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PUTATIVE STEROIDAL PHEROMONES IN THE ROUND GOBY, *Neogobius melanostomus*: OLFACTORY AND BEHAVIORAL RESPONSES

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Abstract—To identify potential hormonal pheromones of the round goby (*Neogobius melanostomus*), a species recently introduced to the Great Lakes, we used electro-olfactogram (EOG) recording to examine olfactory responsiveness to more than 100 steroids and prostaglandins. *Neogobius* detected free and conjugated 18-, 19- and 21-carbon steroids, but did not detect prostaglandins. EOG cross-adaptation, used to determine if *Neogobius* can discriminate the detected compounds at the sensory level, suggested that the detected steroids act on four classes of olfactory receptor mechanisms named (according to the most potent ligand for each): estrone, 17β-estradiol-3β-glucuronide, etiocholanolone, and dehydroepiandrosterone-3-sulfate. Although none of the detected steroids induced reproductive behaviors, exposure to steroids from three of the four receptor classes (estrone, 17β-estradiol-3β-glucuronide, or etiocholanolone) increased ventilation rate in males, whereas only etiocholanolone increased ventilation rate in females. Using the ventilation increase as a behavioral bioassay of steroid detection, behavioral cross-adaptation studies in males demonstrated that steroids discriminated at the sensory level are also discriminated behaviorally. These findings suggest the round goby may use steroids as putative pheromones.

Key Words—Round goby, *Neogobius melanostomus*, sex pheromones, steroid, electro-olfactogram, cross-adaptation, ventilation, sexual dimorphism.

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INTRODUCTION

Water-borne steroids, prostaglandins, and their metabolites are potent stimulators of reproductive physiology and behavior in a number of fish species (Sorensen and Caprio, 1998; Sorensen and Stacey, 1999). Moreover, electro-olfactogram (EOG) recording studies (e.g., Sorensen et al., 1995), which seek evidence for hormonal pheromones by examining olfactory response to numerous steroid and prostaglandin compounds, indicate that these fish pheromones are widespread among freshwater fishes and are present in some euryhaline marine forms (Stacey and Cardwell, 1995, 1997). The majority of studies on fish hormonal pheromones have focused on ostariophysin species from the orders Cypriniformes, e.g., goldfish, *Carassius auratus* (Sorensen and Stacey, 1999); common carp, *Cyprinus carpio* (Irvine and Sorensen, 1993; Stacey et al., 1994a); tinfoil barb, *Puntius schwanenfeldi* (Cardwell et al., 1995); loach, *Misgurnus anguillicaudatus* (Kitamura et al., 1994), and Siluriformes, e.g., African catfish, *Clarias gariepinus* (van den Hurk and Resink, 1992), but there also is evidence for hormonal pheromones in several Salmoniformes, e.g., Atlantic salmon, *Salmo salar* (Waring and Moore, 1997); arctic char, *Salvelinus alpinus* (Sveinsson and Hara, 1995); brook trout, *Salvelinus fontinalis*, and brown trout, *Salmo trutta* (Essington and Sorensen, 1996). In contrast, with the exception of the pioneering work of Colombo et al. (1977, 1982) in the black goby (*Gobius jozo*), reports of pheromonal androgens in the urine of male yellowfin Baikal sculpins (*Cottocomephorus grewingki*) (Katsel et al., 1992), a brief description of EOG responsiveness to steroids in a cichlid (*Haplochromis burtoni*) (Robison et al., 1998), and indications that preovulatory Eurasian ruffe (*Gymnocephalus cernuus*) release a novel conjugated steroid with behavioral actions on males (Murphy et al., 2000), nothing is known of hormonal pheromone systems in perciform fishes, a diverse and ecologically important group both in freshwater and marine ecosystems.

In this study, we report the results of EOG and behavioral studies indicating the use of steroidal pheromones by the round goby, *Neogobius melanostomus*, a native of the Caspian and Black seas, which recently has been introduced to the Great Lakes in ship ballast (Crossman et al., 1992; Jude et al., 1992). The objectives of our study were twofold. First, we wished to increase the understanding of perciform pheromones by focusing on a species from a diverse and widely distributed perciform group (Family Gobiidae) that contains many marine taxa and that is suitable for laboratory-based reproductive studies (Moiseyeva and Rudenko, 1976). Second, we hoped that an understanding of hormonal pheromones of *Neogobius* might suggest methods for control of this species in the Great Lakes.

Spawning in *Neogobius* appears to be typical of many gobiids (Miller, 1984). Males migrate from deeper waters to spawning areas in the spring, estab-

lish territories prior to arrival of females, defend a nest site to which females are attracted for spawning, and care for single or multiple batches of eggs (Moiseyeva and Rudenko, 1976; MacInnis and Corkum, 2000). As with other male gobiids [e.g., *Bathygobius soporator* (Tavolga, 1956)], male round gobies use visual displays (coloration changes and posturing) and acoustical signals when courting females (Protosov et al., 1965; Moiseyeva and Rudenko, 1976). In particular, evidence that pheromones play important reproductive roles in other gobies (Tavolga, 1956) and that a conjugated steroid, 5β-androstan-3α-ol-17-one-3-glucuronide (etiocholanolone-glucuronide; ETIO-g), has pheromonal function in *G. jozo* (Colombo et al., 1980, 1982) suggested that, as in many other fish (Stacey and Cardwell, 1995, 1997; Sorensen and Stacey, 1999), EOG studies would be useful in determining if *Neogobius* detects hormonal pheromones.

The round goby has established thriving populations in all of the Great Lakes and might invade the Mississippi River drainage system, with potentially detrimental effects on many North American native fish species and ecosystems (Charlebois et al., 1997). Great Lakes *Neogobius* are of concern because there is evidence they have contributed to population declines of native mottled sculpin (*Cottus bairdi*) and other benthic fishes (Dubs and Corkum, 1996). Round gobies displace native benthic species from optimal spawning habitats, eat their eggs and young, and have a relatively high fecundity since they spawn a number of times in a season (Dubs and Corkum, 1996; Charlebois et al., 1997; MacInnis and Corkum, 2000). Additionally, because round gobies eat zebra mussels (*Dreissena polymorpha*), which can accumulate polychlorinated biphenyls and in turn are preyed upon by sport fish, they might facilitate introduction of toxins into Great Lakes food webs (Ray and Corkum, 1997). Understanding sex pheromones of the round goby could lead to techniques for population monitoring or disruption of spawning.

METHODS AND MATERIALS

Experimental Fish. Sexually mature *Neogobius* were collected between May and August, 1994–1996 by angling and bottom trawl from the St. Clair and Detroit Rivers (Windsor, Ontario, Canada), flown to Edmonton within three days of capture, and maintained in mixed-sex groups (four to six fish) under constant photoperiod (16L : 8D). Fish were held in 70-liter flow-through aquaria supplied with dechlorinated tap water that varied seasonally from 9 to 18°C. Aquaria contained gravel, an air stone, floating artificial plants, and clay flower pots and PVC pipes for shelter. A variety of live, frozen and flake food was provided ad libitum.

Fish were sexed by the shape of the urogenital papilla: the females have a broad, truncated tip with a large pore, and that of the male is long and slen-

der with a minute opening. Both males and females used in the EOG recording and behavior experiments were caught in Windsor in breeding condition. Most females had distended abdomens at capture and discharged eggs into aquaria within two weeks of captivity. Males kept in mixed sex groups periodically exhibited courtship behavior (color changes and territoriality), and occasionally fertilized eggs were found on the ceiling of the PVC tubes, indicating that holding conditions did not inhibit reproductive behavior. Only larger fish (>8 cm in length) were used in both EOG and behavior experiments. All experimental procedures involving fish were in accordance with Canadian Council of Animal Care guidelines.

Electro-olfactogram (EOG) Recording Technique. Sexually mature male and female *Neogobius* were tested for olfactory sensitivity to a variety of odors using EOG recording procedures similar to those described by Cardwell et al. (1995). Larger males and females (>8.0 cm in length) were chosen because it was too difficult to obtain a recording from smaller fish; the incurrent pore was too small for the size of electrodes used. Larger fish were sexually mature at the time of capture (see above) but may have undergone some gonadal recrudescence while maintained in the laboratory. Immediately prior to recording, fish were anesthetized by orally perfusing the gills with dechlorinated tap water containing 0.05% 2-phenoxyethanol (2-PE; Sigma, St. Louis, Missouri), wrapped in wet tissue and secured to a stand placed in an electrically grounded water bath. Fish were fitted with a polyethylene mouth tube that delivered aerated, dechlorinated water (containing 0.05% 2-PE) throughout the entire recording procedure. The temperature of the water bath and the anesthetic water (9–18°C) approximated that of the holding aquaria at the time of testing.

A glass capillary tube (70- to 120-μm tip diameter) filled with gelatin (8% in 0.6% NaCl) bridged Ag–AgCl electrodes filled with 3 M KCl to the olfactory tissue. The tip of the reference electrode was placed in the water bath. The odor delivery tube was positioned next to the excurrent pore of one naris, creating a reversed water flow (in the excurrent pore and out the incurrent pore); initial recordings failed to generate any EOG response if the odor tube was placed near the incurrent pore and the recording electrode was placed in the excurrent pore. In an attempt to facilitate recording, the naris was dissected to expose olfactory receptor cells. However, we were unable to obtain any measurable EOG response from the exposed naris; therefore the naris was left intact for all recordings.

