

RESEARCH ARTICLE

Odorant tuning of olfactory crypt cells from juvenile and adult rainbow trout

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SUMMARY

Teleost fish lack independent olfactory organs for odorant and pheromone detection. Instead, they have a single sensory epithelium with two populations of receptor neurons, ciliated and microvillous, that are conserved among vertebrates, and a unique receptor cell type named the olfactory crypt cell. Crypt cells were shown to be chemosensory neurons that project to specific areas in the olfactory bulb, but their odorant tuning and overall function remain unclear. Reproduction in fish is generally synchronized by sex pheromonal signaling between males and females, but the sensors responsible for pheromone detection remain unknown. In crucian carp, a seasonal variation in the population of olfactory crypt cells and their brain projections pathways, involved in reproduction, led to the hypothesis of a role as sex pheromone detectors. In the present study, morphology and localization of olfactory crypt cells were compared between juvenile and mature rainbow trout of both sexes, and calcium imaging was used to visualize responses of crypt cells from the three groups to common social and food-related odorants, sex hormones and conspecific tissue extracts. Crypt cells from mature trout were found to be larger than those of juvenile specimens, and preferentially localized to the apical surface of the olfactory epithelium. Although a fraction of crypt cells of all groups responded to common odorants such as amino acids and bile salts, cells from mature trout showed a characteristic preference for gonadal extracts and hormones from the opposite sex. These results support an involvement of olfactory crypt cells in reproduction-related olfactory signaling in fishes.

Supplementary material available online at <http://jeb.biologists.org/cgi/content/full/215/10/1740/DC1>

Key words: olfaction, teleost, pheromone, salmonid, chemical sense.

INTRODUCTION

The olfactory crypt cell was first identified and named in an ultrastructural study of teleost fishes (Hansen et al., 1997). Crypt cells were found in several common fishes, including zebrafish, catfish and goldfish, but they are apparently absent from other species, including two types of lungfishes (Hansen and Finger, 2000; Hansen et al., 1999). Their presence in salmonids was first demonstrated by Sandahl and co-workers (Sandahl et al., 2006). Subsequent immunohistochemical studies reported the presence of crypt-cell-like cells in the olfactory epithelium of cartilaginous fish, the elasmobranch *Scyliorhinus canicula* and the skate *Raja clavata* (Ferrando et al., 2006; Ferrando et al., 2007; Ferrando et al., 2010), suggesting that the evolutionary emergence of olfactory crypt cells represents an ancient feature in vertebrate development. Interestingly, thus far crypt cells have been found solely in fishes, as opposed to amphibians or other aquatic animals, suggesting that they are an exclusive attribute of this large and diverse animal group.

Olfactory crypt cells are oval to egg-shaped neurons that are completely surrounded by one or two supporting cells. The association of the crypt cell with its supporting cell(s) is sufficiently strong to withstand mechanical tissue dissociation, and the two cell types are physiologically coupled by gap junctions (Schmachtenberg, 2006). An apical invagination of up to 5 µm depth is the principal characteristic of the neuron. This invagination is bordered by microvilli from the crypt cell and its supporting cell(s), and usually contains both short cilia and microvilli in its inner part. Crypt cells are dispersed in the olfactory epithelium, and are rare

compared with ciliated and microvillous olfactory receptor neurons (Hansen and Finger, 2000; Schmachtenberg, 2006).

To date, little is known about the molecular properties of crypt cells. Immunohistochemical studies in different species suggest that olfactory crypt cells express the G-proteins $G\alpha_o$ and $G\alpha_q$ as well as adenylate cyclase type-III and the glial marker protein S-100, but these proteins may not be expressed in all crypt cells, and are not exclusive markers of this receptor neuron type (Belanger et al., 2003; Germana et al., 2004; Hansen et al., 2004; Hansen et al., 2003; Vielma et al., 2008). An interesting recent study in zebrafish showed that nearly all crypt cells express *ora4*, a single member of the V1R receptor-like *ora* genes, and the G-protein G_{i1b} (Oka et al., 2012; see also Saraiva and Korsching, 2007). However, the physiological implications of this intriguing finding remain unclear.

Crypt cells were shown to project their thin unmyelinated axons to small, restricted patches of the ventral olfactory bulb in catfish (Hansen et al., 2003) and crucian carp (Hamdani and Døving, 2006). The latter study also reported that second-order olfactory bulb neurons, which make synaptic contacts with crypt cells, project to the olfactory cortex primarily through the lateral bundle of the medial olfactory tract, which conveys olfactory information related to reproduction in crucian carp (Hamdani and Døving, 2006; Hamdani and Døving, 2007; Weltzien et al., 2003). Similarly, the medial olfactory tract of male goldfish responds to sex hormones released by females of this species, coordinating spawning between both sexes (Sorensen et al., 1991). Further studies in crucian carp reported a striking dependence of crypt cell density and apical localization

within the olfactory epithelium on the time of year, reaching a peak during the summer spawning season (Hamdani et al., 2008). Together, these antecedents led the authors to propose the hypothesis that crypt cells function as sex pheromone detectors in the olfactory epithelium of fishes.

The importance of pheromonal signaling for the reproduction of fishes has been established for the goldfish, *Carassius auratus*. In this species, hormonally derived sex pheromones precisely coordinate mating, which emerges as a generalized mechanism in the reproductive behavior of fishes (Scott and Sorensen, 1994; Sorensen et al., 1988; Sorensen et al., 1998; Sorensen et al., 2005; Sorensen and Scott, 1994). To establish olfactory crypt cells as pheromone receptors in fishes, it is necessary to demonstrate that they respond specifically to pheromonal compounds at physiological concentrations. Using the patch clamp technique and calcium imaging, we previously showed that crypt cells isolated from the Chilean jack mackerel (*Trachurus symmetricus murphyi*) respond to a blend of amino acids, but not to bile salts or the goldfish pheromones prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one ($17,20P$) (Vielma et al., 2008), providing the first direct evidence for their chemosensory function. However, the comparatively high concentrations of amino acids necessary to evoke responses in that study suggest that these compounds may not be the natural ligands of crypt cells in the jack mackerel. We therefore set out to test a variety of synthetic odorants and natural odor extracts, including putative fish pheromones, to elucidate the chemosensory tuning of olfactory crypt cells. For the present study, we used juvenile and sexually mature rainbow trout, *Oncorhynchus mykiss*, to isolate crypt cells from the olfactory epithelium and test their odorant responsiveness with the calcium imaging technique. Our data provide direct physiological support for the hypothesis that olfactory crypt cells are involved in the signaling of reproduction-related odorants in sexually mature trout.

