Ovarian steroid sulphate functions as priming pheromone in male *Barilius bendelisis* (Ham.)

J P BHATT* and M S SAJWAN

Fish Pheromones and Behaviour Study Unit, Zoology Department, HNB Garhwal University, Srinagar (Gwl) 246 174 India

**Corresponding author (Fax, 91-1388-52174; Email, transmedia@vsnl.com).*

The study reveals that pre-ovulatory females of the fish *Barilius bendelisis* (Ham.) release sex steroids and their conjugates into the water and that a steroid sulphate of these compounds functions as a potent sex pheromone which stimulates milt production in conspecific males prior to spawning. Since males exposed to the purified sub-fraction III of the steroid sulphate fraction have increased milt volume and more spermatozoa with greater motility, the function of this priming pheromone appears to be to enhance male spawning success.

High turbulence and faster water currents render the hillstream ecosystem extremely challenging for chemical communication. Therefore, ovulatory female fish secrete highly water soluble steroid sulphates for rapid pheromonal action in males. Inhibited milt volume in olfactory tract lesioned (OTL) males exposed to the steroid sulphate fraction and 17*a*,20*b*-dihydroxy-4-pregnen-3-one supports the concept that the pheromonally induced priming effect in male fish is mediated through olfactory pathways.

1. Introduction

Recent studies have demonstrated functional chemical communication in various teleost fish species (Liley 1982; Sorensen and Goetz 1993; Sorensen *et al* 1995; Sorensen 1996). The chemicals used in intraspecific communication have been designated as pheromones (Karlson and Luscher 1959). In fish, olfaction is found to play a leading role in mediating the pheromonal response, and pheromones are suspected to elicit attraction and sexual arousal in conspecifics (Colombo *et al* 1982; Van den Hurk and Lambert 1983; Johanson 1984). Previous investigations implicated ovulated ovaries as a production site of the sex pheromones in goldfish (Partridge *et al* 1976), guppy (Crow and Liley 1979), rainbow trout and loach (Honda 1980a,b).

A cholesterol ester and steroid glucuronides have been isolated from the gonads of zebrafish (Algranati and Perlmutter 1981; Van den Hurk and Lambert 1983). Lambert and Resink (1991), Van Weerdt *et al* (1991) and Vermeulen *et al* (1993) obtained steroid glucuronides that accelerate ovarian growth from the testicular tissue of catfish. Recently free and conjugated forms of 17*a*,20*b*-dihydroxy-4-pregnen-3-one (17,20-P) and F-prostaglandins (PGF2*a*) have been reported as pre-ovulatory and post-ovulatory pheromones respectively in goldfish (Stacey *et al* 1989; Van den Kraak *et al* 1989; Kobayashi and Stacey 1993; Sorensen and Goetz 1993; Sorensen *et al* 1995), and are released into water (Scott and Vermeirssen 1994).

There has, however, been a conspicuous lack of information on the role of pheromones in the sexual behaviour of fishes of the Indian subcontinent. Due to fast currents and high turbulence, the streams of the Himalayan region are extremely challenging for chemical communication and still a number of fish species thrive there. Therefore, the present study sought to identify which of the purified ovarian fractions (free, glucuronidated or sulphated steroids) functions as a priming pheromone to synchronize reproductive events in males and females of *B. bendelisis*.

Keywords. Fish; olfactory pathway; priming effect; sex pheromones; steroid sulphate

Abbreviations used: 17,20P, 17*a*,20*b*-dihydroxy-4-pregnen-3-one; PGF2*a*, F-prostaglandins; OTL, olfactory tract lesions.

2. Materials and methods

Barilius bendelisis individuals (8–9 cm, 7–8 g) procured from streams near the University in December–January 1998 were held in a 100 l flow through stock tank under captive conditions (water temperature 13°C, DO 7·2 ppm, CO_2 2·6 ppm, pH 7·6 and photoperiod 10 L : 14 D). Fish were fed with oil cake, rice bran and algae once daily. Based on dimorphic characters (Badola *et al* 1982), males and females were isolated and placed in different aquaria. Fish were divided into two groups: group-I was maintained under captive conditions and group-II was transferred to the ovulation aquaria.

2.1 *Ovulation aquarium*

A 100 l opaque glass aquarium was set on a table and tilted along its axis so that water entered at one end and overflowed from the other. This aquarium was divided by a thin opaque plastic screen allowing water exchange between the two compartments (Sajwan *et al* 1999). All aquaria were well equipped with continuous water-oxygen supply, light, temperature regulation devices as well as gravels and artificial vegetation as spawning substrates.

2.2 *Experimental protocol*

An ovulation aquarium containing 10 fish was put into the photoperiodic chamber on day 1. The temperature of incoming water was increased to 16°C on day 2. The temperature was further raised to 20°C and 25°C on days 3 and 4. In *B. bendelisis*, spontaneous ovulation occurs when mature females are held in a 14 L : 10 D photoperiod and at 25°C under controlled laboratory conditions (Bhatt and Sajwan 1996). Most ovulations (defined as the time when mature oocytes can first be expressed from the gonopore by gentle pressure on the abdomen) occur in the latter half of the following scotophase.

Water samples were collected at 2 h intervals from 1200 h on day 2 (prior to the occurrence of first ovulation). The temperature of aquarium water that held preovulatory females was increased and test fish were checked for ovulation. Water samples were again collected during this ovulatory period at 3 h intervals from 0500 h on day 4 until 1400 h on day 5. These water samples (300 ml) were stored at – 20°C till their use. Six females from each preovulatory and ovulatory stage were sacrificed, the ovaries collected and processed as described below.

