Detailed Materials and Methods

Animals

Marisa cornuarietis used in this study were obtained from stocks bred at Brunel University, UK, which were originally derived from a stock maintained at Prof. Dr. Jörg Oehlmann's lab, Johann Wolfgang Goethe University Frankfurt am Main, Department of Ecology & Evolution, Frankfurt am Main, Germany.

PCR amplification and cDNA cloning

A degenerate primer RT-PCR approach was employed to identify cDNAs encoding putative steroid receptors derived from *M. cornuarietis* total tissue extracts. Total RNA was isolated from reproductively mature female snails using the guanidinium thiocyanate-phenol-chloroform extraction described by Chomczynski and Sacchi (*1*), including a further precipitation step with 4 M lithium chloride. Messenger RNA was isolated from total RNA using the Nucleotrap mRNA purification kit (Macherey-Nagel). First strand cDNA synthesis was performed using Superscript III (Invitrogen) according to the manufacturer's instructions. First round PCR was performed using the degenerate primers 5'-CGC A(A, T)C (A, C, G, T)GG (A, C, G, T)TA (C, T)CA (C, T)TA (C, T)G G(A, C, G, T)G T-3' (sense) and 5'-GAA (A, C, G, T)CC (A, C, G, T)GG (A, G, T)AT (C, T)TT (C, T)TT (A, C, G, T)GC CCA-3' (antisense) at annealing temperatures of 50°C for 10 cycles followed by 55°C for 30 cycles. All degenerate PCR was performed using Platinum *Taq* (Invitrogen). PCR products were resolved on a 1.2% agarose/TBE gel, fragments of the expected size were excised and

purified using the Minelute gel extraction kit (Qiagen), ligated into pDrive vector (Qiagen) and transformed into EZ competent cells (Qiagen). Blue/white screening and colony PCR check were used to select positive clones containing fragments of the expected size. Selected colonies were purified using Qiaprep Miniprep kit (Qiagen) and sequenced using the Amersham Cycle Sequencing kit with ^{32}P. Sequencing revealed that the cloned fragments were putative ER and ERR orthologue transcripts.

To obtain further sequence 3' of an original degenerate fragment encoding a putative ER orthologue transcript, a gene specific sense primer was designed (5'-AAC TGC ACT ATA GAC AAA CAC C-3') and used with a degenerate antisense primer (5'-A(A, G)C ATC TC(C, G) (A, G)(C, T)(C, G) A(A, G)(C, G) AGG TC-3') in PCR with an annealing temperature of 58ºC for 30 cycles. Complete 3' sequence (including stop-codons and 3' untranslated regions) of the putative ER and ERR orthologue transcripts were elucidated by 3' RACE (rapid amplification of cDNA ends). Briefly cDNA was synthesised by Superscript III reverse transcriptase (Invitrogen) using the Invitrogen 3' RACE adapter primer. PCRs were performed using gene-specific primers (ER: 5'-GCA GGA GAG CGC GGC ATT GCT TAC TTC C-3'; ERR 5'- ATG GTG CAG CCC GTG GTA TCG-3') with an Abridged Universal Amplification Primer (Invitrogen) at an annealing temperature of 62°C for 30 cycles. Extended 5' sequence information was attained with the SMART RACE cDNA Amplification kit (Clontech) using gene-specific primers (ER 5'-ACA TTT GCG TAG TCG ACA TGC-3'; ERR 5'- TCC AAC CGC ACT CCT TCT CTA AG-3'. Cloning was performed as described above and sequencing was conducted using the dideoxy method at MWG Biotech laboratories. Different isoforms of 5' putative

ER transcript fragments were isolated using this method including 1 with a putative start codon located further upstream than the other isoforms.

The full translated region of the shorter ER orthologue coding sequence was verified by PCR using the specific primers 5'-TGC TGA TCA CAC TAA TGG CGG-3' (sense) and 5'-GGT GAT CTT GTA GAG CCT TTC TT-3' (antisense) at an annealing temperature of 55°C for 30 cycles. Full ERR coding sequence was also verified by PCR using specific primers 5'- ACC ACT GGG GAC GCT GTG -3' (sense) and 5'- GGA ATC TGC CTC CAG CAT TTC TA -3' (antisense) at an annealing temperature of 60ºC for 30 cycles. Specific PCR was performed using *Pwo* Superyield polymerase (Roche). Fragments of the expected size were gel-purified as described above, ligated into Zeroblunt vector (Invitrogen) and transformed into TOP10 chemically competent cells (Invitrogen). Kanamycin-resistant colonies were checked for the presence of vector containing a DNA insert of the expected size by colony PCR. Positive clones were sequenced by the dideoxy method at MWG Biotech laboratories.

Phylogenetic analyses

Initial structural analyses and BLAST (basic local alignment search tool) queries were performed using the NCBI (National Center for Biotechnology Information) server (http://www.ncbi.nlm.nih.gov). Non-*M. cornuarietis* amino acid sequences included in phylogenetic analyses conducted were obtained via Entrez on the NCBI server. Multiple alignments were performed using Clustal W (*2*). Phylogenetic tree construction was performed by maximum likelihood using PhyML (*3, 4*).

Expression plasmids

The full coding regions of mcER-like (short protein-coding isoform) and mcERR were directionally cloned into pSG5 expression plasmid (Stratagene) by *Eco*R I/*Bam*H I restriction digestion and ligation. Briefly, the mcER-like coding region was isolated by PCR using the primers 5'-TAGgaattcATGCCCAGCCAGTCCCTG-3' (sense) and 5'-CGCggatccCTACACATATGGATTCAT-3' (antisense). McERR coding region was isolated by PCR using the primers 5'3' (sense) and 5'3' (antisense). The sense primers included an *Eco*R I restriction site and the antisense primers included a *Bam*H I restriction site (indicated in lower case). PCR was performed using *Pwo* Superyield polymerase (Roche). Cycling conditions used included an annealing temperature of 65ºC for 30 cycles. Full-length verified clones described above were used as a template for use in PCR.

