The Journal of Experimental Biology 213, 308-317
Published by The Company of Biologists 2010
doi:10.1242/jeb.033142

Olfactory sensitivity to bile fluid and bile salts in the European eel (*Anguilla anguilla*), goldfish (*Carassius auratus*) and Mozambique tilapia (*Oreochromis mossambicus*) suggests a 'broad range' sensitivity not confined to those produced by conspecifics alone

M. Huertas[1], L. Hagey[2], A. F. Hofmann[2], J. Cerdà[3], A. V. M. Canário[1] and P. C. Hubbard[1,*]

[1]Centro de Ciências do Mar, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal, [2]Department of Medicine, University of California, San Diego, CA, USA and [3]Institut de Reserca Técnico-Agroalimentària Research Scientist, CMIMA, 08003-Barcelona, Spain

*Author for correspondence (phubbard@ualg.pt)

Accepted 8 October 2009

SUMMARY

Teleosts have high olfactory sensitivity to bile salts. To assess whether this phenomenon is involved in intra-specific chemical communication alone, or is part of a more 'broad range' sensitivity to bile salts produced by heterospecifics, we investigated possible differences in the odour of bile between the sexes and among different species – the eel (*Anguilla anguilla*), goldfish (*Carassius auratus*) and Mozambique tilapia (*Oreochromis mossambicus*) – using the electro-olfactogram (EOG). We also identified the main bile constituents by liquid chromatography and mass spectrometry. There were marked differences in olfactory response of the eel to thin-layer chromatography fractions of bile from both sexes, and mature and immature conspecifics. Smaller differences were seen in the potency of fractions of bile from male and female goldfish and tilapia. Eels, goldfish and tilapia demonstrated similar olfactory sensitivity to bile from a range of different species, with no apparent correlation between the olfactory potency of bile and a phylogenetic closeness and/or similarity of diet of the donor to the receiver. The three species were able to detect odorants in thin-layer chromatography fractions of heterospecific bile even in the absence of activity in conspecific bile. Eels, goldfish and tilapia responded to both sulphated C_{27} bile salts (5β-scymnol-sulphate and 5α-cyprinol sulphate) and to taurine-conjugated C_{24} bile salts (taurochenodeoxycholic acid, taurolithocholic acid and taurocholic acid), irrespective of whether these bile salts were present in conspecific bile. Together, these results suggest that teleosts have a broad-range olfactory sensitivity to bile salts, with potential roles in both intra-specific chemical communication and in inter-specific interactions.

Key words: eel, goldfish, tilapia, olfaction, bile acids, bile fluid, intra-specific identification, inter-specific identification, chemical communication.

INTRODUCTION

Bile is a multi-functional fluid that acts both as a detergent to aid lipid digestion, and as an excretory fluid for substances that cannot be eliminated by urine (Hofmann, 1999). Bile salts, which are a key constituent of bile, are steroidal compounds with a characteristic fused four-carbon-ring skeleton often found conjugated with glycine, taurine, cysteine or sulphate groups (Goto et al., 2003; Hofmann, 1999; Moschetta et al., 2005). In modern mammals, bile salts are mostly C_{24} carboxylic acids, whereas in primitive mammals, cartilaginous fish and reptiles, C_{27} acids and alcohols are found (Haslewood, 1967; Hofmann and Hagey, 2008; Moschetta et al., 2005).

Many species of fish have been shown to have an acute olfactory sensitivity to bile salts (Døving et al., 1980; Siefkes and Li, 2004; Zhang et al., 2001). Although the biological function of this phenomenon is unclear, with the notable exception of the sea lamprey *Petromyzon marinus* (Li et al., 2002; Polkinghorne et al., 2001; Sorensen et al., 2005), the stability and water solubility of the bile salt molecule makes it an ideal candidate for a role in chemical communication (Rosenthal and Lobel, 2006). We have recently shown that the odour of bile from the European eel (*Anguilla anguilla*) depends not only on the sex, but also changes with sexual maturity (Huertas et al., 2007), suggesting a possible role for bile salts as sex pheromones in teleosts.

Bile salts are unique to vertebrates (Haslewood, 1967), and most bile salts found in the aquatic environment (apart from shorelines) are probably from the faeces of fish (Velez et al., 2009; Zhang et al., 2001). Although most fish have the C_{24} bile acid taurocholic acid, there is sufficient structural variability in the bile salt structures among different fish groups (Haslewood, 1967) to serve as a means of detecting bile salts released by congeners and by other species. For example, behavioural evidence has shown that prey fish are able to recognise potential predators by olfaction (Kristensen and Closs, 2004; Rosenthal and Lobel, 2006). Furthermore, migration could involve olfactory detection of bile salts (Siefkes and Li, 2004).

To test inter-specific sensitivity to bile acids, we used a comparative approach with three teleost species; the European eel (*Anguilla anguilla* Linnaeus 1758, Anguilliformes; hereafter referred to as 'eel'), goldfish (*Carassius auratus* Linnaeus 1758, Cypriniformes) and Mozambique tilapia (*Oreochromis mossambicus* Peters 1852, Perciformes; hereafter 'tilapia'). Not only are these three species phylogenetically diverse, belonging to three different orders ranging from a member of an early-diverging group (the eel) to a more recently diverging group (the tilapia) but also their diets differ, ranging from algae, plants and detritus (tilapia), aquatic invertebrates (tilapia, goldfish and eel) to small fish and other aquatic vertebrates (eel). Thus, potential odour-inducing chemicals present in the bile of these species are likely to be different.

The aims of the present study were twofold. First, we wished to establish whether olfactory sensitivity is either 'fine-tuned' to conspecific derived bile salts or is more 'broad-range', whereby teleosts are able to detect bile salts that they themselves do not produce. This was done using the electro-olfactogram (EOG) (Scott and Scott-Johnson, 2002). Second, we wished to establish whether the odorants present in bile (putative pheromones) are sex dependent. This was accomplished by assessing olfactory sensitivity to thin-layer chromatographic fractions of bile obtained from both genders, followed by an analysis of the composition by liquid chromatography and mass spectrophotometry. Finally, we assessed the olfactory sensitivity of the three species to a range of known C_{24} and C_{27} bile salts tentatively identified as present in their bile.

MATERIALS AND METHODS

Fish and bile fluid samples

Eels (immature males, 105±7 g) were obtained from an eel farm (RAMKO S.L., Tarragona, Spain), transferred to the Universidade do Algarve, kept in dechlorinated freshwater in outdoor tanks (i.e. under natural temperature and photoperiod) and fed daily with commercial extruded pellets (Anguila, DIBAQ, Spain). Goldfish (both sexes, 15±4 g) were taken from a population kept under similar conditions at the Universidade do Algarve and fed once or twice daily with commercial pond-fish food. Tilapia (both sexes, 78±10 g) were taken from a population kept indoors at 27°C under a 12 h:12 h L:D photoperiod and fed a commercial cichlid diet. Bile fluid was collected directly from the gallbladders and stored at –20°C. Bile fluid samples from both sexes of goldfish and tilapia were collected from the above stocks when the fish were killed for use in other studies. Bile fluid from immature and artificially induced mature eels and immature eels (both sexes) and from male Senegalese sole (*Solea senegalensis*, Pleuronectiformes; hereafter 'sole') came from cultured stocks held at IRTA (Tarragona, Spain), being sampled for other reasons (Agulleiro et al., 2006; Huertas et al., 2007; Huertas et al., 2006). Bile fluid from gilthead seabream (*Sparus aurata*, Perciformes; hereafter 'seabream') was taken from four females from stocks kept at the Universidade do Algarve, again being sampled for other reasons. Bile fluid from European flounder (*Platichthys flesus*, Pleuronectiformes; hereafter 'flounder') was taken from wild-caught fish from the River Dee estuary, Cumbria, UK, and held unfed in seawater, at the University of Manchester, UK. The sex of these fish was not determined, as the time of sampling was outside their natural spawning season. For each species or sex, a 'stock' pool of bile fluid was made using an equal volume of bile fluid from four to ten individual fish.

