An ecophysiological investigation of the effects of anthropogenic habitat loss, fragmentation and degradation on the agile antechinus

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Antechinus agilis in a *Eucalyptus* forest fragment in South Gippsland.
Photo: C Johnstone

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Summary

Anthropogenic habitat fragmentation has well-established deleterious effects on populations and communities of native vertebrates, but the mechanisms underlying population decline under fragmentation remain poorly understood. Most studies of vertebrates in anthropogenically-fragmented habitats have focused on population density, demographics or fecundity. Relatively little attention has been given to indices of health status, body condition or physiological stress.

In this study, 30 populations of a small marsupial, the agile antechinus (*Antechinus agilis*), living in anthropogenically-fragmented forest patches were sampled in two years (2007 and 2008). Immediately after sampling in each fragment, a population in a matched control site in similar, but unfragmented forest (a 'pseudofragment') was sampled. Indices of population density (relative abundance), estimated fat reserves (mass-size residuals), health status (erythrocyte variables), parasite load (simplified ectoparasite counts and eosinophil percentages) and chronic physiological stress (total and differential immune cell counts) were examined.

Relative abundances were lower and parasite load indices higher in fragmented than continuous forest. Fragment populations displayed indications of regenerative anaemia, which is related to poor health status and potentially caused by chronic stress, frequent blood loss or heavy parasite loads. Estimated fat reserves were higher in fragment than continuous forest populations. Nonetheless, differential leukocyte counts suggested that chronic physiological stress was greater (i.e. greater neutrophil-to-lymphocyte ratios) (N:L) in populations in fragments.

Anthropogenic fragmentation effects are not often distinguished in the ecological literature from those of co-occurring processes, such as habitat loss and degradation. To investigate the effects of these processes, environmental factors were examined that were

thought to have a potential influence on agile antechinus (e.g. fragment patch core area, proportion of edge habitat, isolation, woody debris abundance, shrub density etc).

Relative abundance of agile antechinus was positively correlated with forest patch core area and native tree-cover within a 0.5 km radius of a study site. Estimated fat reserves, particularly in males, were greater in populations in fragments with a smaller core area, but statistical modelling indicated that the effect was an indirect one: males had greater estimated fat reserves where the abundance of conspecifics was lower, suggesting that this metric was responding to intraspecific competition and *per capita* food availability. Health status, indexed by erythrocyte indicators of regenerative anaemia,was positively associated with greater microhabitat heterogeneity, and abundance of shrubs, logs and native trees other than *Eucalyptus* species. Female abundances were lower in edge habitat (< 60 m from edge) than in fragment interiors (> 80 m from edge), and females had higher chronic stress indicators (N:L) where fragments were more highly dissected by edge habitat. Although parasite load indices and male N:L were higher in fragment than continuous forest sites, the environmental factors responsible were not identified.

The study has demonstrated that anthropogenic habitat fragmentation, loss and degradation can have broadly negative effects on a native vertebrate, not only on its population density, but also in terms of health status and chronic physiological stress. This is a serious concern from a conservation management perspective, because chronic stress has pronounced fitness-reducing effects in vertebrates, including reduced reproductive investment, fecundity and survivorship.

Statement of Originality

Monash University
Monash Research Graduate School

Declaration for thesis partially based on conjointly published work

In accordance with Monash University Doctorate Regulation 17 / Doctor of Philosophy regulations the following declarations are made.

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

The thesis includes one original paper accepted for publication in a peer reviewed journal and three original papers that are in review or preparation for submission to peer-reviewed journals. The core theme of the thesis is the effects of anthropogenic habitat fragmentation (and associated habitat loss and degradation) on populations of agile antechinus using a physiological ecology approach. The ideas, development and writing-up of all the papers in the thesis were principally my responsibility, Christopher Johnstone, the candidate, working within the School of Biological Sciences under the supervision of Associate Professor Alan Lill and Dr Richard Reina.

The inclusion of co-authors on papers in the thesis reflects the fact that the work came from active collaboration among researchers and acknowledges input into team-based research. In the case of chapters three, four, five and six my contribution involved the following:

Thesis Chapter	Publication Title	Publication status	Nature and extent of candidate's contribution
3	Are haematological indicators of stress and poor condition associated with habitat fragmentation in the agile antechinus?	In review	Conception, collection of data set, laboratory procedures, data analysis and writing
4	Impact of anthropogenic habitat fragmentation on population health in a small, carnivorous marsupial	In press	Conception, collection of data set, data analysis and writing
5	Response of the agile antechinus to habitat edge, configuration and condition in fragmented forest	In preparation	Conception, collection of data set, laboratory procedures, data analysis and writing
6	Effects of habitat loss, fragmentation and degradation on mammal abundance and condition: analysis with tree-based statistical modelling	In preparation	Conception, collection of data set, laboratory procedures, data analysis and writing

Signed: ……... Date ……..

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Chapter One

Introduction

1.0 Theoretical overview

Anthropogenic habitat fragmentation is a considerable threat to biodiversity worldwide (Foley et al. 2005). It is generally accepted that anthropogenic fragmentation of a natural vegetation results in levels of species loss that are greater than would be expected from habitat loss alone (Turner 1996). However, despite extensive comparative and experimental field research, computer modelling and discussion, there is still uncertainty concerning the actual mechanisms that underlie aggravation of species loss in fragmented habitats (Ewers & Didham 2005; Fischer & Lindenmayer 2007; Fletcher et al. 2007). It can be argued that even the supposed genetic (inbreeding depression etc) and stochastic (vulnerability to catastrophes) effects of habitat fragmentation, reduction and isolation have not been empirically demonstrated very often (Caughley 1994; Hedrick et al. 1996).

Whereas some species rapidly decline to local extinction after an initial fragmentation event (Newell 1999), others persist in a state of gradual decline for years or decades (Brooks et al. 1999; Pimm et al. 1993). Native species may even show an increase in population density while the biological community of which they are a part suffers a general loss of competitor or partner species (e.g. a co-dependant pollinator and flowering plant) (Ewers & Didham 2005). Effects such as predator or parasite release, invasion by generalists or edge-specialists, matrix subsidies, high forest-field ecotone productivity, altered behaviour of animals in fragmented environments and/or establishments of meta-populations, anthropogenic disturbance (e.g. livestock grazing, over-hunting) and synergistic interactions among threatening processes alter the 'stressfulness' of the environment and add further uncertainty to generalized comprehensive synthesis theories about species' response to anthropogenic fragmentation of their habitat (Ewers & Didham 2005; Fletcher et al. 2007; Hobbs 2001; Lidicker 1999; Saunders et al. 1999).

In an increasingly anthropogenically fragmented terrestrial landscape, long-term persistence of many native species now relies on their capacity to form stable, reproductively viable populations in fragmented habitat (Turner 1996). In order for governments and landowners to make informed choices about conservation measures, there needs to be a fuller understanding of the underlying mechanisms of gradual decline and how these mechanisms can interact (Hobbs 2001).

1.1 Vertebrate chronic physiological stress in fragmented and degraded habitats

Populations of vertebrates that are capable of persisting in fragmented habitat are likely to encounter novel challenges to survival, dispersal and reproduction, including abiotic effects (e.g. changes to environmental flows) (Saunders et al. 1999), increased anthropogenic disturbance (Turner 1996), edge effects (Lidicker 1999; Saunders et al. 1999; Yahner 1988), novel barriers to migration and gene-flow (Berry 2001; Brooker & Brooker 2002; Diffendorfer et al. 1995), invasion by exotic generalists or predators (including 'spill-over' effects) (Lidicker 1999; May & Norton 1996), changes to microclimate (Saunders et al. 1999), anthropogenic disturbance and degradation (e.g. livestock grazing, weed invasion, firewood collection (Hobbs 2001; Knight & Fox 2000) and loss of partner-species (Aizen & Feinsinger 1994; Jennertsen 1988)).

In vertebrates, overt threats to survivorship or reproduction elicit an acute stress response (Simpkiss & Devine 2003; Tsigos & Chrousos 2002), leading to changes in behaviour (e.g. sheltering, escape; Tsigos & Chrousos 2002) and physiology (e.g. increased heart rate, mobilisation of energy substrate stores Sapolsky et al. 2000) that improve an individual's immediate chances of surviving and of eventually producing young. The stress response is primarily governed at a neuroendocrine level via the

hypothalamus-pituitary-adrenal axis (HPA-axis) and autonomic axis (AN-axis) (Sapolsky et al. 2000; Ziegler & Herman 2002) (AN-axis is termed 'neurogenic system' by some authors, e.g. Siegel 1980) (hereinafter HPA-axis and AN-axis mediated stress is termed 'physiological stress', Perdrizet 1997; Selye 1998; Siegel 1980).

Occasional stress responses are essential for life. However, prolonged or frequent acute stress responses can lead to a pathological state of physiological exhaustion termed 'chronic stress' (Sapolsky et al. 2000; Siegel 1980; Simpkiss & Devine 2003; Tsigos & Chrousos 2002), which is associated with reduced overall condition, reproductive output and immunocompetence (Sapolsky et al. 2000).

This study examined whether indicators of chronic physiological stress were higher in populations of the forest-interior adapted dasyurid, the agile antechinus (*Antechinus agilis*, Family: Dasyuridae), living in fragmented (< 300 ha) than in paired unfragmented (> 1000 ha) *Eucalyptus* forest study sites. The overarching aim was to use a large-scale comparative field study approach to examine whether habitat fragmentation might be associated with chronic physiological stress in a free-living vertebrate. If true, then chronic stress could be a factor that is negatively influencing reproduction, survivorship and ultimately persistence at fragmented sites (Martínez-Mota et al. 2007). The study quantified environmental variables (landscape, habitat structure and floristic) to establish whether differences in habitat were evident (e.g. vegetation composition, habitat complexity or heterogeneity); where these would classically be considered likely to negatively impact a vertebrate population (e.g. reduced habitat area or food resources), the study examined possible correlations among the environmental variables and indices of physiological stress.

Physiological stress and overall body condition were evaluated using haematological, mass, morphometric, body condition indices, and parasite variables to address five areas of inquiry.

(1) Do populations living in fragmented habitat have higher haematological indicators of physiological stress and lower indicators of condition than those in similar continuous forest? (Chapter 3)

(2) Do populations living in fragmented habitat have lower indices of fat reserves, lower relative abundance and higher ectoparasite infection level than those in similar continuous forest? (Chapter 4)

(3) Do populations living in *Eucalyptus* forest fragments show differences in relative abundance, body condition indices or haematological indicators of stress that correlate with proximity to forest-field ecotones and/or indices of fragment configuration, isolation and vegetation descriptors? (Chapter 4)

(5) Using tree-based modelling methods, is it possible to generate predictive models of abundance and indices of stress and condition using a suite of habitat variables measured for both fragmented and continuous forest sites? (Chapter 5)

1.2 Study species: the agile antechinus

The genus *Antechinus* is native to Australia and New Guinea (Krajewski et al. 1994). The agile antechinus is a small (adult females 10-26 g, males 15-45 g) nocturnal and scansorial member of this family (Figures 1 and 2). Individuals nest communally in tree-hollows and the diet comprises primarily terrestrial invertebrates, supplemented by some small vertebrates and scavenging from carcasses (Lunney et al. 2001). Pre-1998, the species was included in the brown antechinus (*Antechinus stuartii*) species complex (Dickman et al. 1998) and consequently some early studies ostensibly of brown antechinus were actually conducted on agile antechinus. Agile and brown antechinus are considered to have highly similar life-histories and autecology (Menkhorst & Knight

2004), and authors frequently cite *A. stuartii* studies to support theories concerning *A. agilis* and vice versa.

Fig 1. Agile antechinus. Photo taken in late December 2007. The individual was male and thus was almost certainly a juvenile recently emerged from the maternal tree-nest. Size: approx 90 mm nose to vent. Photo: C Johnstone.

The genus *Antechinus* provides an unusual example of semelparity in a mammal (Braithwaite & Lee 1979). In agile antechinus, all males and most females live for approximately one year and breed just once in that time (Braithwaite & Lee 1979). An annual synchronized 2-3 week breeding rut occurs in the Austral late winter (mid-August in the study area). This is followed by a complete male die-off (Barnett 1973), a life-event linked to loss of body condition, an increase in indicators of physiological stress and the development of a negative nitrogen balance in males (Barnett 1973; Cheal et al. 1976) as well as: 1) involution of immune tissue (in particular, lymphoid tissue) and general immunosuppression (Barker et al. 1978), 2) the activation of a hormone shunt that strips

the body of protein (Woollard 1971), and 3) elevated internal parasite infection level (Beveridge & Barker 1976).

Most females die after weaning their only litter, although a few (~15%) survive to breed in a second year (Braithwaite & Lee 1979; Lee & Cockburn 1985). Sperm storage crypts allow females to ovulate quite late in the breeding season (Shimmin et al. 1999), and gestation is relatively long for a marsupial of this size, approximately five weeks, and includes several periods of slow or arrested development (Naylor et al. 2008). Young remain attached to teats in the mother's pouch for approximately five weeks post-parturition (Figure 2) (Lee et al. 1982) and are nursed until the Austral summer (December in study area).

Fig 2. Agile antechinus. Photos taken in October 2007. The individuals were females and were carrying pouch young, visible on the right.

Photos: C Johnstone.

Dispersal is male-biased (Kraaijeveld-Smit et al. 2002a) and occurs in the Austral late summer (January-February). Females are highly philopatric and typically remain in their maternal home range throughout their life (Kraaijeveld-Smit et al. 2002a) (and see *A. stuartii*; Cockburn et al. 1985b).

Female teat number varies geographically (Cockburn et al. 1983) and is governed genetically (Beckman et al. 2007; Cockburn et al. 1983). The reason for this variation is unknown, but is thought to represent adaptation to local conditions (Beckman et al. 2007). In this study, all females had eight teats (unpublished data). The implication of this is that prior to anthropogenic fragmentation of *Eucalyptus* forests, the environments in which agile antechinus populations were living were reasonably similar across the study area. The species is unusual in that individuals seem to have two distinct spatial ranges, an ~ 0.5-2 ha foraging range and a larger 'social range' of up to 5 ha (known in *A. stuartii*; Lazenby-Cohen & Cockburn 1991).

The species is seldom or never caught in surrounding matrices of grazed paddocks around forest fragments (Bennett 1990a; Holland & Bennett 2009). As such, the species seems unlikely to benefit from a matrix-subsidy, although this possibility cannot be entirely ruled out. The species is considered reliant on canopy cover, in particular that of *Eucalyptus* tree species, which provide hollows for nesting (Sumner & Dickman 1998).

1.3 Habitat fragmentation

Anthropogenically induced habitat loss typically results in fragmentation of the remnant native vegetation (Figure 3) (Fahrig 2003; Fletcher et al. 2007). The result is a landscape level process by which a relatively contiguous natural habitat is degraded into smaller patches scattered in an altered matrix (Foley et al. 2005).

Fig 3. Anthropogenically fragmented *Eucalyptus* forest isolated by a matrix of grazed paddocks. This is a view near Fish Creek in the South Gippsland region where this study was conducted. Photo: courtesy of Gary Wallis

Remnant native vegetation patches are likely to undergo gradual habitat degradation, species loss and alteration of abiotic and biotic processes for years or decades after the initial fragmentation event, so that eventually fragment community-structure may more closely resemble that of the surrounding matrix than that of the contiguous forest from which it was excised (Diamond et al. 1987). Although fragmentation resulting from anthropogenic activity is of high conservation concern, fragmentation processes occur naturally as catastrophes such as bushfires, floods or lava flows can lead to fragmentation of habitat (Ewers & Didham 2005). Topography can led to population effects that resemble those of anthropogenic fragmentation, in particular, isolation, restriction of population size and susceptibility to stochastic events (Brown 1971; Diamond 1977) Initial research and theory on oceanic island archipelagos led to the application of

classical island biogeography theory in habitat fragmentation studies (Gottfried 1979; MacArthur & Wilson 1967; Simberloff 1974; Suorsa et al. 2003; Yahner 1992).

Matrices of cleared fields, crops, timber plantations or mining sites around terrestrial forest fragments are not, however, likely to be as inhospitable as oceans around terrestrial islands (Ewers & Didham 2005). Thus, there has been an increasing trend in the literature away from applying strict island biogeography theory to terrestrial fragmented habitats and towards a more integrated landscape ecology approach (Ewers & Didham 2005; Fahrig 2001; Hanski 1994; Hanski & Gyllenberg 1997; Polis et al. 1997).

1.3.1 Habitat fragmentation in the study area

The study was conducted in 2007 and 2008 in South Gippsland, Victoria, Australia (Figure 4). The region has one of the longest histories of European settlement and agriculture in mainland Australia. Forest clearing for agriculture, gold mining, and timber production was triggered by an 1869 Lands Act allowing settlement, and all the fragments in our study have been isolated by anthropogenic land clearing for > 50 years. The matrix surrounding all *Eucalyptus* fragments in this study was pasture (Figure 3), primarily grazed by dairy and beef cattle, but with some sheep farming. Forest-field ecotones were well defined and fragments were often bounded by fences so that 'edges' were easily identified (Figure 5).

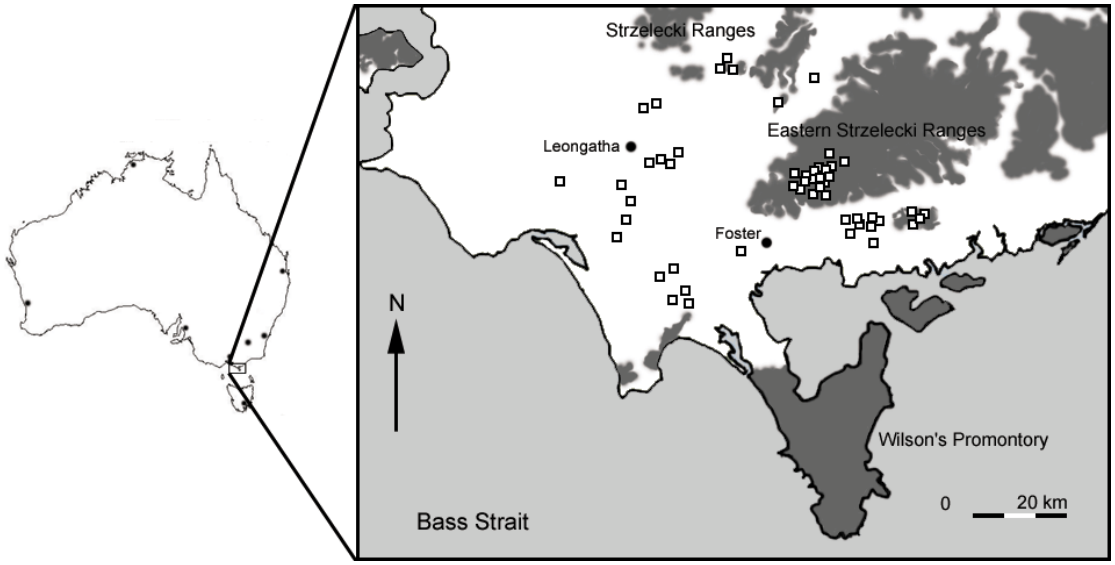

Fig 4. Study region in south-east Australia. White = cleared, agricultural land. Darker shaded areas = tree cover (includes native re-growth, old growth forest and native plantations). Approximate locations of study sites are indicated by white boxes (□). Map based on Victorian Department of Sustainability and Environment (DSE) interactive online maps (http://www.dse.vic.gov.au/).

Species relaxation, the gradual loss of species after fragmentation of habitat, still could be occurring (Saunders et al. 1999), but I have no good evidence for or against this assumption. Fire is an important part of the ecology of the study area. I used available fire history information and local research to choose study sites where there had been no fires for > 30 years, as recent fires could negatively affect abundance in the study species (Catling et al. 1998). However, evidence suggests that abundances of agile antechinus are unaffected by fire history where time since fire is > 20 years (Catling et al. 1998; Claridge et al. 2008), so I consider the > 30 year criterion to be sufficient to avoid any confounding effects.

Fig 5. Example of a forest-field ecotone in the study area.

Photo: C Johnstone

1.3.2 Confounding factors in habitat fragmentation research

As fragmentation is an imprecise concept, the definition of what constitutes a fragment depends to some extent on the taxa being studied and human perceptions of the environment (Ewers & Didham 2005; Fischer & Lindenmayer 2007). Studies differ considerably when defining the area of habitat that constitutes a 'fragment'; even considering only studies that examined vertebrates, fragments have been studied on a scale from ~ 1 ha (e.g. Banks et al. 2005a; Bennett 1990b) up to 'fragments' of > 1000 ha (e.g. Estrada et al. 1993). Fragment shape may also affect a study species, as this determines the amount of habitat exposed to the matrix. Fragments of a similar area may not contain similar areas of suitable core habitat where shape varies (Ewers & Didham 2005; Fletcher et al. 2007).

Clear definition of fragments is not always possible. The process of anthropogenic fragmentation is complex and may result in equally complex landscapes. A tendency among researchers to avoid studying confusing landscapes, in which, for example, there may exist 'mosaic' graduations between several distinct ecologies, can result in over-simplification of theories (Ewers & Didham 2005).

Degree of isolation and time since fragmentation are reported to correlate with species abundance and community structure to varying degrees (Bolger et al. 1997; Brooks et al. 1999; Lawes et al. 2000), but can be difficult to estimate accurately without genetic analysis (Banks et al. 2005b). Differences in ecology and microclimate between fragments also need to be taken into account (Ewers & Didham 2005; Saunders et al. 1999). Plant community composition can vary between fragments (e.g. due to differences in plant succession in 1 m^2 - 500 m^2 experimental plots; Schweiger et al. 2000). Abiotic factors, such as wind-exposure, water-run-off and hours of exposure to sunlight, may affect the magnitude and/or nature of edge effects and environmental degradation post-fragmentation (Saunders et al. 1999). The nature of the matrix also affects fragment ecology and microclimate (Ewers & Didham 2005; Saunders et al. 1999). A matrix of native plantations will be likely to generate very different edge-effects to those where the surrounding matrix consists of crop and an urban matrix will be likely to generate different stressors to one of grazed paddocks or open-cast mining.

Ecological boundaries and novel barriers to dispersal are another level of complexity when considering fragmentation effects on populations. Roads are potential barriers to dispersal for terrestrial species, but individual species' responses to the presence of roads differ (Burnett 1992). Roadside vegetation may provide corridors for dispersal (Bennett 1990a). Animals may be killed on roads (Burnett 1992; Goosem 2000), which can increase stochastic risks for some species and could subsidize scavengers, whether native or exotic. Cryptic barriers to dispersal may also exist (Banks et al. 2005b). For example,

rivers only sometimes represent barriers to dispersal and gene-flow in small mammals--the yellow-footed antechinus (*Antechinus flavipes*) does not appear to experience the Murray River as a dispersal barrier (Lada et al. 2008a), whereas common shrew (*Sorex araneus*) dispersal is restricted by the Rhône river valley (Lugon-Moulin & Hausser 2002). The presence of such barriers cannot be discounted without genetic analysis (Banks et al. 2005b).

When a single species is the focus of study, its response to habitat fragmentation will depend on aspects of its life-history, reproduction and foraging resource requirements, and reproductive strategy. Unexpected interactions with the environment can occur. Exotic species may invade the environment and influence behaviour of native species (e.g. by altered resource use) or lead to evolutionary change (e.g. altered anti-predator defences) (Strauss et al. 2006). Any study that attempts to explain the condition of a species in the context of habitat fragmentation needs to examine as many environmental variables as is feasible in order to reduce the risk of missing important, but unexpected interactions (Hobbs 2001).

1.3.3 Putative mechanisms of species decline and extinction in anthropogenically fragmented habitat

Extinction as a natural event is typically attributed to, (1) demographic stochasticity; (2) environmental change; (3) genetic stochasticity and (4) natural catastrophe (Shaffer 1981). In contrast, a review of all species extinctions in recent history that are thought to have been caused by anthropogenic activity has attributed only four causes, (1) habitat loss and fragmentation, (2) over-exploitation; (3) effects of exotic competitor/predator species and (4) 'extinction chains' (e.g. loss of partner species from the environment) (Caughley 1994; Diamond 1984, 1989; Pimm et al. 1988). More recent authors have added pollution as another potential driver of population decline (Collen et al. 2009; Hilton-Taylor et al.

2009), especially as regards global climate change. Regarding conservation management and genetic effects of habitat change, Caughley (1994) argued that the four cited anthropogenic drivers of decline were important mechanisms underlying species decline, whereas genetic effects (e.g. inbreeding depression) functioned only as *'coup de grace'* for a threatened species (but for a rebuttal see Hedrick et al. 1996).

Habitat fragmentation presumably aggravates some or all of the proposed drivers of decline. A fragmented habitat is more open to anthropogenic exploitation (Turner 1996), and consequently, both over-hunting (Turner 1996) and anthropogenic environmental change (e.g. through livestock grazing) (Hobbs 2001) may result. Invasive exotic species may benefit from a matrix subsidy or increased ease of dispersal (Diamond & Veitch 1981; May & Norton 1996), and 'spill-over' may occur where a mobile species that would be unable to live exclusively in a native habitat patch is able to make forays into fragments (Lidicker 1999). Edge effects, changes in environmental flows, loss of partner species and anthropogenic disturbance may all combine to change the environment of a fragment, rendering it less suitable for a given native species in terms of availability of limiting resources (Saunders et al. 1999). Habitat fragmentation may affect either physiology or behaviour of species so as to put the species more at risk (Fischer & Lindenmayer 2007; Mazerolle & Hobson 2002). Effects of fragmentation on parasite infection levels have also been investigated but the results are equivocal and host / parasite / fragmentation relationships may be taxa or environment specific (Chapman et al. 2006; Püttker et al. 2008). In vertebrate species, an increase in the 'stressfulness' of the environment may result in chronic stress, leading to reduced survivorship and fecundity (Martínez-Mota et al. 2007). Small, isolated populations are inherently susceptible to stochastic events (Hedrick et al. 1996) such as local catastrophes, or stochasticity at the demographic, and genetic levels; fragmentation may lead to 1) increased potential for loss, reduction or subdivision of demographic units; 2) potential loss of sources of

immigration; and 3) more significant impediments to dispersal (Wilcox & Murphy 1985). Where habitat is reduced to increasingly small and isolated fragments, the risk that Allee effects (the inherent positive relationship between population size and rate of population growth) may prevent rapid population recovery even if environmental conditions improve (Courchamp et al. 1999).

1.3.4 Factors that may put a species at risk in anthropogenically fragmented habitat

Animal species that rely on native vegetation show variable responses after fragmentation of their habitat, and this is perhaps a key underlying reason why the literature in this area has remained contentious (e.g. Caughley 1994; Fahrig 2003; Hedrick et al. 1996). Greater vulnerability to habitat fragmentation has been associated with larger body size (largely based on meta-review material but also some evidence from studies addressing this apparent trend; Bennett 1990a; Turner 1996; Wilcox 1980); higher trophic level (Wilcox 1980); reliance on a partner species or a specialist diet (such as frugivory) (Turner 1996); high reliance on native foliage (Bently et al, 2000); inability to utilize the surrounding matrix (Laurence 1994); limited capacity for dispersal or vulnerability in the matrix while dispersing (Pereira et al. 2008) or high site-tenacity (Newell 1999); and sparse or uneven distribution in undisturbed continuous habitat (Turner 1996). Some traits that make a species vulnerable may be ubiquitous in a given taxon, and after fragmentation of native vegetation, entire families or genera may be adversely affected. The evidence suggests that terrestrial mammals are more adversely affected by habitat fragmentation than birds, reptiles or amphibians (Turner 1996). Assuming that a terrestrial ectothermic vertebrate's energy and space requirements are lower than that of a comparable-sized endotherm, then ectotherms such as reptiles and amphibians may be better able to maintain viable population sizes in small isolated habitats (Pough 1980;

Santos et al. 2008; Wilcox 1980). Most birds and bats have better dispersal capabilities than non-flying mammals, and for this reason, the latter should be expected to decline more rapidly after habitat fragmentation and subsequent isolation of populations (Wilcox 1980).

1.3.5 Responses to anthropogenic fragmentation of native vegetation in the genus *Antechinus*

The following discussion of current knowledge concerning the response of the genus *Antechinus* to habitat fragmentation focuses mainly on agile and brown antechinuses. Some discussion of the more distantly related yellow-footed antechinus, swamp antechinus (*Antechinus minimus*) and dusky antechinus (*Antechinus swainsonii*) is included.

Populations of *A. agilis* can persist at relatively high densities in remnant patches of *Eucalyptus* forest within a matrix of grazed fields (Bennett 1990b). The species has been reported in patches as small as 1 ha, marginally above the minimum size of an individual's foraging range (in *A. stuartii*, 0.94ha in males, 0.38ha in females; Lazenby-Cohen & Cockburn 1991, and see Cockburn and Lee 1988). Patch occupancy in forest fragments is more likely in larger fragments (in patches from approx 1 - 82 ha) (Bennett 1990a; Holland & Bennett 2009) and relative abundance (measured in captures per trap night) tends to be higher in fragments with more complex groundcover and understorey (Bennett 1993). Livestock grazing and removal of woody debris has been reported to have a negative effect on forest fragment patch occupancy and/or abundance (by correlation in *A. stuartii*; Knight & Fox 2000) (by experimental manipulation of wood load in *A. flavipes*; Mac Nally & Horrocks 2002) Although only limited breeding has been reported in roadside vegetation strips, *A. agilis* does appear to make use of roadside corridors of native foliage for dispersal. (Bennett 1990a). There is recent evidence that *A. agilis* abundance in a fragmented forest landscape surrounded by grazed paddocks is

better explained by fragment patch area and habitat complexity than by either of these factors alone (Holland & Bennett 2009) and that females may occupy better quality habitat than males (Holland & Bennett 2009).

To date, studies examining relative abundance suggest that *Antechinus* spp. respond primarily to habitat structure and complexity (such as presence of tree-hollows for nesting, understorey density etc; Garden et al. 2007; Kelly & Bennett 2008; Knight & Fox 2000) that can correlate with fragment area, but not to area *per se* (correlation is typically significant but sometimes weak, i.e. a low *r* value (Knight & Fox 2000 though see Bennett 1990a). Cox et al (2003) found that *A. stuartii* increased in abundance with increasing understorey complexity, but not with increasing fragment size. Abundance in *A. agilis* does not appear to correlate well with vegetation species richness, except perhaps negatively (Bennett 1993). A negative relationship between plant species richness and *A. agilis* abundance could be due to reduced abundance at weedy sites or near forest-field ecotones. Lower abundance near forest-field edge habitat than in interior forest has been reported for both *A. agilis* and *A. swainsonii* in wet *Eucalyptus* forest (Berry 2002; Woodward 1995).

Knight and Fox (2000) suggested that *A. stuartii* may be supplementing limited resources in a forest fragment by foraging in the matrix. Cox et al (2003) further argued that in contrast to a strong fragment area-dependence of abundance in the sympatric native Australian rodents *Rattus fuscipes* and *Melomys cervinipes* (which suggested an inability to exploit the surrounding matrix), an apparent lack of relationship between fragment patch area and abundance in *A. stuartii* may indicate the presence of a matrix-subsidy. However, *A. agilis* and *A. stuartii* are seldom or never trapped in grazed paddocks adjacent to occupied forest fragments (Bennett 1990a; Bentley et al. 2000; Laurance 1994) and *A. agilis'* relative abundance is negatively correlated with proximity to forest-field ecotones (Berry 2002; Woodward 1995). This suggests an intolerance of a grazed

field matrix in these two species. As a contrasting example, the more distantly-related yellow-footed antechinus does use grazed fields for foraging and has been reported to increase in abundance in disturbed *Eucalyptus* forest and along forest edges (Bentley et al. 2000; Laurance 1994; Laurance 1997) and for this reason may be more resilient to habitat disturbance than either *A. agilis* or *A. stuartii*.

Several studies have been conducted on *A. agilis* in *Eucalyptus* forest fragments isolated by a matrix of softwood *Pinus radiata* (radiata pine) (Banks et al. 2005b; Lindenmayer et al. 1999). Although an exotic soft-wood plantation matrix represents a very different environment to that where *Eucalyptus* forest fragments are isolated by open fields, these fragmentation studies are worth considering here. As with *Eucalyptus* patches isolated in a matrix of agricultural fields, populations of *A. agilis* can persist at very high abundances in fragmented *Eucalyptus* patches in radiata pine despite individuals being trapped rarely in the surrounding matrix (Lindenmayer et al. 1999). Further investigation in the same landscape by Banks (2005a) suggested that *A. agilis* were not occupying all *Eucalyptus* forest fragments but that the species was always detected in nearby continuous forest. Banks et al. (2005a) also reported that *A. agilis* was influenced negatively by geographic isolation and positively by habitat 'quality' (indexed as plant basal area). Variation in population size (not density) was influenced by the dominant *Eucalyptus* species present at sites and more weakly by patch area and whether a gully was present (gully sites had higher populations). Banks (2005a) showed that the sex ratio was more skewed towards females in fragmented sites than continuous sites, potentially indicating higher male mortality in fragmented environments where dispersal is more dangerous (assuming unity at weaning). Although genetic evidence suggests that *A. agilis* were dispersing through the radiata pine matrix and along corridors of native vegetation and/or riparian habitat (Banks et al. 2005a; Banks et al. 2005b), Banks (2005c) also reported that individuals in *A. agilis* populations in fragments were more closely related than those in matched

continuous forest sites (raising the possibility of inbreeding depression) and that the levels of multiple paternity were lower in *Eucalyptus* forest fragments.

The apparent lack of a repeatable or strong abundance response to fragment patch area reported for *A. stuartii* (Cox et al. 2003; Knight & Fox 2000) and to a lesser extent *A. agilis* (Banks et al. 2005a; Bennett 1990a; Holland & Bennett 2009) remains to be fully explained. In some studies, differing microclimates and consequent differences in environmental factors like understorey complexity, woody debris density and leaf-litter depth (Bennett 1993; Knight & Fox 2000) could have overwhelmed an area-dependant relationship that might otherwise have been observed (Knight & Fox 2000). However, both species show higher site occupancy and/or abundance in continuous forest than in fragments (Banks et al. 2005a; Laurance 1994). The interesting point here is that it is probably not the case that previous studies failed to examine a range of fragment sizes that was sufficiently broad to accurately identify patch-area density relationships. Some studies have included fragments up to 996 ha, very close to the 1000 ha threshold that is used in the current study to define continuous forest (0.3-10 ha (Bennett 1990b); 0.5-498 ha (Knight & Fox 2000) and 0.95-996 ha (Cox et al. 2003); 0.1-62 ha (Banks et al. 2005a)).

If the lack of a repeatable area-dependent relationship for agile/brown antechinus and the forest patches in which the species live is genuine, then several species' traits may contribute to it. The highly scansorial nature of both species and their ability to forage on vertical tree-trunks (personal observation) could mean that fragment area defined by patch boundary does not correspond to habitat area as experienced by these two species. Fischer and Lindenmayer (2007) discusses the problems implicit in assuming that human definitions of habitat and ecotonal boundaries match the experience of a study species. If threatening processes in fragments are related to top-down predation rather than bottom-up food scarcity, the scansorial habit of *A. agilis* could help with evading

terrestrial predators, including the introduced European red fox (*Vulpes vulpes*) and feral cat (*Felis catus*) in fragmented habitat. Key to this argument is the observation that by comparison, the larger (38-170 g) and almost strictly terrestrial, dusky antechinus is only infrequently reported in fragmented habitat and was caught only once in a *Eucalyptus* forest fragment in this study while concurrently being caught frequently in continuous sites with similar forest (personal observation). Another terrestrial antechinus native to the study region, the swamp antechinus (*A. minimus*), is considered threatened in mainland Australia, but is locally common in Tasmania (Menkhorst & Knight 2004) where European red foxes were not present until recently (Menkhorst & Knight 2004). Both foxes and feral cats are known to occur at higher population densities in anthropogenically fragmented landscapes than in continuous forest in Australia, a trend that is thought to be due to subsidy from human activity (e.g. scavenging from road kill) and greater abundances of introduced European rabbits (*Oryctolagus cuniculus*) in pastured landscapes probably providing a dependable food source (Fischer & Lindenmayer 2007; May & Norton 1996).

Other traits that may limit the vulnerability of *A. agilis* and *A. stuartii* to the overall effects of habitat fragmentation are small size, capacity for dispersal through a modified matrix and a relatively generalist diet (Turner 1996). Social nesting (where multiple individuals use the same tree-hollow resource) (Cockburn & Lazenby-Cohen 1992; Lazenby-Cohen & Cockburn 1991) could allow multiple individuals to make use of the same limiting resources. Where reasonably large, stable populations occur in forest fragments, this would likely protect the species from isolation and stochastic effects (Lindenmayer et al. 1999).

The picture of agile and brown antechinus that seems to be presented in the literature is of species that are capable of persisting and reproducing in fragmented habitat, but at lower abundances than in relatively intact continuous forest. It is unknown whether the

lower abundance in forest fragments represents stable, but lower, population density or ongoing gradual decline.

1.4 Body condition estimation in vertebrates

One problematic aspect of examining what are sometimes termed 'performance' (e.g. brood size) or 'condition' (e.g. lipid reserve, aerobic capacity) (Fletcher et al. 2007; Norte et al. 2010; Schulte-Hostedde et al. 2005) is that these terms are sometimes poorly defined in physiological ecology literature. In this study the terms 'body condition' and 'body condition index' refer always and specifically to an estimate of an animals' mass of energy reserves (lipids) derived from residuals of mass (observed values) and skeletal measurements (expected values): a mass-size residual (MSR) (Schulte-Hostedde et al. 2005). This type of index provides values in grams above (good condition) or below (poor condition) the expected mass for each individual. Validation against actual percentage of lipids in dry body mass has repeatedly shown that MSR accurately indexes lipids in small mammal and other vertebrate species (Green 2001; Peig & Green 2009; Schulte-Hostedde et al. 2005). There is debate whether ordinary least squares (OLS), major axis (MA), or reduced major axis (RMA) should be used to generate the residuals (Green 2001; Peig & Green 2009). In theory, because both mass and skeletal measurements are random variables either MA or RMA regression should generate a more accurate estimate of the slope of the relationship (Quinn and Keough 2002). However, OLS are simpler to derive and are not prone to complex interpretation problems that can affect MA and RMA residuals, e.g. the units of MA and RMA residuals are not always readily understandable whereas the units for a body condition index using OLS are grams above or below expected mass. On balance, OLS regression is probably preferable on the grounds of statistical simplicity, although it needs to be

acknowledged that this choice probably sacrifices some accuracy in terms of estimating actual mass of lipids.

1.5 Physiological stress: regulatory processes

In this study 'stress' is defined according to Selye's terminology (Selye 1936, and reprinted in full, Selye 1998). The Selyean study of stress focuses on individual organisms. Stress is framed in the context of (1) stressors: any nocuous agent that threatens survival or reproduction, and (2) the stress response: a mechanism elicited in response to a stressor that improves an individual's chances of evading or acclimating to a stressor (Greenberg et al. 2002; Sapolsky et al. 2000). The vertebrate stress response is mediated through the nervous and endocrine systems, and the response can result in changes at the level of behaviour or physiology, or more commonly a combination of both (Greenberg et al. 2002).

Stress responses can be divided into stressor-specific and more generalized, non-specific responses. Under laboratory conditions specificity of a response is progressively lost as the stressor becomes more severe (Siegel 1980; Tsigos & Chrousos 2002), and it is likely that in the natural environment a similar phenomenon occurs. However, no clear dividing line between specific and generalised stress responses has been proposed and it is generally accepted that a better theoretical model characterizes stress responses on a spectrum rather than into discrete categories (Goldstein 2002; Tsigos & Chrousos 2002; Wingfield & Sapolsky 2003).

1.5.1 Nervous and endocrine mediation

1.5.1.1 Autonomic axis

The autonomic axis provides a rapid nervous response through sympathetic innervation of peripheral organs, importantly, cardiovascular, respiratory, gastrointestinal and renal tissue (Tsigos & Chrousos 2002). Regulation is associated with release of the catecholamine hormones, epinephrine (adrenaline) (secreted primarily by adrenal-medullary tissue) and nor-epinephrine (nor-adrenaline) (secreted primarily at nerve junctions) (Tsigos & Chrousos 2002

1.5.1.2 Hypothalamus-pituitary-adrenal axis

Stimulus in the form of a stressor activates the central nervous system (CNS) and consequent release of corticotropin releasing hormone (CRH) from the hippocampus. CRH triggers release of adrenocorticotropin (ACTH) from the anterior pituitary, and ACTH (and to a lesser extent CRH) triggers release of corticosteroids (in particular glucocorticoids) from the adrenal-cortical tissue (Sapolsky et al. 2000; Tsigos & Chrousos 2002; Ziegler & Herman 2002). It is now broadly accepted that CRH and ACTH elicit and maintain a stress response (Bale et al. 2002), whereas the role of glucocorticoids is more complex and their effects may vary depending on the hormone's concentration in circulating blood (Sapolsky et al. 2000). Possibly, glucocorticoids are not stress hormones *per se*, but in some capacity act as 'anti-stress' hormones (Wingfield & Kitaysky 2002), involved in preventing an over-excitation of the stress response and stimulating the repair of damage.

1.5.2 Acute and chronic stress

Under laboratory conditions increases in the duration or frequency of acute stress can lead to chronic physiological stress (Dhabhar & McEwen 1997). There is debate in the physiological ecology literature as to whether a stressor is an exceptional event that disturbs an animal's allostasis substantially away from an optimal point (McEwen & Wingfield 2003) or whether common or recurrent events such as long-term fasting during moulting (Mortimer & Lill 2007), thermoregulatory expenditure during hot periods of the day (Siegel 1980), or psychosocial conflict (Tamashiro et al. 2005) could also constitute forms of stressors that may elicit a classical HPA-axis mediated response that could assist survival under such circumstances (Mortimer & Lill 2007). Changes in the natural environment that are likely to negatively affect survival and reproduction (for example, an increase in exposure to predators or decrease in abundance of food) have been associated with chronically elevated indicators of physiological stress in wild vertebrates (Boonstra et al. 1998; Hik 1995; Krebs et al. 1995; Zanette et al. 2003). The physiological consequences for an organism of experiencing additional stressors in the wild have been suggested to be synergistic rather than simply additive. In song sparrows (*Melospiza melodia*) (Zanette et al. 2003) and snowshoe hairs (*Lepus americanus*) (Krebs et al. 1995), changes in measurable stress indicators associated with a concurrent decrease in food and an increase in predator activity were greater than would be expected from the observed elevations of stress associated with decreased food or increased predator activity alone.

Chronically elevated physiological stress is associated with several phenomena that are likely to decrease an individual's chances of survival and reproduction in the wild. These include a heightened risk of predation (Møller & Erritzøe 2000), suppression of breeding behaviour (Sapolsky et al. 2000; Siegel 1980; Tsigos & Chrousos 2002; Wasser & Barash 1983), interference with short-term memory and problem-solving faculty (Sapolsky et al.

2000), osteoclastic activity (leeching of Ca^{2+} from bone) (Sapolsky et al. 2000), accelerated telomere shortening (Epel et al. 2004), oxidative stress and neurodegenerative disease (Liu & Mori 1999), decrease in growth rate (Sapolsky et al. 2000; Tsigos & Chrousos 2002), catabolic activity and mobilisation of substrate energy stores (although this should increase at least short term survival chances in some situations, such as in winter) (Sapolsky et al. 2000; Tsigos & Chrousos 2002) and a compromised immune system, specifically an overall decrease in the numbers of leucocytes circulating in the peripheral blood (perhaps specifically in eutherians) (Dhabhar et al. 1996), a decrease in the oxidative burst response of leucocytes (as a function of the capacity of circulating leucocytes to respond to a challenge, e.g. by phorbol myristate acetate, with a respiratory burst *in vitro*) (McLaren et al. 2003), suppression of delayed type hypersensitivity (an antigen specific, cell-mediated immune response) (Dhabhar & McEwen 1997) and involution of lymphoid tissue (atrophy of the thymus in particular, and other lymphoid tissue to a lesser extent) (Sapolsky et al. 2000).

