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REVIEW

Amphibious fishes: evolution and phenotypic plasticity

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ABSTRACT

Amphibious fishes spend part of their life in terrestrial habitats. The ability to tolerate life on land has evolved independently many times, with more than 200 extant species of amphibious fishes spanning 17 orders now reported. Many adaptations for life out of water have been described in the literature, and adaptive phenotypic plasticity may play an equally important role in promoting favourable matches between the terrestrial habitat and behavioural, physiological, biochemical and morphological characteristics. Amphibious fishes living at the interface of two very different environments must respond to issues relating to buoyancy/gravity, hydration/desiccation, low/high O_2 availability, low/high CO_2 accumulation and high/low NH_3 solubility each time they traverse the air–water interface. Here, we review the literature for examples of plastic traits associated with the response to each of these challenges. Because there is evidence that phenotypic plasticity can facilitate the evolution of fixed traits in general, we summarize the types of investigations needed to more fully determine whether plasticity in extant amphibious fishes can provide indications of the strategies used during the evolution of terrestriality in tetrapods.

KEY WORDS: Bimodal breather, Air breathing fishes, Acclimation, Air exposure

Introduction

The majority of fish species are solely aquatic, extracting oxygen (O_2) from the water using the gills, but at least 49 families of fishes have the capacity to extract O_2 from air (Graham, 1997). These air-breathing fishes can be classified as either bimodal breathers, which typically remain aquatic while breathing air at the water surface, or amphibious fishes, which fully emerse and breathe air out of water (Graham, 1976). Here, we define amphibious species as those that naturally spend part of their life on land (Gordon et al., 1968; Graham, 1997). Emersion may be related to feeding and/or foraging, reproduction, predator avoidance, migration, competition, habitat drying and/or poor water conditions (Sayer and Davenport, 1991; Graham, 1997). Correspondingly, there is a tremendous diversity of strategies for coping with life out of water. The duration of emersion and the extent of activity on land vary considerably between amphibious species. In general, fishes that remain quiescent can emerse for long periods (e.g. lungfish), whereas species that are highly active on land move back and forth between water and air frequently (e.g. mudskippers). For recent reviews on some amphibious fish groups, readers are referred to a special issue of *J. Fish Biol.* (vol. 84:3, 2014; see also Martin, 2015; Turko and Wright, 2015).

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Amphibious fishes can survive in two dramatically different environments: air and water. The differences in the physical and chemical properties of these disparate media present challenges that are never encountered by fully aquatic fishes. Amphibiousness has evolved repeatedly among fishes over millions of years, and requires traits that allow these species to maintain homeostasis when moving between water and air. Some authors have debated whether understanding the fixed adaptations for air breathing or terrestriality in extant amphibious fishes can provide insight into the evolution of tetrapods (e.g. Randall et al., 1981; Graham, 1997; Graham and Lee, 2004; Long and Gordon, 2004; Ashley-Ross et al., 2013) – in this Review, we have instead focused on phenotypic plasticity (see Glossary) or traits that are modified to increase performance out of water. In the literature, there are differences in the terminology used to discuss phenotypic plasticity. Physiologists sometimes describe phenotypic plasticity in terms of acclimation – a chronic response to a new set of external conditions in the lab – or acclimatization – a response to a change in the natural environment (Sherwood et al., 2005). Both of these phenomena are typically reversible and could be termed 'phenotypic flexibility' (see Glossary). Ecologists often use the term 'reaction norm' (see Glossary) to describe a set of phenotypes produced by a single genotype under different environmental conditions (Debat and David, 2001), i.e. the reaction to an environment. In this Review, for consistency with the literature, we use 'phenotypic plasticity' to refer to all types of phenotypic change due to the environment.

Here, we discuss examples of plastic traits associated with locomotion, gas exchange, nitrogen excretion, ionoregulation and osmoregulation when fishes move onto land. Most of our discussion of phenotypic plasticity in amphibious fishes concerns phenotypic flexibility, although some aspects of developmental plasticity (see Glossary) are also discussed. The plasticity that allows amphibious fishes to move between water and air is not a perfect solution to optimize the match between phenotype and environment – it has costs and limits, which we have summarized below. Finally, we present evidence that selection on plasticity may lead to the evolution of fixed traits that become established adaptations.

Evolution of amphibious behaviour

Amphibious habits have evolved independently many times in osteichthyan fishes (=Euteleostomi). Excluding tetrapods, which are nested within this group, >200 extant amphibious fishes have been reported, spanning 40 families and 17 taxonomic orders (Fig. 1; Table S1). This is twice the number of species estimated by Long and Gordon (2004), but is probably an underestimate – natural history information is lacking for numerous species, including many nested within highly amphibious clades (Graham, 1997; Hundt et al., 2014; Turko and Wright, 2015). Amphibious habits have also been suggested in bowfin *Amia calva* (Neill, 1950), cichlids (Beebe, 1945; Minshull, 2008) and several other fishes (e.g. Beebe, 1945); these deserve investigation. In some groups, only certain life stages are amphibious – for example, embryonic capelin *Mallotus villosus* or stickleback *Gasterosteus aculeatus* may benefit from increased

> **Glossary**
> **Active plasticity**
> Coordinated changes in multiple gene regulatory pathways or neurochemical/hormonal pathways that produce complex alterations in multiple traits and feedback systems.
> **Aestivation**
> In amphibious fishes, a state where the animal burrows into mud and/or enters metabolic depression.
> **Developmental plasticity**
> Irreversible phenotypic plasticity in response to environmental conditions during early life stages.
> **Genetic assimilation**
> Fixed expression of genes involved in expressing a novel trait at the expense of plasticity.
> **Passive plasticity**
> Physiological or biochemical changes as a direct result of environmental changes in ectotherms over the short term that do not involve homeostatic regulation (e.g. temperature change and body fluid pH).
> **Phenotypic flexibility**
> Reversible phenotypic plasticity in response to environmental changes, acclimation/acclimatization responses.
> **Phenotypic plasticity**
> Biochemical, physiological, behavioural, morphological or life-history traits that change in response to the environment.
> **Reaction norms**
> A set of phenotypes produced by a single genotype in two or more environmental conditions.

O_2 availability or reduced predation risk out of water (Martin and Carter, 2013; Martin, 2014). Other groups – including oxudercine mudskippers (e.g. Ishimatsu et al., 1998, 2007), stichaeid pricklebacks (e.g. Coleman, 1992) and aplocheiloid killifishes (e.g. Wells et al., 2015) – may emerse at all life stages.

In general, the evolution of amphibiousness has allowed fishes to occupy ecological niches unavailable to fully aquatic species (Graham, 1997; Sayer, 2005). The characteristics of these niches vary widely, however, and thus exert different selection pressures. Some amphibious fishes leave the water only very briefly to inhabit generally hospitable aquatic environments. For example, inanga *Galaxias maculatus* (Urbina et al., 2011) and tide pool-inhabiting blennies and gobies (Graham, 1997) use emersion to escape short bouts of aquatic hypoxia. Other species use brief emersions to migrate over land and colonize unoccupied bodies of water (Jordan, 1923; Davenport and Abdul Matin, 1990; Schoenfuss and Blob, 2003). Sometimes, fish don't leave the water, but water leaves the fish. Many intertidal fishes are able to exploit the supralittoral niche by tolerating emersion during low tides (Martin and Carter, 2013). More impressively, *Misgurnus* loaches, *Clarias* catfish and *Protopterus* lungfish can survive for months or even years without water during dry seasons or under drought conditions (Bruton, 1979; Oh and Park, 2009; Mlewa et al., 2011).

How can we explain this remarkable diversity of amphibious species? One hypothesis is that the abiotic factors that drive the evolution of amphibiousness, such as aquatic hypoxia and intermittent periods without water, are common in aquatic systems; thus, many lineages have had the opportunity to invade land independently (Graham, 1997; Graham and Lee, 2004). Furthermore, the colonization of these new adaptive zones by amphibious species may have allowed bursts of speciation in response to reduced competition or opportunities for ecological specialization (Heard and Hauser, 1995; Schluter, 2000). In this model, 'amphibiousness' in its most general sense may represent a key evolutionary innovation – a phenotypic trait that causes

increased species richness in the clade where it is present (Heard and Hauser, 1995; Hunter, 1998). It is not currently possible to rigorously test the key innovation hypothesis, and examples can be cherry-picked to support or reject this idea (see Box 1). However, a notable similarity among the most successful amphibious groups is the use of phenotypic plasticity when switching between environments – perhaps this responsiveness is key to their diversity (Pfennig et al., 2006, 2010; Moczek et al., 2011).

