Differential Gene Expression in the Brain of the African Lungfish, *Protopterus annectens*, after Six Days or Six Months of Aestivation in Air

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Abstract

The African lungfish, *Protopterus annectens*, can undergo aestivation during drought. Aestivation has three phases: induction, maintenance and arousal. The objective of this study was to examine the differential gene expression in the brain of *P. annectens* during the induction (6 days) and maintenance (6 months) phases of aestivation as compared with the freshwater control using suppression subtractive hybridization. During the induction phase of aestivation, the mRNA expression of prolactin (*prl*) and growth hormone were up-regulated in the brain of *P. annectens*, which indicate for the first time the possible induction role of these two hormones in aestivation. Also, the up-regulation of mRNA expression of tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein γ polypeptide and the down-regulation of phosphatidylethanolamine binding protein, suggest that there could be a reduction in biological and neuronal activities in the brain. The mRNA expression of cold inducible RNA-binding protein and glucose regulated protein 58 were also up-regulated in the brain, probably to enhance their cytoprotective effects. Furthermore, the down-regulation of prothymosin α expression suggests that there could be a suppression of transcription and cell proliferation in preparation for the maintenance phase. In general, the induction phase appeared to be characterized by reduction in glycolytic capacity and metabolic activity, suppression of protein synthesis and degradation, and an increase in defense against ammonia toxicity. In contrast, there was a down-regulation in the mRNA expression of *prl* in the brain of *P. annectens* during the maintenance phase of aestivation. In addition, there could be an increase in oxidative defense capacity, and up-regulation of transcription, translation, and glycolytic capacities in preparation for arousal. Overall, our results signify the importance of reconstruction of protein structures and regulation of energy expenditure during the induction phase, and the needs to suppress protein degradation and conserve metabolic fuel stores during the maintenance phase of aestivation.

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Introduction

Lungfishes are an archaic group of sarcopterygian fishes characterized by the possession of a lung opening off the ventral side of the esophagus. Sarcopterygians are recognized as “living fossils” whose evolutionary history dates back to the early Devonian period, some 390 million years ago. They hold an important position in the evolutionary tree with respect to land transition and their close phylogenetic relationships with tetrapods. There are six species of extant lungfishes, four of which (*Protopterus annectens*, *Protopterus amphibius*, *Protopterus aethiopicus* and *Protopterus dolloi*) can be found in Africa. African lungfishes are obligatory air-breathers; they typically inhabit fringing weedy areas of lakes and rivers where dissolved oxygen levels are low, daytime temperatures are high, and seasonal drying is common. During extended periods of drought, they enter into aestivation in mud cocoons [1,2]. Aestivation involves corporal torpor at high environmental temperature with absolutely no intake of food and water for an extended period. Among the four African species, *P. annectens* is known to be the most dependent on aestivation; it normally aestivates for periods of 7–8 months in the wild, and a captive lungfish has emerged from its cocoon after periods of up to seven years [3,4,5,6]. Recently, it has been reported that African lungfishes can be induced to aestivate in completely dried mucus cocoon in plastic boxes or in mud cocoon in the laboratory [7,8,9,10,11,12].

There are three phases of aestivation: induction, maintenance and arousal [1]. As water dries up, the fish hyperventilates and secretes large amounts of mucus which turns into a dry mucus cocoon within 6–8 days. This period constitutes the induction phase of aestivation, during which, the African lungfish has to detect environmental cues and turn them into some sort of internal signals that would instill the necessary changes at the behavioral, structural, physiological and biochemical levels in preparation for the maintenance phase of aestivation. After entering the maintenance phase, the fish has to prevent cell death and degradation of biological structures. At the same time, it has to suppress the utilization of internal energy stores, and to sustain a slow rate of
Gene Expression in the Brain of *P. annectens*

Materials and Methods

Collection and Maintenance of Fish

*Protopterus annectens* (80–120 g body mass) were imported from Central Africa through a local fish farm in Singapore. They were maintained in plastic aquaria filled with dechlorinated freshwater at pH 7.0 and at 25°C in the laboratory. Water was changed daily. No attempt was made to separate the sexes. Fish were acclimated to laboratory conditions for at least 1 month before experimentation. During the adaptation period, fish were fed with frozen fish meat and food was withheld 96 h prior to experiments. Approval to undertake this study was obtained from the Institutional Animal Care and Use Committee of the National University of Singapore (IACUC 035/09).

Experimental Conditions and Tissue Sampling

*Protopterus annectens* were induced to aestivate at 27–29°C and 85–90% humidity individually in plastic tanks (L29 cm × W19 cm × H17.5 cm) containing 15 ml of dechlorinated tap water (adjusted to 0.3% with seawater) following the procedure of Chew et al. [7]. During the induction phase of aestivation, the experimental fish would secrete plenty of mucus between day 5 and day 7 to form a mucus cocoon. Aestivation was hoped that results obtained would shed light on genes that were essential to the initiation, coordination and maintenance of the whole-body aestivation, and genes that were involved in the reduction in metabolism and the protection of biological structures in the brain of aestivating *P. annectens*.

Construction of SSH Libraries

Two sets of forward (up-regulated genes) and reverse (down-regulated genes) SSH libraries for the brain were generated using the PCR-Select™ cDNA subtraction kit (Clontech Laboratories, Inc., Mountain View, CA, USA); one set for fish aestivated for 6 days in air (induction phase) with reference to the freshwater control, and the other set for fish aestivated for 6 months in air (prolonged maintenance phase) with reference to the freshwater control. Two micrograms of poly (A) mRNA from each condition was used for cDNA synthesis. After the first and second strand synthesis, the double stranded cDNA from both groups were digested with Rsa I. A portion of the digested cDNA was ligated with either Adapter 1 or Adaptor 2R, and the rest was saved for subsequent usage as the driver for hybridization. The forward library was generated from the hybridization between adapter-ligated cDNA obtained from fish that had undergone 6 days or 6 months of aestivation in air (tester) and Rsa I-digested cDNA from the control fish kept in freshwater (driver). The reverse library was made the same way, except that the adapter-ligated cDNA from the control in freshwater served as the tester while the Rsa I-digested cDNA from fish aestivated for 6 days or 6 months of aestivation in air acted as the driver. The driver cDNA was added in excess to remove common cDNA by hybrid selection, leaving over-expressed and novel tester cDNAs to be recovered and...
cloned. The PCR amplification of the differentially expressed cDNAs was performed using the Advantage cDNA polymerase mix (Clontech Laboratories, Inc.) and 9002 Applied Biosystems PCR thermal cycler (Life Technologies Corporation, Carlsbad, CA, USA). The primary and secondary PCR amplification of these reciprocal subtractions of cDNA from the control and aestivated fish produced 1 forward and 1 reverse SSH libraries enriched in differentially expressed transcripts.

Differentially expressed cDNAs were cloned using pGEM®-T easy vector system kit (Promega Corporation, Madison, WI, USA), transformed into chemically competent JM109 Escherichia coli (Promega Corporation), and plated onto Luria-Bertani (LB) agar with ampicillin, 3-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and isopropyl β-D-thiogalactopyranoside (IPTG). Selected white colonies were grown overnight in LB broth with ampicillin. The plasmids were extracted using the resin-based plasmid miniprep kit (Axygen Biosciences, Union City, CA, USA). The dried samples were resuspended in water and amplification of the differentially expressed cDNAs was performed using the tBLASTx algorithm [17] and default search parameters. BLAST searches were performed using the tBLASTx algorithm [17] and default search parameters. BLAST searches were conducted to determine the genes that were up- and down-regulated, respectively, in the brain of P. annectens which had undergone 6 days of aestivation (induction phase) in air. A total of 130 genes were identified from these subtraction libraries. Interestingly, many more genes were up-regulated (80 genes; Table 2) than down-regulated (50 genes; Table 3) in the brain of P. annectens after 6 days of aestivation. There were 570 unidentified sequences which could be genes that have yet to be characterized in P. annectens. Tabulin alpha 4a (tabu4a) and some ribosomal protein mRNAs appeared in both forward and reverse

**Relative Quantitative Real-time PCR (qPCR)**

In order to validate the changes observed in the SSH libraries, seven genes were selected for the determination of mRNA expression using quantitative real-time PCR (qPCR). These include pyruvate kinase (ph), fumarate hydratase (fh), glutamine synthetase (gs), phosphofructokinase (pfk), prolactin (prl), Na+/K+-ATPase α2 (nka2), and ferritin heavy chain (fth). Prior to first strand cDNA synthesis, RNA from the brain of fish kept in freshwater, asevitated for 6 days in air or aestivated for 6 months in air were treated separately with ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and ethanol precipitation. The dried samples were resuspended in water and amplification of the differentially expressed cDNAs was performed using the tBLASTx algorithm [17] and default search parameters. BLAST searches were conducted to determine the genes that were up- and down-regulated, respectively, in the brain of P. annectens which had undergone 6 days of aestivation (induction phase) in air. A total of 130 genes were identified from these subtraction libraries. Interestingly, many more genes were up-regulated (80 genes; Table 2) than down-regulated (50 genes; Table 3) in the brain of P. annectens after 6 days of aestivation. There were 570 unidentified sequences which could be genes that have yet to be characterized in P. annectens. Tabulin alpha 4a (tabu4a) and some ribosomal protein mRNAs appeared in both forward and reverse

**Statistical Analysis**

Results for qPCR were presented as means ± standard errors of the mean (S.E.M.). Student’s t-test was used to evaluate the difference between means. Differences with \( P<0.05 \) were regarded as statistically significant.

