Air-breathing and excretory nitrogen metabolism in fishes

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Abstract

During water-land transition, ancient fishes acquired the ability to breathe air, but air-breathing engendered problems in nitrogenous waste excretion. Nitrogen is a fundamental component of amino acids, proteins, and nucleic acids, and the degradation of these nitrogen-containing compounds releases ammonia. Ammonia is toxic and must be removed. Fishes in water excrete ammonia as the major nitrogenous waste through gills, but gills of air-breathing fishes are modified for air-breathing or largely replaced by air-breathing organs. Notably, fishes emerged from water can no longer excrete ammonia effectively because of a lack of water to flush the gills. Hence, ancient fishes that participated in water-land transition must have developed means to deal with ammonia toxicity. Extant air-breathing fishes, particularly amphibious ones, can serve as models to examine adaptations which might have facilitated the emergence of ancient fishes from water. Some of these fishes can actively emerge from water and display complex behaviors on land, while a few can burrow into mud and survive for years during drought. Many of them are equipped with mechanisms to ameliorate ammonia toxicity during emersion. In this review, the mechanisms adopted by air-breathing fishes to deal with ammonia toxicity during emersion were organized into seven disparate strategies. In addition, eight extant air-breathing fishes with distinctive terrestrial behaviors and peculiar natural habitats were selected to describe in detail how these seven strategies could be adopted in disparate combinations to ameliorate ammonia toxicity during emersion.

Keywords: Ammonia, amphibious fishes, gills, lung, urea, water-land transition

1. Introduction

The invasion of land by vertebrates was a salient event of vertebrate evolution. Water-land transition necessitated many important physiological and biochemical adaptations to the terrestrial environment. Particularly, there were profound changes in the respiratory organs, from gills to lungs, to facilitate air-breathing. These adaptations facilitated the migration of fishes to land, leading to the evolution of tetrapods. During water-land transition, fishes had to deal with problems concerning metabolism and excretion of nitrogenous compounds. Nitrogen is a crucial element in biological systems; it is a fundamental component of amino acids, proteins, and nucleic acids. The catabolism of these nitrogen-containing compounds releases ammonia which is toxic and must be removed. Most aquatic animals excrete ammonia as the major nitrogenous waste in water, but fishes emerged from water cannot effectively excrete ammonia due to a lack of water to flush the body surfaces. Hence, ancient fishes involved in water-land transition must have developed ways to defend against ammonia toxicity. Among modern fishes, there are plenty of air-breathing examples, and some of them are amphibious in nature. They have developed mechanisms and strategies to deal with ammonia toxicity during emersion, and therefore can serve as models to examine adaptations which might have facilitated the invasion of the terrestrial habitat by ancient fishes.

2. Ammonia production and excretion in aquatic fishes

Animals digest dietary protein to amino acids. The quantity of amino acids exceeding what is needed for growth and development is degraded because animals cannot store excess amino acids. The majority of amino acids are catabolized in the liver (Campbell, 1991), while some can be broken down in the intestine (Karlsson et al., 2006; Tng et al., 2008). In fishes, a major portion (40-60%) of the dietary nitrogen intake is excreted as nitrogenous wastes within 24 hours (Lim et

al., 2004; Ip et al., 2004c). During fasting, muscle proteins can be hydrolyzed to release amino acid for ATP or carbohydrate production (Houlihan et al., 1995).

In fishes, amino acid catabolism occurs mainly in the liver, and the breakdown of amino acids releases ammonia (See Ballantyne, 2001 for a review). In aqueous solution, ammonia is present in two forms, the molecular NH_3 and the cationic NH_4^+, and the p*K* of the related equilibrium reaction ($NH_3 + H_3O^+ \Leftrightarrow NH_4^+ + H_2O$) is ~9.5. In liver cells, several amino acids can be catabolized by specific deaminases (histidase, asparaginase, serine dehydratase and threonine dehydratase) with the release of NH_3 in the cytosol (Youngson et al., 1982). Nonetheless, ammonia is generally released as NH_4^+ from the α-amino group of various amino acids through transdeamination, which requires the combined actions of cytosolic aminotransferases and mitochondrial glutamate dehydrogenase, in hepatocytes (Walton and Cowey, 1977; French et al., 1981; Ballantyne, 2001). The deamination of glutamate by glutamate dehydrogenase produces NH_4^+ inside the mitochondrial matrix (Campbell et al., 1983). The mitochondrial matrix of some fishes also have glutaminase, which releases NH_3 from the amide-N of glutamine (Ballantyne, 2001). Ammonia produced in the mitochondrial matrix of hepatocytes enters the blood after permeating the mitochondrial and plasma membranes.

In plasma, ammonia is present mainly (>95%) as NH_4^+ because the plasma pH (~7.4) is approximately 1-2 units below the p*K* of ammonia. Ammonia is circulated through plasma to other parts of the body. As ammonia has multiple deleterious effects on many cellular processes (see below), it must be excreted to prevent accumulation (Ip et al., 2001b; Chew et al., 2006b; Ip and Chew, 2010b). Fully aquatic fishes are predominantly ammonotelic in water as they excrete >50% of the nitrogenous waste as ammonia. The primary organ of ammonia excretion in fishes is the gill (Evans and Cameron, 1986; Wilkie, 1997, 2002; Weihrauch et al., 2009), which has large surface area, extensive perfusion by blood, large ventilation rates, small diffusion distances, and close

contact with a voluminous external medium (Evans et al., 2005). Ammonia is largely excreted as NH_3 across the branchial epithelium down a favorable blood-to-water PNH_3 gradient (Wilkie, 1997, 2002; Evans et al., 2005), with or without the participation of transporters/channels (Weihrauch et al., 2009; Wright and Wood, 2009). Rhesus glycoproteins (Rhag, Rhbg and Rhcg) are crucial NH_3 (and NH_4^+) channels in ammonotelic fishes (Nawata et al., 2007, 2010; Nakada et al., 2007a, b; Hung et al., 2007, 2008; Braun et al., 2009). At the gill, erythrocytic Rhag can facilitate NH_3 efflux from erythrocytes into plasma. NH_3 can then permeate the basolateral membrane of the branchial epithelial cells through Rhbg, and exit the apical membrane through a Na^+/NH_4^+ exchange complex. This exchange complex consists of Rhcg, Na^+/H^+ exchangers, vesicular-type H^+-ATPase, and Na^+ channel, which act concertedly to provide an acid trapping mechanism to enhance apical NH_3 excretion (Wright and Wood, 2009). In fact, the outwardly diffusive movement of NH_3 is dependent on acid trapping in the boundary-layer of water by H^+ released through the non-catalyzed or catalyzed (by carbonic anhydrase) hydration of metabolic CO_2 (Wright and Wood, 2009). With acid trapping, NH_3 excreted across the branchial epithelium is converted to NH_4^+ and stays in the external medium. There are indications that Aquaporin 1aa can also act as an ammonia channel in the gill of climbing perch (Ip et al., 2013b).

