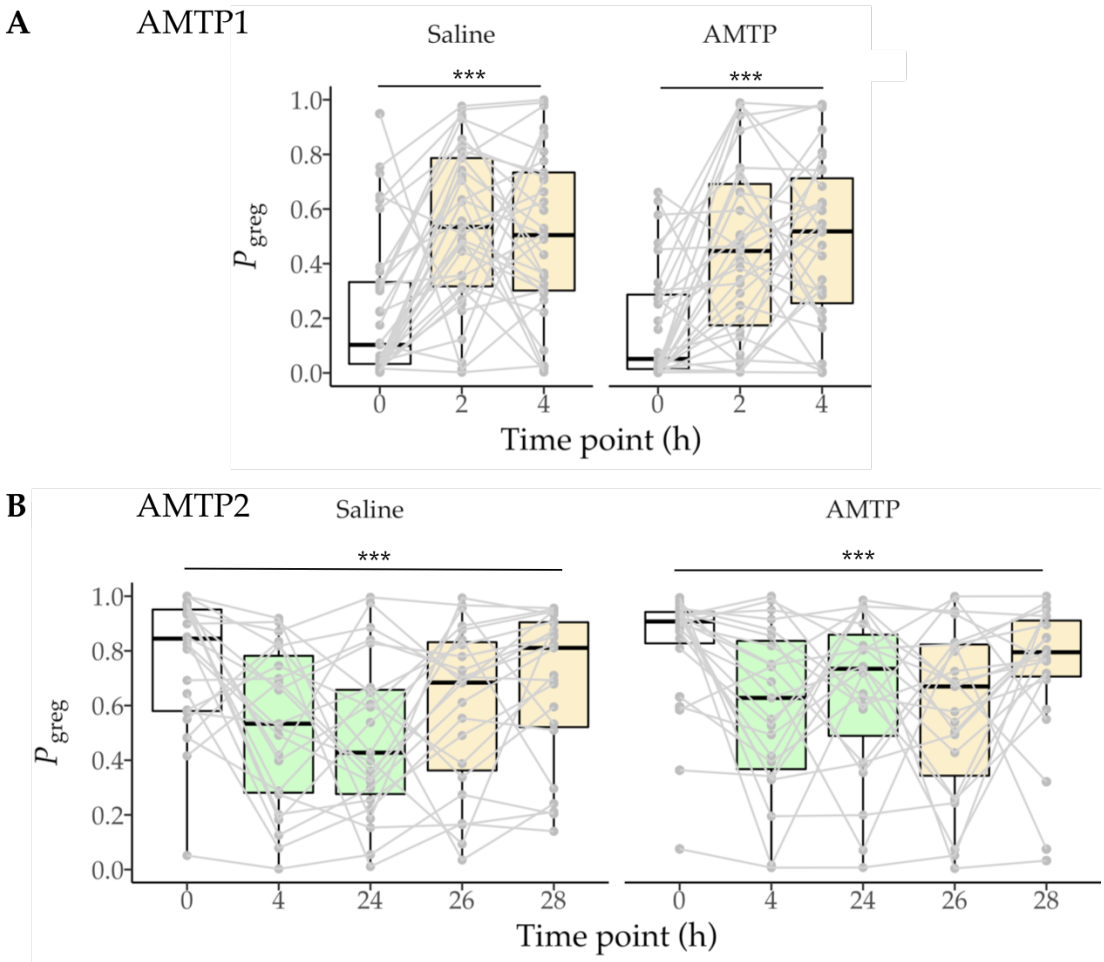


Figure 4.7: No evidence for 5-HT depletion effect on behavioural phase change. (A) AMTP1 experiment: comparison of P_{greg} values over time points of crowding (0h: white bar; 2 and 4 h crowding: orange bars) between solitarious locusts injected with 40 µL 100 mM AMTP ($N = 31$) and saline controls ($N = 33$). (B) As (A) except with gregarious locusts undergoing 4 h and 24 h isolation (green boxes) and then 2 h and 4 h recrowding (orange boxes). Both groups $N = 23$. Each point represents one observation and lines connect observations over time. Each point represents one observation, and lines connect observations over time. Asterisks show evidence for the time point effect in an additive linear mixed effects model (Table 2.4).

Table 4.3: Statistical tests of the effect of AMTP injections and crowding time point on logit values (behavioural gregariousness) in solitarious nymphs. Df = degrees of freedom. Linear model analysis: type 2 ANOVA.

Test	Comparison	Statistic	Df between groups	Df within groups	*p*-value
Logit ~ Time point + Treatment + Time point:Treatment + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	$\chi^2 = 57.88$	2	119	< 0.0001
Linear mixed model	*Treatment* fixed effect	$\chi^2 = 1.33$	1	119	0.2491
Linear mixed model	*Time point:Treatment* interaction	$\chi^2 = 0.16$	2	119	0.9228
Logit ~ Time point + Treatment + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	$\chi^2 = 58.74$	2	122	< 0.0001
Linear mixed model	*Treatment* fixed effect	$\chi^2 = 0.99$	1	122	0.2491
Regression coefficient	2 h vs 0 h	$t = 6.57$	2	N/A	< 0.0001
Regression coefficient	4 h vs 0 h	$t = 6.70$	2	N/A	<0.0001
Regression coefficient	4 h vs 2 h	$t = 0.13$	2	N/A	0.8952
Regression coefficient	AMTP vs Saline	$t = -0.15$	1	N/A	0.2535

4.4.2.2 AMTP2: no evidence for an AMTP effect on solitarisation or regregarisation

To understand the effect of AMTP injections on solitarisation and regregarisation in gregarious nymphs, AMTP and Saline groups received pretreatment before isolating for 24 h and recrowding for 4 h (Figure 4.2B; Figure 4.7B). The median P_{greg} value of the Saline group decreased by 0.41 after 24 h of isolation (24 h vs 0 h) while the AMTP group median P_{greg} value decreased by 0.28 after 4 h of isolation, but recovered, increasing by 0.1 by 24 h (24 h vs 4 h). The median P_{greg} values then increased once more in both groups after 4 h crowding to a final value of 0.81 in the saline control group (increase of 0.38 at 28 h time point) and 0.79 in the AMTP group (increase of 0.16; Figure 4.7B).

Table 4.4: Statistical tests of the effect of AMTP injections, isolation and crowding on logit values (behavioural gregariousness) in gregarious nymphs. Df = degrees of freedom. Linear model analysis: type 2 ANOVA.

Test	Comparison	Statistic	Df between groups	Df within groups	*p*-value
Logit ~ Time point + Treatment + Time point:Treatment + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	$\chi^2 = 26.13$	4	176	< 0.0001
Linear mixed model	*Treatment* fixed effect	$\chi^2 = 0.99$	1	44	0.3200
Linear mixed model	*Time point:Treatment* interaction	$\chi^2 = 2.10$	4	176	0.7175
Logit ~ Time point + Treatment + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	$\chi^2 = 26.41$	4	180	< 0.0001
Linear mixed model	*Treatment* fixed effect	$\chi^2 = 0.99$	1	44	0.3200
Regression coefficient	4 h vs 0 h	$t = -4.23$	4	N/A	< 0.0001
Regression coefficient	24 h vs 0 h	$t = -4.05$	4	N/A	0.0001
Regression coefficient	26 h vs 0 h	$t = -3.68$	4	N/A	0.0003
Regression coefficient	28 h vs 0 h	$t = -1.81$	4	N/A	0.0712
Regression coefficient	24 h vs 4 h	$t = 0.19$	4	N/A	0.8509
Regression coefficient	26 h vs 24 h	$t = 0.37$	4	N/A	0.7086
Regression coefficient	28 h vs 24 h	$t = 2.23$	4	N/A	0.0266
Regression coefficient	28 h vs 26 h	$t = -0.37$	4	N/A	0.3254
Regression coefficient	AMTP vs Saline	$t = 0.99$	1	N/A	0.3254

All statistical details are in Table 4.4. A linear mixed model provided very strong evidence for a crowding effect on gregariousness ($p < 0.0001$), but no evidence for a treatment effect ($p = 0.3200$). There was no evidence for an interaction between the treatment and the time point fixed effects ($p = 0.7175$). Analysis of regression coefficients provided very strong evidence for a solitarising effect of 4 h isolation on gregariousness in both groups ($p < 0.0001$; Figure 4.7B) but no evidence for further solitarisation after another 20 h isolation (24 h vs 4 h: $p = 0.8509$; Figure 4.7B). Regression coefficient analysis also provided weak evidence for a re-gregarising effect

of 4 h re-crowding in both groups (28 h vs 24 h: p = 0.0266; Figure 4.7B). Further regression coefficient analysis revealed very little evidence that 4 h recrowded gregarious locusts (28 h) behaved more solitariously than 0 h in both groups (28 h vs 0 h: p = 0.0712). Further analysis of regression coefficients revealed no evidence for a treatment effect in any time point (p = 0.3254; Figure 4.7B).

4.4.2.1 AMTP3 & 4: no evidence for an AMTP effect on tickle-induced gregarisation

To understand the effect of AMTP on tickle-induced behavioural gregarisation, solitarious nymphs were pretreated with AMTP or saline and tickled for 4 h (Figure 4.3A; Figure 4.8). As the behavioural procedures were identical in AMTP3 and AMTP4, and as there was no evidence for an effect of the experiment in the behavioural results (linear mixed model: *Logit* ~ *Time point* + *Treatment* + *Experiment* + (1|*Animal.ID*); Experiment fixed effect χ^2-value = 2.29; Df(group, residual) = 1,69; p = 0.130), the tickle-induced gregarisation behavioural data were pooled together.

Tickling for 2 h increased the median P_{greg} by 0.52 in the saline control group and by 0.61 in the AMTP group (Figure 4.8). Tickling for a further 2 h (4 h time point) showed no further changes in P_{greg} (Figure 4.8).

All statistical details are shown in Table 4.5. A linear mixed model provided very strong evidence for a tickling effect on gregariousness (p < 0.0001), and very little evidence for a treatment effect (p = 0.0857). There was no evidence for an interaction between the treatment and the tickling time point fixed effects (p = 0.4958). Analysis of regression coefficients provided strong evidence for a gregarising effect of 2 h crowding compared with the 0 h time point in both groups (2 h vs 0 h: p < 0.0001; 4 h vs 0 h: p < 0.0001) but no evidence for further gregarisation after another 2 h crowding (4 h vs 2 h: p = 0.1783). Further analysis of regression coefficients revealed very little evidence for a treatment effect at any time point (p = 0.0900).

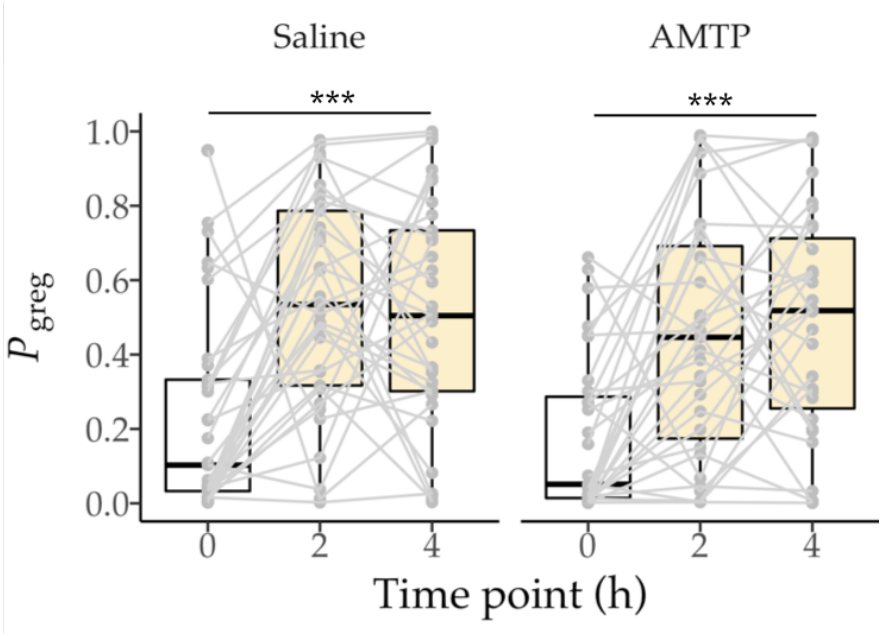


Figure 4.8: No evidence for AMTP effect on tickle-induced behavioural phase change. AMTP3 & 4 experiment: comparison of P_{greg} values over time points of hind leg tickling (0h: white bar; 2 and 4 h tickling: orange bars) between solitarious locusts injected with AMTP ($N = 35$) and saline controls ($N = 38$). Each point represents one observation and lines connect the observations over time. Each point represents one observation, and lines connect observations over time. Asterisks show evidence for the time point effect in an additive linear mixed effects model (Table 2.4).

4.4.2.2 AMTP5: weak AMTP effect on behavioural gregariousness and CNS 5-HT content

4.4.2.2.1 AMTP effects on behavioural gregariousness and CNS 5-HT content

To replicate exactly previously reported effects of AMTP injections on tickle-induced behavioural gregarisation in Anstey et al. (2009), solitarious nymphs were pretreated with AMTP ($N = 30$) or saline injections and tickled for 2 h ($N = 23$; Figure 4.3B). After 2 h tickling in both groups, the median P_{greg} was 0.2 higher in the control group than in the AMTP group (Figure 4.9A) but a Mann-Whitney Test provided very little evidence for a real effect of AMTP on the behavioural gregariousness after 2 h tickling ($p = 0.0899$).

Table 4.5: Statistical tests of the effect of AMTP injections and tickling time point on logit values (behavioural gregariousness) in solitarious nymphs. Df = degrees of freedom. Linear model analysis: type 2 ANOVA.

Test	Comparison	Statistic	Df between groups	Df within groups	*p*-value
Logit ~ Time point + Treatment + Time point:Treatment + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	$\chi^2 = 129.54$	2	142	< 0.0001
Linear mixed model	*Treatment* fixed effect	$\chi^2 = 2.95$	1	71	0.0857
Linear mixed model	*Time point:Treatment* interaction	$\chi^2 = 1.40$	2	142	0.4958
Logit ~ Time point + Treatment + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	$\chi^2 = 130.09$	2	144	< 0.0001
Linear mixed model	*Treatment* fixed effect	$\chi^2 = 2.95$	1	71	0.0857
Regression coefficient	2 h vs 0 h	$t = 9.13$	2	N/A	< 0.0001
Regression coefficient	4 h vs 0 h	$t = 10.48$	2	N/A	<0.0001
Regression coefficient	4 h vs 2 h	$t = 1.35$	2	N/A	0.1783
Regression coefficient	AMTP vs Saline	$t = -1.71$	1	N/A	0.0900

All statistical details are in Table 4.6. The AMTP group showed reduced median 5-HT concentrations compared with the saline control group, with a reduction of 27% in the ganglia, a reduction of 12% in the brain and 27% in the optic lobes (Figure 4.9B). A linear regression model provided weak evidence for a real effect of treatment across the CNS regions ($p = 0.0223$), and very strong evidence for a real effect of region across the treatments ($p < 0.0001$). There was no evidence for an interaction effect of CNS region with AMTP treatment ($p = 0.5600$). Analysis of regression coefficients provided strong evidence that the midbrain contains more 5-HT than the thoracic ganglia across both treatments (Brain vs Ganglia: $p < 0.0001$) and weak evidence that the optic lobes contain more 5-HT than the brain (Optic Lobes vs Brain, $p = 0.0201$; Figure 4.9B). Further analysis of regression coefficients revealed weak evidence that AMTP injections reduce 5-HT concentrations across all regions ($p = 0.0216$; Figure 4.9B).

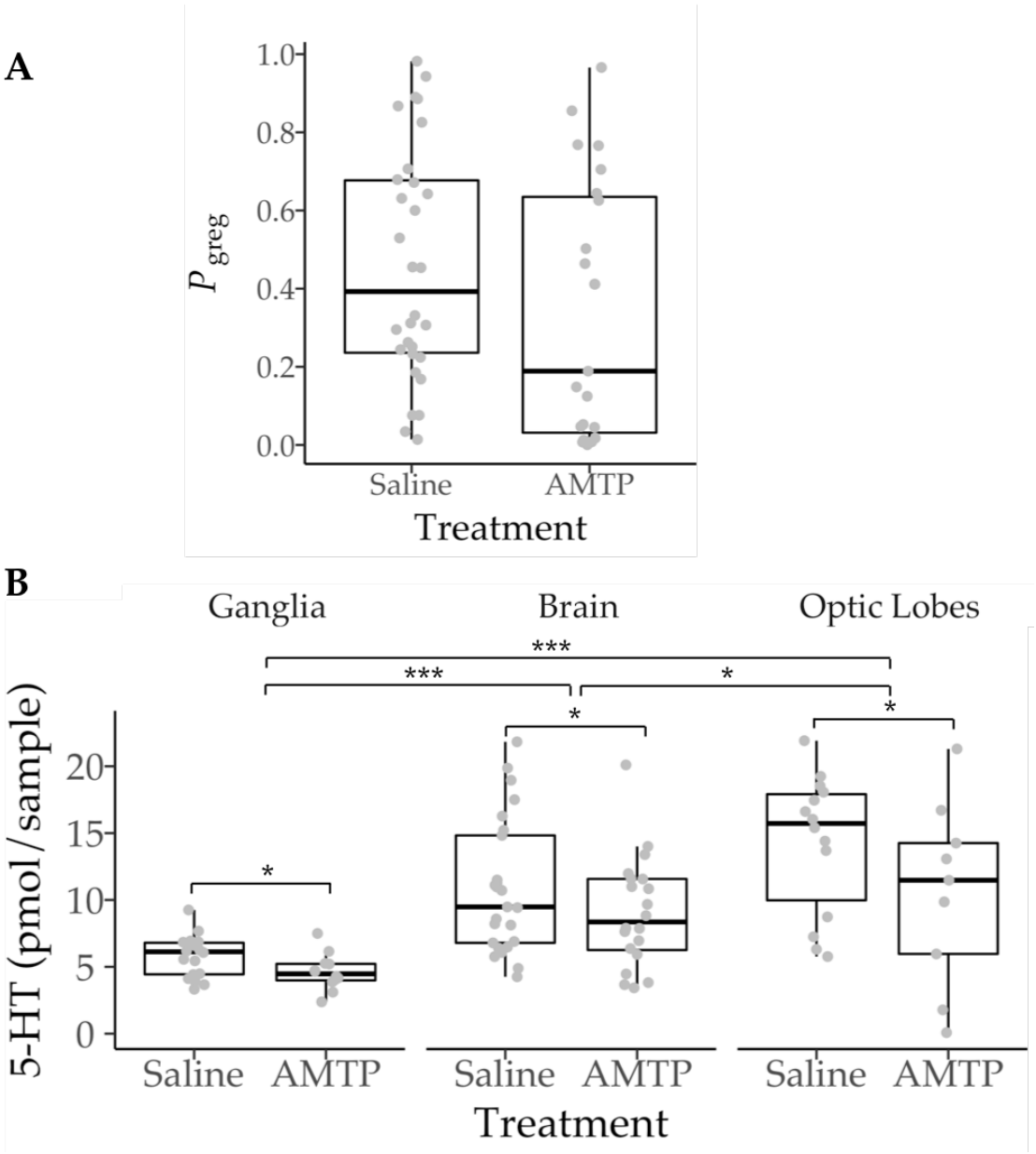


Figure 4.9: Experiment AMTP5: Very little evidence for AMTP effects on tickle-induced behavioural gregarisation or CNS 5-HT concentrations compared to saline controls. (A) P_{greg} values of 5th instar solitarious locusts after 2 h hind leg tickling, one group injected with AMTP ($N = 30$), and the other with saline as a control ($N = 23$). (B) CNS concentrations of 5-HT in AMTP- or saline-injected locusts, for thoracic ganglia (Saline $N = 18$, AMTP $N = 12$), midbrain (Saline $N = 25$, AMTP $N = 22$) and optic lobes (Saline $N = 14$, AMTP $N = 9$). Each point represents one observation. Asterisks show evidence level based on regression coefficients in a linear model (Table 2.4).

Table 4.6: Statistical tests of the effect of AMTP injections and tickling on logit values (behavioural gregariousness) and amounts of 5-HT (Amt_5HT) in the CNS in solitarious nymphs. Df = degrees of freedom. Linear model analysis: type 2 ANOVA and ANCOVA.

Test	**Comparison**	**Statistic**	**Df between groups**	**Df within groups**	***p*-value**
		Logit ~ Treatment			
Mann-Whitney-U	*AMTP vs Saline*	$W = 440$	N/A	N/A	0.0899
		Amt_5HT ~ Region + Treatment + Region:Treatment			
Linear model	*Region* fixed effect	$F = 20.88$	2	92	< 0.0001
Linear model	*Treatment* fixed effect	$F = 5.41$	1	92	0.0223
Linear model	*Treatment*:*Region* interaction	$F = 0.58$	2	92	0.5600
		Amt_5HT ~ Region + Treatment			
Linear model	*Region* fixed effect	$F = 21.07$	2	94	< 0.0001
Linear model	*Treatment* fixed effect	$F = 5.46$	1	94	0.0216
Regression coefficient	Brain vs Ganglia	$t = 4.75$	2	N/A	< 0.0001
Regression coefficient	Optic Lobes vs Ganglia	$t = 6.23$	2	N/A	< 0.0001
Regression coefficient	Optic Lobes vs Brain	$t = 2.37$	2	N/A	0.0201
Regression coefficient	AMTP vs Saline	$t = -2.34$	2	N/A	0.0216
		Ganglia: Logit ~ Treatment + Amt_5HT + Treatment:Amt_5HT			
Linear model	*Treatment* fixed effect	$F = 2.43$	1	26	0.1311
Linear model	*Amt_5HT* fixed effect	$F = 0.21$	1	26	0.6479
Linear model	Interaction effect	$F = 1.09$	1	26	0.3051
		Ganglia: Logit ~ Treatment + Amt_5HT			
Linear model	*Treatment* fixed effect	$F = 2.42$	1	27	0.1313
Linear model	*Amt_5HT* fixed effect	$F = 0.21$	1	27	0.6484
Spearman's Rank	*Amt_5HT* vs *logit*	$\rho = -0.119$	N/A	N/A	0.531
		Brain: Logit ~ Treatment + Amt_5HT + Treatment:Amt_5HT			

Test	Comparison	Statistic	Df between groups	Df within groups	*p*-value
Linear model	*Treatment* fixed effect	$F = 4.75$	1	38	0.0356
Linear model	*Amt_5HT* fixed effect	$F = 0.71$	1	38	0.4037
Linear model	Interaction effect	$F = 0.24$	1	38	0.6298
Brain: Logit ~ Treatment + Amt_5HT					
Linear model	*Treatment* fixed effect	$F = 4.84$	1	39	0.0337
Linear model	*Amt_5HT* fixed effect	$F = 0.73$	1	39	0.3989
Spearman's Rank	*Amt_5HT* vs *logit*	$\rho = 0.181$	N/A	N/A	0.252
Optic Lobes: Logit ~ Treatment + Amt_5HT + Treatment:Amt_5HT					
Linear model	*Treatment* fixed effect	$F = 0.17$	1	18	0.6848
Linear model	*Amt_5HT* fixed effect	$F = 3.19$	1	18	0.0910
Linear model	Interaction effect	$F = 1.39$	1	18	0.2531
Optic Lobes: Logit ~ Treatment + Amt_5HT					
Linear model	*Treatment* fixed effect	$F = 0.17$	1	19	0.6875
Linear model	*Amt_5HT* fixed effect	$F = 3.12$	1	19	0.0932
Spearman's Rank	*Amt_5HT* vs *logit*	$\rho = 0.119$	N/A	N/A	0.596

4.4.2.2.2 Covariance and correlation between CNS 5-HT and gregariousness

Details of the statistical analysis for covariances and correlations between behavioural phase state and 5-HT across the regions of the CNS are shown in Table 4.6. Some samples failed in the processing steps in HPLC sample preparations, so sample sizes varied between regions and treatments for the purposes of correlation analyses between behavioural phase state and 5-HT (thoracic ganglia: Saline $N = 18$, AMTP $N = 22$; midbrain: Saline $N = 22$, AMTP $N = 22$; optic lobes: Saline $N = 13$, AMTP $N = 9$). An ANCOVA and Spearman's Rank correlation test provided no evidence for covariance or correlation between behavioural gregariousness and 5-HT in the thoracic ganglia (ANCOVA $p = 0.6484$; Spearman's Rank $p = 0.531$, Figure 4.10A), midbrain (ANCOVA $p = 0.3989$; Spearman's Rank $p = 0.252$, Figure 4.10B), and very little evidence in the optic lobes (ANCOVA $p = 0.0932$; Spearman's Rank $p = 0.596$, Figure

4.10C). There was no evidence for any interactions between treatment and covariance between logits and 5-HT content in any of the regions (*Treatment:Amt_5HT*: ganglia $p = 0.3051$; midbrain $p = 0.6298$; optic lobes $p = 0.2531$).

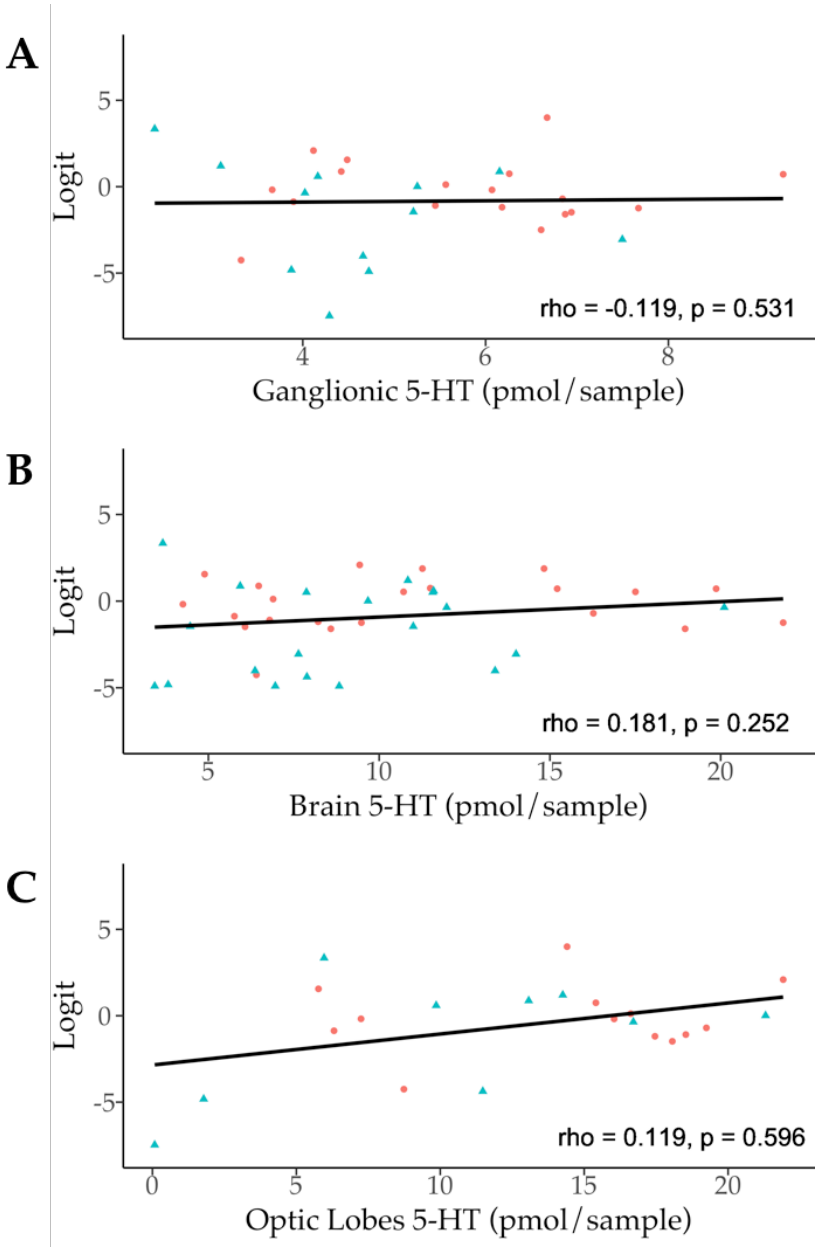


Figure 4.10: Experiment AMTP5: No evidence for a correlation between CNS 5-HT concentrations and behavioural gregariousness (logit-transformed P_{greg}) across solitarious nymphs injected with AMTP (blue triangles) or saline control (orange circles). (A) Individual observations of 5-HT in the thoracic ganglia against behavioural gregariousness, with linear regression fit. Rho (ρ)- and p-values provided from a Spearman's Rank correlation test (Saline $N = 18$, AMTP $N = 22$). (B) and (C) as (A) except with midbrain (Saline $N = 22$, AMTP $N = 22$) and optic lobe (Saline $N = 13$, AMTP $N = 9$) 5-HT concentrations respectively.

4.4.3 Weak evidence for solitarising effect of 5-HT

To understand the effects of 5-HT injections on behavioural gregarisation in solitarious nymphs, four treatment groups of animals were either uncrowded with saline (0 h Saline, $N = 21$), uncrowded with 5-HT (0 h 5-HT, $N = 20$), crowded with saline (2 h Saline, $N = 21$) or crowded with 5-HT (2 h 5-HT, $N = 20$; Figure 4.4).

Details of statistical tests are shown in Table 4.7. There were no changes in median P_{greg} values between the 0 h and 2 h crowded groups (Figure 4.11). Saline-injected locusts of both crowding groups displayed higher median P_{greg} values than those injected with 5-HT (0.15 higher in 0 h Saline than in 0 h 5-HT group; 0.29 higher in 2 h Saline than in 2 h 5-HT group). A linear regression model provided no evidence for an effect of 2 h crowding on behavioural gregariousness ($p = 0.8091$) and there was only weak evidence for an effect of 5-HT injections on behavioural gregariousness ($p = 0.0310$). There was also no evidence for an interaction effect between crowding and 5-HT treatment on behavioural gregariousness ($p = 0.7512$). Analysis of regression coefficients provided weak evidence that the 5-HT injections resulted in less gregarious-like behaviour across both crowding groups (5-HT vs Saline: $p = 0.0310$), and no evidence that crowding changes the behavioural gregariousness in either treatment group (2 h vs 0 h: $p = 0.8091$).

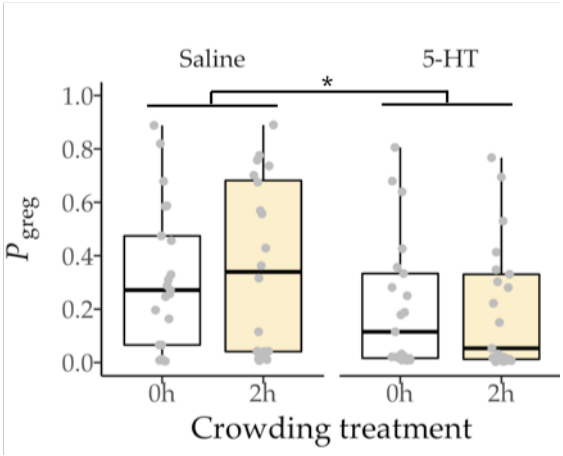


Figure 4.11: Weak evidence for a solitarising effect of central 5-HT injections on behavioural phase state (P_{greg}) in solitarious compared with saline control animals. Animals crowded for 2 h (orange bars) only showed a weak P_{greg} increase compared with uncrowded controls (white bars) in the saline-injected group (0 h saline $N = 21$, 0 h 5-HT $N = 20$) and a decrease in the 5-HT injected group (2 h saline $N = 21$, 2 h 5-HT $N = 20$). Each point represents one observation. Star indicates evidence based on the treatment factor in a linear model.

Table 4.7 Statistical tests of the effect of 5-HT injections and crowding treatment on logit values (behavioural gregariousness) in solitarious nymphs. Df = degrees of freedom. Linear model analysis: type 2 ANOVA.

Test	Comparison	Statistic	Df between groups	Df within groups	*p*-value
Logit ~ Time point + Treatment + Time point:Treatment					
Linear model	*Time point* fixed effect	$F = 0.06$	1	79	0.8102
Linear model	*Treatment* fixed effect	$F = 4.77$	1	79	0.0320
Linear model	*Time point:Treatment* interaction	$F = 0.10$	1	79	0.7512
Logit ~ Time point + Treatment					
Linear model	*Time point* fixed effect	$F = 130.09$	1	80	0.8091
Linear model	*Treatment* fixed effect	$F = 2.95$	1	80	0.0310
Regression coefficient	2 h vs 0 h	$t = -0.24$	1	N/A	0.8091
Regression coefficient	5-HT vs Saline	$t = -2.20$	1	N/A	0.0310

4.4.4 Weak evidence for monoamine-depletion and behavioural effects of reserpine

The aim of these experiments was to establish a monoamine-depletion protocol in *S. gregaria* that would help to reassess the roles of monoamines in behavioural phase change, given the limited efficacy of AMTP in reducing 5-HT content (Section 4.4.2). This section describes the behavioural and neurochemical effects of injecting gregarious and solitarious 5th instar locusts with the vehicle DMSO and the *vat1* inhibitor reserpine.

4.4.4.1 DMSO controls vs saline controls

To understand the behavioural effect of DMSO injections on long-term gregarious nymphs over time, gregarious animals were injected with either saline ($N = 22$) or DMSO ($N = 19$) and kept in the crowd for 48 h (Figure 4.5A). Details of the statistical analysis are shown in Table 4.8. At 24 h after injections, the Saline group had a higher median P_{greg} than the DMSO group by 0.32 (Figure 4.12). After 48 h, the median P_{greg} value of the saline group had decreased by 0.44 compared to the 24 h time point, and the DMSO group median had also dropped by 0.26 (Figure 4.12). A linear regression model provided very little evidence for a real effect of time point after injection on behavioural gregariousness ($p = 0.0920$) and no evidence for a real effect of DMSO on behavioural gregariousness ($p = 0.2033$). There was no evidence for an interaction effect between

time point after injection and DMSO treatment on behavioural gregariousness (p = 0.8258).

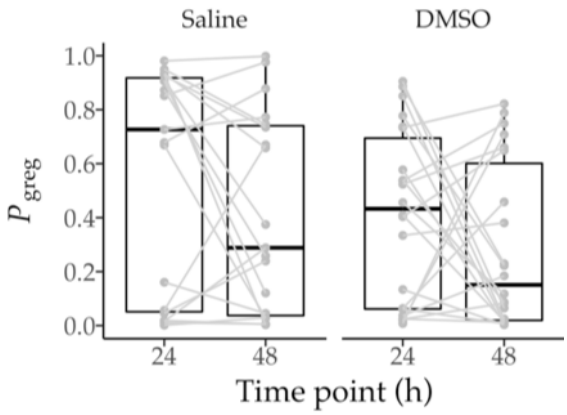


Figure 4.12: No evidence for an effect of DMSO (N = 19) injections on behavioural gregariousness in gregarious 5th instar locusts compared with saline control (N = 22). Comparison of P_{greg} values over time points after injections of either DMSO or control saline in gregarious nymphs. Each point represents one observation and lines connect observations over time.

Table 4.8: Statistical tests of the effect of DMSO injections and time point on logit values (behavioural gregariousness) in gregarious nymphs. Df = degrees of freedom. Linear model analysis: type 2 ANOVA.

Test	Comparison	Statistic	Df between groups	Df within groups	*p*-value
Logit ~ Time point + Treatment + Time point:Treatment + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	F = 2.90	1	39	0.0960
Linear mixed model	*Treatment* fixed effect	F = 1.67	1	39	0.2033
Linear mixed model	*Time point:Treatment* interaction	F = 0.05	1	39	0.8258
Logit ~ Time point + Treatment + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	F = 2.98	2	40	0.0920
Linear mixed model	*Treatment* fixed effect	F = 1.67	1	40	0.2033

4.4.4.2 Weak effect of reserpine injections on behavioural gregariousness

To understand the behavioural effect of two doses of reserpine on gregarious behaviour, gregarious nymphs were injected with 100 µg/g (N = 27) or 50 µg/g (N = 28) reserpine,

or DMSO control (N = 28) and observed 24 h after injection (Figure 4.5B). The drug-injected locusts showed clearly impaired movement in the hind legs and an inability to climb in the behavioural arena, but they still attempted to walk. The median P_{greg} of the 50 µg/g group dropped by 0.23 compared with the DMSO control group whereas the 100 µg/g group showed no change (Figure 4.13A). However, the variation in the drug groups was much larger than in the DMSO control group (Figure 4.13A). Both doses of reserpine led to total depletion of 5-HT (Figure 4.13B) and DA (Figure 4.13C) compared with the DMSO control group (100 µg/g: N = 18; 50 µg/g: N = 16; DMSO control: N = 15).

All details of statistical treatments are shown in Table 4.9. A linear regression model provided very little evidence for a real effect of reserpine injections on behavioural gregariousness (p = 0.0630). Kruskal-Wallis tests provided very strong evidence that reserpine injections depleted both amines (p < 0.0001 for both monoamines).

