Journal of Fish Biology (2004) **65,** 933–946
doi:10.1111/j.1095-8649.2004.00494.x, available online at http://www.blackwell-synergy.com

Behavioural and electrophysiological responses by reproductive female *Neogobius melanostomus* to odours released by conspecific males

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(Received 10 November 2003, Accepted 14 June 2004)

Behavioural and electrophysiological responses of reproductive and non-reproductive female round gobies *Neogobius melanostomus* to water previously occupied by male round gobies (reproductive male water) were compared. Reproductive females spent more time than non-reproductive females in a tank near the input of water conditioned from reproductive males. Also, reproductive females swam significantly faster than non-reproductive females, suggesting that reproductive male odour may have activated spawning behaviour. Olfactory epithelial field potential measurement (electro-olfactogram, EOG) showed that reproductive male water was a potent olfactory stimulus to reproductive females, but not to non-reproductive females. Reproductive females responded significantly more than non-reproductive females to solid-phase (octadecylsilane) extracts of reproductive male water. Also, when these extracts were separated on reverse phase high performance liquid chromatography (HPLC), reproductive females showed noticeably greater responses than non-reproductive females to the fractions that eluted between 30 and 40 min. The behavioural data support the hypothesis that reproductive male round gobies release compounds into the water that attract potential mates. The EOG data indicate that these compounds can be quantitatively extracted from the water and be partially purified by HPLC. The evidence is not sufficient to indicate whether or not the compounds are steroids. The relatively early elution time on HPLC, however, suggests that if these compounds are steroids, then it is more likely that they will be conjugated rather than free steroids.

Key words: behaviour; electro-olfactogram; etiocholanolone-glucuronide; pheromones.

INTRODUCTION

Native to the Ponto-Caspian region of eastern Europe, the round goby *Neogobius melanostomus* (Pallas), a small bottom-dwelling fish, was probably introduced to the Laurentian Great Lakes of North America by ship ballast water (Jude *et al.*, 1992). First reported in the St Clair River in 1990, the fish, a multiple

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spawner, has spread to all five of the Great Lakes (Charlebois *et al.*, 2001). Pheromone signalling may have contributed to the reproductive success of this invading fish species. Round gobies live in benthic habitats where light levels are low and males are often segregated in dark cavities. The parental male round goby maintains and guards a nest into which many females deposit eggs (Wickett & Corkum, 1998; MacInnis & Corkum, 2000). Therefore, chemical communication between males and females may be crucial in regulating and facilitating their reproductive habits.

The round goby responds to putative pheromonal compounds: 18-, 19- and 21- carbon steroids, including 5β-androstan-3α-ol-17-one (etiocholanolone, ETIO) and etiocholanolone-glucuronide (ETIO-g), by increased olfactory epithelial and gill ventilatory activity (Murphy *et al.*, 2001). These findings suggest that sexually mature reproductive round gobies detect and show physiological responses to waterborne steroids through the olfactory sense. The pheromones that stimulate reproductive responses in fishes are often steroidal and prostaglandin compounds associated with gonadal function, or their derivatives (Hara, 1994; Stacey & Sorensen, 2002). In black gobies *Gobius niger* L. exposure to the 5β-reduced androgen, ETIO-g, a metabolite produced by the mesorchial glandular mass of the testis, stimulated attraction and egg deposition by ovulated females, with spent females displaying significantly less attraction (Colombo *et al.*, 1977, 1980). It has not been determined if the ETIO-g is released into water by the reproductive male gobies. van den Hurk & Resink (1992), however, showed that male African catfish *Clarias gariepinus* (Burchell) release steroid glucuronides to which ovulated females are attracted. Behavioural responses of reproductive females to odours released by conspecific reproductive males have been observed in the black bullhead *Ameiurus melas* (Rafinesque) (E.R. Kendle, unpubl. data), sea lamprey *Petromyzon marinus* L. (Teeter, 1980; Li *et al.*, 2002) and Arctic charr *Salvelinus alpinus* (L.) (Sveinsson & Hara, 1995).

Responses of olfactory receptor cells to water previously occupied by conspecifics and to steroidal pheromones have been effectively monitored by recording extracellular field potentials from the olfactory epithelium using electro-olfactogram (EOG) recordings (Sorensen *et al.*, 1987; Frade *et al.*, 2002; Li *et al.*, 2002). Previous studies have shown that olfactory epithelial responses to steroids are not uniform throughout the life cycle of some fishes (Moore & Scott, 1991, 1992; Bjerselius & Olsén, 1993; Irvine & Sorensen, 1993; Cardwell & Stacey, 1995). Therefore, comparison of peripheral sensory events between reproductive and non-reproductive stages may be useful for assessing responses to olfactory sensory stimulation by water that contains reproductive pheromones.