Thin-layer chromatography of bile fluids

Sub-samples of 400 µl of bile from eel, goldfish and tilapia (both sexes and both immature and mature eels) were diluted in 5 ml of distilled water. This was then passed through a solid-phase Isolute 500 mg C18 extraction cartridge (International Sorbent Technology Ltd, Hengoed, UK) and eluted with 4 ml of methanol. Isopropanol (6 ml) was added (Sigma-Aldrich Chemical Co., Madrid, Spain), and the eluate gently mixed for 15 min, centrifuged for 10 min and the supernatant discarded. This step was repeated twice. The resulting pellet was evaporated under nitrogen at 45°C and then re-suspended in 0.5 ml of methanol and stored at –20°C until chromatography. For analysis by thin-layer chromatography, duplicate 50 µl samples were loaded onto paired sets of plates (silica gel plates 60, 0.5 mm, Merck SA, Lisbon, Portugal) and then run using a *n*-butanol:acetic acid:water solvent system (85:10:5) (Sasaki, 1966). One run was set aside to identify bile acids by staining with phosphomolybdic acid (Sasaki, 1966). The other run was used to isolate bile components for EOG recording. Fractions were collected by scraping off 1 cm^2 sections of silica gel, followed by elution in 5 ml methanol. After centrifugation, the supernatant collected and stored at –20°C. A control run was also carried out using 50 µl of methanol alone.

High-performance liquid chromatography analysis of bile

Conjugated bile acids were analysed by reverse-phase HPLC (high-performance liquid chromatography) using a modification of the technique of Rossi et al. (Rossi et al., 1987). An octadecylsilane column (RP C-18; Beckman Instruments, Fullerton, CA, USA) was used with isocratic elution at 0.75 ml min^{-1}. The eluting solution was composed of a mixture of methanol and 0.01 mol l^{-1} KH_2PO_4 (67.4% v/v), adjusted to an apparent pH of 5.3 with H_3PO_4. Bile acids were quantified by measuring the absorbance of their amide bond at 205 nm. Bile acids were tentatively identified by matching their relative retention times with those of known standards.

Nano-electrospray ionization mass spectrometry analysis of bile

Fish bile samples were analyzed using a PE Sciex API III (Alberta, Canada), mass spectrometer modified with a nanoESI (electrospray ionization) source from Protana A/S (Odense, Denmark). The orifice voltage was set at –115 V, and ESI voltage set at –650 V (Chatman et al., 1999). A curtain gas of ultrapure nitrogen was pumped into the interface (at a rate of 0.6 l min^{-1}) to aid evaporation of solvent droplets and prevent particulate matter from entering the analyzer region. Normal-sized palladium-coated, borosilicate glass capillaries from Protana A/S were used for sample delivery. The collision-induced dissociation (CID) experiments were performed with ultrapure argon as a collision gas. The precursor ion spectra were acquired by scanning the first quadrupole, while collisions with argon in the second quadrupole produced ion dissociation. The third quadrupole was used to mass select the fragment ions [mass/charge (*m*/*z*) 97, for sulphated conjugates, *m*/*z* 124 for taurine conjugates]. Spectra were the result of averaging from 50 to 200 scans depending on the number of scans necessary to obtain a signal-to-noise ratio greater than 50.

Bile salts

The sodium salts of taurocholic acid (TCH), taurolithocholic acid (TLC) and taurochenodeoxycholic acid (TCD) were bought from Sigma Aldrich Chemical Co. (Madrid, Spain). The C_{27} bile acids 5α-cyprinol sulphate (5α-cholestane-3,7,12,26,27-pentol-27-sulphate; CYP-S) and 5β-scymnol sulphate (SYS) were isolated and purified from the Asiatic carp (*Cyprinus carpio*), as described by Goto et al. (Goto et al., 2003).

Recording of the electro-olfactogram

The method for recording the EOG from eels, goldfish and tilapia has been described previously in detail (Frade et al., 2002; Hubbard et al., 2002; Huertas et al., 2006). EOGs were recorded from immature male eels only [inducing sexual maturity in captive eels is expensive and time consuming (Huertas et al., 2006)] whereas EOGs were recorded from both male and female goldfish and tilapia and, as no obvious differences in the responses were noted, data from both sexes were pooled. Briefly, the fish were anaesthetized in water containing 100 mg l^{-1} 3-aminobenzoic acid ethyl ester (MS-222; Sigma-Aldrich Chemical Co., Madrid, Spain). They were then immobilized by intramuscular injection of gallamine tri-ethiodide

(Sigma-Aldrich; 3 mg kg^{-1} in 0.9% NaCl) and placed in a padded support with aerated water (containing anaesthetic) pumped over the gills (approx. 100 ml min^{-1} 100 g^{-1}) via a silicon tube placed in the mouth. The olfactory epithelium was exposed by cutting away the section of skin between inhalant and exhalent nares (in the case of the eel), by cutting the small skin flap that overlies the olfactory chamber (goldfish) or by removing the cartilage ring surrounding the single nare (tilapia). The DC potential was recorded using borosilicate micropipettes filled with 0.4% agar in 0.9% NaCl connected to solid-state electronics with Ag/AgCl pellets in 3 mol l^{-1} KCl. The recording electrode was placed close to (but not touching) the raphe between the fourth and fifth anterior lamellae (eel), close to the raphe between the large two posterior lamellae (goldfish) or close to the centre of the olfactory rosette (tilapia). These positions gave the largest and, more importantly, most stable EOG responses to the standard stimuli (see below). The signal was amplified and filtered using either a Grass AC/DC strain gauge amplifier (model CP122, AstroMed, West Warwick, RI, USA; low-pass filter 30 Hz) or a NeuroLog pre-amplifier (model NL102, Digitimer Ltd, Welwyn Garden City, UK) with filter (low-pass 50 Hz, model NL125, Digitimer Ltd). The signal was then digitized (Digidata 1200 or Digidata 1320A, Axon Instruments, Inc., Foster City, CA, USA) and stored on a PC running Axoscope software (Axon Instruments, Inc.). The nostril was continuously irrigated with de-chlorinated tap water, and bile samples were dissolved directly into this water. All surgical and experimental procedures followed the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Treaty Series No. 123) and the Guidelines for the Use of Fishes in Research by the American Fisheries Society (http://www.fisheries.org/afs/docs/policy_guidelines2004.pdf).

Data treatment and statistical analysis

The initial negative peak EOG amplitude was measured (in mV), blank-subtracted and normalized to the previous response to 10^{-3} mol l^{-1} L-asparagine (eels) or 10^{-5} mol l^{-1} L-serine (goldfish and tilapia) as previously described (Frade et al., 2002; Hubbard et al., 2002; Huertas et al., 2006); these responses were recorded at regular intervals (every 15–20 min) throughout the recording session. L-Asparagine was used as a standard stimulus for the eel, as responses to L-serine were smaller and less reproducible in this species than in either the goldfish or tilapia. The order in which different stimuli were given was varied between recording sessions. However, for a given stimulus type, the stimuli were given in order of increasing concentration. At least 1 min was allowed to elapse between successive stimuli. For the concentration–response curves used to differentiate between bile fluids and bile salts, linear regression was carried out on log-transformed, normalized data (Hubbard et al., 2003b). The thresholds of detection were estimated from the intercept of the x axis and slopes were compared by the Fisher test (Zar, 1996) for multiple data. Thresholds of detection of bile and bile acid data were compared by one-way ANOVA followed by the Holm–Sidak test. All data are shown as mean ± s.e.m. (standard error of the mean); in all cases a probability of 0.05 or less was taken to represent statistical significance.

RESULTS

Olfactory sensitivity to bile

The olfactory system of immature male eels proved to be highly sensitive to conspecific bile, giving large amplitude typical fish EOG responses in a strongly concentration-dependent manner down to dilutions of 1:10^7 (Fig. 1) with a calculated threshold of detection of 1:10$^{7.3}$. However, although conspecific bile evoked the largest

