Avoidance of a putative pheromone, 17α,20β-dihydroxy-4-pregnene-3-one, by precociously mature chinook salmon (Oncorhynchus tshawytscha)

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To test the hypothesis that the gonadal steroid, 17α,20β-dihydroxy-4-pregnene-3-one (17α, 20β-P) acts as a pheromone for mature male salmon, we examined the behavioral responses of precociously mature chinook salmon (Oncorhynchus tshawytscha) to this odorant. These males avoided water scented with 17α, 20β-P in a two-choice maze. This avoidance response was not shown by immature chinook salmon of the same age or by mature males to a second steroid hormone, testosterone. Taken together, these results suggest that 17α, 20β-P is a behaviorally relevant odorant for precociously mature male salmon. The avoidance response observed may reflect the sneaker spawning tactic employed by precociously mature fish, as attraction to the odorant was predicted for mature males in general.


Nous avons éprouvé l’hypothèse selon laquelle l’hormone stéroïde gonadique 17α,20β-dihydroxyprénène-4-one-3 (17α, 20β-P) agit comme une phéromone chez les saumons mâles à maturité; nous avons étudié le comportement de Saumons quinnats (Oncorhynchus tshawytscha) à maturité précoce mis en présence de ce composé. Dans un labyrinthe à deux voies, ces mâles évitèrent l’eau parfumée à la 17α, 20β-P. Les mâles immatures du même âge n’avaient pas cette réaction de fuite et les saumons mâles à maturité ne réagissaient pas à une seconde hormone stéroïde, la testostérone. Dans leur ensemble, ces résultats démontrent que l’hormone stéroïde 17α, 20β-P agit sur le comportement des saumons mâles à maturité précoce. La réaction de fuite observée est peut-être reliée à la tactique de fraie furtive employée par les poissons à maturité précoce, puisque, théoriquement, l’hormone a des propriétés attirantes pour tous les mâles à maturité.

[Intégré par la Rédaction]

Introduction

Olfactory cues emanating from conspecifics play an important role in social interactions between fishes including species, sex, kin, and population recognition, schooling, and predator avoidance (Liley 1982; Olsen 1992). For many fishes, odors are also critical during reproduction, with pheromones emanating from both males and females eliciting behavioral and physiological changes that facilitate spawning. For a few species the chemical nature of these pheromones and their role in regulating mate attraction and spawning synchrony are being elucidated (e.g., Sorensen 1992a, 1992b; Stacey et al. 1993). However, the role of pheromones in salmonid reproduction and spawning behavior is still poorly understood (Olsen and Liley 1993; Rouger and Liley 1993).

During the final stages of their homing migration, salmon use olfactory cues to return to their river of origin to spawn. While it has been hypothesized that odors emanating from conspecifics may guide homing (Nordeng 1977), this hypothesis remains controversial (Quinn 1990; Brannon and Quinn 1990). Once salmon reach their spawning grounds, however, such odors may be involved in finding and courting potential mates. Several lines of evidence indicate that salmon on the spawning grounds release and respond to odors from the opposite sex. Mature male salmon are attracted to water scented by females in spawning condition or their ovarian flush (Newcombe and Hartman 1973; Emanuel and Dodson 1979; Honda 1980, 1982; Olsen and Liley 1993). This attraction is specific to ovulating females and experiments using anosmic males indicated that olfaction mediated these responses (Honda 1980, 1982; Olsen and Liley 1993). Odorants emanating from ovulating females were also able to "prime" males for spawning by stimulating production of gonadal steroids and increasing milt production (Liley et al. 1991; Olsen and Liley 1993). However, the chemical nature of the odorant(s) released by spawning females is unknown.