MATERIALS AND METHODS

Animals

Juvenile rainbow trout, *Oncorhynchus mykiss* Walbaum 1792, of 10–12 cm total length, were obtained from the Rio Blanco hatchery in Los Andes (<http://www.pisciculturarioblanco.ucv.cl>) and kept in a dedicated fish facility on the University of Valparaíso campus for up to 3 mo in 1001 tanks with filtered and aerated recirculating water of 16–18°C. Sexually mature male and female trout, with running milt and roe, of 25–30 cm total length were separated at the hatchery and kept on campus in a 2501 tank in filtered and aerated recirculating water at 6–8°C. The experimental procedures were approved by the Bioethics Committee of the University of Valparaíso and are in accordance with the bioethics regulation of the Chilean Research Council (CONICYT). Reagents were purchased from Sigma-Aldrich (Sigma-Aldrich Química Limitada, Santiago, Chile) unless otherwise stated.

Histochemistry and immunochemistry

Trout were killed by decapitation on ice; their olfactory organs were removed and stored in 4% paraformaldehyde, pH 7.4 at 4°C overnight. The tissue was cryoprotected for 24 h in 30% sucrose in phosphate-buffered saline (PBS) and mounted in tissue freezing medium (Tissue-Tek OCT Compound, Sakura Finetek, Torrance, CA, USA). Sections (20 µm) were cut in a cryostat (CM 1900, Leica, Wetzlar, Germany) at –18°C and glued onto poly-L-lysine-coated microscope slides. After washing in PBS, the sections were stained in 0.5% Toluidine Blue for 5 min and mounted in glycerol gelatin.

For western blotting, brain and liver tissue of one adult trout and rat were homogenized in ice-cold non-denaturing lysis buffer containing 1% Triton X-100, 50 mmol⁻¹ Tris-HCl (pH 7.4), 300 mmol⁻¹ NaCl, 5 mmol⁻¹ EDTA and a protease and phosphatase inhibitor cocktail (P8340 and P5726, Sigma-Aldrich). The homogenates were centrifuged at 15,000 g for 15 min at 4°C. The supernatant was collected and the total protein contents were determined with a commercial kit (Qubit Protein Assay Kit, Q33211 and Qubit 2.0 Fluorometer, Q32866, Invitrogen, Carlsbad, CA, USA). The samples were diluted in Laemmli buffer (40 mmol⁻¹ Tris pH 6.8, 7% glycerol, 2.5% sodium dodecyl sulfate, 0.0025% Bromophenol Blue) and denatured by heating to 95°C for 5 min. The wells of a 12% acrylamide-bisacrylamide gel were loaded with 50 and 100 µg of protein together with a molecular weight marker (Kaleidoscope, Bio-Rad, Hercules, CA, USA). Proteins were then transferred to a polyvinylidene difluoride membrane (Amersham Biosciences, Buckinghamshire, UK), visualized by Ponceau Red and blocked in 1% bovine serum albumin (BSA) (BM-0150, Winkler Ltda, Santiago, Chile) with 0.1% Tween-20 in Tris-buffer for 1 h at room temperature. The membrane was incubated at 4°C overnight in the primary antibody, polyclonal rabbit anti-G α_o (sc 387, Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1:500 in blocking solution, followed by incubation in a secondary horseradish peroxidase-coupled anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:2000. Bound immunoglobulins were detected with luminol and H₂O₂ (Supersignal West Pico Chemiluminescent Substrate, Pierce Protein Research Products, Thermo Scientific, Rockford, IL, USA) and photographed in a transilluminator (EpiChemi 3 Darkroom, UVP BioImaging Systems, CA, USA).

For immunohistochemistry, olfactory rosettes were fixed in 4% paraformaldehyde for 1 h at room temperature, cryoprotected and sectioned in a cryostat as described above. After rehydration and washing, the tissue was blocked in 1% BSA (Winkler), 1% non-fat milk, 1% horse serum and 0.3% Triton X-100 (Bio-Rad) for 1 h. The primary rabbit anti-G α_o antiserum was applied at a dilution of 1:250 in blocking solution overnight at 4°C. After three washes, the preparation was incubated in the secondary donkey anti-rabbit Cy-3 conjugated IgG (Jackson ImmunoResearch, diluted 1:400) for 1 h at room temperature, followed by application of DAPI (5 µg ml⁻¹) for 10 min. Finally, the sections were mounted in Fluomount (Dako Industries, Carpinteria, CA, USA). Photographs were taken with a CoolSnap digital camera (Roper Scientific, Ottobrunn, Germany) on a BX-51 microscope (Olympus, Tokyo, Japan) operated by Image-Pro Express software (Media Cybernetics, Silver Spring, MD, USA).

Calcium imaging

After decapitation, the olfactory organs were removed and transferred to cold Ringer's solution, containing (mol l⁻¹): 100 NaCl, 3 KCl, 2 CaCl₂, 1 MgCl₂, 5 HEPES, 10 glucose, pH 7.4 adjusted with NaOH. The sensory area of each olfactory lamella was cut into three to four pieces and digested enzymatically for 30 min at room temperature in type IV collagenase (C5138, Sigma-Aldrich), 0.05% in Ringer's solution. To stop the enzymatic reaction, the tissue was cooled to 4°C and washed three times in Ringer's solution. Subsequently, it was incubated in 5 µmol l⁻¹ Fluo 4-AM (F14201, Molecular Probes), 0.04% pluronic acid F-127 (P6867, Molecular Probes) and 2.5 mmol l⁻¹ probenecid at 4°C for 1 h. To dissociate the olfactory epithelium, the tissue was triturated with a fire-polished Pasteur pipette and the dissociated cells were transferred to the recording chamber, previously coated with a 1:1 solution of 0.025%

concanavaline A and 0.01% poly-L-lysine. Cells settled for 20 min at 4°C and were then washed by continuous bath perfusion with Ringer's solution containing 1 mmol l⁻¹ probenecid. Olfactory crypt cells were identified by their characteristic morphology on an Eclipse FN1 microscope (Nikon, Tokyo, Japan) with a 40× water immersion objective (N.A. 0.8) under Nomarski contrast. Their identity was confirmed by Gα_o immunocytochemistry of dissociated cells in a couple of preparations (not shown). A typical field of view at 400× magnification contained hundreds of unidentified cells and several identifiable crypt cells. Images were recorded every 2 s for 120 s with a Sensicam QE digital camera (Cooke Corp., Romulus, MI, USA). Fluorescence images were analyzed with Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA). Relative fluorescence intensities of the region of interest were obtained by dividing all images through the initial (pre-pulse) image of the series. Two different criteria to define calcium signals as significant responses were applied alternatively: either >10% or >20% fluorescence increase in the region of interest compared with pre-pulse levels during 30 s after stimulus application.