Recent evidence suggests that the observed decrease in peripheral circulating leucocytes during an acute stress response may more accurately represent 'immunomodulation', rather than immunosuppression *per se* (Dhabhar 2002). The changes in leucocytes profiles in circulating peripheral blood post-exposure to an acute stressor is apparently largely due to redistribution of lymphocytes to compartments such as the skin and lymph nodes where a rapid immune response is more likely to be beneficial in the event of injury and infection (Braude et al. 1999; Dhabhar et al. 1995, 1996), although there is also some evidence for movement of neutrophils into peripheral blood from other compartments as well (Davis et al. 2008). Stressor-induced immunomodulation may represent a change from a state of preparedness for contagious disease (non-stressed) to a state of preparedness for infection due to wounding (stressed) (Dhabhar & McEwen 1996).

Despite this, the overall effect of long-term, chronic stress still appears to be immunosuppressive (Lochmiller & Deerenberg 2000; Norris & Evans 2000).

1.5.2.1 Measurable indicators of physiological stress

Circulating levels of glucocorticoids, in particular cortisol (Boonstra et al. 1998) and corticosterone (Clinchy et al. 2004; Spencer et al. 2003), have frequently been used as an indicator of stress in free-living vertebrates. Although useful information can be inferred from glucocorticoid levels, the rate of response to a stressor (such as capture and handling) can be rapid. A measurable increase can occur within a few minutes of exposure to a stressor and can peak at anywhere from ten to 30 minutes later (O'Reilly & Wingfield 2001; Rich & Romero 2005). Trapped wild animals can demonstrate no obvious behavioural stress response, yet undergo a rapid increase in circulating stress hormone soon after trapping (Lynn & Porter 2008). It can therefore be difficult to determine whether glucocorticoid measurements represent baseline or acute stress levels. This is problematic for interpretation because chronically stressed individuals tend to show a higher baseline circulating glucocorticoid concentration, but an attenuated acute stress peak when compared to relatively non-stressed individuals (Dhabhar & McEwen 1997).

Faecal glucocorticoids have been used successfully to measure baseline physiological stress in birds and mammals (Wasser et al. 1997; Wasser et al. 2000), but their use in mammals is problematic because urine interferes with the glucocorticoid assay. Especially for small mammals in live-traps, it is not always possible to determine to what degree faecal matter collected from a trap was contaminated with urine, thus this method was not used here.

Leukocyte profile indicators of physiological stress, specifically neutrophil-to-lymphocyte ratio (N:L) (or equivalent in birds and reptiles) and total white blood cell

count (WBC) have also been used successfully to measure physiological stress in vertebrates (Davis et al. 2008), including marsupials (Baker et al. 1998; Baker & Gemmell 1999; Cheal et al. 1976). The movement of lymphocytes out of peripheral blood, and to some extent of neutrophils into peripheral blood (see above) is the mechanism most commonly cited as underlying elevated measurable N:L ratios in more stressed individuals (Davis et al. 2008; Masello et al. 2009). The association between N:L ratio and physiological stress in vertebrates has been long-established, and physiologists used N:L ratios as a surrogate for stress blood hormone concentrations before assays of hormones were technically feasible (Davis et al. 2008). The volume of blood needed to evaluate differential and total white cell counts can be 5 μL, much less than the > 60 μL that can be required for hormone assays (Davis et al. 2008). Drawing less blood is advantageous when a study species is small, such as the agile antechinus where adult mass can be as little as 10 g.

The rate of N:L response is much slower than that for stress hormones, ranging from half an hour to several hours to perhaps a few days in some species, before measurable differences in N:L occur (Baker et al. 1998; Dhabhar et al. 1994; McLaren et al. 2003). This removes any confounding effects on stress indices by handling stress, although in the present study design, trapping stress may still be a confounding factor. Trapping stress is discussed at length in the relevant results chapters, although, briefly, the best interpretation is probably that given there is no reason to think that trapping times varied among study sites, any observed differences in mean N:L among sites were more likely due to 'background' environmental stress rather than differences in stressfulness of trapping *per se* (i.e. trapping can be treated as a uniform stressor when interpreting the data; Fletcher & Boonstra 2006). This interpretation assumes that no populations were physiologically exhausted by extreme chronic stress to a point where individuals were unable to mount a stress response to trapping (Dhabhar & McEwen 1997). Although the

latter seems unlikely in populations of free-living vertebrates, as any individual unable to respond to a stressor is unlikely to avoid predation, nonetheless it cannot be entirely discounted. Thus N:L in this study is interpreted as a positive index of physiological stress related to the environment, but the results must be treated with some caution.

WBC is a less robust indicator of stress in vertebrates than N:L because WBC variously decreases and increases in different vertebrate species in response to stress. For WBC to be interpretable, the 'normal' range must be known (Davis et al. 2008), even if the possibility that an acute stress response might interfere with the results is slight (i.e. sampling conducted immediately on capture). The best evidence suggests that agile antechinus (and possibly marsupials in general; Baker et al. 1998; Baker & Gemmell 1999) show an *increase* in WBC in response to acute and possibly chronic stress. This is opposite to the 'classical' eutherian response to stress, in which WBC decreases in response to stressors (in rats, Dhabhar et al. 1995, 1996; Dhabhar et al. 1994).

On balance, I considered the advantages of a relatively slow response to acute stress, small blood volume requirements and availability of extensive reference values for comparison (Clark & Adlard 2004), to be sufficient to warrant use of leukocyte indicators as a means to estimate physiological stress in the agile antechinus in this study.

1.6 Erythrocyte indicators of condition

Erythrocyte metrics such as red blood cell counts, mean cell volumes or haemoglobins, haematocrits or haemoglobin in grams per litre of blood have been used for diverse purposes in vertebrate physiological ecology (e.g. Beldomenico et al. 2008a; Nadolski et al. 2006; Norte et al. 2010; Sealander 1962). In this field of research, there are conflicting results whether given indices (e.g. haematocrit; Fair et al. 2007) are valid indicators of health status. Rather than discuss these indices and their possible uses at length here, a

short review style examination of various erythrocyte indices of condition and health is provided in Chapter Two of this thesis. Chapter Two is not currently in the form of a paper ready for publication, but is intended as groundwork for a short review article examining what is a diverse and sometimes contentious area of research.

1.7 Thesis format, approach and expected outcomes

Chapters One and Two of this thesis provide general background and theory and are not in the form of articles for publication. Chapters Three to Six inclusive are a collection of independent papers. Chapter Four has been accepted for publication in a peer reviewed journal, Chapter Three is in submission and Chapters Five and Six are ready for submission. Necessarily, there is some repetition among these chapters as regards study area and species, field and laboratory methodologies and statistical analysis. Chapter Seven presents a general discussion of research questions addressed in the independent papers. Literature cited is provided in a final references section for all chapters. Trapping and sampling were conducted under Monash University Biological Sciences Animal Ethics Committee approvals BSCI/2008/03 and BSCI/2006/05 and Department of Sustainability and Environment permit 10003798.

1.7.1 Original concept and research question: Is it possible that chronic physiological stress, a condition associated with reduced survivorship, reproductive output and success, could be contributing to decline of vertebrates in anthropogenically fragmented habitats?

1.7.2 General research approach: Measure and assess indicators of physiological stress, population density, parasite load and metabolic reserves in populations of a free-living vertebrate species in comparable fragmented and undisturbed forest. Concurrent measurement of environmental variables in study sites so that inferences can be made

regarding what habitat change or landscape level mechanisms could have led to any observed differences in the population health status indicators. By measuring multiple population indices and environmental variables, the study aims to produce a more informative picture of habitat fragmentation effects on the study species.

1.7.3 Expected outcomes: Results from this thesis will be directly relevant to conservation management for the study species in the study area, but should also provide information that will help in the refining and synthesizing of theory concerning the negative effects of anthropogenic habitat fragmentation on vertebrates.

1.7.4 Chapters and specific research questions:

Chapter Two: A short review. What is the evidence for use of erythrocyte indicators of stress and condition in free living vertebrates?

Chapter Three: Are indicators of physiological stress higher in agile antechinus living in fragmented than in continuous native forest?

Chapter Three: Are indicators of population condition (relative abundance, mass-size residuals and ectoparasite count) poorer in agile antechinus living in fragmented than in continuous native forest?

Chapter Four: Do indicators of physiological stress and population condition associate with measurable differences in habitat indices in forest fragments for agile antechinus?

Chapter Five: Can tree-based modelling be used to better understand the relationship between physiological stress and/or population condition with measurable habitat variables?

Chapter Two

Erythrocyte indicators of stress and condition in vertebrate physiological ecology

2.0 Abstract

Erythrocyte indicators of vertebrate health status or physiological condition are used in ecophysiology studies but their application and interpretation are sometimes contentious. Here I present a short review of some evidence for or against the use of these indices. I suggest that evidence for the use of single-parameter condition metrics such as haemoglobin concentration in peripheral blood (Hb) and, possibly even more so, haematocrit (Hct) is equivocal. These indices can be high or low due to various conditions (e.g. dehydration, anaemia, heavy parasite load) and where researchers have attempted validation of Hb and Hct in vertebrate species using body condition indices, the results did not always indicate significant relationships. Theory and empirical evidence better supports the employment of more sophisticated metrics, such as mean cell volume (MCV), mean cell haemoglobin (MCH) and erythrocyte sedimentation rate (ESR), which may be useful for identifying conditions such as semi-starvation stress (MCV), hypothalamus-pituitary-adrenal (HPA-) axis mediated regenerative anaemia (MCV and MCH in conjunction with red blood cell counts) and high infection levels (ESR). Moreover, additional research is needed to determine whether regenerative from non-regenerative anaemia can be reliably distinguished in free-living vertebrates using haematological techniques available to most field biologists. The use of erythrocyte indices of vertebrate health status has promise as methods for inferring information about populations that cannot easily be obtained in other ways (e.g. through hormone or leukocyte indices of stress). However, substantial work is needed before it can be definitively stated that any of the metrics here discussed are reliable in all or even most vertebrate taxa.

Keywords: Body condition, erythrocyte, haematocrit, haemoglobin, health status, red blood cell

2.1 Introduction

Erythrocyte indices of stress and condition are employed in vertebrate ecophysiology studies (e.g. Beldomenico et al. 2008a; Masello & Quillfeldt 2004; Nadolski et al. 2006). The most commonly used indicators, haemoglobin concentration in peripheral circulating blood (Hb) and haematocrit (Hct), are often assumed to correlate positively with body condition but this relationship is frequently not empirically validated in a study species (Fair et al. 2007). Laboratory and field-based evidence suggests that other indices derived from erythrocyte metrics, for example, estimates of mean erythrocyte volume (MCV) or haemoglobin content per cell (MCH), sedimentation rate or even a count of red blood cells per litre (RBC) can be equally or more informative about health status in vertebrates than Hb or Hct (Table 1), yet these variables are not as often used.

This short review focuses on evidence for or against the use of erythrocyte indices of stress and condition in terrestrial vertebrate ecophysiology. The same or similar variables have been used successfully in fish to address ecological questions and examine health status (for example Iwama et al. 1995; for example Wells & Baldwin 1990) but these studies are outside of the scope of this review. Although I endeavoured to review research on diverse taxa, the majority of work in this field has been conducted on birds, and to a lesser extent, mammals. Some work on lizards and snakes has been reported and in these taxa the emphasis has been on parasite-induced stress (e.g. Brown et al. 2006; Schall et al. 1971), but research on erythrocyte condition indices in free-living amphibians is relatively scarce.

In the vertebrate ecophysiological literature there is a considerable disparity between what erythrocyte indicators are typically assumed to index (body condition or aerobic capacity; e.g. Beldomenico et al. 2008a; e.g. Burness et al. 2000; Cuervo et al. 2007) and the conditions these metrics could in theory be used to index (e.g. hypothalamus pituitary

adrenal (HPA-) axis mediated stress, infection or regenerative anaemia; Fisher & Crook 1962; Lewis et al. 2006; Tyler & Cowell 1996).

Erythrocyte variables can increase or decrease as a result of a number of different conditions (e.g. anaemia, dehydration, starvation) (Campbell 1995; Lewis et al. 2006) and the interpretation of such changes must always be treated cautiously or considered in the light of other evidence (e.g. from examination of concurrent hormone or leukocyte samples). If possible, the researcher should also refer to reference ranges provided in general haematology books (e.g. Campbell 1995; Clark & Adlard 2004) or research papers that report 'typical' ranges for a species (e.g. Cheal et al. 1976). However, reference ranges are available for only some species and natural variation in populations can be quite pronounced, at least for some metrics (Fair et al. 2007). Over reliance on generalized reference ranges also can be problematic--comparison among populations where there is variation in genetic background, environmental stress, sampling diel and trapping or handling methods is not always valid. After all, the knowledge that species' physiology can differ in populations living in different environments is an underlying tenet of much of ecophysiological research (Wikelski & Cooke 2006).

The overarching object of this short review is to assess whether there is currently sufficient evidence to support the use erythrocyte indicators of 'condition' in free-living vertebrates.

Table 1. Erythrocyte indicators of stress and condition that are potentially of use in vertebrate ecophysiology. One reference to their use is provided for each indicator.

• RBC	Erythrocyte count (cells/L) (Barnett et al. 1979b)
• Hb	Haemoglobin concentration (g/L) (Norte et al. 2008)
• Hct	Haematocrit (%) (Potti et al. 1999)
• PLC	Platelet count (cells/L) (Nadolski et al. 2006)
• Pct	Platelet(o)crit (%) (Nadolski et al. 2006)
• MCV	Mean erythrocyte corpuscular volume (conventionally fL but often reported as μm^3) (Nadolski et al. 2006)
• MCD	Mean erythrocyte corpuscular diameter (μ) (Sealander 1962)
• MCH	Mean corpuscular haemoglobin (pg) (Colombelli-Négrel & Kleindorfer 2008)
• MCHC	Mean corpuscular haemoglobin concentration (g/dL) (DelGiudice et al. 1990)
• RDW	Erythrocyte distribution width (%) (Nadolski et al. 2006)
• MPV	Mean platelet volume (μm^3) (Nadolski et al. 2006)
• PDW	Platelet distribution width (%) (Nadolski et al. 2006)
• ESR	Erythrocyte sedimentation rate (mm per hour) (Masello & Quillfeldt 2004)
• cESR	Corrected erythrocyte sedimentation rate. The value is corrected for the total amount of sedimentation *possible*. The most accurate corrections probably require reference to tables, but a reasonable estimate is: mm clear plasma after 1 hr settling × 100 / mm plasma after centrifugation of the same sample (Rourke & Ernstene 1930)
• OA	Oxygen affinity (torr / P_{50} [at given temp and pH]) (Swanson 1990)
• OCC	Oxygen carrying capacity (O_2/g of Hb) (Swanson 1990)
• CV & PV	Absolute cell volume and plasma volume (sometimes used as an alternative to Hct). Absolute CV and PV values (mL) can be used to derive relative CV or PV / total body mass ratios (mL/kg) (Shield 1971)
• PCV	Packed cell volume. See haematocrit (Hct)

2.2 Regenerative and non-regenerative anaemia

Anaemia occurs as a result of either reduced numbers of erythrocytes or reduced haemoglobin concentration per erythrocyte (MCHC) (Lewis et al. 2006). Regenerative anaemia occurs where an individual responds to the anaemia by either release of immature erythrocytes from bone marrow or increased erythropoiesis (typically due to hypothalamus-pituitary-adrenal (HPA-) mediated stress, injury or parasite infection; Fair et al. 2007; Fisher & Crook 1962; O'Brien et al. 2001; Tyler & Cowell 1996). Non-regenerative anaemia occurs when the individual is unable to respond to and/or compensate for loss of erythrocytes and this condition can result from nutritional stress, some chronic diseases or toxins (Fair et al. 2007; Tyler & Cowell 1996).

2.2.1 Limitations of hormone and leukocyte stress indices:--Indexing HPA-axis mediated stress (hereinafter 'physiological stress'; Siegel 1980) is usually done using blood hormone (e.g. glucocorticoid) assays or leukocyte metrics (e.g. total neutrophil or lymphocyte concentrations and the ratio of these cell types (N:L) or equivalent in birds and reptiles) (Davis et al. 2008; Romero 2004; Sapolsky et al. 2000). An advantage of using erythrocyte metrics over these more frequently-used indicators of hypothalamus-pituitary-adrenal (HPA-) axis mediated stress is that the rate of change after acute stress is considerably slower than either that for hormones (as rapid as a few minutes; Le Maho et al. 1992) or leukocytes (typically > 1 hr but sometimes as rapid as ½ hr; McLaren et al. 2003). It appears uncommon for erythrocyte indicators to change in response to acute stress (e.g. isolation and restraint) in < 6 hrs and peak responses can occur up to 48 hrs after exposure to a stressor (Oishi et al. 1999; Teague et al. 2007, though see Lynn et al. 2003). Stress hormones and leukocytes have the additional problem that acute responses, which might otherwise be expected to be reasonably similar among individuals (e.g. due to trapping or handling stress), can be attenuated if animals are in a state of physiological

exhaustion resulting from extreme chronic stress (Dhabhar & McEwen 1997). Acute and chronic stress effects on erythrocyte metrics are discussed below, but as far as is currently known, this is not a concern for erythrocyte indicators, although further research is needed.

The evidence in free-living vertebrates is that stressors usually have multiplicative effects on stress indicators such as hormones or differential immune cell metrics (e.g. the effect of high predatory activity and low food availability is greater than would be expected from each stressor measured independently (Boonstra et al. 1998; Chapman et al. 2006; Clinchy et al. 2004; Krebs et al. 1995; Zanette et al. 2003). Consequently, it could be expected that hormone and leukocyte stress indices in a captured free-living vertebrate comprise multiplicative effects of environmental stress and trapping or handling stress. However, evidence from laboratory studies indicate that extreme chronic stress attenuates hormone and leukocyte stress responses (Dhabhar & McEwen 1997). If an individual is already physiologically exhausted when trapped or handled, then its hormone and/or leukocyte values may not be readily interpretable.

The difficulty is that in the absence of other data a field researcher cannot be certain whether a population is chronically stressed. Moreover, because the evidence that chronic stress suppresses acute stress responses is from laboratory studies in which domestic animals (usually *Rattus rattus*) are subjected to stress regimes that may not be representative of stress in wild populations (e.g. hours of isolation and restraint recurrently over days or weeks (Dhabhar & McEwen 1997; Dhabhar et al. 1995), it remains unclear whether free-living vertebrates reach levels of physiological exhaustion where hormone or immune cell stress responses are substantially attenuated without dying (e.g. through predation).

Moreover, N:L ratio, considered a reliable index of chronic physiological stress in vertebrates (Davis et al. 2008), may not always index stress as expected. There are

examples in the literature where numerical domination of neutrophils or heterophils in circulating peripheral blood appear to be due to a physiological mechanism that seemingly increased innate immunity in response to an environment where risk of disease transmission was high (Dufva & Allander 1995; Figuerola & Ferrer 1999; Masello et al. 2009) rather than because of higher environmental stressfulness, as would be the more usual interpretation (Davis et al. 2008). In little penguins (*Eudyptula minor*), adults undergo a protracted moult-fast after the breeding season. During this fast, a time when it could be expected that chronic physiological stress is elevated, the heterophil-to-lymphocyte (H:L) ratio *decreased* (as does female estimated fat reserves, and Hb and Hct in both sexes) (Mortimer & Lill 2007). Although the decreased H:L ratio could in theory be linked to a relative increase in dominance of lymphocyte-mediated specific humoral-immunity (possibly in response to nest parasites), total leukocyte numbers *also* decreased--evidentially the change in H:L was due to loss or trafficking of heterophils out of circulating blood rather than increased lymphocyte concentrations (Mortimer & Lill 2007). Without additional data (e.g. indices of body condition or infection levels, erythrocyte stress metrics or absolute lymphocyte, neutrophil and eosinophil concentrations; Davis et al. 2008; Masello et al. 2009; Wikel 1996) a researcher cannot always be sure whether N:L is high because the animal is stressed or because the animal has elevated innate immunity (Masello et al. 2009).

This line of argument does not diminish the usefulness of hormone and immune cell indices; rather the intention is only to emphasize that all stress and condition metrics have potential drawbacks and the best approach is to measure a suite of relatively independent indicators from which a more informative picture of population stress and condition can be understood.

2.2.2 Acute stress effects on erythrocyte metrics:-- Exposure to acute stress induces changes in erythrocyte metrics characterized by a decrease in RBC, Hct and Hb (Fisher & Crook

1962; Lynn et al. 2003; Oishi et al. 1999), but the changes are thought to be due to changes in fluids and not the erythrocytes themselves (Fisher & Crook 1962; Oishi et al. 1999). Metrics of red blood cell morphology, such as volume or haemoglobin concentration, should not in theory be altered by acute stress and I have found no report of there being so. At least one study has reported elevated Hct in response to trapping stress, but this was attributed to dehydration in the trap (Fletcher & Boonstra 2006).

2.2.3 Chronic stress effects on erythrocyte metrics:-- Exposure to frequent or prolonged stressors (in laboratory work, typically restraint stress), blood loss (either through injury or through blood parasitism) and injection with stress hormones (e.g. adrenocorticotropin (ACTH) and corticosteroids in hypophysectomized rats; Fisher & Crook 1962) stimulates either or both release of immature erythrocytes from bone marrow or increased erythropoesis (as indicated by Fe^{59} incorporation into erythrocytes; Fisher & Crook 1962). The response has been reported in birds, mammals and reptiles (Fisher & Crook 1962; O'Brien et al. 2001; Schall et al. 1971; Teague et al. 2007). Consequent observable changes can include elevated RBC, Hct, Hb and platelet counts (PLC) (after 9 days of treatment; Teague et al. 2007). An interesting illustration of the different effects of chronic and acute stress was the finding that in two species of longspurs, *Calcarius ornatus* and *C. mccownii*, Hct was higher in individuals with higher baseline corticosteroid levels (presumably due to chronic stress) but Hct values decreased after handling (due to acute stress) (Lynn et al. 2003). Because immature erythrocytes (reticulocytes) are larger than mature cells we could also expect an increase in mean cell volume (MVC) (e.g. Fisher & Crook 1962), but in at least one study a decrease in MCV occurred (after 21 days treatment in *R. rattus*; Teague et al. 2007) so MCV may not always be a reliable indicator of the presence of immature red blood cells in all vertebrate taxa. Reticulocytes are less capable of producing haemoglobin than mature red blood cells and they tend to have a lower MCH as a result (Colombelli-Négrel & Kleindorfer 2008;

O'Brien et al. 2001). Thus, prolonged or frequent physiological stress can result in a blood profile typified by high RBC and MCV (and sometimes high Hct and/or Hb), concurrent with low MCH (i.e. a blood profile typical of regenerative anaemia; Colombelli-Négrel & Kleindorfer 2008; Tyler & Cowell 1996). Because regenerative anaemia is probably a generalized emergency state, it could be highly conserved among vertebrate taxa and consequently a potentially useful index of stress. Regenerative anaemia is examined when investigating animal condition in veterinary science (Cowell et al. 1999; Neiger et al. 2002; Tyler & Cowell 1996) but in vertebrate ecophysiology only a few studies have considered the implications of regenerative anaemia in depth (e.g. Colombelli-Négrel & Kleindorfer 2008; O'Brien et al. 2001; Schall et al. 1971). The metric most frequently used to attempt diagnosis of regenerative anaemia in free-living vertebrates has been MCV. There are multiple reports of correlations between higher MCV and poorer indices of condition, reproductive success or habitat quality. Seal et al. (1978) reported that white-tailed deer (*Odocoileus virginianus*) RBC and MCV were different in habitats of differing quality, but did not describe the trends or interpret the differences. In skua (*Cattharacta skua*), adults with higher MCV fledged fewer chicks (Bearhop & Orr 1999). Hibernating black bears (*Ursus americanus*) have higher MCV following food-poor seasons (Hellgren et al. 1993) and female maternal MCV is inversely related to both cub and litter weights (Noyce & Garshelis 1994). Male bushy-tailed woodrats (*Neotoma cinerea*) that had lower MCV had better reproductive success and were preferred by females in mate-choice experiments over males with higher MCV (Weber et al. 2007). Mazerolle and Hobson (2002) reported that male ovenbirds (*Seiurus aurocapillus*) had higher heterophil counts (indicating greater physiological stress) and higher MCV in continuous forest than in forest fragments. The authors attributed the higher heterophil counts to greater stress in male ovenbirds defending the better quality territories in

continuous forest. The observed difference in MCV would seem to support this interpretation.

However, MCV alone may not always be reliable. At least in some vertebrates MCV decreases during semi-starvation or fasting (Stirrat 2003). The advantages and disadvantages of MCV are discussed in further detail below, but the important point remains that MCV, MCH and RBC should be considered together.

Environmental and physiological stress effects on MCV, MCH and RBC remain relatively unexplored fields of research. For this field to progress, we need to determine whether it is possible to accurately differentiate regenerative from non-regenerative forms of anaemia using techniques that are practical in the field. Medical and veterinary diagnosis of anaemia relies on examining MCV and red blood cell distribution width (RDW), a quantitative estimate of the amount of variation in the volume of erythrocytes (Neiger et al. 2002). The 'width' refers to the width of the distribution, not the cells and is calculated: (standard deviation of MCV / mean of MCV) × 100 (i.e. a coefficient of variation multiplied by 100). However, calculating RDW requires measurement of individual cell MCVs using an automatic haematology analyser. These are outside the financial scope of many physiological ecology researchers and are impractical in field laboratories. Nonetheless, I suggest that if the focus of a study is on differences between populations living in different environments (e.g. a comparison of populations living in fragmented and undisturbed forest sites, or sites before and after fire) a population level calculation of RDW may be valid, i.e. calculate total population RDW from MCV estimates for individuals in sites. This approach still requires a field laboratory (for RBC) and would reduce statistical power if individual animals are the sampling unit, but in comparative physiological ecology where the sampling units are sites or treatments, loss of statistical power would not be a concern. This approach hasn't been used in the literature as far as I know, but could easily be trialled.

Examples can be found where physiological ecology studies have reported what appear to be regenerative anaemia blood profiles in populations living in more disturbed or degraded environments, though interpretations are not always provided. For example, brush-tailed possums (*Trichosurus caninus*) had poorer body condition indices and higher RBC in 'peripheral' (fragmented and degraded) than in 'preferred' (undisturbed forest) habitats (Barnett et al. 1979b). Haemoglobin concentration was also higher in 'peripheral' habitat, but analysis of MCV and MCH were not provided (Barnett et al. 1979b), so it is difficult to immediately infer whether the overall difference in Hb between the habitats was proportional to the RBC difference. However, using the data provided by Barnett et al. (1979b) MCH for the two populations can be calculated. The apparent MCH trend was in the expected direction for regenerative anaemia (Means (pg) and *SE* using month as the sampling unit: Males in 'peripheral' = 24.3 ± 1.9 and 'preferred' = 27.1 ± 1.5, Females in 'peripheral' = 27.0 ± 0.4 in 'preferred' = 28.5 ± 0.6). An example of data from one of my own studies where regenerative anaemia appears to have been a factor is shown in Figure 1. The relationship was one where agile antechinus (*Antechinus agilis*) living in fragmented native forest had greater RBC and lower MCH than those in continuous undisturbed forest, but only where the forest fragments were < 30 ha. Note also that RBC and MCH altered seasonally. Such seasonal changes can confound a study if they are not anticipated in the study's experimental design.

An interesting finding in a study on keelback snakes (*Tropidonophis mairii*) was that high haemogregarine parasite loads did not appear to stimulate a compensatory release of immature erythrocytes, but also parasite infection level was not linked to several other indices of host fitness, condition and health (Brown et al. 2006). The implication seems to be that the host-parasite relationship was unusually benign. The presence or absence of a regenerative anaemia response to parasite infection may help determine whether apparently heavy parasite loads are detrimental to host fitness.

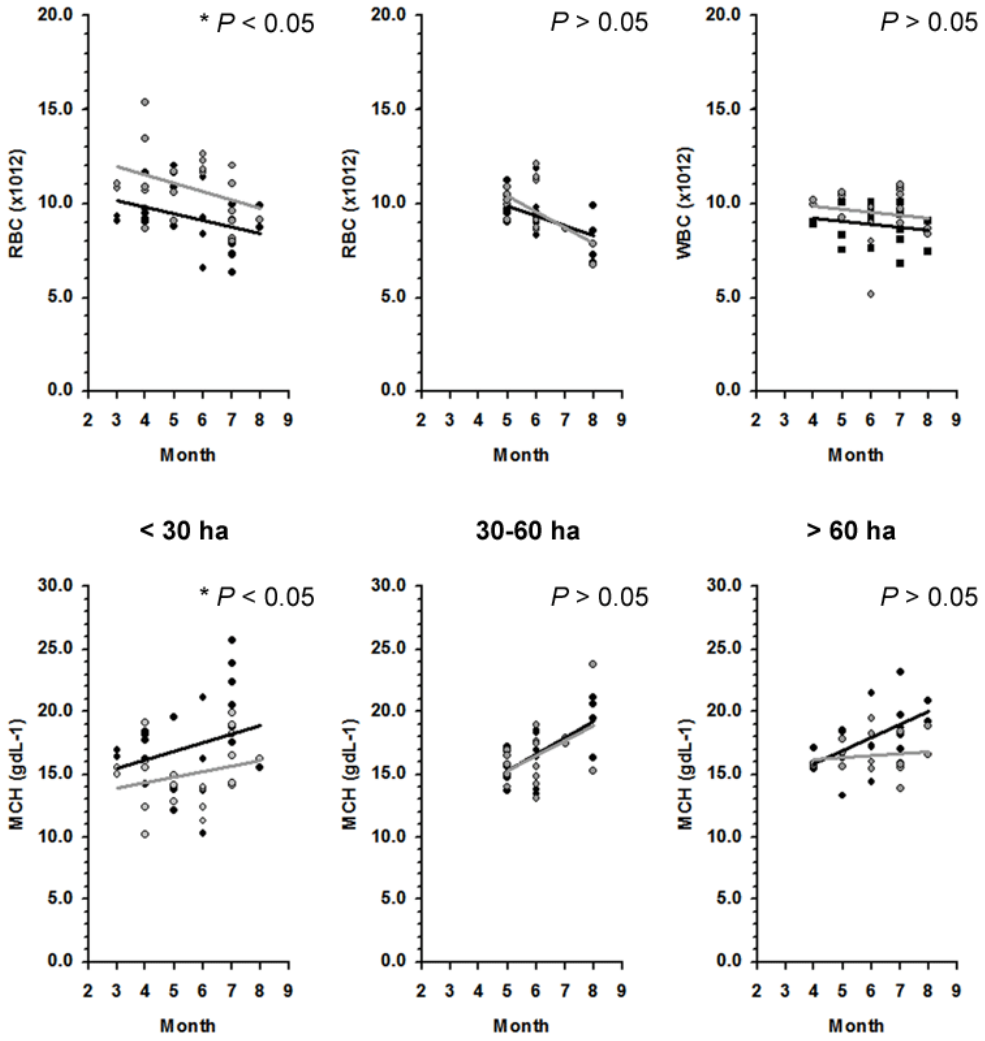

Figure 1. An example of how erythrocyte metrics can be used in physiological ecology. The figure shows a comparison of red blood cell counts (RBC above) and mean cell haemoglobin (MCH below) of populations of a small marsupial (*Antechinus agilis*) living in fragmented and continuous forest. Data are shown for three size classes of fragments, < 30 ha, 30-60 ha and > 60 ha and paired comparative control sites in continuous forest (fragment populations = grey circles and lines; continuous forest populations = black circles and lines; months shown are February [= 2] to September [= 9]). Populations living in < 30 ha fragments had higher RBC

and lower MCH than those living in continuous forest (ANCOVA: * indicates $P < 0.05$). Where fragments were > 30 ha no differences in RBC and MCH between the habitat types was evident. The higher RBC and lower MCH in populations in < 30 ha fragments is evidence that these populations were possibly exhibiting 'regenerative anaemia', the compensatory release of immature red blood cells from bone marrow into circulating blood in response to chronic physiological stress (e.g. in a stressful environment) or blood loss (e.g. from heavy parasite loads).

2.2.4 Thermal effects on erythrocyte metrics:--In temperate environments where seasonal differences in temperature can be marked, RBC, Hb and Hct can increase during winter (Sealander 1962; Swanson 1990). For example, in dark-eyed juncos (*Junco hyemalis*), Hct and oxygen carrying capacity (O_2/g of Hb) were higher in winter than summer, though oxygen affinity did not vary seasonally (Swanson 1990). These changes are usually attributed to thermoregulatory stress, increased thermogenesis, metabolic demands and consequent oxygen requirements (Sealander 1962). In birds, erythrocyte changes during winter could also potentially result from activity associated with establishing breeding status (Fair et al. 2007). However, the effect is not uniform among taxa. In a study of four passerine bird species, Hb and Hct did not vary seasonally, but RBC increased in winter (Breuer et al. 1995). In superb fairy wrens, RBC, Hb and Hct were unchanged in winter but increased in spring, which the authors attributed to increased requirement for oxygen carrying capacity prior to and during the breeding season (Box et al. 2002). That winter was not the typical causative parameter of any change in superb fairy wren RBC, Hb and Hct was attributed to relatively mild winter conditions in the study area (Box et al. 2002). In field voles (*Microtus agrestis*) RBC increased in winter (Beldomenico et al. 2008a), but in another small mammal living in a temperate environment, the agile

antechinus (*Antechinus agilis*) RBC declined from late summer (juveniles emerging from maternal nests) to late winter when the breeding season occurs (agile antechinus are mostly semelparous, typically live for just one year and die after either mating (males) or weaning young (females); Braithwaite & Lee 1979; Cheal et al. 1976). In some taxa the effects of season may be different in different years. Great tit (*Parus major*) Hb and Hct were influenced by an interaction between season and sampling year (Norte et al. 2009), implying that trends in one year probably should not be generalized outside of the sampling time. Seasonal effects on blood parameters also occur in semi-arid environments or tropical areas where dry seasons occur (Stirrat 2003), but these are probably due to nutritional stress (see below). Seasonal effects in a comparative study must be controlled for at the level of experimental design or statistical analysis.

2.3 Haemoglobin and haematocrit

Both Hb and Hct have been used variously as indicators of oxygen carrying capacity (Arnold et al. 1999; Burness et al. 2000; Simmons & Lill 2006), body condition (Cuervo et al. 2007; Masello & Quillfeldt 2004), growth rate (Merino et al. 1999) parasite load (Merino & Potti 1998; Słomczyński et al. 2006) and more generically 'health status' (Ots et al. 1998) and 'physiological performance' (Potti et al. 1999). As condition indices, Hb and Hct are attractive as they require only small blood volumes (< 100 μL) (Campbell 1995) and can be measured in the field using a portable haemoglobinometer or microcentrifuge. However, although using Hb/Hct has some practical advantages, their validity as condition indices is questionable with authors arguing both for and against their use (Bańbura et al. 2007; Cuervo et al. 2007; Fair et al. 2007; Masello & Quillfeldt 2004; Nadolski et al. 2006). The question remains: what, if any, aspects of 'condition' are Hb and Hct indexing and how are these metrics best interpreted?

2.3.1 As indicators of body condition:-- Researchers assume that animals in better condition are able to maintain higher Hb and Hct so that these indicators should have positive associations with body condition, but immediate confusion can arise because 'body condition' is not always well defined in a given study. In some studies body condition is used to mean health status, whereas in others the term specifically means a measure of size or mass of energy stores (fat or protein) indexed against skeletal size measurements (body condition index; Peig & Green 2009; Schulte-Hostedde et al. 2005). Here, I use body condition only to mean estimated energy stores. Given time, the current confusion in the literature over use of the term 'body condition' may resolve itself. Increasingly, researchers are avoiding claims that Hb/Hct are indices of 'body condition' *per se*, but employ more general population wellbeing terminology: 'health status', 'physiological condition' or 'physiological performance' (Bańbura et al. 2007; Norte et al. 2008; Potti et al. 1999). Arguably, these terms are quite vague and their use without an explanation regarding what aspect of condition or health is referred to is probably not helping to clarify an area of research that is beset by conflicting results.

The benefit of higher Hb and/or Hct is assumed to be greater oxygen carrying capacity and therefore potentially a greater ability to delivery oxygen to tissues and meet metabolic demands (Coles et al. 2009), but attempts to validate Hb/Hct and body condition in a given taxa have produced conflicting results. The correlation between Hb or Hct and estimated lipid reserves can be strongly positive (Sánchez-Guzmán et al. 2004; Svensson & Merilä 1996), weakly positive (Cuervo et al. 2007) or non-existent (Burness et al. 2000; Dawson & Bortolott 1997; Nadolski et al. 2006). Results are at times conflicting even within a study or suggest only weak influence of Hb or Hct as a condition indicator. For example, male and female parent great tits' Hct did not correlate with brood size, clutch size, laying date, mean egg weight, mean weight before fledging or number of fledglings,

but Hct was lower in females laying a second brood (Norte et al. 2010); the implication being that Hct was sometimes, but not reliably, an indicator of adult bird condition.

Relationships among Hct/Hb and body condition may be taxon or environment-specific, or plausibly, they may be confounded by age, sex, reproductive status, dehydration or geographic elevation (Fair et al. 2007) (dehydration is a particular problem if animals are kept in traps for extended periods before sampling; Fletcher & Boonstra 2006). A positive relationship between body condition indices and/or mass and Hb has more commonly been reported in birds (Breuer et al. 1995; Sánchez-Guzmán et al. 2004) than in mammals; though as with bird research, some studies of mammals have indicated that Hb can be a useful measure of body condition (Barnett et al. 1979a), but others showed no significant relationship between the two variables (Newson & Chitty 1962; Stirrat 2003). In some studies, differences in Hb or Hct are observed among study sites or treatments, but interpretation is difficult. For example, great tit Hct has been reported to be higher in rural than urban birds, but the authors were uncertain if this was because urban birds were in worse physiological condition or because rural birds had to forage over larger areas for food and so were acclimated to higher levels of physical work (Ots et al. 1998).

As evidenced by contradictory findings among Hb and Hct × body condition validation studies, the use of Hb or Hct alone as an body condition indicator in the sense of 'estimated energy reserves' is equivocal at best. At least some studies have reported positive Hb and/or Hct associations with other condition-related factors (e.g. food availability and survivorship; Bańbura et al. 2007), so perhaps Hb and Hct do index generalized 'health status' in some taxa in some environments--the problem is that any attempt to interpret these metrics in isolation from other condition measures will potentially (and it could be argued *almost certainly*) lead to false conclusions (Fair et al.

2007). Where mass and skeletal measurements are also available, these metrics may be more easily interpreted and should be used in preference.

2.3.2 As indicators of parasite load:-- Both Hb and Hct have been reported to be lower in vertebrates with higher parasite loads (parasite taxa given in square brackets) (Merino et al. 1999 ['ectoparasites': no taxon given]; Potti et al. 1999 [nest mite ectoparasite: Dermanyssus gallinoides]; Słomczyński et al. 2006 ['macro-parasites' and 'micro-parasites' where 'macro' = arthropod ectoparasites, and 'micro' = bacteria and fungi; no species given]) and this is attributed to anaemia caused by blood loss. However, the relationship does not appear to be reliable among host species or parasites. House wrens (*Troglodytes aedon*) showed no relationship between parasite load and Hb/Hct in adults, whilst highly parasitised nestlings of the same species had lower Hb but Hct was unchanged (O'Brien et al. 2001 [blow fly larvae; *Protocalliphora parorum*]); a result attributed to regenerative anaemia (usually characterized by lower Hb per unit of erythrocyte volume; Tyler & Cowell 1996). It could be that regenerative anaemia is a response to moderate parasite infection and that reduction in total blood Hb and/or Hct occurs only when parasite infection levels are so severe that the animal is unable to compensate (i.e. in effect, non-regenerative anaemia). However, in a study on superb fairy-wrens, males in nuptial plumage had higher parasite infection loads than non-nuptial males and females and also had *higher* Hb (Colombelli-Négrel & Kleindorfer 2008) [blood parasite: *Haemaproteus* spp]. The authors theorized that if nuptial males had higher levels of activity, the consequent requirements for greater Hb might have overwhelmed any effect of parasite load on Hb. This would seem to emphasize that measuring Hb alone will not always provide an accurate index of haematophagous parasite numbers and that parasite counts should be undertaken when possible. Measuring Hb is probably more useful if researchers wish to investigate *how* a study population is responding to given levels of parasite infection (i.e.

apparent high parasite burdens do not always have obvious fitness consequences; Brown et al. 2006) [blood parasite: haemogregarine infection].

Experimental manipulation using an anti-parasite treatment (i.e. fumigation of nests or provision of food dosed with anti-intestinal parasite compounds) seem an obvious avenue of research and this approach has been used in some species (Merino & Potti 1998 [blow fly larvae; *Protocalliphora azurea*]; Słomczyński et al. 2006), although more work is needed before general trends can be identified.

2.3.3 As indicators of aerobic capacity:-- Although Hb can be useful for making inferences about blood oxygen transport dynamics, without measurement of rheological properties, blood flow velocity and the degree of erythrocyte aggregation it is difficult to make any definitive interpretation regarding oxygen uptake, release rates or carrying capacity (Maeda 1996; Tateishi et al. 2001; Treacher & Leach 1998). However, accurate measurement of factors such as blood flow velocity, oxygen carrying capacity (O_2/g of Hb) and oxygen affinity (e.g. torr / P50 [at given temp and pH]; Swanson 1990) are not always practical to measure in the field and consequently experimenters often have to rely on best estimates drawn from the measurements that are available, such as Hb. This means that although some inferences about O_2 transport dynamics can be drawn from erythrocyte measurements that are logistically feasible to obtain in the field, cautious interpretation is essential.

Red cell morphology influences O_2 uptake and release rates (Holland & Forster 1966). Experiments with salamander (nucleated), human (not nucleated) and artificial (liposomes containing human haemoglobin) erythrocytes have shown that O_2 uptake rate is faster in smaller erythrocytes (Vandegriff & Olson 1984) which have a greater surface area to volume ratio and shorter diffusion distances (Wells & Baldwin 1990). In a study of O_2 uptake among erythrocytes from several species (approx means: 20 μm^3 [goat], 90 μm^3 [human], 680 μm^3 [bullfrog]), smaller erythrocytes also had a greater initial O_2 uptake

and the differences could not be explained by interspecific haemoglobin kinetic differences (Holland & Forster 1966). A range of 20-680 μm^3 is substantial and it is unclear whether within-species MCV differences are often large enough to have biologically significant effects on oxygen uptake rates. Variables influencing RBC O_2 release rate include MCH, MCHC and Hct (Maeda & Shiga 1994; Vandegriff & Olson 1984), such that higher values of these metrics correlate with slower RBC O_2 release rates. In theory MCV should alter RBC O_2 release rates because of shorter diffusion distances, but this is not supported by empirical evidence (Vandegriff & Olson 1984). The reason why is unknown, but it is possible that stronger effects of MCHC, the haemoglobin concentration in cells, on RBC O_2 release rates could overwhelm any MCV effects.

