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Molecular Aspects of Nitrogen Metabolism in Fishes

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UNIVERSITY OF MIAMI

MOLECULAR ASPECTS OF NITROGEN METABOLISM IN FISHES

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Molecular Aspects of Nitrogen Metabolism in Fishes (August 2009)

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Molecular aspects of nitrogen metabolism in vertebrates is an interesting area of physiology and evolution to explore due to the different ways in which animals excrete nitrogenous waste as they transition from an aquatic to a terrestrial lifestyle. Two main products of nitrogen metabolism in fishes are ammonia and urea. Ammonia is produced during protein catabolism and build up of ammonia is toxic. Some aquatic vertebrates convert ammonia into a less toxic compound urea via *de novo* synthesis through the ornithine-urea cycle (O-UC). Five enzymes are involved in the O-UC: carbamoyl phosphate synthetase (CPS), ornithine carbamoyl transferase (OCT), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL), and arginase (ARG). An accessory enzyme, glutamine synthetase (GS) also participates in the "fish-type" O-UC.

Teleosts excrete ammonia passively over their gills into the aquatic environment. The teleost, *Opsanus beta*, has been shown to increase urea production after 48 hours of crowding. This thesis explored how crowding stress affected nitrogen metabolite levels of ammonia and urea and O-UC gene expression and enzyme activity in *O. beta*.

Lungfishes while in an aquatic environment avoid ammonia toxicity by releasing excess ammonia across their gills, but when stranded on land they produce urea through the O-UC. Urea production via the O-UC has a metabolic cost of at least four ATP

molecules. This thesis explored the response of a lungfish, *Protopterus annectens*, to six days of aerial exposure and re-immersion conditions by measuring concentrations of O-UC mRNA expression and enzyme activity and nitrogen metabolites ammonia and urea.

CPS acts as the entry point to the O-UC and based on enzymatic studies, most aquatic vertebrates utilize one isoform of this enzyme (CPSIII) while terrestrial vertebrates utilize a different isoform of this enzyme (CPSI). Lungfishes are a particularly interesting group of air-breathing fishes, not only because of their link to the origins of tetrapods, but also because CPS I may have originated within this group. Both CPS III and CPS I have been enzymatically described within this group. This thesis uses phylogenetics to investigate how CPS nucleotide sequences in lungfishes evolved compared to other vertebrates.

ACKNOWLEDGMENTS

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Chapter 1. General Introduction

Ammonia and urea as metabolites for nitrogen metabolism

An interesting way to examine vertebrate transition from an aquatic to a terrestrial lifestyle is by exploring the required changes in nitrogen metabolism and excretion. In vertebrates there are three main end products for nitrogen metabolism: ammonia, urea and uric acid (Campbell et al., 1987; Griffith, 1991; Wood et al., 1995; Wright, 1995). Ammonia is the primary end product in aquatic vertebrates while terrestrial vertebrates form urea and uric acid (Griffith, 1991; Wright, 1995). Since the quantitative contribution of uric acid to nitrogen metabolism is small in fishes compared to other metabolites (Mommsen and Walsh, 1992), this thesis will focus on ammonia and urea.

The ability to deal with excess nitrogen is a requirement for all living organisms because the build up of ammonia is toxic (Anderson, 2001; Ip et al., 2001; Korsgaard et al., 1995) and therefore organisms must find a way to excrete excess ammonia. The mode of action for ammonia toxicity is not well understood (Korsgaard et al., 1995; Mommsen and Walsh, 1992; Walsh, 1998), however it has been shown that ammonia can interfere with energy metabolism and neurological functions (Ip et al., 2001; Korsgaard et al., 1995; Randall and Tsui, 2002). Ammonia within the body is in the form of NH_4^+, while aqueous ammonia exists as either NH_3 or NH_4^+ with the majority being NH_3 (Chew et al., 2006; Ip et al., 2001; Mommsen and Walsh, 1992; Walsh, 1998; Wood et al., 1995). In fishes, NH_4^+ is capable of substituting for other cations like K^+ and H^+ affecting ionic balance through Na^+/K^+ ATPase, $Na^+/K^+/2Cl^-$ cotransporter, and Na^+/H^+ exchanger (Ip et al., 2001; Ip et al., 2004b; Walsh, 1998; Wilkie, 2002; Wright, 1995). In

nerve cells, NH_4^+ can replace K^+ in potassium ion channels, affecting membrane potential (Ip et al., 2001; Korsgaard et al., 1995; Randall and Tsui, 2002). The toxic effects of ammonia result in multiple symptoms eventually leading to convulsions, coma and death (Chew et al., 2006; Ip et al., 2001; Randall and Tsui, 2002; Wright, 1995).

In fishes, soluble ammonia diffuses down a partial pressure gradient from the liver to the plasma, to the gills and finally to the external aqueous environment without any metabolic input (Anderson, 1995b; Ip et al., 2001; Korsgaard et al., 1995; Wilkie, 2002). Alternatively, terrestrial vertebrates avoid ammonia toxicity by converting it to less toxic compounds like urea (Chew et al., 2006; Ip et al., 2001; Ip et al., 2004a; Ip et al., 2004b; Janis and Farmer, 1999; Mommsen and Walsh, 1992; Randall and Tsui, 2002; Sayer, 2005). Urea is produced in three pathways in vertebrates: 1. the ornithine-urea cycle (O-UC); 2. the uricolysis pathway; 3. the argininolysis pathway (Anderson, 1995b; Mommsen and Walsh, 1992; Walsh, 1998; Wood et al., 1995; Wright and Land, 1998). This thesis focuses on molecular aspects of the O-UC and does not address the uricolysis or the argininolysis pathways directly.

The only source of *de novo* synthesis of urea in fishes is through the O-UC and in vertebrates it requires the metabolic input of at least four molecules of ATP (Mommsen and Walsh, 1991; Mommsen and Walsh, 1992; Walsh, 1998; Wood et al., 1995). The enzymes involved in the O-UC are carbamoyl phosphate synthetase (CPS) (EC 6.3.4.16), 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 arginase (ARG) (3.5.3.1) (Anderson, 1995b; Anderson, 2001; Griffith, 1991; Korsgaard et al., 1995; Mommsen and Walsh, 1991; Wood et al., 1995). In fishes, glutamine synthetase (GS) (EC 6.3.1.2),

an enzyme on an accessory pathway to the O-UC, is also involved in urea synthesis (Anderson, 1995b; Anderson, 2001; Griffith, 1991; Korsgaard et al., 1995; Walsh, 1998; Wood et al., 1995; Wright and Land, 1998). Changes in the utilization of the enzymes of the O-UC and their compartmentalization have occurred as vertebrates transitioned from the aquatic to the terrestrial environment (Anderson, 1995b; Mommsen and Walsh, 1989; Mommsen and Walsh, 1991). However, CPS is of particular interest due to it's change in substrate utilization during the production of urea via the O-UC during the water-land transition (Lindley et al., 2007).

In the mammalian O-UC (summarized in Figure 1.1), the CPS I isoform utilizes NH_4^+ as a nitrogen substrate in the presence of HCO_3^- and 2 ATPs resulting in the production of carbamoyl phosphate (Anderson, 1995b; Anderson, 2001; Mommsen and Walsh, 1991; Takiguchi and Mori, 1995; Wood, 1993). The CPS reaction requires a co-factor, N-acetyl glutamate (N-AcGlu), and is localized in the mitochondrial matrix Takiguch. In mammals, OCT is also located within the mitochondrial matrix (Anderson, 1995b; Mommsen and Walsh, 1991; Takiguchi and Mori, 1995) while the next three enzymes of the O-UC cycle (ASS, ASL and ARG) are located in the cytosol (Anderson, 2001; Mommsen and Walsh, 1991; Takiguchi and Mori, 1995). Therefore, citrulline must be transported through the mitochondrial membrane and the production of urea occurs in the cytosol (Griffith, 1991; Mommsen and Walsh, 1992). For ornithine to be recycled back into the O-UC, ornithine must then be transported back into the mitochondria (Griffith, 1991; Mommsen and Walsh, 1992). In mammals, GS is cytosolic and does not participate in the O-UC cycle but instead acts to mop up excess ammonia not utilized by the mammalian O-UC (Mommsen and Walsh, 1989).

In the "fish-type" O-UC (summarized in Figure 1.2), GS is localized within the mitochondrial matrix (Anderson, 1995b; Mommsen and Walsh, 1989; Mommsen and Walsh, 1991) and forms glutamine in the presence of NH_3, glutamate and ATP. The resultant glutamine is then used by CPS III as a nitrogen substrate, and similar to mammals, this reaction also requires N-AcGlu as a co-factor and is localized in the mitochondrial matrix (Anderson, 1995a; Anderson, 1995b; Mommsen and Walsh, 1991). In teleost fish and mammals, OCT is present in the mitochondrial matrix and ASS and ASL are found in the cytosol (Anderson, 1995b; Mommsen and Walsh, 1991). However, unlike mammals, ARG is located in the mitochondrial matrix in teleosts (Anderson, 1995b; Mommsen and Walsh, 1989; Mommsen and Walsh, 1992). Therefore, in teleosts citrulline is transported out of the mitochondrial matrix but arginine is transported back into the mitochondria during the production of urea and urea is produced within the mitochondrial matrix (Anderson, 1995b; Griffith, 1991; Mommsen and Walsh, 1992). Ornithine is recycled back into the O-UC from within the mitochondrial matrix and urea must be transported out of the mitochondria (Anderson, 1995b; Mommsen and Walsh, 1992), likely by a type of urea transporter (Walsh et al., 1994a).

It has been proposed that fishes excrete urea when pathways of ammonia excretion are blocked (Mommsen and Walsh, 1989; Walsh, 1997). In Lake Magadi tilapia (*O. grahami*), ammonia excretion into external waters is blocked by the high pH (10.0) and the buffering capacity of the lake (Wood et al., 1994). Since this fish is unable to excrete ammonia, it must excrete all of its nitrogenous waste as urea (Wood et al., 1994). Interestingly, in another ureotelic teleost, the gulf toadfish (*Opsanus beta*), urea excretion is not affected by changes in pH (Walsh et al., 1989). Ureotely is also

induced in fishes with increased ammonia levels in the surrounding waters (Ip et al., 2003; Saha et al., 2003) and gulf toadfish increase urea production after 5 mM ammonium chloride exposure for 24 to 48 hours (Wang and Walsh, 2000). Air exposure has also been shown to induce ureotely in amphibious fishes because the fish no longer have access to water which can act a sink for excess ammonia (Ip et al., 2001; Loong et al., 2005; Wood et al., 2005). Toadfish that were air exposed for 8 to 12 hours increased both plasma ammonia and urea levels and were found to increase their overall urea excretion (Walsh et al., 1990). To avoid ammonia toxicity air-exposed lungfishes increase their overall urea synthesis and decrease their ammonia production (Chew et al., 2004; Chew et al., 2003; Ip et al., 2005b; Loong et al., 2005; Loong et al., 2008).

Proposed evolution of carbamoyl phosphate synthetase

Carbamoyl phosphate synthetase results in the production of carbamoyl phosphate from a series of chemical reactions outlined in Figure 1.3 (Anderson and Meister, 1966; Holden et al., 1999; Lim and Powers-Lee, 1996; Thoden et al., 1997; Thoden et al., 2002; van den Hoff et al., 1995). These reactions progress quickly (Holden et al., 1999; Thoden et al., 1997) and the net result is the production of carbamoyl phosphate from one molecule of bicarbonate, two molecules of ATP, and either one molecule of glutamine or one molecule of ammonia depending on the type of CPS (Anderson and Meister, 1966; Holden et al., 1999; Hong et al., 1994; Thoden et al., 1999).

Carbamoyl phosphate synthetase is involved in two metabolic pathways: pyrimidine biosynthesis and production of arginine/urea (Figures 1.1 and 1.2) (Anderson, 1995a; Anderson, 1995b; Anderson, 2001; Eroglu and Powers-Lee, 2002; Holden et al.,

1999; Wood et al., 1995). Three isozymes make up the CPS protein family and the different isozymes vary in the pathway utilized, the nitrogen substrate that is utilized, what allosteric effectors regulate the enzyme, the co-factors required for activation, and the types of organisms in which they are found (Anderson, 1995a; Anderson, 1995b; Anderson, 2001; Campbell and Anderson, 1991; Holden et al., 1999; Hong et al., 1994; Saha et al., 1999).

The most common isoform is CPS-II, and it is present in all organisms for the pyrimidine biosynthesis pathway (Anderson, 1995a; Anderson, 2001; Holden et al., 1999; Hong et al., 1994). Glutamine acts as the nitrogen substrate for CPS-II (Anderson, 1995a; Anderson, 1995b; Anderson, 2001; Holden et al., 1999; Hong et al., 1994). Most prokaryotes only have a single form of this enzyme and it is used for both pyrimidine biosynthesis and for arginine synthesis (Anderson, 1995a; Anderson, 1995b; Holden et al., 1999; Hong et al., 1994). In prokaryotes, ornithine and inosine monophosphate (IMP) act as positive allosteric effectors for the arginine pathway and uridine monophosphate (UMP) acts as a feedback inhibitor. Uridine 5'- triphosphate (UTP) acts as a feedback inhibitor in all other organisms when using CPS-II via the pyrimidine pathway (Anderson, 1995a; Hong et al., 1994). In higher eukaryotes, phosphoribosyl pyrophosphate (PRPP) acts as an allosteric activator for the CPS-II involved in the pyrimidine pathway (Anderson, 1995a; Hong et al., 1995; Hong et al., 1994; Lim and Powers-Lee, 1996; McCudden and Powers-Lee, 1996).

CPS-I is found mainly in terrestrial vertebrates (Anderson, 1995a; Anderson, 1995b; Anderson, 2001; Felskie et al., 1998; Hong et al., 1994; Kong et al., 1998; Lim and Powers-Lee, 1996; McCudden and Powers-Lee, 1996) although three studies have

found CPS-I in fishes, specifically the stinging catfish, *Heteropneustes fossilis* (Saha et al., 1997), the air-breathing walking catfish, *Clarias batrachus* (Saha et al., 1999) and two species of lungfish, *Protopterus annectens* and *Protopterus aethiopicus* (Mommsen and Walsh, 1989). As described above, CPS-I is involved in the O-UC, is localized to the mitochondrial matrix of the liver, uses ammonia as its aminating substrate (Anderson, 1995a; Anderson, 1995b; Hong et al., 1994; van den Hoff et al., 1995) and has N-AcGlu as a positive allosteric effector (Anderson, 1995a; Anderson, 1995b; Felskie et al., 1998; Hong et al., 1994; Kong et al., 1998; van den Hoff et al., 1995).

CPS-III has been described in invertebrates and fishes, including the coelacanth (Anderson, 1995a; Anderson, 1995b; Anderson, 2001; Felskie et al., 1998; Hong et al., 1994; Kong et al., 1998; Mommsen and Walsh, 1989). As described above CPS III is involved with the O-UC, is localized to the mitochondrial matrix (Anderson, 1995a; Anderson, 1995b; Anderson, 2001; Hong et al., 1994), uses glutamine as its' nitrogen donor, and has N-AcGlu as a positive allosteric effector (Anderson, 1995a; Anderson, 1995b; Anderson, 2001; Felskie et al., 1998; Hong et al., 1994; Kong et al., 1998). Contrary to Mommsen and Walsh (1989), recent enzymatic studies found CPS-III in the lungfish species, *Protopterus dolloi* (Chew et al., 2003), *P. annectens*, and *P. aethiopicus* (Loong et al., 2005). Interestingly, both CPS I and CPS III has been found in *C. batrachus* (Saha et al., 1999; Saha et al., 2007).

The ancestral proteins for CPS are proposed to be a glutamine amidotransferase (GAT), another protein of unknown origin, and a kinase protein (Anderson, 1995a; Hong et al., 1994; Lawson et al., 1996; van den Hoff et al., 1995). The protein of unknown origin fused with the GAT to form glutaminase, while at the same time the kinase protein

underwent a duplication event to become a synthetase. These two new proteins fused to form prokaryote CPS II with the glutaminase moiety forming the N-terminus and the synthetase portion forming the C-terminus (Anderson, 1995a; Hong et al., 1994; Lawson et al., 1996; van den Hoff et al., 1995). The prokaryote CPS then underwent two types of modifications each resulting in the formation of a new isoform of CPS in eukaryotes. The cytosolic form of CPS-II resulted from the fusion of the prokaryote form of CPS-II with two more proteins dihydro-orotase and aspartate transcarbamylase to the C-terminal end. The mitochondrial form of CPS acquired a mitochondrial signal on the N-terminal end of the prokaryote CPS-II and also acquired an N-AcGlu binding site in the C-terminal end of the enzyme to form CPS-III in invertebrates and fishes (Anderson, 1995a; Hong et al., 1994; van den Hoff et al., 1995). Finally, in terrestrial vertebrates, CPS-III lost its glutamine binding site to become CPS-I (Anderson, 1995a; Hong et al., 1994; van den Hoff et al., 1995).

Phylogenetic analyses generally agree with the proposed evolution of the CPS family, showing that a CPS I/III clade likely arose from CPSII (Figure 1.4) (Lindley et al., 2007). Most studies however, do not show that CPS I arose from CPS III but rather show both as separate clades arising from the same common ancestor (Cammarano et al., 2002; Lawson et al., 1996; van den Hoff et al., 1995; Zhou et al., 2000). What is interesting about the phylogenetic data is that CPS I forms a terrestrial clade, whereas CPS III forms an aquatic clade but this may be an artifact of the gene tree reflecting the true nature of the species tree.

Messenger RNA expression and protein production

The levels of protein synthesized in the cell is affected by transcription, translation and protein stability (Lewin, 2004; Weaver, 2008). Therefore, changes in enzyme activity may result from changes in mRNA expression, post-transcriptional and post-translational changes within the cell. Messenger RNA (mRNA) expression is regulated by transcription factors including enhancers, activators, and repressors (Levine and Tjian, 2003; Lewin, 2004; Weaver, 2008). Measurements of mRNA expression represent not only mRNA production but also mRNA decay; therefore, the level of mRNA expression represents the steady-state level of expression (Mata et al., 2005). Enzyme activity can also be affected by the rate of protein translation which is regulated by mRNA decay and small RNA binding proteins that bind to the 3' untranslated region of the transcript and slow down translation (Gebauer and Hentze, 2004; Mata et al., 2005; Weaver, 2008). Furthermore, protein translation can be affected by altering the phosphorylation state of initiation factors for a particular protein (Dever, 2002; Gebauer and Hentze, 2004; Mata et al., 2005; Weaver, 2008) and the ribosomal density or number of ribosomes per transcript (Brockmann et al., 2007; Mata et al., 2005). Since protein translation depends on mRNA produced during transcription, we would expect mRNA expression levels to be correlated with protein levels; however, the *in vivo* protein half-life also affects whether these two entities are correlated (Brockmann et al., 2007; Greenbaum et al., 2003). Furthermore, it has been suggested that on a global scale, mRNA expression is only partly correlated with protein expression (Brockmann et al., 2007).

Since urea production occurs via the O-UC, we would expect to find reports of studies examining the relationships between urea concentrations and activity or mRNA expression of the O-UC enzymes. Many studies in mammalian systems have examined enzyme activity and mRNA expression of the O-UC enzymes relating O-UC expression to hormones, diet and age related factors, but little information is available relating O-UC mRNA expression to urea levels (Grofte et al., 1998; Morris et al., 1987; Nebes and Morris, 1988; Takagi et al., 2008; Ulbright and Snodgrass, 1993). In fishes, despite many studies on O-UC enzyme activity relating to urea production, there is a dearth of information relating O-UC mRNA expression and urea levels, especially in ureotelic fishes (Iwata et al., 2000; Kong et al., 2000).

Thesis objectives

Three research areas are explored in this thesis relating to nitrogen metabolism and the genes involved in the O-UC cycle in fishes.

The objective of the second chapter is to determine the mitochondrial isoform of CPS utilized by lungfishes (transitional fishes inhabiting both terrestrial and aquatic environments) and to determine the relationships lungfishes CPS to other CPS enzymes. The second chapter uses multiple phylogenetic analyses on sequences from a small fragment of CPS isolated from the liver of five species of lungfishes. A longer fragment is also analyzed for one species of lungfish, *P. annectens*, to determine if the region of the small fragment of CPS influenced the placement of the lungfishes clade within a particular clade in the CPS protein family. The results of chapter two suggest that all

lungfishes possess CPS I and that the origins of CPS III do not fall within the lungfishes clade.

The objective of the third chapter is to perform a comprehensive analysis of hepatic nitrogen metabolites, mRNA expression, and activity of O-UC enzymes in the West African lungfish, *P. annectens* exposed to emersion conditions and re-immersion conditions. The goal was to determine if there was any relationship(s) of ammonia and/or urea concentrations to mRNA expression and enzyme activity of the O-UC enzymes. A further objective of the third chapter was to determine if there was any relationship of O-UC enzymes' mRNA expression with enzyme activity of O-UC genes. The results of chapter three suggest that urea production is increased in hepatic tissue of aerially exposed *P. annectens* via a coordinated induction of both mRNA expression and enzyme activity of O-UC genes.

The objective of the fourth chapter was to examine the impact of crowding stress on concentrations of ammonia, urea, mRNA expression, and activity of the O-UC enzymes in the liver of a teleost fish, the gulf toadfish (*O. beta*). To accomplish this we manipulated cortisol levels by crowding toadfish for two days and one week to determine if stress had any impact on the above factors. We also looked at the relationship between mRNA expression and enzyme activity for the O-UC genes in the toadfish liver and the relationships between plasma cortisol concentrations and nitrogen metabolite concentrations. The results of chapter four showed that there was a coordinated induction of enzyme activity for the O-UC genes associated with a rise in both cortisol levels and urea production, but there was no similar induction of mRNA expression. Enzyme activity was correlated to urea concentrations indicating that enzyme activity is a better

predictor of urea production in toadfish. We also saw evidence that CPS acted as a control point for urea production for both mRNA expression and enzyme activity.