Challenges of life in and out of water

Amphibious animals must cope with two very different environments. The late French physiologist Pierre Dejours seconded the view of Bliss (1979) that the contrasting characteristics of air and water impose limitations on design and function (Dejours, 1988). Dejours (1988) said that, 'For the physiologist there are some good reasons to divide the animal kingdom into aquatic and terrestrial animals'. Aquatic animals live in a hydrating (freshwater) or dehydrating (seawater), buoyant, dense medium with relatively low O_2 content, whereas terrestrial animals must resist the force of gravity in a highly dehydrating environment with comparatively more O_2 available. What two settings could be more opposite? Below, we discuss some of the challenges associated with life in and out of water and then review how phenotypic plasticity allows amphibious fishes to meet these challenges.

Each of the phenotypic changes we document below involves behavioural, physiological and/or biochemical plasticity of

> **Box 1. Testing the key innovation hypothesis**
> Incomplete natural history information about many species nested within clades of generally amphibious fishes, considerable phylogenetic uncertainty and a potentially problematic extant species bias (Hunter, 1998) currently limit our ability to rigorously test the key innovation hypothesis. These methodological considerations are important, because there is no clear relationship between the evolutionary origins of amphibiousness and species richness. In support of the key innovation hypothesis, a single evolution of amphibiousness probably occurred within the Oxudercinae, and these amphibious mudskippers outnumber those in the aquatic sister genus *Pseudapcryptes* 30 to 2 (Murdy, 1989, 2011). Similarly, the 22 amphibious species of the swamp eel family Synbranchidae are in sharp contrast to the monospecific aquatic sister genus *Macrotrema* (Rosen and Greenwood, 1976; Graham, 1997), and the highly amphibious Galaxiid subfamily Galaxiinae contains 48 species compared with 2 species in the sister Aplochitoninae (Begle, 1991). Amphibious killifish lineages also tend to contain higher diversity than their sister groups (Turko and Wright, 2015), as do amphibious blenny clades (Hundt et al., 2014). These examples of species-rich amphibious groups include fishes that remain active out of water (e.g. mudskippers, killifish), as well as those that remain quiescent (e.g. swamp eels, blennies), suggesting that patterns of speciation are not strongly influenced by amphibious habits. Support for the key innovation hypothesis is not universal, however. We found >20 cases of lone amphibious species nested within fully aquatic clades. Perhaps the most striking example is the highly amphibious salamanderfish *Lepidogalaxias salamandroides*, which belongs to a monotypic family (and probably even order) as the sister clade to the ~16,000 other species of Euteleostei (Li et al., 2010; Betancur-R et al., 2013). Clearly, evolving amphibiousness is not enough to drive an adaptive radiation. How, then, can we explain the radiations of many amphibious groups such as mudskippers, killifishes or synbranchid eels? One hypothesis is that the ability to use phenotypic plasticity when switching between media is critical to the success of these lineages (Pfennig et al., 2006, 2010; Moczek et al., 2011).
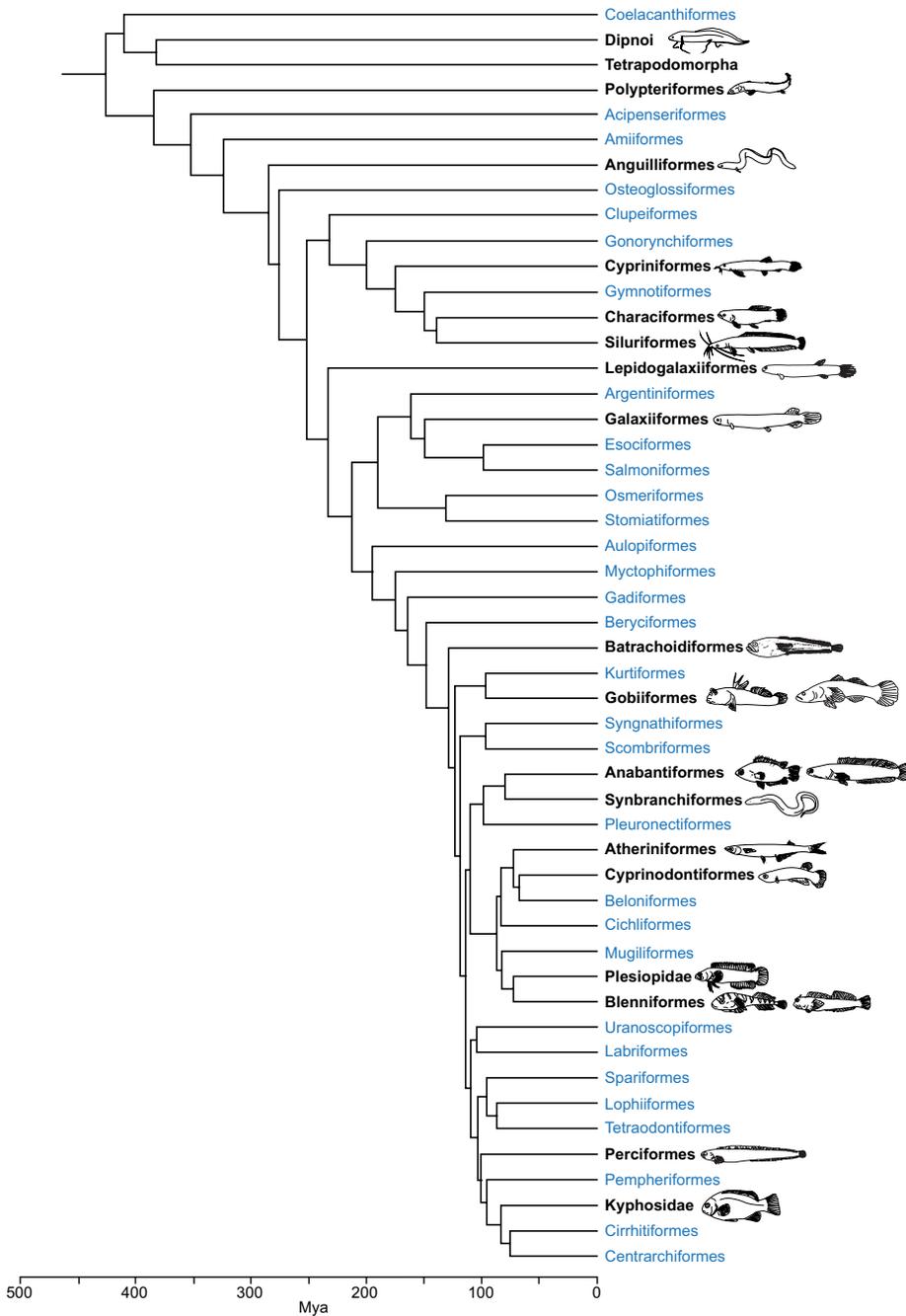
Fig. 1. Amphibious fishes are present in many osteichthyan orders, suggesting multiple evolutionary origins of the ability to survive out of water. Lineages that contain at least one amphibious species are highlighted in bold and accompanied by a sketch of a representative amphibious species from that group; fully aquatic groups are labelled in blue. A list of the >200 amphibious species used to create this tree is available in Table S1. The scale bar indicates time since divergence (Mya, millions of years ago). Many lineages have been omitted for simplicity. Relationships and divergence time estimates are modified from Betancur-R et al. (2013).

neurohormonal pathways, genes, proteins and/or metabolites, plus associated changes in cells and tissues. We have attempted to provide examples of several types of plasticity observed across physiological systems – locomotion, ion and water balance, gas exchange, pH regulation, metabolism and nitrogen excretion – with some example species that display the same trait plasticity. These examples come with a caveat, however. So little research has been conducted on the ~200 amphibious species, that we have only scratched the surface on the diversity of phenotypically plastic responses across this broad group of fishes. Much of our knowledge is limited to a few clades (e.g. blennies, gobies, killifishes, lungfish), and future work on the plasticity of other amphibious groups is required to determine the generality of these responses. Explicit comparisons of species with similar habits [e.g. active emersion or

aestivation (see Glossary)] in phylogenetically distant groups will be especially useful.

