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Structure and Function of the Axillary Organ of the Gulf Toadfish, *Opsanus beta* (Goode and Bean)

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ABSTRACT. The structure of the axillary organ of a batrachoidid species, the gulf toadfish (*Opsanus beta* Goode and Bean 1879), has been examined and several simple experiments designed to elucidate its function performed. Electron microscopy (EM) studies revealed cells and structures suggesting secretory and iono regulatory roles (e.g., abundant intracytoplasmic secretory particles, rough endoplasmic reticulum, sparse Golgi bodies, indented epithelial cells with microvilli, numerous endocytotic vesicles, etc.). Our physiological experiments allowed us to reach several conclusions: the organs do not excrete significant quantities of urea relative to other areas of the fish (head and gills), the organs do not secrete a substance that is toxic to a teleost test fish (*Gambusia affinis*), the secretions do not induce short-term modifications in locomotory activity of other gulf toadfish (e.g., by pheromonal means) and the secretions do not inhibit the growth of several species of microorganisms in culture. The function of the organ and its secretions remains unknown, representing a fertile area for research on structure and function in comparative physiology. COMP BIOCHEM PHYSIOL 119A;1:17–26, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. Axillary organ, gulf toadfish, *Opsanus beta*, pheromones, anti-microbial compounds, toxins

INTRODUCTION

In the family Batrachoididae (toadfishes and midshipmen), there are approximately 69 species in 19 genera and 3 subfamilies (12). Several species of the subfamily Batrachoidinae possess an axillary foramen, a pore located between the body wall and the pectoral fin. In the family in general, the pelvic fins are typically small in size and located in the "jugular" position anterior to the pectoral fins, which are large and used extensively during routine locomotion. The presence or absence of this axillary foramen (and other "glandular" pores) has been a very useful characteristic for systematic studies [e.g., (3)], but the anatomy and function of the structure has not been especially well studied.

The earliest reported description of the axillary pore is by Louise B. Wallace (15) of the Marine Biological Laboratory, who demonstrated in *Batrachus* sp. that a glandular structure (the axillary gland) underlies the pore. The genus *Batrachus* was later reclassified into several genera and species, but it is likely that Wallace was examining the abundant local oyster toadfish, *Opsanus tau*, whose distribution extends from Maine to Central Florida (13). The gland is described as a pouch-shaped sac with numerous infoldings of epidermal tissue and is typically divided into several chambers. Through a variety of staining methods, Wallace established that at least one of the six cell types she described had vacuoles. The gross morphology of the gland and the presence of vacuoles strongly suggested a secretory function. At the time, through the work of a French scientist, Alphonse Bottard (2), it was thought that many members of the family were venomous, so Wallace reported that "the results gained from a few simple experiments with the living fish, do not seem to favor the idea of a poisonous secretion," although she presented no data. She concluded on the description of the gland by saying, "My work, however, has not been sufficient to determine the nature of the secretion which still offers an interesting problem to the physiologist."

A second early reference to the axillary gland was made by Gill (7), who described the secretory cells as being "club-shaped, cylindrical and uncommonly large (0.275 mm

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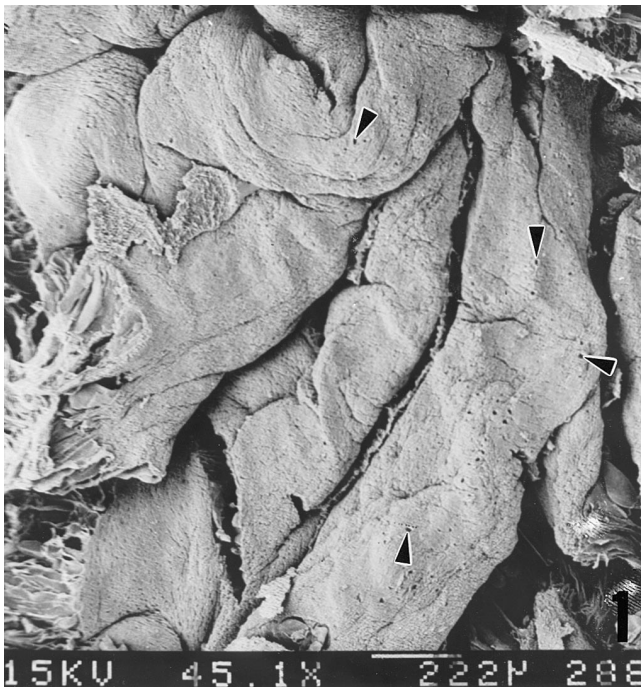


FIG. 1. SEM showing the highly folded internal surface of the axillary organ of the gulf toadfish (*Opsanus beta*). Arrowheads, epithelial cell pore. ×45.

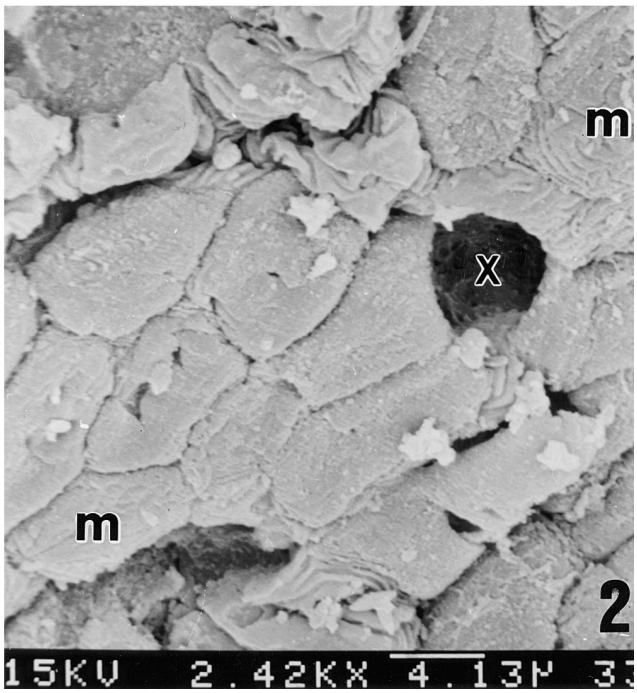


FIG. 2. Close up of the surface of the axillary organ of *Opsanus beta* showing microridged epithelial cells (m) and epithelial cell cavitation (x). ×2400.

