Water temperature and pH influence olfactory sensitivity to pre-ovulatory and post-ovulatory ovarian pheromones in male *Barilius bendelisis*

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The attractive response and sexual activity elicited by pre-ovulatory steroid sulphate and post-ovulatory 15K-PGF pheromones are greater in wild caught tubercular males and immature males which express breeding tubercles on the snout (at 12–13 days post androgen implant) than in non-tubercular and non-androgen implanted males of freshwater fish *Barilius bendelisis*. This shows that circulatory androgens exert an activational effect on olfactory receptors of male fish. Wild caught tubercular males and androgen implanted juvenile males exhibit a high responsiveness to steroid sulphate at the water temperature and pH which fish experience during the pre-spawning phase. The male's sensitivity to 15K-PGF is almost equally high at the water temperature and pH which they experience in wild during the both pre-spawning and spawning periods. This suggests that the differential olfactory sensitivity to the two classes of pheromones in androgen implanted males is due to the varied temperature and pH of water, and that during the breeding season the male's olfactory sensitivity to PGF pheromone is more widespread than to the steroidal pheromone. An increased and decreased olfactory sensitivity in mature males to sex pheromones and L-alanine respectively during the breeding phase is in agreement with the hypothesis that pheromonal stimuli dominate over feeding stimuli to promote spawning success.

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1. Introduction

Certain gonadal hormones and/or their metabolites (prostaglandins and C18, C19, C21 steroids) function as sex pheromones in some freshwater teleosts (Stacey and Sorensen 1991; Stacey *et al* 1994). Female goldfish (*Carassius auratus*) release the pre-ovulatory steroidal pheromone, 17*a*,20*b*-dihydroxy-4-pregnen-3-one (17*a*,20*b*-P) (Stacey *et al* 1989). This oocyte maturation inducing steroid (Nagahama *et al* 1983) stimulates the production of plasma gonadotropin and milt in male (Dulka *et al* 1987). The post-ovulatory ovary releases metabolites of prostaglandins (15K-PGF) which stimulate sexual behaviour in the male (Sorensen *et al* 1988). Both these pheromones act through olfactory pathways (Sorensen *et al* 1989).

An electro-olfactogram (EOG) study has shown sex and maturity differences in peripheral olfactory responses to the steroid pheromone in *Salmo solar* (Moore and Scott 1991) and to the prostaglandin pheromone in teleost fish (Sorensen and Goetz 1993). Recently Cardwell *et al* (1995) documented that in male *Puntius schwanenfeldi*,

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Abbreviations used: 17*a*,20*b*-P, 17*a*,20*b*-dihydroxy-4-pregnen-one; EOG, electro-olfactogram; 11-KA, 11-Keto-androstenedione; MT, 17*a*-methyltestosterone.

EOG response to a putative sex pheromone (15K-PGF) was greater in individuals with breeding tubercles. Development of these tubercles is androgen dependent in fish (Liley and Stacey 1983). This observation suggests a functional relationship of androgen with the peripheral olfactory receptor response. However, none of the previous studies indicate the possible role of sex specific olfactory responses and also the environmental correlates of olfactory sensitivity to two different classes of sex pheromones is not well understood in fish. During the course of present study we noticed the reddish body colour and prominent tubercles on the snout of field caught male *Barilius bendelisis* throughout the breeding season (February mid to August). The temperature and pH of water vary in the pre-spawning and spawning period of this fish.

The aim of the present investigation was to determine whether the male's olfactory sensitivity increased by circulatory androgens would be similar for the pre-ovulatory steroid sulphate and post-ovulatory 15K-PGF during the breeding period or whether the increased olfactory sensitivity to these two different class of sex pheromones depends upon the temperature and pH of water which fish experience during pre-spawning and spawning periods in the wild.

2. Materials and methods

Mature males of *Barilius bendelisis* (Hamilton) were collected during the pre-spawning (February–March, $n = 108$, body weight 7 g) and spawning (July–August, $n = 107$, body weight 9·2 g) phase from a nearby stream 'Khanda gad'. In both cases, field caught fish were maintained in a flowthrough tank at 16·5°C, pH 8·3 and 23°C, pH 7·4; this mimic the male in the pre-spawning and spawning period respectively. Each time, the subjects were fed with commercial fish food twice daily.

All specimens caught from the wild were males, since they exhibited enlarged fan shaped dorsal and ventral fins and reddish body colour (Badola *et al* 1982). The maturity was confirmed by hand stripping a minute amount of milt from the genital pore of these field caught males. Tubercular individuals were isolated from the non-tubercular ones and were placed in two different tanks till they were employed for tests.