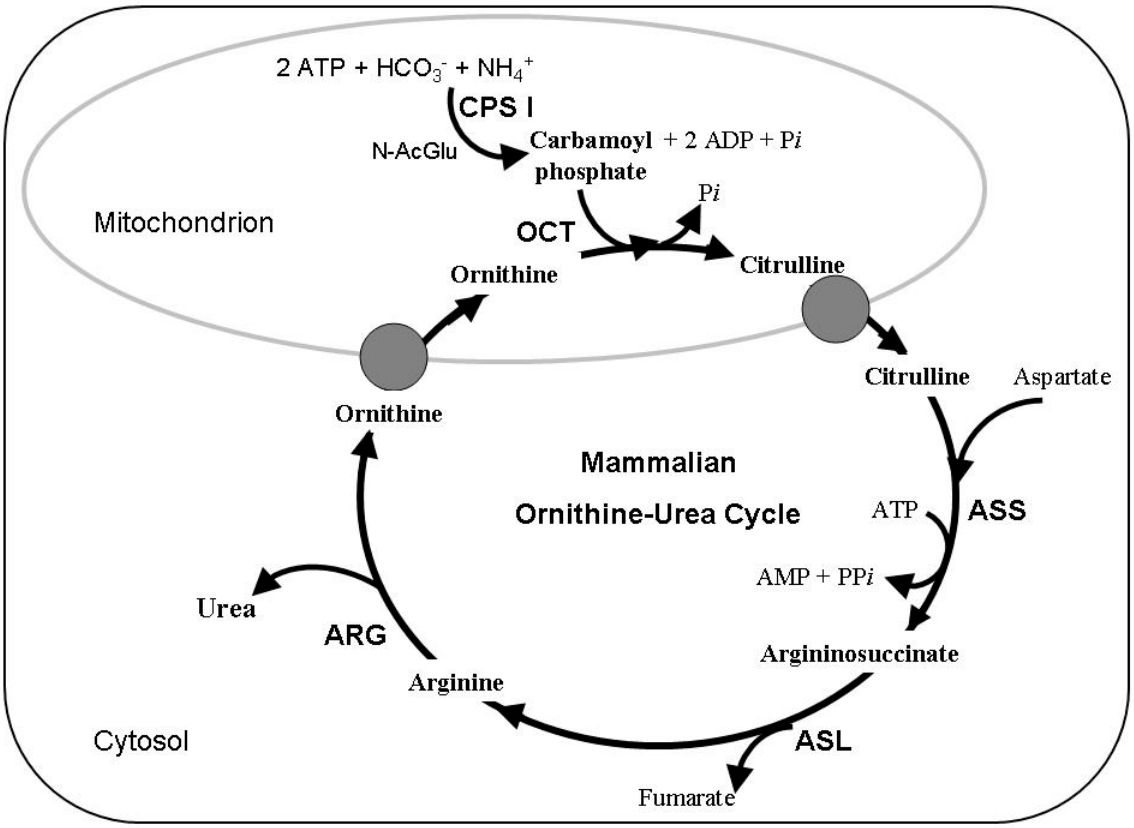


Figure 1.1 Illustration of the ammonia-dependent ornithine-urea cycle from the hepatic tissue of a terrestrial vertebrate. The bold and capitalized lettering shows the five O-UC enzymes involved in this cycle: CPS I, carbamoyl phosphate synthetase I; OCT, ornithine carbamoyl transferase; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase; ARG, arginase. Adapted from urea cycle by Holden et al. 1999 and from Anderson 2001.

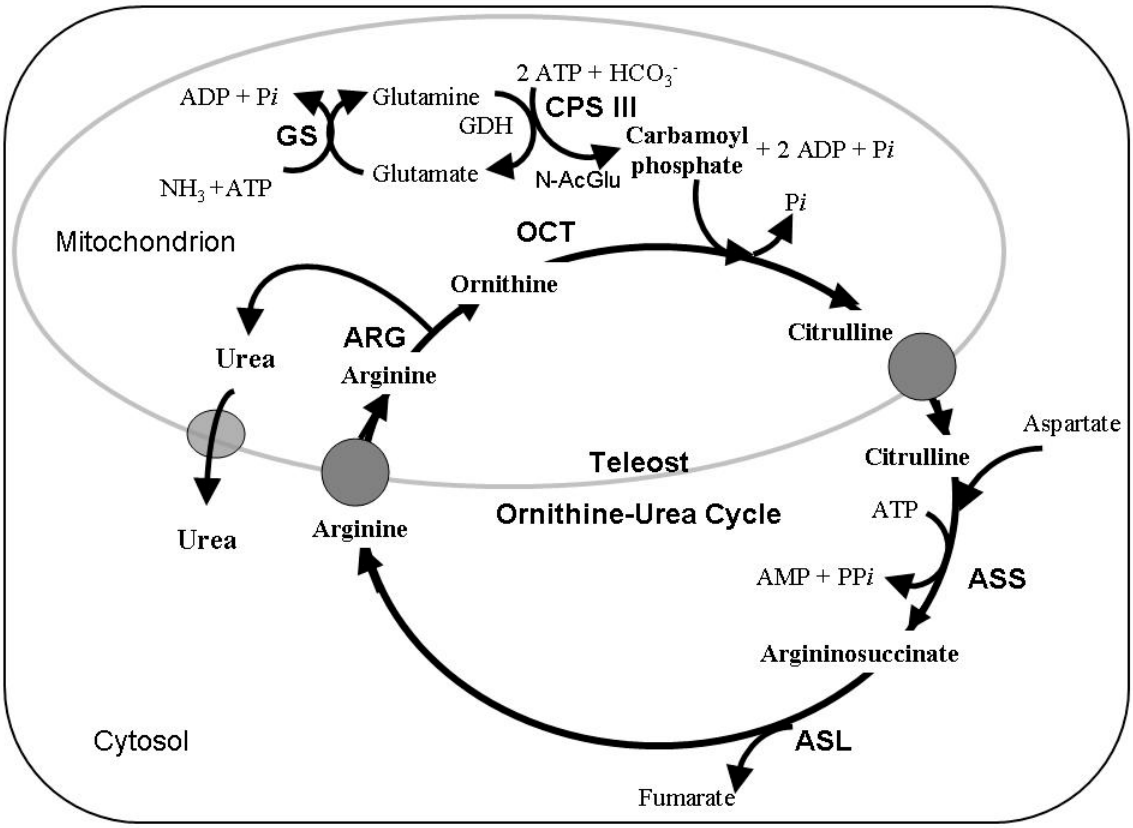


Figure 1.2 Illustration of the glutamine-dependent ornithine-urea cycle from the hepatic tissue of an aquatic teleost. The bold and capitalized lettering shows the five O-UC enzymes involved in this cycle: CPS III, carbamoyl phosphate synthetase III; OCT, ornithine carbamoyl transferase; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase; ARG, arginase. In the "fish-type" O-UC an accessory pathway involved in the formation of glutamine is also present and therefore shows the participation of GS, glutamine synthetase for this pathway. GDH, glutamine dehydrogenase. Adapted from urea cycle by Holden et al. 1999, Anderson 2001, and Anderson 1980.

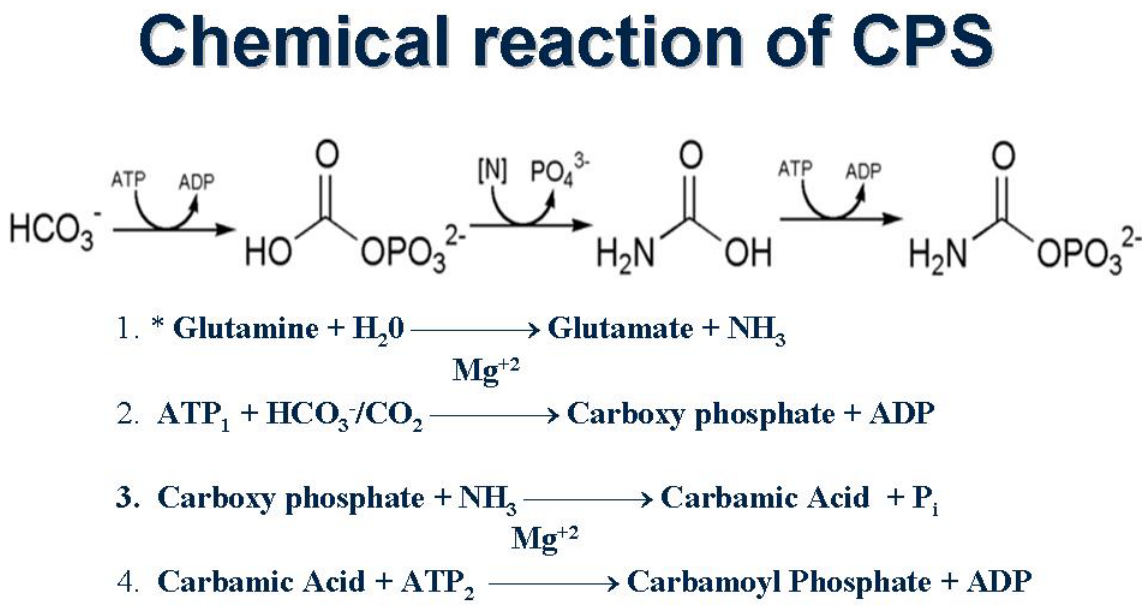
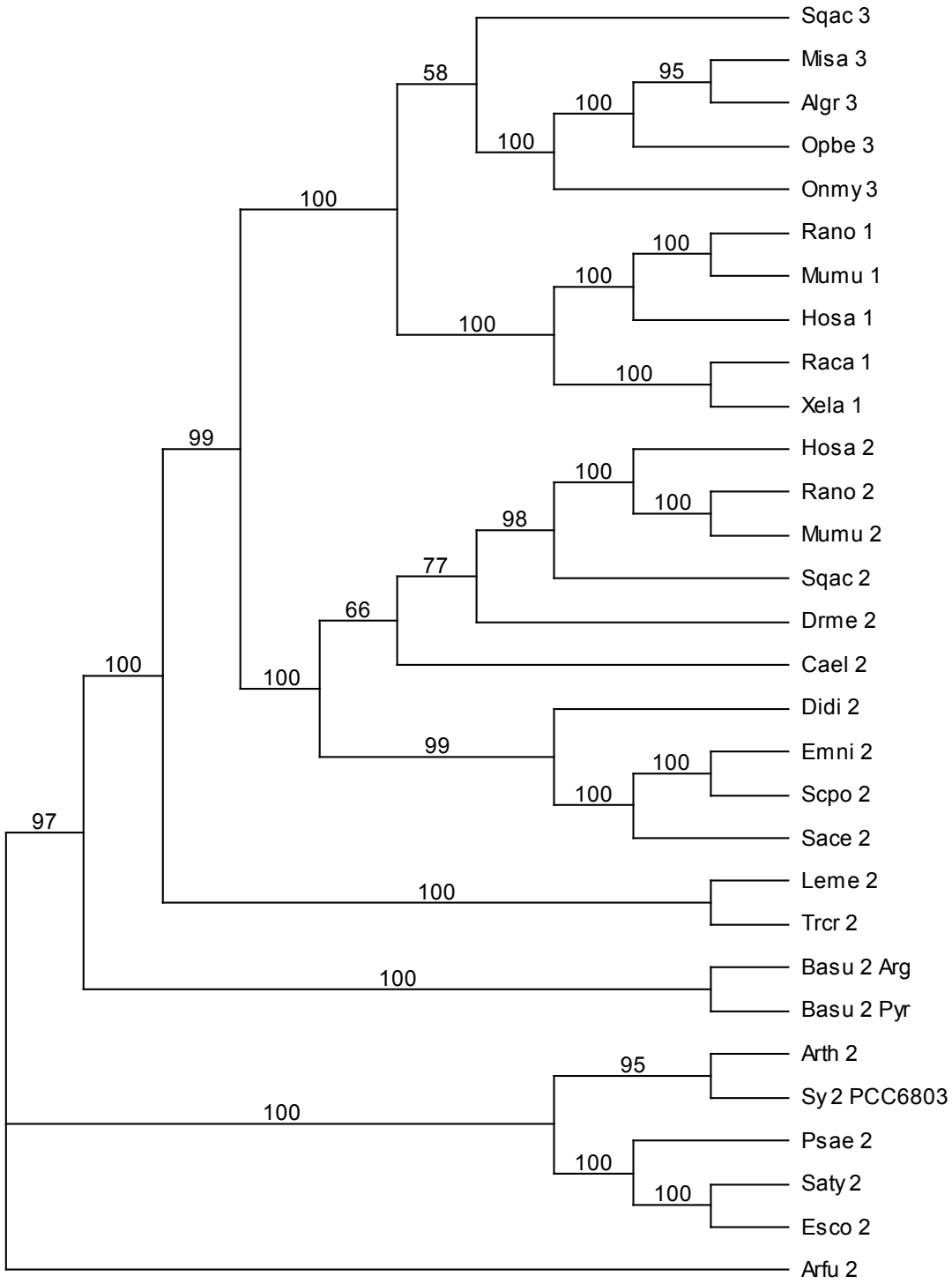


Figure 1.3 The net reaction catalyzed by carbamoyl phosphate synthetase using either NH_3 or glutamine as a nitrogen donating source [N] to produce carbamoyl phosphate. Partial reactions involved in achieving the overall net reaction are also shown. (Adapted from Thoden et al. 1997). * This reaction only necessary if glutamine serves as the nitrogen donor.

Figure 1.4 Maximum likelihood tree constructed from concatenated nucleotide sequences of all the isoforms of CPSase. *Archaeoglobus fulgidus* was used as an outgroup taxa. Species used in this analysis, identified by a four-letter symbol, and GenBank accession numbers are given below: The type of CPSase (I, II or III) is numerically identified as 1, 2 or 3, respectively. Some accession numbers include genomic nucleotide sequence for more than the CPSase-specific gene and these sequences were edited to reflect only the exon portions of the CPSase-specific product. More than one accession number listed for a single species indicates that partial CPSase products were combined to give complete sequence information for the entire CPSase gene of that organism. **CPSase 3 isoforms** - *Squalus acanthias*, Sqac 3 (L31362); *Micropterus salmoides*, Misa 3 (AF006491); *Oncorhyncus mykiss*, Onmy 3 (U65893); *Alcolapia grahami*, Algr 3 (AF119250); *Opsanus beta*, Opbe 3 (AF169248); **CPSase 1 isoforms** - *Homo sapiens*, Hosa 1 (NM_001875); *Rattus norvegicus*, Rano 1 (NM_017072); *Rana catesbeiana*, Raca 1 (U05193); *Xenopus laevis*, Xela 1(DQ179105); *Mus musculus*, Mumu 1 (XM_129769**); CPSase 2 isoforms** - *Homo sapiens*, Hosa 2 (NM_004341); *Squalus acanthias*, Sqac 2 (U18868); *Mus musculus*, Mumu 2 (XM_131981); *Rattus norvegicus*, Rano 2 (XM_343027); *Dictyostelium discoideum*, Didi 2 (X14633, X55433); *Drosophila melanogaster*, Drme 2 (X04813); *Trypanosoma cruzi*, Trcr 2 (AB005063); *Leishmania mexicana*, Leme 2 (AB005062); *Caenorhabditis elegans*, Cael 2 (NM_063437); *Emericella nidulans*, Emni 2 (AF112473); *Arabidopsis thaliana*, Arth 2 (NM_113690, NM_102730); *Saccharomyces cerevisiae*, Sace 2 (M27174); *Schizosaccharomyces pombe*, Scpo 2 (X81841); *Bacillus subtilus* (subsp. subtilis str. 168*)*, Basu 2 (NC_000964); *Synechocystis* PCC6803 Sy 2 (D90916, D64002); *Pseudomonas aeruginosa PA01,* Psae 2 (NC_002516); *Salmonella typhimurium LT2,* Saty 2 (AE008696); *Escherichia coli* K12, Esco 2 (NC_000913); *A. fulgidus DSM 4304,* Arfu 2 (NC_000917). Phylogenetic tree modified from Lindley et al., 2007.

Figure 1.4


Sqac 3
Misa 3
Algr 3
Opbe 3
Onmy 3
Rano 1
Mumu 1
Hosa 1
Raca 1
Xela 1
Hosa 2
Rano 2
Mumu 2
Sqac 2
Drme 2
Cael 2
Didi 2
Emni 2
Scpo 2
Sace 2
Leme 2
Trcr 2
Basu 2 Arg
Basu 2 Pyr
Arth 2
Sy 2 PCC6803
Psae 2
Saty 2
Esco 2
Arfu 2
58
95
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100
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100

Chapter 2. Phylogenetic Aspects of Carbamoyl Phosphate Synthetase: a Transitional Enzyme in Transitional Fishes

Carbamoyl phosphate synthetase (CPS) is responsible for the formation of carbamoyl phosphate from glutamine or ammonia, bicarbonate and ATP. There are three different isoforms of CPS that play vital roles in two evolutionary pathways, pyrimidine biosynthesis (CPS II) and arginine/urea biosynthesis (CPS I and CPS III). Gene duplication has been proposed as the evolutionary force creating this gene family with CPS II likely giving rise to CPS I and III clade. In the evolutionary history of this gene family it is still undetermined when CPS I diverged from CPS III.

CPS I has been described mainly in terrestrial air-breathing tetrapods with respect to its role in urea formation, whereas CPS III has been described mainly in aquatic fishes. Transitional organisms such as lungfishes are of particular interest because they are capable of respiring via gills and with lungs and therefore can be found in both aquatic and terrestrial environments. Notably, enzymatic characterization of the mitochondrial CPS isoforms in this transitional group has not led to clear conclusions. In order to determine which CPS isoform is present in transitional animals, we examined partial sequences for CPS amplified from five species of lungfish and compared it to CPS isoforms from other fish and mammals.

Reverse transcription-PCR (RT-PCR) and sequencing of the CPS isoforms in five species of lungfish was used to evaluate where the split between CPS I and CPS III had occurred. It was determined that for the CPS isoforms, lungfishes formed a monophyletic clade within the CPS I and separate from the CPS III clade of other vertebrates. This would imply that the lungfish CPS is derived from CPS I and is likely

to have a physiological function similar to CPS I. This finding is important because it supports the hypothesis that lungfish use a urea cycle similar to terrestrial air-breathing vertebrates.

Background

Carbamoyl phosphate synthetase (CPS) is an essential enzyme found in all classes of organisms and is involved in pyrimidine biosynthesis and the ornithine-urea cycle (O-UC)/ arginine biosynthesis (Anderson, 1995a; Holden et al., 1999). The evolution of the CPS gene family provides a novel way to examine questions related to nitrogen metabolism as vertebrates transitioned from an aquatic to a terrestrial lifestyle. Nitrogen metabolism in fishes has two primary endpoints, ammonia and urea (Griffith, 1991; Walsh, 1998; Wood, 1993; Wright, 1995). Ammonia toxicity is a biological consequence of nitrogen metabolism that both aquatic and terrestrial organisms must overcome (Anderson, 2001; Ip et al., 2001; Korsgaard et al., 1995). Fish avoid ammonia toxicity by excreting excess ammonia to the external aqueous environment (Ip et al., 2001; Korsgaard et al., 1995; Wilkie, 2002), whereas terrestrial vertebrates use a strategy of metabolizing ammonia to urea via the O-UC and then excrete urea (Ip et al., 2001; Ip et al., 2004a; Ip et al., 2004b; Janis and Farmer, 1999; Mommsen and Walsh, 1992; Randall and Tsui, 2002; Sayer, 2005). Ammonia is utilized during the formation of carbamoyl phosphate via CPS directly (or indirectly via glutamine synthetase (GS)) for both of the metabolic processes.

There are three different isoforms of CPS: one isoform, CPS II, is involved in pyrimidine synthesis, while the other two isoforms, CPS I and CPS III, are involved with

nitrogen metabolism via the O-UC. All isoforms result in the production of carbamoyl phosphate but they differ in their nitrogen substrate, allosteric effectors, co-factors and their cellular location (Table 2.1).

The proposed evolution of the CPS protein family is that CPS I/ CPS III in eukaryotes likely arose from a CPS II-like protein and CPS I arose from CPS III (Anderson, 1995a; Hong et al., 1994; van den Hoff et al., 1995). It has been proposed that the prokaryote CPS II formed from a fusion of a glutamine amidotransferase domain (GAT) and two synthetase domains (Anderson, 1995a; Hong et al., 1994; van den Hoff et al., 1995). In eukaryotes a mitochondrial signal and N-acetyl glutamate (NAG) binding site were added to CPS I and CPS III and later terrestrial vertebrates lost the glutamine binding site (Anderson, 1995a; Hong et al., 1994; van den Hoff et al., 1995). Phylogenetic studies of all CPS isoforms revealed that the CPS II isoform is basal to the CPS I/III clade (Lawson et al., 1996; Lindley et al., 2007; van den Hoff et al., 1995; Zhou et al., 2000). Hong et al. (1994) suggested that CPS III represents an evolutionary intermediate between CPS II and CPS I and an analysis of the evolution of urea synthesis in vertebrates by Mommsen and Walsh (1989) indicated that the origins of CPS I may have arisen within the lungfish group. Loong et al. (2005) suggested that CPS I was not derived from CPS III before the evolution of extant lungfishes. Other studies suggested that the CPS I and CPS III isoforms arose as a duplication event from the same common ancestor (Lawson et al., 1996; Lindley et al., 2007; van den Hoff et al., 1995; Zhou et al., 2000).

CPS I and III have been described in animals and are involved in the O-UC (Anderson, 1995a; Anderson, 1995b; Anderson, 2001; Hong et al., 1994). Both CPS I

and III are localized to the mitochondrial matrix and require NAG as a co-factor, but CPS I utilizes ammonia as a nitrogen donor while CPS III utilizes glutamine (Anderson, 1995a; Anderson, 1995b; Hong et al., 1994; van den Hoff et al., 1995). While enzymatic studies have described CPS I mainly in terrestrial vertebrates, CPS III has been described mainly in invertebrates and fishes, including the coelacanth (Anderson, 1995a; Anderson, 1995b; Anderson, 2001; Felskie et al., 1998; Hong et al., 1994; Kong et al., 1998; Mommsen and Walsh, 1989).

Although most enzymatic studies have described the CPS III isoform in fishes, two studies of air-breathing fishes, specifically the walking catfish, *Clarius batrachus* (Saha et al., 1999) and two species of lungfishes, *P. annectens* and *P. aethiopicus* (Mommsen and Walsh, 1989) reported the use of ammonia and NAG in the enzymatic reaction for CPS, indicating that these fishes use the CPS I isoform to catalyze the reaction. Recent studies on O-UC enzyme activities in lungfishes described CPS III activity in *P. dolloi*, *P. annectens*, and *P. aethiopicus* and those authors also showed that both glutamine and ammonia can act as the nitrogen substrate for CPS III, although the activity is much less when the nitrogen substrate utilized is ammonia (Chew et al., 2003; Loong et al., 2005).

The dipnoans or dual air-breathers represented by lungfishes are an ideal group for examining the evolution of nitrogen metabolism and specifically CPS evolution due to the unique aquatic and terrestrial environments which they inhabit. Lungfishes are seasonally exposed to dry periods where they no longer have access to water to excrete their excess nitrogenous wastes and they must find ways to reduce ammonia levels (Graham and Lee, 2004). Some lungfishes overcome this problem by decreasing overall

ammonia production during their exposure to land and increasing their *de novo* synthesis of urea via the O-UC (Chew et al., 2004; Ip et al., 2004a; Ip et al., 2005a; Loong et al., 2005). Increased urea production also has the added benefit of decreasing water loss (Griffith, 1991).

Lungfishes are members of the Sarcopterygii or lobe-finned fishes which are thought to be the first among the extant vertebrates to transition from water to land. These fishes are important because Sarcopterygians are the most likely ancestor for the origin of tetrapods, and lungfishes have been proposed to be the closest living species to tetrapods (Brinkmann et al., 2004a; Meyer and Zardoya, 2003; Takezaki et al., 2004). Lungfishes form a monophyletic group within Sarcopterygii (Schultze and Campbell, 1987) and without their link to the origin of tetrapods, the lungfish group would be a relatively obscure group of fishes (Forey, 1987). There are only six extant species of lungfishes, represented by three families and three genera, all of which reside in fresh water (Brinkmann et al., 2004a; Graham, 1997; Nelson, 2006).

