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RESEARCH ARTICLE

Olfactory sensitivity of the marine flatfish *Solea senegalensis* to conspecific body fluids

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ABSTRACT

Chemical communication is better understood in freshwater fish than marine fish. The Senegalese sole (*Solea senegalensis*) is a marine flatfish wherein one of the problems in aquaculture is the poor reproductive performance of hatchery-bred males. Is chemical communication involved in the reproduction of this species? Urine, intestinal fluid and mucus samples were taken from adult fish (wild-caught and hatchery-bred) over the spawning season (March–May), and assessed for olfactory potency using the electro-olfactogram (EOG). The effect of stimulation of the olfactory system with adult female urine on circulating luteinizing hormone (LH) levels was also tested in males. Intestinal fluid and urine were potent olfactory stimuli for both juvenile and adult conspecifics, evoking large-amplitude, concentration-dependent EOG responses, with thresholds of detection at approximately $1{:}10^6$. However, the amplitude of the response to urine depended on the sex and state of maturity of both the donor and the receiver. Most olfactory activity could be extracted by C18 solid-phase cartridges. Urine from mature females evoked a slight, but significant, increase in circulating LH levels in mature males 30 min after exposure. Furthermore, the olfactory potency of urine differed between wild-caught and hatchery-bred fish; however, contrary to expectations, urine from wild-caught females was less potent than that from hatchery-bred females. Taken together, these results strongly suggest that faeces- and urine-released odorants are involved in reproduction in the Senegalese sole, and establish a basis for further investigation into pheromonal communication in marine teleosts.

KEY WORDS: Pheromone, Reproduction, Urine, Faeces, Marine, Olfaction

INTRODUCTION

Many (if not all) teleosts use pheromones to regulate reproductive physiology and behaviour, in a variety of different ways (Stacey, 2015; Wyatt, 2014). However, the chemical identity and exact biological roles of such pheromones have been clearly demonstrated only in a few freshwater species, such as the goldfish (*Carassius auratus*; Dulka et al., 1987; Sorensen et al., 1988), masu salmon (*Oncorhynchus masou*; Yambe et al., 2006) and Mozambique tilapia (*Oreochromis mossambicus*; Keller-Costa et al., 2014). Marine fish have received much less attention (Hubbard, 2015); the black goby (*Gobius niger*) is the only example wherein the chemical identity of a pheromone is known (Colombo et al., 1980). This is surprising given the economic importance of many marine species, both wild-caught and in aquaculture.

Urine is a common vehicle for pheromones (Wyatt, 2014). However, due to osmoregulatory constraints, urine production is much lower in marine than freshwater fish (reviewed in Marshall and Grosell, 2006). To overcome this, marine fish may simply accumulate urine in the bladder for release during the appropriate social context, e.g. the plaice (*Pleuronectes platessa*, a marine flatfish) can retain urine in the bladder for up to three days (Fletcher, 1990). Alternatively, other body fluids, such as intestinal fluid or mucus, may be involved; the intestinal fluid of mature gilthead seabream (*Sparus auratus*) has a much higher olfactory potency than its urine (Hubbard et al., 2003) and, in female Mozambique tilapia, this potency depends on reproductive status (Miranda et al., 2005). Similarly, the olfactory potency of eel (*Anguilla anguilla*) mucus depends on both the sex and maturity of the donor (Huertas et al., 2007). Given the wide range of reproductive strategies of teleosts, any or all of these mechanisms may be operating, but research is needed to clarify this.

Flatfish (order Pleuronectiformes) are almost exclusively marine. The European aquaculture industry has recently invested in the culture of one such flatfish, the Senegalese sole (*Solea senegalensis* Kaup 1858). However, reproductive bottlenecks hinder its culture; reproduction relies on wild-caught brood-stock (Dinis et al., 1999), as breeders reared in captivity only spawn unviable eggs (Agulleiro et al., 2006; Guzmán et al., 2009). In particular, it appears that hatchery-bred males do not complete courtship behaviour (Carazo, 2013; Mañanós et al., 2007; Martín, 2016). Our working hypothesis is that the fault may lie in the chemical communication system. Normally, the male sole selects a female and rests his head on her body for approximately 15 min (Carazo et al., 2016); the female may or may not respond but the male will encourage her to swim to the surface with him following (i.e. as a male and female pair) where gamete release occurs. Thus, chemical cues may be involved in both mate choice and spawning; the close contact between males and females during pair formation may facilitate the exchange of chemical information.

In addition, the anatomy and biology of flatfish suggest functional asymmetry in the olfactory system (Doldán et al., 2011; Kasumyan, 2004; Prasada Rao and Finger, 1984). In the Senegalese sole, higher sensitivity for conspecific-derived odorants was found in the upper (right) olfactory epithelium, and for prey-derived odorants, higher sensitivity was found in the lower (left) epithelium (Velez et al., 2005, 2007, 2011, 2009). This asymmetry may not be confined to differential receptor expression, but could extend to transduction pathways (Velez et al., 2013) and neuronal

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processing in the olfactory bulb and above. Thus, this species may prove to be a valuable model for the comparison of neural detection and processing of two different functional classes of odorants: sex pheromones and kairomones (prey-related odorants in this case).

The aims of the present study were threefold. Firstly, to establish which body fluids may be involved as vehicles for odorants involved in chemical communication during reproduction in the Senegalese sole; does the olfactory potency of urine, intestinal fluid or mucus depend on reproductive status and/or sex? Secondly, we examined a possible endocrine function of exposure to female urine on the circulating levels of luteinizing hormone (LH) in males. Finally, we tested whether there may be differences in the strength of urinary signals between wild-caught and hatchery-bred fish.

MATERIALS AND METHODS

Fish care and experimentation complied with the guidelines of the Portuguese legislation for the use of laboratory animals under a 'Group-1' licence issued by the Veterinary General Directorate of the Ministry of Agriculture, Rural Development and Fisheries of Portugal. All electro-olfactograms (EOGs) were recorded from the upper (right) olfactory rosette of captivity-bred Senegalese sole (hereafter 'sole').

