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SYMPOSIUM

Hormonal Prostaglandin $F_{2\alpha}$ Mediates Behavioral Responsiveness to a Species-Specific Multi-component Male Hormonal Sex Pheromone in a Female Fish

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Synopsis Although hormonally-derived female sex pheromones have been well described in approximately a dozen species of teleost fish, only a few male sex pheromones have been characterized and the neuroendocrine underpinnings of behavioral responsiveness to them is not understood. Herein, we describe a study that addresses this question using the goldfish, *Carassius auratus*, an important model species of how hormones drive behavior in egg-laying teleost fishes. Our study had four components. First, we examined behavioral responsiveness of female goldfish and found that when injected with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), a treatment that drives female sexual receptivity, and found that they became strongly and uniquely attracted to the odor of conspecific mature males, while non-$PGF_{2\alpha}$-treated goldfish did not discern males from females. Next, we characterized the complexity and specificity of the male pheromone by examining the responsiveness of $PGF_{2\alpha}$-treated females to the odor of either mature male conspecifics or male common carp odor, as well as their nonpolar and polar fractions. We found that the odor of male goldfish was more attractive than that of male common carp, and that its activity was attributable to both its nonpolar and polar fractions with the later conveying information on species-identity. Third, we hypothesized that androstenedione (AD), a 19-carbon sex steroid produced by all male fish might be the nonpolar fraction and tested whether $PGF_{2\alpha}$-treated goldfish were attracted to either AD alone or as part of a mixture in conspecific water. We found that while AD was inactive on its own, it became highly attractive when added to previously unattractive female conspecific water. Lastly, in a test of whether non-hormonal conspecific odor might determine species-specificity, we added AD to water of three species of fish and found that while AD made goldfish water strongly attractive, its effects on other species holding water were small. We conclude that circulating $PGF_{2\alpha}$ produced at the time of ovulation induces behavioral sensitivity to a male sex pheromone in female goldfish and that this male pheromone is comprised of AD and a mixture of body metabolites. Because $PGF_{2\alpha}$ commonly mediates ovulation and female sexual behavior in egg-laying fishes, and AD is universally produced by male fishes as a precursor to testosterone, we suggest that these two hormones may have similar roles mediating male–female behavior and communication in many species of fish.

Introduction

Although it is well established that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) produced in association with ovulation (Takahashi et al. 2018) mediates female sexual receptivity in many, and perhaps all egg-laying teleost (bony) fishes (Stacey 1981; Stacey et al. 2003; Juntti et al. 2016; Sorensen et al. 2018), how it does so is not understood. Evidence that $PGF_{2\alpha}$ has multiple roles mediating and coordinating reproduction in egg-laying female fish takes several forms. Production of this lipid has been shown to be closely linked to ovulation in many species of female fish (Takahashi et al. 2018). Further, where examined in variety of egg-laying species including the goldfish (*Carassius auratus*) and related carps, circulating levels $PGF_{2\alpha}$ have been shown to rise dramatically at the

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time of ovulation (Sorensen et al. 2018), the same time at which females also become sexually receptive to mature males (Stacey and Liley 1974; Stacey 1981). Additionally, when $PGF_{2\alpha}$ is injected into sexually-inactive nonovulated females (or males) to elevate circulating levels of this lipid, they rapidly become fully receptive to mature male conspecifics (Stacey 1976, 1981; Kobayashi and Stacey 1993; Kawaguchi et al. 2014; Juntti et al. 2016) and start releasing a $PGF_{2\alpha}$-based sex pheromone that stimulates male activity, leading to spawning (Sorensen et al. 1988, 2018). Sexual activity in ovulating female carp/goldfish can also be blocked with F prostaglandin (PGF) synthethase inhibitors (Sorensen et al. 2018) and a receptor for $PGF_{2\alpha}$, PTgfr, has recently been found in the forebrain of an African cichlid, *Astatotilapia burtoni* (Juntti et al. 2016). However, while blocking expression of this PTgfr receptor causes *A. burtoni* females to become unresponsive to males (Juntti et al. 2016), how it mediates responsiveness to males in this African cichlid and presumably other egg-laying fishes including the carps and goldfish, is presently unknown.

Although the neuroendocrine basis of $PGF_{2\alpha}$'s action(s) mediating sexual receptivity in ovulated egg-laying female fishes is unknown, it almost certainly includes facilitating recognition of sexually-mature male conspecifics with whom they must mate (Stacey 1976, 1981; Butler et al. 2019). This presumably involves multiple sensory systems including olfaction (pheromones), vision, audition, and touch with precise roles and relative importance varying greatly by species, life history, and habitat. Indeed, androgenic hormones are known to mediate sexual responsiveness of a variety of male fishes to female cues. In particular, androgenic hormones are known to enhance how both peripheral and central components of male fishes' olfactory systems detect female sex pheromones in many cyprinids (including goldfish) as well as several other taxonomic groups (Cardwell et al. 1995; Belanger et al. 2010; Ghosal and Sorensen 2016). Similar examples of androgenic hormones upregulating peripheral neural sensitivity to acoustic, electrical, and visual cues have also been clearly documented in a variety of other fish species (Brantley et al. 1993; Dunlap et al. 1998; Liang et al. 2020). In contrast, the effect(s) of $PGF_{2\alpha}$ on female fish sensory system(s) is only known from a single study of retinal sensitization in the retina of *A. burtoni*, clear-water tropical cichlid that nest-guards (Butler et al. 2019), yet females of many species rely very heavily on pheromones and other cues, suggesting they too are likely affected.

