The Journal of Experimental Biology 208, 2037-2043
Published by The Company of Biologists 2005
doi:10.1242/jeb.01584

Olfactory discrimination of female reproductive status by male tilapia (*Oreochromis mossambicus*)

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Accepted 9 March 2005

Summary

The current study investigated whether discrimination of sexual status of female tilapia by males is mediated by olfaction. Size-matched groups of female tilapia were assigned as pre- or post-ovulatory according to the time since their last ovulation (15–19 days pre-ovulatory, *N*=7; 1–3 days post-ovulatory, *N*=8). Female-conditioned water and body fluids (urine, bile, faeces and plasma) were assessed for olfactory potency in males by recording the electro-olfactogram (EOG). Water extracts, urine and faeces from pre-ovulatory females all evoked significantly larger amplitude EOGs in male fish (*N*=6), with correspondingly lower thresholds of detection, than those from post-ovulatory females. Plasma and bile evoked very large amplitude EOGs in males but with no differences between the two groups of females. Anosmic males (*N*=6) did not behave differently towards pre- or post-ovulatory females, while sham-operated males (*N*=6) showed a marked increase in urination rate towards pre-ovulatory females. We conclude that the ability of male tilapia to discriminate between females of differing reproductive status is mediated by odorants released into the water, probably *via* the urine and faeces, by pre-ovulatory females.

Key words: chemical communication, behaviour, reproduction, courtship, fish, cichlid, pheromone.

Introduction

Chemical communication is widely believed to be important during reproduction in teleosts. However, given the number and diversity of reproductive strategies shown by this division, the number of species that have been investigated in any depth is very small. The most-studied is the goldfish (Stacey and Sorensen, 2002), where females release three pre-ovulatory steroid hormone-derived pheromones in their urine, which are detected by the olfactory system of the males. This, in turn, evokes a number of physiological responses, including increases in milt volume and sperm motility. During ovulation, the females release prostaglandins in their urine, which then induces spawning behaviour in the males.

Given the range and complexity of reproductive strategies in cichlids (Barlow, 1991; Keenleyside, 1991), it is surprising that the phenomenon of chemical communication has not been equally well investigated in this group. Ample evidence suggests that it is important in kin recognition (Barnett, 1982; Russock, 1990; Vives, 1988) and male–male aggression (Giaquinto and Volpato, 1997), but the chemicals involved, and their routes of release, are unknown. Other studies have suggested that sex steroids may also have pheromonal roles (Oliveira et al., 1996; Robinson et al., 1998; Rocha and Reis-Henriques, 1996, 1998) during courtship, but this has not been tested directly. The Mozambique tilapia (*Oreochromis mossambicus* Peters 1852) can spawn repeatedly throughout the year. Reproductively active females have a regular ovulatory cycle of 15–20 days (Coward and Bromage, 2000), which is normally only interrupted by successful spawning; they are maternal mouth-brooders, and mouth-brooding females delay their next ovulatory cycle until the fry are released. Males establish a hierarchy where dominant individuals develop a characteristic black colouration and aggressively defend a nest and associated area. We have previously shown that female tilapia have high olfactory sensitivity to substances released by males, particularly in the urine (Frade et al., 2002), and that males increase their rate of urination as part of their courtship display (Almeida et al., 2005). Furthermore, urine from territorial males is a more potent odorant than that from non-territorial males (Frade et al., 2003). It seems likely, therefore, that, in contrast to the goldfish, territorial male tilapia are sending chemical signals to the females. However, in a given population, the number of males displaying territoriality varies; a few remain territorial on a permanent basis, a similar number remain subordinate whilst a third group display intermittent territoriality (Oliveira and Almada, 1996). Preliminary observations suggest that these males become territorial when one or more of the females becomes pre-ovulatory; i.e. ready to spawn. This observation

suggests that males are able to assess the reproductive status of females and alter their behaviour accordingly (Almeida et al., 2005). It is possible that pre-ovulatory females release substances into the water, which the males then detect by olfaction, and it is this chemical information that stimulates courtship behaviour and territoriality in the males. This is the hypothesis tested by the current study.