Buoyancy, gravity and movement on land
Water is 800 times denser and 60 times more viscous than air (Dejours, 1976, 1988; Table 1). Thus, aquatic animals must cope with greater frictional resistance (due to viscosity) and expend more work to combat inertia (due to density) relative to terrestrial animals. These physical factors have energetic and mechanical consequences for movement, which generally requires more effort in water than in air (Graham, 2006), affecting activities including feeding, locomotion and respiration. In water, some fishes can attain neutral buoyancy using a gas-filled swim bladder; by floating, they minimize energy expenditure at rest (McNeill Alexander,

Table 1. Properties of water and air and the physiological consequences

	Water	Air	Consequence
1. Viscosity	60	1	Reduced buoyancy in air requires greater body support
Density	800	1	
2. Water availability	FW hydrating/SW desiccating	Highly desiccating	Desiccation in air
3. O_2 and CO_2 diffusivity	1	8000	O_2 availability greater in air
O_2 solubility	1	30	
CO_2 solubility	30	30	CO_2 retention and acidosis in air
4. NH_3 solubility	700	1	NH_3 excretion is easier in water
5. Ionic environment	Variable	–	Ion balance in air is challenging

Adapted from Dejours (1975, 1988). Values are ratios. FW, freshwater; SW, seawater.

1993) (although many small, marine amphibious fishes lack swim bladders; Martin, 2015). Once a fish emerses, the lack of buoyancy and increase in apparent gravity would compress tissues. Thus, terrestrial and amphibious animals tend to have more robust musculoskeletal systems (Ashley-Ross et al., 2013).

Amphibious fishes have adopted a diverse array of terrestrial movement strategies despite lacking appendages and generally having body forms much more suited for swimming (e.g. Harris, 1960; Ellerby et al., 2001; Swanson and Gibb, 2004; Sayer, 2005; Gibb et al., 2011; Ashley-Ross et al., 2013; Pronko et al., 2013; Close et al., 2014; Pace and Gibb, 2014; Standen et al., 2014; Bressman et al., 2015; Flammang et al., 2016). One strategy is to rely on fixed adaptations that are a compromise for movement in air and water. This is seen in mudskippers, which make short but frequent forays onto land (Harris, 1960). Another strategy is to express plastic locomotory behaviour and morphology. If amphibious fishes emerse for days to weeks, does the musculoskeletal system become stronger in response to the increased apparent gravity? Does terrestrial exercise in fishes (e.g. mudskippers on a treadmill; Jew et al., 2013) lead to the same training effect as observed with swimming (McClelland, 2012)? Do fishes need to balance trade-offs when adjusting locomotor performance for aquatic versus terrestrial locomotion and, if so, how do lag times in the induction and reversal of these responses influence the scope of plasticity in fishes with different amphibious habits (Fig. 2)?

Although we cannot currently answer many of these questions, we recently discovered reversible plasticity in locomotory function in the mangrove rivulus *Kryptolebias marmoratus*. Jumping performance on land improved dramatically in air-acclimated fish; they had lower lactate levels and increased aerobic muscle cross-sectional area compared with control fish held in water (E. Brunt, A.J.T., G. R. Scott and P.A.W., submitted). The stimulus for these changes is unknown, but is likely to be partly associated with the increase in apparent gravity, as *K. marmoratus* are quiescent on

land, which should minimize any training effect. Further work is necessary to understand how reversible plasticity in locomotory behaviour and morphology in amphibious fishes that emerse for prolonged periods may lead to functional improvements on land and whether mechanisms are conserved across species.

Some amphibious fishes modify terrestrial locomotion if raised for months out of water. For example, when juvenile Senegal bichirs (*Polypterus senegalus*) were exposed to a moist terrestrial habitat for 8 months, they developed differently shaped clavicle and cleithrum bones. These changes provided more space for the pectoral fins to move compared with water-reared cohorts, partly explaining their more efficient locomotion on land (Standen et al., 2014). There appeared to be no trade-offs with respect to aquatic locomotion; the air-raised bichirs swam just as well. Examination of the fossil record revealed that this developmental plasticity in bichir paralleled macroevolutionary changes apparent in early tetrapods (Standen et al., 2014). These findings raise intriguing questions about how similar plastic responses in ancient amphibious fishes might have played a role in the evolution of fixed locomotory traits in tetrapods (see 'Developmental plasticity', below).

Water availability and ion balance

Water is abundant in aquatic environments; freshwater promotes hydration whereas seawater is a desiccating medium (Table 1). Water availability is variable and potentially very low in terrestrial habitats. Desiccation on land represents a major challenge for the evolution of terrestrial life. Thus, land-dwellers must have mechanisms to reduce water loss and/or obtain sufficient water. Aquatic fishes either gain water passively across the gills (freshwater) or actively drink (seawater) to replace lost water, but once emersed, these mechanisms are unavailable to fishes. Nevertheless, marine amphibious fishes may have had water conservation mechanisms (owing to the desiccating nature of
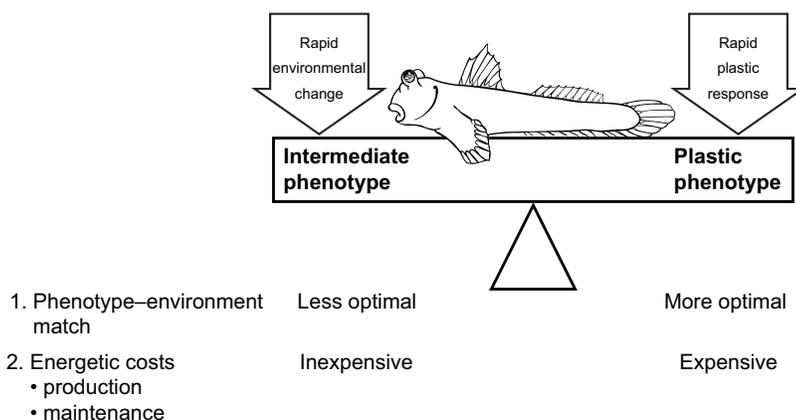

Fig. 2. The trade-offs between expressing plastic or non-plastic, intermediate traits when amphibious fish move from an aquatic to a terrestrial habitat. Theory predicts that phenotypically plastic responses to an environmental change (e.g. moving from water to land) will evolve when the environmental change is longer in duration than the time required for physiological, biochemical and/or morphological alterations (Padilla and Adolph, 1996; Alpert and Simms, 2002; Gabriel et al., 2005; Stomp et al., 2008). Plastic responses provide a more optimal match to the new environment, despite the high energetic costs incurred to produce and maintain the phenotype. Alternatively, intermediate phenotypes that may be less optimal in a particular environment, but do well enough across environments, will be selected for in situations where there is frequent or rapid environment change. See 'Plasticity – costs and limits', below, for more details.

seawater) that preadapted them to life out of water. Amphibious fishes may be tolerant of some water loss for intervals when they emerse (Horn and Gibson, 1988; Evans et al., 1999), but many have strategies to remain in positive water balance (e.g. Litwiller et al., 2006; Wilkie et al., 2007; Frick et al., 2008a).

Behavioural strategies are often used to limit water loss and maintain hydration in fish out of water. One strategy is to seek refuge in moist terrestrial habitats, such as beneath rocks or vegetation, or inside rotting logs or other structures (Table 2). Some amphibious fishes seek margins (thigmotaxis) or group with conspecifics to reduce their surface area, whereas others (e.g. mudskippers) moisten their body surfaces by rolling in mud. In mudskippers, hormones associated with social behaviours and osmoregulation (arginine vasotocin and isotocin) have even been implicated in the propensity of these fish to emerse (Sakamoto et al., 2015).

The cutaneous surface is an important site of water and ion balance in emersed fishes. Mucus secreted onto the cutaneous surface may inhibit cutaneous water loss under some conditions; for example, lungfish secrete a thick mucus coating that hardens into a protective cocoon during aestivation (Smith, 1930; Sturla et al., 2002). Excess mucus production by the skin has been reported in a few other species out of water, but this is not ubiquitous (Table 2).

