Identity of a tilapia pheromone released by dominant males that primes females for reproduction

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Running title:

Male tilapia sex pheromone

Summary

Knowledge of the chemical identity and role of urinary pheromones in fish is scarce, yet necessary to understand the integration of multiple senses in adaptive responses and the evolution of chemical communication [1]. In nature, Mozambique tilapia (*Oreochromis mossambicus*) males form hierarchies and females mate preferentially with dominant territorial males which they visit in aggregations or leks [2]. Dominant males have thicker urinary bladder muscular walls than subordinates or females and store large volumes of urine which they release at increased frequency in the presence of subordinate males or pre-ovulatory, but not post-spawned, females [3-5]. Females exposed to dominant male urine augment the release of the oocyte maturation-inducing steroid 17α,20β-dihydroxypregn-4-en-3-one (17,20β-P) [6] and spawn in the vicinity of males artificially scented with urine from dominant males (own unpublished results). Here we isolate and identify a male Mozambique tilapia urinary sex pheromone as two epimeric (20α- and 20β-) pregnanetriol 3-glucuronates. We show that both males and females have high olfactory sensitivity to the two steroids which cross adapt upon stimulation. Females exposed to both steroids show a rapid, 10-fold increase in production of 17,20β-P. Thus, the identified urinary steroids prime the female endocrine system to accelerate oocyte maturation and, possibly, spawning synchrony. Tilapia are globally important as a food-source and an invasive species, with devastating impact on local freshwater ecosystems [7, 8]. Knowing the chemical identity of the cues that mediate reproduction may lead to the development of tools for population control [9-11].

Results and Discussion

Bioassay guided fractionation of male urine samples

The Mozambique tilapia olfactory response to male urine and C18-SPE solid phase extracts of urine is positively correlated to social status (dominance index, DI) of the donor (Figure 1D). The dominance index DI of the donor males was assessed by daily observing the donor males dominant (aggressive displays, circling or mouth-to-mouth fights, courtship towards females, nest digging, dark colour) and subordinate (submissive displays, fleeing, light grey colour) behaviours [3, 5]. Urine samples were subsequently collected from dominant ($DI \geq 0.8$), intermediate ($0.16 < DI < 0.8$) and subordinate males ($DI \geq 0.16$). Bioassay-guided fractionation of dominant male urine extracts by high performance liquid chromatography (HPLC) with light scattering detection (ELSD), revealed one fraction (fraction A) with particularly strong olfactory potency (Figure 1A and 1E, [5]). Fraction A did not absorb ultraviolet radiation indicating absence of chromophores. Fraction A was also present but at lower intensity (Figure 1B and 1C) in urine from intermediate and subordinate individuals. Furthermore, peak area of fraction A taken from different individuals correlated positively with the amplitude of the olfactory responses of the recipients (Figure 1E; Spearman correlation, $r_s = 0.939$, $P < 0.001$) and with the donor DI (Figure 1A-C and 1F; Spearman correlation, $r_s = 0.743$, $P < 0.001$).

Identification, structure elucidation and quantification

Ultra-performance liquid chromatography coupled high-resolution mass spectrometry (UPLC-HRMS) of fraction A revealed the presence of two compounds with similar retention times (Figure S1A) and molecular ions at *m/z* 511.2908 and 511.2912, in negative ionization polarity (Figure S1B and C), indicating a neutral mass of the

compounds of 512 Da. The indicated molecular formula $C_{27}H_{44}O_9$ (Δppm 1.292) fits both compounds. Detailed ESI-MS^n studies in the positive polarity (Figure S1D-F) further revealed that these compounds contain a hexose acid moiety attached to a steroid backbone (aglycone). The consecutive losses of water observed after serial fragmentations indicated the presence of at least three oxygen atoms bound to the aglycone unit.

The proton-nuclear magnetic resonance (^{1}H NMR) spectrum confirmed the presence of two closely related compounds, i.e. stereoisomers (Figure 2C), as suggested by UPLC-HRMS. Comparison of the ^{1}H NMR spectrum of fraction A with the spectra of the standards 5β-pregnan-3,17,20β-triol (**2**; Figure S3A) and 5β-pregnan-3,17,20α-triol (**3**; Figure S3A) preliminarily identified the aglycone moiety of the urinary steroids as 5β-pregnan-3α,17,20-triols (Figure S2A-C). The combined use of ^{1}H-^{1}H and ^{1}H-^{13}C shift-correlated 2D NMR allowed full structural and stereochemical elucidation of the two steroid conjugates as the sodium salts of epimeric 5β-pregnan-3α,17α,20α-triol-3α-glucuronate (**14**, Figure 2D) and 5β-pregnan-3α,17α,20β-triol-3α-glucuronate (**10**, Figure 2E). To confirm their chemical structure and test for bioactivity, both isomers were synthesized from the precursor 3α,17-dihydroxy-5β-pregnan-20-one (**1**, Figure S3A) via a slightly modified previously described route [12] (Figures S3A-C). Retention times on liquid chromatography-mass spectrometry (LC-MS) (Figure 2F) and all spectral data for the synthetic and natural compounds were identical (Figure 2A-C and Figure S2).

Quantification by LC-MS of **14** and **10** in individual urine samples (mean ± SEM) unveiled, respectively, 2.1 ± 1.3 and 25.2 ± 8.6 µM in subordinate (N = 6) and 12.8 ± 3.8 and 229.5 ± 64.4 µM in dominant (N = 7) males. Furthermore, as with urine, urine extract and fraction A, the dominance index was positively correlated to the

urinary concentration (Figure 2G) of **10** (Spearman correlation, r_s = 0.790, P < 0.0001, N = 19) and **14** (Spearman correlation, r_s = 0.550, P = 0.0145, N = 19).

Olfactory sensitivity to the identified and synthesized compounds

Stimulation of the olfactory epithelium with increasing concentrations (10^{-11} M to 10^{-5} M) of the two synthetic pregnanetriol 3-glucuronates (Figure 3C) produced sigmoidal concentration-response curves in males (Figure 3A) and females (Figure 3B). The thresholds of detection were around 10^{-9} M and plateaus were reached at about 10^{-6} M. Interestingly, for both males and females compound **10** had a significantly lower (mean ± SEM) apparent EC_{50} (27.55 ± 7.76 nM) and maximum response I_{max} (1.8 ± 0.1) than compound **14** (88.18 ± 11.68 nM and 1.99 ± 0.12; two-way repeated measures ANOVA followed by Holm Sidak test; P < 0.001 and P = 0.019). The lower apparent EC_{50} indicate a higher affinity of **10** than **14** for the olfactory receptor(s). The similar apparent Hill-coefficients of about 1 for both isomers (%*R*: **14** 1.1±0.1 and **10** 1.2±0.1) are consistent with a 1:1 binding ratio to the receptor(s), with no cooperativity. No statistically significant correlation was found between the gonadosomatic index (size of the ovaries relative to body size; range 0.39% to 7.63%) and EC_{50} or I_{max} values of compound **10** (20β-epimer; Spearman correlation, N= 11, r_s = -0.491, P = 0.116 and r_s = 0.255 and P = 0.433, respectively) or **14** (20α-epimer; Spearman correlation, N= 11, r_s = -0.591, P = 0.051 and r_s = 0.109 and P = 0.734, respectively). This indicates that the olfactory sensitivity of females to the two identified steroids is independent of their reproductive condition.

There was no olfactory response to the aglycones of **2** and **3**, even at 10^{-6} M. Also, D-glucuronic acid sodium salt alone or a mixture of the aglycone steroids and D-glucuronic acid sodium salt at 10^{-6} M was not detected, neither were sulphated or C-17-

or C-20-glucuronidated androgens or progestogens [see also 13]. Similar olfactory sensitivity to **10** and **14** was only found to structurally related C-3α glucuronidated steroids (e.g. 3α,17-dihydroxy-5β-pregnan-20-one 3α-glucuronate or etiocholan-3α-ol-17-one 3α-glucuronate), which demonstrates that C-3α glucuronidation is essential for the olfactory response to the two isomers and suggests an olfactory receptor mechanism specific for 3α-reduced 3-glucuronates.

Whether the two compounds share common olfactory receptor mechanisms was tested by cross-adaptation in which the olfactory epithelium is first adapted to one isomer and then stimulated with the second [14]. In compound **10** adapted olfactory epithelium (Figure 3D), the response to compound **14** was the same as the self-adapted control, indicating (a) shared receptor mechanism(s). In contrast, adaptation to **10** or **14** failed to reduce the response to the bile acid taurochenodeoxycholic acid (TCD) to below 80% of the unadapted TCD alone, indicating independent receptor mechanisms. Reciprocal adaptation of **10** to **14** (Figure 3E) strongly reduced the response, unlike TCD, which further support (a) common olfactory receptor mechanism(s) for the two isomers. The difference in response of **10** compared to the self-adapted control may reflect the higher affinity of **10** for the olfactory receptor as indicated by the lower apparent EC_{50}. These results are consistent with **10** and **14** acting through (a) shared olfactory receptor(s) specific to 5β-reduced 3α-glucuronidated steroids and distinct from that of TCD.

Can females use this sensory information to assess the social status of the male? Compounds at urinary concentrations of **10** and **14** would be maximally detected by the olfactory system and the receiver would not be able to perceive the difference between the two (Figure 3). However, as the urine is released to the water it is diluted allowing for differential perception. For example, a 1/10,000 v/v dilution of dominant male urine

evokes strong olfactory [13] and endocrine responses in females [6]. Such a dilution would contain 23 nM of compound **10,** which is close to the EC_{50} value and lies on the steepest (i.e. linear) part of the EOG concentration response curve, while dilution of subordinate male urine would bring it to near or below detection limit. Furthermore, dilution of subordinate male urine would bring compound **14** necessarily to below the limit of detection. The effect of urine dilution together with the dynamics of urine release by dominant males [5] suggests a possible mechanism for females to distinguish dominant from subordinate males by sensing pregnanetriol 3α-glucuronate concentration (see also below).