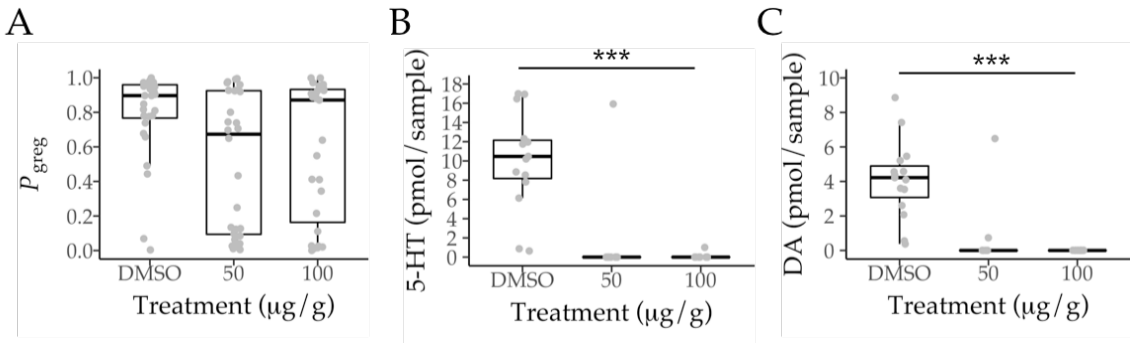


Figure 4.13: Very little evidence for ganglionic monoamine depletion effects on behavioural gregariousness or ganglionic 5-HT concentrations compared to saline controls. (A) P_{greg} values of gregarious nymphs 24 h after injection with either 100 µg/g (N = 27) or 50 µg/g (N = 28) reserpine, or DMSO control (N = 28). (B) As (A) except showing ganglionic concentrations of 5-HT between the three treatments (100 µg/g: N = 18; 50 µg/g: N = 16: DMSO control: N = 15). (C) As (B) except showing ganglionic concentrations of DA between the three treatments (100 µg/g: N = 18; 50 µg/g: N = 16; DMSO control: N = 15). Each point represents one observation. Asterisks show evidence for a group effect in a Kruskal-Wallis test (Table 2.4).

Table 4.9: Statistical tests of the effect of reserpine doses on logit values (behavioural gregariousness), amounts of 5-HT (Amt_5HT) and amounts of DA (Amt_DA) in the thoracic ganglia of gregarious nymphs. Df = degrees of freedom. Linear model analysis: type 2 ANOVA.

Test	Comparison	Statistic	Df between groups	Df within groups	*p*-value
		Logit ~ Treatment			
Linear model	*Treatment* fixed effect	$F = 2.86$	2	80	0.0630
		Amt_5HT ~ Treatment			
Kruskal-Wallis test	*Treatment* effect	$\chi^2 = 38$	2	N/A	< 0.0001
		Amt_DA ~ Treatment			
Kruskal-Wallis test	*Treatment* effect	$\chi^2 = 38$	2	N/A	< 0.0001

4.4.4.3 Very little evidence for the effect of reserpine injections on behavioural gregarisation

To investigate the effect of reserpine dose on behavioural gregarisation and monoamine concentrations, solitarious were injected with either 50 µg/g reserpine (reserpine group; $N = 21$) or DMSO control (DMSO group; $N = 15$), observed 24 h later and crowded for 4 h before final observations (Figure 4.5C). Animals injected with control DMSO and crowded for 4 h increased their median P_{greg} values by 0.45 compared with 0 h crowding (Figure 4.14A). However, animals injected with 50 µg/g reserpine did not change their median P_{greg} value after 4 h crowding (Figure 4.14A). DMSO-injected locusts at both crowding time points had a slightly higher median P_{greg} value than those injected with reserpine at 0 h (difference of 0.10) and a much higher median after 4 h crowding (difference of 0.51).

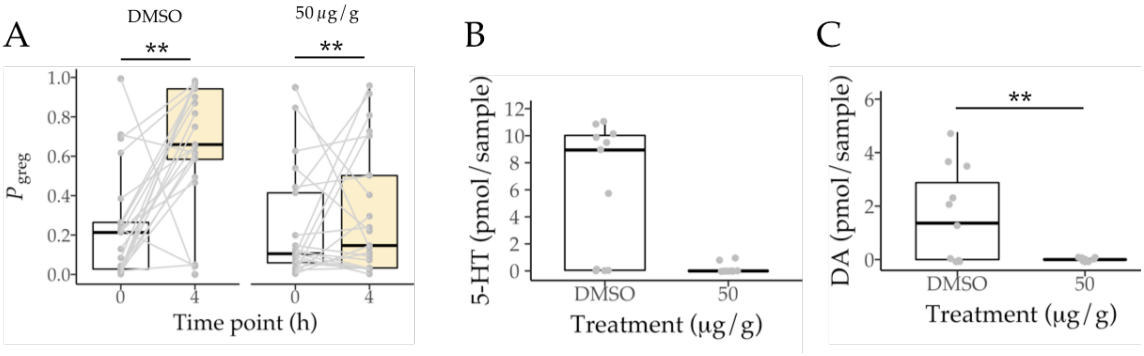


Figure 4.14: Very little evidence for ganglionic monoamine depletion effects on crowd-induced behavioural gregarisation or ganglionic 5-HT concentrations compared to saline controls. (A) P_{greg} values of 5th instar solitarious locusts 24 h after injection with either 50 µg/g reserpine (N = 21), or DMSO control (N = 21) at 0 h and 4 h crowding (orange boxes). (B) As (A) except showing ganglionic concentrations of 5-HT between the two treatments (50 µg/g: N = 10; DMSO control: N = 11) after the 4 h crowding time point. (C) As (B) except showing ganglionic concentrations of DA between the two treatments (50 µg/g: N = 10; DMSO control: N = 11). Each point represents one observation and lines connect observations over time. Asterisks show evidence for a crowding effect based on an additive linear mixed model (A) and a treatment effect (C; Table 2.4).

All statistical details are in Table 4.10. A linear regression model provided moderate evidence for a real effect of 4 h crowding on behavioural gregariousness in this study (p = 0.0053) and there was very little evidence for a real effect of reserpine injections on behavioural gregariousness (p = 0.0501). There was very little evidence for an interaction effect between crowding and reserpine treatment on behavioural gregariousness (p = 0.0567). Analysis of regression coefficients provided very little evidence that the reserpine injections resulted in less gregarious-like behaviour across both crowding groups (reserpine vs DMSO; p = 0.0571), and moderate evidence that 4 h crowding increases the behavioural gregariousness in each treatment group (4 h vs 0 h; p = 0.0080). As a trend for an interaction was present, analysis of the interaction regression coefficients in the interaction model provided very little evidence that the reserpine group gregarised less than the DMSO group (4 h-0 h reserpine vs 4 h-0 h DMSO; p = 0.0639).

Table 4.10: Statistical tests of the effect of reserpine injections and crowding time point on logit values (behavioural gregariousness), amounts of 5-HT (Amt_5HT) and amounts of DA (Amt_DA) in the thoracic ganglia in solitarious nymphs. Also compares the effect of reserpine on logit values between gregarious (Greg) and crowded solitarious (Sol) nymphs. Df = degrees of freedom. Linear model analysis: type 2 ANOVA.

Test	Comparison	Statistic	Df between groups	Df within groups	*p*-value
Logit ~ Time point + Treatment +Time point:Treatment + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	$\chi^2 = 8.02$	1	40	0.0046
Linear mixed model	*Treatment* fixed effect	$\chi^2 = 3.96$	1	40	0.0465
Linear mixed model	*Time point:Treatment* interaction effect	$\chi^2 = 3.63$	1	40	0.0567
Regression coefficient	4 h vs 0 h: reserpine vs DMSO	$t = -1.91$	1	N/A	0.0639
Logit ~ Time point + Treatment + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	$\chi^2 = 3.84$	1	41	0.0053
Linear mixed model	*Treatment* fixed effect	$\chi^2 = 7.76$	1	41	0.0501
Regression coefficient	4 h vs 0 h	$t = 2.79$	1	N/A	0.0080
Regression coefficient	50 µg/g vs DMSO	$t = -1.96$	1	N/A	0.0571
Amt_5HT ~ Treatment					
Mann-Whitney U test	Reserpine vs DMSO	$W = 147$	1	N/A	0.0597
Amt_DA ~ Treatment					
Mann-Whitney U test	Reserpine vs DMSO	$W = 116$	1	N/A	0.0065
Logit ~ Phase + Treatment + Phase:Treatment					
Linear model	*Phase* fixed effect	$F = 5.84$	1	94	0.0176
Linear model	*Treatment* fixed effect	$F = 13.17$	1	94	0.0005
Linear model	*Phase:Treatment interaction*	$F = 0.09$	1	94	0.7618
Logit ~ Phase + Treatment					
Linear model	*Phase* fixed effect	$F = 5.89$	1	95	0.0171
Linear model	*Treatment* fixed effect	$F = 13.30$	1	95	0.0004
Regression coefficient	Greg vs Sol	$t = 2.43$	1	N/A	0.0171

Test	Comparison	Statistic	Df between groups	Df within groups	*p*-value
Regression coefficient	Reserpine vs DMSO	$t = -3.65$	1	N/A	0.0004
Mann-Whitney U test	Reserpine: Greg vs 4 h Sol	$W = 380$	N/A	N/A	0.0841

Large variation was observed in 5-HT and DA ganglionic content (5-HT: Figure 4.14B; DA: Figure 4.14C respectively). Both doses of reserpine resulted in total depletion of both monoamines compared with the DMSO control group (Figure 4.14B and C). Due to the variation in the DMSO group, Mann-Whitney U tests provided very little evidence for a real treatment effect on ganglionic 5-HT concentration ($p = 0.0597$) and moderate evidence for a real treatment effect on ganglionic DA concentrations ($p = 0.0065$).

To understand if reserpine affects the behaviour of crowded solitarious locusts proportionally more than long-term gregarious locusts (i.e. understand the role of monoamines in gregarious behaviour acquired from short-term vs long-term crowd stimuli), the behavioural gregariousness of the 4 h crowded solitarious 50 µg/g reserpine ($N = 21$) and DMSO control groups ($N = 21$; Figure 4.14A) were analysed with that of the long-term gregarious 50 µg/g reserpine ($N = 28$) and DMSO control groups ($N = 21$; Figure 4.13A). The median P_{greg} value of control DMSO crowded solitarious was lower than that of the long-term gregarious group by 0.24, and the median P_{greg} value of the reserpine crowded solitarious was lower than that of the long-term gregarious group by 0.51, though the gregarious group shows large behavioural variation (crowded solitarious in Figure 4.14A; long-term gregarious in Figure 4.13A).

All statistical details are shown in Table 4.10. A linear regression model provided no evidence for an interaction between the phase and treatment ($p = 0.7618$). The model provided strong evidence for a real effect of reserpine treatment on behavioural gregariousness ($p = 0.0004$) and weak evidence for an effect of phase ($p = 0.0171$). Analysis of regression coefficients provided very little evidence that long-term gregarious locusts showed more gregarious-like behaviour than 4 h crowded locusts across both drug treatment groups (Greg vs Sol: $p = 0.0571$), and strong evidence that 50 µg/g reserpine decreased the behavioural gregariousness in each phase (reserpine vs

DMSO: $p = 0.0004$). A Mann-Whitney U test between the gregarious and solitarious reserpine groups also provided very little evidence for a phase effect on behavioural gregariousness when lacking monoamines ($p = 0.0841$).

4.5 Discussion

4.5.1 Inconclusive effects of blocking DA receptors with fluphenazine

The experiments investigating the effect of fluphenazine on behavioural gregariousness over time were confounded by an unexpected change in the behavioural phase state of both treatment groups during the initial observations (Figure 4.6). This confounding effect thus masked any potential effect of fluphenazine injections on behavioural phase maintenance or phase transition.

Since solitarious locusts apparently gregarised, and gregarious locusts apparently solitarised, it is unlikely that the changes were due to physiological or pathological reactions to repeated injections and handling, or an effect of age. One potential explanation for this behavioural change in the groups is a familiarity response to repeated assays in the behavioural arena. Insects react to familiar stimuli differently to novel stimuli - such as bees behaving differently towards familiar vs unfamiliar conspecifics (Kukuk, 1992) – and contextual familiarity is used for navigation in foraging insect species such as ants (Baddeley et al., 2012; Collett et al., 2013; Wystrach et al., 2011). Novelty may have a distinct salience for locusts of each phase. A behaviourally active gregarious locust may lose its activity (appear to solitarise) in response to familiar surroundings, whereas an inactive solitarious locust may become more active as it becomes familiar with the initially novel environment. This hypothesis has major implications for repeated measures behavioural experiments carried out in *S. gregaria*, so it was tested explicitly in Chapter 5 of this thesis.

Due to the confound encountered in this experiment, the experimental design requires adjustments to minimise this occurrence in future. The long progression of the time points was initially hoped to minimise familiarity responses to the behavioural assay encountered previously in this PhD project (not shown). This was unsuccessful, and implies that future experiments should minimise the experimental lengths so as to avoid confounding effects of repeated exposure to handling, injections and the behavioural arena. Thus, future experiments investigating this question would best use shorter time spans, with fewer behavioural observations for best effects.

4.5.2 Limited 5-HT depletion and behavioural effects of AMTP injections

Chapter 3 of this thesis cast doubt onto the role of 5-HT in behavioural gregarisation in *S. gregaria* by finding neither an increase of ganglionic 5-HT content with gregarisation nor correlations between ganglionic 5-HT and measures of gregarisation, conflicting with previous work (Anstey, 2012; Anstey et al., 2009; Rogers et al., 2004). However, the depletion of 5-HT using AMTP in previous work was presented as further evidence for a role of 5-HT in behavioural gregarisation (Anstey, 2012; Anstey et al., 2009). AMTP was thus employed here to re-examine the role of 5-HT in behavioural gregarisation and re-gregarisation of 5th instar solitarious locusts. This section of the discussion breaks down the findings and discusses their implications for the role of 5-HT in behavioural phase change.

4.5.2.1 Very little evidence for AMTP effect on CNS 5-HT content and behavioural gregarisation

The initial experiments in this chapter (AMTP1-4) repeated and extended the AMTP injection protocol of Anstey et al. (2009). The extension was an attempt to maximise 5-HT depletion, because previous work found no effect of AMTP injections on behavioural solitarisation using the original protocol (Stettin, 2014). In the present work, the injection protocol's efficacy was not confirmed using HPLC. The behavioural results from experiments AMTP1 (Figure 4.7) and pooled AMTP3 & AMTP4 (Figure 4.8) indicated that even this extended protocol was insufficient to affect the behavioural gregariousness of solitarious locusts undergoing 4 h crowding or tickling, thus disagreeing with the published results (Anstey et al., 2009). The previous study only reported the effect of AMTP injections on tickle-induced gregarisation, with a sample size of just 10 saline controls and 13 AMTP-injected locusts. However, a PhD thesis from the same group found no evidence for an effect of AMTP injections on crowd-induced gregarisation, with larger sample sizes of 27 saline-injected and 31 AMTP-injected (Anstey, 2012). Compared with the published tickling sample sizes, experiments reported in this chapter had larger sample sizes of 23 in the Saline group and 30 in the AMTP group. The work of Anstey (2012) along with the results from this chapter together imply that the effect of AMTP on crowd-induced behavioural gregarisation is considerably more modest than reported – or even absent. These results left two options for interpretation: first, that the AMTP is efficacious in depleting 5-HT and that behavioural gregarisation is independent of 5-HT action, or second, that the injection

regime of AMTP failed to deplete 5-HT in the CNS. This question was therefore addressed by measuring 5-HT content using HPLC quantification following AMTP treatment.

Experiment AMTP5 aimed to replicate both the behavioural results of the published experiment (Anstey et al., 2009) and also the reported HPLC confirmation of the efficacy of 5-HT depletion (Anstey, 2012). AMTP injections again had only a weak effect on behavioural gregariousness, and resulted in less than 30% depletion in CNS 5-HT amounts, indicating that the injection regime is not efficacious (Figure 4.9B). In the present work there was no correlation between gregariousness and 5HT content of the CNS (Figure 10). This echoes the conclusions of Chapter 3, that 5-HT does not have a role in behavioural phase change.

There are two minor caveats to consider in comparing the present results to those of Anstey and colleagues. First, the logistic regression model used in this thesis to generate P_{greg} estimates was not identical to the one used previously, for example lacking the 'grooming' behavioural factor (Anstey, 2012) and using an automated behavioural quantification step (Stettin, 2014). These differences, however, have no important bearing on the ability of the model to correctly estimate an animal's behavioural phase state. Second, the HPLC quantification did not use an internal DHBA calibration that would nominally permit correction for standard and sample degradation. Nevertheless, because the treatment groups were always run in the same sequence in each HPLC session, and sessions were always comparable in duration, potential degradation effects will have been well managed and thus negligible.

One important observation in these experiments, and in the thesis as a whole, is that the median P_{greg} of solitarious locusts in the Leicester colony after 4 h tickling or crowding is rarely higher than 0.6, compared with the > 0.8 median P_{greg} values reported previously (Anstey, 2012; Anstey et al., 2009; Rogers et al., 2014). This suggests that solitarious locusts used in this thesis are not fully gregarious by this time point compared with the animals used in the previous group. This is likely down to inbreeding effects in lab strains as discussed in Chapter 3 (Berthier et al., 2010). While it could be argued that this potential strain difference reduces effect sizes for drug actions, Chapter 3 demonstrated

that the strain difference does not explain a lack of a role of 5-HT in gregarisation. The fact that behaviour does change with gregarising stimuli in the Leicester strain, albeit not completely, indicates that this strain is suitable for these analyses, and that the AMTP procedures used by Anstey et al. (2009) are ineffective.

4.5.2.2 No AMTP effect on regregarisation

In light of the limited efficacy of AMTP discussed in Section 4.4.2, the finding that AMTP injections had no effect on regregarisation of gregarious locusts isolated for 24 h requires cautious interpretation (Figure 4.7B). One consideration of this experiment is that the median P_{greg} values in this experiment never fell below 0.43 (roughly the same as a 4 h crowded solitarious locust). Unlike behavioural gregarisation, solitarisation in *S. gregaria* is reported to require multiple days of isolation to have a robust decrease in behavioural gregariousness (Roessingh and Simpson, 1994). The reason for selecting a 24 h isolation period was because it appeared to show dramatic neurochemical changes in Rogers et al. (2004), unlike the longer-term isolation periods. With longer isolation periods, it could have been argued that the gregarious nymphs had fully solitarised to the point of ceasing the potential of "regregarisation" rather than simply "gregarisation". To extend this experiment, it will be interesting to compare regregarisation for animals treated with a set of isolation periods, and use HPLC to quantify neuromodulators over these processes.

4.5.3 Weak effect of 5-HT on behavioural gregariousness

The weak evidence for a 5-HT-induced reduction in behavioural gregariousness was unexpected considering the debate in the literature regarding the efficacy of thoracic 5-HT injections in *S. gregaria*. Both central injections into the haemocoel and topical application of 5-HT to the thoracic ganglia were reported to increase the behavioural gregariousness of 5th instar solitarious locusts (Anstey, 2012), though only the topical application of 5-HT to the thoracic ganglia combined with an enzymatic desheathing of the ganglia was published from this group (Anstey, 2012; Anstey et al., 2009). This became the subject of discussion in later work from another group which found no effect of central injections of 5-HT (Tanaka and Nishide, 2013). Anstey and colleagues subsequently published a meta-analysis of their existing data (Rogers et al., 2014), which confirmed (albeit with highly unbalanced sample sizes) the lack of effect of central injections of 5-HT on behavioural gregariousness in the absence of ganglionic

desheathing. The outcome thus appeared to be that 5-HT is unable to cross the ganglionic blood-brain-barrier. Other reports, however, provide evidence for behavioural effects of DA injections on behavioural gregariousness in adult *S. gregaria* and *L. migratoria* nymphs (Alessi et al., 2014; Ma et al., 2011), casting doubt on this assertion that the CNS is protected from injections of monoamines into the thoracic haemocoel in the absence of enzymatic pretreatments. Elevated levels of 5-HT in the haemolymph are rapidly cleared via excretion and neuronal reuptake in *Periplaneta americana* (Sloley and Downer, 1990) and *Rhodnius prolixus*, which shows clearance from 20 nM to approximately 7 nM in 20 min (Lange et al., 1989). Thus, it remains remote that 5-HT injections can reach the CNS before large-scale clearance from the haemolymph. Observed effects on behaviour in my experiment and in other studies using such injections may reflect secondary effects of 5-HT or other monoamines on peripheral tissues such as the gastro-intestinal system, which influence the health and activity of the locust in ways that are unrelated to phase state. To fully answer the question of whether 5-HT injections increase 5-HT concentrations in the CNS, HPLC quantification is required at the same time as injections, which was not included in this thesis due to time constraints.

4.5.4 Strong effect of reserpine on behavioural gregariousness and CNS monoamine content

The drug reserpine is a nonspecific vesicular monoamine transporter inhibitor which depletes 5-HT, DA and OA in the CNS. It has previously been employed to investigate the roles of monoamines in the behaviour of insects including the cockroach *Periplaneta americana*, the butterfly *Inachis io* and cricket *Gryllus bimaculatus* (Sloley and Owen, 1982; Stevenson et al., 2000; Vieira et al., 2007). Given that this is the first reported behavioural study of *S. gregaria* employing reserpine, it was thus necessary to establish a protocol that would effectively deplete monoamines without causing high mortality.

4.5.4.1 Variable effects of monoamine depletion in behavioural gregariousness in gregarious locusts

Both doses of reserpine consistently achieved total depletion of DA and 5-HT in the thoracic ganglia of gregarious locusts. OA was not quantified in these samples, though it is plausible that it was also depleted alongside the other monoamines as indicated in Robertson (1976). There was large behavioural variation 24 h after injection with

reserpine, with some individuals showing high P_{greg} values in spite of containing almost no monoamines in their thoracic ganglia (Figure 4.13). This suggests that, at most, depletion of DA and 5-HT only partially reduces behavioural gregariousness in long-term gregarious 5th instar locusts. Reserpine-treated gregarious locusts exhibited lethargy, an inability to climb, and a lack of movement in the hind legs. However, they still walked in the behavioural arena. These observations are similar to those made in previous work, where crickets were lethargic after a dose of reserpine but still carried out stereotypical behaviours such as ritualistic fighting (Stevenson et al., 2000). The doses used in the present study were based on effective doses in previous work (Sloley and Owen, 1982; Stevenson et al., 2000; Vieira et al., 2007), but recent work in the University of Leicester group reveals that doses 1000 times smaller are still sufficient to achieve total monoamine depletion (De Keyser, R., unpublished, 2017). This experiment established a monoamine depletion protocol and suggests that CNS monoamines play only a small role in the expression of gregarious behaviour in long-term gregarious 5th instar locusts. This appears to support results from Rogers et al. (2004), who found no differences in CNS 5-HT, DA or OA content between long-term gregarious and solitarious nymphs, and suggested that monoamine action is most important in behavioural phase transition, rather than the maintenance of phase behaviour. However, they did find larger amounts of 5-HT and DA in solitarious adults than in gregarious. Since this would create the expectation that solitarious behaviour could be acquired only with increases to monoamines, the lack of evidence for a monoamine depletion effect on behavioural gregariousness may support this finding. However, the behavioural variation shown in the drug-injected groups makes such an interpretation difficult.

4.5.4.2 Behavioural gregarisation reduced by monoamine depletion in solitarious locusts

Though only very weakly supported statistically, reserpine-injected locusts did not gregarise compared with DMSO-injected controls (Figure 4.5C), suggesting that the presence of monoamines is required for rapid behavioural gregarisation, which would agree with the consensus (Cullen et al., 2017).

The finding that reserpine injections reduced the behavioural gregariousness of both 4 h crowded solitarious and long-term gregarious nymphs equally is intriguing as it suggests

that the expression of gregarious behaviour from 4 h crowding is mechanistically similar to the expression of gregarious behaviour induced by generations of crowding (i.e. the maintenance of gregarious behaviour). In other words, both phases require the presence of monoamines to behave gregariously in the arena. These findings provide little support for the hypothesis that labile neuronal mechanisms modulated by monoamines are required for the rapid behavioural plasticity in short-term behavioural gregarisation, but that the long-term expression of gregarious phase behaviours does not require monoamine function (Anstey et al., 2009; Ott et al., 2012; Rogers et al., 2004).

These results expand on the previous experiment comparing reserpine-injected gregarious nymphs with controls. The seeming contradictory claims made in each section are likely due to the high behavioural variation shown by the reserpine-injected gregarious locusts, and complicate the interpretation of the effect of monoamines on the expression of gregarious behaviour. One reason for this may be that the logistic regression model used to calculate P_{greg} in this thesis is strongly influenced by one exploration factor in the behavioural arena: fractal dimension of the movement trajectory. Exploratory behaviour is presumably facilitated by unimpaired locomotion, so this assay may have difficulty in distinguishing between changes in behavioural gregariousness and changes in locomotion and activity; this has been the subject of considerable debate previously (Rogers et al., 2014; Tanaka and Nishide, 2013). The changes in P_{greg} caused by monoamine depletion may therefore be due at least in part to changes in locomotion, rather than phase state *per se*. Future experiments should seek to analyse additional non-locomotory behaviours that might more clearly define the (lack of) effect of monoamine depletion on behavioural phase state.

4.5.5 Conclusions

Now that the role of 5-HT in behavioural gregarisation is in question, the establishment of a robust monoamine depletion protocol using reserpine in this thesis provides a useful tool for the re-examination of the importance of monoamines in the expression of gregarious behaviour, and provides no evidence for a mechanistic difference between behavioural gregariousness acquired from short-term crowding and that expressed in long-term gregarious animals. Furthermore, these experiments using non-specific depletion of 5-HT, DA and (most likely) OA also highlight the importance of

distinguishing locomotory effects of pharmacological interventions from true effects on behavioural gregariousness in the Desert Locust.

5 Phase-specific responses to repeated stimulus exposure in the Desert Locust

5.1 Summary

Experiments from Chapter 4 using a behavioural assay suggested potential phase-specific changes in behaviour as a result of repeated arena assays. Thus, I aimed to investigate this effect further, and identify potential neophobic or neophilic tendencies of either phase in this arena and another behavioural paradigm. First, I investigated the effects of repeated injection, handling and repeated arena assays on apparent behavioural gregariousness. Gregarious and solitarious 5th instar locusts were treated with either one behavioural assay (at 96 h), two behavioural assays (at 0 h and 96 h time points), three behavioural assays with daily handling (0 h, 48 h and 96 h), or behavioural assays with daily injections of 5 µL locust saline (0 h, 48 h and 96 h). There was strong evidence for an increase in behavioural gregariousness between the 0 h and 96 h time point in all repeated assay groups in solitarious locusts, but not gregarious locusts. This result implied that repeated assays were sufficient to increase behavioural gregariousness. To assess whether this effect requires a crowd stimulus in the arena, I ran repeated arena assays in 5th instar solitarious and gregarious locusts in the presence or absence of a crowd stimulus in the arena, with time intervals between assays of 2 h, 24 h or 48 h. There was strong evidence for an increase in exploratory behaviour over 2 h intervals, but not in longer intervals. Furthermore, this effect did not require a crowd stimulus. I also investigated a potential familiarisation effect on adult locusts' odour preferences in a Y-maze behavioural choice paradigm, and whether any effect of familiarity differs between phases. Solitarious and gregarious adult locusts were exposed to lemon or vanilla odours for 5 min each day for 3 d and then presented a choice between lemon or vanilla on day 4. Naïve and pre-exposed (familiarised) locusts showed comparable odour preferences. These data indicate a lack of preference for either novel or familiar odours in either phase in *S. gregaria*.

5.2 Introduction

This chapter directly expands upon unexpected results encountered in the fluphenazine experiments of the previous chapter (Section 4.4.1). Those experiments injecting solitarious and gregarious 5th instar Desert Locusts (*S. gregaria*) with locust saline or the drug fluphenazine and then running them through an established arena assay (Roessingh et al., 1993) multiple times increased the behavioural gregariousness of solitarious nymphs and, whereas gregarious locusts showed the opposite trend. These bidirectional behavioural changes in both phases suggested a phase-specific response to an unknown factor, such as daily animal handling, injections, or repeated behavioural assays. One possible explanation for a phase-specific response may be 'familiarity' with the assay procedure (e.g., handling or the arena environment itself), and that such familiarity effects manifest in opposite ways in the two phases.

Habituation and familiarity have been used to assess novelty preferences and learning and memory throughout the animal kingdom. In the context of behaviour, habituation is seen as a decrementing response to repeated stimuli such as the classic gill-withdrawal reflex in *Aplysia californica* (Pinsker et al., 1970). Habituation memory is characterised by its capacity for dishabituation – the loss of habituation by means of a change in the nature of the stimulus (Pinsker et al., 1970; Rankin et al., 2009; Thompson & Spencer, 1966; Thorpe, 1957). Studies of familiarity memory employ novelty preference, habituation and dishabituation behaviours. In infants for example, subjects are given familiarisation pre-trials with a stimulus (e.g. face photograph), and their exploration of the stimulus falls with repeated exposure (i.e. habituates) (Houston-Price and Nakai, 2004; Oakes, 2010; Tighe and Leaton, 2016). When presented with the choice between a novel face and the familiar face, their attention on the novel face is as high as it was with the first presentation of the familiar face (i.e. dishabituation). The higher attention to the novel face is an indication of their novelty preference, though this novelty preference is not present in all animals and all contexts (Houston-Price and Nakai, 2004; Oakes, 2010; Blaser and Heyser, 2015). Thus the difference in response between familiar and novel stimuli infers a familiarity memory, a phenomenon exploited to study

familiarity learning and memory in mammals (Blaser and Heyser, 2015; Souza et al., 2006).

5.2.1 Novelty and familiarity in invertebrates

The reactions of experimental animals to novel and familiar stimuli has been a key tool for investigating object recognition and memory in vertebrate models, but has been studied less in invertebrates (Blaser and Heyser, 2015). Discrimination between familiar and novel stimuli has been most often reported in the context of visual navigation in hymenoptera such as ants, bees and wasps, which use contextual familiarity memory as a key navigational tool. These insects are motivated to identify new landmarks for food sources for their colonies, and have been reported to preferentially explore novel objects in the vicinity of nests and feeders to incorporate them into their spatial representation of the area (Baddeley et al., 2012; Collett et al., 2013; Collett and Collett, 2000; Voss and Zeil, 1998; Wehner et al., 1996; Wystrach et al., 2011; Zhang et al., 1999). Furthermore, object familiarity memories are formed within 1 h exposure to novel stimuli in cockroaches (Durier and Rivault, 2002). Familiarity responses can also be social as well as spatial in eusocial insects, which permits them to recognise nest mates (Kukuk, 1992; Bos and d'Ettorre, 2012). Exploration of novel environments shows intraspecific variation and has been reported to be correlated with intraspecific aggression in ants (Modlmeier et al., 2012; Modlmeier and Foitzik, 2011) and crickets (Kortet and Hedrick, 2007; Wilson et al., 2010).

When considering the precedent for intraspecific differences in neophobia in invertebrates, it is feasible that there are phase-specific differences in neophobia or neophilia in *S. gregaria*. Solitarious and gregarious phases already display opposing behaviours in activity and attraction to conspecifics (Uvarov, 1977). Gregarisation has been suggested to entail habituation to crowd stimuli (Ellis, 1959). For example, solitarious locusts placed into an arena with a crowd stimulus showed high activity and jumping behaviours, possibly in an attempt to retreat from the crowd stimulus, but steadily decreased this escaping behaviour over time in the crowd (Geva et al., 2010). This evidence suggests that locusts may also be able to show differential responses to novel and familiar contexts, which could explain the behavioural changes observed in the previous chapter. Therefore, this chapter aims to reproduce the behavioural changes

observed in Chapter 4, identify if these are present in different phases and time intervals in the established Roessingh (1993) arena, and whether they are reproducible within a separate habituation-dishabituation paradigm modified for this species.

5.2.2 Experimental aims:

1. Investigate the effects of repeated behavioural arena observations, experimenter handling and injection protocols on behavioural gregariousness in gregarious and solitarious 5th instar locusts.
2. Investigate the changes in behavioural exploration over repeated arena assays in the presence or absence of a crowd stimulus in 5th instar solitarious and gregarious locusts, with different time intervals between each behavioural assay.
3. Use a Y-maze arena method to investigate whether adult gregarious locusts prefer to visit novel stimuli and solitarious locusts prefer to visit familiar stimuli.

5.3 Methods

5.3.1 Animal husbandry

All locusts used in this chapter were reared in the long-term gregarious and solitarious lab colonies explained in Chapter 2 of this thesis. Initial experiments were performed on 5th instar nymphs at 2–6 d into the instar stage. Experiments using the Y-maze used adult long-term solitarious and gregarious adults 3-80 d after final moult.

5.3.2 Behavioural arena

Detailed methods for the behavioural assay carried out in this chapter can be found in the General Methods (Section 2.3.2). For behavioural assays using an empty arena only, both chambers of the arena were left empty as controls, and only fractal dimension was measured.

5.3.3 Injections

Details of injections methods and locust saline are described in Section 2.5.

5.3.4 Experiment 1: repeated arena runs vs. handling and injections

To analyse the effect of repeated behavioural assays, experimenter handling and injection procedures on behavioural gregariousness in *S. gregaria*, long-term solitarious and gregarious locusts were assigned into four different treatment groups (shown in Table 5.1 and Figure 5.1). To control for any behavioural effect of days into the 5th larval instar, the first treatment group was designated as age-matched controls (AMC; solitarious N = 25, gregarious N = 8). These animals were run through a single behavioural assay 7 d after moulting (2 d after moult + 96 h). The second group (G1; solitarious N = 19, gregarious N = 9) was intended to control for effects of repeated behavioural assays and was run once 2 d after moulting to the 5th instar (0 h time point), and again 5 d later (96 h). To investigate the effect of animal handling on the behavioural gregariousness, the third group (G2; solitarious N = 19, gregarious N = 10) was run through a behavioural assay at 0 h, 48 h and the 96 h. Each day these locusts were also subjected to handling as used in the injection method (Section 5.3.3), except without presentation of the Hamilton syringe or injection of locust saline. To investigate the effect of injections on behavioural

gregariousness, the final group (G3; solitarious $N = 19$, gregarious $N = 11$) was designed as G2 except with the addition of 5 µL injections of locust saline as in Section 5.3.3. Due to time restrictions, the AMC groups were not run at the same time as the G1-3 groups. In between these treatments, all solitarious locusts were kept in their individual cages and gregarious locusts were kept inside crowding tubs with ~ 30 conspecifics. These holding tubs were placed into holding cages in the gregarious colony to maintain exposure to gregarising stimuli (see Chapter 2 and Chapter 4). Wheat shoots and bran flakes were available to the animals *ad libitum* throughout the holding conditions. All animals were then culled at -10°C.

All behavioural data were analysed in RStudio (v1.0.143, https://www.rstudio.com/) using two separate statistical approaches. The first approach compared groups G1, G2 and G3 only, across 0 h and 96 h only. A linear mixed model was fitted to the logit-transformed P_{greg} values, with the fixed effects *Treatment* and *Time point* and *Treatment:Time point* interaction; and individual *Animal.ID* as a random effect across the time points (e.g. *Logit ~ Time point + Treatment + Time point:Treatment + 1|Animal.ID*). The second statistical approach in this experiment compared the groups G1, G2 and G3 with pristine AMC animals at 96 h in a linear model with just a *Treatment* fixed effect (*Logit ~ Treatment*). For both approaches, linear model fixed effects were analysed by a type 1 analysis of variance (ANOVA), and model coefficients were used to identify trends between factor levels.

Table 5.1: Description of treatments investigating an effect of repeated behavioural arena runs, handling and injection procedures on behavioural gregariousness.

Treatment	Description
AMC	Age-matched; tested at 96h only
G1	Tested at 0h and 96h
G2	Tested at 0h, 48h and 96h; handled daily
G3	Tested at 0h, 48h and 96h; injected with 5 µL locust saline daily

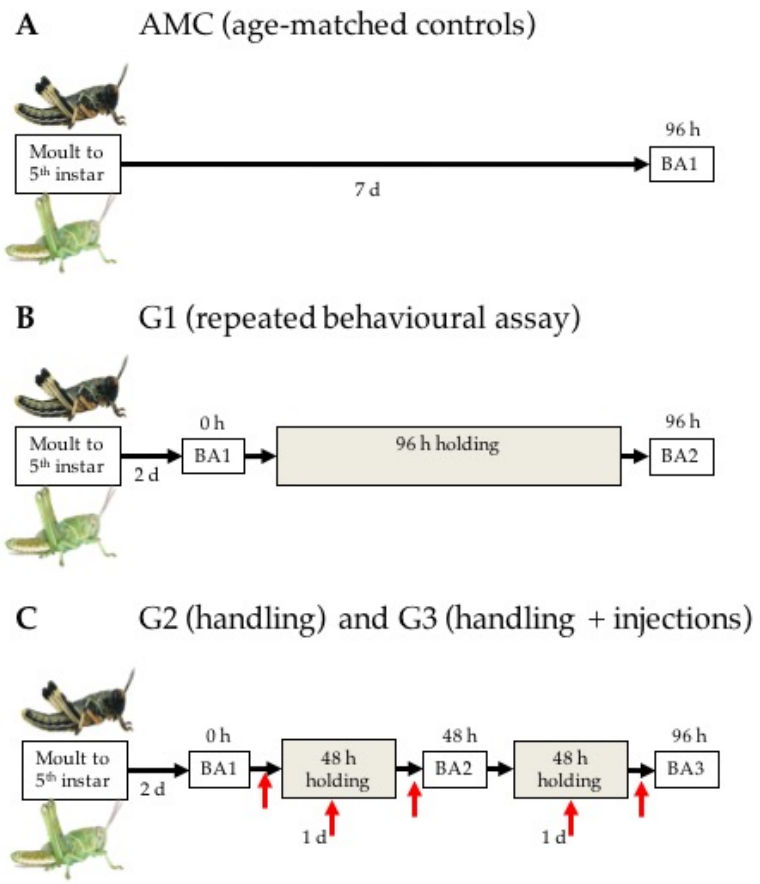


Figure 5.1: Schematic timeline of methods in Experiment 1. (A) AMC group: Solitarious or gregarious 5th instar locusts were run through the behavioural assay (BA) 7 d after moult (time point 96 h relative to the start of the experiments of groups G1-G3). (B) G1 repeated assay group: As AMC, with the difference that locusts were run through the first behavioural assay 2 d after moulting to 5th instar and kept in their phase-specific holding conditions for 96 h until run once more through a behavioural assay (BA2). (C) G2 (handling) and G3 (injection) groups: as G1 with the difference that animals were either injected with 5 µL locust saline under a microscope (G3), or handled under the microscope with no injection (G2) daily (red arrows). As in G1, they were run through a behavioural assay at 0 h (BA1), 48 h (BA2) and 96 h (BA3).