Altering cutaneous and renal water fluxes during emersion is another strategy to retain water. In terrestrial lungfish (*Protopterus dolloi*), whole-body water efflux decreased substantially by 1 week and remained low over 6 months out of water (Wilkie et al., 2007). Similarly, in the non-aestivating *K. marmoratus*, whole-body water efflux was lower during emersion over a hypersaline (45 ppt; mimics seasonal drought) but not hyposaline (1 ppt) substrate (LeBlanc et al., 2010). Follow-up experiments on isolated skin preparations indicated that water influx was significantly enhanced in *K. marmoratus* acclimated to 7 days (not 1 day) of air over a hypersaline substrate, indicating that plastic responses involving skin remodelling (e.g. aquaporins, tight junction proteins) might be important in regulating water balance in emersed fish (Table 2; Q. Heffell, Skin permeability of the amphibious mangrove rivulus *Kryptolebias marmoratus* in response to emersion, MSc Thesis, University of Guelph, Guelph, Ontario, 2016). The reduction in whole-body water efflux in emersed lungfish and rivulus parallels the low epithelial water permeability in terrestrial tetrapods (Gray, 1928). For example, during anuran metamorphosis, the skin becomes keratinized and water turnover rates decline (Burggren and Just, 1992).

In addition to the cutaneous surface, the kidney plays an important role in regulating water balance in animals. In aestivating

Table 2. Reversible plasticity in strategies for ion and water balance in emersed amphibious fishes

Plastic traits in air	Species	Terrestrial period	References
1. Behaviours – seek refuge or margins, ingest water, moisten skin	*Alticus kirki*	4 h	Brown et al., 1991
	Anoplarchus purpurescens	6 h	Barton, 1985
	Pholis ornata	6 h	Barton, 1985
	Cebidichthys violaceus	4–6 h	Horn and Riegle, 1981
	Anoplarchus purpurescens	4–6 h	Horn and Riegle, 1981
	Xiphister mucosus	4–6 h	Horn and Riegle, 1981
	Xiphister astropurpureus	4–6 h	Horn and Riegle, 1981
	Xererpes fucorum	4–6 h	Horn and Riegle, 1981
	Kryptolebias marmoratus	Weeks	Taylor et al., 2008
	Sicyases sanguineus	6 h	Marusic et al., 1981
	Periophthalmodon schlosseri	6 h	Fenwick and Lam, 1988
	Oxyeleotris marmorata	6 h	Fenwick and Lam, 1988
	Periophthalmodon sobrinus	–	Gordon et al., 1968
	Galaxias 'nebula'	12 h	Magellan, 2015
	Parioglossus interruptus	12 h	Hendy et al., 2013
	Periophthalmus modestus	8 h	Sakamoto et al., 2015
2. Mucus production and mucous cells			
Skin	*Kryptolebias marmoratus*	9 days	LeBlanc et al., 2010
	Misgurnus mizolepis	Months	Oh and Park, 2009
	Protopterus aethiopicus	>30 days	Smith, 1930
Gills	*Protopterus annectens*	Weeks	Sturla et al., 2002
3. Water flux	*Kryptolebias marmoratus*	9 days	LeBlanc et al., 2010
	Kryptolebias marmoratus	1–7 days	Q. Heffell, 2016*
	Protopterus dolloi	180 days	Wilkie et al., 2007
	Protopterus dolloi	240 days	Patel et al., 2009
Vasotocin mRNA – brain	*Protopterus annectens*	90 days	Konno et al., 2009, 2010
Aquaporin mRNA – kidney	*Protopterus annectens*	90 days	Konno et al., 2010
4. Ionocytes			
Skin (SW)	*Kryptolebias marmoratus*	9 days	LeBlanc et al., 2010
Gill (FW)	*Kryptolebias marmoratus*	9 days	LeBlanc et al., 2010
Na^+/K^+-ATPase (gill)	*Neochanna apoda*	14 days	Urbina et al., 2014
	Periophthalmus chrysospilos	1 day	Lee et al., 1987
5. Ion flux	*Periophthalmodon schlosseri*	6 h	Fenwick and Lam, 1988
	Oxyeleotris marmorata	6 h	Fenwick and Lam, 1988
	Protopterus dolloi	8 h	Wood et al., 2005

Terrestrial period is the duration of exposure to air.
*Q. Heffell, Skin permeability of the amphibious mangrove rivulus *Kryptolebias marmoratus* in response to emersion, MSc Thesis, University of Guelph, Guelph, Ontario, 2016.
SW, seawater; FW, freshwater.

lungfish (*Protopterus annectens*), the hormone vasotocin may induce the upregulation of vasotocin V_2 receptor and aquaporin mRNA levels in the kidney to control water conservation (Konno et al., 2009, 2010). The V_2 receptor gene shows high homology to amphibian sequences. This is an exciting finding that may be related to the complete shutdown of urine flow during aestivation in *Protopterus* (DeLaney et al., 1977). In the shorter term, renal water efflux was also substantially diminished after 2–6 days out of water in the American eel (*Anguilla rostrata*) relative to that of fish held in water (Butler, 1999). More work is needed to understand the mechanisms regulating decreased urine production and to determine whether these are conserved across diverse amphibious fish species during emersion.

Water balance is closely related to ion balance in fishes. The ionic environment is variable in aquatic habitats (which range from freshwater to hypersaline water), but essential ions may not be readily available on land; thus, ion balance in air may be far more challenging (Table 1). Typically, aquatic fishes obtain ions from the diet and through exchange across the gills via ion-transporting cells or ionocytes (also termed mitochondria-rich cells). The kidney plays a smaller role in ionic regulation, reabsorbing univalent ions in freshwater fishes and secreting divalent ions in seawater fishes (Marshall and Grosell, 2006). In emersed amphibious fishes, branchial ion exchange is typically eliminated, and the cutaneous surface – and possibly the kidney – becomes more important in ion balance.

Ion flux across the skin and the presence of cutaneous ionocytes have been documented in amphibious fishes (e.g. Yokota et al., 1997; LeBlanc et al., 2010; Itoki et al., 2012; Cooper et al., 2013). In *K. marmoratus* acclimated to air on a hypersaline surface, skin ionocyte cross-sectional area increased, although ionoregulation was not perfectly maintained, as whole-body Na^+ levels were 30% higher relative to those of control fish in water (LeBlanc et al., 2010). The rate of ion flux may also be modulated to regulate ion balance on land. For example, in marine *Periophthalmodon schlosseri* and freshwater marble goby, *Oxyeleotris marmorata*, held out of water for 6 h, Ca^{2+} homeostasis was maintained by a large decrease in cutaneous Ca^{2+} efflux to almost zero (Fenwick and Lam, 1988). Over longer periods of emersion, however, other species show little change in whole-body flux rates for Na^+ and Cl^- (*P. dolloi*, Wilkie et al., 2007; *K. marmoratus*, LeBlanc et al., 2010).

The role of the kidney in ion regulation has not been examined in any species of amphibious fishes to our knowledge. During the evolution of terrestrial vertebrates, ion regulation shifted from the skin and kidneys in amphibians to the kidneys and salt glands in birds and reptiles. In amphibious fishes, it would be interesting to know whether renal responses to terrestrial acclimation are similar to the mechanisms of ion regulation in terrestrial vertebrates.

Gas exchange and metabolism

O_2 and CO_2 diffuse much faster in air relative to water, and O_2 is much more soluble in air (Dejours, 1976, 1988; Table 1). Air holds $\sim$210 ml O_2 l^{-1}, whereas water holds only 5–6 ml O_2 l^{-1} (depending on salinity) at 25°C (Graham, 2006). Thus, the respiratory systems of air breathers do not have to be quite so efficient as those of water breathers. Aquatic O_2 levels also tend to be more variable than in terrestrial environments. Amphibious fishes typically inhabit shallow waters, tide pools or transient aquatic habitats that can vary from hyperoxic (due to algal or plant photosynthesis) to hypoxic (poor mixing, organismic respiration, low flow) either daily or seasonally (e.g. Bridges, 1988; Graham, 1985; Martin and Bridges, 1999; Currie et al., 2010). In addition, respiratory challenges in stagnant waters may be exacerbated by

high levels of CO_2 or H_2S, which interfere with O_2 binding and transport (Dehadrai and Tripathi, 1976; Abel et al., 1987).

