BIOLOGY OF REPRODUCTION 39, 1039–1050 (1988)

F Prostaglandins Function as Potent Olfactory Stimulants That Comprise the Postovulatory Female Sex Pheromone in Goldfish[1]

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ABSTRACT

This study establishes that ovulated female goldfish release F type prostaglandins (PGFs) to the water where they stimulate male spawning behavior and comprise the goldfish postovulatory pheromone. We first demonstrated that ovulated and prostaglandin-injected female goldfish release immunoreactive PGFs to the water. Next, using electro-olfactogram recording (EOG), we determined that waterborne prostaglandins function as potent olfactory stimulants for mature male goldfish. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and its metabolite 15-keto-prostaglandin $F_{2\alpha}$ (15K-$PGF_{2\alpha}$) were the most potent prostaglandins; the former had a detection threshold of 10^{-10} M and the latter a detection threshold of 10^{-12} M. Studies of prostaglandin-injected fish indicated that PGF metabolites are an important component of the pheromone. Cross-adaptation experiments using the EOG demonstrated that goldfish have separate olfactory receptor sites for $PGF_{2\alpha}$ and 15K-$PGF_{2\alpha}$ that are independent from those that detect other olfactory stimulants. Finally, we established that male goldfish exposed to low concentrations of waterborne PGFs exhibit reproductive behaviors similar to those elicited by exposure to the odor of ovulated fish. Together with our recent discovery that a steroidal maturational hormone functions as a preovulatory "priming" pheromone for goldfish, these findings suggest that hormones and their metabolites may commonly serve as reproductive pheromones in fish.

INTRODUCTION

Recently ovulated goldfish, like many teleost fish, release a pheromone(s) that rapidly stimulates male spawning behavior (Partridge et al., 1976; Liley, 1982; Stacey et al., 1986). The behavioral or "releasing" actions of this pheromone contrast with the physiological or "priming" actions of another pheromone that is released by female goldfish prior to ovulation. We recently discovered that preovulatory goldfish release the oocyte maturational steroid hormone, 17α,20β-dihydroxy-4-pregnen-3-one (17,20P), to the water where it functions as a pheromone. Waterborne 17,20P stimulates the endocrine system of mature males via their olfactory system to increase milt (sperm and seminal fluid) production by the time of spawning (Stacey and Sorensen, 1986; Dulka et al., 1987; Sorensen et al., 1987). In this study, we hypothesized that the goldfish postovulatory pheromone (the specific chemical signal that differentiates ovulated from nonovulated

Accepted July 13, 1988.
Received March 1, 1988.
[1] This study was supported by the Alberta Heritage Foundation for Medical Research (Fellowship to P. W. S.), the Department of Fisheries and Oceans Canada (Contract# FP430-6-9064/01-1SF to P. W. S. and T. J. H.), the Natural Sciences and Engineering Council of Canada (Grant# A7576 to T. J. H. and #A2903 to N. E. S.), and the National Science Foundation (Grant#DCB-8517718 to F. W. G.).
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goldfish) is likely to be a hormone or hormone-like compound closely associated with ovulation itself. 17,20P is a poor candidate for the postovulatory pheromone because its synthesis and release are declining by the time of ovulation (Dulka et al., 1987), and because waterborne 17,20P has only minor effects on male behavior (Sorensen, unpublished results).

Kittredge et al. (1971) first suggested that aquatic organisms are likely to have evolved to use hormones and their metabolites as sex pheromones because they represent pre-existing and relevant chemical cues whose detection need only involve an externalization of internal hormone receptor mechanisms. More recently, Doving (1976) suggested that this possibility might also apply for teleost fish. Although several studies have since implicated steroidal pheromones in fish (Colombo et al., 1982, van den Hurk and Lambert, 1983; Lambert et al., 1986; van den Hurk et al., 1987; Stacey et al., 1987), no study, with the exception of ours on the goldfish preovulatory pheromone, has simultaneously demonstrated pheromone release, olfactory sensitivity, and a relevant biological response (i.e., a neuroendocrine reflex or sexual arousal). Similarly, no previous study has identified a fish postovulatory pheromone.

We suspected for several reasons that the goldfish postovulatory pheromone could consist of prostaglandins (PGs). First, circulating and ovarian levels of F type prostaglandins (PGFs) increase in goldfish (and other teleosts) at the time of ovulation (Bouffard, 1979: Cetta and Goetz, 1982; Goetz, 1983), probably reflecting a role in modulating follicular rupture (Dennefors et al., 1983; Goetz, 1983). Second, circulating prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), apparently the most abundant PGF in goldfish (Bouffard, 1979; Stacey and Goetz, 1982; Goetz, 1983), acts as a hormonal signal that triggers female spawning behavior in goldfish through direct actions on the brain (Stacey and Peter, 1979). Third, non-ovulated goldfish injected with $PGF_{2\alpha}$ not only exhibit normal female spawning behavior (Stacey, 1976; Stacey and Goetz, 1982), but also release an odor that elicits male reproductive behaviors identical to those elicited by the odor of ovulated females (Sorensen et al., 1986). Spawning males do not distinguish between naturally ovulated females and $PGF_{2\alpha}$-injected females (Stacey, 1981).

In particular, we believed that $PGF_{2\alpha}$ metabolites might be an important component of the postovulatory pheromone because, where studied in mammals, PGs have been found to be rapidly metabolized and excreted (Samuelsson et al., 1975; Hoult and Moore, 1977; Granstrom and Kindahl, 1982). Although little is known about PG metabolism in fish, both the short duration of the spawning response elicited by $PGF_{2\alpha}$ injection (Stacey and Goetz, 1982) and the fact that indomethacin (a cyclo-oxygenase inhibitor) treatment quickly blocks spawning in ovulated females (Stacey, 1976) indicate that goldfish also metabolize $PGF_{2\alpha}$ rapidly.

Several predictions arose from our hypothesis that prostaglandins and/or their metabolites function as the goldfish postovulatory pheromone. First, recently ovulated and $PGF_{2\alpha}$-injected goldfish should release PGFs to the water. Second, the olfactory sense of mature males should detect waterborne prostaglandins because olfactory ablation blocks the responsiveness of goldfish to pheromones (Partridge et al., 1976; Stacey and Kyle, 1983). Third, $PGF_{2\alpha}$-injected goldfish should release a potent olfactory stimulant(s) that is detected by olfactory receptors that also respond to prostaglandins. Fourth, those olfactory receptors that respond to waterborne prostaglandins should be relatively specific and not respond to other odorants. Fifth, mature male goldfish exposed to physiological concentrations of waterborne PGFs should exhibit the same reproductive behaviors elicited by exposure to water from ovulated females. This study sought to test these predictions using a variety of techniques, including radioimmunoassay of water samples, electrophysiological recording, and behavioral observation.

