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Effects of crowding on ornithine–urea cycle enzyme mRNA expression and activity in gulf toadfish (*Opsanus beta*)

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SUMMARY

The gulf toadfish (*Opsanus beta*) is a facultatively ureotelic fish that excretes primarily urea under conditions of crowding or confinement. To examine the relationship between ammonia production, urea production and the ornithine–urea cycle (O–UC) enzyme activity and mRNA expression, we subjected toadfish to two-day and seven-day crowding regimes. Plasma cortisol levels were measured and liver tissue was assayed for ammonia and urea concentrations. Liver glutamine synthetase (GS), carbamoyl phosphate synthetase III (CPS), ornithine carbamoyl transferase (OCT) and arginase (ARG) activities were also measured. Quantitative PCR was utilized to determine liver GS, CPS, OCT, ARG, argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) mRNA expression. Hepatic ammonia concentrations decreased with increased duration of crowding whereas liver urea and circulating cortisol levels increased. An elevation in enzyme activity with increased duration of crowding was observed for all four O-UC enzymes examined. By contrast, mRNA expression was variable for the O–UC enzymes and only CPS and ASS had mRNA expression levels that were elevated in crowded fish. These results suggest that the activities of O–UC enzymes are better predictors for urea production than O–UC enzyme mRNA expression levels.

Key words: nitrogen metabolism, transcription, carbamoyl phosphate synthetase, glutamine synthetase, ornithine carbamoyl transferase, argininosuccinate synthetase, argininosuccinate lyase, arginase, urea, ammonia, liver, cortisol.

INTRODUCTION

The main waste products of nitrogen metabolism in fishes are ammonia and urea (Wood, 1993). Most fishes are ammoniotelic, excreting their excess nitrogenous wastes primarily as ammonia into their surrounding waters whereas some fishes are ureotelic, producing urea *via* the ornithine–urea cycle (O–UC) and excreting ≥50% of their nitrogenous waste as urea (Anderson, 1995; Ip et al., 2001). The vertebrate O–UC is comprised of five enzymes: carbamoyl phosphate synthetase (CPS) (EC 6.3.4.16) at the entry point to the O-UC cycle, followed by ornithine carbamoyl transferase (OCT) (EC 2.1.3.3), argininosuccinate synthetase (ASS) (EC 6.3.4.5), argininosuccinate lyase (ASL) (EC 4.3.2.1) and lastly arginase (ARG) (3.5.3.1) (Anderson, 1995; Mommsen and Walsh, 1991). In fishes, there is one important accessory enzyme, glutamine synthetase (GS) (EC 6.3.1.2) that supplies the fish-type of CPS (III) with glutamine as a nitrogen source (Anderson, 1995; Anderson, 2001).

The gulf toadfish (*Opsanus beta*) is a facultative ureotelic fish with a fully functional O–UC. In its natural environment, the toadfish is believed to excrete waste nitrogen both as ammonia and urea but ureotely can be induced by inhibiting the excretion of ammonia through air exposure or submersion in high levels of environmental ammonia (Walsh et al., 1990; Walsh and Milligan, 1995; Walsh et al., 1994). Crowding/confinement treatments also induce ureotely in the gulf toadfish and therefore it has been proposed that the switch to ureotely in toadfish is in response to stress (Hopkins et al., 1995; Walsh et al., 1994). Hopkins et al. showed that the acute response to up to two hours of crowding treatment was a 2–4-fold increase in circulating cortisol concentrations, with cortisol returning to control levels after 24 h (Hopkins et al., 1995). At the same time, urea becomes the primary nitrogenous waste between 24 h and 48 h of crowding treatment and remains so for seven days (Walsh et al., 1994).

The increase in urea excretion in response to stress is believed to be due to an upregulation of O–UC enzymes. However, little is known regarding their regulation in toadfish. A few studies have addressed the issue; an increase in GS activity has been measured after 24 h of crowding, suggesting that the 'fish-type' O–UC is activated at this time (Hopkins et al., 1995; Walsh et al., 1994). However, other studies focusing on the effects of crowding on the activities of other O–UC enzymes are contradictory. For example, Walsh et al. (Walsh et al., 1994) found that OCT activity was elevated in fish crowded for 96 h but a follow-up study by Walsh and Milligan (Walsh and Milligan, 1995) did not find an increase in OCT activity in unfed, confined toadfish.

With respect to mRNA expression of O–UC enzymes, a 5-fold increase in hepatic GS mRNA expression and up to a 10-fold increase in CPS mRNA expression has been measured in confined toadfish using ribonucleic acid protection assays (RPAs) (Kong et al., 2000). However, the mRNA expression of the other O–UC enzymes in response to crowding has not been investigated. Furthermore, little is known about how O–UC enzyme activity in toadfish relates to enzyme mRNA expression. In rats treated with dexamethasone (a synthetic glucocorticoid), hepatic O–UC enzyme activities were found to vary with the relative abundance of mRNA using dot-blot and quantitative northern blot analyses (Morris et al., 1987). However, recent studies on O–UC enzymes in fish have also shown that despite increases in their mRNA levels, there was no

corresponding increase in O–UC enzyme activities (Iwata et al., 2000; Kong et al., 2000). Greenbaum et al. suggest that poor correlations of mRNA levels with protein levels are due to complicated post-transcriptional mechanisms and differential *in vivo* protein half-lives (Greenbaum et al., 2003).

The objective of this study was to undertake a more in-depth analysis of the mRNA expression and activity of O–UC enzymes in the toadfish liver. Our goal was to determine the relationships between hepatic ammonia levels, urea levels, O–UC enzyme activity and mRNA expression and to investigate how crowding affects these relationships. Furthermore, we aimed to investigate the relationship between mRNA expression and activity of the O–UC enzymes in toadfish liver. We therefore measured hepatic ammonia and urea concentrations, plasma cortisol levels and O–UC enzyme activities and mRNA expression in toadfish that were uncrowded, crowded for two days and crowded for seven days.

MATERIALS AND METHODS

Experimental animals

Gulf toadfish, *Opsanus beta* (Goode and Bean), were caught by local shrimp fishermen from Biscayne Bay, FL, USA, between May and July 2007. Mature toadfish (0.036–0.191 kg) were kept in an outdoor tank at the shrimpers' dock with flow-through aerated seawater for up to 24 h before being transferred to the laboratory wet lab facilities. In the laboratory, toadfish were transferred to glass aquaria (45 or 80 l) with flowing, aerated seawater. Fish were treated with malachite green (final concentration 0.05 mg l^{-1}) in formalin (15 mg l^{-1}) (AquaVet, Hayward, CA, USA), as described by Walsh and Milligan (Walsh and Milligan, 1995), to prevent parasitic infection by *Cryptocaryon irritans*. Toadfish were acclimatized in these aquaria for at least seven days prior to the start of the experiment. The fish were fed squid weekly, and food was withheld four days prior to the start of the experiment and throughout the remainder of the experiment.

