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RESEARCH ARTICLE

# Olfactory sensitivity to steroid glucuronates in Mozambique tilapia suggests two distinct and specific receptors for pheromone detection

**Tina Keller-Costa[1,2], Adelino V. M. Canário[1] and Peter C. Hubbard[1,*]**

## ABSTRACT

Cichlids offer an exciting opportunity to understand vertebrate speciation; chemical communication could be one of the drivers of African cichlid radiation. Chemical signals mediate key aspects in the lives of vertebrates and often are species specific. Dominant male Mozambique tilapia [*Oreochromis mossambicus* (Peters 1852)] release a sex pheromone, 5β-pregnan-3α,17α,20β-triol 3-glucuronate and its 20α-epimer, via their urine. The objective of this study was to assess the sensitivity, specificity and versatility of the olfactory system of *O. mossambicus* to other steroids and their conjugates using the electro-olfactogram. *Oreochromis mossambicus* was sensitive to several 3-glucuronidated steroids, but did not respond to prostaglandins, unconjugated steroids or 17- or 20-conjugated steroids. Stimulation of the olfactory epithelium with increasing concentrations (1 pmol $l^{-1}$ to 10 μmol $l^{-1}$) of 5β-pregnan-3α,17α,20β-triol 3-glucuronate, 5β-pregnan-3α,17α,20α-triol 3-glucuronate, 3α,17α-dihydroxy-5β-pregnan-20-one 3-glucuronate, etiocholanolone 3α-glucuronate and 17β-estradiol 3-glucuronate produced characteristic sigmoidal concentration–response curves. However, tilapia were most sensitive to 17β-estradiol-3-glucuronate, which also had the lowest apparent $EC_{50}$ and maximal response amplitude. Cross-adaptation and binary mixture experiments suggested that 5β,3α-reduced pregnan- and androstan-3-glucuronates share (a) common olfactory receptor(s), whereas 17β-estradiol 3-glucuronate is detected via (a) distinct olfactory receptor(s). In conclusion, the Mozambique tilapia has evolved high olfactory sensitivity and specificity to 3-glucuronidated steroids through two distinct olfactory receptor types; one detecting a male sex pheromone and a second detecting 17β-estradiol 3-glucuronate, a putative female-derived signal. However, *O. mossambicus* differs markedly in its olfactory perception from the more recently derived East African cichlid *Astatotilapia burtoni*, suggesting that chemical communication could, indeed, be involved in speciation.

**KEY WORDS: Pheromone, Steroid, Olfaction, Receptor, Chemical communication, Cichlid**

## INTRODUCTION

Sex steroids and their conjugates can be potent odorants for teleost fishes and, in some species, have been identified as sex pheromones, facilitating the location and choice of suitable mates, and/or triggering endocrine changes in conspecifics that prompt gonadal maturation and improve fertility to enhance reproductive success (Stacey, 2010; Stacey and Sorensen, 2005). Pheromones may be composed of a single or multiple component(s) and are detected by olfactory receptors from which the signal is relayed to specific brain areas that integrate the information and trigger the appropriate behavioural and/or endocrine response. A simple and reliable method to study olfactory sensitivity in freshwater fishes, and to explore whether different odorants are detected by separate or shared receptors, is the electro-olfactogram (EOG) (for general review see Scott and Scott-Johnson, 2002). In EOG cross-adaption tests, the response amplitude to one test odorant is measured prior to adaptation and then again during adaptation to a second odorant. If the 'test' and 'adapting' odorant act through independent olfactory receptor sites, the response to the test odorant during adaptation should be unaffected, i.e. not greatly reduced, compared to the signal measured prior to adaptation (Caprio and Byrd, 1984; Cole and Stacey, 2006; Sorensen et al., 1995). In binary mixture tests, receptor sites are separate if the EOG response to a mixture of two odorants is approximately the sum of the responses to the individual odorants given alone. Conversely, EOG responses to the mixture that are smaller or equivalent to those at twice the concentration of either odorant indicate (a) shared olfactory receptor(s) (Cole and Stacey, 2006). In goldfish (*Carassius auratus*), for example, EOG recordings from cross-adaptation and binary mixture tests established that the pre- and post-ovulatory pheromones, released by females, are detected by conspecific males with high sensitivity through separate olfactory receptors (Sorensen et al., 1988; Sorensen et al., 1995). The preovulatory pheromone includes free and sulphated 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P), acting via different receptors (Sorensen et al., 1995). The post-ovulatory goldfish pheromone, by contrast, consists of F-type prostaglandins, mainly $PGF_{2\alpha}$ and 15K-$PGF_{2\alpha}$, which too have distinct olfactory receptor sites (Sorensen et al., 1988).

Within the Perciformes, the largest teleost order (Order Summary for Perciformes, www.fishbase.org), studies on olfactory sensitivity to, and pheromonal function of, hormonal steroids are scarce and derive from a few representatives of the Gobiidae (Colombo et al., 1980; Corkum et al., 2008; Murphy et al., 2001; Tierney et al., 2013) and Cichlidae (Cole and Stacey, 2006; Hubbard et al., 2014; Keller-Costa et al., 2014). Cichlids are an extremely diverse taxon with currently 1670 described species ('List of Nominal Species of *Cichlidae*', www.fishbase.org), mostly native to Africa, and adaptation of the sensory and signalling systems to different environmental conditions has been suggested as an important driver in African cichlid radiation (Seehausen et al., 2008). Focus so far has mainly been on the evolution of colour polymorphism that is linked to light heterogeneity in the habitat (Seehausen et al., 2008) alongside specialisation for particular trophic niches (Greenwood, 1991). Divergent selection on chemical communication systems may, however, constitute an additional speciation factor. Nevertheless,

[1]Centre of Marine Sciences (CCMAR), Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal. [2]Departamento de Biologia, Universidade de Évora, Apartado 94, 7002-554 Évora, Portugal.

*Author for correspondence (phubbard@ualg.pt)

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**List of abbreviations**

| | |
|---|---|
| 15K-PGF$_{2\alpha}$ | 15-keto-prostaglandin F$_{2\alpha}$ |
| 17,20β-P | 17α,20β-dihydroxypregn-4-en-3-one |
| 20one-P-3-G | 3α,17α-dihydroxy-5β-pregnan-,20-one 3α-glucuronate |
| 20α-P-3-G | 5β-pregnane-3α,17α,20α-triol 3α-glucuronate |
| 20β-P-3-G | 5β-pregnane-3α,17α,20β-triol-3α-glucuronate |
| BW | body weight |
| E2-3,17-diG | 17β-estradiol 3,17-diglucuronate |
| E2-3-G | 17β-estradiol 3-glucuronate |
| E2-3-S | 17β-estradiol 3-sulphate |
| EOG | electro-ofactogram |
| ETIO-3-G | 5β-etiocholan-3α-ol-17-one 3α-glucuronate |
| ETIO-3-S | 5β-etiocholan-3α-ol-17-one 3α-sulphate |
| PGF$_{2\alpha}$ | prostaglandin F$_{2\alpha}$ |
| SAC | self-adapted control |
| SL | standard length |
| TCD | taurochenodeoxycholic acid |

knowledge of the identity, perception and functions of chemical signals across cichlids is limited, with the exception of two maternal mouth-brooders, *Astatotilapia burtoni* and *Oreochromis mossambicus* (Mozambique tilapia). *A. burtoni* has olfactory sensitivity to several hormonal steroid conjugates (Robison et al., 1998) with five distinct olfactory receptor sites, classified according to the type and position of the conjugate in the steroid (Cole and Stacey, 2006). Unfortunately, it is not yet known whether *A. burtoni* synthesises or releases any of these steroid conjugates and, if so, what their pheromonal function may be. Male *O. mossambicus* use urinary signals to mediate aggression between males and, during courtship, to prime females to spawn (Barata et al., 2008; Barata et al., 2007; Huertas et al., 2014; Keller-Costa et al., 2012). Dominant male urine contains high concentrations of 5β-pregnane-3α,17α,20β-triol-3-glucuronate (20β-P-3-G) and of its α-epimer (20α-P-3-G), which stimulate the females' endocrine system and oocyte maturation (Keller-Costa et al., 2014). Both steroids evoke large olfactory responses mediated by (a) common receptor(s). In contrast, steroids known to be present in the blood and urine of *O. mossambicus* males, including 11-ketotestosterone, 17α,20β-dihydroxypregn-4-en-3-one and their glucuronate and sulphate conjugates (Oliveira et al., 1996; Rocha and Reis-Henriques, 1996), are not detected by the olfactory epithelium (Frade et al., 2002). However, it is unknown whether prostaglandins or other steroids, including steroids that are structurally related to the urinary pregnanetriol-3-glucuronates, are detected and, if so, how many different receptor sites are involved. Such insights are necessary to assess the olfactory steroid receptor diversity in African cichlids and therefore to address the hypothesis that chemical signal diversification is a driver for African cichlid radiation.