MATERIALS AND METHODS

Prostaglandin Release by Ovulated and Prostaglandin-Injected Goldfish

We first sought to determine whether ovulated female goldfish release PGFs to the water. Mature vitellogenic females (Ozark Fisheries, Stoutland, MO) were induced to ovulate using an established protocol (Stacey et al., 1979). Briefly, they were moved from stock tanks (14°C, 16L:8D photoperiod, lights on at 0800 h) into 70-l flow-through aquaria (20°C; 16L:8D) containing aquatic vegetation (spawning substrate) at 2100 h on Day 1; on the morning of Day 3, they were checked for ovulation by applying gentle pressure to their abdomens. Ovulated fish were

divided into 2 groups: one group was kept isolated from males ("ovulated") and the other was allowed to spawn with males for several hours until they had released all their eggs ("spawned-out"). Nonovulated, ovulated, and spawned-out fish were then placed into individual glass jars containing 1.5 l of dechlorinated, aerated water. After 2 h, 10 ml of water was collected from each jar and frozen.

To determine if $PGF_{2\alpha}$ is released/excreted by $PGF_{2\alpha}$-injected fish, nonovulated female goldfish were injected i.m. with either 10 μg $PGF_{2\alpha}$ in 10 μl of saline buffer (an amount equivalent to that used to evoke female spawning behavior and pheromone release [Stacey and Goetz, 1982; Sorensen et al., 1986]), or buffer alone, placed into jars, and their water was collected and frozen 2 h later. Water samples from both the ovulation and $PGF_{2\alpha}$-injection experiments were later thawed, acidified to a pH of 3.5 with 0.1 N HCl, and split into 2 aliquots, each of which was extracted with 5 ml of ethyl acetate. The ethyl acetate was evaporated under a stream of nitrogen at 37°C and assayed for $PGF_{2\alpha}$ ("immunoreactive PGF") using an established protocol (Cetta and Goetz, 1982). Siliconized glassware was used for sample collection, extraction, and radioimmunoassay. Extraction efficiency for $PGF_{2\alpha}$ was estimated to be greater than 90%. The antiserum employed cross-reacts 100% with $PGF_{2\alpha}$; 24% with $PGF_{1\alpha}$; 0.01% with PGE_1, PGE_2, and PGA_2; and 0.23% with 15-keto-prostaglandin $F_{2\alpha}$, a metabolite of $PGF_{2\alpha}$.

Olfactory Sensitivity of Male Goldfish to Waterborne Prostaglandins

The olfactory sensitivity of mature male goldfish (fish with tubercles and from which sperm could be expressed; Ozark Fisheries) to waterborne prostaglandins was determined by recording electro-olfactogram (EOG) responses. The EOG is a multiunit transepithelial voltage transient recorded from the surface of the olfactory epithelium and reflects olfactory receptor potentials (Ottoson, 1971; Getchell, 1974). A protocol previously used to record goldfish olfactory responses was employed (Sorensen et al., 1987). Briefly, mature male goldfish were immobilized with an i.m. injection of Flaxedil (gallamine triethiodide; 3 mg/kg body wieght; Rhone-Paulene Pharmacie, Montreal Que., Canada), placed on a stand, and their gills and nares were perfused with dechlorinated 11°C water. These animals were not anesthetized because tranquilizing doses of anesthetic destroy fish olfactory epithelia and reduce olfactory sensitivity (Lewis et al., 1985). Five-second pulses of odorants were introduced into the flow perfusing the nares by using an apparatus designed to minimize pressure and temperature fluctuations (Evans and Hara, 1985). The EOG response was recorded differentially using Ag/AgCl electrodes (WPI Type EH-lF) bridged to saline/gelatin-filled glass pipettes (tip diameter 60–80 μm). The recording electrode was positioned just above the olfactory epithelium, and the reference electrode was placed on the skin surface (noninvasive procedures). As in previous experiments (Sorensen et al., 1987), the recording electrode was positioned at the location that produced a maximal response to the 10^{-5} M L-serine standard and a minimal response to a "blank" water (no odorant added) control. The fish were grounded. Electrical signals were amplified by a DC-preamplifier (Grass 7Pl) and displayed on a pen recorder.

Test solutions were prepared by dissolving prostaglandins (Cayman Chemical Co., Ann Arbor, MI) in glass-distilled methanol at 1 mg/ml and placing 17 μl into 50 ml of dechlorinated water to create a 10^{-6} M dilution that was then progressively diluted to create a concentration series. Because methanol was detectable in 10^{-6} M PG solutions (7×10^{-4} M methanol), responses to a methanol control were tested in conjunction with each series of solutions. A blank water control (no odorant added) and an amino acid standard (10^{-5} M L-serine; Sigma Chemical Co., St. Louis, MO) were also tested at the beginning and end of each concentration series. L-Serine was chosen as the standard because its potency as an odorant for goldfish is representative of other L-amino acids and because we used it as a standard in earlier studies (Sorensen et al., 1987). Amino acids are important components of food odors and are potent olfactory stimulants for fish (Caprio, 1984; Hara, 1986). Each test stimulus was tested 3 times, and 2 min were allowed between stimuli for recovery. Responses were averaged and then expressed as a percentage of the response elicited by the most recent 10^{-5} M L-serine after responses to the most recent blank water control had been subtracted. Responses to 10^{-6} M solutions were corrected to remove that portion of the response attributable to methanol. Responses were tested to all 5 prostaglandins ($PGF_{1\alpha}$, $PGF_{2\alpha}$, $PGF_{3\alpha}$, PGE_1, and PGE_2) found in fish (Stacey and Goetz, 1982).

Pheromone Release by $PGF_{2\alpha}$-Injected Fish and Olfactory Sensitivity to $PGF_{2\alpha}$ Metabolites

To test the possibility that female goldfish release PGF metabolites we determined the olfactory potency of water collected from $PGF_{2\alpha}$-injected fish and compared its potency to that of $PGF_{2\alpha}$ added directly to the water. Twenty-five-gram females with nonvitellogenic ovaries were injected i.m. with either 10 μg $PGF_{2\alpha}$ (the dose used earlier to measure immunoreactive PGF release) or 10 μl buffered saline, rinsed, and placed into beakers containing 1.5 l of dechlorinated water (11°C) for 45 min. EOG responses of mature males to these water samples were recorded using the established protocol. Because undiluted water evoked extremely large responses, both water samples were diluted 10 and 100 times with dechlorinated water for testing. Responses to saline- and $PGF_{2\alpha}$-injected fish water were expressed relative to a 10^{-5} M L-serine control and were compared using paired *t*-tests.