In this study, the hypothesis that reproductive male round gobies release a pheromone that attracts reproductive females was tested. A laboratory experiment was conducted to compare the movement of reproductive and non-reproductive females to water previously occupied by reproductive males (reproductive male water) and control (untreated) water. Chemical characterization of the pheromone was initiated by monitoring EOG activity in male water before and after extraction with solid-phase octadecylsilane (C18) cartridges and then after fractionation on reverse phase high performance liquid chromatography (HPLC).

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS AND SAMPLE COLLECTION

All animal collection, maintenance and experimental procedures were in accordance with the Canadian Council on Animal Care and the Ontario Animals for Research Act guidelines. Round gobies were collected during the spring, summer and autumn of 2002, and the spring and summer of 2003 by angling in the Canadian waters of the upper Detroit River (42°22′ N; 82°59′ W) and by trawling with the Ontario Ministry of Natural Resources in Lake Erie, near Pelee Island (41°51′ N; 82°37′ W) and near Port Dover (42°42′ N; 82°10′ W). Following transport to wet laboratory facilities at the University of Windsor (Department of Biological Sciences), the round gobies were maintained under constant photoperiod (16L : 8D) in holding tanks (205 l) with aerated, 18° C flow-through dechlorinated tap water, gravel and PVC tubing for shelter. All round gobies were fed Nutrafin® fish flakes. Fish were placed in one of four holding tanks designated for reproductive males, non-reproductive males, reproductive females or non-reproductive females. The size range of the reproductive and non-reproductive females used in this study was 75 to 117 mm (total length, L_T, $n = 28$ reproductive females, $n = 37$ non-reproductive females), 85 to 105 mm L_T for non-reproductive males ($n = 20$) and 110 to 235 mm L_T for reproductive males ($n = 20$).

All round gobies were sexed by the shape of their urogenital papilla (Miller, 1984). The reproductive males were identified by secondary sexual characteristics, black nuptial colouration and swollen cheeks (MacInnis & Corkum, 2000). Males lacking these traits were classified as non-reproductive. Following experimental procedures (behavioural analyses, EOG and collection of conditioned water), the animals were euthanized by MS-222 overdose, and the gonado-somatic index (I_G) was measured from $I_G = 100 M_G M^{-1}$, where M_G is the gonad mass and M the total mass of the fish. The mean ± S.E. I_G of 10 reproductive and 10 non-reproductive males was $2{\cdot}21 \pm 0{\cdot}14$ and $0{\cdot}27 \pm 0{\cdot}06$, respectively. There was a significant difference in the I_G between these two groups of males (t-test, d.f. = 18, $P < 0{\cdot}001$). Females with swollen abdomens were classified as reproductive. The mean ± S.E. I_G of reproductive ($n = 26$) and non-reproductive females ($n = 24$) was $11{\cdot}80 \pm 0{\cdot}42$ and $2{\cdot}90 \pm 0{\cdot}28$, respectively. Although there was a significant difference in the I_G for these two groups of females (t-test, d.f. = 47, $P < 0{\cdot}001$), there was no significant difference in the mean L_T between reproductive and non-reproductive females (t-test, d.f. = 47, $P > 0{\cdot}05$). The ovaries of reproductive females contained eggs with mean ± S.E. diameters of $2{\cdot}16 \pm 0{\cdot}14$ mm, values similar to oocyte diameters in histological preparations within 1 week of spawning (Kulikova, 1985). The oocytes contained a prominent germinal vesicle, visible under a dissecting microscope following fixation in Zamboni's fixative (2% paraformaldehyde, 1·5% picric acid, 0·1 M phosphate buffer, pH 7·4) at 4° C, and clearing in an ascending glycerol series (Pankhurst, 1985).

BEHAVIOURAL ASSAY

A laboratory experiment was conducted to determine if reproductive and non-reproductive females responded to odours of water conditioned by reproductive males (reproductive male water). In these experimental tests, a female round goby was added to a tank ($0{\cdot}90 \times 0{\cdot}28 \times 0{\cdot}28$ m) with 20 l of 18° C aerated dechlorinated water. A shelter was provided at one end of the tank for the female and water (either dechlorinated control water or reproductive male water) was added at the opposite end of the tank. To obtain reproductive male water, a reproductive male was held in 1 l of dechlorinated aerated tap water for 4 h. Water (500 ml) with or without male odour was placed in an intravenous bag, secured above the tank. The flow (6 ml min^{-1}) from the bag to the tank was regulated by a valve on a Tygon® delivery tube; outflow was removed from the opposite end of the tank at the same rate. After a female round goby was acclimated to the tank for 30 min, control water was added for 15 min followed by stimulus (control or male) for 30 min. There were four replicates for reproductive females and five replicates for non-reproductive females for reproductive male water and control water. Each fish was used