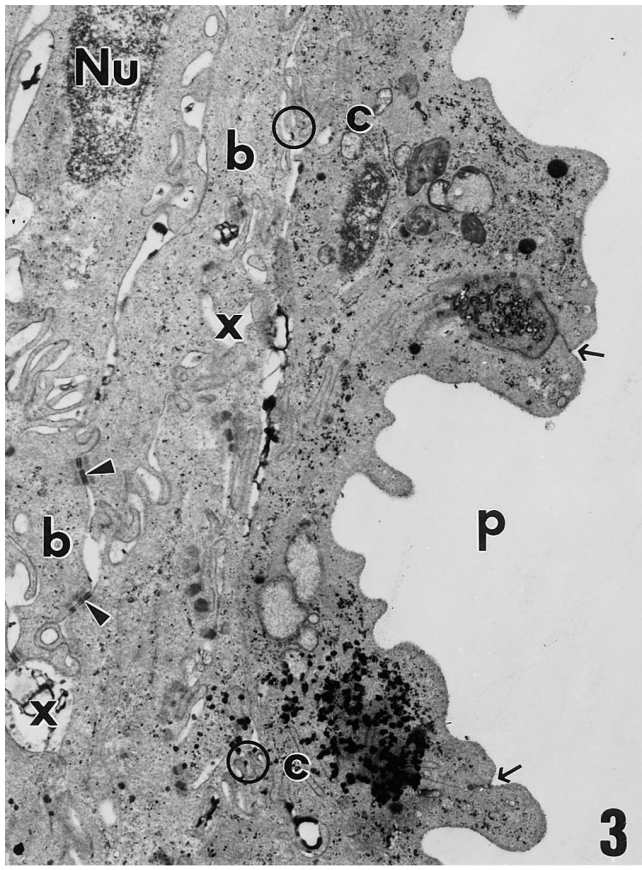


FIG. 3. TEM of the epithelium of the axillary organ of *Opsanus beta* showing intracytoplasmic and extracellular electron dense secretory granules in both the surface epithelial cells, the more deeply situated filament cells and in the intercellular spaces. c, pavement cells; b, filament cells; Nu, nucleus of a filament cell; circles, intercellular spaces; p, epithelial cell cavitation; arrows, surface epithelial cell tight junctions; arrowheads, desmosomes; x, intercellular spaces some of which contain electron dense secretory material. ×18,800.

long)," and further referred to the contents as "either a fine-grained or a clear yellow substance . . .". The batrachoidid axillary gland then disappeared from the literature until the 1960s when it was used as a systematic character, and its structure was examined more intensely by electron microscopy (EM) in *O. tau* by Vernick and Chapman (14). For this species, they described three basic cell types: mucous, epithelial and clavate, with a large central vacuole and clear evidence for Golgi secretion into the vacuoles. The epithelial and mucous cells overlayed the clavate cells, and it was not clear if the secretory materials of the clavate cells passed through the overlying epithelial cells (via a transcellular pinocytotic route) or if the clavate cells secreted their contents directly to the lumen. One other published abstract (8) confirmed the morphological observations of Vernick and Chapman (14) for *O. tau* and also made the statement

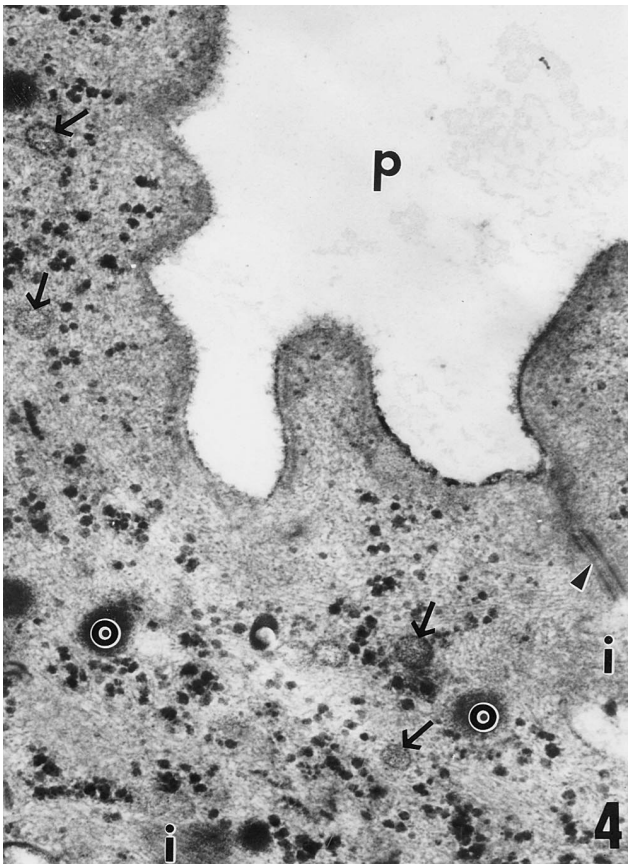


FIG. 4. Close up of an invagination of a surface epithelial cell of the axillary organ of *Opsanus beta* showing small and large (o) electron dense granules, secretory bodies (arrows), myofibrils (i) and surface epithelial cavitation (p). Arrowhead, intercellular junction. ×70,540.

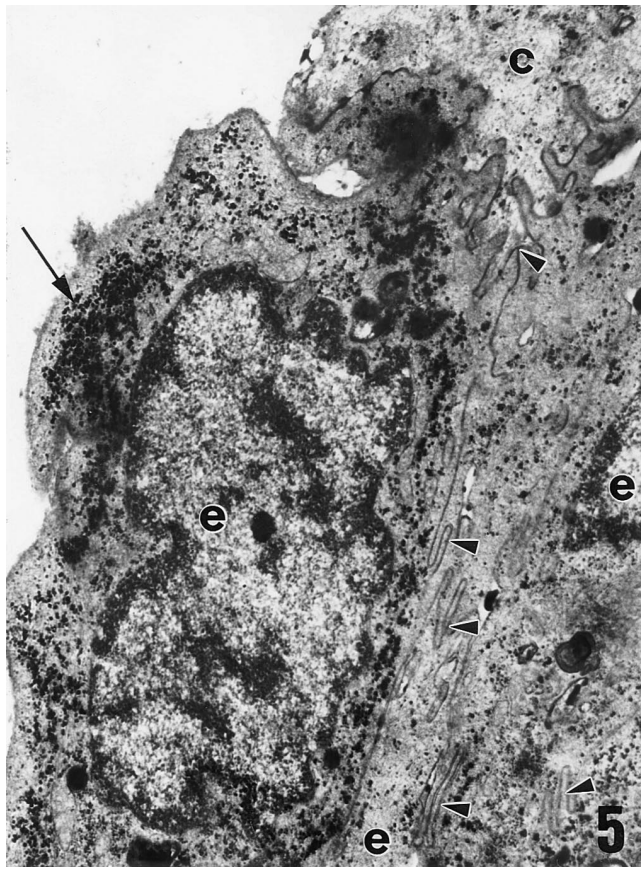


FIG. 5. Ovoid surface epithelial cells (e) of the axillary organ of *Opsanus beta* that are characterized by abundant perinuclear electron dense granules (arrow) and secretory bodies. Arrowheads, interlocking cell junctions; c, pavement cell. ×37,600.

