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Male three-spined sticklebacks *Gasterosteus aculeatus* make antibiotic nests: a novel form of parental protection?

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The egg nest of male three-spined sticklebacks *Gasterosteus aculeatus* is constructed with a glue-like secretion that this study demonstrates has antimicrobial properties. Glue collected from reproductively active males decreased the growth rate of bacteria and opportunistic fungi, and eggs were more likely to mature and hatch after exposure to their father's glue. This phenomenon may represent a direct physiological contribution from a male towards protecting his offspring from pathogens, and if so is a novel form of parental protection.

Key words: antimicrobial; paternal care; pathogen; stickleback glue.

INTRODUCTION

Parasites and pathogens are a prevalent challenge for all organisms. Due to their potential effect on reproductive success, parents employ a diversity of tactics to protect their offspring. For example, at the earliest stages of the reproductive process, mate choice influences offspring immunocompetence, because attraction between parents is influenced by the compatibility or complement of the parental major histocompatibility complex (MHC) classes (Wedekind *et al.*, 1995; Reusch *et al.*, 2001; Ekblom *et al.*, 2004; Richardson *et al.*, 2005). More direct forms of protection also come into play, for example, the transfer of antibodies from mothers to their offspring. Indeed, in vertebrates that gestate, offspring are protected through maternal antibodies during pregnancy and lactation, possibly for years (Roitt, 1997). Similarly, in various egg laying species, mothers deposit antibodies and other protective compounds in their eggs shortly before laying (Smith *et al.*, 1994; Swain *et al.*, 2006). Parents also indirectly look after their offspring with environmental manipulations: birds add vegetation or fungi that contain

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anti-parasitic compounds to their nests (McFarland & Rimmer, 1996; Lozano, 1998), wood ants use solidified conifer resin to inhibit the growth of microorganisms in their nests (Christe *et al.*, 2003) and some species of frog secrete foam that may protect their eggs from pathogens (Cooper *et al.*, 2005).

The full range of mechanisms that enhance offspring resistance to pathogens, from mate choice to environmental manipulation, are possible in nest-building fishes, in which it is typically the male that constructs the nest and provides the care. Mate choice and reproductive behaviour in three-spined sticklebacks *Gasterosteus aculeatus* L. has been studied from the classical ethological investigations of Tinbergen (1951) to the present (Boughman *et al.*, 2005), with female choice apparently focused on body colouration, the vigour of a courtship dance and possibly nest quality (Milinski & Bakker, 1990; Rowland, 1994; Barber *et al.*, 2001*a*). Nests are constructed by reproductively active males, which excavate a site, collect vegetation and bind this with a sticky glue-like secretion produced by the kidney and stored in the urinary bladder (Wootton, 1976). This secretion is dominated by the glycoprotein 'spiggin' (Jakobsson *et al.*, 1999). Following nest construction, males court females to spawn, and once fertilized eggs are in the nest, an intense phase of paternal care begins. The male defends the eggs from predators, and fans them to ensure aeration and the removal of wastes.

Although *G. aculeatus* and other fish eggs may have acquired protection through the transfer of maternal immune molecules, with no active immune system fish eggs would appear to be highly vulnerable to infection. Indeed, *G. aculeatus* clutches often succumb to bacterial infections (Barber & Arnott, 2000). A vigilant male that removes dead or diseased eggs is likely to provide a safer environment for the remainder of his clutch (Wootton, 1976, 1984). Alternatively, a male may be able to add material to the nest that can protect the eggs from microbial infection. Some species of fishes are known to secrete proteins with antimicrobial functions in the mucus on their skin (Fernandes *et al.*, 2002). It was hypothesized that the glue-like secretion used in nest building may protect the clutch by inhibiting local bacterial growth. Thus, the antimicrobial properties of *G. aculeatus* glue were investigated.