Throughout a recording session (maximum duration of 3 hr), the naris was continuously perfused with dechlorinated tap water (background) until a computer-controlled solenoid switched the background solution to a test solution for 2 sec. The amplified signals (Grass P-18 DC amplifier) were digitized (National Instruments Lab-PC A/D converter), and the response was recorded for 10 sec from initiation of the odor pulse. After recording was completed, the gills were perfused with tap water without anesthetic until the fish began venti-

lating and could be returned to its aquarium. A number of fish were retested in a maximum of two additional EOG recording sessions (allowing at least one week between sessions), each of which was for a different cross-adaptation experiment (see EOG Cross-Adaptation below). There was no evidence that the EOG response was affected by prior recordings, insofar as response magnitudes remained equivalent on subsequent tests.

When an anesthetized fish had been mounted in the water bath for 30–45 min, recording began by determining olfactory response to 10^{-5} M L-alanine. If the response to L-alanine was greater than 3 mV (prestimulus voltage to peak stimulus voltage), testing with hormonal compounds was initiated. If the response was less than 3 mV, the recording electrode and/or odor tube were repositioned until a minimum 3-mV response was obtained or the recording was terminated and the fish recovered.

During the course of a recording, the sensitivity by 10^{-5} M L-alanine was monitored frequently to ensure the stability of the recording. As well, to determine if mechanical artifacts contributed to the EOG recording, the naris was exposed occasionally to 2-sec pulses of the same dechlorinated tap water (background water) that chronically irrigated the naris and was used for dilution of test odors (see Tested Odors below). Data (base to peak voltage differences) are presented as an absolute response in mV with response to background water (if any) subtracted. Sensory adaptation during recording was minimized by using a short exposure time (2 sec) and allowing 1–2 min between exposures to test solutions.

Tested Odors. The olfactory epithelium of *Neogobius* was exposed to 10^{-8} M solutions of eight prostaglandins (prostaglandins E_1, E_2, $F_{1\alpha}$, $F_{2\alpha}$, and $F_{3\alpha}$; 15-keto-prostaglandin E_2; 15-keto-prostaglandin $F_{2\alpha}$; 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$) and 114 steroids (Table 1) to determine compounds that consistently induced EOG response. Steroids and prostaglandins selected for testing included all those reported to be olfactory stimulants or to have pheromonal activity in other fish (Stacey and Cardwell, 1995; Sorensen and Stacey, 1999), as well as a number of related compounds, metabolites, and conjugates. Steroids and steroid conjugates were purchased from Sigma (St. Louis, Missouri) and Steraloids (Newport, Rhode Island) or received as gifts from Dr. A. P. Scott (Lowestoft), and Dr. J. G. D. Lambert (Utrecht). Prostaglandins were purchased from Cayman Chemical Company (Ann Arbor, Michigan). Because, in initial tests, prostaglandins failed to induce an EOG response in any fish, they will not be considered further in this section.

The 10^{-5} M L-alanine (Sigma, St. Louis, Missouri) used to monitor EOG recording preparations was prepared as a 10^{-2} M stock solution in double-distilled deionized water, stored in glass scintillation vials at 4°C and diluted in background water at the time of EOG recording. Olfactory response to 10^{-6} M L-alanine was also recorded, as this concentration was used in behavior tests.

TABLE 1. STEROIDS TESTED IN EOG RECORDING, GROUPED ACCORDING TO STRUCTURAL SIMILARITY AND INCLUDING ABBREVIATIONS

5α-Androstan (unconjugated): 5α-androstan-3,17-dione; 5α-androstan-3β-ol-17-one (epiandrosterone; EPIANDR); 5α-androstan-17β-ol-3-one.

5α-Androstan (conjugated): 5α-androstan-3β-ol-17-one-3β-glucuronide (epiandrosterone-glucuronide; EPIANDR-g); 5α-androstan-3α-ol-17-one-3α-SO_4 (androsterone-sulfate; ANDR-s).

5β-Androstan (unconjugated): 5β-androstan-3,17-dione (etiocholan-3,17-dione; ETIO-3,17-dione); 5β-androstan-3α-ol-17-one (etiocholanolone; ETIO); 5β-androstan-3β-ol-17-one.

5β-Androstan (conjugated): 5β-androstan-3α,17β-diol-3α-glucuronide; 5β-androstan-3α,17β-diol-17β-glucuronide; 5β-androstan-3α,11β-diol-17-one-3α-glucuronide (etiocholanolone-glucuronide; 11β-ETIO-g); 5β-androstan-3α-ol-17-one-3α-glucuronide (etiocholanolone-glucuronide; ETIO-g)

4-Androsten (unconjugated): 4-androsten-11β,17β-diol-3-one; 4-androsten-3,17-dione (androstenedione; AD); 4-androsten-11-keto-17β-ol-3-one (11-ketotestosterone); 4-androsten-11-keto-3,17-dione; 4-androsten-11α-ol-3,17-dione; 4-androsten-11β-ol-3,17-dione; 4-androsten-17α-ol-3-one; 4-androsten-17β-ol-3-one (testosterone; T).

4-Androstene (conjugated): 4-androsten-17β-ol-3-one-17β-glucuronide (testosterone glucuronide); 4-androsten-17β-ol-3-one-17β-SO_4 (testosterone sulfate).

5-Androsten (unconjugated): 5-androstene-3β,17β-diol; 5-androstene-3β-ol-17-one (dehydroepiandrosterone; DHEA).

5-Androsten (conjugated): 5-androstene-3β-ol-17-one-3β-glucuronide (dehydroepiandro sterone-glucuronide; DHEA-g); 5-androstene-3β-ol-17-one-3β-SO_4 (dehydroepiandrosterone-sulfate; DHEA-s).

Estratriens (unconjugated): 1,3,5(10)-estratrien-3α,17α-diol (17α-estradiol); 1,3,5 (10)-estratrien-3α,17β-diol (17β-estradiol; E2); 1,3,5 (10)-estratrien-3α-ol-17-one (estrone; E1); 1,3,5 (10)-estratrien-3α,16α,17β-triol (estriol; E3).

Estratriens (conjugated): 1,3,5 (10)-estratrien-3α,17β-diol-3α-glucuronide (17β-estradiol-3α-glucuronide; E2-3g); 1,3,5 (10)-estratrien-3α,17β-diol-17β-glucuronide (17β-estradiol-17β-glucuronide; E2-17g); 1,3,5 (10)-estratrien-3α,17β-diol-3α-SO_4; 1,3,5 (10)-estratrien-3α,17β-diol-17β-SO_4); 1,3,5 (10)-estratrien-3α,17β-diol-di-SO_4; 1,3,5 (10)-estratrien-3α-glucuronide-17β-SO_4; 1,3,5 (10)-estratrien-3α-SO_4-17β-glucuronide

Estrens (unconjugated): 4-estren-17β-ol-3-one

5α-Pregnan (unconjugated): 5α-pregnan-3,20-dione; 5α-pregnan-3β,17α-diol-20-one; 5α-pregnan-3β,20α-diol; 5α-pregnan-3β,20β-diol; 5α-pregnan-17,21-diol-3,20-dione; 5α-pregnan-17-α-ol-3,20-dione; 5α-pregnan-3β-ol-20-one; 5α-pregnan-3β,11β,17α,21-tetrol-20-one; 5α-pregnan-3α,17α,20β-triol; 5α-pregnan-3β,17α,20β-triol; 5α-pregnan-11β,17,21-triol-3,20-dione; 5α-pregnan-3α,17α,21-triol-20-one; 5α-pregnan-3β,17α,21-triol-20-one

5β-Pregnan (unconjugated): 5β-pregnan-3α,20β-diol (3α,20β-5βP); 5β-pregnan-3β,20β-diol; 5β-pregnan-17α,21-diol-3,20-dione (3β,20β-5βP); 5β-pregnan-3α,17α-diol-20-one (3α,17α-5βP); 5β-pregnan-3β,17α-diol-20-one; 5β-pregnan-3,20-dione (5βP); 5β-pregnan-17α-ol-3,20-dione; 5β-pregnan-3α,11β,17α,21-pentol; 5β-pregnan-3α,11β,17α,20β,21-pentol; 5β-pregnan-3α,17α,20α,21-tetrol; 5β-pregnan-3α,17α,20β,21-tetrol; 5β-pregnan-3α,11β,17α,21-tetrol-20-one; 5β-pregnan-3α,17α,20β,21-tetrol-11-one; 5β-pregnan-3α,17α,20α-triol; 5β-pregnan-3α,17α,20β-triol (3α,17α,20β-5βP); 5β-pregnan-3α,17α,21-triol-11,20-dione; 5β-pregnan-11β,17α,21-triol-3,20-dione; 5β-pregnan-3α,17α,21-triol-20-one; 5β-pregnan-3β,17α,21-triol-20-one

