The following supplement accompanies the article

Habitat-associated intraspecific variation in behavior and stress responses in a demersal coral reef fish

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Supplement. Additional methodological detail

Histological confirmation of gonadal sex

After 24 h fixation in 4% paraformaldehyde and storage in 70% ethanol at 4°C, gonadal tissues were dehydrated in a graded ethanol series. Ethanol was removed from the tissues with toluene. Tissues were then paraffin infiltrated overnight at 60°C before being embedded in paraffin. Gonads were sectioned (10 µm) by rotary microtome, and 3 sections spaced at distances 100–400 µm apart were obtained from each gonad and mounted onto albumin-coated glass slides. Slides were then stained with hematoxylin and eosin, and the resulting tissues were photographed with a digital camera (SPOT RT KE, Diagnostic Instruments, Inc.) attached to an Olympus BX-60 light microscope operating in brightfield mode. The sex of each bicolor damselfish was confirmed by visual identification of spermatogenic or oogenic cells (Leino et al. 2005).

Isolation and sequencing of partial cDNA sequences

Using scuba, divers collected 2 adult male bicolor damselfish (standard lengths: 45.30 mm and 65.05 mm) by hand net (SlicDive Inc.) on 14 November 2006 from the fringing coral reefs of Curaçao, the Netherlands Antilles, in the southern Caribbean Sea. Each fish was euthanized in tricaine methanesulfonate (MS-222) (Argent Chemical), and the whole brain was immediately dissected and placed in RNAlater (Ambion, Inc.) at 4°C for 24 h before being stored at –20°C. Total RNA was extracted from the brains of the fish using TRI Reagent (Molecular Research Center) with bromochloropropane as the phase separation reagent, and then quantified by spectrophotometry (NanoDrop 1000, NanoDrop Technologies). RNA quality was confirmed by electrophoresis of the RNA on a 0.8% agarose gel.

Total RNA was reverse transcribed in a 20 µl reaction by first incubating 2 µg of total RNA template with 0.5 µl random hexamer (10 mM), 1 µl dNTPs (10 mM) and 8.43 µl water at 65°C for 5 min. The mixture was placed on ice for 1 min, and then 4 µl of 5× First Strand Buffer was added, along with 2 µl of 0.1 M Dithiothreitol, 1 µl of RNase inhibitor, and 1 µl of Superscript III reverse transcriptase (SuperScript III Reverse Transcription kit, Invitrogen). The mix was then incubated under a thermal profile of 25°C for 10 min, 42°C for 50 min, and 70°C for 5 min, before being stored at –20°C.

PCR was performed using degenerate primers designed to consensus regions of cDNA sequences from other teleost fishes (Table S1). Degenerate primer PCR was performed in 50 µl reactions containing 36.6 µl water, 5.0 µl 10× buffer, 3.0 µl of 25 mM $MgCL_2$, 0.4 µl

GoTaq DNA polymerase (5 U μl^{-1}), 1.0 µl of 10 mM dNTPs, 1.0 µl each of forward and reverse primers (50 µM), and 2.0 µl of cDNA template. The following thermal profile was used: 95°C for 2 min, 35 cycles of 95°C for 30 s, 51°C for 30 s, 72°C for 1 min, and then 72°C for 5 min. When examination on a 1.2% agarose gel revealed a band of predicted size, the cDNA was purified (QIAquick PCR purification Kit, Qiagen Inc.) and sequenced on an ABI PRISM 3100 Genetic Analyzer using Big Dye Terminator Cycle Sequencing Kit v 3.1. The resulting sequences were then aligned using Sequencher v. 4.8 (GeneCodes) and their identities were confirmed by BLASTX searching against known teleost sequences provided in GenBank.