Juveniles of male *B. bendelisis* (age 11–12 month, length 116 mm, weight 5·2 g) were taken from the laboratory reared stock for the experiments. The male of this species attains maturity at the age of 15 month and length 123 mm (Dobriyal and Singh 1990). These juveniles were maintained under captive conditions (water temperature 13°C, pH 7·9, free CO_2 2·7 ppm, DO 7·4 ppm) till they were required for the experiments.

2.1 *Preparation of odourant's solution*

Since the steroid sulphate fraction isolated from the pre-ovulatory ovarian extract of *B. bendelisis* was proved to be potent olfactory stimulants in male conspecific (Bhatt and Sajwan 2001), a 1% stock solution (1 mg/100 ml distilled water) of this purified fraction was prepared and stored at 4°C prior to use. For testing, the stock solution was diluted in aquarium water to make 10^{-7} concentration (Sajwan *et al* 1999).

15-Keto-prostaglandin (15K-PGF, Sigma, USA) which functions as post-ovulatory pheromone in fish (Cardwell *et al* 1995) was dissolved in ethanol (1 mg/100 ml) and stored at – 20°C. Aliquots were diluted in distilled water at 10^{-5} M concentration and then stored at 4°C before use. This stock solution (100 µl) was further diluted in 100 ml of aquarium water to make 10^{-8} M solution.

The stock solution (10^{-2}) of an amino acid, L-alanine (from Sigma) was prepared in distilled water and aliquots were diluted in background water at 10^{-5} M concentration for the testing. L-alanine has been determined as a food odour to the teleost fish (Caprio 1984).

2.2 *Androgen treatment to fish*

Juvenile (gonads immature) males of *B. bendelisis*, anaesthetized with 0·02% 2-phenoxy-ethanol, were implanted (i.p.) with 17*a*-methyltestosterone (MT = 0·15 mg/g body wt, $n = 48$) and with 11-Keto-androstenedione (11KA – 0·15 mg/g body wt, $n = 47$). Androgens were implanted (i.p.) with beeswax, procured from the local bees culture farm. A control group of juveniles ($n = 48$) was implanted with beeswax alone. The androgen implanted subjects were left overnight for recovery in 100 litre aquaria.

2.3 *Sexual behaviour*

To determine whether androgens increase the peripheral olfactory sensitivity in male fish to the ovarian pheromones, the sexual acts (chasing, nudging activities) were recorded in tubercular and non-tubercular males as well as in androgen implanted and non-androgen implanted immature males. *B. bendelisis* male chased and nudged (male puts its mouth/head on female gonopore) the ovulatory female of PGF_{2a} injected female under captive conditions (Bhatt and Sajwan 1996).

2.4 *Experiment I: Attractive response in wild caught tubercular and non-tubercular males to female sex pheromones*

Attraction tests were carried out in 150 litres aquaria which were divided into two equal halves by vertical line

drawn on their front windows. One day before each test, tubercular (n = 8) and non-tubercular (n = 8) males procured from wild during pre-spawning and spawning periods were placed each time in two different observation aquaria to adapt them to new environment. The temperature and pH of water was maintained similar to what the test fish experienced in wild during pre-spawning (temp. 16·5°C, pH 8·3) and spawning (temp. 23°C, pH 7·4) phase. On the day of the experiment, first a control test was run in which odour free water (blank water) was inserted through two drip sets simultaneously from the left and right corners of an aquarium at about 5 cm below the aquarium water level. After 1 or 2 min, the number of fish and time spent by them in both halves of the aquarium were scored at 30 s intervals for 15 min. Scoring at 30 s interval for 15 min led to 30 scores of number of fish in each half of the aquarium for each batch of 8 fishes. The test experiments on a batch of fish followed the control experiments on that batch of fish immediately only if the fish did not show an obvious preference in the control test for one of the aquarium half. In the test experiments, 300 ml solution of each of L-alanine (food odour), steroid sulphate (pre-ovulatory pheromone) and 15K-PGF (post-ovulatory pheromone) were released separately into the different aquaria at 20 ml/min flow rate for 15 min period from one of the corners of the aquarium where the overall mean number of fish was less during the control tests. Each time an equal volume of blank water was simultaneously administered from the other corner of the aquarium. Scoring was as in the control tests. Each test included 7 replicates with different batches of fish in a paired control and test experiments. Tests were conducted on a fixed hour of a day to avoid diurnal variations.