Where oxygen transport is the focus of a study, at least Hb, Hct, MCV, MCH and MCHC should be examined and other metrics should be included wherever possible. For example, during nestling development in short-tailed shearwaters (*Puffinus tenuirostris*), a trend towards reduced MCV and more ellipsoidal red blood cells occurred with age, and both of these changes could feasibly improve oxygen uptake and eventual delivery (Arnold et al. 1999).

2.3.4 Haemoglobin-Haematocrit residuals:--Elsewhere, I and coauthors of a paper have suggested a novel erythrocyte measure of stress, Haemoglobin-Haematocrit residuals (HHR), that we think may be a better health index than either metric alone and can still be obtained relatively easily from a blood sample in the field (Johnstone et al. *in preparation*). When used as an indicator of condition we found no convincing relationship for female agile antechinus, but males had higher HHR where microhabitat heterogeneity was higher and in study sites where there was a greater dominance of woody debris, shrubs and native trees with fissured bark. As the antechinus is a ground and tree foraging insectivore (Sumner & Dickman 1998) that has a breeding system involving

intense male/male competition (larger males typically sire more offspring; Kraaijeveld-Smit et al. 2002b; Kraaijeveld-Smit et al. 2003), it is plausible that male HHR was higher in sites where foraging resources were more abundant. The principal is similar to Mass-Size residuals (MSR), which are used to estimate lipid reserves from mass and one or more skeletal measurement (Peig & Green 2009; Schulte-Hostedde et al. 2005). An HHR index estimates the amount of haemoglobin per unit of packed cell volume. A high HHR should reflect erythrocytes that have high levels of haemoglobin per unit volume of cells, whereas a low HHR would indicate lower haemoglobin by weight per volume of cells, and possibly a higher percentage of reticulocytes and the occurrence of regenerative anaemia. In the body condition literature there is debate over whether ordinary least squares (OLS), major axis (MA), or reduced major axis (RMA) regression should be used to generate the residuals (Green 1989; Peig & Green 2009). In theory, because both Hb and Hct are random variables, either MA or RMA regression should generate a more accurate estimate of the slope of the relationship (Quinn & Keough 2002). However, OLS residuals are simpler to derive and their units are more readily interpretable, i.e. the units for MSR using OLS residuals are grams above or below the individual's expected mass. On balance, OLS regression is probably preferable on the grounds of statistical simplicity, especially if the researcher is not familiar with how to derive MA and RMA residuals.

2.4 Nutritional stress

A potentially underused feature of red blood cell physiology is that MCV reportedly decreases in at least some mammal species during nutritional stress under laboratory and field conditions (Stirrat 2003). This response has been observed during human 'starvation trials' (cited in: Shield 1971) and the same phenomenon may be responsible for reports

that packed cell volume can decrease during starvation, e.g. where absolute blood cell volume decreased but absolute plasma volume remained unchanged (e.g. in nutritionally stressed quokka (*Setonix brachyurus*); Shield 1971). Some authors have termed the reduction in MCV a 'semi-starvation' response (Stirrat 2003), implying that the stress must be substantial. In agile wallabies (*Macropus agilis*), MCV was reportedly lower during the dry season when food abundance is low (Stirrat 2003) and in black bears (*Ursus americanus*) MCV is reduced during hibernation (Hellgren et al. 1993). In at least one study, high MCV was reported to correlate positively with longer survivorship (in *Parus major*; MCV measured in 12-day-old nestlings and survivorship was until fledging; Nadolski et al. 2006). Shield (1971) thought that there was sufficient evidence to claim that a common environmentally induced trend was one in which reduced food intake associates with lower body temperatures, reduced metabolic rates and decreased total blood cell volumes in mammals. In contrast, undernourished white-tailed deer had a high Hct and RBC (attributed to associated dehydration and haemoconcentration) but MCV did not vary in any readily interpretable manner (DelGiudice et al. 1990). Subsequent 'refeeding' to a high protein/high fat diet after semi-starvation treatment decreased Hct and RBC, but MCV was not significantly altered (DelGiudice et al. 1990).

Because MCV should in theory increase in vertebrates with regenerative anaemia (Tyler & Cowell 1996), high or low values of MCV on their own have only partial usefulness. I have stated already that RBC and MCH should also be examined (and in any instance MCV is calculated from RBC), and where researchers examine nutritional stress, it could be that a body condition index based on mass and skeletal measurements will always be preferable. To our knowledge, the underlying mechanism is unknown, but if we assume its adaptive value is to reduce the metabolic cost of erythrocyte maintenance at a time when food supply is limited, the effect on blood cell morphology appears counterintuitive. The relative metabolic cost of maintaining a greater number of smaller

erythrocytes is higher than for maintaining fewer larger cells due to higher blood viscosity and hence a reduced blood flow rate (Arnold et al. 1999; Wells & Baldwin 1990). The only means by which a reduction in MCV could reduce metabolic costs would be if there were a concurrent decrease in RBC. Nutritionally stressed vertebrates down-regulate and/or cease erythropoiesis, presumably to conserve energy (Fair et al. 2007). If erythropoiesis were slowed for long enough, erythrocyte population aging should in theory result in a higher proportion of smaller mature cells. If using MCV as an index of nutritional stress it would be pertinent to consider the life-span of the erythrocyte cell in a study species, i.e. if erythrocyte replacement is very slow, periods of short nutritional stress lasting only days or weeks are unlikely to effect MCV (red blood cell average life spans in mammals 51-175 days; in birds 28-35 days; in a turtle 600-800 days; in a toad 700-1400 days; Altland & Brace 1962; Degernes et al. 1999; Kurata et al. 1993; Rodnan et al. 1957). The tendency is for species of a given taxa (e.g. mammals) that have smaller mass to have shorter red blood cell life-spans (e.g. mice = 51, dog = 75, cattle = 175 days; Kurata et al. 1993). This is a conjectural explanation for the association between starvation and reduced MCV, and experimental validation and investigation is required.

2.5 Infection and Disease

Erythrocyte sedimentation rate (ESR) is used extensively in human health (Lewis et al. 2006), but is underutilized in vertebrate physiological ecology. The metric is established by storing freshly sampled blood at room temperature vertically for an hour and recording the proportions of sediment cell and plasma (mm sedimentation per hour) (Lewis et al. 2006). The rate of sedimentation is influenced by the size and concentration of erythrocytes in the sample, composition of plasma proteins, plasma viscosity and

erythrocyte morphology (Lewis et al. 2006). Sedimentation is typically faster at higher temperatures, so maintaining the same temperature when using ESR is important (Lewis et al. 2006). Proteins, in particular immunoglobulins and fibrinogen as well as other acute-phase proteins (e.g. haptoglobin, ceruloplasmin etc), accelerate sedimentation rate because they cause erythrocyte clumping (the formation of rouleaux; Lewis et al. 2006), whilst albumin inhibits the process (Lewis et al. 2006). Consequently ESR is a useful but non-specific index of inflammation and infection; severe disease correlates with faster ESR, where 'disease' refers to bacterial and viral infections, some examples of which include influenza, pulmonary tuberculosis and acute toxic hepatitis in humans, and also to non-infectious diseases such as multiple sclerosis and Hodgkin's lymphoma (Rourke & Ernstene 1930). Few vertebrate physiological studies have used ESR, but where they have the results are interesting. Thus adult and juvenile burrowing parrots (*Cyanoliseus patagonus*) had a higher ESR associated with lower body condition indices, and ESR was also higher in males with short tail feathers (Masello & Quillfeldt 2004). In blue tits (*Parus caeruleus*), birds with higher indexed fat reserves had slower ESR.

Rourke and Ernstene (1930) conducted an extensive validation of ESR in humans and concluded that it should be corrected (cESR) by subsequently centrifuging the sample and calculating cESR as a proportion of the total blood cell compaction possible (cESR = mm clear plasma after 1 hr settling × 100 / mm plasma after centrifugation of the same sample). The cESR metric has not been employed in any physiological ecology study as far as I know, and its potential use seems worth investigating.

2.6 Platelet indices

Platelet measurements are widely used in medical research, the primary metrics of importance being the platelet count (PLC), platelet(o)crit (Pct), mean platelet volume

(MPV) and platelet distribution width (PDW) (for an explanation see red blood cell distribution width, above) (Wiwanitkit 2004). Of these, only PLC is easily calculated using standard haematological methods in the field and the remaining metrics require an automated haematology analyser (Wiwanitkit 2004). Platelet metrics are used to diagnose disorders such as leukaemia (Lewis et al. 2006) and could have use in at least some vertebrate ecophysiological studies (for an example see Nadolski et al. 2006).

2.7 Conclusion

The use of erythrocyte indicators of stress and condition in vertebrate physiological ecology has the potential to yield informative results that would be difficult or impossible to obtain in other ways. However, considerable work is still needed to validate erythrocyte responses to environmental stressors, both in the laboratory and in natural environments. Although it does not appear that erythrocyte indicators are likely to be confounded by the interplay between acute stress (trapping and handling) and chronic physiological exhaustion and stress, this also remains to be confirmed. I argue that the tendency to rely on either Hb or Hct alone as indices of body condition, 'physiological performance' or 'health status' needs to be circumscribed and more sophisticated metrics, such as HHR, MCV, MCH and ESR, should be adopted more commonly, as they are much more likely to yield unequivocal results.

Monash University

Declaration for Thesis Chapter Three

Declaration by candidate

In the case of Chapter Three, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Conception, collection of field data and samples, laboratory work, data analysis, major writing	90

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Alan Lill	Writing and editing	5
Richard Reina	Writing and editing	5

Candidate's Signature		**Date** 21/09/2010

Declaration by co-authors

The undersigned hereby certify that:

1. the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
2. they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
3. they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
4. there are no other authors of the publication according to these criteria;
5. potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
6. the original data are stored at the Department of Biological Sciences, Monash University, Clayton and will be held for at least five years from the date indicated below:

		Date
Signature 1		21/09/2010
Signature 2	[redacted]	21/09/2010

Are haematological indicators of stress and poor condition associated with habitat fragmentation in the agile antechinus?

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Running headline: Stress and condition in a fragmented habitat

Abstract

Although the vertebrate stress response is essential for survival, frequent or prolonged stress responses can result in chronic physiological stress, which is associated with a suite of conditions that can impair survivorship and reproductive output. Anthropogenic habitat fragmentation and degradation are potential stressors of free-living vertebrates that may result in such chronic stress.

Haematological indicators of stress and condition were compared in agile antechinus (*Antechinus agilis*) populations in 30 forest fragments and 30 undisturbed, continuous forest sites (pseudofragments) in southeastern Australia over two years.

In peripheral blood, the total leukocyte count was lower and the neutrophil/lymphocyte ratio and percentage of eosinophils in the total leukocyte population higher in fragment than pseudofragment populations, indicating that fragment populations were probably experiencing higher levels of stress hormone-mediated and/or parasite infection-related chronic physiological stress.

The total erythrocyte count and haematocrit were higher and mean erythrocyte volume and haemoglobin content lower in fragment than pseudofragment populations, suggesting that fragment populations were in poorer condition. Whole blood haemoglobin concentration and mean cell haemoglobin concentration were similar in fragment and pseudofragment populations.

We suggest that where anthropogenic activity results in habitat fragmentation and degradation, chronic stress could potentially contribute to a decline in agile antechinus populations. The broader implication is that chronic stress could be both symptomatic of, and contributing to, decline of some vertebrate populations in anthropogenically fragmented and degraded habitats.

Key-words: *Antechinus agilis*, neutrophil/lymphocyte ratio, physiological stress, repeatability, small mammal

Introduction

A large body of research shows that fragmentation can cause the decline of animal populations and communities (Fazey et al. 2005; Fischer & Lindenmayer 2007), but research has tended to focus on population-level variables, such as population density, survival and reproduction. Davis, Maney & Maerz (2008) argued strongly for the use of haematological indicators of stress and condition in vertebrate ecology and there is a growing interest in whether such indicators could be increased by anthropogenic habitat fragmentation and degradation (Mazerolle & Hobson 2002; Suorsa et al. 2004). This approach could provide an early warning of a population's likely decline by helping to identify stressors before a population size decrease is evident (Wikelski & Cooke 2006).

Recent studies have also examined whether stress mediated by the hypothalamus-pituitary-adrenal (HPA) axis (hereinafter 'physiological stress') could itself be directly contributing to the decline of vertebrate populations in fragmented or degraded habitats (Martínez-Mota et al. 2007). The rationale is that prolonged or frequent stress responses can result in a disease state ('chronic stress') (Martínez-Mota et al. 2007) characterised by behavioural and physiological conditions that are potentially deleterious for survival and fecundity (Sapolsky et al. 2000; Siegel 1980; Tsigos & Chrousos 2002). These conditions include immunomodulation (Dhabhar & McEwen 1997), decreases in growth (Santos et al. 2000) and reproductive rates (e.g. fewer offspring, smaller eggs, altered parental behaviour) (Wingfield & Sapolsky 2003), increased rates of molecular aging (Epel et al. 2004; Liu & Mori 1999) and impairment of memory and cognition (McEwen & Sapolsky 1995). Chronic stress could thus be both symptomatic of, and contribute to, population decline in fragmented and degraded habitats.

Studies of birds and amphibians variously support (Suorsa et al. 2004) or conflict with (Homan et al. 2003; Mazerolle & Hobson 2002) the contention that chronic stress might be greater in vertebrate populations living in degraded than in relatively undisturbed habitats. Little research with this focus has been conducted on mammals, but Martínez-Mota *et al.* (2007) reported that faecal corticosterone concentrations (an index of nominal 'baseline' stress) were higher in howler monkey (*Alouatta pigra*) populations in fragmented than in unfragmented habitat.

The native agile antechinus (*Antechinus agilis,* Marsupialia:Dasyuridae; Dickman) (Fig. 1) (Dickman, Parnaby, Crowther *et al.*, 1998) is a nocturnal, carnivorous marsupial which is locally common and whose autecology (Banks et al. 2005a; Kraaijeveld-Smit et al. 2002a) and ecophysiology (Naylor et al. 2008) are well documented. It is an interesting and important species in which to explore the effects of habitat fragmentation and degradation on stress because it is restricted to *Eucalyptus* forest in south-east Australia (Sumner & Dickman 1998) which historically has been extensively fragmented. Although the agile antechinus can persist in fragmented habitat for some time, its patch occupancy rate is lower where forest fragments are smaller (Bennett 1990a). The population density of the closely-related brown antechinus (*Antechinus stuartii*) can be reduced through fragmentation of its forest habitat by agriculture (Knight & Fox 2000), suggesting that *A. agilis* might ultimately also be vulnerable.

Fig. 1. Agile antechinus.

We used several commonly employed haematological indicators of stress and condition to determine whether agile antechinus living in forest fragments in SE Australia experience greater chronic stress (as indicated by leukocyte variables) and have poorer condition (as indicated by erythrocyte variables) than conspecifics inhabiting similar unfragmented forest.

Materials and methods

Study species

The agile antechinus (females 10-26 g, males 15-45 g) is a scansorial insectivorous marsupial that nests communally in tree-hollows. The species is largely semelparous, having a synchronized breeding rut and a complete annual adult male 'die-off' in late August (Barnett, 1973). Most females also die after weaning their only litter, but a few survive to breed in a second year (Braithwaite & Lee, 1979). Agile antechinus are unusual in having two distinct spatial ranges, a 1-2 ha foraging range and a larger 'social range' of up to 5 ha (Lazenby-Cohen & Cockburn 1991). Pre-1998, the species was included in the brown antechinus (*A. stuartii*) species-complex (Dickman et al. 1998).

Study area and sites

The study was conducted in an area of South Gippsland, Victoria, Australia, approximately centred on the coordinate 38°37'S, 146°10'E (Fig. 2). Forest fragments were > 2 km from unfragmented forest (defined as continuous tracts of forest > 1000 ha), situated within a matrix of grazed fields and had sharp forest-field ecotones. Habitat similarity among study sites was achieved by restricting all sites to stands of forest composed of three Ecological Vegetation Classes (EVC) (Davies et al. 2002) ('Lowland Forest', 'Wet or Damp Forests [Wet]' and 'Wet or Damp Forests [Damp]'). The EVC categories are defined according to a system developed by the Department of Sustainability and Environment, as described in Davies et al. (2002). The dominant trees in all sites were *Eucalyptus* species. During 2007 and 2008 when the study was conducted, the mean monthly rainfall in the study area was 73 mm and mean monthly maximum and minimum ambient temperatures were 17.7°C and 7.8°C, respectively (Australian Bureau of Meteorology 2009).

Agile antechinus populations respond to habitat complexity (Holland & Bennett 2009; Knight & Fox 2000) and *Eucalyptus* forest fragments are typically more anthropogenically-degraded and therefore less complex than continuous forest (Mac Nally et al. 2000). In this study we assume that 'fragmentation' effects could be due either to fragmentation *per se* or are co-correlated and associated with habitat degradation. For a related study we concurrently recorded landscape configuration (fragment area, degree of isolation), topography (slopes, altitudes), floristic and habitat complexity variables at occupied sites and the effects of these environmental features on agile antechinus stress and condition are examined elsewhere.

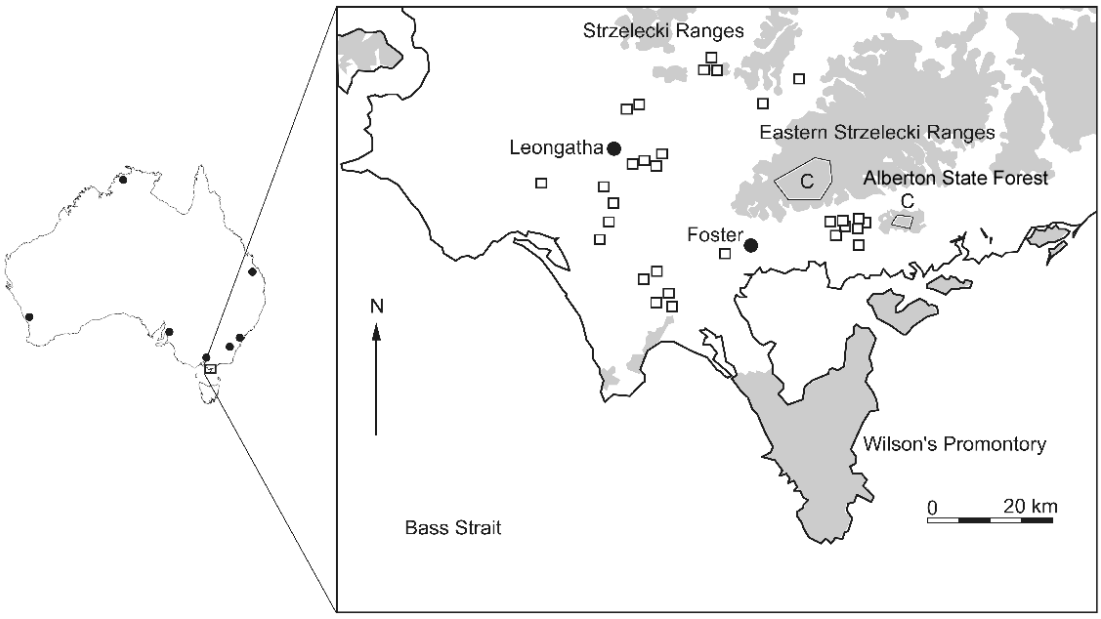

Fig. 2. Study region in South Gippsland, south-east Australia. White = cleared agricultural land. Darker shaded areas = tree cover (includes native re-growth, old growth forest and native plantations). Approximate locations of fragment study sites are indicated by white boxes (□). Comparative control sites (pseudofragments) were situated within the areas delineated by a heavy line and labelled 'C'. Map based on Department of Sustainability and Environment (DSE) interactive 'Forest-Explorer Online' maps (http://www.dse.vic.gov.au/).

Each of the 30 forest fragments in the study (range in area 6.6 to 298.6 ha) was paired with a 'control' site (termed a pseudofragment; Mac Nally & Bennett 1997) of the same area and shape that was randomly situated (except for avoiding overlap among pseudofragments) in relatively undisturbed, unfragmented forest of similar composition in either the Eastern Strzelecki State Forest and National Park or Alberton State Forest (Figs 2 and 3). Extensive clearing in the area for agriculture and forestry meant that unfragmented forest suitable for 'control' sites was restricted to these two locations and consequently pseudofragments collectively were more constrained than fragments in their spatial distribution. However, no agile antechinus captured in a pseudofragment was

ever re-captured in another pseudofragment. Fragment-pseudofragment experimental designs are common in fragmentation studies and are critical in separating the effects of fragmentation and degradation from those of habit loss (Mac Nally & Bennett 1997).

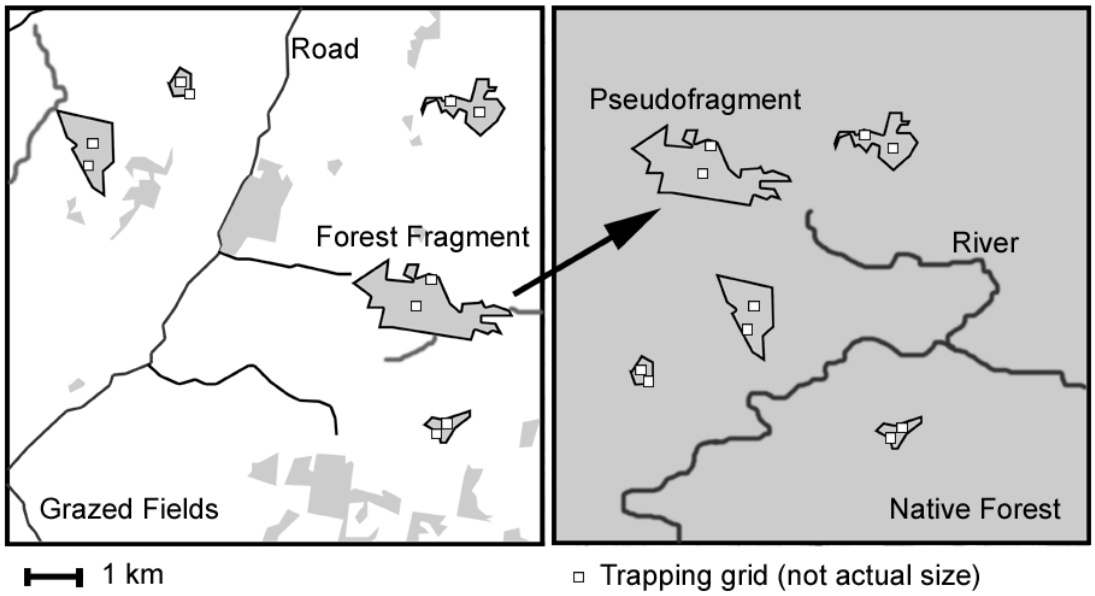

Fig. 3. A theoretical example of a Fragment-Pseudofragment experimental design using actual fragments in the study area. Pseudofragments are arranged randomly, except for avoiding overlap in an area of continuous habitat (Mac Nally *et al.*, 1997). The spatial arrangement of data collection sites (trapping grids) were kept as similar as possible in fragments and pseudofragments.

Live-trapping of agile antechinus

All animal work conformed to the relevant ethical standards and legal requirements. Trapping was conducted from early April to early August, 2007 and late March to early August, 2008, the austral autumn and winter. Sampling periods avoided two potentially confounding life-history events, dispersal (January/February) and the breeding rut and subsequent male 'die-off' (mid-late August). Trapping was conducted for three consecutive nights in each fragment and for the following three nights in its paired

pseudofragment. Trapping for a fragment-pseudofragment pair was completed in eight successive days. As there was likely to be seasonal variation in some haematological indicators (Cheal et al. 1976), this temporal pairing of sampling was essential. The position of each trapping grid in a fragment was replicated as closely as possible in the paired pseudofragment using a GPS (accuracy 6 m).

Weatherproofed, 33 × 10 × 10 cm live-traps (Elliott Scientific™, Australia) supplied with bedding and bait (oats, peanut butter, water and vanillin) were set in two grids per site, one < 60 m from the edge and the other in the interior (> 80 m from the edge). A fragment grid comprised 21 traps in a 40 × 120 m area. Due to higher capture rates on pseudofragment grids, the number of traps in a comparably-sized grid was reduced to nine traps in 40 × 120 m. Traps were set ≤ 3 h before dusk and animals were released ≤ 3 h after dawn. Typically, two trapped agile antechinus were blood-sampled per grid per trapping day (i.e. up to 6 per grid and 12 per study site over three days). These individuals were the first two found in traps each day that were not recaptures.

Sampling and measurements

At capture, a < 1 mm diameter disc of pinna tissue was removed to facilitate identification on recapture. From each sampled animal ~ 100 μL of blood were collected by capillarity in a heparinised microhaematocrit tube after puncturing one of the two lateral veins near the base of the tail with a 27 gauge needle. Blood samples that could be affected by leukocyte trafficking (Dhabhar et al. 1994) were collected first. After blood sampling, we collected mass and morphometric measurements (for constructing a body condition index), ectoparasite counts and faecal material for endoparasite assessment; these data, as well as a formal analysis of relative abundances, are reported elsewhere.

Whole blood haemoglobin concentration (Hb) (± 0.1 grams per litre [$g \bullet L^{-1}$]) was determined immediately with a Hemocue 201+ haemoglobinometer (Hemocue®,

Ängelholm, Sweden). All other blood samples were kept chilled and stored for ≤ 10 h before laboratory analysis. On average, blood collection was completed in about 8 min after removal of an antechinus from a trap and never took > 15 min.

Haematocrit (Hct) (%) was determined by centrifugation for 3 min at 12,700 g. Measuring Hct twice in the same sampling event in each of 52 individuals indicated a measurement error of 6%. To make concurrent white (WBC) ($\times 10^{11}$ cells per litre) and red blood cell (RBC) ($\times 10^{12}$ cells per litre) counts, five μL of blood were diluted with Natt and Herrick's solution at a ratio of 1:200 (Campbell 1995). Counting was conducted under 400 × magnification using an improved Neubauer haemocytometer (Blau Brand, Germany). Two samples per individual were used for all total cell counts and measurement error was 12% for WBC and 9% for RBC. Blood smears made by the pull-wedge method (Lewis et al. 2006) were stained with May-Grünwald-Geimsa stain (Lewis et al. 2006). Differential leukocyte counts were obtained by making visual sweeps from the 'head' to the 'tail' of each smear. They always comprised > 200 leukocytes and were used to determine the ratio of neutrophils to lymphocytes (N:L) and % Eosinophils (% E) (eosinophils as a percentage of all leukocytes). The same experimenter made all leukocyte measurements. Although WBC are sometimes difficult to interpret (Davis et al. 2008), total neutrophil and lymphocyte concentrations derived from total and differential leukocyte counts may be more useful than N:L alone (Masello et al. 2009), and therefore we also derived and analysed these cell concentrations. Mean red cell volume (MCV, in femtolitres [fL]), mean cell haemoglobin content (MCH, in picograms per cell, [pg●cell^{-1}]) and mean cell haemoglobin concentration (MCHC, in grams per decilitre, [g●dL^{-1}]) were calculated from the measured erythrocyte variables (Lewis et al. 2006). A valid indicator of local environmental conditions should be less varied within than among study sites i.e. should have a positive repeatability (R), the ratio of variation among sites to variation within and among sites (Lessells & Boag 1987). Each haematological indicator's R was

calculated using the method of Lessells *et al.* (1987). This method calculates repeatability using *F* distribution mean squares for within and among treatments and a group size 'mean' adjusted to account for uneven designs (the wider the spread of group sizes, the lower the adjusted mean).

Controlling for leukocyte trafficking

In some vertebrates, changes in leukocyte concentrations in peripheral blood occur within 30 min of exposure to an acute stressor, such as handling (Dhabhar et al. 1995). However, little is known about leukocyte trafficking in marsupials and a rapid, acute trafficking response could potentially have confounded attempts to measure baseline WBC, N:L and % E in agile antechinus. Therefore we incorporated a small validation test in which agile antechinus were live-trapped and WBC, N:L and % E were determined from a blood sample taken immediately after removal from the trap (0 min). Further determinations were made from a second sample taken either 10 (n = 7), 20 (n = 6) or 30 min (n = 7) later. The prediction was that if handling caused acute stress, these variables would be observably higher (N:L and WBC) or lower (% E) in the 10, 20 or 30 min treatment than at 0 min (Davis et al. 2008).

Trapping stress was also a concern, but was more difficult to test. Determining 'baseline' stress requires instant-kill trapping, with a researcher nearby to take blood samples immediately (Fletcher & Boonstra 2006), but this was impractical for our study area and species. We address the trapping stress issue in more detail later in the Discussion, but the following three points are pertinent here. In a study conducted on *A. agilis* in similar environments, mean time in a trap was probably ~ 8 hrs (i.e. the species may be a dusk forager) and time spent in traps did not vary significantly among study sites (Tara Bramwell, personal comm.); many trapped antechinus in the present study

were therefore probably experiencing a trapping stress response but trapping can probably be considered a uniform stressor (Fletcher & Boonstra 2006).

Data analysis

Statistical analysis was conducted with R (2.8.1, R Core Development Team) (version 2.8.1 http://www.r-project.org/). Data were checked for normality and homoscedasticity and N:L was $\log_{10}$ transformed to achieve normality. No other transformations were needed. Linear mixed effects models (LMEM) (Crawley 2007) ('lme' in package 'nlme') were used to compare the 0 and10 min, 0 and 20 min and 0 and 30 min values in the leukocyte trafficking test and to examine effects of forest fragmentation on blood variables (fragmentation had two levels, fragment or pseudofragment). Given the strikingly different life histories of male and female agile antechinus (Barnett 1973), they were treated as separate populations and 'sex' was included in the models as an explanatory factor (two levels, male or female). To avoid pseudoreplication, the factors sampling year, month, forest block, site and trapping grid were included as random effects in the models; 'forest block' accounted for pseudofragment location, so that for 'control' sites in unfragmented forest, n = 2 (Fig. 2). The interaction term fragmentation × sex was never significant ($P > 0.129$) and so it was removed from all models to facilitate interpretation of main effects (Engqvist 2005). Final models were validated graphically (Zuur et al. 2009) using ordinary and standardized residuals. Residual spread was similar for fragments and pseudofragments in all indicators. Effect sizes, where given, estimate the slope of a relationship (for continuous predictor variables) or the difference between the slopes (for factors).

Results

Blood samples were collected from 549 agile antechinus in 30 forest fragments and 30 pseudofragments. In 2007, 73 males and 61 females were sampled in fragments and 109 males and 52 females in pseudofragments. In 2008, fragment samples comprised 70 males and 55 females and pseudofragment samples 80 males and 49 females.

Repeatability and leukocyte trafficking

All blood variables had positive repeatability (i.e. variation was greater among than within study sites), and the ranking from highest to lowest was WBC, (log) N:L, RBC, MCV, % E, MCH, Hb, Hct and MCHC (Table 1). No significant variation was apparent for WBC or (log) N:L among any of the post-handling treatments (0-10 min, 0-20 min and 0-30 min) in the leukocyte trafficking test (Table 2). For % E, there was no significant effect for 0-10 min and 0-30 min, but it was significantly lower ($P = 0.019$) in the 20 min than the 0 min treatment (Table 2).

Table 1. Repeatability of blood variables in agile antechinus (*Antechinus agilis*). Repeatability (R) was calculated using *F* distributions and the method recommended in Lessels and Boag (1987). Also shown are the number of individual agile antechinus (*n*) and the adjusted mean number of individuals per site (n_o). The number of sites was 60 for all variables. *P* was < 0.001 (*) for all blood variables. Where R is positive, an indicator was less variable within sites than among sites. Higher R values indicate better repeatability within sites (i.e. WBC had the highest repeatability and MCHC the lowest). Abbreviations for blood variables given in Materials and methods.

Variable	*n*	n_o	*F*	(*df*)	R
N:L	551	9.17	7.88	(59,491)*	0.429
WBC	548	9.11	10.23	(59,488)*	0.503
% E	551	9.17	3.47	(59,491)*	0.212
RBC	548	9.11	4.00	(59,488)*	0.248
Hb	520	8.65	2.78	(59,460)*	0.171
Hct	525	8.73	2.39	(59,465)*	0.137
MCV	525	8.73	3.44	(59,465)*	0.218
MCH	520	8.65	3.01	(59,460)*	0.189
MCHC	513	8.53	1.94	(59,453)*	0.099

Table 2. Results of the leukocyte trafficking validation test in *Antechinus agilis*. Means and standard errors for leukocyte variables are given for agile antechinus sampled at 0 min (n = 20), 10 min (n = 7), 20 min (n = 6) or 30 min (n = 7) after removal from a trap. *t* and *P*-values from the linear mixed effects model are for pair-wise comparisons between blood samples taken at 0 min post-removal from a trap and samples taken at 10 min, 20 min and 30 min. Degrees of freedom were 17 for all comparisons. * $P < 0.05$. Abbreviations for blood variables given in Materials and methods.

Time since removal from trap (minutes)	Mean ± SE	t-value	*P*-value
WBC ($x10^{11}$ cells●L^{-1})			
0	1.88 ± 0.11		
10	1.91 ± 0.21	0.483	0.635
20	1.97 ± 0.18	0.161	0.874
30	2.30 ± 0.24	1.897	0.075
(log)N:L			
0	1.38 ± 0.22		
10	1.65 ± 0.47	0.996	0.333
20	0.85 ± 0.22	-1.879	0.078
30	1.26 ± 0.21	-0.200	0.844
% E			
0	0.016 ± 0.01		
10	0.012 ± 0.01	-0.560	0.583
20	0.008 ± 0.01	-2.584	0.019*
30	0.015 ± 0.01	0.130	0.898

Stress and condition in fragment and pseudofragment populations

(i) *Leukocyte variables*

Total leukocyte count was significantly (1.2 ×) higher in males than females and in pseudofragments than fragments (1.3 ×). Although (log)N:L did not differ between the sexes (Table 4), females in 2007 showed an apparently different N:L trend to those of females in 2008 or males in either year (Table 3). Therefore, we partitioned the sexes and re-applied the LMEM: males had (25%) higher (log)N:L in fragments than pseudofragments (effect size = 0.11 ± 0.04, *df* = 30, *t*-value = 3.08, *P* = 0.004) but female (log)N:L was not different in the two habitat types (effect size = 0.05 ± 0.06, *df* = 29, *t*-value = 0.95, *P* = 0.348). Partitioning the sexes was further warranted when we examined total neutrophil and lymphocyte concentrations. Males had marginally (1.1 ×) higher neutrophil concentrations than females (effect size = 1.4 ± 0.69 ($\times 10^{10}$), *df* = 431, *t*-value = 1.98, *P* = 0.049) and lymphocyte concentrations were influenced by the interaction term SEX × FRAGMENTATION (*df* = 430, *t*-value = -2.67, *P* = 0.008). When partitioned by sex, male but not female lymphocyte concentrations were (1.4 ×) higher in pseudofragments (male: effect size = 5.2 ± 1.3 ($\times 10^{11}$), *df* = 31, *t*-value = -3.89, *P* < 0.001; female: effect size = 0.3 ± 0.2 ($\times 10^{10}$), *df* = 29 , *t*-value = -1.56, *P* = 0.129). Percentage eosinophils was significantly higher in males than females and in fragment than pseudofragment populations (1.2 × in both cases) (Tables 3 and 4).

(ii) Erythrocyte variables

There was no difference in RBC between the sexes, but RBC was higher (by 10%) in fragment than pseudofragment populations (Tables 3 and 4). Whole blood haemoglobin concentration did not differ between the sexes or between fragment and pseudofragment populations. There was no Hct sex difference, but it was significantly (1.1 ×) higher in fragment than pseudofragment populations. There was no difference in MCV or MCH between the sexes, but MCV was significantly larger (1.1 ×) and MCH significantly higher (1.1 ×) in pseudofragment than fragment populations. Mean cell haemoglobin concentration did not differ between the sexes or habitat types.

Table 3. Means and standard errors of haematological variables for fragment and pseudofragment (comparative control) populations of *Antechinus agilis* differentiated by study year and sex. Abbreviations for blood variables given in Materials and methods. For N:L, means were calculated from the means of ratios rather than ratios of neutrophil and lymphocyte means.

	Fragment	Pseudofragment
	Mean ± s.e	Mean ± s.e.
WBC ($x10^{11}$ cells●L^{-1}):		
Females 2007	2.82 ± 0.22	3.71 ± 0.21
Females 2008	3.05 ± 0.22	3.11 ± 0.16
Males 2007	3.25 ± 0.18	4.64 ± 0.20
Males 2008	3.27 ± 0.21	3.54 ± 0.18
N:L:		
Females 2007	0.71 ± 0.08	1.10 ± 0.22
Females 2008	1.3 ± 0.14	0.94 ± 0.13
Males 2007	0.92 ± 0.10	0.67 ± 0.05
Males 2008	1.20 ± 0.11	0.93 ± 0.10
Neutrophils ($x10^{11}$ cells●L^{-1}):		
Females 2007	1.05 ± 0.12	1.56 ± 0.16
Females 2008	1.51 ± 0.14	1.20 ± 0.13
Males 2007	1.31 ± 0.11	1.66 ± 0.12
Males 2008	1.52 ± 0.14	1.44 ± 0.12
Lymphocytes ($x10^{11}$ cells●L^{-1}):		
Females 2007	1.65 ± 0.12	1.95 ± 0.13
Females 2008	1.42 ± 0.10	1.72 ± 0.12
Males 2007	1.73 ± 0.11	2.72 ± 0.13
Males 2008	1.48 ± 0.11	1.83 ± 0.96
% E:		
Females 2007	0.019 ± 0.002	0.013 ± 0.001
Females 2008	0.020 ± 0.002	0.018 ± 0.002
Males 2007	0.023 ± 0.002	0.018 ± 0.001
Males 2008	0.027 ± 0.002	0.023 ± 0.002

Continued on next page

Table 3 continued.

	Fragment	Pseudofragment
	Mean ± s.e	Mean ± s.e.
RBC (x10^{12} cells●L^{-1}):		
Females 2007	10.90 ± 0.31	9.92 ± 0.31
Females 2008	9.04 ± 0.22	8.71 ± 0.21
Males 2007	10.29 ± 0.29	9.16 ± 0.21
Males 2008	9.61 ± 0.21	8.77 ± 0.22
Hb (g●L^{-1}):		
Females 2007	150.9 ± 1.6	150.6 ± 2.4
Females 2008	152.1 ± 2.5	153.1 ± 2.5
Males 2007	153.2 ± 1.8	150.0 ± 2.0
Males 2008	158.5 ± 2.2	152.8 ± 1.6
Hct (%):		
Females 2007	0.48 ± 0.01	0.47 ± 0.01
Females 2008	0.49 ± 0.01	0.48 ± 0.01
Males 2007	0.49 ± 0.01	0.47 ± 0.01
Males 2008	0.50 ± 0.01	0.48 ± 0.01
MCH (pg●$cell^{-1}$):		
Females 2007	46.1 ± 1.6	49.8 ± 1.7
Females 2008	55.7 ± 1.6	56.6 ± 1.5
Males 2007	50.9 ± 2.1	54.0 ± 1.5
Males 2008	54.1 ± 2.0	56.7 ± 1.4
MCV (fL):		
Females 2007	14.6 ± 0.5	16.0 ± 0.6
Females 2008	17.1 ± 0.4	18 ± 0.5
Males 2007	16.0 ± 0.6	17.0 ± 0.4
Males 2008	16.6 ± 0.3	18.4 ± 0.4
MCHC (g●dL^{-1}):		
Females 2007	31.9 ± 0.4	32.2 ± 0.4
Females 2008	30.9 ± 0.5	32.2 ± 0.5
Males 2007	31.8 ± 0.4	31.9 ± 0.3
Males 2008	31.8 ± 0.4	32.4 ± 0.3

Table 4. Results of linear mixed effects models applied to blood variables. Sampling year, month, forest block, site (fragment or pseudofragment) and trapping grid were included as random factors. The effect sizes are for males (versus females) and fragments (versus pseudofragments) (e.g. males had higher N:L than females and fragment populations had higher N:L than pseudofragment populations). *t* tests compared males and females (379 degrees of freedom) and fragment and pseudofragment populations (31 degrees of freedom). Interaction terms of $p < 0.05$ were deemed significant. For all non-significant interaction terms, $p > 0.129$; these interactions were removed from the analyses and not included in this summary of results. * $P < 0.05$. Abbreviations for blood variables are in Methods and materials.

	Effect size	SE	*t*-value	*P*-value
N:L				
Sex (M)	0.006	0.026	0.226	0.822
Fragmentation (F)	0.078	0.038	2.045	0.049*
WBC				
Sex (M)	5.0×10^{10}	1.2×10^{10}	4.239	< 0.001*
Fragmentation (F)	-6.6×10^{10}	2.4×10^{10}	-2.719	0.011*
% E				
Sex (M)	0.005	0.001	3.457	0.001*
Fragmentation (F)	0.005	0.002	2.305	0.028*

Continued on next page

Table 4 continued.

	Effect size	SE	*t*-value	*P*-value
RBC				
Sex (M)	-1.7×10^{11}	1.8×10^{11}	-0.927	0.355
Fragmentation (F)	11.4×10^{11}	3.2×10^{11}	3.538	0.001*
Hb				
Sex (M)	1.286	1.417	0.908	0.365
Fragmentation (F)	2.603	2.149	1.211	0.235
Hct				
Sex (M)	0.004	0.005	0.897	0.371
Fragmentation (F)	0.014	0.006	2.242	0.032*
MCV				
Sex (M)	1.529	1.133	1.349	0.178
Fragmentation (F)	-4.504	1.820	-2.475	0.019*
MCH				
Sex (M)	0.469	0.345	1.361	0.174
Fragmentation (F)	-1.692	0.497	-3.401	0.002*
MCHC				
Sex (M)	0.081	0.287	0.284	0.777
Fragmentation (F)	-0.466	0.411	-1.135	0.265

Discussion

Repeatability of haematological values

In ecological studies, *R* for condition indicators seldom exceeds 0.8, even when the sampling unit is the individual (Lessells & Boag 1987) or where variation is examined among siblings in a brood (e.g. Nadolski et al. 2006). Here we used individuals as repeated samples in a site and so we expected R values to be relatively low. The R value > 0.4 for N:L and WBC in *A. agilis* thus suggests that these variables responded in a repeatable way to local environmental conditions and therefore had potential as condition indicators. Variables with R between 0.2 and 0.39 (% E, RBC and MCV) were probably also responding in a repeatable way to local conditions, but should be viewed more circumspectly. However, there was relatively weaker support for repeatability of response to local conditions for the remaining variables (R < 0.2; MCH, Hb, Hct and particularly MCHC).

Handling and trapping stress

There were no significant differences in WBC and N:L among the post-handling treatments in the trafficking test and only one (0-20 min) for % E. Eosinophil concentrations decrease under acute stress in most vertebrates (Davis et al. 2008), but have rarely been investigated in marsupials. This variable decreased in the trafficking test, but the decline occurred > 15 min after removal of animals from the trap when all blood-sampling had finished. It seems unlikely that handling stress affected our results for this or any leukocyte variable.

The best available evidence is that (unlike the situation in eutherians) in marsupials WBC *increases* after exposure to a stressor (Baker et al. 1998; Baker & Gemmell 1999).

Our validation study WBC trended linearly, but non-significantly, higher at 10, 20 and 30 min post-removal from traps. This has interesting implications for interpreting WBC in marsupials; the argument that WBC is unreliable because a high value would represent lower HPA-axis stress *or* a response to infection is potentially invalid in this taxon. Nonetheless, it is unknown in the study taxa whether WBC increases due to *both* acute and chronic stress, or whether WBC 'baseline' levels are higher or lower in more stress individuals; for these reasons we refrain from any WBC interpretation but use WBC and differential immune cell counts to examine total lymphocyte and neutrophil concentrations instead. However, repeating the test with larger sample sizes or the inclusion of samples at 40 or 60 min could be warranted.

