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Extreme olfactory specificity of male goldfish to the preovulatory steroidal pheromone 17α,20β-dihydroxy-4-pregnen-3-one

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Summary. 1. To determine the specificity of the goldfish (*Carassius auratus*) olfactory system to the reproductive pheromone 17α,20β-dihydroxy-4-pregnen-3-one (17α,20βP), and to determine if related sex steroids might also function as pheromones, electro-olfactogram (EOG) responses were recorded from mature male goldfish.

2. Of the 24 steroids tested, 17α,20βP was the most stimulatory. It had a detection threshold of 10^{-12} *M* and at a concentration of 10^{-8} *M* elicited an EOG response 3 times that elicited by 10^{-5} *M* L-serine

3. 17α,20β,21-triol-4-pregnen-3-one, a metabolite of 17α,20βP, was the only other highly stimulatory steroid. Its threshold was 10^{-11} *M*.

4. In cross-adaptation experiments EOG responses to all 21-carbon steroids were inhibited during adaptation to 17α,20βP: responses to this pheromone are transduced by a single receptor/transduction mechanism.

5. To verify the biological relevance of EOG recording whole animal responsiveness was determined by measuring blood gonadotropin. When goldfish were placed into homogeneous steroid solutions endocrine responsiveness strongly correlated with EOG recording. However, when steroids were added to aquaria containing fish, responses were less specific indicating that transient wisps of steroids trigger endocrine responses.

6. Although the extreme sensitivity and specificity of the goldfish olfactory system to 17α,20βP gives it the potential to serve as a highly specific cue, realization of this potential is probably determined by the dynamics of pheromone exposure.

Key words: Goldfish – Gonadotropin – Olfaction – Pheromone – Steroid

Introduction

Electro-olfactogram (EOG) recording from the olfactory epithelium of goldfish, *Carassius auratus*, has established 17α,20β-dihydroxy-4-pregnen-3-one (17α,20βP) as the most stimulatory odorant described in a fish (Sorensen et al. 1987). 17α,20βP has both endocrinological and pheromonal functions in this species. It is synthesized by the ovaries of female goldfish in response to surging gonadotropin (GtH) levels approximately 12 h prior to ovulation (and spawning) and promotes oocyte final maturation (Nagahama et al. 1983; Scott and Canario 1987). Subsequently, 17α,20βP is released to the water where it functions as a priming pheromone for males (Dulka et al. 1987a; Stacey et al. 1989). Male goldfish exposed to low concentrations (10^{-10} *M*) of 17α,20βP experience: 1) a rapid (within 15 min) increase in blood GtH accompanied by increased steroidogenesis (Dulka et al. 1987a), 2) a small increase in sexual (behavioral) arousal (Sorensen et al. 1989), and 3) an increase in milt (sperm and seminal fluid) production within several hours of exposure and in time for spawning (Stacey and Sorensen 1986; Dulka et al. 1987a). Because the rate of 17α,20βP synthesis and release drops dramatically at the time of ovulation (and spawning) (Stacey et al. 1989), this steroid is considered a preovulatory primer pheromone. A second pheromone comprised of F prostaglandins (PGF) is later released by ovulated, sexually active female goldfish to stimulate male sexual behavior (Sorensen et al. 1988).

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Abbreviations: EOG electro-olfactogram; *L-Ser* L-serine; *P* progesterone; *17αP* 17α-hydroxyprogesterone; *17α,20βP* 17α,20β-dihydroxyprogesterone; *17α,20β,21P* 17α,20β,21-trihydroxyprogesterone; *A* androstenedione; (abbreviations for steroids referred to infrequently are in Table 1); *C19* 19-carbon; *C21* 21-carbon; *GtH* gonadotropin; *ANOVA* analysis of variance; *RIA* radioimmunoassay

Responses to the 17α,20βP and PGF pheromones appear to be mediated by the olfactory system. Not only has recording from the olfactory epithelium (EOG) established the sensitivity of this tissue to these compounds (Sorensen et al. 1987, 1988), but olfactory ablation (whether achieved by blocking the nasal passages [Partridge et al. 1976] or sectioning the medial olfactory tracts [Stacey and Kyle 1983; Stacey and Sorensen 1986; Kobayashi et al. 1986; unpublished results]) completely eliminates behavioral and endocrinological responsiveness to these pheromones. Although it is possible that the terminal nerve (cranial nerve 0), which runs within the medial olfactory tracts, is responsible for pheromonal responsiveness (see Demski and Northcutt 1983; Kyle et al. 1987), we have been unable to record any changes in the firing rate of terminal nerve cell bodies in goldfish exposed to 17α,20βP but have recorded changes in the firing rates of their mitral cells (Fujita and Sorensen, unpublished results). It is also unlikely that responses to 17α,20βP are caused by its direct absorption from the water because this steroid is released by ovulatory females (and pheromonally active) at concentrations two orders of magnitude below that found in the blood of mature males (Stacey and Sorensen 1986; Dulka et al. 1987a; Stacey et al. 1989), and goldfish do not accumulate 17α,20βP when it is introduced into the water in low concentrations (Dulka, unpublished results).

The discovery that goldfish use an unmodified steroidal hormone as a pheromone raises many questions. Because either 17α,20βP or a closely-related steroid functions as the oocyte maturational hormone in a variety of fishes (Scott and Canario 1987), it is reasonable to ask whether and how a sex steroid can function as a species-specific pheromone (Sorensen and Stacey 1990). One possible answer is that fish metabolize steroidal hormones in different, species-specific manners and that the olfactory mechanisms which detect these compounds have sufficient specificity to distinguish between closely-related metabolites. Although steroid metabolism and release have been studied in only a few species of fish (the goldfish, [Yano and Ishio 1978a, b, c; Van Der Kraak et al. 1989]; the black goby, *Gobius jozo* [Colombo et al. 1980]; the African catfish, *Clarias gariepinus* [Schoonen and Lambert 1986; Schoonen 1987]; and the zebra danio, *Brachydanio rerio* [van den Hurk et al., 1987]), there are indications of species-specific differences in hormone metabolic pathways. Olfactory specificity to a sex pheromone has yet to be directly examined in a fish or any other vertebrate.

Hormonal pheromones could also be species-specific if they were comprised of several components. Indeed, the zebra danio sex attractant (van den Hurk and Lambert 1983) and the goldfish postovulatory pheromone (Sorensen et al. 1988) are both thought to have two components. Two related pieces of evidence lend credence to this possibility for the goldfish preovulatory pheromone. First, ovulatory goldfish release as much 17α-hydroxyprogesterone (17αP; 17α,20βP's precursor) and 17α,20β-dihydroxy-4-pregnen-3-one glucuronide (a conjugated metabolite) as they do 17α,20βP (Van Der Kraak et al. 1989; Stacey et al. 1989). Second, although not as potent as 17α,20βP, water-borne 17αP and its precursor progesterone (P) stimulate sperm production in male goldfish (Stacey and Sorensen 1986) and EOG studies indicate that P is an olfactory stimulant (Sorensen et al. 1987).