To date, sex pheromones identified in fishes have been primarily steroid hormones involved in gonadal maturation or their metabolites (Liley 1982; Sorensen 1992a). Therefore, we hypothesized that reproductive steroid hormones would also be the most likely salmonid sex pheromones. Furthermore, since pheromonal compounds that attract males are apparently only present at significant levels in ovulating females, we hypothesized that the most likely pheromone candidates would be hormones that are expressed at high levels at or near the time of ovulation and spawning readiness. While the hormonal regulation of gonadal development is complex, involving many steroidal and nonsteroidal compounds, plasma concentrations of two reproductive hormones, gonadotropin (GtH) and 17α, 20β-dihydroxy-4-pregnene-3-one (17α, 20β-P), rise dramatically in females at the time of spawning (Scott et al. 1983; Ueda et al. 1984; Dye et al. 1986; Liley et al. 1986b). The steroid 17α, 20β-P has been implicated as the major hormone responsible for final oocyte maturation (Goetz 1987) and acts as a potent pheromone mediating spawning synchrony in the goldfish, Carassius auratus (Dulka et al. 1987).

In this study we tested whether 17α, 20β-P acts as a pheromone for male chinook salmon (Oncorhynchus tshawytscha) by measuring the behavioral responses of mature males to water scented with 17α, 20β-P as they returned to the University of Washington hatchery (UW) to spawn. To determine if these responses were important in reproductive behavior, and not a general phenomenon for all life stages, we compared the responses of mature and immature male salmon to this odorant.
To examine the specificity of responses to 17α,20β-P, we also tested a second steroid hormone, testosterone, which is a potent attractant for mature male Atlantic salmon (Salmo salar) parr (Moore and Scott 1991; Moore 1991).

Methods

Experimental animals
Precociously mature male chinook salmon (i.e., mature in the first year only 3–6 months after release) were collected from the UW hatchery pond from mid-October to early November in 1990 (150 fish, mean weight ± SE 61.90 ± 2.85 g) and 1991 (300 fish, 165.40 ± 3.83 g). These males were used because their small size facilitated behavioral testing. Experiments with large adult UW chinook males were not performed because of the limited number available and the cost of the odorants that would be required for testing large fish. Chinook salmon generally mature at 3 or 4 years of age but in recent years up to 50% of the UW chinook salmon have matured precociously. Immature UW chinook salmon of the same age as the mature fish were reared at the UW hatchery until smolting, when they were transferred to another freshwater facility in the Lake Washington watershed. On November 9, 1990, 200 of these immature salmon (mean weight 124.69 ± 3.39 g) were transferred to UW for testing. All test fish were maintained in an outdoor concrete raceway in water pumped from the Lake Washington Ship Canal for up to 1 week prior to testing. Mature fish were not fed prior to testing but immature fish were fed to satiation once a day up to 12 h before testing. Forty-eight hours before testing, 30–50 fish were transferred to circular flow-through holding tanks in the laboratory for acclimation in Ship Canal water on a natural photoperiod. In 1990, 98 immature and 89 zero-age maturing male chinook salmon were tested for their responses to 17α,20β-P and control water. In 1991, 272 mature males were tested for responses to 17α,20β-P, testosterone, and control water.

Experimental apparatus
Fish were tested for water preference in two fiberglass two-choice tanks (140 × 52 cm) similar to those described by Quinn and Busack (1985). To ensure that the current characteristics were equivalent in each arm, water was spliced through identical V-notched walls from a small reservoir at the head of each arm. Tanks were filled to a depth of 8.0 cm and received a constant flow of 5.0 L/min in each arm. All preference tests were conducted using untreated Ship Canal water. To facilitate maximum mixing, stock solutions of test odorants were introduced into a test arm via a remotely operated peristaltic pump as the water spliced through the V-notch. Stock solutions of 17α,20β-P (Sigma Chemical Co.) and testosterone (UW Pharmacy Services) were made the day of testing by dissolving 1.0 mg of 17α,20β-P or 0.37 mg of testosterone in 1.5 mL of methanol, and subsequently, diluting into 300 mL of distilled water to a final concentration of 1 × 10⁻⁵ M steroid. “Control water” solution consisted of 1.5 mL of methanol per 300 mL of distilled water. Stock solutions were metered into one arm of the maze at 5.0 mL/min to give a final concentration in the odor arm of 1 × 10⁻⁸ M steroid.