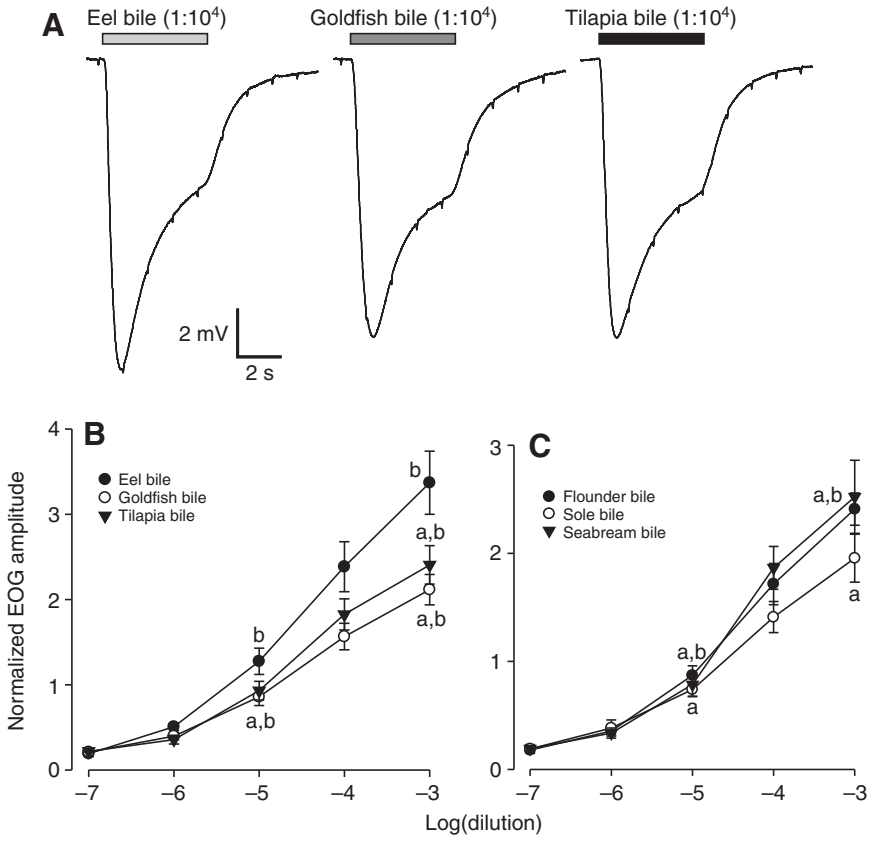

Fig. 1. Olfactory sensitivity to conspecifc and heterospecific bile fluid in the eel. (A) Typical electro-olfactogram (EOG) traces recorded from an immature eel in response to bile fluid from eels, goldfish and tilapia (all diluted 1:10,000). The small, regular peaks (approx. 1 Hz) were assumed to be due to the heart-beat, a regular characteristic when recording from eels. A downward deflection of the trace is negative. (B) Semi-logarithmic plot of pooled normalized EOG amplitudes recorded in immature male eels in response to dilution of bile fluid from eels, goldfish and tilapia. (C) Semi-logarithmic plot of pooled normalized EOG amplitudes recorded in immature male eels in response to dilution of bile fluid from flounder, sole and seabream. Data are mean ± s.e.m. (N=6). Values with different letters indicate significantly different response amplitudes to bile fluid (at the same dilution) among all six different types (ANOVA, P<0.05).

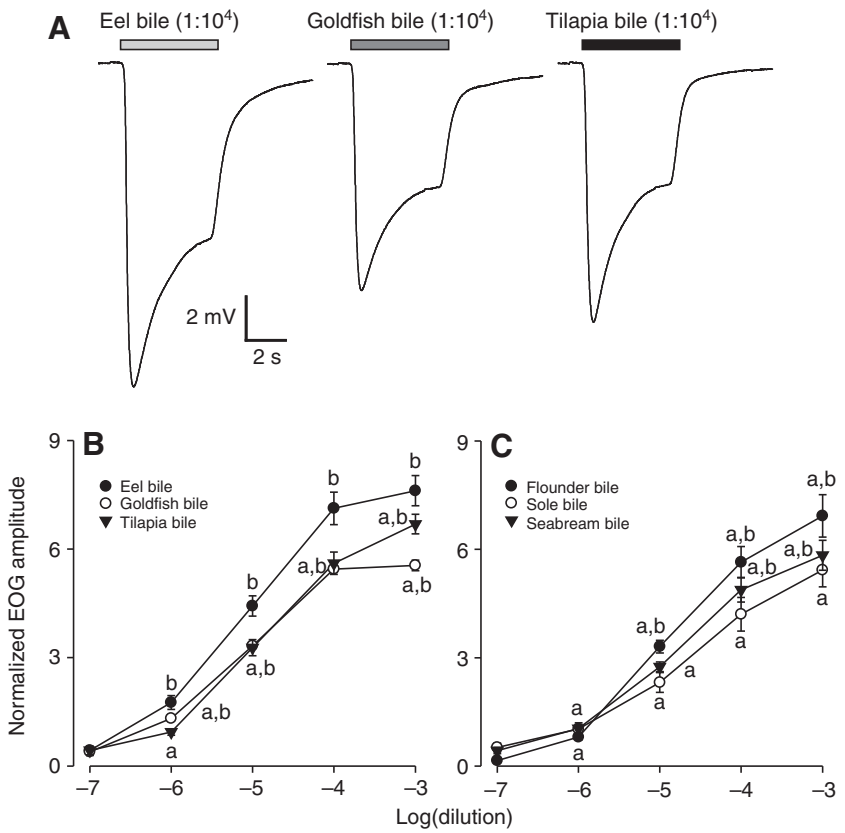

Fig. 2. Olfactory sensitivity to conspecifc and heterospecific bile fluid in the goldfish. (A) Typical electro-olfactogram (EOG) traces recorded from a goldfish in response to bile fluid from eels, goldfish and tilapia (all diluted 1:10,000). A downward deflection of the trace is negative. (B) Semi-logarithmic plot of pooled normalized EOG amplitudes recorded in goldfish in response to dilution of bile fluid from eels, goldfish and tilapia. (C) Semi-logarithmic plot of pooled normalized EOG amplitudes recorded in goldfish in response to dilution of bile fluid from flounder, sole and seabream. Data are mean ± s.e.m. (N=6). Values with different letters indicate significantly different response amplitudes to bile fluid (at the same dilution) among all six different types (ANOVA, P<0.05).

amplitude EOGs, heterospecific bile evoked similar strongly concentration-dependent, large amplitude EOGs with similar estimated thresholds of detection (Fig. 1B,C); goldfish $1{:}10^{7.4}$, tilapia $1{:}10^{7.3}$, flounder $1{:}10^{7.2}$, sole $1{:}10^{7.4}$, seabream $10^{7.1}$. There was no apparent correlation between the olfactory response and either the phylogenetic relatedness of the different species or the similarity of diets.

The olfactory epithelium of goldfish also showed high olfactory sensitivity to conspecific bile fluid, giving large amplitude typical fish EOG responses in a strongly concentration-dependent manner and with an estimated threshold of detection of $10^{7.4}$ (Fig. 2). However, at the higher concentrations tested ($1{:}10^4$ and $1{:}10^3$), the responses reached an apparent maximum, suggestive of olfactory receptor neuron saturation. Again, the olfactory epithelium of goldfish proved to be highly sensitive to the bile fluids of the other species (Fig. 2B,C) with similar thresholds of detection; eel $1{:}10^{7.5}$, tilapia $1{:}10^{7.4}$, flounder $1{:}10^{7.1}$, sole $1{:}10^{7.7}$, seabream $1{:}10^{7.1}$. Contrary to expectations, both eel and tilapia bile evoked significantly higher amplitude EOG responses than did conspecific bile. There did not appear to be a maximum response reached to the bile fluids of the three marine species. Again, there appeared to be no relationship between the sensitivity to a species bile fluid and phylogenetic relatedness and/or diet. Indeed, of the six species, the goldfish (a stenohaline freshwater fish) is only likely to encounter conspecifics and eels.

Tilapia also proved to have high olfactory sensitivity to conspecific bile fluid (Fig. 3) with an estimated threshold of detection of $1{:}10^{7.0}$. Again, all EOG responses were typical in form for fish. Nevertheless, the bile fluid of other species also proved to be equally potent; eel $1{:}10^{7.2}$, goldfish $1{:}10^{7.8}$, flounder $1{:}10^{7.1}$, sole $1{:}10^{7.3}$, seabream $1{:}10^{7.3}$. In the case of the goldfish bile, however, the responses to the highest concentrations again appeared to reach a maximum even though the amplitude was significantly less than those of the responses to eel or tilapia bile (Fig. 3B). The responses to eel bile also tended to be higher than those to the other species. Very little difference was seen in the concentration–response curves of the bile fluids of the marine fish (Fig. 3C).

Thin layer chromatography

The extracts of eel, tilapia and goldfish bile fluid showed different staining patterns on the thin layer chromatography plate, suggesting a different composition of bile salts in the different species. Furthermore, there were marked differences in the pattern of staining between the bile fluid of eels of either sex and between mature and immature eels (Fig. 4); inter-sex differences were much less marked in goldfish and tilapia bile. There were faint spots in the last fraction (fraction 12) of extracts from mature eels of both sexes, which were not present in those of immature eels. There were densely staining spots in fractions 4 and 5, and 6 and 7 of extracts from mature males, which corresponded to a taurine conjugated C_{27} trihydroxy bile acid (4 and 5) and to a taurine conjugated C_{27} dihydroxy bile acid (6–7). Both spots were weak or absent in extracts from females. Extracts from mature males showed spots in fraction 5 (a mixture of 5α- and 5β-scymnol sulphate), fraction 7 (a mixture of 5α- and 5β-bufol sulphate), and fraction 8 (taurocholic acid). By contrast, those from females had significantly larger spots in fraction 8 (taurocholic acid), whereas extracts from mature females also had a larger proportion of fraction 7 (mixture of 5α- and 5β-bufol sulphate). Eel bile also had staining on the origin (i.e. fraction 0; substances that failed to migrate); this staining was not seen in bile from the other species.