2.2a *Extraction–fractionation procedure*: The isolated ovaries were rinsed with distilled water, crushed and homogenized. The homogenate was transferred into a stoppered tube into which distilled dichloromethane (2 ml) and distilled water (2 ml) were added. To remove the protein contents from the homogenate, 20 ml ethanol : acetone (1 : 1, v/v) was added drop by drop and then centrifuged at 1500 *g* at room temperature for 30 min. This deproteinized ovary extract (2 ml) was mixed with 5 ml dichloromethane. After shaking and centrifugation (10,000 *g*), the lower organic layer was removed as 'free steroid fraction-F'. The residue was mixed with dichloromethane (5 ml), centrifuged and evaporated at reduced pressure. On adding 1 ml ethanol, 100 µg *b*-glucuronidase and 1 ml acetate buffer (pH 4·8), this extract was left overnight at 37°C. After cooling, the residue was re-extracted with dichloromethane, evaporated and reconstituted in the freezer to obtain 'glucuronide fraction-G'. The aqueous residue was combined with 4 ml *n*-butanol and evaporated. The dried residue was mixed with a drop of distilled water and 2 ml of trifluoro-acetic acid. This fraction was left overnight at room temperature and then $NaHCO_3$ (1 ml) was added to it. The organic phase was removed and again extracted in 2 ml ethyl acetate to obtain the 'sulphate fraction-S'.

2.2b *Preparation of subfractions*: Each of the three ovarian fractions was passed through borosil glass columns (500 × 10 mm) packed with florisil (60–100 mesh-fluka) and the subfractions were collected with the aid of the mini-fraction collector.

2.2c *Chromatographic analysis*: Ovary fractions and subfractions dried overnight were examined on 25 DC-plastikfolin 20 × 20 cm kieselgel 60 F_{254} two-dimensional TLC plates (E Merck) activated in an airtight oven at 100–110°C for 30 min. The cooled plates were spotted with fresh ovarian fraction and known sex steroid (Sigma). Different solvent systems such as chloroform : methanol (98 : 2), ethyl acetate : benzene (1 : 9), benzene : hexane (8 : 2) and acetone : hexane (3 : 7) were used as a mobile phase. The TLC plates were now dried and passed through iodine vapours in order to develop the spots of sex steroids and ovarian fractions. These spots were examined under UV light and their R_f value was calculated.

2.2d *GC analysis*: Polyfunctional steroids in many instances will not directly resolve by gas chromatographic separation. Therefore, trimethylsilyl and methoxine derivatives were prepared. The steroids were dissolved in dichloromethane, dried under a nitrogen stream and *O*-methylhydroxylamine hydrochloride (200 µl) in 2% pyrimidine was added. After vortexing for 10 min, the mixture was heated at 100°C to convert the keto group into a methoxine group. The mixture was evaporated, *N*-trimethyl silylimidazole added to it and heated at 100°C. This mixture was combined with acetonitrile (3 × 200 µl) and hexane (2 ml). The steroid-containing hexane fraction

was evaporated and the residue was dissolved in hexane (10 ml). Two µl of this solution was injected with a Hamilton microsyringe into the injection port of the glass column (1·8 m × 2 mm, coated in 100–120 mesh chromosorb) fitted in the oven of the GC.

Nitrogen was used as a carrier gas whose flow rate was maintained at 3·5 ml/min. A mixture of hydrogen and oxygen was used for flame ionization. The temperature on the injection port was set at 250°C while the oven temperature was maintained at 160°C for 1 min. This temperature was raised to 190°C (15°C/min) and after 0·5 min it was elevated to 220°C (2°C/min). Five minutes later the temperature was increased by 1°C/min. The eluted components of ovarian fractions were collected on FID and recorded as various peaks.

2.2e *Stock solutions of sex steroid*: Steroid conjugates 17,20P glucuronide and 17,20P sulphate directly dissolved in distilled water, but due to insolubility in water, free steroid 17,20P was pre-dissolved in ethanol. Stock solutions were made up of 10^{-5} M by dissolving 1 mg of free steroid in 1·5 ml distilled ethanol and 0·5 ml of this solution was mixed with 100 ml of distilled water. The molecular weight of these steroids ranged from 287 to 365 (Scott and Vermeirssen 1994). The stock solutions of the steroid conjugates were made up to 10^{-4} M. The test solutions of desirable concentration (10^{-4} to 10^{-10} M) were made in dechlorinated water. The final concentrations of these steroids in aquarium water could not be determined.

2.3 *Experiment I: Milt volume in isolated males and males in contact with mature female*

Five males and five ovulatory females were put into two different compartments of an ovulation aquarium at 2300 h. A perforated opaque plastic screen dividing the aquarium ensured water exchange between two compartments of the aquarium. Another aquarium containing only males served as a control. This experiment included six trials. At the end of each trial, males were anaesthetized in 0·02% phenoxy-ethanol and a light gentle pressure was applied around the urinogenital pore. The oozing milt was aspirated into a micropipette. Test males were stripped for milt at 3 h intervals until 1100 h on the following day.

2.4 *Experiment II: Milt volume in males exposed to the water which previously held pre- and post-ovulatory females*

Water samples (300 ml) collected at 3 h intervals from the pre- and post-ovulatory female holding aquaria were inserted into different aquaria containing males (n = 6) at 0900 h at a flow rate of 20 ml/min until 1900 h. Simultaneously, in a control test six males were treated with blank water. All test males were maintained in captivity at 25°C and a photoperiod of 14 L : 10 D. These conditions are similar to those experienced in the wild during the spawning phase. At the end of each trial, males were anaesthetized in the 0·02% 2-phenoxy ethanol and then stripped of milt at 2 h intervals from 1100 h to 1900 h and the milt volume was measured.