"The secretion is antibacterial." The results of Hamlett and Schwartz (8) were not published further, to our knowledge.

Although some batrachoidids from a different subfamily (Thalassophryninae) are known to be venomous and direct injections of concentrated toxin of *Thalassophyrne* can kill pigs and chickens, virtually nothing is known of its potency or mechanism of action. In humans, the stings can cause intense radiating pain, swelling and redness but are generally not considered serious and not fatal (9). There is one study of putative ichthyotoxins from *O. tau* (11) in which the general body mucous secretions of *O. tau* were harvested by daily agitation of fish in distilled water for 5 min and then the mucus was extracted in organic solvents. When the organic extracts of the daily secretions of six fish (equivalent to 1 mg crude toxin identified as 3-octanone) were dissolved in 2 ml ethanol and added to 250 ml of sea water, two individuals of silversides (*Menidia menidia*) were killed in 37 and 71 min, whereas one control survived 120 min (11). 3-Octanone has also been identified as an ant alarm pheromone (5). We know of no other experimental data on the function of the glandular secretions in the toadfish.

We undertook the following study to determine if the morphology of the axillary organs in the gulf toadfish, *Opsanus beta* (distributed from central Florida throughout the Gulf of Mexico through the Texas coast) (13), shows similarities or differences to the gland in *O. tau*. Furthermore, we wished to test several preliminary hypotheses on the function of the organ. First, although our previous experiments, which occluded axillary organ secretion, suggested that they were not involved in urea excretion (19), we wished to examine this possibility by an experimental protocol that allowed the organ to actively secrete. We also tested several other functions for the secretions, namely that they have anti-microbial activity, are poisonous to other species of fish and have pheromonal properties that might influence the behavior of other conspecifics.

MATERIALS AND METHODS

Microscopy

Specimens of the gulf toadfish (*O. beta*; 74–290 g) were captured by roller trawl in Biscayne Bay, Florida in March

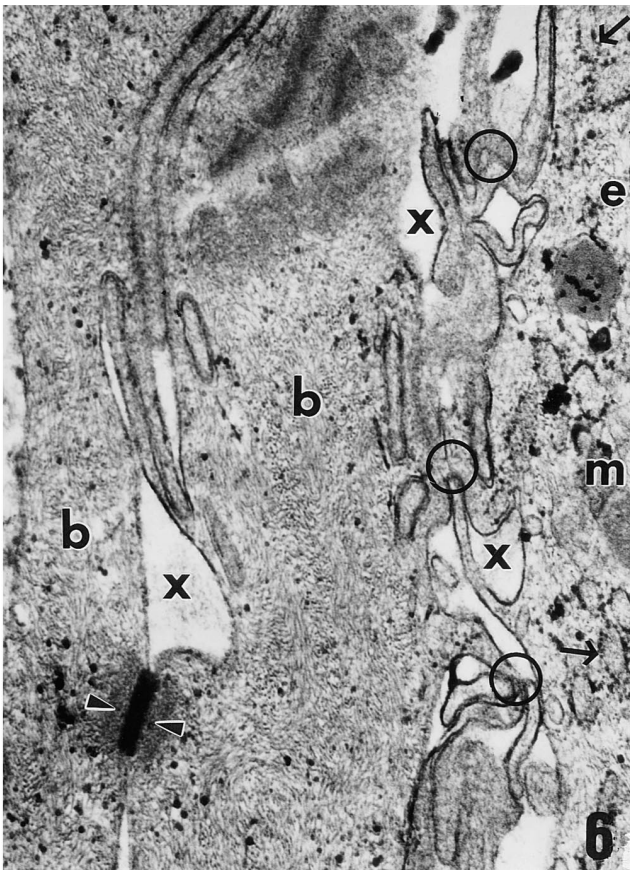


FIG. 6. A view of the intercellular interdigitations (circles) between a surface epithelial cell (e) and filament cells (b). x, intercellular spaces; arrowheads, desmosomes; arrows, rough endoplasmic reticulum; m, mitochondrion. ×43,400.

