Available online at www.sciencedirect.com
SCIENCE DIRECT
Comparative Biochemistry and Physiology, Part A 144 (2006) 196–202
CBP
www.elsevier.com/locate/cbpa

Olfactory sensory input increases gill ventilation in male round gobies (*Neogobius melanostomus*) during exposure to steroids

Rachelle M. Belanger [a,c], Lynda D. Corkum [a], Weiming Li [b], Barbara S. Zielinski [a,*]

[a] *Department of Biological Sciences, University of Windsor, Windsor, ON, Canada N9B 3P4*
[b] *Department of Fisheries and Wildlife, Michigan State University, East Lansing, MI 48824, USA*
[c] *Department of Biological Sciences, Bowling Green State University, Bowling Green, OH 43403, USA*

Received 30 May 2005; received in revised form 18 February 2006; accepted 21 February 2006
Available online 28 February 2006

Abstract

In teleostean fish, ventilation increases have been observed in response to low dissolved oxygen levels, visual stimuli, and gustatory cues. However, olfactory sensory input may also stimulate gill ventilation rate. We investigated whether olfactory sensory input mediates gill ventilation responses, as suggested by the observation that steroidal compounds detected by the olfactory system elicited increases in opercular activity in the perciform teleost, the round goby (*Neogobius melanostomus*). Close parallels between gill ventilation and olfactory responses, led us to conduct an empirical study that used two different olfactory sensory deprivation techniques to seek a causal relationship between olfactory epithelial activity and hyperventilation. Chemical lesion of olfactory sensory neurons or mechanical occlusion of the nasal cavities inhibited gill ventilation responses of reproductive male round gobies to estrone (1,3,5(10)-estratrien-3-ol-17-one) and to ovarian extracts. This direct evidence demonstrates the role of olfactory sensory input for the gill ventilation response to putative reproductive pheromones and may represent an important regulatory mechanism for odorant sampling during pheromone communication.

Keywords: Copper sulfate; Estrone; Fish; Gill ventilation; Nasal occlusion; Olfactory sensory neurons; Ovarian extract; Round goby

1. Introduction

In teleostean fish, opercular movement allows for the flow of water over the gill arches where gas exchange takes place. Oxygen receptors on the gills serve to modulate gill ventilation (Smatresk, 1990; Burleson and Milsom, 1993) such that the rate of gill ventilation increases as oxygen levels decrease (Gee and Gee, 1991). Gill ventilation responses may be driven by alternate sensory modalities. For example, the visual effects of predators override gill chemoreceptors in the flathead gray mullet, *Mugil cephalus,* and gill ventilation rates increase following this visual stimulation (Shingles et al., 2005). This hyperventilation allows the fish to acquire adequate oxygen as it descends in the water to avoid predators. In channel catfish, *Ictalurus punctatus*, the taste of amino acids stimulates gill hyperventilation during feeding (Valentinčič and Caprio, 1994). Finally, a "sniffing" mechanism, driven by opercular movement may facilitate odor detection in flounder species, *Lepidopsetta bilineata* and *Platichthys stellatus* (Nevitt, 1991). Accessory nasal sacs, caudal to the olfactory chamber in many Acanthoptergii fish (including gobies and flounder; Burne, 1909; Sinha and Sinha, 1990; Belanger et al., 2003; Hansen and Zielinski, in press) undergo a pumping action that coincides with the opening and closing of the mouth (Nevitt, 1991). When the mouth opens, muscles that regulate gill motion contract and water is drawn into the anterior nostril. When the mouth closes and the gill muscles relax, water is expelled from the posterior nostril (Johnson and Brown, 1962).

Recent studies of the round goby have demonstrated increased gill ventilation frequency during exposure to synthetic steroids (putative reproductive pheromones), with threshold values closely matched between olfactory epithelial (electro-olfactogram) and gill ventilation responses (Murphy

* Corresponding author. Tel.: +1 519 253 3000x2726; fax: +1 519 971 3609.
E-mail address: zielin1@uwindsor.ca (B.S. Zielinski).

1095-6433/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpa.2006.02.027

et al., 2001; Murphy and Stacey, 2002). The 18-carbon steroid, estrone (1,3,5(10)-estratrien-3-ol-17-one), evoked large ventilation increases in males, in methyl testosterone treated females, and smaller responses in untreated females (Murphy et al., 2001; Murphy and Stacey, 2002); however, a causal relationship, using sensory deprivation, was not demonstrated. Alternatively, gill ventilation rate increases may be mediated by non-olfactory sensory receptors, such as chemoreceptors on the gills (Milsom et al., 2002; for review see Perry and Gilmour, 2002), taste receptors in the mouth (Valentinčič and Caprio, 1994) or non-nasal chemoreceptors (Kotrschal et al., 1997).

In the present study, we compare the effect of olfactory sensory deprivation using chemical lesion with copper sulfate and mechanical obstruction (nasal occlusion) on estrone-induced gill ventilation in the round goby, *Neogobius melanostomus*. Although copper sulfate has been repeatedly used to induce specific degeneration of olfactory sensory neurons (OSNs) in fish (e.g., Julliard et al., 1993, 1996; Beyers and Farmer, 2001), this treatment does not eliminate all OSNs (Saucier and Astic, 1995). We complemented the copper sulfate lesioning technique by occlusion of nasal passageway with an inert substance. A similar strategy was used by Wisby and Hasler (1954) and Jahn (1969) in their studies on olfactory input in salmonid homing migration. The use of both of these techniques allowed us to effectively determine if olfactory input stimulates gill ventilation. If steroid mediated gill ventilation increase is stimulated through olfactory-sensory mechanisms, we expect to inhibit responses in the male round goby exposed to estrone and female gonadal extracts following olfactory sensory deprivation.