Hormonal pheromones could also be rendered species-specific by the behavioral mechanism(s) which determines the nature of pheromone exposure. For example, we have calculated that the small quantity of 17α,20βP released by female goldfish, and dilution caused by active swimming, make it likely that males must be within several cm of the odor source to be exposed to supra-threshold pheromone concentrations (Sorensen and Stacey 1990). Only conspecifics attracted by visual (or other) cues are likely to be so close.

This study was designed to answer 4 questions related to pheromone function in goldfish: 1) How specific is the olfactory system to 17α,20βP? 2) Do all steroids which induce EOG responses act on the same olfactory receptors, or in other words, could the preovulatory pheromone be comprised of several components with independent actions? 3) Does endocrine (whole animal) responsiveness to waterborne steroids mimic the specificity and sensitivity suggested by EOG recording? and 4) Do male goldfish respond to rapidly dissipating pheromonal odor plumes? Because all teleost fish appear to use similar steroidal hormones these findings may well be relevant for understanding pheromone function in other species of fish.

Materials and methods

Animal maintenance. Common goldfish, *Carassius auratus*, of the comet variety and 10–15 cm in total length were obtained from Grassyforks Fisheries Co. (Martinsville, IN) in October and November 1986. Mature males were selected on the basis of 3 criteria: 1) the presence of expressible milt (sperm and seminal fluid); 2) the presence of pectoral fin tubercles, a sexually dimorphic feature; and 3) sexual responsiveness as gauged by willingness to spawn with females injected with prostaglandin $F_{2\alpha}$ (Stacey 1981). These fish were divided into two stocks which were then maintained in 2000 l flow-through aquaria (16 °C; 16:8 LD photoperiod) and fed Nutrafin flaked food (R.C. Hagen, Montreal) ad libitum. One stock was used for both the electrophysiological experiments and the bioassays of pheromone specificity, and the other was used to bioassay the dose-response relationship to 17α,20βP. Fish were sacrificed after the EOG experiments and found to have an average gonadosomatic index (gonad weight/whole body weight × 100) of 2.6 ± 0.31 ($n = 15$).

Electro-olfactogram (EOG) recording. Procedures followed those used by Sorensen et al. (1987) with the exception that recording was 'differential' rather than 'single-ended'. Fish were immobilized with an intramuscular injection of Flaxedil (gallamine triethiodide; 3 mg/kg body weight), secured to a stand in a flow-through trough, and their gills perfused with 11 °C dechlorinated water flowing through a tube placed in their mouths. An olfactory rosette was then exposed by cutting away overlying skin and perfused with water at a rate of 10 ml/min. The same water was used to perfuse the gills and naris, maintain the fish, and formulate the odorants. The water bath containing the fish was grounded. Because tranquilizing doses of anesthetic have been reported to de-

Table 1. Compounds tested

Abbreviation	Common name	Chemical name
5-Pregnen 21-Carbon Steroids (Pregnenolone-like Compounds)		
1. PREG	Pregnenolone	5-Pregnen-3β-ol-20-one
2. 17αPREG	17α-Hydroxypregnenolone	5-Pregnen-3β,17α-diol-20-one
3. 3β,17α,20βPREG	5-Pregnen-3β,17α,20β-triol-3-one	5-Pregnen-3β,17α,20β-triol
4-Pregnen 21-Carbon Steroids (Progesterone-like Compounds)		
4. P	Progesterone	4-Pregnen-3,20-dione
5. 17αP	17α-Hydroxyprogesterone	4-Pregnen-17α-ol-3,20-dione
6. 20αP	4-Pregnen-20α-ol-3-one	4-Pregnen-20α-ol-3-one
7. 20βP	4-Pregnen-20β-ol-3-one	4-Pregnen-20β-ol-3-one
8. 17α,20βP	17α,20β-Dihydroxyprogesterone	4-Pregnen-17α,20β-diol-3-one
9. 17α,20αP	17α,20α-Dihydroxyprogesterone	4-Pregnen-17α,20α-diol-3-one
10. 17α,20β,21P	17α,20β,21-Trihydroxyprogesterone	4-Pregnen-17α,20β,21-triol-3-one
11. 11β,17α,20β,21P	4-Pregnen-11β,17α,20β,21-tetrol-3-one	4-Pregnen-11β,17α,20β,21-tetrol-3-one
A-Ring Reduced 21-Carbon Steroids (Pregnan Compounds)		
12. 17α(5βR)	5β-Pregnan-17α-ol-3,20-dione	5β-Pregnan-17α-ol-3,20-dione
13, 3α,17α(5βR)	5β-Pregnan-3α,17α-diol-20-one	5β-Pregnan-3α,17α-diol-20-one
14. 3α,17α,20β(5βR)	5β-Pregnan-3α,17α,20β-triol	5β-Pregnan-3α,17α,20β-triol
15. 3α,17α,20β(5αR)	5α-Pregnan-3α,17α,20β-triol	5α-Pregnan-3α,17α,20β-triol
16. 3β,17α,20β(5αR)	5α-Pregnan-3β,17α,20β-triol	5α-Pregnan-3β,17α,20β-triol
17. 3α,6α,17α(5βR)	5β-Pregnan-3α,6α,17α-triol-20-one	5β-Pregnan-3α,6α,17α-triol-20-one
18. 3α,17α,20β,21(5βR)	5β-Pregnan-3α,17α,20β,21-tetrol	5β-Pregnan-3α,17α,20β,21-tetrol
Corticosteroids (4-Pregnen 21-Carbon Steroids with Hydroxyl Groups at the 11 or 21 Position)		
19. 17α,21P	11-Deoxycortisol	4-Pregnen-17α,21-diol-3,20-dione
20. 11β,17α,21P	Cortisol	4-Pregnen-11β,17α,21,triol-3,20-dione
21. 11β17αP	21-Deoxycortisol	4-Pregnen-11β-17α-diol-3,20-dione
22. 21P	11-Deoxycorticosterone	4-Pregnen-21-ol-3,11,20-trione
19-Carbon Steroids (Androgens)		
23. A	Androstenedione	4-Androsten-3,17-dione
24. T	Testosterone	4-Androsten-17β-ol-3-one

stroy fish olfactory epithelium (Lewis et al. 1985) these animals were not anesthetized.

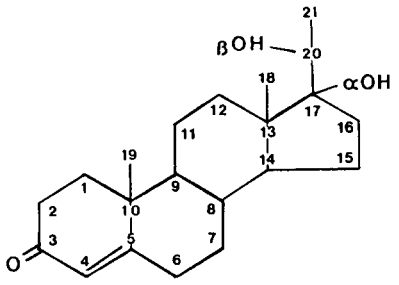
EOG responses were recorded using Ag-AgCl electrodes (type EH-1S; WPI Instruments, New Haven, CT) filled with 3 *M* KCl and bridged to saline gelatine-filled (8%) glass capillaries (tip diameter 60–80 μm). Electrical responses were amplified by a DC-preamplifier (Grass 7P1) and displayed on a pen recorder (Grass 7B polygraph). One electrode was positioned immediately above the olfactory epithelium and the other was placed lightly on the skin surface near the perfused naris. The recording electrode was positioned to yield minimal responses to the 'blank' (no odor added) control and maximal responses to the 'standard' odorant, 10^{-5} *M* L-serine. L-Serine was chosen as the standard because its potency is representative of L-amino acids (Sorensen et al. 1987) – which are traditionally considered to be potent olfactory stimulants in fish (Hara 1975; Caprio 1984; Hara 1986) – and because it was used as the standard in earlier studies (Sorensen et al. 1987, 1988).