Experimental protocol

We sought to examine the impact of crowding stress on toadfish and therefore had three treatments. Uncrowded toadfish were held in outdoor 6000 l tanks (10 fish per tank) that had flow-through, sand filtered seawater and PVC tubes for shelters for seven days at ambient temperature. These tanks were exposed to natural light and temperature conditions and were filled with seagrass (*Thalassia testudinum*), mimicking the natural toadfish habitat. Crowded toadfish were transferred to 6 l plastic tubs (10 fish per tub) in the laboratory that had flow-through, aerated seawater and PVC tubes for shelters and were maintained at ambient temperature for two or seven days (McDonald et al., 2009; Walsh and Milligan, 1995). Crowded fish were maintained under a 12 h:12 h light:dark photoperiod regime. It should be noted that the two extremes of our treatment groups, namely fish that were uncrowded for seven days and fish that were crowded for seven days, had food withheld for the same period.

After treatment, blood was sampled from each fish for cortisol analysis by caudal puncture using a 23-gauge needle and a 1.0 ml syringe rinsed with heparinized saline (50 IU ml^{-1}). Approximately 100 µl blood was extracted from each fish, plasma was separated by centrifugation (10,000 ***g*** for one minute), immediately frozen in liquid nitrogen and stored at –80°C for later analysis of plasma cortisol levels. Toadfish were then anesthetized with 2.0 g l^{-1} of MS-222 (tricane methanesulfonate), weighed, sexed and liver tissue was extracted, immediately frozen in liquid nitrogen and stored at –80°C for later analyses of enzyme activities and mRNA expression levels.

Ammonia, urea and cortisol assays

A sub-sample of frozen liver tissue was deproteinized for ammonia and urea analyses using the methods of Kun and Kearney (Kun and Kearney, 1971) as modified by Wang and Walsh (Wang and Walsh, 2000). Ammonia concentrations of liver tissue were measured using a colorimetric assay (Raichem, San Diego, CA, USA) in which the amount of ammonia present in a sample is equivalent to the amount of nicotinamide adenine dinucleotide phosphate (NADPH) oxidized in the presence glutamate dehydrogenase (GLDH). Urea concentrations were measured using the diacetyl–monoxime method outlined in Rahmatullah and Boyde (Rahmatullah and Boyde, 1980). Cortisol levels were measured in blood plasma using a commercial ^{125}I radioimmunoassay kit (MP Biochemicals, Solon, OH, USA) with standards diluted to the same protein range as toadfish plasma.

Ornithine–urea cycle enzyme activity

For the analysis of O–UC enzyme activities, approximately 100 mg of liver was homogenized on ice in a 2.0 ml centrifuge tube using an IKA Ultra Turrax® T8 Homogenizer (Willmington, NC, USA) in 4× volume of homogenization buffer (20 mmol l^{-1} K_2HPO_4, 10 mmol l^{-1} Hepes, 0.5 mmol l^{-1} EDTA, 1 mmol l^{-1} dithiothreitol, in 50% glycerol, pH 7.5 at 25°C). Homogenates were centrifuged at 16,000 ***g*** for five minutes at 4°C in an Eppendorf® 5415D centrifuge (Westbury, NY, USA), and the supernatant was used directly or diluted as needed according to previously described methods (Barber and Walsh, 1993; Kajimura et al., 2006; Walsh et al., 1994).

RNA extractions and cDNA synthesis

RNA was extracted from 200 mg of tissue using the chaotropic extraction protocol outlined by Whitehead and Crawford (Whitehead and Crawford, 2005). The RNA solution was then further purified using the RNeasy® mini protocol for RNA cleanup (Qiagen, Valencia, CA, USA). Purified RNA was quantified using the NanoDrop ND-1000 spectrophotometer (Montchanin, DE, USA). RNA was diluted to 500 ng µl^{-1} and treated with Turbo DNase (Ambion, Austin, TX, USA) to remove any traces of DNA from the extraction process. First strand cDNA synthesis was performed using Superscript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Approximately 1 µg of Turbo DNase (Ambion)-treated RNA was denatured at 65°C for five minutes with 100 ng of Random Hexamers and 10 mmol l^{-1} dNTP mix in a total volume of 10 µl. The denatured RNA was placed on ice for later use. Reverse transcription (RT) was performed in 25 µl reactions containing the denatured RNA, 1× PCR buffer [20 mmol l^{-1} Tris-HCl (pH 8.4) and 50 mmol l^{-1} KCl], 0.01 mol l^{-1} DTT, 2.5 mmol l^{-1} $MgCl_2$, 0.4 mmol l^{-1} each dNTP, 40 units RNaseOUT™, 50 units Superscript™ III RT and ddH_2O (double-distilled H_2O). The RT reaction was incubated at 42.0°C for 1 h, 70.0°C for 15 min and then cooled to 4.0°C. The cDNA product was stored at 4.0°C. Unused purified RNA and Turbo DNase-treated RNA was precipitated out of solution by adding 0.1× volume 3 mol l^{-1} sodium acetate and 2.5× volumes 100% ethanol and stored at –20°C.

Gene specific DNA amplification, RT-PCR and quantitative PCR

Degenerate primers were designed, using zebrafish (*Danio rerio*) sequence in Oligo 6.7, to amplify DNA product for OCT, ASL, ASS and Elongation factor-1 α (EF1α) (Table 1) in the gulf toadfish by aligning available vertebrate sequences from Genbank and looking for regions of conserved sequences in the coding regions of the target genes. Primers designed to amplify ARG I in rainbow trout (*Oncorhynchus mykiss*) (Wright et al., 2004) were used to amplify a portion of the ARG I gene in gulf toadfish. GS and CPS

Table 1. Primers used to amplify DNA product for the ornithine–urea cycle (O–UC) gene in gulf toadfish (*Opsanus beta*) and gene specific primers designed for quantitative (qPCR) used in this study

