

# Antimicrobial egg cleaning by the fringed darter (Perciformes: Percidae: *Etheostoma crossopterygum*): implications of a novel component of parental care in fishes

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Broad-spectrum antimicrobial compounds have recently been identified in the epidermal mucus of fishes and probably serve as a first line of defence against microbial pathogens. Because of the ubiquitous nature of fungi and bacteria in aquatic systems, defence against these pathogens should be required throughout the lifespan of fishes, including the egg stage. We conducted experiments on *Etheostoma crossopterygum* (Percidae: *Catonotus*), the fringed darter, to determine if the presence of a guarding male inhibits microbial colonization of eggs. Based on results from a combination of in-stream experiments, *in vitro* microbial assays, and morphological characteristics and behaviour of breeding males, we propose that antimicrobial egg cleaning by the guarding male is an effective component of parental care in these fish. Although innate antimicrobial compounds have been identified in a variety of organisms ranging from insects to vertebrates, integration of these compounds into a species's reproductive life history has been identified only in a small number of insect species. The results from this study not only indicate that *E. crossopterygum* males provide a novel form of vertebrate parental care, but also have implications regarding the evolution of parental care in fishes and transitional evolutionary stages from no parental care to male parental care.

**Keywords:** nest guarding; parental care; *Etheostoma crossopterygum*; egg cleaning; antimicrobial compounds

## 1. INTRODUCTION

Fishes display a remarkable variety of parental care, with forms of parental investment quite different from other vertebrate groups (Breder & Rosen 1966; Perrone & Zaret 1979; Gross & Sargent 1985). Nest guarding is the most common parental behaviour exhibited among families of fishes, with males more likely than females to be the guarding sex (Blumer 1979). This bias may be due to the greater net fitness advantage to males resulting from guarding (Gross & Sargent 1985). In any case, this parental presence presumably inhibits predation and decreases the amount of debris on the eggs, provides oxygen and assists in the removal of waste (Moyle & Cech 1996).

Male guarding behaviour and the evolution of parental care in fishes are well studied (Balshine-Earn & Earn 1998; Goodwin *et al.* 1998; Lindstrom 2000; St Mary *et al.* 2001). Male guarding in externally fertilizing species has been proposed to be the result of an evolutionary transition from no parental care to male parental care (Gross & Sargent 1985). However, this transition does not automatically ensure parental care to the offspring. In some cases, an intermediate stage is evident in which the male gains some benefit from remaining at the nest while the offspring realize no benefit from the male's presence (Gross & Sargent 1985; Clutton-Brock 1991).

Although a considerable amount of effort has been directed towards understanding reproductive behaviour in fishes (Breder & Rosen 1966; Perrone & Zaret 1979; Baylis 1981; Gross & Sargent 1985), almost no attention has been given to the relationship between parental care, microbial infection and egg viability. This is surprising considering that all organic surfaces in aquatic systems are covered by biofilms that are composed primarily of heterotrophic bacteria and fungi (Lock *et al.* 1984). Localized growth inhibition of these microbes should be essential to egg survival.

Recent studies have identified the presence of antimicrobial compounds in the epidermal mucus of a variety of fishes (e.g. *Oncorhynchus mykiss* (Austin & McIntosh 1988); *Cyprinus carpio* (Cole *et al.* 1997); *Pleuronectes americanus* (LeMaitre *et al.* 1997); *Morone saxatilis* × *M. chrysops* hybrid (Silphaduang & Noga 2001)). These species are members of different orders (Salmoniformes, Cypriniformes, Pleuronectiformes and Perciformes, respectively), suggesting that the presence of antimicrobial compounds in fish mucus is not phylogenetically constrained and such compounds may be ubiquitous among fishes. These compounds have been proposed to serve as a first line of defence against pathogens (Boman 1995). Because nest-guarding fishes are in contact or close proximity to their eggs, antimicrobial compounds in fish mucus may serve an adaptive purpose by preventing microbial colonization of eggs.