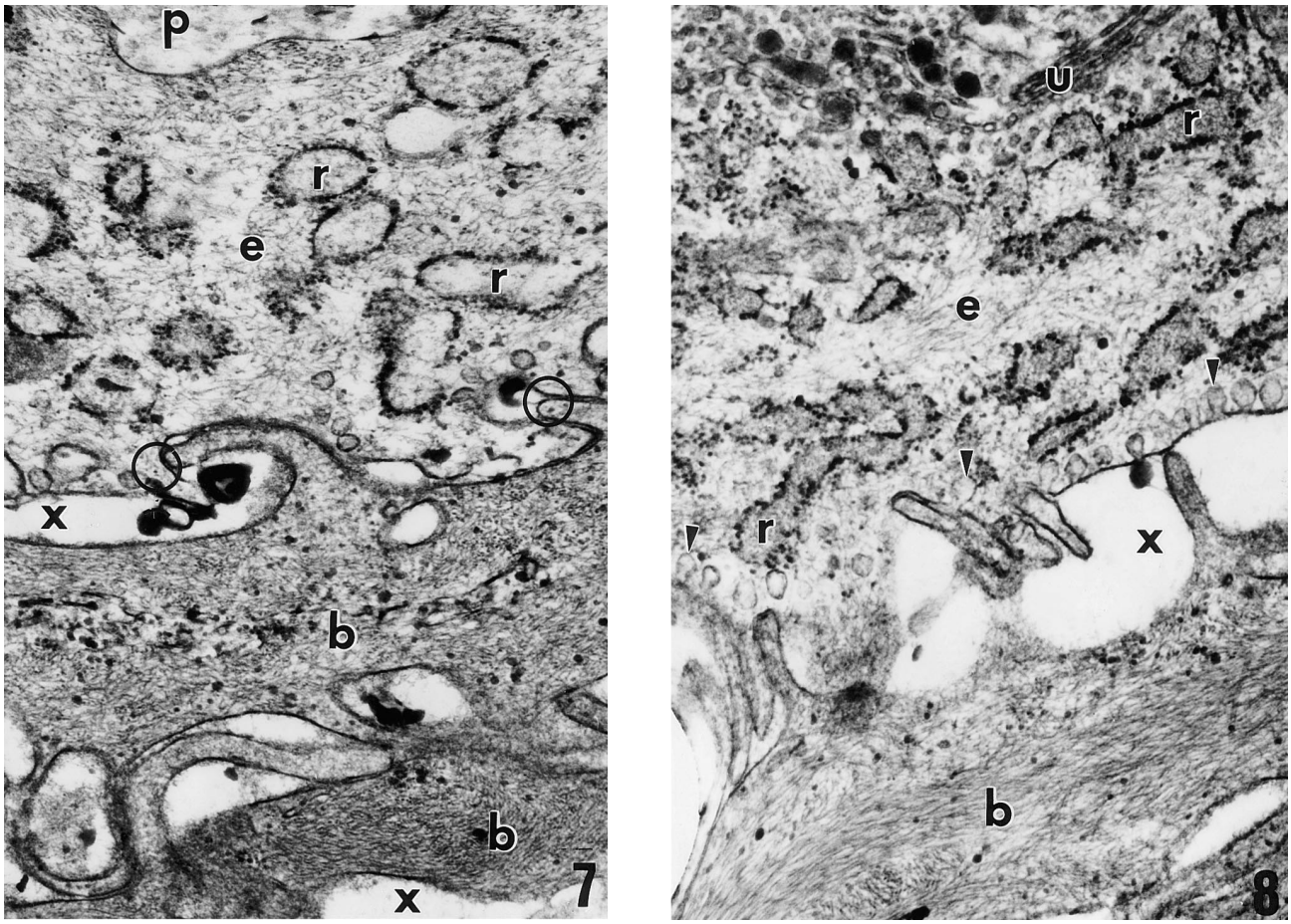
1995. The fish spent no more than 24 hr in a large outdoor holding tank under ambient conditions, after which they were transferred to the laboratory as previously described (19) and not fed. Fish were kept in the laboratory no more than 1 week before use. After stunning the fish by a blow to the head, the axillary organs were carefully dissected out. For transmission electron microscopy (TEM), the organ was diced into small pieces that were immersed in 2.5% glutaraldehyde buffered in sodium cacodylate (pH 7.4, osmolarity 350 mOsm) and kept at 4°C for 2 weeks. For scanning electron microscopy (SEM), the organ was cut open, spread out and immersed in the fixative. This was done to ensure satisfactory fixation of the internal surface. The samples for TEM were postfixed in 2% osmium tetroxide, dehydrated in graded concentrations of ethanol and propylene oxide before infiltration and embedding in araldite. Ultrathin sections were cut, counterstained with uranyl acetate and lead citrate and examined on a JEOL 1200EX electron microscope. The tissues for SEM were dehydrated by seven daily changes of absolute alcohol, critical point dried in liquid carbon dioxide, sputter coated with gold-palladium complex and viewed on an ISI-DS 130 dual-stage scanning electron microscope.

Physiological Studies

SERIES I: UREA EXCRETION. In the first experimental series, conducted in March 1995 and February 1996, cannulae were implanted in the axillary pores of fish that had previously been crowded (e.g., six fish in an 80-l aquarium with flowing sea water) to induce ureagenesis (19). The fish were lightly anesthetized with 0.5 g/l Tricaine methanesulfonate, and a plastic insert (a 1-cm length of rigid plastic tubing, i.d. 2.5 mm, with a 5 mm o.d. flange at the end) was sewn into the skin of each axillary pore with a purse suture. The insert was then overlain with a piece of dental dam through which the catheter passed and the dam glued to the skin surrounding the pore with cyanoacrylate glue. The dilated end of a 40-cm Bard all-purpose urethral catheter (size 12 Fr, elastic rubber; Davol, Inc.) was stretched around the plastic insert. The fish were then placed in a plastic tub with 2 l static sea water, and the catheters were filled with sea water and led to test tubes outside the tub 3 cm below tub water level. At the end of 24 hr, water in the tub and catheters was sampled and ammonia and urea determined as previously described (19). Flow rates were determined from the volume of fluid entering the test tube, but this sample and the volume in the catheter were combined (2–3.5 ml per side) for urea and ammonia analyses.

SERIES II: TOXICITY. In the second experimental series, conducted in August 1995, organ secretions were harvested by inserting a cotton swab into the axillary foramen of netted but unanesthetized toadfish and holding the swab there for 10 sec. Secretions from each pore of a fish were harvested and the fish was not used again for these experiments. Later experiments (series IV) revealed that this procedure resulted in about 30 ml of secretion per fish. The two cotton swabs from a single fish were then immediately immersed into a 100-ml beaker containing 40 ml fresh water and an individual mosquito fish (0.5–1 g; *Gambusia affinis*), a popular toxicity test organism cultured from a local population (1). The behavior of the fish was observed for the first 30 min and thereafter examined at longer intervals for 24 hr. Control fish were exposed to swabs dipped in sea water.

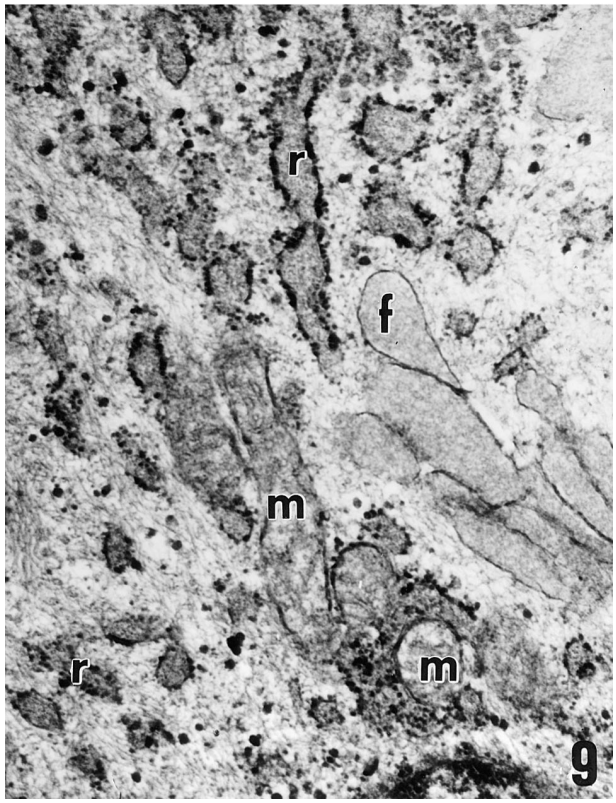
SERIES III: PHEROMONAL ACTIVITY. This experiment, carried out in September 1995, was designed to determine if the axillary organ secretions had pheromonal activity that caused an observable change in behavior of other toadfish. Individual toadfish (donors) were isolated in a 60-l aquaria with flow-through sea water at ambient conditions, and axillary organ secretions were harvested as in series II. Previously, a second set of individual toadfish (test subjects) had been acclimated (48 hr) to plastic tubs (8 l volume) containing a section of polyvinyl chloride (PVC) pipe for shel-