Constant volumes (0.5 ml) of olfactory stimulants were delivered to the perfused naris using an apparatus which maintained constant pressure and temperature (see Evans and Hara 1985; Sorensen et al. 1987). Stimulus duration was 5 s and although dye tests indicated that odorant concentration was diluted approximately 50%, no correction was made for this in the data analysis. Each odor was tested 3 times and a 2 min break was allowed between exposures to permit complete recovery (Sorensen et al. 1987).

Olfactory stimulants used for EOG recording. Because initial experiments indicated that ovulatory goldfish may release many hormones and hormonal metabolites (Van Der Kraak et al. 1989) we decided to test the olfactory potency of a wide variety of possible precursors and metabolites of 17α,20βP. 24 sex steroids were chosen and are listed in Table 1 with their abbreviations, which will be used throughout this paper. A schematic representation of 17α,20βP is shown in Fig. 1 whose legend also briefly explains steroid nomenclature and hormone synthesis pathways.

Steroids were purchased from Sigma Chemical Co. (St. Louis, MO) and Steraloids Inc. (Wilton, NH). Stock solutions were made up at a concentration of approximately 10^{-5} *M* by dissolving 1 mg of each steroid in 1.5 ml of glass-distilled methanol and placing 0.5 ml of this solution into 100 ml of distilled water. Because the molecular weights of these steroids ranged from 287 to 365 actual stock solution concentrations theoretically ranged from 0.91–1.16×10^{-5} *M* with 18 of the 24 solutions within 5% of 1.00×10^{-5} *M*, the concentration of the 17α,20βP stock solution. Given the inherent difficulties of accurately weighing out such small quantities of compounds and repeatedly diluting them, and our interest in log molar relationships alone in this study, we felt that this was an acceptable level of error.

A control solution containing the amount of methanol (1.25×10^{-1} *M*) used in the 10^{-5} *M* steroid stock solutions was also made up in distilled water. A 10^{-3} *M* stock solution of L-serine (Sigma Co., St. Louis, MO) was made up in distilled water only. Fresh stock solutions were made up at least once every 3 weeks and were stored at 4 °C. Aliquots of stock solutions were subsequently diluted with dechlorinated water to formulate the test odorants (Sorensen et al. 1987). Test solutions and a 'blank' water control (no added odorants) were made up no more than 30 min prior to testing and stored in an 11° C water bath. Glassware was thoroughly washed in a detergent, rinsed with tap water, then methanol,



17α,20β-dihydroxy-4-pregnen-3-one

(17α,20βP)

Fig. 1. 17α,20β-dihydroxy-4-pregnen-3-one (17α,20βP). 17α,20βP is a 21 carbon (C21) 4-pregnen (it has a double bond between carbons 4 and 5) steroid with two hydroxyl (OH) groups, one of which is attached to carbon 17 in the α confirmation, and the other is attached to carbon 20 in the β confirmation. 5-pregnen steroids such as pregnenolone (PREG) have a double bond between carbons 5 and 6 and no double bond between carbon 4 and 5. 4-pregnen steroids can be reduced to 5α- and 5β-pregnan steroids which have no double bond and whose hydrogen atom at position 5 either points down (5α) or up (5β) from the plane of the molecule. Steroids with 19 carbons (C19) such as androstenedione (A) and testosterone (T) lack carbons 20 and 21 but use the same numbering scheme as C21 steroids. Fish steroid synthesis pathways are generally thought to proceed from pregnenolone (PREG) to progesterone (P) to 17α-hydroxyprogesterone (17αP) to 17α,20β-dihydroxyprogesterone (17α,20βP) (Suzuki et al. 1981; Schoonen and Lambert 1986; Scott and Canario 1987) and occasionally to 17α,20β,21P (Trant et al. 1986). In the African catfish 17α,20βP is metabolized by reduction to 5α- and 5β-pregnan steroids, the addition of hydroxyl groups at various locations of the molecule, and conjugation with glucuronic acid (a sugar group) at position 3 and/or 20 (Schoonen 1987). Abbreviation scheme used in Table 1 is based on that employed by Scott and Canario (1987)

and finally distilled water. To confirm that we were testing the intended concentrations (i.e. there was no problem with solubility) 10^{-6} *M* solutions of 8 steroids (PREG, P, 17αP, 17α,20βP, 11β,17α,21P, 17α,21P, T, A) were prepared from week-old stock solutions and analyzed by high pressure liquid chromatography. These solutions were found to be within 10% of their intended concentrations.

Protocol for determining structure-activity relationship using EOG recording. Each fish was first tested with blank water, 10^{-8} *M* and 10^{-7} *M* methanol controls, 10^{-5} *M* L-serine standard, and 10^{-8} *M* 17α,20βP to ensure recording quality and establish a baseline level of responsiveness. With the exception of 17α,20βP and 17α,20β,21P which were tested last to minimize contamination, odors were generally tested in the order listed in Table 1. Odorants were tested in order of increasing concentration and the apparatus thoroughly rinsed between individual odorants. L-Serine standard and blank were tested every 4–5 odorants to ensure that response level remained constant. Each fish was tested with only 10–12 odorants so that individual preparations lasted no more than 6 h. After several tests it became apparent that the detection thresholds of several compounds fell below 10^{-8} *M*. Subsequently, the range of concentrations tested for these compounds was increased; P and 17αP were tested at concentrations of 10^{-10} *M* to 10^{-7} *M* and both 17α,20βP and 17α,20β,21P tested at concentrations of 10^{-13} *M* to 10^{-7} *M*.

Protocol for determining olfactory specificity using EOG recording (cross-adaptation). To determine whether the EOG responses elicited by these sex steroids were transduced by a common mechanism, cross-adaptation experiments were performed. In cross-adaptation the response to a test odorant is first measured and then the olfactory epithelium is perfused with an adapting odorant into which pulses of the test odorant made up in the adapting odorant are added (see Caprio and Byrd 1984). Reductions in EOG responses during adaptation reflect the extent to which the olfactory receptor mechanism responsible for responses to the adapting stimulus cross-reacts to the test odorant. P, 17αP, and 17α,20β,21P were chosen as test odorants because of their potency. Androstenedione (A) was also chosen because, although it did not evoke large responses, the fact that it elicited any response at all was surprising given its major structural differences from 17α,20βP (it has only 19 carbons).

17α,20βP was used as the adapting stimulus because it was the most stimulatory compound. It was tested at 10^{-8} *M* because this concentration evoked near maximal EOG responses indicating possible receptor site saturation. Adaptation was first verified by testing 10^{-8} *M* 17α,20βP (adaptation should abolish responses to equimolar concentrations of itself) and responses to 10^{-5} *M* L-serine standard and a blank were tested as controls (adaptation to a steroid would not be expected to affect responses to these stimuli). Test stimuli were initially tested at 10^{-7} *M*, a concentration ten times that of the adapting stimulus. If responses to test odors persisted during adaptation they were tested at equimolar concentrations with the adapting stimulus (10^{-8} *M*).