2.5 *Experiment II: Responsiveness in androgen implanted immature males to female sex pheromones*

Tests were conducted in 100 litre aquaria divided into two equal halves by a vertical line drawn on front windows. Androgen implanted (n = 7) and non-androgen implanted juvenile males were placed into two different observation aquaria 12 h before the experiments started. Two test solutions (300 ml of each) i.e. either L-alanine and blank water, or steroid sulphate and blank water, or 15K-PGF and blank water were injected simultaneously from the left and right corners of the aquarium at 20 ml/min flow rate for 15 min period. Both androgen implanted and non-androgen implanted groups of fish were exposed to the test solutions under pre-spawning (temp. 16.5°C, pH 8·3) and spawning (temp. 23°C, pH 7·4) conditions. The test procedure was similar as in experiment I except that in this experiment the number of fish and time spent by them in both halves of an aquarium were scored at 20 s intervals for 15 min. Scoring 20 s intervals for 15 min led to 45 scores of number of fish in each halves of the aquarium for each batch of 7 fishes. Each test was performed with 8 trials each time with a fresh batch of fish in a paired control and test experiments.

2.6 *Experiment III: Behavioural responsiveness in androgen implanted juvenile males and tubercular males to the ovarian pheromones*

To determine whether androgen implant increases the olfactory sensitivity in male fish to the ovarian pheromones and whether expression of tubercles has any relationship with the pheromonal response in males, we investigated the effects of exposure to the pre-ovulatory steroid sulphate and post-ovulatory 15K-PGF pheromones on male's sexual behaviour. In cyprinids (Sorensen and Goetz 1993) and in *B. bendelisis* (Bhatt and Sajwan 1996), PGF-injected and ovulated females holding water triggered the male courtship activity. During the course of this study two components of courtship i.e. chasing and nudging were selected to be recorded. *Chasing*: analogous to following which includes a definite orientation of male towards females. In this action, sexually aroused male butt against the ovipore of female. *Nudging*: in this action, the physical contact between a sexually aroused male and stimulus female (male puts its mouth most frequently at the gonopore of female) takes place (Sorensen *et al* 1988).

Before androgen implant, a pair of juvenile males (n = 2) was placed in each of the two different observation aquaria overnight to make the fish familiar to its new environment. Two hundred ml of each of steroid sulphate and 15K-PGF solution was released separately into these two different aquaria for 10 min period. The number of chases and nudges displayed by non-androgen implanted and androgen implanted males prior to exposure as well as on exposing them to each of steroid sulphate and 15K-PGF solutions for 10 min were noted on a check sheet divided in 10 min sections from both fish groups. This experiment included 8 replicates each time with a new batch of fish.

2.7 *Statistics*

Analysis of data for attraction test was done by method of Van den Hurk and Lambert (1983). Since the independence of 30 s and 20 s scores depended upon a subjective impression, the mean number of fish in an odourant solution receiving half of an aquarium over 30 scores (exp. I) 45 scores (exp. II) in any 15 min obser-

vance was used as the recorded variable for each batch of 7 and 8 fishes respectively. This led to 7 and 8 pairs of means for control and test for 7 and 8 batches of fish in test experiments and respective control tests were drawn/calculated by applying Student's *t*-test.

Data on courtship activity (chasing, nudging) were analysed by Mann–Whitney *u*-test. The mean values of number of chases and number of nudges were sum up (denoted as courtship act in text) each time, and a significant difference between pre and post exposure values was calculated by applying Student's *t*-test.

3. Results

3.1 *Attractive response in wild caught tubercular males to the ovarian pheromones*

3.1a *Under pre-spawning condition (water temp. 16×5°C, pH 8×3)*: It was evident from figure 1 that simultaneous exposure to L-alanine (food odour) and blank water (odour free water), both tubercular and non-tubercular males exhibited greater attraction to L-alanine than to blank water. The attraction to L-alanine was little higher in non-tubercular males ($P < 0{\cdot}01$) than in tubercular males ($P < 0{\cdot}05$). On the other hand, the tubercular and non-tubercular males if simultaneously exposed either to steroid sulphate and blank water or to 15K-PGF and blank water, the non-tubercular males showed an insignificant ($P > 0{\cdot}05$) while tubercular males exhibited a high ($P < 0{\cdot}01$, $0{\cdot}001$) attractive response to the pheromonal solutions as compared to the blank water. It was interesting to note that under pre-spawning conditions, the attractive response in tubercular males was almost equal for pre-ovulatory steroid sulphate and post-ovulatory 15K-PGF.