2. Materials and methods

2.1. Experimental animals

Round gobies (*N. melanostomus*) were collected during the spring, summer, and fall (2000 to 2002) by angling in the upper Detroit River and by trawling in the western basin of Lake Erie. Round gobies were sexed by the shape of the genital papilla (Miller, 1984). Males and females were housed separately in the laboratory. Fish were maintained under constant photoperiod (16L:8D) in holding tanks (205 L) with aerated 18 °C flow-through dechlorinated tap water, gravel, and PVC shelters. Fish were fed Nutrafin® fish flakes and zebra mussels, *Dreissena polymorpha*. All animals were maintained in accordance with Canadian Council on Animal Care and the Ontario Animals for Research Act guidelines.

Reproductive status was confirmed by post mortem evaluation of the gonadosomal index (GSI) (Strange, 1996). Reproductive round goby males (mean ± standard error (S.E.) GSI values: 1.38 ± 0.08%) were black and had swollen cheeks. Reproductive males, used in this study, differed from non-reproductive round goby males, which had a significantly lower GSI value, were mottled gray, and had cheeks that were never swollen.

2.2. Gill ventilation assay

Individual reproductive male round goby were examined for gill ventilation responses to odors by observing a change in the frequency of opercular movement. Reproductive males were used in this study because the reproductive status of round goby has been shown to affect behavioral responsiveness to conspecific odors; only reproductive female gobies are attracted to conspecific odors (Gammon et al., 2005). An increase in the opercular beat frequency was observed within 4 min of odorant application which is consistent with previous studies that demonstrate an increase in ventilation rate within 1 and 10 min after odor presentation (Murphy et al., 2001).

Accordingly, a reproductive male fish was placed in an 8-L aquarium containing 5.5-L dechlorinated water (18°C), an air stone, and a "nest" constructed from ceramic tile (10 cm wide × 15 cm long × 5 cm deep) with a single Plexiglas® transparent side to allow for recording of the goby within the "nest". The aquarium was surrounded by Styrofoam®, with an opening on one side where a camera lens was positioned. Video images of fish ventilation were obtained using a Panasonic WVBL604 high-resolution video camera and Panasonic AG6050 time-lapse video recorder. Each fish was acclimated in the tank overnight, and the assays were conducted the following day. Behavioral assays were conducted with the test fish being located within the nest and remaining there for the duration of the trial. The opaque barriers surrounding both the nest and the aquarium prevented the test animals from visualizing the addition of the test odorant over the air stone (located behind the nest) by the experimenter (adapted from Murphy et al., 2001; Gammon et al., 2005). Preliminary dye tests showed a thorough mixing of the test solutions within 4 min of application, as observed in previous behavioral studies of round goby (e.g., Murphy et al., 2001; Gammon et al., 2005).

Gill ventilation was recorded over a 15-min pre-test period before the odorant was added, and for a 30-min test period after the odorant was added. The values for percentage change in basal ventilation were determined by comparing ventilation rate prior to odorant application, to the ventilation rate following odorant application. Previous studies have shown that basal ventilation rates are stable in the round goby (Murphy et al., 2001; Murphy and Stacey, 2002). In this study, we averaged basal ventilation rate values sampled at 5, 10, and 15 min prior to odorant application. This average was used to calculate the percent increase in basal ventilation at 4 min post-odorant application. The selection of the 4-min sampling period was based on the timing results in our dye diffusion tests, and on the ventilation responses observed by Murphy et al. (2001, Fig. 4) and Murphy and Stacey (2002, Fig. 1).

2.3. The effect of copper sulfate on gill ventilation

Copper sulfate ($CuSO_4$; Fisher Scientific) is a known chemical agent that causes anosmia in fish (Hara et al., 1976; Hansen et al., 1999), and results in specific degeneration of mature and immature OSNs (Cancalon, 1982; Julliard et al., 1993) within 18 h of treatment. We tested each of 10 male round

gobies for gill ventilation response to 10^{-8} M estrone (1,3,5 (10)-estratrien-3-ol-17-one (E1274, Sigma-Aldrich, USA); 10^{-3} M stock in 95% ethanol, diluted to 10^{-8} M in dechlorinated water), after a procedural control (osmic) and following nasal infusion of a copper sulfate solution (anosmic). Fish that were subjected to two consecutive procedural control treatments did not show aversive effects from excessive anesthesia. First, animals were subjected to the procedural control in which each fish was anesthetized with 0.5% MS-222, wrapped in wet paper towelling, and the gills were continually perfused for 10 min with the aerated MS-222 solution. A 23-gauge needle was positioned into each posterior naris for the 10 min and secured with Play-doh®. Each needle was connected to an empty 60 cm^3 syringe via plastic tubing. At this time, an innocuous aqueous solution was not passed through the nasal passage in order to avoid high-pressure delivery of a fluid, which would mechanically damage the cilia of the olfactory epithelium. Previous electro-olfactogram recordings have demonstrated loss of OSN responsiveness following mechanical damage to the epithelium (Scott and Brierley, 1999). The use of this procedural control allowed us to compare responses of "intact" olfactory tissue to lesioned (anosmic) tissue. The gill ventilation response to 10^{-8} M estrone was recorded 18 h later. The same fish was then treated with copper sulfate by a procedure modified from Winberg et al. (1992). Each animal was anesthetised, wrapped in wet paper towelling, the copper sulfate solution (30 mL of 10 mg $CuSO_4$/L dechlorinated water) was directed from the syringe reservoir and 23-gauge needle into each naris for 10 min, and gill ventilation responses to estrone were tested 18 h later. We did not passively drip $CuSO_4$ solution into the nasal cavity because low pressure flow would not access all of the OSNs. These cells line the dorsal surface (as well as the ventral surface) of the olfactory chamber in the round goby (Belanger et al., 2003). The two consecutive trials (procedural control and copper treatment) were conducted on the same fish within 48 h of each other and data were analysed using a Wilcoxon test.