TABLE 1. CONTINUED

5β-Pregnan (conjugated): 5β-pregnan-3α,17α-diol-20-one-3α-glucuronide (3α,17α-5βP-3g)
4-Pregnen (unconjugated): 4-pregnen-11β,17α-diol-3,20-dione; 4-pregnen-11β,21-diol-3,20-dione; 4-pregnen-16α,17β-diol-3,20-dione; 4-pregnen-17α,21-diol-3,20-dione; 4-pregnen-17α,20α-diol-3-one; 4-pregnen-17α,20β-diol-3-one; 4-pregnen-20β,21-diol-3-one; 4-pregnen-17α,21-diol-3,11,20-trione; 4-pregnen-3,20-dione (progesterone); 4-pregnen-11α-ol-3,20-dione; 4-pregnen-11β-01,-3,20-dione; 4-pregnen-17α,-ol-3,20-dione; 4-pregnen-21-ol-3,20-dione; 4-pregnen-20α-ol-3-one; 4-pregnen-20β-ol-3-one; 4-pregnen-11β,17α,20β,21-tetrol-3-one; 4-pregnen-11β,17α,21-triol-3,20-dione; 4-pregnen-11β,17α,21-triol-3,20-dione (cortisol); 4-pregnen-14α,17β,21-triol-3,20-dione; 4-pregnen-17α,20β,21-triol-3,11-dione; 4-pregnen-17α,20α,21-triol-3-one; 4-pregnen-17α,20β,21-triol-3-one; 4-pregnen-3,11,20-trione
4-Pregnen (conjugated): 4-pregnen-11β,21-diol-3,20-dione-21-SO_4; 4-pregnen-17α,21-diol-3,20-dione-21-SO_4; 4-pregnen-17α,20β-diol-3-one-20β-glucuronide; 4-pregnen-17α,20α-diol-3-one-20α-SO_4; 4-pregnen-17α,20β-diol-3-one-20β-SO_4; 4-pregnen-21-ol-3,20-dione-21-glucuronide; 4-pregnen-20β-ol-3-one-20β-SO_4; 4-pregnen-11β,17α,21-triol-3,20-dione-21-glucuronide; 4-pregnen-11β,17α,21-triol-3,20-dione-21-SO_4; 4-pregnen-17α,20β,21-triol-3-one-21-SO_4; 4-pregnen-17α,20β-diol-3-one-20β-PO_4
5-Pregnen (unconjugated): 5-pregnen-3β,17α-diol-20-one; 5-pregnen-3β,21-diol-20-one; 5-pregnan-3β-ol-20-one (pregnenolone); 5-pregnen-3β,17α,21-triol; 5-pregnen-3β,17α,21-triol-20-one
5-Pregnen (conjugated): 5-pregnen-3β,21-diol-20-one-3β-SO_4; 5-pregnen-3β,21-diol-20-one-21-SO_4; 5-pregnen-3β-ol-20-one-3β-glucuronide; 5-pregnen-3β-ol-20-one-3β-SO_4

All other test odors were first prepared as 10^{-3} M stock solutions in 99% ethanol (free and sulfated steroids, prostaglandins) or 50 : 50 ethanol–water (glucuronated steroids), and stored at −20°C. Working solutions (10^{-6} M) were prepared in duplicate by diluting stock solutions in double-distilled, deionized water and stored in glass scintillation vials at 4°C. Test solutions were prepared immediately before recording by diluting appropriate amounts of the working solutions in 100 ml of background water (Cardwell et al., 1995). Ethanol control solutions were prepared in the same manner but contained no steroid or prostaglandin.

In initial recordings, five gobies (two males, three females) were exposed to 10^{-8} M test solutions of all the compounds listed above. Compounds that did not elicit a response from any fish were considered undetectable and were not tested again. A compound was considered detectable if, in more than one fish, it induced a voltage change greater than that induced by the background solution. Additionally, to ensure that a response to a compound was not due to contamination of the test solution, each detected steroid was tested on the same fish with a new test solution. Following these intial recordings, only detected steroids were used in further studies. After additional fish were tested with detectable steroids, male and female EOG responses to detected steroids were compared (*t*-test) (Wilkinson, 1994).

EOG Concentration–Response Studies. To compare olfactory potency of detected steroids, concentration–response relationships were determined for six detected steroids (estrone, E1; estradiol-3-glucuronide, E2-3g; etiocholan-3α-ol-17-one, ETIO; androsterone-sulfate, ANDR-s; dehydroepiandrosterone-sulfate, DHEA-s; dehydroepiandrosterone-glucuronide, DHEA-g) that, at 10^{-8} M, elicited consistent and large responses (greater than 20% of the response to 10^{-5} M L-alanine) and were thought likely to operate through separate olfactory receptor mechanisms. Criteria for determining compounds that may act through separate olfactory receptor mechanisms were arbitrary and based on studies in goldfish showing that 19- and 21-carbon steroids act through different receptor mechanisms and that some free and conjugated steroids also act through separate mechanisms (Sorensen et al., 1995). The concentration–response relationship of ETIO-g also was determined because, even though at 10^{-8} M it induced an EOG response less than 20% of that induced by 10^{-5} M L-alanine, it has been proposed to have pheromonal function in *G. jozo* (Colombo et al., 1980, 1982).

Concentration–response tests began at 10^{-12} M and increased by log molar increments to 10^{-8} M, with 10-min intervals between each exposure. Concentration–response tests for each selected steroid were performed on six fish (four females, two males). Response magnitudes (millivolts) were analyzed by ANOVA and Tukey multiple comparison test ($\alpha = 0.05$) (Wilkinson, 1994).

EOG Cross-Adaptation Studies. These studies compared the EOG response to a test compound before and during adaptation to an adapting compound, under the assumption that, if the EOG response to the test compound is unaffected by adaptation, it is acting through an olfactory receptor mechanism separate from that mediating response to the adapting compound (e.g., Caprio and Byrd, 1984; Sorensen et al., 1995). The design of the standard cross-adaptation procedure makes the assumption that a brief exposure to an odor will not influence EOG response to that odor when presented a short time later. However, interpretation of a cross-adaptation experiment will be confounded if a tested odor induces a smaller response on the second presentation, even in the absence of a tonically delivered adapting odor. The extent of this phenomenon in *Neogobius* was evaluated by a sequential exposure test.

Six fish (four females, two males) were exposed sequentially to 2-sec 10^{-8} M pulses of all steroids used for the cross-adaptation studies, and then retested with 2-sec 10^{-8} M pulses of the same steroids 30 min later: response magnitude to 10^{-5} L-alanine remained constant throughout. Differences between response magnitudes induced by the first and second exposures were analyzed by Wilcoxon matched-pairs test (Wilkinson, 1994). As well, for each steroid, the magnitude of the EOG response induced by the second exposure was converted to a percentage of the response induced by the first exposure (% IR = percentage of initial response), and the mean % IR used in analysis of the cross-adaptation experiment (see below). Steroids that failed to induce a second response in this

sequential exposure test were eliminated from all cross-adaptation studies (see Results).

Our cross-adaptation procedure was similar to that described by Sorensen et al. (1995). The naris first was exposed sequentially to 2-sec 10^{-8} M solutions of all of the detected compounds to establish initial preadaptation responses. Then, during adaptation to a 10^{-7} M solution of the adapting compound, the 10^{-8} M test steroids were administered again and in the same sequence. Test steroids were used at 10^{-8} M because many of them did not induce an EOG response at lower concentrations.

Five adapting steroids (E1, E2-3g, DHEA-g, ETIO, and DHEA-s) were chosen based on structure and detection threshold and on the results of preliminary cross-adaptation experiments (data not shown) that indicated which compounds operate through separate olfactory receptor mechanisms. Cross-adaptation experiments for each adapting steroid were conducted on six fish (four females, two males).

To monitor the quality of the EOG recording during the cross-adaptation procedure, the naris was exposed to 10^{-5} M L-alanine at the beginning and end of each cross-adaptation test, and after every fourth steroid exposure. As it was difficult to maintain a recording on *Neogobius* for the time required for a cross-adaptation experiment (>3 hr), only results of cross-adaptation tests in which L-alanine responses remained relatively stable throughout the recordings (approximately 50% of the tests) were used for analysis and presentation.

To reduce the potential for adaptation during the preadaptation testing, the compounds known to have the smallest response magnitudes were tested first. As well, compounds of similar structure were not tested in sequence to reduce the possibility of adaptation by sequential exposure to compounds that might operate through the same receptor mechanism. Some steroids that induced small responses at 10^{-8} M in initial testing (see Detected Odors in Results) failed to induce a response in the pretest of the cross-adaptation, and therefore, were not tested during the cross-adaptation phase.

Two liters of 10^{-7} M adapting steroid solution were prepared immediately before beginning cross-adaptation pretesting, held in a running bath of background water to maintain temperature, and used both to adapt the naris and to prepare dilutions of test steroids for cross-adaptation testing. Immediately following preadaptation application of 2-sec pulses of all the test steroids, the naris was exposed to the adapting steroid until the induced response reached a stable plateau (usually within 3 min) and then exposed to 2-sec pulses of the test steroids dissolved in adapting solution. The magnitude of the response induced during adaptation was converted to %IR and, for each steroid, the difference between %IR in cross-adaptation and %IR in the sequential exposure test was analyzed by Mann-Whitney *U* test.

Odor-Induced Ventilation Increase. Individual male or nonovulated female

gobies displayed no overt reproductive response to bolus addition of single detected steroids (final water concentration 10^{-8} M). However, a number of detected steroids did induce a transitory increase in ventilation frequency (opercular movements per minute) that formed the basis of a consistent and quantitative behavioral bioassay. Although the biological significance of odor-induced ventilation increases in *Neogobius* is unclear, the fact that in some benthic fish water flow through the naris changes in synchrony with ventilation (Nevitt, 1991) suggests increased ventilation might facilitate odor detection or processing.