Analysis of EOG data. The magnitude of each EOG response was measured from the baseline to the peak of each phasic displacement and converted to mV using the amplifier's calibration scale. Each set of triplicate responses was then averaged and the most recent (averaged) blank water response subtracted. With the exception of the cross-adaptation data, responses were calculated as percentages of the most recent L-serine response. EOG data were generally standardized in this manner to reduce inter-individual variability caused by differences in electrode size and placement.

First, to determine whether the methanol carrier was eliciting a response, responses to each of the methanol controls were compared with responses to blank water using a *t*-test. Responses to the steroids and their methanol control were next compared using a one way analysis of variance (ANOVA). Because both ANOVAs were significant ($P \leq 0.01$), we were then able to compare individual steroid and methanol responses using the Newman-Keuls procedure (Winer 1971). This procedure adjusts probability (critical) values downward to take into consideration multiple comparisons. Because these experiments had many possible comparisons (24!), this procedure, while technically correct and quite conservative, had little power (i.e. it did not find differences significant unless they were quite large).

Because it could not be assumed that responses to L-serine would not be influenced by adaptation to 17α,20βP, cross-adaptation data were analyzed in mV rather than relative to L-serine. Absolute responses to each compound were analyzed by comparing pre-adaptation values to values obtained during adaptation using matched-pairs *t*-tests (Winer 1971).

Protocol for determining whether whole animal (endocrine) responsiveness to water-borne 17α,20βP reflects olfactory sensitivity. Initial experiments to test biological (endocrine) sensitivity to water-borne 17α,20βP followed our established protocol of adding aliquots of steroid stock solutions to the tanks already containing fish to create desired final dilutions (Stacey and Sorensen 1986; Dulka et al. 1987a). We chose not to test the effects of focally administering odorants to restrained males because of earlier results indicating that stress reduces endocrine responsiveness to pheromones (Sorensen, unpublished results). Groups of 3 male fish were placed into flowing, well-aerated, 70 l aquaria (250 ml/min; 20 °C; 16L:8D) and at the next scotophase (the natural time of pheromone release by ovulatory females; Stacey et al. 1989) appropriate amounts of a 17α,20βP stock solution (0.1 mg of ster-

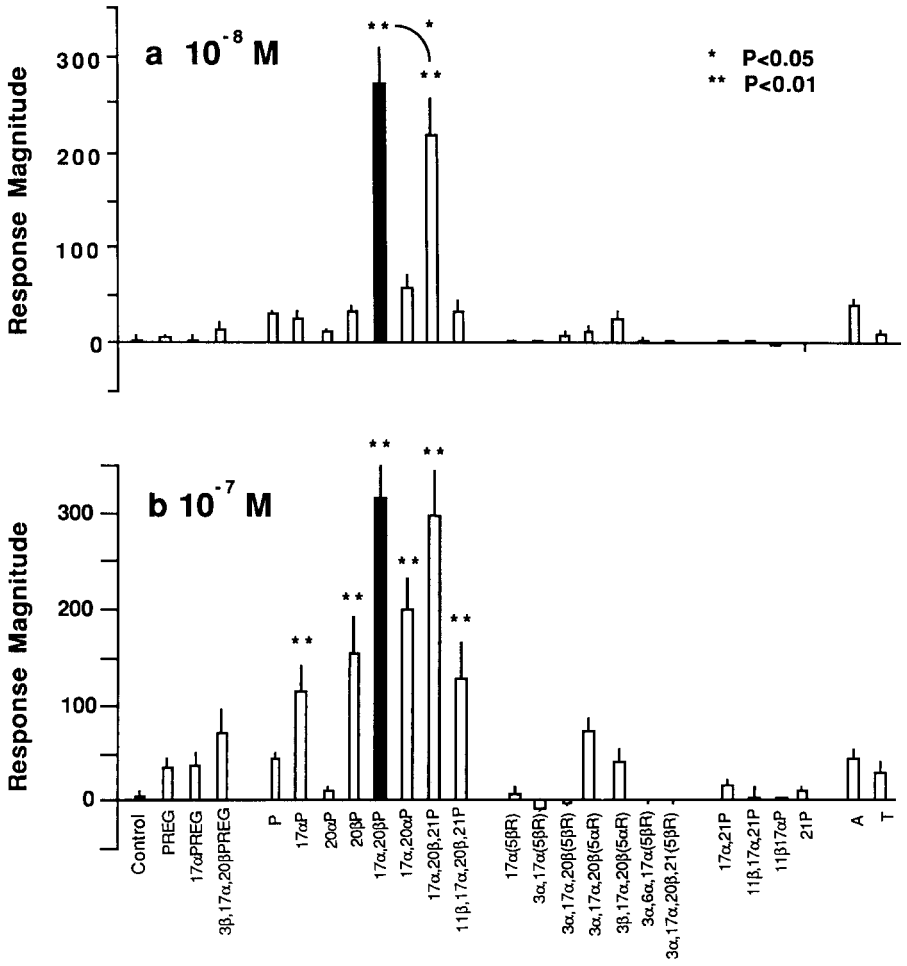


Fig. 2. a Electro-olfactogram (EOG) responses of 24 sex steroids when tested at a concentration of 10^{-8} *M* using mature male goldfish ($n=7$ or 8). Response magnitude is represented as a percentage of the EOG response elicited by 10^{-5} *M* L-serine standard. Vertical bars, standard error. Unless connected by a line, asterisks (** $P \leq 0.01$; * $P \leq 0.05$) designate significant differences between the steroid response and methanol control. Steroid abbreviations described in Table 1. **b** EOG responses of 24 sex steroids when tested at a concentration of 10^{-7} *M* on the same mature male goldfish ($n=7$ or 8) used to test 10^{-8} *M* concentrations. Response magnitude is represented as a percentage of the EOG response elicited by 10^{5} *M* L-serine standard. Vertical bars, standard error. Asterisks (** $P \leq 0.01$) designate significant differences between steroid responses and methanol control. Steroid abbreviations described in Table 1

oid per ml of ethanol; 3.3×10^{-4} *M*) were added to each aquarium to create final concentrations ranging from 10^{-13} *M* to 10^{-9} *M* in log molar steps. Ethanol alone (0.025 ml; the amount added in association with the 10^{-9} *M* 17α,20βP test group) was added to one set of aquaria as a control. To ensure rapid odorant dilution, steroid and ethanol solutions were added by injecting them into the vigorous bubble-streams created by each aquarium's airstone. Ethanol was used as a carrier because we were concerned that methanol might have toxic effects. Like methanol, ethanol is a poor olfactory stimulant for goldfish and is not detectable at the concentration used to make 10^{-7} *M* steroid stock solutions (Sorensen, unpublished results). Each treatment group contained at least 12 individuals (4 tanks).

Sixty min after steroid addition, fish were removed from their aquaria, anesthetized (2-phenoxyethanol; Syndel Laboratories, Vancouver, British Columbia), and blood samples taken from their caudal vasculature. Blood samples were kept on ice until clotted (several hours), centrifuged and serum GtH subsequently measured by radioimmunoassay (RIA) according to established protocol (Hontela and Peter 1978). GtH titres were analyzed by ANOVA after log transformation and then individual comparisons performed using Newman-Keuls follow-up tests (Winer 1971).