While many fish live in shallow, clear waters and rely on vision, many do not, and these species appear to commonly rely on pheromones to mediate many types of social interactions, including sex (Stacey and Sorensen 2002; Sorensen 2015; Stacey 2015). Evidence from dozens of teleost fishes shows their sex pheromones to be species-specific, potent, and detected at very low concentrations (Stacey 2015). With one possible exception (Yambe et al. 2006), all teleost sex pheromones have been found to be comprised of mixtures of relatively common hormones and their derivatives (Sorensen and Stacey 1999; Stacey and Sorensen 2002; Stacey 2015). Mixtures of 21-carbon progestational (C21) and 19-carbon androgenic (C19) steroids have frequently been implicated as priming cues (cues that primarily effect physiological systems), while PGFs have commonly been described as releasing pheromones (pheromones with behavioral actions) (Stacey and Sorensen 2002; Stacey 2015). However, the majority of these "hormonal pheromones" are largely known from either studies of male behavior or neural sensitivity and there is limited information on the complete chemical identity of natural signals (the complete sets of chemicals that naturally released and discerned by fish). Further, only a few examples of male-derived hormonal pheromones that stimulate the behavior of ovulated females have been characterized (Stacey 2015).

An important overarching question about hormonal pheromones that remains to be resolved is whether and how mixtures of common hormones and their derivatives can be species-specific (Sorensen and Stacey 1999; Stacey 2015). Clearly, it makes sense for fishes to have evolved to detect mature individuals using the hormones they produce because these cues are "honest" and abundant, but hormonal compounds are also conserved so there is little variety and species information. One possible solution to this challenge is that mature fish may have evolved to perceive mixtures of hormonal products within background of body metabolites that all fish release as the latter cues are diverse and found in complex mixtures, and thus capable of imparting species information (Sorensen and Baker 2015). Evidence supporting this possibility comes from a study of the PGF-derived female sex pheromone in the common carp, *Cyprinus carpio*, and its close relative the goldfish. Mature male carp only respond fully to the odor of PGFs released by ovulated female conspecifics when it is introduced in a background of common carp body odor (Lim and Sorensen 2011). The possibility that body metabolites such as nitrogenous metabolites impart species

information is also supported by observations that juveniles of many fish discern each other's odors (Sisler and Sorensen 2008), and in the case of the goldfish this species-identify odor is comprised of a common set of unidentified polar and nonpolar metabolites released by all of its life stages (Levesque et al. 2011). Because the olfactory system of fish detects dozens of polar and nonpolar odorants and mixtures thereof, this possibility seems particularly reasonable (Sorensen and Caprio 1998). Sex pheromones that contain mixtures of hormones and nonhormonal metabolites have been tentatively termed "pheromonal complexes" (Sorensen et al. 2000; Levesque et al. 2011; Lim and Sorensen 2011; Sorensen and Baker 2015). However, whether and how complex mixtures of hormonal and nonhormonal body compounds might be used by species other than the common carp is unknown.

The goldfish is an excellent model to examine hormone and pheromone function and identity in fishes. It is a member of the Cyprinidae, a family of approximately 1300 freshwater fishes which contains the carps https://www.fishbase.in/search.php. Like thousands of fishes, the goldfish is a seasonal scramble-spawning species, in which males compete for access to ovulated sexually active in open waters females as they release (lay) eggs onto spawning substrate (plants), generally in turbid, dark waters (Kobayashi et al. 2002; Stacey 2015). Identifying mature male and female individuals is of paramount importance to all fish yet many, like the goldfish are not sexually dimorphic in appearance (i.e., color and shape). Instead, they rely heavily on sex pheromones to mediate sexual interactions (Stacey and Kyle 1983), and are known to use three female sex hormonal pheromones and a male pheromone to do so (Kobayashi et al. 2002; Stacey and Sorensen 2002; Stacey 2015). While dozens of gonadal steroids are produced and released by ovulatory female goldfish (Sorensen and Scott 1994), three are of special importance: androstenedione (AD; the C19 steroidal precursor of testosterone and estrogen), 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP, the maturation-inducing hormone and a C21 steroid), and 17α,20β-dihydroxy-4-pregnen-3-one-20-sulfate (17,20βPS), which are released in overall increasing amounts and as a changing mixture until the time of ovulation (Scott and Sorensen 1994). Each of these steroids is detected with high sensitivity and specificity by the goldfish olfactory system, and together this mixture serves as a priming pheromone with changing endocrine and behavioral actions on exposed males (Dulka et al. 1987; Stacey 1991, 2015; Sorensen and Scott 1994; Poling et al. 2001). Notably, AD is also released by mature male goldfish in the absence of other sex steroids which detect it and use it to mediate competitive interactions (Poling et al. 2001; Sorensen et al. 2005). Later at the time of ovulation, and when their circulating $PGF_{2\alpha}$ levels are high, ovulated female goldfish, like many other species, release a PGF pheromone and allow males to approach them (Sorensen et al. 2018). Receptive females enter floating plants while urinating more frequently, where males then line up side-by-side with them and attempt to spawn (Kobayashi et al. 2002). That female recognize males by odor is suggested by the observation that they urinate more frequently in waters containing males (Appelt and Sorensen 2007) and that olfactory-ablated females do not mate as quickly (Stacey and Kyle 1983; Kawaguchi et al. 2014; Shinohara and Kobayashi 2020); however, this male releaser pheromone has not yet been identified. The possibility that circulating $PGF_{2\alpha}$ might mediate responsiveness to a male releasing sex pheromone in ovulatory females has also not yet been investigated in either the goldfish or any other fish species.

The present study asked whether and how $PGF_{2\alpha}$ might mediate female sexual receptivity to a male sex pheromone in female goldfish, an important model species, by asking four questions:

(1) Do male goldfish release a sex pheromone that is discerned by sexually-active $PGF_{2\alpha}$-injected females?
(2) If there is a male pheromone, is it species-specific, perhaps owing to its containing both nonpolar (i.e., potentially hormonal) and as well as polar compounds, similar to the PGF pheromone complex?
(3) If there is a male pheromone and it contains a nonpolar fraction, is AD one of its primary components?
(4) Finally, if there is a male sex pheromone, is it species-specific because of non-hormonal bodily metabolites which synergize the actions of AD?