2.4. G-protein immunocytochemistry for assessing the effect of copper sulfate on olfactory sensory neurons

G-proteins are coupled to olfactory receptor proteins on the cell membrane of OSNs (e.g., Jones and Reed, 1989). In teleosts, the G-proteins, $G_{\alpha o}$ and $G_{\alpha olf}$, are located on microvillar and ciliated OSNs, respectively (Belanger et al., 2003; Hansen et al., 2003). We assumed that $G_{\alpha olf}$ and $G_{\alpha o}$ immunolabeling was lost following copper induced OSN degeneration (Julliard et al., 1993, 1996; Starcevic and Zielinski, 1997). Accordingly, three procedural control treated specimens and five copper sulfate (10 mg/L) treated round gobies were examined for $G_{\alpha olf}$ immunocytochemistry. The peripheral olfactory organ was fixed in Zamboni's fluid (2% paraformaldehyde, 1.2% picric acid in 0.1 M phosphate buffer), the tissue was cryoprotected through an ascending sucrose series (10%, 20%, and 30% in 0.1 M phosphate-buffered saline (PBS), pH = 7.4), placed in M-1 cryo-embedding matrix (Thermo Shandon, Fairlawn, NJ), and sectioned using a Microm HM-500 OM cryostat (Heidelberg, Germany). The tissue sections were applied to microscope slides (Fisherbrand Colorfrost/Plus Microscope Slides, Fisher Scientific, Pittsburg, PA, USA), cold fixed in acetone for 10 min, rehydrated in 0.1 M PBS, and incubated overnight in polyclonal primary antibodies, raised in rabbit against the G-proteins $G_{\alpha o}$ and $G_{\alpha olf}$ (diluted 1:1000, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The following morning, the sections were rinsed in 0.1 M PBS, incubated in anti-rabbit IgG conjugated to 488 nm absorbing Alexa dye (diluted 1:100; Molecular Probes, Eugene, OR, USA), rinsed again, and mounted with Vectashield® (Vector Labs, Burlingame, CA, USA). Fluorescence microscopy and image analysis performed on a Zeiss Axioskop FS (post-acquisition software, Northern Eclipse) showed degeneration of the OSNs, 18 h following the copper sulfate treatment (Fig. 1). In fish that received the procedural control treatment, the

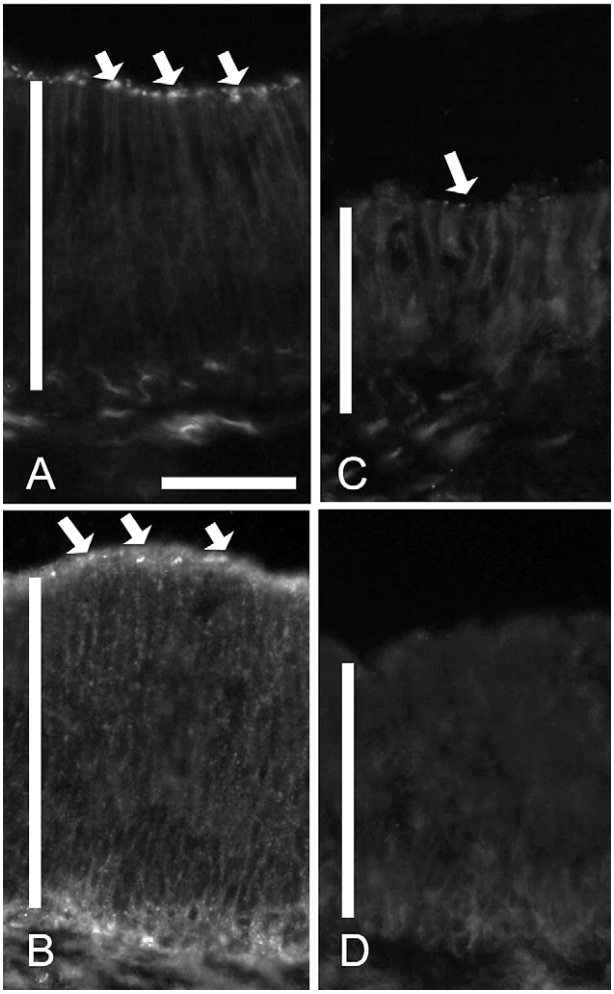

Fig. 1. The effect of copper sulfate (10 mg/L) on G-protein immunoreactivity in the olfactory epithelium. The horizontal scale bar, shown in (A) is 50 μm. (A and C) show $G_{\alpha olf}$ immunoreactivity, and (B and D) show $G_{\alpha o}$ immunoreactivity. The vertical bars show the thickness of the olfactory epithelium. (A and B) In untreated olfactory epithelium, the olfactory knobs of olfactory sensory neurons are $G_{\alpha olf}$-immunoreactive (arrows in A) and $G_{\alpha o}$-immunoreactive (arrows in B). (C and D) Eighteen hours following treatment with copper sulfate (10 mg/L), the thickness of the olfactory epithelium was reduced. Immunoreactivity against $G_{\alpha olf}$ (C) and $G_{\alpha o}$ (D) was faint. An arrow in C shows a faint $G_{\alpha olf}$ immunoreactive olfactory knob.