**Results**

**Induction Phase (6 days) of Aestivation**

Two subtracted libraries, forward (Table 2) and reverse (Table 3), were constructed to determine the genes that were up- and down-regulated, respectively, in the brain of P. annectens which had undergone 6 days of aestivation (induction phase) in air. A total of 130 genes were identified from these subtraction libraries. Interestingly, many more genes were up-regulated (80 genes; Table 2) than down-regulated (50 genes; Table 3) in the brain of P. annectens after 6 days of aestivation. There were 570 unidentified sequences which could be genes that have yet to be characterized in P. annectens. Tabulin alpha 4a (tabu4a) and some ribosomal protein mRNAs appeared in both forward and reverse

**Table 1. Primers used for quantitative real-time PCR on fumarate hydratase (fh), ferritin heavy chain (fth), glutamine synthetase (gs), Na+/K+-ATPase α2 (nka2), phosphofructokinase (pfk), pyruvate kinase (pk) and prolactin (prl), with β-actin as the reference gene, from the brain of Protopterus annectens.**

| Gene       | Primer sequence (5’ to 3’) |
|------------|----------------------------|
| fh (JZ347458) | Forward (5’T-GTAGAACGACCTACATCCAC-3’) |
| fh (JZ347374) | Reverse (5’-GCTTGACCCTACTGACAACT-3’) |
| gh (GCAATCTGATCCTGTCAC-3’) |
| nka2 (JZ347474) | Forward (5’-AGACATCTGCGAAGCTTC-3’) |
| pk (JZ347497) | Reverse (5’-CATTACGCCCTCTTCAACCA-3’) |
| pk (JZ347493) | Reverse (5’-CGACATTGTCGCTTACCC-3’) |
| prl (JZ347487) | Forward (5’-CAACAGTTCACATCTACATCT-3’) |
| prl (JZ347487) | Reverse (5’-CTTACATTGACTGCGCCAAG-3’) |
| β-actin     | Reverse (5’-CAGTCTTGCGCTTCCAAAT-3’) |
| β-actin     | Reverse (5’-CAAGTCTGCGCTTCCAAAT-3’) |

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# Table 2. Known transcripts found in the forward library (up-regulation) obtained by suppression subtractive hybridization PCR from the brain of *Protopterus annectens* aestivated for 6 days in air with fish kept in freshwater as the reference for comparison.

| Group and Gene | *P. annectens* accession no. | Biological processes | E-value | No of clones |
|----------------|------------------------------|----------------------|---------|--------------|
| **Apoptosis**  |                              |                      |         |              |
| Plasminogen activator inhibitor 1 RNA-binding protein | JZ347480 | Regulation of anti-apoptosis | 3.00E-07 | 1 |
| **Carbohydrate metabolism** |                         |                      |         |              |
| Enolase | JZ347451 | Glycolysis | 4.00E-31 | 1 |
| Fructose-bisphosphate aldolase C | JZ347457 | Glycolysis | 4.00E-91 | 3 |
| Fumarate hydratase | JZ347458 | TCA cycle | 5.00E-30 | 2 |
| Pyruvate kinase | JZ347493 | Glycolysis | 9.00E-35 | 1 |
| **Cell cycle and proliferation** |                  |                      |         |              |
| BRCA2 and CDKN1A interacting protein | JZ347437 | Cell cycle and DNA repair | 2.00E-57 | 1 |
| Growth hormone precursor | JZ347464 | Positive regulation of growth | 0 | 2 |
| RAN, member RAS oncogene family | JZ347497 | Cell cycle | 3.00E-173 | 1 |
| Secreted acidic cysteine rich glycoprotein | JZ347521 | Regulation of cell proliferation | 1.00E-65 | 4 |
| **Lipoprotein, fatty acid and cholesterol homeostasis and transport** |                  |                      |         |              |
| c11or2 homolog | JZ347439 | Lipid transport | 8.00E-56 | 1 |
| **Ion binding and transport** |                         |                      |         |              |
| ADP/ATP translocase 2 putative | JZ347428 | Membrane transport | 8.00E-50 | 5 |
| Mitochondrial ATP synthase beta subunit | JZ347472 | ATP synthesis | 9.00E-35 | 5 |
| Sec61 beta subunit | JZ347520 | Membrane transport | 1.00E-64 | 2 |
| Solute carrier family 20, member 1b | JZ347524 | Phosphate transport | 3.00E-17 | 1 |
| Solute carrier family 25 alpha, member 5 | JZ347526 | Membrane transport | 1.00E-51 | 1 |
| **Iron metabolism and transport** |                  |                      |         |              |
| Apoferritin higher subunit | JZ347429 | Iron binding | 2.00E-88 | 1 |
| **Nitrogen metabolism** |                         |                      |         |              |
| Glutamine synthetase | JZ347462 | Glutamine biosynthetic process | 2.00E-23 | 2 |
| **Nucleic acid binding and transcription** |                  |                      |         |              |
| Brain abundant, membrane attached signal protein 1 | JZ347436 | Negative regulation of gene-specific transcription | 8.00E-18 | 2 |
| Breast carcinoma amplified sequence 2 | JZ347438 | RNA splicing | 1.00E-83 | 2 |
| Ctr9, Paf1/RNA polymerase II complex component | JZ347445 | Histone monoubiquitination | 0 | 1 |
| DEAH (Asp-Glu-Ala-His) box polypeptide 15 | JZ347449 | RNA splicing | 6.00E-07 | 1 |
| H3 histone, family 3B | JZ347379 | Nucleosome assembly | 3.00E-74 | 1 |
| High mobility group protein-1 | JZ347466 | Positive regulation of transcription | 9.00E-16 | 1 |
| Histone H2A.Z putative mRNA | JZ347467 | Nucleosome assembly | 3.00E-97 | 3 |
| p68 RNA helicase | JZ347477 | ATP dependent helicase activity | 7.00E-06 | 4 |
| Poly(A) binding protein, cytoplasmic 1 | JZ347481 | RNA splicing | 2.00E-54 | 2 |
| Polymerase (RNA) II (DNA directed) polypeptide J | JZ347482 | Transcription | 2.00E-36 | 3 |
| Small nuclear ribonucleoprotein E | JZ347523 | RNA splicing | 2.00E-64 | 2 |
| SRA stem-loop-interacting RNA-binding protein, mitochondrial precursor | JZ347527 | Regulation of transcription | 8.00E-38 | 1 |
| Tripartite motif protein 28 | JZ347537 | Regulation of transcription | 5.00E-19 | 1 |
| Y box binding protein 1 isoform 2 | JZ347547 | mRNA splicing | 6.00E-61 | 2 |
| **Protein degradation** |                         |                      |         |              |
| Proteasome (prosome macropain) subunit alpha type 4 | JZ347489 | Proteolysis | 5.00E-95 | 1 |
| **Protein synthesis, transport and folding** |                  |                      |         |              |
| 40S ribosomal protein S2 | JZ347424 | Translation | 3.00E-65 | 2 |
| 40S ribosomal protein S30 | JZ347425 | Translation elongation | 8.00E-37 | 4 |
| Amyloid beta (A4) precursor protein | JZ347354 | Protein transport | 6.00E-48 | 5 |
| Eukaryotic translation elongation factor 1 alpha 2 | JZ347371 | Translation | 4.00E-25 | 40 |
| Group and Gene | P. annectens accession no. | Biological processes | E-value | No of clones |
|----------------|-----------------------------|----------------------|---------|--------------|
| Eukaryotic translation elongation factor 1 beta 2 | JZ347452 | Translation | 9.00E-54 | 1 |
| Eukaryotic translation initiation factor 3, subunit 3 gamma | JZ347454 | Translation | 2.00E-28 | 1 |
| Lipocalin | JZ347384 | Protein transport | 1.00E-23 | 28 |
| Prefoldin subunit 2 | JZ347483 | Protein binding | 1.00E-31 | 1 |
| RAB36, member RAS oncogene family | JZ347495 | Protein transport | 5.00E-11 | 1 |
| Ribosomal protein L12 | JZ347505 | Translation | 7.00E-74 | 5 |
| Ribosomal protein L3 fragment 1 | JZ347500 | Translation | 2.00E-35 | 2 |
| Ribosomal protein L35 | JZ347509 | Translation | 7.00E-41 | 2 |
| Ribosomal protein L41 | JZ347510 | Translation | 3.00E-21 | 1 |
| Ribosomal protein L4-like | JZ347502 | Translation | 5.00E-133 | 2 |
| Ribosomal protein L5 | JZ347503 | Ribosome assembly | 0 | 1 |
| Ribosomal protein L7a-like | JZ347392 | Ribosome biogenesis | 9.00E-78 | 8 |
| Ribosomal protein S12 | JZ347516 | Translation | 3.00E-36 | 2 |
| Ribosomal protein S27a | JZ347519 | Translation | 5.00E-97 | 3 |
| Ribosomal protein S2e | JZ347511 | Translation | 1.00E-75 | 1 |
| Ribosomal protein S3 | JZ347512 | Translation | 0 | 2 |
| Ribosomal protein S4 | JZ347514 | Translation | 0 | 8 |
| Transmembrane protein 11 | JZ347536 | Protein binding | 2.00E-17 | 2 |
| **Response to stimulus** | | | | | |
| Cold-inducible RNA-binding protein | JZ347442 | Response to stress | 4.00E-33 | 3 |
| Ganglioside-induced differentiation-associated protein 1-like 1 | JZ347460 | Response to retinoic acid | 2.00E-29 | 2 |
| **Signaling** | | | | | |
| Prolactin | JZ347487 | Lactation, positive regulation of cell proliferation | 5.00E-14 | 15 |
| RAC/CDC42 exchange factor | JZ347496 | Regulation of Rho protein signal transduction | 2.00E-39 | 1 |
| RAS-like, family 11, member B | JZ347498 | Signal transduction | 1.00E-25 | 2 |
| Reticulon 1-C.1 | JZ347499 | Signal transduction | 2.00E-04 | 1 |
| **Structural** | | | | | |
| Tubulin alpha-1 chain putative | JZ347538 | Cell structure | 9.00E-170 | 9 |
| Tubulin, alpha 4a | JZ347416 | Cell structure | 0 | 11 |
| Tubulin, beta | JZ347540 | Cell structure | 7.00E-44 | 1 |
| Tubulin, beta 5 | JZ347541 | Cell structure | 5.00E-164 | 1 |
| **Others** | | | | | |
| ATG7 autophagy related 7 homolog | JZ347431 | Autophagy | 2.00E-44 | 1 |
| Cardiac muscle alpha actin 1 | JZ347440 | Unclassified | 2.00E-07 | 1 |
| Cell cycle associated protein 1b | JZ347441 | Unclassified | 2.00E-12 | 2 |
| Creatine kinase, mitochondrial 1 | JZ347444 | ATP binding | 3.00E-150 | 1 |
| Dynamin 1-like | JZ347450 | GTP catabolic process | 2.00E-26 | 6 |
| Fumarylacetoacetate hydrolase | JZ347459 | Cellular amino acid metabolic process | 8.00E-10 | 2 |
| Glucose regulated protein, 58 kDa | JZ347461 | Unclassified | 1.00E-40 | 1 |
| HMP19 protein | JZ347468 | Unclassified | 6.00E-43 | 2 |
| Myosin, light chain 1, alkali; skeletal, fast, | JZ347473 | Muscle contraction | 4.00E-27 | 1 |
| Nasopharyngeal epithelium specific protein 1 | JZ347475 | Unclassified | 1.00E-46 | 2 |
| Ornithine decarboxylase antizyme | JZ347476 | Cell differentiation | 2.00E-137 | 3 |
| Prolyl 4-hydroxylase, alpha polypeptide II | JZ347488 | Unclassified | 1.00E-25 | 2 |
| Quinoid dihydropteridine reductase | JZ347494 | Metabolic process | 2.00E-19 | 1 |
| Stromal cell derived factor receptor 1 | JZ347529 | Unclassified | 1.00E-39 | 6 |
subtraction libraries, indicating that they could be false positives or they encode for different isoforms of the same protein.