Thus, the objectives of this study were, firstly, to assess olfactory sensitivity of *O. mossambicus* to steroids; secondly, to establish, by

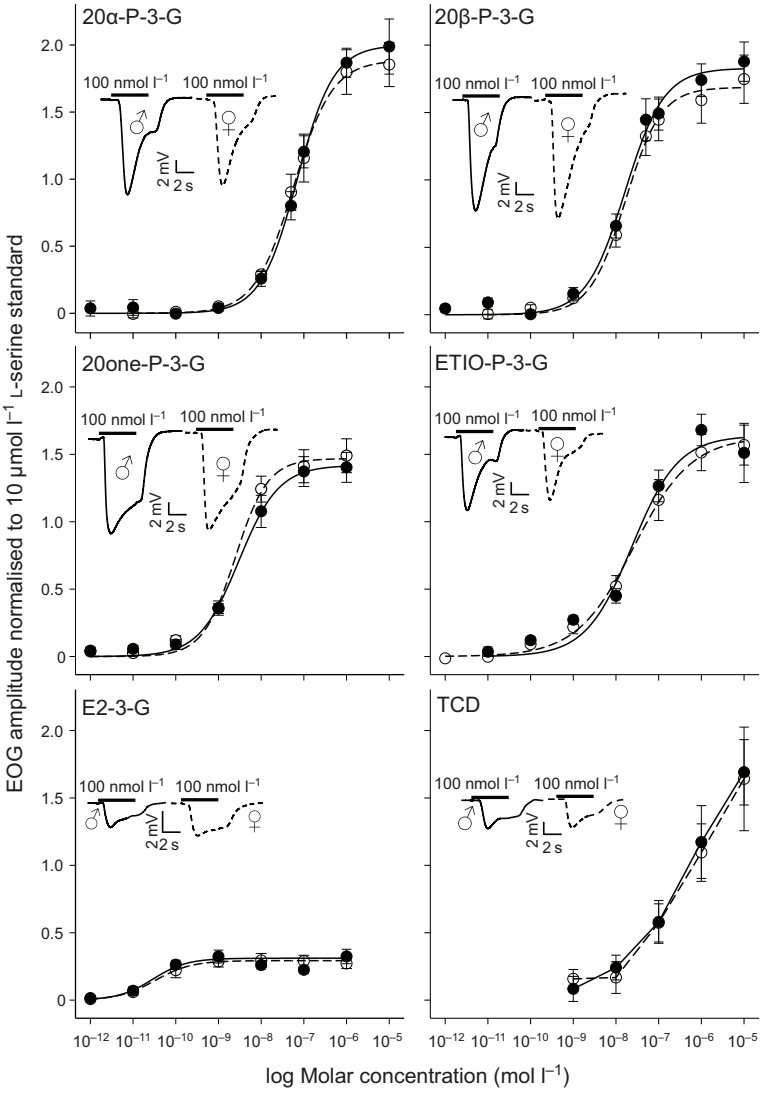


**Fig. 1. EOG concentration response profiles.** Normalised (to 10 µmol l$^{-1}$ L-serine response) EOG concentration–response curves (semi-logarithmic plot, mean ± s.e.m.) for the male tilapia sex pheromone, 20α-P-3-G and 20β-P-3-G, and other steroid 3α-glucuronates and a bile acid (TCD). Responses of males (*N*=8–14; filled circles) and females (*N*=10–14; open circles) are shown. A sigmoidal (three-parameter Hill) curve was fitted to the response profiles to the steroid 3α-glucuronates of both sexes (males, solid; females, dashed line). The *x* axes (odorant concentration) are all the same as the lowest two graphs. Representative EOG traces of a male (solid line) and a female (dashed line), recorded at 100 nmol l$^{-1}$ odorant concentrations, are presented as insets.

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**Table 1. Steroids tested in in this study**

| Chemical class | Chemical group | Compound name | Abbreviation | Detection threshold[a] | Saturation[a] | Conc. receptor tests[b] |
|---|---|---|---|---|---|---|
| Bile acid | 24 carbon | Taurochenodeoxycholic acid | TCD | 10 nmol $l^{-1}$ | – | 10 µmol $l^{-1}$ |
| Prostaglandins (PG) | 20 carbon | Prostaglandin F2α | PGF2α | Insensitive[c] | – | – |
| | | Prostaglandin 15keto-F2α | 15k-PGF2α | Insensitive[c] | – | – |
| Unconjugated steroids | 21 carbon | 5β-pregnan-3α,17α,20β-triol | 20β-P | Insensitive[c] | – | – |
| | | 5β-pregnan-3α,17α,20α-triol | 20α-P | Insensitive[c] | – | – |
| | | 3α,17α-dihydroxy-5β-pregnan-20-one | 20one-P | Insensitive[c] | – | – |
| | 19 carbon | Etiocholan-3α-ol-17-one | ETIO | Insensitive[c] | – | – |
| | 18 carbon | 17β-estradiol | E2 | Insensitive[c] | – | – |
| 3-sulphated steroids | 19 carbon | Etiocholan-3α-ol-17-one 3-sulphate | ETIO-3-S | 100 nmol $l^{-1}$ [d] | – | – |
| | 18 carbon | 17β-estradiol 3-sulphate | E2-3-S | Insensitive[c] | – | – |
| 17-sulphated steroids | 19 carbon | Testosterone 17-sulphate | T-17-S | Insensitive[c] | – | – |
| 20-sulphated steroids | 21 carbon | 17α,20β-dihydroxy-4-pregnen-3-one 20-sulphate | 17,20β-P-20-S | Insensitive[c] | – | – |
| 3-glucuronidated steroids | 21 carbon | 5β-pregnan-3α,17,20β-triol-3-glucuronate | 20α-P-3-G | 1 nmol $l^{-1}$ | 1 µmol $l^{-1}$ | 1 µmol $l^{-1}$ |
| | | 5β-pregnan-3α,17,20α-triol-3-glucuronate | 20β-P-3-G | 1 nmol $l^{-1}$ | 1 µmol $l^{-1}$ | 1 µmol $l^{-1}$ |
| | | 3α,17-dihydroxy-5β-pregnane-20-one-3-glucuronate | 20one-P-3-G | 100 pmol $l^{-1}$ | 1 µmol $l^{-1}$ | 1 µmol $l^{-1}$ |
| | 19 carbon | Etiocholan-3α-ol-17-one-3-glucuronate | ETIO-3-G | 1 nmol $l^{-1}$ | 1 µmol $l^{-1}$ | 1 µmol $l^{-1}$ |
| | 18 carbon | 17β-estradiol-3-glucuronate | E2-3-G | 10 pmol $l^{-1}$ | 1 µmol $l^{-1}$ | 10 nmol $l^{-1}$ |
| 17-glucuronidated steroids | 19 carbon | Testosterone 17-glucuronate | T-17-G | Insensitive[c] | – | – |
| | 18 carbon | 17β-estradiol 17-glucuronate | E2-17-G | Insensitive[c] | – | – |
| 20-glucuronidated steroids | 21 carbon | 17α,20β-dihydroxy-4-pregnen-3-one 20-glucuronate | 17,20β-P-20-G | Insensitive[c] | – | – |
| 3,17-diglucuronidated steroids | 18 carbon | 17β-estradiol 3,17-diglucuronate | E2-3,17-diG | 100 nmol $l^{-1}$ [d] | – | – |

[a]Read estimates based on mean concentration response curves. [b]Concentration used in electro-olfactogram (EOG) cross-adaptation and binary mixture tests. [c]*O. mossambicus* does not have any olfactory sensitivity to this steroid. [d]Responses not consistent and EOG amplitudes small, therefore this steroid was excluded from further concentration–response, cross-adaptation and binary mixture tests. Conc., concentration.

cross-adaptation and binary mixture tests, whether steroid odorants act via shared or independent olfactory receptors; and thirdly, to compare these results to findings from *A. burtoni* (Cole and Stacey, 2006), a more recently derived African cichlid.