5.3.5 Experiment 2: Changes in exploratory behaviour in response to repeated arena assays and crowd stimuli in the behavioural arena

To investigate whether solitarious and gregarious locusts change their exploratory behaviour over repeated arena assays differently between empty arenas or those with crowd stimuli, all animals were run through the behavioural P_{greg} assay. The behavioural assays carried out in the absence of a crowd stimulus simply used both side chambers of the arena as empty. Solitarious locusts were assayed at 0 h, 2 h and 4 h (2 h intervals) to replicate the schedule in the pharmacological experiments in Chapter 4 (Experiment 2i; Figure 5.2A; with crowd stimulus (Crowd group) N = 26 across time points, without

crowd stimulus (Empty group) $N = 28$ across time points). Other time intervals used for solitarious locusts were 24 h (Experiment 2ii; Figure 5.2B; time points 0, 24, 48, 72 h; Crowd $N = 37$ each time point; Empty $N = 13$ across time points except for 72 h with 8) and 48 h (Experiment 2iii; Figure 5.2C; 0, 48 and 96 h; Crowd $N = 16$ each time point; Empty $N = 33$ across time points except for 96 h with 8). These intervals were based on time points within the 48 h used in Experiment 1 and the earlier fluphenazine experiments in Chapter 4. As gregarious locusts showed no evidence for behavioural changes over 96 h or 48 h time points in Experiment 1, they were run through behavioural assays with just one time interval of 24 h (Experiment 2iv; Figure 5.2B; Crowd $N = 28$ across time points; Empty $N = 26$ across time points). Some animals did not finish the full 72 or 96 h time course in the solitarious locust cohorts due to technical issues. The experiments without the crowd stimulus were run ~ 1 month after the experiments with the crowd stimulus.

The fractal dimension of the trajectory of the locust in the arena served as a metric for exploratory behaviour. The Empty and Crowd groups of each time interval were jointly analysed in a linear mixed model with *Time point*, *CrowdStim* and the *Time point*:*CrowdStim* interaction as fixed effects and individual *Animal.ID* as a random effect across the time points (*Fractal Dimension ~ Time point + CrowdStim + Time point*:*CrowdStim* + 1|*Animal.ID*). To investigate an effect of the crowd stimulus on exploratory behaviour across phases in their first time in the behavioural arena, all data from the 0 h time point of Experiment 2 were jointly analysed in a linear model with *CrowdStim, Phase* and their interaction as fixed effects (*Fractal Dimension ~ Phase + CrowdStim + Phase*:*CrowdStim*).

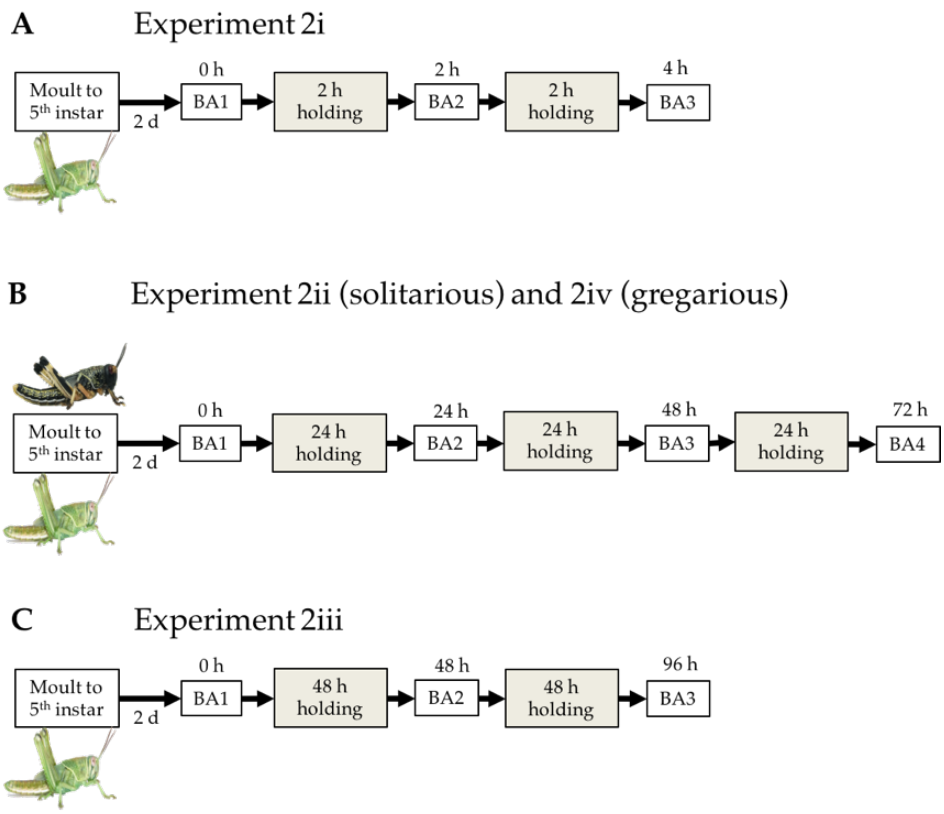


Figure 5.2: Schematic timeline of methods in Experiment 2. (A) Experiment 2i: Solitarious 5th instar locusts were run through the behavioural assay (BA) 2 d after moult (time point 0 h). One group were run through a behavioural assay containing a crowd stimulus in one of the side chambers, and the other group were run through behavioural assays lacking a crowd stimulus. After the first behavioural assay, solitarious locusts were held in their isolated holding cages for 2 h before being run through another behavioural assay (2 h time point). This step was repeated for the final 4 h time point. (B) Experiment 2ii: As (A) except with 24 h intervals between behavioural assays and finishing at 72 h. Experiment 2iv: as Experiment 2ii except with gregarious 5th instar locusts held in crowding tubs. (C) Experiment 2iii: As (A) except with a gap of 48 h in between behavioural assays and finishing at 96 h.

5.3.6 Experiment 3: Y-maze arena

In contrast with the continuous behavioural data of an established arena, I decided to investigate a phase-specific reaction to novel stimuli in a binary Y-maze arena setup. A Y-maze is a useful behavioural assay that provides test animals with only two choices for exploration, providing a binary choice paradigm that is simpler than the Roessingh arena assay (1993). It has been used to test visual recall in bees (Zhang et al., 1999), naïve odour preferences in the fruit fly (Simonnet et al., 2014) and odour conditioning in rats (Johnson et al., 1995). In order to test whether locusts show contextual familiarity responses in a this choice paradigm, part of this chapter moves away from the established behavioural assay (Roessingh et al., 1993) and uses a Y-maze paradigm recently developed in the locust. This paradigm utilises two distinct odours (lemon and vanilla)

that have been used to demonstrate appetitive and aversive conditioning in *S. gregaria* (Simões et al., 2011, 2012, 2013). As 70% of locusts tended to move towards the vanilla odour and 30% towards the lemon odour in this paradigm (Simões et al., 2011, 2012, 2013), any changes from this naïve preference therefore could be inferred as changes in odour salience. This paradigm was thus used to assess the effect of familiarising a locust with an odour on its choices in the Y-maze.

A Y-maze was designed using specifications in previous work (Simões et al., 2011, 2012, 2013) and lit from above with a lamp with an E27 15W energy saving bulb (Figure 5.3Ai). The arena was covered with a clear plastic lid to allow visual observation and a closed ventilation system. A wooden beam (2 cm diameter) was suspended 11 cm above the floor of the arena for the locust to walk on. To allow constant release of lemon or vanilla odours within the Y-maze setup, vanilla extract or lemon extract (both Nielsen-Massey brand, Leeuwarden, the Netherlands) was placed into small glass vials (1.5 cm diameter x 4 cm height; Figure 5.3Aii). The plastic lids for the vials were pierced to make a hole to allow a string wick (secured with tape) to release the odour gradually. These vials were placed into 15 mL Falcon tubes and these tubes were placed outside of each test arm. The odours within the Falcon tubes were ventilated through the arena by connecting them with an aquarium pump (APS50, Tetratec) via a T-shaped inlet tubing attachment with identical length arms. Outlet tubing then connected the Falcon tubes with the ends of the Y-maze arms. An electrical fan attachment (5 cm diameter) was connected behind the main arm to ensure adequate directional ventilation of the arena from the two odours. On each session, adult locusts were placed into a modified opaque Sarstedt tube with bungs removed to allow ventilation (2 cm diameter, 10 cm length). A foam disc lid was then placed on the opposite end of the tube and the tube was left on the workbench for 5 min to allow the animal time to recover from handling stress. The tube was then attached to the wooden beam by means of a Velcro attachment on the extreme end of the main arm of the Y-maze and the lid removed immediately. Once the tube was opened inside the arena, the test locusts was observed for 5 min, or until it moved past the halfway point of one of the test arms, before removal from the arena. The odours used in each arm were selected according to the stage of the experiment and treatment, described in the next section in more detail.

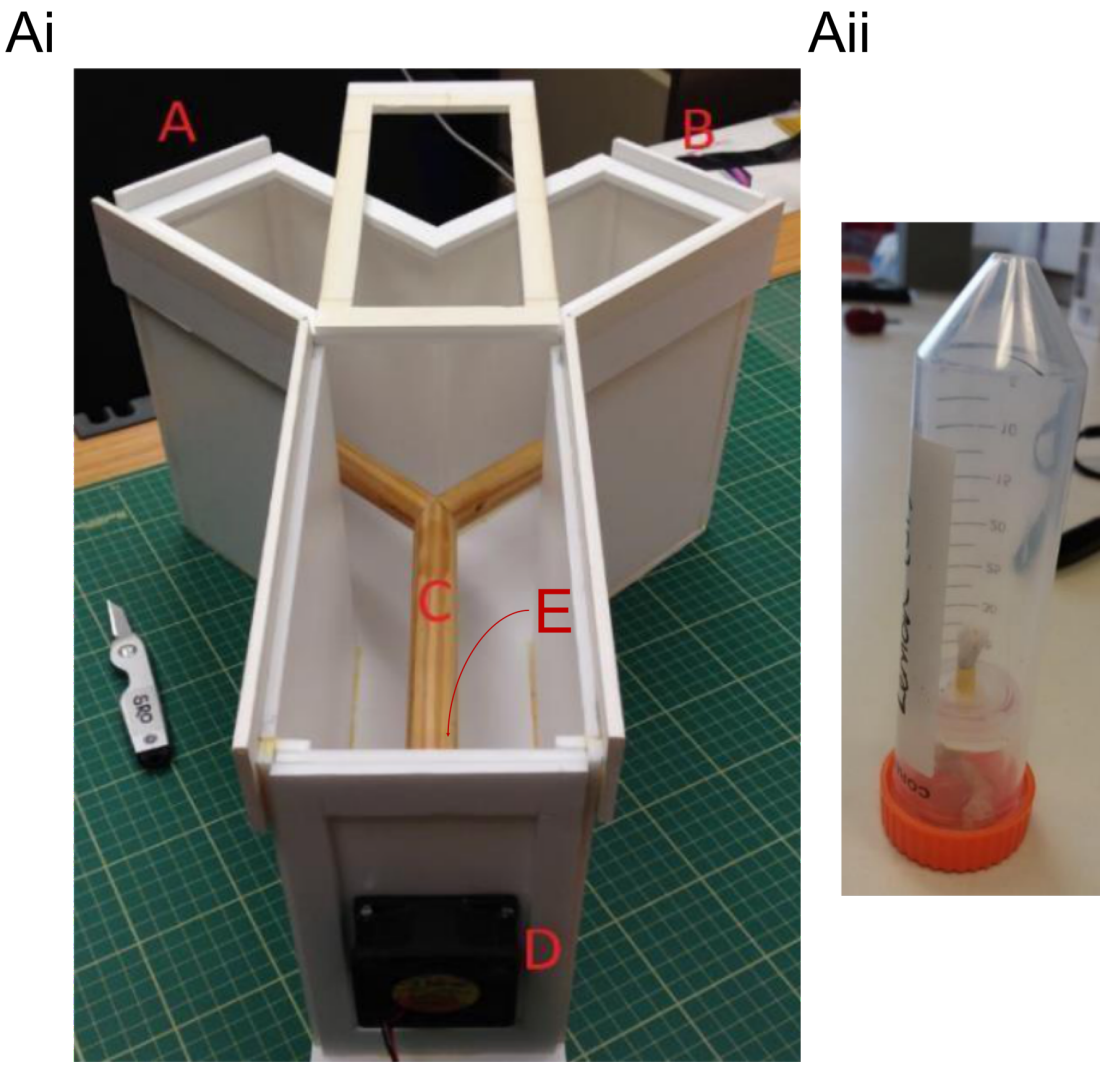


Figure 5.3: Y-maze and odour release designs. (Ai) Image of the Y-maze structure with the two test arms (A and B), suspended wooden beam through the arms (C), an extraction fan to ventilate the arena with the odours (D) and the Velcro attachment for mounting the locust holding tube on the main arm (E; Velcro attachment behind fan). Image created by Ott, S. (2015) and modified by Purton, S. (2015). (Aii) Falcon tube holding glass vial containing either vanilla essence or lemon essence. Image created by Purton, S. (2015).

5.3.6.1 Naïve Y-maze assay

A total of 80 gregarious and 21 solitarious adult locusts were run through the Y-maze arena assay just once to investigate innate odour preferences. The odours presented on each test arm of the Y-maze were alternated so that the first 50% of animals that day received lemon odour on the left arm (setup LV) and the latter 50% of animals received vanilla odour on the left side (setup VL). The binary choice (or lack of choice) made by each test locust was recorded by the observer.

5.3.6.2 Odour pre-exposure Y-maze assay

A total of 81 gregarious and 80 solitarious adult locusts were run through the Y-maze arena assay after sessions of pre-exposure to one of the odours in both arms. On the first pre-exposure session, the test locusts were released into the arena and presented with either vanilla (gregarious locusts) or lemon (solitarious locusts) in both arms for 5 min before removal from the arena (0 h time point). The test locusts were then returned to either solitarious or gregarious holding cages for 24 h. This pre-exposure procedure was repeated twice more (24 h and 48 h time points). On the 72 h time point, the test locusts were presented with a choice between vanilla and lemon, with alternating LV and VL setups as in the naïve choice experiment. Wheat shoots and bran flakes were available to the animals *ad libitum* throughout the holding periods.

5.3.6.3 Statistical analysis

Only locusts that moved to one of the test arms were included in the statistical analysis. In order to account for side bias in the test locusts, the odour choice variable was fitted with a logistic regression model in RStudio, using the fixed effects *Trial* (naïve or pre-exposed), *Vanilla side* (the position of the odour, left (VL) or right (VL) arm), the *Phase* of the locust, and the interaction between *Trial* and *Phase* (*Odour choice ~ Vanilla side + Trial + Phase + Trial:Phase*). The *Trial*:*Phase* interaction of the model would indicate phase-specific differences in the effect of pre-exposure (familiarity) on odour preferences. The *Vanilla Side* effect would indicate a side bias in the Y-maze independent of any other factors. The *Trial* effect would signify changes across the phases between naïve and pre-exposed odour choices. The *Phase* effect would identify phase-specific odour preferences irrespective of familiarity. Orthogonal contrasts in the model were analysed in R using the *summary* function.

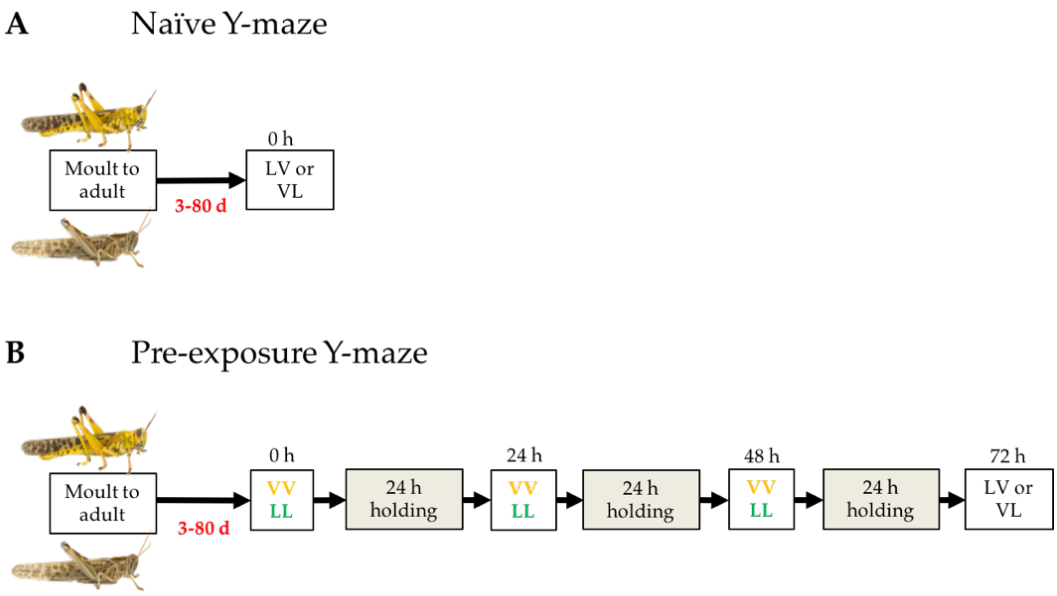


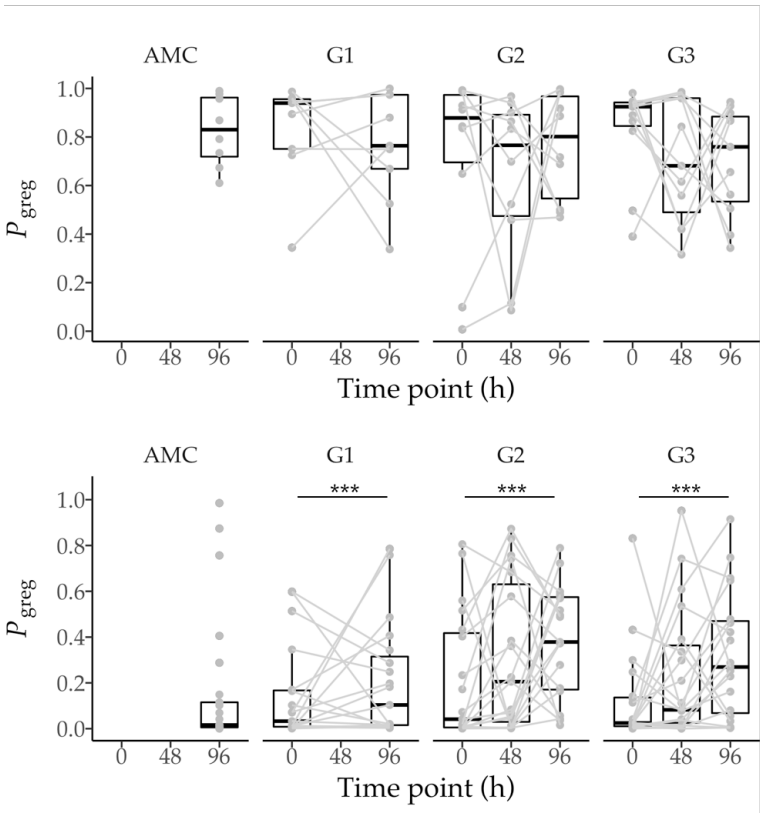
Figure 5.4: Schematic timeline of methods in Experiment 2. (A) Naïve Y-maze: Solitarious or gregarious adult locusts were run through a Y-maze arena 3-80 d after moulting to adults. They were placed into the Y-maze with each arm treated with a test odour of lemon or vanilla, alternating with lemon on the left (LV) and vanilla on the left (VL) for each trial. Locusts moving to an arm of choice were recorded and then removed. Locusts failing to make a choice were removed after 5 min. (B) Pre-exposure Y-maze: Solitarious or gregarious adult locusts were run through a Y-maze arena 3-80 d after moulting to adults. They were placed into the Y-maze with both arms treated with a test odour of either lemon (LL; green) for solitarious locusts or vanilla (VV; orange) for gregarious locusts for 5 min. They were returned to their colony conditions (isolated holding cages for solitarious locusts; crowding tubs with ~ 30 conspecifics for gregarious locusts) for 24 h. This was repeated the next day. On the final time point (72 h) they were given the same choice trial as in (A).

5.4 Results

5.4.1 Experiment 1: weak evidence for behavioural change in response to repeated arena runs, handling or injections

5.4.1.1 Gregarious

Gregarious nymphs were assayed in four different treatment groups with different combinations of repeated behavioural arena observations (AMC $N = 8$; G1 $N = 9$; G2 $N = 10$; G3 $N = 11$), experimenter handling and injection protocols to investigate the effect of these factors on behavioural gregariousness. Though minor drops in median P_{greg} of up to 0.24 were observed (Figure 5.5A), there was no evidence for an effect of time point ($p = 0.7059$; Table 5.2) or treatment on behavioural gregariousness ($p = 0.6602$; Table 5.2) between the 0 h and 96 h time points of G1, G2 and G3. There was also no evidence for an interaction between the treatment and the assay time point fixed effects ($p = 0.5600$; Table 5.2). A linear model comparing the behavioural gregariousness of all repeated measures groups with the AMC group at the 96 h time point also provided no evidence for a treatment effect ($p = 0.5700$; Table 5.2).



Treatment	Description
AMC	Age-matched; tested at 96h only
G1	Tested at 0h and 96h
G2	Tested at 0h, 48h and 96h; handled daily
G3	Tested at 0h, 48h and 96h; injected with 5 µL locust saline daily

Figure 5.5: Weak evidence for an effect of repeated arena runs, handling and injections on behavioural gregariousness. (A) Comparison of P_{greg} over time points 0, 48 and 96 h between gregarious 5th instar locusts given 1 of 4 treatments (table on right; AMC $N = 8$, G1 $N = 9$, G2 $N = 10$, G3 $N = 11$ across time points). Each point represents one observation and lines connect individual observations over time. (B) As (A) except with 5th instar solitarious locusts undergoing the same treatments (AMC $N = 25$, G1 $N = 19$, G2 $N = 19$, G3 $N = 19$ across time points). Asterisks show evidence for a time point effect in a linear mixed model (Table 2.4).

5.4.1.2 Solitarious

At 48 h, G2 ($N = 19$) and G3 ($N = 19$) groups showed small increases in median P_{greg} values from their 0 h time point (G2 median increased by 0.17, G3 median increased by 0.05). At the 96 h time point, G1 ($N = 19$), G2 and G3 showed higher median P_{greg} values than at the 0 h time point (96 h vs 0 h: G1 increased by 0.07, G2 increased by 0.34, G3 increased by 0.24; Figure 5.5B). The AMC ($N = 25$) group showed solitarious behaviour at the 96 h time point (Figure 5.5B).

Table 5.2: Statistical tests of the effect of animal handling, injections and behavioural assays on logit values (behavioural gregariousness) in gregarious nymphs between 0 h and 96 h time points. Further model comparing G1, G2 and G3 with age-matched controls (AMC) at 96 h time point. Df = degrees of freedom. Linear model analysis: type 1 ANOVA.

Test	Comparison	Statistic	Df between groups	Df within groups	*p*-value
Logit ~ Time point + Treatment + Time point:Treatment + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	$F = 0.14$	1	27	0.7081
Linear mixed model	*Treatment* fixed effect	$F = 0.42$	2	27	0.6641
Linear mixed model	*Time point:Treatment* interaction	$F = 0.59$	2	27	0.5600
Logit ~ Time point + Treatment + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	$F = 58.74$	1	29	0.7059
Linear mixed model	*Treatment* fixed effect	$F = 0.99$	2	27	0.6602
96 h time point only, with AMC group: Logit ~ Treatment					
Linear model	*Treatment* fixed effect	$F = 0.69$	3	34	0.5700

Details of the linear regression models used are shown in Table 5.3. A linear mixed model comparing the behavioural changes over the 0 h and 96 h time points between G1, G2 and G3 provided strong evidence for an effect of time point on behavioural gregariousness ($p = 0.0001$) but not for a treatment effect on behavioural gregariousness ($p = 0.2400$). There was no evidence for an interaction between treatment and assay time point ($p = 0.3158$). Across all treatment groups, analysis of regression coefficients provided strong evidence for an increase in behavioural gregariousness between 96 and 0 h ($p = 0.0001$; Figure 5.5A).

All statistical details are shown in Table 5.3. A linear model comparing the behavioural gregariousness of all repeated measures groups with the AMC group at the 96 h time point provided moderate evidence for a treatment effect ($p = 0.0022$). Analysis of regression coefficients in the linear model provided no evidence that repeated assays increase gregariousness compared with AMCs (G1 vs AMC: $p = 0.1758$), but moderate

to strong evidence that handling and injections increase behavioural gregariousness compared to AMCs (G2 vs AMC: $p = 0.0004$; G3 vs AMC: $p = 0.0058$). The regression coefficients provided weak evidence that additional daily animal handling increases the animal's gregariousness more than just repeated arena assays at 96 h (G2 vs G1 $p = 0.0331$) but no evidence that additional handling and injections were more effective than repeated arena assays (G3 vs G1 $p = 0.1732$), nor that injections were more effective than handling (G3 vs G2 $p = 0.4293$).

5.4.2 Experiment 2

5.4.2.1 Experiment 2i: Strong evidence for behavioural changes over 2 h repeated assays in solitarious nymphs

Solitarious nymphs were run through the established behavioural assay with and without a crowd stimulus (*CrowdStim* variable; Section 5.3.2) to assess changes in their exploratory behaviour (fractal dimension) over repeated exposure 2 h apart. The group with the crowd stimulus in the arena (Crowd group; $N = 28$) showed more exploratory activity than the group lacking a crowd stimulus in the arena across all time points (Empty; $N = 28$; difference of 0.15 at 0 h, 0.29 at 2 h, and 0.26 at 4 h; Figure 5.6A). Both groups increased their exploratory behaviour over the 2 h and 4 h time points compared to 0 h, with the 4 h time point showing an increase in fractal dimension of 0.16 between 4 h and 0 h in the Empty group and 0.26 in the Crowd group (Figure 5.6A).

A linear mixed model provided no evidence for an interaction between the crowd stimulus and time point fixed effects ($p = 0.6929$; Table 5.4). There was strong evidence for an effect of time point ($p = 0.0002$; Table 5.4) and the presence of the crowd stimulus ($p < 0.0001$; Table 5.4).

Table 5.3: Statistical tests of the effect of animal handling, injections and behavioural assays on logit values (behavioural gregariousness) in solitarious nymphs between 0 h and 96 h time points. Further model comparing G1, G2 and G3 with age-matched controls (AMC) at 96 h time point. Df = degrees of freedom. Linear model analysis: type 1 ANOVA.

Test	**Comparison**	**Statistic**	**Df between groups**	**Df within groups**	***p*-value**
Logit ~ Time point + Treatment + Time point:Treatment + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	$F = 17.60$	1	54	0.0001
Linear mixed model	*Treatment* fixed effect	$F = 1.47$	2	54	0.2400
Linear mixed model	*Time point:Treatment* interaction	$F = 1.18$	2	54	0.3158
Logit ~ Time point + Treatment + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	$F = 17.49$	1	56	0.0001
Linear mixed model	*Treatment* fixed effect	$F = 1.47$	2	54	0.2400
Regression coefficient	96 h vs 0 h	$t = 4.18$	3	N/A	0.0001
96 h time point only, with AMC group: Logit ~ Treatment					
Linear model	*Treatment* fixed effect	$F = 5.32$	3	78	0.0022
Regression coefficient	G1 vs AMC	$t = 1.37$	3	N/A	0.1758
Regression coefficient	G2 vs AMC	$t = 3.68$	3	N/A	0.0004
Regression coefficient	G3 vs AMC	$t = 2.84$	3	N/A	0.0058
Regression coefficient	G2 vs G1	$t = 2.17$	3	N/A	0.0331
Regression coefficient	G3 vs G1	$t = 1.38$	3	N/A	0.1732
Regression coefficient	G3 vs G2	$t = -0.80$	3	N/A	0.4293

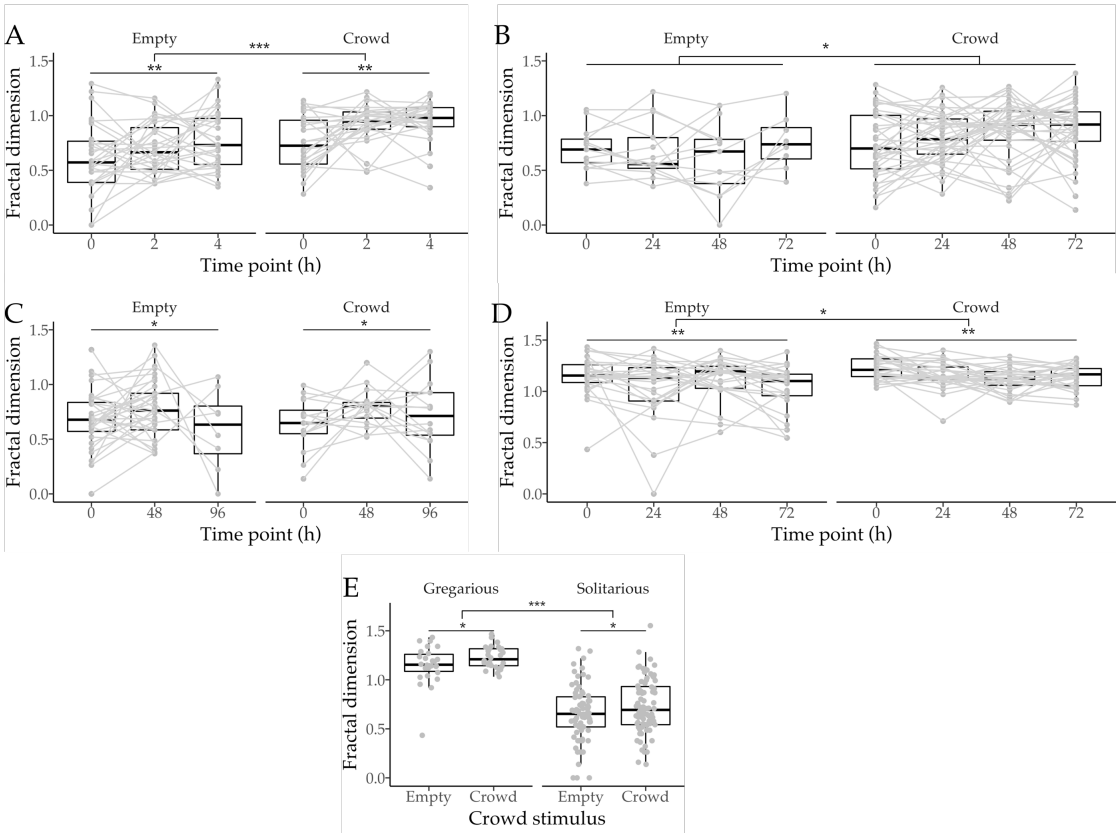


Figure 5.6: Evidence for an effect of repeated arena runs and the presence of a crowd stimulus in the arena on exploratory behaviour (fractal dimension of the trajectory) in solitarious and gregarious locusts. (A) Comparison of fractal dimension values over time points 0, 2 and 4 h between solitarious 5th instar locusts in the presence of a crowd stimulus in the arena (Crowd; $N = 28$ each time point) or with both chambers left empty (Empty; $N = 26$ each time point). Each grey point represents one residual (animal at that time point) and grey lines represent change in animal's behaviour over time. (B) As (A) except with assays at time points 0, 24, 48 and 72 h (Crowd $N = 37$ each time point; Empty $N = 13$ across time points except for 72 h with 8). (C) as (A) and (B) except with assays at time points 0, 48 and 96 h (Crowd $N = 16$ each time point; Empty $N = 33$ across time points except for 96 h with 8). (D) as (B) except with gregarious 5th instar locusts (Crowd $N = 28$ each time point; Empty $N = 26$ across time points). (E) Comparison of all 0 h assays in solitarious and gregarious nymphs, without and without crowd stimuli (Solitarious Empty $N = 74$, Crowd $N = 79$; Gregarious Empty $N = 27$, Crowd $N = 28$). Asterisks show evidence for a time point, crowd stimulus effect (A-D) or a phase effect based on linear models (E; Table 2.4).

Table 5.4: Statistical tests of the effect of behavioural assay time point and the crowd stimulus (CrowdStim) on fractal dimension (exploratory behaviour) in solitarious nymphs between 0 h, 2 h and 4 h. Df = degrees of freedom. Linear model analysis: type 2 ANOVA.

Test	Comparison	Statistic	Df between groups	Df within groups	*p*-value
Fractal dimension ~ Time point + CrowdStim + Time point:CrowdStim + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	$\chi^2 = 17.60$	2	104	0.0002
Linear mixed model	*CrowdStim* fixed effect	$\chi^2 = 15.90$	1	104	< 0.0001
Linear mixed model	*Time point:CrowdStim* interaction	$\chi^2 = 0.73$	2	104	0.6929
Fractal dimension ~ Time point + CrowdStim + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	$\chi^2 = 17.49$	1	105	0.0002
Linear mixed model	*CrowdStim* fixed effect	$\chi^2 = 1.47$	2	105	< 0.0001
Regression coefficient	2 h vs 0 h	$t = 3.01$	2	N/A	0.0033
Regression coefficient	4 h vs 0 h	$t = 4.01$	2	N/A	0.0001
Regression coefficient	4 h vs 2 h	$t = 1.00$	2	N/A	0.3206
Regression coefficient	Crowd vs Empty	$t = 3.99$	1	N/A	0.0001

5.4.2.2 Experiment 2ii: No evidence for behavioural changes over 24 h repeated assays in solitarious 5th instar locusts

To investigate the effect of daily behavioural assays over 24 h gaps, Experiment 2i was modified except with 24 h intervals rather than 2 h. The two *CrowdStim* groups showed similar exploratory activity at 0 h (Figure 5.6B) but the Crowd group ($N = 37$ all time points) showed an increase in exploratory behaviour by the 72 h time point compared to their first time in the arena, whereas no substantial changes were observed in the Empty group ($N = 13$ all time points except for 8 at 72 h; Figure 5.6B). A linear mixed model provided no evidence for an interaction between *crowd stimulus* and *time point* ($p = 0.2811$; Table 5.5). There was no evidence for an effect of time point ($p = 0.1855$; Table 5.5) but weak evidence for an effect of the presence of the crowd stimulus ($p = 0.0460$; Table 5.5).

Table 5.5: Statistical tests of the effect of behavioural assay time point and the crowd stimulus (CrowdStim) on fractal dimension (exploratory behaviour) in solitarious nymphs between 0 h, 24 h, 48 h and 72 h. Df = degrees of freedom. Linear model analysis: type 2 ANOVA.

Test	Comparison	Statistic	Df between groups	Df within groups	*p*-value
Fractal dimension ~ Time point + CrowdStim + Time point:CrowdStim + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	$\chi^2 = 4.90$	3	139	0.1792
Linear mixed model	*CrowdStim* fixed effect	$\chi^2 = 3.90$	1	139	0.0484
Linear mixed model	*Time point:CrowdStim* interaction	$\chi^2 = 3.82$	3	139	0.2811
Fractal dimension ~ Time point + CrowdStim + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	$\chi^2 = 4.82$	3	142	0.1855
Linear mixed model	*CrowdStim* fixed effect	$\chi^2 = 3.98$	1	142	0.0460
Regression coefficient	Crowd vs Empty	$t = 2.00$	1	N/A	0.0517

5.4.2.3 Experiment 2iii: no evidence for behavioural changes over 48 h repeated assays in solitarious nymphs

This experiment was as 2ii but with 48 h intervals. The two *CrowdStim* groups showed similar exploratory activity at all time points (Crowd $N = 16$; Empty $N = 33$ except for 96 h with $N = 8$; Figure 5.6C). Both groups showed minimal increases in exploratory behaviour at the 48 h time point compared to their first time in the arena (48 h vs 0 h: Crowd group median difference = 0.15; Empty group median difference = 0.08; Figure 5.6C) but this increase was absent by 96 h (Figure 5.6C). A linear mixed model provided weak evidence for an effect of time point ($p = 0.0468$; Table 5.6), no evidence for an effect of the presence of the crowd stimulus ($p = 0.8050$; Table 5.6) and no evidence for an interaction between the crowd stimulus and time point fixed effects ($p = 0.3676$; Table 5.6).