Phylogenies created for extant lungfishes based on morphological characteristics, including skull structure, dentition, urogenital systems and external gills, place the Australian lungfish, *N. forsteri*, as the most ancestral species (Lundberg, 1993; Marshall, 1987; Miles, 1977; Tokita et al., 2005; Wake, 1987). The South American lungfish, *L. paradoxa* and the African lungfishes, the *Protopterus* species, are classified as the sister taxa and these two groups represent the most derived condition for the lungfishes (Lundberg, 1993; Marshall, 1987; Miles, 1977; Tokita et al., 2005; Wake, 1987). Molecular studies have also found similar familial relationships for living lungfishes, with Ceratodontidae being ancestral to Lepidosirenidae and Protopteridae, which are

sister groups (Brinkmann et al., 2004a; Brinkmann et al., 2004b; Hedges et al., 1993; Mallatt and Winchell, 2007; Meyer and Dolven, 1992; Tokita et al., 2005; Zardoya and Meyer, 1996).

With this background in mind, we performed phylogenetic analyses of a CPS fragment isolated from the liver of five of the six extant species of lungfishes in order to determine both the type of CPS isoform(s) present in lungfishes as well as the phylogenetic relationships of CPS with respect to lungfish. We also performed phylogenetic analyses on a larger fragment of CPS in *P. annectens* that spanned both the GAT domain and the synthetase domain.

Materials and methods

Tissue collection and preservation

P. dolloi, *P. annectens*, *P. aethiopicus* and *L. paradoxa* were imported from Africa to Singapore by Dr. Y.K. Ip at the National University of Singapore and were acclimatized in the laboratory for one month in plastic aquaria filled with water containing 2.3 mmol L^{-1} Na^{+}, 0.54 mmol L^{-1} K^{+}, 0.95 mmol L^{-1} Ca^{+2}, 0.08 mmol L^{-1} Mg^{+2}, 3.4 mmol L^{-1} Cl^{-} and 0.6 mmol L^{-1} HCO_3^{-}, at pH 7.0 and 25 °C (Loong et al., 2005). Water was changed daily, fish were fed frozen bloodworms during their acclimatization and a 12h: 12h light: dark cycle was maintained. *N. forsteri* was imported to Macquarie University in Sydney, Australia from Queensland, Australia by Dr. Jean Joss and kept in a 1700 L fiberglass tank in fresh water (0 ppt) on a natural light cycle. Fish were euthanized by a blow to the head and liver tissue was excised and

immediately placed in RNA*later*® (Ambion®) at a ratio of 4:1 RNA*later*®: tissue and then frozen and stored at –80.0 °C.

RNA extractions

Chaotropic RNA extractions were carried out from liver tissue (100 mg) isolated from the five species of lungfishes using the methods of Whitehead and Crawford (2005). RNA was purified using the RNeasy® mini protocol (Qiagen) and quantified and the quality checked by using the NanoDrop ND-1000 spectrophotometer. RNA was re-precipitated in 1/10 volumes 3 M sodium acetate and 2.5 volumes 100% ethanol and stored at -20°C.

Amplification of small fragments of CPS gene in five species of lungfish

CPS primers "2" and "3" (Table 2.2) designed by Korte et al. (1997) were used to amplify CPS product from hepatic tissue of the five species of lungfishes. A modified Qiagen OneStep RT-PCR protocol was utilized for one step RT-PCR reactions where both RT reaction and PCR reaction performed in the same tube. Each One-Step RT-PCR reaction was performed in 25 μL and utilized approximately 500 ng of total RNA, 1X Qiagen OneStep RT-PCR Buffer (includes 2.5 mM $MgCl_2$), 0.4 mM dNTPs, 1.0 μL Qiagen OneStep RT-PCR enzyme mix, 0.6 μM of each primer and RNase-free water. Touchdown RT-PCR was performed as follows: One cycle of 45°C for 30 minutes (RT reaction); one cycle of 95°C for 15 minutes to activate HotStarTaq (Qiagen); 2 cycles of 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 2 minutes, with a step down of 1°C during the annealing phase every two cycles until the annealing temperature dropped to

50°C; and 30 cycles of 94°C for 30 seconds, 50°C for 1 minute, and 72°C for 2 minutes. PCR products were visualized on a 1X TAE agarose gel using GelStar® nucleic acid stain (Cambrex) and stored at -20°C.

Amplification of large fragment of CPS gene in *P. annectens*

Primers specific for CPS I and CPS III isoforms in vertebrates were designed that successfully amplified previously identified CPS I or CPS III fragments in mouse (*Mus musculus*) or gulf toadfish (*Opsanus beta*), respectively (Table 2.2). One-step RT PCR was performed as above using all possible combinations of primers A-F given in Table 2.2. Rapid amplification of cDNA ends (RACE) was performed on RNA extracted from the liver tissue of *P. annectens* according to the protocol provided with the Marathon™ cDNA amplification kit (BD Biosciences Clonetech, PaloAlto, CA) using CPS gene specific primers (GSP) G and H (Table 2.2) designed from *P. annectens* the 550 bp CPS sequence amplified above (Table 2.2). PCR products were visualized on a 1X TAE agarose gel using GelStar® nucleic acid stain (Cambrex) and stored at -20°C.

Cloning and sequencing

To verify the identity of PCR products as CPS, appropriate sized products produced by RT-PCR were purified, cloned and sequenced. Zymoclean™ gel DNA recovery kit (Zymo Research) was used to purify DNA product from the agarose gel and the TOPO® TA cloning reaction protocol (Invitrogen) was followed to clone purified PCR product into chemically competent *E. coli* cells. The *E. coli* mixture (200 μL) was spread on LB-plates containing kanamycin and X-gal and incubated overnight at 37° C. Colonies with inserts were blue/white screened and any colonies with an insert were

inoculated into LB broth and incubated at 37° C overnight. To screen LB broth for insert, a PCR reaction was set up in 50 μL volume that contained 1μL of LB broth, 1X PCR buffer (10 mM Tris-HCl (pH 9.0), 50 mM KCl, and 0.1% Triton® X-100), 1.5 mM $MgCl_2$, 0.2mM dNTP, 0.3 mM each M13 forward and reverse primers, 2.5 units of Promega Taq polymerase and sterile water. Cycles were as follows: One cycle of 94°C for 10 minutes, 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. PCR products were visualized in a 1X TAE agarose gel using GelStar® nucleic acid stain (Cambrex) and only products containing the cloned insert were sequenced.

Cloned PCR product was purified using a magnetic bead system with Agencourt® AMPure® kit. Applied Biosystems BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used to sequence purified clones. Sequencing reactions were performed in 10 μL volumes with 0.5 μL BigDye® v3.1, 2.15 μL BigDye® v3.1 sequencing buffer (5X), 1.0 μL primer (3.2 μM), 1.0 μL AMPure® purified PCR product of clone, and 5.35 μL sterile water. Sequencing cycles were as follows: 40 cycles of 94°C for 15 seconds, 50°C for 20 seconds, and 60°C for 4 minutes. The sequencing product was cleaned with Agentcourt® CleanSEQ® (Agentcourt®) to remove the dye-terminators from the sequencing reaction. The cleaned sequencing product was eluted in 45 μL Tris (0.5 mM, pH 7.5) and 40 μL of this product was run on a 48 capillary ABI 3730 DNA Analyzer.

Sequence analyses and phylogenetics

A BLAST search was performed in Genbank to confirm the identity of the PCR product sequence as an isoform of CPS (Altschul et al., 1990). Other vertebrate CPS

sequences were retrieved from Genbank and aligned using amino acid sequence alignment with CLUSTALW (Version 1.81) (Thompson et al., 1994) and later adjusted by eye in the BioEdit Sequence Alignment Editor (Version 7.0.5.2) (Hall, 1999). A phylogenetic tree was generated for the deduced protein sequences for both the small and large partial fragments of CPS (See Table 2.3 for Accession numbers) using parsimony analysis in PAUP* Version 4.01b 10 (Swofford, 2002) using stepwise addition with the TBR branch swapping algorithm. Clade support was tested using the bootstrap command (1000 replicates). Putative ancestral amino acid sequences were identified for internal nodes and apomorphies identified within the protein tree using the describe tree options within the PAUP* 4.01b 10 program (Swofford, 2002). Phylogenetic trees were also generated for the large fragment nucleotide data using maximum parsimony and for the small fragment nucleotide data using maximum parsimony and maximum likelihood with the starting tree using stepwise addition with the TBR branch swapping algorithm in PAUP* 4.01b 10 (Swofford, 2002). Trees based on nucleotide alignments were run unweighted and weighted (2:4:1 by codon position) and all trees were bootstrapped (n=1000). *Caenorhabditis elegans* CPS II sequence was used as an outgroup for these analyses. Using the computer program Mr. Bayes 3.1 (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003), a phylogenetic tree was also generated for the small fragment of CPS using Bayesian inference with the General Time Reversible model with a proportion of invariable sites and a gamma-distributed rate variation across sites.

Results

Sequence of small fragments for CPS

One-step RT-PCR with primers numbers 2 and 3 from Korte et al. (1997) resulted in approximately 550 base pair (bp) band for *P. dolloi*, *P. annectens*, *P. aethiopicus*, *L. paradoxa* and *N. forsteri* that correspond to the sequence of the CPS III isoform of *S. acanthias* between positions 1888 and 2400 (Genbank accession number L31362). Nucleotide composition for each of the lungfishes CPS fragments were as follows (%A, %C, %G, %T): *P. dolloi* (27.24, 20.73, 23.58, 28.46), *P. annectens* (26.95, 21.19, 23.05, 28.81), *P. aethiopicus* (26.83, 20.73, 23.37, 29.07), *L. paradoxa* (27.04, 21.80, 23.69, 27.46), *Neoceratodus forsteri* (27.03, 20.73, 23.58, 28.66). CPS I isoforms had an average percent GC content of 47.28 ± 0.47 while CPS III isoforms had an average percent GC content of 52.95 ± 0.76. Both were significantly different from the average for lungfishes' sequences which had a percent GC content of 44.47 ± 0.22. Terrestrial vertebrates' sequences had an average percent GC content of 49.33 ± 0.93 while sequences from teleosts had an average percent GC content of 52.08 ± 1.13 and again both were significantly different from the lungfishes sequences. A comparison of the protein sequence of the CPS fragments within the lungfishes clade showed only three amino acid changes within the Protopterus family, seven changes between the Protopterus family and *L. paradoxa*, three changes between the Protopterus family and *N. forsteri*, and five changes between *L. paradoxa* and *N. forsteri*.

Sequence of large fragment of CPS in *P. annectens*

Four larger partial fragments of CPS were obtained from *P. annectens* using the following combinations of degenerate primers from Table 2.2 (approximate size given in brackets): A and B (1000 bp), E and F (1600 bp), A and F (1000 bp), and B and E (1600 bp). All other degenerate primer combinations failed to amplify CPS fragments in lungfishes, even those that amplified CPS fragments from mouse or toadfish. A 2800 bp partial CPS fragment was also amplified for the 5' RACE reaction using the GSP primer H. Alignment of these fragments revealed overlapping identical sequences all from the same CPS isoform. The combined larger fragment of CPS *in P. annectens* was 2028 bp in size and corresponded to the sequence of the CPS III isoform of *S. acanthias* between positions 868 and 2896 (Genbank accession number L31362). Nucleotide composition for the larger CPS fragment of *P. annectens* was 30.42 %A, 19.80 %C, 23.56 %G, 26.22 %T).

Phylogenetic analyses

Small fragments analyses

Maximum parsimony analysis of the 174 deduced amino acid sequences of the small CPS fragments resulted in 61 sites that were informative. Amino acid analysis revealed the three different CPS isoforms clades that had moderate bootstrap support for the CPS II clade and strong bootstrap support for a CPS I/III clade (Figure 2.1). The CPS II clade was basal to the CPS I/III clade and the CPS I isoforms were separated from CPS III isoforms but there was not strong bootstrap support. Lungfishes were located within the same clade as the CPS I isoforms but formed a separate branch which was highly

supported by bootstrap analysis (Figure 2.1). The putative ancestral amino acid sequences were identified for four internal nodes and showed that the ancestral state of the CPS II clade (node A) differed from the ancestral state for the CPS I/III clade (node B) by 35 amino acids. The ancestral protein sequence of the CPS I/III clade (node B) differed by five amino acids from the ancestral protein to CPS I clade (node C) and by three amino acids for the ancestral protein of the CPS III clade (node D).

Phylogenetic analyses of the nucleotide sequences of the CPS isoforms were performed on a total of 522 characters and 269 were parsimony-informative. Weighted parsimony for the DNA data resulted in trees similar to the protein tree, which also separated the CPS isoforms into three distinct groups. However, weighted parsimony of the nucleotide data had better species resolution within a clade in comparison to the protein data (Figure 2.2). Within the CPS II clade there was a separation of aquatic and terrestrial vertebrates with the exception of *S. acanthias*, which formed its own branch within the CPS II group. CPS I and CPS III formed a single clade off of CPS II which was further separated into two branches, one representing the CPS I isoforms and one representing the CPS III isoforms; this was also supported in the protein phylogeny. All CPS III isoforms were present in the same clade with the exception of *S. acanthias*, which arose on its own branch separate from CPS I or CPS III. All of the CPS I isoforms were present in the same clade. The lungfish species arose within the CPS I clade and *L. paradoxa* was basal to the *Protopterus* family. Unweighted parsimony analysis of DNA data (data not shown) did not cluster any of the lungfishes CPS sequences with the CPS III clade, but instead the lungfishes CPS isoforms formed their own branch within the CPS I vertebrate group.

Maximum-likelihood analysis of DNA data produced trees with similar topologies both weighted (Figure 2.2) and unweighted (data not shown) and separated the CPS isoforms. The CPS II clade was basal to the CPS I/CPS III clade (Figure 2.3). As seen in the parsimony analyses, there was a separation of aquatic and terrestrial organisms within the CPS II clade with the exception of *S. acanthias*, which again was located on its own branch within the CPS II group. All of the CPS I isoforms were located within one clade and the lungfishes clade again formed monophyletic group within this clade. *S. acanthias* CPS III isoform was basal to the CPS I group and was not part of the CPS III clade. The rest of the CPS III isoforms formed their own clade separate from the CPS I clade.

Bayesian analysis of the nucleotide data resulted in a tree with a topology that was similar to the weighted parsimony and weighted maximum likelihood (Figure 2.4) where the CPS II clade was basal to the CPS I/III clade. Bayesian analysis also separated aquatic and terrestrial vertebrates within the CPS II clade, but again the CPS II isoform of *S. acanthias* formed it's own branch within this clade. This analysis also separated the CPS III isoforms from the CPS I isoforms except for the type III isoform of *S. acanthias*, which like the parsimony analyses formed it's own branch within the CPS I/III clade. Again the lungfishes clustered on their own branch within the CPS I group.

All phylogenetic analyses of the small fragment supported that the CPS I/III clade arose from CPS II and the monophyletic placement of the lungfishes clade basal to the rest of the tetrapods within CPS I.

Large fragment analyses

Maximum parsimony analysis of the deduced amino acid sequence for the large fragment of CPS resulted in 361 sites that were parsimony-informative. As seen in the small fragment analyses amino acid analysis separated the CPS isoforms into three clades but with 100 percent bootstrap support for the CPS II and CPS I/III clade. Again the CPS II clade was basal to the CPS I/III clade and the CPS I isoforms were separated from CPS III isoforms except for *S. acanthias*. *P. annectens* was located within the CPS I clade basal to the terrestrial vertebrates (Figure 2.5).

Maximum parsimony analyses of the nucleotide sequence for the large fragment of CPS was performed on a total of 2028 characters and 1288 were parsimony-informative. Weighted parsimony for the DNA data resulted in trees similar to the protein tree, which also separated the CPS isoforms into three distinct groups (data not shown) and produced a phylogenetic tree similar to Figure 2.5 but with lower bootstrap values.

Discussion

The phylogenetic analyses in our study showed that the weighted nucleotide trees, the Bayesian tree, and the protein trees formed a CPS I/III clade that was derived from the CPS II clade. These findings agree with the proposed CPS evolution of CPS I/III from CPS II (Anderson, 1995a; Hong et al., 1994; Lindley et al., 2007; van den Hoff et al., 1995). Nucleotide trees separated the CPS isoforms into their own clades with the exception of the CPS III isoform of *S. acanthius*, which formed its own branch separate from CPS I or CPS III in the parsimony tree and in the Bayesian tree, and formed its own

branch within the CPS I clade in the maximum likelihood tree. These results agree with the phylogenetic analyses of Lindley et al. 2007 where *S. acanthius* CPS III did not cluster with other CPS III isoforms and it was speculated to result from the different physiological roles of urea production in elasmobranchs compared to teleosts.

In the analyses of the small CPS fragment, a single lungfish CPS group basal to the higher vertebrates within CPS I clade was supported with a bootstrap value of 100 for all nucleotide based trees in this study and a probability of 100 percent for the Bayesian tree. Lungfishes are thought to form a monophyletic group separate from other tetrapods (Schultze and Campbell, 1987) as are amphibians (Hedges et al., 1990) and the phylogenies in the current study support that theory. Furthermore the placement of the lungfish CPS isoforms with the other terrestrial vertebrates and not with the aquatic vertebrate species leads us to speculate that, despite the seasonal aquatic condition of this transitional group of fishes, their pattern of urea formation via the O-UC is more related to their terrestrial existence. This pattern was also supported by the phylogenetic analyses of the larger fragment of CPS in *P. annectens*.

Despite the polyploidy condition known for the African lungfish (*P. dolloi*) (Le Comber and Smith, 2004), only one CPS isoform was isolated for all of the lungfishes that clustered with the CPS I and degenerate primers failed to amplify any other CPS product. We expected, based on previous enzymatic studies, to potentially amplify both a CPS I and a CPS III isoform in *P. dolloi*, *P. annectens*, and *P. aethiopicus* (Chew et al., 2003; Loong et al., 2005; Mommsen and Walsh, 1989) but found only a CPS I-like isoform for the fragment amplified from liver tissue. When examining the genomes of *Takifugu rubripes* and *Tetradon nigroviridis*, only two isoforms of CPS were present,

CPS II (involved in pyrimidine biosynthesis) and a CPS I-like mitochondrial form (involved in O-UC), suggesting that there is only one mitochondrial CPS enzyme in fishes. Perhaps the CPS sequences in lungfishes represent transitional proteins able to utilize both ammonia and glutamine as a substrate. This is supported by the results in chapter three of this thesis where both CPS I and CPS III activity was detected in liver tissue of *P. annectens*. Fishes have that have CPS III have been shown to utilize both ammonia and glutamine as a nitrogenous substrate (Casey and Anderson, 1983; Lindley et al., 1999; Saha et al., 1997) but CPS I only utilizes ammonia (Anderson, 2001; Lindley et al., 2007). By identifying the structural components of the glutamine binding site and altering one amino acid in CPS I of *Rana catesbeiana*, Saeed-Kothe and Powers-Lee (2003) demonstrated that it was possible to change the substrate use in CPS I from ammonia to glutamine. It may be possible that the structural components of the glutamine-binding sites are maintained in lungfish thereby allowing it to utilize both glutamine and ammonia as a substrate.

Using the trees based on the small fragment nucleotide data we found that within the lungfishes clade the two Protopterus species, *P. aethiopicus* and *P. annectens* clustered on the same branch and *P. dolloi* formed its own branch basal to but outside of the branch leading to the other two Protopterus species and agrees with the phylogeny by Tokita et al. (2005) that showed the same branching pattern. However, the most primitive lungfish *N. forsteri* also formed a branch just outside the *P. aethiopicus*/ *P. annectens* group and the *N. forsteri* / Protopterus cluster is supported by a bootstrap value of 100 in the DNA trees generated from this study. *L. paradoxa* forms its own branch basal to the *N. forsteri* / Protopterus clade and this branching order does not conform to

the proposed evolution of lungfishes (Tokita et al., 2005), therefore suggesting that this tree represents the gene evolution and not the species evolution. However, a recent deuterostome phylogeny based on 28S and 18S rRNA sequences produced a tree with the same structure as the trees in this study but when only vertebrate sequences were considered, the tree looked more similar to the traditional trees with *N. forsteri* ancestral to *L. paradoxa*/ *Protopterus* clade (Mallatt and Winchell, 2007).

Another finding in our data was the difference in percent GC content within the nucleotide sequences between the aquatic vertebrates, terrestrial vertebrates and lungfishes. Previous studies on percent GC content have shown that poikilothermic vertebrates have a lower GC content in their genome than the homioithermic vertebrates (Bernardi and Bernardi, 1990), but this type of GC-bias was not apparent for the CPS isoform fragments in this study. This study showed that the lungfishes and terrestrial vertebrates had lower GC content than the other aquatic vertebrates for the small CPS fragment analyzed and this study may indicate that the type of air-breathing influences the isoform of CPS utilized. Due to the GC bias, a phylogenetic analysis and ancestral reconstruction was performed using the deduced CPS protein sequences. The protein trees had the same general structure as the nucleotide data indicating that the GC-bias did not affect the separation of the CPS isoforms. Analysis of the nucleotide changes resulting in amino acid changes from the ancestral node of CPS I/III to the CPS I and CPS III ancestral nodes did not show that the GC-bias affected these amino acid changes. We suggest analyzing the sequences of more transitional air-breathing fishes in order to determine if this type of GC-bias in CPS isoforms is consistent in air-breathing vertebrates.

To date, there has been only one phylogenetic study which attempted to determine the structure of the relationships amongst of all of the lungfish species (Tokita et al., 2005) and this is likely due to the limited access to this obscure group of fishes. As is the case with previous lungfish phylogenetic studies, our study was also missing *P. amphibicus*. In order to better understand the phylogeny of the lungfishes within the lungfish clade, we suggest that future studies should attempt to include all six species of lungfishes.

CPS Isoform	Nitrogen Substrate	Allosteric Effector	Cellular Location	Biochemical Pathway
CPS I	Ammonia	N-acetylglutamate	Mitochondrial Matrix	O-UC/Arginine
CPS II	Glutamine	UTP (-), PRPP (+)	Cytosolic	Pyrimidine Biosynthesis
CPS III	Glutamine	N-acetylglutamate	Mitochondrial Matrix	O-UC/Arginine

Table 2.1. Differences in the CPS isoforms found in higher vertebrates (Modified from P. M. Anderson 1995a).

Primer Name	Nucleotide position in *Squalus acanthias*	CPS Type	Primer Sequence – 5' to 3'
A	1480df[a]	I	CCA TCA CCC CTC AGT TTG TNA
B	2499dr[a]	I	GCC ATA ACC TCT CCH ACA CTY T
C	3289dr[a]	I	TGC CCC CCA ACT GAT AYR ATG
D	477 f[a]	III	GTA TAG GCA CTG GAA CTC TGT
E	886df[a]	III	AGC CTG TGT TTG GTA TCW GTA HGG
F	2743dr[a]	III	GCT TGT ATA GGA ACC AYT TGT C
G	2086f-GSP1	III	TTC CAT AAC CTG AGA GAG ACT GCC
H	2349r-GSP2	III	CTA CCT ACT TGA CCA GTT GTG CCT
I	*Korte 2 dr	III	CAT IAC YTC ICC IAC ISW YTT CAT
J	*Korte 3 df	III	TGG AAI GAR RTI GAR TAY GA

Table 2.2. Vertebrate CPS primers designed to amplify CPS I or CPS III, developed from an alignment of all available CPS sequences in Genbank. Primer names are based on positional information from CPS III in *S. acanthias*, d = degenerate, f = foreward, r = reverse, GSP = gene specific primer used in RACE. [a]These primers were tested against toadfish and mouse to ensure that a CPS type specific product was amplified (Laberge unpublished). * Primers designed by Korte et al. 1997 used in this study.