## RESULTS

### Detected steroids and EOG concentration response tests

Mozambique tilapia consistently responded to 3-glucuronidated steroids (Fig. 1), but they did not give EOG responses to representatives of 17- or 20-glucuronidated or sulphated steroids. Nor did they respond to any unconjugated steroids, E2-3-S or prostaglandins, even at concentrations as high as 1 µmol $l^{-1}$ (Table 1). ETIO-3-S and E2-3,17-diG induced small EOG responses, yet only at high concentrations (100 nmol $l^{-1}$ and 1 µmol $l^{-1}$). However, the latter responses were not consistent and were not pursued further.

Sigmoidal concentration–response curves were obtained using all 3-glucuronidated steroids (Table 1), and no differences were found between the responses of male and female recipients (Fig. 1). The detection threshold was lowest for E2-3-G (10 pmol $l^{-1}$), followed by 20one-P-3-G (100 pmol $l^{-1}$), and for the majority, the threshold was around 1 nmol $l^{-1}$. The EOG response amplitudes to 20α-P-3-G, 20β-P-3-G, 20one-P-3-G and ETIO-3-G increased rapidly before reaching an apparent maximum at around 1 µmol $l^{-1}$, which suggests saturation of the olfactory receptors (Fig. 1). For E2-3-G, both the EOG amplitude and saturation concentration (1 nmol $l^{-1}$) was much lower. Accordingly, the E2-3-G apparent half-maximal effective concentration $EC_{50}$ (mean ± s.e.m.; male, 0.07±0.02 nmol $l^{-1}$; female, 0.14±0.08 nmol $l^{-1}$) and apparent maximal olfactory response $I_{max}$ (male, 0.38±0.05; female, 0.34±0.04) were lower than the apparent $EC_{50}$ and $I_{max}$ values of all the other 3-glucuronidated steroids (Fig. 2A,B). As for the other steroids, 20α-P-3-G (male, 89.62±16.15 nmol $l^{-1}$; female, 86.3±18.74 nmol $l^{-1}$) and ETIO-3-G (male, 54.38±18.4 nmol $l^{-1}$; female, 40.74±10.92 nmol $l^{-1}$) had similar and the highest apparent $EC_{50}$ values, followed by 20β-P-3-G (male, 25.72±9.73 nmol $l^{-1}$; female, 30.12±12.92 nmol $l^{-1}$) and 20one-P-3-G (male, 4.78±1.01 nmol $l^{-1}$; female, 3.67±0.71 nmol $l^{-1}$; Fig. 2A). The apparent $I_{max}$ values in response to 20α-P-3-G, 20β-P-3-G and ETIO-3-G were similar and nearly twice that of the response to 10 µmol $l^{-1}$ L-serine (Fig. 2B). The apparent $I_{max}$ values of male (but not female) responses to 20one-P-3-G were lower than that in response to 20α-P-3-G. The apparent Hill coefficients were close to 1 for all steroids, suggesting a simple 1:1 binding ratio to the olfactory receptors with no cooperativity.

The concentration–response curve for taurochenodeoxycholic acid (TCD) showed a rapid increase of EOG amplitudes at supra-threshold (around 10 nmol $l^{-1}$) concentrations, without reaching an apparent maximum up to 10 µmol $l^{-1}$ (Fig. 1); TCD was expected to act via a distinct olfactory receptor and was therefore used as a control in cross-adaptation and binary mixture tests.

### EOG cross-adaptation tests

To confirm that the continuous perfusion with and sequential exposure to steroids during cross-adaptation did not desensitise the olfactory epithelium, responses to the steroids were again recorded after a 10 min wash-out and compared to the initial unadapted responses. No reduction in the EOG responses was observed for any of the tested steroids, regardless of the concentration. However, significant increases in the mean EOG response amplitudes were noted for some steroids, i.e. 20β-P-3-G (~27%) and 20one-P-3-G (~26%) at 1 µmol $l^{-1}$ and 20α-P-3-G (22%) at 10 nmol $l^{-1}$ concentrations (paired *t*-tests, $P$=0.045, $P$=0.021 and $P$=0.046, respectively). Increasing EOG response magnitudes over time are a widely observed phenomenon; because responses to test steroids during adaptation were compared only with the initial responses recorded before cross-adaptation, the increase

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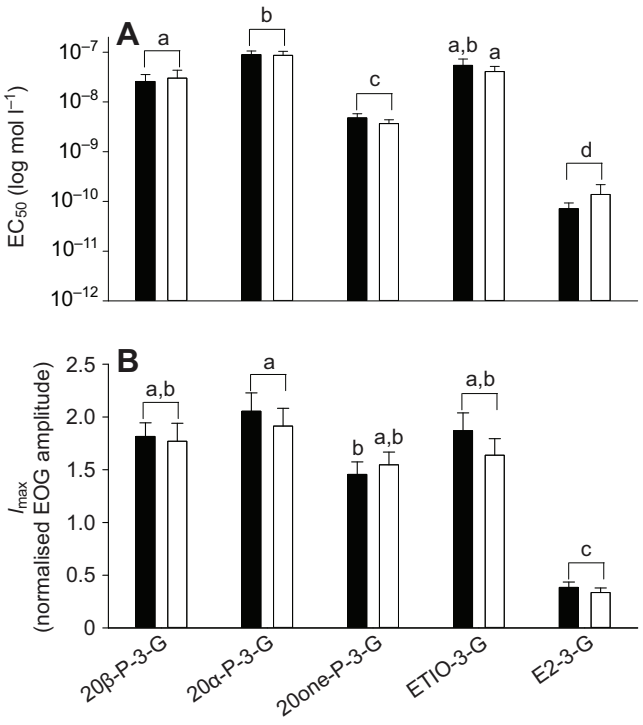


**Fig. 2. Comparison of apparent $EC_{50}$ and $I_{max}$ values.** Data (mean ± s.e.m.) were calculated from sigmoidal concentration–response curves of the male tilapia sex pheromone 20α-P-3-G and 20β-P-3-G, and other steroid 3α-glucuronates. Apparent (A) $EC_{50}$ values (in log mol $l^{-1}$) and (B) $I_{max}$ values for males ($N$=7–14; black bars) and females ($N$=10–14; open bars) for each steroid were similar. The different letters above the bars indicate significant differences ($P$<0.001) among the steroids, two-way ANOVA followed by Holm–Sidak *post hoc* test. $F$ and $P$ values were as follows: apparent $EC_{50}$ values in – sexes: $F_{1,103}$=0.131, $P$=0.781; steroids: $F_{4,103}$=177.968, $P$<0.001; interaction between sexes and steroids: $F_{4,103}$=0.257, $P$=0.905. Apparent $I_{max}$ values in B – sexes: $F_{1,103}$=0.686, $P$=0.409; steroids: $F_{4,103}$=34.280, $P$<0.001; interaction between sexes and steroids: $F_{4,103}$=0.397, $P$=0.810. All data were of equal variance.

noted here for some steroids should not influence any conclusions drawn.