Table 5.6: Statistical tests of the effect of behavioural assay time point and the crowd stimulus (CrowdStim) on fractal dimension (exploratory behaviour) in solitarious nymphs between 0 h, 48 h, and 96 h. Df = degrees of freedom. ANOVA = type 1.

Test	Comparison	Statistic	Df between groups	Df within groups	*p*-value
Fractal dimension ~ Time point + CrowdStim + Time point:CrowdStim + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	$F = 3.30$	2	69	0.0427
Linear mixed model	*CrowdStim* fixed effect	$F = 0.07$	1	47	0.7977
Linear mixed model	*Time point:CrowdStim* interaction	$F = 1.01$	2	69	0.3676
Fractal dimension ~ Time point + CrowdStim + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	$F = 3.20$	2	71	0.0468
Linear mixed model	*CrowdStim* fixed effect	$F = 0.06$	1	47	0.8050
Regression coefficient	48 h vs 0 h	$t = 2.36$	2	N/A	0.0208
Regression coefficient	96 h vs 0 h	$t = 0.06$	2	N/A	0.9505
Regression coefficient	96 h vs 48 h	$t = -1.78$	2	N/A	0.0793

5.4.2.4 Experiment 2vi: no evidence for behavioural changes over 24 h repeated assays in gregarious 5th instar locusts

This experiment was as 2ii except with gregarious nymphs rather than solitarious. The Empty group ($N = 26$) showed slightly lower exploratory activity than the Crowd ($N = 28$) group at all time points except for the 48 h time point (Empty vs Crowd: median difference of -0.06 at 0 h, -0.01 at 24 h, +0.06 at 48 h, and -0.07 at 72 h; Figure 5.6D). Both groups showed a small decrease in exploratory behaviour at the 72 h time point compared to their first time in the arena (72 h vs 0 h Crowd group median difference = -0.05, Empty group median difference = -0.05; Figure 5.6D). There was moderate evidence for an effect of time point (linear mixed model; $p = 0.0095$; Table 5.7) and weak evidence for an effect of the presence of the crowd stimulus ($p = 0.0207$; Table 5.7). A linear mixed model provided no evidence for an interaction between the crowd stimulus and time point fixed effects ($p = 0.1736$; Table 5.7).

Table 5.7: Statistical tests of the effect of behavioural assay time point and the crowd stimulus (*CrowdStim*) on fractal dimension (exploratory behaviour) in gregarious nymphs between 0 h, 24 h, 48 h, and 72 h. Df = degrees of freedom. ANOVA = type 1.

Test	Comparison	Statistic	Df between groups	Df within groups	*p*-value
Fractal dimension ~ Time point + CrowdStim + Time point:CrowdStim + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	$F = 4.00$	3	156	0.0089
Linear mixed model	*CrowdStim* fixed effect	$F = 5.70$	1	52	0.0207
Linear mixed model	*Time point:CrowdStim* interaction	$F = 1.68$	3	156	0.1736
Fractal dimension ~ Time point + CrowdStim + 1|Animal.ID					
Linear mixed model	*Time point* fixed effect	$F = 5.70$	3	159	0.0095
Linear mixed model	*CrowdStim* fixed effect	$F = 4.00$	1	52	0.0207
Regression coefficient	24 h vs 0 h	$t = -2.77$	3	N/A	0.0061
Regression coefficient	48 h vs 0 h	$t = -2.12$	3	N/A	0.0352
Regression coefficient	72 h vs 0 h	$t = -3.14$	3	N/A	0.0020
Regression coefficient	48 h vs 24 h	$t = 0.66$	3	N/A	0.5129
Regression coefficient	72 h vs 24 h	$t = -0.36$	3	N/A	0.7200
Regression coefficient	72 h vs 48 h	$t = -1.01$	3	N/A	0.3117
Regression coefficient	72 h vs 48 h	$t = 2.38$	3	N/A	0.0207

5.4.2.5 Pooled 0h time point between phases: Very little evidence for more exploratory behaviour in arenas with a crowd stimulus

Data from the 0 h time point of all experiments and both phases in Experiment 2 were analysed jointly to investigate the effect of the presence of a crowd stimulus in the behavioural arena on the exploratory activity of the two phases. The exploratory activity of both phases was slightly higher in arenas containing a crowd stimulus (Crowd vs Empty: median difference of +0.06 in gregarious phase (Empty $N = 27$; Crowd $N = 28$), +0.04 in solitarious phase (Empty $N = 74$; Crowd $N = 79$; Figure 5.6E). Unsurprisingly,

locusts in the gregarious phase showed more exploratory behaviour than solitarious locusts overall (gregarious vs solitarious: median difference of +0.50 in Empty group, +0.52 in Crowd group; Figure 5.6E). A linear regression model provided no evidence for an interaction between *crowd stimulus* and *phase* ($p = 0.941$; Table 5.8). There was weak evidence for more exploratory behaviour in the presence of the crowd stimulus ($p = 0.0457$; Table 5.8) and very strong evidence for a phase effect ($p < 0.0001$; Table 5.8).

Table 5.8: Statistical tests of the effect of phase and the crowd stimulus (*CrowdStim*) on fractal dimension (exploratory behaviour) in nymphs. All data pooled from the 0 h time point of Experiment 2. Df = degrees of freedom. Linear model analysis: type 1 ANOVA.

Test	Comparison	Statistic	Df between groups	Df within groups	*p*-value
Fractal dimension ~ Phase + CrowdStim + Phase:CrowdStim					
Linear model	*Phase* fixed effect	$F = 150.03$	1	203	< 0.0001
Linear model	*CrowdStim* fixed effect	$F = 4.02$	1	203	0.0463
Linear model	*Phase:CrowdStim* interaction	$F = 0.01$	1	203	0.9414
Fractal dimension ~ Phase + CrowdStim					
Linear model	*Phase* fixed effect	$F = 150.76$	1	204	< 0.0001
Linear model	*CrowdStim* fixed effect	$F = 4.04$	1	204	0.0457
Regression coefficient	Solitarious vs Gregarious	$t = -12.28$	1	N/A	< 0.0001
Regression coefficient	Crowd vs Empty	$t = 2.01$	1	N/A	0.0457

5.4.3 Experiment 3: No evidence for familiarisation response in Y-maze

To investigate a phase-specific reaction to novel odour stimuli in a Y-maze arena, gregarious and solitarious adult locusts were pre-exposed to a Y-maze arena ventilated with lemon and vanilla odours respectively for 5 min each day for 3 d. The next day their odour choices were recorded when presented with lemon or vanilla odour, and compared with those given the choice under naïve conditions. There was a clear bias towards the left arm of the Y-maze in the naïve (Left side choice: $N = 61$ (60.4%); Right side: $N = 40$ (43.6%)) and familiarity trials (including pre-exposure trials; Left side choice: $N = 326$

(56.2%); Right side: $N = 254$ (43.8%)) for both phases. However, since the side of each odour presentation was alternated in a balanced design, inferences could still be made regarding odour preferences.

Naïve gregarious locusts (with no odour pre-exposure) presented with the choice between lemon and vanilla odour showed no odour preference (naïve trial; lemon $N = 40$ (49%); vanilla $N = 41$ (51%); Figure 5.7A). After 2 trials of pre-exposure to lemon odour, 43% gregarious adults chose the lemon odour ($N = 34$) and 57% chose the vanilla odour ($N = 45$; Figure 5.7A).

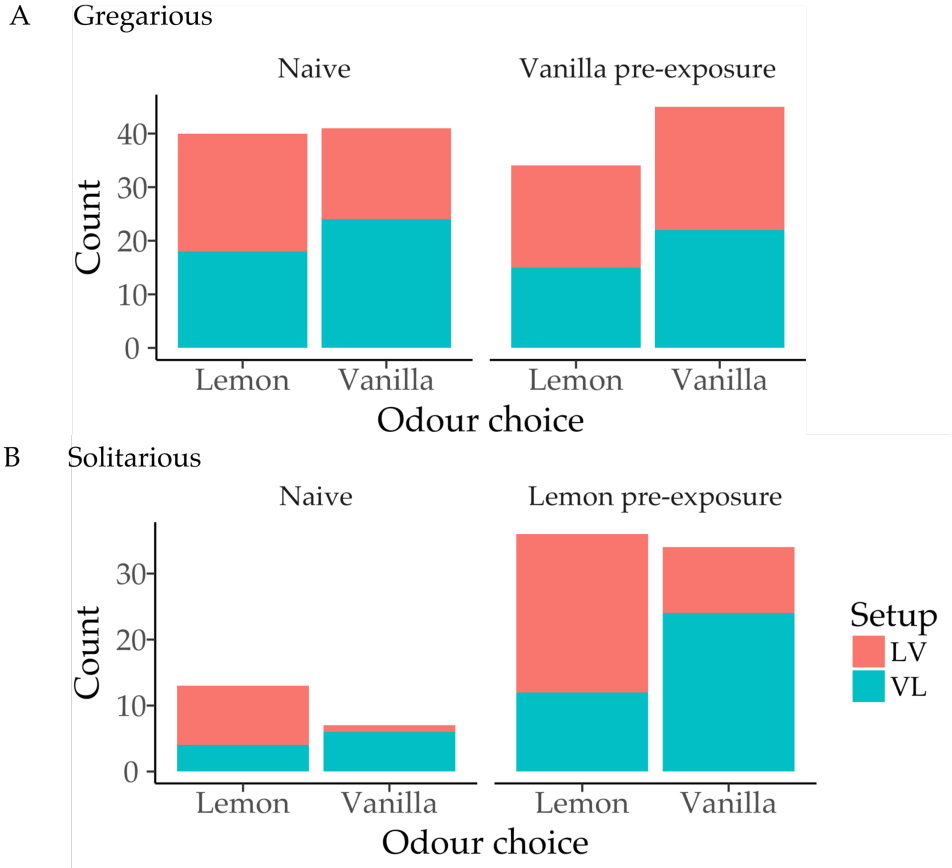


Figure 5.7: No evidence for an effect of odour pre-exposure on odour preference in solitarious or gregarious adult locusts. (A left) Naïve gregarious locusts showed no odour preference (lemon $N = 40$ (49%); vanilla $N = 41$ (51%)) and weakly enhanced preference for vanilla after 2 sessions of vanilla pre-exposure (A right) (lemon, $N = 34$ (43%); vanilla $N = 45$ (57%)). (B left) Solitarious locusts showed a slight preference for lemon odour (lemon $N = 13$ (65%)); vanilla $N = 7$ (35%)) and no odour preference after lemon pre-exposure (B right; lemon $N = 36$ (51%); vanilla $N = 34$ (49%)). Both phases, particularly the solitarious locusts, showed a bias for the left side of the Y-maze (more lemon choices in setups with lemon in the left arm (LV), more vanilla choices with vanilla in the left arm (VL).

A total of 20 solitarious locusts out of 21 assayed selected an arm within 5 min in the naïve choice trial. The locust that failed to move onto the test arms was removed from the analysis. Solitarious adult locusts presented with the choice between a lemon and vanilla odour in the absence of pre-exposure trials showed a 65% preference for lemon odour (lemon $N = 13$; vanilla $N = 7$ (35%); Figure 5.7B). After 3 trials of pre-exposure to vanilla odour, 70 solitarious locusts out of 80 assayed selected an arm within 5 min in the 72 h time point. The locusts that failed to move onto the test arms were removed from the analysis. Of those that moved into a test arm, 51% chose the lemon odour ($N = 36$) and 49% chose the vanilla odour ($N = 34$; Figure 5.7B).

Analysis of coefficients in a logistic regression model (Table 5.9) provided no evidence that repeated exposure changes odour preferences in a phase-specific manner (*Trial:Phase* interaction $p = 0.6745$; Table 5.9). There was also no evidence that the pre-exposure changes odour choice in any locust (*Trial* $p = 0.1596$; Table 5.9). The model provided very little evidence that solitarious locusts choose lemon odours more than gregarious locusts (*Phase* $p = 0.0956$; Table 5.9). There was moderate evidence that the side of the odour affects odour choice (i.e. side bias; *Vanilla side* $p = 0.0013$; Table 5.9).

Table 5.9: Table of logistic regression model fit for fixed effects on side choice in the Y-maze setup. Df= degrees of freedom.

Odour choice ~ Vanilla side + Trial + Phase + Trial:Phase				
Fixed effect	**Estimate**	**Standard Error**	***z*-value**	***p*-value**
Intercept	-0.097	0.157	-0.618	0.5364
Vanilla side	0.419	0.131	3.208	0.0013
Trial	0.221	0.157	1.406	0.1596
Phase	0.262	0.157	1.67	0.0956
Trial:Phase	-0.066	0.157	-0.420	0.6745

5.5 Discussion

5.5.1 Experiment 1: solitarious locusts exhibited behavioural changes over time, but gregarious locusts did not

The gregarious animals in this experiment showed no evidence of changing behaviour over repeated arena assays nor with repeated injections or handling. The lack of evidence for an effect observed in the gregarious animals was surprising, as treatment G3 was designed to be identical to that of the control group of the fluphenazine experiment, which showed a dramatic loss in behavioural gregariousness over the 96 h time period. Despite the sample sizes being lower than in the fluphenazine experimental control group, it is also clear that decreases in behavioural gregariousness were not as large in this experiment, since the lowest median values in this experiment were 0.76 (Figure 5.5A), whereas the lowest median P_{greg} was 0.31 in the saline experimental control group of the fluphenazine experiment. The reason for the large difference between effect sizes is unclear, and may be the result of chance, or an abnormal batch of animals used in the previous chapter.

The solitarious locusts undergoing this experiment similarly showed a more modest increase in behavioural gregariousness over time than in the fluphenazine experiment of the previous chapter. For comparison, the highest median P_{greg} value of the saline control group then was 0.57, and the highest median of this experiment was of 0.38 in group G2 at 96 h (Figure 5.5B). These differences in effect sizes between both phases across both experiments suggest that, while there remains a sign of increases in behavioural gregariousness over time, it is not as dramatic as that seen in the previous chapter, again possibly due to random day or batch effects. It is also important to note that the increases in behavioural gregariousness seen in the previous experiments were already prominent by the 48 h time point and showed only modest increases thereafter. In contrast, the two groups run at 48 h in this experiment only showed changes at 96 h, particularly in G3 (Figure 5.5B). Another important trend shown here is that the strong evidence for an increase in behavioural gregariousness between 0 h and 96 h in solitarious locusts appears consistent across the treatment groups, indicating that one behavioural assay is sufficient to increase behavioural gregariousness even 96 h after, and that the addition of extra handling or injections is not required to change behavioural gregariousness. This result

implies a form of learning taking place within the behavioural arena context that could indicate a loss of cryptic behaviours typical to solitarious locusts (Uvarov, 1977). The question remained whether or not this is a feature specific to behavioural gregariousness, for example, that these animals showed familiarisation towards the crowd stimulus, or if this is a general response to familiarity in all contexts. This led to the next set of experiments investigating behavioural changes in response to repeated arena exposure in the presence and absence of a crowd stimulus.

5.5.2 Experiment 2: Subtle changes in exploratory behaviour with repeated behavioural assays

Repeated behavioural assays reduced the exploratory behaviour of solitarious nymphs, however this was only apparent at intervals of 2 h with and without a crowd stimulus present, whereas larger intervals were insufficient to change the exploratory behaviour of solitarious or gregarious nymphs. As the Empty arena setup lacked a crowd stimulus (by definition), the time on stimulus side behavioural metric was meaningless, precluding the use of P_{greg}. The decision to use the exploratory variable fractal dimension as the comparison metric in this study was based on the fact that the logistical regression model used to calculate P_{greg} is based primarily on fractal dimension (see Chapter 2), thus this was an established phase-related behaviour that was still valid in both contexts.

In solitarious locusts, short-term repeated behavioural assays evoked the strongest increases in exploratory behaviour over time in both contexts (Figure 5.6A), whereas 24 h intervals were less consistent, with only the crowded arena showing increased exploration (Figure 5.6B). The increases were not consistent by 48 h intervals, however, it was interesting to see slight upwards trends in exploration at the 48 h time point (Figure 5.6C). This trend suggests that short-term familiarity responses were taking place within 2 h, whereas longer intervals showed weak long-term familiarity responses of the context. These changes were potentially strengthened in the presence of a crowd stimulus, but still present without. The presence of the crowd stimulus weakly increased exploratory behaviours overall in solitarious locusts, and this seemed consistent across time, however, only 2 of the 3 solitarious experiments (2i and 2ii) found robust evidence for this effect. Experiments using gregarious locusts every 24 h indicated slight decreases in exploratory activity over time (Figure 5.6D), however, the changes observed were only

subtle in this case. As Experiment 1 had not indicated any changes in gregarious behaviour, only 24 h time intervals were used in this phase, however, in future it would be interesting to investigate the effect of short-term repeated behavioural assays on the behavioural exploration of gregarious locusts, given the strong short-term effects observed in solitarious animals. It is difficult to define whether the stronger response at 2 h intervals is a true familiarisation response to the context, or also due to short-term nonassociative habituation to stressful animal handling associated with the assay. To answer this question, it would be useful to attempt a dishabituation procedure over 2 h intervals, for example, by placing animals into a different context each time (Rankin et al., 2009).

The evidence that both phases show more exploration in the presence of a crowd stimulus when entering the behavioural arena for the first time is surprising (Figure 5.6E), as their classic behaviours include low activity and the avoidance of conspecifics, which might imply more exploratory behaviour without the aversive stimulus of conspecifics (Roessingh et al., 1993; Uvarov, 1977, 1966). Geva et al. (2010) found high activity levels of solitarious locusts when first placed in a crowd, which steadily reduced over time to more gregarious levels. This could imply therefore that solitarious locusts exhibit more exploratory behaviour in the presence of conspecifics in order to seek an escape as compared with an empty arena. However, the fact that gregarious locusts exhibit the same trend, and that the effect size is subtle at best, cast doubt on this explanation. More similar results to this chapter were reported by Despland (2001), who noted that solitarious animals increased their turns and walking speed in the presence of olfactory stimuli, and gregarious nymphs increased their walks. This was suggested to be an excitatory effect of odourants from conspecifics. Any effect of odour was difficult to interpret in the present experiment because the arena assay was carried out in the solitarious colony holding room, which may have allowed wheat smells or locust odours to permeate the arena (though there were 20 air changes per hour, see Chapter 2 for details). Furthermore, the arena was not consistently wiped down after use until later on in this project. Other caveats to these experiments are that the crowd-stimulus arenas and empty arena experiments were run separately in different animal batches, and were not fully balanced, thus it cannot be ruled out that the trends originated by chance alone. For future investigations, it is recommended that experiments are carried out in a more balanced

and time-controlled assay, in a room separated from animal husbandry-related stimuli such as wheat odours, and ideally with an identical separate behavioural arena dedicated to observations in the absence of a crowd stimulus for the experimental period.

Over time in these experiments, there was little evidence for a behavioural response in 5th instar locusts to re-exposure to an empty arena over 24 h and 48 h intervals. To understand whether these potential changes were a robust event that is identifiable across different behavioural paradigms and developmental stages of the locusts, it was necessary to test for the presence of behavioural familiarisation responses in a paradigm with clear binary outcomes and simpler non-gregarising contextual stimuli in adult locusts.

5.5.3 Experiment 3: No familiarisation responses or odour preference in the Y-maze

As there was no evidence that either phase changed their odour preference between the naïve and the pre-exposed trials, this experiment fails to support the hypothesis that either phase prefers either novel or familiar contextual stimuli when given the choice. One potential biological explanation for this result may be that, as a mobile, migratory insect, *S. gregaria* has no permanent territory, in contrast to eusocial insects such as ants, which use familiar environmental cues as a key navigational tool (Baddeley et al., 2012; Collett et al., 2013; Wystrach et al., 2011). As both solitarious and gregarious locusts migrate using basic cues such as wind direction (Farrow, 1990; Kennedy, 1951), it is feasible that they have no evolutionary requirements for a preference for or against novel stimuli. However, there were also caveats in this experiment. First, adult insects were chosen to maintain consistency with previous work establishing the Y-maze paradigm, which had also reported clear olfactory distinction between, and preferences for, vanilla odours and lemon odours (Simões et al., 2011, 2012, 2013). While finding no evidence for familiarisation responses in adult locusts, this does not rule out differences in odour preferences and familiarising responses between 5th instar nymphs and adult gregarious locusts. Second, it is possible that the odours presented were not sufficiently intense to be distinguishable in the Y-maze arena. The lack of an innate odour preference in the naïve trial conflicts with earlier evidence that suggested that 70% animals in this paradigm move towards vanilla and 30% move towards lemon (Simões et al., 2011, 2012, 2013). The discrepancy here could be explained by differences in odour brands, or

potentially cross-contamination when switching the odour vials during the trials. Further differences between the paradigm carried out in this chapter and those of before were that the assay took place in the presence of wheat odours in a room with rapid air changes (see animal husbandry, Chapter 2, Section 2.1), with no caloric restriction. It is possible that wheat odours were thus contaminating the ventilation system and confusing the issue. In future experiments, it is recommended that this experiment take place with clean air away from the animal colony and with more stringent controls on odour contamination between the test arms. It would also be of interest to investigate whether or not 5th instar locusts show similar trends and also whether housing locusts with the odour allows a stronger familiarisation response, if present.

The experiments of this chapter attempted to unravel the factors involved in the large-scale behavioural changes observed in the fluphenazine experiments of Chapter 4. They provided evidence that this behavioural change was due to an effect of repeated behavioural assays, though the effect size indicates this is only partial at most. At intervals of 2 h, the presence of a crowd stimulus was not required for increases in exploratory behaviour over repeated arena observations, though experiments using longer intervals showed very little evidence for a familiarity effect. Furthermore, the Y-maze experiment found no evidence for a familiarity response to odour stimuli within a binary paradigm in adult locusts. At long intervals, therefore, any familiarisation responses in repeated arena assays are too subtle to exert substantial confounding effects in experimental designs. This chapter therefore provides some support for the use of repeated behavioural assays with the same locusts over time in subsequent behavioural experiments, though cautious interpretation is advised at short intervals (e.g. 2 h). This in turn informs the more efficient extraction of behavioural data from each individual animal without the fear of a confounding effect of repeated behavioural assays.

6 Identification of activity-related immediate-early genes in the Desert Locust

6.1 Summary

Immediate-early genes (IEGs) have been extensively used as markers of neuronal activity in paradigms exploring stress, behavioural challenges, and learning tasks (Guzowski et al., 2005). However, these tools have not been used in the Desert Locust *S. gregaria*, particularly during its rapid behavioural phase transition. With the aim of understanding IEG messenger RNA (mRNA) transcription during behavioural phase transition in *S. gregaria*, this chapter used a previously assembled transcriptome to produce nucleotide sequences of the well-studied IEGs Early Growth Response (*EGR*), *FOS*, Hormone receptor-like 38 (*Hr38*) and Jun-Related Antigen (*JRA*), using query sequences from the model organisms *Drosophila melanogaster* and *Mus musculus*. Primers for Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) amplification were then designed and validated based on these sequences. Solitarious 5th instar locusts were then either crowded with conspecifics for 30 min or left uncrowded for 30 min before snap freezing in liquid nitrogen. Their RNA was then extracted from dissected and pooled brain and thoracic ganglia tissue. RT-qPCR was used to compare the IEG expression between crowded and control locusts. Further comparisons of long-term gregarious and long-term solitarious locusts were also carried out using quantification in the transcriptome. There was no evidence for IEG expression differences between either short- or long-term crowding in any IEG. More detailed examinations using microarray or RNA-seq approaches in comprehensive neuronal activity assays may reveal appropriate IEG targets for use in creating neuronal activity maps in *S. gregaria*.

6.1 Introduction

6.1.1 Neuronal circuitry mediating behavioural phase change in the desert locust

The neuronal and neurochemical basis of behavioural phase change in the locust has received much recent attention in *S. gregaria* (for examples see Alessi et al., 2014; Anstey et al., 2009; Roessingh et al., 1998; Rogers et al., 2003, 2004, 2014; Rogers and Ott, 2015; Tanaka and Nishide, 2013; Chapters 3 & 4) and *L. migratoria* (Guo et al., 2011, 2013, 2015; Ma et al., 2011). However, there is very little known regarding the neural connections between sensory stimuli and behavioural plasticity during phase change. Although electrophysiological recordings are able to reveal neuronal components of behavioural circuitry, they are unable to visualise multiple neuronal populations across the CNS (Burrows, 1996; Watanabe et al., 2017). Unlike the popular model organism *D. melanogaster*, methods for carrying out histological investigations into neuronal populations involved in particular behaviours or learning and memory in non-model species are relatively undeveloped (Guven-Ozkan and Davis, 2014; Watanabe et al., 2017). Thus, new tools are required to better understand the anatomical and neuronal basis of behavioural phase change in the locust.

6.1.2 Immediate-early genes – Established proxies for neuronal activation

One common tool for monitoring upregulation of neuronal activity has been the quantification and localisation of the expression of immediate-early genes (IEGs) in the CNS (Guzowski et al., 2005). IEGs encode a wide range of proteins, including transcription factors, growth factors and scaffolding proteins (Lanahan and Worley, 1998), and show a low basal expression, but are upregulated in response to sustained neuronal activity such as in the induction of synaptic plasticity in both vertebrates and invertebrates (Alberini et al., 1994; Bailey and Chen, 1983; Montarolo et al., 1986; Morgan and Curran, 1989; Sanyal et al., 2002; Cole et al., 1989). The upregulation of IEG transcription is characteristically rapid and transient, peaking at 30-60 min after stimulus presentation and returning to basal expression levels within 1–2 h (Bahrami and Drabløs, 2016; Cole et al., 1989; Greenberg and Ziff, 1984), making them useful markers for recent neuronal activation. This approach has been successfully applied in rodents (Guzowski et al., 2005), songbirds (Velho et al., 2005; Mello and Clayton, 1994; Moorman et al., 2011), fish (Boyer et al., 2013; Randlett et al., 2015; Baraban et al.,

2005; Wai et al., 2006) and invertebrates such as honeybees (Alaux and Robinson, 2007; Lutz and Robinson, 2013; Ugajin et al., 2013; McNeill and Robinson, 2015; Ugajin et al., 2012; Kiya et al., 2008) and fruit flies (Chen et al., 2016; Fujita et al., 2013). This tool could thus be used to investigate the anatomical basis of behavioural phase change in *S. gregaria*, with a focus on creating neuronal activity maps in the locust CNS during this process. This section briefly describes IEGs most commonly used in previous work and their potential use as neuronal activity markers in *S. gregaria*.

6.1.2.1 Early Growth Response *(EGR)*

The gene *EGR (EGR1–3* in mammals; *stripe* in *D. melanogaster*) encodes the nuclear phosphoprotein Krox24 (Bahrami and Drabløs, 2016). It has an important role in long-term synaptic plasticity (Guzowski et al., 2001; Knapska and Kaczmarek, 2004), controls cell proliferation functions in non-neuronal cells (Liu et al., 2001; Zhang et al., 2003), and is rapidly upregulated in response to neuronal activity in both rats (Cole et al., 1989; Crispino et al., 1998) and the fruit fly *D. melanogaster* (Chen et al., 2016; Guan et al., 2005). It has been used as a neuronal activity marker in the honeybee *Apis mellifera* (Lutz and Robinson, 2013; Sommerlandt et al., 2016; Ugajin et al., 2013) and in the cricket *Gryllus bimaculatus* (Watanabe et al., 2017). While there has been no characterisation of this gene within *S. gregaria,* an *EGR* sequence has been uploaded to NCBI for *L. migratoria* (AHB17949.1), indicating that *EGR* is conserved in locusts.

6.1.2.2 FOS

One common IEG employed as a neuronal activity marker in mammalian studies is *FOS* (or *c-fos*, Bahrami and Drabløs, 2016), a member of the Fos transcription factor family which includes *FOS*, fos-related antigens 1 and 2 (*FRA-1* and *FRA-2* respectively), and *FosB*. *FOS* and other members of the Fos protein family are transcribed in the cell in response to stimuli such as growth factors, cytokines and heat stress, and reaches maximal expression increase by 30-60 min after cell stimulation (O'Donnell et al., 2012). Its protein product forms a subunit of the active transcription factor activator protein (AP-1), formed through heterodimerisation of FOS around the leucine zipper domain with a protein of another family of transcription factor: the Jun transcription factor family (*JUN*, *JUNB*, *JUND* in mammals; Healy et al., 2013). This proto-oncogene mediates a wide range of transcription-dependent processes, including long-term synaptic plasticity and

learning (Kaczmarek, 1993), cell proliferation, differentiation and survival (Kovary and Bravo, 1991; O'Donnell et al., 2012). Visualisation of the expression of Fos family proteins has been carried out to assess neuronal activity in many vertebrate models, for example following stressful stimuli (Borelli et al., 2006; Sandner et al., 1993; Sharp et al., 1991), aversive conditioning paradigms (Campeau et al., 1991; Herry et al., 2008; Nakagawa et al., 2003; Swank and Bernstein, 1994), appetitive conditioning (Tronel and Sara, 2002) or spatial learning (Guzowski et al., 2001). The *FOS* gene, also known as Fos-related antigen (*FRA*) in invertebrates or *kayak* in *D. melanogaster* (Perkins et al., 1988; Sanyal et al., 2002), has been less employed as an activity marker in invertebrate work but has been reported to undergo upregulation in response to cold stress for 2 h in larvae of the wax moth *Galleria mellonella* (Cymborowski, 1996). Increases in *FOS*-like immunoreactivity have also been reported in response to agonistic behaviour, electrical stimulation of the antennae or injections of picrotoxin, a receptor antagonist of the inhibitory neurotransmitter gamma aminobutyric acid (GABA) in *G. bimaculatus* (Ghosal et al., 2010; Watanabe et al., 2017). *FOS*-like immunoreactivity increases in response to nociceptive stimuli in another cricket species *Acheta domesticus* (Renucci et al., 2000). There was, however, no activity-related upregulation of *kayak* in the brain of *D. melanogaster* after neuronal stimulation paradigms (Chen et al., 2016). This evidence suggests that there are species differences in the activity-related expression of the *FOS* gene and others in the Fos transcription factor family.

6.1.2.3 Hormone receptor-like in 38 (*Hr38*)

The IEG *Hr38* is an insect-specific member of the Nuclear Hormone Receptor family (NR4; Hwang et al., 2014; Sluder and Maina, 2001). Other members of the family include nuclear receptor related 1 (*Nurr1* in mammals; Bassett et al., 2004; Crispino et al., 1998) and nerve growth factor-induced clone B (Bassett et al., 2004). *Hr38* has a range of functions in *D. melanogaster* such as carbohydrate metabolism (Ruaud et al., 2011), cuticle development (Kozlova et al., 2009) and phagocytosis (Stroschein-Stevenson et al., 2005). Expression of *Hr38* is rapidly upregulated, peaking at 60-90 min and returning to basal levels 240 min after neuronal depolarisation in *D. melanogaster* (Chen et al., 2016; Fujita et al., 2013; Guan et al., 2005) as well as pheromonal exposure in the silk moth *Bombyx mori* (Fujita et al., 2013).

6.1.2.4 Jun-related antigen (*JRA*)

As mentioned in Section 6.1.2.2, proteins of the Jun transcription factor family can assemble into homodimers or heterodimers with the Fos family to form AP-1, with long-lasting effects on neuronal function, such as in long-term synaptic plasticity (Healy et al., 2013; Kaczmarek, 1993; Sanyal et al., 2002). The *JUN* gene (jun-related antigen (*JRA*) in invertebrates) has been less widely used than *FOS* as a neuronal activity marker in mammals, however one study visualised members of the Jun family in the rat brain after sexual behaviour, noting localised increases depending on the protein and brain region (Flanagan-Cato and McEwen, 1995). *JRA* has also been used to characterise neuronal activity during alarm pheromone exposure and presentation of a sucrose stimulus in bees (Alaux and Robinson, 2007; McNeill and Robinson, 2015; Sommerlandt et al., 2016). Although *JRA* showed less dramatic activity-related changes in *D. melanogaster* (Chen et al., 2016; Guan et al., 2005), there remains the potential for a *S. gregaria* orthologue to show IEG-like activity in behavioural phase change.

6.1.3 Sequence discovery and quantification of candidate IEGs in *S. gregaria*

S. gregaria orthologues for the four IEGs discussed in Section 6.1.2 are not published, so the first aim of this chapter was to confirm their presence in *S. gregaria*. An existing transcriptome assembled in a previous PhD project (Shand, 2015) was used to identify protein and nucleotide sequences of *S. gregaria* orthologues of *EGR, FOS, Hr38* and *JRA*. This transcriptome was constructed using brains and thoracic ganglia from long-term solitarious and gregarious adult locusts. Although basal IEG expression is generally expected to be almost negligible in neurons in the absence of sustained depolarisation (Bahrami and Drabløs, 2016; Guzowski et al., 2005), sufficient transcription was expected across whole tissue samples to allow the assembly of at least partial sequences of these IEG transcripts for quantification. Query protein sequences from *D. melanogaster* and the mouse, *M. musculus*, were obtained from Flybase and NCBI were BLASTed against the assembled transcriptome in order to identify similar transcripts in *S. gregaria,* and these were then used to design target-specific primers for subsequent quantification.

IEG expression was quantified in the pooled brain and thoracic ganglia of *S. gregaria* using real-time quantitative polymerase chain reaction (RT-qPCR) amplification. There

was insufficient time to explore localised expression changes by *in-situ* hybridisation. However, as a previous study used qPCR to show overall increases in antennal *Hr38* expression after pheromonal stimulation (Fujita et al., 2013), it seemed reasonable to assume that this technique would detect potential widespread changes in neuronal activity caused by behaviourally gregarising stimuli. IEG expression in the CNS was compared between uncrowded solitarious locusts and those given 30 min of exposure to a conspecific crowd stimulus, which is sufficient to initiate behavioural gregarisation (Roessingh et al., 1998; Roessingh and Simpson, 1994; see also Chapters 3 & 4). The aim was to determine whether any of the four target IEGs undergo upregulation within the first 30 min of behavioural gregarisation. Identifying such a change would allow further region-specific and anatomical approaches to localise the neuronal circuitry activated during behavioural phase change. In addition to the 30 min crowding stimulus, quantitative analysis of the transcriptome itself was used to investigate long-term phase differences in expression of these IEG targets. This would help to understand the effects of long-term crowd living on the expression of these IEG targets.

6.1.4 Aims:

- Use a transcriptome assembled in previous work to identify sequences for four candidate IEGs in *S. gregaria*: *EGR*, *FOS*, *Hr38* and *JRA*.
- Design qPCR primers specific to the IEGs of interest and confirm their specificity with Sanger Sequencing.
- Compare transcript levels of candidate IEGs between uncrowded and 30 min crowded 5th instar solitarious locusts.
- Use the assembled transcriptome to compare the transcript levels of candidate IEGs between long-term phases.

6.2 Methods

6.2.1 Animal husbandry

See Chapter 2 for details. Primers were optimised in 5th instar gregarious locusts; 5th instar solitarious locusts were only used to investigate the effect of crowding on IEG transcript expression, and balanced for males and females in each treatment group.

6.2.2 Crowding treatments

Solitarious 5th instar nymphs were crowded as described in Chapter 2, with modifications to reduce handling prior to the snap-freezing stage. One hour prior to snap-freezing, locusts were labelled with a black non-toxic permanent marker pen to facilitate individual identification, and the food was removed from their holding cages; 30 min prior to snap-freezing, the locusts were placed into cylindrical holding cages made of metal wire mesh (8 cm D × 10 cm L) with 6 mm × 6 mm mesh size to allow the sight and smell of conspecifics. Solitarious locusts in the crowding group were all placed into a single holding cage and long-term gregarious 5th instar nymphs were added to make ten locusts inside the holding cage. This holding cage was then placed in a transparent plastic tub (25 cm L × 15 cm W × 15 cm D) with ~ 30 conspecifics (mixed male and female) in order to maximise exposure to gregarising stimuli. Control solitarious locusts were placed in identical individual wire mesh holding cages and carried to a separate holding room away from the solitarious colony to minimise confounding effects of odorants from wheat. They were placed onto a bench top and the sight of their neighbouring experimental animals was blocked using opaque cardboard rectangles (10 cm W × 20 cm L). After 30 min, the metal holding cages containing both groups were plunged into liquid nitrogen for ~5 s to snap freeze the experimental animals. Tissue samples were then stored at -80°C for 4 weeks until use.

6.2.3 RNA extraction

6.2.3.1 Extraction

Samples were removed from -80°C storage and kept on dry ice immediately prior to dissection. Watchmakers' forceps were passed through a Bunsen burner flame immediately prior to dissection. The midbrain and the prothoracic, mesothoracic and

metathoracic ganglia were dissected out on a Peltier-cooled platform, to maintain tissue at approximately -10°C, and pooled together into a sterile 1.5 mL microcentrifuge tube kept on dry ice. Samples were then processed using a commercial RNA extraction kit (RNEasy Micro Kit, Qiagen, UK, 74034). Using this kit, a solution of β-mercaptoethanol (BME; M6250, Sigma) and Buffer RLT (3.5 µL and 350 µL respectively per sample) was mixed and added to the sample tubes. The samples were then vortexed for 60 s to homogenise the tissue. They were then spun in a centrifuge (5415 R, Eppendorf, UK) at 13,200 RPM for 3 min. The supernatants were transferred to a gDNA spin column provided in the kit and spun for 30 s at 10,000 RPM to remove genomic DNA contamination. The sample flow-throughs from the columns were then treated with 350 µL 70% ethanol (in RNAse-free water), placed into an RNEasy MinElute column and then spun for 30 s at 10,000 RPM. 700 µL Buffer RW1 was then added to the columns holding the sample RNA and the columns were spun again for 30 s at 10,000 RPM. The same step was repeated, except with 500 µL Buffer RPE. 500 µL ethanol was then added to the column and the sample was spun for 2 min at 10,000 RPM. The spin column was then left open and spun at 13,200 RPM for 5 min to dry the membrane. Finally, 14 µL RNAse-free water was added to the membrane of the column and then the samples were spun at full speed for 1 min to elute the RNA. The concentrations and purity of the RNA samples were then quantified using a NanoDrop 3000 spectrophotometer. The samples were then stored at -80°C until RNA integrity analyses and cDNA synthesis steps.