2.5 *Experiment III: Competitive priming effect of purified ovarian fractions in males*

To determine the efficacy of different ovary fractions and subfractions in priming the reproductive physiology of males, the following tests were conducted.

2.5a *Test 1*: Various concentrations (3, 2, 1, 0·5 and 0·25%) of the ovarian fractions were tested in male fish initially to find out the dose-response relationship. Based on maximal response, 1% (1 mg fraction per 100 ml water) solution of each fraction was applied in test males.

2.5b *Test 2*: Eight males in each of the different aquaria were maintained at 25°C and 14 L : 10 D photoperiod 5 h before the test started. Males were stripped of milt and placed back in the aquaria. This was considered as pre-test milt volume. A 500 ml solution of each ovarian fraction (F, G, S) was released at 2200 h into different aquaria containing males. These males exposed to ovary fractions were stripped of milt at 3 h intervals till 0100 h the following day. The milt volume, sperm motility, and sperm density were compared in pre- and post-treatment male groups.

2.5c *Test 3*: Ten males were placed in each of the five aquaria at the temperature (25°C) and photoperiod (14 L : 10 D) they experienced in the wild during the spawning period. Test males were stripped of milt and were returned to the aquaria. One percent stock solutions of subfractions I, II, III, IV and V were prepared and stored in the refrigerator till use. The stock solution of each subfraction was diluted (10^{-5} concentration) in background water (aquarium water). A volume of 500 ml of each solution was inserted into different aquaria containing males. After 12 h, these males were stripped of milt. The milt volume, sperm motility and sperm density were compared in pre- and post-treatment male groups.

2.6 *Experiment IV: Comparative efficacy of sex steroids in enhancing the milt volume in test males*

Six mature males were put in each of the different aquaria under 25°C and 14 L : 10 D photoperiod conditions 5 h

prior to the start of the experiment. After stripping initial milt, males were returned to the aquaria. A 200 ml volume (10^{-5} M) of each sex steroid solution (17,20P, 17,20P-G and 17,20P-S) was added to the male-holding aquaria at 2300 h. Fish were then anaesthetized with 0·02% phenoxy ethanol and stripped of milt at 2 h intervals until 1100 h the following day. Milt volume, sperm density and sperm motility were compared between pre- and post-treatment groups of test males.

2.7 *Experiment V: Effect of olfactory tract lesions on pheromonally induced milt volume*

Mature males were divided into two groups. In group-I, males were anaesthetized with 0·02% phenoxy ethanol and their paired olfactory tracts were exposed by cutting a three-sided bone flap through the cranium with a dental saw. Both left and right olfactory tracts were cut. The cranial cavity was filled with teleost saline (Bhatt and Sajwan 1996) and the bone flap was returned to its normal position by gently pushing its free margins beneath the edge of the skull (Dulka and Stacey 1991). Such olfactory tract lesions (OTL) males were left overnight for recovery.

In group-II males, the region between two nostrils was cauterized with MS 222 (0·01%). These SHAM-operated males received no further treatment to their olfactory tracts and thus served as controls. Six males each from the OTL and SHAM groups were exposed to the steroid sulphate fraction of the pre-ovulatory ovarian extract and 17,20P (10^{-5} M) for 24 h. Milt volume was then measured in the OTL and SHAM groups.

2.8 *Sperm motility*

The milt from males was collected in capillary tubes using suction. These tubes containing milt were placed in a deep freezer at – 20°C for 10 min. The milt was diluted with spermatic fluid and the spermatozoa were quickly activated by reduction in ionic concentration (Defraipont and Sorensen 1993). A drop of activated and diluted milt was then put on a glass slide which was covered with a cover slip and placed under a microscopic (× 400). Generally 30–50 sperm were present in the microscope's field of view. The relative motility of sperm was scored according to criteria established by De W Kruger *et al* (1984), i.e. spermatozoa motile with flagellar movement = 5, most spermatozoa motile and a few vibrating in loco = 4, most spermatozoa immotile and a few with progressive motion = 3, most spermatozoa vibrating in loco and a few motile = 2, most spermatozoa immotile and occasionally one or two vibrating = 1, spermatozoa immotile = 0 (motility score = 0–5). The motility of sperm was scored within sixty seconds.

2.9 *Sperm density*

The relative number of spermatozoa was estimated by counting the number of sperm cells in the microscope's