It was also determined if glue antimicrobial efficacy was related to the mate choice characteristics of *G. aculeatus*. Female preference for the carotenoid-based red throat and belly of males appears to confer indirect benefits; redder males produce fry that are more resistant to parasite infection (Barber *et al.*, 2001*b*). There are, however, direct benefits too; redder males can build superior nests and locate these in good locations (*i.e.* well aerated sites and hidden from predators; Kraak *et al.*, 2000). The present study, therefore, tested if the degree of male redness was linked to the direct beneficial effects of antimicrobial glue.

MATERIALS AND METHODS

EXPERIMENT 1: THE ANTIBACTERIAL PROPERTIES OF MALE GLUE

Thirty-four male *G. aculeatus* were collected from the Inverleith Pond (55°58′ N; 4°20′ W), Scotland, in the autumn and then brought into breeding condition over a 4 month period in 20 l aquaria in a climate and light controlled room that imitated seasonal changes

during the breeding season. Fish were fed to excess on bloodworms *Chironomous* sp. and were provided with nest building material (a Petri dish containing sand and lengths of cotton thread), as well as visual, though not physical, access to gravid females. This environment should help to stimulate glue production. The fish were studied in two batches ($n = 20$ and $n = 14$) with a 2 day period separating the batches.

Collection of glue

Glue was harvested from all 34 males. Individual fish were sacrificed and the urinary bladder was removed, cleaned of adhering soft tissues and measured with callipers. Two measurements of the bladder, the vertical and horizontal diameters, were obtained and averaged. The bladder wall was pierced using a gauge 25 needle, and the contents were flushed out by injecting 0·6 ml of isoton (Beckman Coulter, Fullerton, CA, U.S.A.). This extract was collected in a 1·5 ml Eppendorf tube and stored at −80° C until used in antibacterial assays. At this time a section of kidney was also excised from 11 randomly chosen fish to act as a control tissue. To verify that glue had been extracted from the urinary bladders, the extract was run through a sodium dodecyl sulphate polyacrylamide gel. The most intense band was found in the 203 kDa range, which is the correct size for spiggin, the most abundant protein in *G. aculeatus* nest building glue (Jakobsson *et al.*, 1999).

Antibacterial assays

Antibacterial activity was quantified by comparing the growth rate of the common fish pathogen *Pseudomonas fluorescens* (strain SB W25) in the presence or absence of the glue. Separate assays were run for each fish. To prepare the *P. fluorescens*, an overnight culture was grown in Luria broth (LB) at 27° C. Additional LB was then added to attain a starting optical density of 0·1 at 650 nm in a spectrophotometer. This culture was divided into 3 ml aliquots for experimental treatments: control samples had 0·6 ml of isoton added to the LB ($n = 12$), whilst the samples treated with glue had 0·6 ml of fish glue extract added to the LB. For an additional set of controls ($n = 11$), 0·6 ml of kidney extract was added to a 3 ml culture. Cultures were then grown for 41·5 h at 10° C, a temperature chosen to reflect typical conditions in the natural habitat from which the *G. aculeatus* came. Every 4 h, the growth rate of the bacteria was estimated by measuring the optical density of the culture at 650 nm in a spectrophotometer. This wavelength was chosen through a process of calibration that determined a wavelength that provided a linear response over the expected densities of the bacterium.

For analysis of bacterial growth, the optical density of each culture was recorded over the 41·5 h period to calculate the area under the bacterial growth curve. The area under the curve approach offers a straightforward measure of bacterial growth that avoids problems with repeated measures (Crowder & Hand, 1990). ANOVA was used to study bacterial growth differences between the glue from the different fish, which was then used to study the relationship between redness and antibacterial activity. After averaging the three bacterial growth measurements taken per tissue (glue or kidney) from each fish, the optical densities of the controls (*P. fluorescens* grown in LB alone) were subtracted (separately for each batch) to obtain a standardized measurement of growth relative to that obtainable by bacteria in the absence of any putative growth inhibiting or enhancing factor in the fish tissues. The statistical model (ANOVA) incorporated treatment (a two level fixed factor, kidney or urinary bladder extract), batch and fish (classified as a random effect).