Materials and methods

Pre- and post-ovulatory females and collection of body fluids

Adult tilapia (*Oreochromis mossambicus* Peters 1852) were kept in family groups (one male with four or five females) in fibreglass tanks (approximately 250 litres) with a glass window and sand substratum. Water temperature was kept at 27°C and the fish were fed once a day with commercial cichlid food (Nutrafin basix®; Rolf C. Hagen, Inc., Montreal, Canada). Under these conditions, the fish spawned regularly but the fertilized eggs were removed from the mother's mouth and incubated elsewhere to maintain the ovulatory cycle. All fish were tagged, and daily observations determined the cycle length of individual females, thus allowing prediction of the next ovulation. Only regularly cycling females that had cycle lengths of 9–19 days (mean, 15 days) were used. Females were designated 'pre-ovulatory' on the day prior to their predicted ovulation and 'post-ovulatory' one or two days after their last ovulation. As fish were used in experiments, they were replaced in the family tanks by fish of the same size from a stock tank. Pre-ovulatory and post-ovulatory females were isolated in a 20-litre glass tank with dechlorinated tapwater (at 27°C) for two hours. A two-litre sample of the water was then passed through a solid-phase C18 extraction cartridge (Sep-Pak®; Waters, Milford, MA, USA) according to the manufacturer's instructions, using water and methanol (10 ml) as the weak and strong solvents, respectively. The methanol was then evaporated to dryness and the solid residue re-dissolved in 1 ml of methanol. The female was then lightly anaesthetized [in water containing 50 mg l^{-1} 3-aminobenzoic acid ethyl ester (MS-222)] and a blood sample taken from the caudal vein into a heparinized syringe. This was immediately centrifuged and the plasma taken off and frozen at –20°C. Where possible, a urine sample was also taken. The fish was then killed by a sharp blow to the head and samples of faeces and bile fluid were taken (Frade et al., 2002). The ovaries were weighed and the gonado-somatic index (GSI) calculated. A random sample of 10 eggs was also taken and weighed and their diameters measured under a binocular microscope. Although the masses (pre-ovulatory, 115±22 g; post-ovulatory, 96±18 g; N=7) and standard lengths (pre-ovulatory, 152±10 mm; post-ovulatory, 145±7 mm; N=8) of the two groups were similar, the GSI of pre-ovulatory females (4.66±0.74%; N=7) was over three times that of post-ovulatory females (1.37±0.13%; N=8, t=4.37, P<0.001). This appeared to be due chiefly to a large increase in the size of the cohort of eggs undergoing final maturation (Fig. 1). The faeces samples were weighed and vortexed briefly with three times their mass of distilled water. These were then centrifuged and the supernatant removed and frozen. Equal volumes of each body fluid from pre-ovulatory or post-ovulatory females were pooled (N=7). However, due to difficulty in obtaining urine from females (compared with males), the pools of urine were derived from only three individuals in each pool. The pools were mixed, divided into aliquot samples and frozen. Equal volumes of the eluates of the water samples from pre-ovulatory or post-ovulatory females were also pooled. All samples were kept at –20°C until use. Prior to assay in an EOG experiment, samples were diluted in charcoal-filtered dechlorinated tapwater (the same water used to irrigate the nostril of the male; see below).