2. Constraints imposed by air-breathing on nitrogenous waste excretion in fishes

Some fishes adopt air-breathing as an adaptive response to live in aquatic habitats where dissolved PO_2 is low (Graham, 1997). Many of them have degenerate gills compensated with accessory breathing organs (Graham, 1977). The gill morphology and morphometry of these fishes are modified (Low et al., 1988, 1990; Graham, 1977) in pursuance of reducing the loss of O_2 taken up by the air-breathing organs to the hypoxic water. However, these modifications may also interfere with ammonia excretion in water, as degenerate gills impede branchial ammonia excretion. With accessory breathing organs and degenerate gills, many air-breathing fishes practice

bimodal breathing during immersion. When they hold air in the buccal cavity without ventilation under water, the rate of branchial ammonia excretion would be drastically reduced. With air-breathing capability, some air-breathing fishes can survive passively in air during short periods of emersion, while others acquire the ability to actively emerge from water and make excursion onto land. A few of them can even burrow into mud to avoid desiccation as the external media dry up. When out of water, air-breathing fishes are confronted with problems of ammonia intoxication. The lack of water to flush the branchial and cutaneous surfaces leads to a build-up of excreted ammonia in the external unstirred-layer of water and disrupts the normally outwardly directed PNH_3 gradient. This would hinder ammonia excretion, leading to the accumulation of endogenous ammonia in the body.

3. Deleterious effects of ammonia

Ammonia is toxic to fishes because of multiply reasons. At the organismal level, it causes hyperventilation, hyper-excitability, coma, convulsions and, eventually, death of the fish (Hillaby and Randall, 1979; McKenzie et al., 1993). At the branchial level, NH_4^+ can affect certain transporters and hinder ionoregulation by replacing K^+, and interfere with the operation of Na^+/K^+ -ATPase and $Na^+:K^+:2Cl^-$ co-transporter (Wilkie, 1997; Person-Le Ruyet et al., 1997). It can also replace H^+ in Na^+/H^+ exchanger and affect acid-base balance (Randall et al., 1999). At the cellular level, ammonia can interfere with energy metabolism, as it can impair the tricarboxylic acid cycle (Arillo et al., 1981), by inhibiting pyruvate dehydrogenase, isocitrate dehydrogenase, or α-ketoglutarate dehydrogenase (Cooper and Plum, 1987). Ammonia can also activate phosphofructokinase I and stimulate glycolysis in fishes (Kloppick et al., 1967). In the central nervous system, ammonia asserts its acute effects through the activation of certain channels or transporters (Binstock and Lecar, 1969) and disruption of electrochemical gradients (Cooper and

Plum, 1987). For instance, NH_4^+ can substitute for K^+ to activate the background K^+ channel and affect the resting membrane potential (Binstock and Lecar, 1969).

In mammalian brain, acute ammonia toxicity is attributable to glutamatergic dysfunction, glutamine accumulation leading to astrocyte swelling, and activation of N-methyl-D-aspartate (NMDA) receptors (Brusilow, 2002; Felipo and Butterworth, 2002; Rose, 2002; Albrecht and Norenberg, 2006; Albrecht et al., 2010). The excessive activation of NMDA receptors in response to ammonia (Hermenegildo et al., 1996; Kosenko et al., 1999) can lead to oxidative stress, neuronal degeneration, and death of neurons (Miñana et al., 1996). Nitrosative/oxidative stress and mitochondrial permeability transition also contribute to ammonia neurotoxicity (Reddy et al., 2009; Bemeur et al., 2010; Görg et al., 2010; Häussinger and Görg, 2010; Görg et al., 2013). As mitochondrial permeability transition involves the opening of a pore in the inner mitochondrial membrane, it would lead to the collapse of ionic gradients resulting in mitochondrial dysfunction. It can also enhance the entry of cytosolic glutamine into the mitochondrion, and cause an increase in the production of ammonia through the glutaminase reaction in the mitochondrial matrix of astrocytes (Albrecht and Norenberg, 2006). Although ammonia may also cause oxidative stress in fish brains (mudskipper; Ching et al., 2009), many of these mechanisms proposed for ammonia toxicity in mammalian brains have not been confirmed in fishes, and air-breathing fishes apparently have higher brain ammonia tolerance than mammals(Wee et al., 2007; Tng et al., 2009; Ip and Chew, 2010b; Ip et al., 2005a, 2013a).

4. Strategies adopted by air-breathing fishes to defend against ammonia toxicity during emersion

Many extant air-breathing fishes have high tolerance for both emersion (in air) and environmental ammonia in water, but the strategies utilized to defend against ammonia toxicity under these two different conditions are not exactly the same (see Ip et al., 2001b, 2004a, 2004b;

Chew et al., 2006b, Ip and Chew, 2010b, Chew and Ip, 2014 for reviews). For instance, some of the strategies adopted to deal with environmental ammonia (e.g. lowering environmental pH and reducing NH_3 permeability of the skin) may not be applicable to fishes emerged from water. Specifically for emersion, extant air-breathing fishes generally display seven ammonia-defense strategies (Fig. 1), with four major themes including reduction in ammonia production, continuous excretion of ammonia, detoxification of ammonia, and tolerance of high levels of internal ammonia. All these strategies can be found in the invertebrate world, albeit not necessarily related directly to ammonia defense or emersion. Many invertebrates are known to regulate the rate of proteolysis when confronted with salinity or dehydration stresses (Gilles and Pequeux, 1981). They can also accumulate free amino acids, including alanine, glutamate, glutamine, glycine, proline and serine, during salinity acclimation (Livingston, 1985). Crustaceans (Greenaway, 1991) and certain land snails (Campbell, 1973) are known to volatilize ammonia. Land snails and earthworms possess carbamoyl phosphate synthetase, and increase the synthesis and accumulation of urea during aestivation or fasting (Campbell, 1973; Hiong et al, 2005). As these strategies were developed before the evolution of vertebrates, they were readily available for ancient and extant air-breathing fishes to adopt in various combinations to defend against ammonia toxicity during emersion.

4.1. Reduction in protein degradation and amino acid catabolism

This is a strategy that would lead to a reduction in ammonia production during emersion. Amino acids can be produced from proteolysis or through *de novo* synthesis. The steady state concentration of an amino acid is maintained by the balance between the rate of its production and the rate of its degradation. Changing in one or both of these two rates may lead to a change in the steady state concentration of the amino acid. Many air-breathing fishes can decrease the rate of amino acid catabolism in general, which would decrease the rate of ammonia production and

impede the build-up of ammonia in their bodies during emersion (Fig. 1). These include mudskippers (Lim et al., 2001; Ip et al., 2001c), marble goby (Jow et al., 1999), four-eyed sleeper (Ip et al., 2001a), weather loach (Chew et al., 2001), swamp eel (Tay et al, 2003; Chew et al., 2005a), and African lungfishes (Chew et al., 2004; Loong et al., 2005; Ip et al., 2005d). Despite decreases in the degradation of amino acids, the total free amino acid contents may remain relatively unchanged in multiple tissues of some of the air-breathing fishes. This is due to a reduction in the rate of proteolysis, which reduce the release of amino acids and decrease their rate of catabolism (Fig. 1).

4.2. Partial amino acid catabolism forming alanine

This is another strategy that would result in a reduction in ammonia production during emersion, particularly in relation to amino acid catabolism. Certain amino acids can be converted to glutamate; they include arginine, glutamine, histidine and proline. Through the transamination reaction catalyzed by alanine aminotransferase, glutamate can react with pyruvate to produce α-ketoglutarate and alanine without the release of ammonia (Ip et al., 2001c, Chew et al., 2003c). The α-ketoglutarate produced can be channelled into the tricarboxylic acid cycle and catabolized partially to malate. Malate can be directed out of the tricarboxylic acid cycle and turned into pyruvate by malic enzyme. This would provide a continuous supply of pyruvate to sustain the transamination reaction catalyzed by alanine aminotransferase to form α-ketoglutarate and alanine (Chew et al., 2003c). Overall, this metabolic pathway facilitates the partial catabolism of carbon chains derived from certain amino acids without producing ammonia. As ammonia is not released and then converted back to alanine, partial amino acid catabolism cannot be regarded as a mechanism for ammonia detoxification (Fig. 1). For this pathway to work, the kinetic properties of glutamate dehydrogenase must be modified in order to avoid the consumption of α-ketoglutarate for glutamate formation. As partial amino acid catabolism allows certain amino acids

to be used as an energy source without releasing ammonia, it is a major strategy adopted by fishes which are active on land. These include giant mudskipper (Ip et al., 2001c), climbing perch (Tay et al., 2006), and small snakehead (Chew et al., 2003c).