olfactory epithelium was approximately 100 μm thick, and $G_{\alpha olf}$ and $G_{\alpha o}$-immunoreactivity was intense in apical puncta, at the site of OSN cilia and microvilli (Fig. 1A, B). Eighteen hours following intra-nasal treatment with copper sulfate, the height of the olfactory epithelium decreased to approximately 70 μm; $G_{\alpha olf}$ and $G_{\alpha o}$-immunoreactive puncta were rarely seen in the apical region of the olfactory epithelium (Fig. 1C, D). Decrease in olfactory epithelial thickness has been previously shown to accompany the loss of OSNs following copper sulfate treatment (e.g., Julliard et al., 1993). Loss of G-protein labeling implies an accompanying loss of the function of G-protein activity and of olfactory signal transduction. The presence of infrequent weakly labeled G-protein immunoreactive apical puncta following copper treatment (Fig. 1C, D) confirms previous observations of OSNs that have been spared cell death following copper treatment (Saucier and Astic, 1995; Beyers and Farmer, 2001). Thus, olfactory transduction will still be present, but minimal in these individuals.

2.5. Nasal occlusion

We hypothesized that nasal occlusion would prevent odorant access to OSNs. We selected a hydrophilic vinyl polysiloxane, a nontoxic dental impression material (Reprosil®, DENTSPLY International Inc., Milford, Delaware), for occluding the nasal cavity as this procedure had been shown previously to fill the entire nasal passageway (Belanger et al., 2003). The procedure entailed anesthetizing a fish with 0.5% MS-222 followed by the injection of the Reprosil® into the posterior nares until the nasal cavity was filled. It filled the lumen of the nasal cavity, including the anterior and posterior nares, the accessory nasal sacs and the olfactory chamber adjacent to the peripheral olfactory organ (Belanger et al., 2003). To confirm that the entire nasal cavity was plugged, some fish were euthanized by MS-222 overdose, and the nasal cast was removed as previously described (Belanger et al., 2003). A procedural control, including all of the steps for plugging the nose, excluding the Reprosil® injection was performed on fish. As with the copper sulfate lesioning, an innocuous solution was not passed through the nasal cavity in order that the epithelium remained intact for purposes of experimentation. These fish were designated as untreated, osmic animals. Each of 10 round gobies with nasal occlusion and 10 different gobies (the procedural control without nasal occlusion) were tested for gill ventilation responses to 10^{-8} M estrone. Comparisons between osmic and anosmic males were made after exposure to estrone using a Mann–Whitney *U*-test.

2.6. Ventilation responses of reproductive males to extracts from gonadal and muscular tissue

Because extracts from ovarian (gonadal) tissue are known to contain estrogenic steroids in other fishes (e.g. *Danio rerio*, van den Hurk and Lambert, 1983; *Barilius bendelisis*, Bhatt et al., 2002), we assumed that estrogenic steroids also occur in female round gobies. Specifically, we assumed that gonadal extracts of reproductive female round gobies were a putative source of estrone that would evoke gill ventilation increases. Accordingly, we obtained and compared extracts from round goby female ovarian tissue with muscle tissue (non-steroidogenic) from the same specimen. The sensory source for the gill ventilation responses to these tissue extracts was determined by testing the effect of olfactory sensory deprivation through Reprosil® nasal occlusion. For this, five reproductive females (with extended abdomens) were injected in the peritoneum with 5 units/g fish weight human chorionic gonadotropin (HCG; C1063, Sigma-Aldrich, Milwaukee, WI) to ensure gonadal maturation (Kulikova, 1985) and slightly higher yields of gonadal metabolites (Arbuckle et al., 2005). Twelve to 18 h after HCG injection, the females were euthanized. Gonadal tissue was removed and weighed (1.8–3.8 g, $n=5$) and an equal weight of muscular tissue also was taken. Since females were injected with HCG, it was necessary to test an equal amount of homogenized female HCG injected muscle tissue for ventilation responses in males to rule out the possibility that HCG was eliciting a response. Maturation status was confirmed by GSI. Values >10% indicate ovaries with mature oocytes (Kulikova, 1985). The GSI values (14.3%±1.01%) for the HCG injected round goby females indicated final oocyte maturation.

Gonadal and muscular tissue from each fish was homogenized separately on ice with a glass homogenizer (2.0 g tissue in 10 mL of 95% ethanol). The diluted homogenate (1 mL homogenate in 99 mL dechlorinated water) was exposed to male round goby to test for changes in ventilation. The percentage change in basal ventilation of reproductive male round goby was compared among osmic ($n=15$) and anosmic ($n=15$) fish exposed to extracts of gonadal tissue and osmic fish ($n=10$) exposed to muscular tissue. Mechanical olfactory sensory deprivation using Reprosil® was used to render round goby males anosmic for these trials. A one-way ANOVA (Student–Newman–Keuls post-hoc test) was performed to compare the mean percentage basal ventilation of reproductive male round gobies among the three treatments. Responses to the carrier (containing ethanol) were conducted and evaluated similarly to the test odors and no ventilation increases were observed ($p<0.05$).