Biological function of the identified compounds

Having established that both male and female tilapia are sensitive to **10** and **14** we tested whether the two synthetic steroids are sufficient to emulate the priming effect of male urine on maturation-inducing steroid production by females [6]. Pre- and post-spawned females were exposed separately to five different test stimuli: **i**) dominant male urine (1:10,000 v/v in tank water), **ii**) the corresponding C18-SPE extract (i.e. the steroids containing urine fraction), **iii**) the aqueous flow-through of C18-SPE, **iv**) 50 nM of a 4:1 mixture of the two isomers **10** and **14** and **v**) methanol control. Before stimulation, females in the five trials (N = 8) released 17,20β-P to the water at similar rates (mean ± SEM; 136 ± 11.1 ng kg^{-1} h^{-1}; Figure 4). One hour after applying the stimulus, females exposed to dominant male urine showed a highly significant increase (nearly 10-fold), in the release rate of 17,20β-P (1282 ± 247.7 ng kg^{-1} h^{-1}; Figure 4), in agreement with previous observations [6]. Similar increases in 17,20β-P release rates were also measured in females stimulated with the C18-SPE urine extract (1324 ± 250.5 ng kg^{-1} h^{-1}) and with the synthetic steroid mix (1970± 434.3 ng kg^{-1} h^{-1}). In contrast, no significant change in 17,20β-P release rates were seen when the stimulus was the

aqueous C18-SPE urine flow-through or methanol control. The slightly larger release rate of 17,20β-P after stimulation with the synthetic steroids as compared to raw urine or urine extract (Figure 4) may be related to the higher concentration of **14** and **10** in the synthetic steroid mixture (96.1 µM and 413.1 µM) than in the dominant male urine pool (17.7 µM and 263 µM). These results demonstrate that the identified pregnanetriol glucuronates are responsible for the observed priming effect of urine from dominant tilapia males. Steroidal pheromone mediated priming also exists in goldfish, in which females release the maturation-inducing steroid 17,20β-P (primarily via the gills) and its sulphate (primarily via the urine) to stimulate gonadotrophin secretion, milt production and sperm motility in males [15-17]. Although 17,20β-P and 17,20α-P (including their sulphate and glucuronate conjugates), which are produced in the testis [18, 19], are at higher concentration in urine of dominant Mozambique tilapia males than in subordinates [20], the species lack olfactory sensitivity to these steroids [13]. We suggest that the urinary pregnanetriol-3α-glucuronates **10** and **14** from dominant tilapia males are honest signals, carrying information to the female about the male`s reproductive performance, i.e. sperm-quality. Also, in the same way as urine (own unpublished results) the two pregnanetriol glucuronates **10** and **14** may have a releaser (i.e. behavioural) effect on female mate choice and spawning decision. Given that **10** is correlated with DI, is present at much higher concentrations and has higher olfactory potency than **14**, we predict that **10** is the main biologically active component. However, the relative contributions of both steroids remain to be investigated.

Although reproductive pheromones in teleosts have been the focus of several studies during the past two decades, only in goldfish (*Carassius auratus*) and the masu salmon (*Oncorhynchus masou*) [21] their identity is known and their biological functions defined. Yet in both of the aforementioned species, females are the signalling

sex and mating strategies and compounds released are different from the Mozambique tilapia. The current study presents not only the first chemical identification of a cichlid sex pheromone, but also the first sex pheromone from a teleost with a mating system in which territorial males signal to females, and that is strongly driven by female mate choice. Fish are the largest group of vertebrates and can use diverse signals for communication; visual cues, sound, electrical fields and chemicals. The chemical identification of a sex pheromone in male tilapia urine that primes the female reproductive system and possibly promoting spawning synchrony will stimulate further research into chemical communication and behaviour in particular and how different sensory information is integrated. This should shed light on the role of chemical communication on inter- and intra-sexual selection, and how the diversity of urinary pheromone signalling in freshwater fishes has shaped reproductive strategies, social structure and evolution.

Method summary

Animals – Mozambique tilapia were maintained in accordance with specific legislation for the use of laboratory animals under a "Group-1" license issued by the Ministry of Agriculture, Rural Development and Fisheries of Portugal. Mature male Mozambique tilapia, held in mixed sex groups, were tagged and observed daily to assess their social rank or dominance index (DI) and collect urine samples [3, 5] from dominant ($DI \geq 0.8$), intermediate ($0.16 < DI < 0.8$) and subordinate males ($DI \geq 0.16$).

Chemical identification – Urine was extracted with C18- solid phase cartridges, fractionated by HPLC, and identified using mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy as detailed in the supplemental experimental procedures. Compounds **10** and **14** were chemically synthesized from the precursor

3α,17-dihydroxy-5β-pregnan-20-one via a slightly modified previously described route [12] as detailed in the supplemental experimental procedures.

Electrophysiology - Electro-olfactograms and cross-adaptation tests were recorded as previously described [5] and detailed in the supplemental experimental procedures.

Hormone measurement - 17,20β-P release to the water was measured by radioimmunoassay as previously described [6] and detailed in the supplemental experimental procedures.

Author Contributions

T.K.-C., P.C.H., E.N.B. & A.V.M.C. conceived the study. T.K.-C. isolated pheromones. T.K.-C. & P.C.H. carried out electrophysiology. T.K.-C., P.C.H. & A.R. conducted endocrine experiments. J.P.S. carried out structure elucidation - MS. C.P & B.S. carried out structure elucidation - NMR. C.P. & Y.N. carried out steroid synthesis. T.K.-C., P.C.H., J.P.S., C.P., B.S. & A.V.M.C. wrote the paper.

Supplemental Information

Supplemental Information includes Supplemental Data, Supplemental Experimental Procedures and Supplemental References and can be found with this article online.

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Figure legends

Figure 1. The HPLC peak area in the most active urine fraction A and olfactory responses to male urine, urine extract and fraction A increase with male social rank in tilapia.

(**A-C**) Representative C18-HPLC chromatograms of urine extracts from a dominant (**A**), intermediate (**B**) and subordinate (**C**) tilapia male. An evaporative light scattering detector (ELSD), universal for non-volatile compounds, was used for peak detection. The olfactory most active peak was collected between 63-66 min as fraction A (as indicated by the horizontal bar above the peak). Chenodeoxycholic acid (CDC) was included as internal standard. For liquid chromatography-based ESI-MS analyses of HPLC fraction A see Figure S1. (**D**) Mean (N = 6) EOG responses normalised to 10^{-5} M L-serine of 3 males and 3 females elicited by raw urine (grey circles), the corresponding C18-SPE urine extract (open squares) and corresponding HPLC fraction A (black triangles) at 1:10,000 v/v dilution from 17 tilapia donor males of different social rank given by the dominance index. Olfactory responses to all stimuli were positively correlated with the social rank of the donor male (Spearman correlation; urine: r_s = 0.537, P = 0.0258; urine extract: r_s = 0.591, P = 0.0124; HPLC fraction A: r_s = 0.784, P <0.0001 (**E**) Regression analysis (solid line) of mean EOG responses normalised to 10^{-5} M L-serine of 3 males and 3 females elicited by fraction A (black triangles) at 1:10,000 v/v dilution from the same 17 males of different social rank as a function of HPLC peak areas (relative to 0.4 mM CDC standard). Dashed lines indicate 95 % confidence interval (R^2 = 0.9697, P < 0.0001). (**F**) HPLC peak areas (relative to 0.4 mM CDC standard) of fraction A of urine extracts from the same 17 tilapia males as a function of

the dominance index. Both variables are strongly positively correlated (Spearman correlation, $r_s = 0.743$, $P = 0.0002$).

Figure 2. Two isomers of 5β-pregnanetriol 3α-glucuronate are present in the most active male urine fraction.

(**A-C**) ^{1}H NMR spectra (500 MHz, MeOH-d_4) of (**C)** HPLC fraction A, (**A**) synthetic sodium 5β-pregnan-3α,17α,20β-triol 3α-glucuronate (**10**) and (**B**) synthetic sodium 5β-pregnan-3α,17α,20α-triol 3α-glucuronate (**14**). To the right of the full ^{1}H NMR spectra, extensions of representative spectral regions are shown. Signal H-20α in (**B**) overlaps with the signal of an impurity (*) of the methyl glucuronate of **14**. The large singlet signal at δ_H1.9 in (**C**) represents the methyl group of acetate. For assignment of relevant signals, see also Table S1 and partial HSQC NMR spectra in Figure S2. Chemical synthesis of both pregnanetriol 3-glucuronates from the aglycone precursor 5β-pregnan-3α,17α-diol-20-one is presented in Figure S3. (**D, E**) Structures of the two principal components of Mozambique tilapia male urine extract: 5β-pregnan-3α,17α,20α-triol 3-glucuronate (**14**) and 5ß-pregnan-3α,17α,20β-triol 3-glucuronate (**10**). Arrows indicate the epimeric centre. (**F**) LC-MS traces of raw Mozambique tilapia urine (dashed black) diluted fifty times; 5β-pregnan-3α,17α,20α-triol-3α-glucuronate (**14**, solid grey) and 5β-pregnan-3α,17α,20β-triol-3α-glucuronate (**10**, solid black) at 10 µM in negative ionization polarity. (**G**) Correlation between male social status (dominance index) and urinary concentration of the sex pheromone. Urinary concentrations in µM (common logarithm scale) of **14** (open triangles) and **10** (black triangles) as a function of the dominance indices of the donor males. The dominance index was positively correlated to the urinary concentration of **10** (Spearman correlation, $r_s = 0.790$, $P < 0.0001$, $N = 19$) and **14** (Spearman correlation, $r_s = 0.550$, P

= 0.0145, N = 19). Note that for some males concentrations were below LC-MS detection limit of 1 µM for **14** (5 males) and 8 µM for **10** (2 males). Values assigned were 0.5 x detection limit.

Figure 3. Synthetic compounds 10 and 14 evoke strong olfactory responses in females and males.

(**A,B**) Normalised (to response amplitude of 10^{-5} M L-serine standard) EOG concentration-response curves (mean ± SEM) recorded from males (**A,** N = 14) and females (**B,** N = 10) for the synthetic **14** (empty circles) and **10** (black circles). Triangles on the curves indicate respective mean apparent EC_{50} values. (**C**) Typical EOG responses to synthetic **14** and **10** at 10^{-7} M recorded from a tilapia male. (**D,E**) EOG cross-adaptation. Relative EOG response (mean + SEM) to 10^{-6} M of **14** (empty bar, N =10), to 10^{-6} M of **10** (black bar, N = 10) and 10^{-5} M TCD (grey bar, N = 6) as percentage of the initial response (% R_I) to these compounds during 10^{-6} M adaptation to either **10** (**D**) or **14** (**E**). SAC = self-adapted control. Different letters over the bars indicate significant differences at $P < 0.05$: One-way RM ANOVA followed by the Holm-Sidak post-hoc test, $F_{2,14} = 223.3$, $P < 0.001$ (**D**), $F_{2,14} = 95.1$, $P < 0.001$ (**E**).