4.3. Active ammonia excretion

This strategy facilitates the continuous excretion of ammonia during emersion. A priori, the most effective way to avoid ammonia toxicity in fishes during emersion is to excrete ammonia continuously despite the lack of water to flush the branchial and cutaneous surfaces (Fig. 1). As ammonia is continuously excreted into a thin layer of external water, its concentration would build up therein and impede the diffusive ammonia efflux. Therefore, to achieve continuous ammonia efflux, the fish must have the ability to actively excrete ammonia against unfavourable $P\mathrm{NH_3}$ and $\mathrm{NH_4^+}$ gradients. With such an ability, the fish would be able to maintain low concentrations of internal ammonia and prevents the brain from ammonia intoxication. However, only a few air-breathing fishes with modified gill structures or accessory air-breathing organs are capable of active ammonia excretion. These include giant mudskipper (Randall et al., 1999; Ip et al., 2004d; Chew et al., 2003a, 2007; Chew et al., 2014, 2015), climbing perch (Tay et al., 2006; Loong et al., 2012a; Ip et al., 2012a, b) and African sharptooth catfish (Ip et al., 2004e).

4.4. Volatilization of ammonia This is another strategy which would facilitate the continuous excretion of ammonia during emersion. Terrestrial ammonotely is uncommon among vertebrates, but some air-breathing fishes can volatilize $\mathrm{NH_3}$, and hence excrete ammonia continuously, during emersion (Fig. 1). While the temperate intertidal blenny, *Blennius pholis*, can excrete 8% of the total ammonia as $\mathrm{NH_3}$ gas during emersion at 13˚C (Davenport and Sayer, 1986), several tropical air-breathing teleosts, including weather loach, can volatize a substantial amount of ammonia at temperatures close to 30^{o}C while on land (Rozemeijer and Plaut, 1993; Frick and Wright, 2002; Tsui et al., 2002). In the tropics, the humidity and temperature are high, which are

conducive for the excretion of NH_3 into the film of water covering the body surface and the volatilization of NH_3 in sizable quantities. Furthermore, at high temperatures, the ammonia equilibrium constant (pK_{amm}) is lowered resulting in a larger fraction of NH_3 at a given pH, which would accelerate NH_3 volatilization.

4.5. Glutamine synthesis

This strategy involves the detoxification of endogenous ammonia to glutamine. NH_4^+ can react with glutamate with the hydrolysis of ATP to form glutamine catalysed by glutamine synthetase (Campbell and Anderson, 1991; Fig. 1). Glutamate can be synthesized *de novo* from α-ketoglutarate and NH_4^+ in the presence of NADH (one mole of NADH is equivalent to three moles of ATP), catalyzed by glutamate dehydrogenase. Taken together, the formation of one mole of glutamine removes two moles of ammonia. Contrary to alanine formation, the production of glutamine is energetically intensive. One mole of ATP is required for the production of every amide group of glutamine via glutamine synthetase. If ammonia detoxification starts with NH_4^+ and α-ketoglutarate and ends in glutamine, every mole of ammonia detoxified would result in the hydrolysis of two moles of ATP-equivalent (Ip et al., 2001a). Whether glutamine production begins with glutamate or α-ketoglutarate is dependent on the subcellular localization of glutamine synthetase. In fishes, glutamate dehydrogenase is a mitochondrial enzyme (Campbell, 1973). Hence, to detoxify the ammonia released intracellularly through transdeamination to glutamine or urea, glutamine synthetase must be present inside the mitochondrion. However, ammonia may enter the cells under certain conditions, especially when the blood ammonia concentration is high. In order to detoxify the infiltrated NH_3, glutamine synthetase must be located in the cytosol instead.

In fish brains, glutamine formation plays a major role in ammonia detoxification (Levi et al., 1974; Arillo et al., 1981, Dabrowska and Wlasow, 1986; Mommsen and Walsh, 1992; Peng et al., 1998), and glutamine synthetase is localized to the cytosol of brain cells to protect them from

ammonia circulating in the blood (Korsgarrd et al., 1995). The activity of glutamine synthetase in fish brains are high, and brain glutamine synthetase has high affinity to ammonia with binding constant in the micromolar range (Mommsen and Walsh, 1991; Peng et al., 1998; Ip et al., 2001b). Hence, fish brains often show the largest increases in glutamine concentration in response to ammonia toxicity. However, marble goby (Jow et al., 1998), four-eyed sleeper (Ip et al., 2001a; Anderson et al., 2002), weather loach (Chew et al., 2001) and swamp eel (Tay et al., 2003; Chew et al., 2005a) can detoxify endogenous ammonia to glutamine in non-cerebral tissues during emersion. As these fishes mostly remain quiescent on land, the energy demand for muscular activity is small, which may have enabled them to exploit glutamine synthesis as a means to detoxify ammonia. Unlike urea, the glutamine accumulates in tissues and can be used for the syntheses of other compounds, like purines, pyrimidines, and mucopolysaccharides, when water becomes available again.

4.6. Urea synthesis

This is another strategy adopted by a few air-breathing fishes to detoxify endogenous ammonia. Urea is less toxic than ammonia. It can be produced through the ornithine-urea cycle, the routine turnover of arginine by argininolysis, and the degradation of uric acid through uricolysis. Of these, only the ornithine-urea cycle takes part in ammonia detoxification through *de novo* urea synthesis. In general, urea is a minor component of nitrogenous wastes in fishes, but the ability to excrete urea does not denote the possession of a functional ornithine-urea cycle. Fishes are described as ureogenic only when they possess a functional ornithine-urea cycle with at least a low rate of urea synthesis. Fishes which excrete >50% of nitrogenous waste as urea-N are regarded as ureotelic. Ureogenic fishes are not necessarily ureotelic. Only marine (Anderson, 1995) and euryhaline elasmobranchs (Tam et al., 2003; Ip et al., 2003) and very few teleosts (Randall et al., 1989; Iwata et al., 2000) are ureogenic and ureotelic in water. African lungfishes

289 are ureogenic, but they are ammonotelic in water under normal circumstances (Chew et al., 2003b;
290 Lim et al., 2004). The majority of teleosts are non-ureogenic and non-ureotelic. Some normally
291 ammonotelic fishes may increase urea excretion or become transiently ureotelic after feeding (Ip
292 et al., 2004c; Lim et al., 2004; Chew et al., 2006a). During immersion, urea produced is excreted
293 through facilitated urea transporters across the branchial epithelium (McDonald et al., 2006).

294 Ureogenic fishes possess carbamoyl phosphate synthetase III which uses glutamine as a
295 substrate (Anderson, 1995; Loong et al., 2012b, c). Overall, the synthesis of one mole of urea
296 requires the hydrolysis of five moles of ATP in ureogenic fishes. As urea synthesis in fishes is
297 energy intensive, many air-breathing teleosts do not adopt ureogensis as a major strategy to
298 ameliorate ammonia toxicity during emersion. They include mudskippers (Lim et al., 2001),
299 marble goby (Jow et al., 1999), four-eyed sleeper (Ip et al., 2001c), weather loach (Chew et al.,
300 2001), small snakehead (Chew et al., 2003c), African sharptooth catfish (Ip and Chew,
301 unpublished results), swamp eel (Tay et al., 2003), mangrove killifish (Frick and Wright, 2002)
302 and central mud minnow (Currie et al., 2010). By contrast, African lungfishes (sarcopterygians),
303 possess a functional ornithine-urea cycle and utilize ureogenesis as an essential mechanism to
304 defend against ammonia toxicity on land (Chew et al., 2003b, 2004, 2005b; Ip et al., 2005d, Loong
305 et al., 2005, 2007, 2008b; Fig. 1).