6.2.3.2 RNA integrity analysis

Initial analysis of RNA integrity utilised an Agilent RNA 6000 kit, with an Agilent 2100 Bioanalyser (Agilent Biotechnologies, Germany). Although this method is more sensitive and quantitative for the assessment of RNA integrity than gel electrophoresis, the ribosomal profile of *S. gregaria* is incompatible with current computational templates, complicating the process (Shand, 2015). Only 50% of trial samples received acceptable RNA Integrity Numbers (RINs) of 9–10, the rest failed to obtain a RIN from the process. Subsequent assessments were therefore carried out using gel electrophoresis.

Before commencing gel electrophoresis, RNase*Zap*™ RNase Decontamination Solution (ThermoFisher, UK, AM9780) was sprayed onto plastic gel chambers, tanks and combs prior to use and all pipette tips were autoclaved to avoid sample contamination. To mix

the agarose gel, 2% agarose powder (A9539, Sigma) was added to TAE solution (40 mM Tris (10376743, Sigma), 20 mM acetic acid (10394970, Sigma), and 1 mM EDTA (D/0650/50, Sigma) in distilled water, pH = 8.0) and heated to boiling until total dissolution of the agarose. 2 µL ethidium bromide dye (10 mg/mL, E1510, Sigma) was added to the solution and it was then left to cool. Masking tape was used to form walls on a clear plastic chamber and a clear plastic comb was inserted into ready-made slots in the chamber wall. 50 mL agarose gel was poured into the holding chamber and left to set for 30 min. The gel was then removed from the holding chamber and the comb was carefully removed to expose the wells. The set gel was placed into the electrophoresis tank and submerged with TAE buffer, leaving a thin layer of buffer over the top of the wells. After this, 2 µL of Hyperladder 1 Kb (Bioline, UK, BIO-33053) was pipetted into the left-most well of a row. Each sample was then mixed with 2–3 µL loading dye from the Hyperladder 1 Kb kit and pipetted into an individual well, 1 µL per well. A current was passed through the electrophoresis tank at 60 V for 50 min to ensure adequate band separation. The gel was then removed from the electrophoresis tank and imaged using a camera, UV scanner and visualiser (GelVue UltraViolet Transilluminator, Syngene). All RNA samples from solitarious animals selected for cDNA synthesis showed clear, separated bands, indicating high integrity.

6.2.4 cDNA synthesis

A commercial kit was used to carry out cDNA synthesis from extracted RNA samples (Tetro cDNA synthesis kit, Bioline, UK, 65042). A mastermix of the kit reagents (Oligo $(dT)_{18}$ Primer Mix, 32 µL; Random Hexamer Primer Mix, 32 µL; dNTP Mix 10mM Total, 32 µL; RNase Inhibitor, 32 µL; 5x Reverse Transcriptase (RT) Buffer, 128 µL) was prepared to reduce pipetting errors. For each sample, one reaction was prepared with the RT enzyme and one without the enzyme as a no-RT control in the qPCR reaction. For each reaction, 9 µL of mastermix was pipetted into a well on a 96-well qPCR plate. For the RT reactions, 1 µL RT was added to this mix, or 1 µL diethyl pyrocarbonate (DEPC)-treated water for the no-RT controls. Depending on the RNA sample concentration, volumes of sample added to the reaction were scaled to add 0.15 ng RNA material to each reaction. DEPC-treated water was then added to fill the volume to 20 µL total per well. The well was then placed into a T3 Thermocycler (Biometra, Germany) and incubated at the following temperatures (Table 6.1).

Table 6.1: cDNA synthesis steps in thermocycler in chronological order.

Step #	Temperature (°C)	Time (min)
1	25	10
2	45	30
3	85	5
4	4	Until removal from thermocycler (5-15 min)

6.2.5 Transcriptome assembly and transcript expression quantification

A transcriptome assembled in previous work (Shand, 2015) was queried for identification of contigs containing candidate IEGs. This transcriptome was assembled from the brains, optic lobes and thoracic ganglia of long-term solitarious adult locusts (Cambridge lab colony) and long-term gregarious adult locusts (Leicester lab colony). Extracted mRNA from those tissues was sequenced into 1.3×10^9 reads (25–75 bp long) and the transcriptome was assembled from these reads using three assemblers: Trinity, Trans-Abyss and Bridger. Duplicate contigs were then removed to create the reduced final assembly.

With the assembled transcriptome, Tophat (https://ccb.jhu.edu/software/tophat/index.shtml) and Cufflinks (https://cole-trapnell-lab.github.io/cufflinks/) software were used to quantify the abundance of different contigs (Shand, 2015; Trapnell et al., 2012). Tophat was used to map the sequence reads to the assembled transcriptome and Cufflinks was used to analyse the mapped reads, outputting a transcript's expression as Fragments Per Kilobase of transcript per Million mapped reads (FPKM), or the proportion of a sample's reads mapped to that transcript. The differential expression of the gene between phases was analysed using the Cufflinks package CuffDiff. The resulting test statistics and *p*-values were then used to assess the evidence for differences in gene expression between long-term phase locusts. Separate comparisons were carried out in males and females in pooled brain and optic lobe samples. Comparisons between thoracic ganglia did not separate out the sexes. Poorly expressed contigs (i.e. FPKM < 0.3) were excluded from quantitative analysis due to biological and technical noise. *P*-values were corrected

for multiple comparisons to *q*-values across every contig in the transcriptome using the False Discovery Rate correction in the CuffDiff package (Shand, 2015).

6.2.6 Gene discovery

Protein sequences of candidate IEGs were obtained from the National Centre for Biotechnology Information (NCBI) database. All protein sequence queries for candidate IEGs were obtained from *D. melanogaster*. The sequences were then BLASTed against the locust transcriptome with a cutoff *q*-value of 0.00001 (Section 6.2.5), and the six closest contig matches were selected for subsequent retrieval of the nucleotide sequence from the transcriptome. The retrieved nucleotide sequences were then translated into peptide sequences with identified open reading frames using an online translation tool (http://web.expasy.org/translate/). These translated peptide sequences were aligned with each other using the online sequence alignment tool Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The region in full agreement for all 6 contigs was BLASTed back into NCBI against the reference organism and against all organisms to ensure the correct target was present. The nucleotide sequence of this region was then retained for primer design. When possible, full nucleotide sequences of the transcript were reconstructed and analysed for protein domains using the online tool SMART (http://smart.embl-heidelberg.de/smart) (Letunic et al., 2015). Scale diagrams of the protein lengths and common domains between IEGs in *S. gregaria, D. melanogaster* and *M. musculus* were constructed using the online tool Illustrator of Biological Sequences (IBS; http://ibs.biocuckoo.org/online.php) (Liu et al., 2015).

6.2.7 Primer design

The final 300 base pairs of the aligned nucleotide sequence from Section 6.2.6 were entered into the online primer design tool OligoPerfect™ Designer (https://tools.thermofisher.com/content.cfm?pageID=9716) to automatically design qPCR primers with the design specifications found in Table 6.2. The first primers from the resulting options were selected and ordered from Sigma (UK).

Table 6.2: Specifications for primer design. bp = base pairs

Condition	Minimum	Optimum	Maximum
Primer size (bp)	20	24	26
Melting temperature (°C)	60	63	65
G/C nucleotide ratio	40	50	60
Product size (bp)	70	100	120
Experimental conditions	Salt concentration = 50 mM	Primer concentration = 50 nM	

For the housekeeping reference genes *RP49* and *EFa148D*, primers were ordered according to designs validated and published previously in qPCR for *S. gregaria* (Van Hiel et al., 2009).

6.2.8 PCR

The efficacy and specificity of the primers was validated by PCR (two biological replicates per IEG). A commercial kit was used to assemble the mastermix (MyTaq™ DNA Polymerase, BIO-21105, Bioline, UK). Following the kit specifications, the mastermix was mixed using 5 µL 5× MyTaq Reaction Buffer, 0.5 µL MyTaq DNA polymerase, 0.5 µL each direction of 20 µM primers, 4 µL cDNA template and 14.5 µL nuclease-free water (ddH_2O). The mix was then incubated on a T3 thermocycler (Biometra, Germany) according to specifications on the MyTaq kit manual.

Five µL of the PCR products were run on agarose gels to examine the size of the products. A gel was prepared using the same procedure as in Section 6.2.3.2, except using 1 µL ethidium bromide per 50 mL gel, and 5 µL PCR product in each well. In addition, Hyperladder 50 bp (BIO-33054, Bioline, UK) was used instead of 1 kb due to the small size of the amplicon sequences (< 110 bp).

Table 6.3: PCR reaction thermocycler steps in chronological order

Step #	Temperature (°C)	Time
1	95	2 min
2	95	30 s
3	60	30 s
4	72	45 s
Repeat steps 2–4 34 times		
5	10	Until removal from thermocycler (overnight)

The remaining 20 µL PCR product was subsequently purified by isopropanol (IP) precipitation. The PCR products were transferred to sterile 1.5 ml tubes and 80 µl of 75% IP (in ddH_2O) was added to each tube. The samples were vortexed for 5 s, incubated at room temperature for 15 min, and then spun in a centrifuge (5415 R, Eppendorf, UK) at 4,500 RPM for 30 min at 13°C. The sample tubes were gently inverted onto tissue paper to drain the IP solution. After this, 150 µL of the same IP solution was added to each tube. The tubes were then inverted 3 times to mix the solution and spun again at 4,500 RPM for 2 min. The tubes were drained onto lab tissue once more and the step was repeated. Samples were air dried on a heat block (Grant, M6250) at 58°C for 10 min. The pellets were then re-suspended in 15 µL ddH_2O and the DNA concentrations were quantified using a NanoDrop 3000 spectrophotometer.

6.2.9 Cloning

Two IEGs of interest (*Hr38* and *JRA*) could not be sequenced using only the PCR product purification procedure, so their PCR products were cloned to improve the sequencing outcomes (Section 6.2.10). All pipette tips and tubes in this procedure were sterilised to prevent contamination.

6.2.9.1 Preparing the PCR Product

Samples of cDNA selected for cloning were run through a PCR reaction as in Section 6.2.8, but scaled down to 10 µL total reaction volume. Gel electrophoresis was conducted to confirm a successful reaction as in the same section, but with 5 µL Hyperladder and

2 µL PCR product in the wells to allow approximate quantification of the product. The products (measuring approximately 60 ng/band) were then diluted 1:2 in ddH_2O.

6.2.9.2 Bacterial culture plate preparation

To prepare plate culture media, LB medium (200 mL ddH_2O + 5 g LB powder (L1704, High salt, Melford, UK)) and LA medium (200 mL LB medium + 2.3 g agar (AGA01, Formedium, UK)) were mixed and then autoclaved. Plates were then prepared in sterile conditions (Bunsen burner flame inside fume hood, sprayed with 70% ethanol). LA-kanamycin plates were prepared in Petri dishes (9 cm diameter) with a stock solution of 200 mL LA buffer + 500 µL kanamycin stock (50 mg/ml, 11815-024, Gibco) + 200 µL Xgal stock (20 mg/ml, BIO-37035, Bioline) for blue-white colour plasmid insertion screening. Once set, the plates were transferred to a sterile workbench with a Bunsen burner flame active.

6.2.9.3 Ligation reaction

The StrataClone PCR Cloning kit (240205, Agilent Biotechnologies, UK) was used to insert the PCR product into plasmids. In brief, the ligation reaction mixture was prepared from 3 µl StrataClone Cloning Buffer, 2 µl of PCR product (5–50 ng, 1:2 diluted from before) and 1 µl StrataClone Vector Mix. The reaction mixture was gently mixed by repeated pipetting, incubated at room temperature for 5 min and placed on ice. For each ligation reaction, one tube of StrataClone SoloPack competent cells was thawed on ice, and 1 µL of the cloning reaction mixture was gently added. The mixture was then incubated on ice for 20 min, heat-shocked to 42°C in a water bath for 45 s, and transferred back on ice for 2 min. Subsequently, 250 µL LB medium pre-warmed to 42°C was added to each reaction mixture. The competent cells were then left to recover for 1 h at 37°C with agitation (Sanyo Orbital Incubator, 200 RPM).

6.2.9.4 Bacterial cell culture

After the 1 h incubation in Section 6.2.9.3, 50 µl or 100 µl of the incubated transformation mixture was pipetted onto the colour-screening plates prepared in Section 6.2.9.2. A sterile glass spreader was used to spread the mixtures onto the plate medium. The plates were then incubated overnight at 37°C to allow colony growth. The following morning, the plates were moved to room temperature for 4 h for optimal colony growth. The plates

were then checked for bacterial colonies. Using the Xgal colour-coding system, blue-coloured colonies were avoided whereas white colonies expressing the plasmid were selected. The plastic dish area surrounding these selected colonies was then marked with a permanent pen. A sterile pipette tip was then used to collect the selected white colonies and dropped into sterile glass vials containing 5 mL LB buffer + 5 µL kanamycin. These vials were then labelled using a pen and masking tape and then incubated overnight at 37°C with agitation (200 RPM).

6.2.9.5 Plasmid DNA isolation

DNA extraction from the colonies was carried out using the Plasmid DNA ISOLATE kit (Bioline, UK, BIO-52055). Briefly, 1.3 mL of the overnight cell culture from the end of Section 6.2.9.4 was pipetted into a sterile 1.5 mL centrifuge tube and spun on a centrifuge at full speed for 1 min. The supernatant was discarded; the bacterial cell pellet was then re-suspended in 250 µL of Resuspension Buffer and vortexed using a vortex mixer for 5 s. After this, 250 µL Lysis Buffer was added and the mixture was mixed by inverting 6 times. The lysis was then ceased by the addition of 350 µL Neutralisation Buffer and inverting 6 times. The samples were then spun on the centrifuge at 13,200 RPM for 10 min. The supernatant was then gently poured into the kit spin column with provided collection tube, and the column was then spun at 10,000 RPM for 1 min. 500 µL Wash Buffer AP was then added to the column and it was spun at the same settings. The same step was then repeated with 700 µL Wash Buffer BP. The column was then spun at 13,200 RPM for 2 min and then placed into a 1.5 mL centrifuge tube. After this, 50 µL ddH_2O was added to the column, left to incubate at room temperature for 1 min, and then the column was spun at 10,000 RPM for 1 min to elute the plasmid DNA. The concentration and purity of the final DNA sample were quantified using a NanoDrop 3000 spectrophotometer and the samples were then stored at 10°C for 1 d until sequencing.

6.2.10 Sequencing

Samples were sent off for Sanger sequencing at GATC, Germany (https://www.gatc-biotech.com/en/expertise/sanger-sequencing). Briefly, PCR products of the genes *EGR* and *FOS* (5 µL, 20-80 ng/µL) were pipetted into sterile 1.5 mL microcentrifuge tubes. After this, 5 µL of 5 µM forwards or reverse primer was added. The samples were then

transported to GATC at room temperature. The same procedure was used for plasmid DNA products of the genes *Hr38* and *JRA* except that their starting concentration was 80-100 ng/μL. Sequencing results were received within 4 working days. Once received, the sequencing results were aligned with the expected amplicon sequence to confirm the correct primer target.

6.2.11 qPCR

6.2.11.1 Plate setup

Once primer specificity was established, solitarious samples were run on a qPCR reaction to investigate expression levels of the four activity-related IEGs – *EGR*, *FOS*, *Hr38* and *JRA* – after 30 min crowding compared with 30 min without crowding. The 2 Housekeeping genes (HKGs) Ribosomal protein 49 (*RP49*) and Elongation factor 1α (*EFa48D*) were used as stable reference genes (Van Hiel et al., 2009). The SensiFAST™ SYBR® No-ROX Kit (Bioline, UK, BIO-98005) was used to form mastermixes for the RT-qPCR reaction. A robot (CAS1200, Corbett Robotics) was used to pipette the mastermix, primers and cDNA templates into wells on a 96-well qPCR plate. In brief, the mastermix was made up of 10 μL 2 × SensiFAST™ SYBR® No-ROX mix and 0.8 μL each of 10 μM forwards and reverse primers. In sample wells, 1 μL of cDNA template (from Section 6.2.4) and 7.4 μL ddH_2O were added to the mastermix. In the no-RT control wells (to check for genomic contamination), the cDNA template was replaced with no-RT control cDNA samples from Section 6.2.4. In water control wells (to check for primer dimer formation or water contamination), the cDNA template was replaced with ddH_2O. All biological samples were run in triplicate, with one well for the no-RT control sample, and each gene used a single water control well. All biological samples for each gene were run on a single plate, with one gene target per plate. All plates were run through the RT-qPCR reaction on the same day.

6.2.11.2 RT-qPCR reaction

Prepared qPCR plates were placed in a thermocycler fluorescence detector (CFX Connect Real Time System; BioRad). The qPCR reaction mixtures were cycled through temperatures shown in Table 6.4. SYBR green fluorescence was measured over 40 cycles. Threshold cycle (C_t) values were derived from the amplification curves using

BioRad CFX software. The C_t values for all wells were exported from the CFX software as .csv files.

Table 6.4: qPCR reaction thermocycler steps in chronological order

Step #	Temperature (°C)	Time
1	95	3 min
2	95	20 s
3	60	30 s
4	72	20 s
Repeat steps 2-4 39 times		
5	95	10 s
6	50	5 s
7	95	1 min

6.2.11.3 Calculations

Normalised relative gene expression values were calculated in RStudio v1.0.143 (RStudio.org) following Hellemans et al. (2007). In brief, a mean value per animal sample (k) per gene (j) was calculated from C_t values of triplicate wells (Ct_{jk}). The mean of Ct_{jk} values from the uncrowded control group was calculated to obtain a reference C_t (Ct_{ref}) for each gene. The Relative Quantity of each gene per animal sample (RQ_{jk}) was then calculated using:

$$RQ_{jk} = E^{Ct_{ref} - Ct_{jk}} \qquad \text{Eqn 6.1}$$

where E is the amplification factor of the gene primers (assumed to be 2).

The Normalising Factor for each animal sample (NF_k) was calculated by finding the geometric mean of the RQs of the HKGs *RP49* and *EFa48D*. The Normalised Relative Quantity (NRQ) of each gene in each animal sample was then calculated using:

$$NRQ_{jk} = \frac{RQ_{jk}}{NF_k}\ , \qquad \text{Eqn 6.2}$$

These NRQ values were then used in subsequent statistical analysis for differences between the crowded and uncrowded treatment groups. The coefficient of variation of the HKGs was also calculated following Hellemans et al. (2007) to confirm stable expression across the plates.

Amplification factors and efficiencies of the gene primers were calculated by running RT-qPCR on a 1:2 dilution series of pooled gregarious brains, with dilutions consisting of 1, 1:2, 1:4 and 1:8 in triplicate wells. A linear model was fitted in RStudio to the mean C_t values from each triplicate with the log dilution series as the predictor variable, and the slope of the linear fit was calculated (Hellemans et al., 2007). The base for exponential amplification (E) and efficiency of the primers were calculated using Eqn 6.3 and Eqn 6.4.

$$E = \log_{10}\left(\frac{-1}{slope}\right) \quad \text{Eqn 6.3}$$

$$Efficiency\,(\%) = E - 1, \quad \text{Eqn 6.4}$$

Unexpectedly, the calculated efficiencies of the IEG and HKG gene primers were unreliable, ranging from 92% to 256%, potentially due to amplification interference from primer dimerisation. For this reason, it was decided to assume an efficiency of 100% for all gene primers in subsequent calculations.

6.2.11.4 Statistical analysis

Sample sizes were $N = 7$ in the uncrowded control group and $N = 9$ in the crowded group. Median RNA concentrations and NRQs were compared between treatment groups using Mann-Whitney U tests in RStudio.

6.3 Results

6.3.1 RNA extraction

Sixteen of the 23 solitarious 5th instar CNS samples (7 uncrowded control and 9 crowded) gave mRNA yields that were sufficient for subsequent cDNA synthesis. The median RNA concentrations were 55.80 ng/µL (uncrowded control) and 45.10 ng/µL (crowded 30 min). There was no evidence for a difference between the yields in the two treatment groups (Mann-Whitney U test: W = 20, $p = 0.25$).

6.3.2 Gene Discovery

6.3.2.1 EGR

The *EGR* nucleotide sequence was assembled from the two most complete contigs showing a strong alignment with the *D. melanogaster* stripe isoform A query protein sequence (Table 6.5). The combination of the two contigs covered a full ORF flanked with a 5' and 3' UTR. The full reconstructed sequence was 5263 nucleotides long, and the ORF was 1770 nucleotides long. The protein encoded by the ORF was 589 amino acids long (Table 6.6) and, when BLASTed against *D. melanogaster* and *M. musculus* in NCBI, aligned closely with stripe isoform C (55% *S. gregaria* query covered, 59% identity, $e = 4\times10^{-97}$) and two *EGR* family proteins in *M. musculus* (*EGR1,* NP_031939.1: 23% *S. gregaria* query covered, 74% identity, $e = 4\times10^{-66}$; *EGR3,* NP_061251.1: 19% *S. gregaria* query covered, 84% identity, $e = 1\times10^{-67}$). The predicted *S. gregaria* protein showed three C2H2 Zinc Finger domains highly conserved across *D. melanogaster* stripe isoform A (NP_524395.2) and *M. musculus* EGR1 (EDK97120.1) (Figure 6.1). A coiled coil domain in the *D. melanogaster* sequence, however, was not identified in the *S. gregaria* protein or in the *M. musculus* protein. The closest protein sequence alignments between the *S. gregaria* sequence and *D. melanogaster* stripe isoform A (FBgn0003499) covered 140 identical AA residues (AA residues 998–1137 in *D. melanogaster*, 376–515 in *S. gregaria,* $e = 1\times10^{-86}$; upper bracketed line in Figure 6.1). The closest protein sequence alignments between *S. gregaria* and *EGR1* in *M. musculus* (EDK97120.1) covered 89 AA residues within this 140-residue region (AA residues 411–499 in *S. gregaria,* 320–408 in *M. musculus*: 100% *S. gregaria* query covered, 94% identity, $e = 7\times10^{-58}$; lower bracketed line in Figure 6.1). The strongest hits in the NCBI database were other C2H2 Zinc Finger Domain-containing proteins in other organisms such as

Zinc Finger Protein of *Oryctes borbonicus* (83% *S. gregaria* query covered, 50% identity, e = 1×10^{-150}) and dendritic arbor reduction protein 1 of *Aedes aegypti*（80% *S. gregaria* query covered, 46% identity, e = 2×10^{-119}). Curiously, the *S. gregaria* EGR protein sequence did not show a BLAST hit with the reported sequence for a EGR protein in *L. migratoria* on NCBI (AHB17949.1) – which is certainly a mis-named *JUN* (see Discussion).

Table 6.5: Table of IEG queries and the longest matching contigs within the existing transcriptome. Queries sequences are taken from *D. melanogaster* in the National Centre for Biotechnological Investigations (NCBI.com).

Query	NCBI ID	Contig ID	Query E-value vs transcriptome	Contig length (nucleotides)
EGR (*stripe* isoform A)	NP_524395.2	k41.R4699496 (partial)	0.55×10^{-86}	2180
		k51.J1550316 (partial)	0.1×10^{-82}	4335
FOS (*kayak*)	NP_001027579.1	k51.J1575726	0.3×10^{-12}	4382
Hr38 (isoform D)	ACZ94310.1	k51.R1562459	0.1×10^{-164}	2887
JRA	NP_476586.1	comp33188_seq0	0.4×10^{-43}	1922

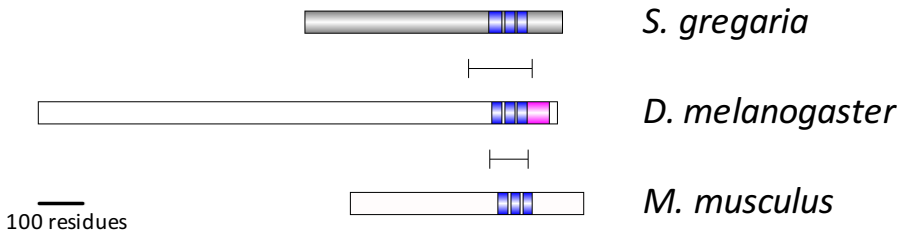


Figure 6.1: Scale representation of EGR protein sequences of *S. gregaria* (grey, top), *D. melanogaster* (stripe isoform A, white, middle) and *M. musculus* (EGR1; white, bottom). Blue regions indicate conserved C2H2 Zinc Finger domains. Pink region indicates a coiled coil domain. Bracketed lines span sections of sequence with closest alignment between *S. gregaria*, *D. melanogaster* (top bracketed line) and *M. musculus* (bottom bracketed line).

Table 6.6: Open Reading Frame (ORF) protein sequences and lengths for identified IEGs.

Target IEG	ORF length (amino acid residues)	Protein sequence
EGR1	589	MIMDGVETLAPHPPLLHPHPLFLPAESAAAAAVAAAAHHFNVLSFDCLYSGKDAVAGAAGSPSSPATAASAAASSTV PAPLAAEPAAPEPQPGDLNTPVTTSGDIPSFFGPSTVVEPPPITGSLDPEELSLEPSAAGTPSAATASPALVDAKPEARLSP EPGTSAAPAPSSAPASASAATPDEESSNTAGSTMYPGSQGRVSYRGIFTATASDQWLTDKLAQQQQQQQQQQQQQQQ FFGLAPPQSYPGSPASGAYEDVRTQQQQQAELLGLSMDNLPLKQPPGYPSCVSDVSQQPELYGRPSGKYPWLEQQEY AAAAAAGPSGLVPKQEPPTSAAAAVVAAAAAAVFGGDVQQPPSAQGNGGEQTQRPSQAYSPQVQLAEYNPSTSKGH EILSQVYQQSNVPLKLVPVKPRKYPNRPSKTPVHERPYACPVENCDRRFSRSDELTRHIRIHTGQKPFQCRICMRSFSRS DHLTTHIRTHTGEKPFSCDVCGRKFARSDEKKRHAKVHLKQRMKKESKLVTSTSTAQQQQQQQQQQQQQQQQQAL HHHHHLSHHHHGHPHGHHGHAHGHASTSGMQQVEDALALPVTTSL
FOS	373	MEPIDLSSLIPPELWFQQFVTLDGLNSGVPTRTTATLTPTTLRNIEQTLIDLECEVSSHQNEAGFVPPVVQPVSQVGGTY MSLLESKPAWQADVGQQAARPTVVTTASVLPTTVATTATIPVTAANVTAAPRRNVGGRRPTKNTGISPEEEERRRVRR ERNKMAAARCRKRRLDHTNALLQETEGLEQKKQGLQLEIQQLTQQKEDLEFLLEAHQRHCRLNGTAGSCSPLDVKPF DYRPPTSVSEEMCVKRETDEDPHGPPPAKRLAPEPSLPGPVAGTGGVAKPPRPNSLPVAAAFTPAAGTLKVAASVSEV AGVPVTTPSAGMPFNFDSLMEGGTGLTPVSAPLIPSCSSQQRNSADLASPDAVPAKLVSL
Hr38	623	MLLLQTQAPFGSSSFADLLSAPYSEETGALSEDIDPFPDVVFQTAPEPPAPAPAPAPAPAPLPSFQETYSPRYPHQAAAA AGPYKMDDDCYNVAPPYNHHQQHQPHHHHHQVAGASPYEFAPSPHPQHAPFAPYFPPAGAPPPPQQPPQPSPGAAAA AGFDPLVCHESYHFPSELHVAATALSPRQRRASLPLQRSESTTSSSSESPKLRVLTAAGPPPSASSSASSSPGAGPGPEAL VAPPASVAAAAVAAASAQPPPPPAAPGPSGSRAGPAGSAAPPSPSQLCAVCGDTAACQHYGVRTCEGCKGFFKRTVQ KGSKYVCLADKACPVDKRRRNRCQCRFQKCLAVGMVKEVVRTDSLKGRRGRLPSKPKSPQESPPSPPVSLITALVRA HVDTSPDLASLDYSQYREPSPTEPVISEAEKVQQFYNLLTTSVDVIRAFADKIPGFTDLCREDQELLFQSASLELFVLRL AHRTRPDDTKLTFCNGVVLDKQQCQRSFGDWLHAIMEFCQSLHAMDIDISAFACLCALTLVTERHGLKEPHKVEQLQ MKIISSLRDHVTYNAEAQRKPHYFSRLLGKLPELRSLSVQGLQRIFYLKLEDLVPAPPLIENMFVASLPF
JRA	292	MVRNCAVPRMETTFYEEAAAYQQRLGDVNHLKRSLTLDLNSVRSASNAKRPRFNASVTNGMVGGPPVLSSPDLNLL KIGSPELEKLIIAQSGLVTTAPTPTPSQILFPPTVTEEQEMYARGFVDALNELHTSDSSQMSAGLDVPVTSSNGVTYTTLE SHAGGGGGHVIQQTTFPRLSSYGVVMSPPEVVVKDEPQTVPSLGSSPPLSPINMECQEKIKLERKRQRNRIAASKCRRR KLERISRLEEKVKQLKGENSELGSVVHKLKEQVCHLKEQVMDHVHSGCQIMVSSHF

6.3.2.2 FOS

The transcript for the gene *FOS* was located in several contigs, flanked with non-coding UTRs. Three different putative isoforms of the protein sequence were found within these contigs, which differed in their first 40 AA residues but showed high homology with FOS-like protein sequences in the NCBI database. One isoform (contig k51_J1575726; Table 6.5) was selected for closer analysis. The ORF was 1,119 nucleotides and 373 amino acids long (Table 6.6). When BLASTed against *D. melanogaster* and *M. musculus* in NCBI, the protein sequence showed the closest homology with *D. melanogaster kayak* (isoform D, NP_001027580.1; 68% *S. gregaria* query covered, 33% identity, e = 3×10^{-38}) and members of the *M. musculus* Fos transcription factor family (*FRA-1* (AAC52888.1), 22% *S. gregaria* query covered, 60% identity, e = 3×10^{-13}; *FOS* (NP_034364.1), 26% *S. gregaria* query covered, 50% identity, e = 1×10^{-12}). The closest alignment between the *S. gregaria* predicted protein and *D. melanogaster* kayak isoform A (NP_001027579.1) and *M. musculus* FOS was in a 25-residue region within a Basic Region Leucine Zipper domain (BRLZ; AA residues 255–280 in *D. melanogaster*; 148–172 in *S. gregaria*; 134–159 in *M. musculus*; *S. gregaria-D. melanogaster*: 100% *S.*

gregaria query covered, 100% identity, e = 3× 10^{-20}; *S. gregaria-M. musculus*: 100% *S. gregaria* query covered, 76% identity, e = 4× 10^{-12}; Figure 6.2 bracketed lines). The strongest protein hits in the NCBI database were FRA A isoform in *G. bimaculatus* (BAX36487.1; 95% *S. gregaria* query covered, 61% identity, e < 1× 10^{-250}) and FOS protein in the termite *Zootermopsis nevadensi*s (BAX36487.1; 95% *S. gregaria* query covered, 62% identity, e = 3× 10^{-177}). The strongest protein sequence hit in *L. migratoria* was the confusingly named EGR1 (AHB17949.1; 39% *S. gregaria* query covered, 24% identity, e = 8 × 10^{-9}).

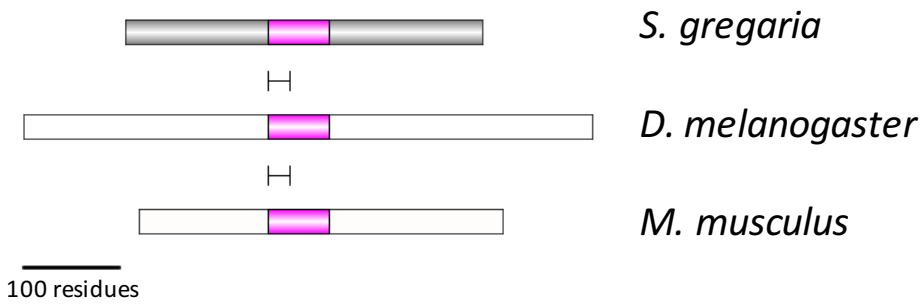


Figure 6.2: Scale representation of FOS protein sequence of *S. gregaria* (grey, top), *D. melanogaster* (FOS isoform A, white, middle) and *M. musculus* (FOS, white, bottom). Pink region indicates a Basic Region Leucine Zipper domain. Bracketed lines represent sections of sequence with closest alignment between *S. gregaria*, *D. melanogaster* (top bracketed line) and *M. musculus* (bottom bracketed line).

6.3.2.3 Hr38

The transcript of *S. gregaria Hr38* was located in several contigs, flanked with non-coding UTRs. The longest contig containing the full sequence was k51.R1562459 (Table 6.5), with an ORF length of 1869 nucleotides (623 AA residues; Table 6.6). When BLASTed against *D. melanogaster* and *M. musculus* in NCBI, the protein sequence showed the closest homology with *D. melanogaster* Hr38 (NP_001163024.1; 82% *S. gregaria* query covered, 67% identity, e < 1 × 10^{-250}) and *M. musculus* nuclear receptor subfamily 4 group A member 2 (*Nurr1*; NP_038641.1; 60% *S. gregaria* query covered, 67% identity, e < 1 × 10^{-250}). The predicted *S. gregaria* protein showed two conserved domains with *D. melanogaster* Hr38 isoform D protein (ACZ94310.1), namely C4 Zinc Finger in Nuclear Hormone Receptors (ZnF_C4, Figure 6.3 blue) and a Ligand Binding

Domain of Hormone Receptors (Figure 6.3 green). The region with the closest alignment between *S. gregaria* and *D. melanogaster* was at AA residues 745–1078 (*D. melanogaster*) and 285–623 (*S. gregaria*; 82% *S. gregaria* query covered, 67% identity, $e < 1 \times 10^{-250}$; Figure 6.3). The two regions of closest alignment between *S. gregaria* and *M. musculus* (NP_038641.1) were *M. musculus* AA residues 263–374 (100% *S. gregaria* query covered, 92% identity, $e = 1 \times 10^{-101}$) and 555–598 (84% *S. gregaria* query covered, 81% identity, $e = 1 \times 10^{-22}$), corresponding to AA residues 275–386 and 567–610 in *S. gregaria* (Figure 6.3 bracketed lines). The strongest hits in the NCBI database (confirmed and complete entries only) were nuclear receptor subfamily 4 group A member 2 isoform X1 in the ant *Pseudomyrmex gracilis* (XP_020279543.1; 100% *S. gregaria* query covered, 60% identity, $e < 1 \times 10^{-250}$) and nuclear receptor subfamily 4 group A member 2 in the mosquito species *Aedes aegypti* (XP_021701958.1; 95% *S. gregaria* query covered, 63% identity, $e < 1 \times 10^{-250}$). The closest match in *L. migratoria* was ecdysone receptor (AAD19828.1; 64% *S. gregaria* query covered, 29% identity, $e = 1 \times 10^{-48}$).

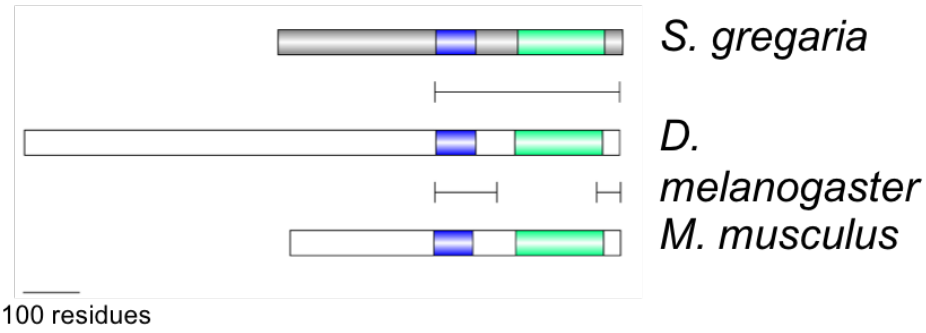


Figure 6.3: Scale representation of Hr38 protein sequence of *S. gregaria* (grey, top), *D. melanogaster* (Hr38 isoform A, white, middle) and *M. musculus* (nuclear receptor subfamily 4 group A member 2, white, bottom). Blue region indicates a C4 Zinc Finger in Nuclear Hormone Receptors (ZnF_C4) domain. Green region indicates a Ligand Binding Domain of Hormone Receptors. Bracketed lines represent the section of sequence with closest alignment between *S. gregaria*, *D. melanogaster* (top bracketed line), and *M. musculus* (bottom bracketed line).