Ventilation frequency was monitored in aerated 100-liter, flow-through test aquaria (90 cm long × 30 cm wide × 38 cm high) that were opaque on the ends and one side, with one side left clear for observation; a gravel substrate was provided and one PVC tube (12 cm length; 6 cm diameter) was placed with one end facing the observation side. Fish usually remained within the PVC tube, enabling ventilation frequency to be monitored readily. To minimize visual disturbance during observations, fish were observed through a small hole cut in an opaque cloth curtain that blocked the fish's view of the experimenter. Test odors and control solutions were injected into the test aquaria (at the water surface directly over the PVC tube) through silastic tubing fed through a hole in the cloth curtain and anchored to each aquarium. Injections of dye showed that an odor plume should reach the PVC tube within one min and disperse evenly throughout the aquarium within 3 min.

A single male or nonovulated female (>8 cm in length) was placed in each test aquarium at least five days before testing and exposed to different odors on consecutive days. On the morning of each test day, water flow was turned off 2 hr before an experiment, the fish was tested with only one odor, and water flow was resumed. In each test, a fish was observed for 12 min prior to odor addition (pre-exposure period), a test solution was then injected, and the fish was observed for an additional 24 min (test period). Ventilations were counted continuously for 1 min during every 3-min interval before and after addition of an odor, yielding 4 min of pre-exposure ventilation and 8 min of test ventilation. Six males and six females were tested with each odor.

Ventilation frequency of individual males and females was monitored in response to addition of ethanol vehicle (1 ml), eight steroids that induced an EOG response (E1, E2, E2-3g, ETIO, ETIO-g, DHEA-s, DHEA-g, and ANDR-s), one steroid that did not induce an EOG response (testosterone-glucuronide; T-g), and L-alanine. Steroid odors were tested either individually or in combinations by injecting either 100 μl or 1 ml of a 10^{-3} M steroid-ethanol solution to create 10^{-9} and 10^{-8} M concentrations after dispersal. L-Alanine (10 ml of a 10^{-2} M aqueous solution) was added to create a 10^{-6} M final concentration.

To determine if basal (pre-exposure) ventilation of males and females differed, pre-exposure ventilation frequencies of males and females were compared by unpaired *t*-test (Wilkinson, 1994), using for each fish the grand mean

of pre-exposure frequencies from all odor tests. Ventilation frequency differences between pre-exposure and test periods were analyzed separately by sex and odor, comparing the mean (per minute) frequency of the four pre-exposure samples and the mean of the frequencies observed during minutes 4 and 7 of the test period (Wilcoxon test) (Wilkinson, 1994). All ventilation data from the test period are presented as a percentage of the mean basal (pre-exposure) ventilation frequency.

Ventilation Concentration–Response Studies. Concentration–response relationships were examined for those steroids (E1, E2-3g, and ETIO) that increased ventilation frequency of male fish when tested at 10^{-8} M. The protocol was identical to that already described, except that the final concentrations in the test aquaria ranged from 10^{-12} M to 10^{-8} M and were prepared by diluting appropriate volumes of 10^{-3} M steroid solutions into 100 ml of double-distilled, deionized water, and then adding 1 ml of this solution to the test aquarium. Testing of each steroid began at 10^{-12} M, and increased in log molar increments on consecutive days. Six males were tested, all receiving the same treatment each day.

For each steroid, differences in test ventilation frequency due to odor concentration were analyzed by Friedman's nonparametric ANOVA (Wilkinson, 1994), using for each fish the increase in ventilation during the test period (mean of minutes 4 and 7 after odor addition). Dunnett's test (Zar, 1984) was used to determine threshold by comparing the response (mean of minutes 4 and 7 after odor addition) of the lowest concentration tested (10^{-12} M) to each of the higher concentrations.

Behavioral Cross-Adaptation Studies. Behavioral cross-adaptation using ventilation as the behavioral response was conducted to determine if *Neogobius* can behaviorally discriminate among steroids that might act through separate olfactory receptor mechanisms, as indicated by the results of our EOG cross-adaptation studies (see EOG Cross-Adaptation Studies in Results). Unlike EOG cross-adaptation, behavioral cross-adaptation did not involve addition of the test odor prior to applying the adapting odor, because there was no means for rapidly removing the test odor prior to applying the adapting odor. Thus, the purpose of the behavioral cross-adaptation experiment was to determine if adaptation eliminates the ventilatory response to the test steroid, rather than to compare responses to a test steroid prior to and during adaptation.

In behavioral cross-adaptation, a fish was observed for a 12-min pre-exposure period, immediately exposed to an adapting steroid (either 10^{-8} M E1 or ETIO), observed for a 24-min adapting period, immediately exposed to a test steroid (10^{-9} M), and observed for a further 24-min test period. Ventilation frequency was monitored continuously every third minute; six male fish were tested in each treatment group. The adapting steroid was expected to increase ventilation, whereas the test steroid was expected to increase ventilation only if it acts

through a different receptor mechanism than the adapting steroid (Murphy and Stacey, 1999).

Differences in ventilation frequencies in the pre-exposure, adapting, and test periods were analyzed by Friedman's nonparametric ANOVA (Wilkinson, 1994) and pairwise differences were analyzed by Dunnett's test (Zar, 1984) using mean frequencies for the pre-exposure period and for minutes 4 and 7 of the adapting and test periods.

RESULTS

Response to Test Odors. L-Alanine consistently induced an EOG response in *Neogobius* both at 10^{-5} M (Figure 1) and 10^{-6} M (mean ± SEM = 3.1 ± 0.6 mV; $N = 8$). Response magnitude to 10^{-5} M L-alanine was similar ($P > 0.05$; t test) in males and females (Figure 1). There were no significant mechanical artifacts in the EOG recordings, responses to background water being <0.1 mV in all cases. Neither the ethanol solvent in 10^{-8} M test solutions (up to 0.001%) nor any of the eight tested prostaglandins induced EOG responses in any fish. However, of 114 tested steroids (Table 1), 19 met the criterion of detection by inducing an EOG response >0.1 mV in more than one fish (Figure 1). Because there was no indication that EOG responses of males and females differed in magnitude (Figure 1), EOG results from both genders were pooled in concentration–response and cross-adaptation studies.

At a concentration of 10^{-8} M (the highest concentration tested), a diverse array of conjugated and unconjugated 18-, 19-, and 21-carbon steroids induced EOG responses; those induced by the 18-carbon steroids generally were the largest (Figure 1). The detected 19-carbon steroids form a diverse group, including the 4-androstene (AD), 5-androstene (DHEA-g, and DHEA-s), 5α-androstan (ANDR-s, and EPIANDR-g), and 5β-androstan (ETIO, ETIO-g, 11β-ETIO-g, and ETIO-3,17-DIONE) configurations. In contrast, all the detected 21-carbon steroids were variations and conjugates of the 5β-pregnan moiety.

EOG Concentration–Response Studies. All concentration–response profiles increased in magnitude with concentration once detection threshold was reached (Figure 2). Although it was clear that some fish responded to 10^{-11} M E1, E2-3g, ETIO, and ETIO-g, group responses to steroids were not significantly different ($P < 0.05$) than responses to background water until 10^{-9} M for E1, E2-3g, and ETIO, and 10^{-8} M for DHEA-g, ANDR-s, ETIO-g, and DHEA-s (Figure 2).

EOG Cross-Adaptation Studies. When steroids intended for use in the cross-adaptation studies were delivered as 2-sec 10^{-8} M pulses 30 min apart (sequential exposure study), there was a trend for many steroids to induce smaller EOG responses on the second presentation, although the reduction was significant ($P < 0.05$; Wilcoxon test) only for DHEA-g (Figure 3A). Although few

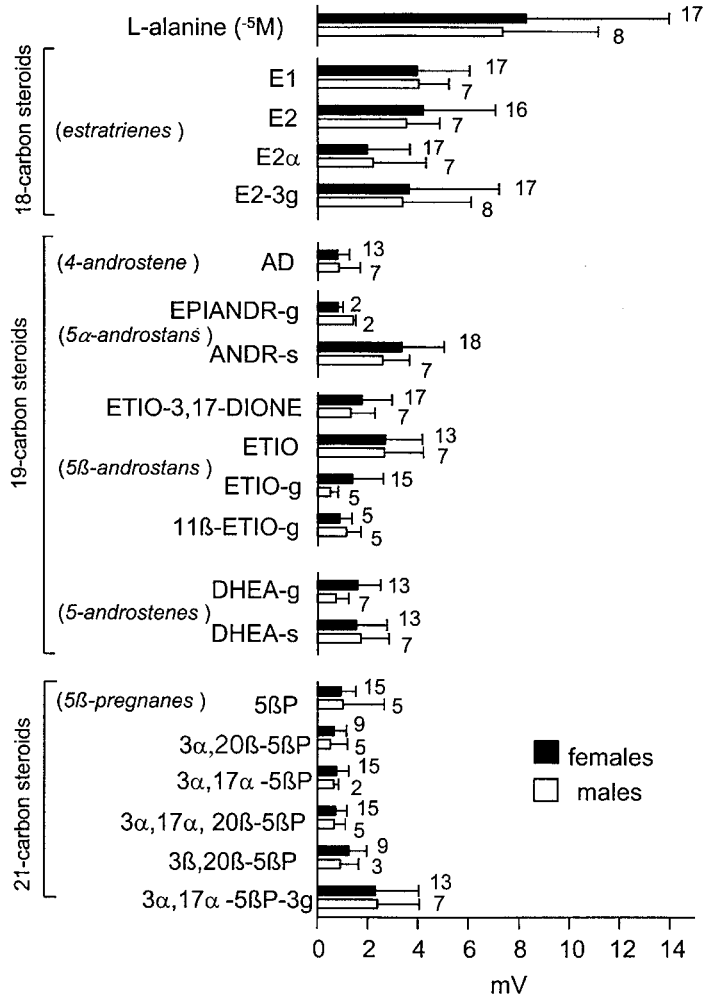

FIG. 1. Comparison of male and female EOG response magnitudes (mean + SE) to detected steroids (10^{-8} M) and amino acid standard. Sample size indicated beside bars. See text and Table 1 for full names of steroids.