306 **4.7. Tolerance of high levels of internal ammonia**

307 Some air-breathing fishes adopt the strategy to accumulate and tolerate high concentrations
308 of ammonia in their tissues during emersion (Fig. 1), although ammonia is not always evenly
309 distributed within their bodies. Some of them accumulate high levels of ammonia in the muscle,
310 while others can tolerate relatively high concentrations of ammonia in the brain (see below). How
311 the cells and tissues, especially those in the brain, of these fishes ameliorate the deleterious effects
312 of ammonia is enigmatic at present. They might have developed K^+-specific K^+ channels, K^+-

specific Na^+/K^+-ATPase, and/or special NMDA receptors. Also, they might be able to tolerate the build-up of glutamine better than those of other fishes and mammals. Mammalian brains can tolerate only ~1 μmol g^{-1} of ammonia, beyond which encephalopathy would develop (Cooper and Plum, 1987). Hence, air-breathing fishes with high brain ammonia tolerance, like mudskippers (>14·μmol·g^{-1} of brain ammonia; Ip et al., 2005a) and swamp eel (>3 μmol g^{-1} of ammonia in the brain; Tay et al., 2003; Chew et al., 2005a; Tng et al., 2009), are ideal specimens for studies on mechanisms of ammonia defence in the central nervous system, a feat which mammals have apparently lost during evolution.

5. Eight air-breathing fishes which display various combinations of the seven strategies to deal with ammonia during emersion

Air-breathing fishes can adopt the above-mentioned seven strategies in various combinations to defend against ammonia toxicity during emersion. The combination adopted can be correlated to the behavior of the fish, the frequency and duration of terrestrial exposure, and/or the nature of the environment in which it lives. To underscore the flexibilities of adaptations to emersion, eight air-breathing fishes with distinct terrestrial behaviors and diverse natural habitats are selected to highlight how these seven strategies can be adopted in disparate combinations to ameliorate ammonia toxicity. Among these eight fishes, the spectrum of behaviour ranges from brief and frequent periods of intense activity on land (mudskippers) to infrequent but long duration of passive survival in mud (African lungfishes). Mudskippers are amphibious gobies which move actively on mudflats of estuaries during low tides. They build burrows in mud when the tide is low, and display uncommon feeding, territorial as well as courtship behaviors on mudflats. The small snakehead and the four-eyed sleeper are non-amphibious and would prefer to stay in water. However, they can be exposed to air occasionally when trapped in crevices, especially during dry seasons or when the tide is low. While on land, the small snakehead would try to wriggle its way

back to water, but the four-eyed sleeper would stay passively with minimal activity awaiting the return of water. The weather loach may encounter seasonal drought, during which it would burrow into soft mud and stay inside the mud for up to several months until the drought is over. Lastly, the African lungfishes can be exposed to drought for long periods in their natural habitats, and they can uniquely undergo aestivation inside a mucus cocoon in mud for up to four years without food and water.

5.1. Two mudskippers

Mudskippers (Class: Actinopterygii, Order: Perciformes, Family: Gobiidae) are amphibious and euryhaline fishes commonly found in mangrove swamps in estuaries of the tropical Indo-Pacific. They make burrows in the mudflats during low tides, and certain mudskipper species would stay inside them during high tides. The giant mudskipper, *Periophthalmodon schlosseri*, and the Boddart's goggle-eyed mudskipper, *Boleophthalmus boddarti*, display different behaviors despite sharing the same macro-habitat. When compared with *P. schlosseri*, *B. boddarti* appears less well adapted to land, and its burrows are found on the lower regions of the mudflats. Individuals of *B. boddarti* are usually seen on the mudflats at low tide; as the tide rises, they retreat into the burrows and remain submerged until the tide ebbs. Indeed, the branchial morphology and morphometry in *B. boddarti* are comparable to those of other water-breathing teleosts (Low et al., 1990, 1988), and it is a facultative air-breather. As for *P. schlosseri*, the burrows are found on high ground far away from the water's edge. At high tide, individuals of *P. schlosseri* typically swim along the water's edge, or hunt for food on land. The gills of *P. schlosseri* exhibit unusual modifications for air-breathing; their gill filaments are branched and adjacent secondary lamellae of the same filament are fused to form numerous fenestrae (Low et al., 1990, 1988). These branchial modifications of *P. schlosseri* facilitate active ammonia excretion into water trapped inside the fenestrae during emersion (see below), but they render aquatic respiration ineffective.

As a result, *P. schlosseri* displays bradycardia in water (Kok et al., 1998), and it can be suffocated by prolonged immersion without adequate aeration.

While terrestrial exposure leads to decreases in rates of ammonia and urea excretion in *P. schlosseri* and *B. boddarti*, the levels of ammonia accumulation in their tissues are low, indicating that they can reduce ammonia production through decreased amino acid catabolism (Lim et al., 2001). This should theoretically result in an increase in the total free amino acid concentrations in certain tissues. However, exposure to terrestrial conditions in constant darkness leads to decreases in the total free amino acid concentrations in the liver and plasma of *P. schlosseri* and in the muscle of *B. boddarti* (Lim et al., 2001; Ip et al., 2001c). Therefore, it can be deduced that these two mudskippers decrease rates of proteolysis and amino acid catabolism simultaneously while they remain quiescent in constant darkness due to the lack of visual stimulations. The decrease in proteolytic rate must be greater than the decrease in the rate of amino acid catabolism; only then, would there be decreases in the steady-state concentrations of various free amino acids and consequently lowering the total free amino acid concentrations. The slight accumulation of ammonia in the tissues and organs of these two mudskippers indicates that the decrease in the rate of nitrogenous excretion is still greater than the decrease in the rate of ammonia production.

Although decreases in protein and amino acid catabolism represent an effective strategy to slow down the internal build-up of ammonia, it hinders the utilization of amino acids for ATP production. Hence, it may not be a useful mechanism for amphibious fishes which are active on land. To overcome this, *P. schlosseri* adopts the strategies of reduction in amino acid catabolism in conjunction with partial amino acid catabolism leading to the formation of alanine. This allows the giant mudskipper to use proteins and amino acids as energy sources to support movement on land without producing ammonia. When exposed to terrestrial conditions under a dark:light regime, contents of several essential amino acids, including isoleucine, leucine, proline, serine,

lysine and valine, increase in the tissues of *P. schlosseri*. Specifically, the concentration of alanine increases significantly in the muscle, liver and plasma, accompanied by a rise in the total free amino acid concentrations in the muscle and plasma (Ip et al., 1993; Ip et al., 2001c). As the physical activity of *P. schlosseri* is high in light due to visual stimulations, it can be deduced that *P. schlosseri* mobilizes amino acids through increased proteolysis to support physical activity on land under a dark:light regime (Ip et al., 2001c).