Figure 4. Male urine and the synthetic pregnanetriol 3-glucuronates increase the release of the oocyte maturation inducer 17,20β-P in females.

Release rates (mean ± SEM) of 17,20β-dihydroxypregn-4-en-3-one ($ng.kg^{-1}.h^{-1}$) of eight female tilapia during 1 h before (black bar) and 1 h after (grey bar) exposure to the following stimuli diluted 1:10,000 v/v: conspecific dominant male urine; C18-SPE male urine extract; C18-SPE male urine flow-through; 4:1 mixture of synthetic steroid glucuronates **10** and **14** or methanol (control). All females had similar 17,20β-P release

rates before any stimulus was added. Females significantly increased 17,20β-P release after stimulation with either urine, urine extract or the synthetic steroid mixture (***P < 0.001), but not aqueous urine flow-through or the methanol control. Different letters above bars indicate significant differences in 17,20β-P release rates after stimulation, comparing the effect of raw male urine to the other stimuli; two-way RM ANOVA followed by the Holm-Sidak post-hoc test: F = 14.222, P < 0.001 (stimulus); F = 41.104, P < 0.001 (time); F = 10.898, P < 0.001 (interaction stimulus x time).

Figure 1
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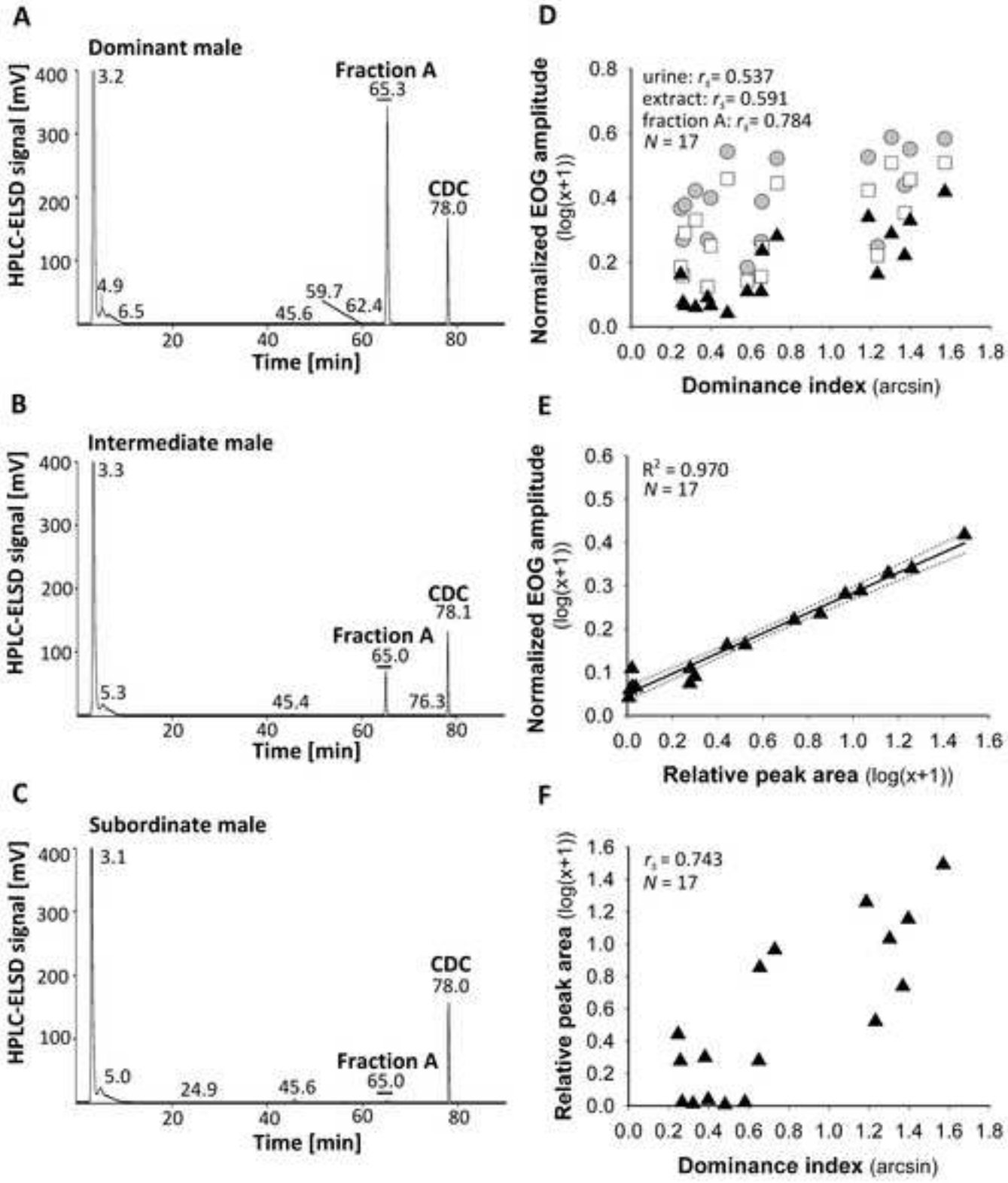

Figure 2

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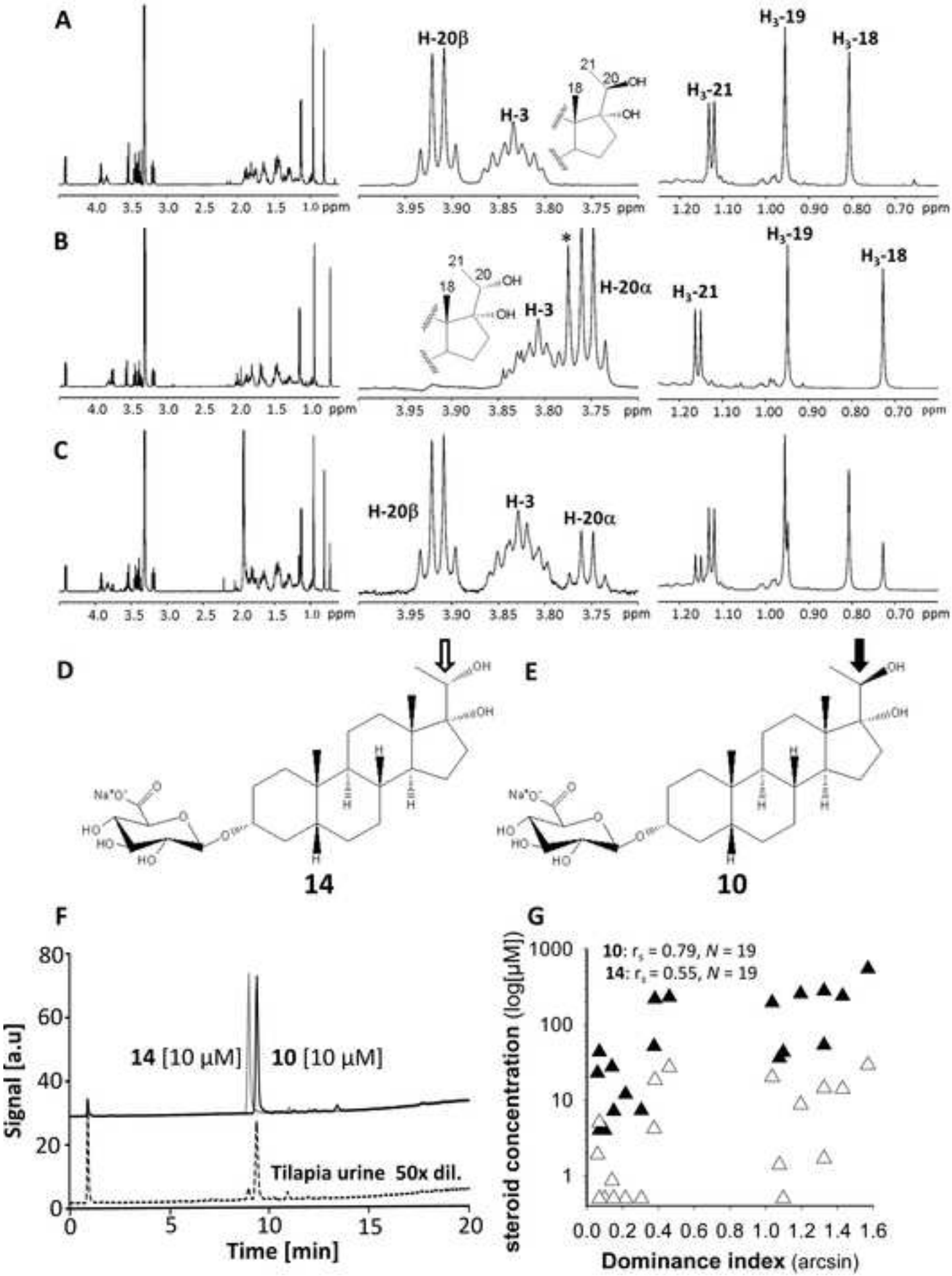