The results of the EOG cross-adaptation studies at 1 μmol $l^{-1}$ (10 μmol $l^{-1}$ TCD) confirmed that the two male tilapia urinary steroids 20α-P-3-G and 20β-P-3-G act through (a) shared receptor(s) (Fig. 3). They further suggested that 20one-P-3-G, as well as the androstane ETIO-3-G are detected by the same olfactory receptor(s), hereafter called '3G-R-I', referring to the position of the glucuronate group on the steroid. 20β-P-3-G and 20one-P-3-G consistently reduced the EOG responses to all test steroids (except TCD) during adaptation to a point that they were not significantly different from the self-adapted control (SAC). Some slight anomalies were found, however, with 20α-P-3-G and ETIO-3-G as adapting steroids; both reduced the response to 20one-P-3-G only partially, to a level still significantly different from the SACs. A less-pronounced response reduction was also observed when 20β-P-3-G was adapted to 20α-P-3-G. In contrast, responses to TCD could not be reduced below 80% of the unadapted response, regardless of the adapting steroid. Surprisingly, however, when the olfactory epithelium was adapted to TCD, the mean responses to all administered test-steroids were reduced by at least 57%, although they remained significantly higher (except for ETIO-3-G) than that of the SAC.

Given the distinct concentration–response curve of E2-3-G, we hypothesised that this steroid acts via a receptor other than 3G-R-I. To test this, cross-adaptation tests including E2-3-G, 20α-P-3-G and 20β-P-3-G were performed at concentrations of 10 nmol $l^{-1}$, as the EOG amplitudes in response to the three steroids were similar at this concentration, as opposed to those at 1 μmol $l^{-1}$ (Fig. 1). E2-3-G did not reduce the responses to 20α-P-3-G or 20β-P-3-G below 70% of the initial response during adaptation (Fig. 4). Reciprocal adaptation of the olfactory epithelium to 20α-P-3-G or 20β-P-3-G confirmed these results, as responses to E2-3-G were consistently much higher than the SACs and were generally closer to the initial response. This indicates that the Mozambique tilapia is able to distinguish E2-3-G from other 3-glucuronidated pregnanes and androstanes via (a) distinct olfactory receptor(s), hereafter called '3G-R-II' (Fig. 6).

### EOG binary mixture tests

The mean independent component index ($I_{CI}$) and mixture discrimination index ($I_{MD}$) of the binary mixture comprising 20α-P-3-G and 20β-P-3-G were around 0.5 and 1.0, respectively, and consistent with the cross-adaptation studies, strongly indicating that there is a shared olfactory receptor mechanism. The mean $I_{CI}$ and $I_{MD}$ values for 20α-P-3-G or 20β-P-3-G mixed with either 20one-P-3-G or ETIO-3-G were statistically similar to those in response to the 20α-P-3-G and 20β-P-3-G mix (Fig. 5). The mean 'within-group' $I_{CI}$ and $I_{MD}$ values were 0.49 and 0.99, respectively. This is consistent with the cross-adaptation tests; 20one-P-3-G and ETIO-3-G are detected by the 3G-R-I, as are the urinary pheromonal steroids 20α-P-3-G and 20β-P-3-G.

Mean $I_{CI}$ values for 20α-P-3-G or 20β-P-3-G mixed with E2-3-G were generally closer to 1 and significantly different ($P$<0.001) from the 20α-P-3-G and 20β-P-3-G mix, indicating that E2-3-G is detected by a different receptor type, 3G-R-II. The mean $I_{MD}$ values for the 20β-P-3-G and E2-3-G mix, and the 20α-P-3-G and E2-3-G mix were above 1, which further supports the notion of receptor independence. Yet, only for the 20α-P-3-G and E2-3-G mix (but not the 20β-P-3-G and E2-3-G mix) was the $I_{MD}$ statistically different from that of the 20α-P-3-G and 20β-P-3-G mix.

Both the $I_{CI}$ and $I_{MD}$ values of TCD mixed with 20α-P-3-G, 20β-P-3-G or E2-3-G were close to 1 ($I_{CI}$) or clearly above 1 ($I_{MD}$) and significantly different ($P$<0.001) from the 20α-P-3-G and 20β-P-3-G mix, supporting our assumption that TCD acts via a separate receptor type, and consistent with the cross-adaptation studies. The mean 'across-group' $I_{CI}$ and $I_{MD}$ values were 0.77 and 1.45, respectively, and significantly larger than the mean 'within-group' $I_{CI}$ and $I_{MD}$ values (Mann–Whitney rank sum tests, $P$=0.002).

## DISCUSSION

This study demonstrates that the Mozambique tilapia possesses a high olfactory sensitivity to several 3-glucuronidated steroids, which it detects via two distinct olfactory receptor mechanisms; 3G-R-I selects C21 and C19 5β,3α-reduced steroids, whereas 3G-R-II selects C18 aromatic steroids (Fig. 6). However, given the limited range of steroids tested, we cannot exclude the possibility that other steroids may also be detected.

### Cross-adaptation tests

EOG responses not only confirmed the sensitivity of females and males to the previously identified male tilapia sex pheromone components 20α-P-3-G and 20β-P-3-G, but they also show that structurally related 3-glucuronidated pregnane(s) and androstane(s) produce similar concentration–response curves and act via the same olfactory receptor type 3G-R-I. Some slight anomalies, however, were observed during cross-adaption studies that used 20α-P-3-G or ETIO-3-G as the adapting steroid and 20β-P-3-G and/or 20one-P-3-

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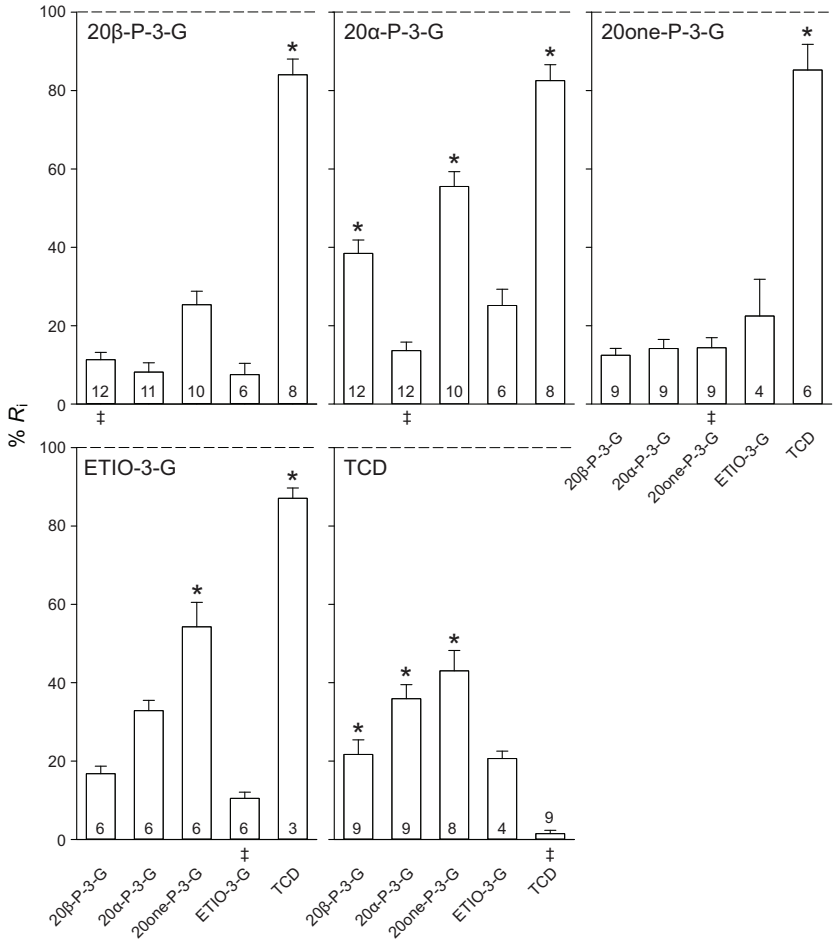