Indeed, after exposure to terrestrial conditions in light for three hours followed with three minutes of exercise, ammonia and alanine concentrations increase in the muscle of *P. schlosseri*, indicating the mobilization of certain amino acids through partial amino acid catabolism (Ip et al., 2001c). However, the glycogen content remains unchanged in spite of a small increase in the lactate concentration in the muscle of these experimental fishes. For *P. schlosseri* forced to exercise for three minutes after 24 h of exposure to terrestrial conditions in a dark:light regime, there is an even greater accumulation of alanine in the muscle (Ip et al., 2001c). Hence, the efficiency of partial amino acid catabolism during exercise on land is dependent on the preceding period of terrestrial exposure. Through partial amino acid catabolism and the resulting alanine formation, *P. schlosseri* can reduce its dependency on glycogen for energy supply, and sustain a high metabolic rate for an extended period on land (Kok et al., 1998).

When kept out of water, *P. schlosseri* can continuously excrete ammonia into the water trapped in the fenestrae of the branchial inter-lamellar fusions. No other fish is known to have intrafilamentous interlamellar fusions like *P. schlosseri*. After 24 hours of exposure to terrestrial conditions, the ammonia concentration in water samples (pH 6.3) collected from the branchial surface increases to ~30 mmol l^{-1} (Chew et al., 2007). As the plasma ammonia concentration is low (0.6 mmol l^{-1}), both P_{NH3} and NH_4^+ concentration gradients are acting inwards across the branchial epithelium under such circumstances. Hence, it can be concluded that *P. schlosseri* is

capable of actively excreting NH_4^+ through certain branchial ionocytes (mitochondrion-rich cells) into the fenestrae during emersion. The ionocytes in the gills of *P. schlosseri* are uniquely isolated from one another by filament-rich cells (Wilson et al., 1999). It is possible that these filament-rich cells provide contractions to help release the fenestral water and to facilitate water renewal.

During active NH_4^+ excretion, NH_4^+ can enter certain branchial ionocytes through the basolateral Na^+:K^+:$2Cl^-$ cotransporter 1a by replacing K^+ (Chew et al., 2015). Unlike the uphill K^+ movement, the intracellular ammonia concentration is low, and the transport of NH_4^+ across the basolateral membrane involves a downhill NH_4^+ electrochemical gradient. As increased transport of Na^+, NH_4^+ and $2Cl^-$ into the cell would alter the transmembrane Na^+ gradient, there must be an increase in branchial activity of Na^+/K^+-ATPase with decreased NH_4^+ affinity (Chew et al., 2014), so as to maintain intracellular Na^+ and K^+ homeostasis. Indeed, the *Na^+/K^+-ATPase α-subunit* isoforms cloned and sequenced from the gills of *P. schlosseri* possesses K^+ binding sites with much higher affinity to K^+ than NH_4^+ (Chew et al., 2014). Therefore, Na^+/K^+-ATPase is unlikely to be involved directly in the transport of NH_4^+ from the blood into the branchial ionocyte of *P. schlosseri* during active NH_4^+ excretion, although active NH_4^+ excretion can be inhibited by the Na^+/K^+-ATPase inhibitor, ouabain (Randall et al., 1999). Actually, the inhibition of Na^+/K^+-ATPase by ouabain leads to the dissipation of the Na^+ electrochemical potential gradient, which necessarily hinders the operation of Na^+:K^+:$2Cl^-$ cotransporter 1a to transport NH_4^+ across the basolateral membrane (Chew et al., 2014). As active NH_4^+ excretion in *P. schlosseri* can be inhibited by amiloride (Randall et al., 1999) which is a Na^+/H^+ exchanger inhibitor, active excretion of NH_4^+ across the apical membrane of branchial ionocytes probably involves Na^+/H^+ (NH_4^+) exchangers (Randall et al., 1999; Wilson et al., 2000).

While out of water, the rate of active NH_4^+ excretion must be greater than the rate of NH_3 back-flux; only then, would there be a continuous excretion of ammonia into the branchial water.

The branchial water collected from *P. schlosseri* after 24 hours of emersion has a pH of 6.3, indicating that H^+ is excreted into the water in the fenestrae (Chew et al., 2007). In fact, *P. schlosseri* has the ability to lower the pH of the external medium, especially in an alkaline medium and/or in the presence of environmental ammonia (Ip et al., 2004d; Chew et al., 2003a). The continuous excretion of H^+ into the water inside the fenestra decreases the pH therein (Ip et al., 2004d) and prevents NH_4^+ from dissociating into NH_3 and H^+. This would avoid a back flux of NH_3 through NH_4^+-trapping despite the high concentration of ammonia (32 mmol l^{-1}) therein (Ip et al., 2004d). It would be more advantageous for *P. schlosseri* to adopt NH_4^+-trapping, rather than to alter the fluidity of the branchial epithelial surface to decrease NH_3 permeability, as its major respiratory organ is the gill and it does not have any accessory breathing organ (Low et al., 1988). A reduction in the fluidity of the branchial epithelium would have the undesirable effect of decreasing the permeability of the epithelium to O_2 and hinder respiration. At present, the origin of the H^+ for NH_4^+-trapping is unknown, but it may involve the hydration of CO_2 by carbonic anhydrase producing H^+ and HCO_3^- inside the ionocyte. H^+ can be excreted through the apical membrane to trap the excreted NH_4^+. Separately, HCO_3^- can be transported through a basolateral Cl^-/HCO_3^- exchanger into the blood, which can explain why the blood pH becomes atypically more alkaline in *P. schlosseri* exposed to terrestrial conditions (Kok et al., 1998).

With the intraperitoneal injection of ammonium acetate (8 μmol g^{-1}) into *P. schlosseri* followed with 24 hours of terrestrial exposure, a major portion (33%) of the injected ammonia is excreted from the head region, probably through the gills, after six hours (Chew et al., 2007). Ammonia concentration builds up quickly in the small amount of water inside the fenestra and reaches an extraordinarily high concentration of ~90 mmol l^{-1} (Chew et al., 2007). Hence, *P. schlosseri* can indeed excrete a high load of ammonia effectively through active NH_4^+ excretion on land, contributing in part to its high terrestrial affinity.

458 **5.2. Small snakehead**

459 The small snakehead, *Channa asiatica* (Class: Actinopterygii, Order: Perciformes, Family:
460 Channidae), is an obligate air-breathing freshwater teleost found in Asia, including Taiwan,
461 Southern China and Sri Lanka. It is a predaceous fish residing in slow-flowing streams and in
462 crevices near riverbanks. It may experience bouts of aerial exposure during dry seasons. While out
463 of water, it is unable to move around using its pectoral fins, and does not display any special
464 behavior. At the start of the emersion, it may attempt several times to struggle back to water
465 through eel-like body movements. If unsuccessful, it would turn motionless and remain quiescent
466 with occasional body movements. The reduction in nitrogenous excretion is completely balanced
467 by the nitrogenous accumulation in its tissues during 48 hours of terrestrial exposure (Chew et al.,
468 2003c). Hence, different from mudskippers (Ip et al., 2001c), it does not adopt the strategy of
469 reduction in rates of proteolysis and amino acid catabolism during emersion. Yet, after 48 hours
470 of terrestrial exposure, the concentration of alanine increases 4-fold, from 3.7 to 12.6 μmol g^{-1}, in
471 the muscle (Chew et al., 2003c), and the accumulated alanine accounts for 70% of the deficit in
472 ammonia excretion. Hence, *C. asiatica* practises partial amino acid catabolism, which allows the
473 utilization of certain amino acids as energy sources and, at the same time, minimizes ammonia
474 accumulation during emersion. To facilitate alanine formation during terrestrial exposure, there
475 are significant decreases in the aminating activities of glutamate dehydrogenase from the muscle
476 and liver (Chew et al., 2003c). However, unlike *P. schlosseri*, *C. asiatica* is incapable of increasing
477 the rate of partial amino acid catabolism to sustain locomotor activities on land (Chew et al.,
478 2003c), which may explain why it can only afford short bursts of muscular activity to try to get
479 back to water during emersion.

