ABSTRACT

This study aimed to sequence and characterize two pro-coagulant genes, coagulation factor II (f2) and fibrinogen gamma chain (fgg), from the liver of the African lungfish Protopterus annectens, and to determine their hepatic mRNA expression levels during three phases of aestivation. The protein abundance of F2 and Fgg in the liver and plasma was determined by immunoblotting. The results indicated that F2 and Fgg of P. annectens were phylogenetically closer to those of amphibians than those of teleosts. Three days of aestivation resulted in an up-regulation in the hepatic fgg mRNA expression level, while 6 days of aestivation led to a significant increase (3-fold) in the protein abundance of Fgg in the plasma. Hence, there could be an increase in the blood-clotting ability in P. annectens during the induction phase of aestivation. By contrast, the blood-clotting ability in P. annectens might be reduced in response to decreased blood flow and increased possibility of thrombosis during the maintenance phase of aestivation, as 6 months of aestivation led to significant decreases in mRNA expression levels of f2 and fgg in the liver. There could also be a decrease in the export of F2 and Fgg from the liver to the plasma so as to avert thrombosis. Three to 6 days after arousal from 6 months of aestivation, the protein abundance of F2 and Fgg recovered partially in the plasma of P. annectens; a complete recovery of the transcription and translation of f2/f2 in the liver might occur only after refeeding.

KEY WORDS: Blood circulation, Haemostasis, Prothrombin, Thrombin, Thrombosis, Fibrin

INTRODUCTION

Lungfishes belong to an archaic group of freshwater fishes that are categorized under class Ostechthyes, subclass Sarcopterygii (lobed-finned fishes), and possess primitive lungs for air breathing. They hold a very important evolutionary position in the tree-of-life (lobed-finned fishes), and possess primitive lungs for air breathing.

There are six species of extant lungfishes worldwide. Of these, four (Protopterus aethiopicus, Protopterus amphibius, Protopterus annectens and Protopterus dolloi) are found in Africa. African lungfishes are obligate air breathers, and can undergo aestivation in subterranean mud cocoons for as long as 4 years during drought (Smith, 1930; see Ip and Chew, 2010; Ballantyne and Frick, 2010, for reviews). Aestivation is a state of torpor often associated with arid conditions at high environmental temperature and without food or water intake for an extended period. It is characterized mainly by physical inactivity and low metabolic rate (Pinder et al., 1992; Hasiotis et al., 2007; Withers and Cooper, 2010). In nature, aestivation can occur inside a subterranean mud cocoon. In the laboratory, African lungfishes can be induced to aestivate in dried-mucus cocoons in air (Chew et al., 2004; Ip et al., 2005; Loong et al., 2005, 2007, 2008a,b, 2012a,b). There are three phases of aestivation: induction, maintenance and arousal. During the induction phase, the aestivating fish detects environmental cues, turns them into internal signals, and makes the required biochemical, physiological, structural and behavioural changes for aestivation. It hyperventilates and secretes plenty of mucus. The mucus will turn into a cocoon after 6–8 days. The fish enters into the maintenance phase of aestivation when it is encased in a dried-mucus cocoon, with a complete cessation of feeding and locomotor activities. The aestivating lungfish must prevent cell death, preserve biological fuels and structures, and sustain a slow rate of waste production during the maintenance phase of aestivation. The lungfish can be aroused from aestivation with the addition of water. After struggling out of the cocoon, it swims sluggishly to the water surface to gulp air. Upon arousal, it re-hydrates, excretes the accumulated waste products and begins to feed after 7–10 days. Distinctive changes in cardiorespiratory properties and blood circulation are hallmarks of the aestivation process, where the lungfish’s ventilation rate, heart rate and mean blood pressure are altered rapidly. During the induction phase, the lungfish hyperventilates and the frequency of lung breaths increases rapidly from ~5 to ~30 breaths per hour (Fishman et al., 1986). Within 1–2 months (the maintenance phase) of aestivation, hyperventilation gradually slows down until it reaches the original frequency of ventilation. Simultaneously, the heart rate also decreases from ~25 to ~10 beats min⁻¹ (Delaney et al., 1974; Fishman et al., 1986, 1992). Following the decrease in heart rate, the mean blood pressure in the systemic circulation declines from ~30 to ~14 mmHg (Delaney et al., 1974; Fishman et al., 1986). Hence, there must be a decrease in blood flow in the vascular system of the...
aestivating lungfish due to the reduction in cardiac output and the resultant drop in blood pressure. While blood is maintained in a fluid state under physiological conditions, the haemostatic system has evolved to react rapidly to seal up injured blood vessels with blood clots (Colman, 2006). Specifically, thrombosis entails the formation of a blood clot (thrombus) in a blood vessel. Theoretically, decreased blood circulation would predispose the aestivating lungfish to intravascular thrombosis. However, it is apparent that thrombosis does not transpire during the maintenance phase of aestivation despite a decrease in blood flow; if it did, the lungfish would succumb during the aestivation process. Therefore, adaptive changes must occur in the haemostatic system of the aestivating lungfish to avoid thrombus formation. However, apart from haematological profiling studies (Johansen et al., 1976; Delaney et al., 1976; Ikechukwu and Obinnya, 2010), there is a dearth of information on the blood coagulation system of African lungfishes and its relationship to aestivation.

The blood coagulation system in fishes is fundamentally similar to that in mammals (Jagadeeswaran and Sheehan, 1999; Jagadeeswaran et al., 2000; Davidson et al., 2003a,b; Manseth et al., 2004). It involves a cascade of reactions where the coagulation factors are zymogens of serine proteases, and each factor sequentially activates the downstream coagulation factor by proteolytic cleavage (Furie and Furie, 1988). Eventually, the reactions converge onto a common pathway that triggers the activation of thrombin to cleave fibrinogen (a blood-borne glycoprotein composed of three pairs of non-identical polypeptide chains) into fibrin monomers, which are cross-linked to form a fibrous clot. The coagulation factor 2 (F2) gene encodes prothrombin, which is predominantly produced by the liver and circulated in the bloodstream. Prothrombin is essential for the formation of blood clots (haemostasis). When an injury occurs whereby blood vessels are damaged, prothrombin is converted to its active form, thrombin. Thrombin then converts fibrinogen into fibrin, the primary protein that makes up blood clots. The gamma component of fibrinogen is encoded by fibrinogen gamma chain (fgg). Blood clots seal off damaged blood vessels and prevent further blood loss after an injury.