The key problem underlying trapping stress relates to the interplay between acute stressors and chronic stress in vertebrates. In a sense, it is not that acute stressors interfere with reading baseline levels (stress is dynamic and the concept of 'baseline' stress is probably erroneous anyway; McEwen & Wingfield 2003), but rather that there is experimental evidence that vertebrates can reach levels of chronic physiological stress where their normal acute stress response is attenuated (Dhabhar & McEwen 1997; Dhabhar et al. 1995). That is, physiological exhaustion due to chronic stress may confound responses to trapping and handling which are otherwise expected to be reasonably similar among individuals. Severely chronically-stressed individuals may have a lower mean N:L, for example, than healthy individuals if an acute stress response has been experienced by both groups < 8 hrs before blood sampling (e.g. an encounter with a predator, psychosocial stress and, pertinently, trapping and/or handling; Dhabhar & McEwen 1997; Dhabhar et al. 1995).

However, data supporting the notion that acute stress responses are diminished by chronic stress are from laboratory studies in which domestic animals (usually *Rattus rattus*) were subjected to stress regimes (usually restraint, repeated daily over several

weeks; Dhabhar & McEwen 1997; Dhabhar et al. 1995) that are unlikely to be suitable representations of stressors encountered by free-living vertebrates. It is unclear whether generalizing these results is valid. To our knowledge, this level of stress-induced physiological exhaustion has not been reported in a free-living vertebrate, and it remains unclear whether free-living vertebrates frequently (or ever) experience extreme physiological exhaustion without dying (e.g. through predation).

The evidence from free-living vertebrates is that HPA-axis responses to stressors are more typically multiplicative than diminishing i.e. stress associated with predation activity and food scarcity is greater than would be expected from either stressor alone (Boonstra et al. 1998; Chapman et al. 2006; Clinchy et al. 2004; Krebs et al. 1995; Zanette et al. 2003). If this is the case in our study species, the interpretation would be that a higher N:L is associated with higher levels of environmental stressors and/or longer times in a trap. The only reason to suppose that mean times in a trap differed among sites is that trap saturation occurred in some pseudofragments i.e. on average, the pseudofragment animals were probably in traps longer than those in fragments. As our hypothesis was that fragments were the more stressful of the two habitat types, trap saturation in pseudofragments should have increased Type II error, which is not ideal but which we consider acceptable (i.e. N:L in control sites was potentially overestimated).

Finally, we found that the erythrocyte and N:L results were only in sensible agreement if N:L was interpreted as a positive index of stress; in vertebrates, chronic injection with stress hormones or exposure to stressors triggers erythropoiesis and release of immature erythrocytes from bone marrow and consequently an elevated RBC (Fisher & Crook 1962; Teague et al. 2007). However, this erythropoietic response appears to be too slow to be confounded by trapping stress (Teague et al. 2007).

Some erythrocyte variables (Hct, Hb, RBC) can be altered in < 8 hrs after acute stress and/or injection with stress hormones (Fisher & Crook 1962; Fletcher & Boonstra

2006; Oishi et al. 1999), but this is thought to be due to changes in fluids (probably plasma and/or water release into blood from other compartments), not erythrocytes (Fisher & Crook 1962; Oishi et al. 1999), and there is no evidence in the literature of a diminishing effect of chronic stress on acute erythrocyte responses to stress (i.e. as far as is known, chronically stressed and healthy individuals have similar fluid responses to acute stress). Thus the interpretation of erythrocyte values is probably not confounded by trapping stress, and MCH, MCV and MCHC are certainly not affected. Erythropoiesis due to repeated blood loss, heavy parasite load, exposure to stressors or stress hormone injection causes a blood profile termed 'regenerative anaemia' (Colombelli-Négrel & Kleindorfer 2008; Tyler & Cowell 1996). Immature erythrocytes are less capable than mature cells of producing haemoglobin (Lewis et al. 2006) and thus chronically-stressed vertebrates often show blood profiles with a lower than average MCH and an elevated RBC. In our study, N:L was higher, MCH was lower, and RBC higher in fragment than pseudofragment populations. The implication is therefore that N:L was acting as a positive index of stress. Assessing a range of haematological indicators rather than one or two allows a more informative interpretation of the data.

Although N:L ratio is clearly not a 'baseline' measure in this study, after considering the evidence we think that interpreting it as a positive index of stress is realistic. However, we acknowledge that this is one of two possible interpretations, the other being that populations in undisturbed, continuous forest sites were physiologically exhausted by chronic stress, such that their N:L response to trapping was attenuated (i.e. they were incapable of mounting effective stress responses to trapping).

Leukocyte counts and habitat fragmentation

Fragment populations had a significantly lower WBC, but a higher % E and N:L (in males), than pseudofragment populations. Strong eosinophilia is associated with

metazoan ecto- and endoparasite (Rothenberg 1998; Wikel 1996) infection in vertebrates. Higher % E in fragment populations could have potentially been a response to higher parasite infection levels in fragmented forest. Higher parasite burdens in fragment populations would not be surprising if the animals were generally in poorer condition (Beldomenico et al. 2008b). The relationship between habitat fragmentation and parasite load has been documented in some other mammals (e.g. Chapman et al. 2006; Püttker et al. 2008), but no overall trend has emerged. The relationship may be taxon-specific or susceptible to the influence of parasite release processes (Kruess & Tscharntke 1994).

The N:L ratio is considered a reliable indicator of chronic physiological stress in vertebrates (Davis et al. 2008; Salvante 2006) and has been used to measure stress in some other marsupials (Baker et al. 1998; Baker & Gemmell 1999). Regardless of any trapping stress effects, the difference in N:L between the two environments clearly showed that environmental differences were associated with differences in stress. Two interpretations of this trend are possible: **(1)** the higher N:L recorded in fragments suggests that residents were more chronically stressed than conspecifics in pseudofragments. However, on partitioning the sexes, we found that the association between fragmentation and higher N:L was only present in males. Given the different life histories of the *Antechinus* sexes, it is possible that certain environmental stressors disproportionately affected one of the sexes. Male *A. agilis* have a pre-breeding requirement to accumulate substantial fat stores (Kraaijeveld-Smit et al. 2003) and fragmentation effects on microhabitat complexity, foraging opportunities and(or) food abundance that affect the sexes differently could be profitable avenues of research in this species. Dispersal is male-biased in agile antechinus (Kraaijeveld-Smit et al. 2002a), and this also could be a factor contributing to stress in males. If dispersing males cross novel ecotones (forest-field boundaries) or enter the surrounding grazed matrix where predation risk is high, chronic stress could result. There is also evidence that females may

occupy better habitat than males (Holland & Bennett 2009), and if so, females may be avoiding the more stressful environments in fragmented landscapes (e.g. females may be avoided edge habitat). **(2)** Possibly the lower N:L in pseudofragment populations indicated chronic physiological exhaustion and an attenuated peak N:L response to trapping.

However, WBC was two orders of magnitude above baseline reference values for this species (Clark & Adlard 2004) and was *greater* in pseudofragment populations (marsupials probably have an *elevated* WBC after acute stress; Baker & Gemmell 1999). The implication is that pseudofragment populations showed a *more* pronounced WBC response to trapping than did *A. agilis* in fragments, making the 'physiological exhaustion' interpretation quite unlikely. However, if one does accept 'physiological exhaustion' in undisturbed forest as plausible, then two possible underlying causes could be crowding and competition for food (Johnstone et al. 2010). Differential leukocyte concentrations suggested that males had higher innate immunity than females (higher neutrophil numbers), but that neutrophil numbers were not different between study environments. Male and female lymphocyte concentrations responded differently to fragmentation. Whilst females showed no lymphocyte response to fragmentation, male lymphocyte concentrations were lower in fragments. Trafficking of lymphocytes away from blood to compartments such as the skin and lymph nodes is the mechanism usually evoked to explain N:L responses to stress (Davis et al. 2008; Dhabhar et al. 1995).

In our study area, reduced structural complexity (Fischer & Lindenmayer 2007), livestock grazing (Knight & Fox 2000), loss of trees with suitable nesting-hollows (Cockburn & Lazenby-Cohen 1992), reduced woody debris density (Mac Nally & Horrocks 2002), increases in interspecific competition levels (Banks & Dickman 2000) or invasive predator activity (particularly of European red foxes (*Vulpes vulpes*) and feral cats (*Felis catus*) (Stokes et al. 2004) could all have been contributing to the 'stressfulness' of

forest fragments. Over-activation of the acute stress response by such an array of stressors has the potential to generate chronic stress and greater susceptibility to disease and reduce reproductive output and lower survivorship (Sapolsky et al. 2000).

Erythrocyte variables and habitat fragmentation

The erythrocyte variables examined provide information on condition other than immunocompetence and should be largely unaffected by trapping and handling stress. The interpretation of the trends recorded for these variables is necessarily less straightforward than that for differential leukocyte counts because low and high values may both indicate the presence of various pathologies (Lewis et al. 2006) and wider study and verification of these variables as condition indicators is needed. Nonetheless, some inferences about condition can be drawn from the observed trends on the basis of what is known broadly about vertebrate haematology.

RBC and Hct were higher and MCH and MCV lower in fragment than in pseudofragment populations. Elevated RBC has been linked to poor body condition indices in marsupials, especially in those living in poorer quality habitats (Barnett et al. 1979b). Increased erythrocytes per unit volume of whole blood can occur because of (a) a reduction in plasma volume (e.g. through dehydration) (Hainsworth et al. 1968), (b) enhanced erythropoiesis (Dorshkind 1990) or (c) the release of immature erythrocytes (reticulocytes) from bone marrow (Colombelli-Négrel & Kleindorfer 2008). Dehydration typically results in a higher Hct, which was observed in fragment populations. Chronic injection with stress hormones, exposure to stressors or blood loss can trigger enhanced erythropoiesis and release of reticulocytes (Teague et al. 2007) in vertebrates, which can alter both RBC and erythrocyte morphometrics. This results in a blood profile termed 'regenerative anaemia' (Tyler & Cowell 1996). Blood parasite infection level can have a similar effect because haematozoa lyse erythrocytes (O'Brien et al. 2001). Many

vertebrates respond to stress, injury or blood loss with increased erythrpoiesis and a compensatory release of immature erythrocytes (reticulocytes) into the peripheral blood circulation (Colombelli-Négrel & Kleindorfer 2008; Teague et al. 2007), where their presence can increase RBC and sometimes Hct. Reticulocytes are less capable than mature erythrocytes of producing haemoglobin and so tend to have a lower MCH (and sometimes MCHC) (Colombelli-Négrel & Kleindorfer 2008). Populations in forest fragments therefore exhibited a RBC, Hct and MCH profile that could reflect a high level of regenerative anaemia. Blood parasites (particularly *Babesia* spp.) can reach very high concentrations in *A. agilis* (Barker et al. 1978) (we did not record *Babesia* occurences on blood smears, but the slides have been retained for possible re-examination to this end). That either dehydration, environmental stress, injury or haematozoan infection level (or all) might explain the higher RBC in fragment populations requires empirical verification, but the higher RBC and Hct and lower MCH do suggest that fragment populations were in poorer condition overall than those in pseudofragments.

The lower MCV in fragment populations of antechinus could not have been caused by the possible presence of large numbers of reticulocytes, as they are the same size as, or slightly larger than, mature erythrocytes (Lewis et al. 2006). We have no good explanation for the observation, though interestingly, at least one study on the effects of frequent and prolonged stress (restraint) on a mammal (*Rattus rattus*) reported a similar pattern to what we observed, elevated RBC (after 9 days of stress treatment) but reduced MCV (after 21 days of stress treatment) (Teague et al. 2007).

Some studies of marsupials have indicated that Hb can be a useful measure of body condition (Barnett et al. 1979b), but others showed no significant relationship between the two variables (Stirrat 2003). Despite the differences in RBC and Hct, we found no significant disparity in Hb between fragment and pseudofragment populations.

Presumably, Hb influence on blood oxygen carrying capacity was similar in both types of site.

Conclusion

The most plausible interpretation of our data is that they showed that *A. agilis* populations living in fragments exhibited signs of greater environmental stress (higher male N:L and possibly an attenuated WBC peak response in both sexes), stress caused by metazoan parasite infection (higher % E) and poorer condition (higher RBC and Hct, lower MCV and MCH) than comparative pseudofragment populations. This suggests that there was a positive association between habitat fragmentation and the intensity of environmental stress in *A. agilis*, possibly operating indirectly through the associated level of habitat degradation, invasive predators etc. Food supplementation, predator exclusion, parasite infection experiments and habitat manipulation could help to clarify this causal nexus.

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Monash University

Declaration for Thesis Chapter Four

Declaration by candidate

In the case of Chapter Four, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Conception, collection of field data and samples, laboratory work, data analysis, major writing	90

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Alan Lill	Writing and editing	5
Richard Reina	Writing and editing	5

Candidate's Signature		**Date** 21/09/2010

Declaration by co-authors

The undersigned hereby certify that:

1. the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
2. they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
3. they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
4. there are no other authors of the publication according to these criteria;
5. potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
6. the original data are stored at the Department of Biological Sciences, Monash University, Clayton and will be held for at least five years from the date indicated below:

		Date
Signature 1		21/09/2010
Signature 2	[redacted]	21/09/2010

Impact of anthropogenic habitat fragmentation on population health in a small, carnivorous marsupial

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Abstract

Habitat fragmentation is a major cause of population reduction and loss, and increasing evidence suggests that effects of fragmentation on populations vary as a function of the life history and autecology of species. We investigated the effect of anthropogenic habitat fragmentation on several indicators of population health in the agile antechinus (*Antechinus agilis*), a small Australian marsupial with an unusual life history. We examined relative abundance, body condition (mass size residuals, MSR), and ectoparasite load. Abundance was 2.3-fold higher in continuous than in fragmented *Eucalyptus* forest, and ectoparasite loads were higher in fragmented than in continuous forest in March, April, May and July, but not in June or August. Unexpectedly, MSR was also higher in what would usually be considered the less favorable fragmented habitat than in continuous forest (means: fragmented + 0.6 g, continuous -0.9 g). Other body condition indicators did not differ consistently between fragmented and continuous forest populations. Results suggest that an apparently common and secure small mammal species could be declining in anthropogenically fragmented and degraded habitat. Although habitat fragmentation has been associated with nutritional stress in vertebrates, food availability probably was not contributing to the lower abundance of agile antechinus in habitat fragments. The findings indicate that care is needed when generalized expectations about the response of a species to anthropogenic habitat fragmentation are used to inform conservation management.

Keywords.--abundance, *Antechinus*, anthropogenic, body condition, degradation, ectoparasite, fragmentation

Introduction

Ecological and conservation biology studies on small mammals have used various measures of population well-being (health), including population density (Johnson 2007), individual growth rates (Karels et al. 2000), body condition indices (BCI--Schulte-Hostedde et al. 2005), parasite loads (Barnard et al. 2003), wound or injury occurrence (Dunford 1977; Singleton 1989) and percentage of females lactating or successfully weaning young (Karels et al. 2000). These indicators are typically assumed to be correlated with physical environmental factors, most commonly habitat quality (Johnson 2007). When examining vertebrate populations in multiple sites with differing levels of disturbance, fragmentation, or degradation (Chapman et al. 2006; Keesing 1998; Lada et al. 2007; Nupp & Swihart 1996; Suorsa et al. 2004), measuring several independent rather than one indicator of population health can provide a more comprehensive and informative description of the response a species has to disturbance.

Multiple factors can contribute to population decline in fragmented habitat, including isolation, habitat degradation, the creation of novel ecotones, an increase in anthropogenic disturbance (e.g., grazing by livestock and hunting), invasion by generalist species, stochastic threats, and Allee effects (i.e., where an inherent positive relationship exists between population density and per capita growth rate--Ewers & Didham 2005; Fischer & Lindenmayer 2007; Hobbs 2001; Saunders et al. 1999). Increasing evidence suggests that effects of fragmentation on population health vary as a function of life history, autecology, diet (specialists are more severely affected than generalists), and body size (larger taxa are more severely affected than smaller taxa--Debinski & Holt 2000; Ewers & Didham 2005; Turner 1996), and therefore the use of single indicator or model species to gauge community well-being in complex natural ecosystems is contentious (Carignan & Villard 2002; Lindenmayer 1999).

Life history in the genus *Antechinus* is very unusual, involving a rare case of mammalian semelparity (Braithwaite & Lee 1979). An annual synchronized female estrus is followed by stress-hormone mediated senescence and mortality of all males in late winter (Barnett 1973; Braithwaite & Lee 1979), leaving populations comprised solely of pregnant females. The breeding rut is competitive, with larger males experiencing better breeding success than smaller ones, and multiple paternity within litters is common (Kraaijeveld-Smit et al. 2002b; Kraaijeveld-Smit et al. 2003). Only a small proportion of females live to breed in a second year (Wood 1970).

The nocturnal agile antechinus (*Antechinus agilis*) is native to southeastern Australia (Dickman et al. 1998; Sumner & Dickman 1998) and restricted to native *Eucalyptus* forest for its foraging, nesting, and breeding (Banks et al. 2005c; Shimmin et al. 2002; Sumner & Dickman 1998). The species was considered to be part of the brown antechinus (*A. stuartii*) species complex until 1998 (Dickman et al. 1998), and some earlier studies of *A. stuartii* apparently were conducted on *A. agilis*. Agile and brown antechinus are unusual in that these species appear to use two distinct ranges, a foraging range (< 3 ha) and a social range (< 5 ha--Cockburn & Lazenby-Cohen 1992; Lazenby-Cohen & Cockburn 1991). Individuals will move over 500 m outside their foraging range to visit communal nests, and the smaller foraging and larger social ranges do not always overlap (Lazenby-Cohen & Cockburn 1991). This has led to the suggestion that the agile and closely related brown antechinus are unique among mammals in that individuals use a larger habitat area for social interaction than foraging (Lazenby-Cohen & Cockburn 1991).

The presence of agile antechinus in Southeast Australia presents an opportunity to study a species, with a well-documented autecology, that possesses many traits (e.g. restriction to forest, reluctance to cross the surrounding matrix, and a specialized life-history) usually considered to reduce fitness in circumstances of anthropogenic habitat fragmentation (Turner 1996). Although populations of *A. agilis* do persist in some

anthropogenically fragmented forests (Bennett 1990a), the species is generally thought to be negatively affected by habitat fragmentation (Banks et al. 2005a; Bennett 1990a). We compared measures of population density (relative abundance), lipid reserves (body condition indices), and ectoparasite load (simplified parasite counts) in populations living in anthropogenically fragmented and relatively undisturbed, continuous *Eucalyptus* forest (fragments and continuous forest always were separated by > 2 km of agricultural fields). We predicted that where native tree cover was fragmented anthropogenically rather than continuous, agile antechinus would have a lower relative abundance, poorer body condition, and higher ectoparasite load.

Materials and methods

Study area, sites, and population.--The study was conducted in 2007 and 2008 in South Gippsland, Victoria, Australia (Fig. 1). The region has one of the longest histories of European settlement and agriculture in mainland Australia. Forest clearing for agriculture, gold mining, and timber production was triggered by an 1869 Lands Act allowing settlement, and all the fragments in our study have been isolated by anthropogenic land clearing for > 50 years. The matrix surrounding all *Eucalyptus* fragments in this study was pasture, primarily grazed by dairy and beef cattle, but with some sheep farming. Species relaxation, the gradual loss of species after fragmentation of habitat, still could be occurring (Saunders et al. 1999), but we have no good evidence for or against this assumption. Fire is an important part of the ecology of the study area. We used available fire history information and local research to choose study sites where there had been no fires for > 30 years, as recent fires could negatively affect abundance in the study species (Catling et al. 1998). However, evidence suggests that abundances of agile antechinus are unaffected by fire history where time since fire is > 20 years (Catling

et al. 1998; Claridge et al. 2008), so we consider the > 30 year criterion to be sufficient to avoid any confounding effects.

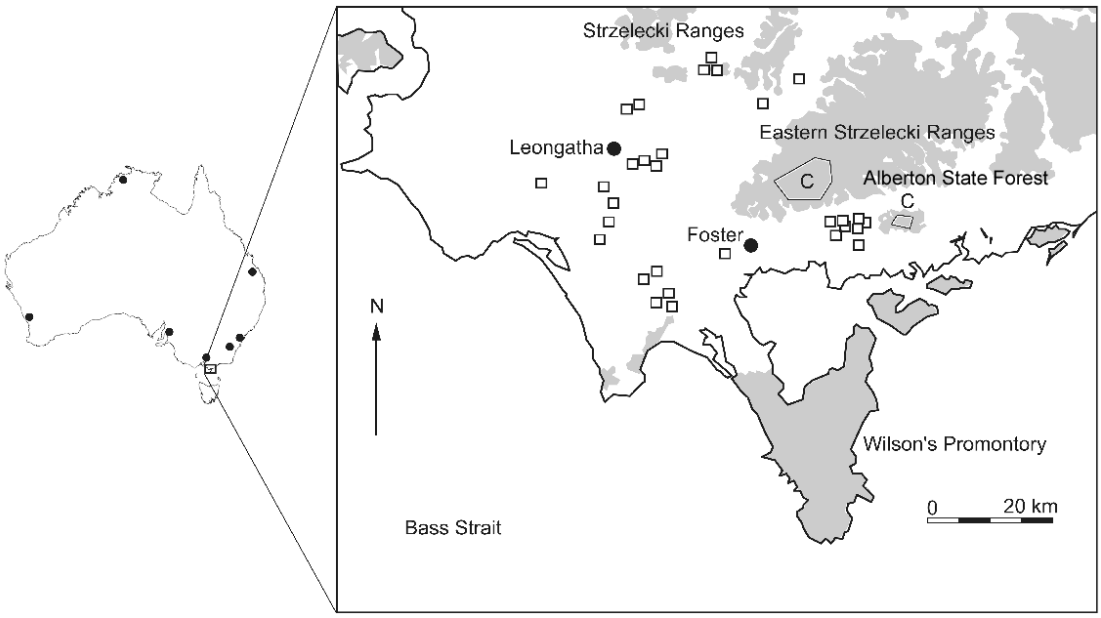

Fig. 1.--Study region in South Gippsland, Southeast Australia. White areas represent cleared agricultural land, and gray areas represent native tree cover. Approximate locations of fragment study sites are indicated by white boxes (□). Comparison pseudofragment sites were situated within the 2 areas delineated by a heavy line and labelled C. Map is based on the Department of Sustainability and Environment interactive forest-explorer online maps (Forest-Explorer Online maps, http://www.dse.vic.gov.au).

Live trapping was carried out from April-August 2007 and March-August 2008. Teat number varies among female agile antechinus and is known to be genetically regulated and associated with local environmental conditions (Beckman et al. 2007; Cockburn et al. 1983). However, homogeneity of teat number in our study area (all 8 teats--C. P. Johnstone, pers. obs.) strongly suggested that all sites were environmentally similar prior to fragmentation of the native vegetation by land clearing for agriculture.

A control pseudofragment in an area of relatively similar, undisturbed, continuous forest was matched for size and shape to each fragment (Fig. 2) (Mac Nally & Bennett 1997). Pseudofragments were nonoverlapping but were otherwise randomly distributed in suitable continuous forest. Trapping in a fragment was immediately followed by trapping in the paired pseudofragment. All forest fragments and pseudofragments studied were occupied by agile antechinus, ensuring that estimates of relative abundance were not confounded by including unoccupied sites. The 30 fragments were 4.8 to 293.6 ha in area and were dispersed in an anthropogenically disturbed agricultural landscape 2.1 to 38.6 km from any area of continuous forest (defined as > 1,000 ha of continuous native tree cover; Fig. 1). Fragments were within an area bounded by the coordinates 38°35'26"S 145°41'41"E, 38°21'55"S 146°06'10"E, 38°37'19"S 146°28'20"E, and 38°45'12"S 146°01'33"E. All pseudofragments were situated in an area bounded by the coordinates 38°28'03"S 146°18'45"E, 38°36'50"S 146°32'40"E, 38°34'35"S 146°19'12"E, and 38°32'41"S 146°16'26"E.

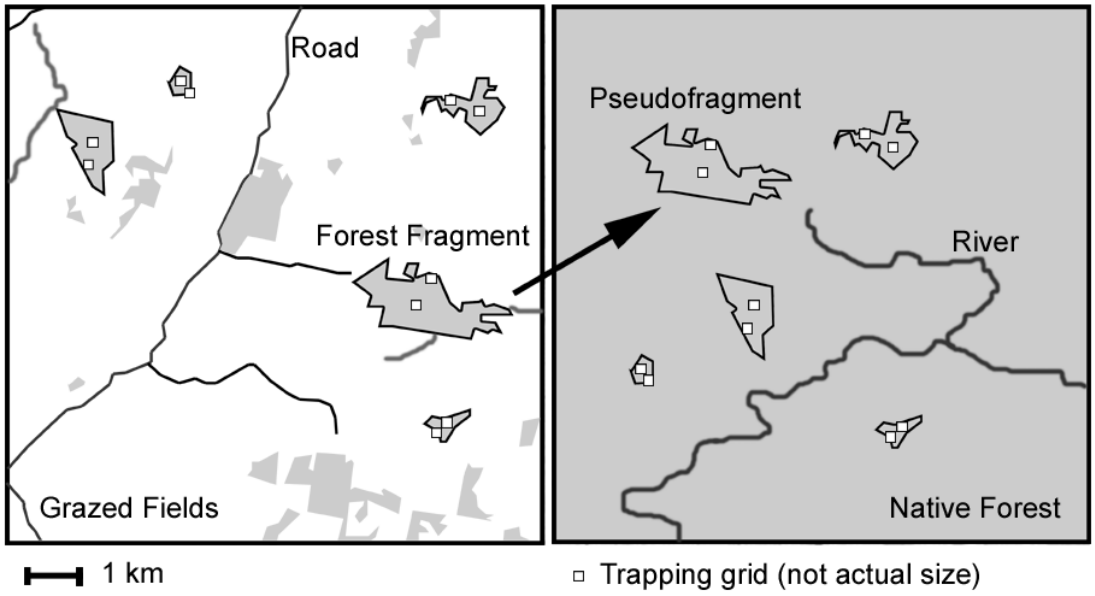

Fig. 2.--Hypothetical example of a fragment-pseudofragment design. Trapping in a fragment was followed immediately by trapping in the paired pseudofragment. In landscape ecology studies this design (or similar) is needed to separate the effects of habitat fragmentation from habitat loss (Mac Nally & Bennett 1997).

Possible spatial autocorrelation of study sites is a concern in research on the effects of fragmentation. The underlying problem is that where landscapes have a level of anthropogenic fragmentation that is suitable for study, typically only a few areas of continuous habitat suitable for control sites remain. Consequently, comparative control sites often must be restricted to a few large blocks of habitat (usually reserves). In the present investigation fragments were always > 500 m from another study fragment, and pseudofragments always had > 1 km of native forest buffer between them. Although we cannot completely discount the possibility that spatial autocorrelation confounded some aspects of our results, an examination of site longitudes and latitudes suggested that fragments and pseudofragments probably were not substantially different in their spatial distribution (General Linear Model: Latitude $F_{1,58} = 1.58$, $P = 0.217$; Longitude $F_{1,58} = 0.56$, $P = 0.459$). Some agile antechinus could have dispersed from other neighboring

fragments or continuous forest to the study sites prior to sampling, but sampling commenced approximately 2 months after the annual male-biased dispersal (Cockburn et al. 1985b) in January-February, which should have allowed time for immigrants to acclimate to local conditions.

Native tree cover was identified using online native vegetation cover maps (1: 75,000) available from the Victorian Department of Sustainability and Environment (DSE; Forest-Explorer Online maps, http://www.dse.vic.gov.au/). Habitat similarity among study sites was achieved by restricting sites to stands of forest composed of the 3 Ecological Vegetation Classes (EVC--Davies et al. 2002), Lowland Forest, Wet or Damp Forests (Wet), and Wet or Damp Forests (Damp). Most sites contained a mixture of the first two EVCs, but some also contained small amounts of Riparian Forests or Woodlands or Rainforests.

Trapping protocols.--Trapping and data collection were approved by Monash University Biological Sciences Animal Ethics Committee (permits BSCI/2008/03 and BSCI/2006/05) and conducted under Department of Sustainability and Environment permit 10003798. All research involving live animals followed the guidelines approved by the American Society of Mammalogists (Gannon & Sikes 2007). Trapping was conducted with weather-proofed Elliott ™ live traps (Elliott Scientific, Upwey, Victoria) baited with a mixture of rolled oats, peanut butter, water, and artificial vanilla essence. A plastic tube wrapped in bedding provided an additional insulated refuge for trapped animals. Trapping was conducted overnight for 3 successive days at each fragment and pseudofragment. Trapping in a pseudofragment was started the day after trapping in its paired fragment to incorporate a temporal control in the study. All trapping in a fragment-pseudofragment pair was completed in 8 days (2× trap-setting days and 6× trap nights). In each fragment one trapping grid was placed < 60 m from the edge and a

second in the interior (> 80 m from the edge). The spatial positioning of trapping grids in a fragment was replicated as closely as possible in its paired pseudofragment.

Relative abundance.--Due to differences in morphology, physiology, and behavior (Barnett 1973; Cockburn & Lazenby-Cohen 1992; Marlow 1961), males and females were treated as separate populations for all measured variables. In pseudofragments trapping rates of agile antechinus and mammalian by-catch were much higher than expected (up to 100% of traps were occupied per night), and therefore trapping effort was scaled down to reduce trap mortality. In each fragment 2 trapping grids of 21 traps each were used, both being arranged in 3 lines of 7 (20 m × 20-m spacing). Pseudofragment grids had 9 traps in 3 lines of 3 (60 m × 20-m spacing). Thus pseudofragment grids covered the same area as fragment grids but had fewer traps. Varying trapping effort can confound comparative measures of abundance (Lada et al. 2007), but we used a binomial distribution of trapping rate (Lada et al. 2007) to allow for the discrepancy. A trapping success was defined as the capture of one agile antechinus in a trap at a site on any given night, but the binomial variable was based only on the first night of trapping at a site, with each trap set being defined as a trial (i.e., the number of trials was 42 for fragments and 18 for pseudofragments). By using data only from the first night, the possibility that learning could confound results was reduced. Binomial variables were calculated with the Excel ™ (Microsoft, Redmond, WA) function BINOMDIST and used a 0.5 probability of success. Cumulative distribution functions were used rather than probability mass functions. The use of a binomial variable gave more weight to sites that had a greater trapping effort (Lada et al. 2007); i.e., the fragments. Trapping rate (TR) is the binomial variable, and it is considered to be a measure of relative abundance (a surrogate for population density). We predicted that the relative abundance of agile antechinus would be greater in undisturbed pseudofragments than in more degraded fragments.

Body condition indices.--All captured individuals were sexed by visual inspection. Typically, 6 individuals were sampled for body mass, morphometrics, and ectoparasite variable(s) in each grid over the 3 trapping days, making a total of 12 individuals per study site. The measurements taken were: mass (g to the nearest 0.5 g) and nose-vent length (NV, in mm to the nearest 0.1 mm). All measurements were taken by the same researcher. Repeated measurements are important for reducing the incidence of Type II error when constructing small mammal body condition indices (Blackwell et al. 2006). However, when taking body measurements on individuals, we took blood samples for a related research project. As handling was already lengthy (up to 15 min) and potentially stressful, we decided that it was preferable to reduce handling time by taking single measurements. This meant that comparing BCI in the two habitat types entailed a greater risk of obtaining a false negative result. However, sample sizes of $\leq$12 individuals per site and the use of site means in the analyses should have helped to mitigate the error associated with using single measurements. BCI was calculated as the residuals of body mass as a function of NV (mass size residuals, MSR, in g--Schulte-Hostedde et al. 2005). Ordinary least-squares regressions were used to generate residuals for MSR (Schulte-Hostedde et al. 2005).

Ectoparasite load.--Permanently attached mites (order Acariformes) and ticks (order Parasitiformes) on both ears of each antechinus were counted, but we did not adjust for intensity of parasite infection (i.e., larger parasitiforms, such as *Ixodes* spp., were not weighted differently to smaller acariform species). Restricting the count to the ears reduced handling time and lessened the risk that parasites might be obscured by fur and overlooked.

Data analysis.--Data were analyzed using R 2.8.1 (R Development Core Team 2010). They were checked for normality and homoscedasticity. The binomial abundance variable, TR, was $\log_{10}$-transformed (logTR) to achieve normality, but no other transformations were needed. Analyses of variance (ANOVA) were applied to predictor (environmental) and response (abundance, BCI, and ectoparasite load) variables to identify significant relationships. ANOVA predictor factors were: sampling year (2007 and 2008), month (months were allowed to vary from calendar months by 1-3 days, so fragment/pseudofragment pairs were kept in the same month), sex (2 levels), and fragmentation (2 levels). Where the assumption of homogeneity of regression slope of linear models was violated, data were separated so that fragmentation, the factor of primary interest, could be examined (i.e. a *post hoc* simple main effects test; Engqvist 2005; Quinn & Keough 2002). This treatment of interaction terms in linear model analyses avoids using the more involved Wilcox modification of the Johnson-Neyman procedure and is preferable if data can be partitioned easily (Engqvist 2005). Simple main effect testing has the usual problems associated with multiple testing, but we used a Sequential Bonferroni adjustment of *P*-values to account for this where needed (Quinn & Keough 2002). We followed the recommendation of Quinn and Keough (2002) that simple main effects tests use the residual sum of squares and degrees of freedom from the full ANOVA. Pseudoreplication was avoided by using site means in analyses, unless otherwise stated. Values are shown as means and standard errors (*SE*). Where means for months are given, they are based on all sites sampled in the particular month. Simple main effects testing by month was necessary to examine possible seasonal differences in ectoparasite counts. However, only 2-4 fragments were sampled in a given month. Thus, to obtain a reasonable estimate as to whether biologically significant differences between fragment and continuous forest populations could have occurred in a given month, analyses of ectoparasite data at this level used individual animals as the sampling unit.

Results

In 2007 181 male and 196 female adult agile antechinus were captured in 15 forest fragments and 248 males and 146 females in 15 pseudofragments. The corresponding numbers for 2008 were 178 males and 183 females and 201 males and 93 females, respectively. In 2007 73 males and 60 females and 110 males and 52 females were sampled for morphometrics, body mass, and ectoparasites in fragments and pseudofragments, respectively. The corresponding numbers for 2008 were 70 males and 55 females and 81 males and 50 females, respectively (Table 1).

Relative abundance.--Relative abundance was significantly smaller in fragments (TR < 0.001 ± < 0.001) than in pseudofragments (TR = 0.077 ± 0.023) (Tables 1 and 2). We observed no difference in logTR between the sexes, but logTR was significantly greater in 2007 (TR = 0.058 ± 0.022) than 2008 (TR = 0.022 ± 0.011). The interaction term YEAR×MONTH was significant in this analysis, but when the data were partitioned by year, logTR was not affected by the month in which sampling occurred in either 2007 ($F_{1,108}$ = 2.70, P = 0.103) or 2008 ($F_{1,108}$ = 2.79, P = 0.097).

Body condition: mass size residuals.--Mean MSR was greater in fragments (0.62 ± 0.44 g) than in pseudofragments (-0.61 ± 0.29 g; Tables 1 and 2). It also was greater for males (0.87 ± 0.41 g) than females (-0.91 ± 0.31 g), but no significant differences were found between years. The interaction term YEAR×MONTH was significant in this analysis; when the years were analyzed separately, MSR increased in 2007 from -1.61 ± 0.59 g (April) to 3.16 ± 4.32 g (August; $F_{1,108}$ = 16.64, P = < 0.001) but did not vary during the sampling period in 2008 ($F_{1,108}$ = 0.81, P = 0.370). Although our results imply a negative relationship between relative abundance and body condition in free-living agile antechinus, this was significant only for males (females: n = 55, $F_{1,108}$ = 1.57, P = 0.214; males: n = 59, $F_{1,108}$ = 9.67, P = 0.004).

Ectoparasite load.--Although mean ectoparasite counts per individual were higher in fragments (4.60 ± 1.41), than in pseudofragments (1.68 ± 0.44) (Table 1), this finding was confounded by a significant FRAGMENTATION×MONTH interaction term (Table 2). When the 2 habitat types were analyzed separately, pseudofragment populations showed no seasonal variation in ectoparasite counts ($F_{1,108}$ = 0.22, P = 0.639) whereas ectoparasite counts in fragment populations decreased from March (13.2 ± 7.2) to August (1.7 ± 0.4; $F_{1,108}$ = 19.65, P = < 0.001). Partitioning data by month revealed a possible trend where fragment populations had higher ectoparasite infection counts than pseudofragments in March and May, but ectoparasite counts were the same or similar in both habitat types in April, June, July and August (March: $F_{1,22}$ = 11.13 P = 0.018; April: $F_{1,65}$ = 5.18 P = 0.104; May: $F_{1,146}$ = 7.90 P = 0.030; June: $F_{1,135}$ = 3.75 P = 0.110; July: $F_{1,126}$ = 4.52 P = 0.105; August: $F_{1,54}$ = 1.79 P = 0.186).

Table 1.--Means, *SE*s, and ranges for mass, morphometric, trapping rate, and ectoparasite variables for *Antechinus agilis* in fragmented and pseudofragmented forest. Means and *SE*s are shown for trapping rate (unmodified %TR) averaged over 3 trapping nights, binomial distribution of trapping rate (logTR binomial, first night only), mass (g), mass size residuals (MSR), and nose-to-vent length (NV). Ectoparasite values do not include one site pair, which was excluded from analyses due to ectoparasite count in the fragmented part of the pair being an outlier (an order of magnitude higher than the mean for all sites).

	FRAGMENT	PSEUDOFRAGMENT
	Mean ± *SE*	Mean ± *SE*
Unmodified %TR		
Females 2007	0.11 ± 0.03	0.22 ± 0.04
Females 2008	0.08 ± 0.02	0.11 ± 0.02
Males 2007	0.10 ± 0.02	0.26 ± 0.04
Males 2008	0.08 ± 0.01	0.21 ± 0.03
logTR binomial		
Females 2007	-7.36 ± 1.00	-2.21 ± 0.38
Females 2008	-8.86 ± 0.85	-3.46 ± 0.31
Males 2007	-6.79 ± 0.71	-1.78 ± 0.31
Males 2008	-8.25 ± 0.47	-2.17 ± 0.32

Continued on next page

Table 1 continued.

	FRAGMENT	PSEUDOFRAGMENT
	Mean ± *SE*	Mean ± *SE*
Mass (g)		
Females 2007	16.83 ± 0.58	16.88 ± 0.65
Females 2008	16.82 ± 0.55	16.71 ± 0.56
Males 2007	25.19 ± 1.65	20.88 ± 0.65
Males 2008	25.23 ± 1.09	24.15 ± 0.73
NV (mm)		
Females 2007	79.43 ± 1.48	80.80 ± 1.46
Females 2008	79.22 ± 0.89	79.50 ± 1.06
Males 2007	88.21 ± 1.70	85.05 ± 1.00
Males 2008	87.98 ± 0.96	88.99 ± 0.95
MSR (g)		
Females 2007	-0.65 ± 0.65	-1.53 ± 0.90
Females 2008	-0.52 ± 0.51	-0.82 ± 0.41
Males 2007	1.76 ± 1.33	-0.40 ± 0.56
Males 2008	1.96 ± 0.69	0.20 ± 0.39
Ectoparasite count		
Females 2007	2.70 ± 0.78	0.49 ± 0.20
Females 2008	6.24 ± 3.93	3.06 ± 1.42
Males 2007	2.83 ± 0.84	0.54 ± 0.10
Males 2008	6.37 ± 3.70	2.47 ± 0.80

Table 2.--ANOVA results for *Antechinus agilis* population well-being (health) indicators as a function of the predictor variables Fragmentation (fragment or pseudofragment), Sampling Year (2007 or 2008), Sex, and Month. Values have been averaged by study site and sex, and the sexes have been treated as separate populations. All nonsignificant interactions were removed from the final analyses. All *d.f.* are the same for all factors within a given *F*-test. * indicates $P < 0.05$.

logTR	$F_{1,108}$	P
FRAGMENTATION	188.49	< 0.001
YEAR	8.50	0.004*
SEX	3.46	0.066
MONTH	0.08	0.774
YEAR × MONTH	8.67	0.004*
MSR	$F_{1,108}$	P
FRAGMENTATION	7.44	0.007*
YEAR	0.68	0.411
SEX	13.59	< 0.001*
MONTH	10.86	0.001*
YEAR × MONTH	7.67	0.007*
Ectoparasite count	$F_{1,108}$	P
FRAGMENTATION	4.77	0.031*
YEAR	4.66	0.033*
SEX	0.00	0.959
MONTH	7.23	0.008*
FRAGMENTATION × MONTH	12.48	0.001*

Discussion

Consistent with our initial prediction, relative abundance of agile antechinus was significantly lower in anthropogenically fragmented *Eucalyptus* forest than in undisturbed, continuous forest. This difference was evident for both sexes, at all sampling times, and in both study years. The likelihood is that direct (e.g., barriers to dispersal) and/or indirect (e.g., habitat degradation) factors were causing lower recruitment and/or poorer survival in anthropogenically fragmented habitat (Fig. 3). We do not know whether the difference in abundance between the two habitat types was stable, or if a gradual, ongoing decline in abundance was occurring in fragments. As extinction of vertebrate populations after anthropogenic fragmentation of habitat can take years or decades (Diamond et al. 1987; Pimm et al. 1993), it can be particularly difficult to detect in an apparently common and secure species (i.e., one which has a broad distribution across multiple sites where it is often locally common) such as the agile antechinus (Menkhorst & Knight 2004). It is thus a serious and often overlooked conservation concern.

Two potentially confounding aspects relate to the finding that relative abundance of agile antechinus was lower in fragments than in pseudofragments. First, we had no reason to anticipate the trap saturation that occurred in the pseudofragments, which meant that population density was probably underestimated in those sites. This saturation could have led to either of two false conclusions: (1) that fragment and pseudofragment abundances were similar, when pseudofragment abundance was actually greater, or (2) that abundance was greater in fragments, when it was actually similar in both types of site. However, abundance of agile antechinus was greater in pseudofragments than in fragments as initially predicted, so the only possible effect of trap saturation in the pseudofragments was simply a reduction in the apparent magnitude of this detected disparity.

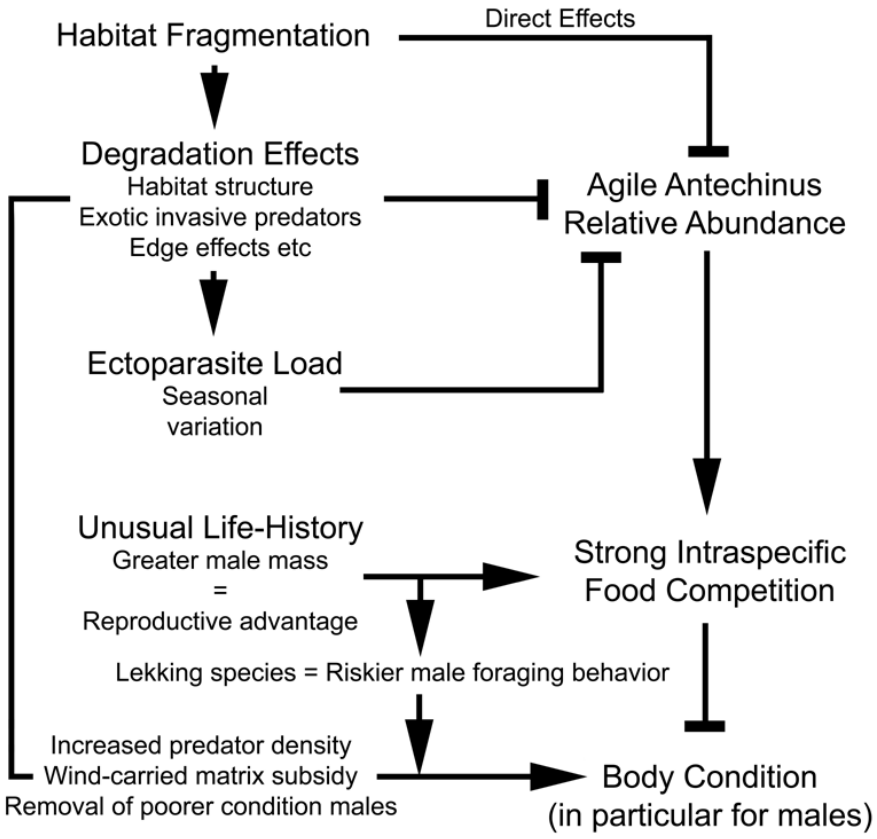

Fig. 3.--Conceptual flow diagram of possible relationships among environmental variables, life-history traits, and population well-being (health) indicators for *Antechinus agilis*. Positive relationships are indicated by an arrow. Negative relationships are indicated by a line. The line at the left margin connects two sets of degradation effects.