Odorants

A mixture of glycine and the L-isomers of the amino acids arginine, asparagine, aspartate, citrulline, cysteine, cystine, glutamate, glutamine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, taurine, threonine, tyrosine and valine was prepared as food odor, to a final summed concentration of 1 mmol l⁻¹ in Ringer's solution. The bile salts/acids glycocholic acid, glycodeoxycholic acid, sodium taurocholate, taurodeoxycholic acid, sodium glycochenodeoxycholate, sodium taurochenodeoxycholate and deoxycholic acid were applied at a final summed concentration of 0.1 mmol l⁻¹ as the social odor mix. Additionally, conspecific natural bile of juvenile trout was used as social odor source. Bile was collected from three juvenile trout, pooled, filtered and stored at -20°C until its use, diluted 1:1000 in Ringer's solution. Skin extract from juvenile trout was applied as a social and/or alarm odor stimulus. It was prepared by pulverizing 1 g of skin in liquid nitrogen followed by resuspension in 3 ml H₂O and centrifugation at 15,000 g for 15 min at 4°C. The supernatant was aliquoted, stored at -20°C and applied at a dilution of 1:250 in Ringer's solution. 17α-Methyltestosterone (dissolved in ethanol and diluted to 10 μmol l⁻¹ in Ringer's solution), testicular extract and seminal fluid (both from male reproductive trout, diluted 1:100 in Ringer's solution) were used as male odorants. Two testicles of a reproductive male trout, 15 g each, were washed in PBS, pulverized in liquid nitrogen and resuspended in 50 ml H₂O. The suspension was centrifuged at 15,000 g for 15 min at 4°C, and the supernatant was aliquoted and stored at -20°C. Seminal fluid was collected from three reproductive male trout, pooled, diluted 10 times in Ringer's solution and centrifuged at 800 g for 20 min at 4°C. The supernatant was aliquoted and stored at -20°C until its use at a final dilution of 1:1000 in Ringer's solution.

4-Androstene-3,17-dione (4-AD), 17,20P (both dissolved in DMSO), 17β-estradiol (EST) and PGF_{2α} were diluted in Ringer's solution to 1 μmol l⁻¹ each. These hormones and conspecific ovarian extract from female reproductive trout (diluted 1:100) were applied as female odorants. Ovarian extract was prepared as the testicular extract, but only one ovary of 25 g was used from a reproductive female trout close to spawning. All odorants applied in the calcium imaging experiments were freshly prepared from stock solutions each day. Stimuli were applied by adding 200 μl of odorant solution to the Ringer's carrier flow perfusing the recording chamber, at t=10 s after the start of the recording. A specially designed open

glass connector (a gift of J. Caprio, Department of Biological Science, Louisiana State University, Baton Rouge, LA, USA), approximately 10 cm of tubing ahead of the recording chamber, was used for odorant addition, effectively eliminating any possibility of mechanical stimulus artifacts. The volume of the recording chamber with immersed objective was 1 ml, and the flow rate was 8 ml min⁻¹. The maximum odorant concentration reaching the cells was measured by photon transmittance during dye perfusion of the recording chamber, and by osmometry after application of 3 mol l⁻¹ KCl, revealing that peak concentrations were reached after 10–20 s, with an average fourfold dilution of the original stimulus solution. Ringer's solution without odorants and the solvents DMSO (0.02%) and ethanol (0.1%) were applied as negative and vehicle controls, and KCl was applied as positive control at a concentration of 50 mmol l⁻¹ in the recording chamber.

Statistical analysis

To compare crypt cell size between juvenile and adult male and female trout, data were analyzed for normality with the Kolmogorov–Smirnov test, followed by Kruskal–Wallis and Dunn's *post hoc* test. One-way ANOVA and Tukey's *post hoc* test were applied to test the statistical significance of olfactory responses to each odorant stimulus compared with the control, and for the analysis of odorant selectivity ($P \leq 0.05$). Statistical calculations were performed in GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). All bar graphs display means ± 1 s.d.

RESULTS

Olfactory crypt cell localization and morphology in the rainbow trout

To confirm the presence of crypt cells in the olfactory epithelium of rainbow trout and to comparatively analyze their morphology and distribution in juvenile and mature specimen, cryosections of the olfactory epithelium were stained with Toluidine Blue or labeled with an antiserum against the G-protein Gα_o, which has been established as a useful marker of olfactory crypt cells in teleosts (Hansen et al., 2003) (Fig. 1A). The antibody appeared to label all crypt cells in the olfactory epithelium, in addition to a subset of microvillous olfactory receptor neurons, which could easily be differentiated by morphological criteria. Specificity of the immunoreaction was supported by western blot, on which the antibody marked a specific band at the expected molecular weight of approximately 40 kDa. Gα_o immunoreactivity identified crypt cells in the olfactory epithelia of juvenile and reproductive male and female trout. However, the density of crypt cells was too low to allow a comparative quantification.

Olfactory receptor neurons are known for their constant renewal and relatively short lifespan (Costanzo and Graziadei, 1983; Hansen et al., 1999; Zeiske et al., 1992). To be functional, receptor neurons are expected to have direct access to the lumen of the nasal cavity, which for crypt cells implies their localization in the apical section of the olfactory epithelium. A previous study reported a seasonal variation in the position of crypt cells in the olfactory epithelium of the crucian carp, correlating their peak surface localization with the reproductive summer season (Hamdani et al., 2008). To relate crypt cell function to sexual maturity, the position of crypt cells within the olfactory epithelium was compared between juvenile and mature male and female specimens (Fig. 1Bi,ii). Although a portion of crypt cells localized to the middle or basal part of the olfactory epithelium in all examined specimens, the percentage of apical crypt cells was significantly higher in sexually mature male (73±4%, $N=65$ cells from three fish) and female (80±7%, $N=45$ cells from three