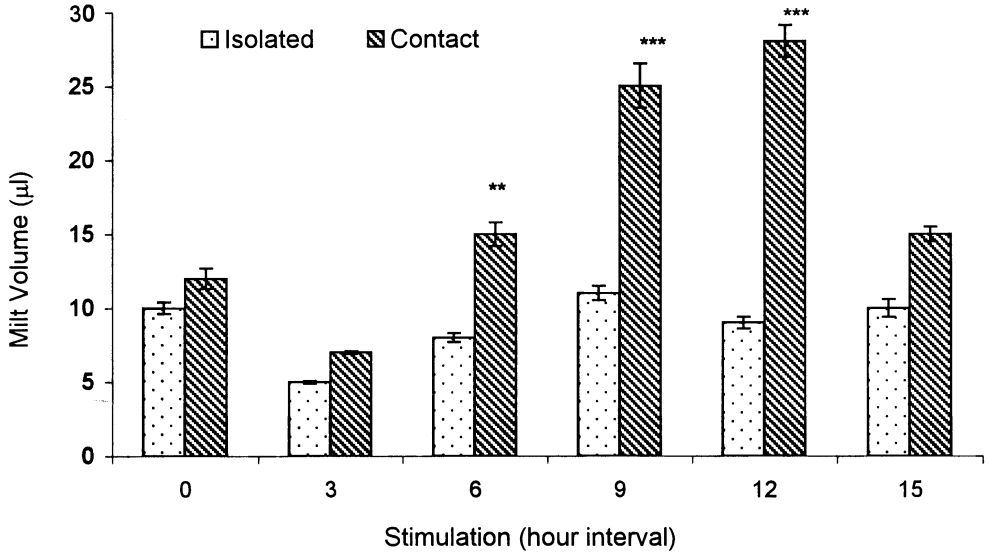

Figure 1. Milt volume in isolated males and males in contact with females. Mean (± SEM) stripped milt volume (µl ± SEM) repeatedly sampled at 0, 3, 6, 9, 12 and 15 h intervals in all male groups isolated from females or in males in contact with ovulatory females. Significant difference refers to isolated males vs males in contact with females applying student's *t*-test. The experiment includes 6 trials and 36 males stripped for milt each time. $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

field of view and quantified on a scale from 1–4 (1 = less than 10 cells, 2 = 10–20 cells, 3 = 20–50 cells, 4 = more than 50 cells).

2.10 *Statistics*

Because data on milt volume, sperm density and sperm cell motility were normally distributed, parametric statistics were used for all analyses. In this study two factors, i.e. exposure hours and odour treatment, were considered. Each experiment was accomplished with multiple trials and the mean values (± SEM) of all above parameters were obtained from the tests. Significant differences in mean values between control and odour-treated males groups for each parameter (milt volume, sperm density, sperm motility) were derived applying student's *t*-test.

3. Results

Long photoperiods and warm temperature regimes trigger gonadal development in the study model. Large males exhibit black-reddish body colour with prominent tubercles on the snout during the breeding stage. Subfraction III of the steroid sulphate fraction constituted a single spot on the TLC plates and single peak on GLC whose retardation factor, R_f (0·26) and retention time, R_t (16·52) were close to the R_f (0·26) and R_t (16·75) of a known sex steroid 17,20P.

It is evident from experiment I (figure 1) that the milt volume increased significantly ($P < 0·001$) in males which remained in contact with ovulatory females as compared to isolated males. An increase in the milt volume in the former male group was noticed within 6 h ($P < 0·05$) and the milt level attained a peak within 12 h of exposure.

Results of experiment II (figure 2) indicate that the milt volume rose three-fold in males exposed to the water which previously held pre-ovulatory females in contrast to the males exposed to the water that held post-ovulatory females. In the former male group, increase in milt volume was found within 6 h and the level of milt reached a peak within 12 h of exposure.

The results of experiment III (test 2) showed a three-fold increase in milt volume in males exposed to the sulphate fraction within 2 h of exposure ($P < 0·001$) (figure 3) and the level of milt remained elevated until 18 to 24 h of exposure. Sperm density and sperm motility were significantly greater ($P < 0·01$) in male groups exposed to the sulphate fraction.

It is apparent from test 3 of experiment III (figure 4) that the subfraction III of the steroid sulphate fraction significantly increased ($P < 0·001$) milt volume, sperm density and sperm motility in males on 6 h of exposure.

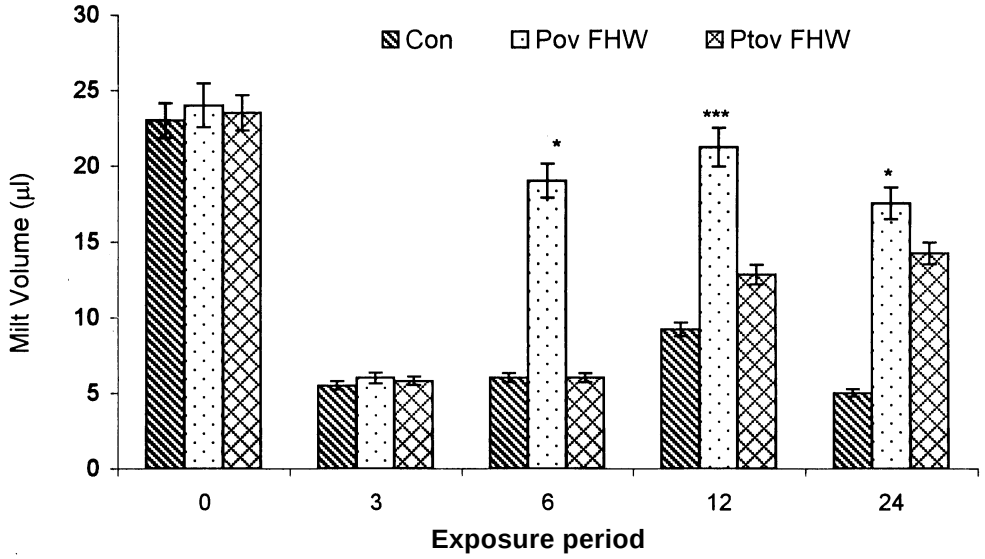

Figure 2. Milt volume in males exposed to the water that held pre-ovulatory and post-ovulatory females. Milt volume (µl ± SEM) in male *B. bendelisis* exposed to blank water (control, $n = 30$), pre-ovulatory female holding water ($n = 30$) and post-ovulatory female holding water ($n = 30$) during 24 h exposure period. This experiment was performed with 5 trials. A significant difference refers to control vs treatment male groups applying student's *t*-test $*P < 0·05$; $***P < 0·001$. Con, Control (blank water); Pov FHW, pre-ovulatory female holding water; Ptov FHW, post-ovulatory female holding water.