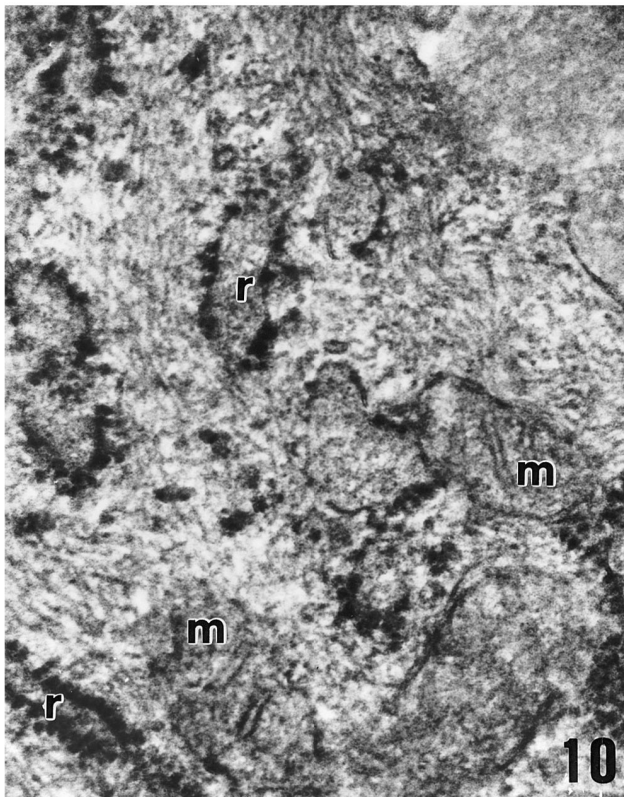


FIGS 7 and 8. Views of the interface between a surface epithelial cell (e) and filament cells (b). Circles, microvillous-like interdigitations; r, rough endoplasmic reticulum; p, epithelial cell invagination; x, intercellular spaces; u, Golgi body; arrowheads, endocytotic vesicles. Note the profuse microfibrillar network in both the surface epithelial and the filament cells. Fig. 7, ×75,850; Fig. 8, ×84,200.

ter with flowing sea water. Twelve hours before secretion harvesting from the donors, the flowing water to the test subjects was turned off and allowed to drain to a volume of 2 l. Immediately after harvesting, the two swabs were placed in the tub approximately 2.5 cm from the mouth of a test toadfish, which was typically in the PVC shelter with its head pointed out. Control fish were exposed to similarly placed swabs that had been dipped in sea water. The activities of the fish were observed for the next 10 min and then periodically for a total of 1 hr. At the end of 1 hr, both donor fish and test fish were anesthetized with an overdose of Tricaine methanesulfonate (3 g/l) and their sex was determined. All combinations of male/female and donor/test fish were examined.

SERIES IV: ANTIMICROBIAL ACTIVITY. Toadfish were collected in October 1995 and axillary organ secretions were harvested by netting the fish, gently inserting the plastic tip of an automatic pipetter (Pipetteman P20) into the foramen and drawing off as much fluid as possible. The secretions from several toadfish were pooled (150 ml) in an Eppendorf tube and diluted to 300 ml with distilled water and then passed through a 0.2-mm syringe filter (Acrodisk) to ensure sterility and frozen at −80°C for no more than 1 week. Cultures from single colonies of a fish pathogen (*Yersinia ruckeri*) and three strains of *Escherichia coli* (XL1 Blue, DH5a and XLOLR) were plated onto standard plastic Petri dishes (100 × 15 mm) with LB medium. *Synechococcus*, a unicellular cyanobacterium, is a predominant microbe occurring in certain subtropical environments. An aerobic nitrogen-fixing strain, Miami BG 043511, isolated from the group was used for these tests. Agar medium for the strain culture was prepared by adding 2.5% (v/v) agar into a combined nitrogen-free artificial seawater medium A–N (10) with NaCl concentration of 18 g/l, $NaHCO_3^-$ concentration of 2.5 g/l and 10 mM of HEPES buffer. Before autoclaving, the medium was adjusted to a final pH of 7.6. The sterilized medium was distributed into a Petri dish in a room lit with





FIGS 9 and 10. Views of the ultrastructure of the surface epithelial cells. f, secretory granules; r, rough endoplasmic reticulum; m, mitochondria. Fig. 9, ×81,400; Fig. 10, ×89,500.

ultraviolet light and was inoculated by using a previously sterilized Pasteur pipet to remove 0.5 ml of the strain culture from a culture tube that was spread evenly over the plate with a triangle-shaped glass loop. The strain in the dish was grown at 27 ± 1.0°C under continuous illumination of 150 mE m^{-2} s^{-1} for 3 days before injecting organ extraction. Two sterile filter papers (6 mm diameter) were then placed onto the top of the seeded media, and 15 ml of either the thawed diluted secretion or 50% sterile sea water was sterile pipetted onto the filter paper. The cultures were then incubated at 22°C (*Y. ruckeri*) or 37°C (all others) and examined after 24 hr.

