
A male sexually dimorphic trait provides antimicrobials to eggs in blenny fish

Eva Giacomello, Daniela Marchini and Maria B Rasotto

Biol. Lett. 2006 **2**, 330-333
doi: 10.1098/rsbl.2006.0492

Supplementary data

["Data Supplement"](#)

<http://rsbl.royalsocietypublishing.org/content/suppl/2008/12/08/2.3.330.DC1.html>

References

[This article cites 20 articles, 2 of which can be accessed free](#)

<http://rsbl.royalsocietypublishing.org/content/2/3/330.full.html#ref-list-1>

Article cited in:

<http://rsbl.royalsocietypublishing.org/content/2/3/330.full.html#related-urls>

Email alerting service

Receive free email alerts when new articles cite this article - sign up in the box at the top right-hand corner of the article or click [here](#)

To subscribe to *Biol. Lett.* go to: <http://rsbl.royalsocietypublishing.org/subscriptions>

A male sexually dimorphic trait provides antimicrobials to eggs in blenny fish

Eva Giacomello^{1,*}, Daniela Marchini²
and Maria B. Rasotto¹

¹Department of Biology, University of Padova, Via U. Bassi 58/B, 35131 Padova, Italy

²Department of Evolutionary Biology, University of Siena, Via A. Moro 2, 53100 Siena, Italy

*Author for correspondence (eva.giacomello@unipd.it).

Predation and microbial infections are the major causes of natural mortality for early life stages of oviparous species. The parental traits reducing the effects of predation are rather well described, whereas antimicrobial mechanisms enhancing offspring survival are largely unexplored. In this paper, we report that a male sexually dimorphic trait, the anal glands, of the redlip blenny (*Ophioblennius atlanticus atlanticus*) and the peacock blenny (*Salaria pavo*), two fish species with paternal egg care, produce a mucus enriched with antimicrobial substances. Histological and histochemical analyses showed that the anal glands of these species are characterized by the massive presence of mucus-secreting cells. Anal gland extracts, from both the hydrophilic and the hydrophobic protein fraction, exhibited a lysozyme-like activity. Field observations demonstrated that redlip blenny males, while performing egg care, rub the anal region over the nest internal surface, probably facilitating the transfer of mucus to eggs. These results strongly indicate that this sexually dimorphic trait is involved in egg defence against microbial infections.

Keywords: Blenniidae; sexually dimorphic trait; parental care; antimicrobial activity; anal glands

1. INTRODUCTION

In oviparous animals, the benefits of parental care and the quality of the sex performing egg care have mainly been described in relation to the morphological and/or behavioural traits reducing the effects of predation, while almost no attention has been given to the relationship between parental investment and microbial infection (Clutton-Brock 1991). Indeed, although many antimicrobials have been identified in different animal taxa (Brogden 2005), their possible role in reproduction has been poorly investigated and primarily related to the maternal transfer of immune factors to eggs (Yousif *et al.* 1994; Marchini *et al.* 1997).

The epidermal mucus of fishes plays a significant role in their protection against pathogenic infections and antimicrobials have been found associated with and released from the epidermal mucus-secreting cells of several species (Cole *et al.* 1997; Ellis 2001). Recently, in a species performing paternal care, the skin

The electronic supplementary material is available at <http://dx.doi.org/10.1098/rsbl.2006.0492> or via <http://www.journals.royalsoc.ac.uk>.

mucus has also been demonstrated to protect developing eggs (Knouft *et al.* 2003). Considering the widespread occurrence of male parental care in fish (Clutton-Brock 1991), if the adult epidermal mucus can protect the eggs deposited on the substrate against pathogens, morphological and/or behavioural traits facilitating or enhancing the transfer of mucus to eggs should be expected in the breeding males of many species.