**Fig. 3. EOG cross-adaptation studies.** Relative EOG response (mean + s.e.m.) to 1 µmol $l^{-1}$ steroid 3α-glucuronates (or 10 µmol $l^{-1}$ TCD) expressed as a percentage of the initial unadapted response (% $R_I$) to the same 1 µmol $l^{-1}$ steroid (10 µmol $l^{-1}$ TCD) delivered before cross-adaptation. ‡Self-adapted control (SAC). Numbers in bars indicate sample size. *Significant differences from the SAC ($P$<0.05). Kruskal–Wallis ANOVA on ranks followed by Dunn's method, multiple comparisons versus SAC as the control group. 20β-P-3-G: $H$=28.951, degrees of freedom d.f.=4, $P$<0.001. 20α-P-3-G: $H$=38.624, d.f.=4, $P$<0.001. 20one-P-3-G: $H$=16.136, d.f.=4, $P$=0.003. ETIO-3-G: $H$=23.243, d.f.=4, $P$<0.001. TCD: $H$=26.903, d.f.=4, $P$<0.001.

G as test odorant; responses were reduced but not to the extent of those of the SAC. This may be explained by the lower apparent $EC_{50}$ values obtained for 20β-P-3-G and 20one-P-3-G than for 20α-P-3-G and/or ETIO-3-G. When two odorants compete for the same receptor site, but one odorant has a higher affinity (as indicated by the lower apparent $EC_{50}$), it is likely to replace the other odorant at the receptor binding site, thereby giving a partial olfactory response.

Cross-adaptation tests reveal further that E2-3-G is detected through a separate olfactory receptor, type 3G-R-II, producing a markedly different concentration–response curve.

The bile acid TCD was expected to act through a separate olfactory receptor type from that of the steroid conjugates tested. Consistent with our previous work (Keller-Costa et al., 2014), the response to TCD was never reduced below 80% of the initial response, regardless

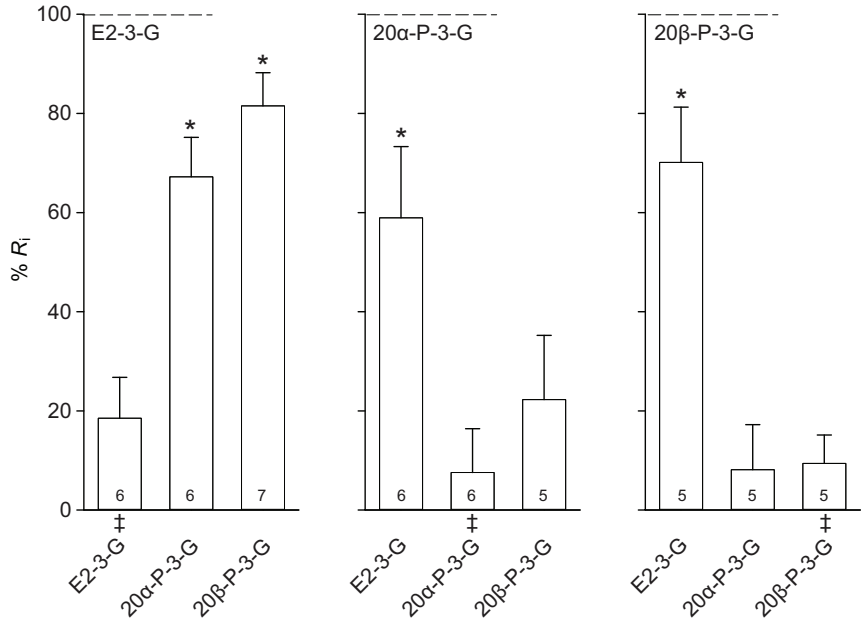


**Fig. 4. EOG cross-adaptation studies involving 17β-estradiol-3-G (E2-3-G).** Relative EOG response (mean + s.e.m.) to 10 nmol $l^{-1}$ steroid conjugates expressed as a percentage of the initial unadapted response (% $R_I$) to the same 10 nmol $l^{-1}$ steroid delivered before cross-adaptation started. ‡SAC. The numbers in the bars indicate sample size. *Significant difference from the SAC ($P$<0.05). Kruskal–Wallis ANOVA on ranks followed by Dunn's method, multiple comparisons *versus* SAC as the control group. E2-3-G: $H$=11.523, d.f.=2, $P$=0.003. 20β-P-3-G: $H$=9.420, d.f.=2, $P$=0.009. 20α-P-3-G: $H$=6.371, d.f.=2, $P$=0.041.

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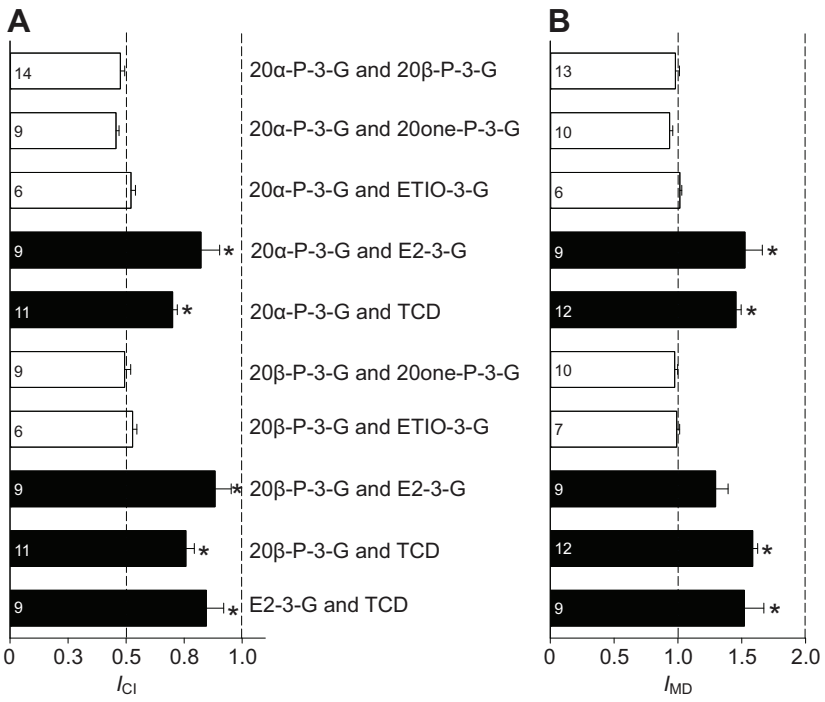


**Fig. 5. Results of EOG binary mixture tests.** (A) Independent component ($I_{CI}$) and (B) mixture discrimination ($I_{MD}$) indices (mean + s.e.m.) calculated from binary mixture tests. Open bars ('within-group'): values for $I_{CI}$ (~0.5) and $I_{MD}$ (~1) indicate that the steroids in the mixture interact with (a) common receptor(s). Black bars ('across-group'): values for $I_{CI}$ (~1) and $I_{MD}$ (>1) indicate that the steroids in the mixture act on different receptors. The numbers in the bars indicate sample size. *Significant differences. Kruskal–Wallis ANOVA on ranks followed by Dunn's method, multiple comparisons *versus* the 20α-P-3-G and 20β-P-3-G mix as control group. $I_{CI}$ in A: $H$=67.6369, d.f.=9, $P$=<0.001; $I_{MD}$ in B: $H$=62.672, d.f.=9, $P$<0.001.

of the adapting odorant. Surprisingly, however, with TCD as the adapting odorant, responses to the test steroids were considerably reduced (by 50% or more), although never as low as those of the SAC. It is possible that TCD may act as partial agonist, or antagonist, at the 3G-R-I receptor sites when present at high concentrations.