6.3.2.4 *JRA*

The transcript for the *S. gregaria* gene *JRA* was located in several contig matches to the query sequence (Table 6.5) and flanked with non-coding UTRs. The longest contig containing the full sequence was comp2996_seq0, with an ORF length of 876 nucleotides (292 AA residues; Table 6.6). When BLASTed against *D. melanogaster* and *M. musculus* in NCBI, the protein sequence showed the closest homology with *D. melanogaster* JRA isoform A (NP_476586.1; 86% *S. gregaria* query covered, 41% identity, e = 1×10^{-55}) and *M. musculus* JUND isoform D (NP_001273873; 74% *S. gregaria* query covered, 49% identity, e = 5×10^{-72}). The protein showed conserved BRLZ domains with the query *D. melanogaster* Jun isoform A (NP_476586.1) and *M. musculus* c-jun (1411298A) proteins (Figure 6.4 pink). The closest alignment between *S. gregaria* and *D. melanogaster* was between AA residues 204–248 (*D. melanogaster*) and 208–252 (*S. gregaria*; 100% *S. gregaria* query covered, 82% identity, e = 1×10^{-30}). The closest alignment between *S. gregaria* and *M. musculus* was AA residues 208–252 (*S. gregaria*) and 247–291 (*M. musculus*; 100% *S. gregaria* query covered, 100% identity, e = 1×10^{-31}; Figure 6.4 bracketed lines). The strongest hits in the NCBI database were with the same *EGR1* sequence in *L. migratoria* as with *FOS* (AHB17949.1; 100% *S. gregaria* query covered, 98% identity, e < 1×10^{-250}), transcription factor AP-1 in *Z. nevadensis* (XP_021915872.1; 100% *S. gregaria* query covered, 75% identity, e = 8×10^{-148}) and *JRA* in *G. bimaculatus* (BAX36489.1; 100% *S. gregaria* query covered, 69% identity, e = 1×10^{-138}).

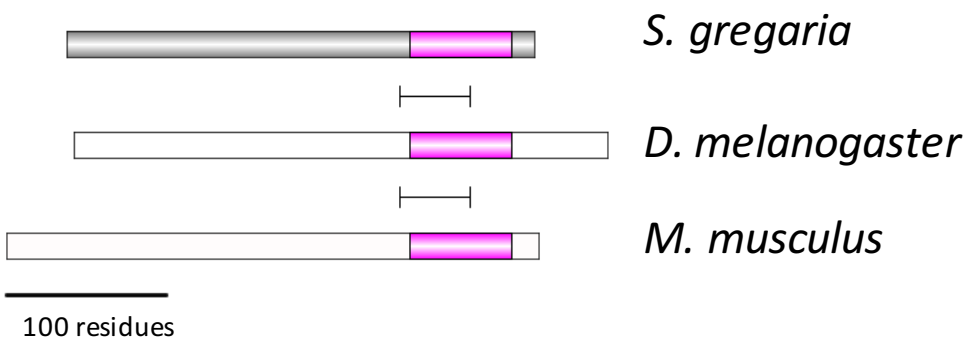


Figure 6.4: Scale representation of JRA protein sequence of *S. gregaria* (grey, top), *D. melanogaster* (JRA isoform A, white, middle) and *M. musculus* (Jun protein, white, bottom). Pink region indicates a Basic Region Leucine Zipper domain. Bracketed lines represent sections of sequence with closest alignment between *S. gregaria*, *D. melanogaster* (top bracketed line) and *M. musculus* (bottom bracketed line).

6.3.3 Primer validation

Primers were targeted within the final 300 bp regions of the four IEG nucleotide sequences. Their characteristics are shown in Table 6.7. The primers were tested in trial PCR reactions using cDNA from 5th instar gregarious locusts. The resulting electrophoresis bands indicated a clean amplification that matched the expected target size (Figure 6.5). All four IEG primer pairs also gave faint bands approximately 50 bp in size, possibly indicating primer dimerisation. However, as the primer dimer amplification was only taking place after ~ cycle 40 in the absence of template in RT-qPCR (data not shown), this was deemed acceptable for the amplification of cDNA template with these primers. Cloning of the PCR products and Sanger Sequencing by Biotech GATC (data not shown) confirmed successful amplification of the target sequence. The primers were therefore deemed suitable for use in RT-qPCR.

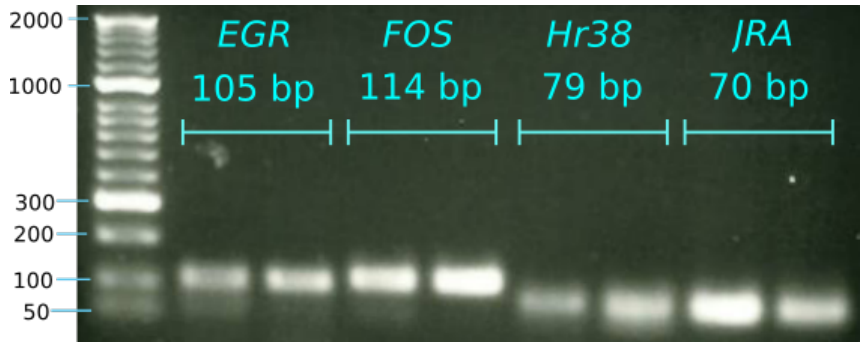


Figure 6.5: Confirmation of primer specificity for IEG targets. Gel electrophoresis of IEG primer PCR products, with a DNA ladder (left, numbers in base pairs) to assess the size of the amplified target. Each IEG was run in duplicate wells, with one biological sample (5th instar gregarious locust) per well. Numbers in blue are the target sizes.

Table 6.7: Primer design details for each IEG identified.

Gene	Target size	Primer direction	Melting temperature (°C)	Sequence (5'3')
EGR	105	Forwards	67.5	CTTCAGCTGCGACGTCTGTG
		Reverse	66.4	ATGAAGAAGGAGAGCAAGCTGGT
FOS	114	Forwards	66.9	TGATTCTCTTATGGAAGGGGGAAC
		Reverse	66.2	GGAACAGCATCTGGAGAAGCTAAG
Hr38	79	Forwards	65.9	GGAAACTTCCTGAACTTCGATCAC
		Reverse	66.7	GGAACAAGATCTTCCAGCTTCAGA
JRA	70	Forwards	66.1	GAGAAAATAGCGAGCTAGGCAGTG
		Reverse	66.1	ACCTGTTCTTTTAGATGGCACACC

6.3.4 No evidence for short-term crowding effect on IEG expression

Overall, there was no evidence for an effect of 30 min crowding on the expression of the IEGs analysed in this experiment (Figure 6.6). Median *EGR* expression for the crowded group was approximately 25% lower than in the control group (Table 6.8, Figure 6.6A). However, a Mann-Whitney U test revealed no evidence for this difference. Other differences between treatment groups gene expression were minimal (Table 6.8, Figure 6.6B-D), and Mann-Whitney U comparisons provided no evidence for differences between the two groups. One apparent outlier sample in the *Hr38* crowded group showed an NRQ value of 3.43, more than three times that of the median value of 1.04. Raw C_t values indicated that the most abundant target in the wells was the HKG *EFα48D*, with *Hr38* showing the lowest abundance (Table 6.9). A fraction (21%) of No-RT control and water control wells revealed amplification, with C_t values ranging from 37.9 to 39.9 (not shown).

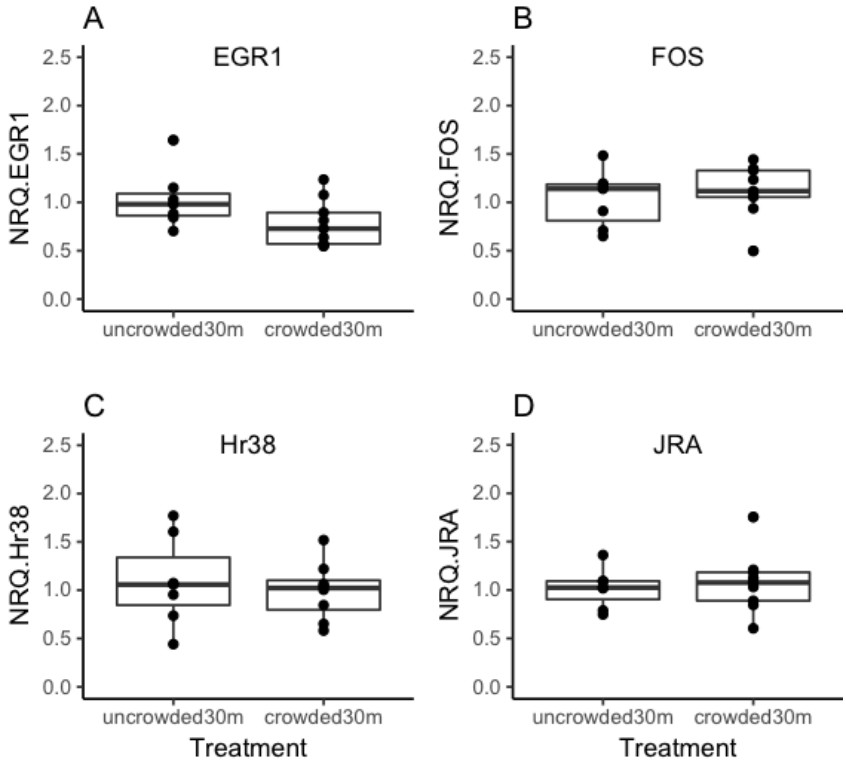


Figure 6.6: No evidence for changes in the CNS expression of four IEGs over 30 min crowding in solitarious locusts. Normalised Relative Quantity (NRQ) gene expression levels for 6 target genes for solitarious 5th instar locusts either crowded for 30 min (crowded30m, $N = 7$) and control locusts kept isolated for 30 min (uncrowded30m, $N = 9$). Genes shown are IEGs *EGR* (A), *FOS* (B), *Hr38* (C) and *JRA* (D). Not shown is an outlier in the crowded30m group in *Hr38* (C) that has an NRQ value of 3.43.

Table 6.8: Statistical analysis of gene expression in solitarious 5th instar locusts exposed to 30 min crowding, or left uncrowded for 30 min.

Gene	Uncrowded median NRQ	Crowded median NRQ	Mann-Whitney U *W*-value	*p*-value
EGR	0.98	0.73	47	0.11
FOS	1.14	1.11	27	0.68
Hr38	1.06	1.04	32	1.00
JRA	1.03	1.08	26	0.61

Table 6.9: Raw C_t values of amplification in HKGs and IEG targets, arranged in order of transcript abundance (median C_t in uncrowded control group) from highest to lowest.

Gene	Uncrowded median C_t	Crowded median C_t
EFα48D	25.19	25.24
RP49	26.93	26.94
FOS	31.11	30.58
JRA	31.83	31.54
EGR	33.43	33.66
Hr38	35.71	34.81

6.3.5 No evidence for long-term phase effect on IEG abundance in transcriptome

6.3.5.1 EGR

Analysis of IEG expression in the existing transcriptome revealed no clear effect of long-term crowding on IEG expression. The two contigs used for reconstruction of *EGR* showed low abundance, with the highest expression of only 3.02 FKPM for contig k51.J1550316 in solitarious female brain samples (Table 6.10). In all samples, contig k41.R4699496 failed to reach an adequate abundance for statistical comparisons. Contig k51.J1550316 showed little difference in expression between the two phases in any comparison, with fold-changes of 0.93-1.30, and was absent from the thoracic ganglia samples.

Table 6.10: Gene expression analysis of *EGR*-associated transcripts across different comparisons. Expression = transcript expression. Sol = solitarious, Greg = gregarious.

Comparison	Contig ID	Sol expression (FKPM)	Greg expression (FKPM)	Fold-change (Greg/ Sol)	*q*-value
Male (brain)	k41.R4699496	0.26	0.30	1.16	Abundance too low
Female (brain)	k41.R4699496	0.24	0.32	1.30	Abundance too low
Thoracic ganglia	k41.R4699496	0.34	0.21	0.62	Abundance too low
Male (brain)	k51.J1550316	3.46	3.52	1.10	1.00
Female (brain)	k51.J1550316	3.03	2.80	0.93	0.87

6.3.5.2 *FOS*

The contig containing the full *FOS* sequence was not available in the quantification output, so an alternative contig with the same *kayak* query score was selected (c211433_g1_i1, e-value = 1×10^{-13}; Table 6.11). Across the brain and thoracic ganglia, *FOS* expression showed expression values of 7.1-10.9 FKPM, suggesting a relatively low abundance overall. Gregarious locusts showed 17-42% more *FOS* expression than solitarious locusts, however, statistical analysis provided no evidence for a real effect.

Table 6.11: Gene expression analysis of *FOS*-associated transcripts across different comparisons. Expression = transcript expression. Sol = solitarious, Greg = gregarious.

Comparison	Contig ID	Sol expression (FKPM)	Greg expression (FKPM)	Fold-change (Greg/ Sol)	*q*-value
Male (brain)	c211433_g1_i1	7.14	10.32	1.42	0.15
Female (brain)	c211433_g1_i1	7.70	10.97	1.42	0.61
Thoracic ganglia	c211433_g1_i1	7.96	9.32	1.17	0.94

6.3.5.3 *Hr38*

All contigs for *Hr38* showed low expression levels, with none reaching 0.6 FKPM. The contig k51.R1562459 showed a maximum expression value of 0.52 FKPM (Table 6.12) in the brains of male solitarious 5th instar locusts. The thoracic ganglia showed negligible expression of *Hr38*. Abundance was also too low to reliably compare the two phases, which showed differences of 12-13% across the two phases in the brain, but no change in the thoracic ganglia.

Table 6.12: Gene expression analysis of *Hr38*-associated transcripts across different comparisons. Expression = transcript expression. Sol = solitarious, Greg = gregarious.

Comparison	Contig ID	Sol expression (FKPM)	Greg expression (FKPM)	Fold-change (Greg/ Sol)	*q*-value
Male (brain)	k51.R1562459	0.52	0.45	0.87	Abundance too low
Female (brain)	k51.R1562459	0.20	0.23	1.12	Abundance too low
Thoracic ganglia	k51.R1562459	0.03	0.03	1	Abundance too low

6.3.5.4 JRA

The contig containing the full *JRA* sequence was not available in the quantification output, so the longest alternative contig with a similar JRA protein sequence query score was selected (c173173_g1_i1, e-value = 1×10^{-43}; Table 6.13). The expression of this contig was relatively high, with a minimum expression value of 24.18 FKPM in the thoracic ganglia of female gregarious locusts. Gregarious locusts expressed 29-47% more *JRA* in the brain than did solitarious locusts, but 16% less than solitarious locusts in the thoracic ganglia. Statistical analysis provided no evidence for a difference between the two phases.

Table 6.13: Gene expression analysis of *JRA*-associated transcripts across different comparisons. Expression = transcript expression. Sol = solitarious, Greg = gregarious.

Comparison	Contig ID	Sol expression (FKPM)	Greg expression (FKPM)	Fold-change (Greg/ Sol)	*q*-value
Male (brain)	c173173_g1_i1	29.46	37.79	1.29	0.87
Female (brain)	c173173_g1_i1	27.38	40.40	1.47	0.13
Thoracic ganglia	c173173_g1_i1	29.00	24.18	0.84	0.94

6.4 Discussion

In this chapter, the nucleotide sequences of four IEGs were extracted from a previously assembled transcriptome. Primers were generated for these four IEGs and their expression was analysed using RT-qPCR. Crowding solitarious 5th instar locusts for 30 min was insufficient to change the expression of any of the four IEGs compared with uncrowded controls. Furthermore, no evidence was found for long-term phase-specific differences in IEG expression.

6.4.1 *S. gregaria* IEG protein sequences show conserved regions with *D. melanogaster* and *M. musculus* proteins

6.4.1.1 EGR

The putative sequence for *S. gregaria* EGR protein was reconstructed in this chapter and closely aligns with stripe isoforms in *D. melanogaster* and *EGR3* and *EGR1* proteins in *M. musculus*. Furthermore, the closest alignments within this protein sequence with *D. melanogaster* and *M. musculus* were within conserved C2H2 Zinc Finger domains, common to the EGR protein family amongst others. The finding that close matches to this sequence in NCBI included distinct C2H2 Zinc-Finger domain-containing proteins such as Dendrite arbor protein 1 may question whether or not this sequence belongs to the EGR family. However, its high homology with the EGR orthologue *AmEGR* in *Apis mellifera*, used in previous work as an activity marker (Lutz and Robinson, 2013; Ugajin et al., 2013), and stripe (Chen et al., 2016) provides reasonable basis to investigate an IEG-like activity-related upregulation of this sequence.

6.4.1.2 FOS

Three transcript variants for the *S. gregaria FOS* gene were retrieved from the transcriptome in various contigs with at least two contigs showing each different variant. The three variants differed in their first 40 AA residues, and were otherwise identical. This finding could imply different isoforms, as regions within the first 40 AA residues of all variants were matching with other species in NCBI (not shown). Another possibility is that different splice variants have been detected in this transcriptome. In mammals, an alternative different splice variant of *FosB*, *ΔFosB*, is thought to influence the half-life of expressed FRA proteins, and by extension, the neuronal function (Kovács,

1998). The *D. melanogaster* orthologue *kayak* has more recently been found to show different splice variants (Hudson and Goldstein, 2008). Although an intriguing avenue for future work in *S. gregaria*, this result also indicates that primers designed for the final region of the protein will amplify multiple variants in the sample, complicating subsequent RT-qPCR quantification. However, in the scenario that one variant is activity-related and others are not, this would likely only dilute an increase in the variant, rather than hide it. As they all aligned equally with the query sequences from *D. melanogaster* and *M. musculus*, one variant was selected for further homology analysis. Further BLAST searches on NCBI revealed that this sequence matched closely with *FOS* family proteins in other species, as well as *D. melanogaster* and *M. musculus*, indicating that this is the correct target for a *S. gregaria* orthologue of the FRA protein. Furthermore, the presence of the conserved BRLZ domain in this sequence adds evidence for the functional similarity of this sequence to that of the Fos protein family in other species. Due to its widely established use in neuronal activity marker in vertebrates (Guzowski et al., 2005), it can be suggested as a potential IEG candidate for this chapter. Although a previous study found no activity-related expression of *kayak* in the brain of *D. melanogaster* (Chen et al., 2016), and although it has not been widely used in invertebrate studies as an activity marker, it is unclear if this lack of activity-related expression was applicable to all invertebrate orthologues of FOS proteins, thus this protein was reserved for targeting in qPCR.

6.4.1.3 Hr38

The protein sequence for Hr38 protein was extracted from one contig in the transcriptome. Its close homology with the query sequences as well as orthologues of other species indicates that this is the *S. gregaria* orthologue of the *Hr38* gene. The conserved protein domains ZnF_C4 and Ligand Binding Domain of Hormone Receptors also add evidence for the functional similarity of this sequence to that in the other model organisms. Activity-related *Hr38* expression has previously been shown in *D. melanogaster* and the silkmoth *Bombyx mori* (Chen et al., 2016; Fujita et al., 2013), indicating its potential utility in locust species.

6.4.1.4 JRA

The protein sequence for *S. gregaria JRA* was extracted from one contig in the transcriptome, showing close homology with *JRA* in *D. melanogaster*, and a member of the Jun family in *M. musculus*. The close alignments in the BRLZ protein domain also imply functional conservation within the different orthologues. The fact that this protein matches best with AP-1 and JRA protein sequence entries from other species also strengthens evidence for its orthologue identity for *JRA*. The finding that it best matches a protein sequence labelled EGR1 in *L. migratoria* at the same time as matching Jun-related proteins in several other species suggests that the entry for *L. migratoria EGR1* (AHB17949.1) is mistakenly named, and in fact is the *L. migratoria* orthologue of the *JRA gene*. Although not found to be activity-related in *D. melanogaster* (Chen et al., 2016), this gene has been used previously as an activity marker in *A. mellifera* (Alaux and Robinson, 2007; McNeill and Robinson, 2015; Sommerlandt et al., 2016), indicating a potential for neuronal activity-related expression in *S. gregaria* as well.

6.4.2 IEG expression is relatively low in the assembled transcriptome

All target IEGs in this chapter, with the exception of *JRA*, showed low transcript abundance, to the point of some failing to meet the abundance threshold for statistical analyses between the two long-term phases. *Hr38* was particularly poorly expressed, with a FKPM of almost 0 in the thoracic ganglia. This was reflected in the higher C_t values of the *Hr38* reactions in the RT-qPCR (Table 6.9). It was interesting to observe that the relative abundance of the four IEGs was preserved across transcriptomic and qPCR quantification, implying a stable basal expression of these genes. The low abundance found in the transcriptome agrees with consensus regarding low basal expression of IEGs in CNS tissue (Bahrami and Drabløs, 2016; Guzowski et al., 2005).

6.4.3 No evidence for short-term or long-term phase effects on IEG expression

6.4.3.1 No evidence for an effect of long-term crowding on four IEG orthologues in *S. gregaria*

From overall comparisons between long-term phases in the transcriptome assembled previously (Shand, 2015), there was no evidence for phase-specific expression of any of the target IEGs. Crowded environments provide a complex set of sensory stimuli, which has already been suggested to explain morphometric brain differences in the gregarious

phase compared with the solitarious phase of *S. gregaria* (Ott and Rogers, 2010). This also increases the necessity for avoiding aversive and likely stressful stimuli such as collisions in flying adults (Matheson et al., 2004) and the threat of cannibalism in marching bands (Bazazi et al., 2008). The IEGs *FOS* and *EGR*1 have been shown to be responsive to acute (Cullinan et al., 1995; Sandner et al., 1993) and chronic stressful stimuli in rats (Sharp et al., 1991), but remain transient even during the chronic stimulus. However, some neuronal populations were observed to display chronic upregulation of *FOS* even 5 d the onset of chronic stress (Brown and Sawchenko, 1997). Thus the question addressed in this section was: does the complex crowd environment of gregarious locusts provide stressful stimuli to the individual locust, and if so, does this induce chronic upregulation of IEG expression detectable across the CNS?

The lack of evidence for an upregulation in IEGs from the transcriptome suggests that either (1) chronic upregulation of IEGs caused by the stressful environment is not detectable across whole CNS tissues and only takes place in different cell populations, (2) the upregulation of IEGs in response to the stressful environment is only transient and is activated only in situations of stress within the crowd setting or (3) chronic crowd stimuli are not sufficiently stressful to induce IEG upregulation in *S. gregaria*. The first interpretation implies that a histological approach is required to locate IEG expression in response to chronic crowding stimuli. The second interpretation indicates that IEGs undergo only transient upregulation in response to specific stimuli in this context (Bahrami and Drabløs, 2016). The third interpretation is intriguing as this would have major implications for the neuroscience of swarming behaviour in *S. gregaria*. OA, a known stress-related neurohormone in arthropods (Verlinden et al., 2010), shows little difference in CNS concentrations between long-term gregarious and long-term solitarious 5th instar nymphs (Rogers et al., 2004). Long-term gregarious locusts have increased transcription of stress related heat shock proteins (Badisco et al., 2011). This may allow a normalising compensatory response to the increased stressors of the crowd, and keep other stress-sensitive mechanisms such as IEGs and OA sensitive to new stressors. This question merits further work to understand the physiology of crowding conditions on *S. gregaria*, such as investigating the physiological effect of acute stressors on chronically crowded locusts compared with solitarious locusts.

6.4.3.2 No evidence for an effect of 30 min crowding on four IEG orthologues in *S. gregaria*

The RT-qPCR experiments provided no evidence for upregulation in the four candidate IEGs during 30 min crowding of solitarious locusts. One IEG candidate, *EGR*, even exhibited a 25% decrease in expression in the crowded group compared with the uncrowded controls, though this effect was not supported statistically. This suggests that these four IEGs are not suitable for assessing neuronal activity in *S. gregaria* during crowd-induced gregarisation. The four IEGs were selected based on their use in previous work (Alaux and Robinson, 2007; Chen et al., 2016; Fujita et al., 2013; Guzowski et al., 2001; Lutz and Robinson, 2013; McNeill and Robinson, 2015; Sommerlandt et al., 2016), with the hypothesis that at least one of these orthologues in *S. gregaria* would demonstrate robust upregulation with exposure of a solitarious locust to a crowd stimulus. However, it is important to remember that this experiment relied on several major assumptions at the outset. This section re-examines the assumptions of this experiment in light of the results obtained.

The first assumption of this experiment was that this procedure resulted in widespread neuronal activation that would enable the detection of activity-related expression of the IEGs. Previous work has assessed the activity-related expression of candidate IEGs by using non-physiological methods (Fujita et al., 2013; Chen et al., 2016). To confirm the activity-related expression of IEGs in the CNS of *S. gregaria*, it would be useful to assess their expression in response to widespread neuronal activity induced by, for example, the GABA receptor antagonist picrotoxin, as has been tested in recent work in *G. bimaculatus* (Watanabe et al., 2017).

The second assumption of this experiment was that 30 min crowding was sufficient to elicit behavioural gregarisation. Unlike in previous chapters of this thesis, the behavioural gregariousness of the crowded locusts was not quantified due to time restraints. There was therefore no confirmation of a salient effect of 30 min crowding on the behavioural phase state of the treatment group compared with controls. Future experiments may benefit from including a confirmation experiment in a fraction of the cohort. Furthermore, it will be useful to understand the effects of different gregarising procedures such as tickling the hind leg, a potent gregarising stimulus in *S. gregaria*

(Anstey et al., 2009; Roessingh et al., 1998; Rogers et al., 2003), which would provide evidence for a gregarisation-specific IEG response to behavioural gregarisation.

The third assumption of this experiment was that any IEG expression induced by crowd-induced behavioural gregarisation would be the same well-defined rapid and transient IEG upregulation as expected in events involving strong neuronal activation (Guzowski et al., 2001, 2005). At the outset, behavioural gregarisation was expected to induce the same IEG expression as shown in other non-associative stimulus presentation paradigms such pheromonal exposure in *Bombyx mori* (Fujita et al., 2013), and acute and chronic stress paradigms in mammals (Cullinan et al., 1995; Sandner et al., 1993; Sharp et al., 1991; Umemoto et al., 1997; Bonaz and Rivest, 1998), which are shown to follow the same classical IEG expression patterns as during a discrete behaviour (Kiya et al., 2008; Ugajin et al., 2012) or associative learning paradigm examined in other work (Guzowski et al., 2001; Sandner et al., 1993; Swank and Bernstein, 1994). However, it is possible that gregarisation differs from these presentations by being a continuous, cumulative and labile process (Roessingh et al., 1998; Rogers et al., 2003; Simpson et al., 2001). Although 30-60 min after a stimulus is widely found to be the time point at which IEG expression is maximal (Bahrami and Drabløs, 2016; Guzowski et al., 2005), 30 min crowding is unlikely to demonstrate robust behavioural gregarisation in 5th instar solitarious animals, as typically behavioural gregarisation is only observed after 1–4 h crowding in *S. gregaria* (Anstey et al., 2009; Bouaïchi et al., 1995; Roessingh and Simpson, 1994; Rogers et al., 2014). It could thus be speculated that IEG upregulation in the non-associative plasticity of behavioural phase transition has a different temporal and spatial distribution in the CNS than in other forms of plasticity studied to date.

A fourth assumption of this experiment was that IEG expression changes would be sufficiently expressed in specific regions to be detectable in the total tissue sample. The decision to pool together the brain and three thoracic ganglia was intended to compromise between time constraints and achieving coverage of a wide range of CNS tissue. However, the lack of effect observed in these samples could, in principle, be explained by highly cell- or region-specific expression changes that were masked by 'noise' from non-responsive regions. In *D. melanogaster* antennae, however, there is a general increase in overall *Hr38* expression in response to stimulation by a female pheromone

(Fujita et al., 2013), which suggests that masking by non-responsive neurons may not be a significant problem. A genome-wide study of activity-related expression in *D. melanogaster* observed many-fold activity-related IEG increases for *Hr38* and *stripe* in whole brains. However, the different methods used to activate neurons in these tissues were not representative of physiological neuronal activity underlying behaviour or plasticity (Chen et al., 2016). Other studies have undertaken RT-qPCR quantification only in specific brain regions, such as the antennal lobes (Alaux and Robinson, 2007), mushroom bodies (Lutz and Robinson, 2013) and a range of other brain regions (McNeill and Robinson, 2015). Further work investigating IEG responses in the separate regions of the *S. gregaria* CNS during crowding would be useful to answer this question.

A fifth assumption in this experiment was that confounding stimuli such as animal handling prior to the crowding period and holding conditions were insufficient to maximise IEG expression in both the control group and treatment group, masking any crowd stimulus (i.e. differential) effects. Increases in *FOS* in response to handling have been reported in rats (Campeau et al., 1991), however, an element of animal handling cannot be eliminated from experiments like this. To reduce exposure to handling, the experimental design for the present work used a wire mesh holding cage within the crowd tub in order to reduce handling stress at the time of snap-freezing. However, the action of labelling and placing the solitarious animals into the mesh cages 30 min before the crowding period will likely have elicited a stress response. Furthermore, control solitarious locust mesh cages were partitioned to prevent visual stimuli, but they were not isolated from the odour of other locusts in the experimental cohort. Any effect of this additional olfactory stimulus was expected to be negligible due to the minimally gregarising effects of olfactory stimuli alone (Roessingh et al., 1998), particularly as the animals spent only 30 min in the mesh cages in the presence of only 2–3 other animals in a room with a very high air exchange rate (20 air changes per hour). A possible additional confound is that the animals were able to see the investigator moving, which may elicit a predator-induced stress response. Although it seems unlikely that these confounds could lead to coincidentally similar levels of IEG expression in four different IEGs in the treatment groups any repetition of these experiments would benefit from the inclusion of a pristine solitarious control group that are snap-frozen straight from their holding cage.

If all of the assumptions above were correct, then an important conclusion from this experiment would be that IEGs responding to a crowding stimulus in *S. gregaria* do not correspond to those responding to learning events in other vertebrate and invertebrate species. Several transcripts in addition to *Hr38* and *stripe* were identified as being activity-related in *D. melanogaster* (Chen et al., 2016), providing options for a further search for responsive IEGs to behavioural phase change. Otherwise, identification of hitherto unidentified IEGs upregulated during short-term exposure to a crowding stimulus would require a more extensive exploratory analysis of candidate IEGs in *S. gregaria,* using microarray or RNA-seq techniques for example. Overall, while this experiment has found no evidence for activity-related expression of four IEGs in response to a crowd stimulus, there remain a number of important questions and refinements to this technique that require addressing prior to discounting IEGs from consideration in the assessment of mechanisms underlying behavioural phase change.

7 General Discussion

This project began with the aim of investigating the role of the monoamine 5-hydroxytryptamine (5-HT) in behavioural phase change in the Desert Locust, *Schistocerca gregaria*. It has provided novel findings with important implications for future work. This chapter discusses key questions in the field that are addressed by my results in the preceding chapters.

7.1 Is the P_{greg} model a suitable indicator of behavioural phase state?

The majority of behavioural assays in this thesis use a P_{greg} logistic regression model to assess behavioural phase state. Since its first publication (Roessingh et al, 1994), this metric has been widely used in behavioural assays as it provides a single measure incorporating multiple phase-related behaviours. A meta-analysis indicated that behaviours selected for the model used by Anstey et al. (2009; walk speed, rest time fraction, grooming frequency and time spent on the stimulus side) all change at similar rates during behavioural gregarisation. This is in contrast to the argument that a single behavioural metric can drive the change in P_{greg}, adding evidence to the reliability of P_{greg} in behavioural phase change experiments (Rogers et al., 2014; Tanaka and Nishide, 2013). However, it is clear that the locomotor function of the locusts plays a large role in determining the measured P_{greg} in both this assay and those prior (Rogers et al., 2014; Tanaka and Nishide, 2013; and see review Cullen et al., 2017), which may have been compromised in locusts injected with reserpine and 5-HT in Chapter 4. Furthermore, the fluphenazine experiments of Chapter 4 indicate changes in P_{greg} that arose in the absence of experimental phase-changing treatments. This begs the question: 'How reliably can a change in P_{greg} be interpreted as a change in behavioural phase state as opposed to a difference in locomotor function or a familiarity effect to an arena?'. The lack of evidence for a difference in P_{greg} between reserpine-injected gregarious locusts and controls (Figure 4.13) suggests that the core gregarious behaviours of the model are preserved in spite of the locomotor impairment. Furthermore, the results of Chapter 5 indicate that familiarity effects of repeated arena assays at intervals of 48 h are not affecting the P_{greg} values observed in other experiments with repeated observations. Chapter 5 also found

no evidence that fractal dimension, the main driving factor of the P_{greg} model in this thesis, changes after repeated arena assays with intervals of 24 h or longer. These two outcomes of this thesis thus support the use of the P_{greg} metric as a reliable indicator of behavioural phase state, particularly for work using repeated observations.

7.2 What mechanisms control behavioural phase state in *S. gregaria*?

7.2.1 The role of monoamines in behavioural phase change

Chapters 3 and 4 found no evidence that 5-HT is the key driver of behavioural phase change in *S. gregaria*, and it was unlikely that the methodology, maternal line, nor animal strain could explain this conflict. Thus, alternative mechanisms may include other monoamines. The neurochemicals DA and OA have also been reported to undergo dramatic changes in expression during behavioural gregarisation (Rogers et al., 2004), though the outcome of Chapter 3 indicates that these findings may be more up for debate than previously assumed. These two neurochemicals are important for behavioural plasticity in multiple invertebrate species (examples: Agarwal et al., 2011; Awata et al., 2016; Burke et al., 2012; Stevenson et al., 2005; Stevenson and Rillich, 2016), and have already been suggested to mediate other aspects of locust phase change in addition to short-term behavioural gregarisation (Alessi et al., 2014; Ma et al., 2011, 2015; Verlinden et al., 2010). In Chapter 4, reserpine was used to strongly deplete monoamines, and there was very little evidence that this prevented behavioural gregarisation in solitarious *S. gregaria*, indicating a need for further reinvestigations of monoamine roles in behavioural gregarisation in *S. gregaria*.

7.2.2 Roles of synaptic processes in behavioural gregarisation

As the results of Chapters 3 and 4 of this thesis cast doubt onto the roles of monoamines in behavioural phase change, this also highlights the need to investigate other potential avenues of investigation, such as synaptic plasticity. One key intracellular signalling molecule in synaptic plasticity, PKA was reported to be required for behavioural gregarisation over 2 h crowding (Ott et al., 2012). Downstream effects of PKA activation include rapid transcriptional or translational changes at the neuronal level, which consolidate changes in neuronal connectivity through protein synthesis and long-term synaptic remodelling (Kandel, 2001). Transcriptomes published for *L. migratoria*

revealed changes in gene expression accompanying changes in behavioural gregariousness over 64 h of phase-changing stimuli (Guo et al., 2011; Wang et al., 2014). It is important to note, however, that 64 h crowding was still insufficient to fully gregarise solitarious *L. migratoria* in these studies (Guo et al., 2011). The more rapid time scale of behavioural gregarisation in *S. gregaria* may preclude this mechanism, which generally accompanies long-term memory formation laid down over longer time frames (Kandel, 2001). Stettin (2014) found no evidence that pharmacologically blocking gene transcription or translation affects behavioural gregarisation after 2 h crowding in *S. gregaria*. Previous studies have largely relied upon CNS-wide manipulation of synaptic plasticity to assess the role of specific factors, when it is likely that at least some key elements underlying behavioural phase change are localised to specific neuronal networks. Due to a lack of appropriate tools for this purpose in *S. gregaria*, this exciting possibility has largely been unexplored.

7.3 What is the future of the Desert Locust phase change model?

7.3.1 Answering the 'monoamines question'

The novel findings in this thesis that conflict with earlier work demonstrate that studies on the Desert Locust require more methodological developments to best exploit this powerful model for behavioural plasticity. The use of HPLC to quantify tissue amounts of monoamines is precise, but limited in understanding the actions of neurochemicals at the anatomical and cellular level. Similarly, the use of broad spectrum pharmacological compounds to manipulate monoamine function, while simple to apply, is limited in specificity. These limitations mean that conflicts such as the one between my work and Anstey et al. (2009) and Rogers et al. (2004) are difficult to resolve using these techniques alone.

Studies by Guo et al. (2013), Ma et al. (2011) and Ott et al. (2012) use RNAi to knock down the expression of target genes in the neuronal cell signaling pathways in *L. migratoria* and *S. gregaria*. This has the advantage of manipulating neuronal physiology more specifically than pharmacological compounds, and was used to test the involvement of specific monoamine receptors (Guo et al., 2013), key monoamine synthesis enzymes (Ma et al., 2011) and cell effector molecules downstream of monoamine receptor

activation (Ott et al., 2012). Combining this approach with qPCR-quantified receptor expression as did the *L. migratoria* studies may pinpoint the specific actions of monoamines during behavioural phase change.

7.3.2 What genetic tools could tell us

To understand what tools could be available for studies linking neuronal populations, monoamine functions, neuronal activity and behavioural plasticity, one only needs to observe modern molecular techniques in *D. melanogaster*. Using specific mutant lines, genomic promoter regions and gene editing techniques in this model organism, it is possible to selectively express target genes (e.g. Green Fluorescent Protein) in neuronal populations, and make these inducible by heat or light treatments, leading to new insights into the roles of these neuronal populations in behaviour (reviewed in Owald et al., 2015). If these techniques were in place for locusts, this would allow unprecedented glimpses into the neuronal basis of behavioural phase change. For example, inducing a knockdown of monoamine release specifically in 5-HT neurons of the thoracic ganglia during behavioural gregarisation could provide strong evidence for the importance of 5-HT in the thoracic ganglia during phase change. However, this possibility remains a long way off for locusts, as molecular techniques are in their infancy for these insects. In fact, the complete sequenced genome of *S. gregaria* is still pending (Cullen et al., 2017). The publication of the complete genome of *L. migratoria* (Wang et al., 2014), and a report of the use of the CRISPR/CAS9 technique in this species (Li et al., 2016) provides groundwork for similar achievements in *S. gregaria*, but much work is still required to develop these genetic tools in locusts.