Results obtained from the forward library (Table 2) demonstrated for the first time that the mRNA expression of certain genes related to signaling (prl), cell cycle and proliferation (growth hormone [gh]), and transcription in general were up-regulated in the brain of P. annectens after 6 days of aestivation in air. A number of ribosomal genes that was involved in protein synthesis were also up-regulated. Other up-regulated genes included those involved in carbohydrate metabolism (enolase [eno]), fructose-bisphosphate aldolase C (alddc), andb, andiron metabolism. The fewer genes that were down-regulated in the brain of P. annectens after 6 days of aestivation in air (Table 3) included genes involved in cell cycle, protein synthesis, protein degradation, ion binding and transport (nhk2 and Na+/K+-ATPase beta1a [nhk1a]), and oxidation reduction [cytochrome c oxidase subunit Vla polyptpe 1 (cox6a1)]. There was also a down-regulation in the mRNA expression of some genes related to carbohydrate metabolism (prl and glyceraldehyde 3-phosphate dehydrogenase [gpdh]).

Maintenance Phase (6 Months) of Aestivation

Forward (Table 4) and reverse (Table 5) libraries were also constructed to reflect the genes that were up- and down-regulated, respectively, in the brain of P. annectens after 6 months of aestivation in air. Unlike the brain of P. annectens that had undergone 6 days of aestivation in air, only a total of 81 genes were identified from these subtraction libraries. Again, the forward library showed that more (63) genes were up-regulated (Table 4) while the reverse library showed that only 18 genes were down-regulated (Table 5). Out of the 1000 sequences obtained, 458 sequences were unidentified and they could again be genes that have yet to be characterized in P. annectens. Ribosomal protein L19 appeared in both forward and reverse subtraction libraries, indicating that it could be a false positive or it could be encoding for different isoforms of the same protein in the 2 libraries.

Unlike the induction phase, some genes involved in protein synthesis, iron metabolism, carbohydrate metabolism (e.g., pk), and lipid metabolism appeared in the forward library (Table 4) indicating that their mRNA expression were up-regulated in the brain of P. annectens after 6 months of aestivation in air. As for the reverse library (Table 5), genes that were down-regulated included prl and others involved in cell structure, iron metabolism (e.g., fth), and nucleic acid binding.

Confirmation of Up- or Down-regulation of Selected Genes using qPCR

In agreement with the SSH results of the brain of P. annectens after 6 days of aestivation in air, there were significant increases in the mRNA expression of pk, gs, fh, and prl (Fig. 1A-D) and significant decreases in the mRNA expression of gpdh (Fig. 1E) and nhk2 (Fig. 1F). Also, there was a significant increase in the mRNA expression of pk (Fig. 1G) in the brain of P. annectens after 6 days of aestivation in air and significant decreases in the mRNA expression of prl (Fig. 1H) and fth (Fig. 1I) in corroboration of the SSH results.

Discussion

More Genes are Up-regulated than Down-regulated during the Induction and Maintenance Phases of Aestivation

Surprisingly, many more genes were up-regulated than down-regulated in the brain of P. annectens during the induction and maintenance phases of aestivation, although one would expect exactly the opposite in a situation of metabolic down-regulation [19]. From the physiological point of view, aestivation has often been traditionally associated with metabolic depression [20], because conservation of metabolic fuels has been regarded as an important adaptation during long periods of aestivation without food intake. Furthermore, strong global suppression of gene expression is an integral part of metabolic rate depression in various hypometabolic systems that have been studied to date [19]. However, while the association between aestivation and metabolic depression is clearly present in endothermic mammals during aestivation, it is debatable whether it can be universally applied to aestivating ectothermic animals [1]. For instance, whether metabolic depression in turtles is an adaptation to aestivation per se or simply a response to fasting [21,22] remains an open question. In fact, the decrease in oxygen consumption in laboratory-aestivating yellow mud turtle, Kinosternon flavescens, is identical to that of fully hydrated turtles that are fasted for an equivalent period [23,24]. Based on studies mainly on P. aestuarii, it has long been accepted that a profound decrease in metabolic rate occurs in African lungfishes in general during the maintenance phase of aestivation in a mud cocoon or an artificial substratum [25,26], without reference to whether aestivation takes place in hypoxia or normoxia. However, Perry et al. [27] reported that P. dolloi exhibited constant rates of O2 consumption before (0.95±0.07 mmol kg⁻¹ h⁻¹), during (1.21±0.32 mmol kg⁻¹ h⁻¹) and after (1.14±0.14 mmol kg⁻¹ h⁻¹) extended periods (1–2 months) of aestivation in a completely dried mucus cocoon in air (normoxia). Subsequently, Loong et al. [10] obtained results which suggested that metabolic depression in aestivating African lungfish was triggered by hypoxia and not an integral part of aestivation.

In the past, the occurrence of organic structural modifications in aestivating animals has been largely neglected, but to date, aestivation in African lungfishes is known to be associated with structural and functional modifications in at least the heart and the kidney [20,29,30]. Icardo et al. [28] reported that the myocytes in the trabeculae associated with the free ventricular wall of P. dolloi showed structural signs of low transcriptional and metabolic activity (heterochromatin, mitochondria of the dense type) while in water [28]. These signs are partially reversed in aestivating fish (euchromatin, mitochondria with a light matrix), and paradox-
Table 3. Known transcripts found in the reverse library (down-regulation) obtained by suppression subtractive hybridization PCR from the brain of *Protopterus annectens* aestivated for 6 days in air with fish kept in freshwater as the reference for comparison.

| Group and Gene | *P. annectens* accession no. | Biological processes | E-value | No of clones |
|----------------|-------------------------------|----------------------|---------|--------------|
| **Carbohydrate metabolism** | | | | |
| Glyceraldehyde-3-phosphate dehydrogenase | JZ347463 | Glycolysis | 6.00E-22 | 1 |
| Phosphofructokinase | JZ347479 | Glycolysis | 4.00E-163 | 7 |
| **Cell cycle and proliferation** | | | | |
| Protein phosphatase 1 (pp1) gamma 1 | JZ347490 | Cell cycle | 2.00E-172 | 11 |
| SUMO-conjugating enzyme UBC9 | JZ347531 | Cell cycle | 4.00E-147 | 5 |
| **Cell structure** | | | | |
| Actin, gamma 1 | JZ347426 | Cell structure | 3.00E-70 | 4 |
| Transmembrane protein 2 | JZ347535 | Integral to membrane | 1.00E-49 | 2 |
| Tubulin, alpha 4a | JZ347539 | Microtubule-based process | 9.00E-16 | 1 |
| UPF0466 protein C22orf32, mitochondrial | JZ347545 | Integral to membrane | 1.00E-12 | 3 |
| **Complement** | | | | |
| Complement component factor H | JZ347443 | Complement activation | 6.00E-07 | 2 |
| **DNA repair and protection** | | | | |
| Activity-dependent neuroprotector homeobox | JZ347427 | Neuroprotection | 2.00E-16 | 3 |
| Apoptosis-inducing, TAF9-like domain 1 | JZ347430 | DNA repair | 2.00E-16 | 1 |
| **Ion binding and transport** | | | | |
| ATP synthase, H⁺ transporting, mitochondrial Fₒ complex, subunit c-3 | JZ347432 | ATP synthesis coupled proton transport | 1.00E-41 | 1 |
| ATP synthase, H⁺ transporting, mitochondrial Fₒ complex, subunit f | JZ347433 | ATP synthesis coupled proton transport | 6.00E-33 | 2 |
| ATPase, Na⁺/K⁺ transporting, beta 1a polypeptide | JZ347434 | Ion transport | 1.00E-09 | 1 |
| Na⁺/K⁺-ATPase alpha 2 subunit | JZ347474 | Cation transport | 0 | 3 |
| Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3 | JZ347525 | Transmembrane transport | 1.00E-30 | 2 |
| Voltage-dependent anion-selective channel protein 2 | JZ347546 | Anion transport | 8.00E-132 | 1 |
| **Iron, copper metabolism and transport** | | | | |
| Beta-2-globin | JZ347435 | Oxygen transport | 7.00E-05 | 2 |
| **Lipoprotein, fatty acid and cholesterol homeostasis and transport** | | | | |
| Lipoyltransferase 1 | JZ347471 | Protein lipoylation | 9.00E-27 | 3 |
| Sulfotransferase family 4A, member 1 | JZ347530 | Lipid metabolic process | 1.00E-72 | 1 |
| **Nucleic acid binding and transcription** | | | | |
| KH domain containing, RNA binding, signal transduction associated 1b | JZ347469 | RNA binding | 3.00E-05 | 5 |
| Protein phosphatase 1A magnesium-dependent, alpha isoform | JZ347491 | Regulation of transcription | 4.00E-79 | 1 |
| Prothymosin, alpha | JZ347492 | Transcription | 4.00E-24 | 13 |
| Staufen 1 | JZ347528 | Intracellular mRNA localization | 2.00E-35 | 3 |
| Transcription elongation factor B polypeptide 2 | JZ347533 | Regulation of transcription | 6.00E-40 | 1 |
| Translin | JZ347534 | DNA binding | 3.00E-71 | 1 |
| **Protein degradation** | | | | |
| Ubiquitin conjugating enzyme E2 | JZ347544 | Protein degradation | 2.00E-90 | 9 |
| Serpin peptidase inhibitor, clade B (ovalbumin), member 1, like 3 | JZ347522 | Serine type endopeptidase inhibitor activity | 1.00E-08 | 1 |
| **Protein synthesis, transport and folding** | | | | |
| Ribosomal protein L3 fragment 2 | JZ347501 | Translation | 2.00E-28 | 1 |
| DEAD (Asp-Glu-Ala-Asp) box polypeptide 21 | JZ347448 | Ribosomal RNA biogenesis, editing, transport | 4.00E-05 | 4 |
| Eukaryotic translation elongation factor 2 | JZ347453 | Translation | 2.00E-39 | 1 |
cally, aestivation appears to trigger an increase in transcriptional and synthetic myocardial activities, especially at the level of the ventricular septum [28]. In addition, Ojeda et al. [28] demonstrated structural modifications in all the components of the renal corpuscle of aestivating *P. dolloi*. These changes can be reversed after arousal, indicating that the renal corpuscle is a highly dynamic structure capable of modifying its architecture in response to different phases of aestivation. Morphological down-regulation and quick restoration of morphology during the maintenance phase and arousal phase, respectively, also occur in the intestine of *P. annectens* [30]. Thus, aestivation cannot be regarded as the result of a general depression of metabolism, but it involves the complex interplay between up-regulation and down-regulation of diverse cellular activities, and aestivation would logically involve variations in rates of protein degradation and synthesis, reconstructing and regenerating cells and tissues during the induction and arousal phases, respectively, through a rapid protein turnover. Such a proposition is corroborated by the fact that a greater variety of genes were up-regulated than down-regulated in the brain of *P. annectens* during the induction and maintenance phases of aestivation. Whether increased mRNA expression would lead to increases in protein expression through increased translational activities is unclear at present.