Experiment IV (figure 5) showed that the sulphated conjugate of a commercial sex steroid, 17,20P proved potent to significantly increase milt level, sperm density and sperm motility in male *B. bendelisis* ($P < 0{\cdot}001$) within 24 h exposure period compared to control males.

Experiment V (figure 6) reveals that on exposing the ripe males to steroid sulphate fraction and 17,20P, OTL males had significantly lower milt volume compared to the SHAM-treated male group.

4. Discussion

The present study supports the pheromone hypothesis of Gandolfi (1969), Amouriq (1964), Crow and Liley (1979) and Honda (1980a,b) who opined that an inter-sexual attraction mediated by pheromones exists in fish. The sex pheromones appear to originate from the gonads of either sex (Algranati and Perlmutter 1981, Van den Hurk and Lambert 1983). Male

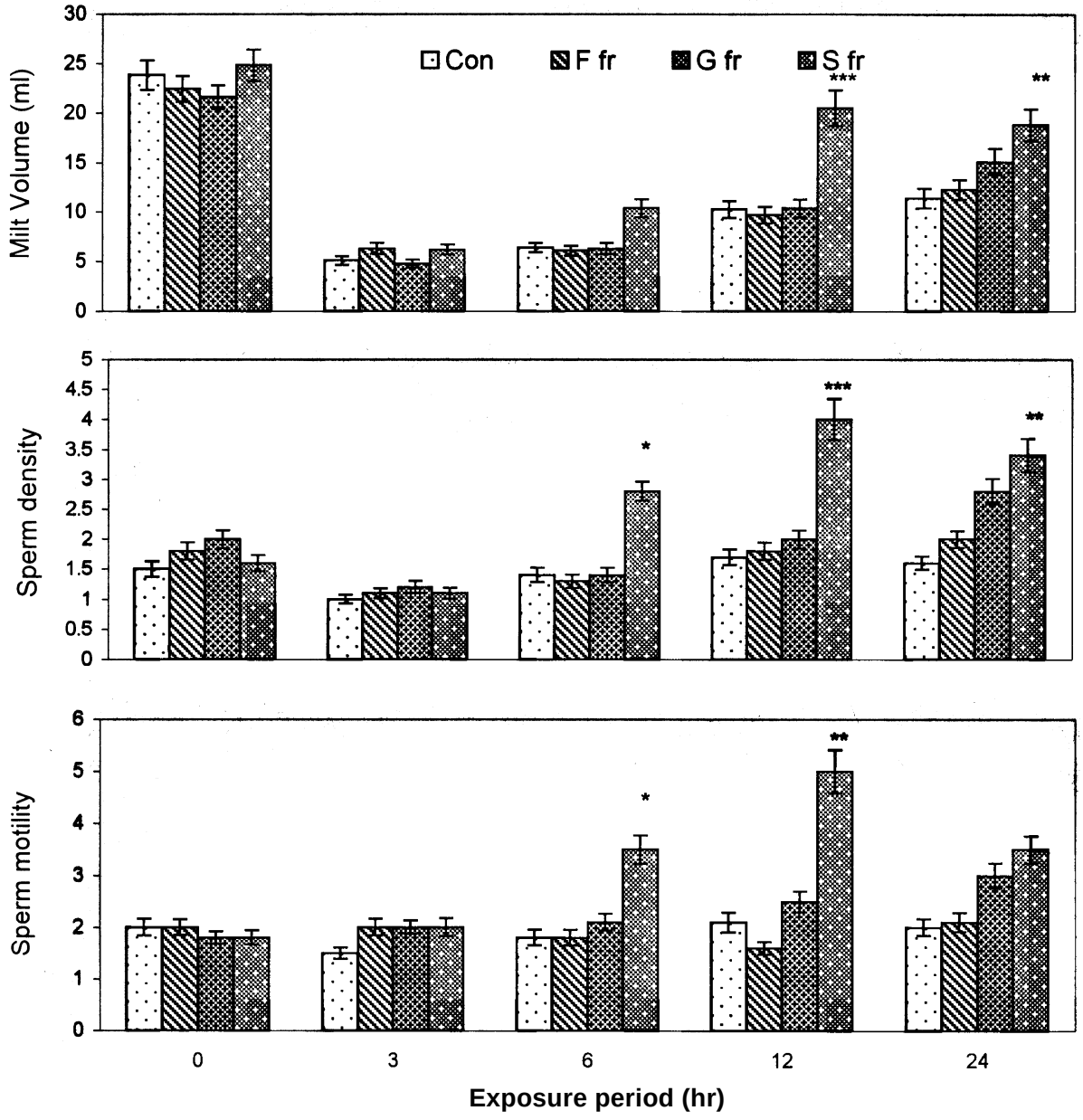

Figure 3. Milt volume (µl ± SEM), sperm cell density and sperm motility in male *B. bendelisis* exposed to control, F, G and S fractions of pre-ovulatory ovarian extract during 24 h exposure period. This experiment includes 5 trials each with 2 males. Significant difference refers to control vs treatment male groups applying student's *t*-test. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$. Con, ethanol control; F fr, free steroid fraction; G fr, glucuronated fraction; S fr, sulphated fraction.

odour induces ovulation in *Gobius jozo* (Colombo *et al* 1980), *Brachydanio rerio* (Van den Hurk *et al* 1987), and *Carassius gariepinus* (Van Weerd *et al* 1991; Lambert and Resink 1991; Vermeulen *et al* 1993). In *Poecilia reitculata* (Johanson 1984), *Salmo gairdneri* (Honda 1980), and *Brachydanio rerio* (Van den Hurk and Sorensen 1993), female odour elicits male sexual behaviour.

