1 **SHORT COMMUNICATION**

2 Copper accumulation by stickleback nests containing spiggin

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28 **Abstract**

29 The three-spined stickleback is a ubiquitous fish of marine, brackish and freshwater ecosystems
30 across the Northern hemisphere that presents intermediate sensitivity to copper. Male sticklebacks
31 display a range of elaborate reproductive behaviours that include nest construction. To build the
32 nests, males bind nesting materials together using a glycoprotein nesting glue, known as ‘spiggin’.
33 Spiggin is a cysteine-rich protein and, therefore, potentially binds heavy metals present in the
34 environment. The aim of this study was to investigate the capacity of stickleback nests to
35 accumulate copper from environmental sources. Newly built nests, constructed by male fish from
36 polyester threads, were immersed in copper solutions ranging in concentration from 21.1-626.6 μg
37 Cu.L^{-1} . Bundles of polyester threads from aquaria without male fish were also immersed in the
38 same copper solutions. After immersion, nests presented higher amounts of copper than the threads,
39 indicating a higher capacity of nests to bind this metal. A significant and positive correlation
40 between the concentration of copper in the exposure solution and in the exposed nests was
41 identified, but there was no such relationship for thread bundles. Since both spiggin synthesis and
42 male courtship behaviour are under the control of circulating androgens, we predicted that males
43 with high courtship scores would produce and secrete high levels of the spiggin protein. In the
44 present study, nests built by high courtship score males accumulated more copper than those built
45 by low courtship score males. Considering the potential of spiggin to bind metals, the positive
46 relationship between fish courtship and spiggin secretion seems to explain the higher amount of
47 copper on the nests from the fish showing high behaviour scores. Further work is now needed to
48 determine the consequences of the copper binding potential of spiggin in stickleback nests for the
49 health and survival of developing embryos.

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51 The spiggin protein in the nest of three-spined sticklebacks binds copper dissolved in the water,
52 providing protection or acting as a source of contamination to embryos and hatched fry.

53 **Keywords:** Aquatic Contamination, Copper, Reproductive Behaviour, Spiggin, Stickleback

54 **1. Introduction**

55 As a consequence of industrialisation, a wide variety of environmental contaminants are released
56 into aquatic environments. Heavy metals are an important group of these compounds that are
57 potentially toxic to aquatic organisms (Clark, 2001), with osmoregulation, reproduction and
58 behaviour being considered as biological endpoints sensitive to disruption (Grossel et al., 2002,
59 Xiao et al., 2015).

60 The three-spined stickleback (*Gasterosteus aculeatus*) is a small teleost fish native to freshwater,
61 brackish and marine environments across the Northern Hemisphere (Wootton, 1976). Male
62 sticklebacks display a range of elaborate reproductive behaviours that involve territoriality, nest
63 construction, courtship and parental care (Van Iersel, 1953), and have become a model organisms in
64 studies of animal behaviour (Hoar, 1962; Sargent, 1982; Rowland, 1989; Bell, 2001). Nests built by
65 males form a focus for courtship (Barber et al., 2001) but primarily serve as functional receptacles
66 for eggs laid by females, providing protection for developing embryos and newly-hatched fry
67 against adverse environmental conditions and predators (Hansell, 2000).

68 Nest building in sticklebacks involves a sequence of activities performed by the male that begins
69 with digging a pit in the sediment, gathering plant material to form a mass, and the formation of a
70 tunnel through this mass for females to pass during spawning (Van Iersel, 1953; Wootton, 1976).
71 Males use an endogenous, hydrophobic glycoprotein nesting glue – named ‘spiggin’ – to bind
72 nesting materials together and stick them to the substrate. This glue is synthesised in the kidney,
73 which enlarges significantly through cell hypertrophy in the run-up to the breeding season (Borg &
74 Mayer, 1995). The glue protein is stored in the urinary bladder and secreted via the cloaca into the
75 nest. Following secretion, spiggin forms highly elastic and adhesive threads (Jakobsson et al.,
76 1999).

77 The protein component of spiggin has a molecular mass of approximately 203 kDa and an amino
78 acid composition that is unusually cysteine-rich (Jakobsson et al., 1999; Seear et al., 2015). The
79 amino acid composition of the spiggin protein does not bear similarities to other proteins that are

80 secreted extracellularly, such as elastin, collagen and silk (Urich, 1994); however a similarly high
81 cysteine composition is observed in some intracellular proteins, including the metallothioneins, a
82 family of cysteine-rich, low molecular weight proteins that are localized to the membrane of the
83 Golgi apparatus. The large number of cysteine residues in metallothioneins is responsible for
84 binding a variety of metal ions (Cobbett & Goldsbrough, 2002), which plays a major role in the
85 defence against metal toxicity (Viarengo et al., 1999; Wood et al., 2012).