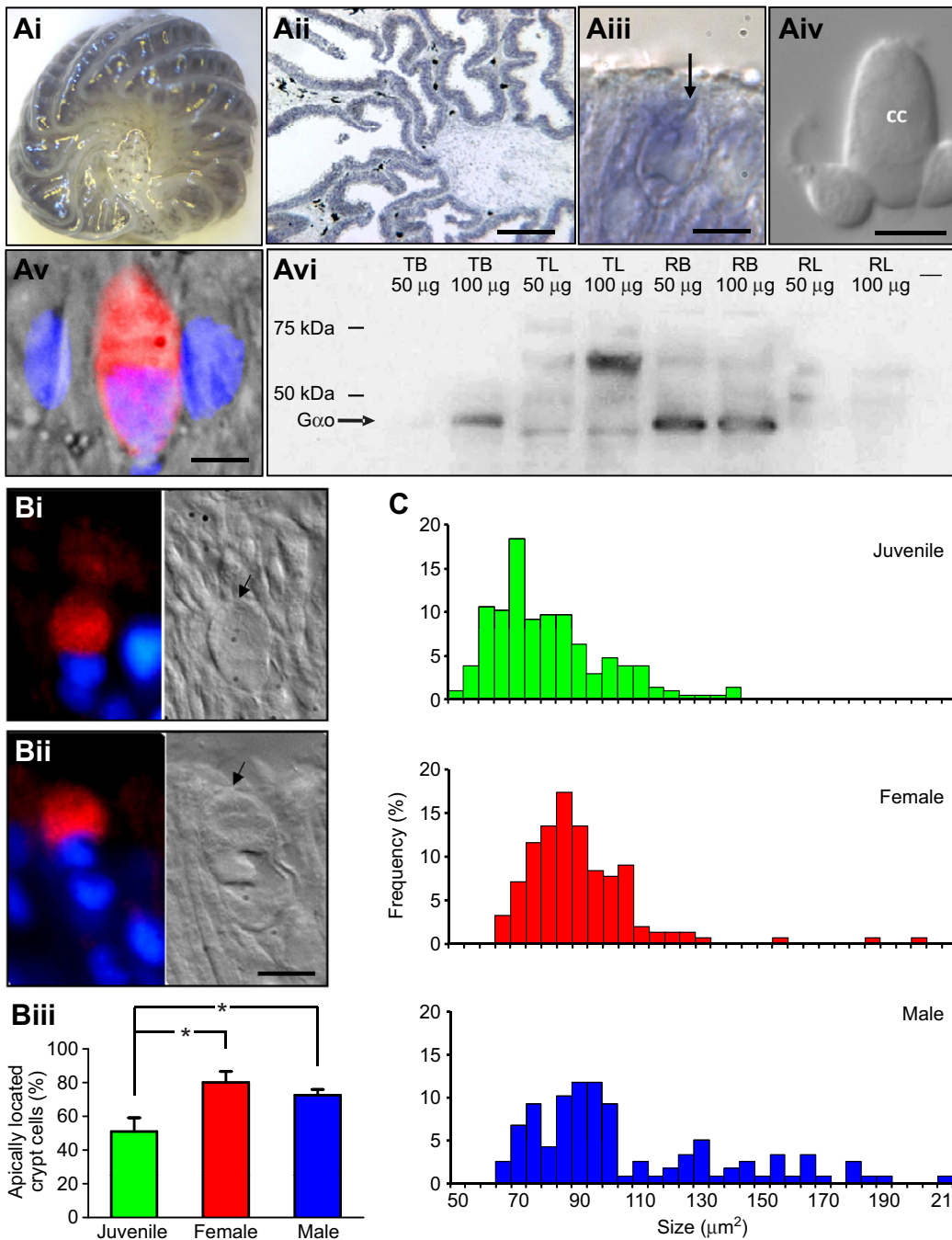


Fig. 1. Olfactory crypt cell identification and morphological characteristics in the rainbow trout, *Oncorhynchus mykiss*. Olfactory rosettes (Ai) were isolated and cryosectioned (Aii) to analyze the localization of crypt cells in the olfactory epithelium (Aiii, arrow), or dissociated for size determination and calcium imaging (Aiv). Crypt cells were G α_o -immunoreactive, which, together with morphological criteria, was used for their identification (Av). The applied antibody, previously characterized in catfish, goldfish and the round goby (Belanger et al., 2003; Hansen et al., 2004; Hansen et al., 2003), marked a specific band around 40 kDa in trout and rat brain tissue (Avi). CC, crypt cell; TB, trout brain; TL, trout liver; RB, rat brain; RL, rat liver. (Bi) Mainly in juvenile trout, crypt cells were regularly found in deeper layers of the olfactory epithelium, without apparent access to the nasal cavity. (Bii) In sexually mature specimens, apical localization of crypt cells was more frequent. Arrows point to crypts. (Biii) Quantitative analysis showed that the difference in the percentage of apically localized crypt cells between immature and mature (male and female) trout was statistically significant (one-way ANOVA, * $P < 0.05$). (C) An analysis of the size of dissociated crypt cells from the three groups revealed that mature male and female trout had their size distribution shifted to the right. Notably, sexually mature male trout expressed some very large crypt cells. Statistical analysis with Kruskal–Wallis and Dunn’s multiple comparison tests revealed a significant size difference between juvenile and mature groups, but not between mature male and female trout. Scale bars, (Aii) 250 µm; (Aiii,iv) 10 µm; (Av,Bi,ii) 5 µm.

fish) fish compared with juveniles ($51 \pm 8\%$, $N=66$ cells from three fish), which established a statistically significant difference between the two age groups (Fig. 1Biii).

Further evidence for a different role of olfactory crypt cells in mature *versus* juvenile trout was obtained from a comparison of their cell size. After dissociation of the olfactory epithelium, crypt cells were photographed and their horizontal dimensions were measured. The resulting size-distribution histograms revealed that the frequency maxima of crypt cells from mature male and female trout are displaced towards larger areas compared with juvenile specimens (Fig. 1C), and the size distribution of the three groups shows a statistically significant difference between juvenile ($N=206$ cells from 10 fish) and mature male ($N=118$ cells from six fish) and female ($N=155$ cells from nine fish) fish, but not between the latter

two groups. These data demonstrate that olfactory crypt cells from sexually mature trout are larger on average, and a higher percentage localizes to the apical olfactory epithelium, gaining direct access to the exterior milieu.

Odorant tuning and response selectivity

Rainbow trout were divided into three groups: juvenile specimens with undetermined sex, mature males and mature females. Freshly isolated olfactory crypt cells from the three groups were loaded with the calcium indicator Fluo-4 AM and stimulated by bath perfusion with common social and food-related odorants, sex hormones and conspecific gonadal extracts. To test the viability of the dissociated crypt cells and the possible occurrence of unspecific responses, the cells were stimulated by KCl, Ringer’s