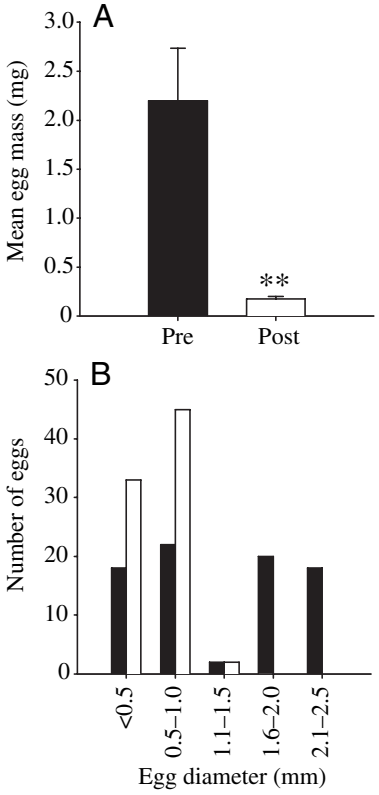

Fig. 1. Comparison of eggs from pre- and post-ovulatory female tilapia. (A) Mean egg mass was significantly higher in pre-ovulatory (Pre; filled bar) than in post-ovulatory females (Post; open bar). Data are shown as means ± S.E.M.; **P<0.01). (B) Frequency distribution of egg diameters from pre-ovulatory (filled bars) and post-ovulatory (open bars) females. Note the presence of larger eggs undergoing maturation in pre-ovulatory females.

Recording of the electro-olfactogram (EOG)

EOGs were recorded from male tilapia exactly as previously described for females (Frade et al., 2002). Briefly, males were anaesthetized by immersion in water containing 100 mg l^{-1} MS-222 and immobilized by intramuscular injection of gallamine triethiodide (3 mg kg^{-1} in 0.9% saline). The fish were then wrapped in a damp paper towel, gently clamped in a purpose-built V-clamp, and aerated water containing

50 mg l^{-1} MS-222 was pumped over the gills *via* a silicon tube placed in the mouth. The ring of cartilage surrounding the single nostril on the right side was cut away. The olfactory rosette was then continually irrigated with dechlorinated, charcoal-filtered tapwater *via* a gravity-fed system (6 ml min^{-1}) that terminated in a small glass tube that was held close to the olfactory rosette. The DC voltage was recorded by glass micropipette electrodes filled with 0.9% NaCl in 4% agar, the recording electrode being placed close to the olfactory epithelium near to the raphe between two adjacent lamellae. The reference electrode was placed lightly on the skin near the nostril and connected to earth *via* the headstage of the amplifier. Odorant-containing water was introduced into this flow *via* a three-way valve for a period of 10 s. The resultant EOG was recorded on a personal computer running appropriate software (Axoscope 1.1; Axon Instruments, Inc., Foster City, CA, USA). The peak amplitude of the EOG was measured, blank-subtracted and normalized (using the response to the 'standard' 10^{-5} mol l^{-1} L-serine) as previously described (Frade et al., 2002). Normalization reduces the variation due to differences in the absolute amplitude of the EOG recorded. This variation may be due to differences in electrode position or conductivity of the water as well as differences in the olfactory sensitivity of individual fish. However, the overall pattern of responses was very similar among different fish. Blanks and standards were run at regular intervals throughout the recording period.

Producing anosmia in males

Males were anaesthetized, immobilized and placed in the V-clamp as described above. The rings of cartilage surrounding both nostrils were removed, and the olfactory epithelium was cauterized with a high-temperature fine-tip cauterizer (model AA11; Aaron Medical Industries, Inc., St Petersburg, FL, USA). A local antiseptic was applied to the wound (Betadine®; ASTA Medica Produtos Farmacêuticos, Lda, Lisbon, Portugal). Sham-operated males were treated in exactly the same way, except with no burning of the epithelium. The fish were allowed to recover for 24 h in isolation. Behavioural experiments began the following day (~48 h after the operation). Operated fish survived well but anosmic fish had difficulty in locating food; they would take pellets dropped into the tank whilst falling but, once on the bottom, the pellets were ignored. Two to three weeks after the experiment, the anosmic fish were killed and the olfactory cavity examined under a binocular microscope; the olfactory lamellae were beginning to regenerate.

