The Journal of Experimental Biology 206, 1523-1533

doi:10.1242/jeb.00251

A second glutamine synthetase gene with expression in the gills of the gulf toadfish (*Opsanus beta*)

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Accepted 21 January 2003

Summary

We characterized the expression of the nitrogen metabolism enzyme glutamine synthetase [GSase; L-glutamate: ammonia ligase (ADP-forming), E.C. 6.3.1.2] in tissues of the gulf toadfish *Opsanus beta* subjected to unconfined (ammonotelic) and confined (ureotelic) conditions. Enzymological results demonstrate that mass-specific GSase activities rank in the order of brain > liver > stomach ≈ kidney > intestine > gill > heart/spleen > muscle. When tissue mass is used to calculate a glutamine synthetic potential, the liver has the greatest, followed by muscle > stomach and intestine, with minor contributions from the remaining tissues. Additionally, during confinement stress, GSase activity increases significantly only in liver (fivefold) and muscle (twofold), tissues that previously showed significant expression of the other enzymes of urea synthesis. Western analyses of samples on SDS gels demonstrated that GSase-specific protein content reflected enzyme activity, and all tissues except muscle had a single, similarly sized GSase subunit of 49.4 kDa; muscle showed staining of two bands of 36.8 and 98.9 kDa, which may possibly result from another gene product or post-translational modification.

RT-PCR and RACE-PCR revealed the presence of a second GSase cDNA from gill tissue that shares only 73% nucleotide and amino acid sequence similarity with the GSase cDNA previously cloned from liver, and that lacks a mitochondrial leader-targeting sequence. RT-PCR and restriction digestion experiments demonstrated that mRNA from the original 'liver' GSase is expressed in all tissues examined (liver, gill, stomach, intestine, kidney, brain and muscle), whereas the new 'gill' form shows expression primarily in the gill. Gill GSase activity shows apparently exclusive expression in the soluble compartment, while other tissues expressing the 'liver' form show both cytoplasmic and mitochondrial activities.

Phylogenetic analysis of a number of GSases demonstrates that the toadfish gill GSase has a greater affinity for a clade that includes the *Xenopus* GSase genes and one of two *Fugu* GSase genes, than it has for a clade containing the toadfish liver GSase and other described teleost GSase genes. The results are discussed in the context of a prior hypothesis on an ammonia-trapping mechanism in the gill of the toadfish.

Key words: glutamine synthetase, toadfish, ureotely, ornithine–urea cycle, ammonia detoxification, *Opsanus beta*, ammonia excretion, gill, ORF, UTR.

Introduction

Glutamine synthetase [GSase; L-glutamate: ammonia ligase (ADP-forming), E.C. 6.3.1.2] is an important enzyme of nitrogen metabolism. In the brain of all vertebrates, astrocytic GSase detoxifies ammonia during the conversion of glutamate to glutamine (at the expense of ATP), a process that is also a step in the recycling of the neurotransmitter glutamate taken up from the synaptic cleft (Cooper, 2001). In the liver of mammals, GSase has a mainly secondary or failsafe role relative to ammonia detoxification: GSase expressed in downstream perivenous hepatocytes scavenges ammonia that has not been detoxified by the ornithine–urea cycle (O-UC) expressed in the upstream periportal hepatocytes (Gebhardt and Mecke, 1983; Jungermann and Katz, 1989), where the first enzyme of urea production, carbamoyl phosphate synthetase (CPSase I) uses primarily ammonia as its nitrogen-donating substrate (for a review, see Meijer, 1995). By contrast, fish species synthesizing substantial quantities of urea *de novo* in an O-UC employ CPSase III, which typically prefers glutamine as nitrogen donor (for a review, see Anderson, 2002). Thus, unlike mammals, this requirement of piscine CPSase III for glutamine intimately links GSase to urea production in fish (Campbell and Anderson, 1991). In fact, in one important fish experimental system for the study of facultative urea production, the gulf toadfish *Opsanus beta*, GSase has emerged

as the main regulatory control point. In this model, the transition between ammonotely and ureotely is modulated by a cortisol-dependent increase in hepatic GSase activity, mRNA and protein levels (Hopkins et al., 1995; Kong et al., 2000), with the cytosolic compartment showing the largest increase in GSase activity (Walsh and Milligan, 1995).

GSase is also an interesting enzyme/gene from the standpoint of molecular evolution, and sequence information is available from representatives of many taxa (Kashiwaga et al., 2001). In recent studies of rainbow trout GSase sequences, at least four GSase encoding genes have been discovered, and these additional GSase genes have presumably resulted from either single-case or genome-wide gene duplications (Murray et al., 2003). Their exact functions are as yet unknown, but increasing evidence points towards differential expression during development (P. Essex-Fraser and P. A. Wright, University of Guelph, personal communication). Prior enzymatic and molecular studies of liver GSase in the gulf toadfish (Walsh, 1996; Walsh et al., 1999) suggested the existence of more than a single GSase gene. The studies of Murray et al. (2003) provide substantial recent impetus and information/approaches useful in the search for additional GSase genes in the toadfish model. Furthermore, if one is to pursue the 5′-flanking region sequence from the inducible liver GSase gene in toadfish to obtain regulatory information, there is an important need to enumerate the GSase genes accurately in this species. Thus, the first goal of this study was to probe the existence of multiple GSase genes in the toadfish.

While liver GSase has received much attention in the toadfish, prior studies have shown substantial expression of other O-UC enzymes in non-hepatic tissues; in particular, CPSase III can show ample expression in fish muscle (toadfish, Julsrud et al., 1998; trout, Korte et al., 1997; Lake Magadi tilapia, Lindley et al., 1999; other fish species, Felskie et al., 1998). Therefore, to attain a more comprehensive view of nitrogen metabolism in the toadfish model, it is desirable to know if GSase activity in toadfish is expressed in other tissues, how GSase subcellular compartmentation compares with liver, and whether activities in other tissues are subject to change by stress. Thus, an additional goal of this study was to obtain information on tissue-specific patterns of GSase activity and gene expression, and to compare these data to the molecular information obtained.