3.1b *Under spawning condition (water temp. 23°C, pH 7×4)*: Figure 2 indicates that the tubercular and non-tubercular males when exposed simultaneously to L-alanine and blank water, as compared to the former, the latter have had a greater attraction to the food odour ($P < 0{\cdot}01$) than to odour free water. The tubercular and non-tubercular males if exposed simultaneously to either steroid sulphate and odour free water or to 15K-PGF and odour free water, tubercular males showed little more attraction to the steroid sulphate ($P < 0{\cdot}05$) and much more attraction to 15K-PGF ($P < 0{\cdot}001$) than to odour

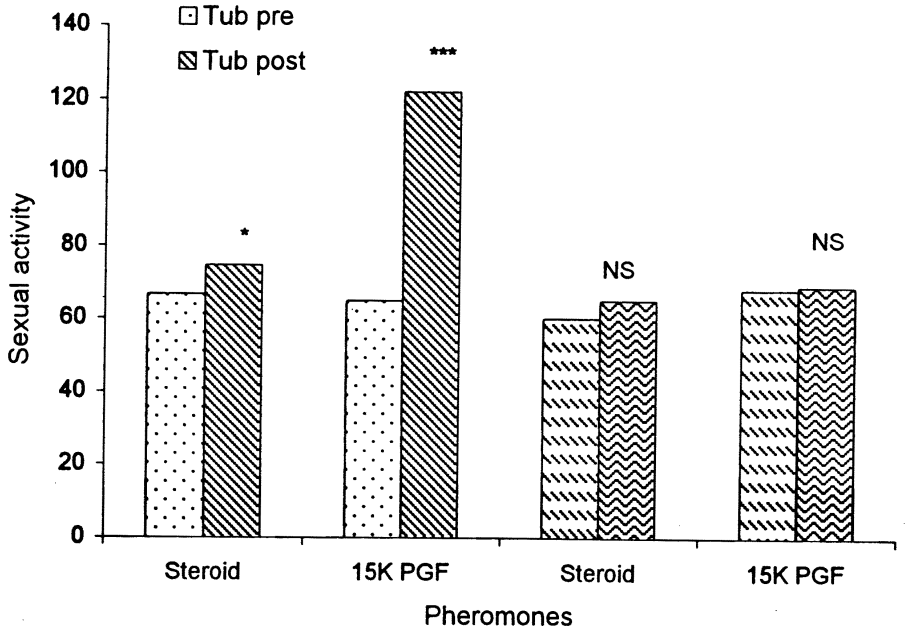

Figure 1. Attractive response to food odour (L-alanine: 10^{-5} M) and pre-ovulatory steroid sulphate (10^{-7} M) and post-ovulatory 15-Keto prostaglandin (10^{-8} M) pheromones in tubercular ($n = 24$) and non-tubercular ($n = 24$) wild caught mature males of *B. bendelisis* under pre-spawning conditions (water temperature: 16·5°C, pH 8·3). Student's *t*-test was applied to obtain difference of attractive response between odourant treatment and control treatment (odour free water) groups of tubercular (Tub) and non-tubercular (Non Tub) males, (NS, Non significant; *significant $P < 0{\cdot}05$; ***highly significant $P < 0{\cdot}001$).