Primer	Accession no.	Sequence (5′→3′)	Product size (bp)
OCT–F[c]	**XM_001334635**[c]	GTCYATTGCCACGATHTTTGA	**582**
OCT–R[c]		TTTCTCGTCTTCTTGBCCCAT	
ASS–F[c]	**NM_001004603**[c]	TATGCTGGTCTGGCTGAAGGA	**939**
ASS–R[c]		ACACTCGGGGTTGARCCAGAA	
ASL–F[c]	**NM_20045**[c]	CAATGAACGCAGGCTSAAGGA	**702**
ASL–R[c]		TAGGTCTTCGTTGTANGTGCT	
ARG*e5f1[a]	**NM_001045197**[a]	TTGGGCTTAGAGAYGTGGAYC	**240**
ARG*e7r2[a]		CCTTCTCTGTARGTNAGTCCTCC	
EF1α–qF[b]	**NM_131263**[b]	AGGTCATCATCCTGAACCAC	**140**
EF1α–qR[b]		GTTGTCCTCAAGCTTCTTGC	
GS–qF[d]	**AF118103**[d]	ACTCGCTGCCATACAAACTT	**184**
GS–qR[d]		TCGTGGATGTTTGAGGTTTC	
CPS–qF[e]	**AF169248**[e]	ATGGCAATCAAAGTCGTTCG	**252**
CPS–qR[e]		CCAGACTAAACTCAAAGCAG	
OCT–qF[f]	**EU704512**	TGAAAAGAGAAGCACCAGAAC	**171**
OCT–qR[f]		ATACACTCGTGCCAAGACAA	
ASS–qF[f]	**EU704511**[f]	CCAATGCCGTTTATGAGGAC	**150**
ASS–qR[f]		TCAAAGCGTCCCTGGTCATT	
ASL–qF[f]	**EU704510**[f]	AGTCGGAACAGTATGGATGC	**173**
ASL–qR[f]		AAACTGCTGCCTGTGCTGTA	
ARG–qF[f]	**EU704513**[f]	TGATTGAAGGCGTACTCCAT	**145**
ARG–qR[f]		TGGATAACGGTGGGGTCAA	

Accession numbers for *Danio rerio* and *O. beta* sequences from which the primers were designed are provided. Abbreviations: Elongation factor 1-alpha (EF1α); glutamine synthetase (GS); carbamoyl phosphate synthetase (CPS); argininosuccinate synthetase (ASS); argininosuccinate lyase (ASL); arginase (ARG); F1 forward primer (F); reverse primer (R); quantitative PCR primer (q).

[a]Primers used from Wright et al. (Wright et al., 2004) designed for *Oncorhynchus mykiss*.

[b]Primers designed by Edward M. Mager (University of Miami) designed for *Pimephales promelas*.

[c]Degenerate primers designed for *O. beta* from conserved nucleotide sequences across multiple vertebrate species, including zebrafish (*D. rerio*), human (*Homo sapiens*), mouse (*Mus musculus*) and rat (*Rattus norvegicus*).

[d]Primers designed from published sequence of *O. beta* in Walsh et al. (Walsh et al., 1999).

[e]Primers designed from published sequence of *O. beta* in Kong et al. (Kong et al., 2000).

[f]Primers designed from sequence of *O. beta* obtained in this study.

sequences were available for gulf toadfish in GenBank for the liver form of GS (Accession number AF118103) and for CPS III (Accession number AF169248).

OneStep real-time polymerase chain reaction (RT-PCR) was performed to amplify OCT, ASS, ASL and ARG using Qiagen OneStep RT-PCR kit (Qiagen). The RT reaction and the PCR reaction were performed in the same tube. OneStep RT-PCR was performed in 25 μl reactions containing approximately 500 ng of total RNA, 1× Qiagen OneStep RT-PCR buffer (includes 2.5 mmol l^{-1} $MgCl_2$), 0.4 mmol l^{-1} dNTPs, 1.0 μl Qiagen OneStep RT-PCR enzyme mix, 0.6 μmol of each primer and RNase-free water. OneStep RT-touchdown PCR was performed as follows: one cycle of 45°C for 30 min; one cycle of 95°C for 15 min; two cycles of 94°C for 30 s, 55°C for 1 min and 72°C for 2 min, with a step down of 1°C during the annealing phase every two cycles until the annealing temperature dropped from 55°C to 50°C; and 30 cycles of 94°C for 30 s, 50°C for 1 min and 72°C for 2 min. PCR products were screened on a 1% agarose gel for appropriately sized products using GelStar® nucleic acid stain (Cambrex, Rockland, ME, USA). All PCR products were stored at –20°C.

PCR product for putative OCT, ASL, ASS and ARG genes amplified from the gulf toadfish were excised from the agarose gel and purified using Zymoclean™ gel DNA recovery kit (Zymo Research, Orange, CA, USA). Purified PCR product was cloned using TOPO® TA cloning kit (Invitrogen) and sequenced both directions using Applied Biosystems BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA. USA). A nucleotide BLAST search against the 'other' databases in GenBank revealed a 71%, 81%, 76% and 71% sequence similarity to *D. rerio* sequences for OCT, ASS, ASL and ARG, respectively. Gene specific quantitative PCR (qPCR) primers (Table 1) were designed from toadfish sequence using Oligo 6.7 to have melting temperatures of 58–60°C, 35–65% GC content and to amplify DNA product between 145 bp and 252 bp.

qPCR was performed in the Mx4000 (Stratagene, La Jolla, CA, USA) with Power Sybr® Green PCR Master Mix (Applied Biosystems) using the following cycles: one cycle of 95°C for 10 min; 55 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 30 s; one cycle of 95°C for 1 min; 41 cycles of 55°C for 30 s and an increase of 1°C per cycle. qPCR was performed on all samples in triplicate and negative controls using no cDNA were run for each gene. Each target gene had a single melting peak for each amplicon and was verified as the correct gene by cloning and sequencing several clones. Amplification efficiencies for qPCR reactions between 89.8% and 93.3% were determined using a standard curve for all O–UC genes of interest and for the housekeeping gene EF1α. The level of EF1α was invariant between each treatment group validating its choice as a housekeeping gene. Fold changes were calculated from the log-transformed C_T (threshold cycle) values and expressed relative to the uncrowded treatment group using a modification of the delta-delta C_T method (Livak and Schmittgen, 2001; Vandesompele et al., 2002).

Data analysis and statistical treatments

All data are reported as means ± s.e.m. (N=number of animals). Levels of urea, ammonia, cortisol, enzyme activity and mRNA

expression were compared between uncrowded and crowded treatment groups. For normally distributed data, significant differences were determined by using a one-way analysis of variance (ANOVA) in Sigma Stat 3.00 (SPSS, Inc., Chicago, IL, USA) followed by a Holm–Sidak *post-hoc* test. If the normality test failed, a Kruskal–Wallis one-way ANOVA on ranks was performed followed by a Dunn's *post-hoc* test. A Dixon's test was applied to all of the mRNA expression data to determine outliers within a treatment. Significance was accepted at $P<0.05$. All data were plotted using Sigma Plot 8.02 (SPSS, Inc.).

Data were log transformed for regression analyses. Data for O–UC enzyme mRNA expression and activity were linearly regressed against concentrations of urea, ammonia and circulating cortisol and against each other to determine whether there were any correlations in the data. Forward and backward stepwise regressions were performed on O–UC enzyme activity and mRNA expression levels using ammonia and urea as the dependent variable. The adjusted R^2 value was calculated and significance was accepted at ($P<0.05$).