Experimental animals and sample collection

The sole from which samples were collected were kept at IRTA (Sant Carles de la Ràpita, Tarragona, Spain) under natural photoperiod and temperature conditions. Sole were fed a diet containing a mixture of dry and fresh food: dry balanced feed (Skretting, Stavenger, Norway), and fresh mussels (*Mytilus edulis*), squid (*Loligo gahi*) and polychaetes (*Nereis virens*). Three developmental stages (juvenile, adult-immature and mature) were used: juvenile fish had undergone metamorphosis but had not passed puberty; adult-immature were fish that had passed puberty but were not mature when sampled; and mature were adult fish that had the presence of sperm or a swollen ovary with vitellogenic oocytes.

Juvenile sole used for EOG recording were obtained and maintained at the field station Ramalhete (Universidade do Algarve, Portugal) under natural photoperiod and temperature. Two weeks before EOG recording the fish were acclimated from a salinity of 35 to 12 ppt in four daily steps. After acclimation, the sole were maintained at 12 ppt, and feeding and behaviour were normal. Adult sole used for EOG recording were obtained from IFAPA–Toruño (Cádiz, Spain) and moved to Ramalhete where they were acclimated for a month before experiments. Adult fish were maintained under natural photoperiod and temperature and acclimated and maintained at a salinity of 10–12 ppt as described above.

Recording the EOG

The method for EOG recording in sole has been previously described in detail (Velez et al., 2005). Briefly, the EOG recording was carried out at 12 ppt to reduce the electrical shunting effect of seawater; the amplitudes of EOGs recorded from marine fish in full seawater are considerably smaller than those of freshwater fish (Silver et al., 1976). The sole were anaesthetised by immersion in water (12 ppt) containing 100 mg l^{-1} MS222 (3-aminobenzoic acid ethyl ester) buffered with 200 mg l^{-1} $NaHCO_3$ and immobilised with 6 mg kg^{-1} gallamine triethiodide (Sigma–Aldrich, Sintra, Spain) injected intramuscularly. Once anaesthetised and immobilised, the fish were wrapped in a damp cloth and placed in a Perspex box with aerated water (containing anaesthetic) pumped over the gills (~100 ml 100 g^{-1} body weight min^{-1}) via a tube inserted into the mouth. The upper olfactory rosette was exposed by cutting the overlying skin and musculature. Stimulus delivery to the olfactory epithelium was similar to that previously described (Hubbard et al., 2002) but the outlet of the stimulus-delivery tube was pulled into a finer point (~0.5 mm) to increase the velocity of the water flow onto the olfactory epithelium; this prevented the build-up of mucus on the olfactory epithelium, a continuous problem in this species. The electrodes were pulled from borosilicate glass tubes, filled with 3 mol l^{-1} NaCl in 1% agar and bridged to solid-state electronics via an Ag/AgCl pellet. The amplifier was a Grass AC/DC strain gauge (CP122; Astro-Med, West Warwick, RI, USA) with low-pass filter set at 30 Hz. The recording electrode was placed at a position that resulted in the largest response to the standard stimulus (10^{-3} mol l^{-1} L-cysteine); this was usually between two lamellae near the middle of the raphe. The reference electrode was slightly touching the skin of the head nearby. The fish was grounded with an Ag/AgCl pellet electrode placed under the head. The recordings were digitised (DigiData 1322A, Axon Instruments, now Molecular Devices, Sunnyvale, CA, USA) and stored on a PC running Axoscope software (Molecular Devices). All stimuli were dissolved directly in charcoal-filtered seawater of 12 ppt. At least 1 min was allowed to elapse between successive stimuli. The stimuli were applied in a varied order, but were presented in order of increasing concentration for concentration–response curves of pooled urine and intestinal fluid. Individual urine samples (1:1000) were tested on three juvenile males and three juvenile females, and three adult males and three adult females, and the arithmetic means of these three normalised responses were used in subsequent analysis. At the end of the experiment the fish were killed with an overdose of MS222 and the GSI (gonado-somatic index; gonad weight/body weight×100) was calculated, and wet mounts of the gonads were fixed and processed for histology.

Does maturity and/or sex of the donor affect the olfactory potency of urine, mucus and intestinal fluid?

To examine the influence of maturity and sex of the donor on the olfactory potency of urine, intestinal fluid and mucus, these fluids were collected on 26 February 2015 to form pools (equal volume from each fish, N=4 fish per pool, one pool per group) from mature males and females and adult-immature males and females. Fish in both groups/pools (mature and adult-immature) were of two origins: hatchery-bred sole (males 1315±671 g; females 1711±497 g; 5 years old) and wild-caught sole (Delta del Ebro, Tarragona, Spain; 434±96 g). For sampling, the fish were removed from the tank and placed (with their eyes covered) on a table. A microscope slide was gently run over the body of the fish and the accumulated mucus was collected with a syringe and stored on ice. The sole were then lightly anaesthetised (45 mg l^{-1} MS222; Sigma–Aldrich, Química, Sintra, Spain), and slight pressure was applied to the abdomen in the area of the urinary bladder; extruded urine was collected with a syringe and kept on ice. Finally, intestinal fluid was also taken by inserting a cannula into the rectum and applying gentle suction. These samples were centrifuged and either the supernatant was collected as individual samples or an equal volume of the supernatant was taken from each sample to form a pool. Sub-samples of these pools (urine and intestinal fluid) were passed through a solid-phase Isolute 500 mg C18 extraction cartridge (International Sorbent Technology Ltd, Hengoed, UK), according to the manufacturer's instructions using water and methanol as the weak and strong solvents, respectively. All samples were stored at −20°C until use. The receivers were eight juvenile sole (N=5 males, N=3 females; 82 ±18 g; 179±21 mm), which were used for recording EOGs with this series of samples (urine, intestinal fluid and mucus).

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Does the reproductive status of the donor and receiver affect the olfactory potency of urine?

