Electronic Supplementary Material

S1. Collection, Extraction and Assay of Hormones

To minimise the impact of diurnal variation in hormone levels [1], we collected all pre-experience hormone samples at the same time of day. At 0930 h, each fish was placed in a glass beaker with 400 ml clean 25 ppt synthetic (Instant Ocean®) sea water for 1 h, after which time the fish were returned to their maintenance containers. Hormones then were extracted from the water samples using a vacuum pump and passing through a C18 solid phase extraction column (Lichrolut RP-18, 500 mg, 3.0 ml; Merck, NJ, USA) fitted to a 24-port manifold. Before use, the columns were first primed with 2 × 2 ml HPLC grade methanol (MeOH) followed by 2 × 2 ml ultrapure water. Columns then were frozen (-80 ℃) until further processing; freeze storage does not impact steroid concentrations [2]. To extract hormones, columns were thawed and purged with 2 × 2 ml washes of ultrapure water. The free fraction of hormone was eluted from the columns by 2 × 2 ml washes with HPLC grade ethyl acetate. The eluted solvent was evaporated at 37 ℃ with a gentle stream of nitrogen (~10 bar), which was passed over the samples through an evaporating manifold (Cole-Parmer, Evap-O-Rac). The resulting hormone residue was re-suspended in 800 µl enzyme-immunoassay (EIA) buffer supplied with the kits, and the samples were stored at -20 ℃ until assay. Cayman Chemicals Inc. EIA kits were used to quantify T and F, following the manufacturer's recommended procedures. Plates were read at 405 nm on an Absorbance Microplate Reader (ELx808™, BioTek, VT, USA). All hormone data are presented as pg/ml. Intra-assay coefficients of variation were (assay plates 1-10): T (7.3%, 5.0%, 5.8%, 4.5%, 2.8%, 7.7%, 5.1%, 10.0%, 5.5% and 5.7%) and F (9.0%, 5.4%, 4.7%, 3.6%, 3.9%, 0.9%, 5.7%, 1.9%, 3.1% and 2.7%). Inter-assay coefficients of variation were 7.9% (T) and 4.8% (F).

Table S2. Sequence of primer pairs used in PCR and Quantitative PCR. In analysis of androgen, glucocorticoid and 5-HT$_{1A}$ receptors and ribosomal protein, the same pair of primers were used both in PCR and qPCR. The range of annealing temperatures was between 61℃-65℃. (Ta: annealing temperature)

receptor genes	Ta (ºC)	product size (bp)	note
androgen Receptor (AR)			
Forward:5'- GGTGATGGTGTTTGCGCTGATA-3'	61	234	PCR & qPCR
Reverse:5'- CAACGGGAATGATGCTGAAAGAG-3'	61		
oestrogen Receptor α (ER$_{\alpha}$)			
Forward:5'- GACTATGCTGCCCCCACCCCT-3'	65	230	PCR
Reverse:5'- TGTAGACGGGTGTTGAGGCGGT-3'	64		
Forward:5'- GCCCCTACTCCTCTCTACAGCAACTC-3'	62	150	qPCR
Reverse:5'- GCTGAGCCTTGGACTGGTGGG-3'	65		
oestrogen Receptor β (ER$_{\beta}$)			
Forward:5'- GTCGGGTCGCCAGTCGCAT-3'	65	290	PCR
Reverse:5'- TGACCGTGCTGCTGGGCTC-3'	63		
Forward:5'- GTCGGGTCGCCAGTCGCAT-3'	65	146	qPCR
Reverse:5'- ATGGTCGGGCTGTAGAAAGAAATGG-3'	63		
glucocorticoid receptor (GR)			
Forward:5'- TGACACCTCCACCCGCCT-3'	61	95	PCR & qPCR
Reverse:5'- TGAACCCTGGCAGAGACTTGG-3'	61		
5-HT1A receptor (5-HT$_{1A}$R)			
Forward:5'-TGGAGAAGCGCCGAGGACA -3'	64	185	PCR & qPCR
Reverse:5'- GCCTTCTCAGTTTTCTTCACGGTC-3'	61		
ribosomal protein L8 (RPL8)			
Forward:5'- TGATAAGCCCATCCTGAAGGC-3'	61	100	PCR & qPCR
Reverse:5'- TGCTCAACAGGATTCATAGCCA-3'	61		