Surprisingly, the results of the previous experiment indicated that biological sensitivity to 17α,20βP clearly exceeded that predicted by EOG recording. Knowing that goldfish exhibit large GtH increases after only 15 min of exposure to 17α,20βP (the shortest time exposure time yet tested; Dulka et al. 1987a), and that natural pheromone exposures are likely to be quite brief, we wondered whether this unexpected level of sensitivity could have been caused by fish responding to steroid solutions prior to their complete dilution. Injecting aliquots of water-soluble dyes into aquaria we estimated that injected steroids probably took less than a min to reach a uniform concentration. To test the possibility that male goldfish were responding to steroid solutions before they were diluted below threshold, we repeated the first experiment with one major modification; steroids were added to empty aquaria, and, after a 5 min break to permit dilution, groups of males were moved into these pre-diluted steroid solutions. Fish were bled 30 min later and their serum GtH analyzed by RIA. The results of this experiment (but not the one preceding it) have been briefly described in a preliminary report (Dulka et al. 1987b).

Protocol for determining whether endocrine responsiveness to waterborne C21 steroids reflects olfactory specificity. To determine whether whole animal specificity to waterborne steroids reflected that of the EOG, and to test whether the method of steroid exposure (addition) influences this relationship, we conducted two more experiments. We elected to test 7 compounds (P, 17αP, 17α,20βP, 17α,20β21P, 3α,17α,20β(5βR), 3α,17α,20β(5αR), and 3β,17α,20β(5αR)) at a concentration of 5×10^{-10} *M*. This concentration was chosen for several reasons: it was used as a standard in earlier experiments (Dulka et al. 1987a), only 17α,20βP and 17α,20β,21P are clearly detectable by EOG recording at this concentration, and we have estimated from natural release rates that males are exposed to this concentration of 17α,20βP (Sorensen and Stacey 1989).

The first experiment tested the effect of adding concentrated steroid stock solutions to aquaria containing fish. Groups of 3 male fish were placed into flowing, well-aerated 70 l aquaria (250 ml/min; 15 °C) and the following scotophase 1 l of either 3×

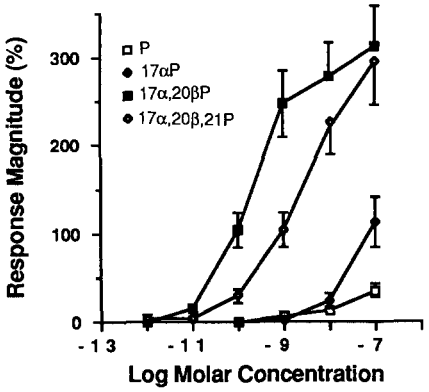


Fig. 3. Semi-logarithmic plot of the concentration-response relationship to progesterone (P), 17α-hydroxyprogesterone (17αP), 17α,20β-dihydroxyprogesterone (17α,20βP) and 17α,20β,21-trihydroxyprogesterone (17α,20β,21P). Responses were recorded from the same preparations ($n=7$–8 mature male fish) used for the experiments shown in Fig. 2a and b. Because their responses to the 10^{-7} *M* and 10^{-8} *M* methanol control solutions were not different from zero and are already shown in Fig. 2a and b, they are not shown here. Average response magnitude is represented as a percentage of that elicited by the standard stimulant 10^{-5} *M* L-serine. Vertical bars, standard error

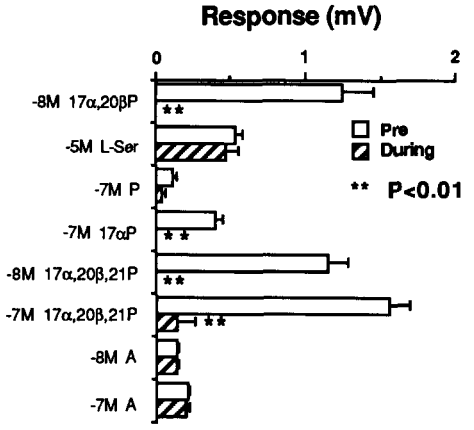


Fig. 4. Cross-adaptation experiment. Electro-olfactogram responses elicited (in mV) prior to adaptation (light-colored bars) and during adaptation (shaded bars) to 10^{-8} *M* 17α,20βP. Odorant abbreviations are as used in the text and defined in Table 1. Concentrations are log molar. 'Pre' and 'During' responses were compared by paired *t*-tests (** $P \leq 0.01$)

10^{-8} *M* steroid or ethanol control solutions were added to the bubble-streams of the aquaria. Steroid solutions were pre-diluted in this manner to hasten dilution. Five groups of fish ($n=15$) were exposed to each treatment. One hour after steroid addition, fish were anesthetized and a blood sample taken for subsequent GtH determination by RIA. After being bled, fish were revived in clean water, returned to their tanks, and 8 h later (the following morning), anesthetized, placed upside down in a foam pad and their milt stripped by applying gentle pressure to their abdomens. Expressed milt was collected in pre-weighed hematocrit tubes by gentle aspiration and subsequently quantified by weighing the full tubes (see Stacey and Sorensen 1986). The individual who performed the stripping was unaware of which treatments the fish received. GtH responses were analyzed after log transformation

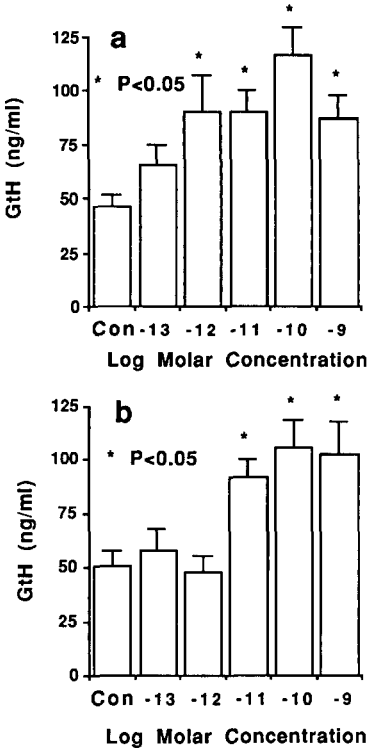


Fig. 5a. Average gonadotropin (GtH; ng/ml of serum) levels of male fish ($n=12$ per treatment) after being exposed to various (log molar) concentrations of 17α,20βP or an ethanol control (Con) for 1 h. In this experiment aliquots of a 3×10^{-4} *M* 17α,20βP solution were added to tanks containing the test fish. Vertical bars, standard error. Average GtH levels compared to the control (* $P \leq 0.05$). **b.** Average gonadotropin (GtH; ng/ml of serum) levels of male fish ($n=18$ per treatment) after being exposed to pre-diluted log molar concentrations of 17α,20βP or an ethanol control (Con) for 30 min. In this experiment fish were added to tanks containing pre-diluted concentrations of 17α,20βP. Vertical bars, standard error. Average GtH levels compared to the control (* $P \leq 0.05$)

by ANOVA and Newman-Keuls follow-up tests (Winer 1971). Milt responses were analyzed by Kruskal-Wallis test followed by a non-parametric analogue of the Newman-Keuls procedure (Marascuilo and McSweeney 1977; Pimentel and Smith 1985).