Behaviour of anosmic and sham-operated males

An anosmic or sham-operated male was injected intramuscularly with 2.4 mg isosulfan blue (Sigma-Aldrich Química, S.A., Sintra, Spain) in 20 µl 0.9% saline (Appelt and Sorensen, 1999) and put back in isolation. The following morning, the behaviour of the male was video-taped for a control period of 45 min, during which the frequency and duration of urine pulses (made visible by the isosulfan blue) were recorded. A pre- or post-ovulatory female was then introduced into the tank and the recording procedure repeated for a further 45 min. The fish were then returned to their family tanks. After a period of at least 24 h, the entire experiment was repeated with a post- or pre-ovulatory female (as appropriate) in place of the previous female. Each male was exposed to a female from a different family tank. Male behaviour was classified as one of the following categories (Baerends and Baerends van Roon, 1950; Oliveira, 1995) and quantified, in terms of duration, using The Observer Video-Pro 4.0 software (Noldus Information Technology, Wageningen, The Netherlands):

immobile – the fish remains immobile either resting on the substrate or within the water column;

swimming – active movement within the water column using the caudal fin for propulsion (but not including the behaviours outlined below);

courtship – circling of the female by the male, inviting the

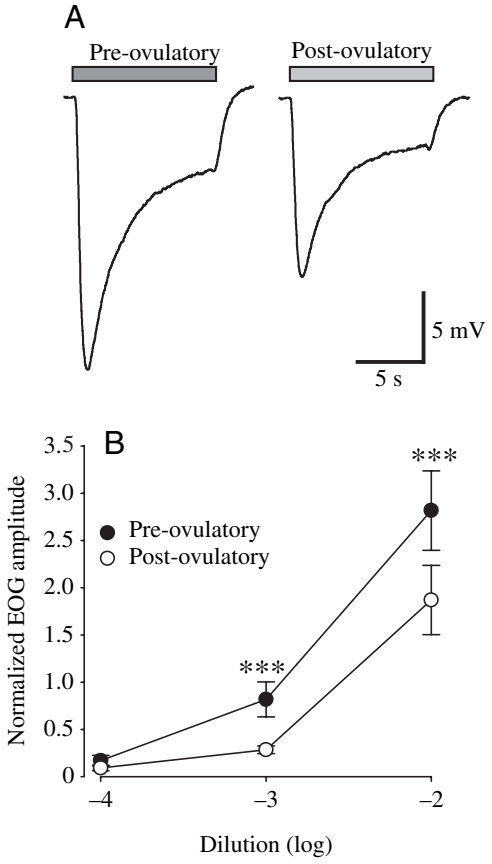

Fig. 2. Electro-olfactograms (EOGs) recorded from male tilapia in response to extracts of female water. (A) Typical EOGs recorded in response to pooled extracts of water (diluted 1:100) from pre-ovulatory (shaded horizontal bar, left panel) and post-ovulatory (shaded horizontal bar, right panel) females. A downward deflection of the EOG trace is negative. (B) Semi-logarithmic plot of pooled normalized EOG amplitudes (N=6) to pooled extracts of female water from pre-ovulatory (filled circles) and post-ovulatory (open circles) females at three dilutions. An equivalent dilution to give the same concentration as the original water sample would be 1:2000. ***P<0.001.