Second and more equivocal, where relative abundance is estimated from trapping rates, it is not always clear to what extent behavior is affecting trapping rate in the target species. Trapping rates of agile antechinus potentially could have been affected by differences in movement rates in the different types of site (Diffendorfer et al. 1995). Anthropogenic habitat fragmentation is often associated with habitat degradation (Knight & Fox 2000; Turner 1996), as was the case in our study area. In particular, fragments on average probably had a less complex habitat structure than

pseudofragments (C. P. Johnstone, pers. obs.). If this resulted in agile antechinus moving less frequently or for shorter distances in fragments than in pseudofragments because the shrub and understorey cover was sparser, it would have reduced their probability of encountering a trap. Differences in feeding motivation also could have affected trapping rates. Thus if food availability limited antechinus population density in pseudofragments, individuals would have been very likely to explore a baited trap. However, if other factors limited population density in fragments, as seems likely, individuals might have been less likely to explore the novel foraging resource provided by a baited trap. Clearly, further research is needed to determine whether such behavioral effects on the trapping rate reduce the resolution of this method of determining the relative abundances of agile antechinus populations in different habitats.

The most powerful measure of body condition, MSR (Schulte-Hostedde et al. 2005), also had a consistent relationship with fragmentation, but it was contrary to our initial prediction. Counterintuitively, agile antechinus had apparently greater lipid stores where habitat was fragmented anthropogenically and degraded. Thus nutritional stress probably was not contributing to the lower relative abundance of agile antechinus in forest fragments. This is not consistent with field studies of agile and other antechinus species that have reported consistent, positive relationships between population density and indicators of food resource abundance, particularly leaf-litter depth and woody debris density (Bennett 1993; Kelly & Bennett 2008; Knight & Fox 2000; Lada et al. 2007). Moreover, Mac Nally and Horrocks (2002) conducted a large-scale experimental manipulation of woody debris load in woodland sites and showed that the relationship between log density and the abundance of yellow-footed antechinus (*A. flavipes*) appeared to be causal.

Leaf litter and fallen timber also add to habitat structure (Garden et al. 2007; Mac Nally & Horrocks 2002), so conceivably their effects on relative abundance of antechinus

are related more to predator avoidance (logs--Stokes et al. 2004) and/or availability of nesting material (leaf litter) or nesting sites (logs--Cockburn & Lazenby-Cohen 1992) than to food resources. We recorded an index of leaf-litter depth and spatial extent (on a scale of 1-5) and the density of logs (per 400 m^2) in our study sites. The observed trend was towards a lower leaf litter index in fragments than in pseudofragments (mean leaf litter index: fragments = 2.7 ± 0.1, pseudofragments = 4.1 ± 0.1) but a higher log density in fragments (C. P. Johnstone, pers. obs.), perhaps due to edge effects and wind-fall (mean log density: fragments = 13.1 ± 1.7, pseudofragments = 9.4 ± 1.3). Thus the lower relative abundance of agile antechinus in fragments could have been related to less leaf litter than in pseudofragments, but if log density had any positive effect on abundance of agile antechinus, it presumably was less influential than other environmental variables. The higher estimated fat reserves of agile antechinus in fragments could have reflected the greater abundance of woody debris in that type of site; manipulation of debris loads might reveal whether a causal relationship is involved (Mac Nally & Horrocks 2002).

Ectoparasite counts for agile antechinus differed among months. In March and May the results agreed with our initial prediction that individuals in fragments would have higher ectoparasite loads than those in continuous forest. Although this would not be surprising if members of fragment populations were in poorer condition (Beldomenico et al. 2008b), they actually had a higher mean MSR than those in pseudofragments, casting some doubt on differences in body condition as an explanation for the disparity in parasite load. Environmental quality possibly was more impaired by habitat degradation or ecological changes in fragments than in pseudofragments (e.g., fewer tree hollows for nesting, poorer quality nesting material, and/or more invasive exotics forming larger parasite reservoirs), such that transmission of parasites was more likely. Regardless, the link between habitat fragmentation and ectoparasite load was not

consistent, because fragment and pseudofragment loads were the same in April, June, July and August. This was apparently due to loads in fragments (but not pseudofragments) decreasing during the trapping season in both years. Why this decrease occurred is unknown.

Negative population density × body condition (PD×BC) relationships have been observed in small mammals during population cycling in subarctic and semidesert landscapes (Korpimäki et al. 2004). However, agile antechinus has been studied extensively, and no suggestion of population cycling exists in the literature. In stable or temperate environments small mammal PD×BC relationships are typically positive or neutral (Keesing 1998; McGuire et al. 1993; Nupp & Swihart 1998; Sale & Arnould 2009; Sale et al. 2009; but see Lada et al. 2008b). Our study area conformed with this stable environment profile, as long-term mean annual rainfall was 800-1,200 mm and mean daily temperature ranged from a minimum of 9-12 to a maximum of just 18-21°C (30 year means--Australian Bureau of Meteorology 2009).

Experimental studies on small mammals using artificially high population densities usually have attributed negative PD×BC relationships to intraspecific competition for limiting resources needed for survival and maintenance (Ostfeld & Canham 1995; Warnock 1965). However, we observed very few wounds on trapped agile antechinus (C. P. Johnstone, pers. obs.) and have no evidence that strong intraspecific fighting affected body condition. Nonetheless, given what is known about semelparous breeding behavior of agile and brown antechinus and metabolic demands during male lek behavior (Lazenby-Cohen & Cockburn 1988; Woollard 1971), it is possible that intraspecific competition for food could have contributed to the negative PD×BC relationship among males. Agile antechinus adult mass can vary considerably within a population, particularly in males (Table 1). In our study, adult male mass varied from 13 g (10 June) to 41 g (26 July), a 3.2-fold difference, and adult female mass from 12 g (25 June) to 26.5 g (24 June),

a 2.2-fold difference (C. P. Johnstone, pers. obs.). Conceivably, individuals could attain quite different prebreeding weights, depending on food availability (Dickman 1989). Females have sperm-storage crypts (Shimmin et al. 1999), and mating order influences paternity success, with larger males and those that mate closer to ovulation siring more offspring (Kraaijeveld-Smit et al. 2002b; Kraaijeveld-Smit et al. 2003). A clear fitness advantage seems to exist for a male to be larger at the outset of the breeding season (due to both lipid and protein reserves). Sufficient metabolic fuel reserves could allow a male to persist with reproductive effort when smaller competitor males are in the early stages of the negative nitrogen balance, development of digestive tract lesion, and escalating parasite loads that eventually cause postbreeding male mortality (Barker et al. 1978; Beveridge & Barker 1976; Woollard 1971). Strong competition for food could result. Brown and agile antechinus occupy defined foraging ranges (Banks et al. 2005a; Lazenby-Cohen & Cockburn 1991), but the possibility that males actually defend foraging territories has not been suggested in the literature and warrants investigation.

Negative PD×BC relationship also could be explained by differences in physical environmental variables in fragmented and continuous forest. Agile antechinus are seldom or never caught in the cleared matrix surrounding occupied *Eucalyptus* forest fragments (Bennett 1990a), but matrix arthropods could have been wind-blown into fragments, providing a super-abundance of prey (spiders in hedgerows--Landis et al. 2000). That wind-carried nutrient, trace element, or subsidies of detritus from the surrounding matrix could affect native vegetation reserves has been advanced by Scott et al. (1999), although in the context of matrix subsidies generally, this possibility has not been widely discussed or studied. If such a subsidy benefited forest fragment populations, food presumably was not the most important limiting resource for population density of agile antechinus, otherwise fragment populations would have reached or exceeded the densities observed in continuous forest.

Another possible explanation for the significant PD×BC relationship was that higher levels of interspecific competition in continuous forest reduced the food resources available to agile antechinus. Certainly, two native, small mammals, the dusky antechinus (*A. swainsonii*; 38-170 g) and the bush rat (*Rattus fuscipes*; 50-225 g), occurred at much higher relative abundances in pseudofragments than in fragments (logTR of *A. swainsonii* in fragments = -6.00 ± 0.08, in pseudofragments = -2.41 ± 0.08; logTR of *R. fuscipes* in fragments = -5.37 ± 0.15, in pseudofragments = -1.68 ± 0.10--C. P. Johnstone, pers. obs.). Both species are competitively dominant over agile antechinus (Banks & Dickman 2000; Dickman 1986), and it is possible that in pseudofragments these competitors reduced foraging opportunities for agile antechinus. Another possible food competitor, the ground-foraging, insectivorous southern brown bandicoot (*Isoodon obesulus*) could have been present in the pseudofragments. Our traps were not large enough to capture adults of this species, but significantly we did not capture any juveniles and saw no indirect evidence (e.g., conical diggings) of bandicoots, so it seems unlikely that they were competing with agile antechinus at any sites. Also, some ground-foraging bird species could have occurred at higher densities in continuous than fragmented forest and reduced the overall availability of invertebrate leaf-litter prey, but we have no data to evaluate this possibility.

Factors other than food availability, such as higher predation rates or fewer nest sites in fragments than pseudofragments, likely limit population density in fragmented forest. Exotic terrestrial predators occur at higher densities in fragmented than continuous forest in Australia-- in particular, European red foxes (*Vulpes vulpes*) and feral cats (*Felis catus*)--May & Norton 1996). More intense browsing pressure from livestock and consequently a sparser shrub understorey in fragmented than continuous forest (Knight & Fox 2000) could enhance both feral terrestrial mammal and native avian predation pressure on antechinus by removing foraging cover (Hobbs 2001; Knight & Fox 2000;

Stokes et al. 2004). Riskier male than female foraging behavior is frequently observed in lek-breeding mammals (Ruckstuhl & Neuhaus 2000) such as the agile antechinus (Lazenby-Cohen & Cockburn 1988); e.g., males have a greater tendency to forage away from shrub cover. High levels of predation could have selectively removed agile antechinus in poorer condition from the fragment populations; however, if this were the only underlying cause of the higher MSR in fragment than continuous forest populations, a shift in the mean, but not the range, of mass and MSR should have occurred, but this was not observed.

The findings indicate that care is needed when generalized expectations about the response of a species to anthropogenic habitat disturbance are used in conservation theory or practice. There may be unexpected effects of anthropogenic habitat fragmentation or degradation on a species. Where relationships of populations and their environments are examined, several independent indicators of health are likely to produce more comprehensive and informative results.

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Monash University

Declaration for Thesis Chapter Five

Declaration by candidate

In the case of Chapter Five, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Conception, collection of field data and samples, laboratory work, data analysis, major writing	90

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Alan Lill	Writing and editing	5
Richard Reina	Writing and editing	5

Candidate's Signature		**Date** 21/09/2010

Declaration by co-authors

The undersigned hereby certify that:

1. the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
2. they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
3. they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
4. there are no other authors of the publication according to these criteria;
5. potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
6. the original data are stored at the Department of Biological Sciences, Monash University, Clayton and will be held for at least five years from the date indicated below:

		Date
Signature 1		21/09/2010
Signature 2	[redacted]	21/09/2010

Response of the agile antechinus to habitat edge, configuration and condition in fragmented forest

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Running headline: Habitat fragmentation effects on *Antechinus agilis*

Abstract

Habitat fragmentation and degradation are serious threats to native communities and ecologies. We studied the response of a small marsupial, the agile antechinus, to several environmental variables in anthropogenically-fragmented *Eucalyptus* forest in south-east Australia. Agile antechinus were more likely to be captured in microhabitats dominated by woody debris than in other microhabitats. Relative abundances of both sexes were positively correlated with fragment core area. Male and female fat stores (indicated by mass-size residuals, MSR) were smaller in larger fragments. A health status indicator, haemoglobin-haematocrit residuals (HHR), did not vary as a function of any environmental variable in females, but male HHR indicated better health where sites' microhabitats were dominated by shrubs, woody debris and trees other than *Eucalyptus*. Females were trapped less often in edge than interior fragment habitat and their physiological stress level, indicated by the neutrophil/lymphocyte ratio in peripheral blood (N:L), was higher where forest fragments had a greater proportion of edge habitat. The latter trend was probably due to lymphopoenia resulting from stress hormone-mediated leukocyte trafficking. Using multiple indicators of population health status allows for a more comprehensive examination of the effects of anthropogenic disturbances, such as fragmentation, on native species.

Introduction

There has been a tendency in the animal ecology and conservation biology literature, and in particular in small mammal studies, for researchers who are examining species' responses to habitat fragmentation to rely on distribution metrics (e.g. occurrence, abundance, density), without much reference to indices of performance (e.g. litter size, survivorship, physiological stress). In a recent review and meta-analysis, Fletcher et al. (2007) noted that in studies of fragment edge and area effects on vertebrates, distribution metrics were almost three times as common in the literature (n = 145) as performance indices (n = 49).

Decline and extinction of vertebrate populations in fragmented habitat is variously attributed to habitat change (loss, degradation, edge effects and isolation), altered species interactions (predation, parasitism etc), changed behaviour (edge avoidance, disrupted dispersal, social relationships or resource-tracking), altered physiology (poor body condition and chronic physiological stress in vertebrates) and stochastic threats associated with small population size (Fischer & Lindenmayer 2007; Fletcher et al. 2007; Martínez-Mota et al. 2007; Ries & Sisk 2004). The area, spatial configuration, isolation and habitat degradation levels of fragments are considered to be the key environmental factors that influence these threatening processes (Diamond et al. 1987; Fischer & Lindenmayer 2007; MacArthur & Wilson 1967; Simberloff 1974; Turner 1996). However, the relative importance of the putative agents of population decline remain unclear and are likely to vary among taxa and landscapes (Fischer & Lindenmayer 2007). Additional research using diverse study areas and species is needed before this possibility can be properly evaluated.

We report elsewhere on performance and distribution differences between agile antechinus (*Antechinus agilis*, Family: Dasyuridae) populations living in fragmented and

continuous *Eucalyptus* forest (Johnstone et al. 2010, *in review*). Here, we compare response metrics of this species to landscape configuration (e.g. fragment area, proportion of edge) and microhabitat variables in an anthropogenically-fragmented landscape in order to identify possible causal relationships. The agile antechinus is the only native mammal carnivore persisting in much of our study area in South Gippsland, south-east Australia (Menkhorst & Knight 2004). The species is considered to be locally common (Menkhorst & Knight 2004) and consequently is not generally the focus of much conservation effort. However, there is a growing sentiment in conservation biology that successful management requires an increased focus on common native species; it would clearly be preferable to prevent future decline rather than wait until common species become threatened before management action is taken.

We examined one distribution metric and three independent performance variables in the agile antechinus: (1) relative abundance (based on trapping rates); (2) mass size residuals (MSR), a well-established measure of fat reserves in small mammals (Schulte-Hostedde et al. 2005); (3) erythrocyte indicators of health status and (4) leukocyte profile indicators of hypothalamus-pituitary-adrenal (HPA) axis-mediated stress (hereinafter: physiological stress; Siegel 1980). This last variable encompassed the neutrophil-to-lymphocyte ratio (N:L) and total neutrophil, lymphocyte and eosinophil concentrations in peripheral circulating blood (Davis et al. 2008). Using these estimates of population health status, we addressed the following questions:

1) Are blood cell indicators of stress or health status correlated with estimated body condition (MSR) in this species?

2) Do agile antechinus in *Eucalyptus* forest fragments preferentially use some microhabitats more than others?

3) Are features of fragmented landscapes, such as edge habitat, fragment area, microhabitat heterogeneity etc., related to agile antechinus' abundance, body condition and blood cell indicators of stress or health status?

Materials and methods

Study area and design

This study was carried out from April to August 2007 and March to August 2008 in South Gippsland, Victoria, Australia (Figure 1). We sampled thirty *Eucalyptus* forest fragments dispersed in an anthropogenically-disturbed, agricultural landscape in an area bounded by the coordinates 38°35'25"S 145°41'41"E, 38°21'55"S 146°06'10"E, 38°37'19"S 146°28'20"E and 38°45'12"S 146°01'33"E. The fragments varied in area from 4.8 to 293.6 ha and were situated 2.1 to 38.6 km from any area of continuous forest (defined as > 1000 ha of continuous native treecover, Figure 1). Habitat similarity among study sites was achieved by restricting sites to stands of forest composed of the three Ecological Vegetation Classes (EVC) (Davies et al. 2002) 'Lowland Forest', 'Wet or Damp Forests (Wet)' and 'Wet or Damp Forests (Damp)'. Most sites contained a mixture of the first two EVCs, but some also contained small areas of the EVCs 'Riparian Forests or Woodlands' or 'Rainforests'.

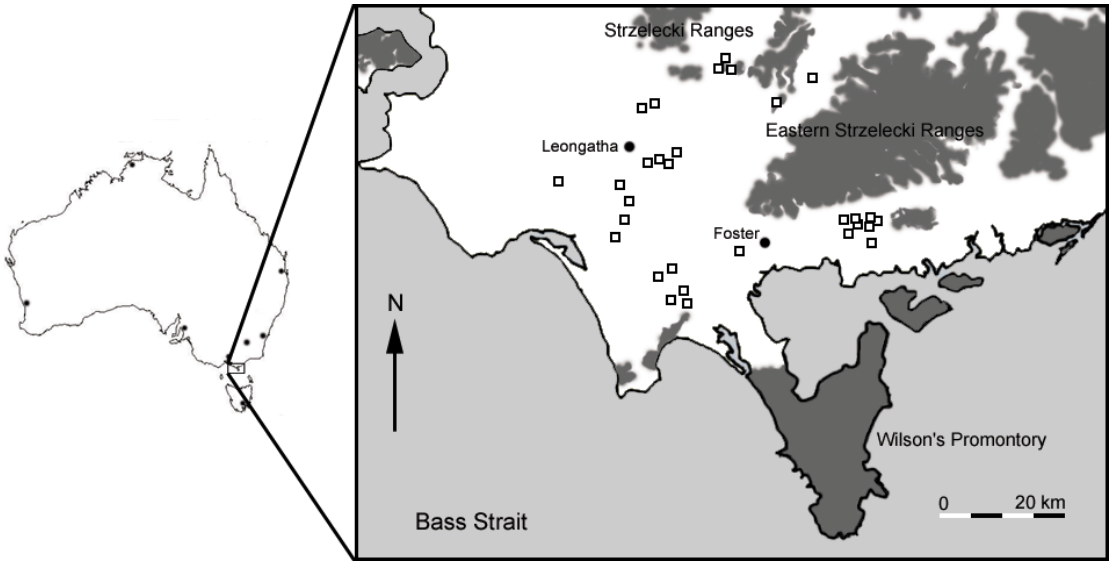

Figure 1. Study region in South Gippsland, south-east Australia. White = cleared agricultural land. Shaded areas = native tree cover (includes native regrowth, old growth forest and native plantations). Approximate locations of fragment study sites are indicated by white boxes (□). Map based on DSE interactive forest-explorer online maps ('Forest-Explorer Online' maps, http://www.dse.vic.gov.au).

Study Species

The agile antechinus is a scansorial, nocturnal species that is restricted to south-eastern Australia. Its diet comprises terrestrial invertebrates, supplemented by some small vertebrates and scavenging from carcasses (Lunney et al. 2001). Home range area can be up to 5 ha, but is more typically 1-3 ha (Cockburn & Lazenby-Cohen 1992; Lazenby-Cohen & Cockburn 1991). Prior to 1998, this species was considered to be part of the brown antechinus (*Antechinus stuartii*) species-complex (Dickman et al. 1998); the two species are very similar in their life-history and morphology (Menkhorst & Knight 2004) and authors frequently cite studies of *A. stuartii* when discussing theories about *A. agilis*' biology and *vice versa*.

Antechinus are unusual mammals because they are mostly semelparous (Braithwaite & Lee 1979). A synchronized breeding rut occurs in the Austral winter (in August in our

study area), followed by senescence and death of the entire adult male population. During the 2-3 week breeding season, male foraging behaviour is reduced and lek behaviour occurs, apparently involving extended periods of 'vigilance' in tree-hollow nests (Kraaijeveld-Smit et al. 2002a; Kraaijeveld-Smit et al. 2002b). A negative nitrogen balance develops in males and eventually they are unable to obtain sufficient food for self-maintenance (Woollard 1971). Sperm storage in females, relatively protracted oestrus (≤ 21 days in captivity) and promiscuous mating behaviour by both sexes contribute to a high level of sexual competition among male agile antechinus, with larger males and those that mate closer to the time of a female's ovulation typically siring more young (Kraaijeveld-Smit et al. 2003; Kraaijeveld-Smit et al. 2002c; Shimmin et al. 1999). After the weaning of young, a male-biased dispersal occurs in the Austral late summer (Cockburn et al. 1985b) (January-February in the study area). Most females die after weaning their only litter (Wood 1970), although a small proportion breed in a second year (~ 15% in A. stuartii; Lee & Cockburn 1985).

Trapping protocols

Trapping employed Elliott ™ live-traps (Elliott Scientific, Upwey, Victoria), baited with a mixture of rolled oats, peanut butter, water and vanillin. Traps were weather-proofed with a plastic bag (150 × 200 mm Prospectors Earth Science, Bella Vista NSW) and provided with bedding (upholsters' lofted wadding) and a plastic refuge tube for the trapped animal. They were set no earlier than 3 h before dusk and checked no later than 3 h after dawn.

One trapping grid was placed in edge (< 60 m from the forest-field ecotone) and one in interior habitat (> 80 m from the forest-field ecotone) in each forest fragment. The two trapping grids had 21 traps each, arranged in three lines of seven (i.e. 21 traps in 4800 m^2). Trapping was conducted for three successive nights in each fragment. We

considered captures per trap night to represent relative abundance and used this as an estimate of population density.

Lipid reserve estimation and haematological methods

All captured individuals were sexed by visual inspection. On each trapping day, the first two 'new' (i.e. not previously captured) agile antechinus captured at the edge and the first two in the interior of a fragment were measured to determine total and differential leukocyte counts, mass (± 0.1 g) and linear distance from nose to vent (NV) (± 0.1 mm). All measurements were taken by the same researcher. A < 1 mm disc of pinna tissue was removed from a unique position to facilitate identification on recapture so that recaptured individuals were not re-sampled.

Blood-sampling was conducted within 15 min of removing an antechinus from a trap. Blood was collected before measuring mass and size in order to reduce the potentially confounding effects of handling stress and consequent leukocyte trafficking on leukocyte counts (Dhabhar et al. 1995; Dhabhar et al. 1994). The volume of blood collected never exceeded 100 μL (~ 0.1 g) and so was unlikely to have markedly affected subsequent measurement of mass. The possibility that trapping and/or handling stress could have influenced our leukocyte measurements (Fletcher & Boonstra 2006) is addressed in the Discussion.

Blood samples were collected by capillarity in heparinised microhematocrit tubes after puncturing one of the two lateral veins near the base of the tail with a 27 gauge needle. Whole blood haemoglobin concentration (Hb) (± 0.1 grams per litre [$g \bullet L^{-1}$]) was determined immediately with a Hemocue 201+ haemoglobinometer (Hemocue®, Ängelholm, Sweden). All other blood samples were stored on ice and processed within 10 h, and no deterioration was observed. Haematocrit (Hct) (± 0.1 mm) (%) was determined by centrifugation for 3 min at 12,700 g. Hb and Hct on their own are

potentially difficult to interpret, as high and low values can be caused by several factors (e.g. anaemia, dehydration, disease) (Lewis et al. 2006). Therefore we derived an index of health status based on Hb/Hct residuals (HHR), following a similar principle to that used for mass-size residuals (see Discussion).

Blood smears for differential leukocyte counts were made by the pull-wedge method (Lewis et al. 2006) and stained with May-Grünwald-Geimsa stain (Lewis et al. 2006). The counts were obtained by making visual sweeps from the 'head' to the 'tail' of each smear under 400 × magnification. Counts comprised > 200 leukocytes and were all conducted by the same person. Population mean neutrophil/lymphocyte ratios were calculated from mean proportions of neutrophils and lymphocytes, as averaging ratios can generate spurious results (Atchley et al. 1976). To make total white blood cell counts (WBC), 5 μL of blood were diluted with Natt and Herrick's solution at a ratio of 1:200 (Campbell 1995). Counting was conducted under 400 × magnification using an improved Neubauer haemocytometer (Blau Brand, Germany). Total neutrophil, lymphocyte and eosinophil concentrations were derived from total and differential leukocyte counts.

Mass-size residuals, a well-supported index of lipid reserves in small mammals (Schulte-Hostedde et al. 2005), were constructed by calculating the residuals of mass as a factor of NV. Ordinary least squares (OLS) regressions were used to generate both HHR and MSR (Schulte-Hostedde et al. 2005).

Relationship of haematotogical variables to body condition

We used linear mixed effect models (LMEM) to examine whether erythrocyte variables, neutrophil, lymphocyte and eosinophil concentrations or N:L ratio were significantly related to the measured body condition index, MSR. In all LMEM, the factor *SITE* (i.e. each fragment) was included as a random effect to avoid pseudoreplication and the covariate *MONTH* (March = 3 to August = 8) was included

because there are biological reasons to expect at least some variation in body condition to be explained by the time of year (Cheal et al. 1976). Final models were validated graphically using ordinary and standardized residuals (Zuur et al. 2009).

Response of agile antechinus to microhabitat, vegetation features and landscape configuration

One investigator (CJ) documented the dominant local microhabitat at all trapping stations using a system of 48 categories. These categories were devised during preliminary fieldwork, with the intention of recording as much variation in microhabitats as possible. We used variable reduction methods (principal component analysis, PCA, and model simplification; Crawley 2007) to reduce the number of categories to seven (Table 1). We derived residuals of trap-nights conducted in a given microhabitat (expected) and number of captures (observed) for each microhabitat for each trapping grid (i.e. from a linear model in which captures in microhabitats was treated as function of trap-nights in microhabitats).

Trapping stations represented pseudo-random samples of microhabitat. Therefore, we constructed vegetation feature indices by applying a PCA to station microhabitat feature occurrences (Table 1). We used PCA axes 1, 2 and 3 (*PC.1*, *.2* & *.3*), which had Eigenvalues > 1, as vegetation descriptors, and these were included as explanatory variables in linear models examining indices of agile antechinus' stress and condition. We were also interested in whether habitat heterogeneity was a factor influencing agile antechinus population health, and so used the complete 48 microhabitat categories to derive a Shannon's heterogeneity index for each site (Tews et al. 2004). This was also included in analyses as a habitat complexity index (*HETEROGEN*). A more detailed breakdown of microhabitat categories is available from the authors on request.

We use the term 'configuration' to encompass both fragment area and spatial configuration (shape and degree of isolation). Fragment configuration data were obtained from online native vegetation cover maps (1: 75,000) from the Victorian Department of Sustainability and Environment ('Forest-Explorer Online' maps, http://www.dse.vic.gov.au), estimated using ImageJ (http://rsbweb.nih.gov/ij/) (measured in pixels and converted to appropriate units). We measured the following fragment variables: (a) largest inside circle (*CORE*, ha), (b) 'nearest neighbour', the distance (m) to the nearest *Eucalyptus* fragment of > 5 ha (*DIST*) and (c) dissection index (*DI*).

The variables *CORE* and *DI* are independent, single parameter shape descriptors (Bowen & Burgess 1981; Gottschlich & Smith 1982; Kincaid & Schneider 1983; McLellan & Endler 1998). For *CORE*, we estimated the largest circle that fitted inside a fragment's perimeter and recorded the circle's area (ha). We consider *CORE* an estimate of unsubdivided fragment area, which we term 'core area'. Dissection Index was estimated by taking the ratio of the perimeter (P) to the square root of the area (A) and scaling the results, so that for a circle $DI = 1.0$ and values > 1.0 are increasingly dissected: $DI = P / (2 \cdot (\sqrt{} (\pi \cdot A))$ (Jaeger 2000).

Data analysis

The responses of the sexes to habitat fragmentation were analysed separately, as the behaviour, morphology and physiology of male and female *Antechinus* differ markedly (Cheal et al. 1976; Marlow 1961; Naylor et al. 2008). All data were analysed with R 2.11.1 (*R* Development Core Team 2010; packages 'nlme', 'MuMIN' and 'hier.part'). Data were checked for normality and homoscedasticity. Relative abundance (RA; captures per trap night) was square-root arcsine-transformed and the ratio variable N:L was $\log_{10}$-

transformed to achieve normality where appropriate, but no other transformations were needed. Pearson's correlation (*r*) was used to identify auto-correlation where necessary.

Linear mixed-effects models (using maximum likelihood) were applied to explanatory factors (*EDGE* response: edge or interior and *MONTH*) and covariates (*DI*, *DIST*, *LIC*, *PC.1, 2, 3* and *HETEROGEN*), and to response variables (RA, MSR, HHR and differential leukocyte parameters of stress) for all subsets model selection using Akaike Information Criterion (AIC) ('dredge' in 'MuMIN'). We checked for correlation structures in the data (sphericity, auto-regression etc.) and included these in the final model where warranted. Restricted maximum likelihood (REML) was used to generate the final models.

Where interactions among factors occur in linear models, they must be interpreted first (Engqvist 2005), one consequence of which is that the main effects are not always interpretable. We use conditioning plots to examine interactions where they have been identified, but provide only provisional interpretations. We used hierarchical partitioning (Mac Nally 1996) to help infer the relative percentage of variation in each response variable that was explained by each predictor variable. In this procedure, if a variable has a total influence of 50% it indicates that it explained 50% of the variation explained by the cohort of explanatory variables used, not 50% of the total variation in the response variable. We report the independent effect (*IE*) explanatory variables and consider variables with *IE* > 25% to have had a potentially important influence on the response variable in question.

Results

We captured 734 agile antechinus over 2700 trap nights at the 30 study sites in 2007 and 2008; 165 males and 131 females were captured at fragment edges and 191 males and 247 females in the interior. Over the two study years, a subset of 263 individuals was

measured for mass, morphometrics and haematological indicators of stress. Of these, 76 males and 45 females were captured in fragment edges and 71 males and 71 females in interiors (Table 2).

Relationship between blood cell variables and body condition

In females, the model including Hct best explained variation in body condition (indexed as MSR). As an AIC difference (ΔAIC) $\geq$ 2 is usually considered reasonable support for a model (Burnham & Anderson 2001), the ΔAICs between models for female Hct and Hb can only be considered marginal (Hct-Hb ΔAIC = 2.0), whereas there is support Hct being a better predictor of MSR (estimated fat reserves) than is HHR (Hct-HHR ΔAIC = 3.4). For males, the model including HHR best explained MSR, but the differences among models were not convincing (HHR-Hb ΔAIC = 0.9 and HHR-Hct ΔAIC = 2.1). None of the individual erythrocyte variables were significantly associated with MSR (all $P > 0.05$). For subsequent analyses we use HHR as a health status indicator, as it is the most readily interpretable of the three erythrocyte variables (Table 3).

There were no significant relationships between any of the female leukocyte variables and the fat reserve index (MSR). In males, lymphocyte and eosinophil concentrations were significantly higher where MSR was greater ($r = 0.20$, $P = 0.015$ and $r = 0.34$, $P = 0.036$, respectively) (Table 3). However, these relationships were somewhat confounded, because all three variables were correlated with *MONTH* during the March-August trapping period (see below) and so were difficult to interpret.

Microhabitat preference

Agile antechinus were more likely than expected to be captured in traps whose local microhabitat was dominated by woody debris than in traps associated with any of the

other six microhabitat categories ($P = 0.033$) (Table 4). No other significant relationships between capture sites and microhabitat characteristics were evident.

Responses of agile antechinus to landscape configuration, edge habitat and vegetation features

(1) Relative abundance

Relative abundance was significantly greater for females in interior than *EDGE* habitat ($r = 0.26$, $P < 0.001$). The variables with the strongest independent effects on female agile antechinus' relative abundance were *EDGE* (29.8%) and *CORE* (34.3%) (Table 5).

Male relative abundances were significantly greater where *PC.2* was higher ($r = 0.19$, $P = 0.022$), although r was small. Core habitat area had a significant effect on male relative abundance ($P < 0.001$), but the relationship was complicated by a significant interaction with *PC.3* ($P = 0.002$). A conditioning plot of *CORE* and *PC.3* suggested that the effect of *CORE* on male relative abundance was generally positive, but that the slope of the effect was less pronounced where *PC.3* was greater (Figure 2). (i.e. male abundance was higher in larger fragments except in fragments where *PC.3* was high). The two most important variables for independently explaining male relative abundances were *CORE* (47.1%) and *MONTH* (15%) (Table 5).

(2) Fat stores

Estimated fat reserves (MSR) in females showed significant associations with habitat *CORE* ($r = -0.27$, $P = 0.001$), *HETEROGEN* ($r = 0.03$, $P = 0.003$), *PC.1* ($r = 0.13$, $P = 0.008$) and *PC.3* ($r = -0.05$, $P = 0.025$), although again most r values were small. The variables with the most important independent effects on female MSR were *CORE* (36.8%) and *HETEROGEN* (16.8%). In males, fat reserves were significantly associated

with the fragment dissection index (*DI)* (r = -0.10, P = 0.034), *CORE* (r = -0.20, P = 0.037) and *MONTH* (r = 0.33, P = 0.002). The interaction term *DI*×*CORE* required interpretation before the main effects were examined (P = 0.059). A conditioning plot of *CORE* and *DI* suggested that the effect of the former on male MSR was generally negative, but that the slope of the effect was less pronounced where *DI* was shallower (Figure 3) (i.e. fat reserves were smaller in agile antechinus in fragments with a greater core area, but only when the fragments also had a higher ratio of edge to interior habitat). The variables that best explained variation in male fat reserves were *MONTH* (42.0%) and *CORE* (16.7%) (Table 5, Figure 3).

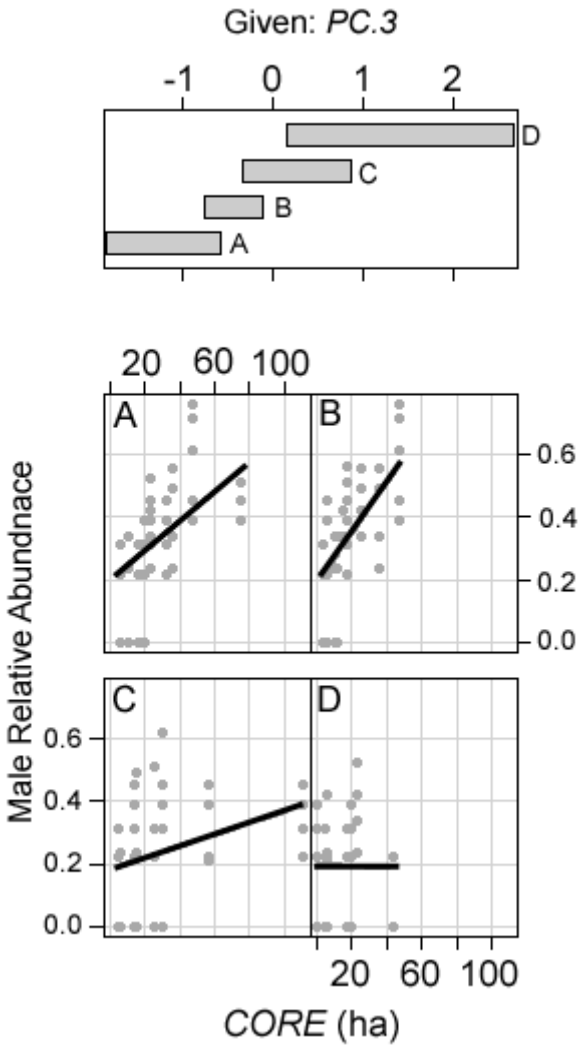

Figure 2. Conditioning plot of *CORE* (ha) given *PC.3* for male relative abundance. The top box shows regions of *PC.3* for which relative abundance is plotted against *CORE*. The overlap in *PC.3* is 25%. Conditioning plots show the range of a response variable (here: male relative abundance) for values of one explanatory variable (here: fragment core area, *CORE*) over given ranges of a second explanatory variable (here: vegetation condition index *PC.3*).

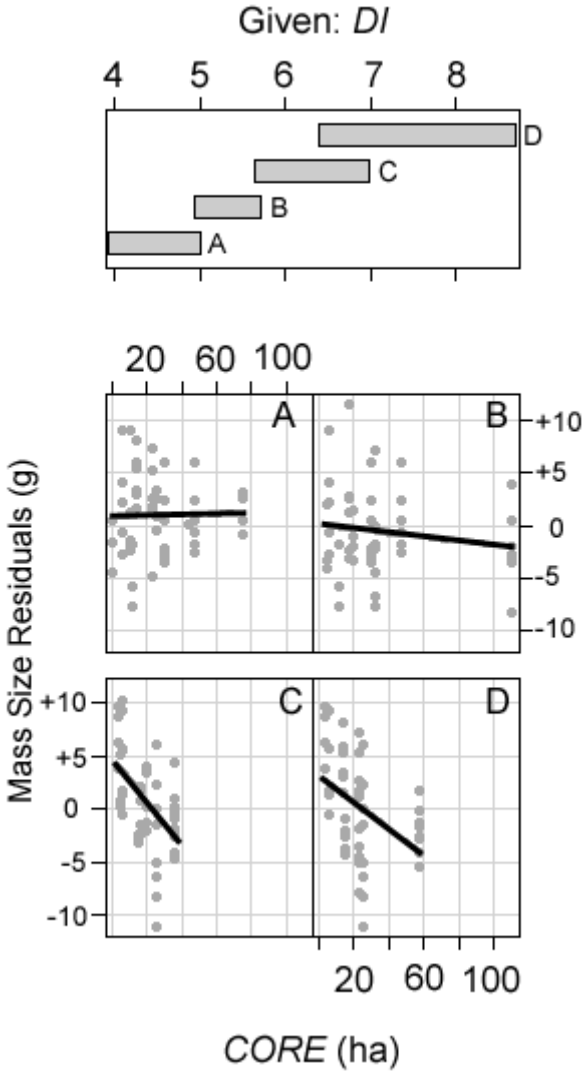

Figure 3. Conditioning plot of *CORE* (ha) given *DI* for male body condition index (MSR). The top box shows regions of *DI* for which MSR is plotted against *CORE*. The overlap in *DI* is 25%.

(3) Haemoglobin/Haematocrit residuals

Female HHR, an index of health status, was not significantly associated with any variable examined. Variation in female HHR was best explained by *HETEROGEN* (27.1%) (Table 5).

In males, *HETETOGEN* (r = 0.07, P = 0.034), *PC.2* (r = -0.15, P = 0.026) and *PC.3* (r = -0.11, P = 0.027) were significantly associated with HHR, although the correlation coefficients were small. The variables that best independently explained variation in male HHR were *PC.2* (19.4%) and *HETEROGEN* (16.2%) (Table 5).

(4) Neutrophil-to-lymphocyte ratio

Female N:L was significantly and strongly associated with *DI* (r = 0.53, P = 0.002). Variation in this stress index was best explained by *DI* (42.6%) and *MONTH* (25.5%). Male N:L was significantly and strongly associated with MONTH (r = 0.53, P < 0.001). For males, the best independent explanatory variables for N:L were *MONTH* (57.2%) and *PC.2* (19.9%) (Table 5).

(5) Leukocyte concentrations

In both sexes, the only significant relationship between an environmental variable and the peripheral blood neutrophil concentration was for *MONTH* (March to August) (females r = 0.52, P < 0.001, independent effect = 63.0%; males r = 0.62, P < 0.001, independent effect = 63.6%) (Table 5).

In females, lymphocyte concentration was significantly associated with *DI* (r = -0.16, P = 0.008), and although *EDGE*, *MONTH*, *PC.1* and *PC.2* were included in the best model, the interaction terms *EDGE*×*PC.1* and *MONTH*×*PC.3* were also included. The relationship between *PC.1* and lymphocyte concentration was positive in both interior and edge habitat, but more pronounced in populations living near forest edges (Figure 4).

The relationship between *PC.3* and lymphocyte concentration was difficult to interpret, as the correlation changed from positive to negative during the sampling period (Figure 4). The independent effects on female lymphocyte concentration were strongest for *PC.3* (22.3%) and *MONTH* (19.6%). The best explanatory variables for male lymphocyte concentration were *PC.1* (20.2%) and *PC.3* (43.3%) (Table 5).

Neither male nor female eosinophil concentration showed a significant relationship with a potential explanatory factor, except for *MONTH* in males ($r = 0.48$, $P < 0.001$). The variables that best independently explained variation in eosinophil concentration were *PC.3* in females (27.1%) and *MONTH* (53.9%) in males (Table 5).

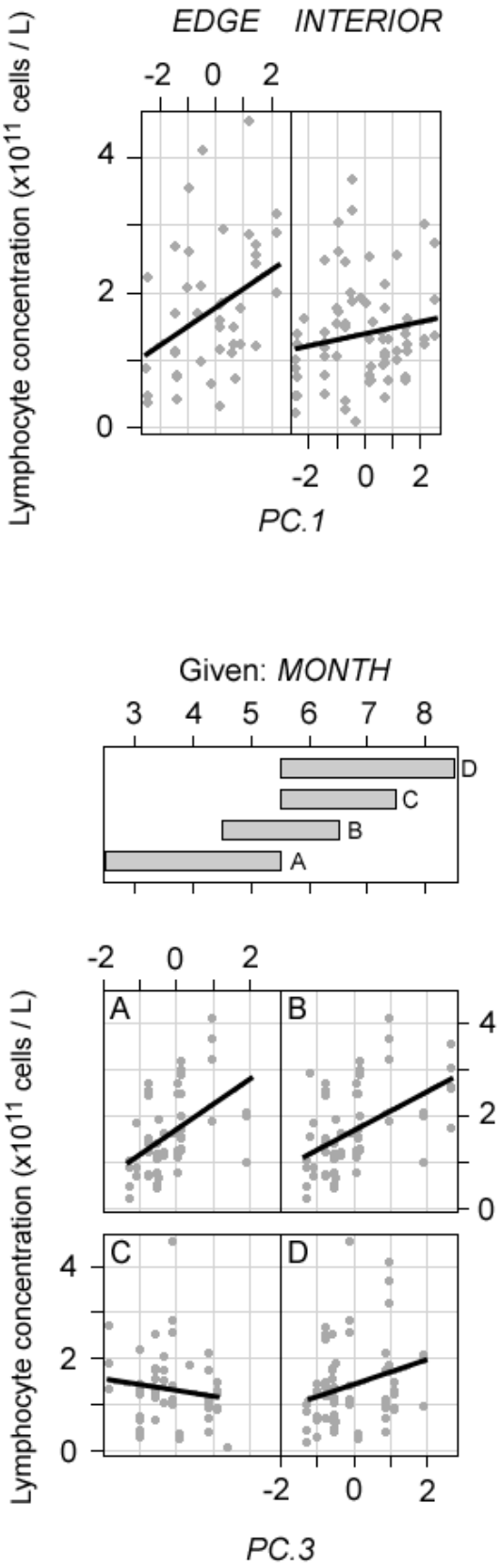

Figure 4. Conditioning plots of *PC.1* given *EDGE* (above) and *PC.3* given *MONTH* (below) for female lymphocyte concentration.