A 2nd experiment was conducted to test the possibility that the results from the first experiment were confounded by fish responding to undiluted steroids. This experiment was essentially the same as the first except that the steroids were prediluted (to 10^{-10} *M*) in 70 l (15 °C) aquaria 5 min before fish were added. Fish were bled after a 1 h exposure, returned to their tanks, and their milt stripped the following morning.

Results

Structure-activity relationships as determined by EOG recording

The average EOG response elicited by 10^{-5} *M* L-serine was 0.53 mV (SEM 0.041; $n=14$), similar to that measured in our previous studies of mature male goldfish (0.53 ± 0.05; Sorensen et al. 1987). Also, as we have

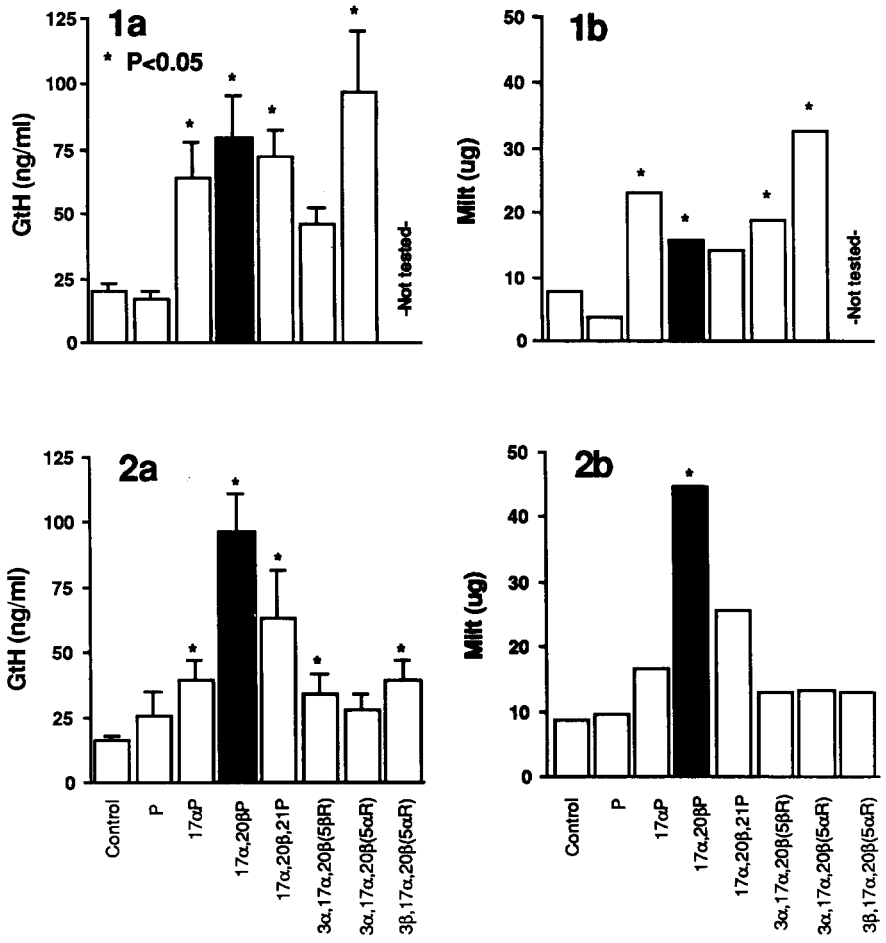


Fig. 6. 1a Average gonadotropin (GtH; ng/ml of serum) levels of male fish ($n=15$ per treatment) after being exposed to various steroids at a concentration of 5×10^{-10} *M* (steroid abbreviations as in text) or ethanol control (Con) for 1 h. In this experiment 1 l of 10^{-8} *M* steroid solution were added to tanks containing the test fish. Vertical bars, standard error. Average GtH levels are compared to the control (*$P\leq0.05$). **1b** Median levels of milt collected from the fish whose GtH values are plotted in Fig. 6-1a. Milt was stripped 8 h after being exposed to 5×10^{-10} *M* steroid solutions or control (7 h after bleeding). Steroid abbreviations as used in the text. Milt levels compared to the control (*$P\leq0.05$). **2a** Average gonadotropin (GtH; ng/ml of serum) levels of male fish (n=15–16 per treatment) after being exposed to various steroids at a concentration of 10^{-10} *M* (abbreviations as in text) or an ethanol control (Con) for 1 h. In this experiment fish were added to tanks containing pre-diluted steroids. Vertical bars, standard error. Average GtH levels compared to the control (*$P\leq0.05$). **2b** Median levels of milt collected from the fish whose GtH values are plotted in Fig. 6-2a. Milt was stripped 8 h after being exposed to 10^{-10} *M* steroid solutions or control (7 h after bleeding). Steroid abbreviations as used in the text. Milt levels compared to the control (*$P\leq0.05$)

found before (Sorensen et al. 1987, 1988), neither of the methanol controls were detectable by EOG recording ($P>0.10$; Fig. 2). EOG responses to all steroids possessed distinct phasic and tonic components and were similar in form.

At a concentration of 10^{-8} *M*, only 17α,20βP and 17α,20β,21P elicited EOG responses significantly greater than methanol control ($P\leq0.01$) and the response to 17α,20βP was larger than that to 17α,20β,21P ($P\leq0.05$; Fig. 2a). At a concentration of 10^{-7} *M* only 6 of the 24 steroids tested elicited responses larger than control ($P\leq0.01$; Fig. 2b). These compounds were: 17αP, 20βP, 17α,20βP, 17α,20αP, 17α,20β,21P, and 11β,17α,20β,21P. Although responses to 17α,20βP and 17α,20β,21P were not different from each other at 10^{-7} *M* ($P>0.05$), both were more stimulatory than the other 4 compounds ($P\leq0.05$) which evoked responses of similar magnitudes.

Concentration-response relationships clearly established 17α,20βP as the most stimulatory compound tested. It had a detection threshold of 10^{-11}–10^{-12} *M* and at a concentration of 10^{-8} *M* evoked an EOG response which was 267% (SEM ± 38.3%; Fig. 3) of that evoked by 10^{-5} *M* L-serine. Olfactory responses to 17α,20βP increased in a sigmoidal fashion and appeared to plateau at approximately 10^{-8} *M*. 17α,20β,21P was slightly less stimulatory than 17α,20βP; it had a threshold of approximately 10^{-11} *M* and appeared to be plateauing at 10^{-7} *M* where it elicited a response of about the same size as that elicited by 10^{-8} *M* 17α,20βP. 17αP and P were considerably less stimulatory than 17α,20β,21P and had thresholds of approximately 10^{-9} *M*. 17αP elicited a larger response than P (Fig. 3).

Olfactory specificity as determined by cross-adaptation

When the olfactory epithelium was adapted to 10^{-8} *M* 17α,20βP, EOG responses to 10^{-7} *M* 17αP, 10^{-8} *M* 17α,20βP, and 10^{-8} *M* 17α,20β,21P were completely eliminated ($P\leq0.01$). Responses to 10^{-7} *M* 17α,20β,21P (which elicited larger responses than 10^{-8} *M* 17α,20βP prior to adaptation) were also greatly reduced ($P\leq0.01$). Although adaptation to 17α,20βP appeared to reduce responses to 10^{-7} *M* P, this difference was not significant. Responses to L-serine and androstenedione (A) were unaffected by adaptation ($P>0.10$; Fig. 4).