General materials and methods

We obtained goldfish from outdoor ponds (Hunting Creek Fish Farm, Thurmont, MD) and separated them into three groups: mature females with large swollen bellies and vitellogenic eggs (40 ± 4.6 g [mean ± SD]; gonadosomatic index [GSI] = $13 \pm 2.4\%$), mature males with pectoral fin tubercles (small protrusions only found on males) and expressible milt (32 ± 4.0 g, GSI = $4.0 \pm 0.3\%$), and juveniles of indeterminate sex (6 ± 1 g; GSI < 0.1%). Common carp were obtained from ponds

(Osage Catfisheries, MI) for use as a heterospecific control and were held for a year in the laboratory until males matured and became spermiated (male GSI = 2.3%; immature fish GSI <0.1%). Black bullhead catfish (*Ameiurus melas*), which were used as another heterospecific control, were wild caught locally as juveniles (GSI <0.1%). All fish were held in 1000 L flow-through tanks supplied with well water (18°C) and on a long day photoperoid (18 L:6 D) and fed *ad libitum*. Prior to testing, mature female goldfish were screened to confirm their responsiveness to $PGF_{2\alpha}$ by injecting them with 50 µg of $PGF_{2\alpha}$ dissolved in 10 µL of physiological saline solution (Lutalyse, Zoetis, NJ; a dose designed to mimic natural levels of $PGF_{2\alpha}$; Sorensen et al. 2018), and placing them with mature males and spawning substrate (floating green yarn balls) in 70 L glass aquaria following established protocols (Sorensen et al. 2018) to ensure they spawned. Over three quarters of all $PGF_{2\alpha}$-injected female fish spawned as did all males.

Female goldfish behavior was assayed following an established protocol to evaluate attraction (Sisler and Sorensen 2008; Lim and Sorensen 2011; Levesque et al. 2011). This assay uses circular two-choice mazes which are 1.5 m in diameter and filled with 300 L of well water to a depth of 20 cm (Fig. 1). They were dimly illuminated with four overhead lights as well as overhead infrared spot lights (850 nm; Vitek, Taiwan) and on a 16 h light:8 h dark photoperiod. Fish distribution was recorded using a low-light camera and DVD recorder while odors were pumped into each end (see below). Following protocol, we tested female goldfish as groups of 5 because they typically live as loose shoals. Prior to testing, fish were held in the 1000-L flow-through tanks. In most instances, we tested 10 groups of 5 but on a few occasions fewer groups were tested when we lacked naive fish.

Each experiment followed the same set of protocols but used different test stimuli. Each type of odor was tested at least seven times using different fish and odors. For each trial, five mature female goldfish were placed into a maze the night before, carefully removed by netting the next morning (09:00 h), injected intramuscularly with 50 µg $PGF_{2\alpha}$ dissolved in 10 µL of saline using a micro-syringe, and placed back into the maze to recover for 30 min before the start of the trial. Pilot experiments using saline injection showed injection itself to have no measurable effect on odor-driven responses to food odor ($P > 0.10$). After the acclimation period, two 15-min tests were conducted, the first of these was a pre-test period during which time blank (untreated) well water was pumped into each side of the maze, while the second was a test period during which time either control blank water or test water relevant to that experiment (e.g., male holding water) was added at 100 mL min^{-1}. Following protocol, test odors were added to the side of the maze (during the test period) that had the fewest goldfish during the control period to create a consistent assay of movement between the two sides (previous experiments show that this ensures fish move evenly across all the regions of the tank; Sisler and Sorensen 2008). Test odors were prepared daily by placing 20 g of fish (1–5 fish) into 1 L of aerated 18°C well water for an hour in a clean plastic bucket (plastics do not absorb/bind steroids or prostaglandins), following established protocols (Sisler and Sorensen 2008; Lim and Sorensen 2011; Levesque et al. 2011). Extracts of fish waters were prepared following established protocols (Levesque et al. 2011) by passing water through an activated C18 resin (Waters Chemical, Milford, MA), and then keeping the filtrate (the polar fraction) and eluting the C18 resin with 5 mL of methanol, drying it to 100 µL under a stream of nitrogen and adding it to 1 L well water (nonpolar fraction).

To evaluate attraction, fish distribution on each side of the maze (test area) or middle neutral zone (where some mixing occurred after 15-min) was noted every 15-s for each trial. The total number of fish counted on each side at the end of the 15-min for each group was then summed by trial (fish in the neutral zone were not counted to avoid ambiguity while providing statistical independence) and the percentage of side use ("PS") calculated, following established procedures (Levesque et al. 2011). To evaluate responses to test odors (or control), the PS on the side receiving the test odor during the test period was then subtracted with the PS on the same side during the pretest period to create a measure of a relative change (attraction) which also normalized data distribution (see Lim and Sorensen 2011; Levesque et al. 2011). Relative attraction values were found to be normally distributed when evaluated for normality (Minitab 19, Minitab LLC, PA). Responses to fish odors and their controls were analyzed by either a two-way (Experiment 1) or one-way ANOVA (Experiments 2–4) and then *a priori* (see details for each experiment below) follow-up tests to compare odors of specific interest using *t*-tests. We report exact *P*-values for *post hoc* comparisons in the text but we only considered comparisons statistically significant if their *P*-values were less 0.05 after performing Bonferroni corrections for multiple comparisons. A synopsis of each experiment and summary of its results is provided below.

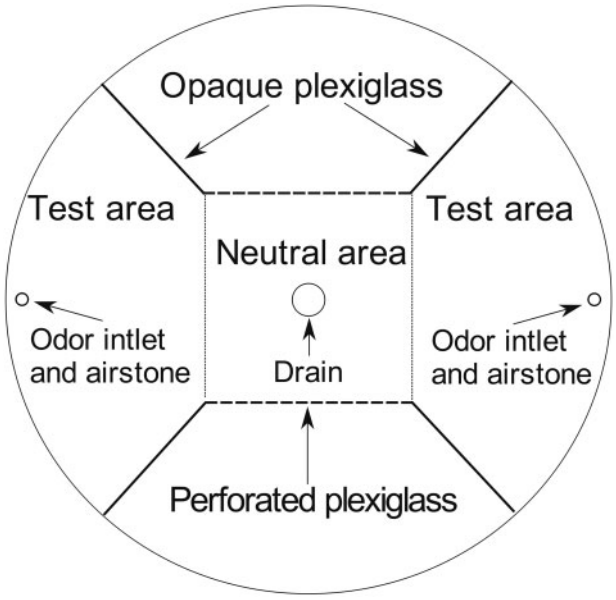

Fig. 1. Diagram of the two-choice maze we used to test responses of female goldfish to various odors. Water was turned off prior to experiments and the presence of fish noted in the test areas on each side every 15-s for 15-min.