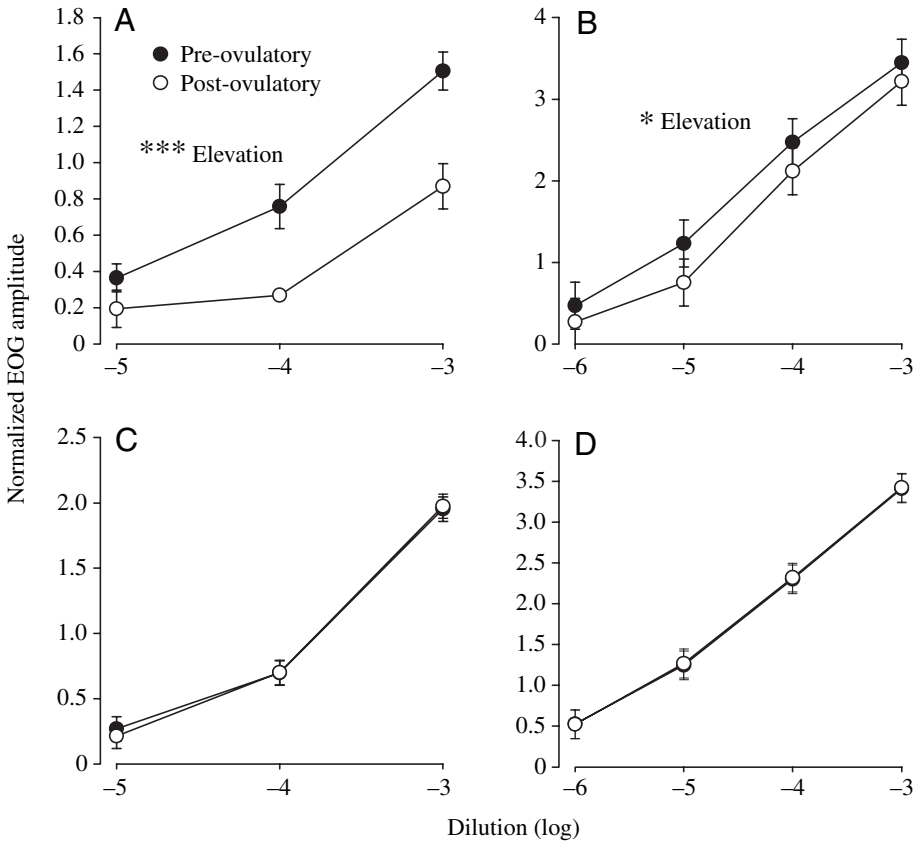

Fig. 3. Semi-logarithmic plots of pooled normalized electro-olfactogram (EOG) amplitudes recorded from male tilapia (N=6) in response to pooled urine (A), faeces (B), plasma (C) and bile fluid (D) from pre-ovulatory (filled circles) and post-ovulatory (open circles) females. Comparison of the regression lines revealed significant differences between the elevations (but not the slopes) of the response to urine from pre- and post-ovulatory females and between the elevations (but not the slopes) of the response to faeces from pre- and post-ovulatory females. No differences were found between the responses to plasma (P=0.845) or bile (P=0.923) from pre- and post-ovulatory females. Estimated thresholds of detection are given in the text. *P<0.05; ***P<0.001.

female to the nest, guiding the female towards the nest, preventing the female from leaving the nest, nest-digging and quivering;

aggression – frontal display (the male remains in front of the female with dorsal and anal fins erect and mouth wide open), lateral display (dorsal and anal fins erect, caudal and pelvic fins fully open, often with dilation of the branchiostegal membrane), biting, butting (rapid swimming towards the female followed by ramming with open mouth) and trapping the female in the corner of the tank;

other – coprophagia and nipping (the fish nips at the surface of the water).

Statistical analysis

Length, mass and GSI of pre- and post-ovulatory females were compared using Student's t-test. Normalized EOG amplitudes in response to water extracts from pre- and post-ovulatory females were compared by Student's t-test for paired samples. Thresholds of detection were estimated by linear regression of normalized (faeces and bile) or normalized and log-transformed (urine and plasma) EOG amplitudes and calculation of the intercept with the x-axis (Hubbard et al., 2003). Linear regressions (slopes and line elevations) were compared between body-fluid stimuli from pre- and post-ovulatory females by Student's t-test (Zar, 1996). The frequency and mean duration of urine pulses released by anosmic or sham males in the presence of a pre- or post-ovulated female were compared by repeated-measures ANOVA followed by the Tukey HSD test. The duration of each behavioural category was compared by ANOVA, setting male type as the independent factor and female type as the repeated factor. In all cases, a P value of less than 0.05 was taken to represent statistical significance. All data are shown as means ± S.E.M.