*Etheostoma* (Perciformes: Percidae), containing approximately 140 species, is the most diverse genus of freshwater fishes in North America (Page 2000). *Catonotus*,

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containing 18 species, is a subgenus of *Etheostoma* in which all species exhibit egg-clustering behaviour characterized by male guarding (Page 1983, 1985; Porterfield *et al.* 1999). Within *Catonotus*, adults are sexually dimorphic during the breeding season. Males are larger and more boldly patterned. During the reproductive period (typically March–May), a male establishes a territory under the cavity of a flat stone. Multiple females will sequentially attach eggs to the underside of the stone that are simultaneously fertilized by the male. This process results in a single layer of up to 1500 eggs (*ca.* 2 mm in diameter) deposited on the nest stone, i.e. on the ‘ceiling’ of the nest cavity directly above the male. The male then remains at the nest until the eggs hatch. Time to hatching is dependent on water temperature and can range from 5 to 10 days among nests across the spawning season (Page 1983), while time to hatching within a specific nest is similar for all eggs.

Although several studies have investigated facets of *Catonotus* reproductive behaviour (Knapp & Sargent 1989; Page & Bart 1989; Lindstrom & Sargent 1997; Page & Knouft 2000; Porter *et al.* 2002), whether *Catonotus* males actually provide parental care is unclear. Because females prefer to deposit eggs in nests already containing eggs (Knapp & Sargent 1989), males may remain at the nest simply to obtain spawnings with females. The benefit of male guarding to eggs is even more uncertain considering that Lindstrom & Sargent (1997) found high levels of filial cannibalism by guarding males.

The objective of this study was to determine experimentally whether mucus from male *E. crossotermum* confers an antimicrobial benefit to guarded eggs. Observations indicate that nests abandoned by the guarding male often contain non-viable eggs with fungal (*Saprolegnia* spp.) and bacterial infection. We predict that because of the behaviour and proximity to the eggs of the breeding male, antimicrobial compounds contained in the male’s mucus will inhibit microbial growth on eggs, increase egg viability, and thus support the hypothesis that species of *Catonotus* provide male parental care.

## 2. MATERIAL AND METHODS

### (a) *In-stream study*

We collected 12 nest stones from Ferguson Creek, Livingston County, KY, USA. At the time of collection, each of the 12 nest stones was guarded by a breeding male and contained from 67 to 1440 eggs. None of the eggs displayed obvious signs of fungal or bacterial infection (i.e. opaque eggs and/or visible hyphae). We collected guarding males with six of the nest stones and each male remained with their respective nest stone throughout the experiment. Males from the remaining six stones were returned to the stream.

For each experimental replicate, an in-stream cage (0.91 m × 0.61 m × 0.46 m) enclosed with 1 mm mesh screen was divided laterally into two equal-sized chambers with a 1 mm mesh screen insert. We placed a nest stone with a guarding male into one chamber, while a nest stone without the guarding male was added to the adjoining chamber. We paired nest stones in each cage to minimize the differences in egg number between nests.

We checked eggs daily for 4 days. During each check, we counted the total number of eggs and the total number of

opaque and/or fungus-covered eggs. We also noted whether or not the male was still guarding the eggs. No eggs hatched during the experiment. A comparison of the percentage of infected eggs versus uninfected eggs among treatments was made using data collected on day 4 (paired *t*-test,  $\alpha = 0.05$ ). Percentage data were arcsine transformed before statistical analyses to achieve normality. Based on daily egg counts, a minimal number of eggs were consumed by guarding males (less than 5%) and our analyses of the percentage of infected eggs assumes that males were not selective regarding which eggs they consumed. Males and nest stones were returned to the stream after termination of the experiment.