RESULTS

The masses of uncrowded toadfish (0.079±0.016 kg), toadfish crowded for two days (0.065± 0.016 kg) and toadfish crowded for seven days (0.083±0.013 kg) were not significantly different from each other. Furthermore, the male:female ratio for each treatment was: uncrowded (4:6), two day crowded (5:5) and seven day crowded (6:4), and the sex of the individuals did not affect any of the parameters measured. Liver ammonia concentrations were highest in uncrowded fish and appeared to decrease with duration of crowding (Fig. 1). Compared with uncrowded fish, liver ammonia levels of fish crowded for seven days were reduced by about 28-fold. A 14-fold difference in liver ammonia was also measured between the two crowding treatments, with fish crowded for two days having significantly higher liver ammonia concentrations than fish crowded for seven days (Fig. 1A). Conversely, urea concentrations in the liver tissue were lowest in uncrowded fish and were elevated by about 7-fold in fish crowded for two days with a further 3-fold increase after seven days of crowding. Similar to liver ammonia levels, fish in the two crowded treatments had liver urea concentrations that were significantly different from each other (Fig. 1B). Circulating cortisol concentrations followed a similar pattern to liver urea levels, being about 5-fold higher in fish crowded for seven days compared with uncrowded fish (Fig. 1C). The two crowded treatments did not have significantly different cortisol levels.

In all four O–UC enzymes assayed (GS, CPS, OCT and ARG), there was a general increase in enzyme activity with increased time of crowding (Fig. 2). Specifically, GS activity in fish crowded for two days was 3-fold higher than in uncrowded fish and remained elevated in fish that were crowded for seven days. CPS activity increased by 2-fold after two days of crowding compared with uncrowded fish, with a 3-fold increase after seven days of crowding. By contrast, OCT and ARG only showed a significant elevation in activity after seven days of crowding (Fig. 2).

One fish from the uncrowded treatment and one fish from the two day crowded treatment were not used for qPCR analyses due to low RNA yield after initial extraction. A Dixon's test for outliers was applied to the relative expression data and found an outlier in the data for three of the six O–UC genes for the seven day crowded treatment; therefore, this particular individual fish was removed from all of the analyses.

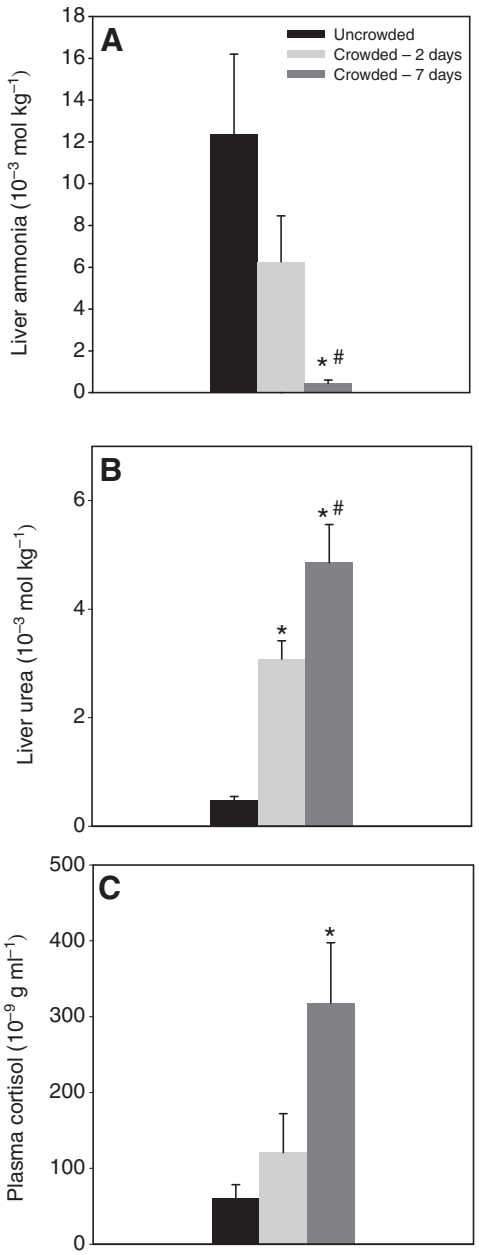

Fig. 1. Liver (A) ammonia levels, (B) urea levels and (C) circulating cortisol levels of gulf toadfish (*Opsanus beta*) that were uncrowded, crowded for two days or crowded for seven days. All treatments $N=9$. Values are means ± s.e.m. *$P<0.05$, significantly different from uncrowded fish, #$P<0.05$, significantly different from fish crowded for two days.

No significant changes were measured in GS, OCT and ARG relative gene expression (Fig. 3). However, the relative mRNA expression of CPS was approximately 4-fold higher in fish crowded for two days and remained elevated in the seven day crowded fish compared with the uncrowded fish. A 3-fold elevation in mRNA expression was also measured in ASS after seven days of crowding compared with both the uncrowded fish and fish crowded for two days, with no measurable difference between the latter two groups. Surprisingly, the relative mRNA expression of ASL showed a 60% reduction in fish crowded for seven days compared with uncrowded fish, with no difference measured in fish crowded for two days.

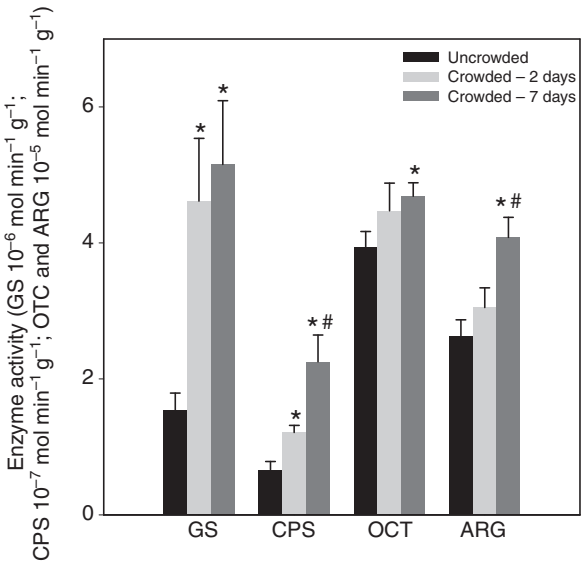

Fig. 2. Enzyme activity measurements for glutamine synthetase (GS), carbamoyl phosphate synthetase (CPS), ornithine carbamoyl transferase (OCT) and arginase (ARG) in liver tissue of gulf toadfish (*Opsanus beta*) that were uncrowded, crowded for two days or crowded for seven days. All treatments N=9. Values are means ± s.e.m. $^{*}P$<0.05, significantly different from uncrowded fish, $^{\#}P$<0.05, significantly different from fish crowded for two days.