Experiments and their results

Experiment 1: Do male goldfish release a sex pheromone that is specifically discerned by sexually-active $PGF_{2\alpha}$-injected females?

Mature female goldfish were injected with $PGF_{2\alpha}$ or not and then their responses to either control well water, juvenile, mature female, or mature male conspecific odor was tested nine times. Both treatment ($PGF_{2\alpha}$) and odor effects as well as interaction were measured using a two-way ANOVA (treatment F-value = 18.32; $P = 0.000054$; odor F-value = 12.86; $P = 0.00000073$; interaction F-value = 2.51; $P = 0.065$; df = 79). Examining the effects of these conspecific odors on each type of female, we compared each with its control using t-tests ($N = 6$) corrected for multiple comparisons. We found that while adding a new blank control water was without measurable effect on $PGF_{2\alpha}$-injected fish when compared with baseline (i.e., tested versus 0; $P = 0.94$), they were strongly attracted to male conspecific odor, spending approximately 30% more of their time in this odor ($P = 0.00017$), but were seemingly unaffected by either the odor of juvenile ($P = 0.63$) or mature female odor ($P = 0.34$). In contrast, control non-treated females were strongly attracted to the odor of all life stages of goldfish and spent between 30% and 40% more time than in each type of holding water (juveniles; $P = 0.0018$; mature females: $P = 0.00087$; mature males: $P = 0.00014$). Blank control had no measurable effect (i.e., ~0%; $P = 0.85$) (Fig. 2).

Experiment 2: If there is a male pheromone, is it species-specific owing to its containing both nonpolar (i.e., potentially hormonal) and as well as polar compounds, similar to the PGF complex?

Mature female goldfish were injected with $PGF_{2\alpha}$ and then exposed to either control well water or one of six other odors including: mature male conspecific (goldfish) or heterospecific (common carp) odor, conspecific or heterospecific male nonpolar eluates, or conspecific or heterospecific filtrates (polar fraction) tested. A one-way ANOVA described a strong treatment effect ($F = 12.72$; $P = 0.0000056$; df = 49). Because we were interested in the effects of species on chemosensory responsiveness, we next performed eight *a priori* comparisons, comparing responses to each odor with its control ($n = 6$), and then with its heterospecific partner ($n = 2$) using t-tests. These comparisons showed that responses were not elicited by control blank water (~0%; $P = 0.66$), and that while whole (unmodified) male goldfish odor elected a strong response of about ~50% ($P = 0.0027$), responses were smaller (~25%) to whole male carp odor ($P = 0.00087$), which was nevertheless larger than its blank control ($P = 0.003$) (Fig. 3). $PGF_{2\alpha}$-injected fish were attracted to both the goldfish nonpolar fraction (extract) (~35%; $P = 0.000451$) and carp nonpolar extract (~30%; $P = 0.00069$), which did not differ from each other ($P = 0.32$). In contrast, the goldfish polar fraction was attractive (~20%; $P = 0.0036$) while the carp polar fraction was not (~5%; $P = 0.44$); responses to these eluates were different when compared ($P = 0.048$) (Fig. 3).

Experiment 3: If there is a male pheromone and it contains a nonpolar fraction, is AD a primary component?

We hypothesized that AD might be the component(s) of the pheromone because this nonpolar sex steroid had already been shown to be released in very large quantities by male goldfish whose females (and males) were known to detect it at picomolar concentrations (Sorensen et al. 2005). As a first step to test this hypothesis, AD was measured in the nonpolar fish extracts (i.e., the amount of AD released by 20 g of fish in 1 L for 1 h; see Experiment 2). We accomplished this using a competitive enzyme immunoassay (11-ANRHU-E01-SLV, Alpco, Salem, NH). The sensitivity of this assay was 1 pg/mL (3.5 pM) and the cross reactivity was 2.8% for androstanedione, 0.2% for testosterone, 0.1% for

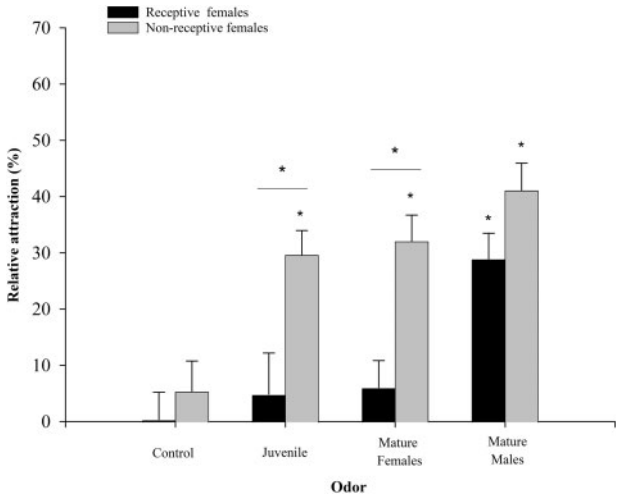

Fig. 2. Changes (mean + SE) in the distribution (i.e., relative attraction) of groups of five mature female goldfish that have either not been treated (non-sexually receptive), or injected with $PGF_{2\alpha}$ (receptive), and then offered the choice of either well water versus well water control, juvenile, mature female, or mature male goldfish holding water in two choice mazes. * denotes statistically significant differences between responses to odor and the matched well water control for that odor ($N = 7$; $P < 0.05$ after correcting for multiple comparisons [see text]). An * with a line denotes significantly different responses between non-injected and $PGF_{2\alpha}$-injected goldfish to those particular odor stimuli ($P < 0.05$; after correcting for multiple comparisons [see text]).

dihydrosterone, 0.1% for epiandrosterone, and less than 0.1% for AD sulfate, androsterone, cortisol, dehydroepiandrosterone sulfate, dihydroandrosterone, 5β-dihydrosterone, 17β-estradiol, estrone, eticholanolone glucuronids, and progesterone. Each sample was run in triplicate. We found that the male goldfish extract contained 61.3 ± 33 ng/L of AD, the mature female goldfish extract had 0.92 ± 0.5 ng/L AD, and the male carp extract had 2.63 ± 1.0 ng/L AD.