EXPERIMENT 2: THE ANTIMICROBIAL PROPERTIES OF MALE GLUE

To further appreciate the antimicrobial properties of fish glue, its effects on the opportunistic microorganisms (largely fungal by gross inspection) that frequently attack and grow on *G. aculeatus* eggs in the laboratory were studied. To isolate these fungi, eggs that had succumbed to infection were crushed, and cultures prepared in LB. Eighteen new fish,

in two batches of nine, which had been brought into breeding condition as above were used for this experiment. In this case, however, excised urinary bladders were weighed and then pulverized using a pestle in an Eppendorf tube with 600 μl of isoton. This solution was then used in the treatments below. As a control tissue, the same procedure was performed on a section of liver that was trimmed to a mass equal to that of the urinary bladder taken from the same fish. Samples were stored at −80° C until needed.

Antimicrobial activity was quantified by comparing fungal growth rate in the presence of the glue or in the presence of liver tissue from each of the 18 fish. To prepare the fungus, an overnight culture was grown in LB at 27° C and additional LB added to attain a starting optical density of 0·1 at 650 nm in a spectrophotometer. This culture was divided into 3 ml aliquots in 20 ml test tubes for experimental treatments. From each fish, either 0·6 ml glue (*i.e.* urinary bladder macerated in isoton) or 0·6 ml control (*i.e.* liver tissue macerated in isoton) extract was added to a tube. Cultures were then grown for 31·5 h at 10° C. Every 4 h, the growth rate of the fungus was estimated by measuring the optical density of the culture at 650 nm. As above, the optical density of each culture over the observation period was used to calculate the area under the growth curve for each fish. ANOVA was then used to study fungal growth differences between the glue and liver treatments. The statistical model thus incorporated treatment (a two level fixed factor, liver or urinary bladder extract), batch and fish (classified as a random effect).

EXPERIMENT 3: DOES MALE GLUE PROTECT EGGS?

Fish rearing

A mixed sex group of non-breeding fish were caught just prior to the onset of their breeding season and brought into the laboratory. The fish were housed at 12° C with a fixed photoperiod of 14L:10D for 4 months prior to the start of the experiment. As males began to develop their secondary sexual characters (red throat and belly, blue iris and increased aggression), they were isolated into individual tanks that contained materials for nest building and were given visual, but not physical, access to gravid female fish. The remaining female fish were fed three times a day on live bloodworms and *Daphnia magna* to encourage egg production.

Establishing split clutches

Once a female was fully gravid and ready to spawn, indicated by the head-up posture in response to a stimulus breeding male, an isolated male with a completed nest was selected. The male was euthanized, and the urinary bladder and paired testes were then dissected out. The testes were placed into a watch glass with 1 ml of distilled water and crushed to release the sperm. Glue was collected by piercing the urinary bladder wall with a gauge 25 needle, and the contents were flushed out into a 1·5 ml Eppendorf tube by injecting 0·5 ml of isoton through the pierced bladder. The volumes of glue collected varied but were typically *c.* 1·0 ml of fluid after flushing 0·5 ml of isoton through the bladder. A female's abdomen was gently squeezed to encourage her to release clutch of eggs into a dampened watch glass. The clutch was split in half using a fine paintbrush and a blunt seeker. Eggs were fertilized in the watch glasses by adding equal volumes of the solution containing the sperm.

The fertilized eggs were then transferred to individual incubators. Each incubator (100 × 60 × 40 mm) contained a 30 mm diameter Petri dish with a 1 mm plastic mesh base. The Petri dish was tilted at a slight angle to allow an air stone to bubble underneath the dish, so that the air bubbles worked their way up the angled mesh base of the dish and then to the surface. The glue and isoton solution was collected in a syringe and washed over one of the halves of each clutch. An equal volume of isoton was washed over the other half of the clutch (which half received the isoton and glue, or only isoton was assigned randomly). Twenty-three pairs of split clutches were prepared in this way (*i.e.* eggs were reared in 46 incubators), each split clutch had a single mother and father, the only difference between the two half clutches was the presence or absence of glue.

