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

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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-pregn-3α,17α,20β-triol 3-glucuronate and its 20α-epimer, via their urine. The objective of this study was to assess sensitivity, specificity and versatility of the olfactory system of O. mossambicus to other steroids and their conjugates using the electro-olfactogram. O. 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 (10^{-12} M to 10^{-5} M) of 5β-pregn-3α,17α,20β-triol 3-glucuronate, 5β-pregn-3α,17α,20α-triol 3-glucuronate, 3α,17α-dihydroxy-5β-pregn-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 androsta-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 much in its olfactory perception from to the more recently derived East African cichlid Astatotilapia burtoni, suggesting that chemical communication could, indeed, be involved in speciation.

Abbreviations:

BW: Body weight
EOG: Electro-olfactogram
E2-3-G: 17β-estradiol 3-glucuronate
E2-3-S: 17β-estradiol 3-sulphate
E2-3,17-diG: 17β-estradiol 3,17-diglucuronate
ETIO-3-G: 5β-etiocholan-3α-ol-17-one 3α-glucuronate
ETIO-3-S: 5β-etiocholan-3α-ol-17-one 3α-sulphate
15K-PGF_{2α}: 15-keto-prostaglandin F_{2α}
20one-P-3-G: 3α,17α-dihydroxy-5β-pregn-20-one 3α-glucuronate
17,20β-P: 17α,20β-dihydroxypregn-4-en-3-one
20α-P-3-G: 5β-pregnane-3α,17α,20α-triol 3α-glucuronate
20β-P-3-G: 5β-pregnane-3α,17α,20β-triol 3α-glucuronate
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<th></th>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>63</td>
<td>PGF$_{2\alpha}$</td>
<td>Prostaglandin F$_{2\alpha}$</td>
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<td>64</td>
<td>SAC:</td>
<td>Self-adapted control</td>
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<tr>
<td>65</td>
<td>s.d.</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>66</td>
<td>s.e.m.</td>
<td>Standard error of the mean</td>
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<tr>
<td>67</td>
<td>SL:</td>
<td>Standard length</td>
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<tr>
<td>68</td>
<td>TCD:</td>
<td>Taurochenodeoxycholic acid</td>
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**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 wherefrom 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-adaptation tests, the response amplitude to one test odorant is measured prior to adaptation and then again during adaptation to a second odorant. If 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 responses to the individual odorants given alone. Conversely, EOG responses to the mixture that are smaller or equivalent to twice the concentration of either odorant indicate (a) shared olfactory receptor(s) (Cole and Stacey, 2006). In goldfish (*Carassius auratus*), for example, EOG recordings including cross-adaptation and binary mixture tests established that the pre- and postovulatory 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 postovulatory goldfish pheromone, on the other hand, consists of F-type prostaglandins, mainly PGF₃α and 15K-PGF₂α, which, too, have distinct olfactory receptor sites (Sorensen et al., 1988).

Within the Perciformes, the largest teleost order (Fishbase, 2014b), 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 1,670 described species (Fishbase, 2014a), 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 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, 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* synthesizes
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 alpha-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-dihydroxyprogren-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 not known
whether prostaglandins or other steroids, including steroids 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 address the hypothesis of chemical signal diversification as a driver for African cichlid
radiation.

Thus, the objectives of this study were, firstly, to assess olfactory sensitivity of *O. mossambicus* to steroids and, secondly, to establish, by cross-adaptation and binary mixture tests, whether steroid odorants act via shared or independent olfactory receptors; and thirdly, to compare these results to findings in *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. Neither did they respond to any unconjugated steroids, E2-3-S or prostaglandins, even at concentrations as high as 1 µM (Table 1, Fig. 6). ETIO-3-S and E2-3,17-diG induced small EOG responses, yet only at high concentrations (10^{-7} M and 10^{-6} M). However, the latter responses were not consistent and were not pursued further.