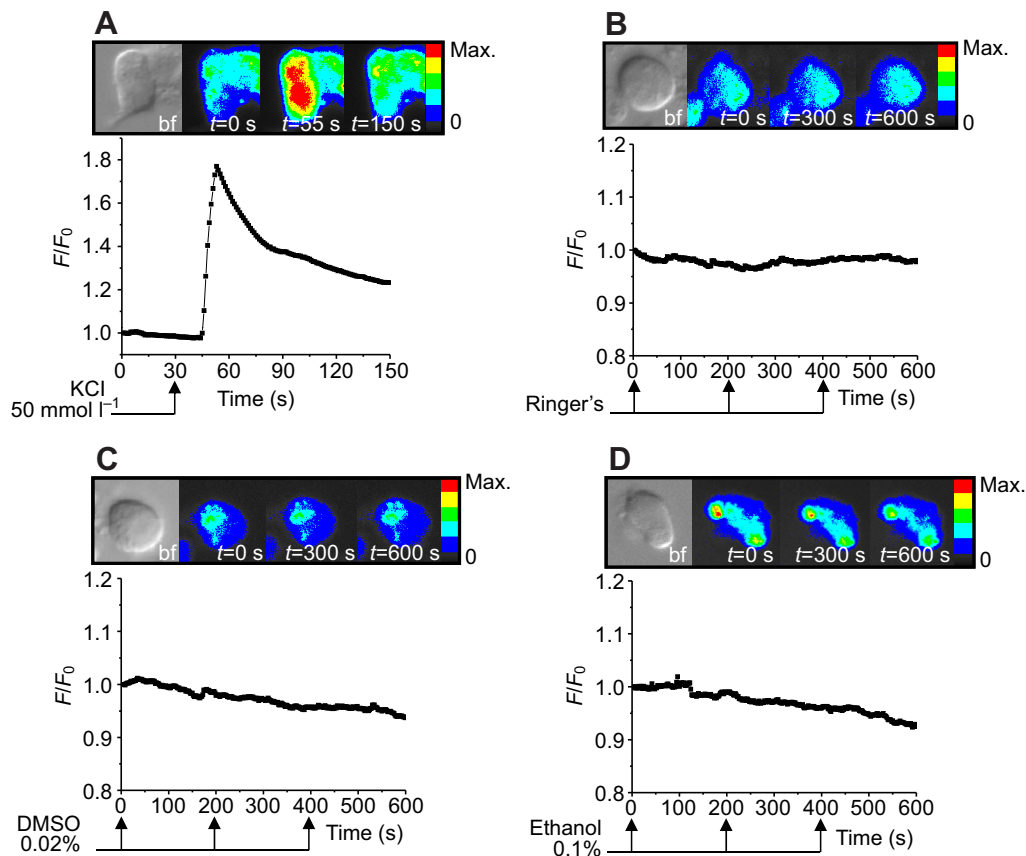


Fig. 2. Controls used in the odorant analysis in rainbow trout. (A) Depolarization of crypt cells with 50 mmol l⁻¹ KCl triggered large and reversible calcium signals (F/F_0), and was used as positive control ($N=20$ cells from three fish). Fluorescence images show calcium signals before, during and after the stimulus. Color scales indicate relative fluorescence intensity. (B–D) Stimulation with pure Ringer's solution ($N=181$ cells from 15 fish) or the solvents DMSO and ethanol ($N=15$ cells from three fish each) at the concentration used for odorant dilution did not elicit significant calcium signals in any tested cell.

solution without odorants, and the solvents DMSO and ethanol. Although KCl perfusion caused strong calcium signals in the vast majority of cells, neither Ringer's solution alone nor DMSO or ethanol triggered significant responses in any tested cell at the concentrations used for odorant dilution (Fig. 2).

Under stimulation with different classes of odorants, crypt cells from juvenile specimens responded to all presented stimuli at a low but statistically significant rate for the 10% criterion of fluorescence increase (supplementary material Table S1), and thus showed no discernable specificity for any class of odorant (Fig. 3A). Calcium signals were rather small in general, which is reflected by the fact that responses to seven out of the 12 stimuli dropped below statistical significance levels for the 20% criterion of fluorescence increase. The situation changed when crypt cells from mature female trout were exposed to the same odorants (Fig. 3B). Responses to the female sex hormones estrogen, PGF_{2 α} and 17,20P were completely absent in this group, whereas females showed significant response rates to testosterone ($N=50$ cells from three fish), testicular extract ($N=66$ cells from four fish) and seminal fluid ($N=54$ cells from three fish). Response frequencies to amino acids and bile salts were also significant for the 10% criterion compared with Ringer's solution as control, and similar to cells from juvenile specimens. Response rates to the remaining odorants were below 5% and not statistically significant (supplementary material Table S2).

Finally, crypt cells from mature male trout presented by far the highest response frequency to female ovarian extract ($N=78$ cells from four fish; Fig. 3C). Also significant for the 10% criterion were response rates to 4-AD ($N=47$ cells from three fish) and 17,20P ($N=47$ cells from three fish), but the remaining odorants did not trigger calcium signals in a significant portion of crypt cells

(supplementary material Table S3). Interestingly, responses to the male sex hormone testosterone were completely absent from the mature male group ($N=33$ cells from three fish). Representative examples of calcium signals and their time courses (right column, Fig. 3) reveal a slow response onset and long time course, which is partly attributed to the slow odorant delivery by bath perfusion, as opposed to the puff pipette stimulation applied in our previous study (Vielma et al., 2008). However, the long time course of the response compared with stimulation with KCl also suggests an intrinsically slow response kinetics and a lack of fast odorant adaptation.

To test how selective individual crypt cells were for different odorants, cells from juvenile, mature male and mature female trout were stimulated in random order successively with each of five odorants of the food- and reproduction-related groups (AA, OE, TE, SF and TEST; Fig. 4). The vast majority of crypt cells from all three groups responded to only one of the tested odorants ($N=57$ cells from 11 fish), whereas only a minor percentage showed calcium signals in response to two or more odorants. This experiment produced no significant differences regarding the odorant tuning width between juvenile, mature male and mature female trout, irrespective of their different response preferences.

The results of the present study indicate a response preference to odorants related to the opposite sex in crypt cells from mature and reproductive rainbow trout. However, crypt cells from juvenile specimens presented uniform response frequencies to all odorants presented, without any apparent preference for a specific odorant group. This suggests that olfactory crypt cells undergo a change of function during sexual maturation, from generalist detectors of different odorant classes to more specialized sensors for reproduction-related odorants.