Materials and methods

Experimental animals

Gulf toadfish *Opsanus beta* (Goode and Bean) were collected from Biscayne Bay, Florida, using a roller trawl by a commercial fisher in June 2001–April 2002. Prior to transfer to holding tanks at the University of Miami RSMAS campus, the fish were dipped in ‘freshwater’ (20 liters distilled water plus 500 ml seawater) for 3 min, and then in Malachite Green/Formaldehyde (0.05 mg and 15 mg l^{-1}, respectively) (Aquavet, Hayward, CA, USA) for 2–4 h to prevent infection by the ciliate *Cryptocaryon irritans*. For 2 weeks the fish were allowed to recover at low densities (see Wood et al., 1995) in 40 liter holding tanks with flow-through, sand-filtered seawater from Biscayne Bay before experimentation. Water temperature was 22–26°C and the natural photoperiod was used. Fish were fed with frozen squid twice a week and food was withheld for 48 h prior to experiments. Some fish continued to be held under these low density conditions (‘unconfined’), while other individuals were transferred to ‘confined’ conditions (several fish in 2–4 liters of seawater) for 48 h to induce ureotely and hepatic GSase activity (Wood et al., 1995; Walsh and Milligan, 1995). Tissues were harvested after fish had been anesthetized with 0.5 g l^{-1} of MS-222 (tricaine methanesulfonate), and either immediately frozen in liquid nitrogen and then placed in a –80°C freezer, or used that day (e.g. for studies of enzyme compartmentation).

Enzyme activities and subcellular compartmentation

For measurement of total GSase activity only, tissues were disrupted either by sonication (liver, brain and kidney) in a micro-ultrasonic cell disruptor (Kontes, Vineland, NJ, USA) or homogenized (all other tissues) with a Polytron (Brinkman, Westbury, NY, USA) in 5 vol. of 50 mmol l^{-1} Hepes, pH 7.4, then centrifuged at 13,000 ***g***. The supernatant was used directly in enzyme assays. For compartmentation studies, tissues were homogenized in an isotonic sucrose buffer using a teflon pestle on glass homogenizer, followed by differential centrifugation exactly following the protocols of Anderson and Walsh (1995). Activities of the marker enzymes for soluble (lactate dehydrogenase, LDH) and mitochondrial (glutamate dehydrogenase, GDH) compartments were measured in all fractions. The transferase assay for GSase (for details, see Walsh, 1996) was always used since the synthetase assay that ultimately measures ADP production has too many competing side reactions (e.g. from ATPases), especially in crude homogenates.

Western blots

Tissues isolated from confined or unconfined toadfish were frozen in liquid nitrogen, thawed and homogenized in 50 mmol l^{-1} Hepes, pH 7.6. The homogenate was centrifuged briefly to remove debris and divided into portions. Protein concentrations of each sample were determined spectrophotometrically using the BCA protein assay kit (Pierce, Rockford, IL, USA). SDS-PAGE 4–15% gradient Tris-HCl gels (Biorad, Hercules, CA, USA) were loaded with 10 μg total protein in each well and electrophoresed. Protein standards used were colored myosin (220 kDa), phosphorylase b (97.4 kDa), BSA (66 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.3 kDa) (Rainbow markers, Amersham, UK). After electrophoresis, gels were electroblotted onto 0.45 μm PVDF membranes at a constant current of 60 mA for 1 h, using a semi-dry electroblotter (Hoeffer, Piscataway, NJ, USA). Blots were blocked overnight in 1% casein solution, then washed thoroughly in phosphate-buffered saline with 0.1% Tween 20 (PBST). Rabbit antibody to a GSase conserved oligopeptide conjugated to keyhole limpet hemocyanin (KLH)

(Acetylcysteinyl-CPRSVGQEKKGYFEDRRPS-Amide, as produced in Anderson et al., 2002) with high homology to amino acids 326–343 of the toadfish liver GSase (see Fig. 3), was diluted from a stock sample in PBST. The blot was allowed to bathe in the primary antibody solution for 1 h, then washed with PBST. Horseradish peroxidase-labeled, anti-rabbit IgG (Amersham) was then diluted in PBST and allowed to bathe the membrane for 1 h. After sufficient washing the blot was detected using the ECL detection kit (Amersham) and light production was captured on XAR-2 film.

Isolation of RNA, mRNA and cDNA synthesis/RT-PCR

Total RNA was isolated from 0.2 g of tissue by homogenization in 1.2 ml phenol-guanidinium thiocyanate (Trizol Reagent, Gibco BRL, Carlsbad, CA, USA) followed by standard chloroform extraction and isopropanol precipitation (Sambrook et al., 1989). mRNA was enriched from total RNA using a mRNA Purification Kit (Qiagen, Valencia, CA, USA) based on oligo(dT) latex binding. cDNA was synthesized using MMLV Reverse Transcriptase and oligo-dT primers (Stratagene, La Jolla, CA, USA). PCR was performed on these cDNAs using primers designed by Murray et al. (2003); specifically we used either their exact primers to the toadfish liver GSase (GS 301 and 501) or degenerate primers designed from sequences for mammalian, piscine, and other GSases (GLUL*e3f1 and 6r1). Locations for these primers are shown in Fig. 1 in the Results. PCR conditions were 94°C for 5 min, followed by 55°C for 3 min, at which point Taq polymerase (Promega, Madison, WI, USA) was added followed by the mineral oil layer in a hot-start protocol, and then 30–35 cycles of 94°C (1 min), 55°C (30 s), 72°C (30 s), followed by one cycle of 72°C (10 min) in a Perkin-Elmer 480 PCR Thermal Cycler using 0.5 ml GeneAmp tubes (Perkin-Elmer, Boston, MA, USA). PCR products (typically in the range 545–570 bp) were separated by gel electrophoresis on 1% agarose gel in TAE buffer (40 mmol l^{-1} Tris-acetate, 1 mmol l^{-1} EDTA, pH 8.0). Bands were either gel-purified or in some cases mixed PCR products used directly, prior to ligation into the plasmid vector pCR 4 TOPO, with the resultant plasmid transfected into chemically competent JM 109 cells (Invitrogen, Carlsbad, CA, USA). Standard blue/white screening on LB kanamycin plates identified colonies with potential inserts, which were then liquid-cultured and the plasmid DNA isolated by the alkaline lysis method (Qiagen). Restriction digests were then performed to ensure the presence of an appropriately sized insert. Insert DNA was sequenced using an automated dideoxy chain-termination sequencing method (Sanger et al., 1977). Generally, PCR reactions were performed in duplicate (from the same template) to exclude PCR errors, and as noted below, multiple samples from each tissue were sequenced to search for novel GSase sequences.