7.3.3 Neuronal networks underlying behavioural phase change

Chapter 6 of this thesis helped to narrow down the search for molecular tools by identifying and assessing the feasibility of using in locusts four IEG targets that have been used for imaging neuronal activity in other invertebrates (Guzowski et al., 2005; Lutz and Robinson, 2013; McNeill and Robinson, 2015; Ugajin et al., 2013; Chen et al., 2016). While the qPCR procedure revealed little response of these four IEGs to 30 min crowding compared with controls, this work lays down the groundwork for subsequent use of the technique in the locust and could help to answer other outstanding issues such

as how monoamines modulate the behaviours underlying phase change. For example, it will now be easier to identify viable IEG targets, develop broad neuronal activity maps of the CNS and combine them with existing maps of the position and connectivity of monoamine-containing cells (Burrows, 1996). In lieu of the genetic tools available to genetic model organisms, this technique will help shed light on neuronal pathways leading from a gregarising stimulus to the resulting behavioural rewiring. Of particular interest would be identifying the exact tactile or chemosensory receptors activating the gregarisation response on the hind leg (Rogers et al., 2003). In addition, unpicking the visual processing pathways that are sufficient to partially gregarise solitarious nymphs is another pursuit that may link the identification and perception of conspecifics with a resultant change in behavioural syndrome (Roessingh et al., 1998).

7.4 Is behavioural phase change a viable target mechanism of swarm control?

As introduced in Chapter 1, locust plagues are a recurring problem for agriculture in affected regions, which collectively cover 20% of the land surface of the planet (Steedman, 1988). Populations are typically controlled with heavy application of non-specific, fast-acting insecticides, which can have damaging effects on desert ecology, though the use of specific slow-acting fungal and pheromonal biopesticides is gaining momentum, particularly in Australia and China (see Cullen et al., 2017; Lecoq, 2010; Peveling, 2001). The concept of a hypothetical 'anti-gregarisation' control agent based on the 5-HT signaling pathway had been discussed in Anstey et al. (2009), while acknowledging that 5-HT signaling is universal to invertebrate and vertebrate organisms. This common mechanism precludes the possibility of specifically manipulating locust gregarisation, however it is possible that downstream effectors, once identified, may provide more species-specific targets (Ott et al., 2012). My work adds to the evidence against a 5-HT-based 'anti-gregarisation' swarm control concept by casting doubt on the finding that 5-HT is sufficient and necessary for behavioural gregarisation. It would be thus premature to focus compound developments on the 5-HT pathway. Furthermore, the initiation of behavioural gregarisation is established to be a rapid process in *S. gregaria* (Roessingh et al., 1994). Even if 5-HT were the underlying initiating mechanism for this process, I have found no evidence that monoamine action is required to maintain gregarious behaviour. Thus, any theoretical 5-HT pathway-based anti-gregarisation compounds would require spraying over solitarious breeding grounds as a preventative

measure immediately prior to conditions promoting gregarisation in the field, an unlikely scenario for developing countries with limited resources.

An intriguing avenue for locust swarm control is manipulation of the behavioural solitarisation process. While Alessi et al. (2014) found a role of DA action in acquisition of solitarious-like behaviour in gregarious adult *S. gregaria*, DA is a near-universal cell-signalling molecule in the animal kingdom similar to 5-HT, thus is similarly impractical for designing control tools specific to locusts. Unlike any hypothetical anti-gregarisation compound, this avenue could be used to target existing hopper bands and adult swarms as well as preventing formation. The resulting disruption of the formations would make them less destructive and more vulnerable to predation.

7.5 Can the locust phase change model be translated across species?

The locust's reversible phase change phenomenon is an extreme example of phenotypic plasticity – which is found across the animal kingdom, particularly in invertebrates (West-Eberhard, 2003). While a direct application to clinical settings is not feasible, the model, by definition, shares fundamental cell signaling and neuronal characteristics with the rest of the animal kingdom, including with human physiology. For example, inferences can be made about the actions of monoamines in behavioural change, investigated in Chapters 3 and 4 of this thesis, which can be compared with their role in behavioural modulation in other invertebrates (Blenau and Baumann, 2001; Libersat and Pflueger, 2004; Kamhi et al., 2017; Vleugels et al., 2015) and vertebrates (Levin, 2006). One key translational link between the locust and other species may be aggression. 5-HT is linked to the expression of aggressive behaviour in solitary invertebrate species such as the stalk-eyed fly (Bubak et al., 2014) and the fruit fly (Alekseyenko et al., 2010, 2014). It is also linked with aggression and dominance in vertebrates such as the vervet monkey (Raleigh et al., 1991) and humans (reviewed in Nelson and Chiavegetto, 2007). OA is also associated with aggression in the cricket (Stevenson et al., 2000; Stevenson and Rillich., 2017) and the fruit fly (Hoyer et al., 2008). While locusts lack a stereotypical aggressive behaviour, Bazazi et al. (2008) observed the importance of cannibalism in nymph migration. Since cannibalism could be presumed to involve more aggressive approaches within the swarm context, the locust provides a useful means for understanding the neurochemical basis of acquiring aggressive behaviours.

The results of Chapter 5 add to the evidence for shared learning mechanisms between *S. gregaria* and other species. The habituation response of solitarious locusts to a behavioural arena with repeated observations at 2 h intervals implies familiarity memory formation, which has been observed in many other animal species (Blaser et al., 2015). This, as well as the evidence for the ability to form appetitive and aversive conditioning memories (Simões et al., 2011, 2012, 2013), points to the locust as being a useful model system for common learning and memory mechanisms in other species. With further development of the IEG quantification method developed in Chapter 6 of this thesis, this model system can help to unravel the neuronal basis of learning and memory, with the additional advantages of the locust having a smaller CNS than vertebrates and a dramatic behavioural polyphenism, which can allow studies into the interactions between polyphenism and other forms of learning and memory.

8 Bibliography

Agarwal, M., Guzmán, M.G., Morales-Matos, C., Díaz, R.A.D.V., Abramson, C.I., Giray, T., 2011. Dopamine and octopamine influence avoidance learning of honey bees in a place preference assay. PLoS ONE 6, e25371. doi: 10.1371/journal.pone.0025371

Alaux, C., Robinson, G.E., 2007. Alarm pheromone induces immediate–early gene expression and slow behavioral response in honey bees. Journal of Chemical Ecology 33, 1346–1350. doi: 10.1007/s10886-007-9301-6

Alberini, C.M., Ghirardl, M., Metz, R., Kandel, E.R., 1994. C/EBP is an immediate-early gene required for the consolidation of long-term facilitation in *Aplysia*. Cell 76, 1099–1114. doi: 10.1016/0092-8674(94)90386-7

Alekseyenko, O.V., Lee, C. and Kravitz, E.A., 2010. Targeted manipulation of serotonergic neurotransmission affects the escalation of aggression in adult male *Drosophila melanogaster*. PloS ONE 5(5), e10806.

Alekseyenko, O.V., Chan, Y.B., de la Paz Fernandez, M., Bülow, T., Pankratz, M.J. and Kravitz, E.A., 2014. Single serotonergic neurons that modulate aggression in *Drosophila*. Current Biology 24(22), 2700-2707.

Alessi, A.M., O'Connor, V., Aonuma, H., Newland, P.L., 2014. Dopaminergic modulation of phase reversal in desert locusts. Frontiers in Behavioral 8, 371. doi: 10.3389/fnbeh.2014.00371

Anstey, M.L., Rogers, S.M., Ott, S.R., Burrows, M., Simpson, S.J., 2009. Serotonin mediates behavioral gregarization underlying swarm formation in desert locusts. Science 323, 627–630. doi: 10.1126/science.1165939

Anstey, M.L., 2012. Serotonin mediates behavioural gregarisation in the desert locust (PhD). University of Oxford.

Asahina, K., 2017. Neuromodulation and strategic action choice in *Drosophila* aggression. Annual Review of Neuroscience 40, 51–75. doi:10.1146/annurev-neuro-072116-031240

Awata, H., Wakuda, R., Ishimaru, Y., Matsuoka, Y., Terao, K., Katata, S., Matsumoto, Y., Hamanaka, Y., Noji, S., Mito, T., Mizunami, M., 2016. Roles of OA1 octopamine receptor and Dop1 dopamine receptor in mediating appetitive and aversive reinforcement revealed by RNAi studies. Scientific Reports 6, 29696. doi: 10.1038/srep29696

Baddeley, B., Graham, P., Husbands, P., Philippides, A., 2012. A model of ant route navigation driven by scene familiarity. PLOS Computational Biology 8, e1002336. doi: 10.1371/journal.pcbi.1002336

Badisco, L., Ott, S.R., Rogers, S.M., Matheson, T., Knapen, D., Vergauwen, L., Verlinden, H., Marchal, E., Sheehy, M.R.J., Burrows, M., Broeck, J.V., 2011. Microarray-based transcriptomic analysis of differences between long-term gregarious and solitarious desert locusts. PLoS ONE 6, e28110. doi: 10.1371/journal.pone.0028110

Bahrami, S., Drabløs, F., 2016. Gene regulation in the immediate-early response process. Advances in Biological Regulation 62, 37–49. doi: 10.1016/j.jbior.2016.05.001

Bailey, C.H., Chen, M., 1983. Morphological basis of long-term habituation and

sensitization in *Aplysia*. Science 220, 91–93. doi: 10.1126/science.6828885

Baraban, S. C., Taylor, M. R., Castro, P. A., Baier, H., 2005. Pentylenetetrazole induced changes in zebrafish behavior, neural activity and c-fos expression. Neuroscience 131 (3), 759–768. doi: 10.1016/j.neuroscience.2004.11.031

Barron, A.B., Søvik, E. and Cornish, J.L., 2010. The roles of dopamine and related compounds in reward-seeking behavior across animal phyla. Frontiers in Behavioral Neuroscience 4,163.

Bassett, M.H., Suzuki, T., Sasano, H., White, P.C., Rainey, W.E., 2004a. The orphan nuclear receptors NURR1 and NGFIB regulate adrenal aldosterone production. Molecular Endocrinology 18, 279–290. doi: 10.1210/me.2003-0005

Bazazi, S., Buhl, J., Hale, J.J., Anstey, M.L., Sword, G.A., Simpson, S.J., Couzin, I.D., 2008. Collective motion and cannibalism in locust migratory bands. Current Biology 18, 735–739. doi: 10.1016/j.cub.2008.04.035

Berthier, K., Chapuis, M.-P., Simpson, S.J., Ferenz, H.-J., Kane, C.M.H., Kang, L., Lange, A., Ott, S.R., Ebbe, M.A.B., Rodenburg, K.W., Rogers, S.M., Torto, B., Vanden Broeck, J., Loon, J.J.A., Sword, G.A., 2010. Laboratory populations as a resource for understanding the relationship between genotypes and phenotypes. Advances in Insect Physiology 39, 1–37. doi: 10.1016/B978-0-12-381387-9.00001-4

Bird, A., 2002. DNA methylation patterns and epigenetic memory. Genes Development 16, 6–21. doi: 10.1101/gad.947102

Blaser, R., Heyser, C., 2015. Spontaneous object recognition: a promising approach to the comparative study of memory. Frontiers in Behavioral Neuroscience 9, 183. doi: 10.3389/fnbeh.2015.00183

Blenau, W., Baumann, A., 2001. Molecular and pharmacological properties of insect biogenic amine receptors: Lessons from *Drosophila melanogaster* and *Apis mellifera*. Archives of Insect Biochemistry and Physiology 48, 13–38. doi: 10.1002/arch.1055

Bonaz, B., Rivest, S., 1998. Effect of a chronic stress on CRF neuronal activity and expression of its type 1 receptor in the rat brain. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 275, R1438–R1449.

Borelli, K.G., Ferreira-Netto, C., Brandão, M.L., 2006. Distribution of fos immunoreactivity in the rat brain after freezing or escape elicited by inhibition of glutamic acid decarboxylase or antagonism of GABA-A receptors in the inferior colliculus. Behavioural Brain Research 170, 84–93. doi: 10.1016/j.bbr.2006.02.015

Bos, N., d'Ettorre, P., 2012. Recognition of social identity in ants. Frontiers in Psychology 3, 83. doi:10.3389/fpsyg.2012.00083

Bouaïchi, A., Roessingh, P., Simpson, S.J., 1995. An analysis of the behavioural effects of crowding and re-isolation on solitary-reared adult desert locusts (*Schistocerca gregaria*) and their offspring. Physiological Entomology 20, 199–208. doi: 10.1111/j.1365-3032.1995.tb00002.x

Boyer, B., Ernest, S., Rosa F., 2013. Egr-1 induction provides a genetic response to food aversion in zebrafish. Frontiers in Behavioral Neuroscience 7, 51. doi: 10.3389/fnbeh.2013.00051

Brader, L., Djibo, H., Faye, F.G., Ghaout, S., Lazar, M., Luzietoso, P.N. and Babah, M.O., 2006. Towards a more effective response to desert locusts and their impacts

on food security, livelihood and poverty. Multilateral Evaluation of the 2003-05 Desert Locust Campaign.

Bridges, R.S., 2015. Neuroendocrine regulation of maternal behavior. Frontiers in Neuroendocrinology, *36*, 178-196.

Brown, E.R., Sawchenko, P.E., 1997. Hypophysiotropic CRF neurons display a sustained immediate-early gene response to chronic stress but not to adrenalectomy. Journal of Neuroendocrinology 9, 307–316. doi: 10.1046/j.1365-2826.1997.00586.x

Bubak, A.N., Renner, K.J. and Swallow, J.G., 2014. Heightened serotonin influences contest outcome and enhances expression of high-intensity aggressive behaviors. Behavioural Brain Research 259, 137-142.

Burke, C.J., Huetteroth, W., Owald, D., Perisse, E., Krashes, M.J., Das, G., Gohl, D., Silies, M., Certel, S., Waddell, S., 2012. Layered reward signalling through octopamine and dopamine in *Drosophila*. Nature 492, 433–437. doi: 10.1038/nature11614

Burrows, M., Newland, P.L., 1993. Correlation between the receptive fields of locust interneurons, their dendritic morphology, and the central projections of mechanosensory neurons. Journal of Comparative Neurology 329, 412–426. doi: 10.1002/cne.903290311

Burrows, M., Newland, P.L., 1994. Convergence of mechanosensory afferents from different classes of exteroceptors onto spiking local interneurons in the locust. Journal of Neuroscience 14, 3341–3350.

Burrows, M., 1996. The neurobiology of an insect brain. Oxford University Press, Oxford, New York.

Burrows, M., Rogers, S.M., Ott, S.R., 2011. Epigenetic remodelling of brain, body and behaviour during phase change in locusts. Neural Systems & Circuits 1, 1–9. doi: 10.1186/2042-1001-1-11

Byrne, J. H., Labar, K. S., LeDoux, J. E., Schafe, G. E., Thompson, R. F., 2014. Learning and Memory: Basic Mechanisms. In: From Molecules to Networks. Elsevier Inc., pp 591-637. doi: 10.1016/B978-0-12-397179-1.00020-8

Camacho, J.P.M., Ruiz-Ruano, F.J., Martín-Blázquez, R., López-León, M.D., Cabrero, J., Lorite, P., Cabral-de-Mello, D.C., Bakkali, M., 2015. A step to the gigantic genome of the desert locust: chromosome sizes and repeated DNAs. Chromosoma 124, 263–275. doi: 10.1007/s00412-014-0499-0

Campeau, S., Hayward, M.D., Hope, B.T., Rosen, J.B., Nestler, E.J., Davis, M., 1991. Induction of the c-fos proto-oncogene in rat amygdala during unconditioned and conditioned fear. Brain Research 565, 349–352. doi: 10.1016/0006-8993(91)91669-R

Chapuis, M. P., Estoup, A., Augé-Sabatier, A., Foucart, A., Lecoq, M., Michalakis, Y., 2008. Genetic variation for parental effects on the propensity to gregarise in *Locusta migratoria*. BMC Evolutionary Biology 8, 37. doi:10.1186/1471-2148-8-37

Chapuis, M. P., Foucart, A., Plantamp, C., Blondin, L., Leménager, N., Benoit, L., Gay, P. E., Bazelet, C. S., 2017. Genetic and morphological variation in non-polyphenic Southern African populations of the desert locust. African Entomology 25, 13–23. doi: 10.4001/003.025.0013

Chen, S., Yang, P., Jiang, F., Wei, Y., Ma, Z., Kang, L., 2010. *De novo* analysis of

transcriptome dynamics in the migratory locust during the development of phase traits. PLoS ONE 5, e15633. doi: 10.1371/journal.pone.0015633

Chen, X., Rahman, R., Guo, F., Rosbash, M., 2016. Genome-wide identification of neuronal activity-regulated genes in *Drosophila*. eLife 5, e19942. doi: 10.7554/eLife.19942

Cole, A.J., Saffen, D.W., Baraban, J.M., Worley, P.F., 1989. Rapid increase of an immediate early gene messenger RNA in hippocampal neurons by synaptic NMDA receptor activation. Nature 340, 474–476. doi: 10.1038/340474a0

Cole, S.H., Carney, G.E., McClung, C.A., Willard, S.S., Taylor, B.J., Hirsh, J., 2005. Two functional but noncomplementing *Drosophila* tyrosine decarboxylase genes: distinct roles for neural tyramine and octopamine in female fertility. Journal of Biological Chemistry 280, 14948–14955. doi: 10.1074/jbc.M414197200

Collett, M., Despland, E., Simpson, S.J., Krakauer, D. C., 1998. Spatial scales of desert locust gregarization. Proceedings of the National Academy of Sciences. 95, 13052–13055. doi:10.1073/pnas.95.22.13052

Collett, M., Collett, T.S., 2000. How do insects use path integration for their navigation? Biological Cybernetics 83, 245–259. doi: 10.1007/s004220000168

Collett, M., Chittka, L., Collett, T.S., 2013. Spatial memory in insect navigation. Current Biology 23, R789–R800. doi: 10.1016/j.cub.2013.07.020

Colquhoun, D., 2014. An investigation of the false discovery rate and the misinterpretation of p-values. Open Science 1, 140216. doi: 10.1098/rsos.140216

Colquhoun, D., 2015. Comment on evidence levels. Available from: http://rsos.royalsocietypublishing.org/content/1/3/140216#comment-1889100957 (Accessed 4 August 2017).

Crispino, M., Tocco, G., Feldman, J.D., Herschman, H.R., Baudry, M., 1998. Nurr1 mRNA expression in neonatal and adult rat brain following kainic acid-induced seizure activity. Molecular Brain Research 59, 178–188. doi: 10.1016/S0169-328X(98)00143-0

Cullen, D.A., Sword, G.A., Dodgson, T., Simpson, S.J., 2010. Behavioural phase change in the Australian plague locust, *Chortoicetes terminifera*, is triggered by tactile stimulation of the antennae. Journal of Insect Physiology, Locust Research in the Age of Model Organisms 56, 937–942. doi: 10.1016/j.jinsphys.2010.04.023

Cullen, D.A., Sword, G.A., Simpson, S.J., 2012. Optimizing multivariate behavioural syndrome models in locusts using automated video tracking. Animal Behaviour 84, 771–784. doi:10.1016/j.anbehav.2012.06.031

Cullen, D.A., Cease, A.J., Latchininsky, A.V., Ayali, A., Berry, K., Buhl, J., De Keyser, R., Foquet, B., Hadrich, J.C., Matheson, T., Ott, S.R., Poot-Pech, M.A., Robinson, B.E., Smith, J.M., Song, H., Sword, G.A., Vanden Broeck, J., Verdonck, R., Verlinden, H., Rogers, S.M., 2017. From molecules to management: mechanisms and consequences of locust phase polyphenism, in: Advances in Insect Physiology. Elsevier, pp. 167–285. doi: 10.1016/bs.aiip.2017.06.002

Cullinan, W.E., Herman, J.P., Battaglia, D.F., Akil, H., Watson, S.J., 1995. Pattern and time course of immediate early gene expression in rat brain following acute stress. Neuroscience 64, 477–505. doi: 10.1016/0306-4522(94)00355-9

Cymborowski, B., 1996. Expression of c-fos-like protein in the brain and prothoracic

glands of *Galleria mellonella* larvae during chilling stress. Journal of Insect Physiology 42, 367–371. doi: 10.1016/0022-1910(95)00117-4

Davenport, A.P., Evans, P.D., 1984. Stress-induced changes in the octopamine levels of insect haemolymph. Insect Biochemistry 14, 135–143. doi: 10.1016/0020-1790(84)90021-0

Davis, R.L., 2011. Traces of *Drosophila* memory. Neuron 70, 8–19. doi: 10.1016/j.neuron.2011.03.012

Despland, E., Collett, M., Simpson, S.J., 2000. Small-scale processes in desert locust swarm formation: how vegetation patterns influence gregarization. Oikos 88, 652–662.

Despland, E., Simpson, S.J., 2000. The role of food distribution and nutritional quality in behavioural phase change in the desert locust. Animal Behaviour 59, 643–652. doi: 10.1006/anbe.1999.1335

Despland, E., 2001. Role of olfactory and visual cues in the attraction/repulsion responses to conspecifics by gregarious and solitarious desert locusts. Journal of Insect Behaviour 14, 35–46. doi: 10.1023/A:1007845528500

Despland, E., Simpson, S.J., 2005. Food choices of solitarious and gregarious locusts reflect cryptic and aposematic antipredator strategies. Animal Behaviour 69, 471–479.

Donk, E.V., Ianora, A., Vos, M., 2011. Induced defences in marine and freshwater phytoplankton: a review. Hydrobiologia 668, 3–19. doi: 10.1007/s10750-010-0395-4

Durier, V., Rivault, C., 2001. Effects of spatial knowledge and feeding experience on foraging choices in German cockroaches. Animal Behaviour 62, 681–688. doi: 10.1006/anbe.2001.1807

Durier, V., Rivault, C., 2002. Influence of a novel object in the home range of the cockroach, *Blattella germanica*. Medical and Veterinary Entomology 16, 121–125. doi: 10.1046/j.1365-2915.2002.00348.x

Ellis, P.E., 1953. Social aggregation and gregarious behaviour in hoppers of *Locusta migratoria migratorioides* (R. & F.). Behaviour 225–260.

Ellis, P.E., 1959. Learning and social aggregation in locust hoppers. Animal Behaviour 7, 91–106.

Ellis, P.E., 1963. Changes in the social aggregation of locust hoppers with changes in rearing conditions. Animal Behaviour 11, 152–160.

Ellis, P.E., 1963b. The influence of some environmental factors on learning and aggregation in locust hoppers. Animal Behaviour 11, 142–151.

Ellis, P.E., 1964. Marching and colour in locust hoppers in relation to social factors. Behaviour 23, 177–192.

Evans, J., Wheeler, D., 2000. Gene expression and the evolution of insect polyphenisms. BioEssays 23, 62–68. doi: 10.1002/1521-1878(200101)23

Farrow, R., 1990. Flight and migration in acridoids, in: Biology of Grasshoppers. John Wiley & Sons, Inc., Chichester, pp. 227–314.

Faure, J.C., 1932. The phases of locusts in South Africa. Bulletin of Entomological Research 23, 293–405. doi: 10.1017/S0007485300004223

Fisher, R.A., 1925. Statistical methods for research workers. Genesis Publishing Pvt Ltd.

Flanagan-Cato, L.M., McEwen, B.S., 1995. Pattern of Fos and Jun expression in the

female rat forebrain after sexual behavior. Brain Research 673, 53–60. doi:10.1016/0006-8993(94)01395

Fujita, N., Nagata, Y., Nishiuchi, T., Sato, M., Iwami, M., Kiya, T., 2013. Visualization of neural activity in insect brains using a conserved immediate early gene, Hr38. Current Biology 23, 2063–2070. doi: 10.1016/j.cub.2013.08.051

Fulton, D., Kemenes, I., Andrew, R.J., Benjamin, P.R., 2008. Time-window for sensitivity to cooling distinguishes the effects of hypothermia and protein synthesis inhibition on the consolidation of long-term memory. Neurobiology of Learning and Memory 90, 651–654. doi: 10.1016/j.nlm.2008.08.006

Fusco, G., Minelli, A., 2010. Phenotypic plasticity in development and evolution: Facts and concepts. Philosophical Transactions of the Royal Society B: Biological Sciences 365, 547–556. doi: 10.1098/rstb.2009.0267

Fuzeau-Braesch, S., David, J.C., 1978. Etude du taux d'octopamine chez *Locusta migratoria* (Insecte, Orthoptere): Comparaison entre insectes gregaires, solitaires et traites au gaz carbonique. Comptes rendus hebdomadaires des seances. Serie D. Sciences naturelles 286, 697–699.

Garthwaite, J., Charles, S.L. and Chess-Williams, R., 1988. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. Nature 336(6197), 385.

Geva, N., Guershon, M., Orlova, M., Ayali, A., 2010. Memoirs of a locust: Density-dependent behavioral change as a model for learning and memory. Neurobiology of Learning and Memory 93, 175–182. doi: 10.1016/j.nlm.2009.09.008

Ghosal, K., Naples, S.P., Rabe, A.R., Killian, K.A., 2010. Agonistic behavior and electrical stimulation of the antennae induces fos-like protein expression in the male cricket brain. Archives of Insect Biochemistry and Physiology 74, 38–51. doi: 10.1002/arch.20360

Glanzman, D.L., 2010. Common mechanisms of synaptic plasticity in vertebrates and invertebrates. Current Biology 20, R31–R36. doi: 10.1016/j.cub.2009.10.023

Goldstein, M.I., Lacher, T.E., Woodbridge, B., Bechard, M.J., Canavelli, S.B., Zaccagnini, M.E., Cobb, G.P., Scollon, E.J., Tribolet, R. and Hopper, M.J., 1999. Monocrotophos-induced mass mortality of Swainson's Hawks in Argentina, 1995–96. Ecotoxicology 8(3), 201-214.

Gotham, S., Song, H., 2013. Non-swarming grasshoppers exhibit density-dependent phenotypic plasticity reminiscent of swarming locusts. Journal of Insect Physiology 59, 1151–1159. doi: 10.1016/j.jinsphys.2013.08.017

Gray, L.J., Sword, G.A., Anstey, M.L., Clissold, F.J., Simpson, S.J., 2009. Behavioural phase polyphenism in the Australian plague locust (*Chortoicetes terminifera*). Biology Letters 5, 306–309. doi: 10.1098/rsbl.2008.0764

Greenberg, M.E., Ziff, E.B., 1984. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature 311, 433–438.

Guan, Z., Saraswati, S., Adolfsen, B., Littleton, J.T., 2005. Genome-wide transcriptional changes associated with enhanced activity in the *Drosophila* nervous system. Neuron 48, 91–107. doi: 10.1016/j.neuron.2005.08.036

Guo, W., Wang, X., Ma, Z., Xue, L., Han, J., Yu, D., Kang, L., 2011. CSP and takeout genes modulate the switch between attraction and repulsion during behavioral phase change in the migratory locust. PLoS Genetics 7, e1001291.

Guo, X., Ma, Z., Kang, L., 2013. Serotonin enhances solitariness in phase transition of the migratory locust. Frontiers in Behavioral Neuroscience 7, 129. doi: 10.3389/fnbeh.2013.00129

Guo, X., Ma, Z., Kang, L., 2015. Two dopamine receptors play different roles in phase change of the migratory locust. Frontier Behavioural Neuroscience 9, 1–13. doi: 10.3389/fnbeh.2015.00080

Guven-Ozkan, T., Davis, R.L., 2014. Functional neuroanatomy of *Drosophila* olfactory memory formation. Learning and Memory 21, 519–526. doi: 10.1101/lm.034363.114

Guzowski, J.F., Setlow, B., Wagner, E.K., McGaugh, J.L., 2001. Experience-dependent gene expression in the rat hippocampus after spatial learning: A comparison of the immediate-early genes Arc, C-fos, and zif268. Journal of Neuroscience 21, 5089–5098.

Guzowski, J.F., Timlin, J.A., Roysam, B., McNaughton, B.L., Worley, P.F., Barnes, C.A., 2005. Mapping behaviorally relevant neural circuits with immediate-early gene expression. Current Opinion in Neurobiology, Neuronal and glial cell biology / New technologies 15, 599–606. doi:10.1016/j.conb.2005.08.018

Hägele, B.F., Simpson, S.J., 2000. The influence of mechanical, visual and contact chemical stimulation on the behavioural phase state of solitarious desert locusts (*Schistocerca gregaria*). Journal of Insect Physiology 46, 1295–1301.

Hartfelder, K., Makert, G.R., Judice, C.C., Pereira, G.A.G., Santana, W.C., Dallacqua, R., Bitondi, M.M.G., 2006. Physiological and genetic mechanisms underlying caste development, reproduction and division of labor in stingless bees. Apidologie 37, 144–163. doi: 10.1051/apido:2006013

Healy, S., Khan, P., Davie, J.R., 2013. Immediate early response genes and cell transformation. Pharmacology & Therapeutics 137, 64–77. doi: 10.1016/j.pharmthera.2012.09.001

Heifetz, Y., Applebaum, S.W., Popov, G.B., 1994. Phase characteristics of the Israeli population of the migratory locust, *Locusta migratoria* (L.) (Orthoptera: Acrididae). Journal of Orthoptera Research 15–20.

Heifetz, Y., Boekhoff, I., Breer, H., Applebaum, S.W., 1997. Cuticular hydrocarbons control behavioural phase transition in Schistocerca gregaria nymphs and elicit biochemical responses in antennae. Insect Biochemistry Molecular Biology 27, 563–568. doi:10.1016/S0965-1748(97)00031-3

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., Vandesompele, J., 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biology 8, R19. doi: 10.1186/gb-2007-8-2-r19

Herry, C., Ciocchi, S., Senn, V., Demmou, L., Müller, C., Lüthi, A., 2008. Switching on and off fear by distinct neuronal circuits. Nature 454, 600–606. doi: 10.1038/nature07166

Hosmer, D.W., Lemeshow, S., Sturdivant, R.X., 2000. 10. Special topics, in: Applied logistic regression. John Wiley & Sons, Inc., pp. 260–351. doi: 10.1002/0471722146.ch8

Hoste, B., Simpson, S.J., Tanaka, S., Zhu, D.-H., De Loof, A., Breuer, M., 2002. Effects of [His7]-corazonin on the phase state of isolated-reared (solitarious) desert

locusts, *Schistocerca gregaria*. Journal of Insect Physiology 48, 981–990. doi: 10.1016/S0022-1910(02)00177-4

Houston-Price, C., Nakai, S., 2004. Distinguishing novelty and familiarity effects in infant preference procedures. Infant Child Development 13, 341–348. doi: 10.1002/icd.364

Hoyer, S.C., Eckart, A., Herrel, A., Zars, T., Fischer, S.A., Hardie, S.L. and Heisenberg, M., 2008. Octopamine in male aggression of Drosophila. Current Biology 18(3), 159-167.

Hoyle, G. and Barker, D.L., 1975. Synthesis of octopamine by insect dorsal median unpaired neurons. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 193(3), 433-439.

Hudson, S.G., Goldstein, E.S., 2008. The gene structure of the *Drosophila melanogaster* proto-oncogene, kayak, and its nested gene, fos-intronic gene. Gene 420, 76–81. doi: 10.1016/j.gene.2008.05.001

Hunt, G.J., 2007. Flight and fight: A comparative view of the neurophysiology and genetics of honey bee defensive behavior. Journal of Insect Physiology 53, 399–410. doi: 10.1016/j.jinsphys.2007.01.010

Hwang, D. S., Lee, B. Y., Kim, H. S., Lee, M.C., Kyung, D. H., Om, A.-S., Rhee, J. S., Lee, J. S., 2014. Genome-wide identification of nuclear receptor (NR) superfamily genes in the copepod *Tigriopus japonicus*. BMC Genomics 15. doi: 10.1186/1471-2164-15-993

Injeyan, H.S. and Tobe, S.S., 1981. Phase polymorphism in *Schistocerca gregaria*: reproductive parameters. Journal of Insect Physiology 27(2), 97-102.

Islam, M., 1997. A preliminary report on the biochemical properties of a maternally-produced gregarizing factor in the desert locust *Schistocerca gregaria* (Forskal). Journal of the Asiatic Society of Bangladesh, Science 23, 111–122.

Janzen, F.J., Phillips, P.C., 2006. Exploring the evolution of environmental sex determination, especially in reptiles. Journal of Evolutionary Biology 19, 1775–1784. doi: 10.1111/j.1420-9101.2006.01138.x

Johnson, B.A., Woo, C.C., Duong, H., Nguyen, V., Leon, M., 1995. A learned odor evokes an enhanced fos-like glomerular response in the olfactory bulb of young rats. Brain Research 699, 192–200. doi: 10.1016/0006-8993(95)00896

Johnson, Z.V. and Young, L.J., 2015. Neurobiological mechanisms of social attachment and pair bonding. Current opinion in behavioral sciences, 3, 38-44.

Kaczmarek, L., 1993. Molecular biology of vertebrate learning: Is c-fos a new beginning? Journal of Neuroscience Research 34, 377–381. doi: 10.1002/jnr.490340402

Kamhi, J.F. and Traniello, J.F., 2013. Biogenic amines and collective organization in a superorganism: neuromodulation of social behavior in ants. Brain, behavior and evolution 82(4), 220-236.

Kamhi, J.F., Arganda, S., Moreau, C.S. and Traniello, J.F., 2017. Origins of Aminergic Regulation of Behavior in Complex Insect Social Systems. Frontiers in Systems Neuroscience 11, 74.

Kandel, E.R., 2001. The molecular biology of memory storage: A dialogue between genes and synapses. Science 294, 1030–1038. doi: 10.1126/science.1067020

Kandel, E.R., Schwarz, J.H., Jessell, T.M., Siegelbaum, S.A., Hudspeth, A.J., 2012. Principles of Neural Science, 5th ed. McGraw-Hill Professional.

Kandel, E.R., Dudai, Y., Mayford, M.R., 2014. The molecular and systems biology of memory. Cell 157, 163–186. doi: 10.1016/j.cell.2014.03.001

Kang, L., Chen, X., Zhou, Y., Liu, B., Zheng, W., Li, R., Wang, J., Yu, J., 2004. The analysis of large-scale gene expression correlated to the phase changes of the migratory locust. Proceedings of the National Academy of Sciences 101, 17611–17615. doi: 10.1073/pnas.0407753101

Kravitz, E.A., 2000. Serotonin and aggression: insights gained from a lobster model system and speculations on the role of amine neurons in a complex behavior. Journal of Comparative Physiology A 186(3), 221-238.

Kennedy, J.S., 1951. The migration of the Desert Locust (*Schistocerca gregaria* Forsk.) I. The behaviour of swarms. II. A theory of long-range migrations. Philosophical transactions of the Royal Society B: Biological Sciences 235, 163–290. doi: 10.1098/rstb.1951.0003

Keverne, E.B., Curley, J.P., 2004. Vasopressin, oxytocin and social behaviour. Current Opinion in Neurobiology 14, 777–783. doi: 10.1016/j.conb.2004.10.006

King, G., Zeng, L., 2001. Logistic regression in rare events data. Political analysis 9, 137–163.

Kiya, T., Kunieda, T., Kubo, T., 2008. Inducible- and constitutive-type transcript variants of kakusei, a novel non-coding immediate early gene, in the honeybee brain. Insect Molecular Biology 17, 531–536. doi:10.1111/j.1365-2583.2008.00821.x

Kleppisch, T., Feil, R., 2009. cGMP signalling in the mammalian brain: role in synaptic plasticity and behaviour, in: CGMP: Generators, Effectors and Therapeutic Implications, Handbook of Experimental Pharmacology. Springer, Berlin, Heidelberg, pp. 549–579. doi: 10.1007/978-3-540-68964-5_24

Knapska, E., Kaczmarek, L., 2004. A gene for neuronal plasticity in the mammalian brain: Zif268/Egr-1/NGFI-A/Krox-24/TIS8/ZENK? Progress in Neurobiology 74, 183–211. doi: 10.1016/j.pneurobio.2004.05.007

Kortet, R., Hedrick, A., 2007. A behavioural syndrome in the field cricket *Gryllus integer*: Intrasexual aggression is correlated with activity in a novel environment. Biological Journal of the Linnean Society 91, 475–482. doi: 10.1111/j.1095-8312.2007.00812.x

Kovács, K.J., 1998. Invited review c-fos as a transcription factor: A stressful (re)view from a functional map. Neurochemistry International 33, 287–297. doi: 10.1016/S0197-0186(98)00023-0

Kovary, K., Bravo, R., 1991. The jun and fos protein families are both required for cell cycle progression in fibroblasts. Molecular and Cell Biology 11, 4466–4472.