Sigmoidal concentration response curves were obtained from 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 pM), followed by 20-one-P-3-G (100 pM) and for the majority was around 1 nM. EOG response amplitudes of 20α-P-3-G, 20β-P-3-G, 20-one-P-3-G and ETIO-3-G increased rapidly before reaching an apparent maximum around 1 µM, which suggests saturation of the olfactory receptors (Fig. 1). For E2-3-G, both the EOG amplitude and saturation (1nM) was much lower. Accordingly, the E2-3-G apparent half-maximal effective concentration EC_{50} (mean ± s.e.m.; ♂ 0.07 ± 0.02 nM; ♀ 0.14 ± 0.08 nM) and apparent maximal olfactory response I_{max} (♂ 0.38 ± 0.05; ♀ 0.34 ± 0.04) were lower than 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 (♂ 89.62 ± 16.15 nM; ♀ 86.3 ± 18.74 nM) and ETIO-3-G (♂ 54.38 ± 18.4 nM; ♀ 40.74 ± 10.92 nM) had similar and highest apparent EC_{50} values, followed by 20β-P-3-G (♂ 25.72 ± 9.73 nM; ♀ 30.12 ± 12.92 nM) and 20-one-P-3-G (♂ 4.78 ± 1.01 nM; ♀ 3.67 ± 0.71 nM; Figure 2 A). Apparent I_{max} of 20α-P-3-G, 20β-P-3-G and ETIO-3-G were similar and nearly twice the response to 10^{-5} M L-serine (Fig. 2B). Apparent I_{max} values of male (but not female) responses to 20-one-P-3-G were lower than 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 co-operativity.

The concentration-response curve for TCD showed a rapid increase of EOG amplitudes at supra-threshold (around 10 nM) concentrations, without reaching an apparent maximum up to 10 µM (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 and sequential exposure with steroids during cross-adaptation did not desensitize the olfactory epithelium, responses to the steroids were again recorded after a 10 minute wash-out, and compared to the initial unadapted responses. No reduction of EOG responses was observed for any of the tested steroids, regardless of the concentration. However, significant increases (mean%) in mean EOG response amplitudes were noted for some steroids, i.e.
20β-P-3-G (~27%) and 20one-P-3-G (~26%) at 10⁻⁶ M and 20α-P-3-G (22%) at 10⁻⁸ M 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; since responses to test steroids during adaptation were compared only to the initial responses recorded before cross-adaptation, the increase noted here for some steroids should not influence any conclusions drawn.

The results of the EOG cross-adaptation studies at 10⁻⁶ M (10⁻⁵ M 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 ‘3G-R-I’ (Fig. 6), referring to the position of the glucuronate on the steroid. 20β-P-3-G and 20one-P-3-G consistently reduced 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. 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, mean responses to all administered test-steroids were reduced by at least 57%, although they remained significantly higher (except for ETIO-3-G) than the SAC (Fig. 6).

Given the distinct concentration response curve of E2-3-G, we hypothesized this steroid to act *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 10⁻⁸ M, as EOG amplitudes of the three steroids were similar at this concentration, rather than at 10⁻⁶ M (Fig. 1). E2-3-G could not reduce the responses to 20α-P-3-G or 20β-P-3-G below 70% 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 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 ‘3G-R-II’ (Fig. 6).

**EOG binary mixture tests**

The mean independent component index (*I*_sci) and mixture discrimination index (*I*_md) of the binary mixture 20α-P-3-G/20β-P-3-G were around 0.5 and 1.0, respectively, and consistent with the cross-adaptation studies, strongly suggesting a shared olfactory receptor mechanism. Mean *I*_sci 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 the 20α-P-3-G/20β-P-3-G mix (Fig. 5). The mean ‘within-group’ *I*_sci 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 $20\alpha$-P-3-G or $20\beta$-P-3-G mixed with E2-3-G were generally closer to 1 and significantly different ($P < 0.001$) from the $20\alpha$-P-3-G/$20\beta$-P-3-G mix, indicating that E2-3-G is detected by a different receptor type, 3G-R-II. Mean $I_{MD}$ values for the $20\beta$-P-3-G/E2-3-G mix and the $20\alpha$-P-3-G/E2-3-G mix were above 1, which further supports receptor independence. Yet, only for the $20\alpha$-P-3-G/E2-3-G mix (but not the $20\beta$-P-3-G/E2-3-G mix) was $I_{MD}$ statistically different from $20\alpha$-P-3-G/$20\beta$-P-3-G mix.

Both $I_{CI}$ and $I_{MD}$ values of TCD mixed with $20\alpha$-P-3-G, $20\beta$-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 $20\alpha$-P-3-G/$20\beta$-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 (Man Whitney rank sum tests, $P = 0.002$).
Discussion

This study demonstrates that the Mozambique tilapia possesses high olfactory sensitivity to several 3-glucuronidated steroids 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. 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 in cross-adaptation with 20α-P-3-G or ETIO-3-G as adapting steroid and 20β-P-3-G and/or 20one-P-3-G as test odorant; responses were reduced but not to the extent of the self-adapted control (SAC). This may be explained by the lower apparent EC50 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 EC50), 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 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 of the adapting odorant. Surprisingly, however, with TCD as the adapting odorant, responses to the test steroids were considerably reduced (50% or more) although never as low as 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