Once a day, all clutches were inspected using a binocular dissecting microscope. The observer was blind to treatment (*i.e.* which clutches were with or without glue). Any dead or diseased eggs were removed. Daily counts were made of eggs lost, and the clutches were followed through to hatching, and the number of fry that successfully hatched was counted. The proportion of eggs lost due to decay each day over a 15 day period that was the entire period where hatching was observed and counted. For analysis, a plot of the area under the curve of proportion hatched and time was calculated. This was carried out for both the glue (+) and glue (−) and then these paired treatments were compared with a Wilcoxon signed-rank test.

QUANTIFICATION OF RED BREEDING COLOURATION

For experiments 1 and 3, male redness was quantified with the aim to test for an association between antimicrobial activity and male colour. Fish were photographed immediately prior to sacrifice by gently placing them into a specially constructed cradle inside a Petri dish. The Petri dish was then held flush against the front glass wall of a clean aquarium, and the fish were then photographed under standardized conditions (Candolin, 1999) using a four megapixel digital camera (Powershot G3, Canon; Tokyo, Japan). Red colouration was quantified from the digital images using the dropper tool in Adobe Photoshop 7 (Adobe Systems Inc., San Jose, CA, U.S.A.). The Adobe RGB colour space represents colour as three components (red, green and blue), each with the potential to range from 0 to 255. Photoshop software is designed for human trichromatic human vision, but it was only used as a reference tool to rank fish according to their red colouration. It was not used to assert what the fish may see themselves. To correct for any discrepancies in lighting at the time the pictures were taken, and to aid meaningful comparison, the colour output of all photographs was first standardized using the background colour of the mount as a reference.

A redness index for each fish was calculated by taking two separate measurements from the throat and the opercular region. For each of these areas, the maximum level of red was calculated. To do this, the three channels (red, green and blue, r, g, b, respectively) were recorded and intensity (i) of red was calculated as $i = r\,(r + g + b)^{-1}$, which can range from zero to 1·0. In this way, the photographs were given a colouration score that was then used to rank the fish from bright red to dull red.

RESULTS

EXPERIMENT 1

Growth of the common aquatic bacterial pathogen *P. fluorescens* was suppressed in the presence of glue extracted from the urinary bladder of reproductively active males compared to bacterial growth in the presence of control (kidney) tissue (Fig. 1; $F_{1,8} = 89{\cdot}1$, $P < 0{\cdot}001$). The experiment incorporated two batches of fish ($n = 20$ and $n = 14$), and there were differences between batches ($F_{1,8} = 37{\cdot}6$, $P < 0{\cdot}001$) but no batch × treatment interaction ($F_{1,8} = 0{\cdot}34$, $P > 0{\cdot}05$).

EXPERIMENT 2

Growth of the fungus that destroys eggs in the laboratory was also inhibited in the presence of fish glue ($F_{1,16} = 48{\cdot}5$, $P < 0{\cdot}001$). The experiment incorporated two batches of fish ($n = 9$ and $n = 9$), and there were no differences between batches ($F_{1,16} = 0{\cdot}8$, $P > 0{\cdot}05$), but there was a batch × treatment