86 The ubiquity and commonness of three-spined sticklebacks across the full spectrum of aquatic
87 habitat types in the Northern hemisphere suggests a relative tolerance of environmental
88 contamination (Sanchez et al., 2008). Gravenmier et al. (2005) characterised the acute toxicity of
89 copper in freshwater to three-spined sticklebacks. On the basis of estimated dissolved copper as a
90 function of water hardness using the biotic ligand model and water hardness normalization
91 techniques, they concluded that the three-spined stickleback shows intermediate sensitivity to
92 copper, being more sensitive than 29 of 78 species, including 16 vertebrate and 13 invertebrate
93 species. Only two studies provide LC50 concentrations based on measured concentrations of total
94 copper: 24-, 48-, 72-, and 96-h LC50 values of 2,780, 1,960, 1,590, and 1,490 $\mu\text{g Cu.L}^{-1}$,
95 (Svesevicius & Vosyliene, 1996) and 382.2, 278.7, 256.6, and 227.2 $\mu\text{g Cu.L}^{-1}$ respectively
96 (Gravenmier et al., 2005). No information could be found regarding the copper sensitivity of
97 embryos or developing fry.

98 Sticklebacks build their nests in shallow, littoral habitats, which are frequently contaminated by
99 heavy metals through direct anthropogenic input, through the direct input of effluents from
100 industrial processes or through the indirect erosion of natural metal-containing substrates or spoil
101 from mining activities. Specifically for copper, natural weathering is considered the most important
102 source to the coast areas (Landner & Lindstrom, 1999). The cysteine-rich spiggin protein that is
103 secreted into the nest may therefore accumulate heavy metals from a range of natural and
104 anthropogenic environmental sources, with potentially two opposing consequences for developing
105 offspring. First, nests containing spiggin may protect embryos and developing fry from heavy

metals if they are bound and removed them the surrounding water. Second, nests containing spiggin might accumulate high concentrations of heavy metals that pose a threat to embryos and developing fry, especially if the glue is subject to biodegradation.

A first step to understanding the potential for the effects of heavy metal contamination to be influenced by the presence of spiggin in the nests of sticklebacks is to compare the levels of accumulation in male-built nests, which contain spiggin, and non-manipulated nesting materials ('thread bundles'), which do not. This study therefore had the following objectives: (1) to investigate the capacity of the nests and thread bundles to accumulate copper from aquatic environmental sources; (2) to test for a correlation between copper accumulation by nests and copper concentration in the surrounding water; (3) to investigate whether inter-male variation in reproductive behaviour correlated with the level of nest copper accumulation.

2. Materials and Methods

Fish husbandry and nest construction

Lab bred adult three-spined sticklebacks *Gasterosteus aculeatus*, derived from numerous natural spawnings, were brought into breeding condition during the Spring of 2014 in laboratory aquaria at the University of Leicester (U.K.) by adjusting photoperiod and temperature regimes to mimic natural springtime levels. Male fish developing characteristic nuptial coloration (Rushbrook and Barber, 2006) were moved from stock holding tanks to individual nesting aquaria (20 x 20 x 40 cm) without visual access to neighbours, to simulate successful territory acquisition. Each aquarium had a gravel substratum, a sponge airlift filter, a plastic plant and 200 of 70 mm-long black polyester threads for nesting material. A 10 cm x 10 cm x 1 cm tray of washed, sieved and sterilised fine sand (i.e. including no organic material) was offered to each stickleback as a nesting site. Every day, until the male completed a nest (determined using criteria in Table 1 of Rushbrook & Barber (2006)), each fish was provided with visual access to a gravid female, presented in a half-filled glass jar introduced into the aquarium, for two separate 10 min presentations. Males were fed daily *ad libitum* a diet of frozen chironomid larvae.

132 The first experiment was performed with five males, with each male building one complete nest. In
133 the second experiment, a group of four new sexually mature males were selected, and each fish
134 built three complete nests. In the second experiment, the courtship behaviour of each male was also
135 scored, using published criteria (Rushbrook & Barber, 2006), on the last day of the first and second
136 periods of nest construction.

137 Fish were held under UK Home Office licenced aquaria, and under conditions that were consistent
138 with national and international guidelines for ethical treatment of animals (ABS/ASAB, 2012). No fish were
139 exposed to heavy metals in these experiments.