480 **5.3 Four-eyed sleeper**

The four-eyed sleeper, *Bostrychus sinensis* (Class: Actinopterygii, Order: Perciformes, class: Actinopterygii, Family: Eleotridae), can be found in the Indo-Pacific, from India to Australia and Taiwan. It inhabits brackish water of the river mouths, and is a facultative air-breather. As it often seeks and stays inside crevices above the water's edge, it may be passively exposed to air by a receding tide. During the first 24 hours of terrestrial exposure, it does not reduce amino acid catabolism but detoxifies endogenous ammonia to glutamine which accumulates in the muscle (Ip et al. 2001a). The reduction in ammonia excretion during such a period is completely accounted for by the accumulated glutamine-N in the muscle. However, there is a much greater discrepancy between the reduction in nitrogenous excretion and the retention of nitrogen in *B. sinensis* after 72 hours of terrestrial exposure (Ip et al., 2001a), indicating substantial reductions in proteolysis and amino acid catabolism after long periods of emersion. In addition, the glutamine content in the muscle returns back to the control level. Hence, it can be deduced that the accumulated glutamine can be metabolized into other nitrogenous compounds via anabolic pathways during long-term terrestrial exposure.

Glutamine synthetase activity is generally below the level of detection in the liver and muscle of non-ureosmotic fishes (Campbell and Anderson, 1991; Mommsen and Walsh, 1992). However, two *glutamine synthetase* genes have been reported for *B. sinensis* and high activities of glutamine synthetase can be detected in its stomach, intestine (foregut and hindgut), liver and muscle (Anderson et al., 2002). Glutamine synthetase is a cytosolic enzyme in the liver of *B. sinensis* (Anderson et al., 2002). The cytosolic compartmentalization of hepatic glutamine synthetase in this fish would facilitate the detoxification of ammonia produced by other tissues and circulated in the blood. Upon entering into hepatocytes, ammonia is converted into the amide-N, and not the amino-N of glutamine. As *B. sinensis* remains quiescent on land, the low energy

demand for muscular activity may provide them with the opportunity to exploit the energy-dependent glutamine synthesis as a means to detoxify ammonia.

5.4. Weather loach

The Oriental weather loach, *Misgurnus anguillicaudatus* (Class: Actinopterygii, Order: Cypriniformes, Family: Cobitidae), inhabits rivers, lakes, ponds, swamps and rice fields, and prefers muddy substratum, in Asia. It is an obligate air-breather, and uses the intestine as an additional respiratory organ (McMahon and Burggren, 1987). Hence, it can live in oxygen-poor waters. It can also burrow in soft mud and survive for several months therein during drought.

During the first 24 hours of terrestrial exposure, *M. anguillicaudatus* can suppress proteolysis and amino acid catabolism to transiently impede ammonia production. Alanine accumulates during this period, indicating the usage of certain amino acids to sustain muscular activities on land through partial amino acid catabolism (Chew et al., 2001). Glutamine begins to accumulate in the muscle and liver of *M. anguillicaudatus* after 24 hours of terrestrial exposure. Simultaneously, ammonia accumulates in all tissues, indicating that increased glutamine synthesis is not the major mechanism of ammonia defense (Chew et al., 2001). Ammonia builds up to very high concentrations in the muscle (~15 μmol g^{-1}), liver (~15 μmol g^{-1}) and plasma (~5 μmol ml^{-1}) after 48 hours of air exposure, and levels off thereafter (Chew et al., 2001). The leveling off of ammonia concentrations is attributable to NH_3 volatilization which occurs only after the build-up of internal ammonia to conducive concentrations (Tsui et al., 2002). On land, *M. anguillicaudatus* can excrete a substantial quantity of ammonia as NH_3, and NH_3 volatilization increases progressively through a 3-day period of terrestrial exposure (Tsui et al., 2002). This indicates that building up of internal ammonia is an essential prerequisite for volatilization to occur.

During terrestrial exposure, the skin of *M. anguillicaudatus* becomes more alkaline (Tsui et al., 2002), and alkalinity would increase the proportion of NH_3 to NH_4^+ in aqueous solution.

Hence, the skin of *M. anguillicaudatus* is a probable site of NH_3 volatilization. In addition, the pH also increases in the liquid covering the mucosal surface of the anterior portion of the digestive tract (Tsui et al., 2002). Notably, cutaneous NH_3 volatilization would be ineffective when the fish burrows into mud which covers the skin; under such circumstances, NH_3 volatilization can only occur through the digestive tract. NH_3 volatilization in *M. anguillicaudatus* exposed to terrestrial conditions is temperature-dependent because the total amount of ammonia excreted is significantly higher at 30ºC than at 25 or 20ºC (Tsui et al., 2002).

After six hours of exposure to air, the ammonia concentration increases from 0.81 to 2.46 μmol ml^{-1} in the plasma of *M. anguillicaudatus* (Chew et al., 2001). The highest concentration (5.09 μmol ml^{-1}) is reached by hour 48. To date, no other fish accumulates such a high level of ammonia in the plasma on land. Usually, the blood ammonia concentration also rises during emersion, butweather loach can uniquely maintain the ammonia concentration in the brain lower than that in the blood after 48 hours of terrestrial exposure (Chew et al., 2001; Tsui et al., 2002). Even then, the brain of *M. anguillicaudatus* must tolerate high concentrations of ammonia and therefore ought to be equipped with effective ammonia defense mechanisms. It has been speculated that *M. anguillicaudatus* may have greater space in the skull to prevent the build-up of intracranial pressure, special mechanisms to maintain the homeostasis of the extracellular glutamate concentration in the brain, and/or NMDA receptors which are less sensitive to fluctuation in glutamate level (see Ip et al., 2004a, b; Ip and Chew, 2010b; Chew and Ip, 2014 for reviews). In addition, the brain of *M. anguillicaudatus* may have Na^+/K^+-ATPase and K^+ channels with high substrate specificity for K^+ to prevent its substitution by NH_4^+ and the disruption of the intracellular K^+ concentration and the resting membrane potential.

5.5. Three African lungfishes

551 Lungfishes can breathe air using primitive ‘lungs’, which are outgrowths of the oesophagus,
552 and survive on land for extended periods during drought. There are six extant lungfish species,
553 four of which are found in Africa. African lungfishes (Class: Sarcopterygii, Subclass: Dipnoi,
554 Order: Lepidosireniformes, Family: Protopteridae) are obligate air-breathers. As their gills do not
555 allow them to breathe exclusively in water, they have to gulp atmospheric air to satisfy their O_2
556 demand during immersion. African lungfishes are ureogenic and possess a functional ornithine-
557 urea cycle in the liver (see Ip and Chew, 2010a; Ballantyne and Frick, 2010; Chew et al., 2016 for
558 reviews). They express carbamoyl phosphate synthetase III which uses glutamine as a substrate
559 (Chew et al., 2003b; Loong et al., 2005; 2012b, c). Despite being ureogenic, African lungfishes
560 are ammonotelic in water, and only become ureotelic briefly after feeding (Lim et al., 2004; Iftikar
561 et al., 2007). African lungfishes can undergo aestivation and enter into a state of torpor in mud
562 cocoons during desiccation (see Ip and Chew, 2010a and Ballantyne and Frick, 2010 for reviews).
563 In mud, they can aestivate for as long as four years (Smith, 1930). There are indications that
564 increased urea synthesis and accumulation may have a physiological role in initiating and
565 perpetuating aestivation in African lungfishes (Ip et al., 2005b, c; see Ip and Chew, 2010a; Chew
566 et al., 2016 for a reviews).