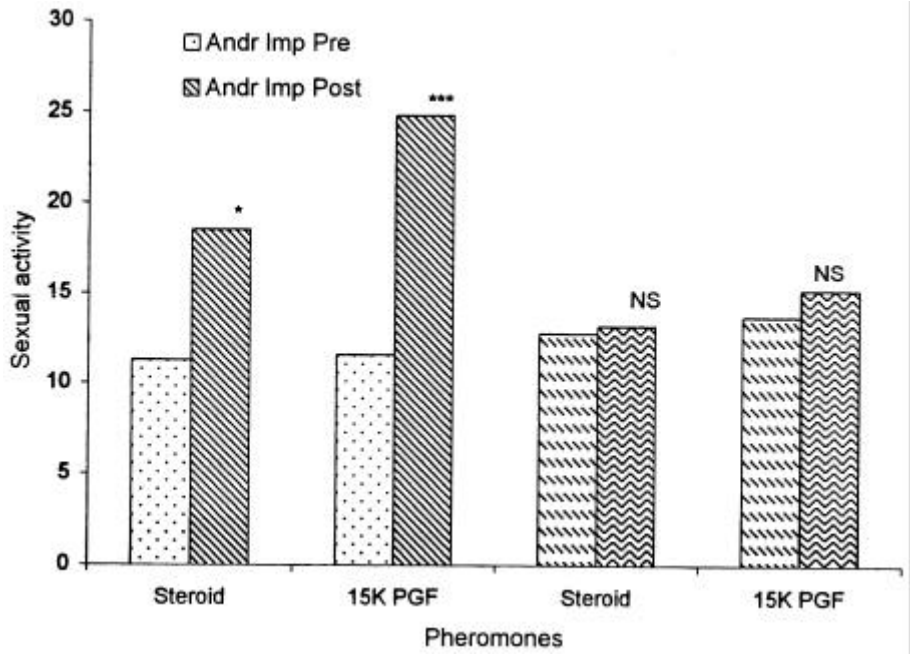

Figure 2. Attractive response to food odour (L-alanine: 10^{-5} M) and pre-ovulatory steroid sulphate (10^{-7} M) and post-ovulatory 15-Keto prostaglandin (10^{-8} M) pheromones in tubercular ($n = 24$) and non-tubercular ($n = 24$) wild caught mature males of *B. bendelisis* under spawning conditions (water temperature: 23°C, pH 7·4). Student's *t*-test was applied to obtain difference of attractive response between odourant treatment and control treatment (odour free water) groups of tubercular (Tub) and non-tubercular (Non Tub) males, (NS, Non significant; *significant $P < 0{\cdot}05$; ***highly significant $P < 0{\cdot}001$).

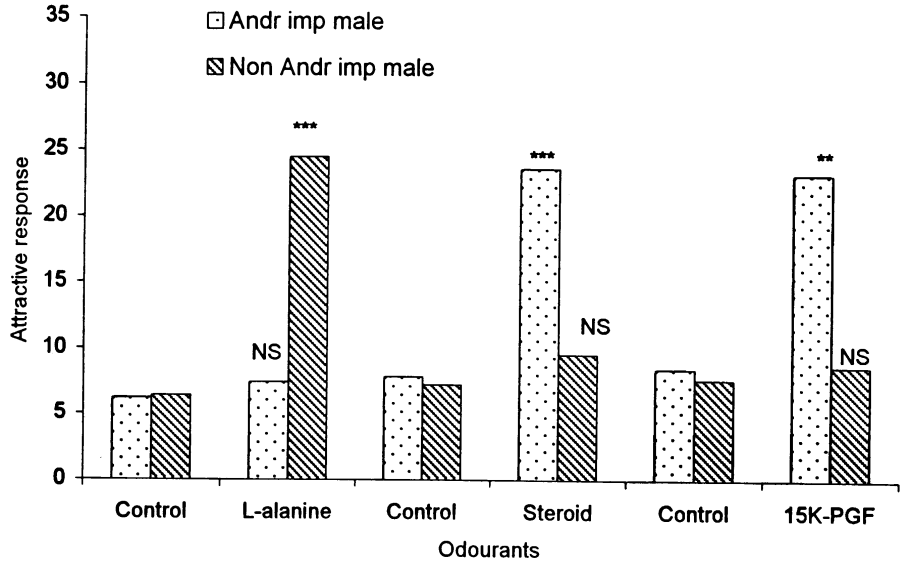

Figure 3. Attractive response to food odour (L-alanine: 10^{-5} M) and pre-ovulatory steroid sulphate (10^{-7} M) and post-ovulatory 15-Keto prostaglandin (10^{-8} M) pheromones in androgen implanted ($n = 21$) and non-androgen implanted ($n = 21$) juvenile males of *B. bendelisis* under pre-spawning conditions (water temperature: 16·5°C, pH 8·3). Student's *t*-test was applied to obtain difference of attractive response between odourant treated males and control males of androgen implanted (Andr Imp) and non androgen implanted (Non Andr Imp) fish, (NS, Non significant; ** more significant $P < 0{\cdot}01$; ***highly significant $P < 0{\cdot}001$).

free water; while non-tubercular males exhibited an insignificant attraction ($P > 0{\cdot}05$) to both of these pheromone solutions as compared to the blank water.

3.2 *Attractive response in androgen implanted immature males to the ovarian pheromones*

3.2a *Under pre-spawning condition (temp. 16×5°C, pH 8×3)*: The dermal tubercles on snout and brilliant red colour on body were expressed in juvenile males of *B. bendelisis* 12–13 days post implant with MT. These secondary sex characters did not appear in 11-KA implanted juveniles.