Species name	CPS Isoform	Sequence Accession Number
Bos taurus	CPS I	XM_587645
Canis familiaris	CPS I	XM_857137
Gallus gallus	CPS I	NM_001045841
Homo sapiens	CPS I	NM_001875
Mus musculus	CPS I	NM_001080809
Rana catesbeiana	CPS I	U05193
Rattus norvegicus	CPS I	NM_017072
Xenopus laevis	CPS I	NM_001095640
Caenorhabditis elegans	CPS II	NM_063437
Danio rerio	CPS II	NM_001009884
Homo sapiens	CPS II	NM_004341
Mus musculus	CPS II	NM_023525
Pan troglodytes	CPS II	XM_001154901
Rattus norvegicus	CPS II	NM_001105710
Squalus acanthias	CPS II	U18868
Takifugu rubripes	CPS II	ENSTRUG00000012246
Tetradon nigroviridus	CPS II	GSTENG00009790001
Alcacius grahami	CPS III	AF119250
Danio rerio	CPS III	XM_678190
Micropterus salmoides	CPS III	AF006491
Oncorynchus mykiss	CPS III	NM_001124709
Opsanus beta	CPS III	AF169248
Squalus acanthias	CPS III	L31362
Takifugu rubripes	CPS III	ENSTRUG00000007058
Tetradon nigroviridus	CPS III	GSTENG00004415001

Table 2.3. Species and accession numbers for CPS sequences used to create phylogenetic trees in this study.

Figure 2.1. Maximum parsimony tree constructed from deduced 174 amino acid sequence of CPS fragments using simple addition with TBR branch swapping. The tree was bootstrapped 1000 times and bootstrap support greater than 50 percent is shown on the branches of the tree. Internal nodes used for ancestral reconstruction analysis are labeled: A, B, C and D. Organisms used to construct this tree include those listed in Table 2.3 with their accession numbers and the five new CPS fragment sequences from *L. paradoxa*, *P. aethiopicus*, *P. annectens*, *P. dolloi*, *N. forsteri*. *C. elegans* CPS II isoform was used as an outgroup.

Figure 2.1.

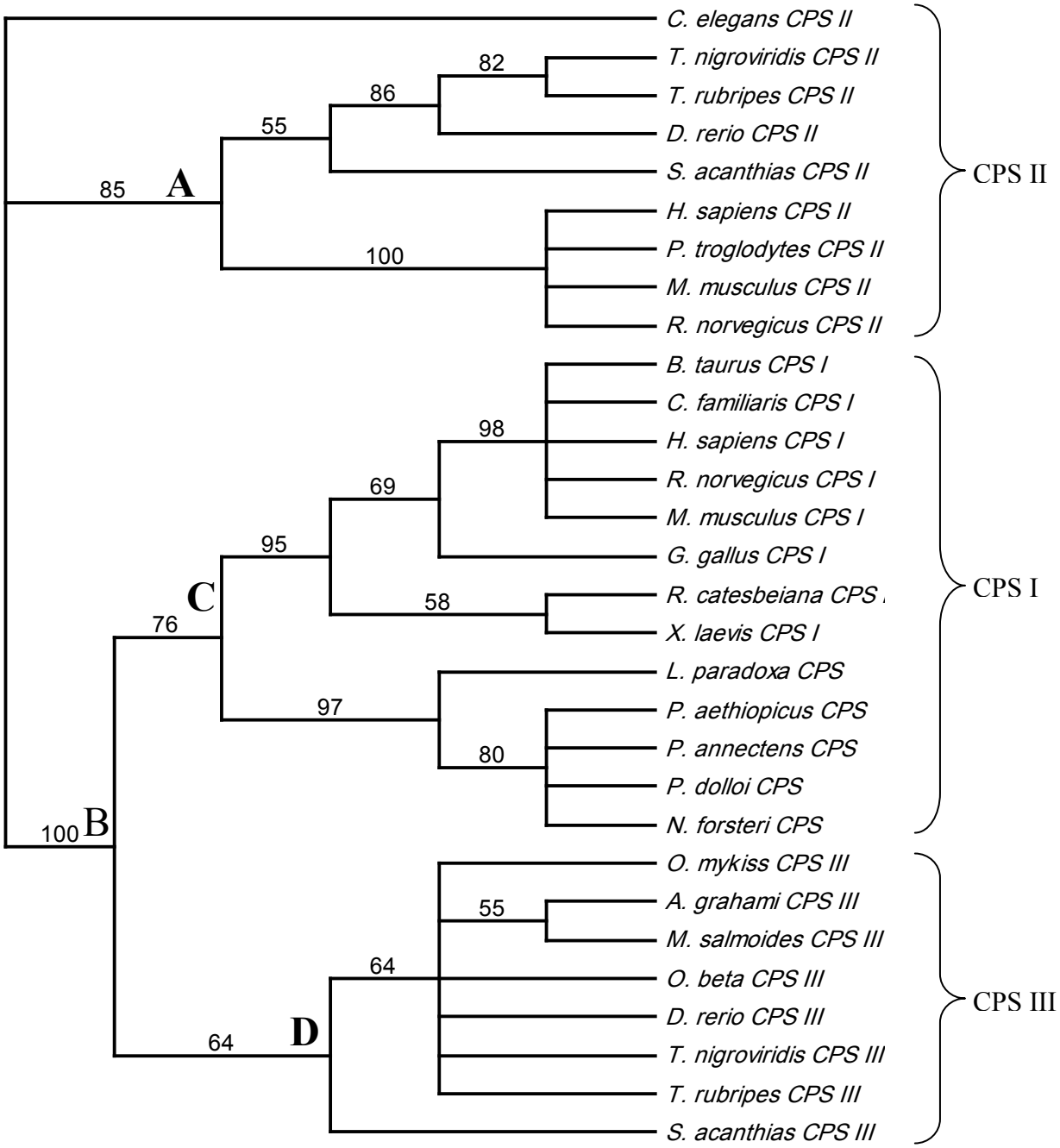

C. elegans CPS II
T. nigroviridis CPS II
T. rubripes CPS II
D. rerio CPS II
S. acanthias CPS II
H. sapiens CPS II
P. troglodytes CPS II
M. musculus CPS II
R. norvegicus CPS II
B. taurus CPS I
C. familiaris CPS I
H. sapiens CPS I
R. norvegicus CPS I
M. musculus CPS I
G. gallus CPS I
R. catesbeiana CPS
X. laevis CPS I
L. paradoxa CPS
P. aethiopicus CPS
P. annectens CPS
P. dolloi CPS
N. forsteri CPS
O. mykiss CPS III
A. grahami CPS III
M. salmoides CPS III
O. beta CPS III
D. rerio CPS III
T. nigroviridis CPS III
T. rubripes CPS III
S. acanthias CPS III
CPS II
CPS I
CPS III
A
B
C
D
82
86
55
85
100
98
69
95
58
76
97
80
100
55
64
64

Figure 2.2. Maximum parsimony tree constructed from 522 bp DNA fragments of CPS using stepwise addition with the TBR branch swapping. Codon positions were weighted 2:4:1. The trees was bootstrapped 1000 times and bootstrap support greater than 50 percent is shown on the branches of the tree. Organisms used to construct this tree include those listed in Table 2.3 with their accession numbers and the five new CPS fragment sequences from *L. paradoxa*, *P. aethiopicus*, *P. annectens*, *P. dolloi*, *N. forsteri*. *C. elegans* CPS II isoform was used as an outgroup.

Figure 2.2.

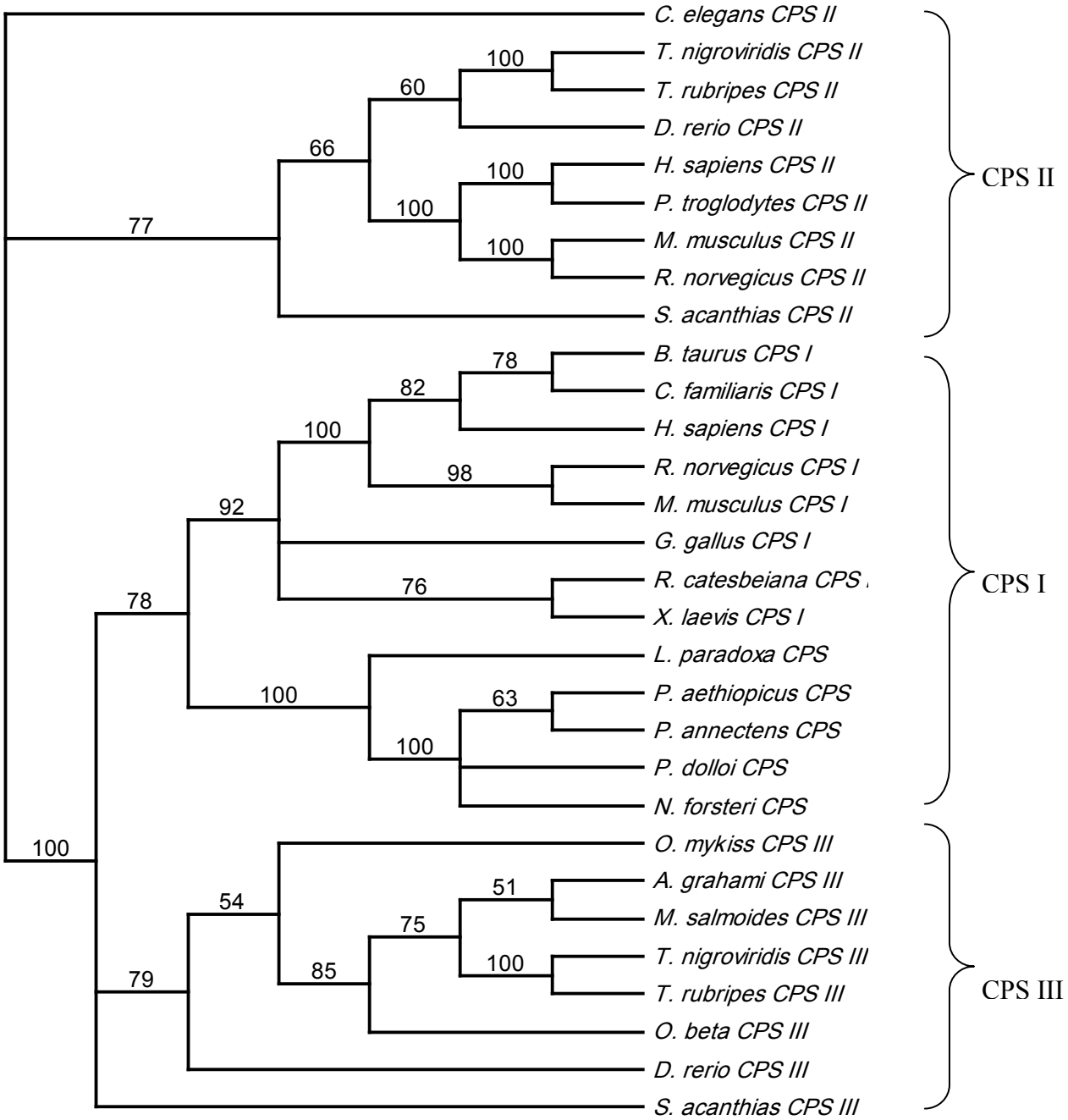

C. elegans CPS II
T. nigroviridis CPS II
T. rubripes CPS II
D. rerio CPS II
H. sapiens CPS II
P. troglodytes CPS II
M. musculus CPS II
R. norvegicus CPS II
S. acanthias CPS II
B. taurus CPS I
C. familiaris CPS I
H. sapiens CPS I
R. norvegicus CPS I
M. musculus CPS I
G. gallus CPS I
R. catesbeiana CPS
X. laevis CPS I
L. paradoxa CPS
P. aethiopicus CPS
P. annectens CPS
P. dolloi CPS
N. forsteri CPS
O. mykiss CPS III
A. grahami CPS III
M. salmoides CPS III
T. nigroviridis CPS III
T. rubripes CPS III
O. beta CPS III
D. rerio CPS III
S. acanthias CPS III
CPS II
CPS I
CPS III
100
60
66
100
100
77
100
78
82
100
98
92
78
76
100
63
100
100
54
75
51
100
85
79

Figure 2.3. Maximum likelihood tree constructed from 522 bp DNA fragments of CPS using stepwise addition with the TBR branch swapping. Codon positions were weighted 2:4:1. The tree was bootstrapped 1000 times and bootstrap support greater than 50 percent is shown on the branches of the tree. Organisms used to construct this tree include those listed in Table 2.3 with their accession numbers and the five new CPS fragment sequences from *L. paradoxa*, *P. aethiopicus*, *P. annectens*, *P. dolloi*, *N. forsteri*. *C. elegans* CPS II isoform was used as an outgroup.

Figure 2.3.

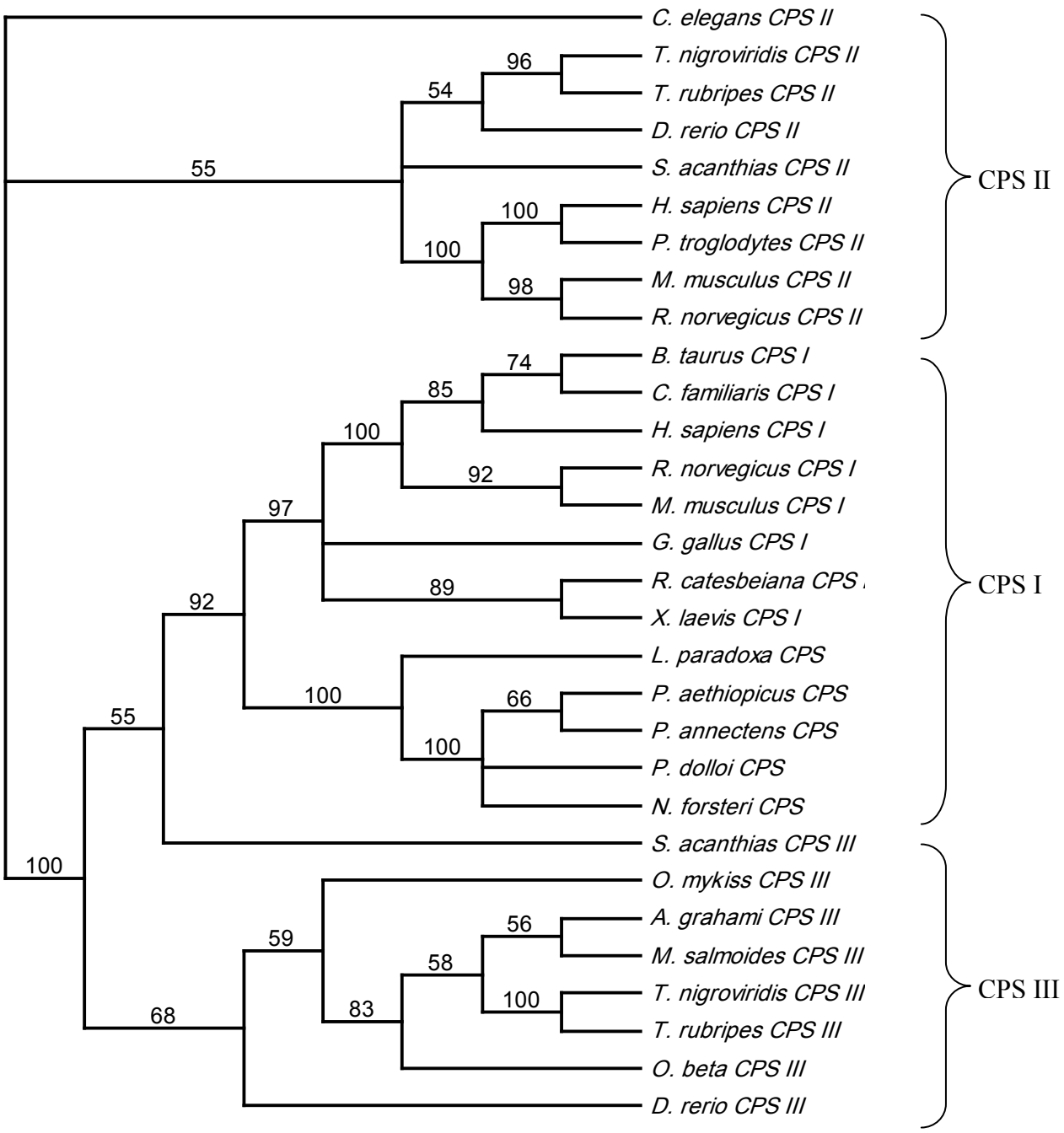

C. elegans CPS II
T. nigroviridis CPS II
T. rubripes CPS II
D. rerio CPS II
S. acanthias CPS II
H. sapiens CPS II
P. troglodytes CPS II
M. musculus CPS II
R. norvegicus CPS II
B. taurus CPS I
C. familiaris CPS I
H. sapiens CPS I
R. norvegicus CPS I
M. musculus CPS I
G. gallus CPS I
R. catesbeiana CPS
X. laevis CPS I
L. paradoxa CPS
P. aethiopicus CPS
P. annectens CPS
P. dolloi CPS
N. forsteri CPS
S. acanthias CPS III
O. mykiss CPS III
A. grahami CPS III
M. salmoides CPS III
T. nigroviridis CPS III
T. rubripes CPS III
O. beta CPS III
D. rerio CPS III
96
54
55
100
100
98
74
85
100
92
97
89
92
100
66
55
100
59
56
58
100
83
68
100
CPS II
CPS I
CPS III

Figure 2.4. Bayesian inference tree constructed from 522 bp DNA fragments of CPS using a general time reversible model with gamma-distributed rate variation across sites and proportion of invariable sites. Values on the tree indicate posterior probabilities given as a percentage. Organisms used to construct this tree include those listed in Table 2.3 with their accession numbers and the five new CPS fragment sequences from *L. paradoxa*, *P. aethiopicus*, *P. annectens*, *P. dolloi*, *N. forsteri*. No outgroup was used in this analysis.

Figure 2.4.

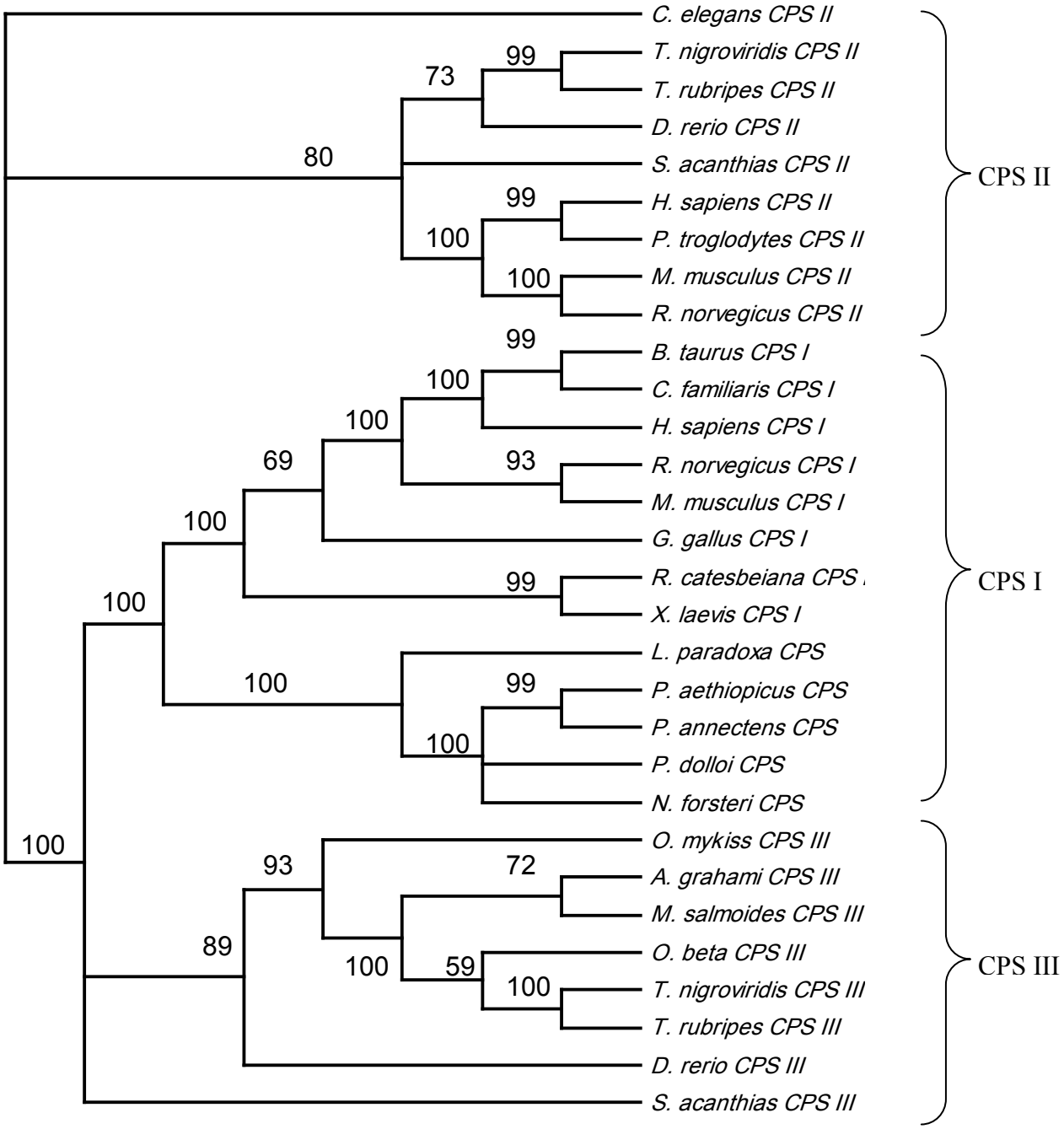
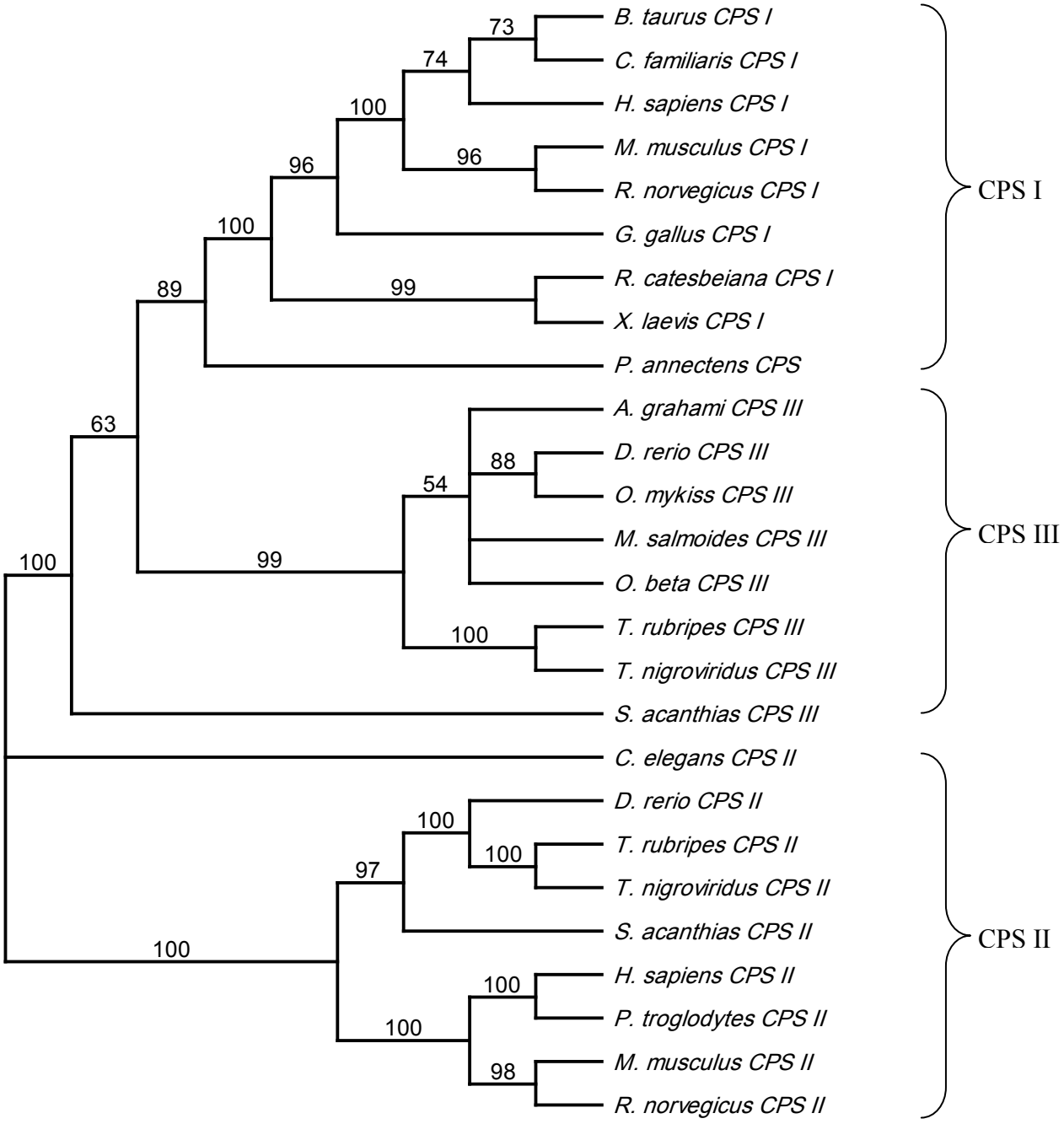