only once. Fish activity was video-taped using a colour camera (Hitachi Denshi VKC-370). Video-tapes were analysed to determine: 1) the time spent in the far half of the tank near the odour source and 2) the mean velocity of the female moving in the tank. A two dimensional image analysis system (Peak Motus® Version 7.2; Peak Performance Technologies Inc., Centennial, CO, U.S.A.) was used to calculate mean velocity of fish movement. Non-parametric Mann–Whitney *t*-tests were used to compare mean velocity of fish movement between reproductive and non-reproductive females in control and reproductive male water.

ELECTRO-OLFACTOGRAM ASSAY

The EOG assays were performed on female round gobies to examine and measure the olfactory epithelial responses of 10 reproductive and 10 non-reproductive females to water previously occupied by male round gobies. The same number of female round gobies (10 reproductive and 10 non-reproductive females) were used when each one of the 50 HPLC fractions was presented to a female round goby randomly (each fraction tested was sampled twice). The preparation of the round gobies for EOG recording was adapted from Murphy *et al.* (2001). Randomly chosen reproductive or non-reproductive females were lightly anaesthetized using a low dosage (0·05%) of 2-phenoxy-ethanol (Sigma, Oakville, ON, Canada), then injected with Flaxedil (gallamine triethiodide 3 mg kg^{-1} body mass, Sveinsson & Hara, 2000). The test female was placed in a perfusion chamber where a tube, placed in the mouth, continually delivered oxygenated, dechlorinated water. Differential EOG responses were recorded using two Ag/AgCl electrodes (type IB 100F-3; WPI Sarasota, FL, U.S.A.) filled with 3 M KCl and bridged to saline gelatin (8%) filled glass capillaries (tip diameter 100 μm). The reference electrode was placed on the skin near the naris. The recording electrode was placed in the anterior nostril and adjusted to maximize responses to the standard odorant 10^{-5} L-alanine (Sigma, Oakville, ON, Canada). Previous immunocytochemical and ultrastructural analysis have shown abundant ciliated and microvillar olfactory sensory neurons in this location (Belanger *et al.*, 2003). Dechlorinated water was continually perfused through this nostril, and test odours were likewise applied to the posterior nostril. The stimulus duration for odorant application was 3 s unless otherwise indicated. The duration for each EOG experiment was 3 to 7 h. Responses were recorded using a Grass pre-amplifier (7P122P), polygraph (Model 79) and a computer assisted data acquisition and analysis system (Polyview, Version 2.5, Grass-Telefactor, West Warwick, RI, U.S.A.). During the course of a recording, the sensitivity to the amino acid standard, L-alanine (10^{-5} M, Murphy *et al.*, 2001) was monitored every 10 tests to assess the quality of the recording. The EOG recordings during exposure to a 3 s pulse of dechlorinated tap water (background water) that normally irrigated the nostril were made to ensure no mechano-stimulation during odorant delivery. Olfactory adaptation was minimized by allowing 2 min between exposures to test solutions.

WATER CONDITIONED BY MALE CONSPECIFICS

Male round gobies (either reproductive or non-reproductive) were kept individually in glass jars containing 1 l of dechlorinated tap water and an air stone for 4 h. The conditioned water was filtered using Whatman paper (size 4, Whatman Inc., Ann Arbor, MI, U.S.A.) to remove any debris and then stored at −20° C until needed, but not >3 months. The olfactory epithelial response of both reproductive and non-reproductive females was tested using conditioned water from reproductive and non-reproductive males. The magnitude (mV) of the female response to male water and to the standard, L-alanine (10^{-5} M, Sigma, Oakville, ON, Canada), were compared.

SOLID PHASE EXTRACTION OF CONDITIONED WATER

Compounds were sequestered from the male conditioned water onto C-18 solid phase extraction (SPE) cartridges (Waters Corp., Milford, MA, U.S.A.) by passing the

conditioned water (1 l) through the SPE cartridge, followed by elution with methanol (5 ml). This preparation was dried in a rotary evaporator and re-dissolved in methanol (1 ml). The methanol solution was added to 1 l of dechlorinated tap water (to reconstitute the original concentration of conditioned water) and presented over the olfactory epithelium of the female round gobies for EOG recordings. A control stimulus of 1 ml methanol diluted in 1 l of dechlorinated water, did not evoke olfactory epithelial responses. The aqueous filtrate of the male water that was passed through the SPE-cartridge, was also presented over the olfactory epithelium to test for the possibility that the stimulatory molecules from the conditioned water did not bind to the SPE cartridge.