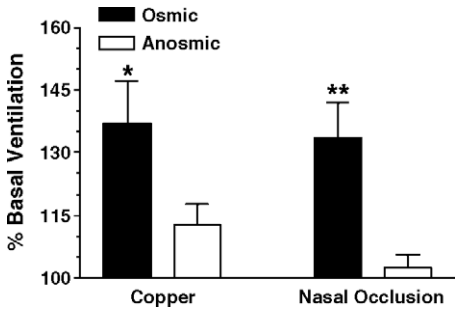

Fig. 2. The effect of 10 mg/L copper sulfate and nasal occlusion on the percentage change in basal ventilation (mean+S.E.) of male round gobies ($n=10$) after the addition of 10^{-8} M estrone compared with the procedural control response recorded 4 min after the addition of the odor. Black bars represent ventilation responses of osmic (procedural control) fish; open bars represent ventilation responses of anosmic (treatment) fish. The symbols, * ($P<0.05$) and ** ($P<0.01$), indicate that there were significant differences in percent basal ventilation between osmic and anosmic fish.

3. Results

3.1. The effect of olfactory sensory deprivation on the gill ventilation responses to estrone

Gill ventilation rates increased in male round gobies during exposure to the putative pheromone estrone (Fig. 2). However, the ventilation response to estrone was significantly diminished following copper treatment (Fig. 2). There was a significant difference (Wilcoxon test, $P<0.05$) in the response (% basal ventilation) between the control and copper treated fish 4 min after the addition of estrone. A small increase in ventilation frequency during the initial 4-min recording of lesioned fish, following estrone application compared with nasal occlusion, may reflect the activity of OSNs that were spared the lethal effects of copper sulfate.

Following nasal occlusion with dental impression material, basal ventilation rate was unaffected when compared to non-occluded individuals. In occluded specimens, frequency of gill ventilation remained unchanged after the addition of estrone to the fish tank (Fig. 2). However, there was a significant difference (Mann–Whitney U-test, $P<0.001$) in the ventilation rate between osmic and anosmic (those with nares occluded with Reprosil®) fish 4 min after the addition of estrone. Thus, olfactory sensory deprivation by two independent techniques, copper sulfate and nasal occlusion, confirmed that gill ventilation rate increases in response to nasal sensory input.

3.2. Ventilation responses of reproductive males to extracts from gonadal and muscular tissue

We compared responses of reproductive males to female gonadal extracts with those obtained from muscle tissue (non-steroidogenic). Results of a one-way ANOVA showed that there were significant differences in percentage of basal ventilation among the three treatments, osmic and anosmic reproductive male round gobies exposed to extracts from reproductive female gonadal tissue as well as, the effect of extracts from muscular tissue ($F_{2,37}=11.44$, $P<0.01$). Responses by osmic males to female gonadal extract showed significant increases in basal ventilation rate after exposure. The post-hoc Student–Newman–Keuls test showed that there was no significant difference in ventilation response between anosmic reproductive male round gobies exposed to extracts of reproductive female gonadal tissue and osmic reproductive males exposed to extracts of muscular tissue (Fig. 3). The extracts of gonadal tissue elicited a noticeable increase in gill ventilation in osmic reproductive males compared with the other treatments (Fig. 3). The absence of this response in anosmic (occluded) reproductive males exposed to extracts of gonadal tissue and in osmic reproductive males exposed to extracts of muscular tissue shows that the change in gill ventilation depends on nasal sensory input. Specificity of gonadal tissue in eliciting this response is demonstrated by failure of extracts from non-gonadal (muscular) tissue from reproductive females to elicit gill ventilation responses in reproductive males (Fig. 3).

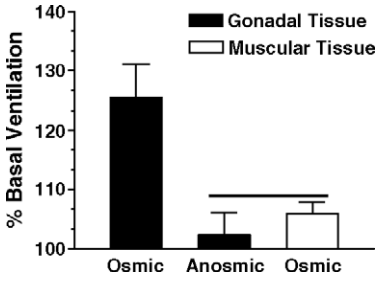

Fig. 3. Percentage change in basal ventilation of osmic ($n=15$) and anosmic ($n=15$) reproductive male round gobies exposed to extracts from gonadal tissue (solid bars) obtained from reproductive females as well as the effect of extracts from muscular tissue (open bar) from these females on osmic ($n=10$) reproductive males. The solid line indicates that there was no significant difference in percentage change in ventilation between anosmic reproductive males exposed to gonadal extracts and osmic reproductive males exposed to extracts from muscular tissue.

4. Discussion

The importance of olfactory sensory input for gill ventilation was demonstrated by the decrease of this response in male *N. melanostomus* following chemical lesion of OSNs with copper sulfate and the complete inhibition following nasal occlusion. These results show that input from the nasal cavity, rather than from taste receptors (Valentinčič and Caprio, 1994) or gill chemoreceptors (Milsom et al., 2002) is required for this assay. The deleterious effect of copper sulfate on the gill ventilation assay is supported by previous observations on the loss of olfactory mediated behavior following copper treatment in rainbow trout (Brown et al., 1982) and Colorado pikeminnows (Beyers and Farmer, 2001). The presence of infrequent $G_{\alpha olf}$ and $G_{\alpha o}$-immunoreactive OSNs remaining in the olfactory epithelium is reflected by the persistence of a small gill ventilation response following copper sulfate treatment. Others have shown that fish retain a slight electro-olfactogram response following copper sulfate treatment (Winberg et al., 1992), suggesting that some OSNs are spared damage (Saucier and Astic, 1995), possibly through glutathione mediated neuroprotection (Starcevic and Zielinski, 1997).