Sensitivity to pheromonal 17α,20β P as determined by endocrine bioassay

When aliquots of the 17α,20βP stock solution (3×10^{-4} *M*) were added to aquaria containing fish, males

in the aquaria which received enough 17α,20βP to create final dilutions of 10^{-12} *M* or greater had significantly increased GtH levels one hour later ($P \leq 0.05$; Fig. 5a). The GtH levels of these males were roughly twice those of the control group and there were no differences ($P > 0.10$) between responding groups. In contrast, when males were placed into aquaria already containing prediluted 17α,20βP, only those fish exposed to concentrations of 10^{-11} *M* or greater had elevated GtH levels ($P \leq 0.05$; Fig. 5b). As with the first experiment, all responding males had GtH levels approximately twice those of the controls; increasing 17α,20βP concentration above threshold did not elicit larger GtH responses.

Specificity of pheromonal responsiveness as determined by endocrine bioassay

When 10^{-8} *M* steroid solutions were added to aquaria containing fish, those fish exposed to 17αP, 17α,20βP, and 3α,17α,20β(5αR) exhibited significant, equivalent increases in both GtH and milt ($P \leq 0.05$; Fig. 6-1a and b). Fish exposed to 17α,20β,21P also had elevated GtH ($P \leq 0.05$) but their milt increases were not large enough to be significant. Conversely, fish exposed to 3α,17α,20β(5βR) had elevated milt ($P \leq 0.05$), but their GtH was not significantly elevated. Of the 6 steroids tested, only P failed to elicit any response.

In contrast, when fish were placed into water containing prediluted 10^{-10} *M* steroid solutions a different pattern of responses was apparent. 17α,20βP elicited a 5-fold increase in GtH which was larger than that elicited by all other steroids including 17α,20β,21P which elicited a 3-fold increase in GtH ($P \leq 0.05$; Fig. 6-2a). 17αP, 3α,17α,20β(5βR), 3β,17α,20β(5αR) also elicited small equivalent increases in GtH ($P \leq 0.05$). Neither P nor 3α17α,20β(5αR) evoked a change in GtH. An increase in milt was observed only in those fish exposed to 17α,20βP ($P \leq 0.05$; Fig. 6-2b).

Discussion

Electro-olfactogram (EOG) recording suggests strongly that the olfactory system of the male goldfish detects the preovulatory female sex pheromone 17α,20βP in a highly specific manner. Of the 24 closely-related sex steroids tested at a concentration of 10^{-8} *M*, only two, 17α,20βP and 17α,20β,21P, evoked EOG responses significantly larger than control, and the responses evoked by 17α,20β,21P were much smaller than those evoked by 17α,20βP. When all 24 steroids were tested at 10^{-7} *M*, a concentration 5 log units above 17α,20βP's detection threshold, only 4 steroids in addition to 17α,20βP and 17α,20β,21P elicited significant responses and all were smaller than those elicited by 17α,20βP. A close examination of the responses elicited by steroids with 21 carbons (C21 steroids) reveals a strong correlation between structural similarity to 17α,20βP and olfactory potency. Four of the 5 steroids (other than 17α,20βP) which elicited significant responses at 10^{-7} *M* differed from 17α,20βP at one position only; the next most potent group of compounds differed at two positions.

The structure-activity relationship of C21 steroids suggests that the olfactory receptor mechanism responding to 17α,20βP recognizes most (if not all) aspects of the steroid molecule. 17α,20βP-like steroids which lack either a 17α OH or 20β OH group (i.e. 20βP or 17αP) or possess them in another configuration (i.e. 20αP, 17α,20αP) evoke a much smaller EOG response than 17α,20βP. Similarly, steroids lacking either a double bond between carbons 4 and 5 or/and a ketone group on the third carbon (compare 17αP to 17α(5βR) and 17α,20βP to all 5α and 5β reduced steroids) are poor olfactory stimulants. Interestingly, 5β reduction appears to cause a greater loss in potency than 5α reduction; the latter configuration is 'flatter' and thus more similar to the unreduced form. EOG response magnitude to C21 sex steroids also drops dramatically when extra hydroxyl groups are added to the 17α,20βP molecule (compare 17α,20βP to 17α,20β,21P to 11β,17α,20β,21P and 17αP to 17α,21P to 11β,17α,21P). In summary, of the nearly two dozen variations on 17α,20βP tested, only one, the addition of a hydroxyl group to carbon 21, (17α,20β,21P), did not radically diminish olfactory potency. We are unaware of any structural reason why the receptor mechanism should recognize this portion of the molecule less specifically than it does the remainder. Although it has yet to be determined whether goldfish produce 17α,20β,21P, this steroid was recently identified as a maturational hormone in the Atlantic croaker, *Micropogonias undulatus* and several other perciform fish (Trant et al. 1986; Thomas 1988). Perhaps goldfish synthesize and release 17α,20βP and 17α,20β,21P at the same time, and evolution has favored an olfactory receptor mechanism which recognizes both because together they constitute a stronger signal.

Several lines of evidence suggest that a single class of olfactory receptors is responsible for the extreme specificity of the olfactory system for C21 steroids. First, the size of EOG responses evoked by C21 steroids correlates strongly with structural similarity to 17α,20βP. Second, the concentration-response relationships of C21 steroids appear to parallel each other with the response curves of those compounds most unlike 17α,20βP being shifted farthest to the right. Third, adaptation to 10^{-8} *M* 17α,20βP completely suppressed responses to 10^{-7} *M* 17αP, and 10^{-8} *M* 17α,20β,21P; and it partially suppressed responses to 10^{-7} *M* 17α,20β,21P and 10^{-7} *M* P. Fourth, all C21 steroids (with the possible exception of P) appear capable of eliciting equivalent GtH responses when supra-threshold concentrations are tested, suggesting that they activate the same neural pathway(s). Final proof that olfactory specificity is attributable to ligand-receptor interactions and not a shared transduction mechanism will require radioligand binding studies using isolated olfactory receptors. This work is in progress and initial results suggest that the receptors are in fact responsible for this specificity (Rosenblum and Sorensen, unpublished results).

This level of specificity appears unprecedented for a fish olfactory system. Amino acid sensitivity has been extensively characterized electrophysiologically, biochemically and behaviorally in several species of fish (Cagan and Zeiger 1978; Hara 1982; Caprio and Byrd 1984; Ohno et al. 1984; Rehnberg and Schreck 1986; Bruch and Rulli 1988; Sveinsson and Hara 1990) and found to be mediated by at least 3 classes of olfactory sites with overlapping sensitivities. Similarly, responses to the goldfish postovulatory pheromone appear to be mediated by 2 classes of receptors which cross-react extensively at concentrations more than 2 log units above their threshold (Sorensen et al. 1988). As more vertebrate pheromone systems are characterized it will be interesting to see whether they share this degree of specificity.