Figure 3
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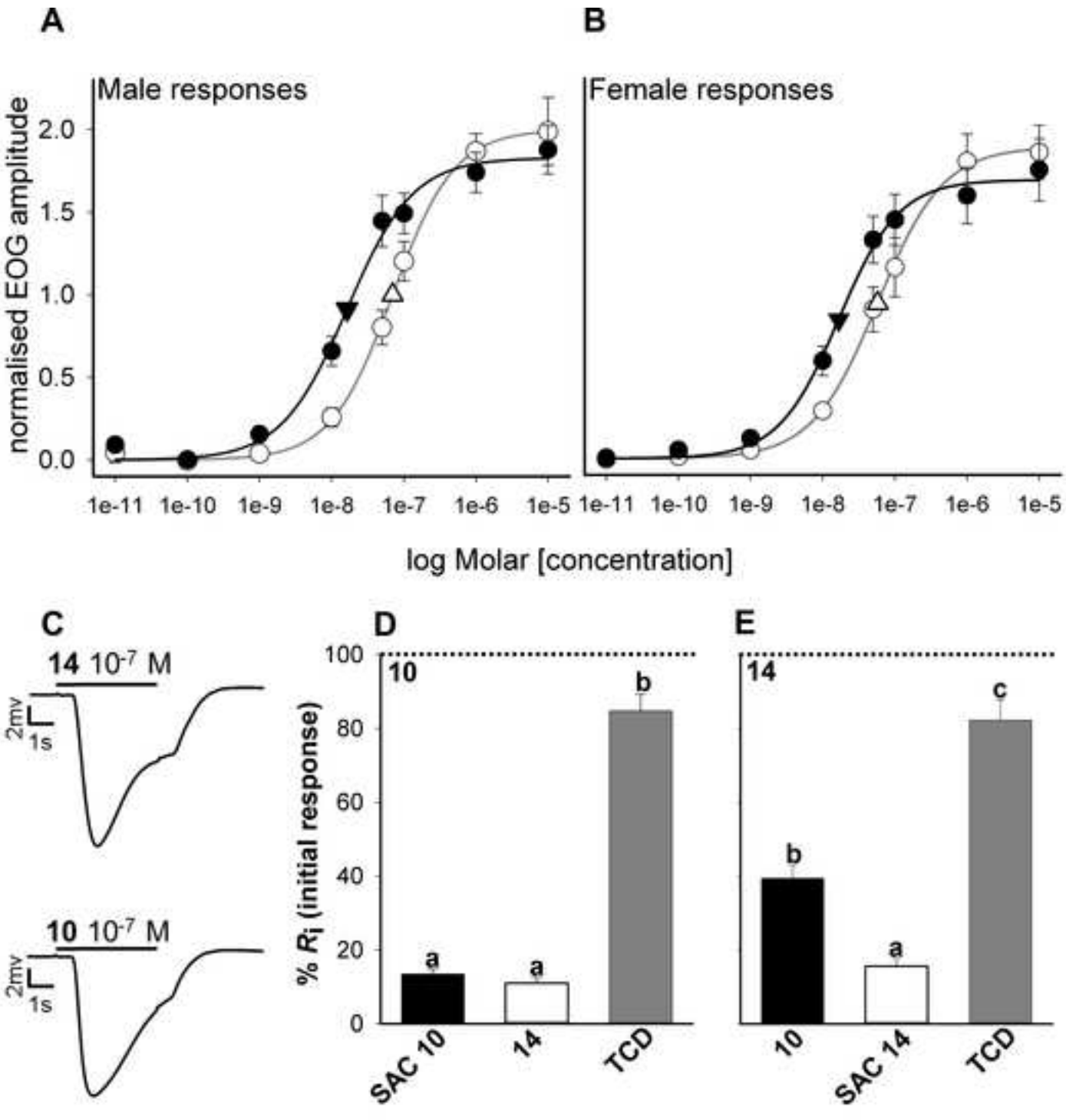

Figure 4
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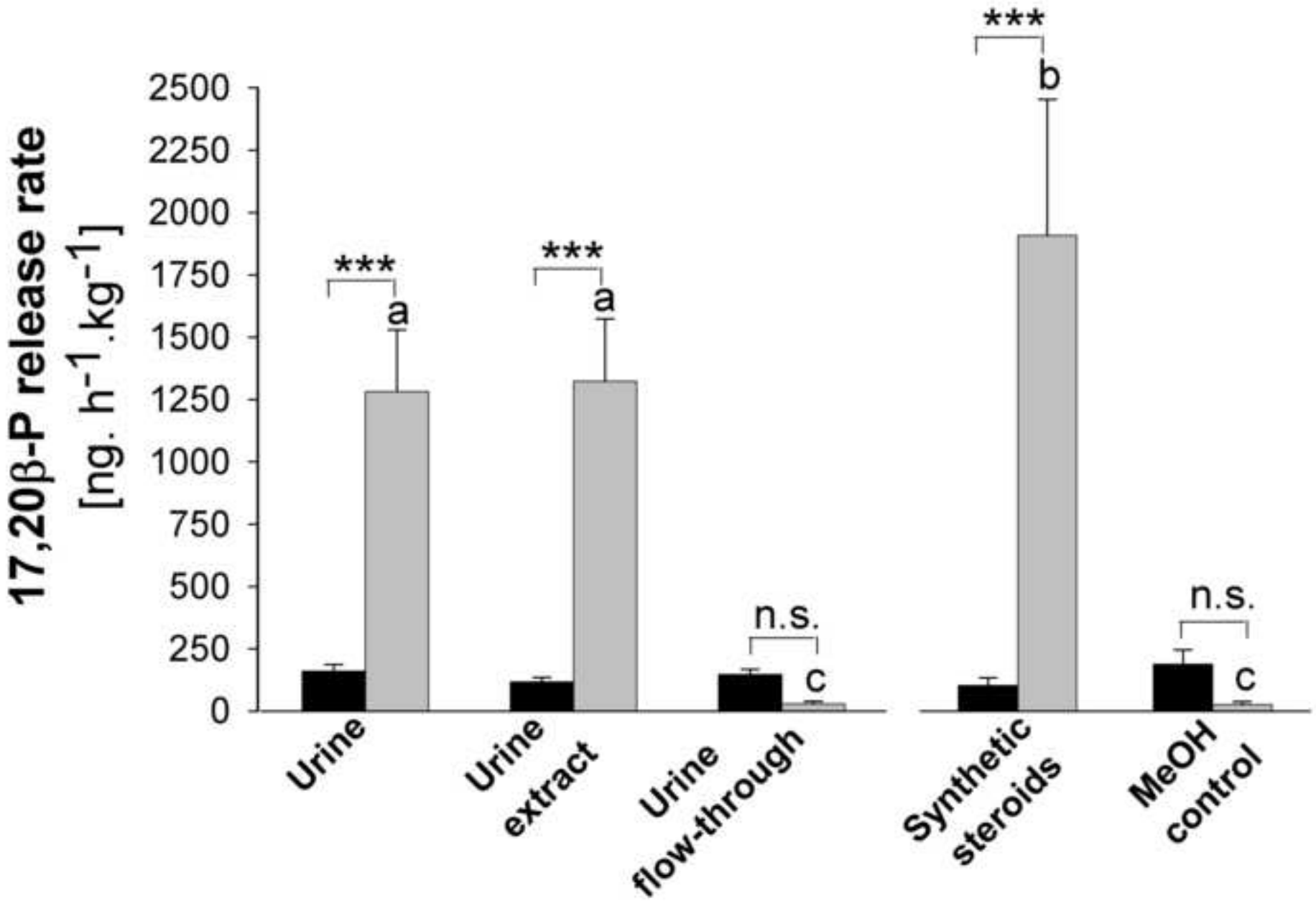


Supplemental Data

(Inventory)

Supplemental figures:

- **Figure S1 related to Figure 1.** Mass spectrometry analysis of the most active urine fraction A.
- **Figure S2 related to Figure 2.** Structure elucidation of the male tilapia sex pheromone using 1D- and 2D-nuclear magnetic resonance spectroscopy.
- **Figure S3 related to Figure 2.** Chemical synthesis of the male tilapia sex pheromone components.

Supplemental table:

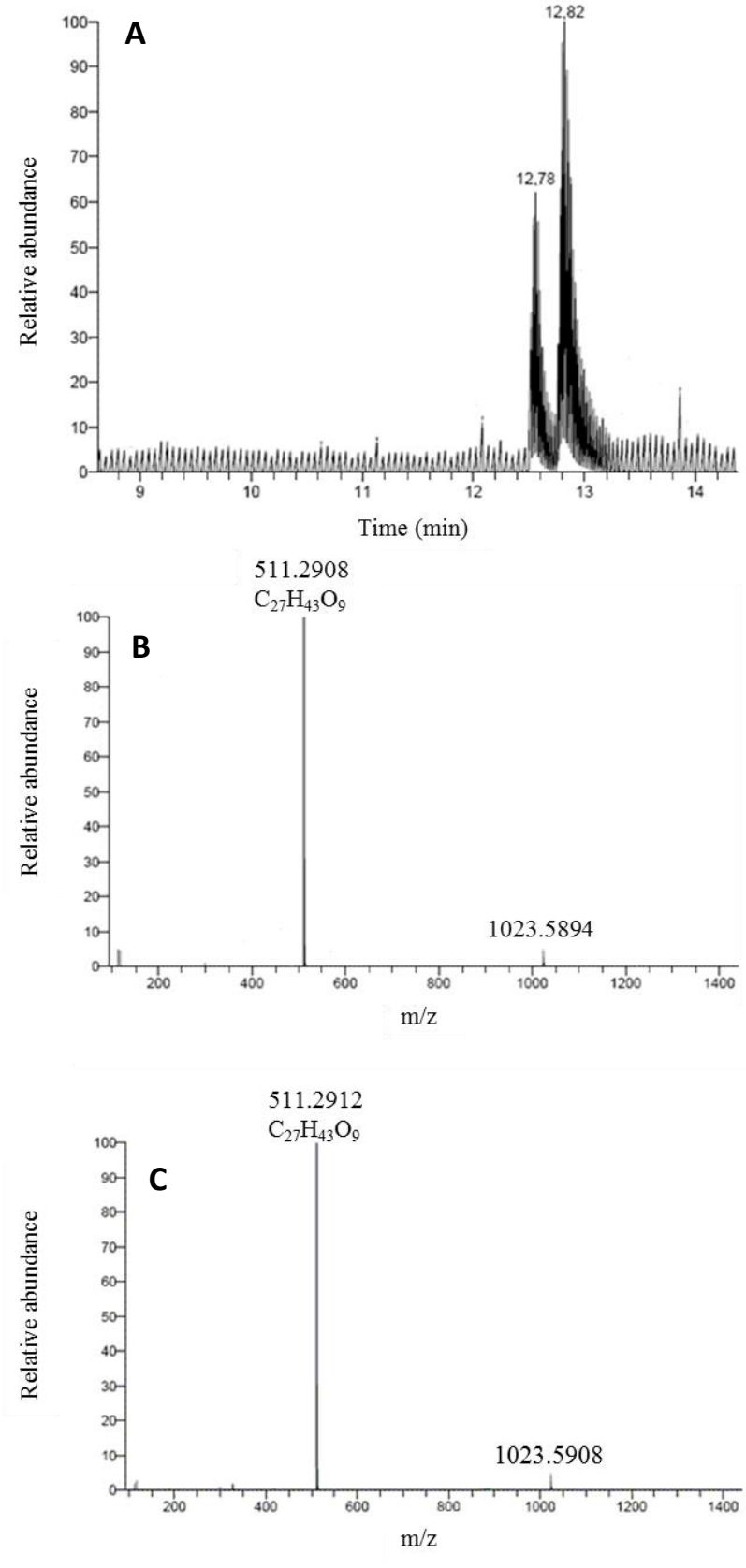
- **Table S1 related to Figure 2.** ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data of synthetic 5ß-pregnan-3α,17α,20α-triol 3-glucuronate (**14**) and 5ß-pregnan-3α,17α,20β-triol 3-glucuronide (**10**) in MeOH-d_4.