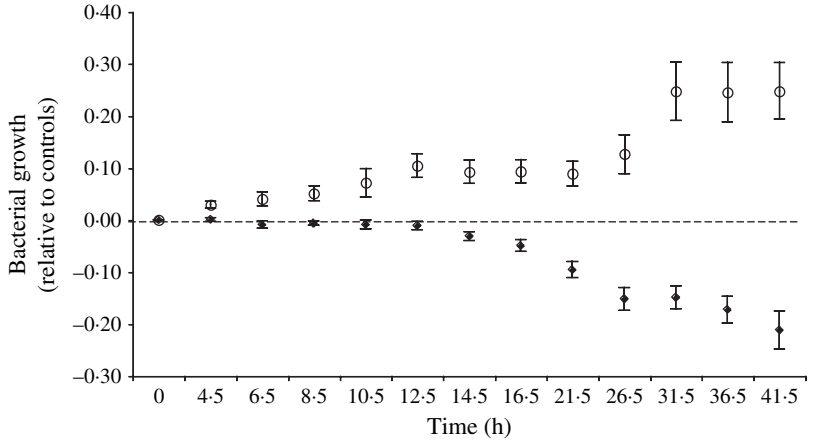

FIG. 1. Relationship between the growth of *Pseudomonas fluorescens* in the presence of nest building glue (◆) and macerated kidney (○) of male *Gasterosteus aculeatus*, illustrating the inhibition of growth by glue extract. Values are mean ± S.E.

interaction ($F_{1,16} = 6{\cdot}4$, $P < 0{\cdot}05$). Whilst the mean amount of growth (area under the growth curve) did not appear to differ substantially (glue: 21·8; control tissue: 20·1), the difference between treatments appears clear when comparing the treatments within each fish: for 17 of 18 fish, growth rates in the control tissue treatment were higher than those in the urinary bladder treatment. A matched-pairs analysis incorporating batch indicated a significant difference between tissues ($t = 6{\cdot}3$, d.f. $= 17$, $P < 0{\cdot}05$).

EXPERIMENT 3

The proportion of eggs hatching over time was higher when exposed to glue (Wilcoxon signed-rank test comparing the split brood for each fish, $z = -75$, $P < 0{\cdot}05$). In total, for eggs not washed over with glue, 466 fry hatched while 1201 succumbed to infection. For eggs exposed to glue, 568 hatched compared to 1141 succumbing to infection. Again, although the mean area under the curve representing the proportion of eggs hatching over time did not appear to differ dramatically (glue+: 6·66 and glue−: 5·87), the difference between the treatments is most evident when viewed as a paired result: 15 of 23 fish showed greater hatching success over time with glue.

MALE COLOURATION

Fish used in the *P. fluorescens* antibacterial assays (Experiment 1) varied in terms of redness, but this was unrelated to the degree to which their glue could suppress bacterial growth [$r^2 = 0{\cdot}018$, $P > 0{\cdot}05$; Fig. 2(a)] or the size of their urinary bladder (where glue is stored) [$r^2 = 0{\cdot}086$, $P > 0{\cdot}05$; Fig. 2(b)]. Similarly, in the split clutch analysis of glue's capacity to reduce egg mortality (Experiment 3), fish redness was again not related to either the level of egg mortality (here egg mortality in the half of the clutch not exposed glue was