Testing procedure
To initiate a trial, an individual fish was placed in the screened downstream section of each tank and allowed to acclimate for 3 min. All observations were made through a slit in a black plastic curtain suspended behind the downstream end of the tanks. One minute before the end of the acclimation period, odorant was introduced into one arm of the tank. Dye tests indicated that odorant reached the downstream section of the tank in less than 1 min and that considerable mixing occurred between the two sides of the downstream portion of the tank when fish were active. After acclimation, the screens were lifted and fish were allowed to swim freely within the tank. The first arm entered by a fish and the final position of each fish (right arm, left arm, or downstream area (no choice)) were recorded for each trial. The time spent by each fish in the left or right arm was also recorded for this 4-min test period. After testing, the mature fish were removed measured (fork length), weighed, and tested for spermatiation by applying gentle pressure to the abdomen. Hatchery-raised immature fish were weighed, measured, and dissected to determine sex and gonadal development. After each trial, tanks were drained, scrubbed with ethanol, and rinsed thoroughly with Ship Canal water, and the odorant supply tube was shifted to the other arm. Only one odorant was tested per day and the test odorant was varied daily to control for change in behavioral responsiveness over the 3- to 4-week testing period. After each day of testing, tanks were drained, scrubbed with ethanol and rinsed overnight with water.

Data analysis
Preference for odorant-scented water was determined by four non-independent measures: the first arm entered by the fish, the position of the fish at the end of the trial, the arm in which the fish spent the most time, and the proportion of time spent in each arm. Fish that entered neither arm during the course of the 4-min trial (no choice) were not included in the preference analysis. Differences in the proportion of fish making no choice in the preference presence of odorant were tested by χ² contingency tables (Zar 1984). To ensure that no bias was associated with the experimental tanks, left- and right-side choice and time preferences in each tank were pooled and compared with χ² tests for independence of the null hypothesis of no preference (Zar 1984). The first and last choice of all fish and the arm in which they spent the most time in response to odorant were compared with the 50:50 distribution expected by chance using the χ² test. Where indicated, pairwise comparisons between experimental groups were tested using the comparisons of proportions test described by Zar (1984). All cases, departures from no preference were tested at a significance level of P = 0.05.

Results
Of the 50 mature zero-age males tested with Ship Canal water versus Ship Canal water plus control water solution only 2 made no choice and the remaining 48 fish displayed no preference for either water source by any of the measurements (Table 1). These results indicated that the carrier solution of methanol – distilled water did not influence the subsequent tests with steroid odorants. For all test groups and all odor treatments, final position did not differ from a 50:50 no-preference response. Responses of mature males to 17α,20β-P were tested in both 1990 and 1991 but there were no differences between years (for all measures of preference and proportion of fish making no choice, P > 0.20) and data were pooled for further analysis. Of the 161 fish tested, 88% were spermatiating. Based on the first arm chosen, the arm in which the fish spent the most time, and the proportion of time spent in each arm, mature males avoided the arm of the maze containing 1 × 10⁻⁴ M 17α,20β-P (Table 1, Fig. 1). Also, these measures differed significantly from the responses of immature males to 17α,20β-P. To test whether avoidance of 17α,20β-P was restricted to mature males, we also tested hatchery-reared UW chinook males of the same age but with little gonadal development. Of the 183 immature fish tested, 98 were males that had had a slight but nonsignificant tendency to prefer the 17α,20β-I arm (Table 1, Fig. 1). No difference was apparent between immature males and females by any of the four measures (P > 0.10 for all measures). In 1991, we also tested the behavioral responses of 149 mature males to 1 × 10⁻⁴ M testosterone. They demonstrated no preference for or avoidance of testos
The absence of competing males is not clear, since precocious for reproductively active male chinook salmon. However, the advantage of such an avoidance response in the behaviorally significant odorant $P_{17\alpha,20\beta}$ suggests that both males and females (Hanson and Smith 1967; Gross 1985).

Avoidance of this steroid may actually avoid high concentrations of $17\alpha,20\beta$-$P$, but attempting to sneak fertilizations (Gebhards 1960; Hanson and Smith 1967; Emanuel and Meyer 1988). Males adopting this sneaking tactic might avoid $17\alpha,20\beta$-$P$. Second, ovarian fluid may contain attractive compounds which overcome the repulsive properties of $17\alpha,20\beta$-$P$. Third, $17\alpha,20\beta$-$P$ may only be attractive as part of a mixture of pheromones released by females. Finally, since experimental evidence for attraction to ovarian fluid has not been established in chinook salmon, the responses of chinook salmon males may differ from other salmonid species tested. This seems unlikely; mature male UW chinook salmon were attracted to water scented by females (Pete 1977). These results are apparently not due to a generalized avoidance by chinook salmon of $17\alpha,20\beta$-$P$ in this testing paradigm. Avoidance of $17\alpha,20\beta$-$P$ was specific for mature males and was not observed for immature males of the same age. This suggests that $17\alpha,20\beta$-$P$ is a behaviorally significant odorant for reproductively active male chinook salmon.