**Induction Phase: Prl and Gh could be Involved in Inducing and Coordinating Aestivation**

PRL, GH and somatolactin are three pituitary hormones whose genes are considered to have evolved from a common ancestral gene [31]. PRL affects a number of physiological processes and among them are the control of mammary gland development, initiation and maintenance of lactation, immune modulation, osmoregulation, control of hypothalamic releasing-inhibiting factors, and behavioral modification. At the cellular level, PRL exerts mitogenic, morphogenic, and secretory activities. In fish, the major function of Prl is osmoregulation in freshwater to prevent the loss of Na⁺ [32], and in terrestrial adaptation [33]. The up-regulation in the mRNA expression of *prl* in the brain of *P. annectens* during the induction phase of aestivation (Table 2) indicate for the first time that Prl might have an important role in the aestivation process, although the mechanism is unknown at present.

A number of endogenous hormonal substances have been proposed to be involved in initiating and maintaining aestivation [34]. These substances are often related to sleep e.g., Gh, somatostatin and arginine vasotocin. In human, GH secretion was associated with sleep and the maximal GH levels occur within minutes of the onset of slow wave sleep [35]. Somatostatin is a cyclic tetradecapeptide that acts as a negative regulator for the secretion of growth hormone. It is synthesized as a large precursor molecule before it is processed into its active form. In the brain of the European hamster, *Cricetus cricetus*, somatostatin levels were reported to be significantly lower in hibernating winter specimens than euthermic winter animals [36]. The novel findings on the down-regulation of *preprosomatostatin 2* (Table 3), together with the up-regulation of *gh* (Table 2) in the brain of *P. annectens* during the

### Table 3. Cont.

| Group and Gene | *P. annectens* accession no. | Biological processes | E-value | No of clones |
|---------------|------------------------------|----------------------|---------|--------------|
| Eukaryotic translation initiation factor 4E-binding protein 2 | JZ347455 | Translation initiation | 4.00E-09 | 9 |
| Procollagen C-endopeptidase enhancer 2 | JZ347468 | Heparin/protein binding | 1.00E-23 | 1 |
| Ribosomal protein L22-like 1 | JZ347506 | Translation | 4.00E-79 | 1 |
| Ribosomal protein L31 | JZ347507 | Translation | 3.00E-88 | 3 |
| Ribosomal protein L34 | JZ347508 | Translation | 6.00E-88 | 1 |
| Ribosomal protein L7a-like | JZ347504 | Ribosome biogenesis | 2.00E-101 | 3 |
| Ribosomal protein S13 | JZ347517 | Ribosome assembly | 5.00E-105 | 1 |
| Ribosomal protein S20 | JZ347518 | Translation | 6.00E-85 | 1 |
| Ribosomal protein S3A | JZ347513 | Translation | 5.00E-146 | 1 |
| Ribosomal protein S9 | JZ347515 | Translation | 4.00E-122 | 10 |

**Response to stimulus**

| Cyclic AMP-regulated phosphoprotein, 21 kDa | JZ347446 | Cellular response to heat | 4.00E-22 | 9 |
| FK506 binding protein 1B, 12.6 kDa | JZ347456 | Regulation of heart rate, response to glucose stimulus | 7.00E-68 | 4 |

**Signaling**

| Guanine nucleotide binding protein (G protein), alpha activating activity polypeptide O | JZ347465 | G-protein coupled receptor protein signaling pathway | 2.00E-04 | 1 |
| Phosphatidylethanolamine binding protein | JZ347478 | Regulation of mitosis and signaling | 4.00E-52 | 1 |

**Others**

| Cytochrome c oxidase subunit Vla polypeptide 1 | JZ347447 | Oxidation reduction | 1.00E-27 | 14 |
| KH domain-containing transcription factor B3 | JZ347470 | Unclassified | 4.00E-10 | 3 |
| Preprosomatostatin | JZ347464 | Unclassified | 5.00E-32 | 1 |
| Preprosomatostatin 2 | JZ347485 | Regulation of cell migration | 3.00E-164 | 5 |
| Telomere associated repeat sequence | JZ347532 | Unclassified | 2.00E-20 | 1 |

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Table 4. Known transcripts found in the forward library (up-regulation) obtained by suppression subtractive hybridization PCR from the brain of *Protopterus annectens* aestivated for 6 months in air with fish kept in freshwater as the reference for comparison.

| Group and Gene | *P. annectens* accession no. | Biological processes                  | E-value   | No of clones |
|----------------|-------------------------------|--------------------------------------|-----------|-------------|
| **Antioxidant** |                               |                                      |           |             |
| Superoxide dismutase | JZ347414          | Antioxidant                          | 5.00E-34  | 1           |
| **Apoptosis**      |                               |                                      |           |             |
| Tumor protein, translationally-controlled 1 | JZ347418          | Anti-apoptosis                        | 2.00E-53  | 2           |
| **Carbohydrate metabolism** |                       |                                      |           |             |
| Alpha enolase-1 | JZ347353          | Glycolysis                            | 1.00E-48  | 1           |
| Enolase 2         | JZ347369          | Glycolysis                            | 2.00E-71  | 4           |
| Glyceraldehyde-3-phosphate dehydrogenase | JZ347377         | Glycolysis                            | 5.00E-161 | 4           |
| Pyruvate kinase   | JZ347390          | Glycolysis                            | 1.00E-56  | 4           |
| **Cell structure** |                               |                                      |           |             |
| Actin-related protein 2/3 complex subunit 3 | JZ347351          | Actin polymerization                  | 4.00E-131 | 2           |
| Beta actin        | JZ347358          | Cell structure                        | 1.00E-174 | 1           |
| Calponin 3, acidic b | JZ347359          | Actomyosin structure organization     | 1.00E-09  | 1           |
| Cofilin-2 putative | JZ347362          | Cell structure                        | 3.00E-18  | 1           |
| Myosin light polypeptide 6 putative | JZ347385          | Muscle filament sliding               | 7.00E-19  | 1           |
| Tubulin beta-1 chain putative | JZ347415         | Microtubule-based movement            | 2.00E-50  | 1           |
| Tubulin, alpha 4a | JZ347416          | Microtubule-based movement            | 0         | 1           |
| Type I keratin isofrom 1 | JZ347419         | Cell structure                        | 1.00E-06  | 8           |
| Up-regulated during skeletal muscle growth protein 5 | JZ347420         | Integral to membrane                  | 8.00E-26  | 2           |
| **Ion binding and transport** |                       |                                      |           |             |
| Adenine nucleotide translocase | JZ347352          | ADP/ATP transport                      | 6.00E-129 | 4           |
| Calretinin putative mRNA | JZ347360          | Calcium ion binding                   | 3.00E-68  | 1           |
| Cysteine and histidine-rich domain (CHORD)-containing, zinc binding protein 1 | JZ347363         | Calcium ion binding                   | 1.00E-28  | 2           |
| voltage-dependent anion channel 2 | JZ347421          | Anion transport                       | 2.00E-69  | 2           |
| voltage-dependent anion channel 3 | JZ347422          | Anion transport, synaptic transmission | 5.00E-72  | 4           |
| **Iron metabolism and transport** |                       |                                      |           |             |
| Ferritin, middle subunit | JZ347375          | Cellular iron homeostasis, iron ion transport | 3.00E-57  | 1           |
| **Lipoprotein, fatty acid and cholesterol homeostasis and transport** |               |                                      |           |             |
| Apolipoprotein O | JZ347355          | Lipid transport                       | 1.00E-22  | 1           |
| **Nucleic acid binding and transcription** |                       |                                      |           |             |
| H3 histone, family 3B | JZ347379          | Nucleosome assembly                   | 1.00E-79  | 4           |
| H3 histone, family 3C | JZ347380          | Nucleosome assembly                   | 8.00E-64  | 1           |
| High mobility group B3b | JZ347381          | DNA binding                           | 1.00E-05  | 1           |
| Histone H3.3 putative | JZ347382          | Nucleosome assembly                   | 1.00E-61  | 1           |
| Polymerase (RNA) II (DNA directed) polypeptide G | JZ347389         | Transcription                         | 4.00E-143 | 1           |
| Splicing factor, arginine/serine-rich 11 | JZ347413          | RNA splicing                          | 6.00E-13  | 1           |
| **Protein degradation** |                       |                                      |           |             |
| Dipeptidylpeptidase 8 | JZ347364          | Proteolysis                           | 2.00E-39  | 2           |
| **Protein synthesis, transport and folding** |                       |                                      |           |             |
| Amyloid beta (A4) precursor protein | JZ347354          | Protein transport                     | 3.00E-69  | 2           |
| Elongation factor 1-alpha putative | JZ347366          | Translational elongation              | 2.00E-78  | 1           |
| Elongation factor 1-beta putative | JZ347367          | Translational elongation              | 3.00E-93  | 2           |
| Eukaryotic translation elongation factor 1 alpha | JZ347372          | Translational elongation              | 2.00E-75  | 3           |
| Eukaryotic translation elongation factor 1 alpha 1 | JZ347370          | Translational elongation              | 4.00E-91  | 7           |
| Eukaryotic translation elongation factor 1 alpha 2 | JZ347371          | Translational elongation              | 3.00E-107 | 32          |
induction phase of aestivation indicate the importance of these genes in the induction of aestivation.