In several species of Blenniidae, a family of demersal spawners with paternal care, males exhibit a peculiar sexually dimorphic trait: a pair of globular structures on the most anterior anal fin rays, the anal glands (Zander 1975). Previous studies have indicated that these organs are well developed in nesting males during the breeding season (Giacomello & Rasotto 2005) but remain rudimentary in males engaged in opportunistic mating tactics (Gonçalves *et al.* 1996; Neat *et al.* 2003). Information on the morphology and physiology of these organs is scanty, however, they have been hypothesized to play a role in pheromone production or in the release of antimicrobials to eggs (Zander 1975). While field studying the mating system of some blennies, it came to our attention that breeding males rub the anal–urogenital region over the nest internal surface during spawning (see electronic supplementary material), a behaviour associated with ejaculation in Gobiidae (Marconato *et al.* 1996).

If the anal glands represent a likely location from which to release a protective mucus onto developing eggs, then it should be expected that (i) they show a high concentration of mucus-secreting cells; (ii) their secretions exhibit antimicrobial activity; and (iii) males perform rubbing behaviour not only during spawning, but also while performing parental care. We tested the predictions (i) and (ii) on the redlip blenny, *Ophioblennius atlanticus atlanticus*, and on the peacock blenny, *Salaria pavo*, two polygynous species whose larger males exhibit larger anal glands (figure 1a) and attain the highest mating success (Oliveira *et al.* 1999; Giacomello 2004). The third prediction was tested, with field observations, in the redlip blenny, where the large shelters used by nesting males facilitate the record of rubbing behaviour.

2. MATERIAL AND METHODS

(a) Field observations

Behavioural observations on 15 nesting males (total length, TL=14.2–22.0 cm) of *O. atlanticus atlanticus* were conducted in Faial Island (Azores, Portugal), by scuba diving, during the breeding season 2003. Repeated 5 min focal observations were made (1–7 of each male), recording the time the male spent rubbing the anal–urogenital region over the nest internal surface and the number of rubbing acts, both during spawning time (6.30–8.30) and outside it (10.00–20.00). The mean rubbing time (\pm s.e.) and frequency of rubbing (\pm s.e., number of rubbing acts per 5 min) were calculated for each individual.

(b) Fish samples

Breeding males of *O. atlanticus atlanticus*, in Faial Island, and *S. pavo*, in the Lagoon of Venice (Italy), were collected in 2004. Animals were anaesthetised with MS222 (Sandoz), measured (TL) and rinsed with filtered seawater to reduce the bacterial contamination on body surface. Anal glands were removed. After surgery all animals fully recovered and were released into the field.

(c) Histology and histochemistry

Anal glands of *O. atlanticus atlanticus* (TL=14.5–21.0 cm, $n=5$) and *S. pavo* (TL=10.9–14.5, $n=2$) were processed and stained in order to reveal mucosubstances as in Neat *et al.* (2003).