For corticotropin-releasing hormone (CRH), nested primers were designed to *Cyprinus carpio* (GenBank accession no. AJ317955), *Danio rerio* (BC085458) and *Oreochromis mossambicus* (AJ011835). The outer and inner nested primers amplified a 396-bp partial sequence of bicolor damselfish CRH provided at GenBank accession no. HM047108. Degenerate primers for CRH receptor 1 (CRH-R1) were designed to consensus regions of CRH-R1 cDNAs from *Ameiurus nebulosus* (AF229359), *Carassius auratus* (AY688837), *Cyprinus carpio* (AJ576244) and *Epinephelus coioides* (AJ820281), and for CRH receptor 2 (CRH-R2) to cDNAs from *Ameiurus nebulosus* (AF229360), *Danio rerio* (XM_681362) and *Oncorhynchus keta* (AJ277158). These primers amplified a 575-bp partial sequence of CRH-R1 (GenBank accession no. HM047110) and a 407-bp sequence of CRH-R2 (GenBank accession no. HM047111) from bicolor damselfish. For CRH-binding protein (CRH-BP), nested degenerate primers were designed to CRH-BP cDNAs from *Cyprinus carpio* CRH-BP1 (AJ490880), *Cyprinus carpio* CRH-BP2 (AJ490881) and *Oncorhynchus mykiss* (NM_001104662 and AY363677), which amplified a 503-bp partial sequence of bicolor damselfish CRH-BP (GenBank accession no. HM047112). Lastly, a 344-bp partial cDNA sequence of urotensin 1 (U-I) from bicolor damselfish (GenBank accession no. HM047113) was amplified using degenerate primers designed to the U-I cDNAs of *Carassius auratus* (AF129115), *Cyprinus carpio* (M11671), *Danio rerio* (NM_001030180), *Oncorhynchus mykiss* (AJ005264) and *Platichthys flesus* (AJ571694). A 754-bp, partial cDNA sequence for elongation factor-1α (EF-1α) was also isolated and sequenced from bicolor damselfish (GenBank accession no. HM047114) using degenerate primers designed to consensus regions of cDNAs for EF-1α from *Carassius auratus* (AB056104), *Oryzias latipes* (NM_001104662), *Pagrus major* (AY190693) and *Seriola quinqueradiata* (AB032900). The resulting partial cDNA sequences were subsequently used to design primers for SYBR green quantitative real-time PCR assays for CRH, U-I, CRH-BP, CRH-R1 and CRH-R2, as well as for EF-1α. The primers used in these assays are presented in Table S2.

Plasma cortisol measurement

Total plasma cortisol was measured by enzyme immunoassay (EIA). Microtiter plates (Corning/Costar Easy Wash Microtiter plates) were coated with 150 µl of rabbit anti-cortisol (polyclonal, Fitzgerald Industries International) at a final concentration of 1:10 000 in coating buffer (0.05 M, carbonate-bicarbonate, pH 9.6). Plates were incubated at 37°C for 3 h and then washed with a 0.15 M NaCl and 0.05% Tween 20 solution using a multi-channel pipette. The wash solution was removed by inverting the plate and tapping dry. Plates were then blocked with 250 µl of EIA buffer (0.1 M phosphate, 0.15 M NaCl, with 0.1% bovine serum albumin) for 30 min, after which this solution was removed, and 150 µl of EIA buffer was added back, along with 2.5 µl of plasma standard or sample and 100 µl of cortisol-horseradish peroxidase conjugate (1:500 000; Fitzgerald Industries International). Plates were incubated on an orbital shaker (30 rpm) for 24 h at room temperature, washed as before, and 200 µl of TMB (3,3',5,5'-tetramethylbenzidine containing 0.01% hydrogen peroxide) (1-Step™ Turbo TMB-ELISA, ThermoScientific) was added to each well. Plates were read (650 nm) at room temperature with shaking until the desired optical density was reached, using a Thermo Multiskan Ascent (ThermoScientific) microplate reader. Then 100 µl of 1.0 M H_2SO_4 was added to each well to stop the color reaction, and an endpoint reading was taken (450 nm). Standards and samples were analyzed with a 4-parameter logistic curve fit ($r^2 > 0.99$). Standard curve for the assay ranged from 2.5 to 500 ng ml^{-1}, and the intra-assay coefficient of variation was 8.6%.

LITERATURE CITED

Leino RL, Jensen KM, Ankley GT (2005) Gonadal histology and characteristic histopathology associated with endocrine disruption in the adult fathead minnow (*Pimephales promelas*). Environ Toxicol Pharmacol 19:85–98

Table S1. Nucleotide sequences of degenerate primers used for isolation of partial cDNAs