We performed a colony-forming unit (CFU) assay to demonstrate that opaque eggs contained a greater number of bacteria than clear eggs. Five clear and five opaque eggs were selected from a nest and placed in separate 1.5 ml microcentrifuge tubes and 250  $\mu$ l of Luria–Bonner broth (LB) was added to both tubes. Eggs were pulverized with a sterile toothpick and vortexed for 1 min; 100  $\mu$ l quantities of serial dilutions ( $10^{-1}$  to  $10^{-6}$ ) from each egg type were then incubated on LB plates for 24 h at 37 °C. After incubation, CFUs were counted and averaged across serial dilutions to determine the number of CFUs per five eggs. This experiment was replicated three times.

### (b) *Collection of epidermal mucus*

To obtain samples of epidermal mucus, we collected 5–12 *E. crossotermum* breeding males from a stream. Epidermal mucus was acquired by gently drawing a sterile spatula across the predorsal nape of each male. We then combined mucus from all males from a particular stream and collection date in a 1.5 ml microcentrifuge tube containing 300  $\mu$ l of 0.1 M potassium phosphate buffer (PPB) pH 7.4. Pooling of samples from multiple males was necessary because of the small amount of mucus that can be obtained from each male. Samples were either used immediately or frozen at –80 °C until required.

### (c) *Microbial assays*

Previous studies of vertebrate antimicrobial compounds indicate that these substances are not specific, with inhibitory effects demonstrated on a broad range of microbes (Zasloff 1987, 2002; Boman 1995; Cole *et al.* 1997; LeMaitre *et al.* 1997; Silphaduang & Noga 2001). Because a limited amount of mucus could be acquired from each *E. crossotermum* male, and relatively small population sizes in each stream limited the number of males that could be captured, we directed antimicrobial assays at bacterial and fungal strains that represented common characteristics of the microbial community.

### (d) *Cytotoxicity assay*

Based on previous studies (Cole *et al.* 1997; LeMaitre *et al.* 1997), the epidermal mucus of fishes appears to serve as a medium for the concentration and, hypothetically, delivery of an antimicrobial agent. We used three separate samples of *E. crossotermum* mucus to determine the effect of mucus on bacterial growth. Samples were collected as described above and contained mucus from five to seven breeding males. Sample A was collected from individuals from Ferguson Creek, Livingston County, KY, USA, on 23 April 2000. Sample B, collected on 16 April 2000, and sample C, collected on 17 April 2000 were acquired from individuals from Big Creek, Union County, IL, USA.

To conduct cytotoxicity assays, we first determined protein concentrations of the three separate samples. Fish mucus is

primarily composed of mucin, which is a glycoprotein (Helfman *et al.* 1997). Because the antimicrobial agent in *E. crossosternum* fish mucus is unknown, we quantified the treatment dose of the antimicrobial agent in the cytotoxicity assay as micrograms of protein in the form of mucin. We determined protein concentration in each sample of mucus + PPB using a Bio-Rad protein concentration assay (microassay procedure). A protein standard (1.36 mg ml<sup>-1</sup> albumin) was used to generate a standard curve by determining absorbance at 595 nm of serial dilutions of the protein standard. We then compared absorbances of dilutions of mucus + PPB to the protein standard curve to calculate protein concentration of the fish mucus samples.

We employed a modified microplate-based *in vitro* bacterial cytotoxicity assay to quantitatively measure the effect of *E. crossosternum* mucus on the growth of *Salmonella typhimurium*. This assay was modified from that published by Kargalioglu *et al.* (2000, 2002). *Salmonella typhimurium* was assayed because it was the standard microbe used to develop the cytotoxicity assay (Kargalioglu *et al.* 2000, 2002); 500 µl of log-phase *S. typhimurium* cells (strain TA100) were grown in 5 ml of LB for 2 h at 37 °C while shaking. The cell titre was adjusted to an optical density (OD) of 0.30 at 595 nm. The cells were exposed to a concentration series of the fish mucus in a total volume of 100 µl per well. A series of wells were prepared with 100 µl of PPB. The negative control wells contained 30 µl of the titred bacterial cell suspension plus 70 µl of PPB. The treatment wells contained 30 µl of the titred bacterial cell suspension and 70 µl of known concentrations of the fish mucus + PPB, and the concentration was determined based on the protein concentration of each sample. The microplate was incubated for 1 h at 37 °C while shaking. After the treatment time, 100 µl of 2× LB was added to each well. At time 0 the initial OD of each well was measured with a Bio-Rad Model 550 Microplate Reader at 595 nm. This provided a time 0 blank reading for each microplate well. The microplate was then placed in a padded holder, incubated at 37 °C, and shaken at 200 r.p.m. for 210 min. The final OD of each well was determined at 595 nm with the microplate reader.