S3. Quantifying receptor gene expression levels

Ten *K. marmoratus* (separate from the study individuals described elsewhere) were decapitated and their brains were removed by microdissection then subsequently pooled, transferred to 400 µl cold TRIzol (Sigma-Aldrich® Co, MO, USA) and homogenised for 40 sec. Following homogenization, 200 µl chloroform was added and the samples were vortexed and incubated at room temperature for 2-3 min. Samples were centrifuged and the aqueous phase was transferred to another tube containing 500 µl of isopropyl alcohol, vortexed and incubated at room temperature for 10 min. The supernatant was removed; 1 ml of 75% ethanol was added to precipitate total RNA followed by centrifugation and removal of all remaining liquid, leaving an RNA pellet. The RNA pellet was dissolved in 50 µl ultrapure water by gentle repeat pipetting. Then, cDNA was synthesised with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., CA, USA). PCR amplified product of *K. marmoratus* AR, ERα/β, 5-HT1AR and GR cDNA generated from *K. marmoratus* tissue cDNA was purified using QIAquick® PCR purification kits, QIAquick® Gel Extraction Kits or MinElute PCR Purification Kits (QIAGEN Inc., CA, USA). PCR cycles were carried out with the following conditions: 40 cycles of 94℃ for 15 sec, 61℃ for 50 sec (annealing) and 72℃ for 30 sec (elongation) and a final 10 min at 72℃. The annealing temperature was adjusted depending on the different primer sets (Table 1). The PCR product was sent off for sequencing (University of Maine DNA Sequencing Facility) using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc., CA, USA), and the sequence was subject to a BLAST search to ensure that the product aligned with the receptor genes.

After targeting the receptor gene sequence by using PCR and RT-PCR, we quantified gene expression using qPCR performed on the Mastercycler® ep realplex System with SYBR (Kapa™ Biosystems, Inc., MA, USA) green according to the manufacturer's instructions. Brains were extracted from the heads stored at -80 ºC and were transferred to 400 µl cold TRIzol and homogenised for 40 sec. Following homogenization, total RNA was extracted as

previously described. Each RNA sample was quantified using a NanoDrop-1000 Spectrophotometer, and adjusted to a final total RNA concentration of 50 ng/μl. cDNA was synthesised with a High Capacity cDNA Reverse Transcription Kit. To run qPCR, 2 μl of ds DNA standards (for AR, ER_{β}, GR and 5-HT_{1A}R gene, concentration of standards were 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} pg/μl; for ER_{α} and RPL8 gene, concentration of standards were 10, 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} pg/μl) and samples were pipetted in duplicate into 96-well PCR plates (twin tec. PCR Plate 96, semi-skirted, wells colourless, Eppendorf, NY, USA). Then, 8 μl mixture (5 μl SYBR® FAST Master Mix 2X Universal, 0.2 μl forward and 0.2 μl reverse 10 μM primers and 2.6 μl ultrapure water) was pipetted into each well containing standards or samples with a multichannel pipette. qPCR cycles were as follows: 95 ℃ for 20 sec and 40 cycles of 95 ℃ for 1 sec and 60 ℃ for 20 sec. Melting curve analysis using Mastercycler® ep realplex System software (Eppendorf, NY, USA) was performed to confirm primer efficiency.

S4. Partial Gene Sequences

Below is a list of sequences for the sex steroid and serotonin receptors generated by PCR and used to develop qPCR primers shown in Table S2. The RPL8 sequence is not shown because we used primers previously designed by Orlando et al. (2006). Embedded within each sequence are the forward (blue) and reverse (red) primers, corresponding to the qPCR primers shown in Table S2; note that the reverse primer sequence reported in Table S2 is the reverse complement of the sequence shown in red below.