O_2 uptake across the gills is limited out of water in most aquatic fishes, because the delicate gill filaments and lamellae collapse and coalesce (Randall et al., 1981). However, in some active amphibious species such as oxudercine mudskippers, which retain water within the opercular chamber and have fortified gills (reviewed by Sayer, 2005), the gill functions very well out of water – better than when fully immersed (Takeda et al., 1999). Other species that remain quiescent out of water actually reduce gill surface area when emersed. For example, *K. marmoratus* reversibly remodel their gills by developing a cell mass between the lamellae (Ong et al., 2007; Table 3), and in aestivating African lungfish the lamellae are thickened and mucus coated (Sturla et al., 2002). Thus, depending on the type of emergence (e.g. quiescent, long-term emersion versus regular active excursions), gill functionality out of water is quite variable across species.

Fishes that use extrabranchial sites of O_2 uptake when emersed have evolved a diversity of air-breathing organs (ABOs), including the gut, cutaneous surface, buccal, pharyngeal or opercular lining, gas bladder and/or lung, and in many cases air gulping is initiated on emersion (Graham, 1997). 'Gulping' air may involve movement of air into an internal organ such as a lung (e.g. tidal air flow in lungfish) or opercular and buccal movements to aerate the epithelial lining of these cavities (e.g. unidirectional flow in intertidal fishes; Martin, 2015). Other amphibious fishes may keep their opercula closed most of the time but occasionally flutter them for variable periods of time (Brown et al., 1991). In contrast, a few species are able to continue to use their gills for air breathing while emersed (e.g. *Synbranchus*, Johansen, 1970; *Periophthalmodon*, Graham, 1997). The sensory systems required to switch the site of gas exchange in emersed amphibious fishes are not well characterized, but this process may involve pathways similar to those described for air-breathing fishes responding to hypoxia or hypercapnia (reviewed by Milsom, 2012). Once the site of respiration is switched from the gills to alternative sites, there are multiple steps along the O_2 cascade from the respiratory surfaces to the mitochondria in the tissues that undergo reversible changes. To increase blood flow and facilitate O_2 transfer across respiratory surfaces, including the cutaneous surface, there are rapid circulatory adjustments (Table 3). It is not clear in various amphibious fishes whether these gas exchange organs are primed and ready for aerial respiration, such as in the mudskipper *P. schlosseri* (Gonzales et al., 2011), or whether angiogenesis occurs in these extrabranchial respiratory surfaces as they acclimate to air exposure, as in the mangrove rivulus *K. marmoratus*; here, there is evidence of angiogenesis in the bucco-opercular lining, skin and fins after 7 days out of water relative to control fish in water (Cooper et al., 2012; Turko et al., 2014).

Following its uptake at the respiratory surface, O_2 must be delivered to the tissues. Generally, heart rate slows after emersion (Table 3). CO_2 retention and the lower blood pH of emersed fishes (see below) both decrease the O_2 affinity of haemoglobin (Hb; Bohr shift) and the blood O_2-carrying capacity (Root shift) (Randall et al., 1981); plasticity of the O_2 delivery system may therefore be necessary to offset the higher blood CO_2 content in air-breathing fishes (Johansen, 1970). Indeed, many adjustments in O_2-carrying capacity and Hb have been described in emersed fishes, including increased Hb–O_2 affinity or cooperativity of Hb binding to O_2, a change in the relative amounts of different Hb isoforms, increased Hb concentration and/or elevated haematocrit (Table 3). Some of these changes also occur during aquatic hypoxia in fishes, raising

Table 3. Reversible plasticity in strategies for gas exchange and metabolism in emersed amphibious fishes

Plastic traits in air	Species	Terrestrial period	References
1. Switching site of respiration			
Gill remodelling	*Kryptolebias marmoratus*	7 days	Ong et al., 2007
	Kryptolebias marmoratus	9 days	LeBlanc et al., 2010
	Kryptolebias marmoratus	Days	Turko et al., 2011
	Protopterus annectens	Weeks	Sturla et al., 2002
	Galaxias 'nebula'	14 h	Magellan et al., 2014
Gulping air	*Trichogaster trichopterus*	1–4 h	Burggren and Haswell, 1979
	Erpetoichthys calabaricus	6–8 h	Pettit and Beitinger, 1985
	Kryptolebias marmoratus	7 days	Turko et al., 2014
	Protopterus aethiopicus	30 min	Delaney and Fishman, 1977
	Protopterus spp.	40 min	Johansen, 1970
Angiogenesis (buccal, pharyngeal, opercular, gut and/or skin)	*Sicyases sanguineus*	1 day	Marusic et al., 1981
	Kryptolebias marmoratus	7 days	Cooper et al., 2012
	Kryptolebias marmoratus	7 days	Turko et al., 2014
Circulatory modifications	*Gillichthys mirabilis*	Minutes	Todd and Ebeling, 1966
	Lepidosiren paradoxa	Minutes	Johansen and Lenfant, 1967
	Protopterus spp.	Minutes	Satchell, 1976
	Kryptolebias marmoratus	3 min	Cooper et al., 2012
2. Transport of O_2 in blood			
Heart rate (bradycardia)	*Sicyases sanguineus*	3 h	Gordon et al., 1970
	Anguilla vulgaris	20 h	Berg and Steen, 1965
	Anabas testudineus	24 min	Singh and Hughes, 1973
	Protopterus aethiopicus	10 months	Delaney et al., 1974
	Leuresthes tenuis	Minutes	Garey, 1962
Heart rate (tachycardia)	*Periopthalmodon australis*	24 h	Kok et al., 1998
	Periopthalmodon australis	Minutes	Garey, 1962
Hb–O_2 affinity, cooperativity, isoforms, concentration, haematocrit	*Protopterus amphibius*	28–30 months	Johansen et al., 1976
	Protopterus aethiopicus	13 months	Delaney et al., 1976
	Hoplosternum littorale	12 h	Johansen et al., 1978
	Galaxias maculatus	6 days	Urbina and Glover, 2012
	Kryptolebias marmoratus	7 days	Turko et al., 2014
3. Utilization of O_2 at the tissues			
Tissue reorganization	*Kryptolebias marmoratus*	14 days	Brunt et al., submitted[‡]
Metabolic reorganization	*Galaxias maculatus*	6 h	Urbina and Glover, 2012
	Anguilla vulgaris	20 h	Berg and Steen, 1965
	Sicyases sanguineus	26 h	Gordon et al., 1970
	Protopterus dolloi	60 days	Frick et al., 2008a
	Protopterus dolloi	60 days	Frick et al., 2008b
	Protopterus dolloi	60 days	Frick et al., 2010
	Gillichthys mirabilis	24 h	Gracey, 2008
4. Metabolic rate			
Increased O_2 uptake	*Erpetoichthys calabaricus**	2 h	Sacca and Burggren, 1982
	Sicyases sanguineus	15–23 h	Gordon et al., 1970
	Neochanna burrowsius	>12 h	Urbina et al., 2014
	Periophthalmodon schlosseri	24 h	Kok et al., 1998
	Periophthalmodon schlosseri	12 h	Takeda et al., 1999
	Alticus kirki	10 min	Martin and Lighton, 1989
Decreased O_2 uptake	*Erpetoichthys calabaricus*	6–8 h	Pettit and Beitinger, 1985
	Anguilla vulgaris	20 h	Berg and Steen, 1965
	Protopterus aethiopicus	60 days	Delaney et al., 1974
	Protopterus aethiopicus	>30 days	Smith, 1930
	Protopterus aethiopicus	240 days	Fishman et al., 1986
	Periopthalmus cantonensis	1 h	Tamura et al., 1976
	Boleophthalmus chinensis	1 h	Tamura et al., 1976
	Galaxias maculatus	>12 h	Urbina et al., 2011
	Alticus kirki	3–4 h	Brown et al., 1992
	Lepidosiren paradoxa	9 h	Johansen and Lenfant, 1967

Terrestrial period is the duration of exposure to air. Hb, haemoglobin.

*Formerly *Calamoichthys calabaricus*.

[‡]E. Brunt, A.J.T., G. R. Scott and P.A.W., submitted.

the possibility that the aquatic hypoxia response is co-opted by amphibious fishes to cope with hypoxaemia in the initial stages of aerial ventilation upon emersion (e.g. Gracey, 2008). However, we have found that this is not the case in *K. marmoratus* (Turko et al., 2014). Here, terrestrial acclimation results in a significant increase in the affinity of Hb for O_2, which is not observed in hypoxia-acclimated *K. marmoratus* in water. An alternative hypothesis is that some amphibious fishes may have co-opted regulatory pathways that maintain acid–base status, and it has been suggested that CO_2 tolerance in fishes may have been a pre-condition for the evolution of air breathing in vertebrates (Ultsch, 1987, 1996; see 'CO_2 retention and acid–base balance', below).