In androgen implanted juveniles (15–16 days post implant), the attractive response to pre-ovulatory steroid sulphate and post-ovulatory 15K-PGF pheromones was far greater ($P < 0{\cdot}01$) than to the odour free water. On the other hand non-androgen implanted juveniles exhibited an insignificant attraction ($P > 0{\cdot}05$) to both these pheromones as compared to the blank water. Under pre-spawning condition, the attractive response of androgen implanted juvenile males was almost equal for both class of pheromones. Androgen implanted and non-androgen implanted immature males if simultaneously exposed to L-alanine and odour free water, the non-androgen implanted juvenile showed remarkably more attraction to the food odour ($P < 0{\cdot}001$) than to the blank water, while androgen implanted fish exhibited an insignificant ($P > 0{\cdot}05$) attraction to L-alanine in contrast to odour free water (figure 3).

3.2b *Under spawning condition (water temp. 23°C, pH 7×4)*: Simultaneous exposure to either steroid sulphate and odour free water or to 15K-PGF and odour free water, the androgen implanted males showed a significant ($P < 0{\cdot}05$) and highly significant ($P < 0{\cdot}001$) attraction to the steroid sulphate and 15K-PGF respectively, in comparison to the odour free water (figure 4). Non-androgen implanted fish did not have any attraction to either of these pheromones. If exposed to L-alanine and blank water, androgen implanted and non-androgen implanted juveniles had an insignificant ($P > 0{\cdot}05$) and much more significant ($P < 0{\cdot}001$) attractive response respectively to the food odour than to the odour free water.

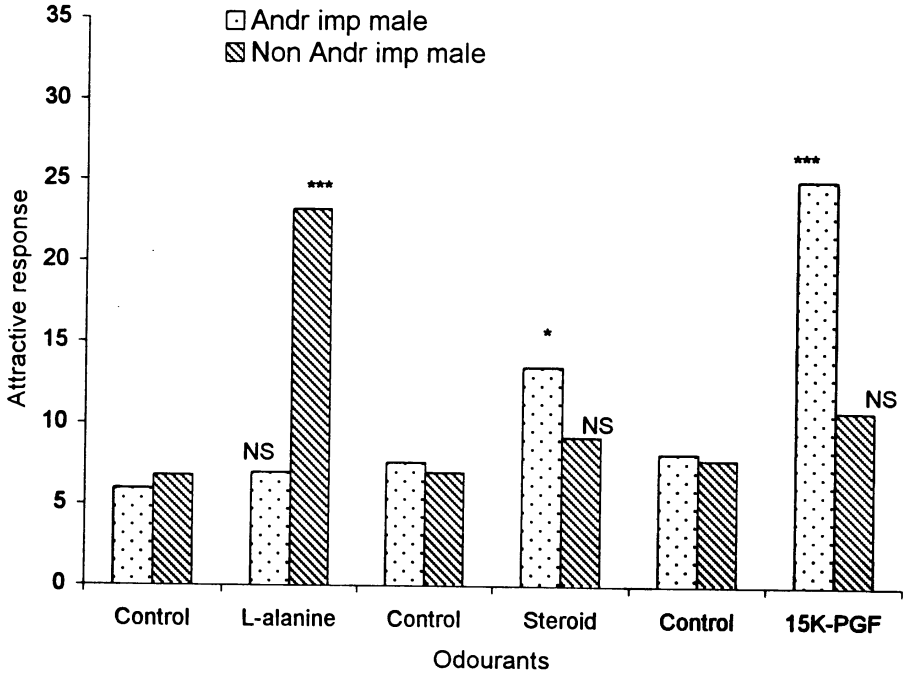

Figure 4. Attractive response to food odour (L-alanine: 10^{-5} M) and pre-ovulatory steroid sulphate (10^{-7} M) and post-ovulatory 15-Keto prostaglandin (10^{-8} M) pheromones in androgen implanted ($n = 21$) and non-androgen implanted ($n = 21$) juvenile males of *B. bendelisis* under spawning conditions (water temperature: 23°C, pH 7·4). Student's *t*-test was applied to obtain difference of attractive response between odourant treated males and control males of androgen implanted (Andr Imp) and non-androgen implanted (Non Andr Imp) fish, (NS, Non significant; *significant $P < 0{\cdot}05$; ***highly significant $P < 0{\cdot}001$).