For each well, we subtracted the blank OD value (time 0 reading) from the OD reading of each specific well after 210 min of incubation. The blank-corrected data for the wells of each concentration of the fish mucus were averaged. The concurrent negative control consisted of bacteria with no fish mucus exposure. The blank-corrected data for the negative control were set at 100%. The blank-corrected data for each fish mucus concentration were converted into a percentage of the negative control, which is the measure of bacterial cytotoxicity.

### (e) *Saprolegnia spp.* inhibition

We conducted an experiment to assess the inhibitory effect of mucus on the growth of *Saprolegnia spp.* (Oomycota). *Saprolegnia spp.* was chosen for this experiment because it is an extremely common freshwater fungus, a common fish fungal pathogen, and also found to be the primary fungal colonizer of eggs in abandoned *E. crossosternum* nests (Wolke 1975; J. H. Knouft and L. M. Page, personal observations). Identification to species in *Saprolegnia* relies on the morphology of the sexual phase. *Saprolegnia* samples acquired from fishes and other aquatic animals typically lack a sexual phase (Hughes 1994), and species-level identification was not possible in this study. Consequently, samples were grouped into the classification of *Saprolegnia spp.*

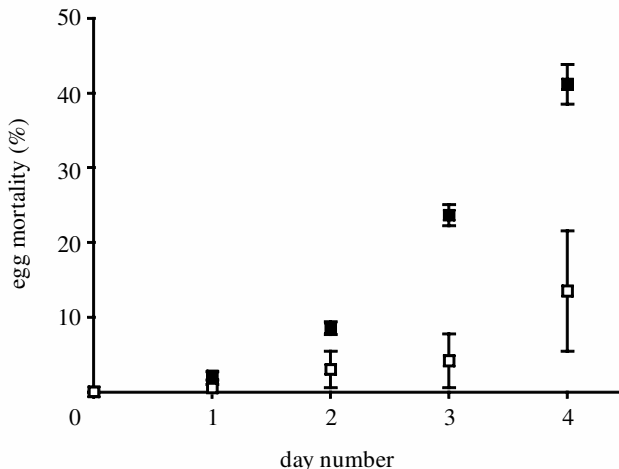


Figure 1. Relationship between presence and absence of a guarding male *Etheostoma crossosternum* and non-viability of eggs in the nest. Open squares: male present; filled squares: male removed.

Using the methods described above, we collected a pooled mucus sample from 12 *E. crossosternum* males from Ferguson Creek, Livingston County, KY, USA. We also collected three nest stones with apparently uninfected eggs and a 1 l sample of unfiltered stream water from Ferguson Creek. Three treatments, each consisting of five replicates, were applied to eggs from each stone. A replicate in treatment A consisted of a 0.5 ml centrifuge tube containing one egg + 120 µl PPB, a replicate in treatment B consisted of a 0.5 ml centrifuge tube containing one egg + 20 µl PPB + 100 µl stream water, and a replicate in treatment C consisted of a 0.5 ml centrifuge tube containing one egg + 20 µl (mucus + PPB) + 100 µl stream water.

We scored each replicate with a rank based on fungal infestation at 0, 8, 16 and 24 h. A score of 1 indicated no apparent fungal infestation. In this case, the egg was nearly transparent. A score of 2 indicated the presence of fungus on the external surface of the egg. At this level, embryos are still living. A score of 3 indicated opaqueness of the egg, suggesting that fungus had invaded the egg, and a score of 4 indicated the presence of both external and internal fungal infestation. Scores of 3 and 4 indicate cases when the embryo is no longer viable. Comparisons among treatments were made separately using data collected at each time point. Because three tests are made on the same dataset, a Bonferroni-corrected  $\alpha$  ( $\alpha'$ ) was used to determine statistical significance of data from each time-point ( $\alpha = 0.050$ , three tests,  $\alpha' = 0.017$ ).