140 *Copper exposure*

141 After a nest had been completed, it was transferred using plastic forceps to a copper immersion
142 solution. Copper solutions were made by diluting a stock solution (nominal concentration of 7.1 g
143 Cu.L^{-1} , as CuCl_2 , Merck) that had been prepared with distilled water and acidified with 1% HNO_3
144 (Merck). In Experiment 1, nests built by males (i.e. containing spiggin) were exposed to a CuCl_2
145 solution of nominal concentration 20 $\mu\text{g Cu.L}^{-1}$, as were bundles of an equivalent number of
146 polyester threads that had never been in contact with the fish and kept in fishless aquaria, under
147 otherwise identical conditions ('thread bundles', i.e. containing no spiggin). In Experiment 2, both
148 nests and thread bundles were exposed to CuCl_2 solutions of nominal concentration 20, 100 or 500
149 $\mu\text{g Cu.L}^{-1}$. In both Experiment 1 and Experiment 2, nests and thread bundles were also exposed to a
150 control treatment of distilled water (dH_2O). For each exposure, the nest or thread bundle was
151 transferred to a beaker containing 200 mL of exposure solution (CuCl_2 solution or distilled water
152 control) and immersed for 2 h. Samples (10 mL) from the different treatments were collected at the
153 beginning of experiment, filtered (0.45 μm mesh filter) and immediately acidified (1% HNO_3) to
154 measure the concentration of dissolved copper. After the exposure period, nests and threads were
155 washed three times in dH_2O for 5 seconds each. The nests and thread bundles were transferred to a
156 Petri dish and dried at 60 °C for 24 h. Nests and thread bundles were subsequently kept in airtight
157 plastic bags and protected from light and humidity until copper quantification.

158 *Copper quantification*

159 Dried nests and thread bundles were weighed, digested in HNO₃ (65% Suprapur ®, Merck) for 24 h
160 and diluted with dH₂O to measure copper. Copper concentrations on the nests and threads and in the
161 water samples (dissolved copper) were measured by atomic absorption spectrophotometry (AAS
162 Analyst 700, Perkin Elmer; copper detection limit 0.0014 µg Cu.L⁻¹). Copper concentrations on the
163 nests and threads are expressed as µg Cu.g⁻¹ dry weight.

164 *Data analysis*

165 Data were expressed as mean ± standard error. For copper accumulation on the nests and thread
166 bundles, a one-way analysis of variance (ANOVA) followed by pairwise Tukey tests, or a Kruskal-
167 Wallis non-parametric ANOVA was used, to detect significant differences between treatments. A
168 Students t-test for independent samples was used to confirm equivalence of measured copper
169 concentrations between nominal 20 µg Cu.L⁻¹ treatments from experiments 1 and 2. Assumptions of
170 data normality and homogeneity of variances required for ANOVA were checked and verified (Zar,
171 1999). Relationships between variables were evaluated using linear regression analysis. In all cases,
172 a significance level of 95% (α=0.05) was adopted.

173 **3. Results and Discussion**

174 *Copper concentrations in exposure solutions*

175 The measured concentration of dissolved copper in the nominal 20 µg Cu.L⁻¹ immersion solutions
176 in Experiments 1 (21.3 ± 0.9 µg Cu.L⁻¹) and Experiment 2 (21.1 ± 4.4 µg Cu.L⁻¹) did not differ
177 statistically (P=0.938, N=9). Nominal concentrations of 100 and 500 µg Cu.L⁻¹ in Experiment 2
178 generated measured concentrations of 44.0 ± 2.5 and 626.6 ± 52.6 µg Cu.L⁻¹ respectively. The
179 measured concentration of dissolved copper in the distilled water controls in both experiments was
180 0.99 ± 0.7 µg Cu.L⁻¹.

181 *Copper accumulation in nests and thread bundles*

182 In Experiment 1, nests exposed to the nominal 20 µg Cu.L⁻¹ solution accumulated a mean of almost
183 three times the copper concentration than thread bundles subject to the same treatment. Thread
184 bundles exposed to the nominal 20 µg Cu.L⁻¹ copper solution developed copper concentrations that

185 were not statistically higher than those kept in distilled water ($P < 0.001$, $N = 12$). In Experiment 2,
186 nests exposed to the nominal $20 \mu\text{g Cu.L}^{-1}$ solution accumulated more copper than the threads,
187 confirming the higher capacity of the nests to bind this metal ($P < 0.004$, $N = 7$) compared to the
188 thread bundles. Combining samples from the nominal $20 \mu\text{g Cu.L}^{-1}$ exposure treatments from
189 Experiments 1 and 2, revealed a highly statistically significant effect ($p < 0.001$, $N = 19$; Figure 1a).
190 In Experiment 2, four new male fish each completed three nests, which were then distributed
191 between copper solutions measuring 21.1, 44.0 and $626.6 \mu\text{g Cu.L}^{-1}$. A significant positive
192 correlation between the concentration of copper in the exposure solution and in the exposed nests
193 was identified ($R = 0.83$, $N = 12$, $p = 0.01$), but there was no such relationship for thread bundles
194 ($R = 0.37$, $N = 12$, $p = 0.11$), reinforcing the finding that copper accumulation is related to the presence
195 of spiggin in nests.