Table 1: Eigenvalues and component loadings from principal component analysis of simplified microhabitat variables. Principal component axes 1-3 of 7 are shown. The dominant microhabitat was recorded at each trapping station as one of 48 microhabitat categories. The categories shown here are simplifications of the field categories and were derived by a model simplification procedure. Trap station microhabitats were treated as pseudo-random samples of the microhabitats in each study site. Bold values are component loadings > +/- 0.40.

	PC.1	*PC.2*	*PC.3*
Eigenvalues	1.55	1.47	1.20
Component loadings:			
DEAD EUCALYPT TREE	-0.17	0.12	**0.76**
EUCALYPT (< 2 m diam.)	**0.48**	0.34	-0.36
EUCALYPT (> 2 m diam.)	-0.39	**0.41**	-0.23
NON-EUCALYPT TREE	**-0.49**	0.31	-0.29
SHRUB	0.13	**-0.63**	-0.18
TEATREE / PAPERBARK	0.38	0.37	0.36
WOODY DEBRIS	**-0.43**	-0.26	0.10

Table 2: Summary of values for stress or condition indicators used in this study. Mean ± standard error of indices of agile antechinus' relative abundance, estimated stored fat (MSR; mass-size residuals) and haematological indicators of stress and condition are shown.

	FRAGMENTS	
	Edge (< 60 m)	Interior (> 60 m)
FEMALES		
Relative abundance:	0.006 ± 0.005	0.037 ± 0.016
MSR (g):	-1.05 ± 0.62	-1.32 ± 0.38
HHR ($g \bullet L^{-1}$)	-0.24 ± 0.62	+0.15 ± 1.78
N:L ratio:	0.822 ± 0.091	1.003 ± 0.158
Neutrophils: ($\times 10^{11} \bullet L^{-1}$)	1.39 ± 0.21	1.30 ± 0.24
Lymphocytes: ($\times 10^{11} \bullet L^{-1}$)	1.82 ± 0.21	1.44 ± 0.13
Eosinophils: ($\times 10^{9} \bullet L^{-1}$)	7.19 ± 1.76	5.64 ± 0.94
MALES		
Relative abundance:	0.004 ± 0.002	0.013 ± 0.010
MSR (g):	+1.58 ± 0.63	+2.13 ± 0.70
HHR ($g \bullet L^{-1}$)	-1.17 ± 1.51	+1.28 ± 2.03
N:L ratio:	0.927 ± 0.103	0.967 ± 0.103
Neutrophils: ($\times 10^{11} \bullet L^{-1}$)	1.42 ± 0.20	1.38 ± 0.14
Lymphocytes: ($\times 10^{11} \bullet L^{-1}$)	1.61 ± 0.15	1.59 ± 0.13
Eosinophils: ($\times 10^{9} \bullet L^{-1}$)	8.02 ± 1.21	9.19 ± 1.43

Table 3. Relationships between blood cell indicators of stress and health status and Mass-Size Residuals (g). Linear mixed effect model results are shown. The *df*, *t*- and *P*-value are from restricted maximum likelihood models (more accurate). The AIC values are from maximum likelihood models (more suitable for comparing models).

MALE	*df*	*t*-value	*P*	AIC
MONTH	26	2.18	0.039	789.8
Neutrophils (cells●L^{-1})	112	1.14	0.257	
MONTH	26	2.98	0.006	784.9
Lymphocytes (cells●L^{-1})	112	2.48	0.015	
MONTH	26	2.23	0.035	786.4
Eosinophils (cells●L^{-1})	112	2.14	0.035	
MONTH	26	3.14	0.004	804.2
(log)N:L ratio	115	-0.89	0.377	
MONTH	26	3.25	0.003	745.7
Hb (g●L^{-1})	105	1.15	0.251	
MONTH	26	3.17	0.004	746.9
Ht	105	-0.40	0.689	
MONTH	26	3.35	0.003	744.8
HHR (g●L^{-1})	105	1.49	0.139	

Continued on next page

Table 3 continued.

FEMALE	*df*	*t*-value	*P*	AIC
MONTH	25	-0.38	0.710	491.1
Neutrophils (cells●L^{-1})	84	-0.15	0.884	
MONTH	25	-0.42	0.681	491.0
Lymphocytes (cells●L^{-1})	84	-0.31	0.759	
MONTH	25	-0.49	0.625	491.1
Eosinophils (cells●L^{-1})	84	0.06	0.952	
MONTH	25	-0.65	0.519	490.7
(log)N:L ratio	84	0.66	0.511	
MONTH	25	-0.43	0.669	479.2
Hb (g●L^{-1})	81	-1.43	0.158	
MONTH	25	-0.28	0.784	477.2
Ht	81	-1.96	0.054	
MONTH	25	-0.43	0.672	480.6
HHR (g●L^{-1})	81	-0.80	0.428	

Table 4. Analysis of the difference between expected (number of traps set) and observed captures of agile antechinus as a function of microhabitat. Means and *SE* are shown for residuals of number of captures (observed) and number of traps set (expected) in each microhabitat category (positive sign = greater than expected and negative sign = less than expected). * $P < 0.05$.

Microhabitat:	Mean ± *SE*	*df*	*t*-value	*P*	
DEAD EUCALYPT TREE	+ 0.01 ± 0.07	180	-0.17	0.866	
EUCALYPT (< 2 m diam.)	- 0.12 ± 0.24	180	-0.43	0.665	
EUCALYPT (> 2 m diam.)	+ 0.07 ± 0.16	180	0.32	0.750	
NON-EUCALYPT TREE	-0.17 ± 0.10	180	0.20	0.842	
SHRUB	-0.14 ± 0.19	180	-0.45	0.651	
TEATREE / PAPERBARK	-0.36 ± 0.07	180	-1.45	0.148	
WOODY DEBRIS	+0.48 ± 0.27	180	2.14	0.033	*

Table 5. Relationships between indicators of population stress, condition and well-being and environmental variables. Pearson's correlation coefficients (*r*), the independent effect of variables from hierarchical partitioning (*IE*), and results of linear mixed effect model (LMEM) fitting are shown. Degrees of freedom, *t*-value and *P*-values are shown for variables that were selected for inclusion in the reduced LMEM using Akaike Information Criterion. * $P < 0.05$.

FEMALES	*r*	*IE*	*df*	*t*-value	*P*	
Relative abundance:						
DI	0.03	0.6				
DIST	0.13	9.3				
EDGE	0.26	**29.8**	144	5.04	< 0.001	*
HETEROGEN.	-0.02	1.6				
CORE	0.27	**34.3**	27	1.91	0.066	
MONTH	0.05	2.7				
PC.1	-0.14	9.6				
PC.2	-0.16	11.1				
PC.3	0.01	1.0				
MSR: (g)						
DI	-0.19	7.5				
DIST	0.10	3.0				
EDGE	0.07	2.7				
HETEROGEN	0.03	16.8	21	3.36	0.003	*
CORE	-0.27	**36.8**	21	-3.77	0.001	*
MONTH	-0.06	0.8				
PC.1	0.13	15.2	21	2.96	0.008	*
PC.2	-0.09	7.3	21	-2.02	0.057	
PC.3	-0.05	9.8	21	-2.41	0.025	*

Continued on next page

Table 5 continued.

FEMALES	*r*	*IE*	*df*	*t*-value	*P*	
Neutrophils:						
DI	0.25	6.8				
DIST	-0.01	0.4				
EDGE	-0.02	0.3				
HETEROGEN.	0.11	1.7				
CORE	0.22	8.7				
MONTH	0.52	**63.0**	25	4.54	< 0.001	*
PC.1	0.03	1.4				
PC.2	0.22	10.3				
PC.3	0.21	7.6				
Lymphocytes:						
DI	-0.16	15.7	21	-2.95	0.008	*
DIST	0.14	2.6				
EDGE	0.21	13.1	84	-2.72	0.008	*
HETEROGEN.	-0.01	2.4				
CORE	-0.07	1.5				
MONTH	0.24	19.6	21	2.86	0.009	*
PC.1	0.24	17.0	21	3.14	0.005	*
PC.2	0.08	5.7				
PC.3	0.29	22.3	21	-2.67	0.014	*
EDGE× PC.1	na	na	84	-2.30	0.024	*
MONTH× PC.3	na	na	21	3.25	0.004	*

Continued on next page

Table 5 continued.

FEMALES	*r*	*IE*	*df*	*t*-value	*P*	
Eosinophils:						
DI	0.08	2.8				
DIST	0.22	21.2				
EDGE	0.02	0.3				
HETEROGEN.	0.00	2.9				
CORE	-0.02	0.7				
MONTH	0.20	15.8				
PC.1	0.14	8.8	23	0.99	0.332	
PC.2	0.18	20.3	23	1.47	0.156	
PC.3	0.26	**27.1**				
PC.1× *PC.2*	na	na	23	0.07	0.943	
(log)N:L ratio:						
DI	0.53	**42.6**	23	3.54	0.002	*
DIST	-0.22	4.5				
EDGE	-0.18	7.8	85	-1.23	0.222	
HETEROGEN.	0.04	0.7				
CORE	0.33	12.0				
MONTH	0.39	**25.5**	23	1.84	0.079	
PC.1	-0.10	1.1				
PC.2	0.13	1.7				
PC.3	-0.08	4.2	23	-1.91	0.069	
HHR: ($g \bullet L^{-1}$)						
DI	-0.1	11.5				
DIST	0.04	2.7				
EDGE	-0.01	0.3				
HETEROGEN.	0.09	**27.1**	23	1.96	0.062	
CORE	0.08	9.7				
MONTH	-0.09	7.3				
PC.1	0.06	19.7	23	1.67	0.108	
PC.2	0.03	1.9				
PC.3	-0.11	19.7	23	-1.63	0.116	

Table 5 continued.

MALES	*r*	*IE*	*df*	*t*-value	*P*	
Relative abundance:						
DI	0.10	3.0				
DIST	0.08	7.7				
EDGE	0.07	2.2				
HETEROGEN.	0.14	4.9				
CORE	0.31	**47.1**	24	5.19	< 0.001	*
MONTH	0.21	15.1				
PC.1	-0.02	0.4				
PC.2	0.19	12.2	24	2.44	0.022	*
PC.3	0.13	7.4	24	-0.97	0.343	
CORE× PC.3	na	na	24	3.53	0.002	*
MSR: (g)						
DI	-0.10	5.5	22	-2.26	0.034	*
DIST	-0.08	3.1				
EDGE	-0.08	2.5				
HETEROGEN.	0.18	15.8	22	1.70	0.104	
CORE	-0.20	16.7	22	-2.22	0.037	*
MONTH	0.33	**42.0**	22	3.46	0.002	*
PC.1	0.04	4.5				
PC.2	0.17	6.7				
PC.3	0.11	3.0				
DI × *CORE*	na	na	22	1.99	0.059	

Continued on next page

Table 5 continued.

MALES	*r*	*IE*	*df*	*t*-value	*P*	
Neutrophils:						
DI	0.15	1.8				
DIST	-0.12	2.3				
EDGE	0.02	0.4				
HETEROGEN.	0.12	1.3				
CORE	0.02	1.2				
MONTH	0.62	**63.6**	26	5.44	< 0.001	*
PC.1	-0.06	0.8				
PC.2	0.35	17.0				
PC.3	0.25	11.7				
Lymphocytes:						
DI	-0.14	15.8				
DIST	0.04	3.1				
EDGE	0.08	7.1				
HETEROGEN.	0.07	5.5				
CORE	-0.05	2.3				
MONTH	0.06	2.2				
PC.1	0.14	20.2				
PC.2	0.00	0.6				
PC.3	0.24	**43.3**	26	1.89	0.070	
Eosinophils:						
DI	0.09	1.7				
DIST	0.07	3.9	24	1.49	0.150	
EDGE	-0.13	3.6				
HETEROGEN.	-0.03	1.9				
CORE	-0.10	1.7				
MONTH	0.48	**53.9**	24	4.11	< 0.001	*
PC.1	0.13	2.9				
PC.2	0.32	19.2	24	1.30	0.206	
PC.3	0.24	11.0				

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Table 5 continued.

MALES	*r*	*IE*	*df*	*t*-value	*P*	
(log)N:L ratio:						
DI	0.24	7.9				
DIST	-0.24	7.1				
EDGE	-0.06	0.9				
HETEROGEN.	0.06	0.7				
CORE	0.02	0.4				
MONTH	0.53	**57.2**	24	4.82	< 0.001	*
PC.1	-0.14	3.9				
PC.2	0.35	19.9	24	1.23	0.229	
PC.3	-0.04	2.0	24	-1.50	0.146	
HHR: ($g \bullet L^{-1}$)						
DI	-0.03	1.6				
DIST	-0.09	10.4				
EDGE	-0.08	7.3				
HETEROGEN.	0.07	16.2	23	2.26	0.034	*
CORE	0.11	10.0				
MONTH	-0.13	14.8				
PC.1	0.02	7.3	23	1.60	0.123	
PC.2	-0.15	19.4	23	-2.38	0.026	*
PC.3	-0.11	12.9	23	-2.36	0.027	*

Discussion

The issue of handling and trapping stress

Indices of stress, such as N:L, can alter sufficiently rapidly to be potentially confounded as baseline measures by the effect of trapping and sometimes even of handling (Delehanty & Boonstra 2009; Fletcher & Boonstra 2006). This is probably *not* true of erythrocyte variables (e.g. HHR), in which it can take as long as 48 h before a peak response to an acute stressor occurs (Oishi et al. 1999). We eliminated the possibility that handling stress affected N:L through a validation trial in which agile antechinus were blood-sampled 0, 10, 20 and 30 min post-removal from a trap (Johnstone et al. *in review*). Detecting trapping stress requires the immediate killing of trapped animals to establish baseline values for each study site (Fletcher & Boonstra 2006), a procedure which would have been both impractical and ethically contentious in our investigation. Therefore arguably the most appropriate interpretation of N:L in the present study is that it probably reflected an additive or multiplicative response (Clinchy et al. 2004; Krebs et al. 1995; Zanette et al. 2003) to a combination of environmental and trapping stress (i.e. variations in N:L were due to variation in the environment and/or in time spent in a trap pre-sampling). Evidence from another study on agile antechinus in similar environments is that time spent in traps did not differ significantly among sites (measured using electronic timers; T. Bramwell pers. comm.). However, although trapping stress has not been widely investigated in small mammals, trapping certainly evoked a stress response in meadow voles (using corticosterone; *Microtus pennsylvanicus*), but its magnitude did not increase as a function of time spent in the trap (i.e. trapping could be considered a uniform stressor; Fletcher & Boonstra 2006). Therefore we interpret N:L here as a positive index of chronic stress (Davis et al. 2008) and assume that differences in N:L among sites are more likely to be due to differences in environmental stress than in mean duration of trap occupancy. This interpretation

assumes that no study population was physiologically exhausted due to extreme chronic stress (Dhabhar & McEwen 1997), which we consider unlikely but cannot entirely discount.

Relationships between blood cell variables and estimated fat reserves of agile antechinus

There was no convincing relationship between any immune cell variable and MSR in female agile antechinus. Male lymphocyte and eosinophil concentrations were higher when body condition indices were higher, but these associations were confounded by correlations between MSR, lymphocyte concentration and eosinophil concentration and time in the study period when trapping occurred (*MONTH)*.

Haematocrit, Hb and HHR explained variation in MSR better than any of the leukocyte variables. In both sexes, HHR was positively correlated with MSR, implying that the amount of haemoglobin per unit of packed cell volume was greater in agile antechinus with larger lipid reserves. Theory and empirical evidence about chronic stress and regenerative anaemia (Teague et al. 2007; Tyler & Cowell 1996) both suggest that HHR is a useful index of health status in vertebrates, although it may not always be strongly related to the size of fuel stores. In vertebrates, blood loss through parasite infection or injury, injection with stress hormones and acute stress causes elevated erythropoiesis and reticulocyte release from bone marrow (Colombelli-Négrel & Kleindorfer 2008; Fisher & Crook 1962; Teague et al. 2007). Reticulocytes are less capable than mature erythrocytes of producing haemoglobin (Lewis et al. 2006), so this process generates a blood profile in which packed cell volume may increase, but the amount of haemoglobin per unit of cell volume decreases (sometimes termed regenerative anaemia; Tyler & Cowell 1996).

MSR has been validated as an estimate of fat stores in several small mammals (Schulte-Hostedde et al. 2005) but not in the agile antechinus, and an empirical

evaluation of the accuracy of MSR as an estimate of lipid reserves in this species could help to clarify the relationship between HHR and MSR.

Effects of microhabitat on capture rates

Capture rates were higher than expected where trapping station microhabitat was dominated by woody debris (logs and fallen branches), so agile antechinus could have been foraging preferentially on or beside fallen timber. Such timber could provide arthropods, such as spiders and beetle larvae, which comprise most of the study species' diet (Lunney et al. 2001), as well as cover from predators (Stokes et al. 2004). Woody debris density contributed to *PC.1* (loading = -0.43), but the latter did not significantly influence agile antechinus' relative abundance in the various study sites. Thus although agile antechinus preferentially used microhabitats dominated by woody debris, fallen timber density *per se* did not affect their relative abundances at sites. In contrast, other authors have found a positive association between *Antechinus* spp.' abundance and/or site occupancy and fallen timber volume and/or density (Bennett 1993; Kelly & Bennett 2008; Mac Nally & Horrocks 2002). Possibly the positive association between woody debris and the capture rate of agile antechinus was caused by movement biases rather than being a direct effect of the debris on survivorship or reproductive success e.g. by non-random movement due to a preference for complex microhabitats where predation risk was lower and food abundance higher (Stokes et al. 2004). This hypothesis could be addressed by (a) trapping agile antechinus and collecting microhabitat information over larger spatial scales (i.e. the equivalent of at least several home ranges and therefore > 10 ha; Lazenby-Cohen & Cockburn 1991), so that the confounding effect of movement into or across trapping grids is reduced (i.e. the problem is the same as for predator exclusion fence experiments where the fenced area is too small to distinguish non-random emigration or immigration from the effects of predator fencing on survivorship;

Diamond 1983), or (b) radio-tracking agile antechinus and documenting their movement patterns through the fragmented landscape (Marchesan & Carthew 2008).

Effects of fragment area on relative abundance

Agile antechinus' relative abundance was positively associated with fragment area. Population densities of brown antechinus also vary with fragment area, but Knight and Fox (2000) suggested that the mechanism may have been an indirect one, in which smaller fragments were more degraded and the resultant lower habitat complexity negatively affected population density. However, in the present study the independent effect of core habitat area on relative abundance was strong (females = 34.3%; males = 47.1%) (Figure 5), implying that a direct effect was operating. Patch occupancy by agile antechinus in another fragmented forest was better explained by a combination of fragment area and vegetation composition than by either variable alone (Holland & Bennett 2009) and other investigations have reported equivocal effects of fragment size on agile antechinus' abundance (Banks et al. 2005a; Bennett 1990a). These varying findings could be attributable to differences in the nature of the environment (e.g. dry *vs* wet sclerophyll, differences in rainfall or competitor species) or the time of year when sampling occurred.

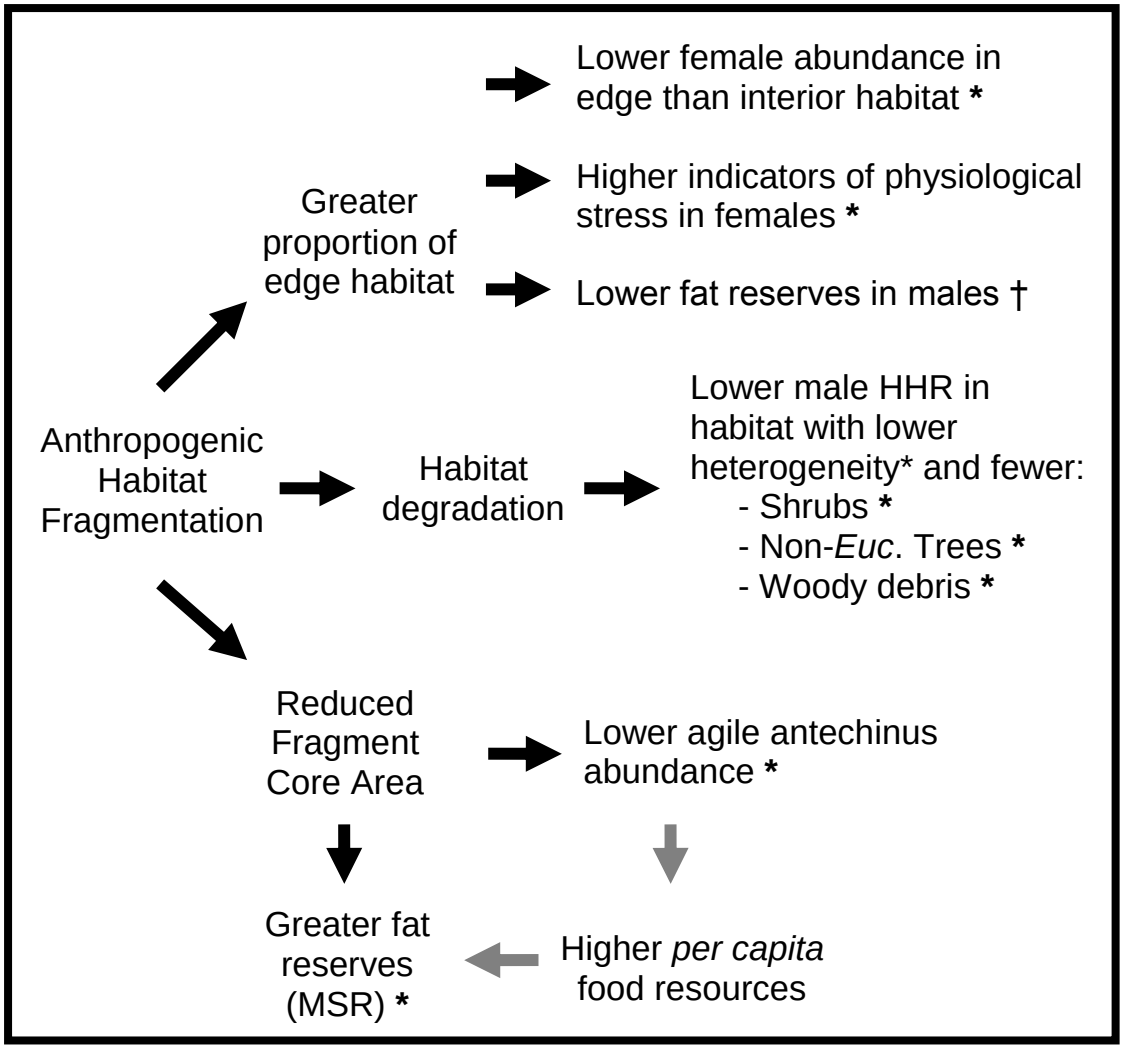

Figure 5. Conceptual flow diagram of the main results. There are well established associations between anthropogenic habitat fragmentation and the creation of novel edge habitat, habitat change and habitat area reduction (Fischer & Lindenmayer 2007). Associations supported by significant findings are indicated by *. Findings that are significant but may be confounded by an interaction are indicated by †. Grey arrows indicate a theoretical mechanism by which an association could be operating.

The lower relative abundance of agile antechinus in small fragments could have been due to higher levels of predator intrusion from the agricultural matrix (Lidicker 1999), altered emigration and/or immigration rates (Gilpin & Diamond 1976; Stamps et al. 1987), greater competition with generalist species (Banks & Dickman 2000; Dickman 1989) or reduced and/or degraded resources (Knight & Fox 2000). Theoretical models predict, and there is evidence to support the occurrence of, proportionally greater emigration from, and reduced immigration into, smaller habitat patches (Fletcher et al. 2007). The rationale here is twofold, namely that dispersers are probably more likely to encounter large than small patches (the 'target effect') (Gilpin & Diamond 1976; Lomolino & Perault 2000) and that patch-dwellers are probabilistically more likely to encounter boundaries in small than large patches, thus increasing the likelihood of emigration (Stamps et al. 1987) given that male agile antechinus have an inherently strong propensity for dispersal (Kraaijeveld-Smit et al. 2002a).

Capture rates of two invasive generalists, the feral house mouse (*Mus musculus*) and black rat (*Rattus rattus*), were low or zero in all study sites (C. Johnstone, unpublished data), so competition with such species was unlikely to have been a factor contributing to the lower relative abundance of agile antechinus in small fragments. On the other hand, bush rat (*Rattus fuscipes*) capture rates were higher and more variable among the study sites (C. Johnstone, unpublished data), and so it is possible that competition for food with this native species could have contributed to variation in agile antechinus' relative abundance (Banks & Dickman 2000).

Effects of habitat structure and heterogeneity abundance and health

In both sexes of agile antechinus, *PC.1*, *PC.2* and *PC.3* had smaller independent effects on relative abundance than did *CORE*. That *CORE* explained variation in relative abundance better than vegetation indices was surprising, given the prevailing opinion that

Antechinus populations are strongly influenced by habitat complexity and structure (Holland & Bennett 2009; Kelly & Bennett 2008; Mac Nally & Horrocks 2002; Stokes et al. 2004; Sutherland & Predavec 1999). The only clear support that we obtained for this predominant view was that *PC.2* was positively associated with male relative abundance, although its independent effect was only 12.2% (compared with 47.1% for *CORE*). The effect of *PC.2* was that male relative abundance was higher where there were more *Eucalyptus* trees of > 2 m in trunk diameter and fewer shrubs. Although large eucalypts could potentially contribute to nest-hollow, leaf litter and woody debris availability, the negative effect of shrub density on agile antechinus' relative abundance was unexpected and its cause enigmatic.

Health status of agile antechinus (indexed by HHR) was associated with certain vegetation characteristics, although the relationship was not very convincing for females. We expect HHR to be greater in individuals in good body condition. Male HHR was higher where microhabitat heterogeneity was greater. Conceivably, heterogeneous habitat provided more foraging and/or nesting opportunities, so that the environment was in a sense generally less stressful. Male HHR was negatively associated with the vegetation descriptors *PC.2* and *PC.3* and *Eucalyptus* densities contributed to both of these indices. Thus males had a poorer health status in forest with denser stands of *Eucalyptus*. As capture rates of agile antechinus were higher in sites with more large eucalypts, it is plausible that there was an indirect effect of social stress or food competition on HHR when male densities were high.

Shrubs, woody debris and trees other than *Eucalyptus* contributed to *PC.2* and *PC.3*, so that a greater dominance of these microhabitat features was associated with better male health. Non-*Eucalyptus* tree species in the study area (e.g. *Cassinia* and *Olearia* spp.) frequently had fissured bark likely to harbour arthropod prey. Higher shrub density could contribute to better body condition in agile antechinus, as small mammals' foraging bouts

are typically longer (Brown 1988) and arthropod abundance higher where shrub cover is denser (Sutherland & Predavec 1999)--although the apparent negative effects of shrub microhabitat site dominance on agile antechinus' abundance renders this interpretation necessarily tentative. Logs and fallen branches could also be promoting better health through increasing foraging resources for antechinus (Knight & Fox 2000).

Edge effects on relative abundance, stress and body condition

The trapping rate of males was not influenced by edges, but female relative abundance was significantly and markedly lower (*IE* = 29.8%) at fragment edges than interiors. Female agile antechinus may generally occupy better quality habitat than males (Holland & Bennett 2009). Predation rates on birds' nests are higher in edge than interior habitat in wet *Eucalyptus* forest (Berry 2002). The same might be true for agile antechinus' tree-hollow nests although admittedly most of Berry's (2002) birds were open-cup nesters whose nestlings were probably inherently more vulnerable than concealed antechinus young. However, if fewer dependent young survive at the edge than in the interior of fragments, this could lead over several generations to successively fewer females living in natal edge habitat because female agile antechinus normally remain in the natal home range throughout life (Cockburn et al. 1985b; Kraaijeveld-Smit et al. 2002a). It is also conceivable that the few dispersing females in the study population were more likely than dispersing males to move away from a forest-field ecotone when it was encountered.

The observed sex difference in relative abundance in fragment edges could also be related to the species' lek breeding system. Spatial segregation of the sexes outside the breeding season is well-known in lek breeding mammals (Ruckstuhl & Neuhaus 2000, 2002). The 'predation risk hypothesis' of sexual segregation in lekking species (Ciuti et al. 2004; Ruckstuhl & Neuhaus 2002) predicts that to maximize their fitness, females should make more use of habitat with a lower predation-risk, whereas males should use habitat

with more abundant foraging resources, even if predation-risk is also higher there (i.e. because good condition males can produce many more young than good condition females, the potential fitness benefits of 'riskier' foraging are different for the sexes) (Ruckstuhl & Neuhaus 2002). Forest-field ecotones are more resource-rich than forest interiors and invertebrate species richness generally declines with distance from edges in forests (Ewers & Didham 2005); however, edge habitats also have higher avian nest predation rates, which may indicate greater predator activity in general (Berry 2002; Ewers & Didham 2005; Paton 1994).

The 'hotspot theory' of lek siting (Bradbury 1981) predicts that during the breeding season, males should aggregate where female traffic is greatest, which is contrary to our observation. Male brown and agile antechinus can move large distances during or before the breeding season (Cockburn & Lazenby-Cohen 1992; Lazenby-Cohen & Cockburn 1988) and it would be interesting to investigate whether males living in edge habitat move into the fragment interior where female population density is higher immediately prior to, or at this time. Equally, we could expect females to forage nearer edges during lactation when metabolic demands are high, though after young detach from the pouch (~ 5 weeks after parturition) the need to return to the maternal nest to nurse may restrict this (Cockburn & Lazenby-Cohen 1992).

Female N:L was significantly higher in fragments that had a greater proportion of edge habitat. Assuming that N:L was a positive index of stress in this study, this finding implied that female agile antechinus found such fragments more stressful than those with relatively more interior habitat. This might not be a direct effect of edges; if females avoided edge habitat, limited availability of core habitat in more dissected fragments could have resulted in crowding, psychosocial stress and competition for nesting sites and food in the interior.

Effects of environmental features on stored lipid reserves

Mass-size residuals, an estimate of stored fat reserves, allow us to make some inferences about whether *per capita* food resources varied among fragments. The most convincing significant relationship between MSR and a landscape variable was the negative association between MSR and core habitat area i.e. in larger fragments, the estimated stored lipid reserves were smaller. The association was strong in both sexes (females = 36.8%; males = 42.0%). Therefore nutritional stress was almost certainly not a factor causing the lower relative abundance of agile antechinus in smaller *Eucalyptus* fragments. One possible explanation for this situation was that inter- and/or intra-specific competition for food was more pronounced in larger fragments. Experimental food provisioning suggests that inter-specific competition between agile antechinus and bush rats can be intense (Banks & Dickman 2000) and the latter were present in many of our study sites. Agile antechinus' relative abundance was also greater in larger fragments, so there is at least a possibility that intra-specific competition is also part of the underlying reason for the observed *CORE* × MSR relationship.

Environmental features affecting immune cell variables

Female N:L was influenced by the proportion of edge habitat in a fragment, but male N:L was not convincingly correlated in a consistent manner with landscape configuration, proximity to forest edge or the vegetation composition indices (*PC.1*, *PC.2*, *PC3*).

Absolute concentrations of leukocytes in peripheral blood can be more informative of population health status than N:L alone (Masello et al. 2009). Neutrophil concentrations in both sexes appeared to be unaffected by any measured environmental variable, but they did respond strongly to seasonality (females = 63.0%; males = 63.6%) i.e. neutrophil concentrations increased during the March (post-dispersal) to August (pre-

breeding season) sampling period. Numerical domination of neutrophils in peripheral blood may reflect greater innate immunocompetence (Dufva & Allander 1995; Figuerola & Ferrer 1999) and presumably the neutrophilia in agile antechinus later in the sampling period (July-August) resulted from neutrophil trafficking, production or release from bone marrow. This could conceivably constitute a form of 'preparation' for breeding and the synchronized breeding rut, during which physical contact among individuals, and hence the risk of disease transmission, probably increased.

Female lymphocyte concentrations responded to a broad set of environmental variables, including interactions between *EDGE* and *PC.1* and *MONTH* and *PC.3*. However, the only unambiguous relationship between female lymphocyte concentration and an environmental factor was that with the proportion of edge habitat in a fragment (*IE* = 15.7%). Trafficking of lymphocytes away from peripheral blood into the skin, lymph nodes and spleen, where they are more likely to be useful in the event of injury, is the most frequently cited mechanism underlying the increased N:L observed in chronically-stressed vertebrates (Braude et al. 1999; Davis et al. 2008; Dhabhar et al. 1995; Dhabhar et al. 1994). Thus it appears likely that lymphopoenia produced the positive association between N:L and edge habitat in female agile antechinus.

Eosinophil concentrations were not convincingly related to any environmental variable. In males, they were higher nearer to the breeding season. As eosinophils are strongly associated with defence against metazoan infections (Rothenberg 1998), this increase could be a 'preparatory' mechanism similar to the neutrophilia discussed above.

Conservation implications

For the conservation management of agile antechinus in the study area, we suggest that preserving forest fragments with large core areas, a high level of microhabitat heterogeneity and a minimum of edge habitat would help to mitigate the negative effects

of habitat fragmentation on this species. This conclusion is in accordance with theories of how anthropogenic habitat fragmentation affects native vertebrates (Fischer & Lindenmayer 2007), although we could only identify negative effects of habitat area reduction, increased patch dissection and lower microhabitat heterogeneity by examining relative abundance and multiple performance metrics.

Acknowledgements

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Monash University

Declaration for Thesis Chapter Six

Declaration by candidate

In the case of Chapter Six, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Conception, collection of field data and samples, laboratory work, data analysis, major writing	90

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Alan Lill	Writing and editing	5
Richard Reina	Writing and editing	5

Candidate's Signature		**Date** 21/09/2010

Declaration by co-authors

The undersigned hereby certify that:

1. the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
2. they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
3. they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
4. there are no other authors of the publication according to these criteria;
5. potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
6. the original data are stored at the Department of Biological Sciences, Monash University, Clayton and will be held for at least five years from the date indicated below:

		Date
Signature 1		21/09/2010
Signature 2	[redacted]	21/09/2010

Effects of habitat loss, fragmentation and degradation on mammal abundance and condition: analysis with tree-based statistical modelling

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Running headline: Tree-based statistical modelling

Abstract

Anthropogenic habitat loss, fragmentation and degradation often co-occur in a landscape and their relative influence on species' health and survival can be difficult to determine. We examined these environmental variables' influence on small mammal relative abundance in a large area (~ 2500 km^2) of south-eastern Australia. For agile antechinus (*Antechinus agilis*), we also examined the association between these variables and two population 'performance' indices, mass-size residuals (MSR) indexing fat reserves and the neutrophil/lymphocyte ratio (N:L) indexing chronic physiological stress. Study sites were in either highly disturbed, fragmented or relatively undisturbed, continuous *Eucalyptus* forest.

We generated tree-based statistical models to identify the relative importance of 36 ecological variables in explaining variation in mammal abundance and performance indices. Habitat loss was most important in explaining small mammal abundance. The models also showed that the habitat area required to support a 'healthy' population was greater in larger species. Habitat fragmentation was the next most important influence on small mammal relative abundance. Habitat degradation, reflected in structural and floristic features, was less important, but explained some variance in relative abundance. For agile antechinus populations, time of year, degree of forest fragmentation and intra-specific competition were important in explaining performance indices. Results indicated that habitat reduction *per se* was a significant threatening process for small mammals. Habitat loss requires at least the same research attention as that currently devoted to anthropogenic habitat fragmentation and degradation.

Keywords: A*ntechinus, fuscipes*, habitat loss, relative abundance, performance indices, conditional inference tree, random forests.

Introduction

Landscape conservation biology is largely concerned with the effects of anthropogenic habitat loss, fragmentation and degradation on native biota. Substantial evidence indicates that anthropogenic habitat fragmentation negatively affects terrestrial vertebrate assemblages and populations (Andrén 1994). Fewer studies have examined the effects of anthropogenic habitat degradation, and habitat loss *per se* has received the least research attention (Fazey et al. 2005), despite a general consensus that it is probably the world's leading cause of native species' decline (Fahrig 1997; Foley et al. 2005). One underlying difficulty here is that habitat loss, fragmentation and degradation often co-occur in a landscape, and thus their independent effects can be difficult to isolate (Fischer & Lindenmayer 2007). Almost half of all mammal species' extinctions in the last 200 years have occurred in Australia (Cardillo & Bromham 2001). Many of Australia's remaining small mammal species are threatened or endangered (Menkhorst & Knight 2004), and the future of species that are currently locally common remains uncertain, as large-scale clearing of native vegetation continues (Gibbons & Lindenmayer 2007).

In 2007 and 2008, we undertook a large-scale (study area ~2500 km^2), comparative study using live-trapping to examine habitat fragmentation effects on populations of the agile antechinus (*Antechinus agilis*), a small marsupial native to south-eastern Australia. Concurrently, we recorded the numbers of other native, small mammals trapped as by-catch and some local environmental variables. The study area has one of the longest histories of native vegetation clearing for agriculture and mining in mainland Australia. Extensive forest clearing was triggered by an 1869 Lands Act which allowed settlement. Consequently, this landscape could provide insights into how comparable, but more recently cleared, areas are likely to change with respect to their mammal fauna in the future.

Vertebrate conservation studies in anthropogenically-disturbed landscapes are typically concerned with comparing a population response variable (e.g. site occupancy or abundance) or performance indices (such as brood size or indices of physiological stress) (Fletcher et al. 2007) with multiple environmental variables in order to help identify possible causal relationships. Experimental field manipulations are useful for subsequent testing of these potential relationships (Mac Nally & Horrocks 2002), but are not always feasible for financial, logistic or ethical reasons (Diamond 1983). Consequently, most large-scale studies use a naturally-occurring experimental 'design' (a 'natural experiment' *sensu* Diamond 1986). However, there may be multiple, correlated, environmental factors that influence a population, and factors may interact, have synergistic effects or partially negate one another (Laurance & Cochrane 2001). The variables measured or indexed may be continuous, ordinal or nominal (or a mixture of these) and data may be non-linear or non-normally distributed. General linear and additive models have difficulty characterizing these sorts of data (Zuur et al. 2007).

Tree-based modelling, an intuitive, easily implemented and interpreted statistical method that copes well with complex data, is underutilized in ecology. It is a tool for examining the relationship between a single response variable and multiple explanatory variables (Quinn & Keough 2002; Zuur et al. 2007). Recently, such models have become popular in medical and genetic research, probably because they tend to be better at predicting known relationships from data than more commonly used methods, such as logistic regression (Nagy et al. 2010). The models produced are predictive and robust to non-linearity, non-normality, autocorrelation and multiple interactions among explanatory variables (Quinn & Keough 2002; Zuur et al. 2007).

We used tree-based models to investigate what were the relative roles of habitat loss, fragmentation and degradation and other environmental variables in determining the study species' abundance or performance indices.

Methods

Study area and species

The study was conducted in a region of south-east Australia whose approximate centre was at 38°37'S, 146°10'E (Figure 1). The dominant canopy trees at all 60 study sites were *Eucalyptus* species, primarily *E. obliqua*, *E. radiata* and *E. regnans*, and habitat similarity was achieved by restricting sites to forest composed of the three Ecological Vegetation Classes (EVC) 'Lowland Forest', 'Wet or Damp Forest (Wet)' and 'Wet or Damp Forest (Damp)' (Davies et al. 2002). Live-trapping was conducted from April to August, 2007 and from March to August, 2008. In these years, mean monthly rainfall was 73 mm and monthly maximum and minimum mean ambient temperatures were 17.7°C and 7.8°C, respectively (Australian Bureau of Meterology 2009).

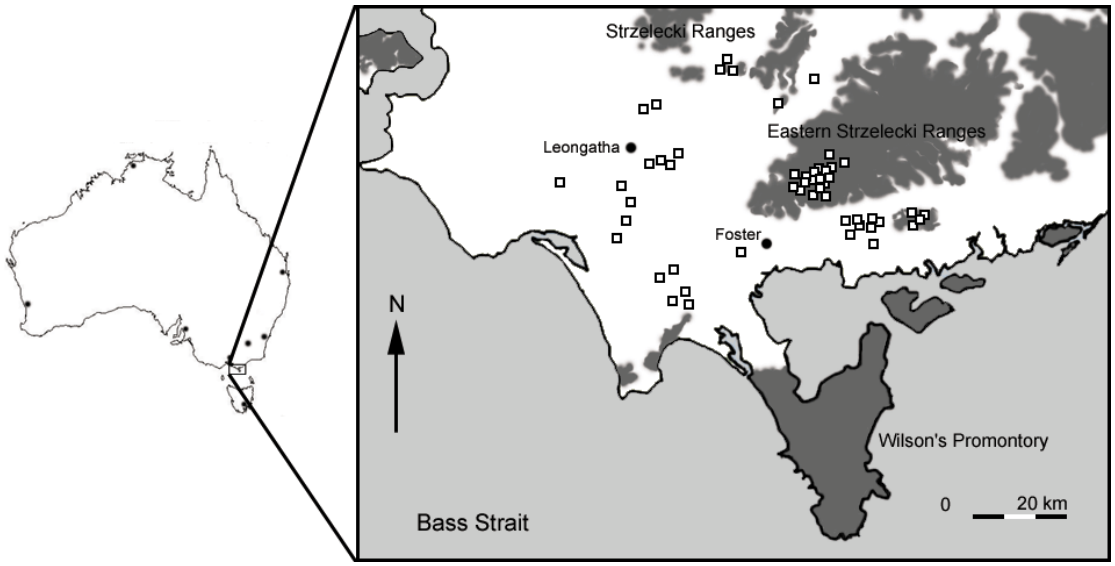

Figure 1. Study region in south-east Australia. White = cleared, agricultural land. Darker shaded areas = tree cover (includes native re-growth, old growth forest and native plantations). Approximate locations of study sites are indicated by white boxes (□).Map based on Victorian Department of Sustainability and Environment (DSE) interactive 'Forest-Explorer Online' maps (http://www.dse.vic.gov.au/).

The two small marsupials (family Dasyuridae) studied were the agile (*Antechinus agilis*) (16-44 g) and the dusky antechinus (*A. swainsonii*) (38-170 g). Agile antechinus are scansorial insectivores (Sumner & Dickman 1998) that forage in leaf litter and on vertical tree trunks (Dickman 1988). They nest communally in tree-hollows in groups of up to 20 individuals (Cockburn & Lazenby-Cohen 1992). Prior to 1998, the species was included in the brown antechinus (*A. stuartii*) species-complex (Dickman et al. 1998). Insectivorous dusky antechinus are largely terrestrial and tend to dig for prey in leaf litter and nest individually or in small family units in burrows (Cockburn & Lazenby-Cohen 1992; Dickman 1988). The eutherian mammal studied in detail was the native bush rat, (*Rattus fuscipes*) (50-225 g). Bush rats nest socially in burrows and typically are herbivorous, but they will opportunistically eat invertebrates. For brevity, hereinafter the three main study species are referred to as *agilis*, *swainsonii* and *fuscipes*, respectively. We also recorded the occurrence as by-catch of the native broad-toothed rat (*Mastacomys fuscus*) and two exotic rodents, the black rat (*Rattus rattus*) and domestic mouse (*Mus musculus*), but their abundances are not analysed here.