Complete inhibition of the gill ventilation response to estrone following olfactory sensory deprivation by nasal occlusion complements the chemical lesioning experiment and supports the importance of olfactory sensory input on the gill ventilation response. Because nasal occlusion mechanically obstructs odorant access to the entire nasal cavity, non-olfactory chemosensory events also were blocked. In amphibians and mammals, trigeminal nerve fibers enter the apical region of the olfactory epithelium where they respond to irritation stimuli (Bouvet et al., 1987; Finger et al., 1990). The terminal nerve, another non-olfactory pathway present in the nasal cavity (Nevitt et al., 1995) has been linked with modulation of OSN activity (Eisthen et al., 2000) rather than chemoreception (Fujita et al., 1991). The existence of these non-olfactory chemosensory and neuromodulatory systems within the nasal cavity

suggests that these may contribute to gill ventilation by modulating OSN function or other aspects of nasal cavity function.

Gonadal source for female putative pheromones was implied from our observation of reproductive male olfactory dependent gill ventilation responses to reproductive female gonadal extracts. The ineffectiveness of muscular tissue from reproductive females in stimulating this response further supports the idea that gonadal tissue contains putative pheromonal compounds. Previous studies have shown that substances originating from the gonad elicit courtship behavior in gobiids (Tavolga, 1956; Colombo et al., 1982; Locatello et al., 2002). Of various internal body fluids from gravid females tested, only ovarian fluid and freshly extruded eggs elicited courtship behavior (fanning and gasping) in male frillfin gobies *Bathygobius soporator* (Tavolga, 1956). Murphy et al. (2001) showed that two ovarian steroids 17β-estradiol and estrone elicited olfactory epithelial voltage transients and gill ventilation responses in the round goby. Estrone is an immediate precursor in ovarian biosynthesis of 17β-estradiol (Hadley, 1992; Specker and Sullivan, 1994) and serum levels of 17β-estradiol are elevated in teleosts prior to spawning (Liley and Stacey, 1983). Therefore, gonadal tissue from reproductive *Neogobius* may contain these unconjugated 18 carbon estrogens or other previously untested compounds. These estrogen compounds do not elicit olfactory activity in the few other perciforms that have been investigated (Sorensen et al., 2004). Furthermore, 18 carbon-unconjugated steroids stimulate olfactory activity in only a few nonperciform fish (Stacey and Sorensen, 2002) and therefore may be specific stimulants of gobies.

The significance of the gill ventilation response is unknown; however, there are two potential explanations in light of the reproductive behavior of these fish, (1) increased oxygen into the blood stream and (2) enhanced accessory nasal sac pumping leading to more water entering the nasal cavity. The increased uptake of oxygen into the bloodstream that results from ventilation may assist in the provision of metabolic energy. However, male round gobies are stationed within nests during the reproductive period (MacInnis and Corkum, 2000), and large expenditures in metabolic energy may not be required when responding to female pheromones. On the other hand, the pumping of the accessory nasal sacs (Belanger et al., 2003) that would increase as a consequence of amplified gill ventilation would allow more water to enter the nasal cavity and OSNs. This would increase contact with putative pheromones that are present in relatively low concentrations in water surrounding the fish. This "sniffing" mechanism may also allow estrone molecules to access specific spatial zones of the olfactory epithelium (Scott-Johnson et al., 2000).

The increased ventilation that accompanies olfactory stimulation by estrone and other putative pheromones is not seen in response to amino acids in the round goby (Murphy et al., 2001). L-Alanine has been determined as a food odor to teleost fish (Caprio, 1984) and is commonly used as a food source odor when examining olfactory sensitivity (Bhatt et al., 2002). Valentinčič and Caprio (1997) postulated that during feeding, the olfactory system detects potent taste stimuli and provides the afferent input for arousal and the release of all feeding activity behaviors, but not increases in gill ventilation.

Our findings clearly show the requirement of olfactory sensory input for gill ventilation response of male round gobies to the putative pheromone, estrone, and to extracts of reproductive female gonadal tissue. Non-gonadal extracts from reproductive females failed to elicit responses. This study demonstrates that the gill ventilation response in the round goby is useful for testing the olfactory recognition of putative pheromones and for investigating the organ of origin for these compounds.

Acknowledgments

This project was supported by NSERC, the Michigan Great Lakes Protection Fund, and the University of Windsor, Faculty of Graduate Studies. We thank Dr. T. Johnson (Ontario Ministry of Natural Resources, Wheatley) and many others for assistance with fish collection and laboratory work.

References

Arbuckle, W.J., Belanger, A.J., Corkum, L.D., Zielinski, B., Li, W., Yun, S.-S., Bachynski, S., Scott, S., 2005. In vitro biosynthesis of novel 5β reduced steroids by the testis of the round goby, *Neogobius melanostomus*. Gen. Comp. Endocrinol. 140, 1–13.

Belanger, R.M., Smith, C.M., Corkum, L.D., Zielinski, B., 2003. Morphology and histochemistry of the peripheral olfactory organ in the round goby, *Neogobius melanostomus* (Teleostei: Gobiidae). J. Morphol. 257, 62–71.

Beyers, D.W., Farmer, M.S., 2001. Effects of copper on olfaction of Colorado pikeminnow. Environ. Toxicol. Chem. 20, 907–912.

Bhatt, J.P., Kandwall, J.S., Nautiyal, R., 2002. Water temperature and pH influence olfactory sensitivity to pre-ovulatory and post-ovulatory ovarian pheromones in male *Barilius bendelisis*. J. Biosci. 27, 273–281.

Bouvet, J.F., Godinot, F., Croze, S., Delaleu, J.C., 1987. Trigeminal substance P-like immunoreactive fibers in the frog olfactory mucosa. Chem. Senses 12, 499–505.