Using suppression subtractive hybridization, Loong et al. (2012b) and Hiong et al. (2015) obtained partial sequences of F2 and Fgg from the liver of P. annectens, and reported possible changes in their hepatic mRNA expression during the three phases of aestivation. Therefore, this study was undertaken to obtain the complete coding cDNA sequences of F2 and Fgg from the liver of P. annectens. It was hoped that the deduced F2 and Fgg amino acid sequences would shed light on the phylogenetic relationship between P. annectens and other animals. Furthermore, efforts were made to determine by quantitative real-time PCR (qPCR) the mRNA expression of F2 and Fgg in the liver of P. annectens kept in freshwater (control) or undergoing the induction phase of aestivation, the maintenance phase or the arousal phase of aestivation. Based on the deduced F2 and Fgg amino acid sequence, custom-made anti-F2 and anti-Fgg antibodies were developed for determination of their protein abundance in the liver and plasma through western blotting. The hypothesis tested was that aestivation would induce changes in mRNA and/or protein expression levels of F2/F2 and fgg/Fgg in the liver during the three phases of aestivation. Specifically, there could be increases in the transcription and translation of F2/F2 and fgg/Fgg in the liver during the induction phase of aestivation, so as to enhance the blood-clotting capacity in preparation for possible physical injuries acquired as the lungfish struggles to get back to water or during burrowing activity. However, during the maintenance phase of aestivation, there could be a decrease in the expression levels of F2/F2 and fgg/Fgg in the liver, and decreases in the protein abundance of F2 and Fgg in the plasma in order to avert thrombosis when confronted with decreased blood flow. During the arousal phase of aestivation, the expression of F2/F2 and fgg/Fgg in the liver would probably recover, at least partially, back to the control levels.

**Note on abbreviations**
As the standard abbreviations of genes/proteins of fishes (http://zfin.org/ cgi-bin/webdriver?MIval=aa-ZDB_home.apg) are different from those of frogs and human/non-human primates (http://www.genenames.org), two different types of abbreviations were adopted in this report. In particular, fish gene symbols are italicized, all in lowercase, and fish protein designations are the same as the gene symbol, but non-italicized with the first letter in uppercase.

**MATERIALS AND METHODS**

**Animals**

*Protopterus annectens* (Owen 1839) (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 water, which was changed daily. No attempt was made to separate the sexes. Fish were fed fresh fish meat and acclimated to laboratory conditions for at least 2 weeks before experiments. This study was performed in accordance with the approved protocol IACUC 035/09 granted by the Institutional Animal Care and Use Committee of the National University of Singapore.

**Experimental conditions and collection of samples**

*Protopterus annectens* kept in freshwater served as controls. Control fish (N=5) were killed with an overdose of neutralized 0.05% MS222 for tissue sampling after food was withheld for 96 h. Blood was collected through caudal puncture into heparinized capillary tubes, then centrifuged at 4000 g at 4°C for 10 min to obtain the plasma, which was stored at −80°C until analysis. The liver was quickly excised, freeze-clamped with aluminum tongs pre-cooled in liquid nitrogen and kept at −80°C. A group of fish (N=35) was induced to aestivate individually at 27–29°C and 85–90% humidity in plastic tanks (L×W×H, 29×19×17.5 cm) containing 15 ml dechlorinated tapwater (made 0.3% with seawater) initially, following the procedure of Chew et al. (2004). It took approximately 6–8 days for the fish to be encased in a brown dried-mucus cocoon. In order to maintain a high humidity (>90%) within the tank, 1–2 ml of water was sprayed onto the side of the tank daily, and the tank was partially covered with a moist towel. Five fish were killed with a strong blow to the head for tissue sampling on day 3 (N=5) and another five on day 6 (N=5), which covered the induction phase of aestivation. After 12 days, five fish were killed for tissue sampling, which represents the early maintenance phase of aestivation (N=5). For the prolonged maintenance phase, five fish were killed after 6 months of aestivation (N=5). The remaining 15 fish, which had already aestivated for 6 months, were aroused from aestivation (the arousal phase) by the addition of 200 ml of water to the tank followed by appropriate levels of mechanical stimulation and breakage of the cocoon. Within a few minutes, the fish swam sluggishly in the water. Another 800 ml of water was then added to cover the fish. One, 3 or 6 days after arousal, fish (N=5 each) were killed with an overdose of neutralized 0.05% MS222 for tissue sampling. Collected samples were kept at −80°C until analysis.

**Total RNA extraction**

Total RNA from liver samples was extracted using Tri Reagent™ (Sigma-Aldrich Co., St Louis, MO, USA), and purified using the RNeasy Plus Mini Kit (Qiagen GmbH, Hilden, Germany). RNA was quantified spectrophotometrically using a Helma tracycell (Helma GmbH & Co. KG, Müllheim, Germany), and its quality was checked electrophoretically.

**Rapid amplification of cDNA ends (RACE) cDNA synthesis**

Total RNA (1 μg) isolated from the liver of *P. annectens* was reverse transcribed into 5’-RACE-Ready cDNA and 3’-RACE-Ready cDNA using a SMARTer™ RACE cDNA Amplification kit (Clontech Laboratories,....
Mountain View, CA, USA). Based on the partial fragments of \( f_2 \) and \( fgg \) obtained from suppression subtractive hybridization PCR by Loong et al. (2012b), specific 5' and 3' RACE primers were designed to obtain the complete sequences of \( f_2 \) and \( fgg \) (Table 1) through RACE-PCR using the Advantage \(^ {\text{TM}} \) PCR kit (Clontech Laboratories). RACE-PCR cycling conditions comprised 1 cycle of 95°C for 20 s followed by 40 cycles of 95°C for 3 s and 60°C for 30 s for 4 min. RACE-PCR products were separated using gel electrophoresis, purified and sequenced. Multiple sequencing was performed in both directions to obtain the full-length cDNA. Sequence assembly and analysis were performed using BioEdit v7.1.3 (Hall, 1999). The cDNA coding sequences of \( f_2 \) and \( fgg \) from \( P. annectens \) have been deposited in GenBank with accession number KJ689301 and KJ689302, respectively.