To investigate if the reproductive condition affects the olfactory potency of urine, individual samples were collected monthly over the spawning season (March–May 2015) from mature males and females (different donors from the previous section). The collection took place on the 17th of each month. Urine samples were collected from 13 hatchery-bred females (1096±384 g) and 11 hatchery-bred males (877±295 g) under natural photoperiod and temperature. During sampling on 17 May 2015, one female was found to have ovulated. The ova and fluid were collected and centrifuged, and the supernatant was removed and stored at −20°C. Urine was collected and stored as described above but no pools were made. The receivers of these samples were 12 juvenile sole (*N*=6 females, *N*=6 males; 48±3 g; 156±6 mm) and 12 adult sole (*N*=6 females, 624 ±65 g; 348±11 mm; GSI 1.05±0.05% and *N*=6 males, 513±100 g; 326±22 mm; GSI 0.04±0.005%). Adult females were in the resting stage of maturity (adult-immature), and the most advanced oocyte stage observed was cortical alveolus with no vitellogenic oocytes present. Males were mature, and motile sperm was obtained from the testes.

Does female urine induce endocrine changes in males?

Five males (532±67 g; 339±2 mm; GSI 0.043±0.004%) were used to assess the effect of olfactory exposure to mature female urine on circulating LH concentration ([LH]). A sperm-quality test was applied to ensure the males were mature and fluent. The fish were anaesthetised, immobilised (with the upper olfactory epithelium exposed) and the olfactory stimulus was delivered as described below for EOG recording; however, electrodes were not placed and no EOG was recorded. The stimulus was urine from a mature female, diluted 1:1000, and delivered to the upper olfactory rosette of the fish. The stimulus was given in 4 s pulses, every 10 s for 3 min (i.e. 18 pulses in total). Three blood samples were taken (via the caudal vein into heparinised syringes): (1) after the fish was set up in the experimental apparatus, as described, and immediately before the stimulus was applied (0 min); (2) at 3 min (immediately after the olfactory stimulation had ended); and (3) at 30 min after the first sample was taken. At the end of the experiment, the fish were killed with an overdose of MS222, and the sex and GSI were checked. The blood samples were immediately centrifuged and the plasma was collected and frozen (−20°C) until assayed for LH using a specific enzyme-linked immunosorbent assay (ELISA) as described by Chauvigné et al. (2016).

Does the source (wild-caught versus hatchery-bred) of the donor affect the olfactory potency of urine?

To explore whether the origin (wild-caught or hatchery-bred sole) of the donor can influence the olfactory potency of urine, individual samples were collected from mature wild-caught sole on 17 May 2015, i.e. 10 females (1500±564 g) and three males (660±239 g), but only one sample was taken in order not to disturb the fish during spawning. EOG responses were recorded from the same sole used above (see Does the reproductive status of the donor and receiver affect the olfactory potency of urine? section) and compared with responses to individual samples from hatchery-bred fish taken at the same time.

Does the reproductive status or sex of the receiver affect the olfactory potency of ovarian fluid?

During sampling on 17 May 2015, one mature female was found to have ovulated. The ova and fluid were collected and centrifuged, and the supernatant was removed and stored at −20°C. EOG recordings were performed with juveniles and adult sole as the receivers (the same used previously with urine samples, 12 juveniles and 12 adults).

Data treatment

EOG amplitudes were measured in millivolts. This was then blank subtracted (EOG amplitude response to the same water used to dilute the stimuli). Data were normalised to the amplitude of the response to 10^{-3} mol l^{-1} L-cysteine (responses to 10^{-3} mol l^{-1} L-cysteine were recorded every 10–15 min). Normality test and one-, two- or three-way repeated-measures ANOVA (as appropriate) followed by Student–Newman–Keuls or Dunnett's *post hoc* tests (circulating [LH]) (SigmaPlot 12, Systat Software, Germany) were used. Data are shown as means±s.e.m., and a significance level of 0.05 was used throughout with two-tailed tests.

RESULTS

Does the maturity or sex of the donor affect the olfactory potency of urine, intestinal fluid and mucus?

Firstly, the response of juvenile sole to conspecific urine was assessed. Pooled urine from mature conspecifics tended to evoke larger amplitude EOGs than that from adult-immature conspecifics, although this did not reach statistical significance (male urine, *P*=0.066, Fig. 1A; female urine, *P*=0.086, Fig. 1B). Nevertheless, the great majority of olfactory activity could be extracted by C18 solid-phase extraction cartridges (eluate versus filtrate, *P*=0.002; two-way repeated-measures ANOVA, *N*=7, Fig. 1C).

When juveniles were exposed to intestinal fluid, pools from mature fish (both male and female) evoked larger amplitude EOGs than pools from adult-immature fish in both males and females (male intestinal fluid, *P*=0.042, Fig. 2A; female intestinal fluid, *P*=0.012, Fig. 2B; two-way repeated-measures ANOVA). However, the threshold of detection was slightly higher for intestinal fluid (around 1:100,000) than for urine (around 1:1,000,000). Mucus from all four groups failed to evoke EOG responses larger than 10% of the standard, even at 1:1000, and thus was not investigated further. These results suggest that life stage and sex of the donor influence the olfactory potency of urine and intestinal fluid but not that of mucus.

Does the maturity or sex of the donor and receiver affect the olfactory potency of urine?

To assess whether the olfactory potency of urine varies over the spawning season, individual samples were collected from mature sole (*N*=11 males; *N*=13 females) and tested on juvenile and adult-immature females and mature males. In juvenile males, urine from mature males and females was equipotent, whereas in juvenile females, the urine from mature males was more potent than that from females (Fig. 3A,B). Significant differences were also found among different months. In juvenile males, urine collected in April (when most spontaneous spawnings occurred) evoked larger amplitude EOGs than urine collected in either March (*P*=0.003) or May (*P*=0.012) from the same fish (Fig. 3A). In juvenile females, a similar pattern was seen (i.e. April urine was most potent).