RESULTS AND DISCUSSION

Microscopy

The paired axillary organs were located on the middle axillary region between the pectoral fins and the thoracic body wall. The opening to the organ was most evident when the pectoral fin was abducted. In the specimens examined, the blind ending cavity was about 2 mm deep on fixed fish. The pea-shaped organ was covered externally by a tough fibrous connective tissue layer, with the surrounding region (i.e., the medial surface of the pectoral fin and the lateral body wall) visibly well vascularized. The epithelial lining of the organ is highly folded (Figs 1 and 2), a feature that should increase the internal surface area over that of a simple saccular organ of the same size. Numerous microscopic cavitations delineated by microridged pavement cells (Figs 1 and 2) were observed. Intercalated between the pavement cells were other epithelial cells, some of which were characterized by luminal microfolds and abundant electron dense granules (Figs 3 and 4) and others were ovoid (Fig. 5) and protruded over the epithelial surface. Both types of cells contained abundant rough endoplasmic reticulum (Figs 6–8), sparse Golgi bodies (Fig. 8), mitochrondria (Figs 6, 9 and 10), dense microfibrillar network (Figs 7 and 8) and on the basal aspect numerous endocytotic vesicles (Figs 8 and 11). Underlying the surface epithelial cells (SECs) were stacks of longitudinally oriented filament cells (FCs) that interdigitated with the surface layer of epithelial cells and with each other through large finger-like projections (Figs 6–8 and 12–14) and joined together across distinct desmosomes (Figs 11–14) and to the SECs across tight junctions (Fig. 12), leaving notably wide intercellular spaces (Figs 7, 8 and 11–14). Both the SECs (see Figs 3–5, 8 and 9) and the FCs (see Figs 6, 7, 13 and 14) had many dark electron dense granules and a meshwork of microfibrils (Figs 6–8 and

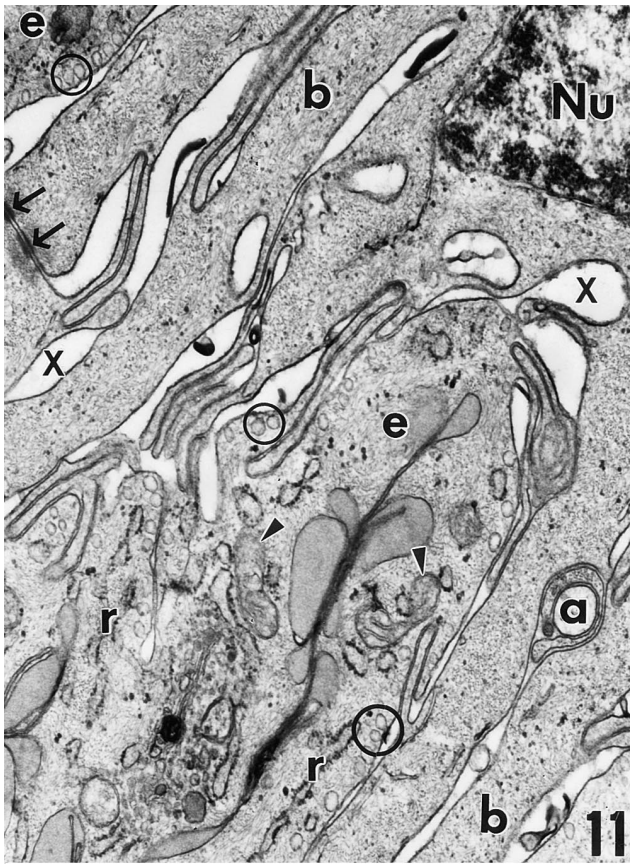


FIG. 11. Stacks of filament cells (b) that surround the surface epithelial cells (e) of the axillary organ of the gulf toadfish, *Opsanus beta*. Circles, endocytotic vesicles between the filament and epithelial cells; arrows, desmosomes between two filament cells; x, intercellular spaces; arrowheads, mitochondria; r, rough endoplasmic reticulum; a, a nerve axon; Nu, nucleus of a filament cell. ×20,200.

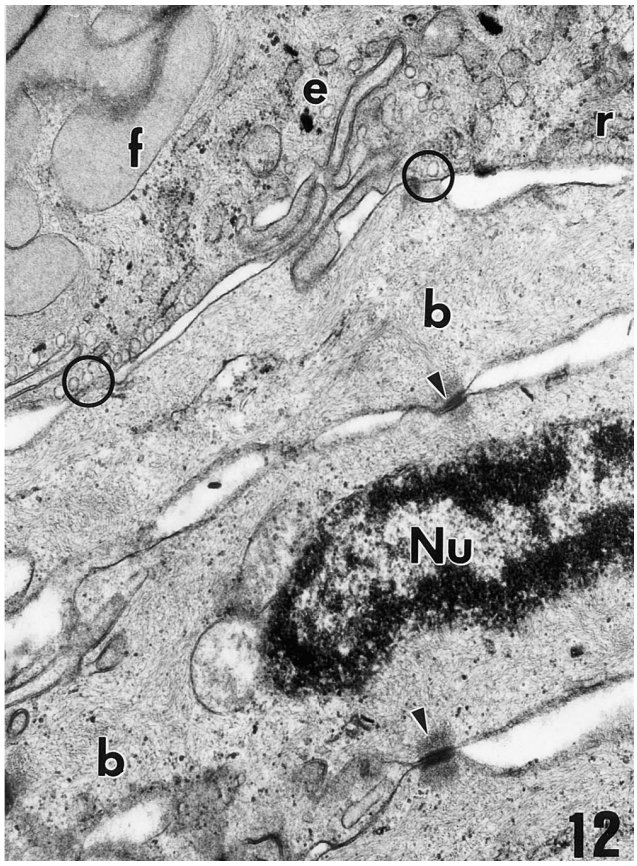
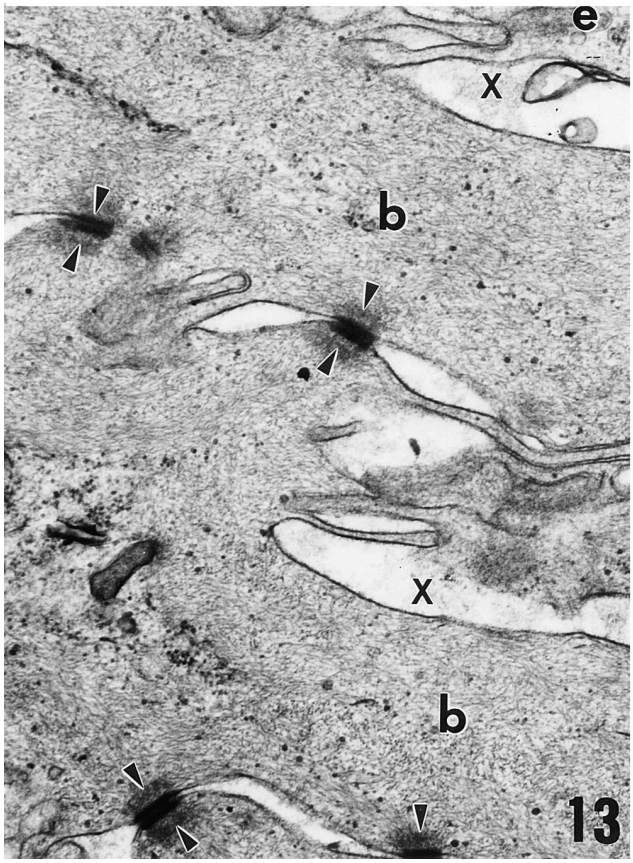