## 3. RESULTS

### (a) *In-stream study*

The presence of a guarding male significantly reduced rates of fungal and/or bacterial infection of eggs (d.f. = 5,  $t_{stat} = -4.879$ ,  $p = 0.0046$ ; figure 1). The elevated mortality on day 4 in the 'male present' treatment is due to a high level of infection (53%) in one replicate. During two out of four nest checks, this male was found away from his nest stone. Consequently the high rate of infection may be due to nest abandonment by this male. Percentage of infected eggs was still low (less than 5%) at the time of abandonment, suggesting that the high rate of infection occurred after abandonment. Infections in the other five replicates on day 4 ranged from 1% to 10%.

Examination of non-viable eggs revealed the presence of both Gram-positive and Gram-negative (*Aeromonas hydrophila*, *Pseudomonas* sp.; identified with Benton-Dickinson Oxi/Ferm Tube II) bacteria as well as high levels of fungal (*Saprolegnia* spp.) infestation. Opaque eggs contained a much greater number of CFUs than clear eggs (mean clear-egg CFUs per five eggs = 1308.3, mean opaque-egg CFUs per five eggs = 767 160.8).

#### (b) Cytotoxicity assay

In all three trials, *S. typhimurium* displayed a dose-dependent cytotoxic response to *E. crossopterum* mucus (least squares linear regression: sample A:  $y = -1.60x + 81.573$ ,  $F_{1,5} = 11.949$ ,  $r^2 = 0.749$ ,  $p = 0.026$ ; sample B:  $y = -0.297x + 102.634$ ,  $F_{1,11} = 67.265$ ,  $r^2 = 0.871$ ,  $p < 0.001$ ; sample C:  $y = -0.410x + 100.869$ ,  $F_{1,10} = 24.153$ ,  $r^2 = 0.729$ ,  $p < 0.001$ ; figure 2). Because we were unaware of the effect of the mucus from each sample before testing, we attempted to include a broad range of doses with a maximum number of concentrations for each sample. Consequently, because different amounts of mucus were collected from each sample group, different numbers of concentrations were used in each trial.

#### (c) *Saprolegnia* spp. inhibition

Because we used eggs from three separate stones in the *Saprolegnia* spp. inhibition study, we conducted an initial test using data collected at 24 h to determine if there was a stone effect across treatments. Neither treatment A (Kruskal–Wallis:  $H_{\text{stat}} = 0.000$ , d.f. = 2,  $p > 0.999$ ), treatment B (KW:  $H_{\text{stat}} = 2.333$ , d.f. = 2,  $p = 0.311$ ), or treatment C ( $H_{\text{stat}} = 0.000$ , d.f. = 2,  $p > 0.999$ ) displayed any differences in response among stones. Therefore, we grouped replicates among stones for subsequent analyses. Kruskal–Wallis tests using data collected at each time-point indicated a significant difference in fungal infestation among treatments at all time points after 0 h (8 h:  $H_{\text{stat}} = 42.429$ , d.f. = 2,  $p < 0.001$ ; 16 h:  $H_{\text{stat}} = 42.308$ , d.f. = 2,  $p < 0.001$ ; 24 h:  $H_{\text{stat}} = 42.857$ , d.f. = 2,  $p < 0.001$ ), with only treatment B exhibiting obvious fungal infestation (figure 3). *Saprolegnia* spp. was the only apparent fungus present on infected eggs at the conclusion of the experiment.