When the same samples were tested on adult-immature fish, however, the peak of olfactory potency in April was less apparent (Fig. 3C,D). Although male and female urine was again equipotent in adult-immature males, stronger EOG responses were given to March samples in adult-immature than in juvenile males (compare Fig. 3A with 3C); March and April samples were equipotent (*P*=0.642) but evoked significantly stronger responses than May

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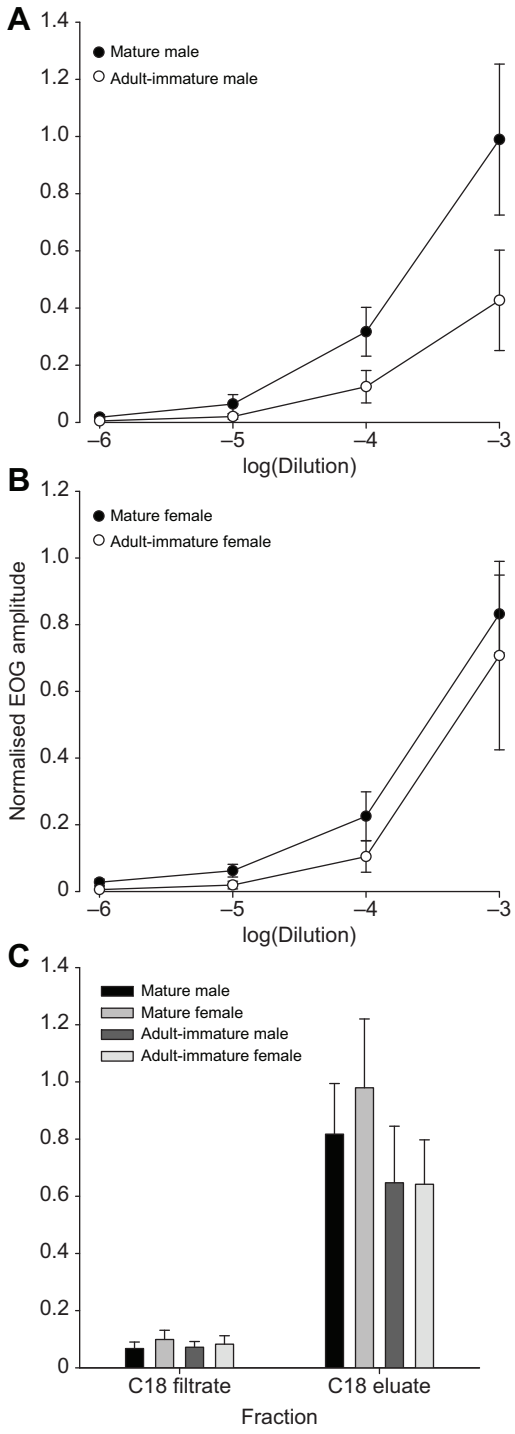

Fig. 1. Olfactory sensitivity to conspecific urine in the sole. Semi-logarithmic plots of normalised amplitude of electro-olfactogram (EOG) responses to dilutions of pools (four donors in each) of urine from mature or adult-immature male (A) and mature or adult-immature female (B) Senegalese sole, recorded from juvenile fish of both sexes. Urine from mature fish tended to evoke larger responses than that from adult-immature fish (male urine, P=0.066; female urine, P=0.086; two-way repeated-measures ANOVA followed by the Student–Newman–Keuls' *post hoc* test, N=8). (C) The normalised amplitude of EOG responses of juvenile sole (both sexes) to 1:1000 dilutions of the filtrate and eluate fractions after the urine pools had been passed through C18 solid-phase extraction cartridges, showing that the majority of the olfactory activity remained in the eluate. The normalised EOG amplitude of the eluate was statistically similar to that of the untreated urine pools (eluate versus filtrate, P=0.002; two-way repeated-measures ANOVA followed by the Student–Newman–Keuls' *post hoc* test, N=7).

samples (P<0.01). Again, the higher potency of male urine (P<0.001) was seen in adult-immature females (Fig. 3D) but, similar to adult-immature males, the March samples were more potent than in juvenile females (compare Fig. 3B with 3D); however, the pattern of response to female urine was similar in juvenile and adult-immature females.

To further investigate the differences in olfactory potency of urine depending on the sex of the donor, and sex and state of maturity of the receiver, concentration–response curves were constructed to individual urine samples taken from one mature male and one mature female in adult-immature and juvenile receivers of both sexes (Fig. 4). Both male and female urine evoked consistently larger amplitude EOGs in male juveniles than in females. However, this only reached statistical significance in females (male urine, P=0.068; female urine, P=0.019; Fig. 4A,B). When tested on adult-immature sole, this difference was less apparent (male urine, P=0.066; female urine, P=0.072; Fig. 4C,D). Moreover, combining all data revealed that differences between the sex of the donor (female, P<0.001; male P<0.001) and between juvenile and adult-