For goldfish of both sexes there was a major band in fractions 4 and 5 (5α-cyprinol sulphate) and a smaller band in fraction 2. In extracts from both sexes there was also a faint band in fraction 9

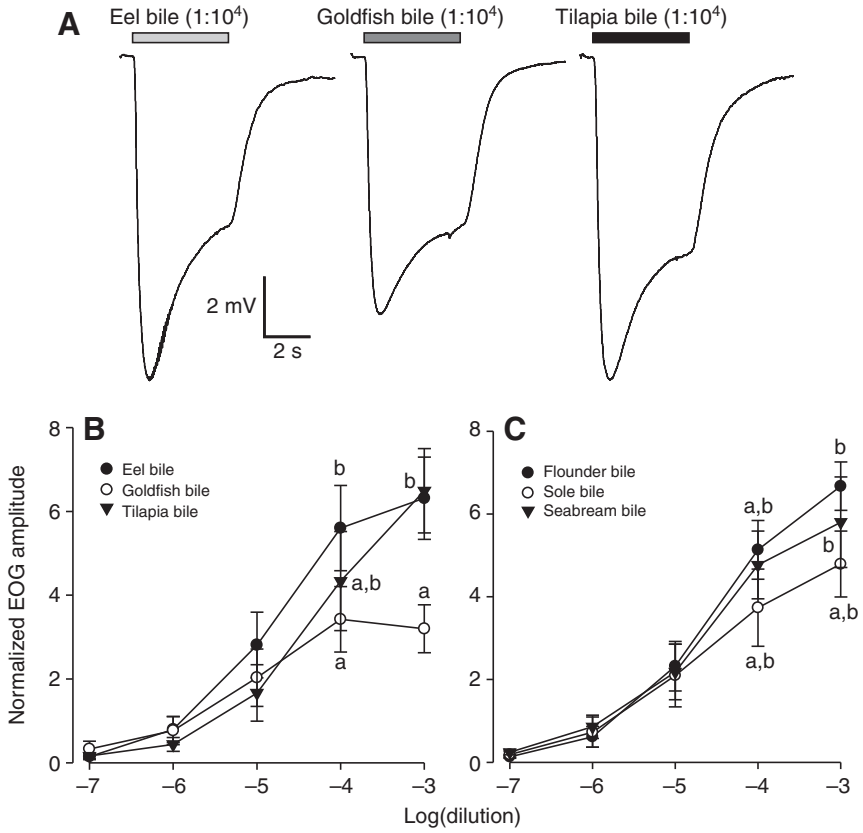

Fig. 3. Olfactory sensitivity to conspecifc and heterospecific bile fluid in the tilapia. (A) Typical electro-olfactogram (EOG) traces recorded from a tilapia in response to bile fluid from eels, goldfish and tilapia (all diluted 1:10,000). A downward deflection of the trace is negative. Note the slight 'ringing' (high frequency oscillations) at the peak of the first trace; a common characteristic when recording large EOG responses (Suzuki et al., 2004). (B) Semi-logarithmic plot of pooled normalized EOG amplitudes recorded in tilapia in response to dilution of bile fluid from eels, goldfish and tilapia. (C) Semi-logarithmic plot of pooled normalized EOG amplitudes recorded in tilapia in response to dilution of bile fluid from flounder, sole and seabream. Data are mean ± s.e.m. (N=6). Values with different letters indicate significantly different response amplitudes to bile fluid (at the same dilution) among all six different types (ANOVA, P<0.05).

(unconjugated 5α-cholic acid) and from females a faint band in fraction 12, which was not evident in males.

For tilapia of both sexes there was a major band in fractions 8–10 (unconjugated cholic acid) and minor bands in fractions 4 and 5 (pentahydroxy C_{27} bile alcohol sulphate) and 2 and 3.

Sensitivity to thin layer chromatography fractions of eel, goldfish and tilapia bile

Eels gave robust EOG responses to fractions 5 to 9 from conspecific bile (Fig. 5). There were, however, differences in the amplitude of responses depending on the sex and maturation state of the donor; fraction 8 from mature females evoked larger responses than the corresponding fractions from males and immature females. Conversely, fractions 5 and 6 from immature males evoked larger responses than those from females. The faint bands on the origin also evoked some responses; the amplitude of these EOG responses evoked were significantly larger with bile from mature males. Fractions 9–12 also evoked some responses, even though little or no staining was seen in these fractions. However, there were no apparent differences between the sexes

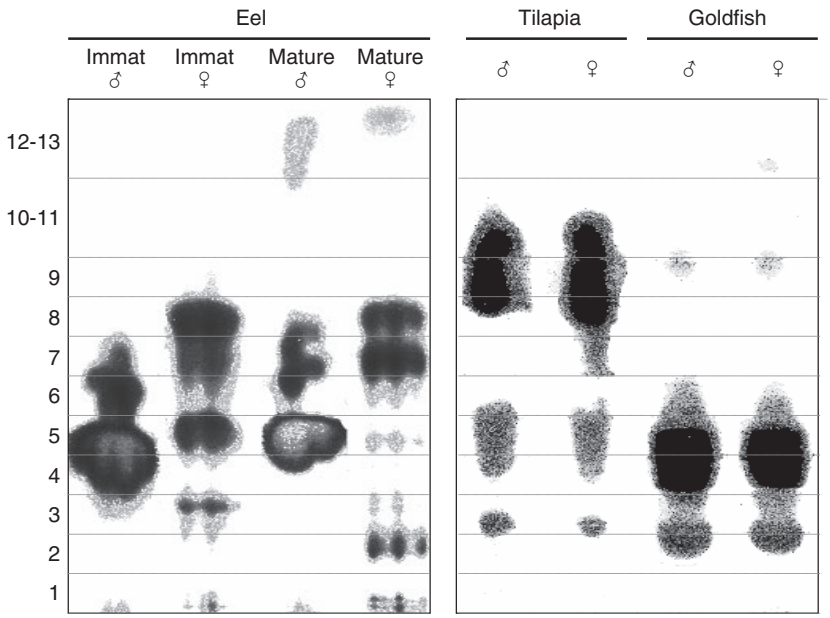

Fig. 4. Thin layer chromatogram of bile fluids from immature and mature eels, tilapia and goldfish (both sexes). The vertical numbering corresponds to the fractions extracted for EOG recording (Fig. 5). For the lanes marked eels, spots in fraction 8 are mostly taurochenodeoxycholic acid; in fraction 7, taurocholic acid; in fraction 6.5, taurine-conjugated dihydroxy C_{27} bile acid(s); in fraction 5 (immature), taurine-conjugated trihydroxy C_{27} bile acids(s); in fraction 5 (mature) 5β-bufol-sulphate. In the lanes marked tilapia, fraction 9 is mostly unconjugated cholic acid. In the lanes marked goldfish, the spot in fractions 4 and 5 is 5α-cyprinol sulphate. The contrast has been digitally enhanced for clarity.