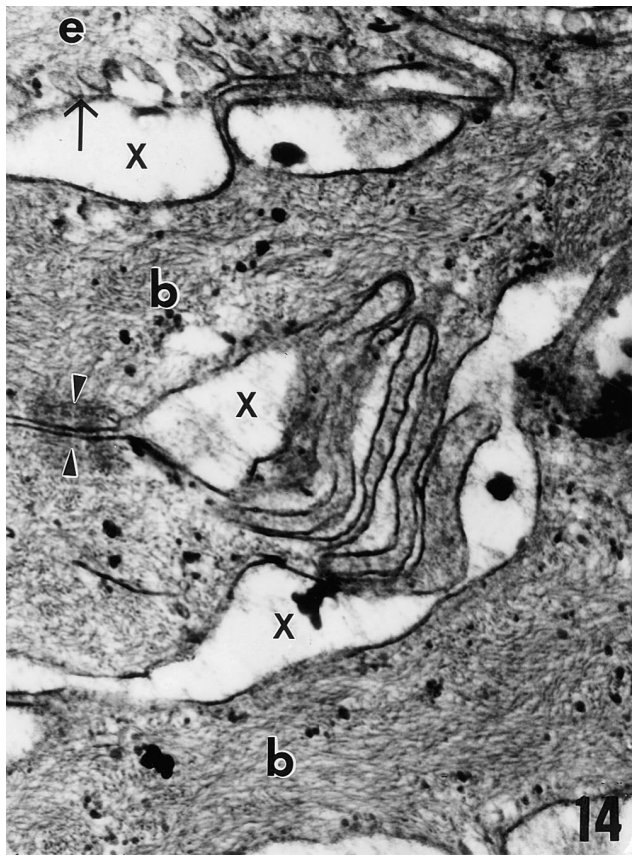
FIG. 12. A surface epithelial cell (e) lying on layers of filament cells (b). Circles, tight junctions between a surface epithelial cell and a filament cell. Note the endocytotic vesicles characteristic of such sites and the wide intercellular spaces between cells. f, secretory bodies; r, rough endoplasmic reticulum; Nu, nucleus of a filament cell; arrowheads, desmosomes between the filament cells. ×80,160.

11–14). Nerve axons were observed between the FCs (Fig. 11) in very close to the SECs.

The ultrastructural characteristics of the axillary organ of *O. beta* are generally similar to those described for *O. tau* (8,9,14,15) but with the following subtle difference. Mucus cells were not observed in the organ of *O. beta* and the immediate subepithelial space was comprised of stacks of longitudinally oriented FCs with very few collagenous elements. The cells termed "clavate subepithelial cells" (14) and the "blebs" (8) of *O. tau*, respectively, are similar to the SECs of *O. beta* and appear to correspond with the endocytotic vesicles observed here.

The gross and ultrastructural morphology of the axillary organ of the gulf toadfish, *O. beta*, was suggestive of secretory and iono regulatory roles. These inferences were based on the unequivocal presence of abundant electron dense secretory material in the epithelial cells, profuse rough endoplasmic reticulum, microvillous amplifications of the cavitations of the surface epithelial cells and numerous endocytotic vesicles on the epithelial cell interfaces. However, from the total absence of goblet mucous cells from the epithelium, the organ appears to secrete no mucus. We speculate that the pore brings the water into contact with the epithelial cells of the axillary organ, the presumably contractile circular filament cells probably effecting a pumping function forcing secretions out of the organ and bringing "fresh" water into contact with the epithelial surface. The evident enervation of the organ (see Fig. 11) may help regulate this process. The well-defined wide intercellular spaces (see, e.g., Figs 6, 7 and 11) may provide storage sites and alternative pathways for transport of the secretory materials to the surface of the organs, especially from the deeply situated FCs. From the particularly profuse vascularization of the surrounding "finpit" area, the secretory function of the organ must be highly tuned with the blood flow. The general ultrastructural morphology of the axillary organ of *O. beta* corresponds with that of the oral epithelium of *Raja clavata* (18) and the fish skin (16,17). However, cells such as sensory cells, multivillous cells, club cells and mucous





FIGS 13 and 14. Close-ups of the filament cells (b) showing the intracytoplasmic myofibrillar network and diffuse electron dense granules. x, wide intercellular spaces; arrowheads, desmosomes; arrow, endocytotic vesicles at the interface between a surface epithelial cell (e) and a filament cell (b). Fig. 13, ×83,450; Fig. 14, ×78,500.

goblet cells, which are abundant in the skin epithelium where mucus is the main secretory material, were lacking from the surface of the axillary organ. Although the cell surface morphology of the axillary organ, especially with respect to presence of pavement-like FCs and cavitation of the SECs, is similar to those cells that line the gills, the epithelial cells lack the numerous mitochondria and profuse microtubular network that characterize the ion-pumping chloride cells of the gills. The epithelial cells of the axillary organ may not have as significant a role in osmoregulation as the chloride cells or may be involved in a totally different ion transfer process. In the oyster toadfish, *O. tau*, a glandular-like internal surface of the axillary organ was observed and fine-grained or clear yellow substance strongly refracting the light and resembling oil was reported (7).

Physiological Studies

As demonstrated previously in studies that occluded the axillary pore openings (19), the organs do not appear to play a role in urea excretion in this species when allowed to be open for flow. Secretion flow rates in these experiments were 0.35 ± 0.13 ml/kg/hr (both sides included), a value on par with urine flow for marine teleosts (6), but the urea excreted through this route in series I constituted only 0.6% of the total (urea and ammonia excretion to the water were 163.4 ± 15.0 [4] mmol urea-N/kg/hr and 103.2 ± 11.3 [4] mmol amm-N/kg/hr; excretion rates from the cannulae were 0.98 ± 0.52 [4] mmol urea-N/kg/hr and 0.54 ± 0.33 [4] mmol amm-N/kg/hr; means ± SE [*n*]).