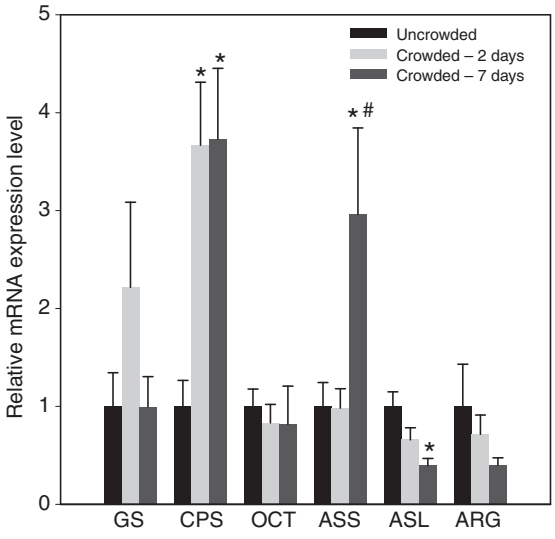

Fig. 3. Relative mRNA expression levels in liver tissue of gulf toadfish (*Opsanus beta*) for glutamine synthetase (GS), carbamoyl phosphate synthetase (CPS), ornithine carbamoyl transferase (OCT), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARG) that were uncrowded, crowded for two days or crowded for seven days. All treatments N=9. Values are means ± s.e.m. $^{*}P$<0.05, significantly different from uncrowded fish, $^{\#}P$<0.05, significantly different from fish crowded for two days.

With the exception of ARG activity *versus* OCT activity, activities measured for the O–UC enzymes were correlated to the activities of other enzymes within the cycle (Table 2). Liver urea concentrations were positively correlated to liver O–UC enzyme activities (Table 2; Fig. 4). Using forward and backward stepwise regressions with liver urea concentration as the dependent variable and O–UC enzyme activity as independent variables, liver urea concentrations could be predicted almost entirely with CPS activity. Liver urea concentrations were also positively correlated to CPS mRNA expression but negatively correlated to ARG mRNA expression (Table 2). Stepwise regression analyses of liver urea concentration against O–UC enzyme mRNA expression confirmed that only CPS and ARG mRNA expression were necessary to predict urea levels.

Both GS and CPS enzyme activities were negatively correlated to liver ammonia concentrations (Table 2) but only CPS activity was necessary to predict liver ammonia concentrations. CPS mRNA expression was also negatively correlated to liver ammonia levels (Table 2), and stepwise regression of liver ammonia against O–UC enzyme mRNA expression indicated that only CPS mRNA expression was necessary to predict liver ammonia concentrations.

Plasma cortisol concentrations were positively correlated to liver urea levels but had no relationship with liver ammonia concentrations (Table 2). Cortisol levels were also positively correlated with liver enzyme activities of GS, CPS and OCT, and CPS mRNA expression but had a negative correlation to OCT mRNA expression (Table 2).

There was little correlation of mRNA expression of the O–UC enzymes to each other within the cycle: CPS mRNA expression was correlated to GS mRNA expression, and ARG mRNA expression was correlated to OCT mRNA expression (Table 2). Only CPS and OCT mRNA expression were correlated to their corresponding enzyme activities (Table 2; Fig. 5).

DISCUSSION

Previous studies have shown that exogenous ammonia exposure, air exposure and crowding conditions induce ureotely in gulf toadfish (Walsh et al., 1990; Wang and Walsh, 2000; Walsh et al., 1994). The present study examined the effects of crowding stress on plasma cortisol concentrations, hepatic ammonia and urea levels, as well as liver O–UC enzyme mRNA expression and activity. Liver urea levels were found to be elevated in crowded toadfish, suggesting an upregulation in the O–UC in these fish. Indeed, all of the O–UC enzymes analyzed showed a coordinated induction in terms of increased activity. However, only two genes, CPS and ASS, showed an upregulation at the level of mRNA expression, suggesting that in terms of the response to crowding stress, induction of the O–UC may be primarily dependent on post-transcriptional changes to existing enzymes.

Levels of mRNA expression have previously been measured in crowded gulf toadfish for only two of the six O–UC enzymes using RPAs (Kong et al., 2000). Supporting findings from this previous study, CPS mRNA expression in toadfish of the present study showed an almost 3-fold increase after two and seven days of crowding compared with uncrowded fish. The suggested sensitivity of toadfish CPS mRNA expression to plasma cortisol levels has also been measured in cultured rat hepatocytes treated with the synthetic glucocorticoid, dexamethasone, in which a 4-fold increase in the transcription of CPS mRNA was measured within 24 h (Ulbright and Snodgrass, 1993). While mRNA levels are affected by transcription factors that can both positively and negatively regulate this process (Weaver, 2005), mRNA expression can also be affected by changes in mRNA stability (i.e. turnover and decay) (Mata et al., 2005; Weaver, 2005). Consequently, the quantity of mRNA measured represents a balance between mRNA production and mRNA stability (Mata et al., 2005). In the case of rat hepatocytes treated with dexamethasone, it was speculated that the increase in

Table 2. Results of linear regressions of the log-transformed data for all parameters compared in this experiment

Parameter measured	GS activity	CPS activity	OCT activity	ARG activity	Urea	Ammonia	Cortisol
GS activity	*	x	x	x	x	x	x
CPS activity	**0.601 (<0.001)**	*	x	x	x	x	x
OCT activity	**0.385 (<0.001)**	**0.286 (0.002)**	*	x	x	x	x
ARG activity	**0.367 (<0.001)**	**0.294 (0.002)**	0.0828 (0.079)	*	x	x	x
Urea	**0.450 (<0.001)**	**0.630 (<0.001)**	**0.192 (0.013)**	**0.169 (0.019)**	*	x	x
Ammonia	**0.191 (0.019)**	**0.228 (0.011)**	0.000 (0.774)	0.112 (0.061)	0.0938 (0.080)	*	x
Cortisol	**0.249 (0.005)**	**0.231 (0.007)**	**0.124 (0.040)**	0.0849 (0.077)	**0.176 (0.017)**	0.000 (0.367)	*
Weight	0.000 (0.950)	0.000 (0.865)	0.000 (0.839)	0.000 (0.913)	0.0685 (0.100)	**0.216 (0.013)**	0.0149 (0.249)

Parameter measured	GS mRNA	CPS mRNA	OCT mRNA	ASS mRNA	ASL mRNA	ARG mRNA	Corresponding enzyme activity
GS mRNA	*	x	x	x	x	x	0.0876 (0.073)
CPS mRNA	**0.166 (0.020)**	*	x	x	x	x	**0.188 (0.014)**
OCT mRNA	0.00891 (0.277)	0.0361 (0.172)	*	x	x	x	**0.178 (0.016)**
ASS mRNA	0.000 (0.742)	0.0339 (0.179)	0.000 (0.633)	*	x	x	n.a.
ASL mRNA	0.000 (0.463)	0.0951 (0.065)	0.075 (0.090)	0.0254 (0.207)	*	x	n.a.
ARG mRNA	0.0276 (0.199)	0.000 (0.699)	**0.187 (0.014)**	0.00201 (0.315)	0.000 (0.467)	*	0.000 (0.723)
Urea	0.000 (0.572)	**0.254 (0.004)**	0.0699 (0.098)	0.0739 (0.092)	**0.140 (0.031)**	0.0854 (0.076)	n.a.
Ammonia	0.0311 (0.201)	**0.316 (0.003)**	0.000 (0.826)	0.0928 (0.081)	0.0862 (0.089)	0.000 (0.905)	n.a.
Cortisol	0.000 (0.801)	**0.121 (0.042)**	**0.138 (0.032)**	0.000 (0.906)	0.000 (0.582)	0.0286 (0.196)	n.a.
Weight	0.0732 (0.093)	0.000 (0.512)	0.000 (0.327)	0.000 (0.763)	0.000 (0.606)	0.0329 (0.182)	n.a.