C. elegans CPS II
T. nigroviridis CPS II
T. rubripes CPS II
D. rerio CPS II
S. acanthias CPS II
H. sapiens CPS II
P. troglodytes CPS II
M. musculus CPS II
R. norvegicus CPS II
B. taurus CPS I
C. familiaris CPS I
H. sapiens CPS I
R. norvegicus CPS I
M. musculus CPS I
G. gallus CPS I
R. catesbeiana CPS
X. laevis CPS I
L. paradoxa CPS
P. aethiopicus CPS
P. annectens CPS
P. dolloi CPS
N. forsteri CPS
O. mykiss CPS III
A. grahami CPS III
M. salmoides CPS III
O. beta CPS III
T. nigroviridis CPS III
T. rubripes CPS III
D. rerio CPS III
S. acanthias CPS III
99
73
80
99
100
100
99
100
100
69
93
100
99
100
100
99
100
100
93
72
89
100
59
100
CPS II
CPS I
CPS III

Figure 2.5. Maximum parsimony tree constructed from deduced 676 amino acid sequence of CPS fragments using simple addition with TBR branch swapping. The tree was bootstrapped 1000 times and bootstrap support greater than 50 percent is shown on the branches of the tree. Organisms used to construct this tree include those listed in Table 2.3 with their accession numbers and the larger CPS fragment sequence from *P. annectens* determined in this study. *C. elegans* CPS II isoform was used as an outgroup.

Figure 2.5


B. taurus CPS I
C. familiaris CPS I
H. sapiens CPS I
M. musculus CPS I
R. norvegicus CPS I
G. gallus CPS I
R. catesbeiana CPS I
X. laevis CPS I
P. annectens CPS
A. grahami CPS III
D. rerio CPS III
O. mykiss CPS III
M. salmoides CPS III
O. beta CPS III
T. rubripes CPS III
T. nigroviridus CPS III
S. acanthias CPS III
C. elegans CPS II
D. rerio CPS II
T. rubripes CPS II
T. nigroviridus CPS II
S. acanthias CPS II
H. sapiens CPS II
P. troglodytes CPS II
M. musculus CPS II
R. norvegicus CPS II
73
74
100
96
96
100
89
99
63
88
54
100
99
100
97
100
100
100
98
CPS I
CPS III
CPS II

Chapter 3. Gene Expression and Enzyme Activities of Ornithine-Urea Cycle Enzymes in the West African Lungfish (*Protopterus annectens*) After Six Days of Aerial Exposure.

The West African lungfish, *Protopterus annectens*, is ammoniotelic under aquatic conditions but becomes ureotelic when the water dries up around it. *P. annectens* avoids ammonia toxicity during aerial exposure by decreasing ammonia production and increasing urea production. The goal of this study was to perform a comprehensive analysis of ammonia and urea production, ornithine-urea cycle (O-UC) enzyme activity and mRNA expression in hepatic tissue of *P. annectens* subjected to aerial exposure for six days followed by re-introduction into freshwater for 24 hours. We also sought to determine if there was any relationship between nitrogen metabolite concentrations and enzyme activity or mRNA expression of the O-UC enzymes. Further, we sought to determine if there was a relationship between O-UC mRNA expression and enzyme activity. Hepatic tissue was assayed for ammonia and urea concentrations. Enzyme activity in the liver was measured for: glutamine synthetase (GS), carbamoyl phosphate synthetase I and III (CPS I and CPS III), ornithine carbamoyl transferase (OCT), and arginase (ARG). Quantitative PCR was utilized to measure messenger RNA expression in the liver for GS, CPS I, OCT, ARG, argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) mRNA expression. Hepatic urea concentrations increased with aerial exposure while ammonia concentrations were unaffected. There was a coordinated induction of both mRNA expression and enzyme activity for the O-UC genes which correlated with urea concentrations with the exception of ARG activity and OCT mRNA expression.

Background

Lungfishes or lobe-fin fishes (Sarcopterygii) are the transitional taxa from wholly aquatic to terrestrial vertebrates (Brinkmann et al., 2004a; Forey, 1987; Meyer and Zardoya, 2003; Takezaki et al., 2004). There are only six living species of lungfishes comprising three families and three genera and all are freshwater species (Graham, 1997; Nelson, 2006). Because of their importance for tetrapod evolution, lungfish are an interesting group of vertebrates to study with respect to early transition onto land due to their ability to survive extended periods in air as the water dries up around them. Some requirements necessary for the water-land transition are: ability to respire in air; ability to maintain a water balance; and ability to deal with nitrogenous wastes (Graham, 1997; Graham and Lee, 2004; Sayer, 2005). For nitrogenous waste, terrestrial existence requires new biochemical and physiological processes.

Ammonia and urea are two of the primary nitrogenous wastes excreted by fishes (Griffith, 1991; Walsh, 1998; Wood, 1993; Wright, 1995). Since build up of ammonia is toxic, lungfishes (and indeed most aquatic species) excrete ammonia passively by diffusion over branchial and cutaneous surfaces (Anderson, 2001; Graham, 1997; Korsgaard et al., 1995; Wood et al., 2005), a process known as ammonioteley. In terrestrial vertebrates passive loss of ammonia is not possible therefore in these animals urea is the typical nitrogenous waste and it is produced via the ornithine-urea cycle (O-UC) requiring the equivalent of four moles of ATP per mole of urea produced (Jackson et al., 1986; Meijer, 1995; Mommsen and Walsh, 1991). In ureotelic fish (fish that produce urea as the primary nitrogenous waste) ammonia is converted to urea because it is less toxic (Ip et al., 2001; Walsh, 1998) at a metabolic cost of five moles of ATP per mole of

urea produced (Mommsen and Walsh, 1991; Mommsen and Walsh, 1992; Walsh, 1998). Early studies on lungfishes have shown that the aquatic Australian lungfish, *Neoceratodus forsteri*, is primarily ammoniotelic with a low rate of urea excretion (Goldstein et al., 1967). However, the African lungfishes, *P. aethiopicus*, *P.annectens* and *P. dolloi* and South American lungfish, *L. paradoxa*, are ammoniotelic under aquatic conditions and switch to ureotely during aestivation (Carlisky and Barrio, 1972; Chew et al., 2004; Ip et al., 2005b; Janssens and Cohen, 1968; Loong et al., 2008). Emersed and aestivating lungfishes avoid ammonia toxicity by decreasing ammonia production and increasing urea synthesis (Chew et al., 2004; Chew et al., 2003; Ip et al., 2005b; Loong et al., 2005; Loong et al., 2008).

In vertebrates there are five enzymes involved in the O-UC pathway: carbamoyl phosphate synthetase (CPS) (EC 6.3.4.16), 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 arginase (ARG) (3.5.3.1) (Anderson, 1995b; Mommsen and Walsh, 1989). During the evolution of urea synthesis from fishes to tetrapods there is a change in the type of CPS isoform utilized by the O-UC from CPS III to CPS I. The primary difference between these two isoforms is the type of nitrogen substrate used during the formation of carbamoyl phosphate. In fishes, glutamine acts as the nitrogen substrate for CPS III whereas in tetrapods ammonia is the substrate for the CPS I reaction (Mommsen and Walsh, 1989). An important enzyme necessary for glutamine production in fishes with CPS III is glutamine synthetase (GS) (EC 6.3.1.2), thus it is an accessory enzyme for the O-UC (Anderson, 1995b; Anderson, 2001).

In response to extended periods of aerial exposure *P. annectens* begins to encase itself in a mucous cocoon which eventually covers the whole surface of the animal's body and impedes ammonia excretion (Loong et al., 2008). During the early phase of aestivation when the fish are exposed to emersion conditions, Loong et al. (2005) showed that *P. annectens* increases hepatic urea concentrations and GS and OTC activities, suggesting the production of urea via the O-UC. The biochemical response of lungfishes is affected by different durations of aerial exposure or different types of environmental exposures (air *versus* mud); there is a general rise in tissue urea concentrations but the activity changes for the O-UC enzymes are dissimilar (Chew et al., 2004; Chew et al., 2003; Ip et al., 2005b; Loong et al., 2005; Loong et al., 2008; Wood et al., 2005). Loong et al. (2005) also found that *P. annectens* had CPS III activity which is in contrast to the study by Mommsen and Walsh (1989) that found the presence of CPS I activity in the liver of *P. annectens*.

To synthesize urea requires the induction of the enzymes of the O-UC. This induction could involve increased mRNA synthesis, more active translation, increased enzyme activity or modulations of enzyme activity by covalent or allosteric regulators (Dever, 2002; Gebauer and Hentze, 2004; Levine and Tjian, 2003; Lewin, 2004; Mata et al., 2005; Weaver, 2008). In gulf toadfish (*Opsanus beta*), with the transition to ureotely, there is a coordinated induction of O-UC enzyme activity but not a coordinate change in mRNA expression suggesting that O-UC activity was a better predictor for hepatic urea concentrations than mRNA expression (Laberge et al., Accepted). In *O. beta* only two of the O-UC enzymes, CPS and OTC, had hepatic enzyme activities that were correlated to mRNA expression (Laberge et al., Accepted). In other fishes, some studies have shown

that despite increases in mRNA expression no corresponding rise in enzyme activity occurred (Iwata et al., 2000; Kong et al., 2000).

The objective of this study was to perform a comprehensive analysis of both mRNA expression and enzyme activity of O-UC genes in hepatic tissue of *P. annectens*. We sought to examine the relationships between hepatic ammonia concentrations, urea concentrations, O-UC mRNA expression and enzyme activity and how six days of aerial exposure or re-immersion treatments affected these relationships. We also sought to determine if there was any relationship between O-UC mRNA expression and enzyme activity in *P. annectens* liver. We therefore measured concentrations of ammonia and urea from liver tissue of *P. annectens* and we measured O-UC mRNA expression and enzyme activity in lungfishes that were immersed in water, subjected to emersion conditions for six days, or subjected to re-immersion after emersion treatment. We found that there was a coordinated induction of the O-UC enzymes for both mRNA expression and enzyme activity in emersed lungfish. Except for ARG activity and OCT mRNA expression, all of the O-UC enzymes were correlated to urea concentrations for mRNA expression and enzyme activity.

Materials and methods

Experimental animals

Specimens of West African lungfish, *P. annectens* (Owen, 1839), weighing 57 – 150 g, were imported from central Africa through a local fish farm in Singapore. Identification of the specimens was performed according to Poll (1961). Lungfish were kept in laboratory wet lab facilities in plastic aquaria (L20.5 cm x W14.5 cm x H6.0 cm) filled

with dechlorinated water containing 2.3·mmol·L^{-1} Na^{+}, 0.54·mmol·L^{-1} K^{+}, 0.95·mmol·L^{-1} Ca^{+2}, 0.08·mmol·L^{-1} Mg^{+2}, 3.4·mmol·L^{-1} Cl^{-} and 0.6·mmol·L^{-1} HCO_3^{-}, at pH 7.0 and at 25.0°C. Water was changed daily. No attempt was made to separate the sexes. Lungfish were acclimated to laboratory conditions for at least one month and fed frozen blood worms during this time. Food was withdrawn 48·hours prior to the experiment and throughout the remainder of the experiment. All treatments performed in this study were under a 12·h: 12·h light: dark regime.

Experimental conditions

Control fish were immersed individually in 1 L of water at 25.0°C in separate plastic tanks (L20.5 cm x W14.5 cm x H6.0 cm) and water was changed daily for 6 days. Individual tanks were not aerated and were covered with plastic lids that allowed the fish to have access to air but prevented the animals from leaving their tanks. For emersion conditions, lungfish were placed in 80 mL of water at 25.0°C in separate plastic tanks (as above) and water was changed daily. This process was repeated over 6 days and the disturbance created by the water change prevented the experimental animal from initiating aestivation during this period. Fish belonging to the re-immersion treatment underwent the protocol for emersion conditions followed by a re-immersion in 1 L of water for 24 hours. At the end of each treatment fish were killed by a strong blow to the head and the liver was quickly excised. A sub-sample of liver tissue was immediately freeze-clamped with liquid nitrogen pre-cooled tongs. Frozen samples were kept at -80°C for use in metabolite and enzyme assays. Liver tissue was also sub-sampled for use in RNA extractions by mincing fresh liver tissue slightly and storing it at 4.0°C in

RNA*later*® solution (Ambion) at a ratio of 5:1 RNA*later*®: tissue. After 24 hours the RNA*later*® infused tissue was transferred to – 80.0°C.

Metabolite and Ornithine-Urea cycle enzyme activity assays

A sub-sample of frozen liver tissue (~200 mg) was deproteinized for ammonia and urea analyses using the methods of Kun and Kearney (1971) as modified by Wang and Walsh (2000). Ammonia level in liver tissue was measured using a Raichem Kit (San Diego, CA) which ascertains the amount of ammonia in a sample by determining the nicotinamide adenine dinucleotide phosphate (NADPH) oxidized in the presence glutamate dehydrogenase (GLDH); the level of ammonia present in the sample is equivalent to the amount of NADPH oxidized. Liver urea concentrations were determined using the diacetyl-monoxime method outlined in Rahmatullah and Boyde (1980).

For the analyses of GS, CPS, OCT and ARG enzyme activities, approximately 100 mg of liver was homogenized on ice in a 2.0 mL microcentrifuge tube using an IKA Ultra Turrax® T8 Homogenizer in 4x volume of homogenization buffer (20 mmol/L 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 25.0ºC). Homogenates were centrifuged for five minutes at 4ºC in an Eppendorf® 5415D centrifuge at 16000 g and the supernatant was used directly or diluted as needed according to previously described methods for determining levels of O-UC enzyme activities (Barber and Walsh, 1993; Kajimura et al., 2006; Walsh et al., 1994b).

GS enzyme activity was measured using a modified γ-glutamyl transferase reaction described by Walsh 1996, specifically the GS reaction mixture contained 60 mM glutamine, 15 mM hydroxylamine-HC1, 20 mM KH_2AsO_4, 0.4 mM ADP, 3 mM $MnCl_2$ and 50 mM HEPES, pH 6.7. Ten µL homogenate was added to 200 µL GS reaction mixture. After 20 minutes the reaction was terminated with the addition of 60 µL Ferric chloride reagent (50 % HC1 : 24 % trichloroacetic acid : 10 % $FeC1_3$). The mixture was centrifuged at 13, 000 g to remove the precipitated proteins and the absorbance of the supernatant was measured at 540 nm with a Molecular Devices Thermomax microplate reader. The amount of γ-glutamyl monohydroxamate formed was determined by comparison to a standard curve of glutamyl monohydroxamate prepared in the same reagents.

CPS enzyme activity for both CPS I and CPS III was assayed based on the colorimetric production of citrulline similar to the methods used by Mommsen and Walsh, 1989 and Kajimura et al., 2006. Specifically the CPS reaction mixture contained 20 mM ATP, 25 mM $MgCl_2$, 5 mM $NaHCO_3$, 5 mM N-acetyl glutamate, 2 mM Dithiothreitol, 5 mM Ornithine, 25 mM Phospho(enol)pyruvate, 1.7 mM UTP, 6 units mL^{-1} pyruvate kinase, 1 unit mL^{-1} ornithine-citrulline transcarbamoylase, 50 mM Hepes, pH 8.0 and 100 mM NH_4Cl (for CPS I) or 20 mM glutamine (for CPS III). Twenty five µL homogenate was added to 250 µL CPS reaction mixture and incubated for 60 minutes and terminated by the addition of 25 µL of 70% perchloric acid. The mixture was centrifuged at 13, 000 g and 50 µL of the supernatant was assayed with a 1 mL of citrulline reagent (1:1 (v:v) mixture of citrulline solution A (200 mg antipyrene in 94 ml H_2O, 6 ml concentrated H_2SO_4, stored in a dark bottle at room temperature) and citrulline

solution B (900 mg diacetyl monoxime in 95 ml H_2O, 5 ml glacial acetic acid, stored refrigerated in a dark bottle) made fresh before use. The citrulline reagent and supernatant mixture was boiled in the dark for 30 minutes, cooled in the dark and 200 µL was transferred to a microplate and the absorbance measured at 412 nm with a Molecular Devices Thermomax microplate reader. The amount of citrulline present was determined by comparison to a standard curve of citrulline prepared with the same reagents.

OCT enzyme activity was also measured using a colorimetric assay for citrulline described by Mommsen and Walsh, 1989 specifically the OCT reaction mixture contained 10 mM ornithine, 10 mM carbamoyl phosphate, and 50 mM Hepes, pH 8.5. Ten µL of homogenate was added to 250 µL of OCT reaction mixture and after 10 minutes was terminated with 25 µL 70% perchloric acid. The mixture was centrifuged at 13, 000 g and 50 µL of the supernatant was assayed with a 1 mL of citrulline reagent as described for the CPS enzyme assays above.

ARG enzyme activity was assayed via a colorimetric assay for the production of urea as described by Mommsen and Walsh, 1989. Specifically the ARG reaction mixture contained 250 mM arginine, 1 mM $MnCl_2$ and 50 mM Hepes, pH 8.0. The homogenate was diluted 1:10 in 5mM $MnCl_2$ in 50 mM Hepes and pre-incubated for 10 minutes. Ten µL of the pre-incubate was combined with 200 µL ARG mixture and incubated for 60 minutes at room temperature and terminated with 20 µL of 70% perchloric acid. Urea concentrations were assayed using the colorimetric diacetyl-monoxime method of Rahmatullah and Boyde (1980).

RNA extractions and cDNA synthesis

RNA was extracted from 200 ng of tissue using the chaotropic extraction protocol described by Whitehead and Crawford (2005). Further purification of the RNA solution was carried out using the RNeasy® mini protocol for RNA cleanup (Qiagen). The concentration of purified RNA was quantified on a NanoDrop ND-1000 spectrophotometer. RNA was further diluted to 500 ng μL^{-1} and treated with Turbo DNase (Ambion) to remove any traces of DNA from the extraction process. First strand cDNA synthesis was carried out using Superscript ^{TM} III Reverse Transcriptase (Invitrogen). Approximately 1μg of Turbo DNase (Ambion) treated RNA was denatured at 65ºC for five minutes with 100 ng of Random Hexamers and 10 mM dNTP mix in a total volume of 10 μL and placed on ice for later use. Reverse transcription (RT) was performed in 25 μL reactions containing the denatured RNA, 1x PCR buffer (20 mM Tris-HCl (pH 8.4) and 50 mM KCl), 0.01 M DTT, 2.5 mM $MgCl_2$, 0.4 mM each dNTP, 40 units RNaseOUT™, 50 units Superscript ™ III RT and ddH_2O. The RT reaction was incubated at 42.0 °C for 1 hour, 70.0 °C for 15 minutes and then cooled to 4.0 °C. The resultant cDNA product was stored at 4.0 °C. Unused purified RNA and Turbo DNase (Ambion) treated RNA was precipitated out of solution by adding 0.1X volume 3M sodium acetate and 2.5X volumes 100% ethanol and stored at -20.0°C.

Gene specific DNA amplification, RT-PCR and quantitative PCR

Alignments of vertebrate nucleotide sequences from Genbank were constructed for GS, CPS, OCT, ASL, ARG and Elongation factor-1 α (EF1α) and sections that were conserved in the coding region of the target gene were used to design primers. The

software package Oligo 6.89 (Molecular Biology Insights) was used to design primers (Table 3.1) that would amplify DNA product, using the appropriate zebrafish (*Danio rerio*) sequence as a template. Primers designed to amplify ASS in gulf toadfish (*Opsanus beta*) in a previous study (Laberge et al., Accepted) were used to amplify a portion of the ASS gene in West African lungfish (*P. annectens*) and the ASL forward primer from the same study was utilized in this study.

OneStep RT-PCR was performed to amplify the genes of the O-UC, GS and EF1α using Qiagen OneStep RT-PCR kit (Qiagen). The RT reaction and PCR reaction were performed in the same tube. One step RT-PCR was performed in 25 μL reactions containing 500 ng of total RNA, 1X Qiagen OneStep RT-PCR Buffer (includes 2.5 mM $MgCl_2$), 0.4 mM dNTPs, 1.0 μL Qiagen OneStep RT-PCR enzyme mix, 0.6 μM of each primer and RNase-free water. One step RT-touchdown PCR was performed as follows: one cycle of 45°C for 30 minutes; one cycle of 95°C for 15 minutes; 2 cycles of 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 2 minutes, with a step down of 1°C during the annealing phase every two cycles until the annealing temperature dropped to 50°C; and 30 cycles of 94°C for 30 seconds, 50°C for 1 minute, and 72°C for 2 minutes. PCR products were screened on a 1% agarose gel for appropriately sized products using GelStar® nucleic acid stain (Cambrex). All PCR products were stored at -20°C.