Next, we tested the behavioral responses of $PGF_{2\alpha}$-injected female goldfish to the quantity of AD we had measured in active mature male goldfish. We tested both low (30 ng/L [~0.1 nmol]; Experiment 3A) and high (1 µg/L; Experiment 3B) concentrations of AD to ascertain the role of concentration which we knew to increase with maturity and male sexual activity (Sorensen et al. 2005). Notably, the high dose is similar to the level of AD previously found to be released by sexually-active mature male goldfish in 1 h (Sorensen et al. 2005). Both AD odors were added to the mazes directly so they were diluted but still fell within the picomolar olfactory threshold of male goldfish for this steroid (Sorensen et al. 2005). In each case, we tested control, mature female goldfish odor (which we knew had only trace levels of AD; 0.1 ng/L, this experiment), AD (dissolved in 10 µL of ethanol; Sigma Chemical Co., St. Louis, MI), AD in mature female water, and odor of mature male goldfish holding water. The results of both experiments (3A and 3B) were then analyzed by one-way ANOVA. Follow-up tests in each experiment compared each odor with its control, and then male odor to AD plus the female fish washing odor ($N = 5$). Experiment 3A (30 ng/L AD) had a significant treatment effect ($F = 10.609$; $P = 0.00002$; df = 59). Further, we found that although neither water control, female odor, or 30 ng/L AD had discernable effects ($P = 0.941$; $P = 0.395$, $P = 0.260$, respectively), both AD plus female odor (~30% attraction; $P = 0.00048$) and male odor did (~40%; $P = 0.00017$) (Fig. 4A). There was no apparent difference between AD plus female odor and male odor ($P = 0.23$).

The results of Experiment 3B using 1 µg AD/L were similar to Experiment 3b which tested 30 ng AD/L and showed a highly significant treatment effect ($F = 8.72$; $P = 0.000071$; df = 36). Follow-up tests then found that while control, female odor, and 1 µg/L AD had no discernable effect ($P = 0.592$; $P = 0.604$, $P = 0.881$, respectfully), AD plus female odor (~20%; $P = 0.024$) and male odor did (~30%; $P = 0.0000079$) (Fig. 4B). There was no apparent difference between AD plus female odor and male odor ($P = 0.10$).

Experiment 4: If there is a male sex pheromone is it species-specific because of non-hormonal bodily metabolites which synergize the actions of AD?

In a final experiment, responses of $PGF_{2\alpha}$-injected female goldfish were tested to the high concentration of AD (1 µg/L) added to the holding waters of mature female goldfish (conspecific), immature common carp, and immature black bullhead catfish (same order, different family of fish) to confirm the role of AD and polar body metabolites as synergistic factor(s) in the male pheromone. ANOVA found a significant treatment effect ($F = 8.2$; $P = 0.0003$). Follow-up tests then compared each odor with the control, and then compared goldfish plus AD to each of the other two stimuli ($N = 4$). We found that while the control and bullhead odor plus AD had no discernable effect ($P = 0.480$; $P = 0.49$), both goldfish plus AD (~25% attraction; $P = 0.003$) and carp plus AD did (~20% $P = 0.079$) with the former being slightly but not significantly larger than the latter ($P = 0.147$) (Fig. 5).

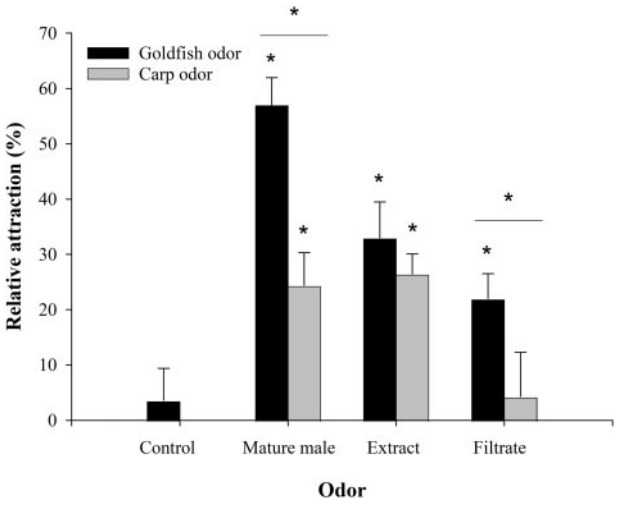

Fig. 3. Changes (mean + SE) in the distribution (relative attraction) of groups of five $PGF_{2\alpha}$-injected female goldfish offered the choice of well water versus well water control, mature male goldfish, mature male common carp odor, mature male water extract of either goldfish or common carp, or mature male odor filtrate of either species ($N = 7$). * denotes statistically significant differences between each odor and its well water control ($P < 0.05$ after correcting for multiple comparisons [see text]). An * with a line denotes differences between responses of $PGF_{2\alpha}$-injected goldfish to goldfish versus carp odors ($P < 0.05$ after correcting for multiple comparisons [see text]).