Supplemental experimental procedures

Supplemental references

Supplemental information

Supplemental data



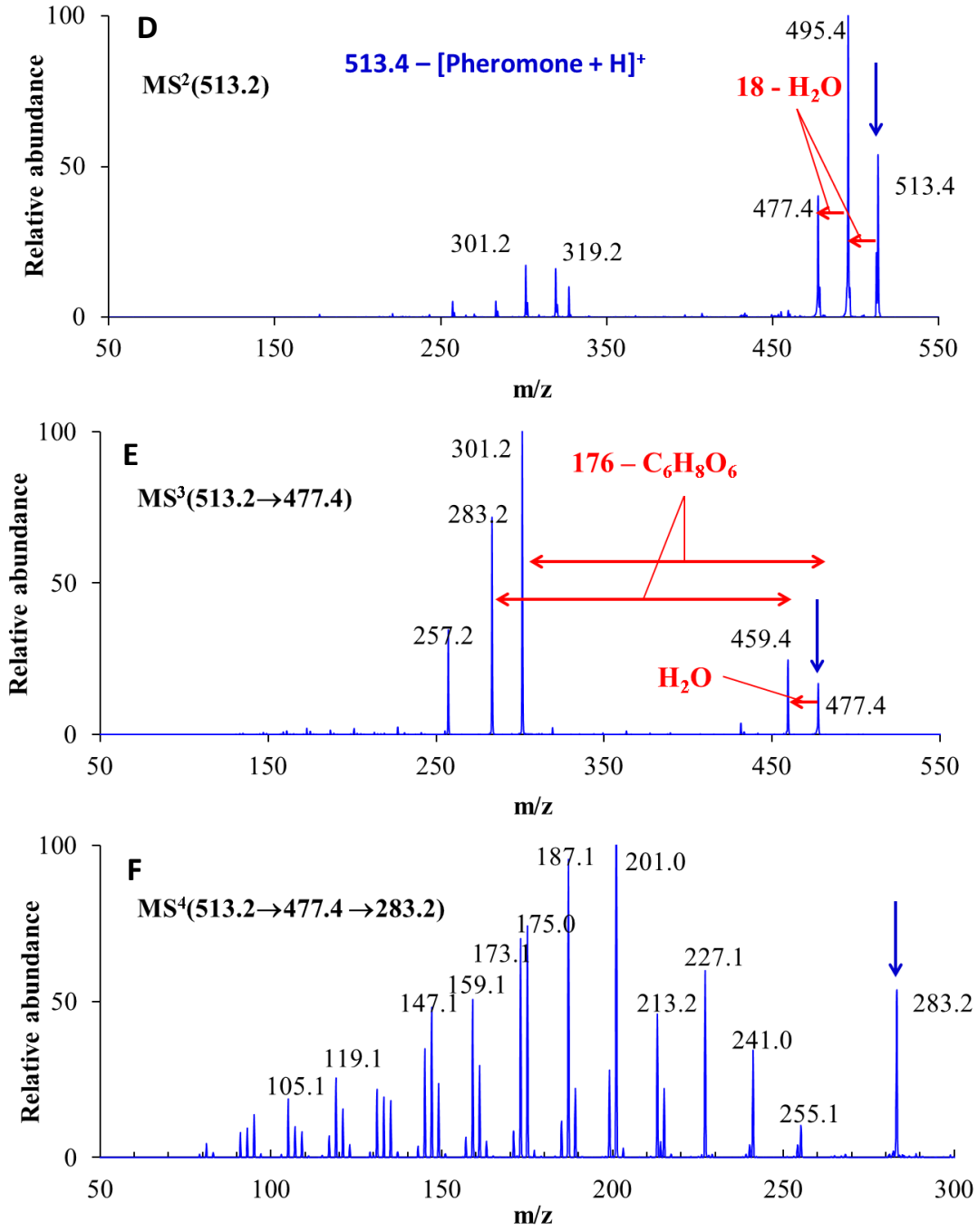
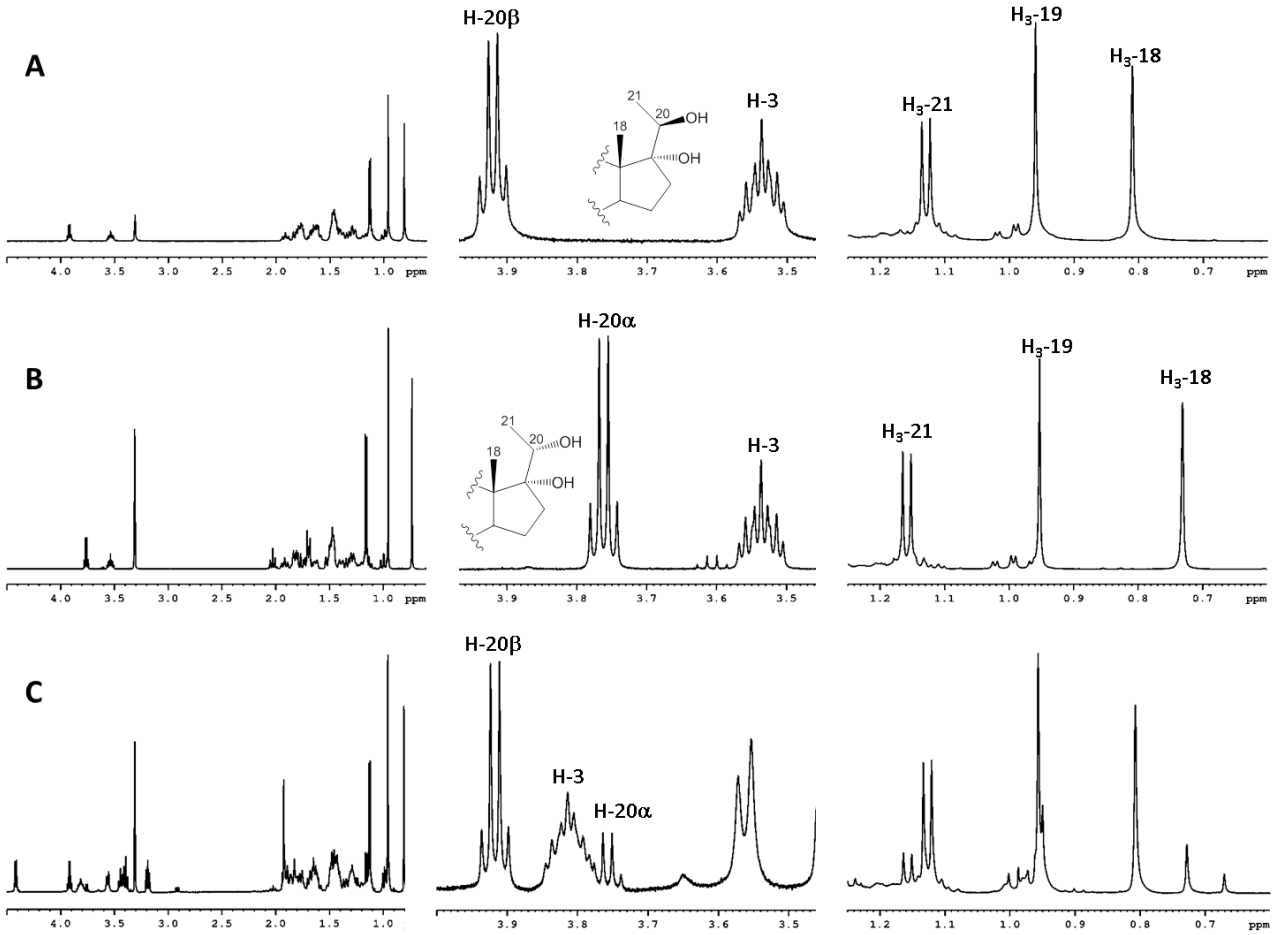


Figure S1 related to Figure 1.

Mass spectrometry analysis of the most active urine fraction A. (**A**) UPLC-ESI-MS of the active urine fraction A. HR-ESI-MS spectra of the peak at (**B**) Rt 12.78 min and (**C**) Rt 12.82 min in the negative polarity. (**D-F**) ESI-MS fragmentation of compounds from the active urine fraction A in positive polarity. (**D**) MS^2 (513.4), (**E**) MS^3 (513→477.4), (**F**) MS^4 (513→477.4→283.1). The vertical blue arrows indicate the fragmented ions. The neutral loss of 176 Da corresponds to a monodehydrated glucuronic acid residue. The remaining structure possesses at least three OH groups. Spectrum (**F**) shows high number of peaks with mass differences of 14 Da, typical of steroids.