**Deduced amino acid sequences and phylogenetic analysis**

The predicted \( f_2 \) and \( fgg \) amino acid sequences were obtained from the \( f_2 \) and \( fgg \) nucleotide sequences using the ExPaSy Proteomic server (http://www.expasy.org/translate/). The amino acid sequences were aligned and compared with selected amino acid sequences from various animal species using BioEdit.

The phylogenetic relationships among \( f_2 \) and \( fgg \) of \( P. annectens \) and those of other animal species were analysed using the neighbour-joining method (NEIGHBOUR) in the PHYLIP phylogeny package (version 3.67) (Felsenstein, 1989), with the inclusion of 100 bootstraps. The respective phylogenetic trees were generated with CONSENSE using 50% majority rule, and plotted with the program TREVIEW. Bootstrap values were indicated at the nodes of the tree branches. The accession numbers of selected \( f_2 \) amino acid sequences (from GenBank) used in the analysis are as follows: *Homo sapiens* \( f_2 \), AA177436.1; *Mus musculus*, AAH13662.1; *Rattus norvegicus*, NP_057213.2; *Xenopus (Silurana) tropicalis*, F2, NP_001015797.1; *Xenopus laevis* F2, NP_001085292.1; *Oncorhynchus mykiss*, F2, CADS9688.1; *Takifugu rubripes*, AA033373.1; *Danio rerio* F2, AAH55596.1; and *Epatatrus stoutii* F2 (as the outgroup), AA21620.2. The accession numbers of selected \( fgg \)/ \( Fgg \) amino acid sequences (from GenBank) used in the analysis are as follows: *Homo sapiens* \( Fgg \), AAB59531.1; *Mus musculus*, NP_598623.1; *R. norvegicus*, FGG, AAH78893.1; *X. laevis* FGG, NP_001080639.1; *Callorhinus milii* Fgg, AFM90043.1; *Plecoglossus altivelis* Fgg, CBX31965.1; *D. rerio* Fgg, NP_998219.1; *Hypophthalmichthys molitrix* Fgg, ADF97606.1; and *Culex quinquefasciatus* Fgg (as the outgroup), EDS27702.1.

**qPCR**

RNA (4 µg) from the liver samples was reverse transcribed using random hexamer primers with RevertAid \(^ {\text{TM}} \) first-strand cDNA synthesis kit (Fermentas International Inc.). qPCR was performed in triplicate using a StepOnePlus \(^ {\text{TM}} \) Real-Time PCR System (Applied Biosystems). The qPCR reactions contained 5 µl of KAPA SYBR \(^ {\text{TM}} \) Master Mix (2×) ABI Prism \(^ {\text{TM}} \) (Kapa Biosystems, Woburn, MA, USA), 0.3 µmol l\(^{-1}\) each of forward and reverse gene-specific qPCR primers (Table 1) and 2 µl of cDNA (equivalent to 1 ng of RNA) or 2 µl of standard in a total volume of 10 µl. Cycling conditions comprised 1 cycle of 95°C for 20 s followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. Data (threshold cycle as \( C_T \)) on the \( y \)-axis and the natural log \( C_T \) on the \( x \)-axis were collected at each elongation step. Melt curve analysis was performed by increasing the temperature from 60°C to 95°C in 0.3°C increments to confirm the presence of only a single product. The PCR products were separated on a 2% agarose gel to verify the presence of a single band.

The method of absolute quantification with reference to a standard curve for each gene was adopted in this study, as it was essential to compare the mRNA expression levels of \( f_2 \) and \( fgg \) in the liver of *P. annectens*. While relative quantitation methods produce fold-change data, they do not facilitate the comparison of gene expression levels. Furthermore, relative quantification requires the incorporation of a reference gene, the expression of which is unaffected by the experimental conditions, but we had difficulties in identifying such a gene as substantial transcriptional changes occur in the liver of *P. annectens* during the three phases of aestivation. In order to determine the absolute quantity of \( f_2 \) and \( fgg \) transcripts in a qPCR reaction, efforts were made to produce a pure amplicon (standard) of a defined region of \( f_2 \) or \( fgg \) cDNA from the liver of *P. annectens* according to the PCR method of Gerwick et al. (2007). PCR was performed with the gene-specific qPCR primers for \( f_2 \) or \( fgg \) with cDNA as a template in a final volume of 25 µl. The cycling conditions comprised an initial denaturation of 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s and one extension cycle of 72°C for 10 min. The PCR product was separated on a 2% agarose gel, excised and purified using a QIAquick gel extraction kit (Qiagen GmbH). The \( f_2 \) and \( fgg \) fragments in the purified product were cloned into pGEM\(^ {\text{TM}}\) Easy vector (Promega Corporation, Madison, WI, USA). The presence of the insert in the recombinant clones was confirmed by sequencing, and the cloned circular plasmid was quantified using a spectrophotometer. The standard cDNA (template) was serially diluted (from \( 10^6 \) to \( 10^2 \) specific copies/2 µl). A standard curve was obtained from plotting threshold cycle (\( C_T \)) on the \( y \)-axis and the natural log of concentration (copies µl\(^{-1}\)) on the \( x \)-axis. The \( C_T \) slope, PCR efficiency, \( y \)-intercept and correlation coefficient \( (r^2) \) were calculated using the default setting of StepOneTM Software v2.1 (Applied Biosystems). Diluted standards were stored at \(-20^\circ \text{C} \). The PCR efficiency for \( f_2 \) and \( fgg \) was 100% and 95.4%, respectively. The quantity of transcript in an unknown sample was determined from the linear regression derived from the standard curve and expressed as copies of transcripts per ng cDNA.