RACE-PCR

5′ and 3′ rapid amplification of cDNA ends (RACE)-PCR was performed to amplify 5′ and 3′ ends from a novel GSase found in gill using the Marathon cDNA Amplification Kit and adaptor-ligated gill cDNA (Clontech, Palo Alto, CA, USA). Gene-specific primers were synthesized based on the known sequences of a 545 bp PCR product (discovered in routine RT-PCR, as described above) and primer sequences for the first round of PCR were: GGSaseGSP1A, antisense gene specific primer for 5′-RACE, 5′-CAAATGCTGCTCCTTCACTG-CCTC-3′ and GGSaseGSP 2A, sense gene specific primer for 3′-RACE, 5′-CTCATCCCAGTGTGCATGTTCAAAG-3′. The sense primers for 5′-RACE and the antisense primers for 3′-RACE were complementary to an adaptor supplied with the kit that was ligated to the 5′ and 3′ cDNA ends. PCR conditions were: 94°C (1 min), followed by 5 cycles of 94°C (30 s), 72°C (4 min), followed by 5 cycles of 94°C (30 s), 70°C (4 min), followed by 25 cycles of 94°C (30 s), 68°C (4 min). Because RACE-PCR can generate multiple bands in the first round, a second round of PCR with nested primers was performed on 1:250 dilutions of the primary PCR reaction products. The gene-specific primers for the second round were: GGSaseGSP1B, antisense primer for 5′RACE, 5′-CTGTTG-CGATGGTTAGTTTCTGCAGGTA-3′ and GGSaseGSP2B, sense primer for 3′-RACE, 5′-TACCTGCAGAAACTAAC-CATCGCAACAG-3′. Adaptor primers were also nested for this second round. Conditions were as in the first round except the last set of cycles was 15 instead of 25. The 5′-RACE reactions produced a band of approx. 420 bp, and the 3′-RACE reactions produced a band of approx. 900 bp. Bands were purified, subcloned and sequenced as above.

Phylogeny of GSases

A protein alignment of 359 amino acids for 25 protein sequences was obtained by using Clustal X using the default settings. The alignment was then refined by eye. Sequences of shorter length were excluded because they are known to affect resolution in phylogenetic reconstruction. A total of 118 sites were constant. The *Drosophila* sequences were used as outgroups. Minimum evolution (ME) reconstructions were conducted in MEGA 2.0 (Kumar et al., 2001), quartet-puzzling was performed in TREE_PUZZLE (Strimmer and Haeseler, 1997) and Bayesian reconstructions in MrBayes (Huelsenbeck and Ronquist, 2001).

A gamma model of evolution was used for ME, and to estimate branch support we performed 10000 bootstrap pseudoreplicates. The JTT model (Jones, Taylor, Thornton model; Jones et al., 1992) was used for quartet-puzzling, with a gamma correction (alpha parameter = 0.55) and eight categories. Amino acid frequencies were estimated from the dataset. 1000 puzzling steps were performed. Exploratory Markov Chain Monte Carlo (MCMC) runs were performed, starting with a JTT model and a gamma correction. Subsequently, we ran the heated MCMC chain for 100000 generations, which was sampled every 100 updates. We discarded 7000 cycles as burn-in before estimating joint posterior probabilities.

Restriction digests

Comparing the sequences of the PCR products for the ‘gill’

and 'liver' forms of the gene generated with the GS 301 and 501 primers, we predicted that the 'gill' form should be cut once by the restriction enzyme *Pvu*II into 137 and 408 bp fragments, and the liver form cut by *Stu*I should produce 299 and 276 bp fragments. Furthermore, *Pvu*II should not cut the liver form, and *Stu*I should not cut the gill form. Therefore, digest experiments were performed with RT-PCR products from 7 tissues of confined and unconfined individuals using *Pvu*II and *Stu*I alone and in combination to identify tissue-specific mRNAs. Buffers and dilutions were as per manufacturer's recommendations (New England Biolabs, Beverly, MA, USA) and digests proceeded overnight at 37°C, after which agarose gels were run on uncut and cut fragments to determine their molecular size.

Results

Enzyme activities, compartmentation and western analysis

GSase activity was assessed in gulf toadfish for several key tissues of general and nitrogen metabolism. These tissues accounted for most of the body mass of the animal (Table 1). Only two significant changes occurred in mass-specific GSase activity upon confinement of the toadfish. These were an approximately fivefold increase in liver GSase activity, as reported previously (Walsh and Milligan, 1995), and a doubling in muscle GSase activity. In confined fish, the mass-specific GSase activity ranked in order of brain > liver > stomach ≈ kidney > intestine > gill > heart/spleen > muscle. However, after accounting for tissue mass and expressing as a percentage of 'whole body' GSase activity, the liver has the greatest glutamine synthetic potential, followed by muscle > stomach and intestine, and minor contributions from the remaining tissues (Table 1).

As in prior studies of enzyme compartmentation in liver of the toadfish (e.g. Anderson and Walsh, 1995; Walsh and Milligan, 1995), the marker enzymes glutamate dehydrogenase (GDH; mitochondrial) and lactate dehydrogenase (LDH; soluble) fractionated mostly as anticipated, with GDH showing only minor contamination of the soluble compartment, and LDH as a minor contaminant of the mitochondrial compartment (Table 2). Exceptions in the present study were the slightly higher proportion of LDH found in the brain mitochondrial fraction, and the lower proportion of GDH present in the mitochondrial fractions of stomach and muscle. We have no ready explanation for the slightly unexpected marker enzyme compartmentation data in the brain. However, in stomach and muscle, this phenomenon might be due to the relative toughness of the tissues leading to a lower harvesting efficiency for intact mitochondria, a suggestion reflected in the higher proportion of GDH in the debris and soluble fractions (Table 2).

For tissues other than gill and liver, GSase activity shows partial distribution in both the soluble and mitochondrial compartments, with a slight bias towards the mitochondrial compartment in the unconfined state (Table 2). Liver GSase compartmentation shows the variable pattern previously demonstrated by Walsh and Milligan (1995), namely that in the unconfined state, enzyme activity is distributed in both mitochondrial and soluble compartments, but that most of the increase in GSase activity upon confinement resides in the soluble compartment (Table 2). Gill GSase compartmentation is notably different from all other tissues in that it appears to be nearly exclusively expressed in the soluble compartment, with fractionation percentages skewed even more towards the soluble compartment than the soluble LDH marker distribution (Table 2).