### Binary mixture tests

The results of binary mixture experiments were generally consistent with those of the cross-adaptation tests. Mean 'within-group' $I_{CI}$ and $I_{MD}$ values were 0.49 and 0.99, even lower than those obtained previously for *A. burtoni* (0.63 and 1.26; Cole and Stacey, 2006) and fitting nearly perfectly to the expected values (<1 and 1) for shared receptor groups. The mean 'across-group' $I_{MD}$ value of 1.45 exceeded the predicted value of 1, suggesting receptor independence. However, the mean 'across-group' $I_{CI}$ value of 0.77 was below the expected value of 1, and lower than 'across-group' values observed from *A. burtoni* (0.94; Cole and Stacey, 2006) and the sea lamprey (0.97; Li and Sorensen, 1997). However, 'across-group' mixtures do not always reach the perfect $I_{CI}$ value of 1, as seen by Caprio et al. (Caprio et al., 1989) upon using amino acid odorants in the channel catfish, *Ictalurus punctatus*. The authors of that study suggest that different receptor site types present on the same receptor neuron may not be as independent as different receptor site types on different neurones, leading to slightly reduced responses in binary mixture tests. It is possible that the 3G-R-I and 3G-R-II receptor types of *O. mossambicus* are present in the same receptor cell. Given the number of discrepancies between the predicted and observed values for cross-adaptation and binary mixture studies, it is possible that multiple receptor subtypes are present, with partially overlapping specificities.

### The olfactory receptor type 3G-R-I detecting the tilapia sex pheromone is specific for 5β,3α reduced 3-glucuronidated steroids

All 3-glucuronidated steroids induced EOG responses of similar amplitude in both males and females, which is consistent with earlier EOG studies in *O. mossambicus* (Keller-Costa et al., 2014) and other teleost species, e.g. *A. burtoni* (Cole and Stacey, 2006), goldfish (Sorensen and Goetz, 1993) or round goby (Murphy et al., 2001). In agreement with previous findings (Frade et al., 2002), the olfactory epithelium of *O. mossambicus* did not respond to unconjugated steroids, nor to a variety of 17- or 20-conjugated steroids, nor to E2-3-S, and it was insensitive to prostaglandins ($PGF_{2\alpha}$ and 15K-$PGF_{2\alpha}$). This suggests that the olfactory receptors for steroid detection in *O. mossambicus* require a glucuronate group at position C3. Structure and three-dimensional orientation of the cyclohexane ring 'A' seem to determine whether the ligand is detected by 3G-R-I or 3G-R-II. However, at least in case of 3G-R-I, some freedom in the functional group or aliphatic chain attached to C17 in cyclopentane ring 'D' of the steroid ligand is possible, although apparently this can affect affinity.

The role of 20one-P-3-G and ETIO-3-G as putative reproductive pheromones has been discussed previously in other teleost species. Testis-derived ETIO-3-G from black goby males (*Gobius niger*) attracts ripe females (Colombo et al., 1980). A similar observation was made of African catfish (*Clarias gariepinus*) males, where the most potent testicular odorant was found to be 20one-P-3-G (Lambert and Resink, 1991). Androstanes and pregnanes with a 5β,3α configuration are also potent odorants for the round goby (*Neogobius melanostomus*, Pallas 1814) (Murphy et al., 2001), and recent studies have demonstrated that round goby males release several conjugated forms of these steroids via their urine (Katare et al., 2011), eventually to attract females (Tierney et al., 2013). However, in the round goby, the olfactory receptors detecting ETIO-3-G appear to be less specific than those in tilapia; several unconjugated androstanes, pregnanes and even androsten, are detected by the same (ETIO-3-G) receptor site (Murphy et al., 2001).

20one-P-3-G and ETIO-3-G are not natural constituents of male tilapia urine (although it is possible that they are released via other routes; T.K.-C., A.V.M.C. and P.C.H., unpublished observations). It remains to be seen whether 20one-P-3-G and ETIO-3-G are able to activate the same signal cascade that triggers the endocrine response in females as 20α-P-3-G and 20β-P-3-G. If so, ETIO-3-G or 20one-P-3-G could be valuable for future research, avoiding the time

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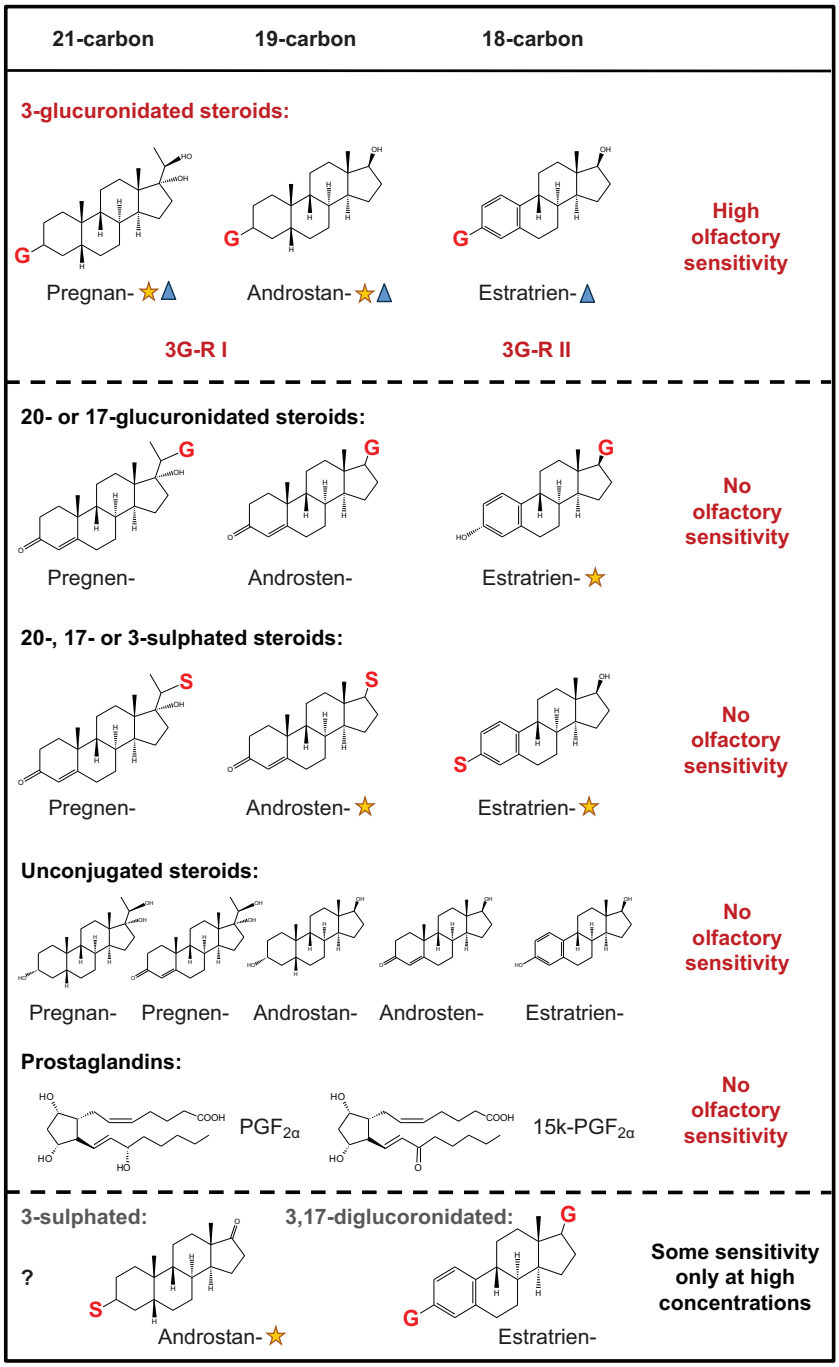


**Fig. 6. Summary of olfactory sensitivity and receptor specificity to steroids in *O. mossambicus*.** The red letters on steroid structures indicate conjugation position; G=glucuronate, S=sulphate. The Mozambique tilapia is highly sensitive to 3α-glucuronidated steroids, and cross-adaptation and binary mixture tests suggest two distinct olfactory receptor types, 3G-R-I and 3G-R-II. Tilapia do not exhibit EOG responses to prostaglandins, unconjugated steroids, 17- or 20-conjugated steroids or E2-3-S (this study) (Frade et al., 2002). Blue triangles indicate steroids that are detected by the phylogenetically close but allopatric relative *O. niloticus* (Hubbard et al., 2014). Yellow stars indicate steroids that are detected by the more recently derived East African cichlid *A. burtoni* (Cole and Stacey, 2006). 15k-$PGF_{2\alpha}$, 15-keto-prostaglandin $F_{2\alpha}$; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$.

intensive and expensive synthesis of 20α-P-3-G and 20β-P-3-G, respectively.