**SDS-PAGE electrophoresis and western blotting**

A commercial firm (GenScript, Piscataway, NJ, USA) was engaged to raise a rabbit polyclonal antibody against amino acids 296–309 (TDGAEETDGGRTS) of the translated amino acid sequence of \( F_2 \) and a rabbit polyclonal antibody against amino acids 376–389 (GGRYSAQAGPDGF) of the translated amino acid sequence of \( Fgg \) from the liver of *P. annectens*. Immunoreactive bands of \( F_2 \) and \( Fgg \) were visualized at the expected molecular mass of 69.3 and 50.5 kDa, respectively.

Western blotting was performed on samples obtained from the control fish and fish that had undergone 6 days, 12 days or 6 months of aestivation, or 3 or 6 days after arousal from 6 months of aestivation. Liver and plasma samples were homogenized three times in five volumes (w/v) of ice-cold buffer containing 50 mM l\(^{-1}\) Tris HCl, (pH 7.4), 1 mM l\(^{-1}\) EDTA, 150 mM l\(^{-1}\) NaCl, 1 mM l\(^{-1}\) NaF, 1 mM l\(^{-1}\) Na\(_3\)VO\(_4\), 1% NP-40, 1 mM l\(^{-1}\) PMSF and 1× HALT\(^ {\text{TM}} \) protease inhibitor cocktail (Thermo Fisher Scientific Inc.) at 24,000 rpm for 20 s each with 10 s intervals using the Polytron PT 1300D homogenizer (Kinematics AG, Lucerne, Switzerland).

### Table 1. Primers used for RACE-PCR and quantitative real-time PCR on coagulation factor II (\( f_2 \)) and fibrinogen gamma chain (\( fgg \)) from the liver of *Protopterus annectens*.

<table>
<thead>
<tr>
<th>Gene/FGG</th>
<th>Accession no.</th>
<th>Purpose</th>
<th>Primer sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( f_2 )</td>
<td>KJ689301</td>
<td>5’ RACE-PCR</td>
<td>CAGAGTATAGCGCGTGCTGCGTCG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3’ RACE-PCR</td>
<td>CGACTACTGCACTCTGAGTGGTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>qPCR</td>
<td>Forward (GATTAGTACCTCGTGCACC)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reverse (ACATCCCTGTGCTACATGGG)</td>
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<td></td>
<td></td>
<td></td>
<td>CTTCCCGGGTGAAATGATCTTC</td>
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<td></td>
<td></td>
<td></td>
<td>GAGAGGATCTTCAACCGGAGAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Forward (GCAAATGGTCAGTACTTCTC)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reverse (TACCCGGACAGTCTTTC)</td>
</tr>
<tr>
<td>( fgg )</td>
<td>KJ689302</td>
<td>5’ RACE-PCR</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>3’ RACE-PCR</td>
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<td></td>
<td>qPCR</td>
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</table>
Switzerland). The homogenate was centrifuged at 10,000 g for 20 min at 4°C. The protein concentration in the supernatant obtained was determined (Bradford, 1976) and adjusted to 5 µg µl⁻¹ with Laemmli buffer (Laemmli, 1970). Samples were heated at 70°C for 15 min, and then kept at −80°C until analysis.

Preliminary experiments showed that the protein abundance of F2 and Fgg in the plasma differed from that in the liver. Therefore, 50 µg of liver protein was loaded for gel separation of F2 and Fgg, but the plasma protein load for F2 and Fgg was 100 and 20 µg, respectively. Proteins were separated by SDS-PAGE (10% acrylamide resolving gel, 4% acrylamide stacking gel) according to the method of Laemmli (1970) using a vertical mini-slab apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were then electrophoretically transferred onto a PVDF membrane using a transfer apparatus (Bio-Rad Laboratories). Blocking of the membrane was performed with 10% skimmed milk in TTBS (0.05% Tween 20 in Tris-buffered saline: 20 mmol l⁻¹ Tris-HCl, 500 mmol l⁻¹ NaCl, pH 7.6) for 1 h. The blocked membrane was incubated with anti-F2 or anti-Fgg antibodies (1:500 dilution in 1% bovine serum albumin in TTBS) overnight at 4°C. The membrane was incubated with goat anti-rabbit alkaline phosphatase-conjugated secondary antibody (1:10,000 dilution; Santa Cruz Biotechnology Inc.) for 1 h, rinsed, and then incubated for 30 min in a solution of 5-bromo-4-chloro-3-indolyl phosphate-p-toluidine salt and nitroblue tetrazolium chloride (Invitrogen, Carlsbad, CA, USA) for colour development. The developed blots were scanned using a CanoScan 9000F Mark II flatbed scanner in TIFF format at 300 dpi resolution. Densitometric quantification of band intensity was performed using ImageJ (version 1.41, NIH), calibrated with a 37 step reflection scanner scale (1×8 in; Stouffer no. R3705-1C). Difficulties were encountered in identifying a reference protein, the expression of which would be unaffected throughout the three phases of aestivation. Hence, results are expressed as arbitrary densitometric units per µg protein, i.e. with reference to the protein concentration, as reported elsewhere for protein abundance of L-gulono-δ-lactone oxidase (Ching et al., 2014), Na⁺K⁺-ATPase (Hiong et al., 2014) and betaine-homocysteine S-methyltransferase (Ong et al., 2015) in *P. annectens* during aestivation.

**Statistical analysis**

Results are presented as means±s.e.m. Statistical analyses were performed using SPSS version 21 (IBM Corporation, Armonk, NY, USA). Homogeneity of variance was verified using Levene’s test. One-way analysis of variance (ANOVA) was performed for all the results, followed by multiple comparisons of means by Tukey’s test (for data with equal variance) or Dunnett’s T3 test (for data with unequal variance). The analysed data were used in the evaluation of the differences between means where applicable. Differences were regarded as statistically significant at P<0.05.