## 4. DISCUSSION

Although previous research has examined male reproductive behaviour in species of *Catnotus* (e.g. Knapp & Sargent 1989; Porter *et al.* 2002), actual identification of male parental care has been elusive. The results from this study confirm that *E. crossopterum* males, and presumably other species of *Catnotus*, provide parental care by reducing the amount of microbial infestation of eggs and consequently increasing egg viability. This reduction in microbial infestation is apparently facilitated by antimicrobial compounds present in the mucus of the guarding male.

Whether the guarding male consumed infected eggs during the in-stream experiment and thus reduced the spread of microbes among the uninfected eggs deserves consideration. Visual observations of the eggs during the experiment, coupled with the results from the microbial assays suggest that this is probably not the major reason

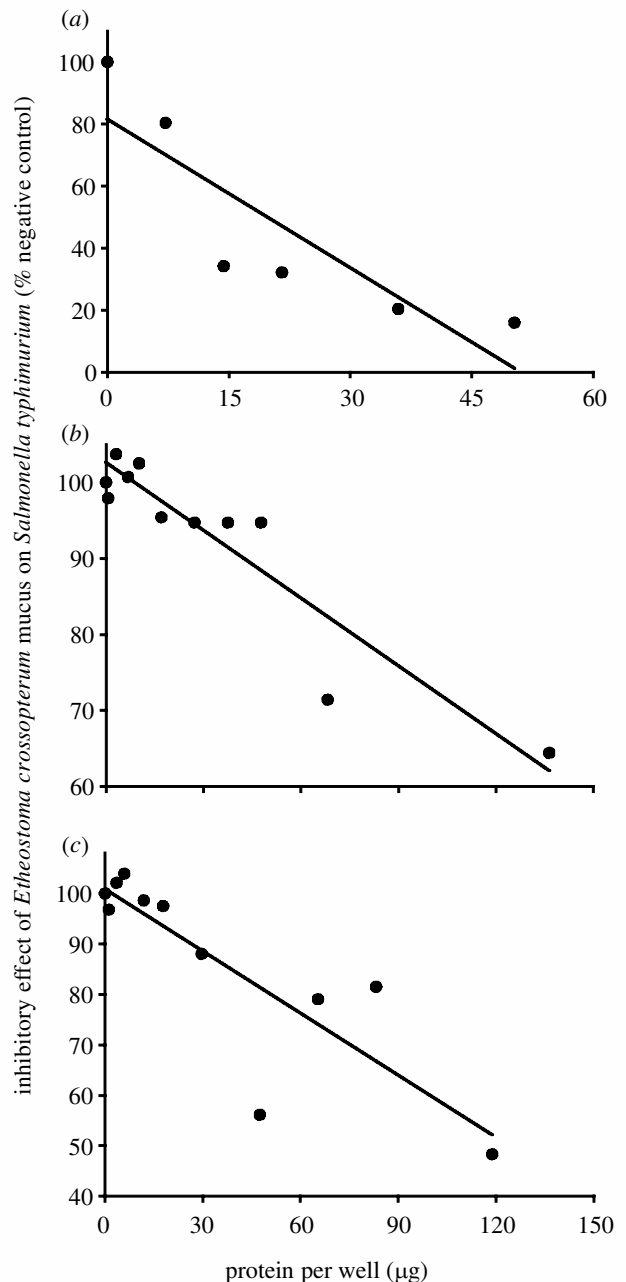


Figure 2. Cytotoxic response of *Salmonella typhimurium* to *Etheostoma crossopterum* mucus. (a) Sample A from Ferguson Creek, Livingston County, KY, USA, collected on 23 April 2000. (b) Sample B from Big Creek, Union County, IL, USA, collected on 16 April 2000. (c) Sample C from Big Creek, Union County, IL, USA, collected on 17 April 2000.

for microbial inhibition. We noted the location of infected eggs in the guarded nests and observed, in subsequent nest checks, that infected eggs were not removed by the male. Single eggs would become infected in the guarded nests but infection would not spread to adjacent, contiguously placed eggs even though eggs were not removed by the male. Localized infections in the unguarded nests would quickly spread to adjacent eggs. This suggests that microbial spread is inhibited by the antimicrobial effect of the mucus and not by removal of eggs by the male.