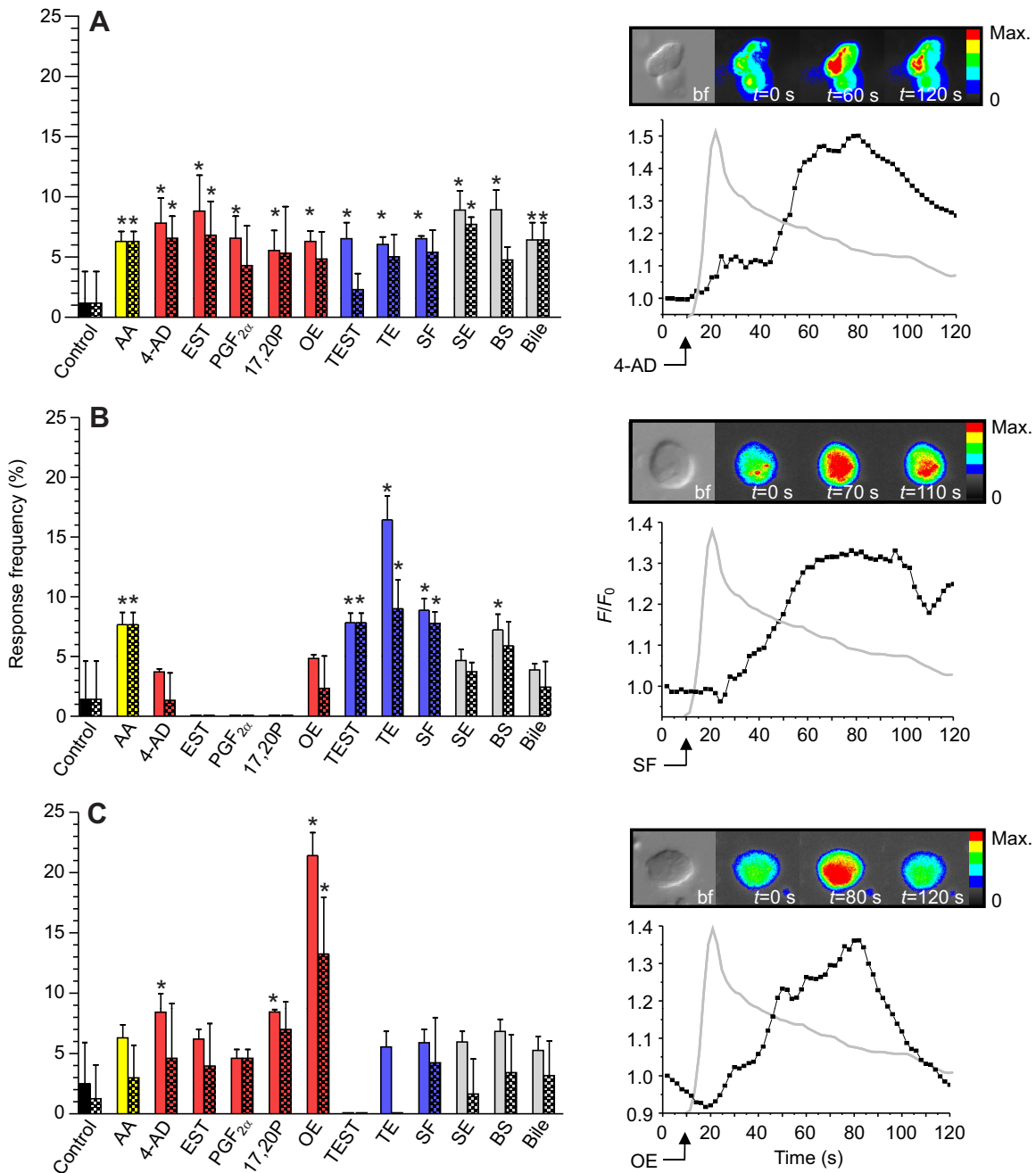


Fig. 3. Olfactory crypt cells of mature trout responded preferentially to odorants related to the opposite sex. (A) Similar percentages of crypt cells from juvenile trout responded to distinct kinds of odorants, without any significant preference. Solid bars represent responses exceeding 10% fluorescence increases; hatched bars represent responses exceeding 20% fluorescence increases compared with the pre-pulse baseline. Asterisks indicate significantly different response frequencies compared with controls (Ringer's solution without odorants; $*P < 0.05$). Right: example of a crypt cell from a juvenile trout responding to 4-androstene-3,17-dione (4-AD) with a strong and long-lasting increase of intracellular calcium. The overlaid grey curve indicates a representative stimulus delivery time course in arbitrary units. bf, brightfield. (B) Crypt cells from mature female trout did not respond to 17 β -estradiol (EST), prostaglandin F_{2 α} (PGF_{2 α}) or 17 α ,20 β -dihydroxy-4-pregnen-3-one (17,20P), but showed a high probability to respond to testicular extract, seminal fluid and 17 α -methyltestosterone. Response rates to these social and food-related odorants were similar to those of juvenile trout. Right: example of a crypt cell from a mature female trout responding to seminal fluid. (C) Response rates to the female hormones 4-AD, 17,20P and ovarian extract were significantly elevated for the 10% criterion in crypt cells from mature male trout compared with Ringer's solution as control, whereas ovarian extract was the only odorant causing significant responses for the 20% criterion. Testosterone did not trigger any responses in this group ($N = 33$). Right: example of a crypt cell from a mature male trout responding to ovarian extract. AA, amino acids; BS, bile salts; OE, ovarian extract; SE, skin extract; SF, seminal fluid; TE, testicular extract; TEST, 17 α -methyltestosterone.

DISCUSSION

Different labeling methods have been applied to mark crypt cells in the olfactory epithelium of fishes: general histological stains such as Toluidine Blue and hematoxylin and eosin (Ferrando et al., 2006;

Schmachtenberg, 2006), retrograde labeling from the olfactory bulb with lipophilic dyes (Hamdani and Døving, 2005; Hansen et al., 2003), fluochrome-coupled plant lectins (Ferrando et al., 2006) and immunohistochemistry against the S-100 protein of as yet unknown

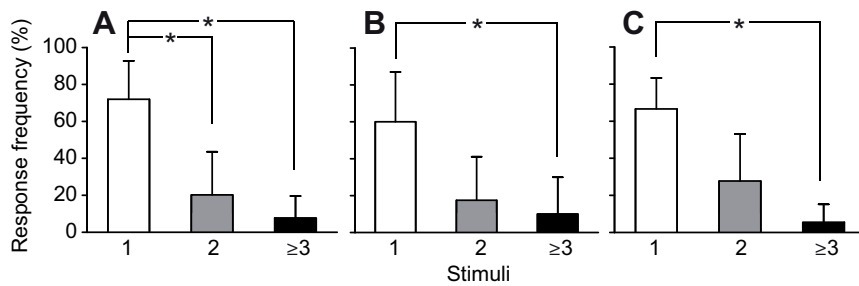


Fig. 4. Percentage of crypt cells from the three groups of rainbow trout that showed responses to only one, two or more stimuli presented individually in random order. Crypt cells from (A) juveniles ($N=29$ from four fish), (B) females ($N=13$ from four fish) and (C) males ($N=15$ from three fish) did not show significant differences in their odorant response selectivity. In all three groups, a significant majority of analyzed cells was selective for only one of the tested compounds (one-way ANOVA, $*P<0.05$).

function (Germana et al., 2004), the growth factor receptor Trk-A (Catania et al., 2003) and the G-proteins $G\alpha_o$, $G\alpha_q$ and G_i1b . $G\alpha_o$ was shown to strongly label crypt cells in channel catfish and goldfish, although it is also expressed in a subset of microvillous olfactory receptor neurons (Hansen et al., 2004; Hansen et al., 2003). Here, we used $G\alpha_o$ to identify crypt cells in the olfactory epithelium of the rainbow trout, where it always labeled their cytoplasm and sometimes the axon. As in other species, some microvillous olfactory receptor neurons were also immunoreactive, but could be easily differentiated by morphological criteria.