An important index for respiratory and metabolic change during emersion is whole-animal O_2 uptake, a proxy for metabolic rate. Metabolic rate is often equivalent during water versus air breathing, but increases in some species during emersion while decreasing in others (reviewed by Graham, 1997; Martin, 2014; Table 3). In general, there are two life-history strategies – fishes that are active out of water for shorter durations show increased or unchanged metabolic rate, whereas those that are inactive on land for longer periods decrease metabolic rate. For example, in marine intertidal species (e.g. mudskippers) that move frequently between water and air and have short sojourns on land, metabolic rate is typically higher out of water (Table 3). However, for fishes that cannot ingest food out of water, prolonged emersion is linked to an overall decrease in energy use, such as the profound depression observed in aestivating lungfish (Smith, 1930; Amelio et al., 2008; Frick et al., 2010; Ballantyne and Frick, 2011; Table 3).

Overall, it is difficult to describe a conserved respiratory response to air breathing in emersed amphibious fishes. The great diversity of lifestyles and energetic demands is matched by the large variation in plastic responses to air. However, the increased reliance on non-branchial structures – particularly skin and lungs – when emersed resembles the developmental trajectory observed in amphibians.

CO_2 retention and acid–base balance

In water, CO_2 solubility is ∼30 times higher than O_2 solubility, but the values of CO_2 and O_2 solubility are similar in air (Table 1). Consequently, water-breathing fishes maintain relatively high rates of ventilation to meet their O_2 requirements, CO_2 in the blood rapidly diffuses into the environment, and the excreted CO_2 is diluted in a large volume of water (Rahn, 1966). Thus, air breathers have total blood CO_2 levels that are typically an order of magnitude higher than those of water breathers (Rahn, 1966; Rahn et al., 1971). In cases where amphibious fishes rely on a lung for gas exchange, the relatively low efficiency of this tidal ventilatory system also contributes to CO_2 accumulation in body fluids (Piiper and Scheid, 1975). In contrast, amphibious fishes that breathe air with gills or ABOs often continue to eliminate CO_2 at similar rates to their aquatic counterparts during short sojourns out of water (Burggren and Haswell, 1979; Pelster et al., 1988; Martin et al., 1993; Graham, 1997; Martin, 2014). For those fishes that do retain CO_2 when air-exposed, blood pH initially decreases (passive plasticity; see Glossary) as a result of a respiratory acidosis (Heisler, 1982) or a combined metabolic and respiratory acidosis (DeLaney et al., 1977; Pelster et al., 1988; Hyde and Perry, 1987; Hyde et al., 1987; reviewed by Shartau and Brauner, 2014). Blood pH will also change in response to air temperature (passive plasticity), decreasing if warmer conditions prevail on land (Martin and Bridges, 1999).

Maintaining acid–base homeostasis out of water without functional gills is a challenge, given that branchial HCO_3^-/Cl^- and Na^+/H^+ (NHE) exchangers and H^+-ATPase are critical for regulating internal pH (Evans et al., 1999). Despite these limitations, plasma HCO_3^- levels were reported to increase gradually over a 7 month period of aestivation in *Protopterus aethiopicus* (DeLaney et al., 1977). In some species, the skin or gut may release acid or base equivalents (see 'Nitrogen excretion', below). Another strategy may be to offset intracellular acidosis by transfer of HCO_3^- from the extracellular fluid (preferential pH_i regulation; Heisler, 1982) or to simply wait until reimmersion to compensate using branchial processes (Hyde and Perry, 1987; Evans et al., 1999). What is not clear – across the diversity of amphibious fishes with different lifestyles – is the degree of plasticity in acid–base transporters and associated regulatory

processes that facilitate acid–base homeostasis during emersion and reimmersion. This is an area ripe for discovery.

The retention of CO_2 in amphibious fishes that remain out of water for prolonged periods parallels the acid–base status of terrestrial vertebrates, as highlighted previously (e.g. Rahn, 1966; Rahn and Garey, 1973; Heisler, 1982). Whether acid–base compensatory mechanisms are conserved across amphibious fishes and air-breathing vertebrates is unknown. However, Shartau and Brauner (2014) have hypothesized that preferential pH_i regulation may have evolved in bimodal-breathing fishes in ion-poor, hypoxic and hypercapnic Devonian swamps to provide extraordinary CO_2 tolerance and a mechanism to cope with the respiratory acidosis that accompanies air breathing. There is much to uncover across a diversity of species in this regard.

Nitrogen excretion

Ammonia (NH_3 or NH_4^+) is formed from the catabolism of proteins and amino acids and is an important nitrogenous waste product in animals. The unionized form NH_3 is relatively toxic (Ip et al., 2001c) and is far more soluble in water than in air (Table 1); therefore, mechanisms to rid the body of nitrogenous waste are often very different between water and air breathers. NH_3 diffuses across the gills of fishes and is diluted in the abundant aqueous medium (Wilkie, 1997). Terrestrial animals convert NH_3 to urea or uric acid, a process that is much more energetically expensive than simply excreting NH_3, but these products can be excreted in far less water (Wright, 1995; Walsh and Mommsen, 2001). Some amphibious fishes reversibly switch from branchial NH_3 excretion to alternative modes of nitrogen excretion while out of water. The signal for switching to a terrestrial mode of nitrogen metabolism and excretion is unknown, but it is likely to be associated with an accumulation of endogenous ammonia, because many of the same mechanisms are shared between fishes exposed to high environmental ammonia in terrestrial and aquatic environments (Chew and Ip, 2014). Here, we have summarized four key reversible changes in nitrogen metabolism and excretion that amphibious fishes adopt when out of water (Table 4): ammonia excretion, ammonia detoxification via urea or via glutamine, and reduction in ammonia synthesis.

One strategy on land is to continue to eliminate ammonia through either active NH_4^+ excretion or the release of NH_3 gas across a non-branchial surface, such as the skin or gut. The dissociation constant, pK, of ammonia is ∼9; thus, alkalinization favours unionized NH_3 formation over ionized NH_4^+. Some amphibious species alkalinize the gut epithelial or cutaneous epidermal surface through diminished H^+ secretion (*K. marmoratus*; Litwiller et al., 2006; Cooper et al., 2013) and/or possibly enhanced HCO_3^- secretion (weatherloach, *Misgurnus anguillicaudatus*; Wilson et al., 2013), and NH_3 is then volatilized into the air. There is increased expression of mRNA encoding cutaneous ammonia transporters – Rhesus (Rh) glycoproteins – in emersed *K. marmoratus* (Hung et al., 2007), presumably leading to enhanced delivery of ammonia to the skin surface. NH_3 excretion by volatization can account for ∼8–40% of total ammonia excretion (Table 4).

Another strategy to eliminate ammonia in air is to excrete NH_4^+. Active NH_4^+ excretion may occur across the fortified gills of the emersed mudskipper *P. schlosseri* (Randall et al., 2004). These fish retain a small volume of branchial water when emersed and, over time, this solution becomes acidified by CO_2 and H^+ excretion. Active NH_4^+ excretion into acidified branchial water in emersed *P. schlosseri* is thought to be the main mechanism of nitrogen excretion (Chew et al., 2007). Although the exact transport mechanisms have not been identified in mudskippers, active

Table 4. Reversible plasticity in strategies for nitrogen excretion in emersed amphibious fishes