Brown, S.B., Evans, R.E., Thomson, B.E., Hara, T.J., 1982. Chemoreception and aquatic pollutants. In: Hara, T.J. (Ed.), Chemoreception in Fishes. Elsevier Scientific, Amsterdam, pp. 363–393.

Burleson, M.L., Milsom, W.K., 1993. Sensory receptors in the first gill arch of rainbow trout. Respir. Physiol. 93, 97–110.

Burne, R.H., 1909. The anatomy of the olfactory organ of teleostean fishes. Proc. Zool. Soc. Lond. 2, 610–663.

Cancalon, P., 1982. Degeneration and regeneration of olfactory cells induced by $ZnSO_4$ and other chemicals. Tissue Cell 14, 717–733.

Caprio, J., 1984. Olfactory and taste in fish. In: Bolis, L., Keyes, R.D., Maddrell, S.M.P. (Eds.), Comparative Physiology and Sensory Systems. Cambridge, New York, pp. 257–283.

Colombo, L., Belvedere, P.C., Marconato, A., Bentivegna, F., 1982. Pheromones in teleost fish. In: Richter, C.J.J., Goos, H.J.Th. (Eds.), Proceedings of the Second International Symposium on the Reproductive Physiology of Fish. Pudoc, The Netherlands, pp. 84–94.

Eisthen, H.L., Delay, R.J., Wirsig-Wiechmann, C.R., Dionne, V.E., 2000. Neuromodulatory effects of gonadotropin releasing hormone on olfactory receptor neurons. J. Neurosci. 20, 3947–3955.

Finger, T.E., Getchell, M.L., Getchell, T.V., Kinnamon, J.C., 1990. Affector and effector functions of peptidergeic innervation of the nasal cavity. In: Green, B.G., Mason, J.R., Kare, M.R. (Eds.), Chemical Senses. Irritation, vol. 2. Marcel Dekker, New York, pp. 1–18.

Fujita, I., Sorensen, P.W., Stacey, N.E., Hara, T.J., 1991. The olfactory system, not the terminal nerve, functions as the primary chemosensory pathway