Transcript	Species Used for Consensus Regions	GenBank Accession Number	Primers Developed	Primer Sequence (5' - 3')
CRH	*Cyprinus carpio*	AJ317955	CRHfor1	CTCAATTT(A/T)(C/T)TCG(G/T)(C/T)ACCAC
	Danio rerio	BC085458	CRHfor2	GTG(A/G)(C/T)TCTGCT(A/C)GTTGCCTT
	Oreochromis mossambicus	AJ011835	CRHrev1	AGCAG(A/G)TG(A/G)AAGGTCAG(A/G)TC(C/T)AGGGA
			CRHrev2	GATGTT(C/T)CCAACTTT(C/G)CCCT
CRH-R1	*Ameiurus nebulosus*	AF229359	CRHRdegFor1	GTCCGHTACAACACCACCAATAA
	Carassius auratus	AY688837	CRHRdegFor2	AAGAGCAAGCTGCA(C/T)TACCACAT
	Cyprinus carpio	AJ576244	CRHRdegRev1	TGAAAGGACTG(G/T)AGGAAAGA(A/G)TT(A/G)AA(A/G)TA
	Epinephelus coioides	AY820281	CRHRdegRev2	TTCCTGTACTG(A/G)AT(C/G)GTCTCTGA(C/G/T)GTG
			CRHRdegRev3	ATCAG(A/C/T)AG(A/G)AC(A/C/G)AGGATCATGGG
CRH-R2	*Ameiurus nebulosus*	AF229360	CRH-R2degFor1	GAGCCG/T)TGGTG(C/T)CG(C/T)CT(C/T)ATAAC
	Danio rerio	XM_681362	CRH-R2degFor2	GGTGAC(C/G)AATTTTTTCTGGAT
	Oncorhynchus keta	AJ277158	CRH-R2degFor3	ATGAC(A/C/T)TA(C/T)TC(C/T)AC(A/C)GACAAG
			CRH-R2degRev1	GGTGA(G/T)GTGGGRA(G/T)GGACAT
			CRH-R2degRev2	AACAGCATGTA(G/T)GTGAT(C/G/T)CC
			CRH-R2degRev4	CCAAACCAGCA(C/T)TGTTC(A/G)TTTTC
CRH-BP	*Cyprinus carpio* (CRH-BP1)	AJ490880	CRHBPfor2	CAG(A/G)GGAGG(A/G)GA(C/T)TTCAT(A/C)AAGGT
	Cyprinus carpio (CRH-BP2)	AJ490881	CRHBPfor3	TTTGATGG(C/G)TGGGTGATGAAGGG
	Oncorhynchus mykiss	NM_001124631 &	CRHBPfor4	AAAC(C/T)CATCAA(C/T)CC(G/T)TTCCCCTG
	CRHBPfor4	AY363677	CRHBPrev2	CACCAT(C/T)CT(C/G)A(C/T)CAC(A/C)GTGTTATC
			CRHBPrev3	CAGTTCCTGTGCTGCTG(G/T)GG
U-I	*Carassius auratus*	AF129115	Uroten1-degF1	ATGAAGCC(C/G/T)GTC(C/T)C(A/C/T)TTG(A/C/G)TCCTGCTC
	Cyprinus carpio	M11671	Uroten1-degF2	TTG(A/C/G)TCCTGCTC(A/C/T)T(A/C/T)(A/G/T)C(C/T)TC (A/C/T)GTC
	Danio rerio	NM_001030180	Uroten1-degF3	TCCTGCTC(A/C/T)T(A/C/T)(A/G/T)C(C/T)(A/T)C(A/C/T)GT (C/T)(C/T)T(A/C)CT
	Oncorhynchus mykiss	AJ005264	Uroten1-degR2	CGCCAT(G/T)T(C/G)GATCAT(A/G)TT(C/T)CT(C/G)AG
	Platichthys flesus	AJ571694	Uroten1-degR3	GTGGAA(A/G)GT(C/G)AGGTCGATGGA
EF-1α	*Carassius auratus*	AB056104	EF1αfor1	GGGAAAGGAAAA(A/G)A(C/T)CCACAT
	Oryzias latipes	NM_001104662	EF1αfor2	CACAT(C/T)AACATCGTGGT(C/T)ATTGGC
	Pagrus major	AY190693	EF1αrev1	C(C/T)TTGAC(A/G)GACACGTTCTT(G/C)A
	Seriola quinqueradiata	AB032900	EF1αrev2	ACGTTGTCACCAGG(A/C/G)(A/G)(C/T)(A/G)GC
β-actin	*Carassius auratus*	AB039726	BAfor1	ATCATGTT(C/T)GAGACCTTCAACACCC
	Cirrhinus molitorella	DQ007446	BArev1	TACTCCTGCTTGCT(A/G)ATCCACAT
	Danio rerio	AF057040	BArev2	GCAATGCC(A/G)GGGTACATGGT
	Spinibarbus denticulatus	DQ656598		
	Rivulus marmoratus	AF168615		
18S	*Cyprinidon variegatus*	EF535030	18Sfor1	CCTGCGGCTTAATTTGACCCAACA
			18Srev1	GACATCTAAGGGCATCACAAGACCT
			18Srev2	TTGCTCAATCTCGTGTGGCTCAAC

Table S2. Nucleotide sequences for primers used in SYBR green quantitative real-time RT-PCR

Transcript	Primer	Sequence (5' - 3')	Amplicon Size (bp)	PCR efficiency (avg. %)
CRH	Forward	GCGGCTTGGAGAGGAGTATTTCAT	121	94.7%
	Reverse	CAGCTGGAGTTGTAACGCTCTGTT		
CRH-R1	Forward	ATGTTCGGAGAGGGCTGCTA	101	94.8%
	Reverse	GGTATACACCAGCCGATGCA		
CRH-R2	Forward	AGCTGAGAAAGTGGGTCTTCCTCT	273	91.4%
	Reverse	TCGCTTTCACGGCTTTCCTGTACT		
CRH-BP	Forward	CATGGTCTTCTTCCGCATCCA	101	94.9%
	Reverse	CTGGTGACTGGGAGATGACATTACA		
U-I	Forward	TGAGCGACAACATCCTGAGGTT	103	98.4%
	Reverse	GTCCTCACCGCCTCATCGT		
EF-1α	Forward	ACAAGTGCGGAGGAATCGACAAGA	366	92.6%
	Reverse	CAACAATGAGCTGCTTCACACCGA		