Considering the results from the cytotoxicity assay, the mucus appears to have antiseptic effects on the bacteria. We conclude that the effect is cytotoxic as opposed to

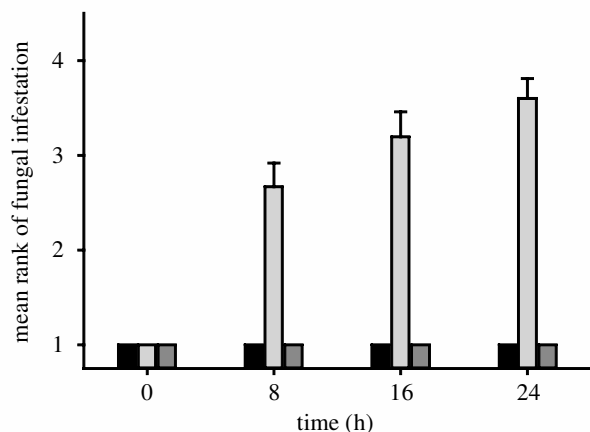


Figure 3. Mean rank of *Saprolegnia* spp. infestation of eggs among treatments over time. Black bars: treatment A, 1 egg + 0.1 M sterile PPB; light grey bars: treatment B, 1 egg + stream water + 0.1 M sterile PPB; dark grey bars: treatment C, 1 egg + stream water + (0.1 M sterile PPB + mucus).

cytotoxicity based on results acquired during assay development and calibration in which treatment wells exhibiting an effect at 210 min had a smaller number of CFUs than treatment wells at 0 min (Kargalioglu *et al.* 2002). It is unclear whether the mucus is fungicidal, inhibits fungal growth or prevents fungal adhesion to the egg surface. Nevertheless, the effect can still be viewed as antifungal given that the presence of the guarding male decreased in-stream fungal growth on eggs and the addition of mucus apparently eliminated fungal growth on eggs in the centrifuge tubes.

The recent discovery of antimicrobial compounds in fish epidermal mucus has revealed a striking line of defence in response to the microbial composition of the aquatic environment (Austin & McIntosh 1988; Cole *et al.* 1997; LeMaitre *et al.* 1997; Ebran *et al.* 2000). If adult fishes benefit from this defence, eggs deposited on the biofilm-covered substrate will also presumably benefit from a defence against microbial pathogens. Results from the bacterial cytotoxicity assay and *Saprolegnia* spp. inhibition study suggest that a compound in *E. crossosternum* mucus exhibits antimicrobial activity. Consequently, the identification of particular morphological and/or behavioural characteristics present in breeding males that facilitate application of mucus to eggs should support antimicrobial egg cleaning as a form of parental care in fishes.

Antimicrobial compounds have been found associated with and dispersed from the epithelial mucus-secreting cells of fishes (Cole *et al.* 1997). Therefore, a higher concentration of mucus cells should increase the amount of antimicrobial compound delivered to the skin surface. In a histological study, Bart & Page (1991) noted that the fleshy pre-dorsal pad displayed only in breeding males had a higher concentration of mucus-secreting cells than the same area in non-breeding males as well as other areas of the breeding male (figure 4). The disproportionately large amount of mucus secreted by breeding males from this area is clearly apparent during handling of these fishes (J. H. Knouft, personal observation). The area occupied by the male under the nest stone is small and contact by

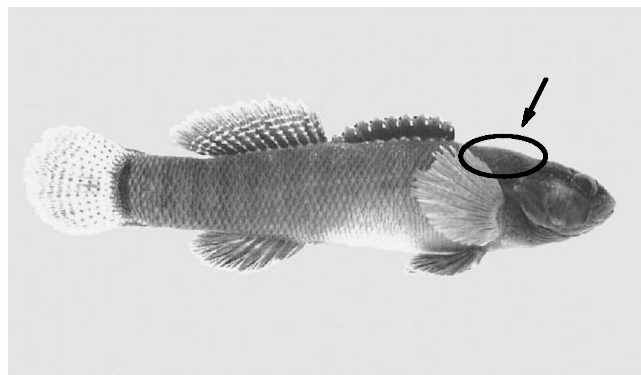


Figure 4. Breeding male *Etheostoma crossosternum*. Oval indicates mucus-cell-rich pre-dorsal pad.