Live-trapping protocol

Trapping was conducted using weatherproofed Elliott live-traps (Elliott Scientific™, Australia) baited with rolled oats, peanut butter, water and vanillin and containing insulating bedding. It was timed to occur between two major *agilis* life-history events, male-biased dispersal (January-February) and the synchronized breeding rut (mid-August-September).

In 2007 and 2008, we conducted 3600 trap-nights in 60 study sites (Figure 1), trapping at 30 sites in each year. Traps were set < 3 hr before dusk and animals released < 3 hr after dawn. Trapping was conducted for three nights at each site and two trapping

grids were used per site. Trapping alternated between sites that were deemed highly disturbed/fragmented and relatively undisturbed/continuous. We used relative abundance, based on capture rate, as an estimate of a species' population density. In disturbed sites, trapping grids comprised three rows of seven traps spaced evenly in a 40 × 120 m area. Capture rates in continuous/undisturbed sites were unexpectedly high and trapping effort was therefore reduced for welfare reasons, so that grids constituted three rows of three traps spaced evenly in a 40 × 120 m area.

Varying trapping effort confounds measures of relative abundance, so to account for this we used a binomial distribution of trapping rate in our analysis (Lada et al. 2007) (BINOMDIST in Microsoft Excel™, where each trap constituted a 'trial' and likelihood of success was 50%). Trap saturation in some continuous/undisturbed sites, possibly as a result of the reduced trapping effort, may have led to underestimation of relative abundance, the effect of which would have been to increase Type II error with respect to the influence of habitat loss, fragmentation and degradation on relative abundance. Trap saturation was unavoidable, but we considered that this underestimation was acceptable, although obviously not ideal.

Agile antechinus' body condition and stress indices

Captured *agilis* were visually sexed and a < 1 mm disc of pinna tissue was removed to facilitate identification on recapture. We took a blood sample and measured body mass (± 0.5 g) and morphometrics (± 0.1 mm) to gauge hypothalamus-pituitary-adrenal axis (HPA)-mediated stress and body condition, respectively (Davis et al. 2008; Schulte-Hostedde et al. 2005). Typically, the first two *agilis* captured on a grid in a trapping night that were not recaptures were sampled (i.e. we obtained 4 samples per day and 12 over the three days of trapping at each site). Blood sampling was conducted first and took < 15 min after removal of animal from a trap. Blood was withdrawn by capillarity into a

heparinised microhaematocrit tube after puncturing one of the two lateral veins at the base of the tail with a 27 gauge needle. We drew ~ 100 μL of blood from each animal; ~ 85 μL were allocated for measuring erythrocyte and leukocyte values reported elsewhere (Johnstone et al. *in preparation*) and the other 15 μL were used to prepare a blood smear (by the pull-wedge method) for estimating the neutrophil-to-lymphocyte ratio (N:L), after staining with May-Grünwald-Geimsa stain (Lewis et al. 2006). N:L is considered an accurate index of HPA axis-mediated chronic stress in vertebrates (Davis et al. 2008). Cell counts were all conducted at 400× magnification by the same experimenter, who counted > 200 cells by visually sweeping the smear from end to end ≥ 5 times, but avoiding the preparation's edges (Lewis et al. 2006). Leukocyte trafficking due to handing and/or trapping stress could conceivably have confounded the estimation of baseline N:L (Dhabhar et al. 1994), but a trafficking validation trial suggested that this did not happen (Johnstone et al. *in review*). Trapping stress was more difficult to assess, because establishing baseline N:L requires instant killing traps (Fletcher & Boonstra 2006) and this killing agile antechinus in the numbers needed would have been logistically difficult and ethically contentious. We present elsewhere a detailed discussion of trapping stress (Johnstone et al. *in preparation*) and we think that the evidence in our study supports a best interpretation where N:L was a positive index of stress. However, we emphasize that the interactions of chronic (environmental) and acute (trapping stress) could have confounded this interpretation (Fletcher & Boonstra 2006). We calculated a body condition index (BCI) using mass-size residuals (MSR) (Schulte-Hostedde et al. 2005) derived from body mass and the nose-to-vent distance for each animal. This index accurately estimates fat stores in many small mammals (Schulte-Hostedde et al. 2005).

Potential explanatory variables

Thirty-six environmental variables which might influence the captured mammals' relative abundance, condition and chronic stress levels were measured (Table 1). Local environmental variables were measured in a 20 × 20 m quadrat positioned randomly in each trapping grid (i.e. there were two measurements per site, unless otherwise stated). We used Simpson's dominance (*Shrub dom.*) and Wilson's evenness (*Shrub even.*) indices derived from species counts of shrubs to characterize differences in shrub assemblages (Smith & Wilson 1996). We also used a single-parameter shape descriptor of the distribution of tree diameters at breast height (DBH) in each site. This was derived from a Weibull distribution (Baker et al. 2005; Horner et al. 2010) and can be used to estimate whether a stand of trees represents recent, medium or old growth forest (Baker et al. 2005). The extent of native tree-cover and degree of forest fragmentation were estimated from online native vegetation maps (1:75,000) (Victorian Department of Sustainability and Environment: 'Forest Explorer Online' www.dse.vic.gov.au) using ImageJ to measure vegetation in pixels which could be converted to hectares (http://rsbweb.nih.gov/ij/).

Table 1. Explanatory variables used in the study. 'Shrubs' were deemed to be any vegetation 60-200 cm tall with a circumference at breast height < 10 cm. 'Trees' were > 200 cm and/or had a circumference at breast height ≥ 10 cm. Large trees had a circumference at breast height ≥ 60 and are an important resource for tree hollow - nesting species in the study area (Cockburn & Lazenby-Cohen 1992). With respect to the predation index, the most likely terrestrial predator to have investigated traps was the introduced European red fox (*Vulpes vulpes*) (Menkhorst & Knight 2004).

Variable	Description
Agilis abun.	Combined male and female *agilis* (log)TR
Altitude (m)	Altitude of trapping grid (estimated with GPS)
Browse	Herbivore browsing surrogate. The percentage of shrubs that were deemed unlikely to be palatable to stock (either prickly bursaria (*Bursaria spinosa, B. lasiophylla*), prickly moses (*Acacia verticillata*) or prickly currant bush (*Coprosma quadrifidia*))
By-catch abun.	By-catch (log)TR e.g. for *swainsonii* this is the combined (log)RA of all non-*swainsonii* small mammal by-catch
DBH % Dead	Percentage of total tree DBH that was from dead trees
DBH % non-Euc.	Percentage of total tree DBH that was not a *Eucalyptus* species. The most common trees in this class were blackwoods (*A. melanoxylon*) and silver wattle (*A. dealbata*)
DBH median	Median tree DBH
DBH shape	The 'shape' parameter of a Weibull distribution of tree DBH

Continued on next page

Table 1 Continued.

Variable	Description
Fragmentation (0.5 km)	Number of polygons needed to 'capture' all native tree-cover within a given radius of the site. Measured at 0.5, 1, 2, 3, 4 and 5 km radii.
Gully	A gully present (1) or absent (0)
Latitude	Latitude of trapping grid (estimated with GPS)
Leaf litter	Visually scored index of leaf litter depth and extent. From shallow and sparse (1) to deep and extensive (5).
Log dens.	Number of logs in 20×20 m quadrat
Longitude	Longitude of trapping grid (estimated with GPS)
Month	Month of the year from March (3) to August (8)
Predation index	A terrestrial predator activity surrogate, derived from evidence that a trap had been disturbed by a terrestrial predator overnight (i. e. either moved > 1 m, had covering plastic bag entirely removed or bearing canine tooth marks).
Ridge	A ridge present (1) or absent (0)
Sex	Sex of captured animal visually determined *(agilis* only)
Shrub dens.	Number of shrubs in 20×20 m quadrat
Shrub dom.	Simpson's species dominance index for shrubs
Shrub even.	Wilson's species evenness index for shrubs
Shrub rich.	Shrub species richness in 20×20 m quadrat
Tree dens.	Number of trees in 20×20 m quadrat
Tree dens. (L)	Number of trees with circumference > 60 cm in 20×20 m quadrat
Tree rich.	Tree species richness in 20×20 m quadrat
Tree-cover (ha)	Total area of native canopy-cover within a given radius of a site (primarily *Eucalyptus* species). Measured at 0.5, 1, 2, 3, 4 and 5 km radii.

Data analysis

Statistical analysis was conducted in R (2.11.1, R Core Development Team 2010), using the packages 'party', 'nlme' and 'bbmle'. Response variables were averaged by trapping grid and, for *agilis*, also by sex. Averaging ratios should be avoided (Atchley et al. 1976) and so N:L ratio site means were derived from differential neutrophil and lymphocyte count means. The distributions of these values were checked for normality and homoscedasticity, and relative abundance (RA) and N:L were $\log_{10}$ transformed to achieve the former distribution. Where linear mixed effects models (LMEM) were used, we included the random effect 'site' to avoid pseudoreplication. Where correlations were examined, they were evaluated with Pearson's correlation coefficients (*r*).

The object of constructing a tree-based model is to recursively split the data and produce a 'tree' of sub-populations and their associated 'risk factors' (Quinn & Keough 2002; Zuur et al. 2007). The model produced resembles an inverted tree, with the first node being the tree's root. Each observation included in our models was either averaged by trapping grid--to test for edge effects--and sex (*agilis*) or by trapping grid only (*fuscipes* and *swasinonii* sexes were not recorded). All possible binary splits of the response variable were assessed for each potential explanatory variable. The aim of each split was to establish groups that had a between-variation as large, and within-variations as small, as possible. The significant advantages of this approach over additive or general linear modelling are that: 1) the number of explanatory variables that can be included is unlimited, 2) interactions among terms can be easily identified and intuitively visualized, 3) the method is not invalidated by auto-correlation of explanatory variables and 4) there is no requirement for linearity and normality in explanatory variables (Quinn & Keough 2002; Zuur et al. 2007). However, 'traditional' tree-based models, termed classification and regression trees (CARTs), have the serious disadvantage that splitting is biased in favour of explanatory variables in which more splitting is possible (Quinn & Keough

2002; Zuur et al. 2007). Moreover, they can easily be over-fitted and require somewhat subjective 'pruning' methods (Quinn & Keough 2002). We therefore used the more recently developed Conditional Inference Tree (CIT) ('party' in 'R') (Hothorn et al. 2006a, 2006b). It is similar to a CART in that it is a form of binary recursive partitioning, but it uses a machine-learning algorithm embedded in a conditional inference framework (Hothorn et al. 2006a). Whereas CART's continue splitting until no further splits are possible, a CIT uses a statistically-determined stopping criterion, an *a priori P*-value, to determine where splitting is no longer valid. We accepted splits where $P < 0.1$ (Nagy et al. 2010). Conditional inference trees are not affected by over-fitting and are unbiased with regard to the types of explanatory variables used (Hothorn et al. 2006a; Strobl et al. 2007).

Tree-based models are robust to autocorrelation of explanatory variables (Zuur et al. 2007), but spatial autocorrelation of the response variable may inflate Type I error, at least in CART's (Segurado et al. 2006). We used Moran's *I* (Legendre 1993) to check for spatial autocorrelation in response variables and found that *agilis* and *swainsonii*' relative abundances were spatially autocorrelated ($P < 0.05$). Therefore, we included latitude and longitude in all subsequent analyses as explanatory variables. This should at least partially account for spatial autocorrelation; if variables are better explained by spatial autocorrelation than environmental factors, we expect this to be reflected in the model produced.

'Random forests' is a tree-based modelling method used for establishing the relative importance of multiple explanatory variables with respect to a single response variable. This iterative method selects a random subset of explanatory variables and constructs a tree from them. This step is repeated until a 'forest' consisting of a pre-determined number of trees is constructed. Using the 'ctree' method in 'party', the relative importance of each explanatory variable was calculated from the number of times it was

used to construct a tree. Because variables are included in trees using an *a priori* conditional inference framework not all variables examined are used in a tree, i.e. those that do not satisfy $P < 0.10$ are omitted. This is a different approach to that used in a CART random forest method, in which relative importance is derived from the variance explained in the out-of-bag sample for each tree. Using CITs, more variables require more trees to achieve 'stability'. We used 4000 trees with the 'ctree' function 'replace' set to 'false' to avoid bias (Strobl et al. 2007). Where a Random Forests approach uses classification trees, the Gini index (Gastwirth 1972) can be used to estimate the most parsimonious sub-set of explanatory variables. However, CITs do not use the Gini index, and consequently, we used a compromise method based on Akaike Information Criteria (AIC) derived from linear mixed models. After determining the relative importance of explanatory variables, we discarded the auto-correlated ones ($r^2 \geq 0.70$) so that in each case only the most important 'tree-cover' and 'fragmentation' variables were retained. We calculated the AIC using maximum likelihood for the entire set of variables using LMEM and included the random effect SITE to avoid pseduoreplication. We then successively discarded the least important variable and recalculated the AIC. We accepted the model with the lowest AIC as representing the most parsimonious subset of explanatory variables and recalculated the results using reduced maximum likelihood. This approach is not ideal as the linear mixed models did not use tree-based models but were fitted using linear relationships between explanatory and response variables. However, we think that it is a sensible approach given that the Gini index cannot be used, and it may have an advantage in that the tree-based models can be 'checked' against linear models.

Results

In 2007, 771 *agilis* (429 males and 538 females), 51 *swainsonii* and 125 *fuscipes* were captured in 1800 trap-nights spread among 30 study sites. All trapped *agilis* were adults.

Morphometrics and body mass were measured for 183 male and 112 female *agilis*; the corresponding numbers for blood sampling were 182 and 113, respectively. During 2008, we captured 655 *agilis* (379 males and 276 females), 14 *swainsonii* and 191 *fuscipes* in the same number of trap-nights and study sites as in 2007. Morphometric and mass data were taken for 151 males and 105 females, whilst blood sampling was conducted for 150 males and 104 females.

Limitations on interpretation

Reporting splits in explanatory variables at precise values can imply a biological significance that is probably not valid. For example, a split in abundance could be reported for native treecover at 75.2 *vs* 75.3 ha, whereas actually there is probably very little difference between these two exact values in terms of environmental conditions. The exact thresholds produced will always depend on the data examined and for this reason the thresholds need to be considered approximations of real relationships only. Obviously this is less of a concern where the explanatory variable is categorical, but least in some instances, the resulting step-functions may not represent a relationship as as accurately as would a model that assumes a continuous relationship (e.g. a LMEM).

Neutrophil-to-lymphocyte ratio and mass-size residuals for *Antechinus agilis*

For the index of physiological stress, (log) N:L, the root of the CIT (Node 1) had 190 observations (Figure 2) and there were four splits ordered as follows:

(1) By *Month,* with populations sampled in March-June having a lower (log) N:L than those sampled in July-August (untransformed means ± s.e. [n] 0.57 ± 0.04 [141] and 0.66 ± 0.12 [71], respectively).

(2) By *Month* within the March-June period, with samples taken in March-April having a lower (log) N:L than May-June samples (0.42 ± 0.03 [40] and 0.76 ± 0.06 [101], respectively).

(3) In the March-April sampling period, *agilis* living in sites with lower overall *agilis* relative abundance ((log)RA ≤ -1) had a higher (log) N:L (0.49 ± 0.04 [24]) than those in sites with a higher relative abundance (0.31 ± 0.03 [16]).

(4) In the May-June samples, (log) N:L was lower in sites where *Fragmentation (1km)* was less pronounced (≤ 7 polygons) (0.64 ± 0.04 [91]) than in highly fragmented sites (1.85 ± 0.28 [10]).

Nodes 4, 5, 7, 8 and 9 were terminal (i.e. did not lead to any further branching).

The root node for the body condition index, MSR, contained 212 observations (Figure 3) and the CIT had two splits:

(1) By *Sex*, with males having a larger MSR than females (0.99 g ± 0.27 [113] and -1.37 g ± 0.26 [99], respectively). Females' MSR was not significantly associated with any explanatory variable.

(2) Male MSR was higher where overall *agilis* relative abundance was lower ((log) RA ≤ -4) (untransformed means ± s.e. (n) for lower and higher relative abundance 3.74 g ± 0.81 [21] and 0.41 g ± 0.24 [92], respectively).

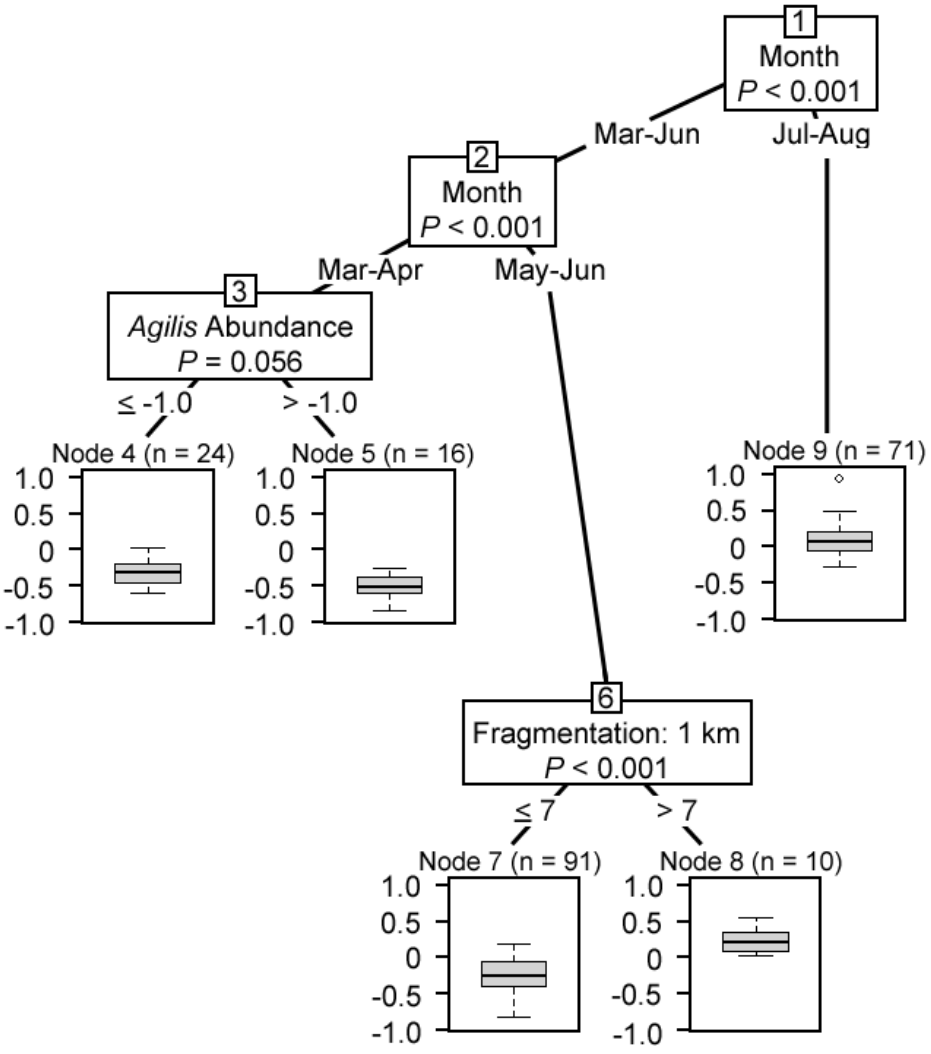

Figure 2. Conditional inference tree for *agilis* (log) N:L. The number of each node is shown in a small box inset in a larger box bearing the relevant explanatory variable's name and associated *P*-value. Categories or numerical ranges for each split are shown immediately below the variable name box. e.g. For Node 1 (*Month*), the split was into populations sampled in March-June *versus* those sampled in July-August. The splitting criterion was $P < 0.1$. Boxplots show medians and upper and lower quartiles for populations for which no further splitting was possible. e.g. *agilis* sampled in May-June were split by habitat fragmentation in a 1 km radius; in sites with low fragmentation indices (≤ 7), median (log) N:L was lower than in sites with a high index of fragmentation (> 7).

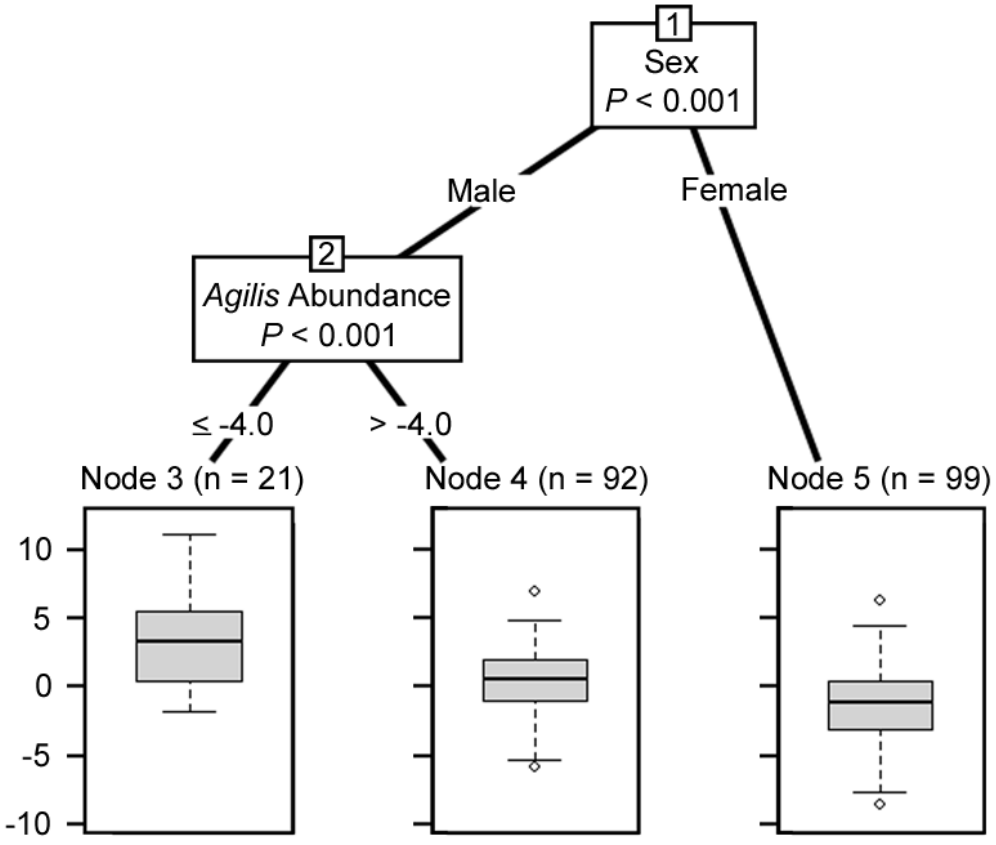

Figure 3. Conditional inference tree for *agilis* MSR. In this example, female MSR was not significantly associated with any environmental variable. Male MSR was greater where abundance of *agilis* was lower (abundance is the log transformed binomial variable and is without units). Other details are as in Figure 3.

Relative abundance of the three study species

(a) *Antechinus agilis*

The CIT for *agilis'* relative abundance ((log) RA) contained 240 observations (Figure 4) and had five splits in the following order:

(1) Its root split was associated with the extent of *Tree-cover (0.5 km)*; sites with 75.2 or fewer ha of native tree-cover had a lower *agilis* relative abundance than sites with 75.3 or more ha of cover (untransformed means and s.e. 0.015 ± 0.005 and 0.157 ± 0.018,, respectively, [n= 120 in both cases]).

(2) In the ≤ 75.2 ha 'branch' of the CIT, *agilis'* relative abundances were lower where tree-cover was 31.5 or fewer ha than in sites where it was 31.6 to 75.2 ha (0.003 ± 0.002 [44] and 0.024 ± 0.008 [76], respectively).

(3) In the higher tree-cover sites, *agilis'* relative abundance was higher where the degree of habitat fragmentation was lower (i.e. ≤ 20 polygons) (means: lower 0.035 ± 0.013 [52] and higher 0.008 ± 0.006 [24]).

(4) In the high native tree-cover 'branch' (> 75.2 ha in 0.5 km) of the CIT, males had a higher relative abundance than females (0.225 ± 0.029 versus 0.088 ± 0.016 [n= 60 in both cases]). Female relative abundance was not associated with any other explanatory variable.

(5) Male relative abundance was lower where *Tree-cover (2km)* was equal to/less than, rather than more than, 1054.3 ha (0.068 ± 0.024 [12] and -0.264 ± 0.033 [48], respectively).

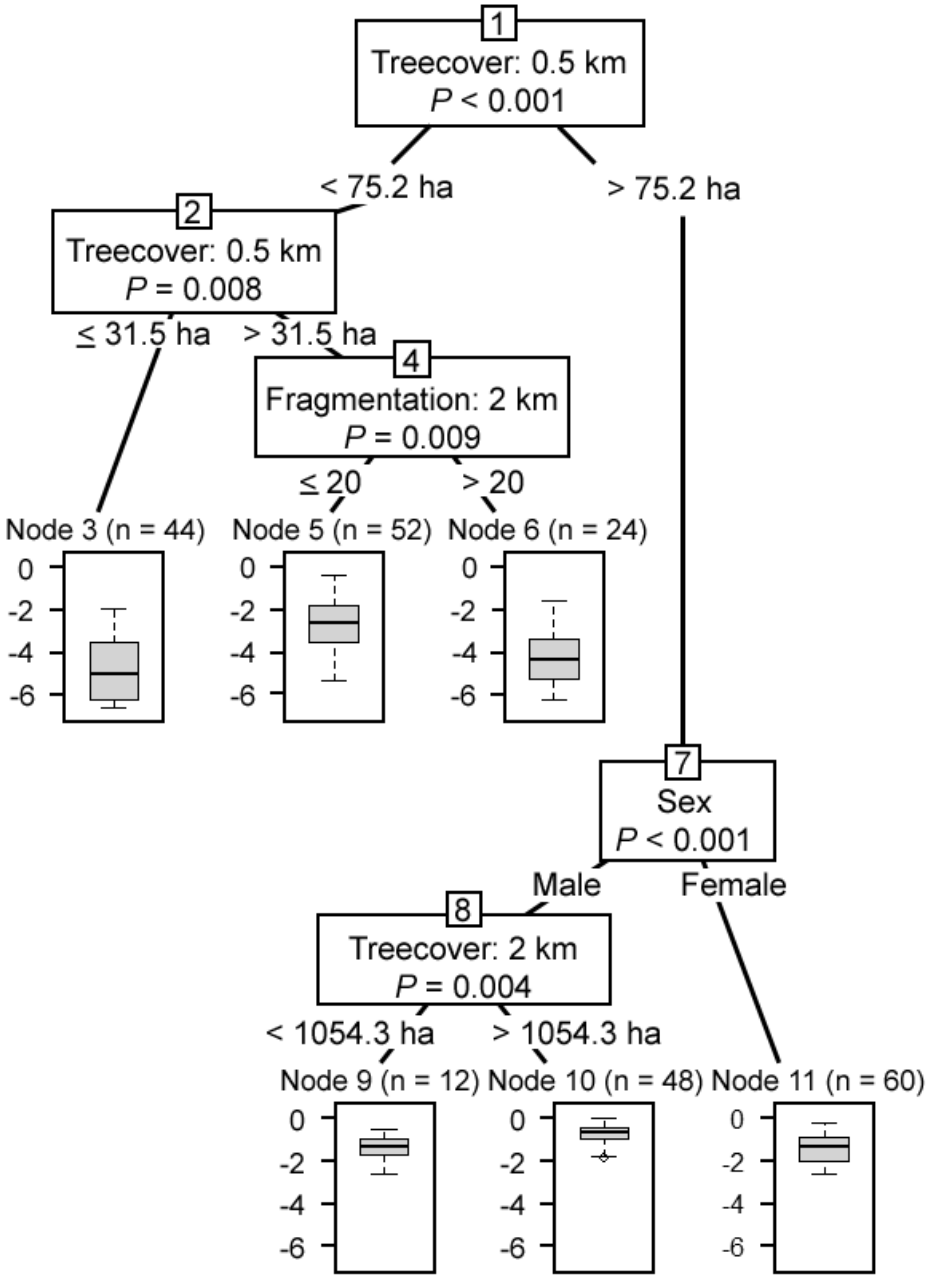

Figure 4. Conditional inference tree for *agilis'* relative abundance. Details are as in Fig 3.

(b) *Antechinus swainsonii*

The CIT for *swainsonii'* relative abundance was based on 120 observations (Figure 5) and had three splits in the following order:

(1) The first split suggested that *swainsonii'* relative abundance was lower where *Tree-cover (2 km)* was equal to/less than 619.8 ha (means $2.2\times10^{-5} \pm 1.5\times10^{-5}$ [60] and 0.021 ± 0.008 [60], respectively).

(2) In the ≤ 619.8 ha tree-cover branch, *swainsonii'* relative abundance was greater in sites ≤ 145°59'4 E than in sites further east ($7.5\times10^{-5} \pm 5.0\times10^{-5}$ [18] and $0.009\times10^{-5} \pm 0.002\times10^{-5}$ [44], respectively).

(3) In the > 619.8 ha tree-cover branch, the relationship was similar in that *swainsonii'* relative abundance was greater in sites ≤ 146°17'5 E than in sites further east (0.085 ± 0.041 [8] and 0.012 ± 0.005 [52], respectively).

(c) *Rattus fuscipes*

The *fuscipes* relative abundance CIT also contained 120 observations (Figure 6) and had three splits ordered as follows:

(1) Where *Tree-cover (3 km)* was 922.5 or fewer ha, *fuscipes'* relative abundance was lower than in sites with greater *Tree-cover (3 km)* (means $12\times10^{-5} \pm 3.9\times10^{-5}$ [62] versus 0.046 ± 0.017 [58]).

(2) In the low tree-cover sites, *fuscipes'* relative abundance was higher at longitudes of 145°59'4 E or less than it was further east ($36\times10^{-5} \pm 12\times10^{-5}$ [18] and $1.9\times10^{-5} \pm 0.9\times10^{-5}$ [44], respectively).

(3) In the > 922.5 ha tree-cover branch, relative abundance was lower where *DBH non-Euc* was equal to/less than, rather than greater than, 20.8% (0.012 ± 0.004 [17] versus 0.107 ± 0.022 [43]).

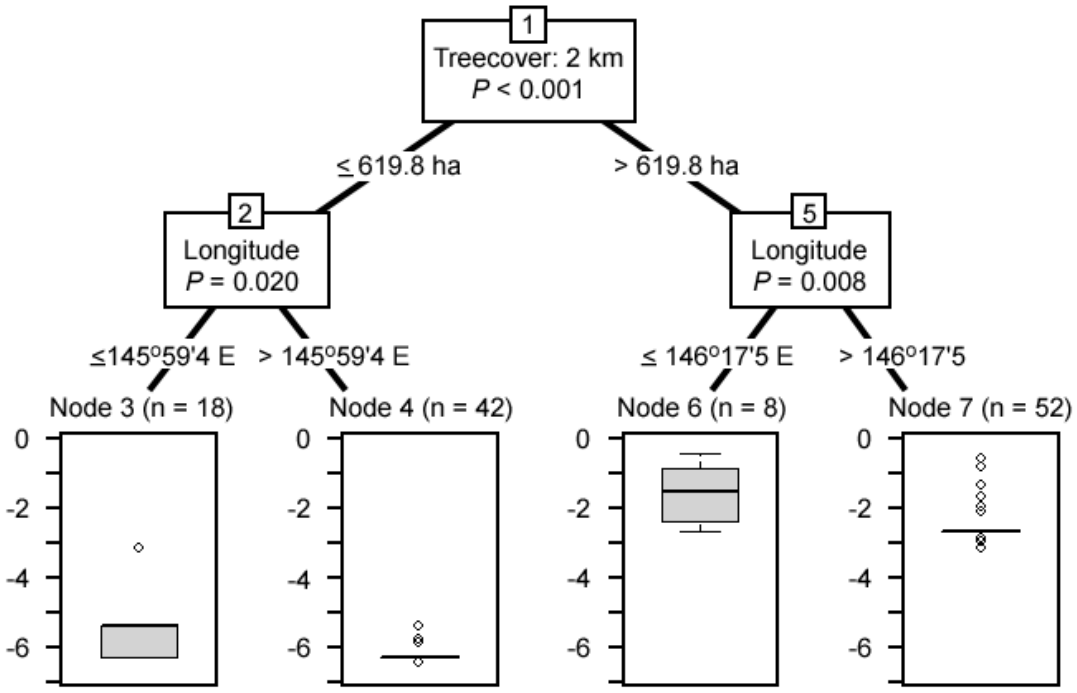

Figure 5. Conditional inference tree for *swainsonii'* relative abundance. *Longitude* is given in degrees. Other details are as in Figure 3.

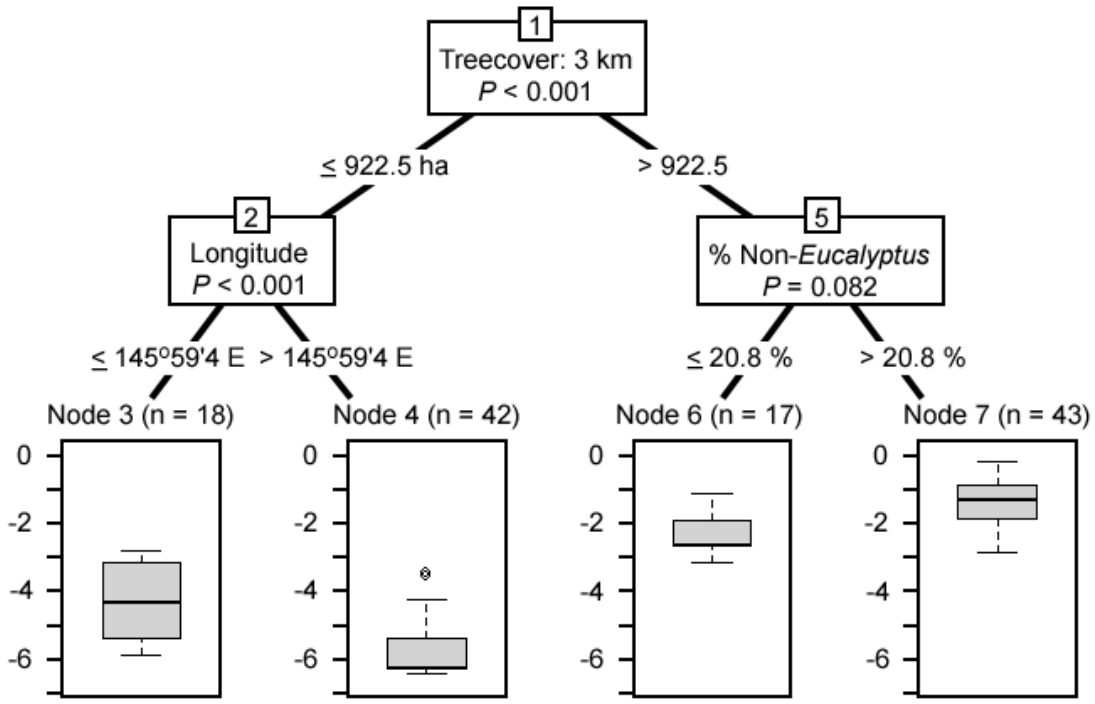

Figure 6. Conditional inference tree for *fuscipes'* relative abundance. Details are as in Figures 3 and 6.

Random forests analysis

A Random Forest procedure was used to determine the relative importance of all explanatory variables (Table 2 and Figure 7) and an iterative AIC model selection procedure to identify the most parsimonious subsets of explanatory variables (Table 3).

Two variables comprised the most influential (from Random Forests) and most parsimonious (from AIC) subset for *agilis* (log) N:L; in order of importance these were *Month* (March-August) and *Fragmentation (5 km)*. MSR was best explained by a subset of ten variables, the most important being *Sex*, *Abun. agili*s and *Month* (Mar-Aug). Seven variables best explained the relative abundance of *agilis*, the three most important being *Tree-cover (0.5 km)*, *By-catch. Abun*. and *Fragmentation (3 km)*. For *swainsonii'* relative abundance, the most parsimonious subset comprised 14 explanatory variables, the three most important being *Tree-cover (2 km)*, *Fragmentation (2 km)* and *By-catch abun*. Eleven variables made up the best subset for explaining *fuscipes'* abundance, the three most influential being *Tree-cover (3 km)*, *Fragmentation (2 km)* and *Leaf litter*.

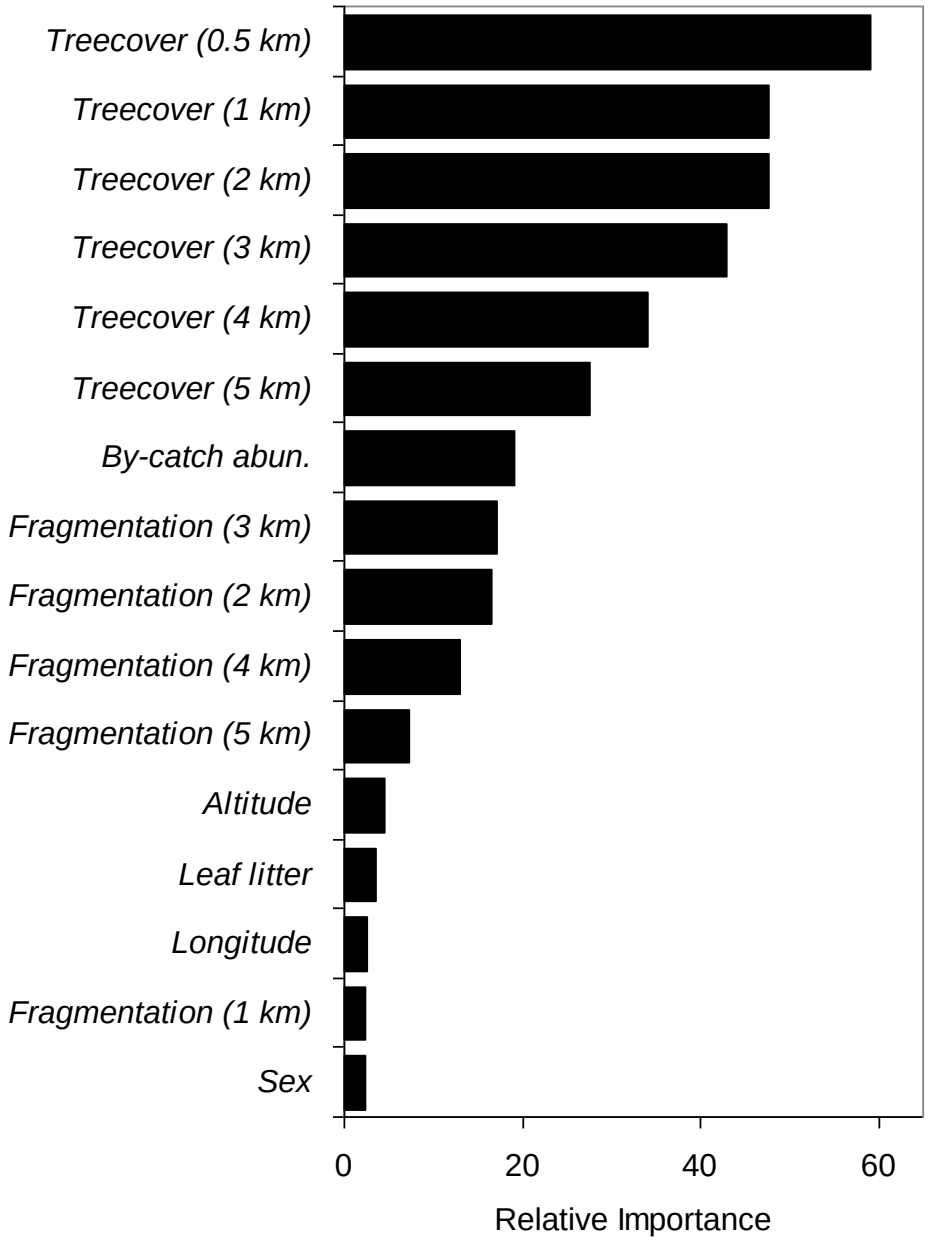

Figure 7. Graphical representation of the relative importance of environmental variables in explaining variation in *agilis'* relative abundance. The x axis does not have units and is a measure of the relative importance of explanatory variables. Nineteen additional variables have relative importance < 1.0 and are not shown.

Table 2. Relative importance of environmental variables (in italics) in explaining the variance in five response variables. Values do not have units and are a function of the number of times an explanatory variable was used to construct a conditional inference tree using a Random Forest procedure. All relative importance values were multiplied × 100 for clarity. Values in bold font represent the most influential and parsimonious subsets, as determined by iterative AIC-based model selection. The most parsimonious subset based on AIC modelling did not always agree with the most important variables as chosen by random forests analysis. This was likely because linear and tree-based models are fundamentally different structures. The difference highlights how different modelling approaches can lead to quite different conclusions.

Explanatory variables	N:L *agilis*	MSR *agilis*	Abun. *agilis*	Abun. *swainsonii*	Abun. *fuscipes*
Agilis abun.	0.01	**20.15**	NA	NA	NA
Altitude	0.16	**3.52**	**4.62**	**4.65**	**4.44**
Browse	0.03	-1.03	0.07	-0.02	-0.19
By-catch abun.	0.06	3.37	**19.02**	**8.42**	**7.41**
DBH % dead	0.02	-0.47	< 0.01	-0.07	0.06
DBH % non-Euc.	0.01	0.98	0.47	**0.86**	**5.31**
DBH median	< 0.01	**5.35**	0.32	< 0.01	0.18
DBH shape	0.01	0.06	0.25	< 0.01	0.16
Fragmentation (0.5 km)	< 0.01	-0.68	0.61	0.08	0.09
Fragmentation (1 km)	0.13	0.95	2.31	6.17	11.51
Fragmentation (2 km)	0.09	-0.07	16.51	**26.30**	**33.10**
Fragmentation (3 km)	0.11	1.56	**17.22**	18.95	24.55
Fragmentation (4 km)	0.14	0.90	13.05	12.25	17.09
Fragmentation (5 km)	**0.20**	0.69	7.24	6.10	7.70

Continued on next page

Table 2 continued.

Explanatory variables	N:L *agilis*	MSR *agilis*	Abun. *agilis*	Abun. *swainsonii*	Abun. *fuscipes*
Gully	< 0.01	-0.68	-0.02	-0.01	< 0.01
Latitude	0.04	0.10	0.16	**0.52**	0.16
Leaf litter	0.01	**14.24**	**3.45**	**4.37**	**7.86**
Log dens.	0.19	0.16	< 0.01	**0.05**	**1.83**
Longitude	0.03	1.67	**2.64**	**4.09**	**5.35**
Month	**1.79**	**16.32**	0.15	**1.01**	**0.70**
Predation index	< 0.01	0.42	0.52	**0.78**	0.37
Ridge	0.01	-0.30	-0.02	0.01	0.05
Sex	-0.01	**91.89**	**2.30**	NA	NA
Shrub dens.	0.01	**3.55**	0.04	-0.01	-0.04
Shrub dom.	0.04	2.44	0.35	< 0.01	0.01
Shrub even.	< 0.01	0.29	0.40	0.03	-0.09
Shrub rich.	0.02	**8.09**	0.02	**0.03**	< 0.01
Tree dens.	0.01	1.67	0.16	**-0.01**	0.05
Tree dens. (L)	0.16	**12.89**	0.17	0.30	**1.36**
Tree rich.	0.07	0.61	0.56	**1.65**	**4.80**
Tree-cover (0.5 km)	0.07	**16.07**	**59.06**	53.43	47.55
Tree-cover (1 km)	0.05	9.58	47.76	53.07	57.89
Tree-cover (2 km)	0.06	2.57	47.61	**112.74**	99.63
Tree-cover (3 km)	0.05	4.19	43.01	100.15	**117.92**
Tree-cover (4 km)	0.05	5.98	34.13	90.56	94.40
Tree-cover (5 km)	0.06	3.85	27.52	71.10	84.20

Table 3. The results of an iterative AIC-based procedure used to select the most parsimonious subsets of explanatory variables (in italics) for each response variable. Importance is the rank allocated to each variable in a Random Forests analysis. Correlation coefficients (*r*) are also given. For *Sex,* the correlation is for male (=1) and female (=0). For *Month*, the correlation is based on March = 3, April = 4 to August = 8. Note that the *r* = 0.01 for *agilis* abundance and Month is correct. If a variable has a high relative importance but weak correlation then the variable is probably acting as a sort of explanatory covariate adding to the explanatory power of other environmental variables.