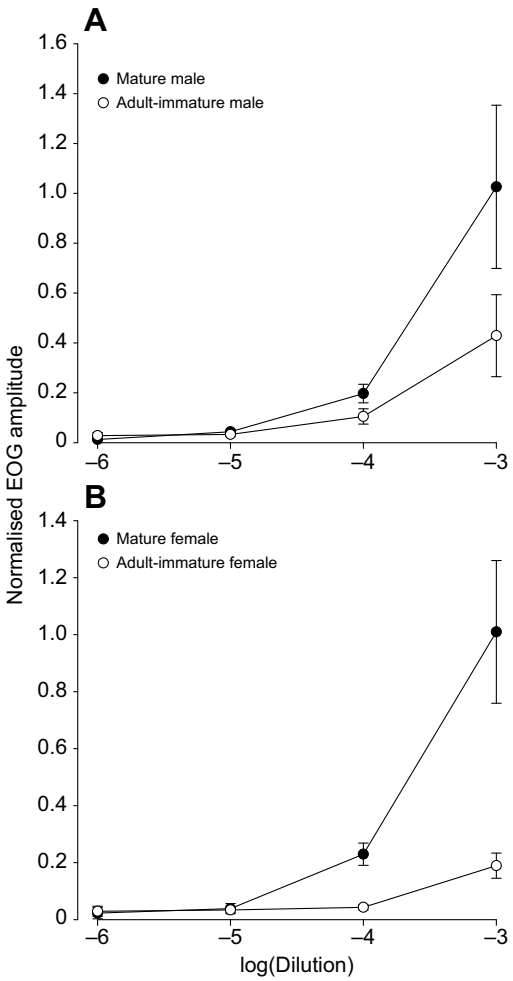

Fig. 2. Olfactory sensitivity to conspecific intestinal fluid in the sole. Semi-logarithmic plots of normalised amplitude of electro-olfactogram (EOG) responses to dilutions of pools (four donors in each) of intestinal fluid from mature or adult-immature male (A) and mature or adult-immature female (B) Senegalese sole, recorded from juvenile fish of both sexes. Intestinal fluid from mature fish evoked larger responses than that from adult-immature fish (male intestinal fluid, P=0.042; female intestinal fluid, P=0.012; two-way repeated-measures ANOVA followed by Student–Newman–Keuls' *post hoc* test, N=7).

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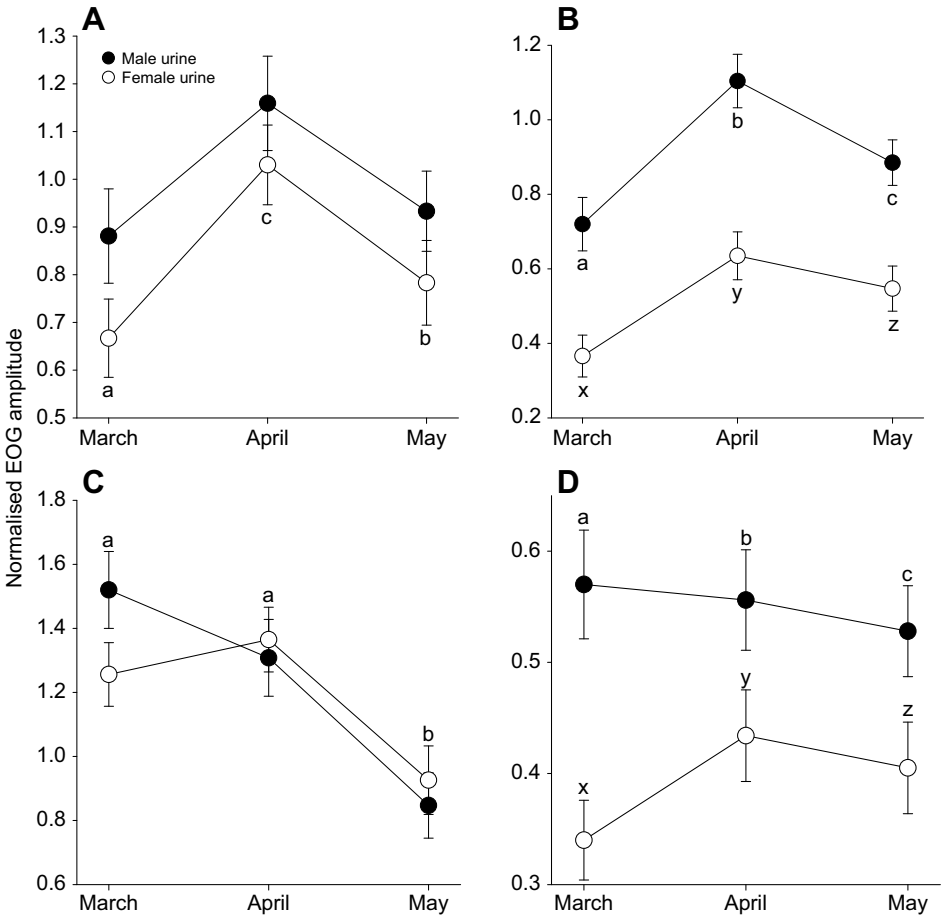

Fig. 3. Olfactory potency of conspecific urine over the spawning season. Normalised electro-olfactogram (EOG) responses to individual urine samples (1:1000 dilution; mean of three independent EOGs) from adult sole over three months of the spawning season from March to May 2015 (the fish spawned more frequently during April). (A) The responses of juvenile males, (B) the responses of juvenile females, (C) the responses of adult-mature males and (D) the responses of adult-immature females (all to the same urine samples). Different letters denote statistical differences [P<0.05; two-way repeated-measures ANOVA followed by Student–Newman–Keuls' *post hoc* test, N=11 (males) or N=13 (females)]. Note that juvenile fish give larger responses to urine taken from adult fish during April (coinciding with spawning) but that male urine evokes larger responses in females than female urine, irrespective of the month. Also, adult fish do not show such a markedly higher response to samples taken during April; however, the larger response to male urine than female urine is maintained in adult females. Interestingly, adult males responded to something in both male and female urine from March that the juvenile males did not.

immature sole were significant (P<0.05; three-way ANOVA). This change with state of maturity or age of the receiver was apparently due to a greater variability of EOG response amplitude of the adults compared with juveniles; this suggests that the process of maturation modulates olfactory sensitivity of females to certain odorants in both mature male and female urine. Together, these results strongly suggest that the olfactory potency of urine depends not only on the sex and state of maturity of the donor but also on the sex and state of maturity (or age) of the receiver.

Does female urine induce endocrine changes in mature males?

To investigate a possible pheromonal role of female urine in the activation of the hypothalamus–pituitary–gonad axis in males, circulating [LH] was measured before and during exposure of the upper olfactory epithelium of males to female urine (Fig. 5). In adult males, exposure of the olfactory epithelium to urine (1:1000) from a mature female (the same urine as in Fig. 4B,D) resulted in a significant increase in circulating plasma [LH] after 3 min that was maintained for at least 30 min (Fig. 5).

Does the source (wild-caught versus hatchery-bred) of the urine donor affect olfactory potency?

Clear differences were seen in the olfactory potency of urine samples taken from wild-caught fish compared with those from hatchery-bred fish. However, contrary to expectations, urine from wild-caught fish was less potent than that from hatchery-bred fish, whether tested on male or female juveniles (Fig. 6). Similar results were obtained from adult fish of both sexes (data not shown). It should be emphasised that the wild-caught fish were kept in the same conditions and fed the same diet as hatchery-bred fish for at least four years before taking the urine samples.