Results of binary mixture experiments were generally consistent with those of the cross-adaptation tests. Mean ‘within-group’ ICI and IMD values were 0.49 and 0.99, even lower than those obtained for A. burtoni (0.63 and 1.26; Cole and Stacey, 2006) and fitting nearly perfectly the expected values (<1 and 1) for shared receptor groups. The mean ‘across-group’ IMD value of 1.45 exceeded the predicted value of 1, suggesting receptor independence. However, the mean ‘across-group’ ICI 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 ICI value of 1, as seen by (Caprio et al., 1989) with amino acid odorants in the channel catfish, Ictalurus punctatus. The authors suggested 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 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 similar amplitude EOG responses 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-, nor to a variety of 17- or 20-
conjugated steroids, nor to E2-3-S and it was insensitive to prostaglandins (PGF₂α and 15K-PGF₂α).
This suggests that the olfactory receptors for steroid detection in O. mossambicus require a
3-glucuronate at position C3. Structure and 3-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 from 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 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 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 they are released via other routes; unpublished observations). It remains to be seen if 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 intensive and expensive synthesis of 20α-P-3-G and 20β-P-3-G,
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 EC₅₀ value suggests high affinity to 3G-R-II. On the other hand, the low apparent Iₘₐₓ may indicate a relatively small number of receptor neurones in the epithelium responding to this odorant. Since 17β-estradiol is produced by the growing follicle, E2-3-G could act as a social cue released by female tilapia, providing information on their reproductive condition. Males are capable of discriminating pre-ovulatory 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).

Pre-ovulatory females release overall more E2 into the water than post-spawn females (Huertas et al., 2014) and urine from pre-ovulatory females contains large quantities (100 - 150 ng.ml⁻¹) of 17β-estradiol (3 and/or 17)-glucuronate (unpublished observations). E2-3-G is also a potent odorant for the round goby, *Neogobius melanostomus*, where it increases ventilation rate (opercula movements per minute) in males, but not females (Murphy et al., 2001). Future investigations will determine if E2-3-G is released by pre-ovulatory tilapia females into their urine and if it functions indeed as a chemical signal.

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

As shown recently, *O. niloticus*, a closely related but allopatric relative of *O. mossambicus* respond to the same types of 3-glucuronidated steroids and both 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 pressure.

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*, too 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 they detect. In addition to 3G-R-I, *A. burtoni* possesses four other independent (putative) receptor sites recognizing 17-glucuronidated-, 3-sulphated-, 17-sulphated- and 3,17-disulphated steroids (Cole and Stacey, 2006). The olfactory sensitivity of *O. mossambicus* to di-sulphated steroids has not been investigated (mainly due to limited commercial availability and 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 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, 1995) 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 it is able to detect. One study reported 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 only one type alone. The reproductive biology and social organization 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 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 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 detected steroids, needs to be explored. Such insights may shed light on the exciting question if 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 (pre-ovulatory) 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” license 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 27ºC under a 12L:12D photoperiod and 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 Inc. (Newport, RI, USA) or Sigma-Aldrich (Spain). The male tilapia sex pheromone components 5β-pregn-3α,17α,20β-triol 3-glucuronate and 5β-pregn-3α,17α,20α-triol 3-glucuronate were synthesized from the precursor 3α,17-dihydroxy-5β-pregn-20-one as described previously (Keller-Costa et al., 2014). All steroids, prostaglandins and the bile acid were dissolved in ethanol or methanol at 10⁻³ M (stock solution) and stored at -20 ºC 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 10⁻⁵ M L-serine to normalize EOG responses was similarly prepared from 10⁻³ M aliquots (in water) stored at -20 ºC.