replicates were conducted, three of the 5β-pregnan compounds (5βP; 3α,20β-5βP; and 3β,20β-5βP) which induced small EOG responses in initial tests (Figure 1) did not induce EOG response when given the second time in the sequential exposure test, and therefore were omitted from subsequent cross-adaptation studies. For each of the remaining 16 detected steroids, the mean % IR during

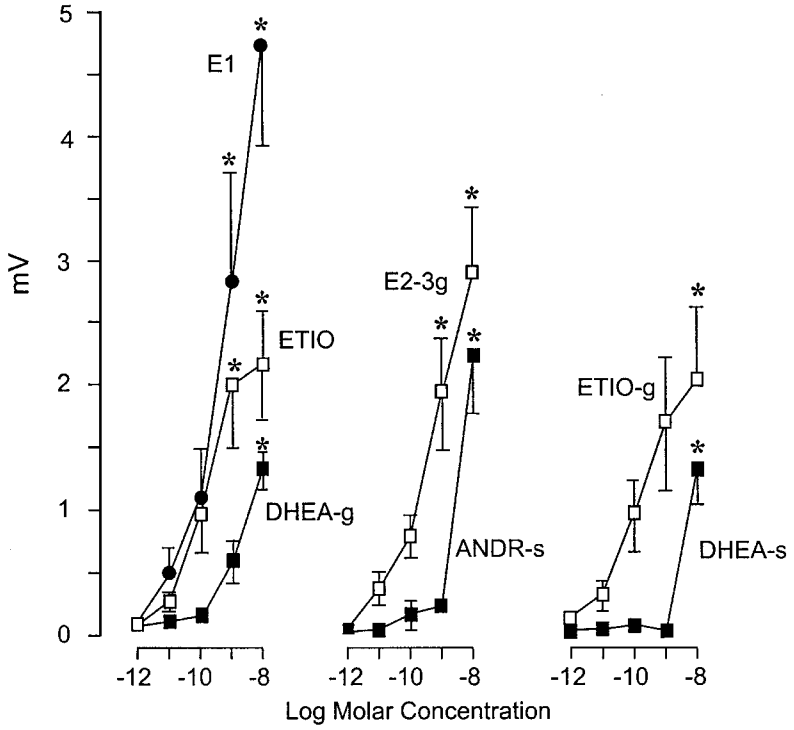

FIG. 2. Concentration response profiles (mean ± SE) for selected steroids. EOG responses expressed in millivolts. $N = 6$ for each steroid. *Response was significantly ($P < 0.05$) greater than response to background water.

the sequential exposure test (Figure 3A) was compared (Mann-Whitney *U* test) to the mean %IR during cross adaptation (Figure 3B–F).

Adaptation to 10^{-7} M ETIO significantly reduced EOG responses to 2-sec 10^{-8} M pulses of a diversity of 4-androsten (AD), 5α-androstan (ANDR-s), 5β-androstan (ETIO; ETIO-3,17-DIONE; and ETIO-g), and 5β-pregnan steroids (3α,17α-5βP; 3α,17α,20β-5βP; 3α,17α-5βP-g) (Figure 3B). Although adaptation to 10^{-7} M DHEA-s also significantly reduced response to ANDR-s (a 5α-androstan), it did not reduce responses to 5β-androstan steroids (e.g., ETIO, and ETIO-g) and produced a different pattern of adaptation of 5β-pregnan steroids than did adaptation to ETIO (Figure 3C).

In marked contrast to the pattern of adaptation to DHEA-s (Figure 3C), adaptation to DHEA-g (Figure 3D) did not affect responses to 5β-pregnanes, significantly reduced response to E2-3g and, rather than reducing response to ANDR-s, reduced response to its 3β isomer, EPIANDR-s (Figure 3D). This pat-

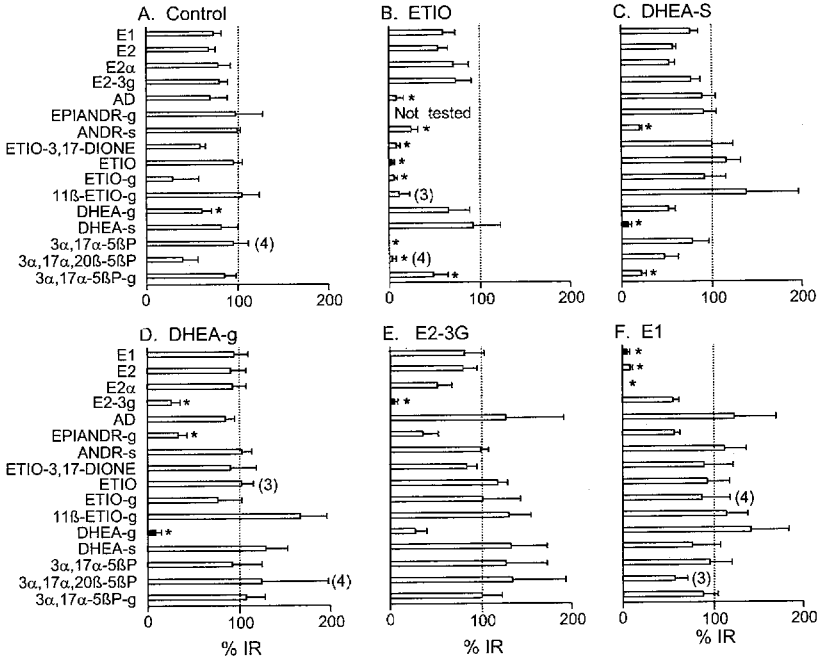

FIG. 3. EOG response to a 2-sec pulse of 10^{-8} M steroid expressed as percent of initial response (% IR) (mean + SE) to either (A) a 10^{-8} M of the same steroid delivered 30 min previously or during adaptation to (B) 10^{-7} M etiocholanolone (ETIO), (C) 10^{-7} M dehydroepiandrosterone sulfate (DHEA-s), (D) 10^{-7} M dehydroepiandrosterone-glucuronide (DHEA-g), (E) 10^{-7} M estradiol-glucuronide (E2-3g), or (F) 10^{-7} M estrone (E1). In B–F, response to adapting steroid indicated by solid bars. $N = 6$ except where indicated beside bars. See text and Table 1 for full names of steroids and text for calculation of % IR. *Response was reduced significantly ($P < 0.05$) by prior exposure (A) or by adaptation (B–F).

tern of adaptation to DHEA-g was similar to that seen during adaptation to E2-3g (Figure 3E), although in the latter case the apparent reductions in response to EPIANDR-s and DHEA-g were not significant.

Finally, adaptation to E1 significantly reduced responses only to the estratriene compounds E1, E2, and E2α (Figure 3F).

Although the results of our cross-adaptation studies (Figure 3) have not fully characterized the olfactory interactions of the steroids detected by *Neogobius*, they indicate the presence of at least four olfactory receptor mechanisms, which, for convenience, we term ETIO, DHEA-s, E2-3g, and E1.

Odor-Induced Ventilation Increase. *Neogobius* did not change their ventila-

tion rate in response to addition of 10^{-6} M L-alanine, the ethanol steroid solvent, 10^{-8} M T-g (a steroid that does not induce EOG response), or 10^{-8} M DHEA-s (a steroid that does induce EOG response) (Figures 4A and B and 5). In contrast, ventilation rate consistently increased in response to ETIO, E1, E2-3g, ANDR-s, and E2 (Figures 4C–E and 5).

Addition of ETIO increased ventilation in both male and female *Neogobius*, the response commencing within the first minute of steroid addition and persisting for approximately 10 min, after which ventilation quickly returned to basal rates (Figure 4C). Similar increases in ventilation were seen in both males and females in response to ETIO-g and ANDR-s (Figure 5), steroids in which EOG responses are significantly reduced during adaptation to ETIO (Figure 3B).

In contrast to the effect of ETIO, addition of E1 and E2-3g increased ventilation of males but not of females (Figure 4D, E). E2, for which EOG response is significantly reduced during adaptation to E1 (Figure 3F), also increased ventilation in males (Figure 5); the effect of E2 on ventilation was not examined in females. Addition of DHEA-g failed to affect ventilation, the apparent trend to increased ventilation being due to an increase in one fish of each sex (Figure 5).

Addition of a mixture of 10^{-9} M E1, E2-3g, ETIO, and DHEA-s induced a significant increase in ventilation, in which the magnitude (Figure 5) and duration (data not shown) were similar to those of its active components.

Behavioral Concentration–Response Studies. The threshold of the ventilation rate response was 10^{-10} M for ETIO and E1 and 10^{-9} M for E2-3g (Figure 6). Ventilation did not increase further at suprathreshold odor concentrations.