WATER EXTRACT FRACTIONATION ON HPLC

The C-18 SPE cartridge-methanol soluble extracts from 10 reproductive males (50 ml total) were combined and dried down in a rotary evaporator. The powder was reconstituted in 100 µl acetonitrile/water/trifluoroacetic acid (28/72/0·01; v/v/v) and loaded onto an analytical reverse-phase HPLC column (Rainin Dynamax Microsorb; 5 µm octadecylsilane; 4·6 mm × 25 cm; fitted with a 1·5 cm guard module). Two pumps delivered solvents through the column at a rate of 0·5 ml min^{-1}. Solvent A was 0·01% trifluoroacetic acid (TFA) in distilled water and solvent B was 70% acetonitrile and 0·01% TFA in distilled water. The column was developed with a gradient of solvent B from 28 to 100% over 50 min, at a flow rate of 4 ml min^{-1} and monitored with a PDA detector (Waters, Model 996). Fractions of 1 min were collected between 18 and 68 min (the range where steroids typically elute). The HPLC fractions were labelled according to the minute at which they eluted. All HPLC samples were diluted 1 : 100 (100 µl of HPLC sample + 9900 µl of dechlorinated tap water). Each of the 50 samples was randomly selected for application during each EOG run. 'Blank' HPLC fractions were tested to ensure that the solvent used during the extraction and HPLC procedures did not account for the olfactory responses observed. The 'blank' was prepared by passing untreated dechlorinated water through the Sep-pak, methanol elution and HPLC fractionation. Fractions from this blank preparation did not stimulate EOG responses from non-reproductive or reproductive females.

DATA ANALYSIS

The amplitude of each EOG response was measured from baseline to the peak and expressed in mV. For calculation of response magnitude, replicates were averaged and then the values were expressed as a percentage response of the most recently applied L-alanine (10^{-5} M) standard. Comparisons between reproductive and non-reproductive female were analysed using non-parametric (Mann–Whitney) *t*-tests. Hotelling's *t*-test (Winer *et al.*, 1991) was used to determine if there was an overall significant difference in mean EOG response of reproductive females to reproductive and non-reproductive males; each HPLC fraction represented a dependent variable.

RESULTS

BEHAVIOURAL ASSAY

Overall, reproductive females spent more time (701 ± 72 s) than non-reproductive females (439 ± 91 s) in the far half of the tank near the odour source when exposed to reproductive male water (Fig. 1). The reproductive females spent significantly more time near the odour source when exposed to reproductive male water compared with control water (Mann–Whitney *t*-test, $P = 0{\cdot}029$). In contrast, there was no significant difference ($P > 0{\cdot}05$) in the time spent by

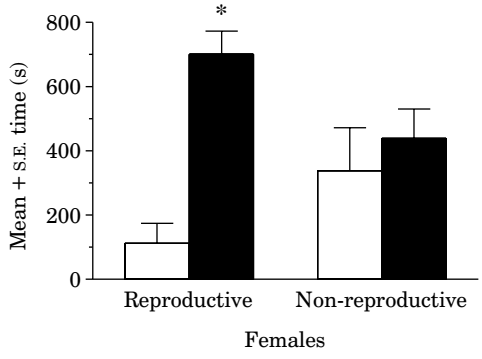

FIG. 1. Mean + S.E. time spent by round goby reproductive and non-reproductive females in response to control water (□) and water conditioned by reproductive males (■). *, a significant difference between the time spent in the far half of the tank near the input source by reproductive females ($n = 4$) when exposed to water conditioned by reproductive males compared with control water (Mann–Whitney t-test, $P = 0{\cdot}029$). There was no significant difference in the time spent near the input source by non-reproductive females ($n = 5$) when exposed to control or reproductive male water.

non-reproductive females near the odour source between reproductive male water and control water (Fig. 1). The reproductive females were significantly more active (swam faster) than non-reproductive females in both control (Mann–Whitney t-test, $P = 0{\cdot}016$) and reproductive male water (Mann–Whitney t-test, $P = 0{\cdot}041$) trials (Fig. 2). During the acclimation period (no inflow) females remained near the shelter. In contrast, females moved throughout the tank during the control and stimulus. The mean swimming speed of reproductive females was significantly higher when exposed to the stimulus (reproductive male water) compared with control treatments (Mann–Whitney t-test, $P = 0{\cdot}029$, Fig. 2).