**RESULTS**

The nucleotide and the deduced amino acid sequences of *f2/F2*

The complete cDNA coding sequence of *f2* from the liver of *P. annectens* consisted of 1836 bp, encoding a protein of 611 amino acids with an estimated molecular mass of 69.3 kDa. The length of *P. annectens* F2 protein was comparable to F2 of other animal species (Fig. 1). An alignment of the deduced amino acid sequence of F2 from *P. annectens* with those from human, mouse, frog, zebrafish and rainbow trout (Fig. 1) indicated a highly conserved region between residues 342 and 630 (according to the alignment in Fig. 1). This conserved region included the N-glycosylation sites for carbohydrate attachment, the cysteine residues responsible for the disulphide interlink between F2 heavy and light chain, and the His-Asp-Ser catalytic triad (histidine 419, aspartate 475 and serine 581) for the charge relay system (Fig. 1). In addition, the identified γ-carboxyglutamate (Gla) domain contained multiple conserved glutamate residues, which are critical for anchoring the protein to the cell membrane via a Ca²⁺-dependent interaction (Huang et al., 2003). However, the Gla domain, together with the two kringle lines indicate the cleavage sites of factor Xa.

![Fig. 1. Molecular characterization of coagulation factor II (F2) of *Protopterus annectens*. A multiple amino acid alignment of F2 of *P. annectens* with F2 sequences from selected vertebrates (*Danio rerio*, *Oncorhynchus mykiss*, *Xenopus tropicalis*, *Mus musculus* and *Homo sapiens*). Identical or strongly similar amino acids are indicated by shaded residues. The γ-carboxyglutamate (Gla) and kringle (KR) domains are underlined. N denotes the N-glycosylation sites, C denotes the charge relay sites and I denotes the interlink sites (disulphide bridge between F2 light and heavy chain). G represents glutamate residues (for Gla formation) and vertical underlines indicate the cleavage sites of factor Xa.](image-url)
domains, which are required for protein structural folding and interaction (Patthy et al., 1984), were only weakly conserved across the different animals. Of particular interest for tetrapods and *P. annectens*, the conserved factor Xa (FXa) cleavage site between residues 327 and 328 revealed that the cleavage was between arginine and tyrosine. By contrast, the cleavage site for fishes was between two tyrosine residues.

**The nucleotide and the deduced amino acid sequences of fgg/Fgg**

The complete cDNA coding sequence of *fgg* from the liver of *P. annectens* consisted of 1311 bp, encoding a protein of 436 amino acids with a calculated molecular mass of 50.5 kDa. An alignment of the deduced amino acid sequence of Fgg from *P. annectens* with those of other animal species (Fig. 2) showed that all the sequences were highly conserved, particularly the glycosylation sites and the cysteine residues that help to form disulphide bridges with the fibrinogen α and β chains (Hoeprich and Doolittle, 1983). Furthermore, the calcium binding domain and the polymerization site required for mediating the cross-linking of fibrin monomers (Olexa and Budzynski, 1981) were conserved in FGG/Fgg across the different animals. Notably, the two plasmin cleavage sites identified in *H. sapiens* were not conserved in FGG/Fgg of other species, suggesting the presence of alternative positions for plasmin action in amphibians and fishes.

**Phylogenetic relationship**

Phylogenetic analyses revealed that the F2 (Fig. 3) and Fgg (Fig. 4) of *P. annectens* were closer to those of tetrapods than those of fishes.

**mRNA expression levels of f2 and fgg in the liver during three phases of aestivation**

The mRNA expression level of *f2* remained unchanged in the liver of *P. annectens* after 3 or 6 days of aestivation (the induction phase) and after 12 days of aestivation (the early maintenance phase; Fig. 5A). However, 6 months of aestivation (the prolonged maintenance phase) led to a significant decrease (by 48%) in the *f2* mRNA expression level in the liver (Fig. 5A). One day after arousal from 6 months of aestivation, there was a further decrease in the mRNA expression level of *f2* (by 50%) in the liver compared with that of fish aestivated for 6 months (Fig. 5B). The mRNA expression of *f2* in the liver of fish aroused for 3 or 6 days remained significantly lower than that of the control fish (Fig. 5B).

**Fig. 2. Molecular characterization of fibrinogen γ chain (Fgg) of *P. annectens*.** A multiple amino acid alignment of Fgg of *P. annectens* with Fgg/FGG sequences from selected vertebrates (*Callorhinchus milii*, *Danio rerio*, *Xenopus laevis*, *Mus musculus* and *Homo sapiens*). Identical or strongly similar amino acids are indicated by shaded residues. N denotes the N-glycosylation sites and I denotes the interlink sites (disulphide bridge between residues 34 and 35). Sα and Sβ represent the sites that form disulphide bridges with fibrinogen α- and β-chain, respectively. Vertical lines indicate the cleavage sites of plasmin.

**Fig. 3. Phenogramic analysis of F2 of *P. annectens*.** A phenogram illustrating the relationship between F2 of *P. annectens* and F2 of selected vertebrates. The number at each branch represents the bootstrap value (max. 100). F2 of *Eptatretus stoutii* was chosen as the outgroup.
The mRNA expression level of fgg was 10-fold higher than that of f2 in the liver of *P. annectens*. There was a transient but significant increase (1.7-fold) in the mRNA expression level of fgg after 3 days of aestivation compared with the control (Fig. 6A). After 6 months of aestivation, the fgg mRNA expression level decreased significantly by 50% compared with the freshwater control (Fig. 6A). The mRNA expression level of fgg in the liver of fish 3 or 6 days (but not 1 day) after arousal from 6 months of aestivation was comparable to that of the control fish (Fig. 6B).

**Protein abundance of F2 and Fgg in the liver during three phases of aestivation**

The protein abundance of F2 remained unchanged in the liver of *P. annectens* after 6 or 12 days of aestivation, but it increased significantly after 6 months of aestivation or 3 days after arousal from 6 months of aestivation as compared with the control (Fig. 7). For Fgg, there was a significant increase in protein abundance in the liver of *P. annectens* after 12 days of aestivation (Fig. 8).