Behavioral Cross-Adaptation. In all four behavioral cross-adaptation experiments that used 10^{-8} M ETIO as the adapting steroid, male *Neogobius* significantly increased ventilation rate when exposed to the adapting odor (Figures 7 and 8A). During adaptation to ETIO, these males did not increase ventilation in response to addition of either 10^{-9} M ETIO or 10^{-9} M ANDR-s (Figures 7A and B and 8A), a steroid in which EOG response is significantly reduced by adaptation to ETIO (Figure 3B). In contrast, males adapted to ETIO significantly increased ventilation in response to addition of 10^{-9} M E1 and 10^{-9} M E2-3g (Figures 7C and D and 8A), steroids in which EOG response magnitude is not reduced by adaptation to ETIO (Figure 3B).

Similar results were obtained in behavioral cross-adaptation experiments that used 10^{-8} M E1 as the adapting steroid (Figure 8B): adapted males did not respond to E2, whose induced EOG response is significantly reduced by E1 adaptation (Figure 3F), but significantly increased ventilation rate in response to steroids (ETIO, E2-3g) whose induced EOG responses are unaffected by E1 adaptation (Figure 3F) (Murphy and Stacey, 1999).

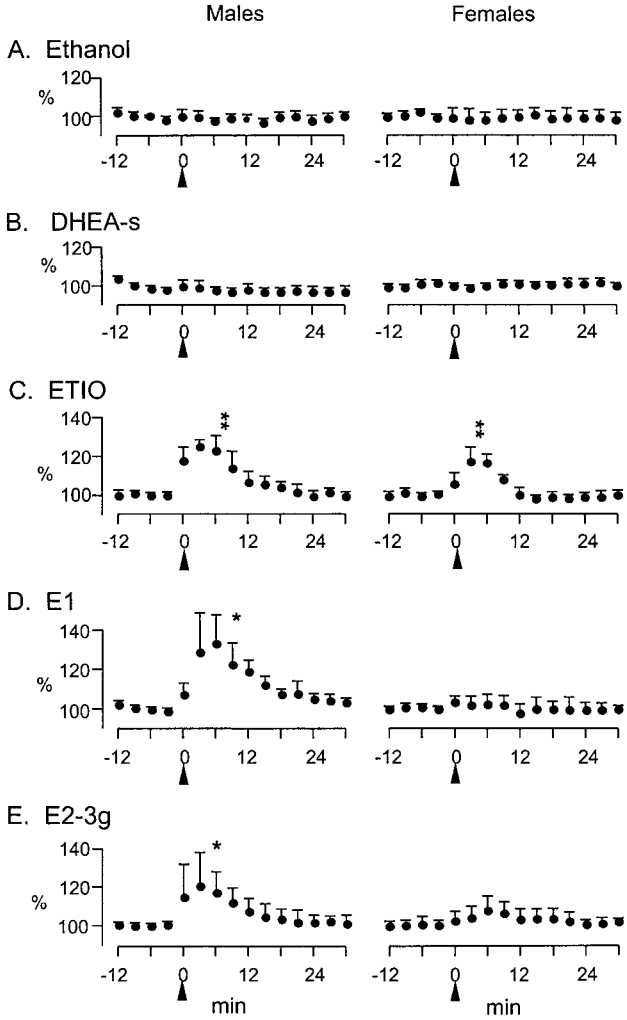

FIG. 4. Percent change in basal ventilation rate (mean + SE) of male and female gobies after addition (arrow) of (A) 1 ml ethanol or exposure to (B) 10^{-8} M DHEA-s, (C) 10^{-8} M ETIO, (D) 10^{-8} M E1, or (E) 10^{-8} M E2-3g. Ventilation rates following addition of test substances were calculated as a percentage of the basal ventilation rate (i.e., the mean ventilation rate in the 12 min preceding additions). $N = 6$ for all groups. Mean ventilation rate during minutes 4 and 7 following test substance addition is significantly different from the basal ventilation rate ($^{*}P < 0.05$; $^{**}P < 0.01$).

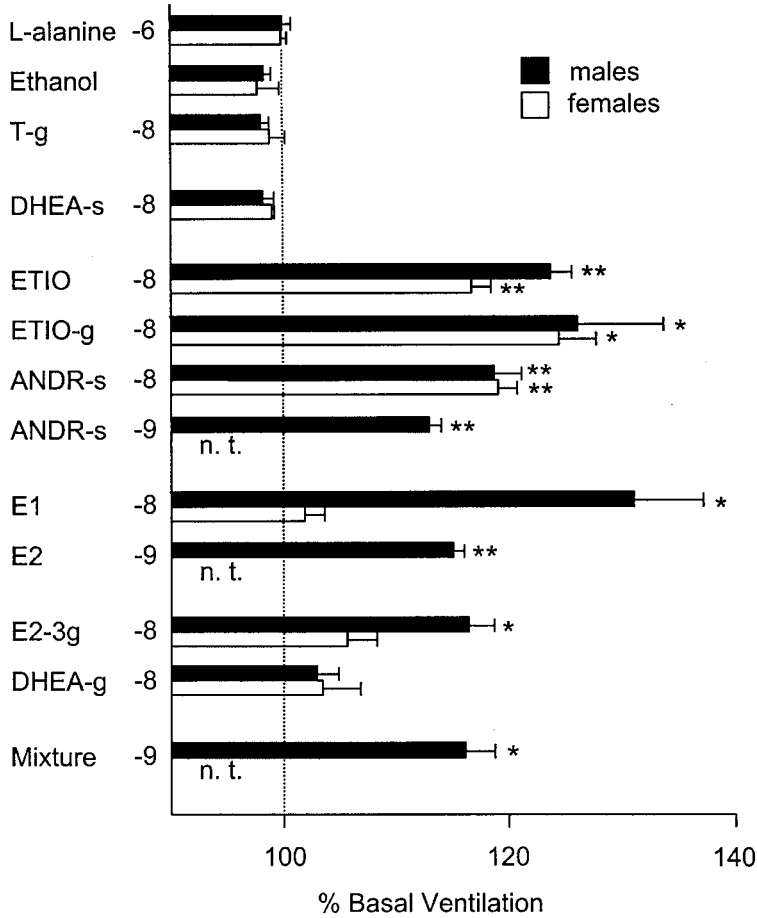

FIG. 5. Percent change in basal ventilation rate (mean + SE) of male and female gobies during minutes 4 and 7 after the addition of single test substances or a mixture consisting of four 10^{-9} M steroids (E1, E2-3g, ETIO, and DHEA-s). Ventilation responses to test substances were calculated as in Figure 4. See text and Table 1 for full names of steroids. $N = 6$ for all groups. n.t. = not tested in females. Mean ventilation rate after addition of a test substance is significantly different from basal ventilation rate ($^{*}P < 0.05$; $^{**}P < 0.01$).

DISCUSSION

The results of this study demonstrate that the round goby, *Neogobius melanostomus*, exhibits olfactory and behavioral responses to steroids, and thus may use released steroids as sex pheromones. The olfactory epithelium of *Neo-*

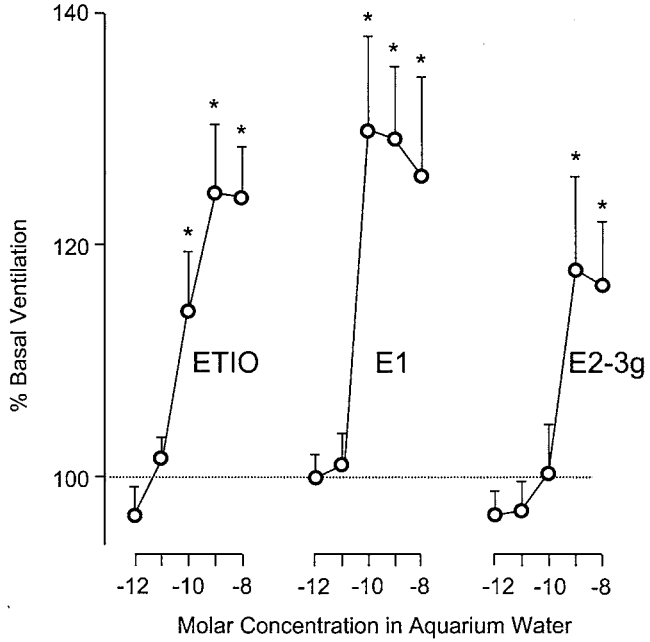

FIG. 6. Percent change in basal ventilation rate (mean + SE) of male gobies during the minutes 4 and 7 after exposure to ETIO, E1, and E2-3g. Ventilation responses to test substances were calculated as in Figure 4. $N = 6$ for all groups. *Mean ventilation rate after addition of a test substance is significantly different from basal ventilation rate ($P < 0.05$).

gobius exhibits EOG responses to a diverse array of steroids (Figure 1), but not to prostaglandins. These findings are consistent with the small number of EOG studies conducted in other perciform species that report a lack of olfactory responsiveness to a range of commercially available prostaglandins and responsiveness to a variety of steroidal compounds (Robison et al., 1998; Stacey et al., 1994b; Stacey and Cardwell, 1995, 1997; Murphy et al., 2000). Our EOG recording studies in *Neogobius* also indicate a lack of sexual dimorphism in detection of steroids (Figure 1). Although the precise reproductive status of the fish used in the recording (i.e., level of gonadal recrudescence) was not determined, other studies on fish report that gender and gonadal maturity have a very minor effect [common carp (Irvine and Sorensen, 1993)] or no effect at all [goldfish (Sorensen et al., 1995), tinfoil barb, *Puntius schwanenfeldi* (Cardwell et al., 1995)] on EOG detection and response magnitude of steroidal odors. By using ventilation increase as a behavioral bioassay, we also find that some detected steroids are perceived by *Neogobius* (Figure 4); unlike olfactory responses, however, behavioral responses to detected steroids is sexually dimorphic (Figure 5).