Recently steroid hormones and/or their metabolites have been identified as sex pheromones in a few fish species. Testicular synthesis of eteocholenolon glucuronide in *Gobius jozo* (Colombo *et al* 1982), steroid glucuronide in *Brachydanio rerio* (Van den Hurk *et al* 1987) and in *Carassius gariepinus* (Vermeulen et al 1993), have been found while ovarian production of oestradiol 17*b*-glucuronide, as well as estradiol glucuronide in *Brachy-*

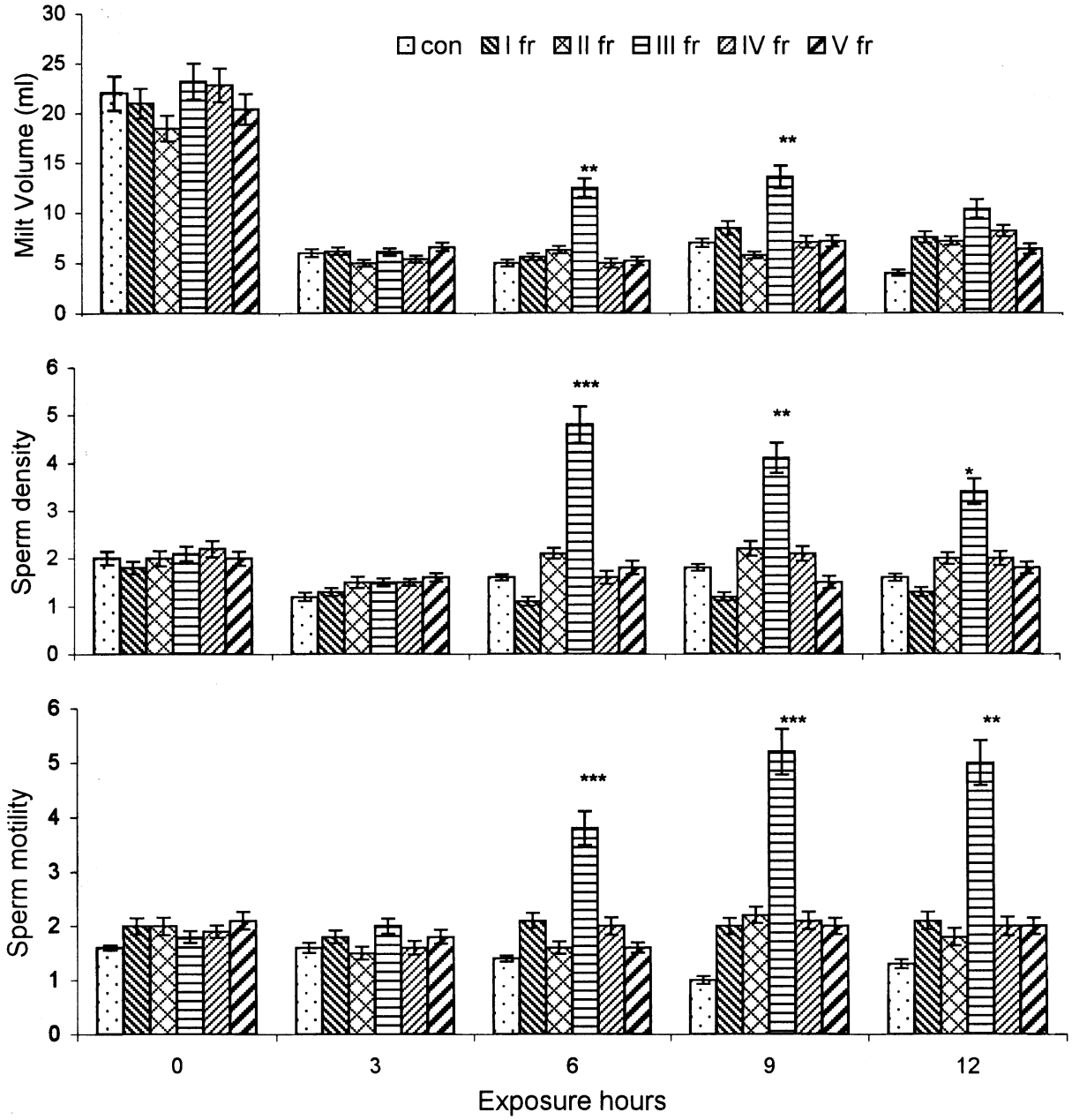

Figure 4. Milt volume (µl ± SEM), sperm density and sperm motility in male *B. bendelisis* exposed to the different sub fractions of S-fraction during 12 h exposure period. The test was performad with 3 trials and 50 males were employed in each trial. Significant difference refers to control vs treatment male groups applying student's *t*-test. **P* < 0·05; ***P* < 0·01; ****P* < 0·001. Con, ethanol control; fr I to V, sub fractions of sulphated fraction of pre-ovulatory ovarian extract.

danio rerio (Van den Hurk and Lambert 1983) and 17,20P in *Carassius auratus* (Dulka et al 1987) have been reported. The steroid glucuronide fraction from the post-ovulatory ovaries elicits a sexual arousal in males of some teleost species (Colombo *et al* 1982).