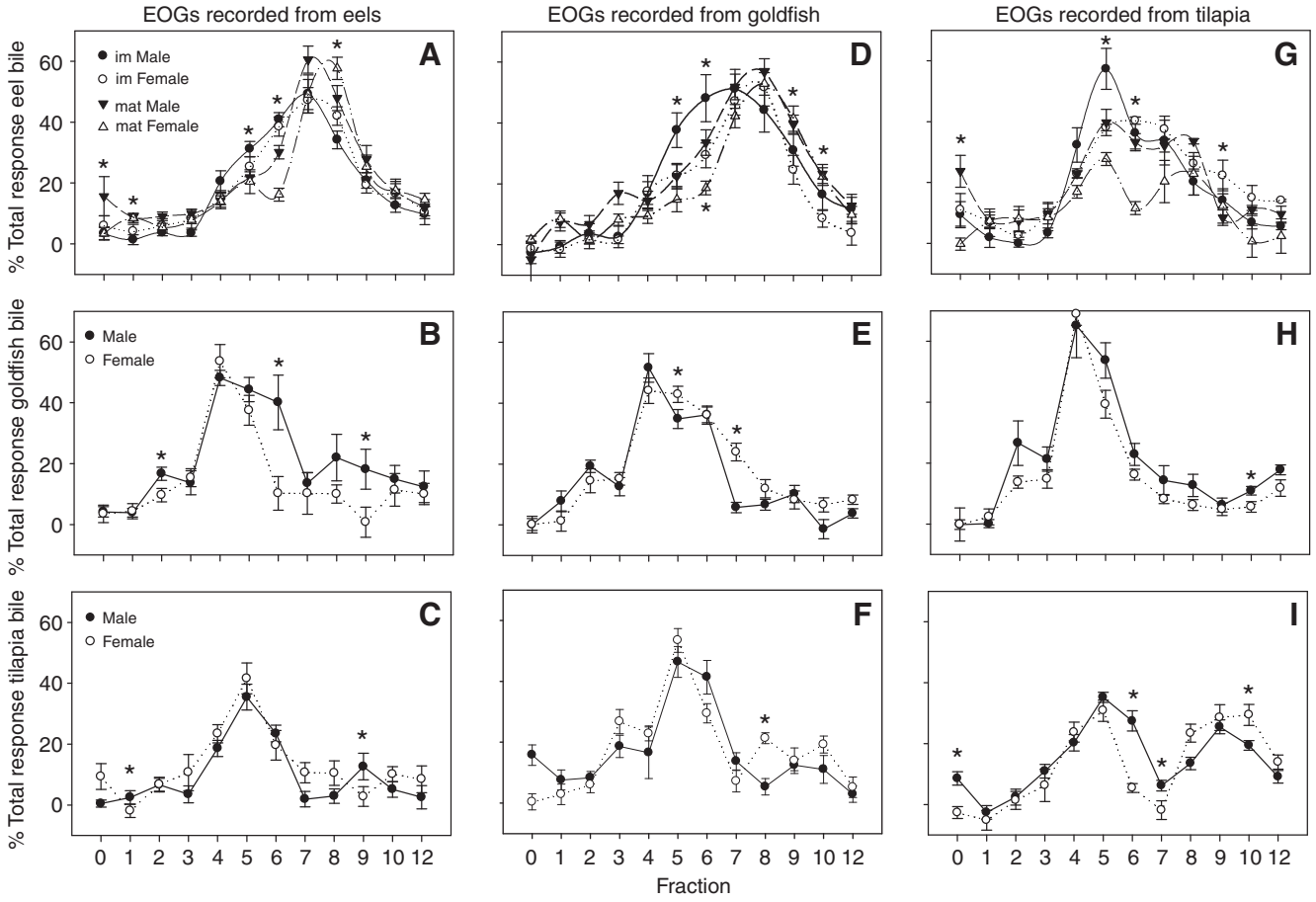

Fig. 5. Olfactory responses of immature male eels, goldfish and tilapia to thin layer chromatography fractions of conspecific and heterospecific bile fluid (corresponding to fractions 1–12 in Fig. 4). Columns (A–C, D–F and G–I) were recorded from eels, goldfish and tilapia, respectively. The sources of bile fluid in row A, D and G were eels of both sexes, both mature and immature (im Male, sexually immature male eel bile; im Female, sexually immature female eel bile; mat Male, sexually mature male eel bile; and mat Female, sexually mature female eel bile). The sources of bile fluid in row B, E and H were male or female goldfish. The sources of bile fluid in row C, F and I were male) or female tilapia. In this figure, all EOG response amplitudes to individual fractions are expressed as a percentage of the response to their respective unfractionated (i.e. total=100%) bile extract (mean ± s.e.m., N=6 or 7). *P<0.05 (ANOVA or Student's t-test, as appropriate) comparing response amplitude to a given fraction among donor sex and state of maturation (eels) or between sex of donor (goldfish and tilapia).

or maturation state, despite the faint bands present in mature, but not immature, eel bile.

Goldfish responded to fractions 2 and 4–6 of conspecific bile. Slight responses were seen with fractions 9 and 12, corresponding to the faint bands on the chromatography plate, but these did not differ between the sex of the donor. Fraction 7 from female bile evoked large EOG responses from males, even though no staining was apparent in Fig. 4.

Tilapia showed a response of similar magnitude to the two main staining bands (fractions 4 and 5 and fractions 8–10) of conspecific bile. EOG amplitudes evoked by fraction 6 from males were larger than those evoked by that of females. Conversely, the larger band in fractions 8–10 from females evoked larger EOG responses than that of males. Although no staining was seen in the thin-layer chromatography plate origin from either sex, fraction 0 of males evoked larger amplitude EOGs than that of females.

In general, eels responded to the same fractions of bile from goldfish and tilapia as did goldfish and tilapia, respectively. However, eels responded better to fractions 6 and 9 from male goldfish than from females. Furthermore, eels responded less to the dense band in tilapia bile than did tilapia themselves. Goldfish responded to the same fractions of eel bile as did eels, including larger responses to fractions 5 and 6 of immature male eel bile and lower amplitude response to fraction 6 from mature female eel bile. However, no response was seen to fraction 0 of mature male eel bile. Similar to the eel, goldfish had larger EOG responses to the smaller band in tilapia bile (fractions 4–6) than to the larger band in fractions 8–10. Conversely, tilapia responded to this fraction (bile from male tilapia also has olfactory activity in this fraction). Again, tilapia had larger responses to fraction 5 from immature male eel bile and markedly lower amplitude responses to fraction 6 from mature female eel bile, whereas the pattern of responses to goldfish bile was similar to that of goldfish themselves.

Bile composition

The striking difference in composition of the bile salts seen by thin-layer chromatography was confirmed using mass spectrometry, as shown in Fig. 6. Eight different chemical structures could be identified: a (m/z 498, taurine-conjugated dihydroxy C_{24} bile acid); B (m/z 514, taurine-conjugated trihydroxy C_{24} bile acid); C (m/z 531, pentahydroxy C_{27} bile alcohol sulphate); D (m/z 540, taurine-conjugated dihydroxy C_{27} bile acid); E (m/z 547, hexahydroxy C_{27} bile alcohol sulphate); F (m/z 556, taurine-conjugated trihydroxy C_{27} bile acid); G (m/z 572, taurine-conjugated tetrahydroxy C_{27} bile acid); and H (m/z 407,