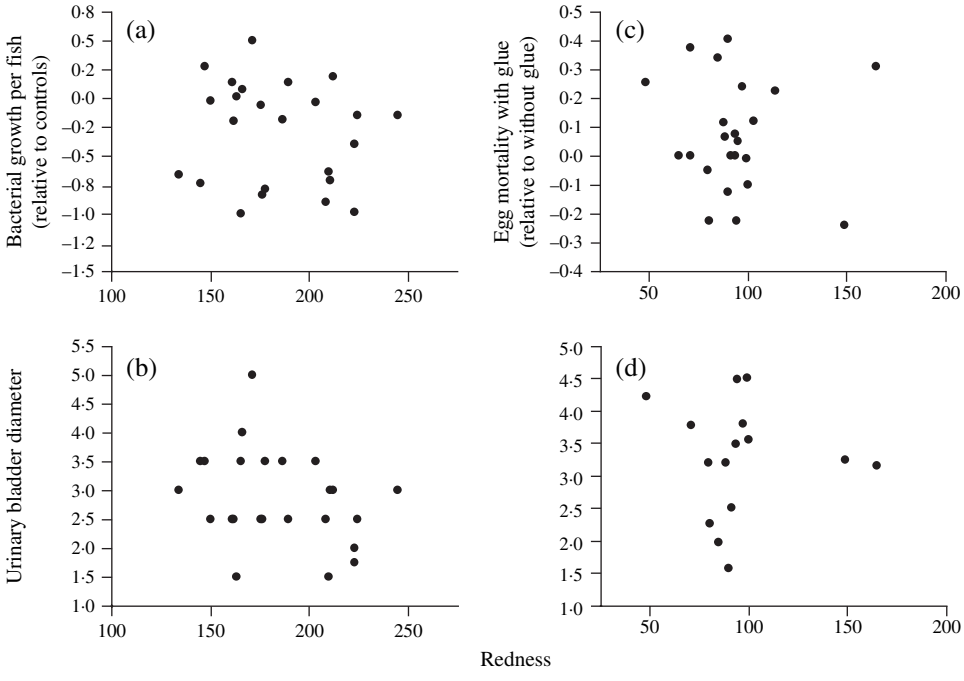

FIG. 2. Relationship between the redness of a male *Gasterosteus aculeatus* and (a) antibacterial activity in its nest building glue, (b) the size of their urinary bladder (for those fish used in the antibacterial activity experiment), (c) the glue's capacity to reduce egg mortality and (d) the size of the urinary bladder (for those fish used in the egg mortality experiment).

subtracted from mortality in the half exposed to glue) [$r^2 = 0{\cdot}004$, $P > 0{\cdot}05$; Fig. 2(c)] or the size of the urinary bladder [$r^2 = 0{\cdot}024$, $P > 0{\cdot}05$; Fig. 2(d)].

DISCUSSION

The antimicrobial properties of the nest-building glue of male *G. aculeatus* was assessed with three independent experiments. Nest-building glue suppressed microbial activity and appeared to enhance reproductive success. Thus, in addition to containing structural proteins important for the construction of nests, *G. aculeatus* glue could be a mechanism by which males protect their offspring from pathogens (Knouft *et al.*, 2003).

In many organisms, it is the female that provides direct protection to her offspring through, for example, the transmission of immune system components into eggs (Galliano *et al.*, 2003). For many taxa, however, paternal care is also vital and is expressed in some remarkable ways, for example through the gathering of specific vegetation for nest building in birds (McFarland & Rimmer, 1996; Lozano, 1998). Delimiting male and female direct contributions to offspring is a focus of sexual selection research, however, there was no evidence that glue quality was associated with male colouration, a characteristic commonly assumed to signal male quality to choosy females (Milinski & Bakker, 1990; Barber *et al.*, 2000). Specifically, before glue was collected, the intensity

of red colouration of the males was determined, but neither the level of antimicrobial activity nor the size of the urinary bladder were related to redness (Fig. 2). Female choice, however, does not always correlate with red colouration in *G. aculeatus* (Bakker & Milinski, 1992; McDonald *et al.*, 1995; Kraak *et al.*, 1999, 2000). Measurements of choosiness other than those based on colouration could yet indicate that the antibacterial quality of nests is indeed a relevant factor for females. Certainly, mate choice in *G. aculeatus* is a complex process that is not fully illuminated by simple measurement of colour; for example, females may gauge male aggression, his courtship zig-zag dance and, of particular relevance to the present result, nest quality (Rowland, 1994; Barber *et al.*, 2001). Additionally, nutritional status is known to influence glue production in sticklebacks (Östlund-Nilsson, 2001; Rushbrook *et al.*, 2007), and it is conceivable that nutritional status affects the quality of the antimicrobial properties of the glue. Energetically, expensive behaviours such as courtship or aggression may act as a physical reflection of these shortfalls.

When half of a split clutch of fertilized eggs was treated with extract from their father's urinary bladder, a greater proportion of fry hatched. Even among those eggs treated with glue, however, many died or became diseased. It would be interesting to determine whether continued application of the glue during the rearing process would help to protect the eggs. Males continue to secrete glue onto the nest material even after they switch from courtship dominated behaviour to a paternal care phase when fertilized eggs are in his nest. Thus, in the natural situation, eggs may gain greater protection by renewed application of glue during their development, a procedure that was not mimicked in the current experiment.