Results

Olfactory responses to 'female water' extracts

Individual female water extracts evoked EOGs of variable amplitude, but the pattern remained constant from male to male. Despite the individual variation, the mean of normalized responses to water extracts from pre-ovulatory females at a dilution of 1:1000 (0.55±0.07; N=6) was significantly higher than that from post-ovulatory females (0.27±0.05; N=6, P<0.01). Furthermore, the amplitudes of responses to a range of dilutions of the pooled extracts from pre-ovulatory females were significantly higher than those from post-ovulatory females (Fig. 2). Neither stimulation with methanol-only controls (1.0%; the highest concentration used in samples) nor control extractions of water (without addition of the fish) evoked EOGs larger than those of blanks (data not shown).

Olfactory responses to female body-fluids

All female body fluids evoked typical fish-form EOGs in male conspecifics, the amplitudes of which were strongly concentration dependent (Fig. 3). Both urine and faeces from pre-ovulatory females evoked EOGs of significantly larger amplitude than those from post-ovulatory females (Fig. 3A,B), whereas the responses to bile fluid and plasma from pre- and post-ovulatory females were essentially identical (Fig. 3C,D). Estimated thresholds of detection were

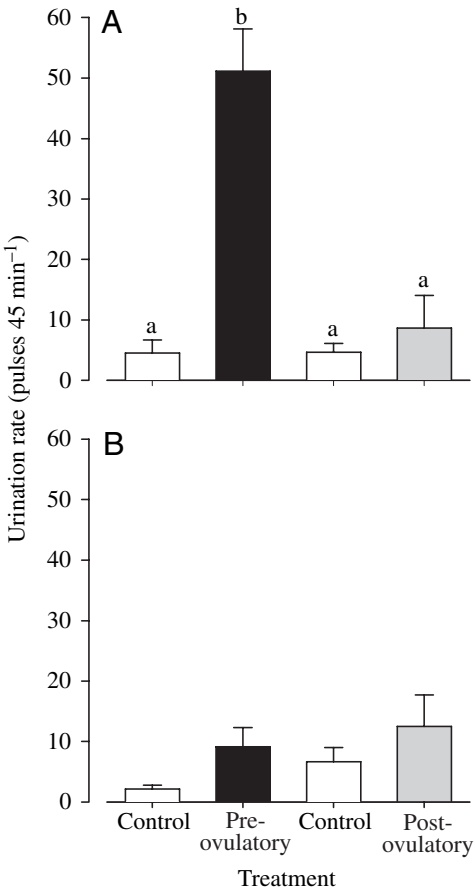

Fig. 4. Urination rates of male tilapia in isolation and in the presence of pre- or post-ovulatory females. Histograms showing the rates of urination of sham-operated (A) and anosmic (B) males in isolation (control; open bars) and in the presence of a pre-ovulatory (black bars) or post-ovulatory (grey bars) female (N=6). Different letters indicate significant differences (P<0.01).