Western blots (Fig. 1) of SDS gels demonstrated that GSases in all tissues except muscle appear as a single subunit band with a molecular mass of approximately 49.4 kDa. This value differs slightly from the 42.3–42.4 kDa previously reported for liver in Walsh (1996), probably due to the slightly different electrophoretic conditions and the use of crude homogenates *versus* purified enzyme. Additionally, the intensity of the staining reflects both the overall differences in activity among tissues, as well as differences in confined *versus* unconfined treatments for liver and muscle. Interestingly, muscle shows a rather different pattern of expression, with two bands at 36.8 and 98.9 kDa, the larger band appearing to increase in expression during confinement. From these initial results, it cannot be concluded if these patterns are related to the twofold increase in GSase activity in muscle and whether they are related to, for example, expression of yet another GSase gene that is muscle-specific, or a post-translational modification that effects the association of GSase subunits in the muscle. Other factors could also be operating, such as antibody cross-reactivity with a related protein in higher abundance than

Table 1. *Glutamine synthetase enzyme activities in selected major tissues from the Gulf Toadfish (*Opsanus beta*) that were either unconfined or confined for 48 h*

	GSase activity		
Tissue	Unconfined	Confined	Percentage of 'whole body' GSase
Liver	4.85±0.72	21.21±1.42*	39.6
Gill	3.56±0.41	2.61±0.25	3.3
Brain	38.05±3.51	38.95±1.50	3.7
Intestine	2.23±0.48	3.84±0.80	9.9
Stomach	9.24±1.16	7.54±1.23	13.0
Muscle	0.14±0.03	0.29±0.06*	28.0
Kidney	5.03±0.57	7.54±2.23	2.3
Heart/Spleen	0.70±0.15	1.07±0.16	0.2

GSase, glutamine synthetase.

Values (μmol min^{-1} transferase activity g^{-1} wet tissue mass) are means ± 1 S.E.M., N=8.

To calculate the percentage of 'whole body' GSase activity, mass-specific activity was multiplied by the mass of the individual tissue then expressed as a percentage of the total activity of all 8 tissues (for confined fish only).

*Significant difference (P<0.05) from unconfined value; Student's t-test.

Table 2. *Subcellular compartmentation of GSase and marker enzymes in various tissues of gulf toadfish* Opsanus beta *that were either unconfined or confined for 48 h*

Treatment/tissue	Enzyme activity (μmol min^{-1} g^{-1} wet tissue)	% of activity in compartment		
		Soluble	Mitochondrial	Debris
Confined				
Glutamine synthetase				
Liver	15.55±1.92	64.8±6.8	13.1±3.0	22.1±5.2
Gill	1.93±0.61	81.8±3.3	3.0±0.8	15.2±3.6
Brain	37.52±1.51	52.7±0.7	29.6±1.7	17.7±1.4
Intestine	3.25	58.7	9.9	31.5
Stomach	4.17	66.8	5.2	28.0
Muscle	0.13	73.8	15.2	11.0
Kidney	1.96	42.4	17.5	40.1
Glutamate dehydrogenase (mitochondrial)				
Liver	11.49±0.88	8.2±0.6	53.0±6.2	38.9±6.5
Gill	5.88±0.98	12.0±4.1	53.9±6.4	34.1±7.4
Brain	1.88±0.28	13.8±1.9	54.5±5.6	31.8±6.3
Intestine	3.95	12.6	57.4	30.0
Stomach	4.22	20.2	36.1	43.6
Muscle	0.97	29.9	23.4	46.7
Kidney	7.27	11.7	52.2	36.1
Lactate dehydrogenase (soluble)				
Liver	6.87±2.07	69.8±4.3	11.9±3.6	18.3±2.3
Gill	32.45±6.97	69.3±3.6	7.8±4.0	23.0±1.9
Brain	47.76±9.02	46.9±2.4	30.4±1.3	22.7±1.6
Intestine	19.95	59.8	8.7	31.5
Stomach	20.86	69.1	3.6	27.3
Muscle	145.29	68.1	1.0	30.9
Kidney	16.95	73.5	4.6	21.9
Unconfined				
Glutamine synthetase				
Liver	4.41	36.0	44.1	19.9
Gill	2.57	82.9	5.2	12.0
Brain	32.46	54.2	30.5	15.3
Intestine	2.95	66.1	14.7	19.2
Stomach	5.48	67.3	5.1	27.6
Muscle	0.17	58.7	8.6	32.8
Kidney	1.47	36.5	25.2	38.4

Values are means ± 1 S.E.M. *N*=4 for liver, gill and brain of confined fish; *N*=1 for other tissues and unconfined treatment.
All samples contained the pooled tissues of 3–5 fish.

GSase (which has the lowest mass-specific enzyme activity of all tissues examined).

GSase cDNA sequences and phylogeny

RT-PCR was performed on mRNA of seven tissues (those listed in Table 1, except for heart/spleen) from each of three individual toadfish using both primer pairs of GS 301/501 and GLUL*3ef1 and 6er1. In all cases except one, the sequences of the 545 bp fragments obtained were identical to that of the appropriate segment of the liver GSase sequence previously published (Walsh et al., 1999). PCR of gill from one individual yielded several subclones of the same 545 bp size, but whose overall sequence was rather different from the liver. This unique sequence for the 545 bp PCR product, which formed the basis for RACE-PCR to obtain a full sequence, can be found between nucleotides 249–791 shown in Fig. 2, except for a 3 bp 'error' that was corrected during sequencing of RACE-PCR products (see more detailed explanation in the final paragraph of Results). Notably, the same gill mRNA sample also yielded additional PCR products whose sequence matched the original liver GSase sequence, suggesting coexpression of the two GSase forms in the gill (see below).