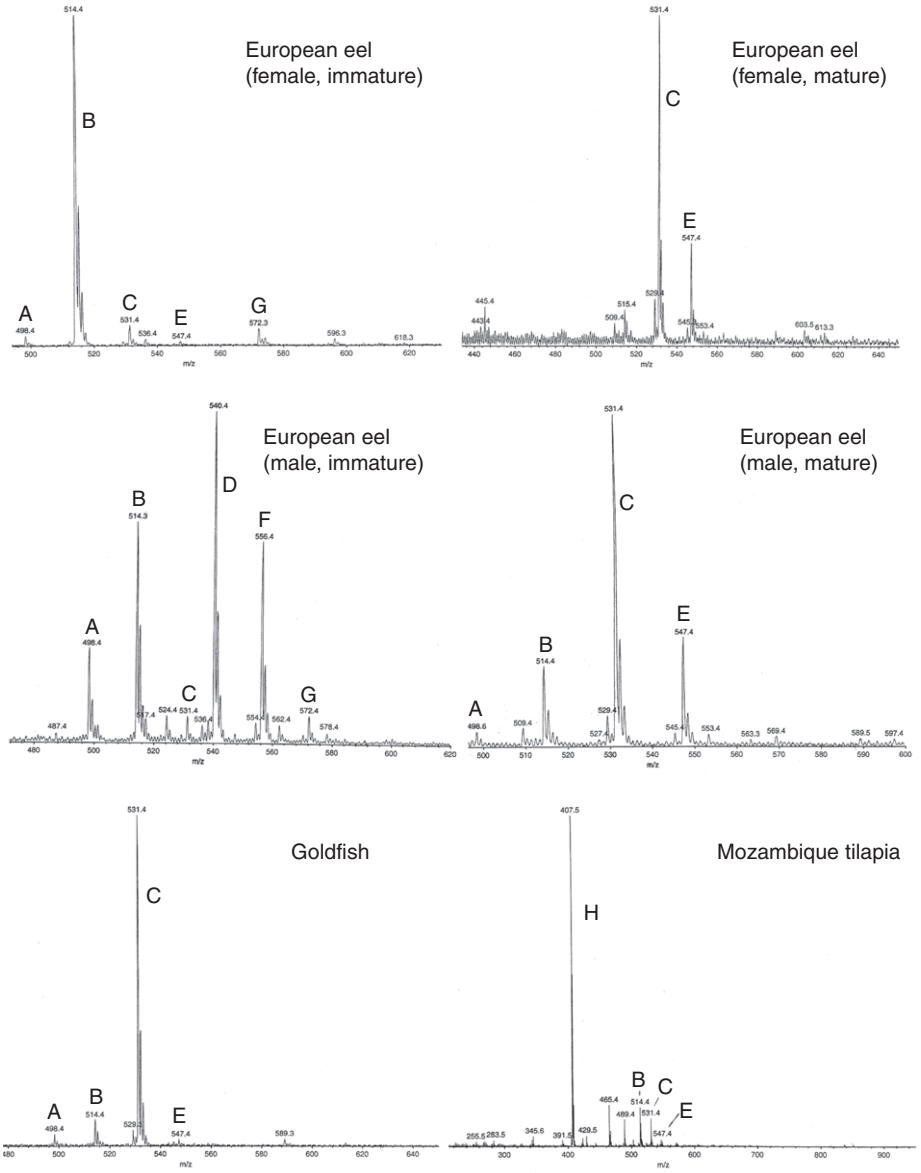

Fig. 6. Mass spectrometry chromatograms of the bile salts of eels, goldfish and tilapia. The two upper panels are from immature and mature female eels; the two middle panels are from immature and mature male eels; the left lower panel is from goldfish; and the right lower panel is from tilapia. Peak identities are as follows: A: *m*/*z* 498, taurine-conjugated dihydroxy C_{24} bile acid; B: *m*/*z* 514, taurine-conjugated trihydroxy C_{24} bile acid; C: *m*/*z* 531, pentahydroxy C_{27} bile alcohols sulphate; D: *m*/*z* 540, taurine-conjugated dihydroxy C_{27} bile acid; E: *m*/*z* 547, hexahydroxy C_{27} bile alcohol sulphate; F: *m*/*z* 556, taurine-conjugated trihydroxy C_{27} bile acid; G: *m*/*z* 572, taurine-conjugated tetrahydroxy C_{27} bile acid; and H: *m*/*z* 407, unconjugated C_{24} bile acid.

unconjugated trihydroxy C_{24} bile acid). As shown in Fig. 6, immature female eel bile contained a single large peak B, which, after sexual maturation, was much reduced and replaced by two peaks C and E in mature eel bile. A similar structural change also occurred in male eel bile, with the immature forms having a series of large peaks A, B, D, F and the adult forms having a lower proportion of A and B, the loss of D and F, and a pair of large new peaks C and E. Goldfish bile is characterized by a single large peak C. Although goldfish and the mature eel bile shared a superficial resemblance based on bile alcohol molecular mass, the identities of their pentahydroxy C_{27} bile alcohol sulphates were quite different. Tilapia bile was different from all of the above mentioned forms in having a single large peak H. Bile of the three marine species (seabream, sole and flounder) contained mainly C_{24} bile acids taurochenodeoxycholic acid and taurocholic acid (Velez et al., 2009) (data not shown).

Olfactory sensitivity to known bile acids

Olfactory responses of the three species to known bile salts at 10^{-5} mol l^{-1} were in general lower than those generated to bile diluted to 1:10^6 (compare Figs 1–3 with Fig. 7). However, EOG amplitudes in response to all bile salts were strongly concentration dependent, allowing an estimation of thresholds of detection (Table 1). The differences between concentration–response curves to bile acids were significant in all three species and some compounds having more olfactory potency than others. In all three species, the sulphated C_{27} bile alcohols generated large responses; similar responses were evoked by taurochenodeoxycholic acid in the goldfish and tilapia. However, all five bile salts evoked robust responses, whether or not a given bile salt was present in that species' bile. Unconjugated forms of each bile salt were consistently less potent than the respective conjugated form (data not shown).

The thresholds of detection of bile salts were in general lower in eel, followed by goldfish and tilapia (Table 1). The range of thresholds of such compounds in the eel extended almost three orders of magnitude (less than 0.1 nmol l^{-1} to over 1 nmol l^{-1}), whereas in the other two species the values are similar (between $10^{-7.5}$ and $10^{-8.5}$ mol l^{-1}).

DISCUSSION

Difference in olfactory potency of bile fluid

The present study demonstrates that fish have a high olfactory sensitivity to both conspecific and heterospecific bile. Olfactory

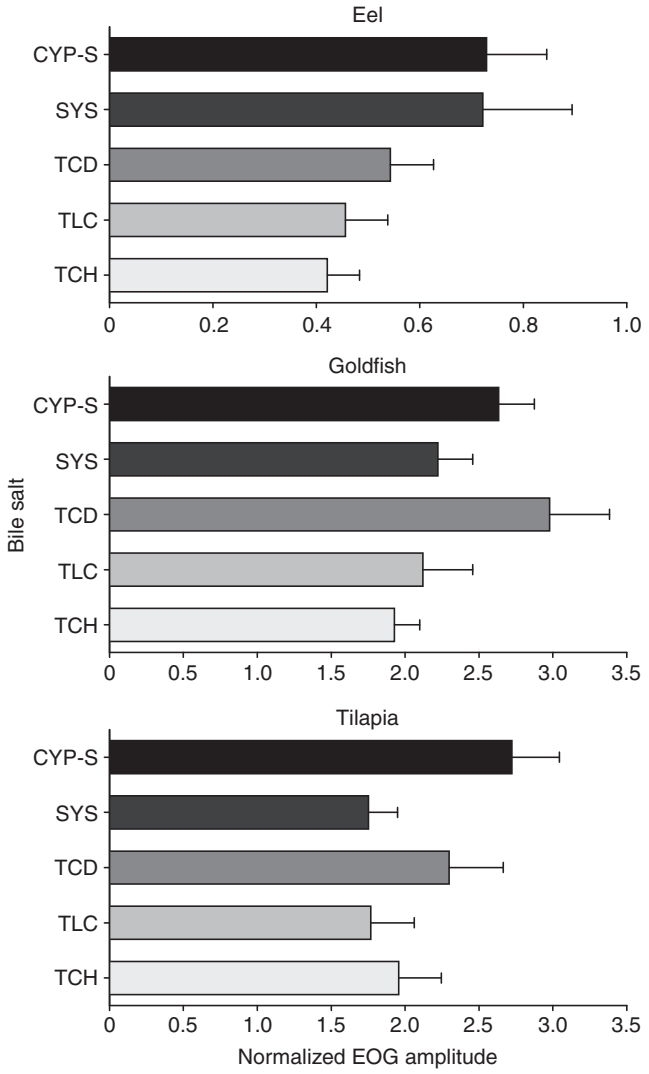

Fig. 7. Normalized EOG responses to five bile salts (10^{-5} mol l^{-1}) tentatively identified as being major constituents of bile fluid from eels, goldfish and tilapia (mean ± s.e.m., N=6 or 7). Note that responses from eels were normalized to those of 10^{-3} mol l^{-1} L-asparagine, whereas those from goldfish and tilapia were normalized to those of 10^{-5} mol l^{-1} L-serine. Thus normalized amplitudes of goldfish and tilapia are directly comparable with each other but not with those of eels.

sensitivity to conspecific bile fluid is consistent with previous findings in the case of the eel (Huertas et al., 2007) and tilapia (Frade et al., 2002; Miranda et al., 2005). The fact that fish could detect bile fluid from different species suggests a potential role for bile salts in interactions between species, including prey detection and/or escape from predators (Rosenthal and Lobel, 2006). However, to test this further, the identity and release rates of those bile salts actually released to the environment must be established. Furthermore, whether some areas of the olfactory epithelia are more sensitive to different bile salts than others was not addressed in the present study. It is possible that the olfactory epithelium shows some degree of functional organization (Hansen et al., 2004), as has been clearly shown in the olfactory bulb (e.g. Fuss and Korsching, 2001). If so, this may mean that the sensitivity to some bile salts may be underestimated in the present study. Nevertheless, the observation that each of the three species is able to detect heterospecific bile salts, as well as conspecifics bile salts, remains valid.

Table 1. Thresholds of detection of bile acids in eel, goldfish and tilapia

Bile acid	Eel	Goldfish	Tilapia
CYP-S	$-8.6^{b,3}$	$-8.2^{a,2}$	$-7.7^{c,1}$
SYS	$-8.4^{a,3}$	$-8.2^{a,2}$	$-7.1^{a,1}$
TCD	$-9.8^{c,3}$	$-8.5^{b,2}$	$-7.2^{a,b,1}$
TLC	$-10.2^{d,3}$	$-9.0^{c,2}$	$-8.4^{d,1}$
TCH	$-8.6^{b,2}$	$-8.5^{b,2}$	$-7.5^{b,c,1}$

CYP-S, 5-cyprinol sulphate; SYS, 5-scymnol sulphate; TCD, taurochenodeoxycholic acid; TLC, taurolithocholic acid; TCH, taurocholic acid.

Concentrations are in mol l^{-1}, expressed as log10. Values with different letters indicate statistically significant differences within a given species, whereas the numbers indicate significant differences among the three species for a given bile salt (ANOVA, P<0.05).