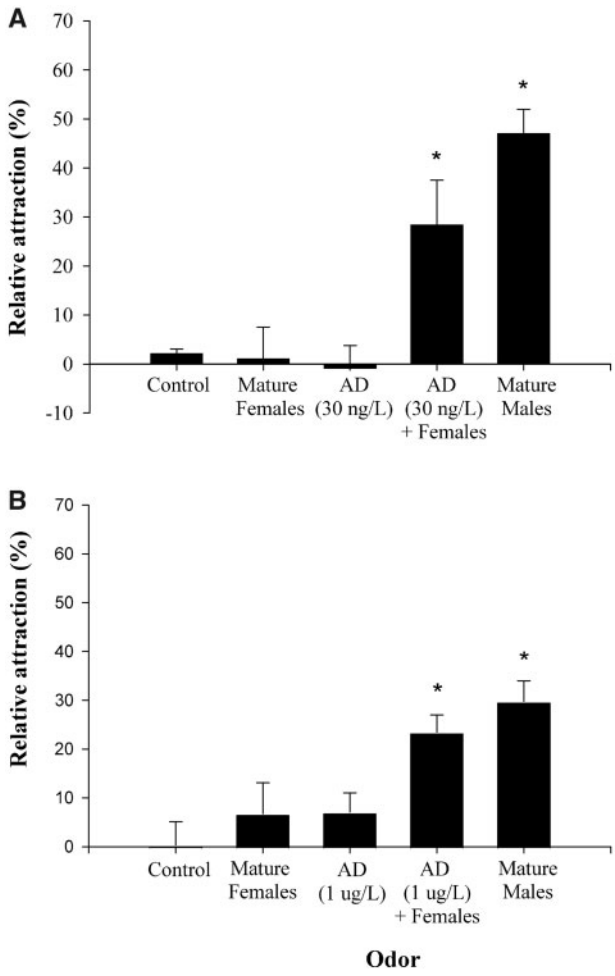

Fig. 4. Changes (mean + SE) in the distribution (relative attraction) of groups of five $PGF_{2\alpha}$-injected female goldfish offered the choice of well water and well water control or female odor, AD (30 ng/mL), AD (30 ng/L) in female goldfish holding water, or mature male odor in two choice mazes (Panel **A**). Panel **B** shows the result of a similar experiment used 1 µg/L of AD ($N = 8$–10). * denotes significant differences between odors and their matched well water control ($P < 0.05$ after correcting for multiple comparisons).

Discussion

This study both identified the first male releasing sex pheromone used by a sexually-receptive female teleost fish and then showed that responsiveness to this signal is mediated by circulating levels of $PGF_{2\alpha}$, a lipid which also mediates ovulation, female behavioral receptivity, and pheromone release (Sorensen et al. 1988, 2018; Juntti et al. 2016). The male sex pheromone was found to be comprised of AD, a common androgen and universal sex steroid precursor, and discerned by $PGF_{2\alpha}$-treated females in the context of polar body metabolites released by its conspecifics. Because many egg-laying female fishes produce and use both $PGF_{2\alpha}$ and AD, and have similar life histories to the goldfish (i.e., many are seasonal scramble spawners), we believe our findings to be of broad significance to teleost fish.

Perhaps our most important finding is that a $PGF_{2\alpha}$ treatment that simulates natural levels of $PGF_{2\alpha}$ found in the blood of ovulated goldfish induces a dramatic shift in their odor preferences, causing them to strongly favor the odor of mature conspecific males and ignore female conspecific odor. This is presumably highly adaptive for a species that needs to routinely identify, and perhaps select, mature male conspecifics in spawning scenarios which typically include large groups of competing fishes in poorly-lit conditions. Similarly, employing hormonal $PGF_{2\alpha}$ to coordinate both male and female behavioral responsiveness with ovulation also seems adaptive. Circulating $PGF_{2\alpha}$ appears to drive a shift in odor preference to mature males. Notably, non-$PGF_{2\alpha}$ injected (non-sexually-receptive) female goldfish did not discern conspecific life stage and were attracted to both male and females odors, confirming the presence of a species-identifying pheromone released by all goldfish that mediates shoaling in non-reproductive individuals (Sisler and Sorensen 2008; Levesque et al. 2011). This may be the first time that $PGF_{2\alpha}$-driven changes in olfactory preferences have been shown in a vertebrate but because $PGF_{2\alpha}$ mediates many aspects for female sexual and parturition

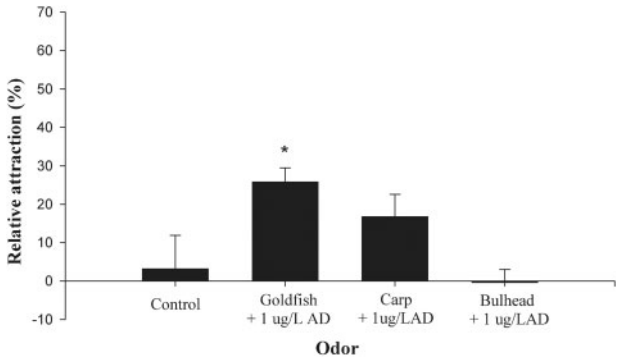

Fig. 5. Changes (mean + SE) in the distribution of $PGF_{2\alpha}$-injected female goldfish offered the choice of well water versus well water control, 1 μg AD/L in female goldfish holding water, 1 μg AD/L in carp holding water, or 1 μg AD/L in black bullhead holding water ($N = 8–10$). * denotes differences between odors and their matched well water control ($P < 0.05$ after correcting for multiple comparisons).

behavior in many vertebrate species, it presumably is common (Stacey 1981; Sorensen et al. 2018; Juntti et al. 2016). This change in olfactory preference appears to reflect an ability to perceive a complex odor rather than an individual cue such as AD.