A
H-20β
H-3
H3-19
H3-18
H3-21
B
H-20α
H-3
H3-19
H3-18
H3-21
C
H-20β
H-3
H-20α
ppm

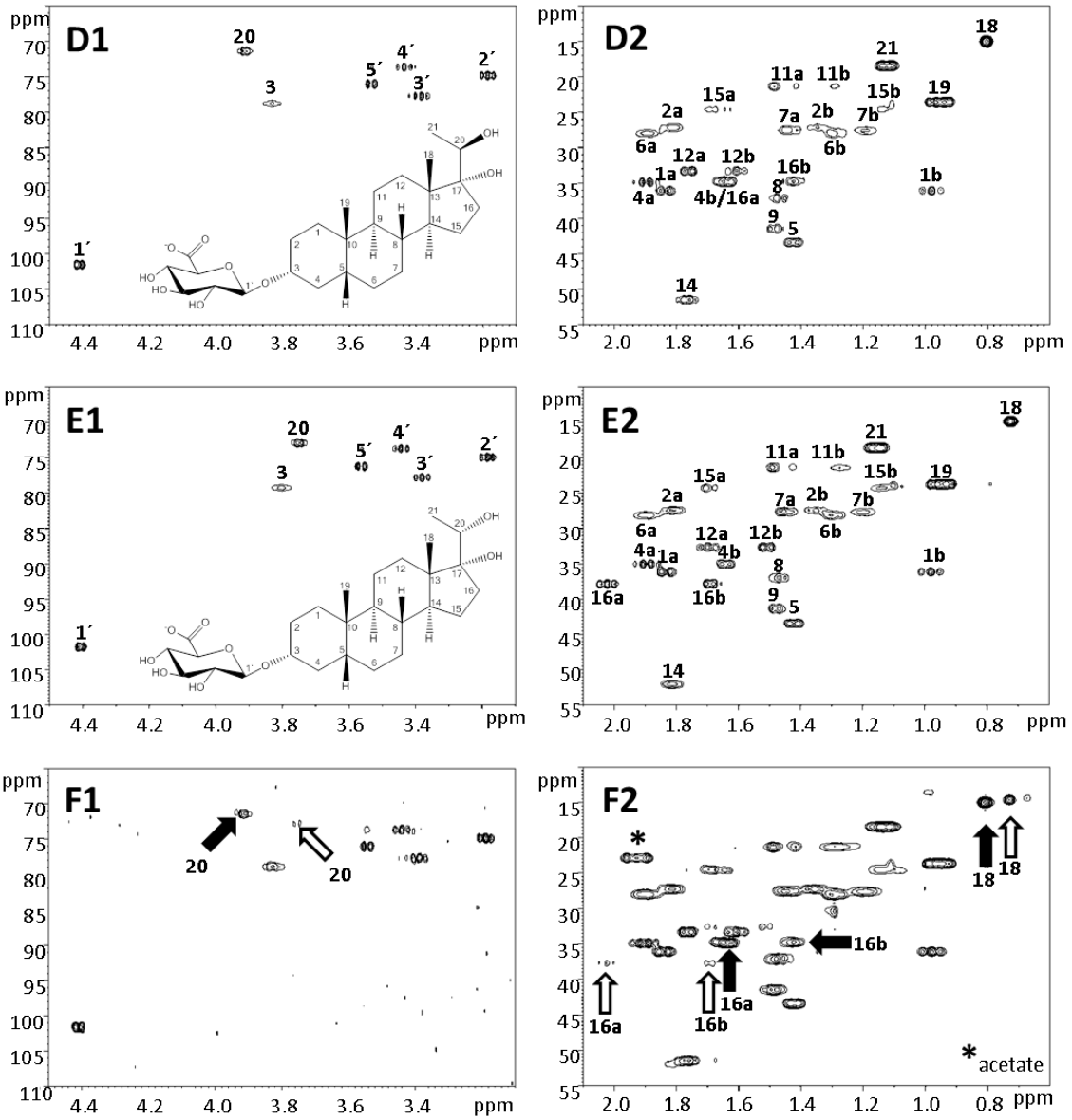
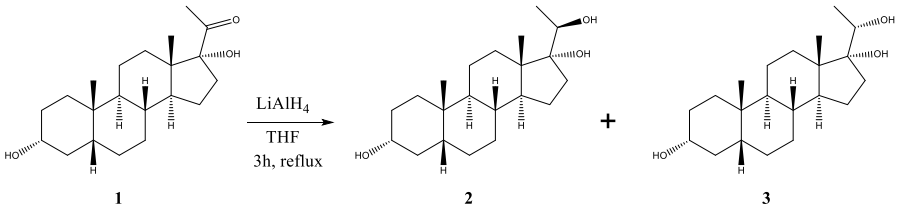


Figure S2 related to Figure 2.

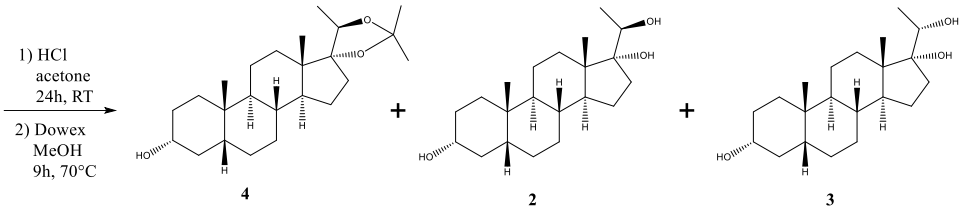
Structure elucidation of the male tilapia sex pheromone using 1D- and 2D-nuclear magnetic resonance spectroscopy. (**A**-**C**) ^{1}H NMR spectra (500 MHz, MeOH-d_4) of (**A**) commercially available 5β-pregnan-3α,17α,20β-triol (Sigma-Aldrich P-8258), identical with **2** (Figure S3A), (**B**) commercially available 5β-pregnan-3α,17α,20α-triol (Fluka 81575), identical with compound **3** (Figure S3A) and (**C**) HPLC fraction A. To the right of the full ^{1}H NMR spectra, extensions of representative spectral regions are shown. Compared to corresponding ^{1}H NMR signals of **10** and **14**, most signals of the aglycones **2** and **3** are unaffected from the missing glucuronate unit. Only H-3 is shifted to the high field due to the unsubsituted OH group. (**D**-**F**) Partial ^{1}H-^{13}C HSQC NMR spectra (500 MHz, MeOH-d_4) of 5β-pregnan-3α,17α,20β-triol-3α-glucuronate (**10**) and 5β-pregnan-3α,17α,20α-triol-3α-glucuronate (**14**). (**D1, D2**) synthetic **10**, (**E1, E2**) synthetic **14** and (**F1, F2**) HPLC fraction A. Arrows (⇑⇑) in **F1** and **F2** indicate signals H/C-16, H/C-18 and H/C-20, respectively, which are relevant to distinguish the two isomers.

A

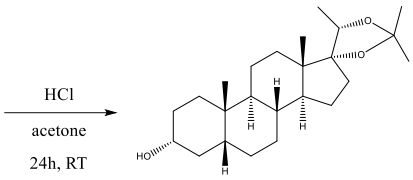


1 **2** **3**

20-β (20-β:20-α=3:2) 20-α

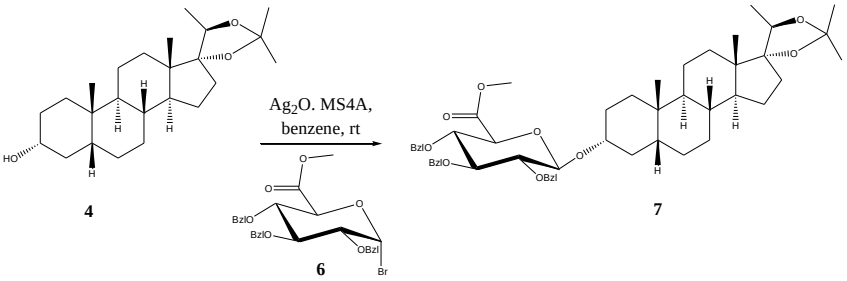


4 **2** **3**

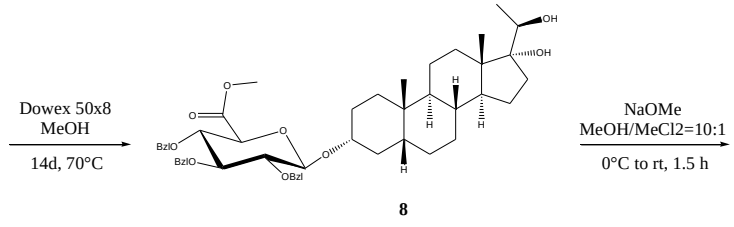


5

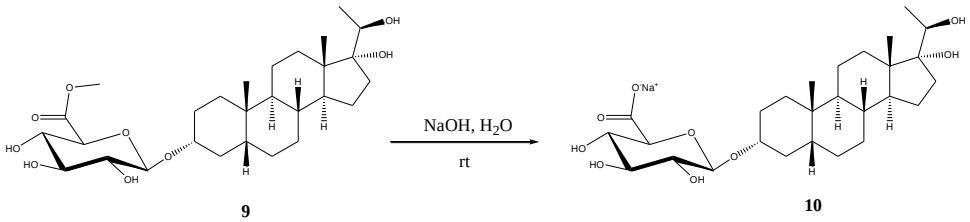
B



4 **6** **7**



8



9 **10**

C

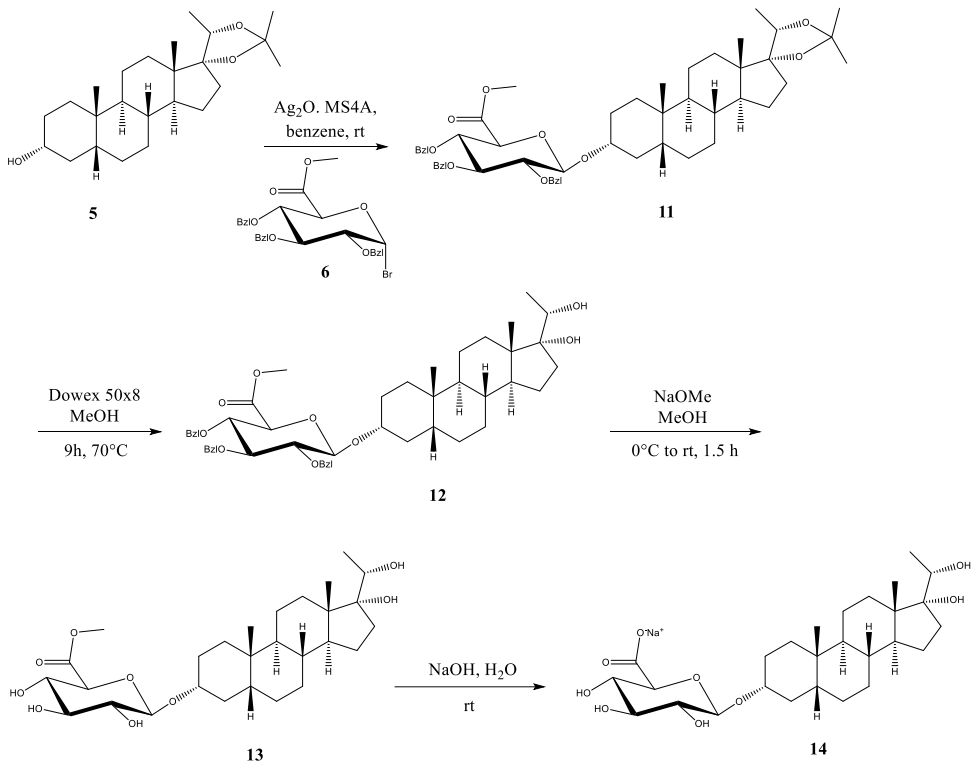


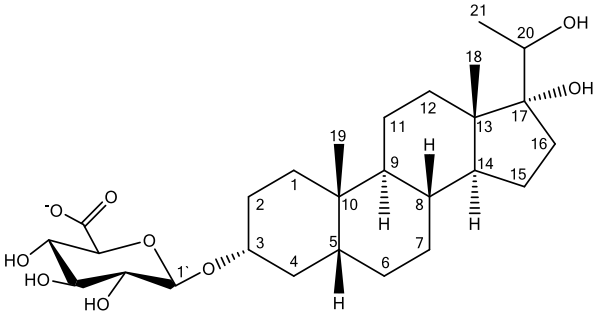
Figure S3 related to Figure 2.