### Tilapia detects a putative social cue from females, E2-3-G, via a distinct olfactory receptor type 3G-R-II

The low detection threshold shows that *O. mossambicus* is highly sensitive to E2-3-G, and the low apparent $EC_{50}$ value suggests a high affinity for 3G-R-II. By contrast, the low apparent $I_{max}$ value may indicate a relatively small number of receptor neurones in the epithelium responding to this odorant. Because 17β-estradiol is produced by the growing follicle, E2-3-G could act as a social cue that is released by female tilapia, providing information on their reproductive condition. Males are capable of discriminating preovulatory *versus* post-spawning females through the smell of the females' urine (Almeida et al., 2005). Moreover, they drastically increase their own urination frequency in the presence of a female that is near ovulation, but not with post-spawn females (Miranda et al., 2005). Preovulatory females release, overall, more E2 into the water than post-spawn females (Huertas et al., 2014), and urine from preovulatory females contains large quantities (100–150 ng $ml^{-1}$) of 17β-estradiol (3 and/or 17)-glucuronate (M. Huertas, O. G. Almeida, A.V.M.C. and P.C.H., unpublished observations). E2-3-G is also a potent odorant for the round goby, *Neogobius melanostomus*, where it increases the ventilation rate (opercula movements per minute) in males, but not females (Murphy et al., 2001). Future investigations will determine whether E2-3-G is released by preovulatory tilapia females in their urine and whether it does indeed function as a chemical signal.

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### Comparison of *O. mossambicus* with a more recently derived African cichlid, *A. burtoni*

As shown recently, *O. niloticus*, like its close but allopatric relative *O. mossambicus*, responds to the same types of 3-glucuronidated steroids, and both fish release similar reproductive signals via their urine (Hubbard et al., 2014). This suggests that, in the allopatric river-dwelling *Oreochromis* (Lowe-McConnell, 1991), reproductive chemical cues have not been subject to differing selective pressures. Comparison of *O. mossambicus* to the more recently derived *A. burtoni*, which in Lake Tanganyika co-occurs with >150 other cichlid species (Greenwood, 1991), suggests that both *O. mossambicus* and *A. burtoni* have one olfactory receptor type for 5β,3α-reduced 3-glucuronidated steroids in common (3G-R-I). But they also show substantial difference in the steroid types that they detect. In addition to 3G-R-I, *A. burtoni* possesses four other independent (putative) receptor sites recognising 17-glucururonidated-, 3-sulphated-, 17-sulphated- and 3,17-disulphated steroids (Cole and Stacey, 2006). The olfactory sensitivity of *O. mossambicus* to disulphated steroids has not been investigated (mainly owing to limited commercial availability and high cost), but this species appears to be largely insensitive to other steroid conjugates. However, it is able to distinguish E2-3-G via a distinct olfactory receptor type (3G-R-II), an ability that *A. burtoni* apparently lacks (Cole and Stacey, 2006). Common to both of these cichlids is that they neither detect prostaglandins nor unconjugated steroids (of those that have been tested). In this they differ substantially from Cypriniformes, such as goldfish (Sorensen et al., 1988; Sorensen et al., 1995) and carp (Lim and Sorensen, 2011; Lim and Sorensen, 2012); Salmoniformes, e.g. Atlantic salmon (Moore and Waring, 1996), brown trout and brook trout (Essington and Sorensen, 1996) and Arctic char (Sveinsson and Hara, 2000); and even the Perciformes, such as the round goby (Murphy et al., 2001). It would be interesting to investigate more cichlid species to establish whether insensitivity to prostaglandins and unconjugated steroids is a general feature of the Cichlidae (Stacey, 2010; Stacey and Sorensen, 2009).

Unfortunately, it is not yet known whether *A. burtoni* releases any of the five steroid types that it is able to detect. One study reports that *A. burtoni* males increase serum testosterone levels in response to a mixture of representatives of the five steroid types (Cole and Stacey, 2003), but not when presented with only one type alone. The reproductive biology and social organisation of *A. burtoni* and *O. mossambicus* are comparable in several ways; both are maternal mouth-brooders and arena spawners (Bruton and Boltt, 1975; Fernald and Hirata, 1977). In both species, males establish hierarchies and increase urination frequency during aggressive encounters with rivals or when courting females (Barata et al., 2008; Barata et al., 2007; Maruska and Fernald, 2012). It is therefore possible that *A. burtoni*, as *O. mossambicus*, releases the steroid types that it detects (or at least some of them) via its urine, playing (a) similar pheromonal role(s) as in the Mozambique tilapia. However, the larger number of receptors suggests greater complexity and/or differences in the meaning of the steroidal 'message'.

Comparison of (only) two African cichlids shows that there is great variability in the types of conjugated steroids that they detect, indicating substantial diversity in olfactory steroid receptors among different species. Clearly, future studies should include more representatives – sympatric and allopatric – from different genera and clades within the Cichlidae to assess whether there is any link between the diversity of steroid receptor types, ecology and phylogeny. In addition, the biological significance of these receptors, i.e. pheromonal function and release routes of the detected steroids, needs to be explored. Such insights may shed light on the exciting question of whether chemical communication could have been among the drivers of African cichlid radiation.

In conclusion, the Mozambique tilapia has evolved high olfactory sensitivity and specificity to 3-glucuronidated steroids. Apparently, two distinct receptor sites are involved; one (3G-R-I) detecting a male sex pheromone (i.e. 20α-P-3-G and 20β-P-3-G) and a second (3G-R-II) detecting 17β-estradiol 3-glucuronide, which may function as a (preovulatory) female pheromone.

## MATERIALS AND METHODS

### Fish

Fish care and experimentation complied with the guidelines of the European Union Council (86/609/EU) and Portuguese legislation for the use of laboratory animals under a 'Group-1' licence issued by the Veterinary General Directorate of the Ministry of Agriculture, Rural Development and Fisheries of Portugal. Sexually mature Mozambique tilapia were raised in captivity from a brood-stock maintained at the University of Algarve (Faro, Portugal). Males and females were kept together in large 500 l stock tanks with a sandy substratum, aerated freshwater at 27°C, under a 12 h light:12 h dark photoperiod and were fed daily with commercial cichlid feed (Sparos Lda, Portugal).

### Odorants

The odorants tested in this study are given in Table 1. Test odorants (steroids, bile acid, L-serine) were purchased from Steraloids (Newport, RI, USA) or Sigma-Aldrich. The male tilapia sex pheromone components 5β-pregnan-3α,17α,20β-triol 3-glucuronate and 5β-pregnan-3α,17α,20α-triol 3-glucuronate were synthesised from the precursor 3α,17-dihydroxy-5β-pregnan-20-one as described previously (Keller-Costa et al., 2014). All steroids, prostaglandins and the bile acid were dissolved in ethanol or methanol at 1 mmol $l^{-1}$ (stock solution) and stored at −20°C until use. Stock solutions were diluted to the appropriate dilution in charcoal-filtered tap-water immediately prior to use in EOG recording (see below). A solution of 10 µmol $l^{-1}$ L-serine to normalise EOG responses was similarly prepared from 1 mmol $l^{-1}$ aliquots (in water) stored at −20°C.