Aside from a single prior study showing that the retina of an African cichlid (*A. burtoni*) becomes sensitized to certain colors after $PGF_{2\alpha}$ treatment (Butler et al. 2019), a role of $PGF_{2\alpha}$ on sensory function has seemingly not previously been shown in a fish. Yet, olfaction is commonly used by fishes to mediate social interactions in dark waters or to confirm information provided by other cues (Brantley et al. 1993; Sorensen and Stacey 1999; Stacey and Sorensen 2002; Sorensen 2015). Although the neural underpinnings of the actions of circulating $PGF_{2\alpha}$ on the goldfish olfactory sense are unclear, we suspect $PGF_{2\alpha}$ elicits changes in forebrain function/odor processing, perhaps the preoptic area, for several reasons. First, the $PGF_{2\alpha}$-induced effect involves a shift in sensitivity to a mixture and olfactory processing of mixtures that occurs in the olfactory bulb and forebrain where primary olfactory receptor neurons converge (Sorensen and Caprio 1998; Olivares and Schmachtenberg 2019). Second, it is consistent with evidence that alpha-adrenergic pathways have a role mediating behavioral responses to $PGF_{2\alpha}$ (Stacey 1984). Third, histochemical studies of *A. burtoni* have identified receptors for $PGF_{2\alpha}$ (PTgfr) and shown an increase in cfos activity in the preoptic area, an important area of sensory integration for spawning (Kyle and Peter 1982; Juntti et al. 2016). This possibility does not preclude the possibility that olfactory receptor neurons expressing AD receptors are upregulated although there is little precedent for such rapid action in this primary sensory system (Stacey 2015). Peripheral olfactory electro-olfactogram (EOG) recording from the goldfish and carp olfactory epithelium show that immature, adult male and female fish are all already sensitive to waterborne AD with no large differences between them although males may be slightly less sensitive (Irvine and Sorensen 1993; Sorensen, unpublished data). Olfactory tract sectioning experiments in $PGF_{2\alpha}$-treated fish suggest the presence of a central inhibitory neural pathway(s) that regulate female responsiveness to a male pheromone (Shinohara and Kobayashi 2020). $PGF_{2\alpha}$-mediated female behavioral sensitivity to water-borne AD may thus have a different etiology than androgenic upregulation of olfactory sensitivity to water-borne PGFs which takes weeks (Ghosal and Sorensen 2016). Because the brain of the goldfish and many other fishes appears to be sexually bi-potential with males also being fully responsive to $PGF_{2\alpha}$ (Stacey 1981; Kobayashi et al. 2002; Saoshiro et al. 2013; Shinohara and Kobayashi 2020), it will be especially interesting to determine its neuroendocrine underpinnings.

The results of the present study are also notable because they have identified key components of what may be the first male pheromone with strong behavioral actions in a teleost fish (Keller-Costa et al. 2014; Stacey 2015). While this type of male releasing pheromone is likely common based on evidence from a few other species including the rainbow trout, *Oncorhynchus mykiss* (Newcomb and Hartman 1973), blenny, *Blennius pavo* (Serrano et al. 2008), zebrafish, *Danio rerio* (Li et al. 2018), black goby, *Gobius niger* (Colombo et al. 1980), round goby, *Neogobius melanostomus* (Murphy et al. 2001) and African catfish, *Clarias gariepinus* (Resink et al. 1989; Van den Hurk and Resink 1992), fathead minnow, *Pimephales promelas* (Cole and Smith 1992), and *A. burtoni* (Robison et al. 1998; Cole and Stacey 2006), our study is the first to identity a key component and show behavioral activity that closely matches that of the natural cue in sexually-receptive females. Our findings confirm earlier observations by both Appelt and Sorensen (2007) and Kawaguchi et al. (2014) suggesting the existence of a male pheromone used by female goldfish. This male pheromone joins three other female hormonal sex pheromones and a male sex pheromone discerned by male goldfish as part of a sophisticated spawning system used by the goldfish and likely other carps to closely coordinate both male and female reproductive systems at both the behavioral and endocrinological levels (Fig. 6).