Because the EOG responses elicited by $PGF_{2\alpha}$-injected fish water were too large to be attributable to $PGF_{2\alpha}$ alone (see Results), we next determined EOG responses of mature males to $PGF_{2\alpha}$ metabolites. The only known study of $PGF_{2\alpha}$ metabolism in fish found that goldfish ovaries produce 15-keto-prostaglandin $F_{2\alpha}$ *in vitro* (Goetz, unpublished results). 15-Keto-prostaglandin $F_{2\alpha}$ (15K-$PGF_{2\alpha}$) and 13, 14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ (13, 14-15K-$PGF_{2\alpha}$) are also the initial metabolites of $PGF_{2\alpha}$ in a variety of mammals (Samuelsson et al., 1975; Hoult and Moore, 1977; Granstrom and Kindahl, 1982). EOG responses of mature males were determined to both of these compounds as well as to arachidonic acid, the precursor of $PGF_{2\alpha}$.

Specificity of Prostaglandin Olfactory Receptors to $PGF_{2\alpha}$-Injected Fish Water, PGFs, and Other Odorants

To verify whether the EOG response to $PGF_{2\alpha}$-injected fish water was attributable to the olfactory receptors responding to PGFs a cross-adaptation experiment was conducted. In cross-adaptation, the EOG response to a test odorant is first measured, and then the olfactory epithelium is perfused with an adapting odorant to which pulses of the test odorant (made up in the adapting stimulus) are added (see Caprio and Byrd, 1984). Reductions in EOG response magnitude to the test odorant during adaptation are interpreted as reflecting the extent to which olfactory receptors responding to the adapting stimulus also respond to the test stimulus. We adapted the olfactory epithelia of male goldfish to either 10^{-8} M $PGF_{2\alpha}$ or 10^{-7} M 15K-$PGF_{2\alpha}$, and tested 10% dilutions of saline- and $PGF_{2\alpha}$-injected female water to see if adaptation to PGFs would selectively reduce responses elicited by $PGF_{2\alpha}$-injected fish water. Responses were analyzed in mV rather than relative to L-serine, because it could not be assumed that responses to the L-serine standard were not influenced by adaptation. Adaptation was initially verified by demonstrating that responses to 10^{-7} M 15K-$PGF_{2\alpha}$ and 10^{-8} M $PGF_{2\alpha}$ were abolished by adaptation to equal concentrations of themselves.

Although the different EOG concentration-response relationships to $PGF_{2\alpha}$ and its metabolites suggested that goldfish have more than one class of olfactory receptors for PGFs, the specificity of these receptors remained to be demonstrated. Therefore, the cross-adaptation experiments were extended to include a variety of PGs as well as representatives of the three established categories of potent olfactory stimulants in fish (Hara, 1986; Sorensen et al., 1987): a representative amino acid (10^{-5} M L-serine), bile acid (10^{-5} M taurocholic acid made up as a sodium salt; Sigma Chemical Co.), and a steroid (10^{-8} M 17,20P; Sigma Chemical Co.). Because the only structural similarity between PGs and amino acids is a terminal carboyxl group, a feature also shared by arachidonic acid—which is a poor olfactory stimulant, and the receptor types proposed for different amino acids are not mutually exclusive (Hara, 1982; Caprio and Byrd 1984), we did not test other amino acids. Last, to determine specifically whether goldfish possess separate classes of olfactory receptors for $PGF_{2\alpha}$ and 15K-$PGF_{2\alpha}$, various concentrations of each compound were tested while a fish was adapted to the other PGF.

Behavioral Responses of Male Goldfish to Waterborne Prostaglandins

Finally, a behavioral experiment was conducted to determine whether male goldfish exhibit reproductive behavior when exposed to waterborne PGFs. Male reproductive behavior is characterized by extensive chasing of females and constant nudging of their ovipores and sides (as if trying to identify the pheromone source; Partridge et al., 1976). If receptive, females allow themselves to be pushed into aquatic weeds where, in a reflexive action, they oviposit in

synchrony with the release of sperm by a companion male(s) (Breder and Rosen, 1966; Partridge et al., 1976; Stacey and Kyle, 1983). Spawning is chaotic with groups of males occasionally pursuing other males and/or nonovulated females (Partridge et al., 1976; personal observations). Single males exposed to the odor of ovulated females or $PGF_{2\alpha}$-injected females become more active, feed less, are attracted to the odor source, and interact with visual images of females (Partridge et al., 1976; Sorensen et al., 1986). Grouped males exposed to these odors exhibit dramatic increases in nudging and chasing; these behaviors are not elicited by exposure to food odor, the odor of nonovulated females, or the odor of waterborne 17, 20P (Sorensen et al., 1986).

The protocol of the behavioral experiments used here is based directly on that used in earlier tests of grouped males (Sorensen et al., 1986). Females were not included in these groups because sexually receptive females would act as an independent pheromone source, and nonreceptive females represent an uncontrolled behavioral variable that we know occasionally elicits some chasing. Tests using grouped males offered a controlled, repeatable means with which we could test whether the behavioral responses elicited by exposure to waterborne PGFs were reproductive behaviors.

Groups of 5 males were held overnight in flow-through 70-l aquaria (20°C; 16L:8D) that contained gravel and aquatic vegetation (spawning substrate). The next morning, swimming activity (number of times fish crossed a vertical midline on the tank glass), feeding activity (number of times fish picked up bottom gravel presumably searching for food), and the number of nudges (social contact) were observed for a 15-min pre-test during which an ethanol control (10^{-6} M or less) was pumped by peristaltic pump into the aquaria through aerators at 10ml/min. A 15-min experimental period started when the input solution was switched to 10^{-8} M $PGF_{2\alpha}$, 10^{-8} M 15K-$PGF_{2\alpha}$, or another ethanol control. Observers were unaware of the treatment given. Although final tank concentration was only 0.4% that of the concentration injected, dye injection indicated that males repeatedly encountered "wisps" of slightly diluted odorant. These testing procedures were repeated using different groups of fish and progressively decreasing concentrations of PGFs until responses were no longer observed. Results were analyzed by the signed-ranks test for dependent samples.