PCR product for putative GS, CPS, OCT, ASS, ASL, ARG and EF1α genes amplified from the West African lungfish were excised from the agarose gel and purified using Zymoclean ™ gel DNA recovery kit (Zymo Research). 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). A nucleotide BLAST search was performed in Genbank against "Other" databases to confirm the identity of the PCR product sequence. Gene specific qPCR primers (Table 3.1) were designed from West African lungfish sequence using Oligo 6.7 to have melting temperatures of 58-62ºC, 35-65% GC content, and to amplify DNA product between 168 bp and 211 bp.

Quantitative PCR was performed in the Chromo4 Four-Color Real-Time PCR System (Bio-Rad Laboratories Inc) with Power Sybr® Green PCR Master Mix (Applied Biosystems) using the following cycles: One cycle of 95°C for 10 minutes; 55 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds; one cycle of 95°C for 1 minute; 82 cycles of 55°C for 30 seconds and an increase of 0.5°C per cycle. Quantitative PCR 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. Standard curves were used to determine the amplification efficiencies between 88.4% and 99.6 % for qPCR reactions for all O-UC genes of interest and for EF1α. The level of housekeeping gene EF1α was invariant between each treatment group. Fold changes of the O-UC genes were calculated from the log-transformed C_T 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 analyses and statistical treatments

All data are reported as mean ± S.E.M. (N=number of animals). Control, emersion and re-immersion treatment groups were compared for levels of urea, ammonia,

enzyme activity and mRNA expression. If the data was normally distributed, significant differences were determined by using a One-way ANOVA in Sigma Stat 3.00 (SPSS Inc.) followed by a Holm-Sidak post-hoc test. If the normality test failed, a Kruskal-Wallis One Way Analysis of Variance 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 $P < 0.05$ and all data was plotted using Sigma Plot 8.02 (SPSS).

Data was log transformed for regression analyses. Data for O-UC enzyme mRNA expression and activity were linearly regressed against concentrations of urea, ammonia 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 concentrations as the dependent variable. The adjusted R^2 value was calculated and significance was accepted at ($P < 0.05$).

Results

A Dixon test for outliers was applied to all analyses in this experiment and found one outlier in the data for each of the control and emersed treatment groups in several analyses; therefore those two fish were removed from all of the analyses.

Urea concentrations were lowest in the control group, increasing more than five fold in the emersed lungfish, and then decreased to almost three fold above controls in the re-immersion group (Fig. 3.1). A two fold decrease in urea levels was seen in the re-immersion treatment after aerially exposed lungfish were re-immersed in water for 24

hours (Fig 3.1). Liver ammonia levels were not significantly affected by any of the experimental treatments and body mass was not significantly different in any of the treatment groups. Furthermore, neither hepatic ammonia concentrations nor lungfish body mass had any significant correlation with enzyme activity, mRNA expression or liver urea concentrations (Table 3.2).

Interestingly, both CPS I and CPS III enzyme activity was detected in all three treatments of lungfish and surprisingly, there was no significant difference in the levels of activity for CPS I and CPS III in each treatment group (data not shown).

There was an increase in enzyme activity levels in all five O-UC enzymes measured (GS, CPS I, CPS III, OCT and ARG) after emersion for six days (Fig. 3.2) with a decrease to control levels after 24 hours re-immersion treatment, except for GS. Activity of GS in emersed lungfish was almost three-fold higher than control lungfish and despite a 60 percent reduction in GS activity in re-immersed lungfish compared to emersed lungfish, GS activity remained 1.7-fold greater in re-immersed lungfish compared to control lungfish. Aerially exposed lungfish increased CPS I activity by almost five-fold after six days of emersion compared to control lungfish. Activity of CPS III in emersed lungfish was 2.8-fold higher than control lungfish. A two-fold increase in OCT activity was observed in emersed lungfish compared to control lungfish. Arginase activity was increased by 1.3-fold in emersed lungfish compared to control lungfish.

Messenger RNA expression for the six enzymes involved in the O-UC increased significantly in all but one (OCT). When emersed, lungfish increase mRNA expression of GS by 8.4-fold, CPS by 5.1-fold, ASS by 7.4-fold, ASL by 5.6-fold and ARG by 3.8-fold compared to control lungfish (Fig 3.3). Surprisingly unlike enzyme activity, not all

mRNA expression levels return to pre-treatment levels with re-immersion. A significant reduction in mRNA expression was seen in the re-immersion group compared to the emersed treatment for CPS (56% decrease) and ASS (77% decrease). No change in relative mRNA expression was seen for OCT for either the emersion treatment or the re-immersion treatment.

Activities measured for the O-UC enzymes were correlated to the activities of other enzymes within the cycle (Table 3.2). Liver urea concentrations were positively correlated with liver O-UC enzyme activities with the exception of ARG (Table 3.2). Forward and backward stepwise regressions using urea as the dependent variable and O-UC enzyme activities as the independent variables showed that only GS and OTC enzyme activities were necessary to predict urea concentrations.

Liver urea concentrations were also positively correlated to mRNA expression for GS, CPS, ASS and ASL and forward and backward stepwise regressions showed that urea concentrations could be predicted from ASL mRNA expression alone. When mRNA expression levels of O-UC enzymes were compared to other enzymes within the cycle we found that eight of these relationships were significantly correlated (Table 3.2). Only GS and CPS I mRNA expression were correlated to their corresponding enzyme activities (Table 3.2.)

Discussion

Lungfish are a unique group of fishes that can respire in aquatic and terrestrial habitats because they possess lung structure(s) and gills (Burggren and Johansen, 1986; Graham, 1997; Tokita et al., 2005). In order to avoid ammonia toxicity, lungfishes are

ammoniotelic under aquatic conditions but switch to ureotely when emersion conditions arise as the water dries up around them. Studies have shown that lungfish ameliorate the toxic effects of ammonia by increasing urea synthesis via the O-UC and decreasing ammonia production (Chew et al., 2004; Chew et al., 2003; Ip et al., 2004a; Ip et al., 2005b; Loong et al., 2005). Chew et al. (2003) showed that *P. dolloi* aerially exposed for six days increased enzyme activity for CPS III, ASS+ASL and GS and suggested that the utilization of ammonia via the O-UC during increased urea synthesis prevented a rise in tissue ammonia concentrations. Exposing *P. annectens*, a congener of *P. dolloi*, to the same aerial conditions resulted in increased rate of urea synthesis and increased enzyme activity of GS and OCT, but not in CPS III (Loong et al., 2005). When Loong et al. (2005) exposed *P. aethiopicus* to similar conditions they found no change in the activity in any of the O-UC enzymes, including CPS III, despite a rise in urea concentration and GS activity. These researchers speculated that the normal capacity of the CPS III enzyme was sufficient to adequately address the rise in urea synthesis and suggested that different lungfishes employed different strategies in regulating their O-UC to defend against ammonia toxicity (Loong et al., 2005).

The present study examined the relationships between hepatic ammonia and urea concentrations to liver O-UC enzyme mRNA expression and activity in *P. annectens* that were treated with aerial exposure for six days (without cocoon formation) and conditions where the fish were re-immersed in fresh water after aerial exposure. Despite no changes in hepatic ammonia concentrations, increases in liver urea concentrations were observed in aerially exposed and re-immersed lungfish suggesting an upregulation of the O-UC in these fish. Surprisingly, all of the O-UC enzymes measured showed a coordinated

induction of enzyme activity with aerial exposure and all but GS activity rapidly returned to control levels after re-immersion. Taken together, these results suggest that the O-UC plays a great role in detoxifying ammonia to urea when lungfish are exposed to aerial conditions and support the findings of Loong et al. (2005) that lungfish rapidly switch back to ammonioteley upon re-immersion in water.

The levels of mRNA expression for the O-UC enzymes have not previously been measured in any lungfish species. Aerially exposed lungfish also showed a coordinated increase in relative mRNA expression for the genes of O-UC except OCT and again there was a return to control levels upon re-introduction to water. Since aerially exposed lungfish increase urea synthesis via the O-UC to defend against ammonia toxicity (Chew et al., 2003; Loong et al., 2005; Wood et al., 2005) one would expect an upregulation in mRNA expression for the genes involved in the O-UC in aerially exposed fish with a decrease in mRNA expression when the fish are re-immersed in freshwater and return to ammonioteley. Indeed 5 of 6 O-UC enzymes had increased mRNA expression which was correlated to urea concentrations.

Carbamoyl phosphate synthetase is considered a control point for the O-UC in many fishes (Anderson and Walsh, 1995; Kajimura et al., 2006; Steele et al., 2001; Todgham et al., 2001) therefore we would expect changes in urea production to be reflected by changes in CPS mRNA expression. There was a concurrent rise in CPS mRNA expression with increased liver urea concentrations and this has been described for CPS III in other fishes (Iwata et al., 2000; Kong et al., 2000; Laberge et al., Accepted) and for CPS I in mammals (Takagi et al., 2008). Similar to the results seen in *O. beta* (Kong et al., 2000; Laberge et al., Accepted), CPS mRNA expression was correlated to

urea concentration indicating that CPS mRNA expression was associated with urea production. After re-introduction into freshwater for 24 hours, urea concentrations and CPS mRNA expression levels fell suggesting a reduction in urea synthesis and a switch back to ammonioteley.

As determined in Chapter 2 of this thesis, CPS mRNA expression measured in this study is for a CPS I-like isoform (Laberge unpublished results). Since CPS mRNA expression was significantly correlated to CPS enzyme activity, indicating that CPS protein abundance was affected by the amount of CPS mRNA expression, we expected the activity of CPS to increase with increased mRNA expression. Indeed we did observe a rise in CPS activity with increased CPS transcript levels, and further CPS activity was highly correlated with urea levels. An increase in CPS activity was also seen in the study by Chew et al. (2003) accompanied by a rise in hepatic urea concentrations in *P. dolloi*, leading those researchers to suggest that increased urea synthesis played a key role in avoiding ammonia toxicity as the water dried up around the fish. However, Loong et al., 2005 using *P.annectens* did not see a rise in CPS III activity in aerially exposed fish despite a 2.2-fold rise in liver urea concentrations and suggested that the activity level of CPS was adequate to handle the rise in urea synthesis. Urea concentrations in our study increased approximately five-fold and likely required a greater amount of active CPS protein to compensate for the increased urea production which may explain the difference in CPS activity. Therefore we suspect in *P. annectens*, like *P. dolloi*, urea synthesis is key to avoiding ammonia toxicity.

It has been suggested that during the evolution of urea synthesis in vertebrates a necessary requirement for the transition from water to land would be a substitution of

CPS III with CPS I (Mommsen and Walsh, 1989). CPS I and III isoforms are localized in the mitochondrial matrix and require N-acetyl-L-glutamate (NAG) as a co-factor; the difference between these two isoforms is that CPS I utilizes ammonia as a nitrogen substrate for urea synthesis, whereas CPS III utilizes glutamine (Anderson, 1995a; Felskie et al., 1998; Saha et al., 1997). Contrary to the study by Mommsen and Walsh (1989) that found CPS I was utilized by *P. annectens* and *P. aethiopicus* during urea synthesis, a recent study by Loong et al. (2005) determined that these two lungfish species utilized CPS III. The present study shows evidence of hepatic CPS I and CPS III activity present at near equal concentrations and this has been observed in other air-breathing fishes (Saha et al., 1999; Saha et al., 1997). This apparent conflict is likely due to the ability of the mitochondrial form of CPS to utilize both glutamine and ammonia as an nitrogenous substrate (Casey and Anderson, 1983; Lindley et al., 1999; Saha et al., 1997). Interestingly, CPS I in *Rana catesbeiana* has the ability to change substrate from ammonia to glutamine by mutagenesis of a single amino acid of at position 258 suggesting that the frog CPS I retains a close link to glutamine-utilizing CPS (Saeed-Kothe and Powers-Lee, 2003). We suggest that the CPS activity observed in this study represents only one isoform of mitochondrial, NAG-dependent CPS present in *P. annectens* capable of using either glutamine or ammonia as its nitrogenous substrate. This suggestion could best be tested by further study of *purified* CPS from lungfish.

Ornithine carbamoyl transferase was the only O-UC enzyme that did not increase in mRNA expression during six days of aerial exposure despite an increase in urea concentration. Similar results have been observed in other vertebrate studies that showed OCT mRNA expression does not increase with increased urea levels (Laberge et al.,

Accepted; Takagi et al., 2008). Gene expression is regulated by transcription factors that act, alone or in concert with other transcription factors, to activate or repress transcription (Chen and Rajewsky, 2007; Weaver, 2008) and therefore OCT mRNA expression may be under transcriptional regulation during urea production and this notion is supported by a strong correlation of OCT mRNA expression with urea levels. As seen in the study by Loong et al. (2005), *P. annectens* increased OCT activity in the aerially exposed lungfish, but OCT mRNA expression was not correlated to enzyme activity suggesting post-transcriptional regulation of OCT. In rats, protein stabilization has been suggested to account for the incongruity between OCT mRNA expression and activity (Ulbright and Snodgrass, 1993). Levels of mRNA expression represents a steady state of mRNA production and transcript stability, therefore decay rates or mRNA half-lives affect the amount of mRNA present (Mata et al., 2005; Weaver, 2008). Similar to the Laberge et al. (Accepted) study, enough OCT mRNA persisted to generate the required OCT activity necessary for the increased levels of urea synthesis and this is supported by the high correlation of CPS activity to urea concentration.

Glutamine synthetase mRNA expression and enzyme activity were also measured due to the importance of GS in an accessory pathway to the O-UC in fishes. As seen in previous studies that examined GS mRNA expression in gulf toadfish, *O. beta*, (Kong et al., 2000; Laberge et al., Accepted; McDonald et al., 2009), we saw a concurrent rise in GS mRNA expression in *P. annectens* with increasing urea levels. We also observed a rise in GS activity comparable to that of Loong et al, 2005 who suggested that the increase in GS activity was due to the use of glutamine as a substrate for the O-UC during urea production. Furthermore, both GS mRNA expression and activity were

correlated with urea levels also suggesting that the glutamine synthetase reaction provided the glutamine substrate necessary for the O-UC in *P. annectens*. It appears that any post-transcriptional changes to GS mRNA expression did not affect protein abundance since GS activity was correlated with mRNA expression for GS.

Similar to GS, there was a simultaneous increase in both ARG mRNA expression and ARG activity in aerially exposed lungfish, but mRNA expression was not correlated to activity indicating post-transcriptional or post-translational regulation of ARG. A further explanation for this disparity may be that total hepatic ARG activity is boosted by the presence of a second ARG isozyme. There are at least two different ARG genes described in vertebrates (Joerink et al., 2006; Morris, 2002; Wright et al., 2004); ARG I is localized mainly to the liver and detoxifies ammonia via the O-UC, whereas ARG II is a ubiquitously expressed at low levels and is involved in ornithine synthesis (Joerink et al., 2006; Morris, 2002; Wright et al., 2004). Since both isozymes are present in the liver of vertebrates, the hepatic activity of ARG may result from the presence of ARG I, ARG II or both enzymes (Morris, 2002). In this study the measurement of mRNA expression specifically targeted the ARG I isozyme due to its involvement in the O-UC and is supported by the correlation of ARG mRNA expression with urea concentrations. Since hepatic ARG activity was not correlated with either ARG mRNA expression or urea concentrations, we suggest that the activity is a result of the combined activities of ARG I and ARG II.

Like the previous study on gulf toadfish, there was increased expression of ASS mRNA with a concurrent rise in hepatic urea levels (Laberge et al., Accepted). Interestingly, the ASS enzyme is active in another metabolic pathway, the citrulline

nitric-oxide cycle (C-NOC) (Husson et al., 2003; Morris, 2002; Yamaguchi et al., 2001); in the teleost fish *O. beta* there was no correlation between ASS mRNA expression and urea production leading the authors to suggest that the rise in ASS mRNA expression was due to its participation in the C-NOC (Laberge et al., Accepted). In contrast, *P. annectens* ASS mRNA expression and urea concentrations were correlated suggesting that ASS is actively participating in the O-UC.

Expression of ASL mRNA also increased significantly in aerially exposed fish and suggests active participation in the O-UC. This suggestion is further supported by the correlation between ASL mRNA and urea concentrations and the step-wise regression that determined that urea concentrations could be predicted by ASL mRNA expression alone. Unfortunately, due to experimental limitations we were unable to determine the enzyme activity levels for ASS or ASL. However, the study by Loong et al., 2005 did not find any significant difference in *P. annectens* aerially exposed for six days compared to freshwater immersed fish using a combined assay for ASS+ASL. Curiously, in *P. dolloi*, there was an increase in ASS+ASL activities that were aerially exposed for six days (Chew et al., 2003). In light of the increased mRNA expression for ASS and ASL, the coordinated induction in activities of O-UC enzymes examined, and the positive correlations of ASS and ASL mRNA expression with the urea concentrations, we would predict that the activities of ASS and ASL should increase during aerial exposure for six days.

Similar to the gulf toadfish study (Laberge et al., Accepted) we saw a coordinated induction of O-UC enzyme activity. A study by Ulbright and Snodgrass (1993) suggested that three different mechanisms permitted a coordinated induction of the O-UC

enzyme activity in rat hepatocytes treated with cAMP and glucocorticoids: mRNA stability, increased transcription and enzyme stabilization. Unlike toadfish, all of the O-UC activities measured in *P. annectens* were correlated with activities of other enzymes within the O-UC and only ARG activity was not correlated with urea production. Stepwise regressions showed that urea concentrations could be predicted by GS and OTC enzyme activity and perhaps this reflects the high level of correlations within the O-UC enzymes. Unlike the toadfish study by Laberge et al. (Accepted), CPS may not act as a rate-limiting enzyme for urea production in *P. annectens* since it was not required to predict urea levels in any of the stepwise regressions. Coordinated induction of O-UC enzyme activity and lack of correlations in mRNA expression lead Laberge et al. (Accepted) to speculate that toadfish enzyme activity was a better predictor of urea production than mRNA expression. Surprisingly, in *P. annectens* there seems to be a coordinated induction of hepatic O-UC mRNA expression during urea production, except OCT and this is supported by the correlation of the O-UC mRNA expression to urea concentrations for all but OCT.

Coordinated induction of both O-UC mRNA expression and enzyme activity in *P. annectens* suggest that both of these cellular processes play a major role in urea production during aerial exposure. However, because only two of the proteins examined, GS and CPS, had enzyme activities that were correlated to their mRNA expression there are likely multiple regulatory mechanisms that regulate that the enzymes of the O-UC throughout the cycle. Furthermore, the lack of correlation between enzyme activity and mRNA expression may be explained by technical variance within the experimental

parameters measured and/or lack of statistical power due to the low number of samples in this study.

Currently little information is available on O-UC enzyme regulation and control for vertebrates and in particular fishes. Therefore we suggest that future studies identify the post-transcription and post-translational mechanisms of O-UC expression and transcription factors associated with this pathway in order to expand our knowledge of how urea production is regulated.

Primer	Accession No.	Sequence (5'→3')	Product Size(bp)
GS- F[c]	**NM_205732**	AGGGCTCCAACAGTGACAT	**545**
GS-R[c]		GCAGCCAGCACCRTTCCA	
CPS-F[a]	**XM_678190**	TGG AAI GAR RTI GAR TAY GA	**512**
CPS-R[a]		CAT IAC YTC ICC IAC ISW YTT CAT	
OTC-F[c]	**XM_001334635**	ACTACAGAGACAGGTTTTGC	**730**
OTC-R[c]		CATTACAGCCATRATNGTCCA	
ASS-F[bc]	**NM_001004603**	TATGCTGGTCTGGCTGAAGGA	**939**
ASS-R[bc]		ACACTCGGGGTTGARCCAGAA	
ASL-F[bc]	**NM_200451**	CAATGAACGCAGGCTSAAGGA	**599**
ASL-R[c]		CTGGGTTCTTCTTCTGRGGCA	
ARG-F[c]	**NM_001045197**	CTGAGTGTTGTNTGGGTKGAYGC	**430**
ARG-R[c]		GTGATGTAAATGCCYTCYCTRTA	
EF1α-F[c]	**NM_131263**	TGAGCGTGAGCGTGGTATC	**1102**
EF1α-R[c]		CAGTCTGCCTTATGTCACG	
GS-qF	Lungfish Seq this study	GTTGGAGTAAAGATAGCAGGA	**176**
GS-qR		AGCACCGTTCCAGTTACCA	
CPS-qF	Lungfish Seq this study	TGTTTCGGGCAAGACTACTG	**197**
CPS-qR		AGAAGGTTGGCACATACGGA	
OTC-qF	Lungfish Seq this study	CCTTGTTTCCTTCCTCCAGA	**178**
OTC-qR		TACCTATCCGACAACCCACT	
ASS-qF[c]	Lungfish Seq this study	TATGCTGGTCTGGCTGAAGGA	**211**
ASS-qR		CAGCAGTTAGCGGTCCTCATA	
ASL-qF	Lungfish Seq this study	CAGAGCAGAAATGTCCAGGT	**171**
ASL-qR		TCTGATTGGCTGGGCTCTC	
ARG-qF	Lungfish Seq this study	GAGATGTTGACGACGGAGAA	**184**
ARG-qR		GCAGAGAAGTCCACACCAT	
EF1α-qF	Lungfish Seq this study	ATGTCTCTGTAAAGGATGTTC	**168**
EF1α-qR		AATCTATGTGAGCAGTATGAC	

Abbreviations: Elongation factor 1-alpha (EF1α); Glutamine synthetase (GS); Carbamoyl phosphate synthetase (CPS); Argininosuccinate synthetase (ASS); Argininosuccinate lyase (ASL); Arginase (ARG); F1forward primer (F); reverse primer (R); Quantitative PCR primer (q).

[a] Primers designed by Korte et al. 1997.

[b] Primers used from Laberge et al. (Accepted)

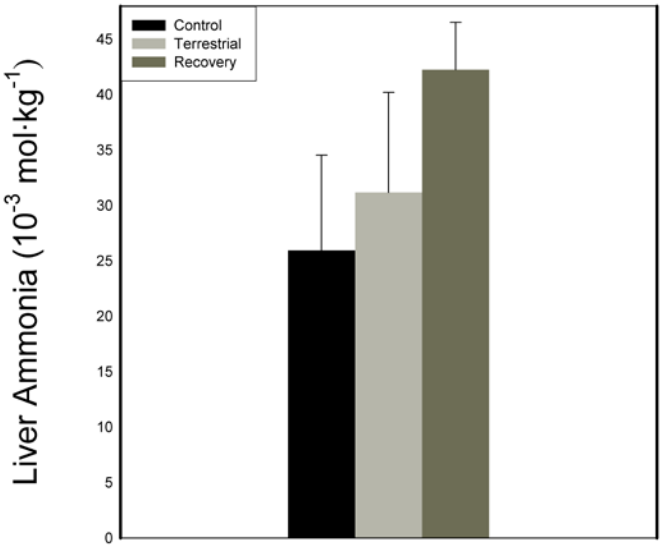
[c] Primers designed from conserved nucleotide sequences across multiple vertebrate species including zebrafish (*Danio rerio*), human (*Homo sapiens*), mouse (*Mus musculus*), and rat (*Rattus norvegicus*).