**Induction Phase: Reduction in Biological Activity of the Brain**

The tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (14-3-3), α polypeptide, γ polypeptide and ε polypeptide are highly abundant in the brain. They are activators of tyrosine and tryptophan hydroxylases, which are also known as tyrosine 3-monooxygenase and tryptophan 5-monooxygenase, respectively [37]. These two hydroxylases are the initial and rate-limiting enzymes in the biosynthesis of dopamine and serotonin, respectively. Both dopamine and serotonin were involved in regulating sleep-waking cycle [38,39]. Moreover, through interaction with more than 100 binding partners, these proteins are crucial for various physiological cellular processes such as signaling, cell growth, division, adhesion, differentiation and apoptosis [37]. The up-regulation of the mRNA expression of tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein γ polypeptide (ywhag) in the brain of *P. annectens* during 6 days of aestivation (Table 2) might lead to the increased production of dopamine and serotonin, which might be important in regulating the biological activity of the brain.

| Group and Gene                                                                 | *P. annectens* accession no. | Biological processes                                      | E-value     | No of clones |
|-------------------------------------------------------------------------------|------------------------------|-----------------------------------------------------------|-------------|-------------|
| Eukaryotic translation elongation factor 1 gamma                               | JZ347373                     | Translational elongation                                   | 2.00E-27    | 3           |
| Glycyl-tRNA synthetase                                                        | JZ347378                     | Glycyl-tRNA aminoacylation, translation                    | 9.00E-68    | 1           |
| Lipocalin                                                                     | JZ347384                     | Protein transport                                          | 1.00E-23    | 7           |
| Ribosomal protein L10                                                         | JZ347394                     | Translation                                               | 2.00E-40    | 1           |
| Ribosomal protein L11                                                         | JZ347395                     | Translation                                               | 4.00E-130   | 6           |
| Ribosomal protein L12                                                         | JZ347505                     | Translation                                               | 6.00E-102   | 13          |
| Ribosomal protein L13                                                         | JZ347396                     | Translation                                               | 2.00E-76    | 3           |
| Ribosomal protein L19 fragment 1                                               | JZ347397                     | Translation                                               | 4.00E-104   | 2           |
| Ribosomal protein L21                                                         | JZ347399                     | Translation                                               | 3.00E-79    | 2           |
| Ribosomal protein L23                                                         | JZ347400                     | Translation                                               | 1.00E-130   | 5           |
| Ribosomal protein L26                                                         | JZ347401                     | Ribosome large subunit biogenesis                          | 6.00E-32    | 1           |
| Ribosomal protein L41                                                         | JZ347510                     | Translation                                               | 3.00E-04    | 10          |
| Ribosomal protein L7a-like                                                    | JZ347392                     | Ribosome biogenesis                                        | 1.00E-104   | 2           |
| Ribosomal protein Large P0                                                    | JZ347404                     | Translational elongation                                   | 2.00E-165   | 1           |
| Ribosomal protein P2                                                         | JZ347405                     | Translational elongation                                   | 4.00E-73    | 2           |
| Ribosomal protein S12                                                         | JZ347410                     | Translation                                               | 2.00E-40    | 5           |
| Ribosomal protein S20                                                         | JZ347411                     | Translation                                               | 1.00E-35    | 5           |
| Ribosomal protein S23                                                         | JZ347412                     | Translation                                               | 7.00E-49    | 5           |
| Ribosomal protein S3                                                          | JZ347406                     | Translation                                               | 0           | 2           |
| Ribosomal protein S4                                                          | JZ347514                     | Translation                                               | 0           | 3           |
| Ribosomal protein S5 putative                                                 | JZ347407                     | Translation                                               | 2.00E-21    | 1           |
| Ribosomal protein S7                                                          | JZ347408                     | Translation                                               | 0           | 4           |
| Ribosomal protein S8                                                          | JZ347409                     | Translation                                               | 2.00E-84    | 1           |
| Ribosomal protein S9                                                          | JZ347515                     | Translation                                               | 0           | 1           |
| **Response to stimulus**                                                       |                              |                                                           |             |             |
| Cold-inducible RNA binding protein                                            | JZ347442                     | Response to stress                                         | 3.00E-29    | 9           |
| FK506 binding protein 1B, 12.6 kDa                                             | JZ347456                     | Regulation of heart rate, response to glucose stimulus     | 1.00E-12    | 4           |
| **Others**                                                                    |                              |                                                           |             |             |
| Ribosomal protein 5S-like protein                                             | JZ347391                     | Unclassified                                              | 1.00E-54    | 12          |
| XMAB21                                                                        | JZ347423                     | Unclassified                                              | 2.00E-33    | 1           |

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Gene Expression in the Brain of *P. annectens*
Induction Phase: Cytoprotection through Increased Expression of Cold Inducible RNA-binding Protein and Glucose Regulated Protein 58

The cold inducible RNA-binding protein (CIRBP) is a member of a glycine-rich RNA binding protein family. It is known to be induced by cold stress [42]. In vitro studies have shown that the CIRBP has cytoprotective effects [43] and regulates neural development. Using cultured neural stem cells, Saito and co-workers [44] reported an increase in mRNA expression for CIRBP and the protein abundance at 32°C compared to those cultured at 37°C. In addition, the knockdown of this gene by RNA interference in neural stem cells showed an increase in apoptotic cell population, indicating the cytoprotective effect of this protein [44]. The up-regulation of cirbp mRNA was also reported when salmon was subjected to hyperosmotic stress during the transition from freshwater to marine environment [45]. Since P. annectens would encounter osmotic and dehydration stresses during the induction and maintenance phase of aestivation, the up-regulation of the mRNA expression of cirbp could enhance its cytoprotective effects in the brain of P. annectens during the aestivation process.

Glucose regulated protein 58 (GRP58) is a member of the protein disulfide isomerase family of proteins that are present mainly, but not exclusively, in the endoplasmic reticulum (ER). It was first identified as a stress protein in response to glucose deprivation and its well-characterized functions include acting as a molecular chaperone to help in the correct folding of glycoprotein [46] and the assembly of the major histocompatibility complex class 1. GRP58 serves as a carrier protein in Alzheimer’s disease to prevent the aggregation of β-amyloids [47]. Hence, the up-regulation of grp58 in the brain of P. annectens during the induction phase (6 days) of aestivation (Table 2) may prevent misfolding of proteins to preserve biological structures.

Table 5. Known transcripts found in the reverse library (down-regulation) obtained by suppression subtractive hybridization PCR from the brain of Protopterus annectens aestivated for 6 months in air with fish kept in freshwater as the reference for comparison.

| Group and Gene                      | P. annectens accession no. | Biological processes                                      | E-value    | No of clones |
|-------------------------------------|----------------------------|-----------------------------------------------------------|------------|--------------|
| Cell structure                       |                            |                                                            |            |              |
| Beta-tubulin                         | JZ347417                   | Cell structure                                            | 2.00E-67   | 1            |
| Myristoylated alanine-rich protein kinase C substrate | JZ347386                   | Cell structure                                            | 2.00E-10   | 3            |
| Ion binding and transport            |                            |                                                            |            |              |
| ATPase, H+ transporting, V0 subunit C | JZ347356                   | ATP synthesis coupled proton transport                     | 4.00E-25   | 4            |
| Basic leucine zipper and W2 domains 1| JZ347357                   | Regulation of transcription                               | 2.00E-05   | 1            |
| Iron metabolism and transport        |                            |                                                            |            |              |
| Ferritin heavy chain                 | JZ347374                   | Cellular iron homeostasis, iron ion transport              | 2.00E-75   | 1            |
| Protein synthesis, transport and folding |                      |                                                            |            |              |
| Coatomer protein complex, subunit gamma 2 | JZ347361                   | Intracellular protein transport, vesicle-mediated transport | 7.00E-23   | 104          |
| DnaJ (Hsp40) homolog, subfamily A, member 1 | JZ347365                   | Protein folding                                           | 4.00E-33   | 1            |
| Elongation factor-1, delta, b        | JZ347368                   | Translation elongation                                    | 4.00E-63   | 1            |
| Ribosomal protein L19 fragment 2     | JZ347398                   | Translation                                               | 2.00E-82   | 3            |
| Ribosomal protein L36a               | JZ347402                   | Translation                                               | 2.00E-62   | 2            |
| Ribosomal protein L38                | JZ347403                   | Translation                                               | 1.00E-32   | 1            |
| Ribosomal protein L8                 | JZ347393                   | Translation                                               | 6.00E-112  | 3            |
| Signaling                            |                            |                                                            |            |              |
| Prolactin                           | JZ347487                   | Lactation, positive regulation of cell proliferation      | 1.00E-20   | 43           |
| Others                              |                            |                                                            |            |              |
| Gephyrin                            | JZ347376                   | Establishment of synaptic specificity at neuromuscular junction | 0         | 1            |
| HN1-like protein                     | JZ347383                   | Unclassified                                              | 2.00E-05   | 4            |
| KH domain-containing transcription factor B3 | JZ347470                   | Unclassified                                              | 4.00E-08   | 146          |
| NADH dehydrogenase 1 alpha subcomplex subunit 13 | JZ347387                   | Electron transport chain                                   | 4.00E-45   | 3            |
| Peptidyl-glycine alpha-amidating monoxygenase A precursor | JZ347388                   | Peptide metabolic process, oxidation reduction            | 3.00E-28   | 1            |

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Induction Phase: Suppression of Proliferation of Brain Cells by Down-regulation of Prothymosin α

Prothymosin α (PTMAα) is a ubiquitously and abundantly expressed small nuclear protein [48,49,50] that is involved in cell proliferation [51] and protection against apoptosis [52,53]. Over-expression of PTMAα accelerates cell proliferation [54,55], whereas inhibition of PTMAα synthesis prevents cell division [56] and induces apoptosis [37]. Consistent with its properties, PTMAα is particularly abundant in tumor cells [58,59].
Mounting evidence suggests PTMA\textsuperscript{a} involvement in transcription regulation \cite{60,61,62}. Thus, the down-regulation of the mRNA expression of \textit{ptma} (Table 3) in the brain of \textit{P. annectens} during the induction phase of aestivation suggests that there could be a suppression of transcription and cell proliferation which is an essential preparation for the maintenance phase of aestivation.