Adjusted R^2 value is listed first with the P-value in brackets. Corresponding enzyme activity parameter represents the activity measured for the enzyme corresponding to its own relative mRNA expression measured. Bold indicates that $P<0.05$ for that regression, × indicates that the regression is already represented elsewhere in the table, *indicates that the parameters listed are identical and therefore no regression performed, and n.a. indicates that there was no appropriate regression for that parameter.

CPS transcription was due to increased stability of CPS mRNA, achieved by inhibiting mRNA degradation (Ulbright and Snodgrass, 1993). In theory, the elevated cortisol levels in crowded toadfish may also be contributing to a decrease in CPS mRNA degradation and therefore an increase in mRNA stability, which could contribute to increased urea production.

In addition to an upregulation in CPS mRNA expression in toadfish that are crowded, an increase in CPS enzyme activity was also measured in crowded fish. This finding was in contrast to previous studies (Walsh and Milligan, 1995; Walsh et al., 1994); however, this may be due to a difference in protocol. In the earlier studies, uncrowded fish were kept individually in 45 l indoor aquaria and had baseline CPS activities of approximately 0.16 μmol min^{-1} g^{-1} liver tissue (Walsh and Milligan, 1995; Walsh et al., 1994). By contrast, uncrowded fish in the present study were kept in a much larger tank in less confined conditions (600 l per fish). These fish had a greater volume of water available to dilute nitrogenous wastes, which would reduce their need for ureotely and may be reflected in their lower CPS activity (0.066 μmol min^{-1} g^{-1} liver). Furthermore, the outdoor tank better simulated the natural toadfish habitat, which appears to have created a lower stress environment for the uncrowded fish in this study compared with previous studies. In the present study we used both laboratory and outdoor environments, which allowed for a much greater difference in the stress levels of uncrowded fish and crowded fish. For this reason, differences may have been measured between toadfish of our present study that may not have been possible in other studies.

The importance of CPS as a regulatory enzyme in urea production is illustrated by the fact that CPS was the only enzyme necessary to predict urea production when stepwise regressions were performed. The fact that CPS mRNA expression levels were correlated to CPS enzyme activity suggests that CPS mRNA stability is probably similar to the *in vivo* protein half-life of CPS. However, a more refined sampling protocol would help tease out the timing of transcriptional and post-transcriptional changes in CPS in response to crowding stress. It is also interesting to note that there was a correlation between the GS mRNA levels and CPS mRNA levels. This correlation may reflect the dependence of the fish-type CPS (III) on GS for its substrate, glutamine (Anderson, 1995; Anderson, 2001; Hong et al., 1994).

In rat hepatoma cells, GS mRNA is elevated in the presence of glucocorticoids (Gaunitz et al., 2002). Furthermore, a glucocorticoid responsive element (GRE) was identified in the first intron of the rat GS gene (Gaunitz et al., 2002). A similar relationship between cortisol and GS is believed to exist in toadfish; Kong et al. (Kong et al., 2000) found a significant increase in GS mRNA expression after 48 h of crowding, and a putative glucocorticoid receptor has been reported for toadfish GS (P.J.W., unpublished results). Therefore, we expected the increased circulating cortisol concentrations measured in fish crowded for seven days to induce toadfish GS mRNA expression. Surprisingly, GS mRNA expression remained similar to that of uncrowded fish and was not correlated to plasma cortisol levels. The reason for this discrepancy is not known; however, the half-life of GS mRNA is estimated to be between 95 min and 8 h (Abcouwer et al., 1996; Feng et al., 1990; Saini et al., 1990; Sarkar and Chaudhury, 1983). Perhaps a transient increase in GS mRNA expression did occur sometime within the seven days of crowding but levels had returned to the uncrowded values by the time liver tissue was sampled. Interestingly, there was a tendency for higher GS mRNA expression in a number of toadfish that were crowded for two days [similar to the Kong et al. (Kong et al., 2000) protocol] that might support this idea.

The increase in GS activity in the present study agrees with previous studies that have shown elevated GS activity with induction of the O–UC after 24 h of crowding (Hopkins et al., 1995; McDonald et al., 2009; Walsh and Milligan, 1995; Walsh et al., 1994; Wood et al., 1995). Like McDonald et al. (McDonald et al., 2009), using a similar crowding protocol, our study showed an increase in GS enzyme activity, plasma cortisol and hepatic urea levels in fish that were crowded. Furthermore, hepatic GS activity was positively