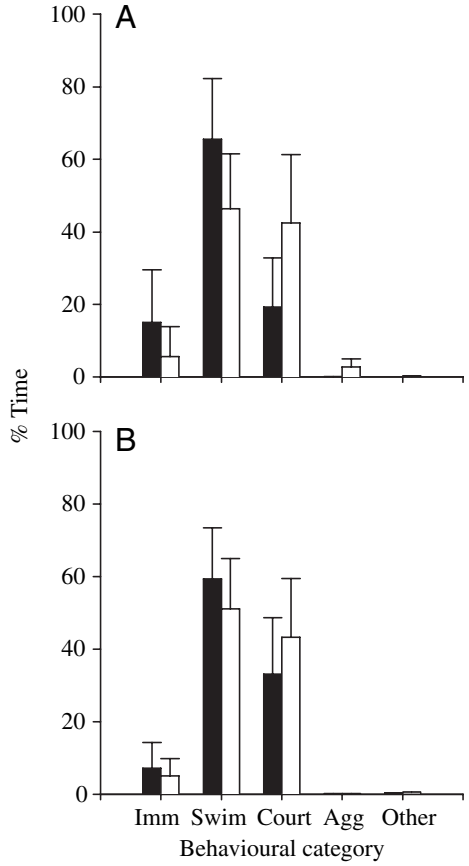

Fig. 5. Effect of anosmia on behaviour of male tilapia in the presence of pre- or post-ovulatory females. Histograms of the proportion of time spent by anosmic (filled bars) and sham-operated (open bars) males in different behavioural categories in the presence of pre-ovulatory (A) and post-ovulatory (B) females. Imm=immobile, Swim=swimming, Court=courtship, Agg=aggression, Other=other types of behaviour (see text for details). Data are shown as means ± S.E.M. (N=6). There are no significant differences between the behaviours of anosmic and sham-operated males.

$1{:}10^{5.6}$ and $1{:}10^{5.1}$ for urine, $1{:}10^{6.4}$ and $1{:}10^{6.1}$ for faeces (pre- and post-ovulatory, respectively), $1{:}10^{6.4}$ for bile and $1{:}10^{5.3}$ for plasma.

Behavioural responses to females in anosmic males

In isolation, male tilapia urinate at a low frequency (on average one pulse every 10 min; Fig. 4), but the urinary bladder is almost always full (A.M., O.G.A., P.C.H. and E.N.B., unpublished observations). Sham-operated males dramatically increased their rate of urination in the presence of pre-ovulatory females but not in the presence of post-ovulatory females (Fig. 4A). Conversely, anosmic males did not increase their rate of urination significantly in the presence of either pre- or post-ovulatory females (Fig. 4B). No significant differences were found between the behaviour exhibited by anosmic and sham-operated males in the presence of pre-ovulatory or post-ovulatory females (Fig. 5), although there was a tendency for sham-operated males to display more courtship behaviour than anosmic males.

Discussion

Olfactory responses to female-derived stimuli

EOGs recorded from male tilapia were very similar in shape and form to those recorded previously from female tilapia (Frade et al., 2002) and many other freshwater fish (Hara, 1994); a sharp initial negative deflection followed by a period of adaptation to approximately 50% of the original deflection (Fig. 2A). After cessation of the stimulus, the voltage returned to baseline level within a few seconds. Extracts of water previously containing pre-ovulatory females evoked larger-amplitude EOG responses in males than those of post-ovulatory females. Although there was considerable variation in the amplitude of response to individual extracts, the pattern of responses in males was highly consistent. This was confirmed by the responses to the pools of the extracts; significantly larger responses were given to the pre-ovulatory pool than to the post-ovulatory pool at all concentrations

tested. Fish are known to release a range of substances into the water, which act as potent odorants to conspecifics. This includes bile acids, steroids and prostaglandins (Dulka et al., 1987; Pinillos et al., 2003; Sorensen et al., 1987, 1988; Zhang et al., 2001), many of which have been shown to possess pheromonal activity (Stacey and Sorensen, 2002). Depending on the chemical characteristics of these substances, their main routes of release are *via* the gills, urine or faeces (Vermeirssen and Scott, 1996, 2001). Thus, the olfactory responses of the males to the female-water extracts are likely to be evoked by a mixture of compounds released *via* different routes.