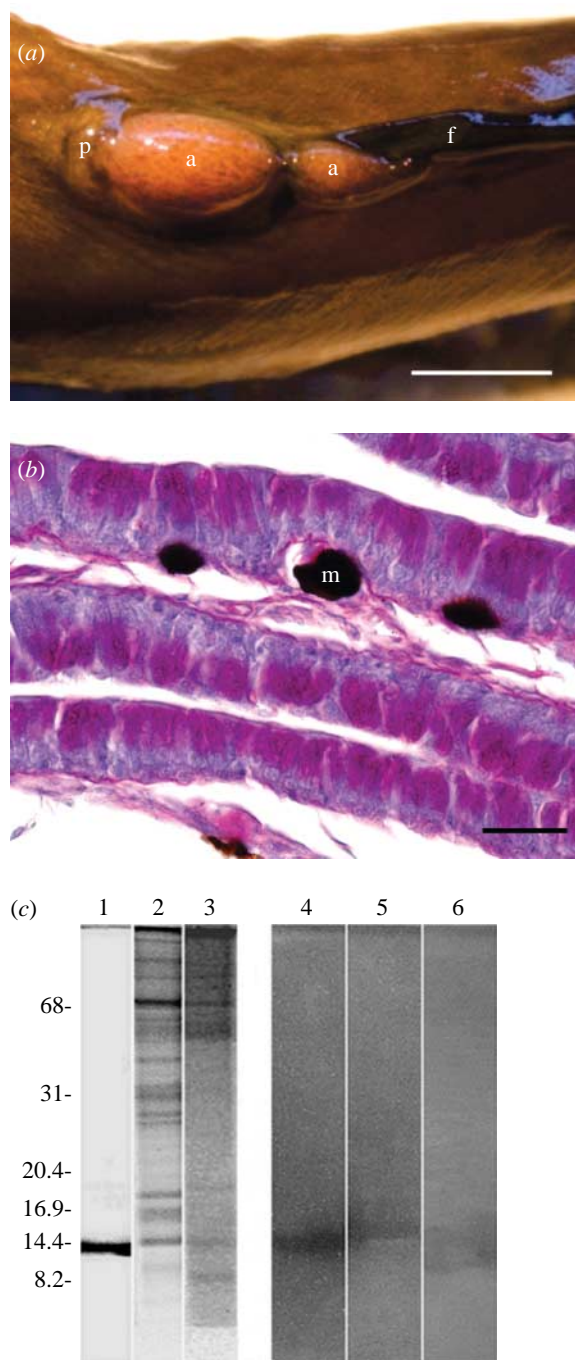


Figure 1. Anal glands of male blennioid fish produce a mucus enriched with antimicrobials. (a) Anal glands in a nesting male of *O. atlanticus atlanticus*. p, genital papilla; a, anal gland; f, anal fin. Scale bar, 0.5 cm. (b) Transverse section of an anal gland of *O. atlanticus atlanticus* (PAS staining). Secreting cells appear pink stained. m, melanophore. Scale bar, 20 μ m. (c) Non-reducing SDS-PAGE (15% polyacrylamide gel) on anal glands extracts. Left panel, SYPRO Ruby stain; right panel, gel overlaid with *M. lysodeikticus*. Lanes 1 and 4, hen egg lysozyme, 1 μ g and 25 ng, respectively; lanes 2 and 5, hydrophobic extract from *O. atlanticus atlanticus*, 16 and 60 μ g, respectively; lanes 3 and 6, hydrophobic extract from *S. pavo*, 8 and 26 μ g, respectively. On the left, the molecular mass of protein markers is expressed in kDa.

(d) Anal gland extracts

After excision glands were immediately frozen and lyophilized. Glands from 1 to 4 animals (4–19 mg dried weight per male) were suspended in 0.3 ml 100 mM Na-phosphate buffer at pH 6.8 (PB) and homogenized with Eppendorf micropestles in ice for 15 min.

The hydrophilic extract consisted of the three supernatants obtained by three runs of centrifugation (all centrifuge steps were performed at 12 000g for 90 min at 4 °C) and resuspension of the pellet with 0.3 ml PB. The pellet underwent 10 runs of resuspension with 1.8 ml PB and centrifugation, discarding supernatants. The pellet was then extracted with 0.1 ml 0.04% Lithium Dodecyl Sulphate (LiDS, Sigma) in PB by magnetic stirring at 4 °C for 2 h and centrifuged. This step was repeated four times. The four supernatants (hydrophobic extract) were pooled together and ultrafiltered with Millipore Microcon YM-3 filters onto which PB was added, so as to obtain a final concentration of LiDS (less than 0.004%) not lytic on *Micrococcus lysodeikticus* (control tests not shown). The protein content of the hydrophilic and hydrophobic extract, determined according to Bradford (1976) using bovine serum albumin (BSA, Sigma) as standard, was about 350 μ g total protein per gland pair in *S. pavo* and 1000 μ g total protein per gland pair in *O. atlanticus atlanticus*, for the former, and about 10 μ g in *S. pavo* and 600 μ g in *O. atlanticus atlanticus*, for the latter.