RACE-PCR of the adaptor-ligated gill cDNA library using primers specific to this new GSase sequence and adaptor primers resulted in approx. 420 bp products at the 5′ end, and predominant approx. 900 bp products at the 3′ end. Sequences

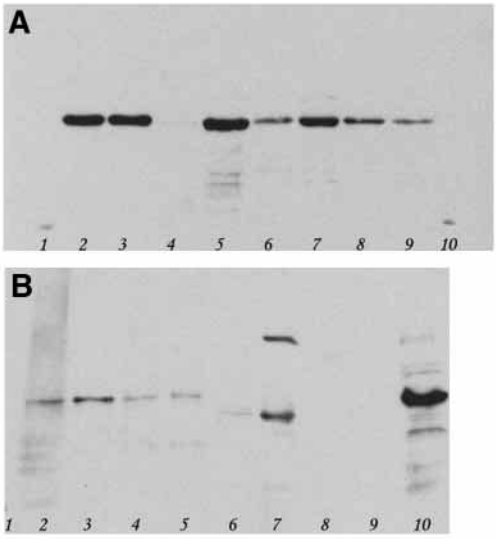

Fig. 1. Western blots of several tissues from unconfined and confined representative individuals of the gulf toadfish *Opsanus beta*. The antibody is to a conserved oligopeptide sequence of GSases (homologous to amino acids 326-343 of the liver Gsase; Fig. 3). (A) Lanes 1 and 10 are molecular mass marker lanes. Even-numbered lanes are samples from unconfined toadfish and odd-numbered lanes from confined toadfish. Samples in lanes 2–3 are derived from brain; lanes 4–5, liver; lanes 6–7, kidney; lanes 8–9, stomach. (B) Lanes 1 and 9 are molecular mass marker lanes, and lane 10 is from unconfined brain (the same sample as in A, lane 2) for cross reference between gels. Even-numbered lanes are samples from unconfined toadfish and odd-numbered lanes from confined toadfish. Samples in lanes 2–3 are derived from intestine; lanes 4–5, gill; lanes 6–7, white muscle. Lane 8 is an empty well.

of several subclones of the 5′-RACE-PCR products consistently yielded the same invariant sequence shown in Fig. 2 (nucleotides 1–385). Interestingly, different 3′-RACE-PCR subclones yielded two sequence variants that differed only by the presence or absence of a 67 bp sequence near the 3′ end, just outside the open reading frame (ORF) (Fig. 2). Presumably, these two variants of the gill GSase are RNA splice variants from the same gene.

The full sequence of the gill GSase (GenBank AF532312) reflects an overall mRNA/cDNA size considerably smaller than the original liver GSase (1263 bp for the gill *versus* 1679 bp for the liver). However, the ORF for the gill yields a protein that is two amino acid residues larger than in the liver (373 *versus* 371 residues) with the substitution appearing near the N terminus (Fig. 3). Note that the gill GSase has no indication of any mitochondrial leader sequence that would target it to the mitochondrial compartment, even when

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CGGCTTCAGACAGAAACCTTCACACAGAGACGATGGCCTCTTCGGTGTCTGTCAGCTCTCGCCTCAACAAGGCGGTGCTGCAGCGGTACCTGAGCCTGCC 100
                                ↳ ORF Start

GCAGCAAGGAAAAACCCAGGTCACCTACATCTGGATCGACGGCACCGGAGAGGGACTTCGAAGCAAGACACGAACTCTGGATAGCGAGCCAAAAAGTGTA 200

GAAGACATTCCTGAGTGGAACTTTGACGGTTCCAGTACGTACCAGGCCGAAGGCTCCAACAGTGATATGTACCTCATCCCAGTGTGCATGTTCAAAGATC 300
                                                  GS501/e3f1                    GSP2A

CGTTTACACTTGACCCCAACAAACTGGTCCTCTGCGAAGTCCTCAAATACAACCGCTTACCTGCAGAAACTAACCATCGCAACAGCTGCAACAAAGTGAT 400
                                                          GSP1B and GSP 2B

GGAGGCAGTGAAGGAGCAGCATTTGTGGTTTGGCATGGAGCAGGAGTACACGCTGTTTGGTACAGATGGACATCCCTATGGGTGGCCCGCCAATGGATTC 500
      GSP1A

CCGGCACCACAAGGCCCCTACTACTGTAGCGTGGGTGCCAACAATGCTTACGGGCGTGACGTAGTGGAGTGCCACTACAAGGCGTGCCTGTATGCAGGAA 600

TCAAAATCTACGGCACAAATGCTGAGGTCATGCCAGCTCAGTGGGAGTTCCAGATCGGCCCCTGTGAGGGCATCGAGATGGGCGACCACCTGTGGGTGGC 700

GCGTTTCTTGTTGCATCGAGTGTGTGAAGATTTTGGCATCATCGCCACCATGGACCCCAAACCAATGAAGGGGAACTGGAACGGTGCTGGCTGTCACACA 800
                                                                                GS301

AATGTGAGCACCAAAGAGATGAGGGAGGAGGGAGGACTGCAATACATCGAGCAGGCCATCGAGAAGTTGAGTAAAAATCATGCCGAGCACATCTGCATGT 900
   e6r1

ATGACCCCCATAAGGGCCAGGATAACATCAGAAGGCTCACGGGGATCCACGAGACCTCCAGCATCCATGACTTCTCTGCTGGCGTGGCCAACCGAGGTGT1000

CCAGTATCCGCATCCCTCGGCATGTGGGGCAGGAGAAACGAGGTTACTTTGAGGACCGCCGCCCGCTGCAAACTGTGACCCGTACGCCGTCACCAAGGTC1100

ATCGCAAGCACCTGTATTCTCAGCACTGACAATAATACACAGTCCCAGCAATAAAAAATGAAGGTCAAACCATGGTCTGTAGCAAACTTGTTTTCGGTGT1200
                                        ORF End↵                              3' Splice Variant

TTGATAAAGTATAAAGAGAGCTGTTTGGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA1263
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Fig. 2. Nucleotide sequence for novel GSase isolated from gill tissue cDNA of the gulf toadfish *Opsanus beta* (GenBank AF532312). The location of various primers used and other features are noted in boldface type (see text for details).