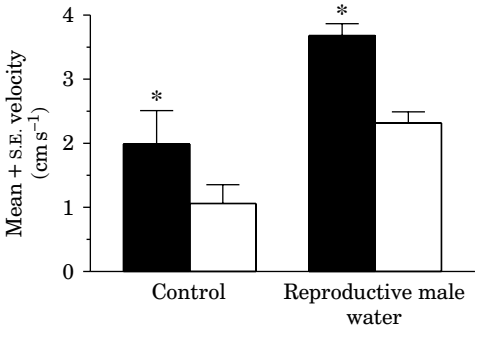

FIG. 2. Mean + S.E. velocity of round goby reproductive females (■) and non-reproductive females (□) during control water and reproductive male water periods in a laboratory tank. *, significant differences in swimming speed between reproductive ($n = 4$) and non-reproductive female ($n = 5$) for control water (Mann–Whitney t-test, $P = 0{\cdot}016$) and reproductive male water (Mann–Whitney t-test, $P = 0{\cdot}041$).

OLFACTORY EPITHELIAL RESPONSES

The largest EOG responses were obtained from reproductive females in response to the application of reproductive male water (Fig. 3). Although, the mean + S.E. olfactory epithelial response by reproductive females to 10^{-5} M L-alanine ($4{\cdot}64 \pm 0{\cdot}64$ mV) was significantly greater than responses by non-reproductive females ($2{\cdot}33 \pm 0{\cdot}35$ mV) (unpaired t-test, d.f. = 30, $P = 0{\cdot}003$), normalization of the EOG values by calculation of relative responses, maintained the reproductive females' elevated response magnitude to reproductive male water. The response magnitude of reproductive females was significantly different between water conditioned by reproductive ($n = 8$) and non-reproductive males ($n = 8$), Mann–Whitney t-test, $P = 0{\cdot}006$ (Fig. 3). No significant differences ($P > 0{\cdot}05$) were detected between these trials by non-reproductive females (Fig. 3).

The methanol eluate of material trapped by C-18 Sep-pak filtration of reproductive male water was stimulatory to the olfactory epithelium of reproductive females, whereas the aqueous filtrate did not have stimulatory properties

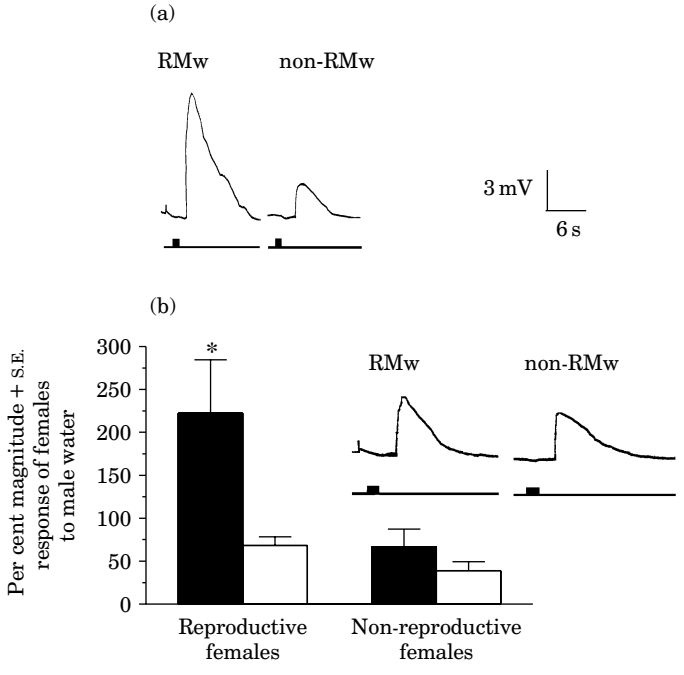

FIG. 3. Male-water evoked responses in round goby females. Representative EOG recordings show responses of (a) reproductive females to reproductive male water (RMw) and non-reproductive male water (non-RMw), and (b) responses of non-reproductive females to RMw and non-RMw. The top tracings show the EOG recordings and bottom lines show the stimulus marker. The small peaks prior to the application of the stimulus in the RMw EOG tracings, show the calibration marker for 1 mV. Per cent magnitude EOG responses of reproductive and non-reproductive female to RMw (■) and non-RMw (□) with reference to the standard L-alanine (10^{-5} M) is also shown. *, a significant difference in mean response of reproductive females exposed to RMw ($n = 8$) compared with non-RMw ($n = 8$) (Mann–Whitney t-test, $P < 0{\cdot}006$). There was no significant difference in the responses by non-reproductive females to RMw ($n = 7$) and non-RMw ($n = 6$).

(Fig. 4). There were significant differences in the olfactory epithelial responses of reproductive females to reproductive and non-reproductive male water (Mann–Whitney *t*-test, $P = 0{\cdot}008$) and to reproductive and non-reproductive male methanol eluate (Mann–Whitney *t*-test, $P = 0{\cdot}008$). These data suggest that organic compounds released by reproductive males evoke strong olfactory epithelial responses in reproductive females.