**Protein abundance of F2 and Fgg in the plasma during three phases of aestivation**

There was a progressive increase in the protein abundance of F2 in the plasma of *P. annectens* during the first 12 days of aestivation (Fig. 9). By day 12, the protein abundance of F2 in the plasma was significantly higher than that of the control fish. However, 6 months of aestivation led to a drastic decrease in plasma F2 protein abundance. Despite being not statistically different from that of the freshwater control, the plasma F2 protein abundance of fish aestivated for 6 months was only 25% of the control values (Fig. 9). The plasma F2 protein abundance returned to the control level in fish 3 or 6 days after arousal from 6 months of aestivation.

The plasma protein abundance of Fgg increased significantly in fish that underwent 6 or 12 days of aestivation, but decreased significantly to 1/6 of the control value after 6 months of aestivation (Fig. 10). Three days after arousal from 6 months of aestivation, the protein abundance of Fgg in the plasma recovered transiently to the control level.

**DISCUSSION**

Roles of F2 and Fgg in blood clot formation

Fibrinogen is a trinodular glycoprotein comprising two symmetrical halves, with each half consisting of three different polypeptide chains, namely α, β and γ chain linked by disulphide bonds (Doolittle, 2010). F2 is the zymogen form of a serine protease that
exhibits pro-coagulant properties by cleaving fibrinogen to form fibrin (Siller-Matula et al., 2011). The released Fgg is one of the subunit polypeptides that is involved in fibrin polymerization and cross-linking by FXIII to form the three-dimensional framework of a blood clot (Günther and Ruppert, 2006). Fibrin polymerization is a complex process that requires specific three-dimensional association of the activated fibrin molecules. Cleavage of fibrinopeptides from fibrinogen exposes the polymerization sites in the central domain of fibrin, which bind to the complementary sites present at the N-terminal region of another fibrin molecule (Doolittle, 1984; Budzynski, 1986). The association of these binding sites results in the formation of half-staggered protofibrils (Fowler et al., 1981; Medved et al., 1990), which are eventually aggregated laterally to form fibres (Mosesson, 2005). The subsequent branching and cross-linking of the fibres then forms an extensive fibrous network known as a clot. F2 is also involved in the activation of the transglutaminase FXIII for fibrin cross-linking, self-generation of more thrombin through the activation of FXI, FVIII and FV, and the stimulation of platelet aggregation via cleavage of the membrane-bound protease-activated receptors (PARs) 1, 3 and 4 (Coughlin, 2005).

### Molecular characterization of F2 and Fgg from *P. annectens*

F2 is only activated to functional thrombin when it forms a protein complex (prothrombinase complex) with platelet phospholipids, factor V and FXa. Hence, F2 contains a Gla domain to carry out Ca$^{2+}$-dependent interactions with the negatively charged platelet phospholipids (Huang et al., 2003). Each glutamate residue within the Gla domain undergoes carboxylation to form Gla residues that are able to bind to Ca${2+}$ (Presnell and Stafford, 2002), and therefore the glutamate residues in the Gla domain of F2 are highly conserved across species including *P. annectens* to preserve its function. In addition, F2 has many interactions with other proteins, which in turn modulate the activity and functional properties of F2. Kringle domains in F2 are known to play important roles in protein and cofactor interactions (Wu et al., 1997), in the binding of mediators such as membranes, other proteins or phospholipids, and in the regulation of the F2 proteolytic activity. As the kringle domains of F2 are not well conserved among various animal species, F2 of different animal species may have variable protein interactions and regulatory control due to the conformational alterations in kringle folds arising from amino acid substitutions (Castellini and Beals, 1987). The activation of F2 is mediated by the cleavage of FXa at two specific sites to release the catalytic domain from the carboxyterminal region of the protein (Bode, 2006). Of note, the FXa cleavage sites in F2 of tetrapods and *P. annectens* are different from the cleavage site in F2 of teleosts, thus showing that the African lungfish is evolutionarily closer to tetrapods than to teleosts. Furthermore, a study by Sun et al. (2000) showed that a single amino

### Fig. 6. mRNA expression levels of fgg in the liver of *P. annectens*

Absolute quantification (copies of transcripts per ng cDNA) of fgg mRNA expression levels in the liver of *P. annectens* during (A) the induction phase (3 or 6 days) and the maintenance phase (12 days or 6 months) of aestivation and (B) the maintenance phase (6 months) and arousal phase (1, 3 or 6 days after arousal from 6 months of aestivation) of aestivation compared with the FW control. Results represent means±s.e.m. (N=5). Means not sharing the same letter are significantly different (P<0.05).