Table 3.1. Primers used to amplify DNA product for the O-UC genes in West African lungfish, (*Protopterus annectens*) and gene specific primers designed for qPCR used in this study. Accession numbers for *Danio rerio* and *P. annectens* sequences from which the primers were designed are provided.

Parameter Measured	**GS Activity**	**CPS 1 Activity**	**CPS 3 Activity**	**OCT Activity**	**ARG Activity**	**Urea**	**Ammonia**
GS Activity	*	X	X	X	X	X	X
CPS1 Activity	**0.585 (<0.001)**	*	X	X	X	X	X
CPS 3 Activity	**0.449 (<0.003)**	**0.585 (<0.001)**	*	X	X	X	X
OTC Activity	**0.474 (0.002)**	**0.712 (<0.001)**	**0.535 (<0.001)**	*	X	X	X
ARG Activity	**0.248 (0.029)**	**0.347 (0.010)**	**0.532 (<0.001)**	**0.376 (0.007)**	*	X	X
Urea	**0.752 (<0.001)**	**0.565 (<0.001)**	**0.316 (0.014)**	**0.598 (<0.001)**	0.143 (0.082)	*	X
Ammonia	0.026 (0.257)	0.000 (0.917)	0.000 (0.974)	0.000 (0.947)	0.000 (0.403)	0.000 (0.621)	*
Weight	0.023 (0.264)	0.054 (0.195)	0.000 (0.402)	0.000 (0.660)	0.000 (0.355)	0.000 (0.360)	0.088 (0.140)
Parameter Measured	**GS mRNA**	**CPS mRNA**	**OCT mRNA**	**ASS mRNA**	**ASL mRNA**	**ARG mRNA**	**Corresponding Enzyme Activity**
GS mRNA	*	X	X	X	X	X	**0.302 (0.016)**
CPS mRNA	0.140 (0.085)	*	X	X	X	X	**0.213 (0.041)**
OCT mRNA	0.000 (0.916)	0.001 (0.330)	*	X	X	X	0.017 (0.280)
ASS mRNA	**0.465 (<0.001)**	**0.428 (0.004)**	0.020 (0.273)	*	X	X	n.a.
ASL mRNA	**0.575 (<0.001)**	**0.359 (0.008)**	0.000 (0.356)	**0.364 (0.008)**	*	X	n.a.
ARG mRNA	0.010 (0.300)	**0.223 (0.037)**	**0.432 (0.003)**	**0.453 (0.003)**	0.140 (0.077)	*	0.076 (0.157)
Urea	**0.309 (0.015)**	**0.289 (0.018)**	0.000 (0.436)	**0.411 (0.004)**	**0.566 (<0.001)**	**0.250 (0.028)**	n.a.
Ammonia	0.000 (0.568)	0.000 (0.971)	0.000 (0.955)	0.000 (0.999)	0.025 (0.258)	0.000 (0.622)	n.a.
Weight	0.000 (0.397)	0.000 (0.731)	0.000 (0.542)	0.000 (0.766)	0.188 (0.053)	0.000 (0.903)	n.a.

Table 3.2. Results of linear regressions of the log transformed data for all parameters measured in this experiment using liver tissue of *Protopterus annectens*. 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 (Note: CPS mRNA is compared to CPS I Activity). Bold font indicates that $p < 0.05$ for that regression, x indicates that the regression is already represented elsewhere in the table, * indicates that the parameters listed are identical and therefore no regression was performed, and n.a. indicates that there was no appropriate regression for that parameter.

A.



B.

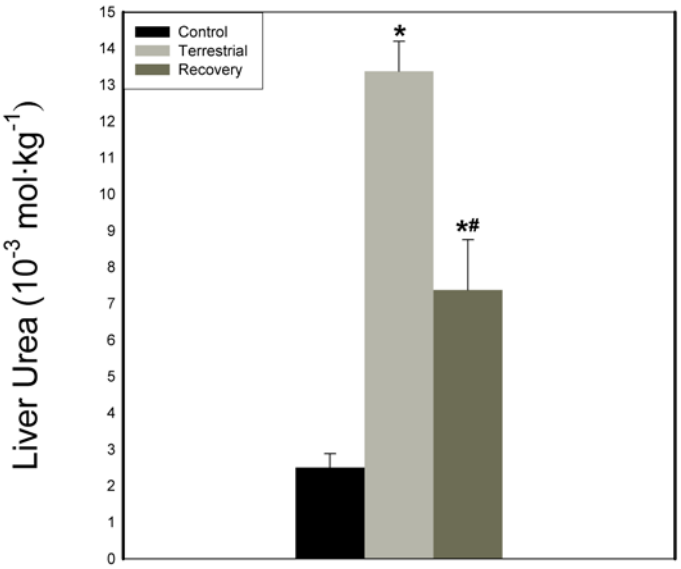


Figure 3.1. Liver (A) ammonia levels and (B) urea levels of West African lungfish (*P. annectens*) for control group (N=5), lungfish emersed for six days (N=5) and lungfish subject to re-immersion for 24 hours (N=6). Values are means ± S.E.M.; $^{*}P < 0.05$, significantly different from control lungfish, $^{\#}P < 0.05$, significantly different from lungfish emersed for six days.

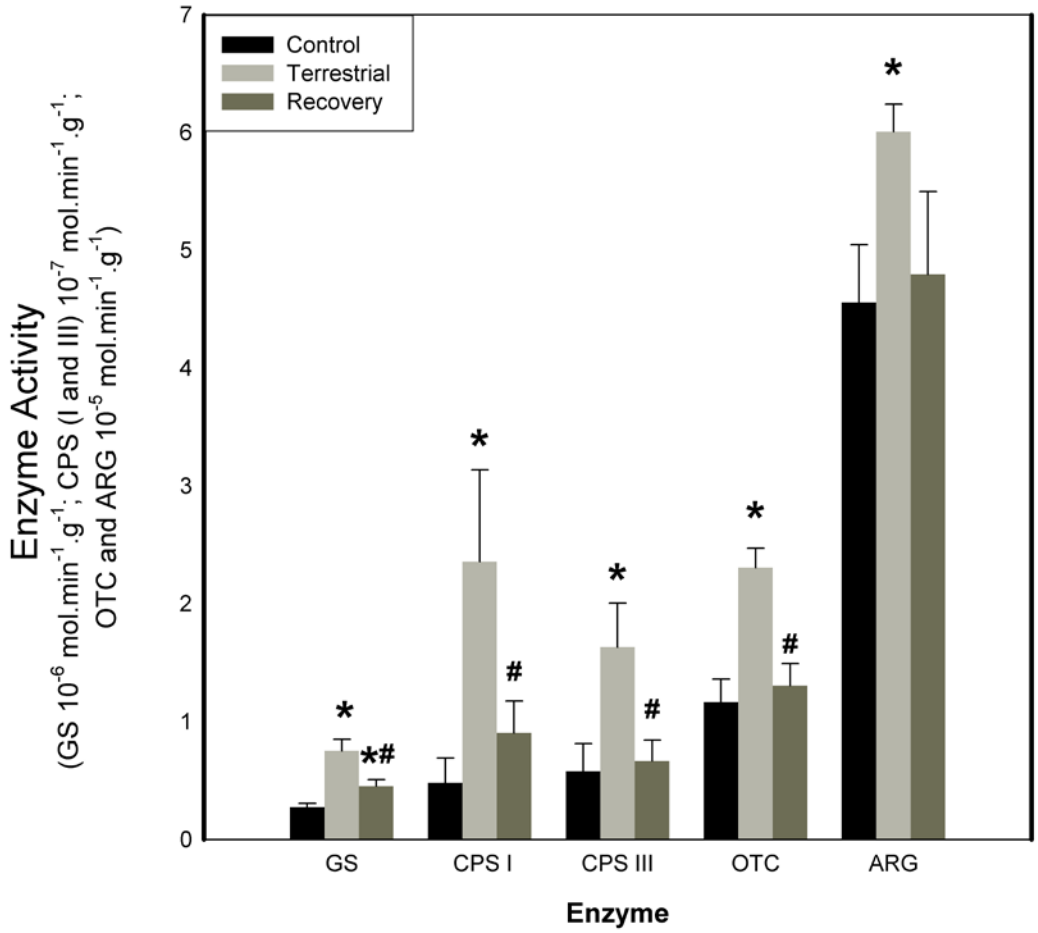


Figure 3.2. Enzyme activity measurements for glutamine synthetase (GS), carbamoyl phosphate synthetase I (CPS I), carbamoyl phosphate synthetase III (CPS III), ornithine carbamoyl transferase (OCT) and arginase (ARG) in liver tissue of West African lungfish (*P. annectens*) for control group (N=5), lungfish emersed for six days (N=5) and lungfish subject to re-immersion in freshwater for 24 hours (N=6). Values are means ± S.E.M.; $^{*}P < 0.05$, significantly different from control lungfish, $^{\#}P < 0.05$, significantly different from lungfish emersed for six days.

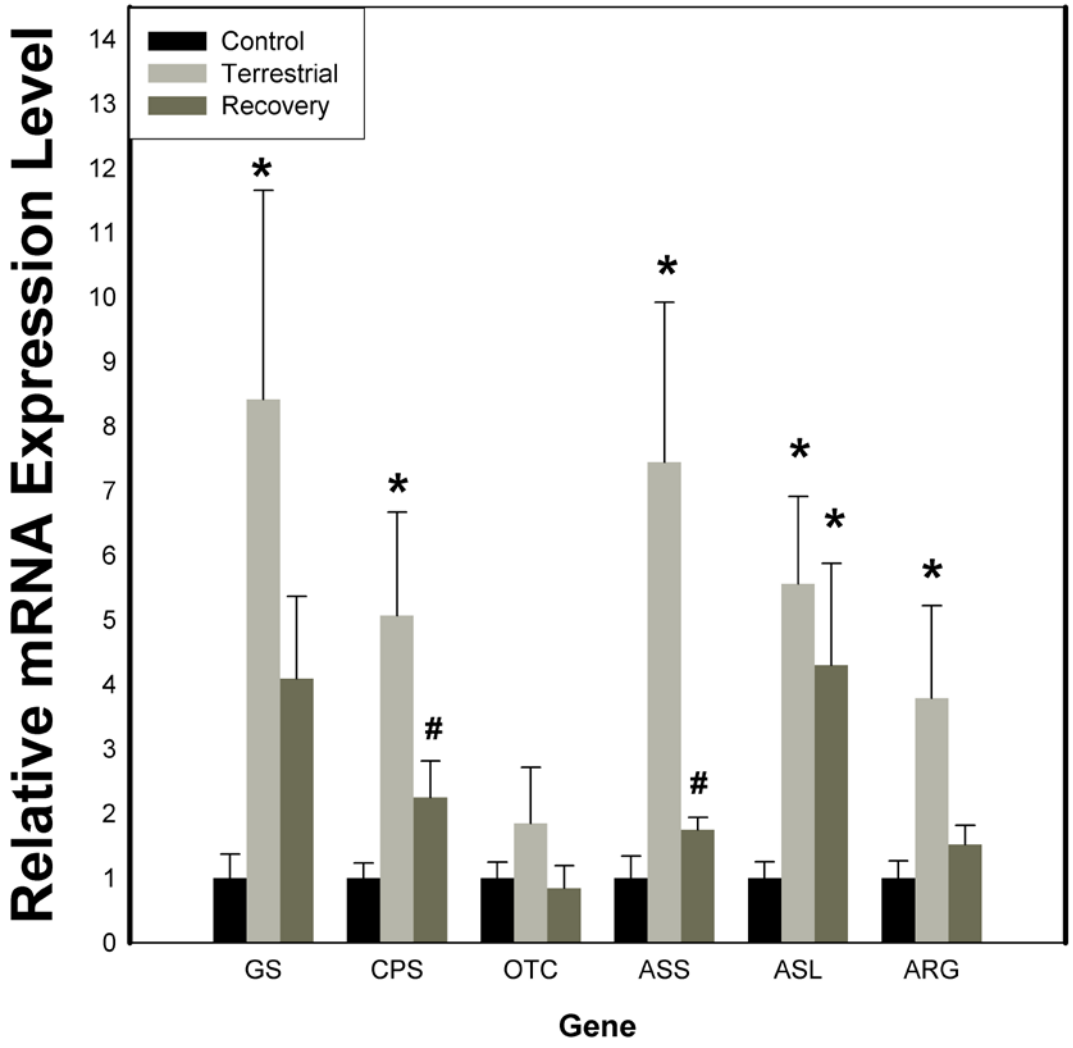


Figure 3.3. Relative mRNA expression levels in liver tissue of West African lungfish (*P. annectens*) for glutamine synthetase (GS), carbamoyl phosphate synthetase I (CPS), ornithine carbamoyl transferase (OCT), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARG) for control group (N=5), lungfish emersed for six days (N=5) and lungfish subject to re-immersion in freshwater for 24 hours (N=6). . Values are means ± S.E.M.; $^{*}P < 0.05$, significantly different from control lungfish, $^{\#}P < 0.05$, significantly different from emersed for six days.

Chapter 4: Effects of Crowding on Ornithine-Urea Cycle Enzyme mRNA Expression and Activity in Gulf Toadfish (*Opsanus beta*)

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 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 while 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. In 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.

Background

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, 1995b; 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, 1995b; 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, 1995b; 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., 1994b). 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., 1994b). Hopkins et al. (1995) showed that the acute response to up to two hours of crowding treatment was a two to four-fold increase in circulating cortisol concentrations, with cortisol returning to control levels after 24 hours.

At the same time, urea becomes the primary nitrogenous waste between 24-48 hours of crowding treatment and remains so for seven days (Walsh et al., 1994b).

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 hours of crowding suggesting that the "fish-type" O-UC is activated at this time (Hopkins et al., 1995; Walsh et al., 1994b). However, other studies focusing on the effects of crowding on the activities of other O-UC enzymes are contradictory. For example, Walsh et al. (1994) found that OCT activity was elevated in fish crowded for 96 hours, but a follow-up study by 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 five-fold increase in hepatic GS mRNA expression and up to a ten-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. (2003) suggest that poor correlations of mRNA levels with protein

levels are due to complicated post-transcriptional mechanisms and differential *in vivo* protein half-lives.

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, Florida, 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 hours 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 (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 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 12h light: 12h 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 usp units mL^{-1}). Approximately 100 μL blood was extracted from each fish, plasma was separated by centrifugation (10000 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 (1971) as modified by Wang and Walsh (2000). Ammonia concentrations of liver tissue were measured using a colorimetric assay (Raichem, San Diego, CA) 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 (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 in 4x volume of homogenization buffer (20 mmol/L 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 25ºC). Homogenates were centrifuged at 16000 g for five minutes at 4ºC in an Eppendorf® 5415D centrifuge 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., 1994b).

RNA extractions and cDNA synthesis

RNA was extracted from 200 mg of tissue using the chaotropic extraction protocol outlined by 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. 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 ^{TM} III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Approximately 1μg of Turbo DNase (Ambion) treated RNA was denatured at 65ºC for five minutes with 100 ng of Random Hexamers and 10 mM dNTP mix in a total volume of 10 μL. The denatured RNA was placed on ice for later use. Reverse transcription was performed in 25 μL reactions containing the denatured RNA, 1x PCR buffer (20 mM Tris-HCl (pH 8.4) and 50 mM KCl), 0.01 M DTT, 2.5 mM $MgCl_2$, 0.4 mM each dNTP, 40 units RNaseOUT™, 50 units Superscript ™ III RT and ddH_2O. The RT reaction was incubated at 42.0 °C for 1 hour, 70.0 °C for 15 minutes 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 0.1X volume 3M sodium acetate and 2.5X 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 4.1) 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 (*O. beta*). GS and CPS 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 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. One step RT-PCR was performed in 25 μL reactions containing approximately 500 ng of total RNA, 1X Qiagen OneStep RT-PCR Buffer (includes 2.5 mM $MgCl_2$), 0.4 mM dNTPs, 1.0 μL Qiagen OneStep RT-PCR enzyme mix, 0.6 μM of each primer and RNase-free water. One step RT-touchdown PCR was performed as follows: one cycle of 45°C for 30 minutes; one cycle of 95°C for 15 minutes; 2 cycles of 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 2 minutes, 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 seconds, 50°C for 1 minute, and 72°C for 2 minutes. PCR products were screened on a 1% agarose gel for appropriately sized products using GelStar® nucleic acid stain (Cambrex). 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). 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. 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 qPCR primers (Table 4.1) were designed from toadfish sequence using Oligo 6.7 to have melting temperatures of 58-60ºC, 35-65% GC content, and to amplify DNA product between 145 bp and 252 bp.

Quantitative PCR was performed in the Mx4000 (Stratagene, La Jolla, CA) with Power Sybr® Green PCR Master Mix (Applied Biosystems) using the following cycles: One cycle of 95°C for 10 minutes; 55 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds; one cycle of 95°C for 1 minute; 41 cycles of 55°C for 30 seconds and an increase of 1°C per cycle. Quantitative PCR 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 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 analyses and statistical treatments

All data are reported as mean ± 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 ANOVA in Sigma Stat 3.00 (SPSS Inc.) followed by a Holm-Sidak post-hoc test. If the normality test failed, a Kruskal-Wallis One Way Analysis of Variance 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).

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. 4.1). Compared to 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. 4.1A). Conversely, urea concentrations in the liver tissue were lowest in uncrowded fish and were elevated by about seven-fold in fish crowded for two days with a further three-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. 4.1B). Circulating cortisol concentrations followed a similar pattern to liver urea levels, being about five-times higher in fish crowded for seven days compared to uncrowded fish (Fig. 4.1C). 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. 4.2). Specifically, glutamine synthetase activity in fish crowded for two days was three-fold higher than in uncrowded fish, and remained elevated in fish that were crowded for seven days.

Carbamoyl phosphate synthetase activity increased by two-fold after two days of crowding compared to uncrowded fish, with a three-fold increase after seven days of crowding. In contrast, OCT and ARG only showed a significant elevation in activity after seven days of crowding (Fig. 4.2).

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 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 that individual fish was removed from all of the analyses.

No significant changes were measured in GS, OCT, and ARG relative gene expression (Fig. 4.3). However, the relative mRNA expression of CPS was approximately four-fold higher in fish crowded for two days and remained elevated in the seven day crowded fish compared to the uncrowded fish. A three-fold elevation in mRNA expression was also measured in ASS after seven days of crowding compared to 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 percent reduction in fish crowded for seven days compared to uncrowded fish, with no difference measured in 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 4.2). Liver urea concentrations were positively correlated to liver O-UC enzyme activities (Table 4.2, Fig. 4.4). 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 4.2). 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 4.2) but only CPS activity was necessary to predict liver ammonia concentrations. CPS mRNA expression was also negatively correlated to liver ammonia levels (Table 4.2) 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 4.2). 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 4.2).

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 4.2). Only CPS and OCT mRNA expression were correlated to their corresponding enzyme activities (Table 4.2 and Fig. 4.5).

Discussion

Previous studies have shown that exogenous ammonia exposure, air exposure and crowding conditions induce ureotely in gulf toadfish (Walsh et al., 1990; Walsh et al., 1994b; Wang and Walsh, 2000). 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 enzyme.

Levels of mRNA expression have previously been measured in crowded gulf toadfish for only two of the six O-UC enzymes using ribonucleic acid protection assays (Kong et al., 2000). Supporting findings from this previous study, CPS mRNA expression in toadfish of the present study showed an almost three-fold increase after two and seven days of crowding compared to 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 four-fold increase in the transcription of CPS mRNA was measured within 24 hours (Ulbright and Snodgrass, 1993). While mRNA levels are affected by transcription factors that can both positively and negatively regulate this process (Weaver, 2008), mRNA expression can also be affected by changes in mRNA stability (*i.e.*, turnover and

decay) (Mata et al., 2005; Weaver, 2008). 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 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., 1994b); 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 $\mu mol \cdot min^{-1} \cdot g^{-1}$ liver tissue (Walsh and Milligan, 1995; Walsh et al., 1994b). In 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 $\mu mol \cdot min^{-1} \cdot g^{-1}$ 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 to 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 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 likely 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, 1995b; 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 and coworkers (2000) found a significant increase in GS mRNA expression after 48 hours of crowding and a putative glucocorticoid receptor has been reported for toadfish GS (Walsh, P.J. unpubl. 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 minutes and 8 hours

(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., 2000 protocol) that might support this idea.

The increase in GS activity in this study agrees with previous studies that have shown elevated GS activity with induction of the O-UC after 24 hours of crowding (Hopkins et al., 1995; McDonald et al., 2009; Walsh and Milligan, 1995; Walsh et al., 1994b; Wood et al., 1995). Like 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 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 to the short half-life of GS mRNA (see above). Therefore, sampling at multiple time points (*i.e.* every two hours for the first 24 hours 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 two-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). 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. Since ASS mRNA expression is not correlated to urea levels, perhaps the upregulation in ASS mRNA expression measured in this 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., 1994b). 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 since 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., 1994b) and no effect of confinement in the other (Walsh and Milligan, 1995). The contradictory results could just be a matter of stressor; both the present study and Walsh et al. (1994) found an upregulation in response to crowding, which is believed to elicit a much more pronounced stress response in toadfish compared to 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). 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; Yu et al., 2001). Arginase 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 amongst 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). 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., 1994b), ARG activity was not significantly different from control fish after two days of crowding. In 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 likely not related to stress and other factors may be contributing to its increase. Arginase 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). Since 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. (2007) 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. 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.

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	

[a] Primers used from 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. 1999.
[e] Primers designed from published sequence of *O. beta* in Kong et al. 2000.
[f] Primers designed from sequence of *O. beta* obtained in this study

Table 4.1. Primers used to amplify DNA product for the O-UC gene in gulf toadfish (*Opsanus beta*) and gene specific primers designed for qPCR used in this study. 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); F1forward primer (F); reverse primer (R); Quantitative PCR primer (q).

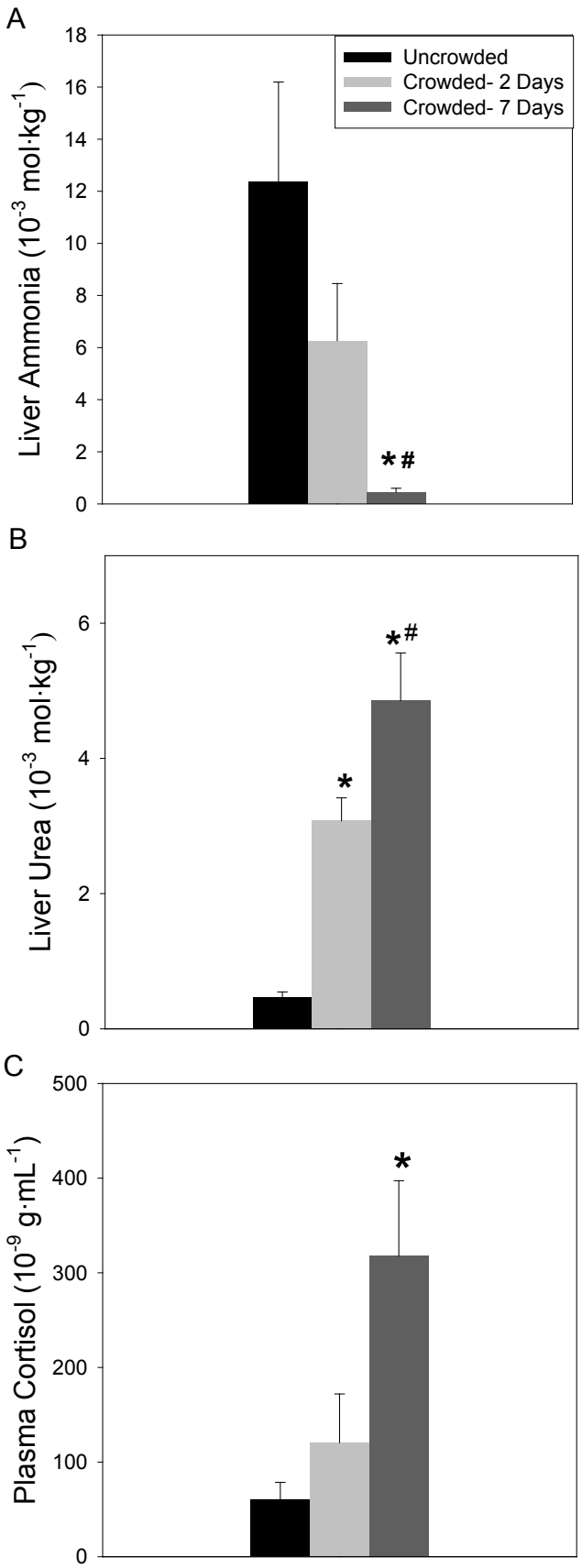
.