Crypt cells were detected with similar frequency in juvenile and adult specimens, but, unexpectedly, a significant portion of crypt cells were located at a depth within the olfactory epithelium without apparent access to the surface. Although several previous studies described a mostly apical localization of crypt cells in the olfactory epithelium and established this as a criterion for their identification (Catania et al., 2003; Ferrando et al., 2006; Hamdani and Døving, 2006; Hansen and Finger, 2000), another recent study reported that the apical position of crypt cells and their overall density depended on the spawning season (Hamdani et al., 2008). In contrast, studies in zebrafish, which express numerous crypt cells, failed to reveal a seasonal fluctuation of crypt cell density (Hansen and Finger, 2000), which might be related to the stable conditions of their natural habitat and their year-round reproductive activity (Spence et al., 2008).

We compared the localization of crypt cells in the olfactory epithelium of juvenile and adult reproductive trout and found that a significantly higher percentage localized to the apical border in sexually mature fish, suggesting a differential role of this receptor neuron depending on the developmental stage. The developmental formation of crypt cells in the olfactory epithelium has been studied in different species, revealing a first appearance as early as 2 days post-fertilization in zebrafish embryos and 2 weeks before hatching in the skate *Raja clavata* (Ferrando et al., 2007), whereas in the turbot *Psetta maxima*, the first crypt cells appear during the metamorphosis from the larval to the juvenile stage (Doldan et al., 2011). In the sturgeon *Acipenser naccarii*, crypt-cell-like cells emerged approximately 2–3 days after hatching but displayed a modified morphology without crypt, which only develops 21 days after eclosion (Camacho et al., 2010).

In the present study, we compared the morphology of dissociated crypt cells from juvenile, adult male and adult female trout under light and fluorescence microscopy. Although the shape of crypt cells was found to be indistinguishable between the groups, crypt cells from sexually mature specimens had a size distribution that was shifted towards larger dimensions. These data suggest that juvenile trout express numerous immature crypt cells. However, as in other species, a significant portion of crypt cells appears to be functional from early developmental stages onwards, supporting a role unrelated to reproductive behavior in sexually immature trout.

The odorants selected for this study represent compounds that were previously shown to have a specific biological meaning in

different fish species. Amino acids are potent odorants that trigger feeding behavior in fish (Ivanova and Caprio, 1993; Valentincic et al., 2000a; Valentincic et al., 1999; Valentincic et al., 2000b). Bile salts, which have been studied in salmonids and lamprey, among others, are considered social and migratory odorants in fishes (Døving et al., 1980; Giaquinto and Hara, 2008; Huertas et al., 2010). The female sex hormones 4-AD, EST, PGF2 α , 17-20P were shown to function as reproductive pheromones in the goldfish (Kobayashi et al., 2002; Sorensen et al., 1988; Sorensen et al., 2005); these hormones activate specific glomeruli in the ventral olfactory bulb of the related crucian carp, and zebrafish (Friedrich and Korsching, 1998; Lastein et al., 2006), and activate the medial olfactory tract of male goldfish (Sorensen et al., 1991). Similarly, the male sex hormone testosterone and some of its derivatives display strong olfactory and/or pheromonal activity in goldfish and other species (Scott and Sorensen, 1994; Sorensen et al., 2005; Stacey and Sorensen, 2005).

Because it is uncertain whether the above compounds have a pheromonal activity in our model, the rainbow trout, we complemented our study with five natural conspecific extracts: skin extract, ovarian extract, testicular extract, diluted seminal fluid and bile. Although skin extract is thought to contain an as yet unknown substance that triggers alarm reactions in fishes (Brown and Smith, 1998; Hamdani and Døving, 2003; Speedie and Gerlai, 2008), seminal fluid as well as ovarian and testicular homogenates are established sources of sex steroids with pheromonal activity in fishes (Honda, 1980; Hubbard et al., 2003; Lambert et al., 1986; Newcombe and Hartman, 1973; Resink et al., 1989; Van den Hurk et al., 1987). Finally, diluted conspecific bile was applied as a natural source of bile salts with high olfactory potency in many species, including the rainbow trout (Giaquinto and Hara, 2008). All of these natural sources of odorants and pheromones were tested in electro-olfactogram recordings of live rainbow trout to confirm their olfactory potency and to adjust their concentrations for the calcium imaging experiments (data not shown).

The present study sought to shed more light on the odorant tuning of olfactory crypt cells and their response specificity. Although we previously demonstrated the chemical sensitivity of crypt cells from the Chilean jack mackerel, *T. s. murphyi*, and their responses to amino acids (Vielma et al., 2008), it seems likely that the natural function of crypt cells goes beyond the detection of amino acids, which were already shown to stimulate both ciliated and microvillous olfactory receptor neurons in different fish species (Erickson and Caprio, 1984; Sato and Suzuki, 2001; Schmachtenberg and Bacigalupo, 2004; Thommesen, 1983; Zielinski and Hara, 1988). Therefore, we applied a broader spectrum of aquatic odorants at final concentrations in the micromolar range, and recorded the responses of a large number of olfactory crypt cells with the calcium imaging technique. In addition, we separated the animals into juvenile, mature male and mature female groups to find evidence for the hypothesized role of crypt cells in

reproductive olfactory signaling (Hamdani and Døving, 2007; Hamdani et al., 2008).

Our main discovery is the observation that crypt cells from juvenile fish displayed no preference for any of the applied odorants, whereas crypt cells from mature specimens of both sexes showed significantly increased response frequencies to putative pheromonal stimuli from the opposite sex, and decreased response rates to reproduction-related odorants from the same sex. This result was robustly obtained irrespective of the application of a more flexible or stringent criterion (10% versus 20% fluorescence increase) for odorant responses. Whereas odorant preference differed between the three groups, response selectivity did not, with a large majority of cells responding to only one of five presented odorants, supporting the notion that individual cells from juvenile and mature trout express the same quantity of odorant receptor types, possibly only one (DeMaria and Ngai, 2010).