RESULTS

Prostaglandin Release

Approximately 20 times more immunoreactive PGF was found in water collected from ovulated females than in water from either nonovulated fish or spawned-out fish who released equivalent, marginally detectable levels (Table 1). High levels of immunoreactive PGF were also found in water samples from those fish injected with $PGF_{2\alpha}$; approximately 2% of the injected $PGF_{2\alpha}$ had been released.

Olfactory Sensitivity of Male Goldfish to Prostaglandins

The olfactory epithelium of male goldfish was acutely sensitive to all PGs tested, especially $PGF_{2\alpha}$ (Fig. 1a). $PGF_{2\alpha}$ had a detection threshold of approximately 10^{-10} M, and at a concentration of 10^{-6} M, where response magnitude appeared to be saturating, it elicited responses more than 3 times those elicited by 10^{-5} M L-serine. Concentration response curves for $PGF_{1\alpha}$, $PGF_{3\alpha}$, and PGE_2, which differ structurally from $PGF_{2\alpha}$ at one position ($PGF_{1\alpha}$ has an extra double bond; $PGF_{3\alpha}$ lacks a double bond; PGE_2 has a ketone group substituted for a hydroxyl group), were similar to the $PGF_{2\alpha}$ curve in shape but shifted to the right by one log unit. PGE_1, which differs from $PGF_{2\alpha}$ at two positions, was the least stimulatory (Fig. 1a). A structural isomer of $PGF_{2\alpha}$, 11β-$PGF_{2\alpha}$, was also considerably less stimulatory than $PGF_{2\alpha}$, and its response curve was similar to that of PGE_1 (data not shown). EOG responses elicited by exposure to PGs had relatively large phasic components and small tonic components, but were fundamentally similar to the EOGs elicited by exposure to other odorants (Fig. 2).

TABLE 1. Immunoreactive F prostaglandin (PGF) (ng) released by female goldfish during a 2-h period (x ± SEM).

Group	Sample size	PGF (ng)
Nonovulators	(10)	1.6 ± 0.19
Ovulated	(5)	23.8 ± 7.50**
Spawned-out	(6)	2.1 ± 0.51
Saline-injected	(2)	2.3 ± 0.48
$PGF_{2\alpha}$-injected	(3)	142.8 ± 21.74+

**$p \leq 0.01$ when compared to nonovulators and spawned-out fish.

+$p \leq 0.05$ when compared to saline-injected fish.

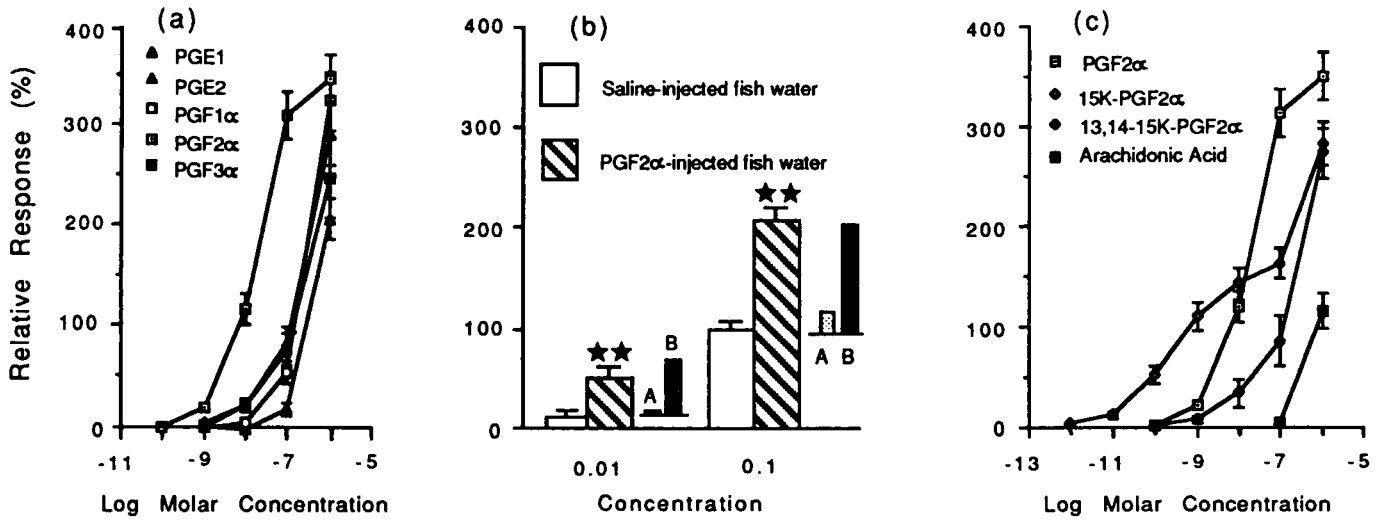

FIG. 1. *(a)* Semi-logarithmic plot of the electro-olfactogram recording (EOG) responses of male goldfish to waterborne prostaglandins (PGs). Average response magnitude is represented as a percentage of that induced by 10^{-5} M L-serine. *Vertical bars* represent standard error. *Abbreviations* are as in the *text*. Sample size is 8–19 fish. *(b)* Average EOG responses (± SEM) of male goldfish to water containing saline- and $PGF_{2\alpha}$-injected female goldfish *(wide bars)* that has been diluted 10 (0.1; n = 17) and 100 times (0.01; n = 7). Responses to saline- and $PGF_{2\alpha}$-injected fish water are compared by paired *t*-tests (**$p \leq 0.01$). Average response magnitude (± SEM) is represented as a percentage of that induced by 10^{-5} M L-serine. The *narrow bars* represent the hypothetical EOG responses that $PGF_{2\alpha}$-injected fish water would have evoked if half the injected $PGF_{2\alpha}$ had been released as either $PGF_{2\alpha}$ *(A)* or its metabolite 15-keto-prostaglandin $F_{2\alpha}$ *(B*; see *Fig. 1c* and *text)*. Their *bases* correspond to the level of responses evoked to control (saline-injected) female water. *(c)* Semi-logarithmic plot of the EOG responses of male goldfish to $PGF_{2\alpha}$, its metabolites and its precursor, arachidonic acid. Average response magnitude (± SEM) is represented as a percentage of that induced by 10^{-5} M L-serine. These data were collected from the same fish used in *Figure 1a* and *1b*, and the values plotted for $PGF_{2\alpha}$ are the same. Sample size is 8–19.