Electro-olfactogram (EOG) recording
The method for EOG recording in tilapia has been described in detail (Frade et al., 2002). Briefly, tilapia were anaesthetized with NaHCO₃-buffered MS222 (3-aminobenzoic acid ethyl ester, Sigma-Aldrich) in water (200 mg.l⁻¹) and immobilized 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 DC voltage signal was amplified (either Neurolog NL102, Digitimer Ltd, Welwyn Garden City, UK or Grass AC/DC strain gauge CP122; Astro-Med, West Warwick, RI, USA) and digitized (Digidata 1322A, Axon Instruments, Inc., 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 10⁻⁹ to 10⁻⁶ M). 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 generated. Mature female (N = 10-14; mean ± s.d.: BW = 44.1 ± 34.7 g; 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 10^{-12} M to 10^{-5} M in log_{10} molar increments (plus 5 x 10^{-8} M) of 4 s odour pulses allowing at least 1 min between exposures. Given the sigmoidal shape of these curves, apparent maximal olfactory response (I\text{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 [3 parameter: y = ax^b/(c + x^b); a = max(y) = I\text{max}; b = Hill co-efficient; c = x50(x,y) = EC_{50}] as mathematical model, in which y is the EOG response and x is the log_{10} stimulus concentration. Two-way (TW) ANOVAs followed by the Holm-Sidak post-hoc method for multiple pairwise comparisons were used to look for statistical differences within I\text{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 10^{-6} M, whereas considerable variation existed from 10^{-9} M to 10^{-7} M concentration (linear part of the sigmoidal curves). Firstly, EOG responses to 4 s pulses of 10^{-6} M 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 10^{-6} M solution of the adapting steroid was then used to perfuse the olfactory epithelium until voltage stabilised (about one minute). Then, a blank was recorded (10^{-6} M adapting steroid in 10^{-6} M adapting steroid). Test solutions (10^{-6} M test steroid in 10^{-6} M adapting steroid) were then administered as 4 s pulses, beginning with the adapting steroid (the self-adapted control (SAC) at 2 x 10^{-6} M). The bile acid TCD at 10^{-5} M was included as a control; it is a potent odorant for tilapia (Huertas et al., 2010), is steroidal in nature, but likely acts via a different olfactory receptor mechanism. Initial EOG responses to the steroids before cross-adaption were blank-subtracted using the response to blank water; same water used to dilute stimuli. EOG responses to the test solutions during adaptation were blank-subtracted using the adapted response to the 1 x 10^{-6} M 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^{-8} M concentrations because response amplitudes of 20α-P-3-G and 20β-P-3-G were roughly comparable to E2-3-G, at 10^{-8} M, whereas enormous differences existed at 10^{-6} M.
For each cross-adaptation dataset, mean % $R_1$ were compared using Kruskal-Wallis-ANOVAs on ranks followed by Dunn’s post-hoc method with multiple comparisons versus the self-adapted control (SAC).

**EOG binary mixture tests**

Odorants at $10^{-6}$ M ($10^{-5}$ M for TCD) were tested at the same concentrations 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 $10^{-5}$ M 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 $10^{-x}$ M, then to steroids A ($R_{2A}$) and B ($R_{2B}$) at twice the concentration ($2 \times 10^{-x}$ M) and finally to a mixture of A and B (each at $10^{-x}$ M) to induce response $R_{A+B}$. The independent component index $I_{CI}$ (1) and the mixture discrimination index $I_{MD}$ (2) were generated as reported earlier (Kang & Caprio, 1991, Li & Sorenson, 1997, Cole & Stacey 2006).

\[ I_{CI} = \frac{R_A + R_B}{R_{2A} + R_{2B}} \]  

(1)

\[ I_{MD} = \frac{R_A + B}{0.5 (R_{2A} + R_{2B})} \]  

(2)

The $I_{CI}$ is predicted to be around 1 in case of independent receptor mechanisms and below 1 (about 0.5) in the case of shared receptor mechanism(s). The $I_{MD}$ is predicted to be 1 in case of a shared receptor and >1 if there is receptor independence. Kruskal-Wallis-ANOVAs on ranks followed by the Dunn’s post-hoc method with multiple comparisons versus a control group ($20\alpha$-P-3-G / $20\beta$-P-3-G mix) were used to compare the binary mixture results.
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Author contributions


Conflict of interest

The authors declare no conflicts of interest.
References


Figures and figure legends

Figure 1. EOG concentration response profiles. Normalised (to $10^{-5}$M L-serine response) EOG concentration-response (CR) 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 (Hill-3-parameter) curve was fitted to the response profiles of 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 $10^{-7}$ M odorant concentrations, are presented as inserts.

Figure 2. Comparison of apparent EC$_{50}$ and $I_{\text{max}}$ 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 nM; log$_{10}(y)+2$ -transformed values) and B) $I_{\text{max}}$ values for males ($N = 7-14$; black bars) and females ($N = 10-14$; open bars) for each steroid were similar. Different letters above bars indicate significant differences ($P < 0.001$) among steroids. Two-Way ANOVA followed by Holm-Sidak post-hoc test. $F$ and $P$ values were as follows: A) apparent EC$_{50}$ values - sexes: $F_{1,103} = 0.131$, $P = 0.781$; steroids: $F_{4,103} = 177.968$, $P < 0.001$, interaction sexes x steroids: $F_{4,103} = 0.257$, $P = 0.905$. B) apparent $I_{\text{max}}$ values - sexes: $F_{1,103} = 0.686$, $P = 0.409$; steroids: $F_{4,103} = 34.280$, $P < 0.001$; interaction sexes x steroids: $F_{4,103} = 0.397$, $P = 0.810$. All data were of equal variance.