### Discussion

We hypothesized that the reproductive steroid hormone, $17\alpha,20\beta$-$P$, might act as a pheromonal attractant for mature male salmon but precocious male chinook salmon avoided water scented with this putative pheromone. These results were unexpected, since mature males are generally attracted to ovarian fluid of ovulating females and this fluid contains high levels of $17\alpha,20\beta$-$P$. For example, $17\alpha,20\beta$-$P$ levels in ovulating coho salmon females may reach concentrations as high as $1 \times 10^{-7}$ M in ovarian fluid (Wright and Hunt 1982). There are several possible explanations for the apparent avoidance of this steroid. First, avoidance of $17\alpha,20\beta$-$P$ may be a consequence of the concentration of the odorant tested and at this concentration both precocious and adult males might avoid $17\alpha,20\beta$-$P$. Second, ovarian fluid may contain attractive compounds which overcome the repulsive properties of $17\alpha,20\beta$-$P$. Third, $17\alpha,20\beta$-$P$ may only be attractive as part of a mixture of pheromones released by females. Finally, since experimental evidence for attraction to ovarian fluid has not been established in chinook salmon, the responses of chinook salmon males may differ from other salmonid species tested. This seems unlikely; mature male UW chinook salmon were attracted to water scented by females (Pete 1977). These results are apparently not due to a generalized avoidance by chinook salmon of $17\alpha,20\beta$-$P$ in this testing paradigm. Avoidance of $17\alpha,20\beta$-$P$ was specific for mature males and was not observed for immature males of the same age. This suggests that $17\alpha,20\beta$-$P$ is a behaviorally significant odorant for reproductively active male chinook salmon.

### Table 1. Responses of mature and immature chinook salmon to water scented with $17\alpha,20\beta$-$P$, testosterone, or control water

<table>
<thead>
<tr>
<th>Odorant tested</th>
<th>First arm entered</th>
<th>Final position</th>
<th>Time preference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Odor</td>
<td>Ctrl</td>
<td>NC</td>
</tr>
<tr>
<td>Mature</td>
<td>Control water</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Mature</td>
<td>$17\alpha,20\beta$-$P$</td>
<td>38</td>
<td>62*</td>
</tr>
<tr>
<td>Immature</td>
<td>$17\alpha,20\beta$-$P$</td>
<td>44</td>
<td>36</td>
</tr>
<tr>
<td>Mature</td>
<td>Testosterone</td>
<td>57</td>
<td>42</td>
</tr>
</tbody>
</table>

Note: Data represent the responses of individual fish: Odor, odor arm; Ctrl, control arm; NC, no choice. Significant departures from a 50-50 no-preference response are indicated as follows: *, $x^2 = 5.76, P < 0.05$; **, $x^2 = 4.00, P < 0.05$.

The ability of $17\alpha,20\beta$-$P$ may reflect differences in the behavioral responses to pheromones by precociously maturing and adult males. Previous studies describing male attraction to females have only tested large adult salmon and not precociously maturing males (Newcombe and Hartman 1973; Emanuel and Dodson 1979; Honda 1980, 1982; Olsen and Liley 1993). On spawning grounds, precocious and larger chinook males employ dramatically different tactics for gaining access to females (Gebhards 1960). Adult males actively court nest-building females and aggressively defend females from other males. Precocious males generally employ sneaking tactics, remaining in the vicinity of females, not engaging in male--male aggression but attempting to sneak fertilizations (Gebhards 1960; Gross 1983; Maekawa and Onozato 1986; Hutchings and Meyer 1988). Males adopting this sneaking tactic might respond differently to pheromonal communication from females. While adult males actively seek ovulating females, precocious males may actually avoid high concentrations of $17\alpha,20\beta$-$P$, because close proximity to females might invite attacks from both males and females (Hanson and Smith 1967; Gross 1985). However, the advantage of such an avoidance response in the absence of competing males is not clear, since precocious
males of other salmonid species will actively court large females in the absence of competitors (Foote and Larkin 1988).