All of the HPLC fractions of the methanol eluate from material trapped by C-18 Sep-pak filtration of reproductive male water elicited an EOG response from reproductive females (Fig. 5). Reproductive females exhibited significantly higher EOG values than non-reproductive female to HPLC fractions obtained from reproductive male water (Hotelling's *t*-test; $P < 0{\cdot}0001$) [Fig. 5(a)]. Application of the HPLC fractions onto the olfactory epithelium of both reproductive

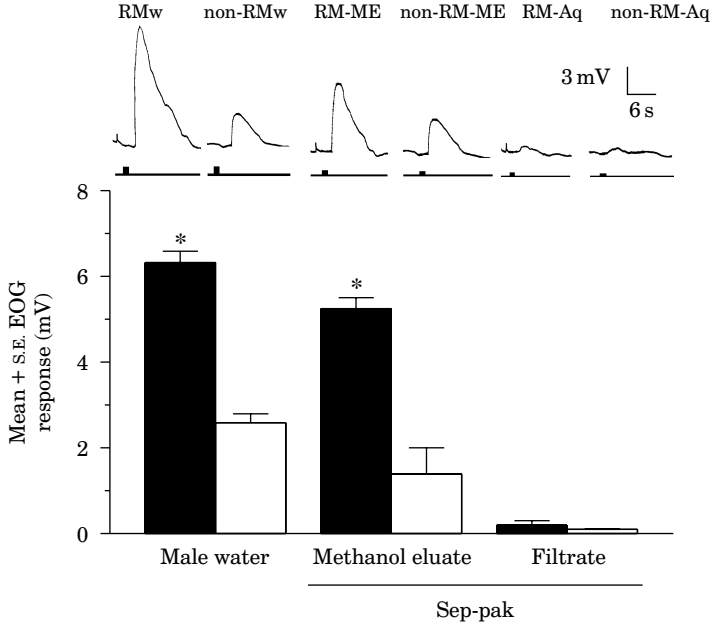

FIG. 4. The effect of C-18 solid phase extraction of round goby male water on olfactory epithelial responses by reproductive females. Representative recordings show olfactory epithelial responses of reproductive females to reproductive male water (RMw), non-reproductive male water (non-RMw), methanol eluate of C-18 solid phase extraction from RMw (RM-ME), methanol eluate of C-18 solid phase extraction from non-RM water (non-RM-ME), aqueous filtrate of C-18 solid phase extraction from RMw (RM-Aq) and aqueous filtrate of C-18 solid phase extraction from non-RMw (non-RM-Aq). The top tracings show the EOG recordings and the bottom lines show the stimulus marker. The small peaks prior to the application of the stimulus, illustrated in the EOG tracings of reproductive male stimuli, show the calibration marker for 1 mV. The mean + S.E. response by reproductive females to male water ($n = 5$), methanol eluate of C-18 solid phase extraction from male water ($n = 5$) and aqueous filtrate of C-18 solid phase extraction from male water ($n = 3$), for RMw (■) and non-RMw (□) is also shown. There were significant differences (*) between the response of reproductive females to males of different reproductive status in both male water (Mann–Whitney *t*-test, $P = 0{\cdot}008$) and methanol eluate of C-18 solid phase extraction from male water (Mann–Whitney *t*-test, $P = 0{\cdot}008$). Females did not distinguish between reproductive and non-reproductive males in the aqueous filtrate.