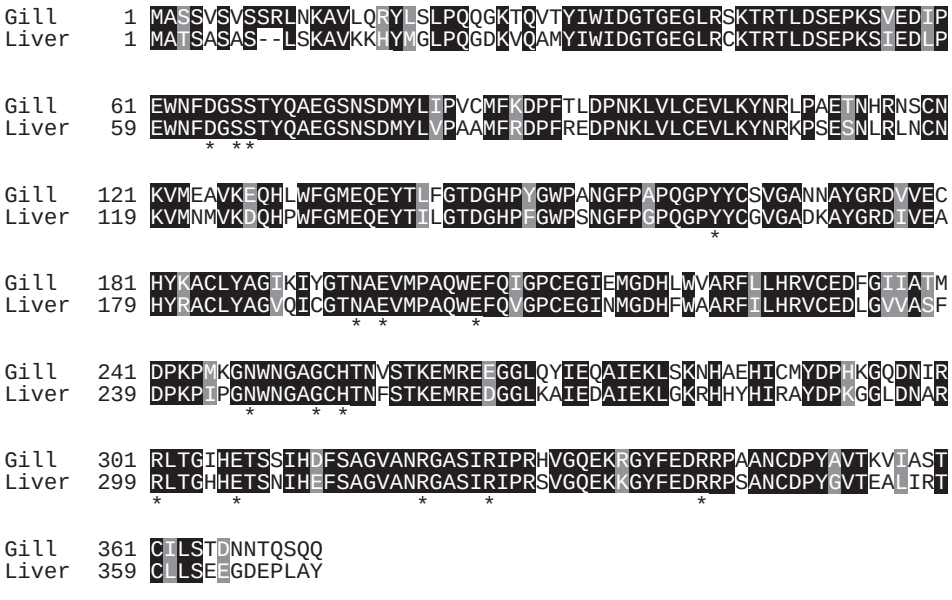

Fig. 3. Amino acid sequence comparison of 'liver' and 'gill' forms of GSase from gulf toadfish *Opsanus beta*. Residues contributing to the active site are noted by an asterisk. Solid shading indicates identical residues; grey shading, similar residues.

nucleotides upstream of the ATG start site (Fig. 1) are read in all three open reading frames. This absence of a leader sequence is in marked contrast to the liver form of GSase, which has previously been shown to possess two presumed ATG translation start sites and a pronounced mitochondrial targeting leader sequence between the two start sites (not shown in Fig. 3).

Although amino acids involved in the catalytic site of GSase are exactly conserved in gill and liver forms (amino acids marked with an asterisk in Fig. 3), the proteins are in fact relatively different in overall amino acid sequence, with only a 73.3% similarity. Furthermore, whereas the liver gene clusters with the majority of fish GSases reported to date, and has a 91.4% and 89.5% similarity to the rainbow trout GS01 and GS02 genes, respectively, the gill form clusters more closely to the *Xenopus laevis* GSases (74.3% similarity) and one of the *Fugu* genes (Fig. 4). Note that the toadfish gill GSase neither has identity with mammalian GSases, nor does it show high similarity with invertebrate and primitive eukaryote GSases, suggesting that it does not originate from, for example, a parasitic or infectious organism colonizing the toadfish gill, or other sources of contamination.

Tissue distribution of the 'gill' GSase

While data from the sequencing of PCR products suggested that gill was the only tissue in which the new GSase was expressed in the toadfish, we wished to test this assumption more carefully in many tissues in a number of individuals from both unconfined and confined conditions. We therefore performed RT-PCR on samples of seven tissues from four individuals from each of two treatments, confined and unconfined (i.e. a subset of half of those individuals used for GSase assays in Table 1), and then subjected these products to *Pvu*II and *Stu*I digestion, as described in Materials and methods. In all samples except those from gill and stomach, only the digestion pattern diagnostic of liver cDNA was found (see sample liver pattern in Fig. 5). In gill samples from all individuals, digestion patterns diagnostic of both liver and gill cDNA were found (see sample gill pattern in Fig. 5). Since the amounts of mRNA/cDNA and numbers of PCR cycles were identical in all samples, these reactions can be considered to be 'semi-quantitative', indicating a more-or-less equivalent expression of the two GSase mRNA forms in gill, and we did not observe any obvious change in these proportions in samples from confined *versus* unconfined fish. Amongst the eight stomach samples examined, only one individual showed slight expression of the gill GSase form (this exception is shown in Fig. 5), which appeared to be minor relative to the liver form, and also minor relative to the expression level of the gill form in the gill tissue. Stomach tissue from this individual was subjected to the entire RT-PCR and digestion procedure two more times to rule out contamination artifacts, and the results were qualitatively the same.

The gill sequence obtained by RACE-PCR differs from that obtained by RT-PCR by three nucleotides at positions 251 (A instead of G) and 263/266 (both T instead of C), and also in fact from the GS 501 primer (the nucleotides in question are underlined in the following primer sequence: GAGGGCTCCAACAGCGACAT). In other words, in order to actually amplify the gill form with GS 501, the primer has to misprime slightly. (Note that the GS 301 primer at the 3′ end is an exact match for both genes, see Fig. 2.) Because of this slight mismatch, we were concerned that the result of the digest experiment, namely the nearly complete 'absence' of expression of the gill form in other tissues, could be the result of not amplifying the 'gill' form of the cDNAs simply due to the slight mismatch and a lower abundance (relative to the liver form) rather than the rare occurrence or 'absence' of the gill template. To test this possibility, we performed PCR on all of the same cDNAs with the RACE-PCR primers GSP2A and GSP1A that are highly specific to the gill form only, and should generate a 152 bp product from the gill form. Confirming the expression pattern above, a significant band of the appropriate size was produced only in gill tissue, whereas only faint bands of an inappropriate size were obtained in other tissues (results not shown). Note that there was no amplification from stomach cDNA.