Plastic traits in air	Species	Terrestrial period (days)	References
1. Continue to excrete ammonia			
NH₃ volatilization (gut)	*Misgurnus anguillicaudatus*	3	Tsui et al., 2002
NH₃ volatilization (skin)	*Kryptolebias marmoratus*	11	Frick and Wright, 2002
NH₃ volatilization	*Blennius pholis*	1	Davenport and Sayer, 1986
Alkalinization (skin)	*Kryptolebias marmoratus*	11	Litwiller et al., 2006
	Kryptolebias marmoratus	1	Cooper et al., 2013
Ammonia transporter mRNA (skin)	*Kryptolebias marmoratus*	1	Hung et al., 2007
NH₄⁺ active excretion (gills)	*Periophthalmodon schlosseri*	1	Chew et al., 2007
2. Detoxification of ammonia via urea			
Urea synthesis (OUC)	*Protopterus aethiopicus*	>30	Smith, 1930
	Protopterus aethiopicus	78–129	Janssens and Cohen, 1968
	Protopterus dolloi	6	Chew et al., 2003
	Protopterus dolloi	21–30	Wood et al., 2005
	Heteropneustes fossilis	30	Saha et al., 2001
Urea transporter mRNA (skin)	*Protopterus annectens*	33	Hung et al., 2009
3. Detoxification of ammonia via	*Periophthalmus cantonensis*	3	Iwata et al., 1981
glutamine (GS)/glutamate (GDH)	*Bostrichthys sinensis*	1	Ip et al., 2001b
synthesis	*Oxyeleotris marmoratus*	3	Jow et al., 1999
	Monopterus albus	1–40	Tay et al., 2003
	Monopterus albus	6	Chew et al., 2005
	Monopterus albus	1	Tok et al., 2011
	Misgurnus anguillicaudatus	3	Chew et al., 2001
	Protopterus dolloi	40	Chew et al., 2004
	Protopterus aethiopicus	6	Loong et al., 2005
	Protopterus annectens	6	Loong et al., 2005
4. Reduced ammonia synthesis and/	*Periophthalmus cantonensis*	2	Iwata, 1988
or altered amino acid catabolism	*Periophthalmodon schlosseri*	1	Lim et al., 2001
	Periophthalmodon schlosseri	1	Ip et al., 2001c
	Bostrichthys sinensis	3	Ip et al., 2001b
	Monopterus albus	6	Tay et al., 2003
	Monopterus albus	40	Chew et al., 2005
	Misgurnus anguillicaudatus	3	Chew et al., 2001
	Kryptolebias marmoratus	10	Frick and Wright, 2002
	Alticus kirki	1	Rozemeije and Plaut, 1993

Terrestrial period is the duration of exposure to air.
OUC, ornithine urea cycle; GS, glutamine synthetase; GDH, glutamate dehydrogenase.

NH_4^+ transport probably involves ammonia transporters (You et al., 2014) and mitochondria-rich cells (Randall et al., 2004).

In other amphibious fishes, ammonia is detoxified by conversion to urea and/or glutamine, relatively non-toxic metabolites that can be stored. Glutamine is the N-donating substrate in the piscine ornithine–urea cycle (OUC), and it is formed from glutamate and NH_4^+ in a reaction catalysed by glutamine synthetase (Anderson, 2001). Although all amphibious fishes synthesize urea to some degree, very few have a complete and functional OUC (Mommsen and Walsh, 1989). The hepatic OUC is induced in lungfish and in the Singhi catfish *Heteropneustes fossilis* during terrestrial sojourns, and urea accumulates in the tissues until water returns (Table 4). During reimmersion of lungfish, the facilitated urea transporter is up-regulated to promote rapid elimination of urea (Wood et al., 2005; Hung et al., 2009; LeMoine and Walsh, 2015). In contrast, a few amphibious species simply store glutamine during air exposure (Table 4); this can be easily degraded by glutaminase to glutamate and NH_4^+ when water returns.

Some emersed amphibious species reduce the rate of ammonia synthesis, presumably to diminish the potential for ammonia toxification (Table 4). The rate of proteolysis and amino acid catabolism is reduced and/or there is a change in the type of amino acids catabolized. For example, partial amino acid catabolism leads to the formation of alanine, allowing the mudskipper *P. schlosseri* to utilize amino acids as fuel during exercise on land (Ip et al., 2001a;

reviewed by Chew and Ip, 2014). Alanine can remain in solution as a harmless compatible osmolyte (Yancey, 2001). The African sharptooth catfish *Clarias gariepinus*, in contrast, appears to show no marked changes in amino acid metabolism or any other parameter that would indicate a plastic response to 4 days of air exposure (Ip et al., 2005) – ammonia levels in the plasma were >3 mmol l^{-1} at the end of the terrestrial exposure, which would be lethal to most fishes. Finally, some species adopt more than one strategy to deal with nitrogenous waste during emersion. For example, the sleeper *Bostrichthys sinensis* initially detoxifies ammonia to glutamine, but longer exposure to air results in a reduction in proteolysis and amino acid catabolism (Ip et al., 2001b).

Of these four plastic strategies adopted by emersed amphibious fishes, the detoxification of ammonia via glutamine and/or urea synthesis parallels the evolution of terrestrial vertebrates most closely. The OUC is essential for nitrogenous waste handling in adult amphibians, as well as in mammals at all life stages, although the N-donating substrate is NH_4^+, not glutamine (Brown et al., 1959; Atkinson, 1995; Meijer, 1995). Nevertheless, glutamine synthesis is highly regulated in terrestrial vertebrates, and transfer of nitrogen in the blood to the hepatic OUC is in the form of non-toxic glutamine rather than NH_4^+ (Nelson and Cox, 2000). Thus, plastic traits for glutamine and urea synthesis in fishes later became fixed adaptations for the handling of nitrogenous waste in tetrapods.

Developmental plasticity

There have been very few studies that have explored the impact of terrestrial rearing on the adult phenotype of amphibious fishes. Many amphibious fishes deposit embryos above the water line, in burrows or in temporary pools prone to desiccation (reviewed by Martin, 1999, 2015; Martin et al., 2004; Ishimatsu et al., 2007; Ishimatsu and Graham, 2011), but not all species show frequent or prolonged episodes of emersion as adults. Regardless, terrestrial rearing often has profound effects on the embryo. In some species, terrestrial incubation induces diapause, a spontaneous and reversible arrest that prevents eggs from hatching into an unfavourable environment. The capacity for embryos to enter diapause seems to have evolved independently six times within African and South American killifishes (Furness, 2015; Furness et al., 2015). Diapause results in a dramatic reduction in metabolic and developmental processes (e.g. annual killifish *Austrofundulus limnaeus*; Podrabsky and Hand, 1999). In other cases, embryos do not enter diapause but hatching is delayed until the appropriate hatching cues are present, even if embryos are hatching competent (e.g. California grunion *Leuresthes tenuis*; Moravek and Martin, 2011; Martin et al., 2011; Martin, 2015). Development may also be accelerated in air, for example in *K. marmoratus* (Wells et al., 2015) and *Fundulus heteroclitus* (Tingaud-Sequeira et al., 2009). Terrestrial incubation in *K. marmoratus* embryos also decreased the energetic costs of development relative to those of embryos reared in water, as shown by lower O_2 uptake and opercular movements and larger yolk reserves (Wells et al., 2015). *Fundulus heteroclitus* embryos in air downregulated aquaporin proteins, a process that may be a protective mechanism to avoid desiccation (Tingaud-Sequeira et al., 2009), and they differentially expressed 806 genes relative to control embryos in water (Tingaud-Sequeira et al., 2013). Thus, plasticity is demonstrated by embryos of many species in response to the rearing environment.

An adaptive view of developmental plasticity suggests that phenotypic changes that occur in response to terrestrial incubation of early stages should result in an enhanced ability of adults to perform or maintain homeostasis during emersion. Unfortunately, few studies to date have considered the long-term consequences of early terrestrial incubation. Irreversible developmental plasticity of locomotory structures and biomechanics that improved terrestrial performance was beautifully demonstrated in the bichir (Standen et al., 2014; see 'Buoyancy, gravity and movement on land', above). In contrast, *K. marmoratus* embryos raised for 30 days in air or water had no detectable phenotypic differences at maturity (Wells et al., 2015). Are subtler measures of physiological and biochemical adjustments required to identify adaptive plasticity that increases the capacity for emersion in adults of this species? Or is developmental plasticity rare in amphibious fishes? Developmental plasticity should evolve when environmental conditions experienced by early life stages are predictive of the environments that adults will face (West-Eberhard, 2003). If the need for adult amphibious fishes to emerse is not correlated with their embryonic rearing environment, then developmental plasticity may not be advantageous – especially if there are trade-offs in function between aquatic and terrestrial environments – and reversible phenotypic flexibility may be more common. More work is needed to understand when these two types of plasticity occur, and how they are controlled mechanistically.

Plasticity – costs and limits

Researchers who have traditionally focused on phenotypic plasticity are interested in morphological, developmental and life-history variability and its impact on fitness and evolution.