567 In the laboratory, African lungfishes can be induced to aestivate in pure mucus cocoons in
568 air or in mud inside plastic containers (Chew et al., 2004; Ip et al., 2005a; Loong et al., 2005,
569 2008a, b). Aestivation comprises three phases. The first 6-8 days are regarded as the induction
570 phase, during which the aestivating lungfish secretes mucus and forms a mucus cocoon. The
571 maintenance phase of aestivation begins when the fish is completely encased in the dried cocoon
572 with no noticeable movement, and this phase can last for at least a year without food and water.
573 With the addition of water, the lungfish can be aroused from aestivation. The aroused lungfish
574 struggles out of the cocoon and swims sluggishly to the water surface to gulp air. It would not feed

575 until 7-10 days later, and will grow and develop normally thereafter. During the induction and
576 arousal phases, aestivating lungfishes have to undergo biochemical, physiological, and structural
577 modifications, which are important facets of the aestivation process. Aestivating African
578 lungfishes rely heavily on protein and amino acid catabolism for the supply of energy and
579 substrates for structural modifications. They must therefore defend against ammonia toxicity
580 during aestivation.

581 By avoiding complete desiccation and preventing cocoon formation, African lungfishes
582 can be exposed to terrestrial conditions without entering into aestivation in the laboratory. The
583 major mechanisms adopted by African lungfishes to deal with ammonia toxicity during terrestrial
584 exposure are increased urea synthesis and decreased ammonia production. During exposure to air
585 for six days, non-aestivating *Protopterus dolloi* displays a significant decrease in the rate of
586 ammonia excretion, but the ammonia contents in the muscle, liver or plasma remain unchanged,
587 indicating an apparent reduction in ammonia production (Chew et al., 2003b). In addition, six days
588 of terrestrial exposure leads to significant increases in urea concentrations in the muscle, liver, and
589 plasma of *P. dolloi*. There are also significant increases in the activities of carbamoyl phosphate
590 synthetase III, argininosuccinate synthetase + lyase, and glutamine synthetase in the liver of *P.*
591 *dolloi* (Chew et al., 2003b), confirming increased urea synthesis has indeed occurred during this
592 period. In comparison, six days of terrestrial exposure has no significant effects on the hepatic
593 carbamoyl phosphate synthetase III activities of *Protopterus aethiopicus* and *Protopterus*
594 *annectens*, and leads to only slight increases in the rates of urea synthesis (Loong et al., 2005).
595 Conversely, terrestrial exposure induces greater degrees of reductions in ammonia production in
596 *P. aethiopicus* and *P. annectens* compared with *P. dolloi*. Thus, there are subtle differences in
597 responses by various species of African lungfish to terrestrial exposure. It would appear that *P.*

598 *aethiopicus* and *P. annectens* depend more on reducing ammonia production than on increasing
599 urea synthesis to ameliorate ammonia toxicity (Loong et al., 2005).

600 With complete desiccation, African lungfishes can be induced to aestivate in air inside a
601 dried mucus cocoon that encases the entire body. In *P. dolloi*, ammonia excretion decreases
602 drastically during the induction phase (the first six days), and comes to a complete stop during the
603 maintenance phase, of aestivation (Chew et al., 2004). This is not attributed simply to a lack of
604 water to flush the branchial and cutaneous surface, as in the case of emersion without entering into
605 aestivation. There are actually changes in the expression levels of ammonia transporters (Rhesus
606 glycoproteins) in the gills of the aestivating lungfish. During the induction phase, the protein
607 abundance of Rhag, but not its transcript level, is down-regulated in the gills of *P. annectens*,
608 suggesting a decrease in the release of ammonia from the erythrocytes to the plasma (Chng et al.,
609 2017b). Furthermore, the branchial transcript levels of *rhbg* and *rhcg* decrease significantly, in
610 preparation for the subsequent shutdown of gill functions. During the maintenance phase, the
611 branchial expression levels of *rhag*/Rhag, *rhbg*/Rhbg and *rhcg*/Rhcg decrease significantly,
612 indicating that their transcription and translation were down-regulated (Chng et al., 2017b). This
613 can be regarded as part of an overall mechanism to shut down branchial functions and to save
614 metabolic energy used for transcription and translation. It can also be regarded as an adaptive
615 response to stop ammonia excretion. In fact, the branchial transcript levels and protein abundance
616 of *aqp1*/Aqp1 and *aqp3*/Aqp3 also decrease significantly during the maintenance phase due to the
617 shutdown of branchial functions and the cessation of volume regulation of branchial epithelial
618 cells, which may reduce the loss of water through branchial epithelial surfaces (Chng et al., 2016).

619 There are apparent decreases in the rate of ammonia production in *P. dolloi* (Chew et al.,
620 2004) and *P. aethiopicus* (Ip et al., 2005a) during the induction phase of aestivation. However, this
621 should not be viewed as a response to suppress ammonia production or to save metabolic reserves

as in the case of six days of emersion without aestivation (Chew et al., 2003b). In fact, the aestivating lungfishes hyperventilate during the first few days of aestivation, indicating the possibility of an increase in metabolic rate. The increased metabolic rate is attributable partially to a rise in metabolic energy required for increased protein synthesis for mucus production and for structural modifications of certain tissues and organs (Ojeda et al., 2008; Icardo et al., 2008, 2012). During the induction phase of aestivation, there is an increase in urea synthesis in *P. dolloi* (Chew et al., 2004) and *P. aethiopicus* (Ip et al., 2005a) not only to detoxify ammonia but to promote the accumulation of urea which may play a role in inducing and perpetuating the aestivation process (Chew et al., 2004; Ip et al., 2005b, c).

Indeed, there are significant increases in urea contents in various tissues and activities of some ornithine-urea cycle enzymes in the liver of *P. aethiopicus* after 46 days of aestivation in air (Ip et al., 2005a). However, a shift in strategy of ammonia defense actually occurs during the maintenance phase of aestivation. Between day 12 and day 46, the rate of urea synthesis decreases progressively, and between day 34 and day 46 (altogether 12 days), the rate of urea synthesis becomes significantly lower than that of the day 0 control (Ip et al., 2005a). This is accompanied by a profound suppression of ammonia production (by 96%) between day 34 and day 46 to lessen the demand for increased urea synthesis. In comparison, the rate of urea synthesis increases 3.8-fold while the rate of ammonia production decreases by 72% in *P. dolloi* during 40 days of aestivation in air. Hence, the ability to reduce ammonia production apparently varies among different African lungfish species. As ammonia excretion comes to a complete halt during the maintenance phase of aestivation, ammonia produced endogenously has to be continuously detoxified to urea despite the reduction in its production. By synthesizing and accumulating the less toxic urea, aestivating African lungfishes can carry out protein catabolism for a long period without being intoxicated by ammonia. Unlike the induction phase of aestivation, the activities of

several ornithine-urea cycle enzymes increase significantly in *P. dolloi* after 40 days of aestivation (Chew et al., 2004).