Kozlova, T., Lam, G., Thummel, C.S., 2009. *Drosophila* DHR38 nuclear receptor is required for adult cuticle integrity at eclosion. Developmental Dynamics 238, 701–707. doi: 10.1002/dvdy.21860

Kukuk, P.F., 1992. Social interactions and familiarity in a communal halictine bee *Lasioglossum (Chilalictus) hemichalceum*. Ethology 91, 291–300. doi: 10.1111/j.1439-0310.1992.tb00870.x

Lanahan, A., Worley, P., 1998. Immediate-early genes and synaptic function. Neurobiology of Learning and Memory 70, 37–43. doi: 10.1006/nlme.1998.3836

Lange, A.B., 2009. Tyramine: From octopamine precursor to neuroactive chemical in insects. General and Comparative Endocrinology, Insect Endocrinology 162, 18–26. doi: 10.1016/j.ygcen.2008.05.021

Lange, A.B., Orchard, I., Michael Barrett, F., 1989. Changes in haemolymph serotonin levels associated with feeding in the blood-sucking bug, Rhodnius prolixus. Journal of Insect Physiology 35, 393–399. doi: 10.1016/0022-1910(89)90113-3

Lecoq, M., 2010. Integrated pest management for locusts and grasshoppers: are alternatives to chemical pesticides credible?. Journal of Orthoptera Research 19(1), 131-132.

Letunic, I., Doerks, T., Bork, P., 2015. SMART: recent updates, new developments and status in 2015. Nucleic Acids Res. 43, D257–D260. doi: 10.1093/nar/gku949

Levin, E. D. (ed.) Basel ; Boston, 2006. Neurotransmitter interactions and cognitive function. EXS 98: Birkhäuser Verlag

Li, Y., Zhang, J., Chen, D., Yang, P., Jiang, F., Wang, X., Kang, L., 2016. CRISPR/Cas9 in locusts: Successful establishment of an olfactory deficiency line by targeting the mutagenesis of an odorant receptor co-receptor (Orco). Insect Biochemistry and Molecular Biology 79, 27–35. doi: 10.1016/j.ibmb.2016.10.003

Libersat, F., Pflueger, H. J., 2004. Monoamines and the orchestration of behavior. BioScience 54, 17–25. doi: 10.1641/0006-3568(2004)054

Liu, J., Grogan, L., Nau, M.M., Allegra, C.J., Chu, E., Wright, J.J., 2001. Physical interaction between p53 and primary response gene Egr-1. International Journal of Oncology 18, 863–870.

Liu, W., Xie, Y., Ma, J., Luo, X., Nie, P., Zuo, Z., Lahrmann, U., Zhao, Q., Zheng, Y., Zhao, Y., Xue, Y., Ren, J., 2015. IBS: An illustrator for the presentation and visualization of biological sequences. Bioinformatics 31, 3359–3361. doi: 10.1093/bioinformatics/btv362

Livingstone, M.S., Harris-Warrick, R.M. and Kravitz, E.A., 1980. Serotonin and octopamine produce opposite postures in lobsters. Science 208(4439), 76-79.

Livingstone, M.S., Tempel, B.L., 1983. Genetic dissection of monoamine neurotransmitter synthesis in *Drosophila*. Nature 303, 67–70. doi: 10.1038/303067a0

Lochmatter, T., Roduit, P., Cianci, C., Correll, N., Jacot, J., Martinoli, A., 2008. SwisTrack - a flexible open source tracking software for multi-agent systems, in: 2008 IEEE/RSJ International Conference on Intelligent Robots and Systems. Presented at the 2008 IEEE/RSJ International Conference on Intelligent Robots and Systems, pp. 4004–4010. doi: 10.1109/IROS.2008.4650937

Lockard, M.A., Ebert, M.S., Bargmann, C.I., 2016. Oxytocin mediated behavior in invertebrates: an evolutionary perspective. Developmental Neurobiology 77, 128-142. doi: 10.1002/dneu.22466

Lovejoy, N.R., Mullen, S.P., Sword, G.A., Chapman, R.F., Harrison, R.G., 2006. Ancient trans-Atlantic flight explains locust biogeography: molecular phylogenetics of *Schistocerca*. Proceedings of the Royal Society B: Biological Sciences 273, 767–774. doi: 10.1098/rspb.2005.3381

Lutz, C.C., Robinson, G.E., 2013. Activity-dependent gene expression in honey bee mushroom bodies in response to orientation flight. Journal of Experimental Biology 216, 2031–2038. doi: 10.1242/jeb.084905

Ma, Z., Yu, J., Kang, L., 2006. LocustDB: A relational database for the transcriptome and biology of the migratory locust (*Locusta migratoria*). BMC Genomics 7, 11. doi: 10.1186/1471-2164-7-11

Ma, Z., Guo, W., Guo, X., Wang, X., Kang, L., 2011. Modulation of behavioral phase changes of the migratory locust by the catecholamine metabolic pathway. Proceedings of the National Academy of Sciences 108, 3882–3887. doi: 10.1073/pnas.1015098108

Ma, Z., Guo, X., Lei, H., Li, T., Hao, S., Kang, L., 2015. Octopamine and tyramine respectively regulate attractive and repulsive behavior in locust phase changes. Scientific Reports 5, 8036. doi: 10.1038/srep08036

Malagoli, D., Ottaviani, E., 2017. Cross-talk among immune and neuroendocrine systems in molluscs and other invertebrate models. Hormones and Behaviour, Neuroendocrine-Immune Interactions: Implications for Integrative and Comparative Physiologists 88, 41–44. doi: 10.1016/j.yhbeh.2016.10.015

Mallon, E.B., Amarasinghe, H.E., Ott, S.R., 2016. Acute and chronic gregarisation are associated with distinct DNA methylation fingerprints in desert locusts. Sci Rep 6, 35608. doi: 10.1038/srep35608

Matheson, T., Rogers, S.M., Krapp, H.G., 2004. Plasticity in the visual system is correlated with a change in lifestyle of solitarious and gregarious locusts. Journal of Neurophysiology 91, 1–12. doi: 10.1152/jn.00795.2003

Mayr, E., 1970. Populations, species, and evolution: An abridgment of animal species and evolution. Harvard University Press.

McCaffery, A.R., Simpson, S.J., Islam, M.S., Roessingh, P., 1998. A gregarizing factor present in the egg pod foam of the desert locust *Schistocerca gregaria*. The Journal of Experimental Biology 201, 347–363.

McNeill, M.S., Robinson, G.E., 2015. Voxel-based analysis of the immediate early gene, c-jun, in the honey bee brain after a sucrose stimulus. Insect Molecular Biology 24, 377–390. doi: 10.1111/imb.12165

Mello, C. V. & Clayton, D. F., 1994. Song-induced ZENK gene expression in auditory pathways of songbird brain and its relation to the song control system. Journal of Neuroscience. 14 (11), 6652–6666.

Menzel, R., Heyne, A., Kinzel, C., Gerber, B., Fiala, A., 1999. Pharmacological dissociation between the reinforcing, sensitizing, and response-releasing functions of reward in honeybee classical conditioning. Behavioral neuroscience 113, 744.

Miller, G.A., Islam, M.S., Claridge, T.D.W., Dodgson, T., Simpson, S.J., 2008. Swarm formation in the desert locust *Schistocerca gregaria*: Isolation and NMR analysis of the primary maternal gregarizing agent. Journal of Experimental Biology 211, 370–376. doi: 10.1242/jeb.013458

Miura, T., 2005. Developmental regulation of caste-specific characters in social-insect polyphenism. Evolution and Development 7, 122–129. doi: 10.1111/j.1525-142X.2005.05014.x

Modlmeier, A.P., Foitzik, S., 2011. Productivity increases with variation in aggression among group members in *Temnothorax* ants. Behavioral Ecology 22, 1026–1032. doi: 10.1093/beheco/arr086

Modlmeier, A.P., Liebmann, J.E., Foitzik, S., 2012. Diverse societies are more productive: A lesson from ants. Proceedings of the Royal Society of London B:

Biological Sciences 279, 2142–2150. doi: 10.1098/rspb.2011.2376

Monastirioti, M., Charles E. Linn, J., White, K., 1996. Characterization of *Drosophila* tyramine β-hydroxylase gene and isolation of mutant flies lacking octopamine. Journal of Neuroscience 16, 3900–3911.

Montarolo, P.G., Goelet, P., Castellucci, V.F., Morgan, J., Kandel, E.R., Schacher, S., 1986. A critical period for macromolecular synthesis in long-term heterosynaptic facilitation in *Aplysia*. Science 234, 1249–1254. doi: 10.1126/science.3775383

Moorman, S., Mello, C. V., Bolhuis, J. J., 2011. From songs to synapses: Molecular mechanisms of birdsong memory. BioEssays. 33 (5), 377–385. doi: 10.1002/bies.201000150

Mosienko, V., Beis, D., Pasqualetti, M., Waider, J., Matthes, S., Qadri, F., Bader, M. and Alenina, N., 2015. Life without brain serotonin: reevaluation of serotonin function with mice deficient in brain serotonin synthesis. Behavioural brain research 277, 78-88.

Morgan, J.I., Curran, T., 1989. Stimulus-transcription coupling in neurons: Role of cellular immediate-early genes. Trends in Neurosciences 12, 459–462. doi: 10.1016/0166-2236(89)90096-9

Mousseau, T.A., Fox, C.W., 1998. The adaptive significance of maternal effects. Trends in Ecology & Evolution 13, 403–407. doi: 10.1016/S0169-5347(98)01472-4

Nakagawa, T., Katsuya, A., Tanimoto, S., Yamamoto, J., Yamauchi, Y., Minami, M., Satoh, M., 2003. Differential patterns of c-fos mRNA expression in the amygdaloid nuclei induced by chemical somatic and visceral noxious stimuli in rats. Neuroscience Letters 344, 197–200. doi: 10.1016/S0304-3940(03)00465-8

Nelson, R.J., Chiavegatto, S., 2001. Molecular basis of aggression. Trends in Neurosciences 24, 713–719. doi: 10.1016/S0166-2236(00)01996-2

Nelson, R.J. and Trainor, B.C., 2007. Neural mechanisms of aggression. Nature Reviews Neuroscience 8(7), 536.

Newland, P.L., Burrows, M., 1997. Processing of tactile information in neuronal networks controlling leg movements of the locust. Journal of Insect Physiology 43, 107–123. doi: 10.1016/S0022-1910(96)00081-9

Newland, P.L., Burrows, M., 1994. Processing of mechanosensory information from gustatory receptors on a hind leg of the locust. Journal of Comparative Physiology A 174, 399–410. doi:10.1007/BF00191706

Nichols, D.E., Nichols, C.D., 2008. Serotonin receptors. Chemical Reviews 108, 1614–1641. doi: 10.1021/cr078224o

Nijhout, H.F., Wheeler, D.E., 1982. Juvenile hormone and the physiological basis of insect polymorphisms. The Quarterly Review of Biology 57, 109–133. doi: 10.1086/412671

Oakes, L.M., 2010. Using habituation of looking time to assess mental processes in infancy. Journal of Cognition and Development 11, 255–268. doi: 10.1080/15248371003699977

O'Donnell, A., Odrowaz, Z., Sharrocks, A.D., 2012. Immediate-early gene activation by the MAPK pathways: What do and don't we know? Biochemical Society Transactions 40, 58–66. doi:10.1042/BST20110636

Orchard, I., Ramirez, J.M. and Lange, A.B., 1993. A multifunctional role for octopamine in locust flight. Annual review of entomology 38(1), 227-249.

Ott, S.R., Rogers, S.M., 2010. Gregarious desert locusts have substantially larger brains with altered proportions compared with the solitarious phase. Proceedings of the Royal Society B: Biological Sciences 277, 3087–3096.

Ott, S.R., Verlinden, H., Rogers, S.M., Brighton, C.H., Quah, P.S., Vleugels, R.K., Verdonck, R., Broeck, J.V., 2012. Critical role for protein kinase A in the acquisition of gregarious behavior in the desert locust. Proceedings of the National Academy of Sciences 109, E381–E387. doi: 10.1073/pnas.1114990109

Owald, D., Lin, S. and Waddell, S., 2015. Light, heat, action: neural control of fruit fly behaviour. Philosophical transactions of the Royal Society B 370(1677), 20140211.

Pener, M., Simpson, S., 2009. Locust phase polyphenism: An update, in: Advances in Insect Physiology. Academic Press, pp. 1–272.

Penick, C.A., Brent, C.S., Dolezal, K., Liebig, J., 2014. Neurohormonal changes associated with ritualized combat and the formation of a reproductive hierarchy in the ant *Harpegnathos saltator*. Journal of Experimental Biology 217, 1496–1503. doi: 10.1242/jeb.098301

Perkins, K.K., Dailey, G.M., Tjian, R., 1988. Novel jun- and fos-related proteins in *Drosophila* are functionally homologous to enhancer factor AP-1. EMBO J 7, 4265–4273.

Peveling, R., Attignon, S., Langewald, J. and Ouambama, Z., 1999. An assessment of the impact of biological and chemical grasshopper control agents on ground-dwelling arthropods in Niger, based on presence/absence sampling. Crop Protection, 18(5), pp.323-339.

Peveling, R., 2001. Environmental conservation and locust control - possible conflicts and solutions. Journal of Orthoptera Research 10, 171–187. doi: 10.1665/1082-6467(2001)010

Pfennig, D.W., Rice, A.M., Martin, R.A., 2006. Ecological opportunity and phenotypic plasticity interact to promote character displacement and species coexistence. Ecology 87, 769–779. doi: 10.1890/05-0787

Pflüger, H. J., Stevenson, P. A., 2005. Evolutionary aspects of octopaminergic systems with emphasis on arthropods. Arthropod Structure & Development, Arthropod Brain Morphology and Evolution 34, 379–396. doi: 10.1016/j.asd.2005.04.004

Pigliucci, M., Murren, C.J., Schlichting, C.D., 2006. Phenotypic plasticity and evolution by genetic assimilation. Journal of Experimenal Biology 209, 2362–2367. doi: 10.1242/jeb.02070

Pinsker, H., Kupfermann, I., Castellucci, V. and Kandel, E., 1970. Habituation and dishabituation of the GM-withdrawal reflex in Aplysia. Science, 167(3926), 1740-1742.

Qi, Y. X., Xia, R. Y., Wu, Y. S., Stanley, D., Huang, J., Ye, G. Y., 2014. Larvae of the small white butterfly, *Pieris rapae*, express a novel serotonin receptor. Journal of Neurochemistry 131, 767–777. doi: 10.1111/jnc.12940

Raleigh, M.J., McGuire, M.T., Brammer, G.L., Pollack, D.B. and Yuwiler, A., 1991. Serotonergic mechanisms promote dominance acquisition in adult male vervet monkeys. Brain Research 559(2), 181-190.

Rankin, C.H., Abrams, T., Barry, R.J., Bhatnagar, S., Clayton, D.F., Colombo, J.,

Coppola, G., Geyer, M.A., Glanzman, D.L., Marsland, S., McSweeney, F.K., Wilson, D.A., Wu, C.-F., Thompson, R.F., 2009. Habituation revisited: An updated and revised description of the behavioural characteristics of habituation. Neurobiology of Learning and Memory, Special Issue: Neurobiology of Habituation 92, 135–138. doi: 10.1016/j.nlm.2008.09.012

Renucci, M., Tirard, A., Charpin, P., Augier, R., Strambi, A., 2000. c-fos-related antigens in the central nervous system of an insect, *Acheta domesticus*. Archives of Insect Biochemistry and Physiology 45, 139–148. doi: 10.1002/1520-6327(200012)45

Rillich, J., Stevenson, P.A., 2014. A fighter's comeback: Dopamine is necessary for recovery of aggression after social defeat in crickets. Hormones and Behavior 66, 696–704. doi: 10.1016/j.yhbeh.2014.09.012

Rind, F.C., Simmons, P.J., 1997. Signaling of object approach by the DCMD neuron of the locust. Journal of Neurophysiology 77, 1029–1033.

Rind, F.C., Simmons, P.J., 1999. Seeing what is coming: Building collision-sensitive neurones. Trends in Neurosciences 22, 215–220. doi: 10.1016/S0166-2236(98)01332-0

Robertson, H. A., 1976. Octopamine, dopamine and noradrenaline content of the brain of the locust, Schistocerca gregaria. Experientia 32, 5, 552-554.

Roessingh, P., Simpson, S.J., James, S., 1993. Analysis of phase-related changes in behaviour of desert locust nymphs. Proceedings of the Royal Society B: Biological Sciences 252, 43–49. doi: 10.1098/rspb.1993.0044

Roessingh, P., Simpson, S.J., 1994. The time-course of behavioural phase change in nymphs of the desert locust, *Schistocerca gregaria*. Physiological Entomology 19, 191–197. doi: 10.1111/j.1365-3032.1994.tb01042.x

Roessingh, P., Bouaïchi, A., Simpson, S.J., 1998. Effects of sensory stimuli on the behavioural phase state of the desert locust, *Schistocerca gregaria*. Journal of Insect Physiology 44, 883–893.

Rogers, S.M., Matheson, T., Despland, E., Dodgson, T., Burrows, M., Simpson, S.J., 2003. Mechanosensory-induced behavioural gregarization in the desert locust *Schistocerca gregaria*. Journal of Experimental Biology 206, 3991–4002.

Rogers, S.M., Matheson, T., Sasaki, K., Kendrick, K., Simpson, S.J., Burrows, M., 2004. Substantial changes in central nervous system neurotransmitters and neuromodulators accompany phase change in the locust. Journal of Experimental Biology 207, 3603–3617.

Rogers, S.M., Harston, G.W.J., Kilburn-Toppin, F., Matheson, T., Burrows, M., Gabbiani, F., Krapp, H.G., 2010. Spatiotemporal receptive field properties of a looming-sensitive neuron in solitarious and gregarious phases of the desert locust. Journal of Neurophysiology103, 779–792. doi:10.1152/jn.00855.2009

Rogers, S.M., Ott, S.R., 2015. Differential activation of serotonergic neurons during short- and long-term gregarization of desert locusts. Proceedings of the Royal Society of London B: Biological Sciences 282, 20142062. doi:10.1098/rspb.2014.2062

Rogers, S.M., Cullen, D.A., Anstey, M.L., Burrows, M., Despland, E., Dodgson, T., Matheson, T., Ott, S.R., Stettin, K., Sword, G.A., Simpson, S.J., 2014. Rapid behavioural gregarization in the desert locust, *Schistocerca gregaria* entails synchronous changes in both activity and attraction to conspecifics. Journal of Insect Physiology 65, 9–26. doi: 10.1016/j.jinsphys.2014.04.004

Roisin, Y., 2000. Diversity and evolution of caste patterns, in: Termites: Evolution, Sociality, Symbioses, Ecology. Springer, Dordrecht, pp. 95–119. doi: 10.1007/978-94-017-3223-9_5

Rossiter, M., 1998. Assessment of genetic variation in the presence of maternal or paternal effects in herbivorous insects, in: Genetic Structure and Local Adaptation in Natural Insect Populations. Springer, Boston, MA, pp. 113–138. doi: 10.1007/978-1-4757-0902-5_6

Rowell, C.H.F., 1971. The orthopteran descending movement detector (DMD) neurones: a characterisation and review. Zeitschrift für vergleichende Physiologie 73, 167–194. doi: 10.1007/BF00304131

Ruaud, A.-F., Lam, G., Thummel, C.S., 2011. The *Drosophila* NR4A nuclear receptor DHR38 regulates carbohydrate metabolism and glycogen storage. Molecular Endocrinology 25, 83–91. doi: 10.1210/me.2010-0337

Sandner, G., Oberling, P., Silveira, M.C., Di Scala, G., Rocha, B., Bagri, A., Depoortere, R., 1993. What brain structures are active during emotions? Effects of brain stimulation elicited aversion on c-fos immunoreactivity and behavior. Behavioural Brain Research 58, 9–18. doi: 10.1016/0166-4328(93)90086-6

Santer, R.D., Rind, F.C., Stafford, R., Simmons, P.J., 2006. Role of an identified looming-sensitive neuron in triggering a flying locust's escape. Journal of Neurophysiology 95, 3391–3400. doi: 10.1152/jn.00024.2006

Sanyal, S., Sandstrom, D.J., Hoeffer, C.A., Ramaswami, M., 2002. AP-1 functions upstream of CREB to control synaptic plasticity in *Drosophila*. Nature 416, 870–874. doi: 10.1038/416870a

Schultz, W., 2016. Dopamine reward prediction-error signalling: a two-component response. Nature Reviews Neuroscience 17(3), 183.

Schwarz, M.P., Richards, M.H., Danforth, B.N., 2007. Changing paradigms in insect social evolution: insights from halictine and allodapine bees. Annual Reviews of Entomology 52, 127–150. doi: 10.1146/annurev.ento.51.110104.150950

Shand, J.D., 2015. The effects of density-dependent polyphenism on circadian biology of the desert locust Schistocerca gregaria. University of Leicester.

Sharp, F.R., Sagar, S.M., Hicks, K., Lowenstein, D., Hisanaga, K., 1991. c-fos mRNA, fos, and fos-related antigen induction by hypertonic saline and stress. Journal of Neuroscience 11, 2321–2331.

Shimoji, H., Aonuma, H., Miura, T., Tsuji, K., Sasaki, K., Okada, Y., 2017. Queen contact and among-worker interactions dually suppress worker brain dopamine as a potential regulator of reproduction in an ant. Behavioural Ecology and Sociobiology71, 35. doi:10.1007/s00265-016-2263-3

Simões, P., Ott, S.R., Niven, J.E., 2011. Associative olfactory learning in the desert locust, *Schistocerca gregaria*. Journal of Experimental Biology 214, 2495–2503. doi: 10.1242/jeb.055806

Simões, P.M., Ott, S.R., Niven, J.E., 2012. A long-latency aversive learning mechanism enables locusts to avoid odours associated with the consequences of ingesting toxic food. The Journal of Experimental Biology 215, 1711–1719.

Simões, P.M.V., Niven, J.E., Ott, S.R., 2013. Phenotypic transformation affects associative learning in the desert locust. Current Biology 23, 2407–2412. doi: 10.1016/j.cub.2013.10.016

Simões, P.M.V., Ott, S.R., Niven, J.E., 2016. Environmental adaptation, phenotypic plasticity, and associative learning in insects: the desert locust as a case study. Integrative and Comparative Biology 56, 914–924. doi: 10.1093/icb/icw100

Simonnet, M.M., Berthelot-Grosjean, M., Grosjean, Y., 2014. Testing *Drosophila* olfaction with a Y-maze assay. Journal of Visualized Experiments 12. doi: 10.3791/51241

Simpson, S., Despland, E., Hägele, B., Dodgson, T., 2001. Gregarious behavior in desert locusts is evoked by touching their back legs. Proceedings of the National Academy of Sciences 98, 3895–3897.

Simpson, S.J., Miller, G.A., 2007. Maternal effects on phase characteristics in the desert locust, *Schistocerca gregaria*: a review of current understanding. Journal of Insect Physiology 53, 869–876. doi: 10.1016/j.jinsphys.2007.05.011

Sitaraman, D., Zars, M., LaFerriere, H., Chen, Y. C., Sable-Smith, A., Kitamoto, T., Rottinghaus, G.E., Zars, T., 2008. Serotonin is necessary for place memory in *Drosophila*. Proceedings of the National Academy of Sciences 105, 5579–5584. doi: 10.1073/pnas.0710168105

Sitaraman, D., LaFerriere, H., Birman, S., Zars, T., 2012. Serotonin is critical for rewarded olfactory short-term memory in *Drosophila*. Journal of Neurogenetics 26, 238–244. doi: 10.3109/01677063.2012.666298

Sloley, B., Owen, M.D., 1982. The effects of reserpine on amine concentrations in the nervous system of the cockroach (*Periplaneta americana*). Insect Biochemistry 12, 469–476. doi: 10.1016/0020-1790(82)90014-2

Sloley, B.D., Downer, R.G.H., 1990. Catabolism and disposition of exogenous 5-hydroxytryptamine in cockroach, *Periplaneta americana*, tissues. Insect Biochemistry 20, 165–171. doi: 10.1016/0020-1790(90)90009-J

Sloley, B.D., 2004. Metabolism of monoamines in invertebrates: the relative importance of monoamine oxidase in different phyla. NeuroToxicology, Monoamine Oxidases: Molecular, Pharmacological and Neurotoxicological Aspects 25, 175–183. doi: 10.1016/S0161-813X(03)00096-2

Sluder, A.E., Maina, C.V., 2001. Nuclear receptors in nematodes: Themes and variations. Trends in Genetics 17, 206–213. doi: 10.1016/S0168-9525(01)02242-9

Smith, T.J., 1990. Phylogenetic distribution and function of arylalkylamine N-acetyltransferase. Bioessays 12, 30–33. doi: 10.1002/bies.950120107

Sneddon, L.U., Taylor, A.C., Huntingford, F.A. and Watson, D.G., 2000. Agonistic behaviour and biogenic amines in shore crabs Carcinus maenas. Journal of Experimental Biology 203(3), 537-545.

Sombati, S. and Hoyle, G., 1984. Generation of specific behaviors in a locust by local release into neuropil of the natural neuromodulator octopamine. Developmental Neurobiology 15(6), 481-506.

Sommerlandt, F.M.J., Rössler, W., Spaethe, J., 2016. Impact of light and alarm pheromone on immediate early gene expression in the European honeybee, *Apis mellifera*. Entomological Science 20, 122–126. doi: 10.1111/ens.12234

Song, H., Wenzel, J.W., 2008. Phylogeny of bird-grasshopper subfamily Cyrtacanthacridinae (Orthoptera: Acrididae) and the evolution of locust phase polyphenism. Cladistics 24, 515–542. doi:10.1111/j.1096-0031.2007.00190.x

Song, H., 2010. Density-dependent phase polyphenism in nonmodel locusts: A minireview. Psyche: a Journal of Entomology e741769. doi: 10.1155/2011/741769

Souza, A.S., Jansen, J., Tempelman, R.J., Mendl, M., Zanella, A.J., 2006. A novel method for testing social recognition in young pigs and the modulating effects of relocation. Applied Animal Behavioural Science 99, 77–87. doi: 10.1016/j.applanim.2005.09.008

Steedman, A., 1988. Locust handbook. Institute, Overseas development Natural Resources: London, UK.

Stettin, K., 2014. Mechanisms driving the acquisition and maintenance of gregarious behaviour in the desert locust. University of Cambridge.

Stevenson, P.A., Dyakonova, V., Rillich, J., Schildberger, K., 2005. Octopamine and experience-dependent modulation of aggression in crickets. The Journal of Neuroscience 25, 1431–1441. doi: 10.1523/JNEUROSCI.4258-04.2005

Stevenson, P.A., Hofmann, H.A., Schoch, K., Schildberger, K., 2000. The fight and flight responses of crickets depleted of biogenic amines. Journal of Neurobiology. 43, 107–120. doi: 10.1002/(SICI)1097-4695(200005)43:2

Stevenson, P.A., Rillich, J., 2016. Controlling the decision to fight or flee: the roles of biogenic amines and nitric oxide in the cricket. Current Zoology 62, 265–275. doi: 10.1093/cz/zow028

Stroschein-Stevenson, S.L., Foley, E., O'Farrell, P.H., Johnson, A.D., 2005. Identification of *Drosophila* gene products required for phagocytosis of *Candida albicans*. PLOS Biology 4, e4. doi: 10.1371/journal.pbio.0040004

Swank, M.W., Bernstein, I.L., 1994. c-fos induction in response to a conditioned stimulus after single trial taste aversion learning. Brain Research 636, 202–208. doi: 10.1016/0006-8993(94)91018-9

Sword, G.A., 2003. To be or not to be a locust? A comparative analysis of behavioral phase change in nymphs of *Schistocerca americana* and *S. gregaria*. Journal of Insect Physiology 49, 709–717.

Symmons, P.M., Cressman, K., 2001. Desert locust guidelines: Biology and behaviour. FAO Rome.

Tanaka, S., Nishide, Y., 2012. Do desert locust hoppers develop gregarious characteristics by watching a video? Journal of Insect Physiology 58, 1060–1071. doi: 10.1016/j.jinsphys.2012.04.005

Tanaka, S., Nishide, Y., 2013. Behavioral phase shift in nymphs of the desert locust, *Schistocerca gregaria*: Special attention to attraction/avoidance behaviors and the role of serotonin. Journal of Insect Physiology 59, 101–112. doi: 10.1016/j.jinsphys.2012.10.018

Thompson, R. F., Spencer, W. A., 1966. Habituation: A model phenomenon for the study of neuronal substrates of behavior. Psychological Review 73(1), 16-43.

Thorpe, W.H., 1957. Learning and instinct in animals. The Eugenics Review 48, 241–242.

Tighe, T.J., Leaton, R.N., 2016. Habituation: Perspectives from child development, animal behavior, and neurophysiology. Routledge. Oxford, England: Lawrence Erlbaum.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., Pimentel, H.,

Salzberg, S.L., Rinn, J.L., Pachter, L., 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nature Protocols 7, 562–578. doi: 10.1038/nprot.2012.016

Tronel, S., Sara, S.J., 2002. Mapping of olfactory memory circuits: region-specific c-fos activation after odor-reward associative learning or after its retrieval. Learn. Mem. 9, 105–111. doi: 10.1101/lm.47802

Ugajin, A., Kiya, T., Kunieda, T., Ono, M., Yoshida, T., Kubo, T., 2012. Detection of neural activity in the brains of Japanese honeybee workers during the formation of a "hot defensive bee ball." PLoS ONE 7, e32902. doi: 10.1371/journal.pone.0032902

Ugajin, A., Kunieda, T., Kubo, T., 2013. Identification and characterization of an Egr ortholog as a neural immediate early gene in the European honeybee (*Apis mellifera* L.). FEBS Letters 587, 3224–3230. doi: 10.1016/j.febslet.2013.08.014

Umemoto, S., Kawai, Y., Ueyama, T., Senba, E., 1997. Chronic glucocorticoid administration as well as repeated stress affects the subsequent acute immobilization stress-induced expression of immediate early genes but not that of NGFI-A. Neuroscience 80, 763–773. doi: 10.1016/S0306-4522(97)00050-X

Uvarov, B.P., Zolotarevsky, B.N., 1929. Phases of locusts and their interrelations. Bulletin of Entomological Research 20, 261–265. doi: 10.1017/S0007485300021234

Uvarov, B.P., 1921. A revision of the genus *Locusta*, L. (= *Pachytylus*, Fieb.), with a new theory as to the periodicity and migrations of locusts. Bulletin of Entomological Research 12, 135–163. doi: 10.1017/S0007485300044989

Uvarov, B., 1966. Grasshoppers and locusts. A handbook of general acridology. Volume I. Anatomy, physiology, development, phase polymorphism, introduction to taxonomy. Cambridge University Press, Cambridge.

Uvarov, B., 1977. Grasshoppers and locusts. A handbook of general acridology. Volume 2. Behaviour, ecology, biogeography, population dynamics. Centre for Overseas Pest Research, London.

Van Hiel, M.B., Van Wielendaele, P., Temmerman, L., Van Soest, S., Vuerinckx, K., Huybrechts, R., Broeck, J.V., Simonet, G., 2009. Identification and validation of housekeeping genes in brains of the desert locust *Schistocerca gregaria* under different developmental conditions. BMC Molecular Biology 10, 56. doi: 10.1186/1471-2199-10-56

Veenema, A.H., Neumann, I.D., 2008. Central vasopressin and oxytocin release: regulation of complex social behaviours, in: Neumann, I.D., Landgraf, R. (Eds.), Progress in Brain Research, Advances in Vasopressin and Oxytocin — From Genes to Behaviour to Disease. Elsevier, pp. 261–276.

Vergoz, V., McQuillan, H.J., Geddes, L.H., Pullar, K., Nicholson, B.J., Paulin, M.G., Mercer, A.R., 2009. Peripheral modulation of worker bee responses to queen mandibular pheromone. Proceedings of the National Academy of Sciences 106, 20930–20935. doi: 10.1073/ pnas.0907563106

Verlinden, H., Vleugels, R., Zels, S., Dillen, S., Lenaerts, C., Crabbé, K., Spit, J., Vanden Broeck, J., 2014. Receptors for neuronal or endocrine signaling molecules as potential targets for the control of insect pests. Advances in Insect Physiology 46, 167–303.

Verlinden, H., Vleugels, R., Marchal, E., Badisco, L., Pflüger, H.-J., Blenau, W., Broeck,

J.V., 2010. The role of octopamine in locusts and other arthropods. Journal of Insect Physiology 56, 854–867. doi: 10.1016/j.jinsphys.2010.05.018

Vieira, R., Mancebo, M.J., Aldegunde, M., 2007. Effects of reserpine and p-chloroamphetamine on 5-HT metabolism and release in the cerebral ganglia of *Inachis io* (Lepidoptera). Journal of physiology and biochemistry 63, 129–141.

Vleugels, R.K., Verlinden, H., Broeck, J.V., 2015. Serotonin, serotonin receptors and their actions in insects. Neurotransmitter 2, e314. doi: 10.14800/nt.314

Voigt, J.P. and Fink, H., 2015. Serotonin controlling feeding and satiety. Behavioural brain research 277, 14-31.

Voss, R., Zeil, J., 1998. Active vision in insects: an analysis of object-directed zig-zag flights in wasps (*Odynerus spinipes*, Eumenidae). Journal of Comparative Physiology A 182, 377–387. doi: 10.1007/s003590050187

Wai, M. S. M. et al. (2006) The pattern of c-fos activation in the CNS is related to behavior in the mudskipper, Periophthalmus cantonensis. Behavioural Brain Research. 167 (2), 318–327. doi: 10.1016/j.bbr.2005.09.018

Walter, M.F., Zeineh, L.L., Black, B.C., McIvor, W.E., Wright, T.R.F., Biessmann, H., 1996. Catecholamine metabolism and in vitro induction of premature cuticle melanization in wild type and pigmentation mutants of *Drosophila melanogaster*. Archives of Insect Biochemical Physiology 31, 219–233. doi: 10.1002/(SICI)1520-6327(1996)31:2

Wang, X., Fang, X., Yang, P., Jiang, X., Jiang, F., Zhao, D., Li, B., Cui, F., Wei, J., Ma, C., Wang, Y., He, J., Luo, Y., Wang, Z., Guo, X., Guo, W., Wang, X., Zhang, Y., Yang, M., Hao, S., Chen, B., Ma, Z., Yu, D., Xiong, Z., Zhu, Y., Fan, D., Han, L., Wang, B., Chen, Y., Wang, J., Yang, L., Zhao, W., Feng, Y., Chen, G., Lian, J., Li, Q., Huang, Z., Yao, X., Lv, N., Zhang, G., Li, Y., Wang, J., Wang, J., Zhu, B., Kang, L., 2014. The locust genome provides insight into swarm formation and long-distance flight. Nature Communications 5, 1–9. doi: 10.1038/ncomms3957

Watanabe, T., Ugajin, A., Aonuma, H., 2017. Whole-brain activity mapping for neuroethological study in the cricket, in: The 8th International Symposium on Adaptive Motion of Animals and Machines. Presented at the AMAM2017, Sapporo, Japan.

Wehner, R., Michel, B., Antonsen, P., 1996. Visual navigation in insects: coupling of egocentric and geocentric information. Journal of Experimental Biology 199, 129–140.

West-Eberhard, M.J., 2003. Developmental plasticity and evolution. Oxford University Press.

Wilson, A.D.M., Whattam, E.M., Bennett, R., Visanuvimol, L., Lauzon, C., Bertram, S.M., 2010. Behavioral correlations across activity, mating, exploration, aggression, and antipredator contexts in the European house cricket, *Acheta domesticus*. Behavioural Ecology and Sociobiology 64, 703–715. doi: 10.1007/s00265-009-0888-1

Winberg, S. and Thörnqvist, P.O., 2016. Role of brain serotonin in modulating fish behavior. Current Zoology 62(3), 317-323.

Wittkopp, P.J., Carroll, S.B., Kopp, A., 2003. Evolution in black and white: genetic control of pigment patterns in *Drosophila*. Trends in Genetics 19, 495–504. doi: 10.1016/S0168-9525(03)00194-X

Woltereck, R., 1909. Weitere experimentelle Unterschungen uber Artveranderung, speizell uber das Wesen quantitativer Artenunterschiede bei Daphniden. Verhandlungen der Deutschen Zoologischen Gesellschaft 110–172.

Wystrach, A., Schwarz, S., Schultheiss, P., Beugnon, G., Cheng, K., 2011. Views, landmarks, and routes: How do desert ants negotiate an obstacle course? Journal of Comparative Physiology A 197, 167–179. doi: 10.1007/s00359-010-0597-2

Yan, H., Simola, D. F., Bonasio, R., Liebig, J., Berger, S. L., Reinberg, D., 2014. Eusocial insects as emerging models for behavioural epigenetics. Nature Reviews Genetics 15, 677–688. doi: 10.1038/nrg3787

Zhang, S. W., Lehrer, M., Srinivasan, M. V., 1999. Honeybee memory: navigation by associative grouping and recall of visual stimuli. Neurobiology of Learning and Memory 72, 180–201. doi: 10.1006/nlme.1998.3901

Zhang, F., Lin, M., Abidi, P., Thiel, G., Liu, J., 2003. Specific interaction of Egr1 and c/EBPβ leads to the transcriptional activation of the human low density lipoprotein receptor gene. Journal of Biological Chemistry 278, 44246–44254. doi: 10.1074/jbc.M305564200