Pheromone Release by $PGF_{2\alpha}$-Injected Goldfish and Olfactory Sensitivity to $PGF_{2\alpha}$ Metabolites

$PGF_{2\alpha}$-injected fish water consistently evoked greater EOG responses than water from saline-injected fish ($p \leq 0.01$ for both 0.1 and 0.01 dilutions), confirming that $PGF_{2\alpha}$ injection evokes the release of a potent olfactory stimulant(s) (Fig. 1b). However, simple release and/or leakage of the injected $PGF_{2\alpha}$ could not explain the magnitude of EOG responses. If half of the $PGF_{2\alpha}$ had been released directly to the water (a liberal estimate; see Table 1) it would have had a concentration of 10^{-8} M. Accordingly, the 0.10 dilution of $PGF_{2\alpha}$-injected fish water would have contained 10^{-9} M $PGF_{2\alpha}$, and EOG responses to it should have been only marginally larger than those elicited by saline-injected fish. Similarly, the 0.01 concentration of $PGF_{2\alpha}$-injected fish water would have contained only 10^{-10} M $PGF_{2\alpha}$, an undetectable concentration (Fig. 1b). These findings suggested that the pheromone released by $PGF_{2\alpha}$-injected fish contained components other than $PGF_{2\alpha}$.

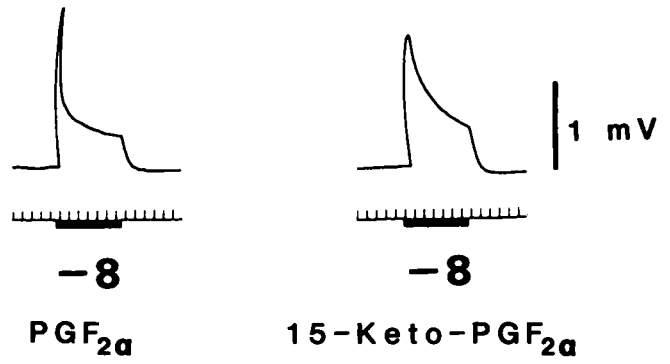

FIG. 2. Electro-olfactogram recording (EOG) responses of a mature male goldfish to 10^{-8} M $PGF_{2\alpha}$ and 10^{-8} M 15K-$PGF_{2\alpha}$. A longer exposure time (30 s) was used to allow the phasic (initial sharp component) and tonic (subsequent plateau) components of the EOGs to be visualized. Responses were recorded on curvilinear graph paper. Time signals, each division = 5s.

EOG responeses recorded to $PGF_{2\alpha}$ metabolites found 15K-$PGF_{2\alpha}$ to be an exceedingly potent odorant with a detection threshold of 10^{-12} M, 100 times lower than that of $PGF_{2\alpha}$ (Figs. 1c and 3). EOG responses to 15K-$PGF_{2\alpha}$ appeared to approach saturation at a concentration of 10^{-9} M, where they evoked responses slightly larger than 10^{-5} M L-serine. The shape of the EOG response to 15K-$PGF_{2\alpha}$ was more rounded (reflecting a slower response) than that elicited by $PGF_{2\alpha}$ (Fig. 2). In theory, if half of the $PGF_{2\alpha}$ injected into female fish had been metabolized and released as 15K-$PGF_{2\alpha}$, the differences between the EOG responses elicited by $PGF_{2\alpha}$-

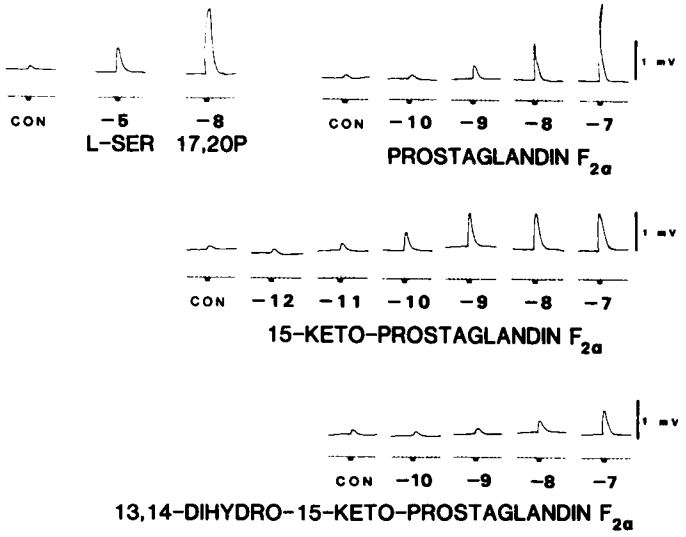

FIG. 3. Representative electro-olfactogram recording (EOG) responses of a mature male goldfish to log molar concentrations of prostaglandin (PG) $F_{2\alpha}$, 15K-$PGF_{2\alpha}$, 13, 14-15K-$PGF_{2\alpha}$, L-serine (L-Ser), 17,20P, and controls (Con; no odor added). Responses to 10^{-6} M PGFs are not shown because a portion of these responses are attributable to methanol carrier. Curvilinear graph paper was used. Time signals, each division = 5s.

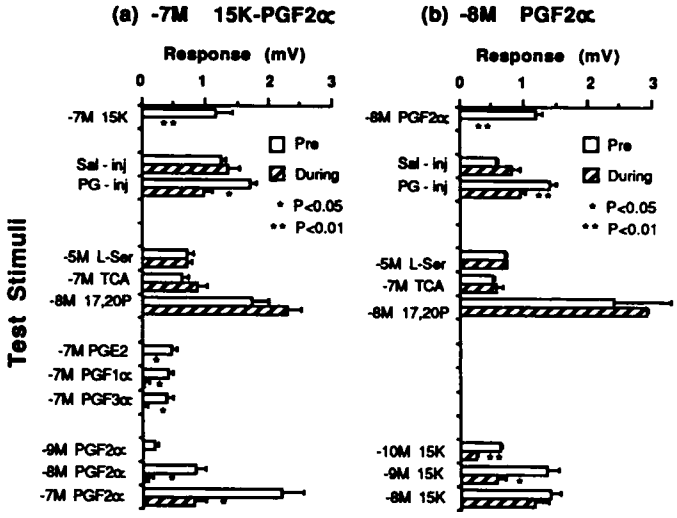

FIG. 4. Electro-olfactogram recording (EOG) responses elicited (in mV) prior to adaptation *(light-colored bars)* and later during adaptation *(shaded bars)* to either *(a)* 10^{-7} M 15K-$PGF_{2\alpha}$, or *(b)* 10^{-8} M $PGF_{2\alpha}$. Stimuli concentrations are log molar. *Abbreviations* are as given in the text with the following exceptions: *Sal-inj* = 0.10 solution (10% dilution) of saline-injected fish water; *PG-inj* = 0.10 solution of $PGF_{2\alpha}$-injected fish water; *L-Ser* = L-serine; *TCA* = taurocholic acid; *15K* = 15K-$PGF_{2\alpha}$. *"Pre"* and *"During"* responses were compared by paired *t*-tests (*$p \leq 0.05$; **$p \leq 0.01$). Sample size ranged from 4 to 6 fish.