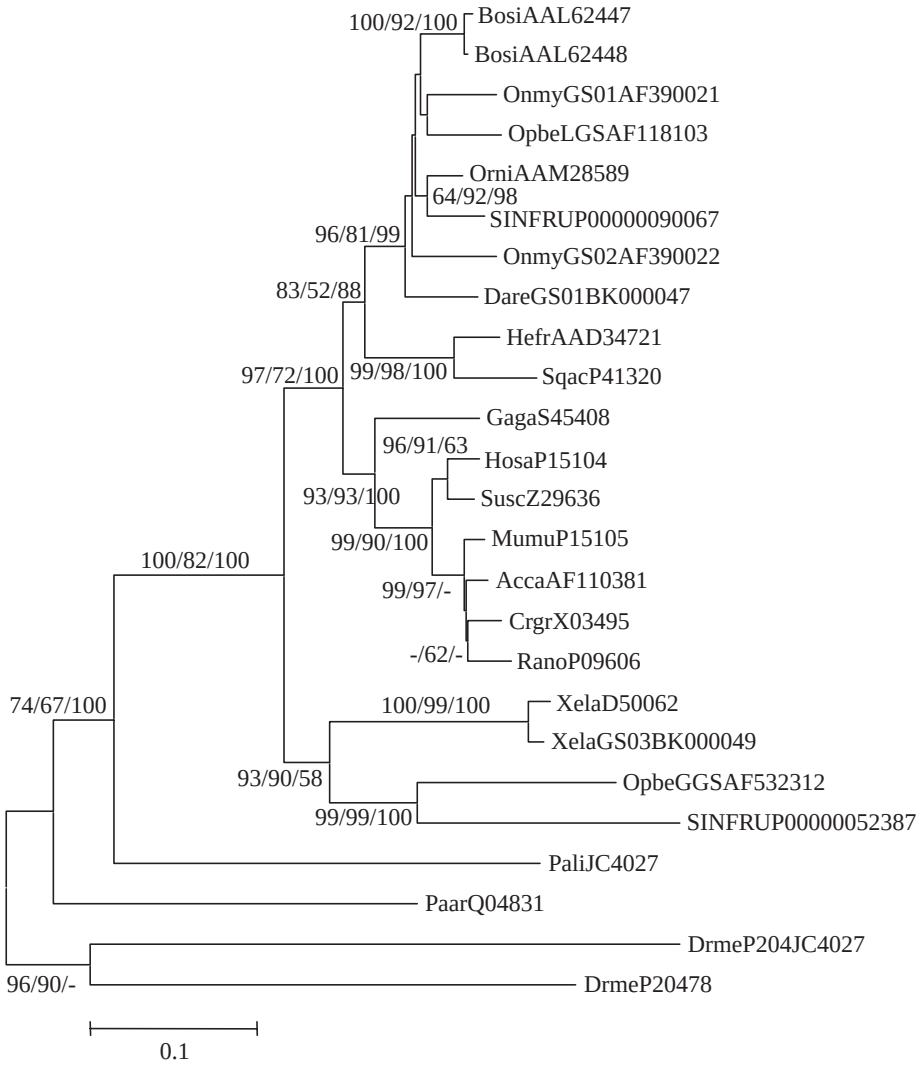

Fig. 4. Minimum evolution phylogenetic tree of several vertebrate GS proteins. *Drosophila* mitochondrial and cytosolic GSase sequences were used as outgroups. Support for nodes (50% majority rule) is depicted on corresponding branches (ME bootstrap support/quartet-puzzling reliability values/Bayesian posterior probabilities are represented from left to right). Dashes indicate that there is no 50% majority rule support for the node under that analysis. Species are generally coded as in Murray et al. (2003), where the four-letter code refers to the first two letters of genus and species, followed by an accession number in either Genbank or SwissProt databases. Species are: Bosi, *Bostrichthyes sinensis* (sleeper); Onmy, *Oncorhynchus mykiss* (rainbow trout); Opbe, *Opsanus beta* (L,G, liver and gill) (gulf toadfish); Orni, *Oreochromis niloticus* (tilapia); SINFRUP, *Takifugu rubripes* (pufferfish); Dare, *Danio rerio* (zebrafish); Hefr, *Heterodontus francisci* (horned shark); Sqac, *Squalus acanthias* (spiny dogfish shark); Gaga, *Gallus gallus* (chicken); Hosa, *Homo sapiens* (human); Susc, *Sus scrofa* (pig); Acca, *Acomys cahirinus* (Egyptian spiny mouse); Crgr, *Cricetulus griseus* (Chinese hamster); Mumu, *Mus musculus* (mouse); Rano, *Rattus norvegicus* (rat); Xela, *Xenopus laevis* (clawed toad); Pali, *Panulirus argus* (spiny lobster); Paar, *Paracentrotus lividus* (sea urchin); Drme, *Drosophila melanogaster* (fruit fly). Scale bar represents proportion of amino acids per site.

Discussion

Transition from ammonotely to ureotely in the gulf toadfish (*O. beta*) appears to involve two key biochemical characteristics: a higher baseline expression of CPSase III and the four enzymes of the O-UC than 'typical' teleosts, as well as an inducible cytosolic form of GSase in the liver, which funnels nitrogen to the O-UC *via* CPSase III (reviewed by Walsh, 1997). Prior studies have shown expression of CPSase III in the toadfish muscle, but relatively low mass-specific enzyme activity (Julsrud et al., 1998). However, an observation that escaped our attention previously is that when tissue mass is taken into consideration, nearly half of the total body CPSase III activity in toadfish is contained in the muscle. The present study shows that this muscle ureogenic potential is almost matched by the nitrogen feeder enzyme GSase, which in muscle comprises 23% of the total body GSase in confined fish (Table 1). Furthermore, we demonstrate that muscle GSase appears to be inducible (twofold) during confinement conditions known to induce ureotely (Table 1). These findings are similar to a recent study of the sleeper *Bostrichthys sinensis*, where ammonia exposure and air exposure induce muscle GSase (Anderson et al., 2002). However, the strategies adopted by the two species are different. While *B. sinensis* retains glutamine as a storage product (Ip et al., 2001), the toadfish does not accumulate excess glutamine during ammonia exposure (Wang and Walsh, 2000) and presumably processes glutamine through the O-UC. Toadfish liver and muscle (as well as the kidney, intestine and brain) express predominantly the originally described 'liver' form of GSase mRNA (see Fig. 5 and Results), but expression of this gene is apparently regulated differentially in these tissues (i.e. fivefold *versus* twofold change after confinement, very different overall expression levels, and possibly very different molecular mass distributions of subunits on western blots). It would be of interest to determine whether the upregulation of liver and muscle GSase, and the apparent constancy of GSase in kidney, intestine and brain, are the result of differential transcription, translation or post-translational modifications.