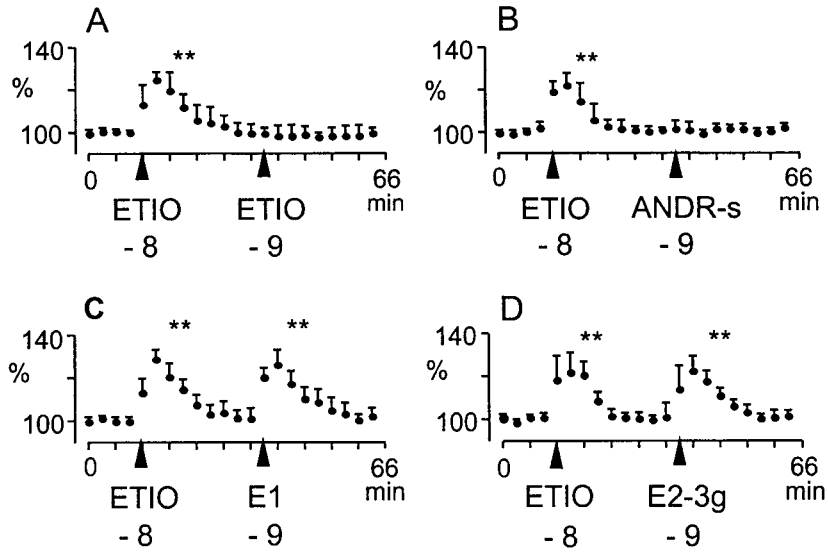

FIG. 7. Percent change in basal ventilation rate (mean + SE) of male gobies in behavioral cross-adaptation studies using 10^{-8} M ETIO (first arrow) as the adapting steroid and 10^{-9} M (A) ETIO, (B) ANDR-s, (C) E1, or (D) E2-3g (second arrow) as the test steroid. Ventilation responses to adapting and test substances were calculated as in Figure 4. $N = 6$ for all groups. **Mean ventilation rate after addition of an adapting or test steroid is significantly different from basal ventilation rate ($P < 0.01$).

Finally, our finding that ETIO-g induces olfactory and behavioral responses in *Neogobius* is consistent with biochemical and behavioral studies (Colombo et al., 1977, 1980, 1982) that indicate a pheromonal function for this steroid conjugate in the black goby, *G. jozo*.

Colombo et al. (1980) showed that ovulated female *G. jozo* are attracted to an ETIO-g source and often induced to oviposit in the absence of a male, whereas preovulatory females, or ovulated females that had completed oviposition, are not. In contrast, we observed no reproductive behavioral responses when isolated *Neogobius* or male–female pairs were exposed to ETIO-g or other detectable steroids (Murphy, 1998). Absence of reproductive behavior response in our studies could have been due to at least four factors. First, absence of female response to ETIO-g and related steroids was likely due to the fact that we were unable to obtain ovulated females for testing. Second, behavioral response to some steroid(s) might require the presence of nonolfactory cues that were not present in our test aquaria. Third, some detected steroids might induce only physiological responses. Fourth, although female *G. jozo* exhibit complex behavioral responses to ETIO-g alone, reproductive behavioral response in *Neogob-*

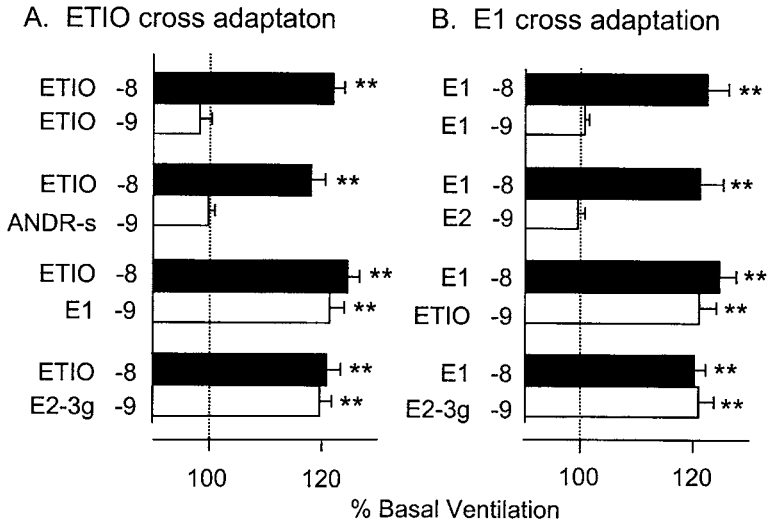

FIG. 8. Percent change in basal ventilation rate (mean + SE) of male gobies in behavioral cross-adaptation experiments using (A) 10^{-8} M ETIO and (B) 10^{-8} M E1 as the adapting steroids, as in Figure 7. Ventilation responses to adapting and test substances were calculated as in Figure 4. $N = 6$ for all groups. **Mean ventilation rate after addition of an adapting or test steroid is significantly different from basal ventilation rate ($P < 0.01$). Note (A) summarized from Murphy and Stacey (1999).

ius might require exposure to steroid mixtures. Nonetheless, we feel that the nature of the steroid-induced olfactory and ventilatory responses reported in this study provide evidence that steroidal compounds may function as reproductive pheromones in *Neogobius*.

Although the function of the ventilation responses to steroid odorants is not clear, the behavior may serve as a "sniffing" mechanism to facilitate odor detection by increasing water flow through the olfactory organ as has been proposed in flounder species (*Lepidopsetta bilineata* and *Platichthys stellatus*) (Nevitt, 1991). Ventilation rate increases may be analogous to tongue flicking observed in many lizard species in response to olfactory stimuli; an increase in the number of tongue flicks in response to novel odors is usually measured as an indication of odor detection in squamate reptiles (Cooper, 1994). Our ventilation rate bioassay requires further clarification on mode of action, as we have not ruled out other mechanisms of detection. For example, there is the small possibility that the steroids could be detected through the taste system or interact with gill processes. Despite these limitations, the ventilation rate measurements closely correspond to the olfactory measurements, and we predict that further investigation will show that this ventilation rate increase in response to steroids

is mediated by the olfactory system. Indeed, odor-induced ventilation could be a valuable behavioral bioassay for examining odor perception in other benthic fishes.

Our EOG cross-adaptation studies indicate that steroids detected by the olfactory organ of *Neogobius* (Figure 1) are discriminated by at least four proposed receptor mechanisms (E1, E2-g, ETIO, and DHEA-s); at least two compounds (ANDR-s and 3α,17α-5βP-g) appear to interact with two of these proposed receptor mechanisms (ETIO and DHEA-s) (Figure 3). Based on results of EOG concentration-response studies (Figure 2), we named the four proposed receptor classes after the most potent steroid odorants, assuming these to be likely natural ligands. However, the steroidal compounds released by *Neogobius* are not known.

Although our EOG and behavioral data indicate *Neogobius* possess multiple olfactory receptor mechanisms for steroidal compounds, using EOG cross-adaptation to link odorants with specific olfactory transduction processes (receptor mechanisms) can be problematic. For example, if olfactory receptors possess multiple binding sites for different odorants, an adapting odor that binds to one site could allosterically affect the binding properties of the other site(s), resulting in nonreciprocal cross-adaptation among compounds thought to share the same transduction mechanism (Caprio and Byrd, 1984). As well, if odorants possess more than one odotope (ligand–receptor binding site) (Shepherd, 1987), EOG cross-adaptation results will be complex. Interpretation of our cross-adaptation results also is confounded by the finding that even a brief (2-sec) pulse of steroid odor can reduce response to that odor when delivered 30 min later (Figure 3A). Therefore, due to the possibility that brief exposure to one compound reduces EOG response to all compounds detected via the same receptor mechanism, we maintained the same order of steroid exposure in our EOG cross-adaptation experiments and compared the reduction of test odorant response magnitude during adaptation to the reduction observed in the control (sequential exposure test; Figure 3A).

Despite these theoretical and practical problems inherent in EOG cross-adaptation studies, our interpretation of the cross-adaptation data is supported by results of the ventilation behavioral bioassays conducted with male *Neogobius*. Although males showed no behavioral response to one of the proposed steroid odorant classes (DHEA-s), they increased ventilation when exposed to the other three (E1, E2-3g, ETIO) (Figures 4 and 6), and did not respond to a steroid (T-g) that failed to induce an EOG response (Figure 5). Most importantly, ventilation responses of males clearly show they discriminate steroids that EOG cross-adaptation results indicate are detected by separate receptor mechanisms and do not discriminate steroids that appear to act through the same receptor mechanism (Figures 7 and 8).

Although behavioral assays have been used to examine discrimination of

amino acid odorants in channel catfish (*Ictalurus punctatus*) (Valentincic et al., 1994), the present study appears to be the first to demonstrate a clear relationship between peripheral (EOG) and central (behavioral) discrimination of steroid odorants. We expect that further behavioral cross-adaptation experiments using detected steroids not employed in this study will clarify the ability of *Neogobius* to discriminate steroid odorants.