Does the reproductive status or sex of the receiver affect the olfactory potency of ovarian fluid?

Juvenile and adult sole of both sexes gave similar responses to the sample of ovarian fluid (Fig. 7). No differences were noted between EOG amplitudes from adult and juvenile fish nor between those from juvenile males and females (P=0.710) or between those from adult males and females (P=0.595). Moreover, response amplitudes to ovarian fluid were smaller than those to urine and intestinal fluid. Although only one sample was taken, these results do not support a pheromonal role for ovarian fluid in the sole.

DISCUSSION

The current study shows clearly that, in the sole, conspecific urine and intestinal fluid are potent olfactory stimuli, with thresholds of detection around $1{:}10^6$ (urine) and $1{:}10^5$ (intestinal fluid); the olfactory potency of urine depends on both the sex and maturity of the donor. This is consistent with, indeed suggestive of, a role for urine in chemical communication during reproduction. Urine has been identified as a vehicle for reproductive pheromones in various freshwater teleosts, such as the goldfish (Appelt and Sorensen, 2007), masu salmon (Yambe et al., 2006) and Mozambique tilapia (Keller-Costa et al., 2014). In the goldfish, the female pre-ovulatory pheromonal steroid, 17α,20β-dihydroxy-4-pregnen-3-one, stimulates spermatogenesis and milt production in males via increased plasma gonadotropin concentrations (Dulka et al., 1987;

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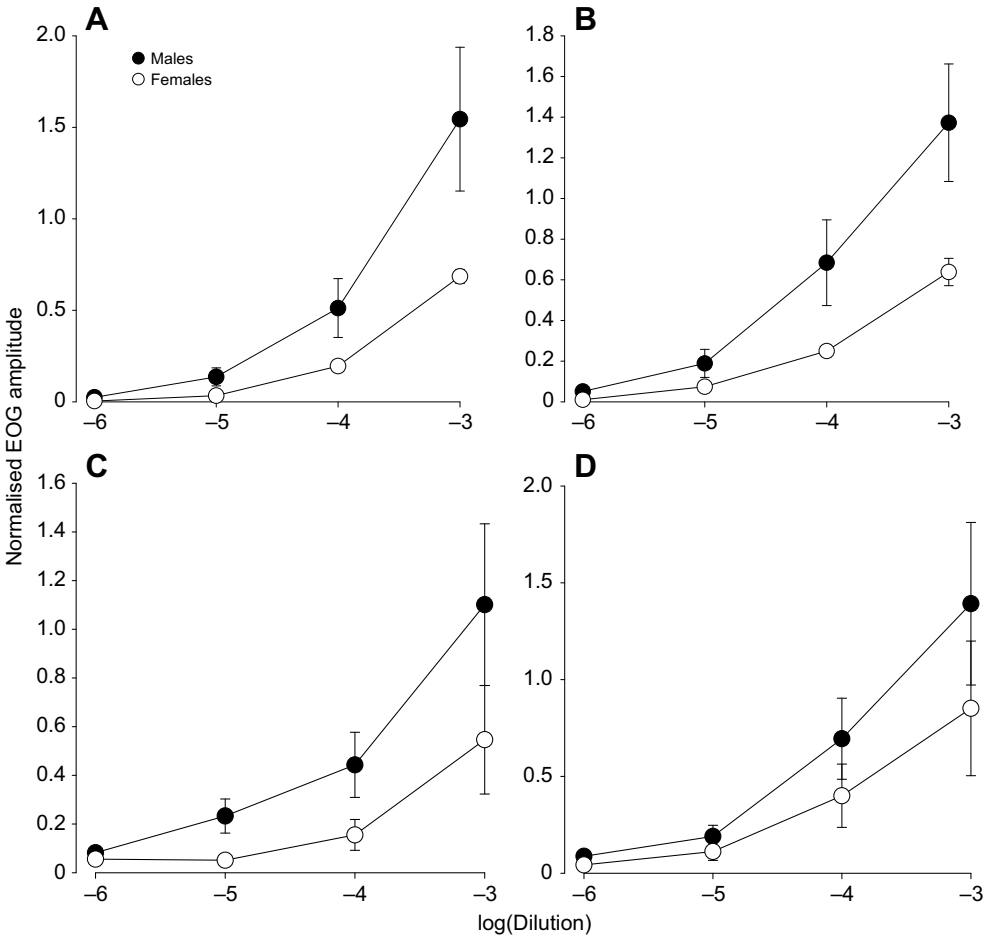

Fig. 4. Olfactory sensitivity to urine as a function of sex and maturity of the receiver. Semi-logarithmic plots of normalised amplitude of electro-olfactogram (EOG) responses to dilutions of individual samples of urine taken from one adult male and one adult female during April. (A) The responses of juvenile males and females to male urine (P=0.068; two-way repeated-measures ANOVA followed by Student–Newman–Keuls' *post hoc* test, N=5) and (B) to female urine (P=0.019; two-way repeated-measures ANOVA followed by Student–Newman–Keuls' *post hoc* test, N=5). (C,D) The responses of adult-immature fish smelling the same male and female urine, respectively (no significant differences between the sexes; two-way repeated-measures ANOVA; males N=11, females, N=6).

Stacey et al., 1989). The present study suggests that urine from mature female sole may play a similar role; a brief exposure of the olfactory system of mature males to female urine provoked a sustained (at least 30 min) increase in plasma [LH]. This is therefore likely to have similar effects on spermatogenesis and milt production in the sole (Chauvigné et al., 2014a,b). Given the apparent difference in urinary odorants between mature and immature males, it is possible that females use this chemical information in mate choice, similar to major histocompatability complex-related odorants in sticklebacks (Aeschlimann et al., 2003; Milinski et al., 2005, 2010) or the anal glands in blennies (Barata et al., 2008). The active components could be extracted using C18 cartridges, suggesting that steroids or prostaglandins may be involved, which compares with other species (reviewed by Stacey, 2015). However, further work is needed to clarify this and identify the compounds responsible.