The urinary bladder extract collected contained a 203 kDa protein that is almost certainly the principle component of fish glue, spiggin. This protein has a well-established role in the structural component of the nest, in that it is sticky and binds the nest material together (Jakobsson *et al.*, 1999). It was not established, however, if spiggin also provides antimicrobial protection, or if another peptide, or even a cellular component in glue performs that role. It even remains possible that urine has antimicrobial properties that play a role in the health of eggs. Nevertheless, the phenomenological observations reported here may now provide the stimulus for research into the molecular compounds that underlie glue's possible antimicrobial effects. In general, secretions such as fish glue or the foam with which amphibians surround their eggs, which may represent a rich source of novel antimicrobial substances. Indeed, the high species diversity of these groups indicates considerable potential for molecular discovery.

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References

Bakker, T. C. M. & Milinski, M. (1992). Female mate choice and male red coloration in a natural three-spine stickleback (*Gasterosteus aculeatus*) population. *Behavioural Ecology* **5,** 74–80.

Barber, I. & Arnott, S. A. (2000). Split-clutch IVF: a technique to examine indirect fitness consequences of mate preferences in sticklebacks. *Behaviour* **137,** 1129–1140.
Barber, I., Nairn, D. & Huntingford, F. A. (2001*a*). Nests as ornaments: revealing construction by male sticklebacks. *Behavioural Ecology* **12,** 390–396.
Barber, I., Arnott, S. A., Braithwaite, V. A., Andrew, J. & Huntingford, F. A. (2001*b*). Indirect fitness consequences of mate choice in sticklebacks: offspring of brighter males grow slowly but resist parasite infections. *Proceedings of the Royal Society B* **268,** 71–76. doi:10.1098/rspb2000.1331.
Boughman, J. W., Rundle, H. D. & Schluter, D. (2005). Parallel evolution of sexual selection in sticklebacks. *Evolution* **59,** 361–373.
Candolin, U. (1999). Male-male competition facilitates female choice in sticklebacks. *Proceedings of the Royal Society B* **266,** 785–789.
Christe, P., Anne Oppliger, A., Bancalà, F., Castella, G. & Chapuisat, M. (2003). Evidence for collective medication in ants. *Ecology Letters* **6,** 19.
Cooper, A., Kennedy, M. W., Fleming, R. I., Wilson, E. H., Videler, H., Wokosin, D. L., Su, T. J., Green, R. J. & Lu, J. R. (2005). Adsorption of frog foam nest proteins at the air-water interface. *Biophysics Journal* **88,** 2114–2125.
Crowder, M. J. & Hand, D. J. (1990). *Analysis of Repeated Measures*. London: Chapman & Hall.
Ekblom, R., Saether, S. A., Grahn, M., Fiske, J., Kålås, J. A. & Höglund, J. (2004). Major histocompatibility complex variation and mate choice in a lekking bird, the great snipe (*Gallinago media*). *Molecular Ecology* **13,** 3821–3828.
Fernandes, J. M. O., Kemp, G. D., Molle, G. & Smith, V. J. (2002). Antimicrobial properties of a histone H2A-like protein from skin secretions of rainbow trout, *Oncorhynchus mykiss*. *Biochemical Journal* **368,** 611–620.
Galliano, M., Minchioyyi, L., Campagnoli, M., Sala, A., Visai, L., Amoreano, A., Pucci, P., Casbarra, A., Cauci, M., Perduca, M. & Monaco, H. L. (2003). Structural and biochemical characterisation of a new type of lectin isolated from carp eggs. *Biochemical Journal* **376,** 433–440.
Jakobsson, S., Borg, B. Haux, C. & Hyllner, S. J. (1999). An 11-ketotestosterone induced kidney-secreted protein: the nest building glue from male three-spined stickleback, *Gasterosteus aculeatus*. *Fish Physiology and Biochemistry* **20,** 79–85.
Knouft, J. H., Page, L. M. & Plewa, M. J. (2003). Antimicrobial egg cleaning by the fringed darter (Perciformes: Percidae: *Etheostoma crossopterum*): implications of a novel form of parental care in fishes. *Proceedings of the Royal Society of London B* **270,** 2405–2411.
Kraak, S. B. M., Bakker, T. C. M. & Mundwiler, B. (1999). Sexual selection in sticklebacks in the field: correlates of reproductive mating, and paternal success. *Behavioral Ecology* **10,** 696–706.
Kraak, S. B. M., Bakker, T. C. M. & Hocevar, S. (2000). Stickleback males, especially large and red ones, are more likely to nest in concealed macrophytes. *Behaviour* **137,** 907–919.
Lozano, G. A. (1998). Parasitic stress and self-medication in wild animals. *Advanced Studies in Behaviour* **27,** 291–317.
McDonald, C. G., Reimchen, T. G. & Hawryshyn, C. W. (1995). Nuptial colour loss and signal masking in *Gasterosteus*: an analysis using video imaging. *Behavior* **132,** 963–977.
McFarland, K. P. & Rimmer, C. C. (1996). Horsehair fungus, *Marasmius androsaceus*, used as nest lining by birds of the subalpine spruce-fir community in the Northeastern United States. *Canadian Field Naturalist* **110,** 541–543.
Milinski, M. & Bakker, T. C. M. (1990). Female sticklebacks use male coloration in mate choice and hence avoid parasitized males. *Nature* **344,** 330–333.
Östlund-Nilsson, S. (2001). Fifteen-spined stickleback (*Spinachia spinachia*) females prefer males with more secretional threads in their nests: an honest-condition display by males. *Behavioral Ecology and Sociobiology* **50,** 263–269.