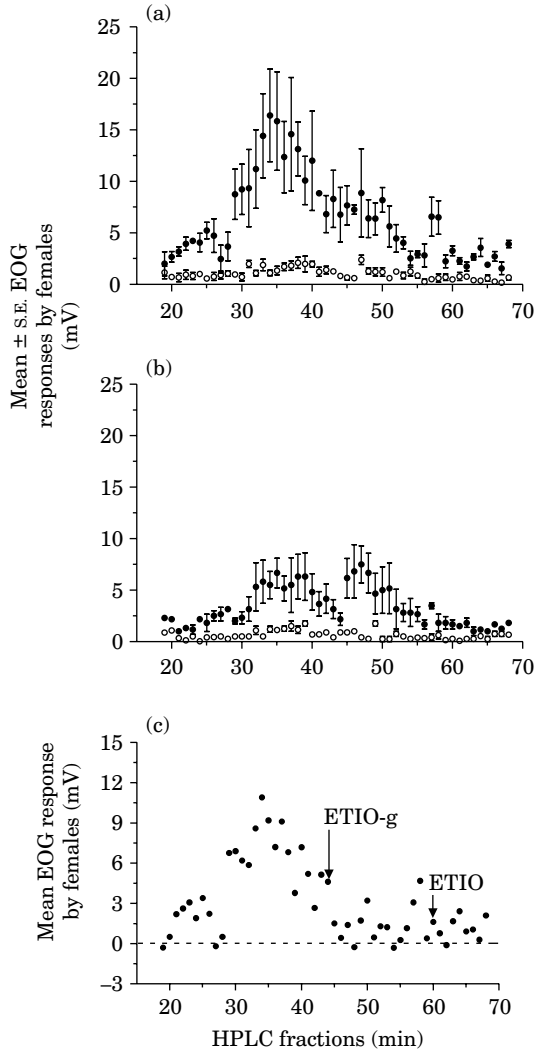

FIG. 5. Olfactory epithelial responses of round goby reproductive (●) and non-reproductive females (○) to HPLC fractions of male water. Mean ± S.E. EOG responses to HPLC fractions of extracted: (a) reproductive (RM) and (b) non-reproductive male (non-RM) water. (c) The difference in mean EOG response of reproductive females between RM and non-RM water. ↓, the expected elution positions of etiocholanolone-glucuronide (ETIO-g) and etiocholanolone (ETIO).

and non-reproductive females revealed lower EOG activity when exposed to non-reproductive male water compared with reproductive male water (Fig. 5). Nevertheless, patterns were consistent in that reproductive females exhibited significantly higher EOG values than non-reproductive females to HPLC fractions obtained from non-reproductive male water (Hotelling's t-test $P < 0{\cdot}0001$) [Fig. 5(b)]. The largest differences in mean EOG activity of reproductive female to reproductive and non-reproductive male came between HPLC fractions 30 and 40 min [Fig. 5(c)].

DISCUSSION

Like other gobiids, reproductive round goby males secure nests to which females are attracted to deposit eggs (Miller, 1984). The nests are located in murky water and dark crevices, yet each male round goby attracts up to 15 females to a nest (MacInnis & Corkum, 2000). Although male round gobies may use a variety of cues (*e.g.* visual, acoustic and olfactory) to attract females to the nest, the present study confirms that reproductive males release odours that elicit an active response which may increase the probability for females to locate then. The reproductive females spent more time than non-reproductive females in the tank near the input source of reproductive male water. Also, reproductive females moved significantly faster than non-reproductive females when exposed to reproductive male water than control water, suggesting that the odour may activate female spawning behaviour. Swimming activity was in general significantly higher in reproductive than in non-reproductive females. Regardless of reproductive status, females were more active when exposed to the reproductive male water than control water. The response of reproductive females to water conditioned by reproductive males in round gobies is similar to the response demonstrated for the preference of reproductive female sea lampreys to spermiating sea lamprey males in a laboratory maze (Li *et al.*, 2002). In a natural habitat, it has been shown that the ovulated female sea lampreys, which display this type of response, have a significantly higher probability to locate spermiating male conspecifics (Siefkes *et al.*, 2003).

The increased olfactory epithelial activity that was observed following stimulation with male water corroborates results from behavioural experiments. The strong responses of reproductive females to reproductive male water, infer strong olfactory sensory neuron generator potentials (Getchell, 1974). The increased olfactory receptor cell activity may stimulate a sequence of neuronal events that lead reproductive females to display increased preference and locomotion responses to the putative male reproductive pheromones, and eventually locate nests with parental males. The unusually large absolute values of the EOG response recorded from reproductive females to stimulation by reproductive male water are surprising, but not unexpected. Similar strong EOG responses were observed in female Mozambique tilapia *Oreochromis mossambicus* (Peters) following stimulation by reproductive male water (Frade *et al.*, 2002).

Unfortunately, the ability of the putative pheromonal compounds to stick to octadecylsilane and to fractionate on HPLC is no proof that they are steroids. Many other types of small organic compounds, including prostaglandins (also shown to be used as pheromones in teleosts, Stacey & Sorensen, 2002), have an equal ability. The evidence that the pheromones are steroids only comes from other studies. For example, when Murphy *et al.* (2001) used EOG recording to examine olfactory responsiveness of round gobies to >100 synthetic steroids and prostaglandins, they found that all the prostaglandins were inactive. They did, however, find 19 steroids that elicited an EOG response. Although they were unable to detect any directed movement in response to any of these EOG-active steroids, Murphy *et al.* (2001) discovered that males markedly increased their gill ventilation rate in response to steroids that had a 5β-reduced A ring (*e.g.* ETIO and ETIO-g). The other line of evidence that the pheromones are