### EOG recording

The method for EOG recording in tilapia has been described previously in detail (Frade et al., 2002). Briefly, tilapia were anaesthetised with $NaHCO_3$-buffered MS222 (3-aminobenzoic acid ethyl ester, Sigma-Aldrich) in water (200 mg $l^{-1}$) and immobilised with 3 mg $kg^{-1}$ gallamine triethiodide (Sigma-Aldrich). They were then maintained in a purpose-built padded 'fish-box', with 100 mg $l^{-1}$ MS222 in aerated water pumped over the gills within a Faraday cage. The olfactory rosette was exposed by cutting away a ring of skin and bone around the nostril, and a glass tube with a constant flow of freshwater (4–6 ml $min^{-1}$) was placed close to the raphe. Stimulus solutions were introduced into this flow by a computer-controlled solenoid valve. Borosilicate glass micropipettes filled with 4% agar in 0.9% NaCl were placed near the centre of the rosette (recording electrode) and lightly in contact with the skin of the head nearby (reference electrode). The direct current voltage signal was amplified (either Neurolog NL102, Digitimer, Welwyn Garden City, UK or Grass AC/DC strain gauge CP122; Astro-Med, West Warwick, RI, USA) and digitised (Digidata 1322A, Axon Instruments, now Molecular Devices, LLC, Sunnyvale, CA, USA). To determine which steroids and prostaglandins *O. mossambicus* detects, 3–6 mature fish were exposed to 4 s pulses of increasing concentrations (from 1 nmol $l^{-1}$ to 1 µmol $l^{-1}$). Odorants that did not evoke olfactory responses, or only inconsistent responses at high concentrations, were excluded from further concentration–response, cross-adaptation and binary mixture studies (Table 1). Consistent responses were obtained from the bile acid TCD, and all tested 3-glucuronidated steroids and EOG concentration–response curves that had been generated. Mature female [$N$=10–14; mean ± s.d.: body weight (BW)=44.1±34.7 g; standard length (SL)=120.3±44.6 mm] and male ($N$=8–14; BW=35.1±11.4 g; SL=106.4±11.5 mm) recipients were exposed to increasing concentrations from 1 pmol $l^{-1}$ to 10 µmol $l^{-1}$ in $\log_{10}$



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molar increments (plus 50 nmol $l^{-1}$) of 4 s odour pulses, allowing at least 1 min between exposures. Given the sigmoidal shape of these curves, apparent maximal olfactory response ($I_{max}$), apparent half-maximal effective concentration ($EC_{50}$) and apparent Hill coefficient values were calculated by fitting a sigmoidal regression curve using the Hill equation [three parameter: $y=ax^b/(c^b + x^b)$; $a=\max(y)=I_{max}$, $b$=Hill coefficient, $c=x50(x,y)=EC_{50}$] as a mathematical model, in which $y$ is the EOG response and $x$ is the $\log_{10}$ stimulus concentration. Two-way ANOVA analysis followed by the Holm–Sidak *post hoc* method for multiple pairwise comparisons was used to look for statistical differences within $I_{max}$ and $EC_{50}$ values.

### EOG cross-adaptation tests

Cross-adaptation studies including 20α-P-3-G, 20β-P-3-G, 20one-P-3-G and ETIO-3-G were performed at saturating concentration because response magnitudes were similar at 1 μmol $l^{-1}$, whereas considerable variation existed from 1 nmol $l^{-1}$ to 100 nmol $l^{-1}$ concentrations (linear part of the sigmoidal curves). Firstly, EOG responses to 4 s pulses of 1 μmol $l^{-1}$ solutions of the steroids were recorded from mature males ($N$=6–12; mean ± s.d.: BW=46.9±18.1 g; SL=116.1±16.4 mm). A 1 μmol $l^{-1}$ solution of the adapting steroid was then used to perfuse the olfactory epithelium until the voltage stabilised (approximately 1 min). Then, a blank was recorded (1 μmol $l^{-1}$ adapting steroid in 1 μmol $l^{-1}$ adapting steroid). Test solutions (1 μmol $l^{-1}$ test steroid in 1 μmol $l^{-1}$ adapting steroid) were then administered as 4 s pulses, beginning with the adapting steroid (the SAC at 2 μmol $l^{-1}$). The bile acid TCD at 10 μmol $l^{-1}$ was included as a control; it is a potent odorant for tilapia (Huertas et al., 2010) and is steroidal in nature but is likely to act via a different olfactory receptor mechanism. Initial EOG responses to the steroids before cross-adaption were blank-subtracted using the response to blank water, the same water used to dilute stimuli. EOG responses to the test solutions during adaptation were blank-subtracted using the adapted response to the 1× 1 μmol $l^{-1}$ adapting steroid blank. EOG responses to the test solutions during adaptation were then converted to a percentage of the initial (unadapted) response (% $R_I$).

Cross-adaptation involving E2-3-G was performed separately on males ($N$=5–7; BW=73.3±19.5 g; SL=135.4±12 mm) at 10 nmol $l^{-1}$ concentrations because the response amplitudes of 20α-P-3-G and 20β-P-3-G were roughly comparable to those in response to E2-3-G, at 10 nmol $l^{-1}$, whereas enormous differences existed at 1 μmol $l^{-1}$.

For each cross-adaptation dataset, mean % $R_I$ values were compared using Kruskal–Wallis ANOVAs on ranks followed by Dunn's *post hoc* method with multiple comparisons *versus* those of the SAC.

### EOG binary mixture tests

Odorants at 1 μmol $l^{-1}$ (10 μmol $l^{-1}$ for TCD) were tested at the same concentrations as those used in cross-adaptation tests on six to fourteen mature males (mean ± s.d.: BW=49.5±22.9 g; SL=117.3±19.3 mm). Tests involving E2-3-G were performed at 10 nmol $l^{-1}$ on nine mature tilapia males (BW=68.7±20.6 g; SL=132.3±13.6 mm). First, fish were exposed consecutively to steroid A (response $R_A$) and B ($R_B$) at $x$ mol $l^{-1}$, then to steroids A ($R_{2A}$) and B ($R_{2B}$) at twice the concentration (2× $x$ mol $l^{-1}$) and finally to a mixture of A and B (each at $x$ mol $l^{-1}$) to induce response $R_{A+B}$. The independent component index $I_{CI}$ (Eqn 1) and the mixture discrimination index $I_{MD}$ (Eqn 2) were calculated as reported previously (Kang and Caprio, 1991; Li and Sorenson, 1997; Cole and Stacey 2006).

$$I_{CI} = \frac{R_{A+B}}{(R_A + R_B)}, \quad (1)$$

$$I_{MD} = \frac{R_{A+B}}{0.5(R_{2A} + R_{2B})}. \quad (2)$$

The $I_{CI}$ is predicted to be around 1 in the case of independent receptor mechanisms and below 1 (approximately 0.5) in the case of shared receptor mechanism(s). The $I_{MD}$ is predicted to be 1 in the case of a shared receptor and >1 if there is receptor independence. Kruskal–Wallis ANOVA analyses on ranks followed by the Dunn's *post hoc* method with multiple comparisons *versus* a control group (20α-P-3-G and 20β-P-3-G mix) were used to compare the binary mixture results.

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#### Competing interests

The authors declare no competing financial interests.

#### Author contributions

T.K.-C., A.V.M.C. and P.C.H. conceived the study. T.K.-C. and P.C.H. performed experiments and data analysis. T.K.-C., A.V.M.C. and P.C.H. prepared the manuscript.

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