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.

Table 4.2. Results of linear regressions of the log transformed data for all parameters compared in this experiment. 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, x 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.

Figure 4.1. Liver (A) ammonia levels, (B) urea levels and (C) circulating cortisol levels of gulf toadfish (*O. 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.

Figure 4.1.


A
Liver Ammonia (10^{-3} mol·kg^{-1})
Uncrowded
Crowded- 2 Days
Crowded- 7 Days
*#
B
Liver Urea (10^{-3} mol·kg^{-1})
*
*#
C
Plasma Cortisol (10^{-9} g·mL^{-1})
*

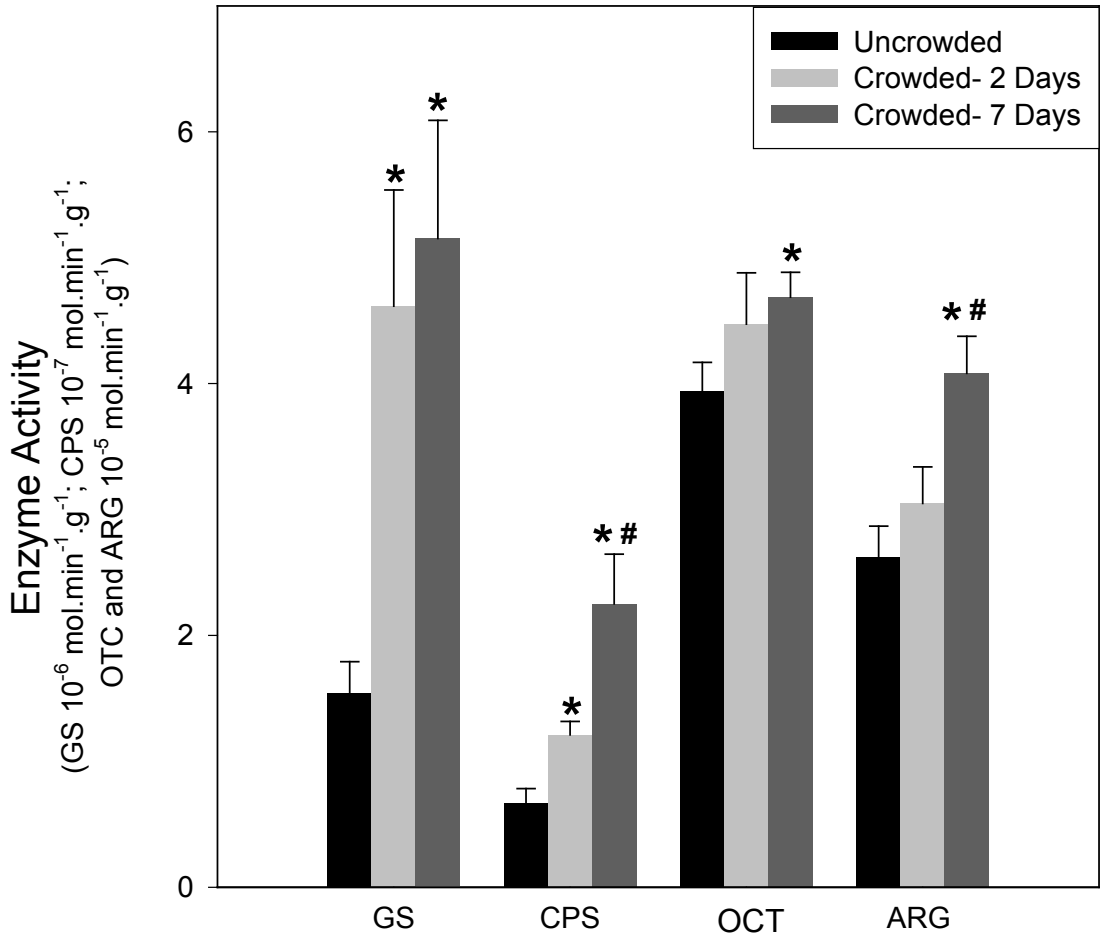


Figure 4.2. Enzyme activity measurements for glutamine synthetase (GS), carbamoyl phosphate synthetase (CPS), ornithine carbamoyl transferase (OCT) and arginase (ARG) in liver tissue of gulf toadfish (*O. beta*) that were uncrowded, crowded for two days or crowded for seven days. All treatments N=9. 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.

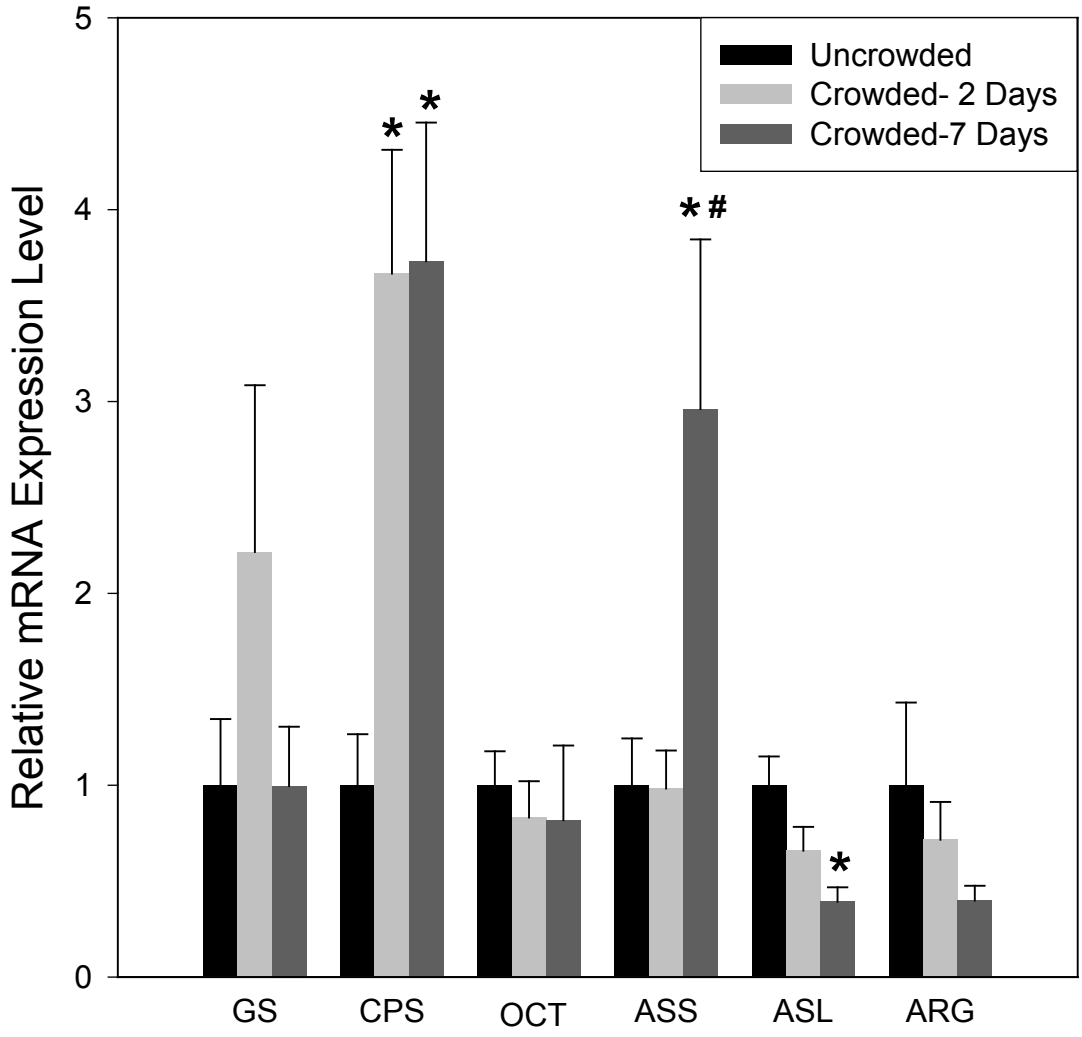


Figure 4.3. Relative mRNA expression levels in liver tissue of gulf toadfish (*O. beta*) for glutamine synthetase (GS), carbamoyl phosphate synthetase (CPS), ornithine carbamoyl transferase (OCT), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARG) that were uncrowded, 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.

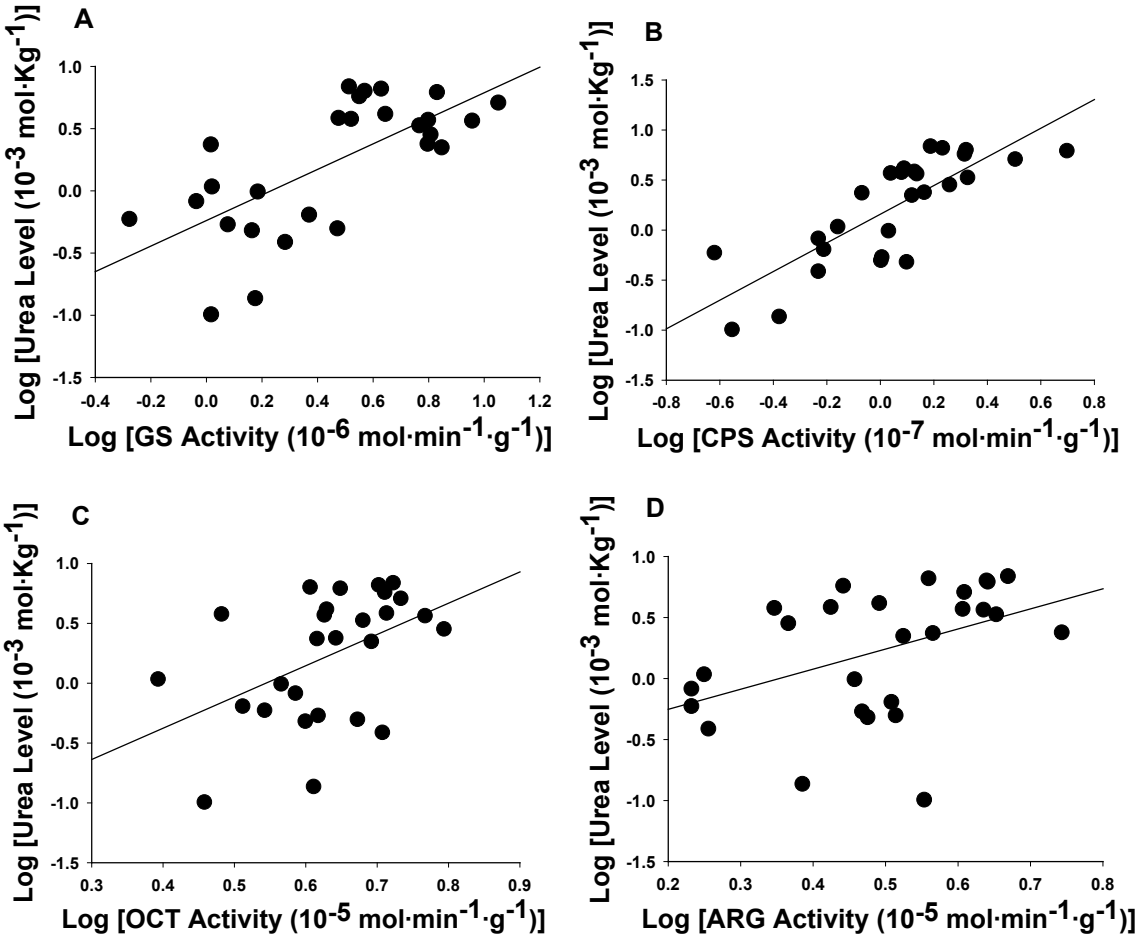


Figure 4.4. Linear regressions of log transformed data for liver urea levels to liver enzyme activities for GS, CPS, OCT and ARG. (A) The equation of the GS regression line and significance of the correlation are y = 1.027x - 0.239, R^2 (Adj) = 0.450, *P* <0.001. (B) The equation of the CPS regression 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.

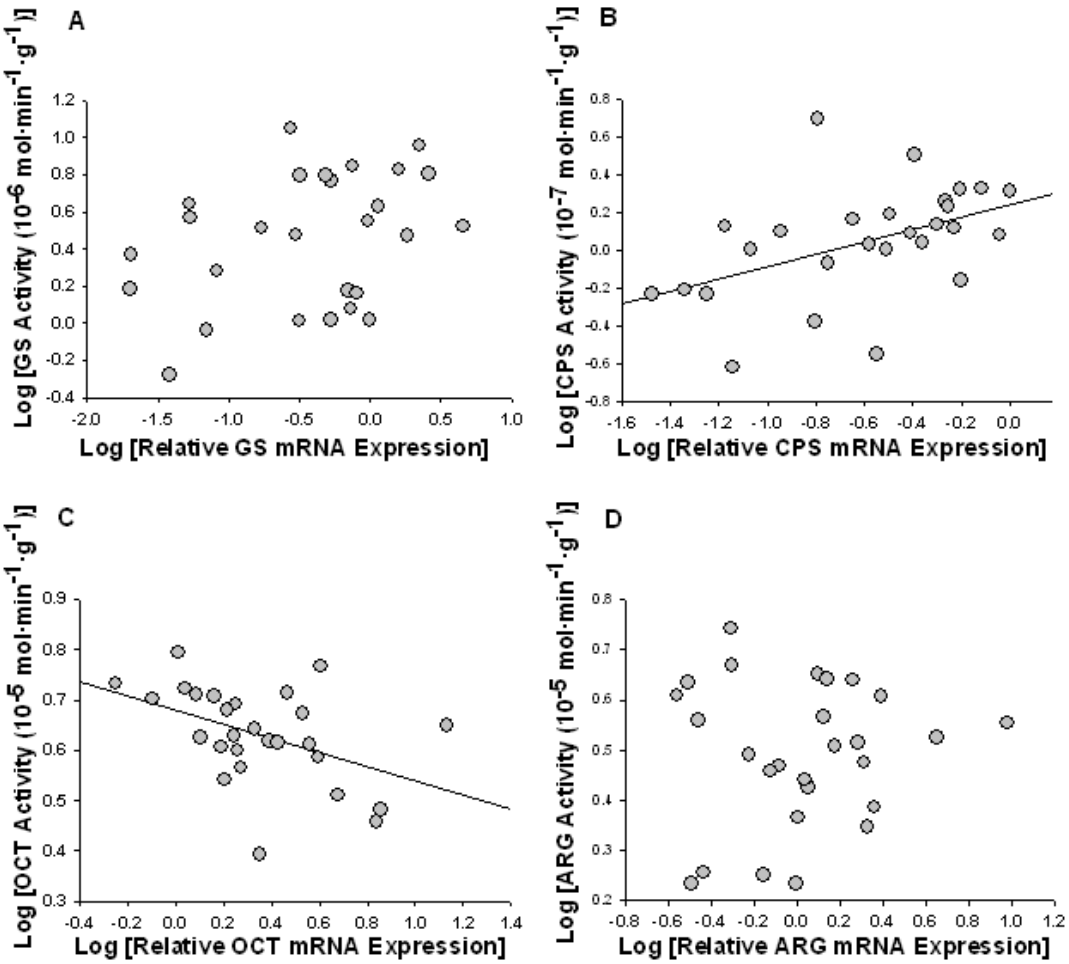


Figure 4.5. Linear regressions of log transformed data for liver mRNA expression and enzyme activity of O-UC enzymes. Only two of the O-UC enzymes tested, CPS and OCT, had a significant relationship ($P < 0.05$) to their mRNA expression. (A) GS mRNA expression versus GS activity, $P = 0.073$. (B) CPS mRNA expression compared to 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) ARG mRNA expression compared to ARG activity, $P = 0.723$.

Chapter 5: Summary and Conclusions

General conclusions

The focus of this thesis was to explore molecular aspects of nitrogen metabolism in fishes with a particular emphasis on the evolution of carbamoyl phosphate synthetase (CPS) and enzyme activity and mRNA expression of ornithine-urea cycle enzymes (O-UC). The information presented in the preceding chapters highlights the differences in nitrogen metabolism with respect to metabolites, O-UC mRNA expression and enzyme activity between two groups of fishes, tetrapod and teleosts.

In chapter two of this thesis I explored the origins of mitochondrial isoforms of CPS type I and type III within the CPS gene family using phylogenetic analyses to determine where the isoforms separated from CPS II. I used nucleotide sequence information obtained from a 522 base pair (bp) fragment of the CPS isolated from hepatic tissue of five species of lungfishes. Multiple phylogenetic analyses of this data showed the CPS I/III clade arose from a CPS II type isoform. This data also showed that a monophyletic lungfish branch was formed within the CPS I clade indicating that the CPS fragment in lungfishes had more shared derived characters with the tetrapod CPS I than with the teleost CPS III. The CPS enzyme is separated into a glutamine amidotransferase (GAT) domain and a synthetase domain with the glutamine binding site located in the GAT domain. Since the 522 bp fragment only represented a portion of the synthetase domain, I also examined a larger nucleotide fragment of CPS (2028 bp) from a representative species of lungfish, *P. annectens* that spanned a portion of both the GAT domain and the synthetase domain. Phylogenetic analyses of this larger fragment resulted in trees with similar structure to those constructed using the smaller CPS

fragments and also placed *P. annectens* within the CPS I clade. Therefore, I concluded that lungfishes possess CPS I as their mitochondrial isoform.

In chapter three of this thesis I explored the impact of two treatments on nitrogen metabolism in the West African lungfish, *P. annectens*: treatment one, six days of aerial exposure and treatment two, six days of aerial exposure followed by 24 hours re-immersion in fresh water. I determined hepatic ammonia and urea concentrations, levels of mRNA expression and activity of the O-UC enzymes in lungfish treated with aerial exposure and re-immersion. There was a coordinated induction of the enzymes of the O-UC both for mRNA expression and activity with a concurrent rise in urea levels which suggests that lungfish switch to ureotely during aerial exposure. When fish were re-immersed into freshwater for 24 hours after aerial exposure there was a rapid return to pre-treatment levels for enzyme activity and mRNA expression suggesting that lungfishes switch to ammonioteley once re-exposed to the aquatic condition. Since enzyme activities of O-UC enzymes were correlated to each other and to urea concentrations, I concluded that the rise in hepatic urea concentrations was directly related to the increased production of the urea cycle enzymes. Despite the high number of the O-UC enzymes being correlated to urea concentrations both for mRNA expression and enzyme activity, mRNA expression was only correlated to enzyme activity for two of the O-UC genes. I therefore suggested that post-transcriptional and post-translational factors and/or technical variance may also have contributed to this lack of correlation.

Surprisingly, I found both CPS I and CPS III activity present in the liver of *P. annectens*. Together with the phylogenetic evidence presented in chapter two, I believe that the CPS I isoform found in lungfish is capable of utilizing dual nitrogen substrates

(NH_3 and glutamine) and therefore suggest that there is only one mitochondrial isoform for CPS present in both aquatic and terrestrial vertebrates.

In chapter four of this thesis I explored the effects of crowding stress on hepatic nitrogen metabolite concentrations, O-UC enzyme activity and mRNA expression and plasma cortisol concentrations in a teleost fish, *Opsanus beta*. Plasma cortisol concentrations, hepatic ammonia and urea concentrations, liver O-UC mRNA expression and enzyme activity were determined. As predicted, there was a decrease in ammonia concentrations with a concurrent rise in urea and plasma cortisol concentrations with increased duration of crowding stress. Surprisingly, there was also a coordinated induction of O-UC enzyme activity that was correlated to urea concentrations. However, there was not a coordinated induction of mRNA expression of the O-UC genes and therefore I concluded that enzyme activity was a better predictor of urea production in toadfish. I also suggested that post-transcriptional and post-translational effects might be affecting the lack of correlations observed between O-UC activity and mRNA expression. I further suggested that the CPS enzyme acted as regulatory enzyme for the O-UC in toadfish since both its mRNA expression and enzyme activity alone could predict urea levels.

Future Directions

It is recommended that future studies on molecular aspects of nitrogen metabolism in fishes focus on continuing certain aspects of this thesis. For a more complete picture on CPS phylogeny in lungfishes I recommend completing the sequence for the CPS genes of

the lungfishes including *P. amphibicus*, which would allow a complete protein tree to be assembled for this significant transitional group. I would not expect the resultant phylogenetic trees to produce great new insights with regards to the monophyletic nature of the lungfishes group but that I would get a more complete CPS protein phylogeny within the Dipnoan clade.

I would also recommend purification of the lungfishes CPS protein and better characterization of this enzyme in these transitional fishes. If the mitochondrial form of CPS in *P. annectens* involved in the O-UC is capable of dual substrate use (ammonia and glutamine) during the production of urea, as I suspect it is, and requires the allosteric effector NAG, then it would be the first CPS I enzyme to be classified this way. This could lead to a change in the way CPS isoforms are classified and suggest that there are only two isoforms of this enzyme, one involved on the O-UC and one involved in pyrimidine biosynthesis.

Future studies on O-UC enzymes in fishes for either mRNA expression or enzyme activity should explore all of the enzymes within the pathway along with GS. At the beginning of this dissertation I naively thought that ASS and ASL did not contribute much to the O-UC. I would include them in any future studies because these studies showed that these enzymes contribute significantly to urea production in some fishes. Perhaps the lack of change in these enzymes in earlier studies, especially ASS, was due to their participation in other metabolic pathways (ie. the citrulline-nitric oxide cycle (C-NOC)) and/or feedback inhibition by urea. I would therefore suggest more research into the interaction of other pathways (C-NOC) with O-UC enzyme expression.

Regulation of gene expression is an area that needs to be explored in fishes and the O-UC. Presently our understanding of what regulates mRNA expression and enzyme activities for the O-UC is in its infancy with respect to the O-UC in fishes with little information available for promoters or transcription factors for O-UC genes. Understanding how RNA stability and protein stability affect gene expression and how the timing of transcription and translation can affect the levels of both enzyme activity and mRNA expression is important to our overall understanding of nitrogen metabolism in fishes.

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