Chemical synthesis of the male tilapia sex pheromone components. (**A**) Synthesis of the epimeric aglycons **2** (20β) and **3** (20α). (**B**) Synthesis of 5β-pregnan-3α,17α,20β-triol 3α-glucuronate sodium salt (**10**). (**C**) Synthesis of 5β-pregnan-3α,17α,20α-triol 3α-glucuronate sodium salt (**14**).

Supplemental table

Table S1 related to Figure 2: ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data of synthetic 5ß-pregnan-3α,17α,20α-triol 3-glucuronate (**14**) and 5ß-pregnan-3α,17α,20β-triol 3-glucuronide (**10**) in MeOH-d_4.

		14			**10**		
No.	**Type**	**δ_C**	**δ_H**	**mult., *J* [Hz]**	**δ_C**	**δ_H**	**mult., *J* [Hz]**
1	CH_2	36.5	1.84/0.98	m/m	36.5	1.84/0.98	m/m
2	CH_2	27.7	1.82/1.35	m/m	27.6	1.82/1.35	m/m
3	CH	79.4	3.81	m	79.2	3.84	m
4	CH_2	35.3	1.90/1.65	m/m	35.3	1.90/1.65	m/m
5	CH	43.8	1.42	m	43.8	1.42	m
6	CH_2	28.5	1.90/1.29	m/m	28.5	1.90/1.29	m/m
7	CH_2	27.9	1.45/1.19	m/m	28.0	1.45/1.19	m/m
8	CH	37.3	1.47	m	37.6	1.47	m
9	CH	41.7	1.48	m	41.8	1.48	m
10	C	36.0			36.0		
11	CH_2	21.7	1.45/1.28	m/m	21.8	1.45/1.28	m/m
12	CH_2	32.9	1.70/1.51	m/m	33.7	1.76/1.61	m/m
13	C	47.5			48.8		
14	CH	52.4	1.81	m	51.9	1.76	m
15	CH_2	24.6	1.67/1.16	m/m	25.1	1.67/1.12	m/m
16	CH_2	38.2	2.03/1.69	m/m	35.1	1.65/1.42	m/m
17	C	87.1			87.1		
18	CH_3	15.1	0.73	s	15.5	0.81	s
19	CH_3	24.0	0.95	s	24.1	0.96	s
20	CH	73.1	3.75	q, 6.4	71.7	3.92	q, 6.2
21	CH_3	18.9	1.16	d, 6.4	18.9	1.13	d, 6.2
1`	CH	102.0	4.41	d, 7.8	101.8	4.41	d, 7.8
2`	CH	75.1	3.19	dd, 7.8/9.0	75.1	3.19	dd, 7.8/9.0
3`	CH	78.0	3.39	dd, 9.0/9.0	78.0	3.39	dd, 9.0/9.0
4`	CH	73.9	3.45	dd, 9.0/9.5	74.0	3.44	dd, 9.0/9.4
5`	CH	76.4	3.57	d, 9.5	76.3	3.54	d, 9.4
6`	C	176.7			177.2		



Structure of the steroidal glucuronates **14** and **10** (epimeric at C-20) with numbering.

Supplemental experimental procedures

Experimental animals and urine collection

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 until used in experiments. Social groups were created in 200 l tanks with five males and five females of similar standard length (SL in mm) and body weight (BW in g; coefficient of variation of BW less than 5 %) as previously described [S1]. Males were tagged (T-Bar anchor FD94, Floy Tag Inc., Seattle, WA, USA) and systematic focal observation of their behaviour carried out daily [S1]. The frequency of submissive displays during agonistic interactions or absence of dark coloration without social interaction and dominant behaviours such as aggression (biting, chasing, lateral displays, circling or mouth-to-mouth fights), nest digging, courtship towards females or dark coloration without social interaction was recorded over five min for each male. A dominance index (DI) ranging from zero to one was calculated for every male each day as the sum of all dominant behaviours and subsequent division by the sum of all dominant and subordinate behaviours. Accordingly, after five days of observation the mean DI was assessed for every male [S1, 2]. Subordinate males had a DI $\leq$ 0.16 and dominant males a DI $\geq$ 0.8, the others were intermediates. After each daily observation, urine was collected from each male by gently squeezing the area immediately above and anterior to the urogenital papilla and collecting the resultant stream of urine into a plastic tube, subsequently stored at -20 °C until further analysis.

Urine extraction and fractionation

Urine samples (pooled over the five observation days) from dominant (mean ± SD; *N* = 6; BW = 150 ± 31 g; SL = 168 ± 11.7 mm), intermediate (*N* = 5; BW = 156 ± 26.9 g; SL = 171 ± 12.3 mm) and subordinate (*N* = 6; 150 ± 42.5g; SL = 170 ± 13.5 mm) males were extracted using solid-phase cartridges (C18, 500 mg, Isolute®, Biotage) and eluted with methanol. Aliquots of each extract (500 µl) were supplemented with 5 µl 4 mM chenodeoxycholic acid (CDC; ≥ 98%, Sigma; the internal standard), dried under nitrogen gas, reconstituted in 55 µl methanol/water [60/40 v/v, containing 0.001% formic acid (FA)] and injected into a HPLC system (Smartline KNAUER, Berlin, Germany) with a C-18 column (3.9 mm x 300 mm; 4 µm particle size; Nova-Pak, Waters). HPLC conditions were as follows: mobile phase was water (MilliQ) and methanol (HPLC-grade), both containing 0.001% FA; 0-4 min isocratic at 15% methanol, 5-91 min linear gradient from 15 % to 100 % methanol, 91-96 min isocratic at 100% methanol; flow-rate: 0.7 ml min^{-1}. The column eluate was first routed to a diode array UV detector (Smartline 2600, KNAUER, Germany), then split off and half routed to an evaporative light scattering detector (Varian 380-LC ELSD, Polymer Laboratories) and the other half collected by an Advantec CHF100SA fraction collector into 30 fractions, each 3 min. ELSD conditions were as follows: nitrogen carrier gas (≥ 98 % purity, 1.6 SLM), 65°C nebulisation temperature, 110°C evaporation temperature. Data were visualized and analysed using the Data-Apex Clarity™ Software. Peak areas were normalized to the peak area of the internal standard CDC. Negative blank fractions, generated by injecting 55µl of 60/40 v/v methanol/water (0.001% FA) under similar conditions, were used to control for uncontaminated and unbiased experimental conditions. All HPLC fractions from each male were kept at -20°C until assessment of olfactory potency.

Identification/Structure elucidation

A large urine pool (~30 ml), collected from various dominant donor males held in social groups (described above) provided the material for subsequent chemical analyses. MS^n studies were

performed on a Bruker Daltonics HCT *ultra* mass spectrometer (Bruker Daltonics, Bremen, Germany). The ionisation was made by electrospray (ESI) in the negative and positive polarities. Typical spray and ion optics conditions were as follows: capillary voltage, 3.5 kV; drying gas (nitrogen), 300ºC at 5 $l.min^{-1}$; nebulizer gas pressure, 20 psi; capillary exit voltage, 130 V; skimmer voltage, 40 V. The LC-MS system was an Agilent Technologies 1200 Series LC coupled to the above described mass spectrometer. Under LC operation the spray and ion optics conditions were the following: ionization, negative ionization polarity; capillary voltage, 3.5 kV; drying gas (nitrogen), 330ºC at 10 $l.min^{-1}$; nebulizer gas pressure, 50 psi; capillary exit voltage, 130 V; skimmer voltage, 40 V. A Hamilton PRP-1 reversed phase LC column (15.0 cm length, 2.1 mm internal diameter, 5 µm average particle diameter), stabilised at 25°C was used. The eluent system was ultra-pure water (A) and acetonitrile (B), both with 0.1% FA. The gradient started with 20% of B, followed by a linear increase up to 80% in 20 min. In a second gradient step an increase up 100 % took place in 5 minutes. A final cleaning step using 100% of B during 5 min was made after each run. The eluent was then allowed to recover the initial conditions (80% of A and 20% of B) in 1 min and then stabilise for additional 6 min before the next run. High-resolution mass spectra were recorded on an UPLC-RLX 3000 system (Dionex) and an Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). UPLC was performed using an Acclaim C18 column (150 x 2.1 mm, 2.2 µm, Dionex, Germany) at a constant flow rate of 300 $\mu l.min^{-1}$ using ultra-pure water with 0.1% FA (solvent A) and acetonitrile with 0.1% FA (solvent B). Isocratic conditions of 50% A and 50% B were used. Full-scan mass spectra were generated in the range of *m/z* 94.00-1400.00, both under negative and positive ionization polarity and analysed with the Xcalibur Qual Browser software (Thermo Scientific).