A second interesting facet of ureotely in toadfish is that urea is excreted in periodic 'pulses' at the gill (Wood et al., 1995, 1997, 1998), presumably through a specific urea transporter (Walsh et al., 2000). However, equally remarkable is the fact

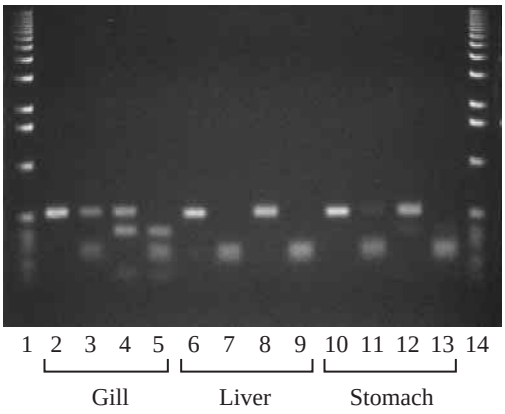

Fig. 5. Relative expression of 'liver' and 'gill' mRNA/cDNA in tissues of gulf toadfish *Opsanus beta*. Representative agarose gel of a restriction digest experiment of mRNAs obtained after RT-PCR with primer pair GS 501 and 301. Lanes 1 and 14 are molecular mass markers, lanes 2–5, gill; lanes 6–9, liver; lanes 10–13, stomach. From left to right, for each tissue: uncut PCR products, PCR products digested with *Stu*I, PCR products digested with *Pvu*II, and PCR products digested with *Stu*I plus *Pvu*II.

that ureotelic toadfish virtually shut down ammonia excretion at the gill, despite continued ventilation of the gill with water and perfusion with blood having typical plasma ammonia values (in the range of several hundred μmol l^{-1}), i.e. a substantial outwardly directed P_{NH3} gradient when factoring in pH values of water and plasma (Wang and Walsh, 2000). Previously it was speculated that gill GSase might play a role in trapping ammonia to minimize its leakage through the gill (Wood et al., 1995), and calculations by Walsh (1997) suggest that there is sufficient gill GSase activity to support this proposed mechanism.

Several findings of the current study may have a bearing on this hypothesis. It seems prudent to conclude that mRNA of the 'gill' form is in low abundance or absent in non-branchial tissues in the toadfish and is expressed nearly exclusively in the gill under the conditions imposed in this study. The gill GSase shows different compartmentation from the form expressed in other tissues in that it appears to have an exclusively soluble/cytosolic distribution. This subcellular localization seems to make 'sense' for an ammonia-trapping function in that localization in the mitochondria might limit the enzyme's ability to contact ammonia efficiently. Additionally, a mitochondrial compartmentation might skew GSase expression towards the mitochondrial-rich cells (MR)/chloride cells, cells that make up only a small proportion of the total gill cell numbers (Perry and Walsh, 1989). Therefore, one would predict expression of GSase in virtually all gill cells. At a molecular level, this predominantly cytoplasmic expression pattern is also consistent with the absence of a mitochondrial leader sequence in the gill gene. However, since at least the mRNA for the liver gene is coexpressed in the gill, the expression of a mitochondrial form of the enzyme is probably minimized by some mechanism. Following the work of Campbell and colleagues on dogfish shark GSase (see Campbell and Anderson, 1991), we have speculated that differential transcription or translation of the liver form of the gene so as to not include the mitochondrial leader sequence can take place (Walsh et al., 1999), and this could limit the expression of the mitochondrial form of the enzyme in the gill. Another interesting possibility is that the two genes are expressed in different cell types (e.g. 'gill' GSase in the pavement cells and 'liver' GSase in the MR cells), speculation that could be tested with gene-specific nucleotide and antibody probes.

Interestingly, gill mass-specific GSase activity does not increase during confinement stress, but it must be kept in mind that the transferase assay does not necessarily measure how the enzyme functions *in vivo*, and its relationship with the synthetase activity can be highly variable (Shankar and Anderson, 1985; Walsh, 1996). It is also possible that even if total GSase activity remains unaltered, the proportion of gill *versus* liver enzymes in the gill might change, and that these enzymes might possess entirely different kinetics with respect to ammonia trapping. Such kinetic differences could also be the result of different susceptibilities of the two proteins to post-translational modifications. A different kinetic variant makes sense for an ammonia-trapping role, since if it were only the cytosolic location that were necessary, this condition could be achieved just by dropping the mitochondrial leader sequence from the liver form of the protein. There is precedent for variable kinetic properties of GSase even among the two forms produced from the toadfish liver gene. Walsh (1996) demonstrated that the cytoplasmic and mitochondrial forms of the enzyme, presumably generated from the same gene with and without the leader sequence, show different substrate K_m values, specific activities, transferase to synthetase activity ratios, pH profiles and susceptibilities to the inhibitor methionine sulfoximine (MSOX).

Clearly the gill GSase gene nucleotide and amino acid sequences are rather different from most other fish GSase genes examined to date (see Murray et al., 2003; Fig. 4). It is not clear if more than two forms of the GSase gene occur in toadfish. On the one hand, since the primers used in this study identified four genes in the tetraploid rainbow trout (Murray et al., 2003), and also amplified genes as divergent as the liver and gill forms in the toadfish, one would expect them to amplify other related GSase genes. On the other hand, the unusual pattern of muscle GSase in western analysis for the toadfish suggests that further analysis is necessary.

Our study also confirms the results of Murray et al. (2003) that the initial gene duplication of GSase in fish probably occurred relatively early in piscine evolution, likely as part of a teleost-specific genome duplication. However, it is clear that something in the selective pressures on toadfish, and perhaps the pufferfish as well, led to a more divergent sequence of the second GSase gene. It is tempting to speculate that it also has a divergent function, one that is closer to that of terrestrial animals that possess CPSase I, namely avid ammonia scavenging. It will be instructive to perform studies of the

biochemistry of purified gill GSase(s), and to examine whether only two 'pure' forms of the enzyme exist with unified subunit composition, or if toadfish 'mix and match' subunits from the two genes in this normally octameric enzyme to express more than two functional proteins. It will also be useful to test the role of gill GSase in ammonia trapping by examining the effects of MSOX on ammonia excretion *in vivo* or in isolated head/gill preparations (Pärt et al., 1999).

In summary, the current studies report on the presence of a second GSase gene in toadfish expressed mainly in the gill, and they have opened another facet of nitrogen metabolism and excretion in this interesting species. Furthermore, when our results are considered in the context of the recent study of Murray et al. (2003), it is clear that further analyses of the GSase genes in additional species should yield interesting evolutionary insights.

This research was supported by grants to P.J.W. from the US National Science Foundation (IBN 0090355) and the National Institute of Environmental Health Science (ES 11005 and ES 05705), and a NSERC Research Grant to T.P.M. M.L.B. was supported through the summer undergraduate fellowship program administered by the Graduate Studies Office of RSMAS. G.D.M. was supported by a NIEHS postdoctoral training grant (ES07320). We thank Drs Alex Ip (National University of Singapore) and Paul Anderson (University of Minnesota) for the generous gift of the GSase antibody and Ellen Busby (University of Victoria) for insights into primer design. We are continually indebted to Jimbo Luznar and his boat captains for providing toadfish specimens.

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