### Fig. 7. Western blotting results for F2 in the liver of *P. annectens*

Protein abundance of F2 in liver kept in FW on day 0 (control), after 6 days (induction phase), 12 days (early maintenance phase) or 6 months (maintenance phase) of aestivation, or 3 or 6 days after arousal from 6 months of aestivation (arousal phase). (A) Example immunoblot of F2. (B) F2 protein abundance expressed as arbitrary densitometric units per 50 μg protein (a.u.). Results represent means±s.e.m. (N=5). Means not sharing the same letter are significantly different (P<0.05).
significantly different (represent means+s.e.m. (Ca²⁺-binding site is also highly conserved in Fgg of the molecular evolution of vertebrate blood coagulation showed that 2009; Amemiya et al., 2013). A study by Davidson et al. (2003a) on Tohyama et al., 2000; Takezaki et al., 2004; Hallstrom and Janke, to be the closest living relatives of tetrapods (Zardoya et al., 1998; comes from molecular phylogenetic studies, which show lungfishes sister group of amphibians (Forey, 1986); support for this view is intermediate form between teleosts and tetrapods. As lungfishes are shared greater similarities with those of amphibians than those of tetrapods. Our analyses revealed that F2 and Fgg from P. annectens may provide molecular clues to the evolutionary relationship of lungfishes to tetrapods and the blood coagulation system in amphibians and teleosts, phylogenetic analyses of blood coagulation proteins from P. annectens may provide molecular clues to the evolutionary relationship of lungfishes to tetrapods and tetrapods, which occurred more than 430 million years ago. As some differences exist between the blood coagulation system in amphibians and teleosts, phylogenetic analyses of blood coagulation proteins from P. annectens may provide molecular clues to the evolutionary relationship of lungfishes to tetrapods and tetrapods. Our analyses revealed that F2 and Fgg from P. annectens shared greater similarities with those of amphibians than those of teleosts. When taken together with phylogenetic information obtained based on the deduced amino acid sequences of other genes, our results lend support to the notion that lungfish is the intermediary form between teleosts and tetrapods. As lungfishes are the closest living sister group of land vertebrates, they would logically possess some genes/proteins that are closer to those of other fishes (e.g. carbamoyl phosphate synthetase III, Loong et al., 2012a; argininosuccinate synthase, Chng et al., 2014) and others that have greater similarity to those of tetrapods (e.g. F2 and Fgg, this study; argininosuccinate lyase, Chng et al., 2014; Na⁺/K⁺-ATPase α-subunit isoforms, Hiong et al., 2014; l-gulono-γ-lactone oxidase, Chng et al., 2014; betaine-homocysteine S-methyltransferase, Ong et al., 2015). Apparent increase in blood-clotting ability during the induction phase of aestivation in P. annectens During the induction phase (after 3 or 6 days) of aestivation in air, the mRNA expression of f2 in the liver of P. annectens remained statistically unchanged despite an apparent decreasing trend.
exposed to a high partial pressure of atmospheric O2. The release of adrenaline from the adrenal gland into the circulation can facilitate microcirculation (Srere et al., 1995) and/or (4) reduction in local hypercoagulability due to the accumulation of pro-coagulant substances, resulting in the formation of intravascular blood clots, which can lead to occlusion of capillaries (Briones, 2009; López and Chen, 2009). A reduction in the blood-clotting capacity to prevent intravascular thrombosis is a common phenomenon in many hibernating and aestivating mammals (Lechler and Penick, 1963; Pivorun and Sinnammon, 1981; Boyer and Barnes, 1999; McCarron et al., 2001). The organs of hibernating mammals are hypo-perfused and are considered to be severely ischaemic (McCarron et al., 2001; Storey, 2003). For instance, cerebral perfusion is known to decrease by approximately 90% (Boyer and Barnes, 1999; McCarron et al., 2001). Thus, any perturbation to blood flow resulting from thrombosis can be lethal to the hibernating animal. Hibernating animals usually employ several strategies to reduce blood clotting: (1) reduction in circulating platelets (Lechler and Penick, 1963), (2) reduction of pro-coagulant factors such as factor V, XIII or IX (McCarron et al., 2001), (3) synthesis of α2-macroglobulin to facilitate microcirculation (Serre et al., 1995) and/or (4) reduction in blood viscosity (Maclean, 1981).

Possible decrease in blood-clotting ability during the prolonged maintenance phase of aestivation in *P. annectens*

In mammals, a reduced blood flow in the vascular system can lead to blood pooling behind venous valves and sinuses. This would cause local hypercoagulability due to the accumulation of pro-coagulant substances, resulting in the formation of intravascular blood clots, which can lead to occlusion of capillaries (Briones, 2009; López and Chen, 2009). A reduction in the blood-clotting capacity to prevent intravascular thrombosis is a common phenomenon in many hibernating and aestivating mammals (Lechler and Penick, 1963; Pivorun and Sinnammon, 1981; Boyer and Barnes, 1999; McCarron et al., 2001). The organs of hibernating mammals are hypo-perfused and are considered to be severely ischaemic (McCarron et al., 2001; Storey, 2003). For instance, cerebral perfusion is known to decrease by approximately 90% (Boyer and Barnes, 1999; McCarron et al., 2001). Thus, any perturbation to blood flow resulting from thrombosis can be lethal to the hibernating animal. Hibernating animals usually employ several strategies to reduce blood clotting: (1) reduction in circulating platelets (Lechler and Penick, 1963), (2) reduction of pro-coagulant factors such as factor V, XIII or IX (McCarron et al., 2001), (3) synthesis of α2-macroglobulin to facilitate microcirculation (Serre et al., 1995) and/or (4) reduction in blood viscosity (Maclean, 1981).

During the maintenance phase of aestivation, the blood flow in the lungfish vascular system is likely to be slow as a result of a decline in blood pressure resulting from a decrease in blood volume and a reduction in cardiac output (Delaney et al., 1974; Fishman et al., 1986, 1992). However, it is important for the aestivating lungfish to maintain continual perfusion of the vital organs such as the heart, lung, brain and liver. Therefore, there must be mechanisms to avoid intravascular thrombosis. Although 12 days (the early maintenance phase) of aestivation resulted in a significant increase in the protein abundance of F2 and Fgg in the plasma of *P. annectens*, this was probably a carryover from the induction phase. In fact, 6 months of aestivation had the opposite effect, and there was a substantial decrease in F2 and Fgg protein abundance in the plasma. The slight but insignificant decrease in mRNA expression levels of *f2* and *fgg* in the liver after 12 days of aestivation is indicative of the initiation of transcriptional changes in the aestivating lungfish. Indeed, after 6 months of aestivation, there