Another explanation is that 17α,20β-P avoidance reflects pheromonal communication between males. As in females, levels of 17α,20β-P increase dramatically in male salmon at the onset of spawning (Ueda et al. 1984; Dye et al. 1986; Liley et al. 1986b). Mature adult males that are subsequently paired with nest-building females exhibit even further elevations in 17α,20β-P levels (Liley et al. 1986a; Liley et al. 1991; Olsen and Liley 1993; Rouger and Liley 1993). These socially induced increases in 17α,20β-P may increase milt production and synchronize spawning (Rouger and Liley 1993) but may also be important for pheromonal signalling between males. Just as physical attributes such as body length and depth act as fitness indicators in establishing dominance among males (Quinn and Foote 1994), olfactory cues such as gonadal hormones may also be involved in status signalling. This might explain the avoidance of high 17α,20β-P concentrations by precocious males since their levels of 17α,20β-P are lower than those of adult males during spawning (Stuart-Kregor et al. 1981; Ueda et al. 1983). Such a system might be particularly important at night, when visual cues are lacking but spawning activity continues (e.g., Hartman 1969).

The spawning tactics of precocious males may also explain the decreased activity and positive rheotaxis (as indicated by the percentage of fish making no choice) of test fish in the absence of 17α,20β-P versus control water. On the spawning grounds, young age groups avoid attacks by limiting activity and remaining inconspicuous to females and larger males (Gebhards 1960). Since both adult males and females may release 17α,20β-P into the surrounding water while courting, limiting activity in the presence of 17α,20β-P may minimize attacks. However, this does not explain the decreased activity levels we also observed in the presence of testosterone.

Precocious male salmon demonstrated no behavioral preference for or avoidance of testosterone. This indicated that the observed responses to 17α,20β-P were not due to a general avoidance of steroids. Precocious males were able to recognize testosterone because the percentage of fish making no choice increased in the presence of this steroid. In contrast to our results, mature Atlantic salmon parr exhibited a strongly positive rheotactic response to water scented with testosterone (Moore 1991). Furthermore, electrophysiological recordings of the olfactory epithelium indicated that testosterone is an extremely potent odorant for mature parr (Moore and Scott 1991). Surprisingly, physiological recognition of testosterone was limited to a narrow time window just prior to spawning. This limited window of sensitivity might explain why we saw no behavioral responses to this odorant, or alternatively, it may reflect differences between the species.

Since we initiated this study several researchers have suggested that the sulphated conjugate of 17α,20β-P, 17α,20β-dihydroxy-4-pregnen-3-one 20-sulphate, which is present in high levels in the plasma and urine of several teleosts (Scott and Canario 1992). may also act as a sex pheromone (Stacey et al. 1993). This compound is a potent odorant for goldfish (Sorenson et al. 1991) and male Atlantic salmon parr (Moore and Scott 1992). While we did not test the responsiveness of precociously mature chinook males to 17α,20β-P-sulphate. Scott et al. (1994) found that mature male rainbow trout neither avoided nor were attracted to this compound.

In summary, precociously maturing egg-chinook salmon avoided water scented with the reproductive steroid hormones 17α,20β-P. This avoidance response was not apparent in immature male or female chinook salmon of the same or mature males tested in the presence of a second steroid hormone, testosterone. Taken together, these results suggest that 17α,20β-P is a behaviorally relevant odorant for precociously maturing male salmon. We hypothesize that mature male salmon would be attracted to 17α,20β-P the avoidance responses we observed were associated with the sneaker spawning tactics employed by precocious fish.

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Wright, R.S., and Hunt, S.M.V. 1982. A radioimmunoassay for 17α,20β-dihydroxy-4-pregnen-3-one: its use in measuring changes in serum levels at ovulation in Atlantic salmon (Salmo salar), coho salmon (Oncorhynchus kisutch), and rainbow trout (Salmo gairdneri). Gen. Comp. Endocrinol. 47: 475–482.