	Importance	*r*
(log) N:L *agilis*:		
Month (March-August)	1	0.61
Fragmentation (5 km)	2	0.21
MSR *agilis*:		
Sex (male)	1	0.39
Agilis abun.	2	-0.26
Month (March-August)	3	0.18
Tree-cover (0.5 km)	4	-0.21
Leaf litter	5	-0.23
Tree dens. (L)	6	0.24
Shrub rich.	7	0.15
DBH median	8	-0.14
Shrub dens.	9	0.08
Altitude	10	-0.06

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Table 3 continued.

	Importance	*r*
(log)RA *agilis*:		
Tree-cover (0.5 km)	1	0.70
By-catch abun.	2	0.64
Fragmentation (3 km)	3	-0.57
Altitude	4	0.43
Leaf litter	5	0.53
Longitude	6	0.21
Sex (male)	7	0.09
(log)RA *swainsonii*:		
Tree-cover (2 km)	1	0.90
Fragmentation (2 km)	2	-0.70
By-catch	3	0.63
Altitude	4	0.59
Leaf litter	5	0.62
Longitude	6	0.36
Tree rich.	7	0.52
Month (March-August)	8	0.01
DBH % Non-Euc	9	0.42
Predation index	10	-0.38
Latitude	11	0.13
Tree dens. (L)	12	-0.35
Log dens.	13	-0.25
Shrub rich.	14	0.07

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Table 3 continued.

	Importance	*r*
(log)RA fuscipes:		
Tree-cover (3 km)	1	0.83
Fragmentation (2 km)	2	-0.69
Leaf litter	3	0.64
By-catch abun.	4	0.59
Longitude	5	0.25
DBH % Non-Euc	6	0.53
Tree rich.	7	0.57
Altitude	8	0.55
Log dens.	9	-0.28
Tree dens. (L)	10	-0.40
Month (March-August)	11	-0.13

Discussion

Variables influencing relative abundance of small mammals

The strongest influences on relative abundance in the three mammal species studied were the extent of native vegetation in the landscape and, to a lesser extent, the degree of fragmentation of native tree-cover (although fragmentation appeared to have a stronger influence on *A. swainsonni* and *R. fuscipes* than on *A. agilis*). Thus habitat loss *per se* explained the patterns of relative abundance of the mammal species better than habitat fragmentation. The results also implied that the effects of habitat loss were not as uninteresting as is often assumed; in a review of publications in conservation biology, only 2% of studies directly examined native vegetation loss as a threatening process (Fazey et al. 2005)--the implication being that most conservation researchers don't consider habitat loss a worthwhile research area. In this study, it appeared that larger-bodied species responded to habitat loss over progressively larger areas, although admittedly this was for a sample of only three species. The relative abundance of *agilis*, the smallest species at 16-44 g, was associated with *Tree-cover (0.5 km)*, that of *swainsonii* (38-170 g) with *Tree-cover (2 km)* and that of *fuscipes*, the largest species at 50-225 g, with *Tree-cover (3 km)*. The intriguing implication of this result is that loss of habitat up to 3 km away from a study site appeared to negatively affect small mammal relative abundance. Significant correlations between a species' body size and the area of habitat required to support a stable population of that species that is not substantially at risk of extinction (what we term here a 'healthy' population) are thought to be widespread (Peters & Raelson 1984; Robinson & Redford 1986), but empirical evidence for them, at least in small mammals, is largely circumstantial e.g. larger areas tend to have more species of a given taxonomic group (Connor & McCoy 1979), that larger species tend to occur at lower densities (Peters & Raelson 1984), that individuals of larger species typically have

larger home ranges (Kelt & Van Vuren 2001) and that larger taxa are often at greater risk of extinction in fragmented habitats (Turner 1996). Moreover, the relationship is potentially confounded by trophic level and variation in landscape productivity (Wright 1983) or distribution of essential 'keystone' habitat structures (roosting sites, water sources, woody debris etc) (Tews et al. 2004), so that it is not always easily identifiable.

The existence of a relationship between population relative abundance and habitat fragmentation was supported for *agilis* by the full CIT model and for all three mammal species by the Random Forests model. For *fuscipes* and *swainsonii*, fragmentation was the second, and for *agilis* the third, most important environmental variable explaining relative abundance. As expected, all of the abundance relationships with fragmentation were negative.

By-catch Abun., *Altitude* and *Leaf litter* also appeared to strongly influence relative abundance in the three mammal species. However, their higher relative abundances where by-catch abundance was higher may simply have reflected a situation in which habitat that was generally less anthropogenically-disturbed benefited the whole native, small mammal assemblage. Such assemblage-level effects of anthropogenic fragmentation and degradation are well known (Andrén 1994). Unexpectedly, higher altitude sites had greater relative abundances of all three species. None of the species are considered alpine specialists and they can be locally common in suitable habitat close to sea-level (Menkhorst & Knight 2004). A possible explanation is that in the study area, higher altitude sites were less accessible to people and thus less anthropogenically-disturbed. On public land, anthropogenic activity (e.g. firewood collection, 4× wheel drive vehicle and motorcycle traffic) appeared to be less intensive at higher altitudes (C. Johnstone, personal observation).

On the other hand, an association between the extent and depth of leaf litter and small mammal abundance has been documented previously in Australia (Dunstan & Fox

1996), although it is usually assumed to relate to foraging resource abundance and 'bottom-up' population regulation (i.e. less leaf litter results in a lower arthropod abundance which in turn results in nutritional stress and lower abundance in omnivorous and insectivorous small mammals) (Dunstan & Fox 1996). However, in reality 'bottom-up' regulation of small mammal population density is generally not well supported empirically (Krebs 2009), and in *agilis* at least, the MSR results are not consistent with this interpretation. Leaf litter extent and depth certainly were associated with *agilis* MSR, but the relationship was *negative* i.e. deeper and more extensive leaf litter was associated with *lower* estimated fat reserves. If *agilis* populations were food-limited, low relative abundance and poor body condition should have been co-correlated and both should have been observed in food-poor sites (Suorsa et al. 2004). This interpretation assumes that MSR is accurately indexing fat reserves, which requires validation. Also, as in most studies on body condition of free-living vertebrates, the interpretation assumes that observed differences in MSR are more likely to be due to food availability than differences in activity. This second possibility is plausible and not often discussed in ecophysiological literature, and it could be investigated using radio tracking of *agilis* in fragments and pseudofragments (Marchesan & Carthew 2008). The reason why leaf litter appeared to influence *agilis'* abundance is therefore unknown. This finding illustrates why it is important to measure performance indicators, such as MSR and N:L, wherever possible, and not rely solely on population density, fecundity or survivorship estimates when addressing ecological questions about possible threatening processes and/or the conservation management of vertebrates. It could be that leaf litter quality in our study was a surrogate for either forest stand health or canopy density or that another unidentified environmental factor was involved. Experimental manipulation of field sites is difficult, but possible, and could be used to address this issue (Mac Nally & Horrocks 2002).

The finding that *agilis* males had higher estimated fat reserves where overall relative abundance of the species was lower indicated the likely occurrence of intra-specific (and possibly even intra-sexual) competition for food. This is not surprising, given that males with greater fuel reserves have a significant reproductive advantage (Kraaijeveld-Smit et al. 2003; Kraaijeveld-Smit et al. 2002c). Perhaps female MSR might reveal a similar trend during a period when energy requirements are higher for females (e.g. during lactation).

Variables influencing the mammalian neutrophil-to-lymphocyte ratio

One of the chief advantages of using tree-based models to infer relationships in ecological studies is that complex interactions among explanatory variables become easier to interpret. Main effects in linear models cannot be interpreted without first interpreting interactions (Zuur et al. 2007), but where three- or four-way interactions occur, most authors eschew interpretation. The tree-based model of the physiological stress index in *agilis*, (log) N:L, illustrated this point well, because it included several complex interactions that would otherwise be difficult to interpret.

The N:L ratio increased from March to August, as reported previously (Cheal et al. 1976). The agile antechinus is a rare example of a largely semelparous mammal (Braithwaite & Lee 1979). Young are weaned in the late Austral summer (January), soon after which there is a male-biased dispersal event (Cockburn et al. 1985b). Adults nest socially until the late Austral winter (August in the study area), when a synchronized, competitive, breeding rut occurs (Lazenby-Cohen & Cockburn 1988) that has features of lek behaviour (Kraaijeveld-Smit et al. 2002b). Successful mating is mostly achieved by the larger males with bigger fat reserves and those that mate closer to the time of female oestrous (Kraaijeveld-Smit et al. 2003; Kraaijeveld-Smit et al. 2002c). After breeding, there is a complete male die-off (Braithwaite & Lee 1979), and after weaning their young, only ~15% of females live to reproduce in a second year (Cockburn et al. 1985a). N:L

increased in the months between the annual dispersal event and the breeding season, possibly reflecting the transition from the warmer summer and autumn months of March and April, with their greater food abundance, to winter (June-August), when thermoregulation is more metabolically-demanding and invertebrate prey abundance is lower (Banks & Dickman 2000). In March and April, N:L was higher where overall *agilis'* relative abundance was lower. Conceivably, where survivorship of sub-adults was lower, acute stressors (such as local predator activity; Stokes et al. 2004) were more intense.

During May and June, *agilis'* N:L was negatively associated with habitat fragmentation. Other authors have investigated the relationship between habitat fragmentation and HPA axis-mediated stress in terrestrial vertebrates with equivocal results (Martínez-Mota et al. 2007; Mazerolle & Hobson 2002; Suorsa et al. 2004). Conceivably the relationship may be either environment- or taxon-specific. We have reported elsewhere evidence of a broad, positive association between habitat fragmentation and a higher N:L in *agilis* (Johnstone et al. *in review*). Interestingly in the present study there was strong evidence of this relationship only during a two-month period between dispersal and the subsequent social re-organization (Cockburn & Lazenby-Cohen 1992). Fragmentation was no longer important in winter (Jul-Aug), a period when psychosocial stress (prior to breeding) or stressors associated with winter conditions were likely to have been more substantial influences on an individual's wellbeing than stressors associated with forest fragmentation i.e. edge effects, novel barriers to dispersal or foraging and a greater abundance of invasive generalists etc. (Fischer & Lindenmayer 2007). Although environmental stressors of wild vertebrates sometimes act synergistically (e.g. stress due to predators and food scarcity is multiplicative, not additive, (Boonstra et al. 1998; Zanette et al. 2003), the unusual breeding system of *agilis* may mean that it is adaptive to avoid the negative and often reproduction-suppressing effects of HPA axis-mediated chronic stress in the breeding

season (Wingfield & Sapolsky 2003). There is evidence for such a physiological mechanism in female *agilis*, as least as far as pertains social stress (Naylor et al. 2008). The situation in males is more complex, as testosterone, cortisol and cortisol binding globulins appear to have cross-regulatory functions that may or may not mitigate the effects of environmental stress at different times in the life-history (Naylor et al. 2008).

Conservation implications

From a conservation perspective, the investigation raised an important cautionary note; the three small mammal species appeared to respond to anthropogenic habitat loss and fragmentation at landscape scales substantially larger than the area that an individual would occupy in its lifetime. Habitat loss up to 3 km associated with differences in small mammal abundance. This study provides no evidence of cause and effect, but plausibly, deforestation up to 3 km from a study site could have had 'flow on' effects that decreased small mammal abundance. Moreover, the effects of large-scale habitat loss and, to a lesser extent, fragmentation influenced relative abundance more than variation in the immediate local environment. For the particular species and area examined here at least, restoration of forest reserves may not increase a species' local abundance if native vegetation levels in the general area are below those needed to sustain 'healthy' populations. The method of analysis used here could be employed to address the question that still concerns conservation managers with respect to reserve area, namely 'How much is enough?' (Fahrig 2001). For *agilis*, which had a higher relative abundance where *Tree-cover (0.5 km)* was ~ 75 ha or more, the establishment and/or management of suitable conservation reserves seems feasible. However, the high-abundance 'thresholds' of ~ 600 ha of forest within 2 km (i.e. ~ 50% tree-cover) and ~ 900 ha within 3 km (i.e. ~ 30% tree-cover) of sites occupied by *swainsonii* and *fuscipes,* respectively, are substantial habitat requirements for small mammals that could be harder to protect. It is of concern

that the three mammal species, considered locally common in some areas (Menkhorst & Knight 2004), are generally not the focus of any conservation effort in the study area, throughout most of which *fuscipes* and *swasinonii* occurred at abundances that were clearly well below the supposed 'norm' recorded in the least-disturbed sites. Small and isolated populations are susceptible to stochastic threats (Fischer & Lindenmayer 2007), but the gradual decline to extinction of populations after fragmentation and degradation of their habitat may take years or decades (Diamond et al. 1987). It is easy to overlook such a cryptic decline until it is too late.

Acknowledgments

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Chapter Seven

Discussion

7.0 Introduction

Anthropogenic habitat fragmentation is well known to be associated with loss of native species from the environment, yet the underlying mechanisms are not well understood (Andrén 1994; Fischer & Lindenmayer 2007; Turner 1996). Through a comparison of agile antechinus (*Antechinus agilis*) populations living in anthropogenically fragmented and relatively undisturbed *Eucalyptus* forest in an area of south-east Australia, this study examined some putative mechanisms that may contribute to the decline of this and possibly other vertebrates after fragmentation of their habitat. In particular, the study examined whether chronic physiological stress may be one such mechanism (Martínez-Mota et al. 2007). If this was the case, the prediction was that indices of physiological stress (e.g. the neutrophil-lymphocyte ratio, N:L) would, on average, be higher in populations living in fragmented and disturbed forest than in those in similar but undisturbed, continuous forest. I also examined a range of other indices of condition and health status (e.g. estimated fat reserves, parasite load, indicators of regenerative anaemia) in order to establish a more comprehensive picture of the health of agile antechinus populations. However, habitat fragmentation is not a simple process and it is often accompanied by habitat modification (e.g. through grazing or loss of other native species; Diamond et al. 1987; Fischer & Lindenmayer 2007). Comparisons of indices of population stress and condition with local environmental variables (e.g. microhabitat heterogeneity) and features of the fragmented landscape (e.g. fragment core area, proportion of edge habitat) were designed to identify some putative environmental mechanisms that could be contributing to poor health status in agile antechinus populations in anthropogenically fragmented habitats.

7.1 Summary of the main findings

The following is a summary of the results presented in this thesis, with some discussion where relevant. Although some results were provided for two other native small mammal species, the bush rat (*Rattus fuscipes*) and dusky antechinus (*Antechinus swainsonii*), this discussion focuses on the results for agile antechinus, as this was the study species of primary interest.

7.1.1 A reminder about abbreviations and chapters

- Hb Haemoglobin (grams per litre)
- Hct Haematocrit (packed cell volume to plasma volume ratio)
- HHR Haemoglobin-haematocrit residuals (index of health status)
- MCH Mean red blood cell haemoglobin
- MCHC Mean red blood cell haemoglobin concentration
- MCV Mean red blood cell volume
- MSR Mass-size residuals (index of estimated fat reserves)
- N:L Neutrophil-lymphocyte ratio (index of physiological stress)
- RBC Red blood cell count (cells per litre)
- WBC White blood cell count (cells per litre)

7.1.1.1 Data chapters

The thesis data chapters hereafter referred to are:

Chapter 3: Are haematological indicators of stress and poor condition associated with habitat fragmentation in the agile antechinus?

Chapter 4: Impact of anthropogenic habitat fragmentation on population health in a small, carnivorous marsupial

Chapter 5: Response of the agile antechinus to habitat edge, configuration and condition in fragmented forest

Chapter 6: Effects of habitat loss, fragmentation and degradation on mammal abundance and condition: analysis with tree-based statistical modelling

7.1.2 Relative abundance

Agile antechinus populations had lower relative abundances in fragmented than in comparable continuous forest (Chapter 4). The underlying correlates appeared to be a smaller forest fragment core area and possibly a negative effect of edge habitat on female abundance (Chapter 5) (Figure 1). Tree-based modelling suggested that the most important effect on relative abundance of agile antechinus was loss of native vegetation cover within a 0.5 km radius of a site (Chapter 6). Of the other examined environmental factors that might feasibly be the focus of conservation management, at least some had important apparent influences on agile antechinus abundance. The three most important were a negative abundance association with fragmentation (2 km radius) and positive associations with leaf litter depth and extent. For dusky antechinus, the most important abundance relationships were a positive association with native treecover extent (2 km radius), negative association of fragmentation (2 km radius) and a positive association with leaf litter depth and extent. Important factors associating with bush rat abundance were a positive relationship with native treecover extent (3 km radius), a negative association with fragmentation (2 km radius) and a positive association with leaf litter depth and extent. It is clear that habitat loss (native treecover), fragmentation and degradation (in particular the loss of leaf litter depth and extent) may have had important independent influences on the three study species. Striking aspects of the findings was the broad agreement among findings for the three species and that habitat loss was consistently more important than habitat fragmentation *per se*. That habitat loss up to 3 km from a site could have been negatively affecting small mammal abundance (in bush rats) was unexpected and a potential concern for conservation of native small mammals in the study area. In the conservation biology literature there is relatively little focus on habitat loss as a factor underlying native vertebrate population decline, whereas the

results here suggest that habitat loss is not only important, but a potentially interesting and informative field of study for conservation management of native small mammal species.

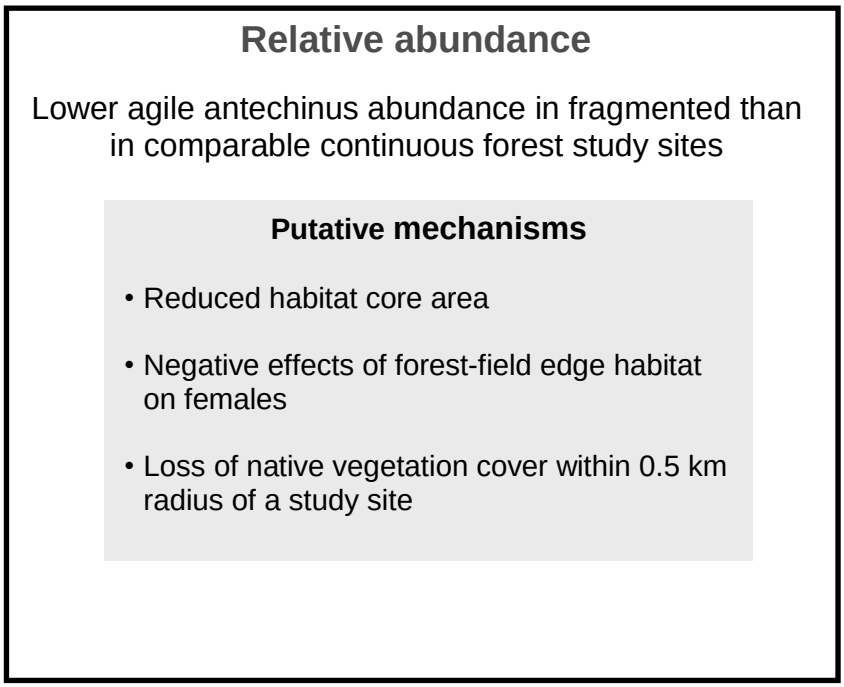

Fig. 1. Summary of some possible effects of anthropogenic habitat fragmentation on agile antechinus' relative abundance.

7.1.3 Estimated fat reserves

An unexpected finding was that estimated fat reserves were, on average, greater in populations living in fragmented than in comparable continuous forest study sites (Chapter 4) (Figure 2).

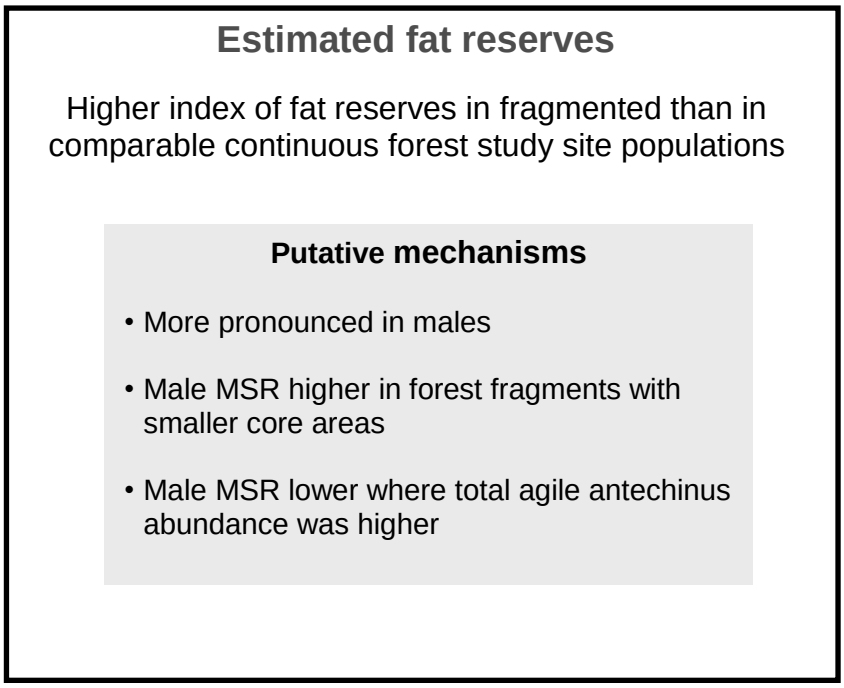

Fig. 2. Summary of anthropogenic habitat fragmentation effects on agile antechinus' estimated fat reserves as indexed by mass-size residuals (MSR).

One immediate implication of this was that nutritional stress probably did not cause the lower agile antechinus abundances in fragmented study sites. The effect was more pronounced in males than in females (Chapters 5 and 6). Male estimated fat reserves were also greater in fragments that had smaller core areas (Chapter 5), a trend that was opposite to the effect of fragment core area on relative abundance. Tree-based, predictive modelling suggested that male estimated fat reserves were best explained by a negative relationship with total agile antechinus relative abundance in a site (Chapter 6). For this reason, it appeared that the effect of habitat fragmentation on fat reserves probably acted indirectly through intraspecific competition. Where agile antechinus relative abundance was lower, presumably *per capita* foraging resources were greater and male estimated fat reserves were consequently greater. However, another possibility is that activity was higher in pseudofragment sites. Plausibly, lower local abundances of

introduced predators could have associated with differences in activity budgets, so that agile antechinus living in undisturbed forest were more active and so had lower fat reserves. As a caveat, ideally MSR needs verification against the actual percentage of lipids in dry mass for the study species. Similar validation of MSR has been done with multiple small mammal species (Peig & Green 2009; Schulte-Hostedde et al. 2005), but if this were undertaken with agile antechinus it would to my knowledge be the first time that MSR is validated in this way in a small marsupial.

7.1.4 Parasite infection indices

A simplified count of ectoparasites was used to estimate external arthropod infection level. One blood metric examined, the percentage of eosinophils in circulating blood, is also probably a functional index of parasite load, as eosinophil abundance is generally strongly and positively associated with metazoan parasite infections (Bignold 1995; Rothenberg 1998). Ectoparasite counts were greater and eosinophil percentages were higher in agile antechinus populations living in fragmented than in continuous forest sites (Chapters 2 and 3). This supports the possibility that metazoan parasite infections were more severe in populations inhabiting forest fragments than continuous forest, but I did not find any significant correlations between these two parasite metrics and any landscape configuration, habitat complexity or vegetation descriptors. Thus the reason for the observed relationship between fragmentation and greater parasite loads in agile antechinus remains unknown. Some possible speculative explanations include: 1) greater abundance of agile antechinus in forest fragments that functioned as disease reservoirs, 2) greater populations of other species in forest fragments such as the European red fox that could have functioned as a cross-species disease reservoir (*cf* possible links or lack thereof between sarcoptic mange in wombats (*Vombatus ursinus*) and red foxes; Martin et al. 1998; May & Norton 1996), 3) frequent re-use of infected nest sites due to scarcity of

this resource in fragments (Cockburn & Lazenby-Cohen 1992) or 4) assuming that individuals in fragments were generally in poorer condition, they may consequentially have been more susceptible to parasite infection (Beldomenico et al. 2008b). Other researchers have investigated effects of habitat fragmentation on parasite loads in vertebrates, but results have been equivocal (Barnard et al. 2003; Chapman et al. 2006; Püttker et al. 2008).

7.1.5 Erythrocyte indices of condition

Erythrocyte metrics were used to index health status in agile antechinus, with particular focus on those that might be used to identify regenerative anaemia (RBC, MCV and MCH; Chapter 3, and HHR; Chapter 5). Populations living in forest fragments had a higher RBC and lower MCH than those in continuous forest (Chapter 3) (figure 3), which was interpreted as possibly reflecting the release of reticulocytes from bone marrow into circulating blood. If correct, this would be a likely indicator of regenerative anaemia, a condition associated with chronic physiological stress, frequent blood loss and/or heavy parasite burdens (Colombelli-Négrel & Kleindorfer 2008; Fisher & Crook 1962). As reticulocytes are larger than mature red blood cells (Lewis et al. 2006), MCV should have been larger in fragment than in control populations if regenerative anaemia was occurring, but this trend was lacking. Interestingly, at least one study on the effects of frequent and prolonged stress (physical restraint) on a mammal (*Rattus rattus*) reported a similar pattern to that which I observed, namely an elevated RBC (after 9 days of stress treatment) but a reduced MCV (after 21 days of stress treatment) (Teague et al. 2007). Comparison of erythrocyte metrics with landscape, habitat and vegetation variables indicated that male HHR were lower in forest fragments in which microhabitat heterogeneity was lower and there were fewer shrubs, logs and native trees that were not *Eucalyptus* species (Chapter 5). This suggested that the erythrocyte indices of health status

in the study species may have been responding to habitat quality and/or complexity, rather than to habitat area, degree of dissection or other landscape level factors.

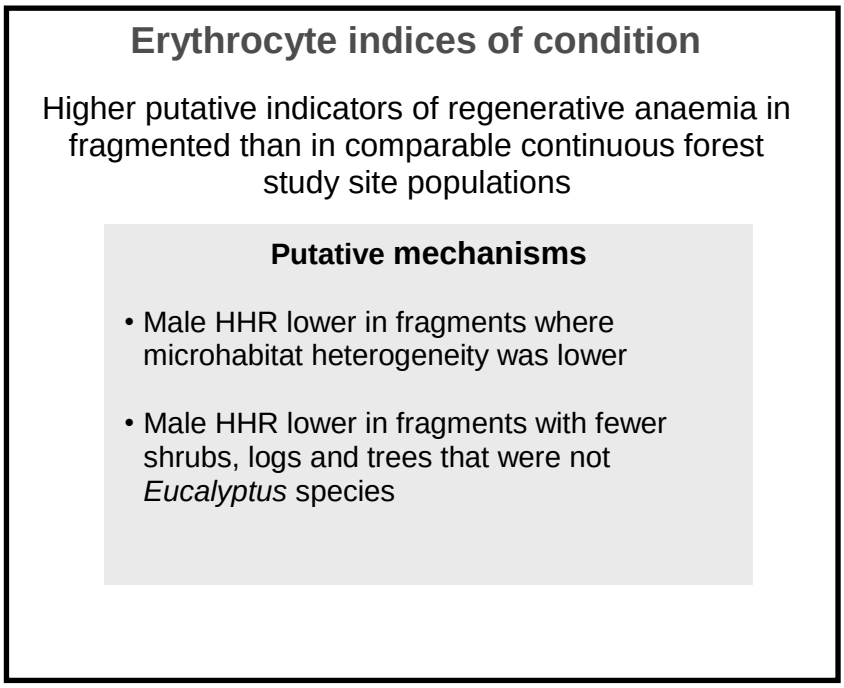

Fig. 3. Summary of anthropogenic habitat fragmentation effects on four erythrocyte indicators of condition in agile antechinus.

7.1.6 Leukocyte indices of chronic stress

A metric considered a good index of chronic physiological stress, N:L (Davis et al. 2008), was higher in agile antechinus living in forest fragments than in those in continuous forest (Chapter 3) (figure 4). This trend was more marked for males than females (Chapter 3). In contrast, WBC was lower in fragment than in continuous forest populations (Chapter 3), but this variable can be difficult to interpret (Davis et al. 2008). However, total and differential leukocyte counts can be used to derive estimates of the concentrations of circulating immune-cell types and these can be more informative than N:L alone (Masello et al. 2009). Neutrophil concentrations did not respond to fragmentation, but male lymphocyte concentrations were significantly lower in fragments

than in comparative continuous forest sites (Chapter 3). Trafficking of lymphocytes away from peripheral blood into compartments such as the lymph nodes, spleen and skin where they will be more useful in the event of injury is the most commonly cited mechanism for the observable positive effect of stress on N:L (Davis et al. 2008; Dhabhar & McEwen 1997; Masello et al. 2009). The implication therefore seems to be that the higher N:L in fragment populations of agile antechinus may have been due to stress-hormone mediated lymphopenia in the peripheral blood. However, male N:L was not convincingly related to any landscape, habitat or vegetation variable in forest fragments. Female N:L was higher in fragments that had a greater proportion of edge habitat. This result was especially interesting, as female relative abundance was also negatively affected by edge habitat (Figure 1). Another intriguing result was that neutrophil concentrations increased during the sampling period from March-August (Chapter 5). This was interpreted as a possible physiological mechanism that increased innate immunity in anticipation of synchronised breeding in August, when risk of disease transmission is most likely maximal.

A difficulty with the interpretation of the leukocyte count results is that captured agile antechinus were almost certainly experiencing a stress response to trapping (Dhabhar & McEwen 1997; Fletcher & Boonstra 2006; Lynn & Porter 2008). The implications of this have been discussed at length in the empirical chapters, and the interpretation provided here is necessary tentative. This confounding element of the study could not have been easily precluded, for ethical and logistic reasons (see Chapter 3 for a full discussion of these). Other independent indicators of population condition (e.g. fat stores, erythrocyte and parasite variables) that were not expected to be affected by time confined in a trap were included specifically because there was concern early in the study that trapping could have rendered leukocyte indicators of stress difficult to interpret.

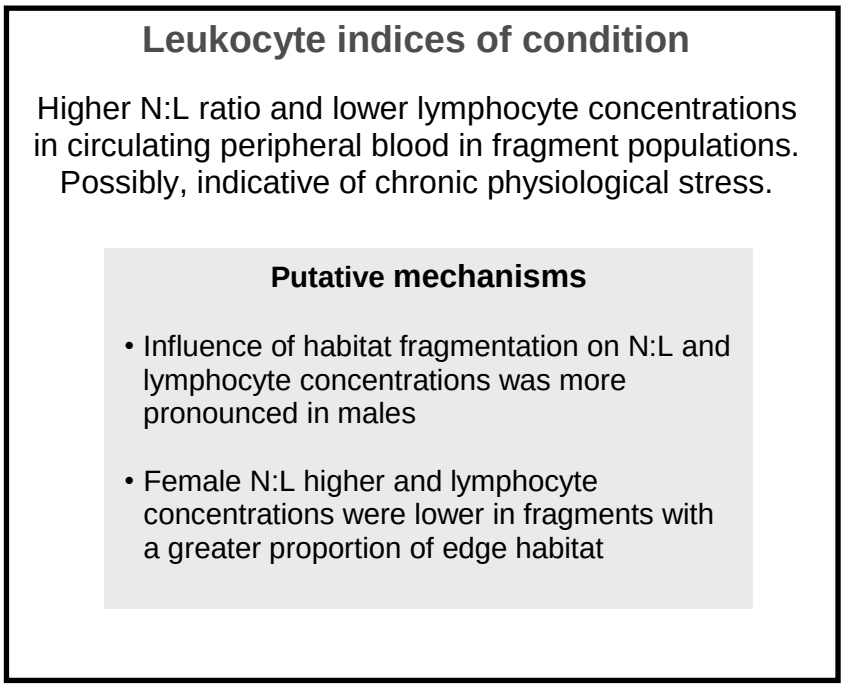

Fig. 4. Summary of anthropogenic habitat fragmentation effects on leukocyte indicators of chronic physiological stress in agile antechinus.

7.2 Synthesis, limitations and future directions

The influence of anthropogenic habitat fragmentation and the associated processes of habitat loss and degradation appeared to have had markedly negative effects on the agile antechinus populations studied. Relative abundances were substantially lower and parasite load indices higher in fragmented than continuous forest and there was evidence that fragment populations were negatively affected by regenerative anaemia (possibly due to chronic stress; Teague et al. 2007). If we can assume that the leukocyte indices of stress were not seriously confounded by trapping stress, there was also evidence

supporting the proposition that chronic physiological stress was greater in populations living in fragmented than continuous forest. This is a conservation management concern because chronic physiological stress can be associated with disease states that reduce reproductive investment, fecundity and survivorship in vertebrates (Sapolsky et al. 2000; Simpkiss & Devine 2003; Tsigos & Chrousos 2002). This finding is central to the study's initial concept. Physiological stress indicators have potential uses as bioindicators that can help us to identify population decline before the effects become obvious at the level of population density (Wikelski & Cooke 2006). Another implication is that conversation management should take stressfulness of the environment into account when planning breeding, restoration or reintroduction programmes to a greater extent than is currently the case. One interesting approach in further research in this field would be to use other less frequently employed methods of identifying stress in vertebrates. For example, long-term chronic stress is associated with accelerated aging processes that can cause contraction of collagen (e.g. in the tail; Austad 1996; Bradley 2003). Accelerated telomere shortening is now reasonably well documented in chronically-stressed humans (Epel et al. 2004), and a novel approach of ecological genetics using telomere indicators of chronic stress to identify environmental stressors is plausible and as yet largely unexplored.

Against prediction, fat reserves in males were higher in fragment than continuous forest populations. The most likely explanation was weaker competition for food in fragments, but it is worthwhile noting that chronic stress is often associated with elevated glucocorticoid levels, increased appetite and elevated rates of lipid deposition (Sapolsky et al. 2000). A highly conjectural, but physiologically plausible explanation for the observed MSR trend was that male agile antechinus in fragments were displaying stress-induced hyperphagia and fat accumulation.

Although the study was designed to be reasonably comprehensive in its examination of the influence of landscape, habitat and vegetation, there remain some intriguing missing pieces to the agile antechinus puzzle. Parasite loads were almost certainly higher in fragment than continuous forest populations, but the results do not provide any clear evidence for the underlying reason. The influence of fragmentation on N:L ratio was considerably more marked in males than in females and yet male N:L did not show any convincing relationship with the measured features of the landscape, habitat heterogeneity or vegetation variables. Males apparently responded by exhibiting physiological stress induced by a feature of the anthropogenically-fragmented environment that was not measured in this study.

Species' distributions are determined by interactions with either or both of the physical environment and other species (Diamond 1986). In this study, the focus was primarily on the former, particularly landscape configuration (e.g. core fragment area or proportion of edges), vegetation characteristics (e.g. plant species richness, woody debris, tree DBH) and topography (e.g. presence or absence of gullies, altitude). The animal species that co-habited the environment with the agile antechinus were not studied in detail. Competition with invasive generalists (the feral house mouse, *Mus musculus* and black rat, *Rattus rattus*) or predation by introduced mammals (feral cats, *Felis catus* and European red foxes, *Vulpes vulpes*) could have influenced agile antechinus' distribution, as could differences in richness or abundance of arthropod prey species among sites. Certainly, foxes occur at higher densities in forest landscapes fragmented by agriculture, probably because they gain a subsidy from human activity (e.g. farming, scavenging from road kill, higher rabbit (*Oryctolagus cuniculus*) densities in pastured landscapes; Fischer & Lindenmayer 2007; May & Norton 1996). I attempted to index terrestrial predator activity at study sites (Chapter 6), but the data were insufficient for a rigorous analysis. There is evidence that when foxes were introduced to Australia, nocturnal avian

predators' (e.g. sooty owls, *Tyco tenebricosa*) diets altered from prey dominated by terrestrial marsupials (e.g. small macropods, such as *Isoodon* or *Potorou*s species) to arboreal/scansorial species, such as the agile antechinus (Bilney et al. 2006). A speculative but plausible theory could be that predation by foxes, subsequent elevated predation pressure by avian predators and loss of shrub cover due to edge effects or human activity (e.g. livestock browsing) could act in concert to negatively affect populations of a small marsupial such as the agile antechinus in an anthropogenically-fragmented habitat. Although this sort of 'higher order' effect of habitat fragmentation (Turner 1996) on native species is difficult to test, some studies have demonstrated that it is possible to distinguish the synergistic effects of different aspects of habitat fragmentation and disturbance (Ewers & Didham 2005; Hobbs 2001).

Most studies that have examined the effects of habitat fragmentation or degradation on a small mammal have focused on the same types of landscape and vegetation variables as those examined here (e.g. Banks et al. 2005a; Holland & Bennett 2009; Kelly & Bennett 2008; e.g. Mac Nally & Horrocks 2002). This approach has provided valuable insight into the ecological processes that could affect native small mammal populations, but clearly effort also needs to be invested in understanding fragmentation influences on relationships among animals. In this connection, surveys of arthropod diversity or abundance, experimental food supplementation, use of camera traps or other methods of surveying terrestrial predator activity and studies on foraging bout duration in small mammals could all provide useful insights (Banks & Dickman 2000; Brown 1988; Sutherland & Predavec 1999; Wilson & Delahay 2001). One possible approach would be to examine 'giving up density', the point when a forager abandons experimentally provisioned food in favour of shelter, as this is often closely linked to local predator activity levels (Brown 1988).

Some of the results obtained here differed somewhat from findings in other studies of *Antechinus* species. Most conspicuously, I found little evidence that habitat complexity or heterogeneity strongly influenced agile antechinus' relative abundance, whereas other authors have reported strong effects of these variables on *Antechinus* species' site occupancy and abundance (Garden et al. 2007; Holland & Bennett 2009; Kelly & Bennett 2008; Knight & Fox 2000). A possible reason for this is differences in the sampling environment. In one study of agile antechinus in a fragmented environment, precipitation in the driest quarter of the year (mm) was reported to be a more important factor determining site occupancy than any measured vegetation, landscape or habitat complexity variable (higher rainfall = greater probability of occurrence; Claridge et al. 2008). Disparities in local climate may have a strong effect on how the agile antechinus responds to other aspects of its environment, such as fragmentation. Agile antechinus are restricted to *Eucalyptus* forest, but have a relatively broad distribution and occur in a variety of native *Eucalyptus* forest types (Sumner & Dickman 1998). This species (or a similarly distributed native vertebrate) could be used as the basis for a comparative study in which the influences of habitat fragmentation in regions with different habitat types or rainfall regimes could be explored. This would go to the heart of current questions about whether theory concerning the relative negative importance of habitat loss, degradation or isolation effects in an anthropogenically-fragmented environment can be generalized geographically for a single species, let alone multiple species (Fischer & Lindenmayer 2007). In the present study area a comparison of fragmentation effects in different ecological vegetation classes (e.g. heathy woodlands, coastal scrubs, wet or damp forest, lowland forest; Davies et al. 2002) would be possible. Assessment of population responses to fragmentation in more geographically diverse sites where agile antechinus occurs, such as in the coastal plains of south-western Victoria (Bennett 1990a), the Otway Ranges (Beckman et al. 2007) and East Gippsland (Claridge et al. 2008), would be

logistically difficult, but potentially very informative about generalized anthropogenic habitat fragmentation effects, or the lack thereof, on this species.

Although comparative studies such as that conducted here have advantages, chiefly that they can be conducted over a large area and in the actual environment of the study species (Diamond 1983), the approach also has disadvantages. Principal among these is that comparative studies can never conclusively demonstrate cause and effect (Mac Nally & Horrocks 2002). To do this, experimental manipulation of field sites is needed; examples include provisioning of woody debris (Mac Nally & Horrocks 2002), food supplementation (Banks & Dickman 2000), addition of artificial shelter from predators (Stokes et al. 2004), anti-parasite treatment (Merino & Potti 1998) and manipulation of litter or brood sizes (Burness et al. 2000). For example, the theory that the higher agile antechinus parasite loads recorded in fragmented habitat could have been caused by a scarcity of tree-hollows and consequent re-use of infected nests could be tested by providing a super-abundance of suitable nest-boxes in some study sites, whilst using non-manipulated sites as controls.

A key aspect of the current study was the idea that measuring several independent indicators of vertebrate stress, condition and population health could provide a more comprehensive understanding of the effects of a threatening process, such as anthropogenic habitat fragmentation, on a native vertebrate species. If the study had been restricted to the more frequently used indicators of population wellbeing, such as abundance, several potentially important effects of the environment on agile antechinus populations would not have been detected. Most of the indicators used in this study have not yet been exploited to their full potential in vertebrate ecology or conservation biology. The use of ecophysiological methods could prove invaluable for conservation management of threatened and declining populations of vertebrates in an increasingly anthropogenically-modified world.

Appendix A: Staining method details

The following material is included so that the staining methods used in this study can be readily replicated.

Natt and Herricks

Natt and Herricks stain is used for concurrent white blood cell and red blood cell counts using fresh blood (Campbell 1995). The dilution is at a ratio of 1:200 blood to stain. In this study 5 μL of blood was diluted in 1 mL of stain in an Eppendorf microcentrifuge tube.

Immediately on addition of the blood, the stain was mixed by shaking for 30 sec and subsequently allowed to rest for 1 min. The solution was immediately drawn off and used to fill a haemocytometer for cell counting. White blood cells are stained a uniform violet whereas red blood cells are acquire paler stain. Cell counting was conducted at 400 × magnification and the concentration of cells per litre was derived taking into account the dilution of blood in stain.

May-Grünwald Giemsa

May-Grünwald Geimsa stain is used in a staining method for differential leukocyte counts using blood smears (Lewis et al. 2006). About 5 μL of fresh blood was applied to the 'head' of a glass slide and this was smeared along the length of the slide using the edge of a second slide (pull-wedge method) so that a thin film of blood was produced. Smears were allowed to air dry in a covered container (to prevent dust settling on the slide) for 24 hr.

Staining commenced with immersion of a slide in methanol for 15 min to fix the preparation. This was followed by 15 immersion in May-Grünwald stain (diluted with equal volume of buffered water) and finally, 15 min immersion in Geimsa stain (diluted with nine volumes of buffered water). The slide was then washed momentarily three times in three beakers of distilled water and then placed in distilled water for 5 min. Each slide was positioned at an angle to dry so that excess liquid would run off.

Final drying took 24 hr and slides were stored in standard slide boxes prior to differential counting of leukocytes on return to the laboratory (400 × magnification). Slides that are fixed and prepared using this method do not need cover slips and will remain usable for at least several months.

Differential counting of leukocytes was conducted by the same individual and all counting was conducted by sweeping from the 'head' to 'tail' of the smear at least five times as differently sized immune cells can be deposited at different positions along the smear. Counting always included > 200 cells. Edges of the smear preparation were avoided as populations of immune cells that are trapping in edges are not always representative of the blood as a whole.

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