3.3 *Behavioural response in tubercular and androgen implanted males to the ovarian pheromones*

Findings of this study indicated that as compared to pre-exposure period (with odour free water) the sexual activity (chasing, nudging) in wild caught tubercular males (figure 5) and androgen implanted juveniles (figure 6) was increased much more ($P > 0{\cdot}001$) by 15K-PGF pheromone than by steroid sulphate ($P > 0{\cdot}05$).

4. Discussion

The actions of pre-ovulatory steroids and post-ovulatory prostaglandin pheromones, which are released sequentially during the pre-ovulatory period of female fish, appear to function relatively independent of each other in a conspecific male (Sorensen *et al* 1989). A sex steroid 17,20-P is detected by olfactory receptors which are different from those which detect bile acid and L-amino acids (Caprio 1984). Similarly, goldfish have separate olfactory receptor sites for PGFs and 15K-PGF that are independent from those detect other olfactory stimulants (Sorensen *et al* 1991). EOG experiments further indicated that these three pheromonal compounds 17,20-P, PGF and 15K-PGF are detected by different receptor mechanisms (Sorensen and Goetz 1993). Recently Cardwell *et al* (1995) demonstrated hormonally induced plasticity in olfactory receptors of fish, *Puntius shwanenfeldi*, to 15K-PGF hormone.

The present study showed that there is a remarkable difference in olfactory sensitivity to steroid and prostaglandin pheromones and food odour (L-alanine) between tubercular and non-tubercular males of a freshwater fish *B. bendelisis*, suggesting an activational effect of androgens on olfactory receptors. Evidently, the breeding tubercles on snout appear on 12–13 days post androgen implant in gonadally immature males. Also, tubercular males and androgen implanted juvenile males are observed to be sexually more active than non-tubercular and non-androgen implanted fish. Though the exact function of such breeding tubercles is not known, it is apparent from this study that only tubercular males are reproductively accessible to the receptive female counterpart. During the sexual acts, tubercular males contact through their mouth the gonopore of sexually receptive female most frequently.

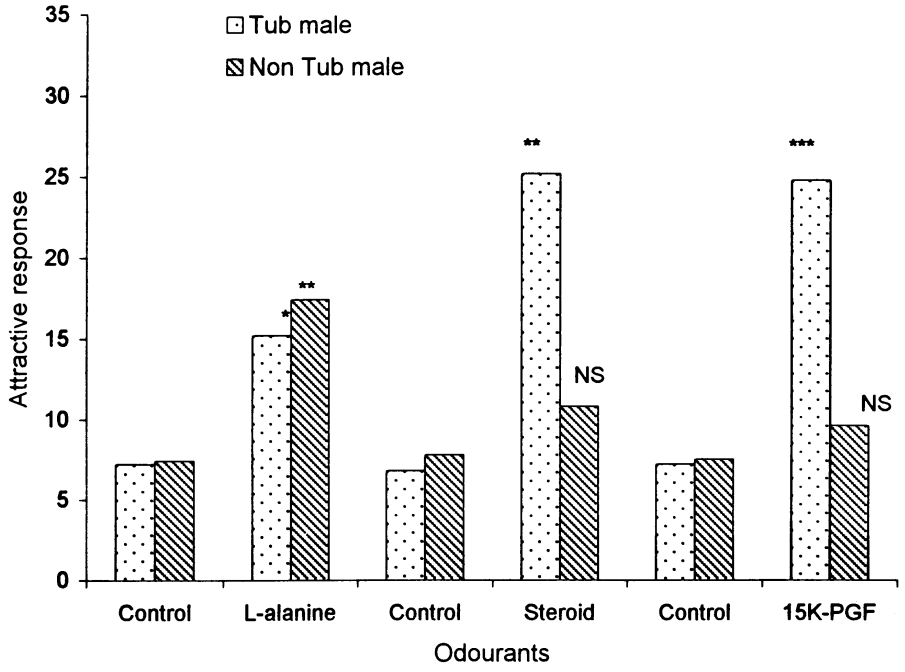

Figure 5. Sexual activity (chasing, nudging) in wild caught tubercular (Tub, $n = 12$) and non-tubercular (Non Tub, $n = 12$) males of *B. bendelisis* during pre-treatment (Pre) and post-treatment (Post) with pre-ovulatory steroid sulphate (10^{-7} M) and post-ovulatory 15-Keto prostaglandin (10^{-8} M) pheromones. Student's *t*-test was applied to derive the difference of sexual activity between pre-treatment (pre) and post-treatment (post) male groups. (NS, Non significant; *significant $P < 0{\cdot}05$; **more significant $P < 0{\cdot}01$; ***highly significant $P < 0{\cdot}001$).