Western blotting results confirmed that 6 days of aestivation had no significant effect on the protein abundance of F2 in the liver. By contrast, there was an up-regulation in *fgg* mRNA expression in the liver on day 3 of aestivation. Notably, the hepatic mRNA expression level of *fgg* was 10-fold higher than that of *f2*. Furthermore, after 6 days of aestivation, there was a significant (3-fold) increase in the protein abundance of Fgg in the plasma, while the liver Fgg protein abundance remained unchanged. Taken together, these results indicate that there could be an up-regulation of the ability to form blood clots in *P. annectens* during the induction phase of aestivation. This also suggests that Fgg could be a more important regulator of blood clot formation than F2 in *P. annectens*. It is apparent that Fgg production in the liver could be up-regulated through increased transcription and translation during the induction phase, and the excess Fgg produced was not retained in the liver, but released to the plasma to enhance the ability of blood clot formation. When the lungfish is stranded on land during a drought, it is exposed to a high partial pressure of atmospheric O2 (*P*O₂), high environmental temperature and desiccation, and perhaps even high UV radiation. Therefore, up-regulation of the ability to form blood clots in *P. annectens* transiently during the induction phase of aestivation could be interpreted as a stress response. Stress is known to cause alterations in the blood-clotting system through the release of adrenaline (Wademeyer et al., 1976; Casillas and Smith, 1977; Smith, 1980; Tavares-Dias and Oliveira, 2009). In fishes confronted with acute stresses, the elevation of circulating adrenaline levels can be high enough to cause widespread intravascular clotting (Woodward et al., 1981). Furthermore, *P. annectens* becomes physically more active during the early induction phase of aestivation, perhaps as it attempts to get back to water. In its natural habitat, the aestivating fish may burrow into the mud and form a subterranean cocoon to slow down the rate of desiccation. The burrowing actions may result in skin abrasion with vascular injury, and it would be essential for effective blood clot formation to prevent infections. In the past, the manifestation of organic structural modifications in aestivating animals has been largely neglected. However, recent reports reveal that aestivation in African lungfishes involves structural and functional modifications in the heart (Icardo et al., 2008), the kidney (Ojeda et al., 2008; Amelio et al., 2008) and the intestine (Icardo et al., 2012). Indeed, there is an apparent increase in metabolic rate in African lungfishes during the induction phase of aestivation. During the first 10 days of aestivation, hyperventilation occurs with a 2- to 5-fold increase in the ventilation rate, and the arterial *P*O₂ increases from 25–40 to 50–58 mmHg (Delaney et al., 1974). Hence, during the induction phase of aestivation, *P. annectens* is unlikely to be confronted with a stagnation of blood flow due to low heart rate and blood pressure, and there is no need to instil anti-thrombotic responses.
was significant down-regulation of the mRNA expression levels of \( f2 \) and \( fgg \) in the liver of \( P.\ annectens \). This supports the hypothesis that pro-coagulant genes could be down-regulated transcriptionally to ameliorate the risk of thrombosis during the maintenance phase of aestivation.

However, why would there be a significant increase in the protein abundance of \( F2 \) and \( Fgg \) in the liver of \( P.\ annectens \) that had undergone 12 days and 6 months of aestivation, respectively? As the transcription of \( f2 \) and \( fgg \) was down-regulated, it is highly unlikely that increased translation would have occurred to increase the production of \( F2 \) and \( Fgg \). A logical explanation is that, in spite of decreases in hepatic \( F2 \) and \( Fgg \) production, a restriction on the export of \( F2 \) and \( Fgg \) to the plasma to reduce the chance of thrombosis during the early maintenance phase of aestivation resulted in their accumulation in the liver. Overall, it can be concluded that long-term aestivation induces changes in the production and export of \( F2 \) and \( Fgg \) in the liver, resulting in decreases in their protein abundance in the plasma of \( P.\ annectens \).

Of note, the gradual decrease in mRNA expression levels of \( f2 \) and \( fgg \) seems to complement the gradual slow down in heart rate during aestivation as reported by Delaney et al. (1974), suggesting homeostatic feedback control in the lungfish. Rubanyi (1993) studied the role of endothelial cells in cardiovascular homeostasis and reported that the activation of endothelial cells could feed back on the regulatory mechanisms of blood coagulation within blood vessels. In addition, Lowe (2005) showed that blood flow exerted shear stresses on the endothelial cells to alter their morphology and function. Thus, it is probable that homeostatic control of blood coagulation occurs in concert with changes in blood flow in \( P.\ annectens \).

Changes in \( f2/fgg \) mRNA expression levels and \( F2/Fgg \) protein abundance during the arousal phase of aestivation in \( P.\ annectens \)

Upon awakening in water, the lungfish quickly rehydrates and excretes the accumulated nitrogenous waste, while heart rate and the blood flow to splanchnic organs return to normal (see Chew et al., 2015 for a review). In fact, arousal is associated with the drastic reversal of physiological and morphological changes in the lungfish. Indeed, the protein abundance of plasma \( F2 \) recovered in \( P.\ annectens \) 3 or 6 days after arousal from 6 months of aestivation, while that of plasma \( Fgg \) returned transiently to the control level 3 days after arousal. In addition, the mRNA expression level of \( fgg \) in the liver of \( P.\ annectens \) returned to a level comparable to that of the control fish 3 or 6 days after arousal. Although the hepatic \( f2 \) mRNA expression level remained significantly lower than the control value during the 6 day arousal period, western blotting results indicate that the release of \( F2 \) accumulated in the liver upon arousal could have contributed to the recovery of plasma \( F2 \) protein abundance. Overall, our result indicated that the complete recovery of the blood-clotting ability in the aroused \( P.\ annectens \) might take longer than 6 days. In general, African lungfishes would begin to feed only 7–10 days after arousal from aestivation. As a complete recovery requires increased synthesis of proteins, it is logical that it would occur only after re-feeding.

Conclusions

Our results indicate for the first time that there can be an increase in the blood-clotting ability, mainly due to changes in hepatic \( fgg/Fgg \) expression levels, in \( P.\ annectens \) during the induction phase of aestivation. The \( fgg \) mRNA expression in the liver is up-regulated after 3 days of aestivation, and the protein abundance of \( Fgg \) in the plasma increases significantly (3-fold) after 6 days of aestivation. By contrast, 6 months of aestivation leads to significant down-regulation in mRNA expression levels of both \( f2/fgg \) in the liver of \( P.\ annectens \). There may also be a restriction on \( F2 \) and \( Fgg \) export from the liver to the plasma, leading to substantial decreases in plasma \( F2 \) and \( Fgg \) protein abundance to suppress thrombosis. Overall, these results reveal that adaptive responses in \( P.\ annectens \) differ during the three phases of aestivation. As there is limited information on processes specific to the induction or arousal phases of aestivation, efforts should be made in the future to identify adaptive responses particular to each of the three phases of aestivation in African lungfishes.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Y.K.I. and S.F.C. conceived and designed the experiments. X.R.T., K.C.H. and M.V.B. performed the experiments. X.R.T., K.C.H., M.V.B. and Y.K.I. analysed the data. W.P.W. worked with the animals. X.R.T. and Y.K.I. drafted the manuscript. K.C.H., M.V.B. and Y.K.I. revised the manuscript.

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