In series II, the axillary organ secretions had no observable toxic effect on *Gambusia affinis* (in 24 hr, there were 0 deaths in six specimens for each of control and treatment groups), a species that is clearly susceptible to typical marine toxins (1). In series III, we observed virtually no movement by toadfish in response to the secretions, either by control or experimental exposures (Table 1). Finally, in series IV, the growth of all microbes was unimpeded by axillary organ secretions or seawater controls. In this type of experiment, if there is antimicrobial activity of a test substance, one expects to see a ring of clear agar and no microbial growth around the filter paper. Indeed, culture streaks went directly up to the border of the filter paper and continued under it in all cases (Fig. 15). One caveat should be added to this conclusion, namely that the freezing of the extracts before testing could have reduced the biological activity of any cold-labile components of the extracts.

Our experiments confirm a secretory role for the axillary

TABLE 1. Effect of toadfish axillary gland secretions on locomotory activity of other toadfish

	Number of fish moving/total donor source		
Test fish	**Male**	**Female**	**Seawater Control**
Male	1*/6	0/6	1†/6
Female	0/6	0/6	0/6

*Test subject retracted ~1 cm from swab.
†Test subject retracted ~2.5 cm from swab.

organ of the gulf toadfish, *O. beta*, and appear to rule out some of the more obvious roles for the axillary organ secretions. In future experiments, design could be improved to include, for example, other marine species in toxicity and anti-microbial tests. Future experimentation could examine other possible roles for the secretions. One possibility is that the secretions lubricate the skin between the frequently used pectoral fins and the body wall. A second possibility is that the organs are supermucous glands designed to trap and remove sediments from these benthic fish (B. B. Collette, National Marine Fisheries Service Systematics Laboratory, personal communication), but the absence of mucous goblet cells from the epithelium appears to rule this out as a primary role of the organ. Organ ablation experiments and detailed histochemical studies of the organ may offer fruitful avenues of research. A third possibility, given the observed secretory rates, is that the organ deals with secretion of specific ions or some other aspect of hydromineral balance. Finally, because the axillary organs in *Opsanus* open to the outside, we and other authors have assumed an exocrine function. Interestingly, some species of the same subfamily (e.g., *Amphichthys cryptocentrus*) (4) have a closed glandular axillary mass, suggesting that an endocrine role may be possible. To echo Wallace's comments of over 100 years ago, our work, however, has not been sufficient to determine the nature of the secretion that still offers an interesting problem to the combined talents of the morphologist and physiologist.

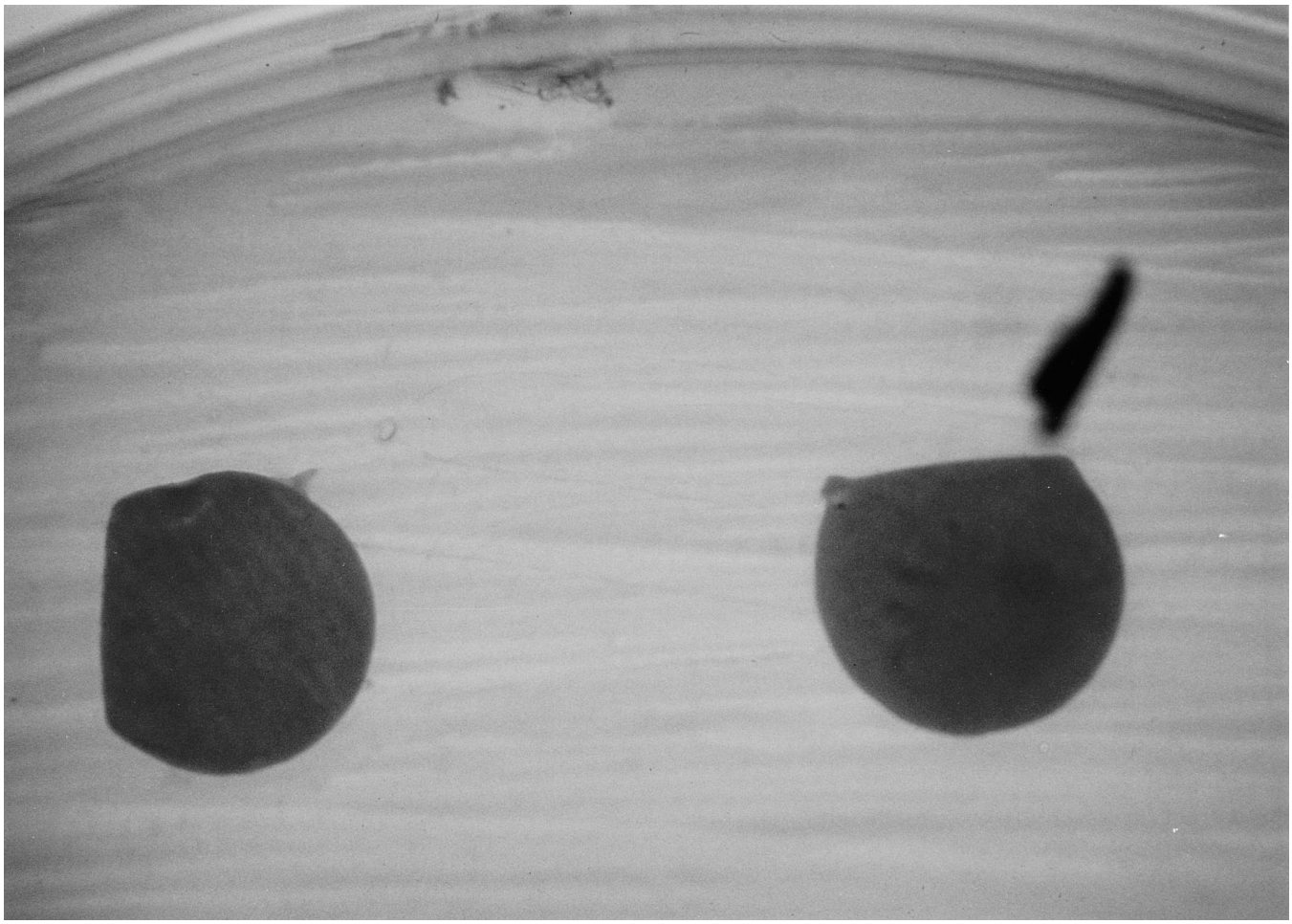


FIG. 15. Portion of agar plate culture of *Yersinia ruckeri* showing filter disks infused with sterile sea water (left) or axillary gland secretion (right).

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