In our study model, *B. bendelisis*, the bright reddish body colour, prominent tubercles on snout and more milt production in males in contact with ovulatory females as compared to the isolated males are important clues indicating that ovulatory females emit chemical signals to stimulate the reproductive behaviour and physiology of the male. The evidence that the milt volume increases three-fold in males exposed to the water which previously held pre-ovulatory females, in contrast to the males

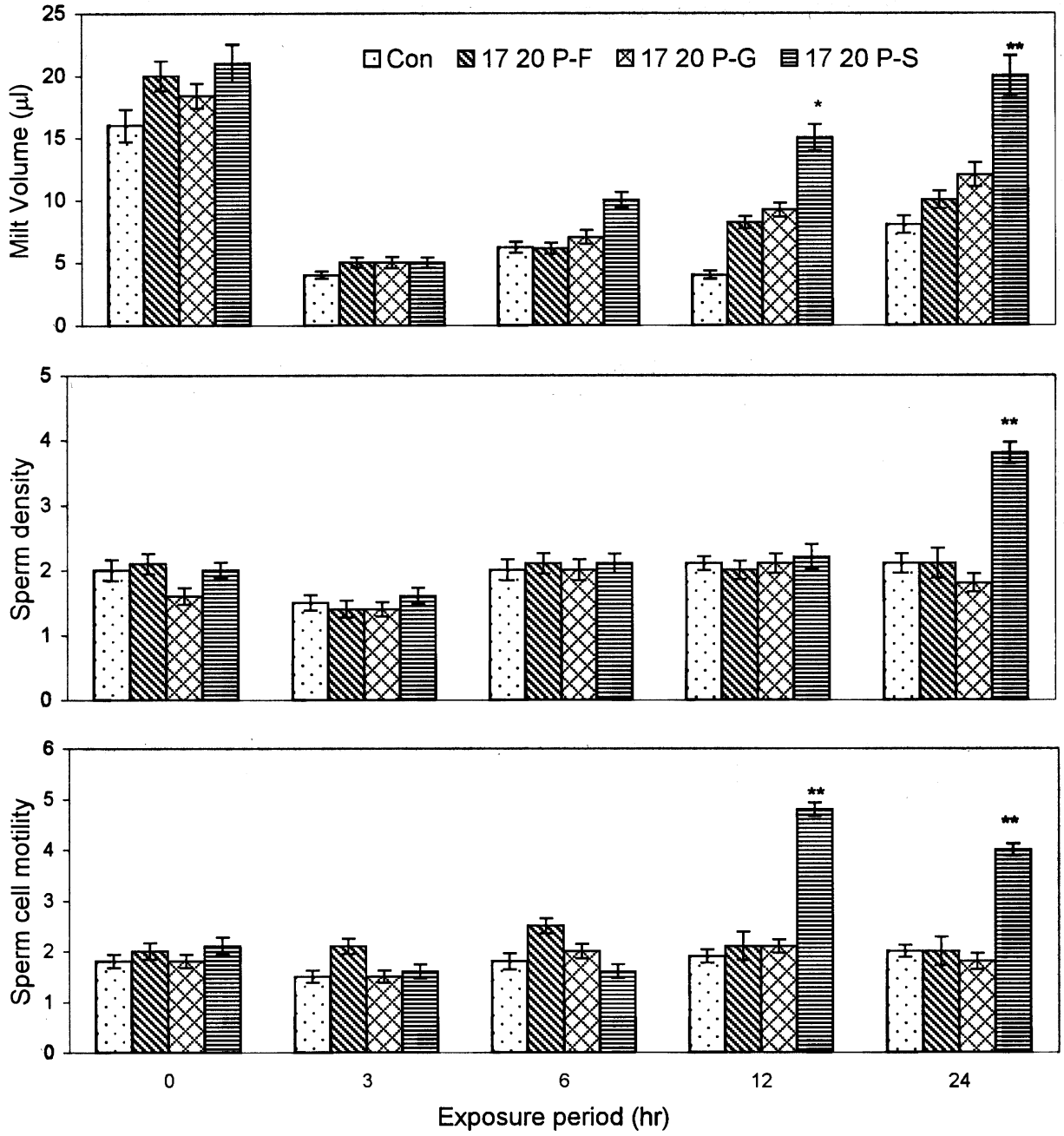

Figure 5. Milt volume (µl ± SEM), sperm density and sperm motility in male *B. bendelisis* exposed to the sex steroid 17,20P including its conjugates for 24 h exposure period. Fish were stripped for milt repeatedly at 3 h intervals. The experiment includes 3 trials and each time 24 males were tested. Significant difference refers to control vs steroid treated males applying student's *t*-test, $*P < 0{\cdot}05$; $**P < 0{\cdot}01$. Con, ethanol; 17,20P = 17*a*,20*b*-dihydroxy-4-pregnen-3-one; G, glucuronide; S, sulphated.

exposed to the water which previously held post-ovulatory females, further suggests that the pre-ovulatory ovarian follicles synthesize sex pheromones and release these into the water where they induce sperm production.

Milt production starts within 6–8 h of exposure in males exposed to the S fraction of pre-ovulatory ovarian extract. The rapid action of sulphated conjugate as compared to the free steroids might be in accordance with the fact that the steroid conjugates happen to be more water soluble than free steroids. Because of high turbulence and fast currents, hillstreams are extremely challenging for chemical communication. Therefore, in such a dynamic aquatic ecosystem, female fish at maximum receptivity release highly water soluble steroid conjugates to form the functional chemical signals to male conspecifics for the temporal synchrony of ovulation with milt production.