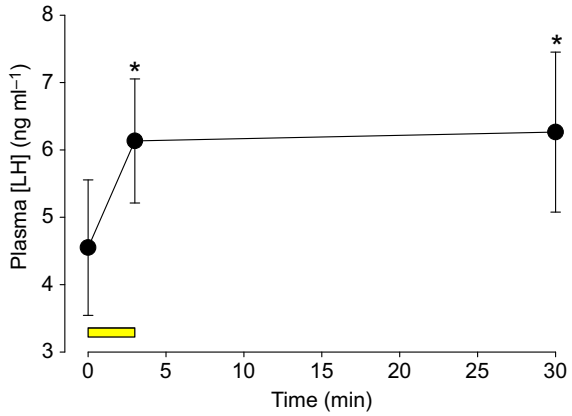

Fig. 5. Effect of exposure to mature female urine on circulating [LH] in adult male sole. The olfactory epithelium of mature male sole (N=5) was exposed to mature female urine (1:1000) in pulses over three minutes (grey horizontal bar). LH was measured in blood plasma taken immediately prior to exposure, immediately after exposure and 30 min after the start of the exposure. *P<0.05; repeated-measures ANOVA followed by Dunnett's *post hoc* test. LH, luteinizing hormone.

Furthermore, olfactory sensitivity to at least some of the urinary odorants also depends on the sex and maturity of the receiver, e.g. mature males have higher sensitivity to (a) component(s) in female urine than females themselves. Thus, the process of sexual maturation may influence the expression of olfactory receptor genes in the olfactory epithelium in the sole, as has been shown in the eel (Churcher et al., 2015), and in cyprinids, olfactory sensitivity is increased by androgens (Belanger et al., 2010; Cardwell et al., 1995; Ghosal and Sorensen, 2016). It is likely, therefore, that during sexual maturation, increased circulating sex steroids evoke a differential expression of pheromone receptors in the olfactory epithelium. To clarify this issue, the urinary pheromones must be identified, as must their respective olfactory receptors, as has recently been achieved for prostaglandin $F_{2\alpha}$ in the zebrafish (Yabuki et al., 2016), and initial studies have begun on gene expression in the olfactory epithelium of the Senegalese sole (Fatsini et al., 2016). This is also important for the comparison of wild-caught and hatchery-bred males (see below).

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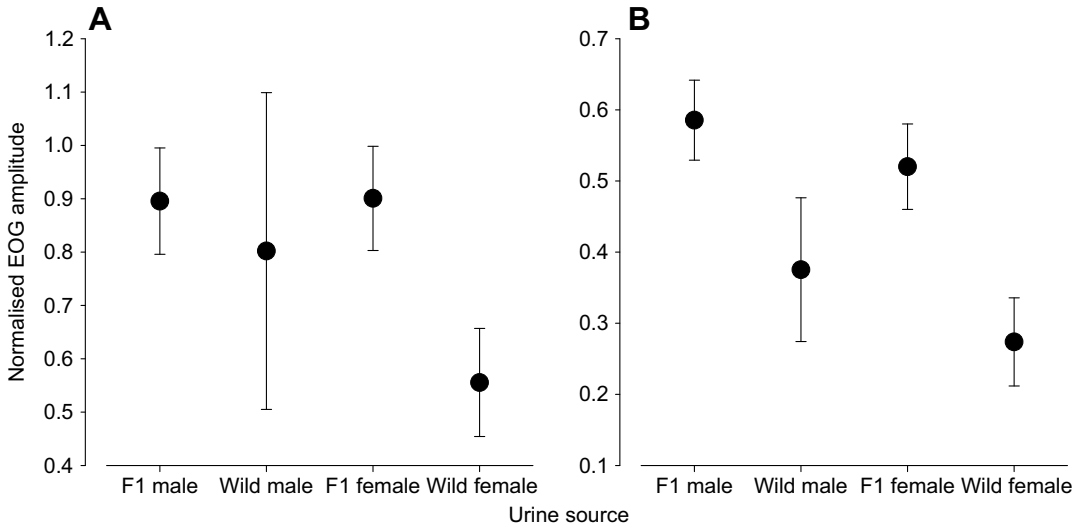

Fig. 6. Comparison of the olfactory potency of urine taken from wild-caught and hatchery-bred sole. Urine samples (diluted 1:1000) from individual hatchery-bred (F1) males (*N*=11) and females (*N*=13) evoked larger amplitude electro-olfactograms (EOGs) than urine from wild-caught males (*N*=3) and females (*N*=11) in both juvenile males (A) and juvenile females (B) but with no differences between the sex of the donor.

As marine fish produce urine at a lower rate than freshwater fish, it is possible that other body fluids, such as intestinal fluid or mucus, may also be a source of odorants involved in chemical communication during reproduction (Hubbard, 2015; Hubbard et al., 2003; Huertas et al., 2007). However, behavioural and physiological measurements of release rates in different social contexts are needed to support this. The current study showed clear differences in the olfactory potency of intestinal fluid between mature and immature sole. Sole continue to feed normally during the spawning season, so this is unlikely to be due to dietary differences. It was previously shown that, in the sole, around 40% of the olfactory activity in intestinal fluid can be explained by bile salts, particularly taurocholic acid (Velez et al., 2009); the identity of the other odorants is still unknown. Olfactory sensitivity to bile salts is widespread among fish (Buchinger et al., 2014), although to date only in the sea lamprey have they been shown to play a pheromonal role (Li et al., 2002; Sorensen et al., 2005). As the previous study (Velez et al., 2009) was carried out with immature fish, it is possible that mature sole produce different bile salts, as has been previously shown in the eel (Huertas et al., 2010). It is also possible that the intestinal fluid of mature sole contains different classes of odorants; future analytical chemistry will determine this. Although we only managed to collect one sample, the fact that the ovarian fluid proved to be less potent than urine, and the olfactory response depended on neither the sex nor maturity of the receiver is, perhaps, counter-intuitive. However, although salmonids have been shown to have a similar olfactory sensitivity to 'urogenital fluid' (assumed to be mostly ovarian fluid; Kitamura and Ogata, 1989), it does not have the same attraction to males as urine from mature females (Olsén et al., 2002), although it may have some attractive properties (Emanuel and Dodson, 1979). Sole spawn in pairs (Baynes et al., 1994; Carazo et al., 2016) with little opportunity for other males or females to participate once the swimming assent has started to liberate gametes and this may be related to the lack of involvement of ovarian fluid in communication. Together, these findings suggest that ovarian fluid is less important than urine in chemical communication in sole, although it may enhance sperm motility (Carazo et al., 2016; Diogo et al., 2010) as in other teleosts (for example, see Elofsson et al., 2006; Rosengrave et al., 2009). Mucus, a highly potent odorant in the European eel (Huertas et al., 2007), was without significant olfactory potency in the sole so may not be important in chemical communication, even though males and females may be in close contact during courtship (Carazo et al., 2016).