mediating responses to sex pheromones in male goldfish. Brain Behav. Evol. 38, 313–321.
Gammon, D.B., Li, W., Scott, A.P., Zielinski, B.S., Corkum, L.D., 2005. Behavioural responses of female *Neogobius melanostomus* to odours of conspecifics. J. Fish Biol. 67, 615–626.
Gee, J.H., Gee, P.A., 1991. Reactions of gobioid fishes to hypoxia: buoyancy control and aquatic surface respiration. Copeia 1991, 17–28.
Hadley, M.A., 1992. Endocrinology, 3rd edition. Prentice-Hall, New York, NY.
Hansen, A., Zielinski, B., in press. Diversity in the olfactory epithelium of bony fishes: development, lamellar arrangement, sensory neuron cell type and transduction components. J. Neurocytol.
Hansen, A., Zippel, H.P., Sorensen, P.W., Caprio, J., 1999. Ultrastructure of the olfactory epithelium in intact, axotomized, and bulbectomized goldfish, *Carassius auratus*. Microsc. Res. Tech. 45, 325–338.
Hansen, A., Rolen, S.H., Anderson, K., Morita, Y., Caprio, J., Finger, T.E., 2003. Correlation between olfactory receptor cell type and function in the channel catfish. J. Neurosci. 23, 9328–9339.
Hara, T.J., Law, Y.M.C., MacDonald, S., 1976. Effects of mercury and copper on olfactory response in rainbow-trout, *Salmo gairdneri*. J. Fish. Res. Board Can. 33, 1568–1573.
Jahn, L.A., 1969. Movement and homing of cutthroat trout (*Salmo clarki*) from open-water areas in Yellowstone Lake. J. Fish. Res. Board Can. 26, 1243–1261.
Johnson, H.E., Brown, J.C.D., 1962. Olfactory apparatus in the black rockfish, *Sebastodes melanops*. Copeia 1962, 838–840.
Jones, D.T., Reed, R.R., 1989. Golf: and olfactory neuron specific-G protein involved in odorant signal transduction. Science 244, 790–795.
Julliard, A.K., Saucier, D., Astic, L., 1993. Effects of chronic low-level copper exposure on ultrastructure of the olfactory system in rainbow trout (*Oncorhynchus mykiss*). Histol. Histopathathol. 8, 655–672.
Julliard, A.K., Saucier, D., Astic, L., 1996. Time-course of apoptosis in the olfactory epithelium of rainbow trout exposed to a low copper level. Tissue Cell 28, 367–377.
Kotrschal, K., Krautgartner, W.D., Hansen, A., 1997. Ontogeny of the solitary chemosensory cells in the zebrafish, *Danio rerio*. Chem. Senses 22, 111–118.
Kulikova, N.I., 1985. The effect of chorionic gonadotropin on growth and maturation of the oocytes of the round goby, *Neogobius melanostomus*. J. Ichthyol. 25, 86–98.
Liley, N.R., Stacey, N.E., 1983. Hormones, pheromones, and reproductive behavior in fish. Fish Physiol. IX B, 1–62.
Locatello, L., Mazzoldi, C., Rasotto, M.B., 2002. Ejaculate of sneaker males is pheromonally inconspicuous in black goby, *Gobius niger* (Teleostei, Gobiidae). J. Exp. Zool. 293, 601–605.
MacInnis, A.J., Corkum, L.D., 2000. Fecundity and reproductive season of the round goby *Neogobius melanostomus* in the Upper Detroit River. Trans. Am. Fish. Soc. 129, 136–144.
Miller, P.J., 1984. The tokology of goboid fishes. In: Potts, G.W., Wooton, R.J. (Eds.), Fish Reproduction, Strategies and Tactics. Academic Press, London, pp. 119–153.
Milsom, W.K., Reid, S.G., Rantin, F.T., Sundin, L., 2002. Extrabranchial chemoreceptors involved in respiratory reflexes in the neotropical fish *Colossoma macropomum*. J. Exp. Biol. 205, 1765–1774.
Murphy, C.A., Stacey, N.E., 2002. Methyl-testosterone induces male-typical ventilatory behavior in response to putative steroidal pheromones in female round gobies (*Neogobius melanostomus*). Horm. Behav. 42, 109–115.
Murphy, C.A., Stacey, N.E., Corkum, L.D., 2001. Putative steroidal pheromones in the round goby, *Neogobius melanostomus*: olfactory and behavioral responses. J. Chem. Ecol. 27, 443–470.
Nevitt, G.A., 1991. Do fish sniff? A new mechanism of olfactory sampling in pleuronectid flounders. J. Exp. Biol. 157, 1–18.
Nevitt, G.A., Grober, M.S., Marchaterre, M.A., Bass, A.H., 1995. GnRH-like immunoreactivity in the peripheral olfactory system and forebrain of Atlantic salmon (*Salmo salar*): a reassessment at multiple life history stages. Brain Behav. Evol. 45, 350–358.
Perry, S.F., Gilmour, K.M., 2002. Sensing and transfer of respiratory gases at the fish gill. J. Exp. Zool. 293, 249–263.
Saucier, D., Astic, L., 1995. Morphofunctional alterations in the olfactory system of rainbow trout (*Oncorhynchus mykiss*) and possible acclimation in response to long-lasting exposure to low copper levels. Comp. Biochem. Physiol. A 112, 273–284.
Scott, J.W., Brierley, T., 1999. A functional map in rat olfactory epithelium. Chem. Senses 24, 679–690.
Scott-Johnson, P.E., Blakley, D., Scott, J.W., 2000. Effects of air flow on rat electro-olfactogram. Chem. Senses 25, 761–768.
Shingles, A., McKenzie, D.J., Claireaux, G., Domenici, P., 2005. Reflex cardioventilatory responses to hypoxia in the flathead gray mullet (*Mugil cephalus*) and their behavioural modulation by perceived threat of predation and water turbidity. Physiol. Biochem. Zool. 78, 744–755.
Sinha, S.K., Sinha, R.K., 1990. Morphology and anatomy of the olfactory organs of the marine fish *Thynnus thunnia* (Cuv. et Val.). Folia Morphol. 2, 169–173.
Smatresk, N.J., 1990. Chemoreceptor modulation of the endogenous respiratory rhythm in vertebrates. Am. J. Physiol. 259, R887–R897.
Sorensen, P.W., Murphy, C.A., Loomis, K., Maniak, P., Thomas, P., 2004. Evidence that 4-pregnen-17,20 β,21-triol-3-one functions as a maturation-inducing hormone and pheromonal precursor in the percid fish, *Gymnocephalus cernuus*. Gen. Comp. Endocrinol. 139, 1–11.
Specker, J.L., Sullivan, C.V., 1994. Vitellogenesis in fishes: status and perspectives. In: Davey, K.G., Peter, R.E., Tobe, S.S. (Eds.), Perspectives in Comparative Endocrinology. National Research Council of Canada, Ottawa, pp. 304–315.
Stacey, N.E., Sorensen, P.W., 2002. Hormonal pheromones in fish. In: Pfaff, D.W., Arnold, A.P., Etgen, A.M., Fahrbach, S.E., Rubin, R.T. (Eds.), Hormones, Brain and Behaviour, vol. 2. Academic Press, New York, pp. 375–434.
Starcevic, S.L., Zielinski, B.S., 1997. Neuroprotective effects of glutathione on rainbow trout olfactory receptor neurons during exposure to copper sulfate. Comp. Biochem. Physiol. C 117, 211–220.
Strange, R.J., 1996. Field examination of fishes, In: Willis, B.E., Willis, D.W. (Eds.), Fisheries Techniques, 2nd edition. American Fisheries Society, Bethesda, pp. 433–446.
Tavolga, W.M., 1956. Visual, chemical and sound stimuli as cues in the sex discriminatory behavior of the gobiid fish *Bathygobius soporator*. Zool. N. Y. Zool. Soc. 41, 49–65.
Valentinčič, T., Caprio, J., 1994. Consummatory feeding behaviour to amino acids in intact and anosmic channel catfish *Ictalurus punctatus*. Physiol. Behav. 55, 857–863.
Valentinčič, T., Caprio, J., 1997. Visual and chemical release of feeding behavior in adult rainbow trout. Chem. Senses 22, 375–382.
van den Hurk, R., Lambert, J.G.D., 1983. Ovarian steroid glucuronides function as sex pheromones for male zebrafish, *Brachydanio rerio*. Can. J. Zool. 61, 2381–2387.
Winberg, S., Bjerselius, R., Baatrup, E., Døving, K.B., 1992. The effect of Cu (II) on the electro-olfactogram (EOG) of the Atlantic salmon (*Salmo salar*) in artificial fresh-water of varying inorganic carbon concentrations. Ecotoxicol. Environ. Saf. 24, 167–178.
Wisby, W.J., Hasler, A.D., 1954. The effect of olfactory occlusion on migrating silver salmon (*Oncorhynchus kisutch*). J. Fish. Res. Board Can. 11, 472–478.