the pre-dorsal pad of the male with the eggs is extremely common. In fact, previous studies have suggested that the male coats the eggs with mucus to reduce his tactile damage to the eggs as well as provide a physical (rather than chemical) barrier against pathogens (Bart & Page 1991). Thus, the delivery of the antimicrobial compounds appears to be facilitated and enhanced by the presence of the mucus-cell-rich fleshy pre-dorsal pad in breeding males.

Considering the results from the in-stream and microbial experiments as well as the morphology and behaviour of breeding males, antimicrobial egg cleaning is probably an effective form of parental care in *E. crossosternum*. This type of parental care is novel largely because of the associated chemical component coupled with the apparently adaptive male pre-dorsal morphology, which permits increased production and application of the epidermal antimicrobial mucus to the eggs. Previous studies have speculated that nest-guarding fishes can mechanically remove debris by fanning eggs and thus prevent microbial colonization (e.g. Côté & Gross 1993); however, such a mechanism has not received strong experimental support. Moreover, it seems unlikely that the minimal flow of water created by fanning would be sufficient to remove attached bacteria and fungal spores, or inhibit the hyphal spreading and growth of fungi that have adhered to the egg surface.

#### (a) *Broader implications of antimicrobial compounds in parental care*

Innate antimicrobial compounds with a broad antimicrobial effect have been identified in a variety of multicellular organisms (Zasloff 2002), ranging from insects (review in Bulet *et al.* 1999) to several groups of vertebrates (e.g. amphibians (Zasloff 1987), fishes (Cole *et al.* 1997) and mammals (Harder *et al.* 1997)). To our knowledge, the integration of these compounds into a species's reproductive life history has only been identified in insects. *Drosophila melanogaster* males transfer antibacterial proteins to their mates, presumably protecting both the female's reproductive tract and the eggs against bacterial infection (Lung *et al.* 2001). Female reproductive glands in the medfly (*Ceratitis capitata*) produce an antimicrobial secretion that is transferred to the egg surface (Marchini *et al.* 1995, 1997). This transfer is apparently the only previously documented case of a species applying an anti-

microbial compound to deposited eggs (Marchini *et al.* 1997).

The few antimicrobial agents structurally identified in the mucus of bony fishes (Osteichthyes) are proteins. It has been proposed that these compounds bind to and essentially dissolve cellular membranes (Pouny *et al.* 1992; Gazit *et al.* 1995; Shai 1995; Ebran *et al.* 1999, 2000; Zasloff 2002). Evidence suggests that an antimicrobial chemical component to parental care may be present in both egg-guarding and egg-dispersing freshwater fish species (e.g. *Pimephales* spp. (Smith & Murphy 1979); *Perca fluviatilis* (Paxton & Willoughby 2000)). Considering the relatively recent identification of antimicrobial compounds in fish mucus and the apparent need for a defence against microbes in aquatic environments, identification of antimicrobial components to reproductive behaviour may eventually be revealed to be a relatively common phenomenon.

D. White and the staff at the Hancock Biological Station were extremely helpful and gracious during our research at their facility. We thank W. Chen for isolating and identifying *Saprolegnia* spp. from experiments during this research, and E. Wagner and Y. Kargalioglu for assistance during the cytotoxicity assays. We also thank P. Levin for assistance with Gram-staining and CFU procedures. S. Phelps provided helpful suggestions regarding experimental design. We also appreciate the comments from L. Harmon, J. Losos and three anonymous reviewers on a previous version of this manuscript. This research was supported by the Illinois Natural History Survey and the Illinois Department of Transportation.

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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.