Putative biological roles of olfactory sensitivity to bile acids between sexes

Our finding that the odour of bile is different between the sexes and the stage of maturation in the eel suggests a role for olfaction in reproduction. A role for pheromonal communication in gonadal maturation has been proposed for the eel (Huertas et al., 2008; Huertas et al., 2006). Significantly, eels seem to have a markedly higher olfactory sensitivity to bile salts than either goldfish or tilapia. Therefore bile salts are good candidates for sex pheromones in this species, as has been previously shown in the sea lamprey (Li et al., 2002; Sorensen et al., 2005). Differences in the composition of the bile fluid between the sexes are also apparent in both the goldfish and tilapia, although they are much less marked than in the eel. Pheromonal communication is known to be important for the goldfish (Kobayashi et al., 2002; Sorensen and Stacey, 2004; Stacey and Sorensen, 2002) and recent work has suggested that it is important in the tilapia (Barata et al., 2007; Miranda et al., 2005). Could bile salts act as pheromones in these species too? Most work has centred on the role of steroids and prostaglandins and their metabolites in goldfish, while work with the tilapia has strongly implicated urine as a vehicle for pheromone release (Almeida et al., 2005; Appelt and Sorensen, 2007; Barata et al., 2008). Both species normally live in freshwater and, as part of their osmoregulatory process, constantly produce large volumes of urine. This may mean that urine predominates as the mechanism for release of pheromones in freshwater fish, whereas intestinal fluid is more important in marine fish (Hubbard et al., 2003a) whereas the gills may also act as a route of release for non-conjugated odorants (Vermeirssen and Scott, 1996). To address this question, the routes and rates of release of olfactory-potent bile compounds needs to be investigated in each species as well as to establish whether males and females release different odorants. There does not always seem to be a perfect match between those bile salts released by a given species and their olfactory potency (Velez et al., 2009; Zhang et al., 2001).

The different bile composition between males and females could be related to their different diets. In the case of the eel [a fish with a clearly different growth strategy between the sexes as females can be 20 times bigger than the males (Davey and Jellyman, 2005)] males have a preference for invertebrates and small fish whereas females prefer bigger fish (Jellyman, 2001). However, during their migration from the rivers and lakes of Europe and North Africa to their spawning grounds in the Sargasso Sea, eels do not feed (Tesch, 2003; van Ginneken et al., 2005). Therefore, changes in the bile salt components of the bile are unlikely to be due to changes in diet and are probably related to sexual maturation.

Bile salts in an evolutionary context

The evolutionary history of bile salts is characterized by a process of progressive oxidation and carbon loss, from sulphated C_{27} bile alcohols to taurine-conjugated C_{24} bile acids (Haslewood, 1967; Hofmann and Hagey, 2008). However, the immature eel is quite unusual, in that its immature form uses taurine-conjugated C_{24} bile acids, but at sexual maturation, it goes through an apparent regression back to C_{27} bile alcohol forms, a process hitherto unseen in any vertebrate group. It is possible to speculate that the evolutionary push behind this change stems from the favourable properties of a C_{27} bile alcohol sulphate as a water soluble odorant. C_{27} bile alcohols are more resistant to microbiological degradation (leading to a longer-lasting signal), are metabolically cheaper to synthesize in quantity (for continuous release) and their conjugate – sulphate – is widely available in seawater (as compared with the non-structural amino acid taurine). In seawater, eels drink as part of their osmoregulatory adaptation to a hyperosmotic environment (Takei et al., 1979), as do marine teleosts in general (Marshall and Grosell, 2006). As a consequence, there is a constant excretion of a watery fluid from the rectum of marine fish (Wilson et al., 2002), even if they do not feed.

Bile acids are not present in invertebrates (Haslewood, 1967). The early evolutionarily divergence of the ancestors of sea lamprey and, later, the eel from the vertebrate lineage, and the observation that all species of fish examined responded to bile salts, suggests that their role as chemical messengers, as well as digestive surfactants, arose very early on in vertebrate evolution. Clearly, the receptor mechanisms for bile salts would also have to have been present; the specificity of such receptors is another facet that needs investigation (Zhang and Hara, 2009).

Olfactory sensitivity to known bile acids

In general, the sulphated C_{27} bile alcohols, 5β-scymnol sulphate and 5α-cyprinol sulphate are equipotent with, or more potent than, the taurine-conjugated C_{24} bile acids, irrespective of whether these compounds are produced by a given species or not. Furthermore it is remarkable that the only C_{24} forms found in large amounts in fish, taurocholic acid and taurochenodeoxycholic acid, evoked the largest responses of the C_{24} bile acids. In general the high sensitivity to C_{27} bile alcohols and the pair of taurine-conjugated C_{24} bile acids is consistent with fish having evolved an olfactory sensitivity to detect bile released by other fish. It is conceivable that olfactory detection of bile acids released by predatory species in conjunction with conspecific bile salts (and possibly other chemical cues), *via* their faeces, may be the mechanism whereby some prey are able to 'chemically label' predators actively preying on that species (Mathis and Smith, 1993).

In summary, fish have a broad range of sensitivity both to bile fluid and to many different bile salts. It appears that bile acids are conveying a chemical message involved in both intra- and inter-species interactions. Based on their ability to alter their bile acid composition, some species of teleosts – notably the eel – may use bile salts as sex pheromones. Many fish live in a low visibility three-dimensional world. It is highly possible that each individual fish is a point source of a bile gradient, which serves as a proximity marker to indicate their location with the same clarity as does a burst of emitted light.

ACKNOWLEDGEMENTS

The authors are grateful to Mr Jon Banks of the School of Biological Sciences, University of Manchester, UK, for collecting flounder bile fluid. This work was supported by Fundação papa a Ciência e a Tecnologia (Portugal) grants POCTI/BSE/38815/2001 and POCTI/BIA-BDE/55463/2004 (funding by the European Union Fonds Social Européen de Dévelopement Régional and the Ministério da Ciência e do Ensino Superior). M.H. was a recipient of a fellowship from the Instituto Nacional de Investigación y Tecnologia Agraria y Alimentaria (Spain).

REFERENCES

Agulleiro, M. J., Anguis, V., Cañavate, J. P., Martínez-Rodríguez, G., Mylonas, C. C. and Cerdà, J. (2006). Induction of spawning of captive-reared Senegal sole (*Solea senegalensis*) using different administration methods for gonadotropin-releasing hormone agonist. *Aquaculture* **257**, 511-524.

Almeida, O. G., Miranda, A., Frade, P., Hubbard, P. C., Barata, E. N. and Canário, A. V. M. (2005). Urine as a social signal in the Mozambique tilapia (*Oreochromis mossambicus*). *Chem. Senses* **30**, i309-i310.

Appelt, C. W. and Sorensen, P. W. (2007). Female goldfish signal spawning readiness by altering when and where they release a urinary pheromone. *Anim. Behav.* **74**, 1329-1338.

Barata, E. N., Hubbard, P. C., Almeida, O. G., Miranda, A. and Canário, A. V. M. (2007). Male urine signals social rank in Mozambique tilapia (*Oreochromis mossambicus*, Peters 1852). *BCM Biol.* **5**, 54.

Barata, E. N., Fine, J. M., Hubbard, P. C., Almeida, O. G., Frade, P., Sorensen, P. W. and Canário, A. V. M. (2008). A sterol-like odorant in the urine of Mozambique tilapia males likely signals social dominance to females. *J. Chem. Ecol.* **34**, 438-449.

Chatman, K., Hollenbeck, T., Hagey, L., Vallee, M., Purdy, R., Weiss, F. and Siuzdak, G. (1999). Nanoelectrospray mass spectroscopy and precursor ion monitoring for quantitative steroid analysis and attomole sensitivity. *Anal. Chem.* **71**, 2358-2363.

Davey, A. J. H. and Jellyman, D. J. (2005). Sex determination in freshater eels and management options for manipulation of sex. *Rev. Fish Biol. Fish.* **15**, 37-52.

Døving, K. B., Selset, R. and Thommesen, G. (1980). Olfactory sensitivity to bile acids in salmonid fishes. *Acta Physiol. Scand.* **108**, 123-131.

Frade, P., Hubbard, P. C., Barata, E. N. and Canário, A. V. M. (2002). Olfactory sensitivity of the Mozambique tilapia to conspecific odours. *J. Fish Biol.* **61**, 1239-1254.

Fuss, S. H. and Korsching, S. I. (2001). Odorant feature detection: activity mapping of structure response relationships in the zebrafish olfactory bulb. *J. Neurosci.* **21**, 8396-8407.

Goto, T., Holzinger, F., Hagey, L. R., Cerrè, C., Ton-Nu, H.-T., Schteingart, C. D., Steinbach, J. H., Shneider, B. L. and Hofmann, A. F. (2003). Physicochemical and physiological properties of 5α-cyprinol sulfate, the toxic bile salt of cyprinid fish. *J. Lipid Res.* **44**, 1643-1651.