Reusch, T. B. H., Haberli, M. A., Aeschlimann, P. B. & Milinski, M. (2001). Female sticklebacks count alleles in a strategy of sexual selection explaining MHC polymorphism. *Nature* **414,** 300–302.
Richardson, D. S., Komdeur, J., Burke, T. & von Schantz, T. (2005). MHC-based patterns of social and extra-pair mate choice in the Seychelles warbler. *Proceedings of the Royal Society B* **272,** 759–767.
Roitt, I. M. (1997). *Essential Immunology*. Oxford: Blackwell Science.
Rowland, W. J. (1994). Proximate determinants of stickleback behaviour: an evolutionary perspective. In *The Evolutionary Biology of the Threespine Stickleback* (Bell, M. A. & Foster, S. A., eds), pp. 297–344. Oxford: Oxford University Press.
Rushbrook, B. J., Katsiadaki, I. & Barber, I. (2007). Spiggin levels are reduced in male sticklebacks infected with *Schistocephalus solidus*. *Journal of Fish Biology* **71,** 298–303.
Smith, N. C., Wallach, M., Petracca, M., Braun, R. & Eckert, J. (1994). Maternal transfer of antibodies induced by infection with *Eimeria maxima* partially protects chickens against challenge with *Eimeria tenella*. *Parasitology* **109,** 551–557.
Swain, P., Dash, S., Bal, J., Routray, P., Sahoo, P. K., Sahoo, S. K., Saurabh, S., Gupta, S. D. & Meher, P. K. (2006). Passive transfer of maternal antibodies and their existence in eggs, larvae and fry of Indian major carp, *Labeo rohita* (Ham.). *Fish and Shellfish Immunology* **20,** 519–527.
Tinbergen, N. (1951). *The Study of Instinct*. Oxford: Oxford University Press.
Wedekind, C., Seebeck, T., Bettens, F. & Paepke, A. J. (1995). MHC-dependent mate preferences in humans. *Proceedings of the Royal Society B* **260,** 245–249.
Wootton, R. J. (1976). *The Biology of the Stickleback*. London: Academic Press.
Wootton, R. J. (1984). *A Functional Biology of Sticklebacks*. London: Croom Helm.