Figure 1. qRT-PCR of selected genes that were differentially expressed based on suppression subtractive hybridization. Relative quantification of mRNA expression (fold change) of (A) pyruvate kinase (pk, JZ347493), (B) glutamine synthetase (gs, JZ347462), (C) fumarate hydratase (fh, JZ347458), (D) prolactin (prl, JZ347487), (E) phosphofructokinase (pfk, JZ347479), (F) Na\textsuperscript{+}/K\textsuperscript{+}-ATPase \textit{a}2 (nka\textit{a}2, JZ347474), (G) pyruvate kinase (pk, JZ347493), (H) prolactin (prl, JZ347487) and (I) ferritin heavy chain (fth, JZ347374), using \textit{\beta}-actin as the reference gene, in the brain of \textit{Protopterus annectens} aestivated for 6 days (d) (A–F) or 6 months (G–I) in air with reference to the those of fish kept in freshwater as control. Results represent mean±S.E.M. (\textit{N}=6). *Significantly different from the corresponding freshwater control (\textit{P}<0.05).

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Induction Phase: A Reduction in Glycolytic Capacity

During the induction phase of aestivation, the mRNA expression of several genes related to carbohydrate metabolism, e.g., eno, aldolc and pfk were up-regulated (Table 2), but there were also down-regulation in the mRNA expression of gapdh and pfk (Table 3) which happened to be the regulatory enzyme of glycolysis. The precise regulation of PFK prevents glycolysis and gluconeogenesis from occurring simultaneously. Therefore judging by changes in the mRNA expression of pfk, which in turns affects one of the regulatory steps in the glycolytic pathway, it would appear that there could be a decrease in the glycolytic capacity in the brain of P. annectens during the induction phase of aestivation. The changes in the mRNA expression of pfk might indicate the need to prevent an increase in gluconeogenesis in relation to a decrease in glycolysis, since the balance between these two processes would be controlled by Pk and phosphoenolpyruvate carboxykinase via the conversion between phosphoenolpyruvate and pyruvate.

The down-regulation in the mRNA expression of gapdh in the brain of P. annectens during the induction phase is consistent with the proposition that there could be a decrease in the glycolytic capacity. However, Gapdh has multiple functions in mammals and some of these may extend to fish. Zheng et al. [63] reported that GAPDH could itself activate transcription. The solute carrier binding protein coactivator in S phase (OC4-S) transcriptional coactivator complex contains GAPDH. GAPDH moves between the cytosol and the nucleus and may thus link the metabolic state to gene transcription [63]. Hara et al. [64] showed that GAPDH initiates apoptosis. GAPDH also appears to be involved in the vesicle transport from the ER to the Golgi apparatus which is part of the shipping route for secreted proteins [65]. Hence, a down-regulation in the mRNA expression of gapdh might also indicate a suppression of transcription, apoptosis and protein secretion, which could be regarded as adaptive responses, in the brain of P. annectens during the induction phase of aestivation.

Induction Phase: Reduction in Metabolic Activity

At present, there is a dearth of knowledge concerning metabolism of the brain of African lungfishes during aestivation, especially concerning the substrates used to supply energy for various metabolic processes. Even if there is a high glycogen store in the brain, it is a limited source of energy store. Hence, it is logical to deduce that the brain would still have to depend on glucose supply from the blood during aestivation. However, aestivating lungfish would have a slower heart beat rate [26,66], and hence a slow rate of blood circulation. Consequently, it would be essential for the brain to reduce its metabolic rate, including the rate of ATP synthesis through oxidative phosphorylation. Indeed, the down-regulation of ATP synthases indicates that ATP synthesis was reduced during aestivation. In general, metabolic rate reduction requires a coordinated suppression of the rate of cellular ATP turnover, including both ATP-generating and ATP-consuming reactions. Hence, the down-regulation of mRNA expressions of nkl92- and staf11a- subunits and voltage-dependent anion channel (vdac) 2 (vdac2) in the brain of P. annectens during the induction phase of aestivation (Table 3) corroborates the proposition that a reduction in metabolic activities in the brain could be essential to aestivation.

Induction Phase: Suppression of Protein Synthesis and Degradation

Protein synthesis is a major energy expense in cells and it will utilise up to 50% of the cellular energy during translation [67,68,69]. Although down-regulation of protein synthesis is a consistent phenomenon in organisms undergoing metabolic depression, translation of genes must become more selective in order to conserve energy but at the same time, allows critical functions to carry on during the subsequent maintenance phase of aestivation. A number of genes related to protein synthesis, transport and folding appeared in the forward and reverse libraries of P. annectens after 6 days of aestivation. Besides various ribosomal proteins, the mRNA expression of eukaryotic translation elongation factor (eef) 1α2 (40 clones) and eef1β2 (1 clone), and eef3;3 (1 clone) were up-regulated (Table 2), while those of eef2 (1 clone) and eukaryotic translation initiation factor 4E-binding protein 2 (9 clones) were down-regulated (Table 3), instead. Overall, these results indicate there could be simultaneous up- and down-regulation in the synthesis of certain proteins. This could be important for the brain to make the necessary preparation to enter into the maintenance phase of aestivation without an over expenditure of energy [1].

SUMO enzymatic cascade catalyzes the dynamic posttranslational modification process of SUMOylation. SUMOylation is a post-translational modification involved in various cellular processes, such as nuclear-cytoplasmic transport, transcriptional regulation, apoptosis, protein stability, response to stress, and progression through the cell cycle [70]. Protein phosphatase 1 (PP1) belongs to a certain class of phosphatases known as protein serine/threonine phosphatases, which control glycogen metabolism, muscle contraction, cell progression, neuronal activities, splicing of RNA, mitosis [72], cell division, apoptosis, protein synthesis, and regulation of membrane receptors and channels [73]. Therefore, the down-regulation of SUMO-conjugating enzyme Ubc9 prevents cell cycle progression at the G2 or early M phase, causing the accumulation of large budded cells with a single nucleus, a short spindle and replicated DNA in Xenopus oocyte [71]. Protein phosphatase 1 (PP1) belongs to a certain class of phosphatases known as protein serine/threonine phosphatases, which control glycogen metabolism, muscle contraction, cell progression, neuronal activities, splicing of RNA, mitosis [72], cell division, apoptosis, protein synthesis, and regulation of membrane receptors and channels [73]. Therefore, the down-regulation of SUMO-conjugating enzyme Ubc9 and pp1 suggests that there could be a suppression of protein degradation in the brain of P. annectens during the induction phase of aestivation. Only then, would there be preservation of protein structure and function during the aestivation process. In addition, the down-regulation of these two genes may be important in arresting the cell cycle and growth during the early stage of aestivation. Together, these adaptive responses would reduce energy expenditure and conserve the limited endogenous energy reserve.

Induction Phase: Defense Against Ammonia Toxicity

Gs catalyzes the formation of glutamine from ammonia and glutamate and the reaction requires ATP, and it is essential for the detoxification of ammonia to glutamine in fish [74,75]. Neural tissues are sensitive to ammonia, and thus Gs activity is high in most fish brains [76,77,78,79]. Four g isoforms have been identified in the rainbow trout Oncorhynchus mykiss [80], which are differentially expressed in the brain and liver of fish exposed to elevated environmental ammonia [81]. At present, it is uncertain whether Gs isoforms are present in African lungfishes. However, it is logical to deduce that Gs could be up-regulated in the brain of P. annectens to detoxify ammonia produced within the brain during aestivation. This is especially important considering the fact that aestivating lungfish would have a decrease in heart rate [26,66], and hence a decrease in blood circulation might slow down the removal of ammonia that was produced endogenously within the brain. Although, it is highly unlikely that amino acids were degraded for energy production in the brain; rather, it is probable that ammonia would be released from amino acid catabolism due to an increased turnover of protein synthesis and
protein degradation during the induction phase to prepare the brain to enter into the maintenance phase of aestivation.

**Maintenance Phase: Prolactin could be involved in Maintaining Whole-body Aestivation**

In contrast to the induction phase, there was a down-regulation in the mRNA expression of *prl* in the brain of *P. annectens* during the prolonged maintenance phase of aestivation (Table 3). A total of 43 clones (out of 500) of *prl* appeared in the reverse library, indicating that there could be a drastic suppression of *prl* expression which was supported by qPCR results. This corroborates the proposition that Prl could be an important part of the signaling mechanisms to induce and maintain aestivation in *P. annectens*.

**Maintenance Phase: Superoxide Dismutase was Involved in Oxidative Defense in the Brain**

Increased synthesis and/or activity of intracellular antioxidant enzymes can protect cellular macromolecules from potentially lethal stress-induced damage. The brain of hibernating squirrel is characterized by increased oxidative stress resistance [82,83,84]. Over-expression of superoxide dismutase (SOD), both mitochondrial (MnSOD) and cytosolic (CuZnSOD), increases oxidative stress resistance [85,86,87]. In addition, over-expression of SODs, glutathione peroxidase (GPx) and catalase (CAT) also act to protect against ischemia associated cytochrome c release from mitochondria, thereby limiting the occurrence of apoptotic cell death [88]. Similarly, Page et al. [89] found that most of the intracellular antioxidant enzymes, including the MnSOD, CuZnSOD, CAT, GPx and glutathione reductase were up-regulated in brain tissue of lungfish that had aestivated for 60 days. Therefore, Page et al. [89] concluded that aestivating *P. dolloi* had enhanced oxidative stress resistance in the brain due to a significant up-regulation of intracellular antioxidant capacity. Results obtained from this study are in agreement with the conclusion of Page et al. [89] because there was an up-regulation of the mRNA expression of *sod1* in the brain of *P. annectens* during the prolonged maintenance phase (6 months) of aestivation (Table 4).