probably steroidal in nature comes from work on the black goby. Colombo *et al.* (1977, 1982) showed that the testes of this species contain unusually high numbers of Leydig cells. These cells are well-known to be involved in the synthesis and secretion of steroids and are concentrated in the region where each testis is suspended from the body wall by lengthwise mesenteries known as mesorchia and hence are termed the 'mesorchial gland' (Colombo & Burighel, 1974). Colombo *et al.* (1977) also showed that the mesorchial gland was capable of transforming radioactive pregnenolone into predominantly conjugated and 5β-reduced steroids. The most prevalent of these was ETIO-g. This steroid was further shown to act as an attractant to gravid females (Colombo *et al.*, 1980). The actual release of this steroid into the water by male black gobies, however, was not demonstrated.

The evidence form the present study suggests that if ETIO-g does have a pheromonal role in the round goby, then the role is likely to be a minor one. The highest amounts of EOG activity were in fractions 30 to 40, while ETIO-g eluted at 44 min.

The HPLC running conditions described in the present paper have been used in several previous studies (Vermeirssen & Scott, 1996; Inbaraj *et al.*, 1997; Moore *et al.*, 2000). In these studies, most of the free steroids expected to be found in fishes (with the exception of cortisol and cortisone, which elute at *c.* 32 and 34 min, respectively) tend to elute in fractions between 40 and 65 min. Most of the conjugated steroids that have been tested, however, (ETIO-g being an exception) tend to elute in fractions between 20 and 40 min. These observations suggest that, if the round goby pheromones are steroidal in nature, then they are likely to be conjugated. As a preliminary step to establishing whether this might or might not be the case, studies have been initiated to determine whether the testis of the male round goby has a similar ability to that of the black goby (Colombo *et al.*, 1977) and the rock goby *Gobius paganellus* L. (Colombo *et al.*, 1970) to synthesize steroids in a conjugated form, and whether any such conjugated steroids might elute in fractions that correspond to the area of high EOG activity.

The approach used in the present study is based upon that pioneered by Li *et al.* (2002). In that particular study, EOG measurements on HPLC fractions of water from reproductive male sea lampreys revealed one dominant and relatively narrow peak of activity that proved an obvious target for analysis and subsequent chemical identification. The rather broad and shallow 'peak' in the present study does not lend itself to the same directed approach. In fact, the broad peak suggests that the reproductive male round goby may use a mixture of compounds, rather than any single compound, as a pheromonal signal. This possibility is reflected by the discovery by Murphy *et al.* (2001) of a large repertoire of synthetic steroidal compounds that serve as odorants to the round goby olfactory epithelium. Such a mechanism would also be consistent with previous findings that mixtures of steroid glucuronides are more effective than single compounds as attractants for male zebrafish *Brachydanio rerio* (Hamilton) (van den Hurk & Lambert, 1983) and female African catfish (Resink *et al.*, 1989).

As mentioned above, Murphy *et al.* (2001) showed that females significantly increased their basal ventilation rate when exposed to 19-carbon synthetic

steroids including ETIO and ETIO-g. The basal ventilation by female round gobies to ETIO was significantly greater in summer than in winter (R. Belanger, unpubl. data), suggesting that females respond more readily to odours during the spawning season. Colombo *et al.* (1980) also showed that only postovulatory female black gobies were induced to move towards ETIO-g that was pumped into a laboratory tank; some females even deposited eggs in the vicinity of the odour source. No response to odour, however, was detected in females after egg deposition (Colombo *et al.*, 1980). Interestingly, parental (*i.e.* nest guarding) black goby males respond to the ejaculate of other parental males, but not to the ejaculate of 'sneaker' males, suggesting that the 'sneaker' male ejaculate is 'pheromonally inconspicuous' (Locatello *et al.*, 2002). 'Sneakers', younger subdominant males, also have undeveloped mesorchial glands, lending further support to the hypothesis that it is probably steroids derived from the mesorchial glands that are used as pheromones by male gobies.

The authors are grateful to J. Green (Department of Chemistry and Biochemistry, University of Windsor) for advice on organic chemistry and for the use of his laboratory facilities, to L. Buchanan (Department of Psychology, University of Windsor) for use of the image analysis system and to S.S. Yun (Department of Fisheries and Wildlife, Michigan State University) for assistance with the HPLC analysis. The technical assistance of C.M. Smith, J. Petruniak, V. Pardalis and K. Yu (University of Windsor) and fish trawling assistance from the Ontario Ministry of Natural Resources staff in Port Dover and Wheatley, is gratefully acknowledged. This research was supported by the Michigan Great Lakes Protection Fund, NSERC and the University of Windsor Faculty of Graduate Studies.

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