(e) Assays for lysozyme-like activity and electrophoreses

Lysozyme is a major component of innate immunity and the standard substrate for assaying its activity is *M. lysodeikticus* cells (Jolles & Jolles 1984). The lysozyme-like activity of anal glands was assayed on plates (Marchini *et al.* 1993) prepared with 0.1% suspension of *M. lysodeikticus* dried cells (Sigma). Mid-dilution samples from hydrophilic and hydrophobic extracts, containing 15–240 or 2.5–160 μ g total protein, respectively, were applied to 2 mm-wells in a volume of 3–10 μ l. Plates were incubated at room-temperature (RT) overnight. Hen egg white lysozyme (LYS, Sigma) was used as positive control. SDS-PAGE was performed in a discontinuous pH gel system (Laemmli 1970), but in non-reducing conditions. Samples were loaded in double in the gel. Runs were carried out at 18 mA for 2 h at 4 °C. After electrophoresis, one part of the gel was stained with SYPRO Ruby gel stain (Molecular Probes), while the other part was renaturated (Saul *et al.* 1990), omitting β -mercaptoethanol. The gel was equilibrated with PB for 10 min at RT and overlaid with a 0.1% suspension of *M. lysodeikticus* in PB plus 0.7% agar (Difco). Low molecular weight proteins (Promega), BSA and LYS were used as molecular mass markers. BSA and LYS were also used as negative and positive control of lytic activity. Acidic native electrophoresis was also performed (Gabriel 1971) and over-layered as above.

3. RESULTS

Nesting males of the redlip blenny rubbed the analurogenital region over the eggs not only during reproductive time (mean rubbing time \pm s.e. 13.42 ± 3.24 s per 5 min; frequency of rubbing \pm s.e. 2.06 ± 0.46), but also while guarding the eggs (mean rubbing time 1.17 ± 0.76 s per 5 min; frequency of rubbing \pm s.e. 0.18 ± 0.11). Anal glands of the parental males of *O. atlanticus atlanticus* and *S. pavo* were constituted by extensive integument folds, characterized by the massive presence of columnar cells with a cytoplasm containing granular material. Histochemical analyses demonstrated that the gland cells contained a mixture of both sulphated and non-sulphated mucins (figure 1b). The mucous secretions of the anal glands exhibited antimicrobial activity, as shown by the analysis of different gland extracts. In both species, 120 μ g of the hydrophilic fraction protein and 40 μ g of the hydrophobic fraction protein was able to produce a lysis halo (5 mm diameter) on *M. lysodeikticus* plates, showing that gland extracts had a lysozyme-like activity. We performed a non-reducing SDS-PAGE, followed by overlaying the gel with *M. lysodeikticus*, with hydrophobic gland extracts, and we observed that the activity was associated with a band of apparent molecular weight of 14 kDa, which is compatible with that of hen egg white lysozyme (figure 1c). This result was supported by the outcome of an acidic native

- Neat, F. C., Locatello, L. & Rasotto, M. B. 2003 Reproductive morphology in relation to alternative male reproductive tactics in *Scartella cristata*. *J. Fish Biol.* **62**, 1381–1391. (doi:10.1046/j.1095-8649.2003.00122.x)
- Oliveira, R. F., Almada, V. C., Forsgren, E. & Gonçalves, E. J. 1999 Temporal variation in male traits, nest aggregations and mating success in the peacock blenny. *J. Fish Biol.* **54**, 499–512. (doi:10.1111/j.1095-8649.1999.tb00631.x)
- Ravi Ram, K. & Wolfner, M. F. 2005 Fates and targets of male accessory gland proteins in mated female *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* **35**, 1059–1071. (doi:10.1016/j.ibmb.2005.05.001)
- Saul, D. J., Williams, L. C., Grayling, R. A., Chamley, L. W., Love, D. R. & Bergquist, P. L. 1990 *CelB*, a gene coding for a bifunctional cellulase from the extreme thermophile *Caldocellum saccharolyticum*. *Appl. Environ. Microbiol.* **56**, 3117–3124.
- Trivers, R. L. 1972 Parental investment and sexual selection. In *Sexual selection and the descent of man* (ed. B. Campbell), pp. 136–179. Chicago, IL: Aldine.
- Yousif, A. N., Albright, L. J. & Evelyn, T. P. T. 1994 *In vitro* evidence for the antibacterial role of lysozyme in salmonid eggs. *Dis. Aquat. Org.* **19**, 15–19.
- Zander, C. D. 1975 Secondary sex characteristics of blennioid fishes (Perciformes). *Publ. Stn. Zool. Napoli* **39**, 717–727.