Hansen, A., Anderson, K. T. and Finger, T. E. (2004). Differential distribution of olfactory receptor neurons in goldfish: Structural and molecular correlates. *J. Comp. Neurol.* **477**, 347-359.

Haslewood, G. A. D. (1967). Bile salt evolution. *J. Lipid Res.* **8**, 535-550.

Hofmann, A. F. (1999). Bile acids: the good, the bad, and the ugly. *News Physiol. Sci.* **14**, 24-29.

Hofmann, A. F. and Hagey, L. R. (2008). Bile acids: Chemistry, pathochemistry, biology, pathobiology, and therapeutics. *Cell. Mol. Life Sci.* **65**, 2461-2483.

Hubbard, P. C., Barata, E. N. and Canário, A. V. M. (2002). Possible disruption of pheromonal communication by humic acid in the goldfish, *Carassius auratus. Aquat. Toxicol.* **60**, 169-183.

Hubbard, P. C., Barata, E. N. and Canário, A. V. M. (2003a). Olfactory sensitivity of the gilthead seabream (*Sparus auratus* L.) to conspecific body fluids. *J. Chem. Ecol.* **29**, 2481-2498.

Hubbard, P. C., Barata, E. N. and Canário, A. V. M. (2003b). Olfactory sensitivity to catecholamines and their metabolites in the goldfish. *Chem. Sens.* **28**, 207-218.

Huertas, M., Scott, A. P., Hubbard, P. C., Canário, A. V. M. and Cerdà, J. (2006). Sexually mature European eels (*Anguilla anguilla* L.) stimulate gonadal development of neighbouring males: Possible involvement of chemical communication. *Gen. Comp. Endocrinol.* **147**, 304-313.

Huertas, M., Hubbard, P. C., Canário, A. M. and Cerdà, J. (2007). Olfactory sensitivity to conspecific bile fluid and skin mucus in the European eel *Anguilla anguilla* (L). *J. Fish Biol.* **70**, 1907-1920.

Huertas, M., Canário, A. V. M. and Hubbard, P. C. (2008). Chemical communication in the genus *Anguilla*: a minireview. *Behaviour* **145**, 1389-1407.

Jellyman, D. J. (2001). The influence of growth rate on the size of migrating female eels in Lake Ellesmere, New Zealand. *J. Fish Biol.* **58**, 725-736.

Kobayashi, M., Sorensen, P. W. and Stacey, N. E. (2002). Hormonal and pheromonal control of spawning behavior in the goldfish. *Fish Physiol. Biochem.* **26**, 71-84.

Kristensen, E. A. and Closs, G. P. (2004). Anti-predator response of naïve and experienced common bully to chemical alarm cues. *J. Fish Biol.* **64**, 643-652.

Li, W., Scott, A. P., Siefkes, M. J., Yan, H., Liu, Q., Yun, S.-S. and Cage, D. A. (2002). Bile acid secreted by male sea lamprey that acts as a sex pheromone. *Science* **296**, 139-141.

Marshall, W. S. and Grosell, M. (2006). Ion transport, osmoregulation, and acid-base balance. In *Physiology of Fishes* (ed. D. H. Evans and J. B. Claiborne), pp. 177-230. Boca Raton: CRC Press.

Mathis, A. and Smith, R. J. F. (1993). Fathead minnows, *Pimephales promales*, learn to recognize northern pike, *Esox lucius*, as predators on the basis of chemical stimuli from minnows in the pike's diet. *Anim. Behav.* **46**, 645-656.

Miranda, A., Almeida, O. G., Hubbard, P. C., Barata, E. N. and Canário, A. V. M. (2005). Olfactory discrimination of female reproductive status by male tilapia (*Oreochromis mossambicus*). *J. Exp. Biol.* **208**, 2037-2043.

Moschetta, A., Xu, F., Hagey, L., van Berge, R., Henegouwen, G. P., van Erpecum, K. J., Brouwers, J. F., Cohen, J. C., Bierman, M., Hobbs, H. et al. (2005). A phylogenetic survey of biliary lipids in vertebrates. *J. Lipid Res.* **46**, 2221-2232.

Polkinghorne, C. N., Olson, J. M., Gallaher, D. G. and Sorensen, P. W. (2001). Larval sea lamprey release two unique bile acids to the water at a rate sufficient to produce detectable riverine pheromonal plumes. *Fish Physiol. Biochem.* **24**, 15-30.

Rosenthal, G. G. and Lobel, P. S. (2006). Communication. In *Behaviour and Physiology of Fish*, vol. 24 (ed. K. A. Sloman, R. W. Wilson and S. Balshine), pp. 39-78. San Diego: Elsevier Inc.
Rossi, S. S., Converse, J. L. and Hofmann, A. L. (1987). High pressure liquid chromatographic analysis of conjugated bile acids in human bile: simultaneous resolution of sulfated and unsulfated lithocholyl amidates and the common conjugated bile acids. *J. Lipid Res.* **28**, 589-595.
Sasaki, T. (1966). Stero-bile acids and bile alcohols LXXXII. Comparative studies on the bile salts of fishes by thin layer chromatography. *J. Biochem.* **50**, 56-62.
Scott, J. W. and Scott-Johnson, P. E. (2002). The electoolfactogram: a review of its history and uses. *Microsc. Res. Tech.* **58**, 152-160.
Siefkes, M. J. and Li, W. (2004). Electrophysiological evidence for detection and discrimination of pheromonal bile acids by the olfactory epithelium of female sea lampreys (*Petromyzon marinus*). *J. Comp. Physiol. A* **190**, 193-199.
Sorensen, P. W. and Stacey, N. E. (2004). Brief review of fish pheromones and discussion of their possible uses in the control of non-indigenous teleost fishes. *N. Z. J. Mar. Freshwater Res.* **38**, 399-417.
Sorensen, P. W., Fine, J. M., Dvornikovs, V., Jeffrey, C. S., Shao, F., Wang, J., Vrieze, L. A., Anderson, K. R. and Hoye, T. R. (2005). Mixture of new sulfated steroids functions as a migratory pheromone in the sea lamprey. *Nat. Chem. Biol.* **1**, 324-328.
Stacey, N. and Sorensen, P. (2002). Hormonal pheromones in fish. In *Non-Mammalian Hormone-Behavior Systems*, vol. 2 (ed. D. W. Pfaff, A. P. Arnold, A. M. Etgen, S. E. Fahrbach and R. T. Rubin), pp. 375-434. London: Harcourt Publishers Ltd./Academic Press.
Suzuki, N., Takahata, M., Shoji, T. and Suzuki, Y. (2004). Characterization of electro-olfactogram oscillations and their computational reconstruction. *Chem. Sens.* **29**, 411-424.
Takei, Y., Hirano, T. and Kobayashi, H. (1979). Angiotensin and water intake in the Japanese eel, *Anguilla japonica*. *Gen. Comp. Endocrinol.* **38**, 466-475.
Tesch, F.-W. (2003). *The Eel.* Oxford: Blackwell Science Ltd.
van Ginneken, V., Antonissen, E., Müller, U. K., Booms, R., Eding, E., Verreth, J. and van den Thillart, G. (2005). Eel migration to the Sargasso: remarkably high swimming efficiency and low energy costs. *J. Exp. Biol.* **208**, 1329-1335.
Velez, Z., Hubbard, P. C., Welham, K., Hardege, J. D., Barata, E. N. and Canário, A. V. M. (2009). Identification, release and olfactory detection of bile acids in the intestinal fluid of the Senegalese sole (*Solea senegalensis*). *J. Comp. Physiol. A* **195**, 691-698.
Vermeirssen, E. L. M. and Scott, A. P. (1996). Excretion of free and conjugated steroids in rainbow trout (*Onchorhynchus mykiss*): Evidence for branchial excretion of the maturation-inducing steroid, 17,20β-dihydroxy-4-pregnen-3-one. *Gen. Comp. Endocrinol.* **101**, 180-194.
Wilson, R. W., Wilson, J. M. and Grosell, M. (2002). Intestinal bicarbonate secretion by marine teleost fish – why and how? *Biochim. Biophys. Acta* **1566**, 182-193.
Zar, J. H. (1996). *Biostatistical Analysis*. New Jersey: Pearson Higher Education.
Zhang, C. and Hara, T. J. (2009). Lake char (*Salvelinus namaycush*) olfactory neurons are highly sensitive and specific to bile acids. *J. Comp. Physiol. A* **195**, 203-215.
Zhang, C., Brown, S. B. and Hara, T. J. (2001). Biochemical and physiological evidence that bile acids produced and released by lake char (*Salvelinus namaycush*) function as chemical signals. *J. Comp. Physiol. B* **171**, 161-171.