and saline-injected fish water would be explained (Fig. 1b). 13,14-15K-$PGF_{2\alpha}$ was a relatively weak olfactory stimulant with a potency less than that of $PGF_{2\alpha}$ and similar to that of L-serine and other amino acids; it could not explain the EOG response elicited by $PGF_{2\alpha}$-injected fish. Arachidonic acid was a very weak olfactory stimulant, with a threshold of approximately 10^{-7} M.

Specificity of Olfactory Receptors to $PGF_{2\alpha}$-Injected Fish Water and PGFs

In the cross-adaptation experiments, neither adaptation to 10^{-7} M 15K-$PGF_{2\alpha}$ nor adaptation to 10^{-8} M $PGF_{2\alpha}$ decreased the EOG responses to saline-injected fish water (Fig. 4). In contrast, adaptation to $PGF_{2\alpha}$ and 15K-$PGF_{2\alpha}$ reduced ($p \leq 0.05$) the responses evoked by $PGF_{2\alpha}$-injected fish water. Adaptation to 10^{-9} M 15K-$PGF_{2\alpha}$ also reduced responses to $PGF_{2\alpha}$-injected fish water, suggesting that 15K-$PGF_{2\alpha}$ was an important constituent of $PGF_{2\alpha}$-injected fish water (data not shown).

In other cross-adaptation experiments, responses to 10^{-5} M L-serine, 10^{-7} M taurocholic acid, and 10^{-8} M 17,20P were not reduced by adaptation to either $PGF_{2\alpha}$ or 15K-$PGF_{2\alpha}$ (Fig. 4). Conversely, EOG responses to $PGF_{1\alpha}$, $PGF_{3\alpha}$, and PGE_2 were all abolished during adaptation to equal concentrations of both 15K-$PGF_{2\alpha}$ and $PGF_{2\alpha}$ (Fig. 4; the data for adaptation to 10^{-7} M $PGF_{2\alpha}$ are not shown), suggesting that responses elicited by these compounds are attributable to olfactory receptors that are most sensitive to $PGF_{2\alpha}$ and 15K-$PGF_{2\alpha}$. Lastly, although adaptation to 15K-$PGF_{2\alpha}$ and $PGF_{2\alpha}$ reduced responses elicited by exposure to lower concentrations of the other PGF, adaptation never eliminated responses to equal concentrations of the other PGF.

Behavioral Responses to Waterborne PGFs

Exposure to 10^{-8} M $PGF_{2\alpha}$ and 10^{-8} M 15K-$PGF_{2\alpha}$ evoked immediate and dramatic increases in swimming and social activity (nudging) of male goldfish, which coincided with decreases in feeding (Fig. 5). Exposure to ethanol control did not affect the behavior of male goldfish. The activity evoked by waterborne PGFs was characterized by chasing, a behavior characteristic of sexual arousal (data not shown). The behavioral thresholds for responses elicited by $PGF_{2\alpha}$ was 10^{-8} M and 10^{-10} M for 15K-$PGF_{2\alpha}$. There was no apparent difference in the nature of the responses elicited by exposure to $PGF_{2\alpha}$ and 15K-$PGF_{2\alpha}$. Responses to both compounds were relatively short in duration,

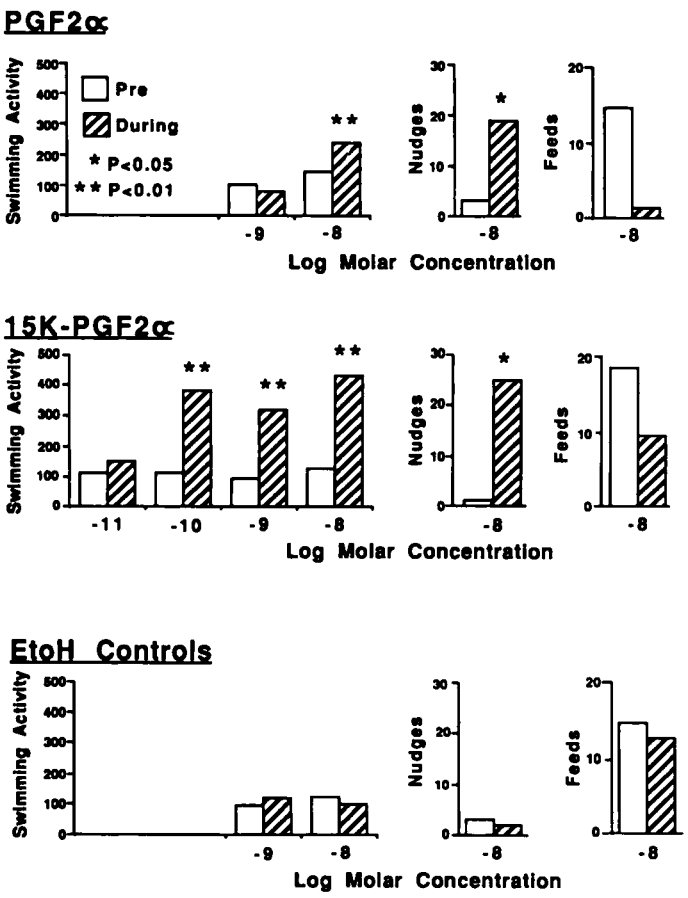

FIG. 5. Behavioral responses of male fish exposed to different concentrations of waterborne prostaglandins ($PGF_{2\alpha}$, 15K-$PGF_{2\alpha}$) and an ethanol control (ETOH). Median response values are shown. Stimuli concentrations are log molar. Because of limited space, data on nudging and feeding is not shown for concentrations less than 10^{-8} M although the same trends were apparent. Results were analyzed by the signed ranks test for dependent samples (*$p<0.05$; **$p<0.01$; n = 11 groups of 5 fish for each concentration).

peaking within 10 min and declining to basal levels within 30 min (data not shown).