Rapidly elevated milt volume and more spermatozoa with greater motility is noticed in males exposed to the purified ovarian steroid sulphate fraction, its isolated sub fraction III and in males exposed to the progesterone metabolites 17,20P. This steroid hormone is known to be synthesized in the gonads of several fish species (Scott and Vermeirssen 1994) and has been found to function as a primer pheromone in male goldfish (Stacey *et al* 1989). The plasma level of 17,20P increases both in female goldfish during the pre-ovulatory period (Peter *et al* 1984) and in males as a consequence of spawning activity (Sorensen *et al* 1995a). The increased amount of this steroid in pre-ovulatory females might be due to its consistent involvement in oocyte maturation (Nagahama *et al* 1983).

The findings of this study strongly suggest that a compound (sub fraction III), isolated from the steroid sulphate fraction of the pre-ovulatory ovarian extract, functions as

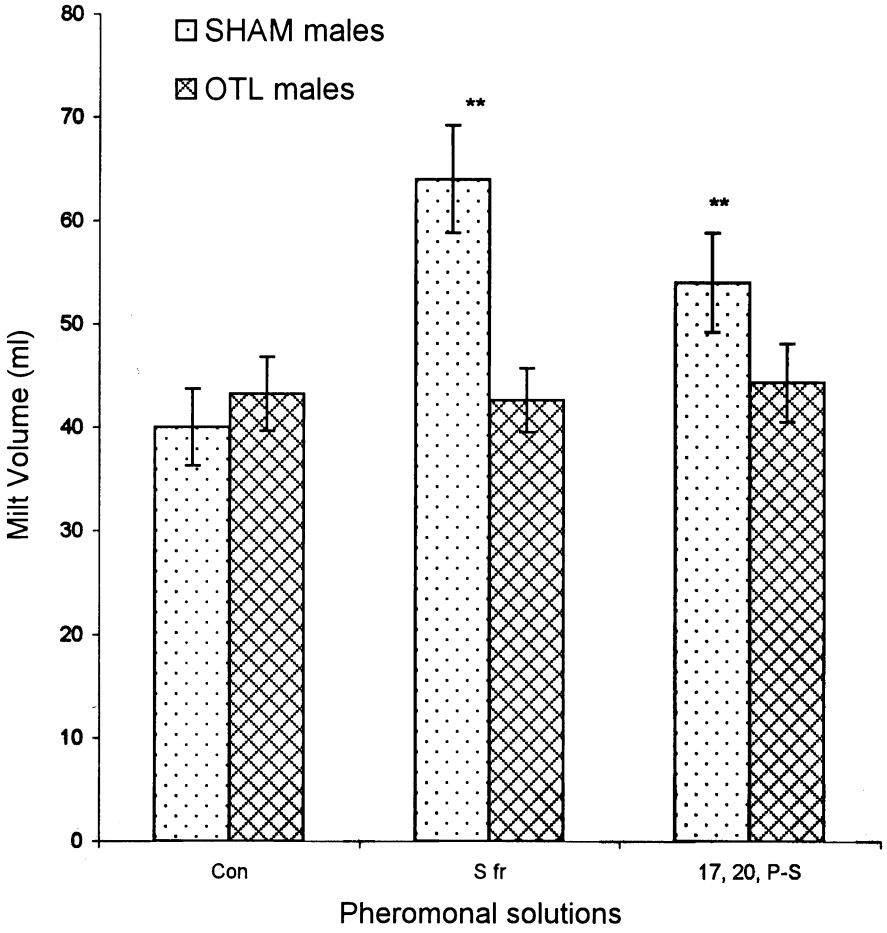

Figure 6. Milt volume (µl ± SEM) stripped from OTL and control (SHAM) male groups exposed to the pre-established primer pheromone 17,20P-S, and ovarian S-fraction. Significant difference refers to OTL vs SHAM males applying student's *t*-test, $^{**}P < 0{\cdot}01$. Con, ethanol control, 17,20P, 17*a*,20*b*-dihydroxy-4-pregnen-3-one; S fr, sulphate fraction.

a reproductive primer pheromone in male *B. bendelisis* like that of the goldfish pheromone 17,20P. It would seem apparent that the release of steroid sulphate which promotes male fertility during spawning should be positively correlated with milt volume, though such a relationship has not been established in externally fertilizing fish species. However, sperm depletion causes decreased male fertility (as measured by the proportion of eggs fertilized) in spawning lemon tetra (Nakutsura and Kramer 1982). Because *B. bendelisis* spawn in large schools in which several males compete for access to the ovulated females, it therefore seems likely that the volume of milt, sperm number and sperm motility may be important determinants of the reproductive success of these fish.

It also emerges from this study that the timing of synthesis and release of steroid sulphates would allow this steroid conjugate to function as a chemical signal to males that ovulation is imminent in the female. The spontaneous ovulation and subsequent spawning in *B. bendelisis* is synchronized with warm temperatures and long photoperiod (Bhatt and Sajwan 1996).

Our finding that OTLs inhibit milt volume in males exposed to the ovarian steroid sulphate fraction provides the evidence that the pheromonally induced reproductive response is mediated by olfaction in fish. The increased milt volume in SHAM (control) males, exposed to ovarian pheromone, further confirms this statement.

Acknowledgements

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Corresponding editor: RENEE M BORGES