Although the males gave robust olfactory responses to individual female body fluids, clear quantitative differences were seen in the amplitude and threshold of detection of the responses to pooled urine and faeces from pre- and post-ovulatory females (Fig. 3A,B). This suggests that pre-ovulatory females are releasing compounds *via* both urine and faeces that post-ovulatory females are not. However, it is also possible that other important compounds are released across the gills. As the responses to dilutions of the plasma from the two groups of females were virtually identical (Fig. 3C), it is unlikely that these compounds are circulating factors, unless they are tightly bound to circulating binding proteins and are therefore unavailable for olfactory detection. Equally, the similarity of the responses to dilutions of the bile fluid from the two groups of females suggests that bile acids, although potent odorants, are not responsible for the difference in olfactory potency of the faeces. Nevertheless, it is possible that bile acids are modified differentially by pre- and post-ovulatory females during their transit down the gut, with the result that pre-ovulatory females release more potent metabolites of bile acids *via* the faeces. Alternatively, a recent study has shown that social status has profound effects on the rate of release of bile fluid into the gut (Earley et al., 2004); subordinate male convict cichlids (*Archocentrus nigrofasciatum*) retain more bile than dominant males, and this is largely independent of food intake. This may well also alter the odour of the faeces. Whether females of different reproductive status also retain bile fluid to differing degrees remains to be tested.

Although the olfactory responses of females to male urine and bile fluid (Frade et al., 2002) are quantitatively similar to those of males to female urine and bile fluid (present study), the thresholds of detection for faeces are over an order of magnitude different ($1{:}10^{4.6}$, male faeces; $1{:}10^{6.4}$ and $1{:}10^{6.1}$, pre- and post-ovulatory female faeces, respectively). At present, the significance of this observation is unclear, although it suggests that females are releasing something *via* the faeces that the males are not.

Behavioural responses of anosmic and sham-operated males

In the presence of pre-ovulatory females, males dramatically increase their rate of urination (Almeida et al., 2003) as well as displaying typical courtship behaviours such as nest-building, quivering and circling the female (Baerends and Baerends van Roon, 1950). We have previously shown that male tilapia behave differently towards pre- and post-ovulatory females (Almeida et al., 2005). The major behavioural difference found was that males urinate much more frequently in the presence of pre-ovulatory than post-ovulatory females. The sham-operated males in the current study also dramatically increased their rate of urination in the presence of pre-ovulatory, but not post-ovulatory, females (Fig. 4A). Preliminary observations suggested that the smell of females alone is not sufficient to evoke these courtship behaviours in males (data not shown). However, anosmic males failed to increase their rate of urination in the presence of either pre- or post-ovulatory females (Fig. 4B). Intriguingly, other behavioural categories, including courtship, were apparently unaffected by anosmia. This may be due, in part, to the experimental design; males often court newly introduced, unfamiliar females whether pre-ovulatory or not. Males in established family tanks, however, only seem to display courtship behaviour when one of the females is close to ovulation (A.M., O.G.A., P.C.H. and E.N.B., unpublished observations). Furthermore, visual and olfactory input may be processed independently by the central nervous system, at least in the context of courtship. During the spawning season, male goldfish are able to discriminate between male and female conspecifics using visual stimuli alone (Thompson et al., 2004). They do not, however, display any courtship behaviour towards the females unless they are in the same tank (i.e. able to smell them too). Taken together, these results suggest that whilst the male tilapia needs to see the female in order to display the full range of courtship behaviours, he will only invest in chemical signalling if he can smell her and judge her ready for spawning.

Conclusions

The current study strongly suggests that pre-ovulatory female tilapia are releasing specific odorants into the water, probably *via* both urine and faeces, and that males base their behavioural response to these females, with respect to urination frequency, on this olfactory information. The identity of these compounds is currently under investigation.

This work was funded by Fundação para a Ciência e a Tecnologia (Portugal) grant number POCTI/BSE/38815/2001 (funding by European Union–FSE/FEDER and Ministério da Ciência e do Ensino Superior). Some of these results have previously been published in abstract form (Almeida et al., 2003, 2005; Miranda et al., 2003).

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