**Maintenance Phase: Regulation of ATP Usage through an Up-regulation of vdac2**

VDACs are pore-forming proteins found in the outer mitochondria membrane of eukaryote. They are the major channels for metabolites to permeate the outer mitochondrial membrane. In mammals, they are known to play an essential role in cellular metabolism. They conduct ATP to bind to several cytosolic carbohydrate kinases, e.g. hexokinase, glyceral kinase and creatine kinase, and thus providing bound kinases with preferential access to mitochondrial ATP [90]. Hence, the up-regulation of *vdac2* and *vdac3* in the brain of *P. annectens* after 6 months of aestivation (Table 4) may reflect on the limited ATP supply by the mitochondria and the importance of transporting ATP to essential kinases on the outer mitochondria membrane to facilitate its usage by the relatively more important metabolic pathways.

**Maintenance Phase: Increased Glycolytic and Protein Synthesis Capacities in Preparation for Arousal?**

Six months of aestivation led to increases in mRNA expression of several enzymes involved in glycolysis (*enol1, enol2, gapdh and pkh*) in the brain of *P. annectens* (Table 4). Unlike the induction phase, both gapdh and pkh no longer appeared in the reverse library. Taken together, these results indicated that the glycolytic capacity in the brain of *P. annectens* varied between the induction and maintenance phases. Simply based on the SSH results, it is difficult to draw a definitive conclusion, but it would appear that there was an up-regulation of the glycolytic capacity during the prolonged maintenance phase of aestivation. Considering that the aestivating fish was in a state of torpor and the importance of conserving endogenous energy stores, this could be viewed as a strategy in preparation for arousal, and an increase in glycolytic capacity might not imply an increase in glycolytic rate.

In comparison with the induction phase, more genes related to protein synthesis, transport and folding appeared in the forward library for lungfish that had entered into the prolonged maintenance phase of aestivation. Specifically, 2 elongation factor 1 (ef1) genes (ef1a and ef1b), and 4 ef1 genes (ef1x, ef1x1, ef1x2 and ef1y) were up-regulated, with 32 clones of ef1x2 appearing in the forward library (Table 4). The increases in mRNA expression of these genes indicate that there could be an increase in the capacity for protein synthesis in the brain of *P. annectens*. This does not necessarily imply an increase in protein synthesis in situ, since the mRNA expression of coatomer protein complex subunit gamma 2 (cop2) was down-regulated with 104 clones (Table 5). Biosynthetic protein transport from the ER via the Golgi apparatus to the plasma membrane is mediated by vesicular carriers [91,92,93]. These vesicles, termed COPI-coated vesicles contain a fuzzy protein coat whose major coat protein components are ADP-ribosylation factor and coatomer, which is a protein complex made up of seven subunits [94,95]. Thus, a down-regulation of the mRNA expression of *cop2* suggested that the transport of protein, even if synthesized, was put on hold during the prolonged maintenance phase of aestivation, probably in preparation for arousal.

**Maintenance Phase: Up-regulation of Histone Expression**

Histones are the chief protein components of chromat in, acting as spools around which DNA winds, and play a role in gene regulation. Five major families of histones (*H*) exist, and *H3* is one of the core histones [96,97]. Acetylation and methylation of different lysine (Lys) and arginine residues in *H3* has been linked to either transcriptionally active or transcriptionally repressed states of gene expression [98], whereas phosphorylation of H3 was initially linked to chromosome condensation during mitosis [99,100]. The phosphorylation of H3 at serine 10 (Ser 10) has an important role in the transcriptional activation of eukaryotic genes in various organisms. Methylation of Lys9 in H3 (H3K9) is a prominent modification that has been implicated in diverse processes, including transcriptional silencing, heterochromatin formation, and DNA methylation [101]. H3 also plays a crucial role in activating the spindle assembly checkpoint in response to a defect in mitosis [102]. Therefore, the up-regulation of mRNA expression of *h3* in the brain of *P. annectens* after 6 months of aestivation (Table 4) indicates that there could be an increase in the capacity of transcription, which could be an adaptive response for increased mitosis for tissue repair upon arousal.

**Conclusion**

We have demonstrated by SSH PCR that during the induction phase of aestivation, several genes in the brain of *P. annectens* such as *prl*, *gh* and *preprosomatostatin 2* could be involved in inducing and coordinating aestivation. There could be a reduction in biological activities of the brain as an up-regulation of *yehat* and a down-regulation of *pehp* in the brain were observed during the induction phase of aestivation. Our results also revealed that several gene clusters were up- or down-regulated in the brain of *P. annectens* after 6 days of aestivation in air. These aestivation-specific genes
could be involved in a reduction in glycolytic capacity, metabolic activities and suppression of protein synthesis and degradation. By contrast there was a down-regulation of pol expression in the brain during the maintenance phase, corroborating the proposition that Pol plays an important role in coordinating aestivation in P. annectens. There was also an up-regulation of sod1 expression in the brain during the maintenance phase of aestivation. Sod is important for oxidative defense, and oxidative defence could be important for life maintenance during the maintenance phase of aestivation. Furthermore, there could be an increase in glycolytic and protein synthesis capacities during the maintenance phase of aestivation, and the up-regulation of h3 indicated an increase in transcription, which could be an adaptive response in preparation for the arousal phase of aestivation.

Author Contributions
Conceived and designed the experiments: YKI SF. Performed the experiments: KCH. Analyzed the data: KCH SF YKI. Contributed reagents/materials/analysis tools: WPW. Wrote the paper: SF CCH YKI. Took care of the animals: WPW.

References
1. Ip YK, Chew SF (2010) Nitrogen metabolism and excreration during aestivation. In: Navas CA, Carvalho JE, editors. Progress in molecular and subcellular biology Vol 49. Aestivation: Molecular and Physiological Aspects. Berlin: Springer-Verlag. 63–94.
2. Ballantyne JS, Frick NT (2010) Lungfish Metabolism. In: Jorgensen JM, Joss J, editors. The biology of lungfishes. New Hampshire: Science Publishers. 305–340.
3. Smith HW (1931) Observations on the African lungfish, Protopterus aethiopicus, and on evolution from water to land environments. Ecology 12: 164–181.
4. Greenwood PH (1986) The natural history of African lungfishes. J Morphol Suppl 1: 163–179.
5. Lomholt JP (1993) Breathing in the aestivating African lungfish, Protopterus aethiopicus. In: Singh BR, editor. Advances in Fish Research: Narendra, New Delhi. 17–51.
6. Johnels AG, Svensson GSO (1954) On the biology of Protopterus annectens. Aestivation: Molecular and Physiological Aspects. Berlin: Springer-Verlag. 63–94.
7. Chew SF, Chan NK, Loong AM, Hiong KC, Tam WL, et al. (2004) Nitrogen metabolism in the African lungfish (Protopterus dolloi) aestivating in a mucous cocoon on land. J Exp Biol 207: 777–786.
8. Ip YK, Yeo PJ, Loong AM, Hiong KC, Wong WP, et al. (2005) The interplay of increased urea synthesis and reduced ammonia production in the African lungfish Protopterus aethiopicus during 46 days of aestivation in a mucous cocoon. J Exp Zool A 303: 354–365.
9. Loong AM, Hiong KC, Lee SML, Wong WP, Chew SF, et al. (2005) Ornithine-urea cycle and urea synthesis in African lungfishes, Protopterus aethiopicus and Protopterus annectens, exposed to terrestrial conditions for six days. J Exp Zool A 303: 1034–1063.
10. Loong AM, Pang CYM, Hiong KC, Wong WP, Chew SF, et al. (2008) Increased urea synthesis and/or suppressed ammonia production in the African lungfish, Protopterus annectens, during aestivation in air or mud. J Comp Physiol B 178: 351–363.
11. Loong AM, Hiong KC, Wong WP, Chew SF, Ip YK (2012) Differential gene expression in the liver of the African lungfish, Protopterus aethiopicus, after 6 days of aestivation in air. J Comp Physiol B 182: 231–245.
12. Loong AM, Gao YR, Chew SF, Ip YK (2012) Molecular characterization and mRNA expression of carbamoyl phosphate synthetase III type A receptor alpha subunit. J Neurosci Res 80: 418–428.
13. Giusi G, Zizza M, Faciolo RM, Chew SF, Ip YK, et al. (2012) Aestivation and hypoxia-related events share common silent neuron trafficking processes. BMC Neurosci 13: 39.
14. Whitehead A, Crawford DL (2005) Variation in tissue-specific gene expression among natural populations. Genome Biol 6: R13.
15. Hall TA (1999) BioEdit: a user-friendly biological sequence editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41: 95–98.
16. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403–410.
17. Johnels A, Svensson GSO (1953) On the biology of Protopterus annectens. Aestivation: Molecular and Physiological Aspects. Berlin: Springer-Verlag. 63–94.
18. Monti JM, Jantos H (2008) The roles of dopamine and serotonin, and of their metabolites, during aestivation in the European hamster (Cricetus crocuta). Brain Res 1216: 123–125.
19. Sakamoto T, Iwata K, Ando M (2002) Growth hormone, prolactin and somatolactin: a structural overview. In: Hochachka PW, Mommsen TP, editors. Biochemistry and molecular biology of fishes: Elsevier Science Publishers, Amsterdam. 39–56.
20. Ayon FG, Kaneko T, Tagawa M, Hasegawa S, Grau EG, et al. (1993) Effects of acclimation to hypertonic environment on plasma and pituitary levels of two prolactins and growth hormone in two species of tilapia, Oreochromis mossambicus and Oreochromis niloticus. Comp Biochem Endocrinol 89: 130–148.
21. Fishman AP, Galante RJ, Winokur A, Pack AI (1992) Estivation in the African lungfish. P Am Philos Soc 136: 61–72.
22. Holl RW, Hartman ML, Veldhuis JD, Taylor WM, Thorner MO (1991) Thirty-second sampling of plasma growth hormone in man: correlation with sleep stages. J Clin Endocrinol Metab 72: 834–861.
23. Messori A, Felschka R, Malacarne V, Monti M, Petr P (1997) The somatostatin system of the brain and hibernation in the European hamster (Cricetus cricetus). Cell Tissue Res 288: 441–447.
24. Berg D, Holzmann C, Riess O (2003) 14-3-3 proteins in the nervous system. Nat Rev Neurosci 4: 752–762.
25. Monti JM, Santos H (2008) The roles of dopamine and serotonin, and of their receptors, in regulating sleep and waking. Prog Brain Res 172: 625–646.
26. Murrell-Rodriguez E, Arias-Carrion O, Sanguino-Rodriguez K, Gonzalez-Arias M, Haro R (2009) Mechanisms of sleep-wake cycle modulation. CNS Neuro Sci Drug Targets 8: 245–253.
27. Zeng L, Inamato A, Rosen MR (2008) Raf kinase inhibitory protein (RKIP): a physiological regulator and future therapeutic target. Expert Opin Ther Targets 12: 1275–1287.
28. Ojika K, Mitake S, Tohdoh N, Appel SH, Otsuka Y, et al. (2000) Hippocampal cholinergic neurostimulating peptides (HCNP). Prog Neurobiol 60: 37–83.
29. Bergner D, Beausoleil D, Bellmarr G (1993) Sequence and expression of a gene encoding a protein with RNA-binding and glycine-rich domains in Brassica napus. Biochim Biophys Acta 1263: 290–295.
30. Icardo JM, Loong AM, Colvce E, Wong WP, Ip YK (2012) The alimentary canal of the African lungfish Protopterus annectens during aestivation and after arousal. Anat Rec 295: 60–72.
31. Loong AM, Hiong KC, Tam WL, Wong WP, Chew SF, et al. (2005) Nitrogen metabolism in the African lungfish (Protopterus dolloi) aestivating in a mucous cocoon on land. J Exp Biol 207: 777–786.
73. Ip YK, Chew SF, Randall DJ (2001) Ammonia toxicity, tolerance, and excretion. In: Wright PA, Anderson PM, editors. Fish Physiology, Vol 20, Nitrigen excretion. Academic Press, San Diego. 109-148.