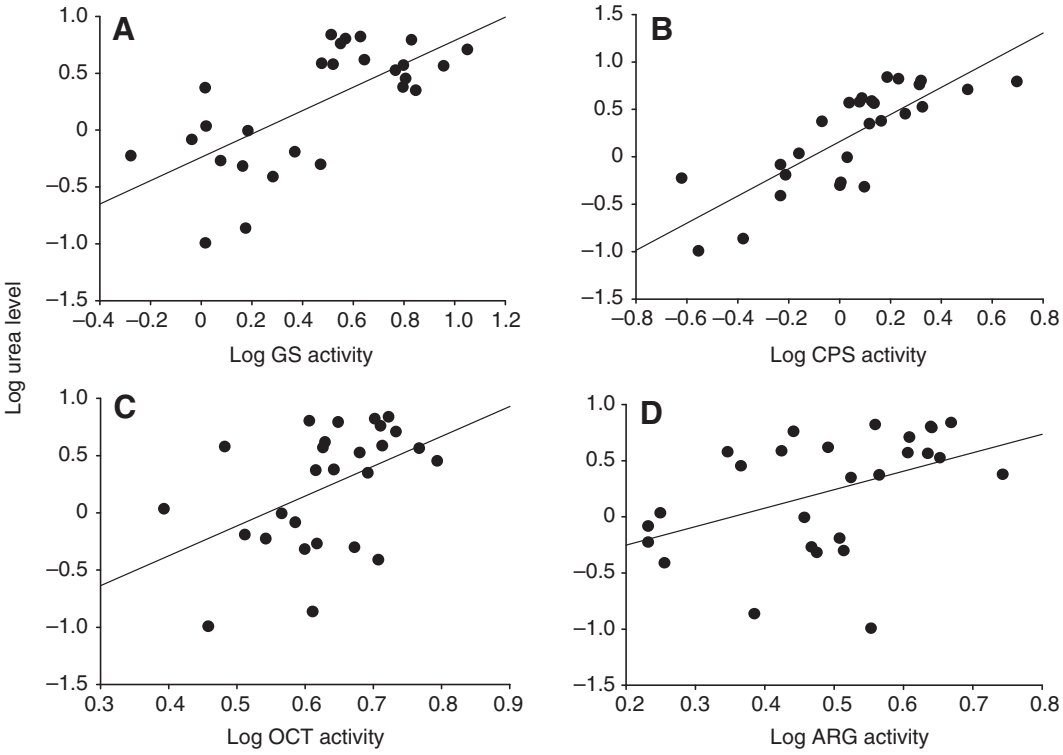

Fig. 4. Linear regressions of log-transformed data for liver urea levels to liver enzyme activities for glutamine synthetase (GS), carbamoyl phosphate synthetase (CPS), ornithine carbamoyl transferase (OCT) and arginase (ARG). (A) The equation of the GS regression line and significance of the correlation are y=1.027x–0.239, R^2 (Adj)=0.450, P<0.001. (B) The equation of the CPS regression line and significance of the correlation are y=1.432x+0.159, R^2 (Adj)=0.630, P<0.001. (C) The equation of the OCT regression line and significance of the correlation are y=2.613x–1.422, R^2 (Adj)=0.192, P=0.013. (D) The equation of the ARG regression line and significance of the correlation are y=1.646x–0.581, R^2 (Adj)=0.169, P=0.019. Units for urea level are 10^{-3} mol kg^{-1}; units for GS activity are 10^{-6} mol min^{-1} g^{-1}; units for CPS activity are 10^{-7} mol min^{-1} g^{-1}; units for OCT activity are 10^{-5} mol min^{-1} g^{-1} and units for ARG activity are 10^{-5} mol min^{-1} g^{-1}.

correlated with plasma cortisol concentrations and with liver urea levels. That GS mRNA expression was not correlated with liver urea concentrations suggests that the pool of GS mRNA was sufficient to upregulate GS protein production or to allow for post-translational modifications. There was no correlation between GS mRNA expression and GS enzyme activity. While the protein half-life of toadfish GS has never been determined, if it is at all similar to mammals, the lack of a correlation between GS mRNA expression and enzyme activity may be explained by the relatively long protein half-life of GS (4–5 days) (Lin and Dunn, 1989) compared with the short half-life of GS mRNA (see above). Therefore, sampling at multiple time points (i.e. every two hours for the first 24 h and then every six hours for the duration of the experiment) may allow for a better resolution of changes in GS activity and mRNA levels.

The present study is the first to examine mRNA expression of ASS, ASL, OCT and ARG in the gulf toadfish. Despite a 2-fold increase in liver ASS mRNA expression in fish crowded for seven days, there was no correlation of ASS mRNA levels to urea levels, indicating that small changes in ASS transcript levels do not amount to measurable changes in urea production. Toadfish ASS mRNA expression was also not correlated with cortisol, despite evidence in starved rats that hepatic ASS mRNA levels may increase in response to dexamethasone (Morris et al., 1987). In fish, there is evidence that ASS is also involved in the citrulline–nitric oxide cycle (C–NOC), which functions in nitric oxide production (Husson et al., 2003; Morris, 2002; Yamaguchi et al., 2001). In carp (*Cyprinus carpio*), cortisol suppresses nitric oxide production (Yamaguchi et al., 2001), which should decrease ASS participation in the C–NOC. Therefore, if C–NOC is subdued in chronically stressed toadfish, a rise in ASS mRNA should occur. As ASS mRNA expression is not correlated to urea levels, perhaps the upregulation in ASS mRNA expression measured in the present study reflects its additional role the C–NOC pathway.

In contrast to ASS, expression of ASL mRNA was reduced in one week crowded fish. The decrease in ASL mRNA expression was negatively correlated with urea levels, supporting earlier studies on ASL enzyme activity that suggested urea acts as a competitive inhibitor to mammalian ASL (Menyhart and Grof, 1977). Unfortunately, due to limits in tissue availability, we did not examine ASS and ASL enzyme activities. However, earlier studies showed that ASS and ASL activity did not to change significantly in fish crowded for four days, despite increases in GS and OCT (Walsh and Milligan, 1995; Walsh et al., 1994). Based on these previous studies, we might not expect an increase in the activity of ASS and ASL in toadfish crowded for two days. However, there seems to be a progression towards increased activity with prolonged durations of crowding. Therefore, it would be consistent if both ASS and ASL activities increased because elevated activities were observed for the other four O–UC enzymes assayed and all were positively correlated with urea levels.

Previous studies on the impact of crowding on OCT activity were conflicting, with an increase in OCT levels in response to four days of crowding in one study (Walsh et al., 1994) and no effect of confinement in the other study (Walsh and Milligan, 1995). The contradictory results could just be a matter of the stressor; both the present study and Walsh et al. (Walsh et al., 1994) found an