African lungfishes can also aestivate in mud, and the ammonia defense strategies appear to be different from those related to aestivation in air. Twelve days of aestivation in air leads to increases in urea synthesis and tissue urea contents, but only minor changes in the ammonia production, in *P. annectens* (Loong et al., 2008b). After 46 days of aestivation in air, the ammonia concentration in the liver decreases significantly, but urea concentration increases in all tissues, indicating a combined strategy of decreased ammonia production and increased urea synthesis to defend against ammonia toxicity. By contrast, 12 days of aestivation in mud leads to only minor increases in tissue urea contents, and the tissue ammonia contents remain unchanged in *P. annectens* (Loong et al., 2008b). More importantly, the tissue urea contents remain unchanged in *P. annectens* after 46 days of aestivation in mud, due to a profound suppression of ammonia production and urea synthesis. Fish aestivated in mud has low blood PO_2 and muscle ATP content, indicating hypoxic exposure (Loong et al., 2008b), and environmental hypoxia could have induced reduction in metabolic rate as well as suppression of ammonia production (Loong et al., 2008a). Apparently, aestivating in mud offers the advantage of a lower dependency on increased urea synthesis to detoxify ammonia.

Urea accumulated in the body of African lungfishes during short term emersion or long-term aestivation can be excreted effectively during arousal in water. After six days of terrestrial exposure, the urea excretion rate increases 22-fold in *P. dolloi* during re-immersion as compared to the control specimen (Chew et al., 2003b). This is the greatest increase in urea excretion amongst fishes during emersion-immersion transition. Upon arousal, urea can be excreted through the gills (28%) and skin (72%) of *P. dolloi* (Wood et al., 2005). The skin of *P. annectens* expresses a putative UT-A type urea transporter, whose transcript level is upregulated upon arousal in water

(Hung et al., 2009). Furthermore, two isoforms of *ut*, *ut-a2a* and *ut-a2b*, have been cloned from the gills of *P. annectens*, and arousal leads to significant increases in the branchial protein abundance of these two isoforms of UT (Chng et al., 2017a). It is essential for the aroused lungfish to regain the ability to excrete ammonia. Indeed, the protein abundance of Rhag, Rhbg and Rhcg in the gills of *P. annectens* recover to the corresponding control levels after one day or three days of arousal from six months of aestivation (Chng et al., 2017b).

7. Summary

Extant air-breathing fishes offer an opportunity to examine adaptations which might have facilitated the invasion of the terrestrial habitat by ancient fishes during evolution. Some air-breathing fishes can emerge from water, others make excursion onto land, and a few can even burrow into mud during drought. During emersion, air-breathing fishes would have difficulties in excreting ammonia. Hence, they are equipped with strategies to ameliorate ammonia toxicity when exposed to terrestrial conditions. There are altogether seven strategies, which include (1) reduction in protein degradation and amino acid catabolism, (2) partial amino acid catabolism forming alanine, (3) active ammonia excretion, (4) volatilization of ammonia, (5) glutamine synthesis, (6) urea synthesis, and (7) tolerance of high levels of internal ammonia (Fig. 1). They can be adopted by air-breathing fishes singly or in combinations, and the combination adopted is generally defined by the behavior of the fish, the frequency and duration of terrestrial exposure, and the nature of the environment in which it lives. Eight air-breathing fishes with distinct terrestrial behaviors and natural habitats have been described in detail to demonstrate how these seven strategies could be adopted in disparate combinations to ameliorate ammonia toxicity when exposed to terrestrial conditions.

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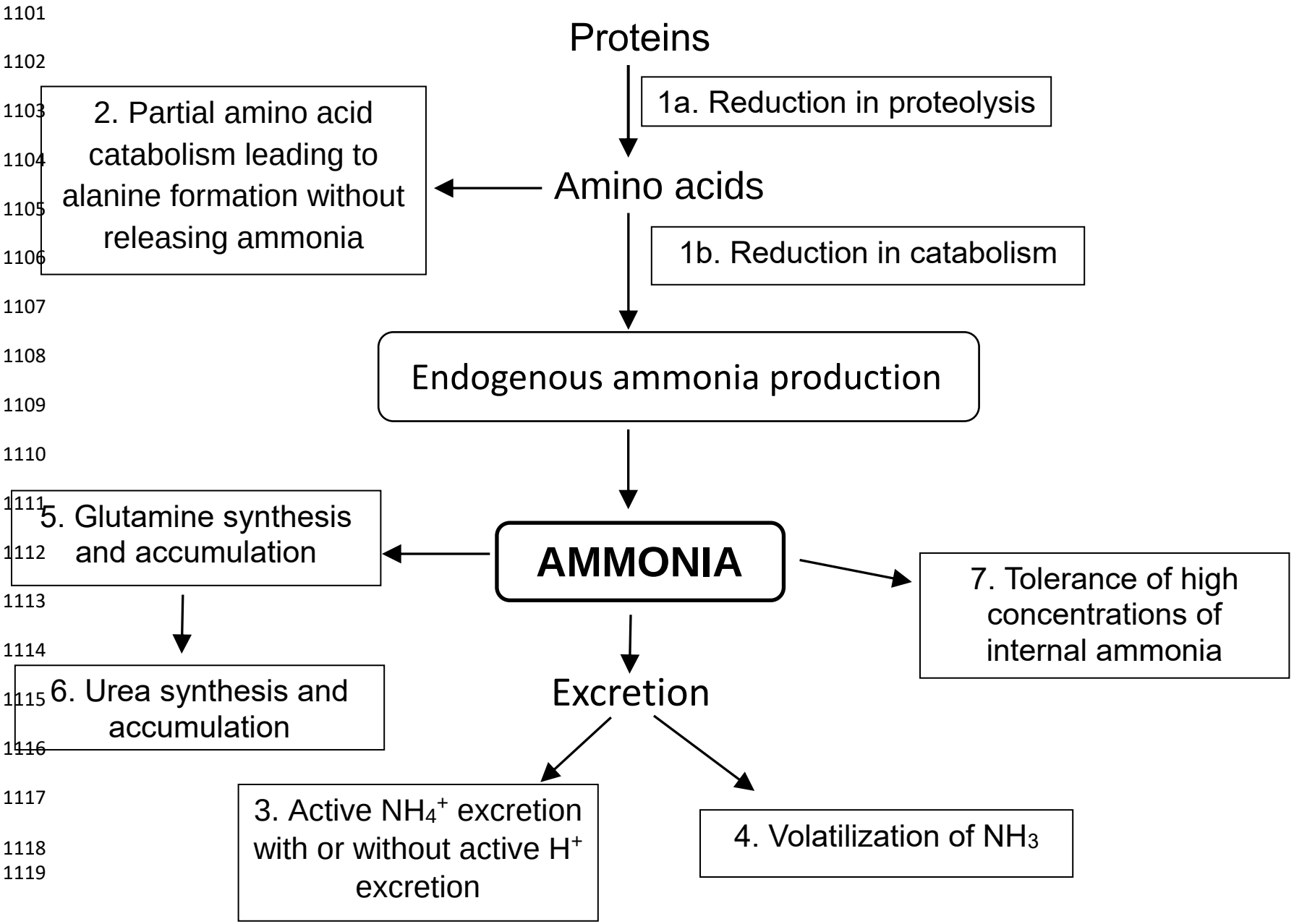
Legend to figures

Fig. 1. Seven strategies employed by air-breathing fishes to defend against endogenous ammonia toxicity during exposure to terrestrial conditions (emersion).

Fig. 1


Proteins
1a. Reduction in proteolysis
2. Partial amino acid catabolism leading to alanine formation without releasing ammonia
Amino acids
1b. Reduction in catabolism
Endogenous ammonia production
5. Glutamine synthesis and accumulation
AMMONIA
7. Tolerance of high concentrations of internal ammonia
6. Urea synthesis and accumulation
Excretion
3. Active NH_4^+ excretion with or without active H^+ excretion
4. Volatilization of NH_3