There are several indications that the goldfish olfactory epithelium may also possess a class of olfactory receptors for steroids with 19 carbons (C19 steroids). Adaptation to 17α,20βP did not suppress responses to the C19 compound androstenedione (A). EOG responses to androstenedione also appeared to be slower and less phasic in character than responses to C21 steroids. Similarly, responses to A appeared to saturate at 10^{-7} *M* in spite of relatively small response size; its concentration-response relationship does not run parallel to and to the right of 17α,20βP. Although the ANOVA did not find the EOG responses elicited by A to be significant, this was almost certainly a statistical artifact caused by this test's lack of power; every time we tested A ($n=8$) it elicited a clear response. Tests of endocrine responsiveness to water-borne A have consistently shown that detectable concentrations of A do not stimulate either GtH release or milt production (Stacey and Sorensen 1986, unpublished results); this steroid does not activate the neural system responsible for 17α,20βP responses. Finally, the equivocal EOG, endocrine, and milt (Stacey and Sorensen 1986) responses to progesterone are interesting because this compound has structural similarities to both A and 17α,20βP (it has 21 carbons yet lacks both the 17α and 20β hydroxyl groups and has a 20 ketone group which is somewhat analogous to the C17 ketone group on A). Possibly this steroid is recognized by both the 17α,20βP and A receptor mechanisms?

As measured by EOG recording, 17α,20βP had a detection threshold of approximately 10^{-12} *M*. Although this threshold is an order of magnitude higher than that reported earlier (Sorensen et al. 1987), the form and magnitude of the 17α,20βP concentration-response curves were similar in both studies. Earlier EOG studies using mature females also reported a lower threshold for P (Sorensen et al. 1987) than that reported here for males. During the course of this study we examined the responses of immature females to P and found them to be no different than those of males (data not shown). We have no explanation for this discrepancy between the two studies but favor the present findings because of the much larger sample size (7 vs. 3 fish).

Endocrine measures of pheromone responsiveness suggest that although EOG recording accurately reflects whole animal sensitivity to pheromones, even brief exposure to supra-threshold pheromone pulses may evoke large responses. When concentrated 17α,20βP was added to tanks of male goldfish a response threshold of 10^{-13}–10^{-12} *M* was indicated, an order of magnitude below that indicated both by EOG recording and by the experiments in which fish were placed into pre-diluted 17α,20βP concentrations. With the exception of the response to 10^{-13} *M* in the first experiment (which was characterized by high variability), all supra-threshold exposures to 17α,20βP elicited equivalent GtH increases in both experiments; response magnitude did not increase with stimulus strength. Exposure time (at least beyond 15 min) also does not seem to influence response magnitude (Dulka et al. 1987a). 17α,20βP appears to act as a neuroendocrine trigger which elevates blood GtH release for at least several hours.

Endocrine and milt responses to C21 steroids also supported the extreme olfactory specificity indicated by EOG recording and confirmed that responses to water-borne C21 steroids are rapid. Although the endocrine and milt responses of male fish exposed to pre-diluted steroid solutions were somewhat more sensitive than predicted by EOG recording, the pattern of responsiveness strongly confirmed EOG specificity. In contrast, when 10^{-8} *M* steroid solutions were added to tanks containing fish, the response appeared almost completely nonspecific, a surprising finding because many of these compounds were barely detectable by EOG at 10^{-8} *M*. Although the precise latency of the endocrine response to 17α,20βP has yet to be determined, these results suggest strongly that it is very brief, perhaps less than a minute. Although the discrepancy between EOG and endocrine sensitivity to C21 steroids could be attributed to the involvement of a non-olfactory sensory system, the complete failure of olfactory tract sectioned animals to respond to 17α,20βP (Stacey and Sorensen 1986; Dulka et al., unpublished results), indicates that, as suggested by other studies (Hara 1975), electrical recording tends to slightly underestimate whole animal sensitivity. Milt production closely paralleled GtH responses in these experiments suggesting that increased sperm production (which may be the most relevant aspect of the pheromone response to the animal) is closely associated with the endocrine response.

This study appears to have demonstrated that a fish's olfactory system can recognize a singular compound of special significance with great sensitivity and specificity. While this specificity could enable the goldfish preovulatory pheromone system to function in a precise, perhaps species-specific manner, the speed of the response means that the dynamics of pheromone dissemination and exposure are likely to determine whether this potential is realized. Although we have calculated that goldfish pheromonal plumes are probably short-lived and only capable of stimulating males within several cm of the donor female, this remains to be demonstrated (Sorensen and Stacey 1990). Certainly the fact that 17α,20βP is synthesized (and likely released) by many fish (Scott and

Canario 1987) would appear to make it unlikely that 17α,20βP per se functions as a species-specific pheromone but rather that it functions as precise indicator of reproductive condition to proximate conspecifics. The answer to this question will only become apparent when we gain an understanding of the hormone metabolism and release in fish. Finally, the potency of both the 17α,20βP (this study) and prostaglandin pheromones (Sorensen et al. 1988) call into question the biological relevance of studying electrophysiological responses to high concentrations of olfactory stimulants which the animal probably never encounters.

In the process of describing the specificity of the 17α,20βP receptor mechanism, this study both confirms the probability that 17α,20βP is the major constituent of the preovulatory pheromone, and raises the possibility that goldfish may use other unrelated steroidal pheromones which are recognized by different mechanisms and have different actions. Clearly, the significance of the androstenedione response must be investigated. Yamazaki and Watanabe (1979) noted that hypophysectomized, estrogen-treated female goldfish released an attractive odor which they suggested to be a pheromone identifying gender. Could it have been related to the A response? It will be especially important to determine whether goldfish detect conjugated steroids. Studies of several fish have implicated glucuronated androgens and progestins as pheromones (Colombo et al. 1980; van den Hurk and Lambert 1983; Resink 1988). Furthermore, recent EOG studies of the African catfish, *Clarias gariepinus* have shown that it detects 5β-pregnane-3α,17α-diol-20-one-3α-glucuronide at concentrations as low as 10^{-11} *M* (Resink 1988). Lastly, we know that goldfish produce conjugated steroids (Yano and Ishio 1978a, b, c; Van Der Kraak et al. 1989; Stacey et al. 1989). Although we have tested several glucuronated androgens and estradiol and found them non-stimulatory (Sorensen et al. 1987), we have been unable to test conjugates of 17α,20βP and related C21 steroids because they are not yet commercially available. The specificity of the 17α,20βP receptor suggests that, if these compounds are recognized, it will be by a different class of receptors.

In conclusion, the extreme specificity of the goldfish olfactory system to 17α,20βP has several implications: 1) water-borne 17α,20βP and possibly its metabolite, 17α,20β,21P, have the potential to serve as highly specific, discrete chemical cues signaling impending ovulation, 2) goldfish do not appear capable of recognizing complex mixtures of C21 steroids although they do appear capable of recognizing C19 steroids, 3) if different species use different classes of sex steroids as pheromones, species-specificity is probably assured, 4) future electrophysiological studies of steroidal pheromones in other teleosts should also expect to encounter extreme olfactory specificity. It becomes increasingly apparent that hormonal metabolites are likely to serve as sex pheromones in many species of fish.

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