Unfortunately, there has been limited 'spill over' of these ideas into comparative mechanistic physiology. A special issue of the Journal of Experimental Biology (vol. 209.12, 2006) provided some advances, with features on the plasticity associated with skeletal muscle, the brain and molecular mechanisms. An excellent small text for senior undergraduates called 'The Flexible Phenotype' (Piersma and van Gils, 2011) goes a long way in integrating concepts of phenotypic plasticity from ecological, physiological and behavioural perspectives. Information often does not travel in the other direction either. For example, Piersma and van Gils (2011) suggested that 'the phenotypic plasticity literature has mostly passed by the staggeringly large body of phenomena related to internal (physiological), external, and seasonally cyclic (moult) and behavioural plasticity, especially in the higher vertebrates'. More integration of these two bodies of literature is needed, and we have made a first attempt below to understand the costs and limits of phenotypic plasticity with respect to amphibious fishes.

There are many examples of active phenotypic plasticity (see Glossary) in amphibious fishes. There are also examples of traits in some species that show little change between an aquatic and terrestrial environment. Why is this? The potential costs of plasticity and limitations associated with generating perfectly matched traits have been debated in the literature (DeWitt et al., 1998; Relyea, 2002; Callahan et al., 2008; van Buskirk and Steiner, 2009; see table 7 in Piersma and van Gils, 2011; Auld et al., 2010; Murren et al., 2015). Briefly, plasticity is costly because it involves energetic costs associated with both (1) building new structures (or molecules), including reversal costs, and (2) maintenance costs for genetic, sensory and physiological or regulatory mechanisms to detect and respond to environmental change (Fig. 2). The first of these represents the energetic cost of initially producing an alternative phenotype (phenotypic cost); it is environment dependent and thus only incurred when plastic changes are expressed. Maintenance costs, the energetic 'tax' paid for the ability to be phenotypically plastic, are incurred in all environments. Theoretically, maintenance costs are more likely than phenotypic costs to restrict the evolution of plasticity to animals in which environmental changes are relatively common and thus the benefits of plasticity can outweigh this expense (Auld et al., 2010). For example, emersed lungfish that switch from ammonia to urea synthesis expend energy to increase the expression of OUC enzymes, but also pay maintenance costs to retain the regulatory machinery to induce this pathway even in water when it is not required. If maintaining this response is very costly, or if emersions are rare and the pathway is not essential to survival, the ability to express plasticity may be lost even if the induced phenotype is beneficial when the fish are on land.

There are also limits to the ability of plasticity to produce phenotypes that are perfectly matched to the environment. For example, developmental plasticity may not be possible if neuroendocrine control systems are unable to respond fully at a particular life stage or if the environmental cues that trigger plastic changes are detected too late in development. In contrast, reversible phenotypic flexibility is mostly limited by the time it takes for a phenotypic response to be expressed and by 'ecological limits' or trade-offs that arise because of pleiotropic genes controlling plastic pathways or the multifunctional roles of tissues and organs (Auld et al., 2010). Lag-time limits to the effectiveness of plasticity may explain why reversible gill remodelling during air exposure, a morphological change that takes days to weeks to express, has evolved in species such as mangrove rivulus (Ong et al., 2007) and lungfish (Sturla et al., 2002) – which remain out of water during

seasonal droughts – but not in mudskippers like *Periophthalmus sobrinus*, which move back and forth between air and water frequently during the day (Gordon et al., 1968). In the mangrove rivulus, the benefits of reduced gill surface area during long periods of emersion must also outweigh the temporary impairment of aquatic respiratory function during the lag time between the return to water and the expression of an 'aquatic' gill phenotype (Turko et al., 2012). More immediate adjustments, such as behavioural plasticity or circulatory changes, should be equally likely to evolve in lungfish, mangrove rivulus and mudskippers, as these changes have short lag times relative to the period of emersion. However, the effectiveness of even relatively fast expressions of plasticity is still limited by ecological trade-offs. For example, although increased skin thickness and mucous cell proliferation would reduce water loss in emersed *K. marmoratus*, these changes are not observed (LeBlanc et al., 2010; Turko et al., 2011). In this case, the effectiveness of plastic changes to conserve water is probably limited by the reliance on the skin for respiration and NH_3 volatilization. A broad comparison of the degree of plasticity across phylogenetically diverse amphibious species with different life histories is needed to determine whether the theoretical predictions in Fig. 2 hold true.

Phenotypic plasticity and genetic evolution

There are many excellent papers on how phenotypic plasticity can lead to the evolution of specialization or adaptations (e.g. Via et al., 1995; West-Eberhard, 2003; Pigliucci et al., 2006; Lande, 2009; Pfennig et al., 2010). Most of the focus is on developmental plasticity. West-Eberhard (2003) proposed that developmental plasticity could lead to genetic assimilation (see Glossary) under some conditions, such as if environments become less variable. The environmentally sensitive developmental genetic pathways that underlie plasticity are not well understood (Pfennig and Ehrenreich, 2014), but understanding these pathways will eventually lead to an understanding of the molecular basis of plasticity and, ultimately, how plasticity influences evolutionary processes. Comparing transcription profiles between aquatic and terrestrially reared fish embryos is a first step (e.g. Tingaud-Sequeira et al., 2013), but it will also be necessary to characterize the potential phenotypic differences at later life stages. By comparing the embryonic stages of multiple amphibious species with different life histories, one could determine whether early exposure to air induces similar regulatory pathways, potentially providing evidence for parallel or convergent evolution. Furthermore, evidence that plasticity in amphibious fishes is regulated by gene expression patterns similar to those of tetrapods (for example, during the metamorphosis of an aquatic tadpole to a semi-terrestrial adult amphibian) would further support the hypothesis that the invasion of land involves the assimilation of inducible, phenotypically plastic traits.

Probably the best example of how phenotypic plasticity may lead to the evolution of fixed traits was demonstrated in the bichir (Standen et al., 2014). As described above (see 'Buoyancy, gravity and movement on land' and 'Developmental plasticity'), the developmental plasticity of locomotory behaviours and skeletal elements in emersed bichir mirrors the evolution of ancestral tetrapods, suggesting that phenotypic plasticity may have played a role in the evolution of more efficient terrestrial locomotion. Similar approaches could be used in other species and for other traits, although one major challenge is that for behavioural, physiological and biochemical plasticity there is no fossil record to help us understand the early stages of tetrapod evolution. However, a comparative approach focusing on developmental plasticity in multiple amphibious fishes and amphibians may provide useful insights.

Acclimatization or phenotypically flexible responses to terrestrial exposure in adult amphibious fishes sometimes also recapitulates the evolution of fixed traits in terrestrial tetrapods, and it is theoretically possible that genetic assimilation can act on these forms of plasticity (Piersma and Drent, 2003). Expressions of plasticity in fishes that frequently emerse could become fixed if the fitness costs of maintaining the ability to be plastic are larger than the benefits of switching phenotypes when returning to water. Alternatively, genetic assimilation could simply occur through genetic drift if an amphibious species spends so much time out of water that there is no fitness cost to losing the ability to express 'aquatic' phenotypes. Thus, a greater understanding of the breadth of phenotypic plasticity in amphibious fishes may eventually lead to a clearer comprehension of the steps along the evolutionary path to terrestrial life.

Conclusions

For over a century, biologists have been describing the remarkable ability of amphibious fishes to survive out of water. The ability to emerse and breathe air allowed ancestral fishes to exploit a new O_2-rich habitat and avoid poor aquatic conditions and aquatic predators. However, there are many negative consequences to breathing air (Table 1); thus, emersion must confer a significant survival advantage. Here, we have provided the first list of extant amphibious fish species, revealing a remarkable evolutionary diversity of form and function across >200 species. These amphibious fishes have met the challenges of life out of water by expressing a variety of plastic traits and relying on fixed adaptations that enhance survival on land. There is a tremendous diversity of life histories, from species that are highly active out of water but return to an aquatic refuge multiple times each day to other species that are quiescent on land and survive there for entire seasons. We understand little about the full extent of phenotypic flexibility and developmental plasticity in amphibious fishes and the underlying regulatory mechanisms. Early rearing in air has been shown to profoundly alter the phenotype of later life stages in *Polypterus* (Standen et al., 2014), but further exploration of developmental plasticity in other species may provide some insights into genetic assimilation and the evolution of terrestriality. Finally, there is inherent value in uncovering the physiological mechanisms (plastic or otherwise) used by amphibious fishes with different life histories and evolutionary origins to cope with emersion, especially given that we only have data on a few taxonomic groups.

Supplementary information

Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.126649.supplemental

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