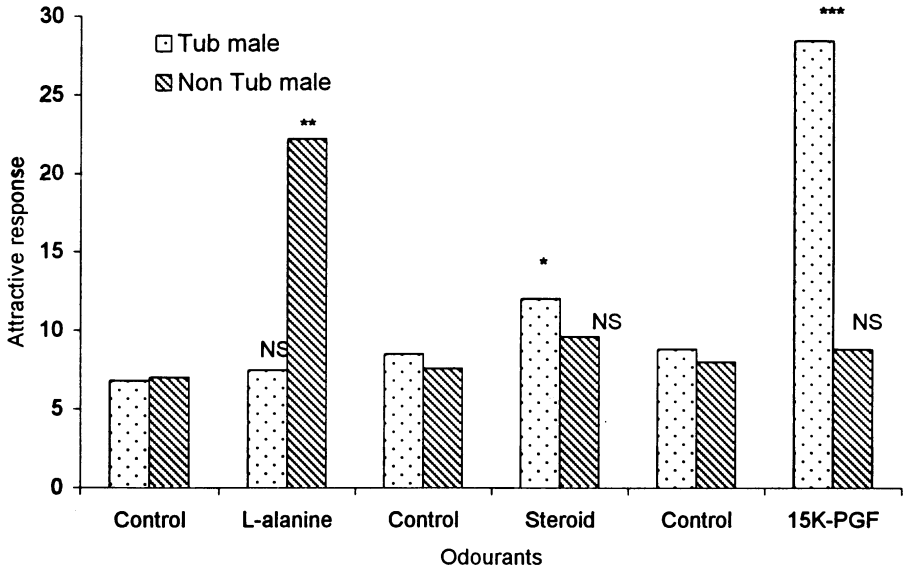

Figure 6. Sexual activity (chasing, nudging) in androgen implanted (Andr Imp, $n = 16$) and non-androgen implanted (Non Andr Imp, $n = 16$) immature males of *B. bendelisis* during pre-treatment (Pre) and post-treatment (Post) with pre-ovulatory steroid sulphate (10^{-7} M) and post-ovulatory 15-Keto prostaglandin (10^{-8} M) pheromones. Student's *t*-test was applied to derive the difference of sexual activity between pre-treatment (pre) and post-treatment (post) male groups. (NS, Non significant; *significant $P < 0{\cdot}05$; **more significant $P < 0{\cdot}01$; ***highly significant $P < 0{\cdot}001$).

Two types of receptor cells (type I and II) recognized in the olfactory epithelium of our study model (Singh and Singh 1987) match with the microvillar and ciliated receptors described on the olfactory epithelium of many other teleost species that serve distinct functions (Dulka 1993). Olfactory receptors for non pheromonal compounds (Breer *et al* 1992) are less sensitive than receptors for sex pheromones (Stacey *et al* 1994) indicating that a different transduction mechanism may be involved.

The striking feature emerging from this study is that the androgen dependent olfactory sensitivity in juvenile males to the two classes of ovarian pheromones is influenced by temperature and pH of medium. Evidently androgen implanted juveniles exhibit a high attraction to sex-steroids only at water temperature and pH which fish experience in wild during the pre-spawning phase; while their sensitivity (attraction, sexual acts) to prostaglandins is equally high at water temperature and pH which fish experience in wild during pre-spawning and spawning periods.

Cardwell *et al* (1995) reported that the effect of androgen on EOG responses in *P. schwanenfeldi* to 15K-PGF was specific without altering responses to steroidal and food (amino acids) odours. Androgens may influence efferent neural fibres from higher brain centres terminating in the olfactory epithelium and specifically increasing responses to 15K-PGF by olfactory receptor neurons. The ultrastructural complexity of olfactory epithelium is correlated with the degree of sexual maturity (Schreibman *et al* 1986), consistent with the hypothesis that reproductive hormones directly influence the olfactory receptor neurons (Cardwell *et al* 1995). Our study indicates that during the breeding season, males olfactory sensitivity to 15K-PGF pheromones is spread from pre-spawning to spawning period while the same to steroidal pheromones is confined to the pre-spawning period, suggesting that the olfactory receptors for prostaglandins might have evolved earlier. The differential sensitivity of olfactory receptor to two classes of pheromones probably reflects a different evolutionary origin of prostaglandins and steroidal pheromone receptors in fish.

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