NMR spectra were measured on a Bruker Avance NMR spectrometer (Bruker-Biospin, Karlsruhe, Germany), operating at 500.13 MHz for ^{1}H and 125.75 MHz for ^{13}C. An inverse triple channel cryoprobe (5 mm) was used. The spectra were recorded in methanol-d_4. Samples were

measured in 5 mm tubes (600 µl methanol-d_4) or 2 mm capillaries (mass-limited samples; 85 µl methanol-d_4). Chemical shifts are referenced to the ^{1}H- and ^{13}C NMR signals of methanol-d_4 [S3].

In the high-field region of the ^{1}H NMR spectrum of the obtained mixture (HPLC fraction A), each of the two compounds displayed two singlets typical of angular methyl groups and a doublet of a methyl group (J ~ 6 Hz) attached to a methine carbon (Figure 2C). As revealed by HMBC, the angular methyl signals are part of the steroid ring system. The combined use of 2D NMR (^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC) correlations, assigned the methyl group doublet to the steroid side chain and also identified the side chain as 2-hydroxyethyl unit attached to the hydroxylated C-17, in both compounds. Direct comparison of the ^{1}H NMR spectrum with pregnane standards (5β-pregnane-3α,17α,20β-triol (**2**; Figure S3A) and 5β-pregnane-3α,17α,20α-triol (**3;** Figure S3A)) allowed preliminary assignment of all ^{1}H resonances of the aglycone moiety, identifying the urinary steroid aglycons as 5β-pregnane-3,17,20-triols (Figure S2A-C).

The doublet of an axial H-1' methine proton (δ 4.41, d, J = 7.8 Hz) characteristic of a proton at the anomeric centre of a β-hexopyranose and signals of four other methine protons, confirmed the hexose unit as already suggested by the MS^n experiments. The absence of hydroxymethylene group signals in the ^{1}H NMR spectrum together with doublet signals of axially oriented H-2'–H-5' ($^3J_{H\text{-}H}$ ~8-9 Hz) and HMBC correlations of H-5' with C-6' (δ 176.6) indicated a glucuronic acid unit. The glycosidation site at 3-OH of the steroid was established by downfield shifts of the H-3 and C-3 resonances observed in the spectra of the steroids as compared to 5β-pregnan-triol standards. H-3 was shifted from δ 3.54 to δ ~3.8 and C-3 from δ 72.5 to δ ~79. Finally, comparing ^{1}H NMR (Figure 2A-C) and 2D NMR spectra (^{1}H-^{1}H COSY, ROESY, TOCSY, HSQC (Figure S2D-F), HMBC and H2BC) of the mixture (HPLC fraction A) with the corresponding spectra of synthetic glucuronate standards allowed full structure elucidation and stereochemical assignment of the two steroid conjugates as the 20-epimers, sodium 5β-pregnan-3α,17α,20β-triol 3α-glucuronate (**10**) and sodium 5β-pregnan-3α,17α,20α-triol 3α-glucuronate (**14**) (see Figures 2 and S2 and Table S1).

Steroid synthesis

The steroidal glucuronates were prepared via a slightly modified route previously described [S4] using 5β-pregnan-3α,17α-diol-20-one (**1**) (Figure S3A) (Sigma-Aldrich) as starting material. Following a standard protocol, reduction with $LiAlH_4$ in absolute THF gave, after hydrolysis with diluted H_2SO_4 and extraction with CH_2Cl_2, a chromatographically inseparable mixture of the 5β-pregnane-3α,17α,20-triols (**2,3**) (20β:20α=3:2 based on ^1H NMR) in quantitative yield. The mixture was subjected to diol protection [S5] followed by a deprotection step using Dowex 50Wx8 [S6], making use of the fact that one of the desired products **4** reacts very slowly. Separation of **4** (20β) by means of chromatography and re-protection of **3** gave the desired diol-protected compound **5** (20α). The average overall yield of **4** and **5** was 84 %. For linking the glucuronic acid to the 20β-steroid (Figure S3B), commercially available benzoyl-protected methyl glucuronate (**6**) was reacted with **4** in the presence of activated molecular sieve (4Å) and freshly prepared Ag_2O in absolute benzene at room temperature. After overnight stirring in darkness the pure product (**7**) was obtained, after chromatography, at a yield of 93%. Subsequently, deprotection of **7** using Dowex 50x8 gave **8** in a yield of 38%. De-benzylation of the glucuronate was accomplished with sodium methylate/sodium hydroxide to give the desired sodium 5β-pregnan-3α,17α,20β-triol 3α-glucuronate (**10**). The overall yield calculated from **1** was 11% and from **4**, 24%. An analogous procedure was used to produce the 20α-configured steroidal glucuronate **14** (Figure S3C). After attachment of the protected glucuronate, the deprotection of the steroidal diol function was accomplished within 9 h to give **12** in a yield of 70% calculated from **5**. Cleavage of the protection groups from the glucuronate proceeded in 42% yield calculated from **12**. The total yield of sodium 5β-pregnan-3α,17α,20α-triol-3α-glucuronate (**14**) calculated from **1** was 7% and from **5**, 29%.

Electro-olfactogram (EOG) recordings

Preparation of animals and recording of the EOG was carried out as previously described [S7]. The DC voltage signal was pre-amplified, filtered (low-pass 50 Hz) and amplified (NL106, Digitimer Ltd: final gain x100 or x1000 depending on the response), and recorded on a PC running Axoscope software (version 9.1, Axon Instruments, Inc., now Molecular Devices, LLC, Sunnyvale, CA, USA). The olfactory potency of raw urine, C18-SPE urine extracts and urinary HPLC fractions from dominant (N = 6), intermediate (N = 5) and subordinate (N = 6) males was assessed on three adult males (mean ± SD; BW = 157.9 ± 19.1 g) and three females (BW = 110.3 ± 15.4 g) at a dilution of 1:10,000 in water (v/v). A screening of all 30 urinary HPLC fractions from selected dominant and subordinate males showed that one fraction (fraction A) contained most olfactory activity and was therefore selected for subsequent EOG recordings to explore in detail the relationship of olfactory potency with peak areas (concentrations). All EOG data were log(x+1)-transformed and correlation (Figure 1D,E) or linear regression (Figure 1F) analysis was performed on pooled data from both sexes since EOG amplitudes of male and female responses were similar.

To investigate the olfactory sensitivity to the two synthesized steroids **10** and **14**, EOG concentration-response curves were generated. Ten females (mean ± SD: BW = 51.8 ± 36.3 g; SL = 131.7 ± 44.9 mm) and 14 males (BW = 35.1 ± 11.4 g; SL = 106.4 ± 11.5 mm) were exposed to increasing concentrations from 10^{-11} M to 10^{-5} M in $\log_{10}$ molar increments (in addition $5x10^{-8}$ M was tested) 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 [3 parameter: $y = ax^b/(c^b + x^b)$; $a = \max(y) = I_{max}$; b = 1 = Hill co-efficient; $c = x50(x,y) = EC_{50}$] as mathematical model, in which y is the EOG response and x is the stimulus concentration. Two-way repeated measures analysis of variance (RM ANOVA) followed by the Holm-Sidak post-hoc method was used to compare EC_{50} values and I_{max} values of

male and female responses to the two synthesized steroids. The steroids 5β-pregan-3α,17α,20α-triol (**3**), 5β-pregan-3α,17α,20β-triol (**2**), 3α,17-dihydroxy-5β-pregnan-20-one 3α-glucuronate, etiocholan-3α-ol-17-one 3α-glucuronate and the sodium salt monohydrate of D-glucuronic acid (all purchased, either from Sigma-Aldrich, Spain or Steraloids, USA) were tested for olfactory potency in the same fish.

Cross-adaptation experiments were carried out to test for the presence of single or multiple receptor mechanisms [S8]. Firstly, EOG responses to 4 s pulses of 10^{-6} M solutions of compounds **14** and **10** were recorded from males (N = 10; mean ± SD: BW = 38.7± 11.6 g SL = 109 ±12.7 mm). A 10^{-6} M solution of the adapting steroid was then used to perfuse the olfactory epithelium until voltage stabilised (about one minute). 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 at 2 x 10^{-6} M). The bile acid taurochenodeoxycholic acid [TCD; 3α,7α-dihydroxy-5β-cholan-24-oic acid N-(2-sulfoethyl)amide, Sigma-Aldrich] at 10^{-5} M was included as a control, since it was expected to act through different receptor mechanisms, and it has been shown to evoke in tilapia large EOG responses of similar magnitude as 10^{-6} M solutions of the above mentioned steroids [S9]. EOG responses to the test solutions during adaptation were converted to a percentage of the initial (unadapted) response (% R_I). For each cross-adaptation dataset, mean % R_I were compared using One-way RM ANOVA followed by the Holm-Sidak post-hoc test.

Hormone measurements

The basic methodology to analyse the endocrine response of females was as previously described [S10]. Groups of four tagged females and one male were kept together in 250 l tanks. A pre- or post-ovulatory (two days prior the predicted ovulation date or three days after the last ovulation) female was placed in a glass tank overnight and moved to an identical tank with clean de-chlorinated tap water (volume normalised to the fish weight 10 $g.l^{-1}$) the next morning. After 1 h, 1 l of water was

collected and C18-SPE extracted (eluted with 5 ml methanol). This volume of water was replaced with clean water and one of the following stimuli was applied to the tank using a micropipette: a volume to give a final dilution of 1:10,000 of **i**) pooled urine of dominant male, **ii**) the corresponding C18-SPE male urine extract, **iii**) the corresponding C18-SPE aqueous flow-through, **iv**) a 4:1 mixture of the two synthesized steroid glucuronates 10 (400 µM) and 14 (100 µM) or **v**) methanol control. After 1 h of stimulation, another litre of water was collected and C18-SPE extracted. At the end of the experiment, females were returned to their original group tank and allowed to undergo another ovulatory cycle before the experiment was repeated using one another stimulus. Thus, each stimulus was tested once in each female and eight females (mean ± SD; BW = 48.2 ± 13.3 g; SL = 112.8 ± 11.7 mm) were used as replicates. C18-SPE methanol extracts from all females were dried under nitrogen gas, re-suspended in radioimmunoassay buffer and assayed for 17,20β-dihydroxypregn-4-en-3-one [S10]. Comparison of 17,20β-P release rates between groups and urine as control was done by Two-way RM ANOVA followed by the Holm-Sidak post-hoc test.

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