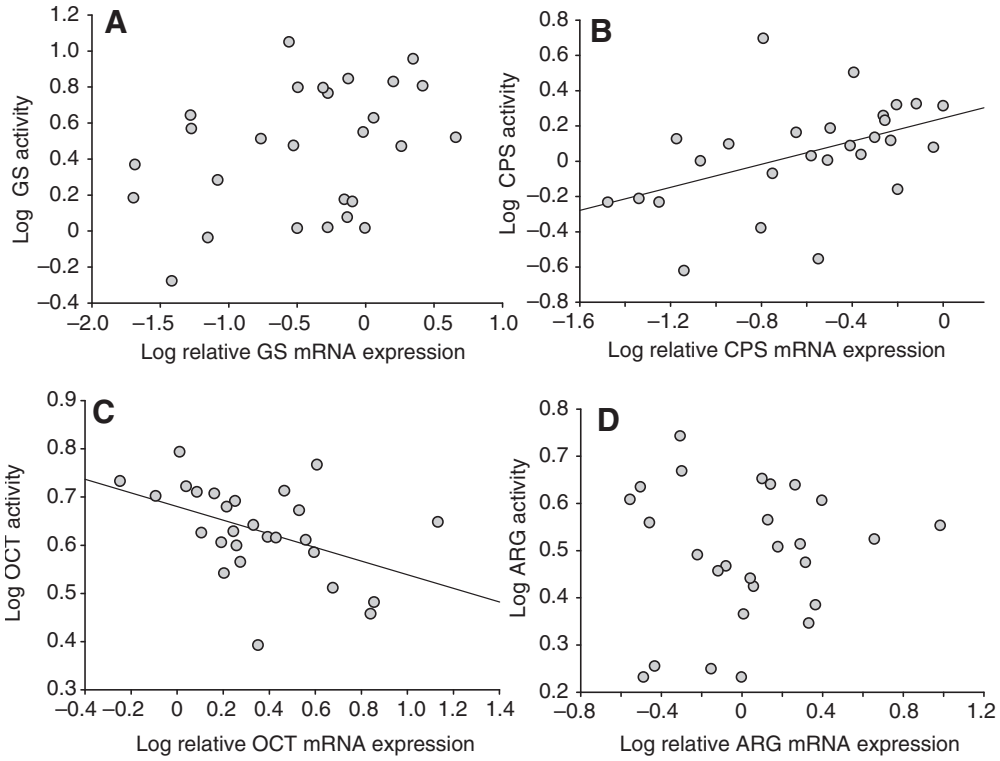

Fig. 5. Linear regressions of log-transformed data for liver mRNA expression and enzyme activity of ornithine–urea cycle (O–UC) enzymes. Only two of the O–UC enzymes tested, carbamoyl phosphate synthetase (CPS) and ornithine carbamoyl transferase (OCT), had a significant relationship (P<0.05) to their mRNA expression. (A) Glutamine synthetase (GS) mRNA expression *versus* GS activity, P=0.073. (B) CPS mRNA expression compared with CPS activity with the equation of the line: y=0.670–0.635, R^2 (Adj)=0.188 and P=0.014. (C) OCT mRNA expression *versus* OCT activity with the equations of the line: y=–1.481x+1.284, R^2 (Adj)=0.178 and P=0.016. (D) Arginase (ARG) mRNA expression compared with ARG activity, P=0.723. Units for GS activity are 10^{-6} mol min^{-1} g^{-1}; units for CPS activity are 10^{-7} mol min^{-1} g^{-1}; units for OCT activity are 10^{-5} mol min^{-1} g^{-1} and units for ARG activity are 10^{-5} mol min^{-1} g^{-1}.

upregulation in response to crowding, which is believed to elicit a much more pronounced stress response in toadfish compared with confinement alone (Sloman et al., 2005; Walsh and Milligan, 1995). The disparity between toadfish OCT mRNA expression and activity in response to stress has also been observed in mammals; rats treated with dexamethasone had no induction of OCT mRNA despite an increase in OCT activity, which was surmised to be due to OCT protein stabilization (Ulbright and Snodgrass, 1993). Interestingly, OCT enzyme activity was correlated with levels of OCT mRNA. Therefore, despite the lack of increase in OCT mRNA levels, enough transcript was available to increase OCT enzyme activity, further indicating OCT protein stabilization during urea production.

Similar to OCT, there was a dichotomy between ARG mRNA expression and activity where transcript levels were not affected by crowding but enzyme activity was; however, unlike OCT, ARG mRNA expression and activity were not correlated. This result could be explained by the fact that there are at least two different ARG genes described in mammals and in teleost fishes that arose from a duplication event (Joerink et al., 2006; Morris, 2002; Wright et al., 2004; Yu et al., 2001). Polyploid fish have undergone further duplications of the ARG genes, resulting in multiple copies of the ARG isoforms (Joerink et al., 2006; Wright et al., 2004). ARG I functions to detoxify ammonia by participating in the O–UC whereas ARG II is thought to play a role in ornithine production (Wright et al., 2004; Yu et al., 2001). In fish, both ARG I and ARG II are found in the liver (Joerink et al., 2006; Wright et al., 2004); thus, total ARG enzyme activity can reflect either ARG I, ARG II or both isozymes (Morris, 2002). However, the predominant isozyme has been shown to differ among teleost fish, i.e. in rainbow trout, the activity of ARG I makes up most of the total ARG enzyme activity whereas in carp, ARG II is the main isozyme (Joerink et al., 2006; Wright et al., 2004). In the toadfish, measurement of liver ARG mRNA expression was specific for ARG I but both ARG I and ARG II may have contributed to the measured ARG activity, which may partially explain why ARG mRNA levels are not correlated to ARG activity.

As in earlier studies that crowded fish for four days (Walsh and Milligan, 1995; Walsh et al., 1994), ARG activity was not significantly different from control fish after two days of crowding. By contrast, fish crowded for one week had a significant increase in ARG activity, suggesting that chronic stress may be necessary to elicit this response. However, ARG activity was not correlated to cortisol levels even when regression analysis was performed on fish crowded for seven days only (data not shown) suggesting that the rise in ARG activity was probably not related to stress and other factors may be contributing to its increase. ARG activity was significantly correlated with liver urea concentrations. Overall urea concentrations can also be affected by arginolysis *via* the degradation of arginine from the diet or hydrolysis of arginine for protein turnover (Wood, 1993). As the fish in this study were not fed for four days prior to treatment, we believe that the arginolysis pathway does not significantly contribute to urea production or to the measured relationship between ARG activity and liver urea concentrations.

Recent studies in fish have found that changes in mRNA expression of O–UC enzymes did not correspond to increases in their corresponding enzyme activities (Iwata et al., 2000; McDonald et al., 2009). Furthermore, a study by Brockmann et al. on a variety of metabolic genes in yeast found that only 20–40% of protein concentrations are related to mRNA expression levels and that post-transcriptional regulation can affect protein translation and degradation (Brockmann et al., 2007). In the present study, only two of the O–UC enzymes, CPS and OCT, had activities that were correlated to mRNA expression. However, there appeared to be a coordinated induction of the O–UC enzyme activities that were correlated to liver urea levels, suggesting that enzyme activity levels are better predictors of liver urea production. In fact, findings of the present study show that the activity of the CPS enzyme alone

can predict urea production, which suggests that CPS is a key regulatory enzyme for the O–UC. To better understand O–UC enzyme regulation, we need to elucidate the mechanisms that underlie their control, including mRNA and protein stability. Future studies should examine transcription factors in the promoter regions of the O–UC genes to determine how the mRNA levels are regulated and post-transcriptional and post-translational control of these enzymes.

LIST OF ABBREVIATIONS

ARG	arginase
ASL	argininosuccinate lyase
ASS	argininosuccinate synthetase
C–NOC	citrulline–nitric oxide cycle
CPS	carbamoyl phosphate synthetase
EF1α	elongation factor-1 α
GLDH	glutamate dehydrogenase
GRE	glucocorticoid responsive element
GS	glutamine synthetase
NADPH	nicotinamide adenine dinucleotide phosphate
OCT	ornithine carbamoyl transferase
O–UC	ornithine–urea cycle
RPAs	ribonucleic acid protection assays
RT	reverse transcription
RT-PCR	real-time polymerase chain reaction

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