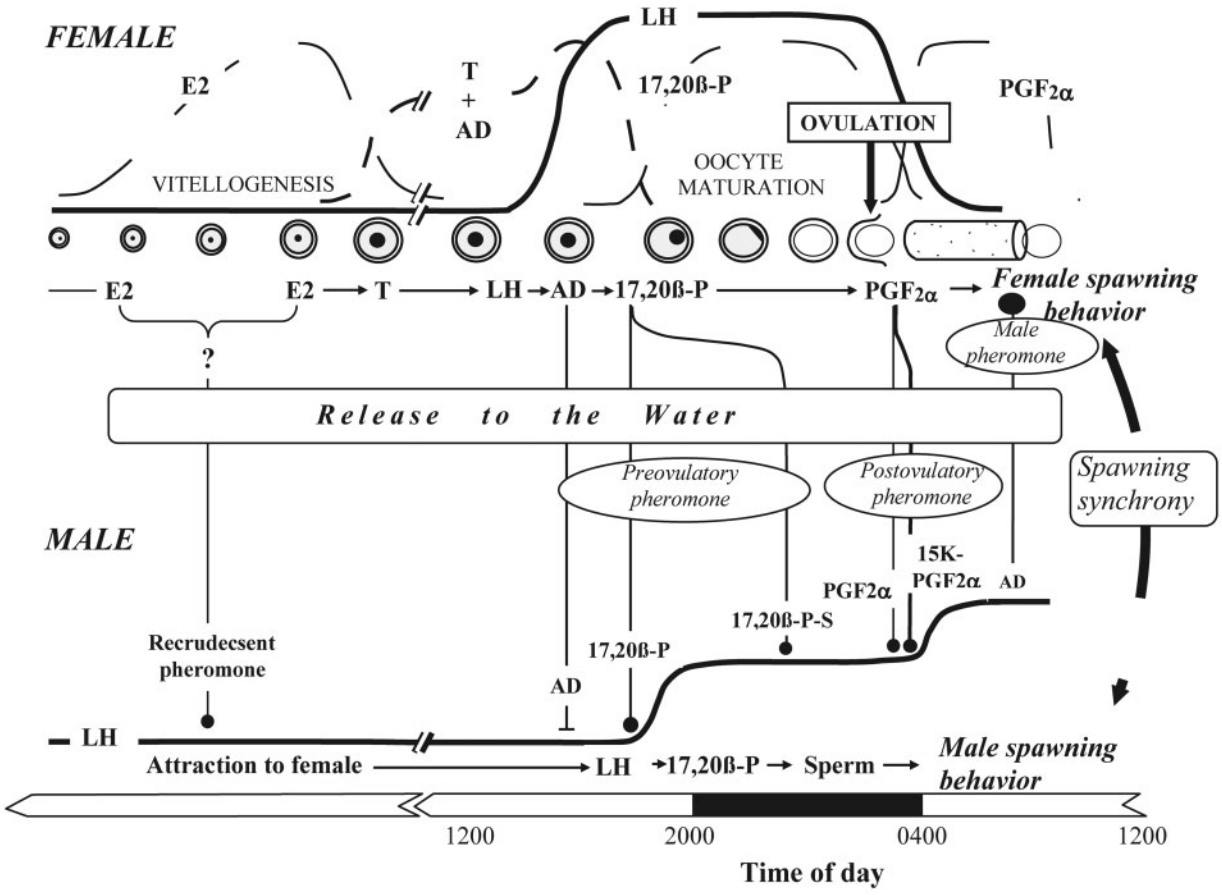

Fig. 6 Schematic representation of the goldfish hormonal pheromone system showing the male pheromone described by this study. The upper half of the figure portrays hormone levels and release in females, the bottom half is males. This figure is based on a schematic concept described by Kobayashi et al. (2002).

It is of special interest that the key component of the goldfish male pheromone perceived by receptive females is AD, a seemingly universal androgenic sex steroid produced and presumably released by both males and female goldfish and likely many other species of fish. Remarkably, AD is found early in the steroidogenic pathways of all vertebrates (Kime 1993) so its production cannot be considered a specialization although its high release rate and detection in goldfish likely are. This common steroid is detected with great sensitivity and specificity by the goldfish and common carp olfactory systems (Sorensen et al. 2005) and is known to be released via the gills (Sorensen et al. 2000). Further, its release is much greater in males than females with release by the former increasing nearly 10-fold at times of high maturity and activity (i.e., spawning males), presumably making it an excellent and "honest" indicator of fertile males (Sorensen et al. 2005). EOG cross-adaption studies in goldfish show it is discerned from other androgens including testosterone while 11-ketotestsoterone is not detected (Sorensen, unpublished results). Remarkably, chemical context appears to be critical to this particular sex steroid pheromone because it is also released by females early in their ovulatory cycle in the presence of 1720βP, whose primary actions on males appear to be inhibitory in this particular chemical context with C21 steroids (Stacey 1981; Sorensen et al. 2000; Sorensen, unpublished results). It also stimulates high levels of aggressiveness among exposed mature male goldfish when present in the absence of other sex steroids (Sorensen et al. 2005). Finally, female fish with high levels of $PGF_{2\alpha}$ respond to AD but only when present with conspecific body metabolites. The goldfish AD-based sex pheromonal odor is thus a complex multi-faceted set of signals which can have multiple behavioral meanings depending on both chemical (both hormonal and nonhormonal) context and receiver identity as well as endocrine state. Because behavioral responses to water-borne AD were generally slightly less than that to mature male goldfish odor, it is possible that other minor hormonal components compliment the actions of

AD in the male odor perceived by $PGF_{2\alpha}$-injected females. Because fish release and detect a variety of androgens including their conjugates (Stacey 1981; Sorensen et al. 2005; Stacey 2015; Li et al. 2018), androgens are likely commonly used as male pheromones similar to the manner we see with the goldfish.

Finally, this study becomes the second show how a major sex pheromone in a teleost fish is perceived within the context of the body odor within which it is found. Remarkably, as with the PGF hormonal pheromone in the carp, both nonpolar and polar body metabolites compliment AD and are necessary for full activity (Lim and Sorensen 2011). As seen in our maze test which used relatively pure water, AD had almost no behavioral activity on its own in $PGF_{2\alpha}$-treated females, even at high concentrations. Its nonhormonal components likely are part of the species-identifying cue/odor previously characterized by Levesque et al. (2011) and shown to release and discerned by life stages. While bile acids are known to be present in the nonpolar fraction and may play a role in the male goldfish sex pheromone, they are not species specific and seem to have little role on their own (Levesque et al. 2011). In contrast, the identity(ies) of the polar component(s) are presently unknown although as seen in the common carp PGF pheromone, they appear to convey key information on species identity (Lim and Sorensen 2011). The mixture of chemical cues released by male goldfish appears to serve several functions, signaling species identity to all conspecifics regardless of sex and maturity (Levesque et al. 2011), and the presence of mature males to both competing males (Sorensen et al. 2005) ovulated females (this study). Each life stage appears to discern different components in the mixture with immature fish seemingly ignoring AD although their olfactory systems detect it. We do not know whether males perceive the odor of AD in the context of polar body metabolites like females, but we do know that their responses to C21 sex pheromones (e.g., 17,20βP and 1720βP-S) are modulated by AD when present (Sorensen and Poling, unpublished results). In all instances, odor context/mixture is thus fundamental to sex pheromone function in mature male and female goldfish. Both goldfish and carp hormonal sex pheromones thus appear to be perceived as part of a complex multi-component odor complex that includes both hormonal information that conveys specific information on sex/reproductive condition and more generic body cues that convey information on species-identity.

In conclusion, our study identifies new, fundamental actions of blood-borne $PGF_{2\alpha}$ in fish which include modulating olfactory perception of a male sex pheromone, while identifying a new male hormonal sex pheromone that is perceived as part of a pheromone complex. This is the second description of a hormonal pheromone complex in fish and suggests that other fish species also use this type of signal. We hope that these findings in the goldfish model will inspire more research in this field at the junction of neuroendocrinology, behavior, and chemical ecology among the more than 36,000 other species of fish, most of which presumably also rely on sex pheromones.

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Data availability

Data available upon request from authors.

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