196 Data from Experiments 1 and 2 was then combined to examine the global relationship between the
197 copper concentration in the exposure solution and the copper concentration that developed in nests
198 and thread bundles following exposure. Combining data in this way is justified because the period
199 of immersion and the nature of the materials being tested was consistent across experiments. To
200 normalise data from across a wide range of copper treatments, all values were log10 transformed
201 prior to data analysis. Among the combined dataset, the copper concentration of nests/thread
202 bundles increased significantly with the concentration of copper in the immersion treatment ($F_{1,39} =$
203 14.38 , $P = 0.001$). However, nests accumulated significantly higher concentrations of copper than
204 thread bundles (ANCOVA, slope: $F_{1,39} = 2.46$ $P = 0.125$; elevation: $F_{1,39} = 25.76$, $P < 0.0005$;
205 Figure 1b). Visual inspection of the data suggested that copper concentration plateaued at
206 immersion concentrations exceeding $10 \mu\text{g.L}^{-1}$, so a further analysis was undertaken, restricted to the
207 region of overlap (i.e. at immersion concentrations $> 10 \mu\text{g.L}^{-1}$). Among these, there was no
208 significant relationship between with immersion concentration and sample concentrations ($F_{1,27} =$
209 0.03 , $P = 0.856$), however, the significant difference in elevation between nests and thread copper
210 concentration remained ($F_{1,27} = 24.6$, $P < 0.0005$).

211 Finally, to investigate whether the amount of nesting glue in nests might be responsible for
212 variation in their copper concentration following immersion, we undertook a preliminary
213 investigation into the relationship between male nesting behaviour and copper accumulation in the
214 nests. In Experiment 2, male courtship behaviour was scored during the first and second period of
215 nest building. Using published criteria, males were scored as exhibiting 'low' or 'high' courtship
216 scores ('LCS' = 0-2 or 'HCS' = 3-5; see Table 1 in Rushbrook and Barber, 2006). Nests built by
217 HCS males accumulated more copper than those built by LCS males ($p < 0.005$; Figure 2).
218 During nest building, males carry the polyester threads to the nest area by mouth and glue them into
219 position using spiggin nesting glue. Sand is also transported by the mouth to the nest site. However,
220 sand used in present experiments were previously sterilised, does not having therefore organic
221 compounds. It is well known that organic coatings as well as Fe/Mn hydrous oxides control the
222 sorptive behaviour of sediments (Lion et al, 1982). Therefore the most parsimonious explanation is
223 that the high cysteine content of the spiggin protein (Jakobsson et al., 1999) is responsible for
224 binding copper ions.
225 Spiggin synthesis in the kidneys of stickleback males occurs in response to androgen levels
226 (Jakobsson et al., 1999). Because secondary sexual characters and reproductive behaviour in
227 stickleback males are also stimulated by androgens, particularly 11KT (Borg, 1994; Borg & Mayer,
228 1995), males with high scores of courtship behaviours typically produce and secrete high levels of
229 the protein spiggin (Macnab et al., 2011). Considering the ability of spiggin to bind metals, a
230 positive relationship between fish courtship and spiggin secretion seems to explain the higher
231 amount of copper on the nests from the fish with elevated behaviour scores. The spiggin protein in
232 the nest of three-spined sticklebacks can therefore bind copper ions dissolved in the water, though
233 understanding the extent to which this provides protection to developing embryos and hatched fry,
234 or acts to increase the source of elevated contamination, would require further work.

235 **Acknowledgments**

236 The authors thank Ceinwen A. Tilley for her assistance during this work. Grasiela L. L. Pinho was
237 in receipt of a postdoctoral fellowship awarded by the Brazilian CNPq (# 245706/2012-7).

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303 **Figure Legends**

304 **Figure 1** Copper accumulation in male-built nests (i.e. containing spiggin) and thread bundles (i.e.
305 containing no spiggin) from Experiments 1 and 2, shown together. (a) Bar charts showing mean +/-
306 SE copper concentrations that developed in nests exposed to copper solution, thread bundles
307 exposed to copper solution and thread bundles exposed to a pure distilled water control. Different
308 letters represent significant differences in copper concentration ($p < 0.001$). (b) Relationship between
309 ($\log_{10}$ transformed) copper concentration of the immersion solution and the concentration of copper
310 in nests (*, solid line) and thread bundles (○, broken line).

311 **Figure 2** Copper accumulation in male-built nests from experiment 2. LCS: fish with low courtship
312 scores (0-2) and HCS: fish with high courtship scores. Different letters represent significantly
313 different nest copper concentrations ($p < 0.005$).

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