Figure 3. EOG cross-adaptation studies. Relative EOG response (mean ± s.e.m.) to $10^{-6}$ M steroid 3α-glucuronates (or $10^{-5}$ M TCD) expressed as percentage of the initial unadapted response (% $R_{I}$) to the same $10^{-6}$ M steroid ($10^{-5}$ M TCD), delivered before cross-adaptation. Sharps # indicate the self-adapted controls (SAC). Numbers in bars indicate sample size. Asterisks * above bars indicate significant differences from the SAC ($P < 0.05$). Kruskal-Wallis ANOVA on ranks followed by Dunn’s method, multiple comparisons versus SAC as control group. 20β-P-3-G: $H = 28.951$, $df = 4$, $P < 0.001$. 20α-P-3-G: $H = 38.624$, $df = 4$, $P < 0.001$. 20one-P-3-G: $H = 16.136$, $df = 4$, $P = 0.003$. ETIO-3-G: $H = 23.243$, $df = 4$, $P < 0.001$. TCD: $H = 26.903$, $df = 4$, $P < 0.001$.

Figure 4. EOG cross-adaptation studies involving 17β-estradiol-3-G (E2-3-G). Relative EOG response (mean ± s.e.m.) to $10^{-8}$ M steroid conjugates expressed as percentage of the initial unadapted response (% $R_{I}$) to the same $10^{-8}$ M steroid delivered before cross-adaptation started. Sharps # indicate the self-adapted controls (SAC). Numbers in bars indicate sample size. Asterisks * above bars indicate significant differences from the SAC ($P < 0.05$). Kruskal-Wallis ANOVA on Ranks followed by
Dunn’s method, multiple comparisons versus SAC as control group. **E2-3-G:** $H = 11.523$, $df = 2$, $P = 0.003$. **20β-P-3-G:** $H = 9.420$, $df = 2$, $P = 0.009$. **20α-P-3-G:** $H = 6.371$, $df = 2$, $P = 0.041$.

**Figure 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 $I_{CI} (\sim 0.5)$ and $I_{MD} (\sim 1)$ indicate that the steroids in mixture interact with (a) common receptor(s). Black bars ('across-group'): values for $I_{CI} (\sim 1)$ and $I_{MD} (> 1)$ indicate that the steroids in mixture act on different receptors. Numbers in bars indicate sample size. Asterisks * above bars indicate significant differences. Kruskal-Wallis ANOVA on Ranks followed by Dunn’s method, multiple comparisons versus the mixture 20α-P-3-G/20β-P-3-G as control group: A) $I_{CI}$: $H = 67.6369$, $df = 9$, $P < 0.001$; B) $I_{MD}$: $H = 62.672$, $df = 9$, $P < 0.001$.

**Figure 6. Summary of olfactory sensitivity and receptor specificity to steroids in O. mossambicus.** 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. It does not exhibit EOG responses to prostaglandins, unconjugated, 17- or 20-conjugated steroids or E2-3-S (this study, Frade et al., 2002). Blue triangles indicate steroids detected by the phylogenetically close but allopatric relative *Oreochromis niloticus* (Hubbard et al., 2014). Yellow stars indicate steroids detected by the more recently derived East African cichlid *Astatotilapia burtoni* (Cole and Stacey, 2006).
Table 1 | Steroids tested in this study.

<table>
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<tr>
<th>Chemical class</th>
<th>Chemical group</th>
<th>Compound name</th>
<th>Abbreviation</th>
<th>Detection threshold</th>
<th>Saturation</th>
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<td>bile acid</td>
<td>24-carbon</td>
<td>taurochenodeoxycholic acid</td>
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<td></td>
<td>prostaglandin 15keto-F2α</td>
<td>15k-PGF2α</td>
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<td></td>
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¹ read estimates based on mean concentration response curves.
² concentration used in EOG cross-adaptation and binary mixture tests.
³ O. mossambicus does not have any olfactory sensitivity to this steroid
⁴ responses not consistent and EOG amplitudes small, therefore this steroid was excluded from further concentration-response, cross-adaptation and binary mixture tests.
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