74. Webb JT, Brown GW (1976) Some properties and occurrence of glutamine synthetase in fish. Comp Biochem Physiol B 54: 171-175.

75. Chakravorty J, Saha N, Ratha BK (1989) A unique pattern of tissue distribution and subcellular localization of glutamine synthetase in a freshwater air-breathing teleost, Heteropneustes fossilis (Bloch). Biochem Int 19: 519-527.

76. Ker K, Chew SF, Lim CS, Kua HS, Kok WK, et al. (1994) The mudskipper Pimelophthalmus schincki and Bilophthalmus buddei can tolerate environmental NH3 concentrations of 446 and 36 µM, respectively. Fish Physiol Biochem 19: 59-69.

77. Wang YX, Wahl PJ (2000) High ammonia tolerance in fishes of the family Cyprinidae (Cypriniformes). Aquat Toxicol 50: 205-219.

78. Murray BW, Bushy ER, Monnsem TP, Wright PA (2003) Evolution of glutamine synthetase in vertebrates: multiple glutamine synthetase genes expressed in rainbow trout (Oncorhynchus mykiss). J Exp Biol 206: 1511-1521.

79. Wright PA, Steele SL, Huitema A, Bernier NJ (2007) Induction of four glutamine synthetase genes in brain of rainbow trout in response to elevated environmental ammonia. J Exp Biol 210: 2901-2905.

80. Landell SL, Kåhn SL, Pizzau TM, Mannijio MG, Torralba JR, et al. (2005) Natural resistance to liver cold ischemia-reperfusion injury associated with the hibernation phenotype. Am J Physiol Gastrointest Liver Physiol 288: G473-G480.

81. Dave KR, Prado R, Raval AP, Drew KL, Perez-Pinon MA (2006) The arctic ground squirrel brain in vivo: effects of injury from cardiac arrest during euthermia. Stroke 37: 1261-1265.

82. Christian SL, Ross AP, Zhao HW, Kristensen HJ, Zhan X, et al. (2008) Arctic ground squirrel (Spermophilus parryii) hippocampal neurons tolerate prolonged oxygen-glucose deprivation and maintain baseline ERK1/2 and JNK phosphorylation despite drastic ATP loss. J Cereb Blood Flow Metab 28: 1307-1319.

83. Murakami K, Kondo T, Epstein CJ, Chan PH (1997) Overexpression of Cu/Zn-superoxide dismutase reduces hippocampal injury after global ischemia in transgenic mice. Stroke 28: 1797-1801.

84. Shi X, Gai I, Ke V, Yang L, Qian S, et al. (2007) Manganese superoxide dismutase protects mouse cortical neurons from chronic intermittent hypoxia-mediated oxidative damage. Neurobiol Dis 28: 206-215.

85. Jiang YC, Perez VI, Song W, Lutgarten MS, Salmon AB, et al. (2009) Overexpression of Mn superoxide dismutase does not increase life span in mice. J Gerontol A Biol Sci Med Sci 64: 1114-1125.

86. Zemlyak I, Brooke SM, Singh MH, Sapolsky RM (2009) Effects of overexpression of antioxidant enzymes on the release of cytokine c and apoptosis-inducing factor in the model of ischemia. Neurosci Lett 453: 182-185.

87. Page MM, Salway KD, Ip YK, Chew SF, Warren SA, et al. (2010) Unregulation of intracellular antioxidant enzymes in brain and heart during estivation in the African lungfish Protopterus dolloi. J Comp Physiol B 180: 361-369.

88. Rostovtseva T, Colombini M (1996) ATP flux is controlled by a voltage-gated channel from the mitochondrial outer membrane. J Biol Chem 271: 28006-28010.

89. Pfeffer SR, Rothman JE (1987) Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. Annu Rev Cell Biol 3: 255-321.

90. Pryer NK, Wuestehube LJ, Schekman R (1992) Vesicle-mediated protein transport in Schizosaccharomyces pombe: a novel role for a GTP-binding protein. Cell 67: 239-253.

91. Serafini T, Orci L, Amherdt M, Brunner M, Kahn RA, et al. (1991) Vesi subunit of Golgi-derived non-clathrin-coated vesicles: a novel role for a GTP-binding protein. Cell 67: 251-259.

92. Fischle W, Wang Y, Allfrey VL (1986) Structure and function of histone chaperones ERp57 and calreticulin bind beta-amyloid. J Biol Chem 261: 9611-9617.

93. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, et al. (2007) Molecular Biology of the Cell. 5th edn. Garland Science, New York.

94. Serafini T, Stenbeck G, Brecht A, Lottspeich F, Orci L, et al. (1991) A coat subunit of Golgi-derived non-clathrin-coated vesicles with homology to the clathrin-coated vesicle coat beta-adaptin. Nature 353: 215-220.

95. Smith MM (1991) Histone structure and function. Curr Opin Cell Biol 3: 429-437.

96. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, et al. (2007) Molecular biology of the cell. 5th edn: Garland Sciences, New York.

97. Fischle W, Wang Y, Allfrey VL (2003) Histone and chromatin cross-talk. Curr Opin Cell Biol 15: 172-183.

98. Wei Y, Mizzen CA, Cook RG, Gorovsky MA, Allfrey GD (1998) Phosphorylation of histone H3 at serine 10 is correlated with chromosome condensation during mitosis in Caenorhabditis elegans. Proc Natl Acad Sci USA 95: 7480-7484.

99. Gurley LR, D’Anna JA, Barham SS, Deaven LL, Tobey RA (1978) Histone phosphorylation and chromatin structure during mitosis in Chinese hamster cells. Eur J Biochem 89: 1-15.

100. Kleinman HS, Horeisz S, Triel RC (1991) Structure and function of histone H3 lysine 9 methyltransferases and demethylases. ChemBiochem 12: 254-263.

101. Luo Y, Jian W, Stavreva D, Fu X, Hager G, et al. (2009) Trans-regulation of PP1 and PP2A-like phosphatases in control of microtubule dynamics during mitosis. EMBO J 16: 5537-5549.

102. Krishnan S, Horowitz S, Trievel RC (2011) Structure and function of histone chaperones ERp57 and calreticulin bind beta-amyloid. J Biol Chem 261: 9611-9617.

103. Wu CL, Shinai AI, Ito GS (1997) Prothymosin alpha promotes cell proliferation in NIH3T3 cells. Life Sci 61: 2091-2097.

104. Dominguez F, Magdalena C, Cancio E, Roson E, Paredes J, et al. (1993) Tissue expression of prothymosin alpha gene in T lymphocytes and leukemic lymphoid cells is tied to lymphocyte proliferation. J Biol Chem 268: 8451-8454.

105. Sburlati AR, Manrow RE, Berger SL (1991) Prothymosin alpha antisense oligomers inhibit lymphoma cell division. Proc Natl Acad Sci USA 88: 253-257.

106. Rajaraman SC, Shull GM, Kohn WK, et al. (1994) The mudskippers Pimelophthalmus schincki and Bilophthalmus buddei can tolerate environmental NH3 concentrations of 446 and 36 µM, respectively. Fish Physiol Biochem 19: 59-69.

107. Wang YX, Wahl PJ (2000) High ammonia tolerance in fishes of the family Cyprinidae (Cypriniformes). Aquat Toxicol 50: 205-219.

108. Murray BW, Bushy ER, Monnsem TP, Wright PA (2003) Evolution of glutamine synthetase in vertebrates: multiple glutamine synthetase genes expressed in rainbow trout (Oncorhynchus mykiss). J Exp Biol 206: 1511-1521.

109. Wright PA, Steele SL, Huitema A, Bernier NJ (2007) Induction of four glutamine synthetase genes in brain of rainbow trout in response to elevated environmental ammonia. J Exp Biol 210: 2901-2905.

110. Landell SL, Kåhn SL, Pizzau TM, Mannijio MG, Torralba JR, et al. (2005) Natural resistance to liver cold ischemia-reperfusion injury associated with the hibernation phenotype. Am J Physiol Gastrointest Liver Physiol 288: G473-G480.

111. Dave KR, Prado R, Raval AP, Drew KL, Perez-Pinon MA (2006) The arctic ground squirrel brain in vivo: effects of injury from cardiac arrest during euthermia. Stroke 37: 1261-1265.

112. Christian SL, Ross AP, Zhao HW, Kristensen HJ, Zhan X, et al. (2008) Arctic ground squirrel (Spermophilus parryii) hippocampal neurons tolerate prolonged oxygen-glucose deprivation and maintain baseline ERK1/2 and JNK phosphorylation despite drastic ATP loss. J Cereb Blood Flow Metab 28: 1307-1319.

113. Murray BW, Bushy ER, Monnsem TP, Wright PA (2003) Evolution of glutamine synthetase in vertebrates: multiple glutamine synthetase genes expressed in rainbow trout (Oncorhynchus mykiss). J Exp Biol 206: 1511-1521.

114. Wright PA, Steele SL, Huitema A, Bernier NJ (2007) Induction of four glutamine synthetase genes in brain of rainbow trout in response to elevated environmental ammonia. J Exp Biol 210: 2901-2905.