>Seq1 [*Kryptolebias marmoratus*] androgen receptor mRNA, partial cds
GAGGATCATTCTGTTCACGAACCAGCAGACGTGCCCCACAACATTTCTCCTAAGG
CGGGTCCGAACTTTAACTCCCAGCTGGTTTTCCTCAACATCCTGGAGTCCATTGAG
CCCGAGGTGGTGAATGCAGGACACGACTGCGGCCAGCCAGACTCTGCTGCCGGC
CTGCTCACCAGCCTGAATGAGCTGGGAGAGAGACAACTGGTCAAAGTGGTCAAA
TGGGCAAAGGGGCTGCCAGGTTTTAGAAACTTGCACGTGGATGACCAGATGACT
GTCATCCAGCAGTCGTGGATGGG**GGTGATGGTGTTTGCGCTGATA**TGGAGGTC
CTATAAGAACGTCAACGGCAGGATGCTTTACTTTGCCCCGGACCTCGTCTTTAAC
GAACACCGGATGCACGTCTCCACCATGTATGANCACTGCATGCGGATGANGCATC
TTTTCCCAGGAANTTTGTGCTGCTGCANAATCACTCANGAAGANNTTCCTCTTGCN
TGAANGGCCCTGCTG**CTCTTTCAGCATCATTCCCGTTG**NAGGG

>Seq2 [*Kryptolebias marmoratus*] oestrogen receptor alpha mRNA, partial cds
TTGACTATGCTGCCCCCACCCCT**GCCCCTACTCCTCTCTACAGCAACTC**CACCAC
TGGGTACTTCTCTGCTCCTCTGGACGCCCACGGACCCCCGTCTAATGGCAGCCTTC
AGTCTCTAGGAAGTGGGCCAACTAGTCCACTTGTGTTTGTG**CCCACCAGTCCAAG
GCTCAGC**CCCTTTATGCATCCTCCTGGTCACTATCTGGAAACCGCCTCAACACCCG
TCTACAAA

>Seq3 [*Kryptolebias marmoratus*] oestrogen receptor beta mRNA, partial cds
GTCGGGTCGCCAGTCGCATCCTGTCCCCGGTCCTCAGCTCCTCCCCTGGTCTGTC
CCACGAACCCAGCCAGCCCATCTGCATCCCCACTCCGTACACCGACCTCGGCCAC
GACTTCCCGC**CCATTTCTTTCTACAGCCCGACCAT**CTTCAGCTACGCCGGTCCGA
GCATTTCGGAGCACCCCTCGGTGCATCAGTCGCTGAGCGCCTCTTTGTTTTGGCCC
GGTCACGGACACGTGGGGGGTTCTGTACCCCTGCACCGCTCCCAGACCCGAGCCC
AGCAGCACGGTCAG

>Seq4 [*Kryptolebias marmoratus*] glucocorticoid receptor mRNA, partial cds

TGACACCTCCACCCGCCTCATGACCACCCTCAACAGGTTGGGTGGCCGGCAGGT
CATCTCTGCTGTCAAGTGGG**CCAAGTCTCTGCCAGGGTTCA**GGAACCTGGACGA
CCAGATGACTCTGCTGCAGTGCTCCTGGCTCTTCCTCATGTCGTTCAGCCTCGGCT
GGAGGTCTTACCAACAGTGCAACGGCAACATGCTCTGCTTCGCGCCGGACCTCGT
CATCAATGAAGAGCGGATGAAGCTGCCCTACATGGCCGAGCAGTTTGAGCAGATG
ATGAAGATTTCCAGCGAGTTCGTGCGGCTGCAGGTGTCCCACGATGAGTACCTGTG
CATGAAGGTCCTGCTGCTGCTCAGCACAGTGCCTAAGGACGGCCTGAAGAGCCAG
GCGGTGTTCGAAGAGATCNNCATGTCGTAAAGAAG

>Seq5 [*Kryptolebias marmoratus*] $5HT_{1A}$ receptor mRNA, partial cds

GCACTTTTGATTTCCGCAACTTGGCTCATTGGTTTCTCCATCTCCNTCCCGCCTATG
TTAGGG**TGGAGAAGCGCCGAGGACA**GGGCGAACCCGGACGCCTGCATGATCAG
CCAGGACCCGGGCTACACGATCTACTCCACGTTTGGAGCTTTTTACATCCCTCTTA
TCCTGATGCTGGTTCTGTACGGGCGGATATTCAGGGCCGCTCGGTTTCGGATTCG
AAA**GACCGTGAAGAAAACTGAGAAGGC**AA

References

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3. Orlando, EF, Katsu Y, Miyagawa S, Iguchi T. 2006. Cloning and differential expression of estrogen receptor and aromatase genes in the self-fertilizing hermaphrodite and male mangrove rivulus, *Kryptolebias marmoratus*. *J. Mol. Endocrinol.* **37**, 353-365. (doi: 10.1677/jme.1.02101).