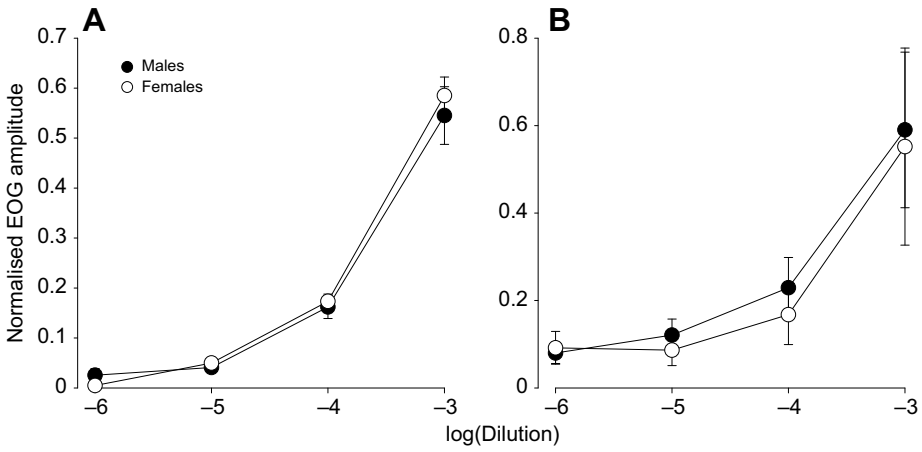

Fig. 7. Olfactory sensitivity to conspecific ovarian fluid. Semi-logarithmic plots of normalised amplitude of electro-olfactogram (EOG) responses to dilutions of one sample of ovarian fluid in juvenile (A) and adult-immature (B) fish of both sexes. No statistical differences were seen between responses from juvenile males and females (*N*=5 for both sexes; *P*=0.710; two-way repeated-measures ANOVA) nor adult males (*N*=11) and females (*N*=6; *P*=0.595; two-way repeated-measures ANOVA).

One of the aims of the current study was to investigate whether the poor reproductive performance of hatchery-bred males could be due, at least in part, to a fault in the reproductive pheromonal system. However, contrary to expectations, the urine from wild-caught sole was less potent than that of hatchery-bred fish. Thus, it seems unlikely (although possible) that wild-caught fish are releasing less of an important pheromonal component than hatchery-bred fish (our original hypothesis). Nevertheless, this finding clearly allows for the possibility that environmental or social cues, found in the natural environment but not in captivity (or *vice versa*), are important for future production of pheromonal cues during spawning. Alternatively, although not addressed in the current study, hatchery-bred fish may not release urinary or intestinal cues in the appropriate context (i.e. a behavioural fault). It is also possible that such cues also modulate neural signalling processing and pheromone receptors in the olfactory epithelium in a similar manner to that of sex steroids mentioned above. Or that the neural processing of the pheromonal message is faulty in hatchery-bred fish. Significantly, mRNA of some olfactory receptors (OR, TAAR and V2R-like) as well as other transcripts related to reproduction (e.g. cytochrome P450), are differentially expressed in the upper olfactory epithelia of wild-caught and hatchery-bred sole (Fatsini et al., 2016). Given the apparent complexity of both the pheromonal message and the respective olfactory receptors in the olfactory epithelium, to address this hypothesis, we need to identify the active components in both male and female urine, their biological roles and their receptors in the olfactory epithelium, then make a full comparison between wild-caught and hatchery-bred males.

In conclusion, the current study has shown that conspecific urine and intestinal fluid, but not ovarian fluid and mucus, may play roles in chemical communication during reproduction in the sole. Moreover, olfactory sensitivity to at least some of the compounds involved depends on both the sex and state of maturity of the receiver, suggesting regulation at the level of the olfactory epithelium. One physiological role of mature female urine may be to increase spermatogenesis and milt production in males via stimulation of the release of LH from the pituitary gland, as has been previously shown for 17α,20β-dihydroxy-4-pregnen-3-one in the goldfish (Dulka et al., 1987). Thus, chemical communication in this marine fish possibly shows much overlap with that of freshwater fish but with some intriguing differences (e.g. a putative role for intestinal fluid). The focus now must be to identify the compounds involved and delineate their biological roles.

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Competing interests
The authors declare no competing or financial interests.

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Conceptualization: E.F., I.C., F.C., J.C., P.C.H. and N.J.D. Methodology: J.C. and P.C.H. Formal analysis: E.F., I.C., F.C. and P.C.H. Investigation: E.F., I.C., F.C. and P.C.H. Resources: F.C., M.M., J.C., P.C.H. and N.J.D. Writing – original draft: E.F., P.C.H. and N.J.D. Writing – review and editing: E.F., F.C., M.M., J.C., P.C.H. and N.J.D. Visualization: E.F. and P.C.H. Supervision: J.C., P.C.H. and N.J.D. Project administration: J.C., P.C.H. and N.J.D. Funding acquisition: M.M., J.C. and N.J.D.

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