Statistically, threshold ventilation response to ETIO and E1 (Figure 6) were lower (more sensitive) than threshold EOG responses (Figure 2); however, such a comparison likely has little biological significance. For example, given our small sample sizes for EOG concentration–response studies ($N = 6$), and the shapes of the concentration-response profiles (Figure 2), the statistical EOG thresholds appear to be conservative estimates of olfactory sensitivity. Moreover, the statistically greater sensitivity of ventilation responses to ETIO and E1 may simply have resulted from fish being exposed briefly to the injected steroid prior to its full dispersal throughout the test aquarium. The low detection thresholds to the steroids also suggest that the odors act through the olfactory system rather than the gustatory system (Sorensen and Caprio, 1998).

Unlike EOG responses to steroids (Figure 1), ventilation responses were clearly sexually dimorphic, males responding to three of the proposed steroid odorant types (E1, E2-3g, and ETIO) and females responding only to ETIO (Figure 4). Male androgenic steroid(s) appears to regulate this sexual dimorphism; females implanted with methyl-testosterone exhibit male-typical ventilation responses to E1 and E2-3g (Murphy and Stacey, unpublished results). The possible functional significance for this behavioral dimorphism is considered below.

Together, our EOG and behavioral data indicate the olfactory organ of *Neogobius* possesses at least four receptor mechanisms for steroid odorants. The proposed E1 receptor responded rather nonspecifically to unconjugated 18-carbon (C_{18}) steroids (E1, E2, and E2α), the nature of the oxygen group at C-17 apparently having little effect on olfactory potency. In contrast, addition of a hydroxyl group on C-16 (estriol; 1,3,5[10]-estratrien-3α,16α,17β-triol), or the addition of a glucuronide or sulfate group at C-3 or C-17 rendered the steroid undetectable by the proposed E1 receptor.

The proposed E2-3g receptor mechanism appears to interact with three seemingly diverse steroids (E2-3g, DHEA-g, and EPIANDR-g; Figure 3D,E). E2-3g and DHEA-g adapted each other, and both reduced the response to EPIANDR-g, although only the effect of DHEA-g adaptation on EPIANDR-g response was significant (Figure 3). Given the structure of the steroids adapted by ETIO (Figure 3B), it is expected that EPIANDR-g also interacts with the proposed ETIO receptor, although this was not examined. The structural requirements for ligands interacting with the proposed E2-3g receptor are unclear, given that it appears to interact with 5α-androstan (EPIANDR-g), 5-androstene

(DHEA-g), and estratriene (E2-3g) compounds. However, both a 5β- or planar glucuronide group at C-3 and an unconjugated oxygen group at C-17 appear important, because estratrienes without a C-3 glucuronide (E2), or with a C-3 sulfate (17β-estradiol-3-SO4) or C-17 glucuronide or sulfate (17β-estradiol-3-glucuronide-17β-SO_4), either did not induce EOG response (Table 1) or were unaffected by E2-3g adaptation (Figure 3). Unfortunately, no 5α-androstan-3α-glucuronides (e.g., androsterone-3α-glucuronide) were tested for interaction with the proposed E2-3g receptor, although ETIO-g, a 5β-androstan with a 3α-glucuronide, was detectable but unaffected by E2-3g adaptation (Figure 3E).

Given that E2-3g increased ventilation in male *Neogobius*, it is surprising that DHEA-g did not (Figure 5). The ineffectiveness of DHEA-g could have been due to the fact that it was a less potent olfactory stimulant than E2-3g (Figure 2). This seems unlikely, however, because ANDR-s, which appeared to be equipotent to DHEA-g in EOG concentration-response studies (Figure 2), increased ventilation when tested at a lower concentration than DHEA-g (Figure 5).

As males gobies are not reported to synthesize C_{18} steroids (Colombo et al., 1977; Asahina et al., 1989), we assume it is C_{18} steroids from females that normally interact with the proposed E1 and E2-3g receptors of males, either to signal that local females are vitellogenic, or to signal gender of females approaching the male's nest. The functional significance of separate receptors for conjugated and unconjugated C_{18} steroids is not known. However, studies on rainbow trout (*Oncorhynchus mykiss*) indicate that free steroids are preferentially released across the gills, whereas conjugates are released in the urine and feces (Vermeirssen and Scott, 1996). If this is the case with *Neogobius*, it is possible that E2-3g is released in concentrated urinary pulses and used by males to detect females at a distance, whereas E1 is released tonically at low concentration and used to identify females at the nest site.

The unconjugated C_{18} steroids detected by *Neogobius* are known to be detected by only a few species of the nonperciform fish (Orders Cypriniformes and Characiformes) that have been studied by EOG recording (Stacey et al., 1994b; Stacey and Cardwell, 1995, 1997). EOG studies indicate unconjugated C_{18} steroids are not detected by any of the small number of perciform species examined (Stacey et al., 1994b; Stacey and Cardwell, 1995, 1997; Robison et al., 1998; Murphy et al., 2000).

There is evidence that conjugated (glucuronated and sulfated) C_{18} steroids have pheromonal function in other species. C_{18} sulfates induce EOG responses in a number of characiform species (Stacey and Cardwell, 1995, 1997), and E2-3g and other glucuronated and sulfated forms induce EOG responses in a perciform, *Haplochromis burtoni* (Robison et al., 1998); however, there has been no attempt to determine pheromonal functions of the detected steroids in any of these species. Van den Hurk and Lambert (1983) proposed that male zebra fish (*Danio*

rerio) are attracted to females by a mixture of ovarian steroid glucuronides that contains 17β-estradiol-17β-glucuronide (E2-17g) and testosterone glucuronide (T-g). However, our EOG studies (Stacey and Cardwell, 1995, 1997), using the suite of steroids tested in *Neogobius*, indicate that zebra fish do not detect these steroid glucuronides, and instead detect one conjugated 4-pregnen compound (17,20β-P-20β-SO_4), which functions as a pheromone in goldfish (Sorensen et al., 1995). These EOG responses in zebra fish are consistent with studies of odor-induced olfactory bulb activity in zebra fish showing that prostaglandin $F_{2\alpha}$ and 17,20β-P-20β-SO_4 induce bulbar activity, whereas E2-17g and T-g do not (Friedrich and Korsching, 1998).

Structural requirements for ligands of the proposed DHEA-s receptor are not clear. A conjugate on C3 evidently is important, because DHEA did not induce an EOG response, but it appears the orientation of the conjugate is not critical, given that DHEA-s (a 5β- steroid) adapted the response to ANDR-s (a 5α- steroid; Figure 3C). Surprisingly, DHEA-s also adapted the EOG response to 3α,17α-5βP-3g. DHEA-s failed to increase ventilation in either sex and is reported to induce EOG response in only one other species, the cichlid *Haplochromis burtoni* (Robison et al., 1998).

The proposed ETIO receptor appears to be relatively nonspecific, since it interacted with a variety of C_{19} and C_{21} compounds, two of which (ANDR-s and 3α,17α-5βP-3g) also interacted with the proposed DHEA-s receptor (Figure 3B). However, the proposed ETIO receptor did exhibit specificity insofar as ETIO adaptation did not reduce EOG responses to 5-androsten compounds (DHEA-g, DHEA-g; Figure 3B) or to any steroids believed to interact with the proposed E2-3g and E1 receptors (Figure 3E,F). Both males and females increased ventilation when exposed to several steroids (ETIO, ETIO-g, and ANDR-s) that interact with the proposed ETIO receptor (Figure 5). Interaction of numerous 5β-reduced steroids with the proposed ETIO receptor is consistent with studies of *G. jozo* showing a preponderance of 5β-reduced products in in vitro incubations (Colombo et al., 1977). However, similar studies of the urohaze goby (*Glossogobius olivaceus*) indicate predominance of 5α-reduction in the testes and seminal vesicles (Asahina et al., 1989). Thus, interaction of the proposed ETIO receptor with a 5α-reduced steroid (ANDR-s) raises questions as to the nature of the natural ligands in *Neogobius*.

Olfactory and behavioral response to ETIO-g by the round goby is consistent with earlier work (Colombo et al., 1977, 1980, 1982) suggesting that this compound is released by male *G. jozo* to attract females and stimulate oviposition. However, given the number and variety of steroids interacting with the proposed ETIO receptor, it is likely that any pheromone acting through this receptor in *Neogobius* would be a mixture of C_{19} and C_{21} compounds. The fact that ETIO-g increases ventilation in both male and female *Neogobius* suggests that this and other steroids interacting with the proposed ETIO receptor induce repro-

ductive responses in both genders. It is possible that male *Neogobius* use ETIO to assess the proximity and reproductive status of neighboring males, whereas females use ETIO for mate search and mate selection, as is likely the case in *G. jozo* (Colombo et al., 1980).

In summary, this study of EOG and behavioral responses to steroidal compounds provides the most extensive data set on putative hormonal pheromones in any perciform species. Although the findings suggest that *Neogobius* has evolved a complex sex pheromone system, further research is required to determine the functions of the putative hormonal pheromones and the natural steroid odorants involved. Such research is warranted not only because *Neogobius* is a tractable species that could serve as a model for pheromone studies of paternal nest-guarding fishes, but also because further understanding might reveal pheromonal techniques to control the spread of this species in the Great Lakes and other North American aquatic ecosystems.

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