DISCUSSION

This study establishes F prostaglandins as a critical component of the goldfish postovulatory pheromone. To our knowledge, this is the first report that fish release PGFs to their environment. Ovulated female goldfish release considerable quantities of immunoreactive PGF to the water and cease releasing these compound(s) shortly after spawning when they are no longer reproductively active. Furthermore, $PGF_{2\alpha}$-injected female goldfish, which are known to release odorant(s) with actions similar to those of the postovulatory pheromone (Sorensen et al., 1986), also release immunoreactive PGF. It is likely that the actual quantity of PGFs released by ovulated fish is much higher than we report here because the antiserum employed had very low cross-reactivity with $PGF_{2\alpha}$ metabolites. An antiserum for 15K-$PGF_{2\alpha}$ compound is currently being developed. Although immunoreactive PGF is found in crude washings of ovulated eggs (Bouffard, 1979; Sorensen P. W., unpublished results), and several studies have suggested that ovulatory fluid is an important source of postovulatory pheromones in fish (see Stacey et al., 1986), urine may be an important contributing source.

This is the first known report of prostaglandins functioning as olfactory stimulants in a vertebrate. Male goldfish appear to possess at least two classes of olfactory receptors for PGFs, one highly specific and sensitive to 15K-$PGF_{2\alpha}$ (or a similar unknown compound), and the other somewhat less specific to $PGF_{2\alpha}$. EOG responses to PGFs were notable because of their large size, low detection thresholds, and tendency to staturate; only 17, 20P represents a more potent odorant in goldfish and responses to amino acids and bile acids do not saturate (Sorensen et al., 1987). Electrical recording from the medial olfactory tracts has confirmed that responses to PGFs are centrally transduced (Sorensen P. W., unpublished results). Neural responses to PGFs are similar to olfactory responses transduced in response to other odorants; there is currently no empirical evidence to suggest that a specialized neural system such as the terminal nerve (Demski and Northcutt, 1982) mediates responses to the postovulatory pheromone.

Because both classes of PGF olfactory receptors function independently of receptors that respond to other known categories of odorants, prostaglandins represent a fourth, previously unsuspected, category of potent olfactory stimulants for fish. Although both cross-adaptation (this study) and mixture experiments (Sorensen P. W., unpublished results) suggest that PGF olfactory receptors specifically recognize $PGF_{2\alpha}$ and 15K-$PGF_{2\alpha}$ at low concentrations, considerable cross-reactivity was evident when concentrations of 10^{-7} M and greater were tested. We believe that the dramatic increase in the EOG evoked by 10^{-6} M 15K-$PGF_{2\alpha}$ in the water was caused by cross-reacting $PGF_{2\alpha}$ receptors. However, because levels of $PGF_{2\alpha}$ in the water and serum of ovulated goldfish (this study; Bouffard, 1979) are less than 10^{-9} M, olfactory specificity should be adequate to distinguish naturally occurring concentrations of PGFs.

This study confirms that $PGF_{2\alpha}$-injected female goldfish release potent odorant(s) which are detected

by those olfactory receptors that respond to PGFs. Although the size of the EOG responses elicited by water from $PGF_{2\alpha}$-injected fish strongly indicated that a $PGF_{2\alpha}$ metabolite (probably 15K-$PGF_{2\alpha}$) is an important component of the pheromone, the pheromone's exact composition requires verification by chemical analysis. The failure of adaptation to PGFs to reduce EOG responses to 17, 20P and the fact that $PGF_{2\alpha}$-injection does not stimulate 17,20P release (Van Der Kraak et al., 1988) strongly suggest that 17,20P is not a component of the postovulatory pheromone. Neither adaptation to 15K-$PGF_{2\alpha}$ nor adaptation to $PGF_{2\alpha}$ decreased the EOG responses to saline-injected fish water, suggesting that the L-amino acids and bile acids commonly found in fish washings (Hara et al., 1984; Doving et al., 1980) could have been responsible for these control responses. Various authors have suggested that these odorants contribute to pheromonal mixtures (Doving et al., 1980; Hara et al., 1984; Saglio and Fauconneau, 1985; Bryant and Atema, 1987). Lastly, although it is clear that $PGF_{2\alpha}$-injected fish release PGFs similar to those released by naturally ovulated fish, and that the actions of these odorants are similar (Sorensen et al., 1986), the exact degree of similarity has yet to be determined by chemical analysis.

Groups of males exposed to waterborne PGFs exhibited the same behaviors observed in males exposed to the odors of ovulated and $PGF_{2\alpha}$-injected goldfish (Partridge et al., 1976; Sorensen et al., 1986). The thresholds for behavioral responses to PGFs (10^{-8} M for $PGF_{2\alpha}$ and 10^{-10} M for 15K-$PGF_{2\alpha}$) were approximately an order of magnitude higher than the thresholds determined by EOG recording, probably due to odor dilution during injection. The effectiveness of our experimental design indicates that repeated brief exposure to wisps of an odor can stimulate strong behavioral responses. Because goldfish spawning behavior is characterized by extensive chasing (Partridge et al., 1976; personal observations), it is likely that males naturally encounter traces of female odor plumes in a manner similar to that tested here. The short duration of the behavioral response to waterborne PGFs could indicate that, as suggested by Partidge et al. (1976), the social context of pheromone exposure is important; behavioral feedback (visual, tactile, etc.) from a receptive female may be essential for a prolonged response. Goldfish may have evolved to ignore the presence of lingering pheromones after spawning has ceased. Unfortunately, our simple bioassay does not allow us to address the specific behavioral/endocrinological relevance of waterborne PGFs, the possibility that the PGFs may have different functions, and the possibility that goldfish also release species-specific and/or gender-specific chemical cues. More sophisticated tests of pheromone function are planned for the future.

It is now apparent that periovulatory female goldfish sequentially release two hormonal pheromones that have important and differing effects on the reproductive physiology and behavior of males. Through their initial actions as hormones and subsequent roles as pheromones, these compounds synchronize female and male reproductive physiology and spawning behavior. Accordingly, the reproductive physiology and behavior of spawning male goldfish can be modeled as a "dual pheromone system" based on the reproductive endocrinology of ovulatory females (Fig. 6).