Together with our findings of larger dimensions and a more apical localization of crypt cells in mature specimen, our data suggest that the population of crypt cells in the olfactory organ of rainbow trout undergoes a functional change during sexual maturation, in which a population of smaller, partly immature crypt cells with receptors sensitive to common odorants is replaced by larger and more apically localized crypt cells expressing, among others, an increased percentage of receptors sensitive to sex hormones and pheromones. This hypothesis is supported by the recent discovery of a V1R-like odorant receptor expressed exclusively in mature zebrafish crypt cells (Oka et al., 2012). However, the claim that only this one odorant receptor is expressed in all crypt cells is difficult to reconcile with our results and current concepts of odorant detection. Thus, there is still need for more research to understand the function of the elusive olfactory crypt cell.

LIST OF ABBREVIATIONS

17,20P	17 α ,20 β -dihydroxy-4-pregnen-3-one
AA	amino acids
4-AD	4-androstene-3,17-dione
BS	bile salts
BSA	bovine serum albumin
CC	crypt cell
EST	17 β -estradiol
OE	ovarian extract
PBS	phosphate-buffered saline
PGF _{2α}	prostaglandin F _{2α}
SE	skin extract
SF	seminal fluid
TE	testicular extract
TEST	17 α -methyltestosterone

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Table S1. Responses of olfactory crypt cells from juvenile trout to the odorants tested in this study

Odorant	Concentration	10% criterion	20% criterion	N (fish)	n (cells)
		Response frequency (%)	Response frequency (%)		
Control	–	1.2±2.6	1.2±2.6	5	77
AA	1 mmol l ⁻¹	6.3±0.8*	6.3±0.8*	6	144
4-AD	1 μmol l ⁻¹	7.8±2.1*	6.6±1.8*	4	89
EST	1 μmol l ⁻¹	8.8±3.0*	6.8±2.8*	4	89
PGF _{2α}	1 μmol l ⁻¹	6.6±1.8*	4.3±3.3	4	89
17,20P	1 μmol l ⁻¹	5.5±1.7*	5.3±3.9	4	89
OE	1:10 ²	6.3±0.9*	4.8±2.3	4	129
TEST	10 μmol l ⁻¹	6.5±1.3*	2.3±1.3	5	170
TE	1:10 ²	6.0±0.6*	5.0±1.8	3	100
SF	1:10 ³	6.5±0.2*	5.4±1.8	3	61
SE	1:10 ²	8.9±1.6*	7.7±0.6*	3	53
BS	100 μmol l ⁻¹	8.9±1.6*	4.8±1.1	3	103
Bile	1:10 ³	6.4±1.4*	6.4±1.4*	4	102

Data are means ± s.d. Asterisks indicate significantly different response frequencies compared with control (Ringer's solution without odorants).

17,20P, 17α,20β-dihydroxy-4-pregnen-3-one; 4-AD, 4-androstene-3,17-dione; AA, amino acid; BS, bile salt; EST, 17β-estradiol; OE, ovarian extract; PGF_{2α}, prostaglandin F_{2α}; SE, skin extract; SF, seminal fluid; TE, testicular extract; TEST, 17α-methyltestosterone.

Table S2. Odorant responses of olfactory crypt cells from sexually mature female trout

Odorant	Concentration	10% criterion	20% criterion	N (fish)	n (cells)
		Response frequency (%)	Response frequency (%)		
Control	–	1.4±3.2	1.4±3.2	5	68
AA	1 mmol l ⁻¹	7.7±1.0*	7.7±1.0*	3	51
4-AD	1 μmol l ⁻¹	3.7±0.3	1.3±2.3	3	81
EST	1 μmol l ⁻¹	0±0	0±0	4	88
PGF _{2α}	1 μmol l ⁻¹	0±0	0±0	3	60
17,20P	1 μmol l ⁻¹	0±0	0±0	4	88
OE	1:10 ²	4.8±0.3	2.3±2.7	4	83
TEST	10 μmol l ⁻¹	7.8±0.8*	7.8±0.8*	3	50
TE	1:10 ²	16.5±2.0*	9.0±2.4*	4	66
SF	1:10 ³	8.9±1.0*	7.8±1.0*	3	54
SE	1:10 ²	4.7±0.9	3.7±0.8	3	83
BS	100 μmol l ⁻¹	7.2±1.3*	5.9±2.0	3	85
Bile	1:10 ³	3.9±0.5	2.4±2.1	3	78

Data are means ± s.d. Asterisks indicate significantly different response frequencies compared with control (Ringer's solution without odorants).

17,20P, 17α,20β-dihydroxy-4-pregnen-3-one; 4-AD, 4-androstene-3,17-dione; AA, amino acid; BS, bile salt; EST, 17β-estradiol; OE, ovarian extract; PGF_{2α}, prostaglandin F_{2α}; SE, skin extract; SF, seminal fluid; TE, testicular extract; TEST, 17α-methyltestosterone.

Table S3. Odorant responses of olfactory crypt cells from sexually mature male trout

Odorant	Concentration	10% criterion	20% criterion	N (fish)	n (cells)
		Response frequency (%)	Response frequency (%)		
Control	–	2.5±3.4	1.3±2.8	5	36
AA	1 mmol l ⁻¹	6.3±1.1	3.0±2.7	3	62
4-AD	1 μmol l ⁻¹	8.4±1.5*	4.6±4.5	3	47
EST	1 μmol l ⁻¹	6.2±0.8	4.0±3.5	3	49
PGF _{2α}	1 μmol l ⁻¹	4.6±0.7	4.6±0.7	3	66
17,20P	1 μmol l ⁻¹	8.5±0.2*	7.0±2.3	3	47
OE	1:10 ²	22.1±3.2*	13.2±4.7*	4	78
TEST	10 μmol l ⁻¹	0±0	0±0	3	33
TE	1:10 ²	5.5±1.3	0±0	4	76
SF	1:10 ³	5.9±1.1	4.2±3.8	3	52
SE	1:10 ²	6.0±0.9	1.7±2.9	3	50
BS	100 μmol l ⁻¹	6.8±1.0	3.4±3.2	3	57
Bile	1:10 ³	5.3±1.2	3.2±2.9	3	59

Data are means ± s.d. Asterisks indicate significantly different response frequencies compared with control (Ringer's solution without odorants).

17,20P, 17α,20β-dihydroxy-4-pregnen-3-one; 4-AD, 4-androstene-3,17-dione; AA, amino acid; BS, bile salt; EST, 17β-estradiol; OE, ovarian extract; PGF_{2α}, prostaglandin F_{2α}; SE, skin extract; SF, seminal fluid; TE, testicular extract; TEST, 17α-methyltestosterone.