Ligand-binding

Binding of [2, 4, 6, 7-^{3}H] 17β-estradiol to mcER-like and mcERR was assessed in triplicate by radio-ligand binding assay using the method described by Routledge et al (*5*). McER-like and mcERR recombinant proteins were synthesised from pSG5-mcER-like and pSG5-mcERR using the TNT T7 Quick-coupled transcription/translation system (Promega). Size and integrity of protein products were checked by parallel protein syntheses incorporating Redivue ^{35}S methionine (Amersham), which were subsequently analysed by 10% SDS-polyacrylamide gel electrophoresis and autoradiography.

Translation products were diluted 20-fold in ligand-binding buffer (20 mM HEPES, 1.5 mM EDTA, 0.1% BSA, 0.25 mM DTT, 10% glycerol) and 50 µl aliquots were incubated for 16 h at 4ºC in the presence of [2, 4, 6, 7-^{3}H] 17β-estradiol (evaporated to dryness in tubes from ethanol stock) over a range of final concentrations from 0.3 to 9.6 nM. Following incubation, free estradiol was removed by addition of 50 µl ice-cold DCC (0.1 g Dextran 70, 1.0 g charcoal (Norit A; Sigma), 4 ml 1 M Tris pH7.4, 0.8 ml 0.5 M EDTA, $H_2$0 to 400 ml). This mixture was incubated on ice for 5 minutes and then separated by centrifugation at 4,000 x g for 2 mins. 80 µl of supernatant was removed, added to 4 ml of Liquiscint (National Diagnostics), mixed and analysed in a TRI-CARB 200A liquid scintillation analyser (Packard). Background radioactivity was assessed by analysis of negative controls, which contained product from transcription/translation procedure performed using empty pSG5 vector. Transcription/translation product from reaction containing pSG5-mouseERα (a gift from Prof. Malcolm Parker) was used as a positive control. Specific protein-specific binding was calculated by subtracting background radioactivity from total measured radioactivity and results were normalised to the maximum [2, 4, 6, 7-^{3}H] 17β-estradiol binding observed to the positive control.

Quantitative and semi-quantitative PCR

Whole albumen gland (female), capsule gland (female), cephalic tentacle, cerebral ganglia, gonad-digestive complex, and penis sheath complex (male) were dissected from individual snails along with sections of foot and lung tissue. All tissue was snap-frozen in liquid nitrogen immediately following dissection. Total RNA was isolated from individual organs from 5 replicate individuals, using Tri-Reagent

(Sigma), according to the manufacturers recommended protocol. All total RNA samples were treated with *DNase I* (Invitrogen) to remove any traces of genomic DNA. To determine absolute amounts of transcripts, RNA standards were synthesised *in vitro* from DNA templates representing a 248-base fragment of the *mcER-like* 3' coding region/3' untranslated region and a 115-base fragment of the *mcERR* 3' untranslated region. The Riboprobe *in-vitro* Transcription System (Promega) was utilized and this included a *DNase I* treatment step. Real-time PCR was carried out using the one-step QuantiTect SYBR Green RT-PCR kit (Qiagen) with an ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Amplification was carried out in 96-well plates, in a total volume of 25 μl containing QuantiTect SYBR Green RT-PCR mix (12.5 μl), 0.5 μM final concentration of each primer, QuantiTect RT Mix (0.25 μl), and RNA standards ($5x10^7 – 5x10^2$) molecules or unknown RNA sample (50 ng). Primer sequences for the *mcER-like* target gene and the standard were 5'-ACT TGC TCA CCG AGA TGC TT-3' (sense) and 5'-CCC AGC CTT GCA GTC TAA AG-3' (antisense). Amplification conditions for *mcER-like* real-time PCR were: reverse transcription for 30 min at 50°C, inactivation for 15 min at 95°C, followed by PCR amplification: 40 cycles of 15 s at 94°C, 30 s at 55°C and 40 s at 72°C. Primer sequences for the *mcERR* target gene and standard were 5'-CAT TTT CCC GTC GAC TCT TG-3' (sense) and 5'-CTG CAT ATC CTC CCT TTG GA-3' (antisense). Amplification conditions for *mcERR* real-time PCR were: reverse transcription for 30 min at 50°C, inactivation for 15 min at 95°C, followed by PCR amplification: 40 cycles of 15 s at 94°C, 30 s at 60°C and 30 s at 72°C. To confirm specificity, PCR products were subjected to melting curve analysis, and subsequent agarose gel electrophoresis and sequence analysis. Each experiment included absolute negative controls (no template), no reverse transcriptase controls

and negative tissue controls (RNA isolated from fathead minnow testis). All determinations were carried out in triplicate. A standard curve of the threshold cycle (C_t) value obtained from the serially diluted RNA standards was compiled. C_t values from the samples were plotted on the standard curve, and from this the number of target RNA copies in each sample was calculated.

Semi-quantitative RT-PCR was performed using the *ER-like* specific primers 5'-ACT TGC TCA CCG AGA TGC TT-3' (sense) and 5'-CCC AGC CTT GCA GTC TAA AG-3' (antisense) and the *ERR* specific primers 5'-TCC GAA GAG TTC ATC ATC AAT CGT-3' (sense) and 5'-AAA GTG GGC TGA AGC GAA ACA A-3' (antisense). Twenty five cycles were performed at an annealing temperature of 60ºC. Individual PCR samples contained equal volumes of 1st strand cDNA synthesized from 500 ng *DNase* I-treated RNA template using methods described above.

References

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