Many important questions remain to be answered about this dual pheromone system. The identities of both pheromones require verification by chemical analysis and both the temporal pattern and method of their release have yet to be determined. It will be particularly important to examine prostaglandin metabolism in goldfish to determine whether the principle metabolite/pheromone is actually 15K-$PGF_{2\alpha}$. Similarly, the significance of a multicomponent postovulatory pheromone also has to be determined. Because 15K-$PGF_{2\alpha}$ saturates at a low concentration, its ability to function as a close-range signal is probably limited; it may act as a long-range cue signifying the presence of an ovulated female(s), while $PGF_{2\alpha}$ functions as a close-range signal identifying the ovulated individual. It is also possible that these pheromones synergize each other's actions. Finally, because males spawning with $PGF_{2\alpha}$-injected females experience rapid elevations in circulating gonadotropic hormone and milt (Kyle et al., 1985), it is possible that one of these PGFs has an endocrine function.

Our discovery that both the preovulatory and postovulatory pheromones in goldfish are hormones with reproductive functions in a variety of fish (Goetz, 1983; Stacey and Goetz, 1982; Scott and Canario, 1987; Goetz et al., 1987) strongly reinforces theoretical arguments that sex hormones and their

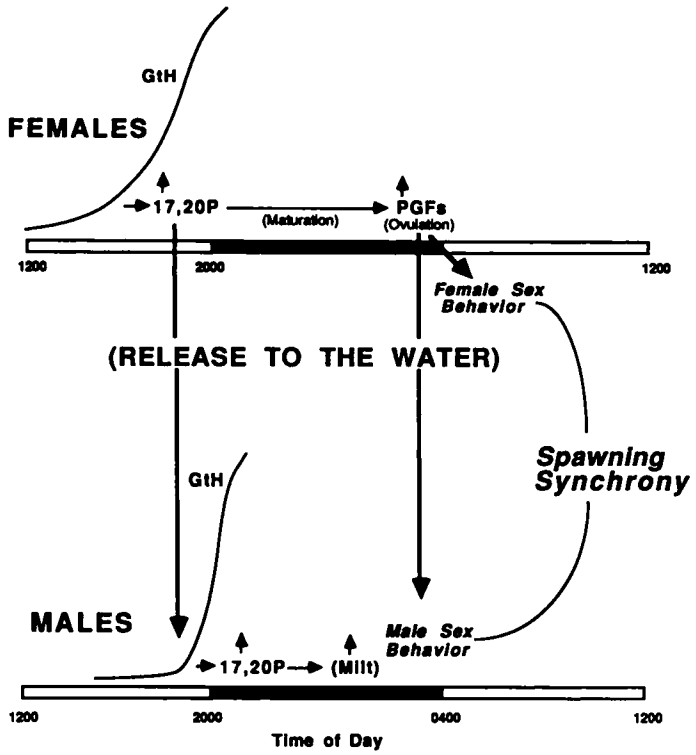

FIG. 6. A model of the dual sex pheromone system employed by goldfish. Environmental cues trigger an ovulatory surge in gonadotropic hormone (GtH) in vitellogenic females in the afternoon in the late spring (Stacey et al., 1979), which subsequently stimulates 17,20P synthesis by the ovary (Kobayashi et al., 1987). Hormonal 17,20P induces final oocyte maturation (resumption of meiosis; Nagahama et al., 1983) and is released to the water where it functions as a preovulatory "priming" pheromone. This pheromone evokes a surge in circulating GtH in males (Dulka et al., 1987), which stimulates the synthesis of testicular 17,20P. Elevated hormonal 17,20P in turn evokes an increase in milt production by the time of ovulation and spawning (Dulka et al., 1987). Later, at the time of ovulation, females produce F type prostaglandins (PGFs) to mediate follicular rupture and to trigger female spawning behavior (Stacey and Goetz, 1982). Circulating PGFs are subsequently metabolized and released to the water where they function as a postovulatory pheromone that stimulates male sexual arousal, effecting spawning synchrony.

metabolites are commonly used as reproductive pheromones. This raises important questions about species-specificity. We believe that because pheromones are greatly diluted under natural conditions selective pressure for the evolution of species-specific pheromones probably only exists among species that spawn in close proximity. Both species-specific (Liley, 1982; Honda 1982a, b; McKinnon and Liley, 1987) and nonspecies-specific (Hunter and Hasler, 1965; Chen and Martinich, 1975; Rossi, 1979; McKinnon and Liley, 1987) pheromones have been reported in fish, although their identities are unknown. Because goldfish olfactory receptors are highly specific for steroids (Sorensen et al., 1987; Sorensen P. W., unpublished results) and prostaglandins (this study), minor variations in either hormonal or metabolic pathways could effectively achieve pheromonal species-specificity where selected for. Species-specific differences in prostaglandin metabolism appear to be common among mammals (Granstrom and Kindahl, 1982). Finally, pheromonal information is probably perceived within the context of other behavioral cues (chemical, auditory, visual, electrical, and tactile) that may also provide essential species-specific information.

We believe it is highly probable that prostaglandins and/or their metabolites are used as postovulatory pheromones by many externally fertilizing teleosts, because spawning in these species coincides with ovulation, and prostaglandins appear to play a fundamental role in modulating ovulation (Dennefors et al., 1983; Goetz, 1983; Stacey et al., 1987). PGFs have already been shown to trigger female sexual behavior in a variety of externally fertilizing species (Stacey and Goetz, 1982; Cole and Stacey, 1984; Liley and Tan, 1985; Villars and Burdick, 1986; Stacey, 1987), and $PGF_{2\alpha}$ injection has been found to elicit pheromone release in the fathead minnow *(Pimephales promelas;* Cole and Smith, 1987*)*. We also believe that externally fertilizing fish whose males and females are closely associated during the preovulatory period, and whose females must spawn soon after ovulation, are likely to use a maturational hormone or metabolite as a preovulatory priming pheromone. Thus, although female sex pheromone systems in fish may differ in detail, it is likely that most species use similar hormonal compounds. It seems ironic that the original definition of a pheromone stated that "unlike hormones ... the substance is not secreted into the blood but outside the body; it does not serve humoral correlation within the organism but communication between individuals" (Karlson and Luscher, 1959). It now appears that, at least in the goldfish, hormones and reproductive pheromones can be one and the same.

ACKNOWLEDGMENTS

We thank K. J. Chamberlain for her help with the behavioral experiments and G. D. Sorensen for her editorial advice. We also thank R. E. Peter for his enthusiastic support of these studies.

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