
Evolutionary and Cardio- Respiratory Physiology of Air-breathing and Amphibious Fishes

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Abstract :

Air-breathing and amphibious fishes are essential study organisms to shed insight into the required physiological shifts that supported the full transition from aquatic water-breathing fishes to terrestrial air-breathing tetrapods. While the origin of air-breathing in the evolutionary history of the tetrapods has received considerable focus, much less is known about the evolutionary physiology of air-breathing among fishes. This review summarises recent advances within the field with specific emphasis on the cardiorespiratory regulation associated with air-breathing and terrestrial excursions, and how respiratory physiology of these living transitional forms are affected by development and personality. Finally, we provide a detailed and re-evaluated model of the evolution of air-breathing among fishes that serves as a framework for addressing new questions on the cardiorespiratory changes associated with it. This review highlights the importance of combining detailed studies on piscine air-breathing model species with comparative multi-species studies, to add an additional dimension to our understanding of the evolutionary physiology of air-breathing in vertebrates.

Keywords : development, evolution, phenotypic plasticity, terrestrialisation, water-to-air transition

1 | INTRODUCTION

The evolution of air-breathing represents one of the most iconic physiological transitions in vertebrate evolution, and it offered an adaptive advantage to fishes by providing the physiological basis for exploiting new ecological niches such as oxygen-poor water bodies and terrestrial environments^{1,2}. Life evolved in water and hence the transition from water- to air-breathing is an inherent part of the physiological ancestry of all terrestrial vertebrates. Changes in breathing medium (water to air) and in apparent gravity associated with movement towards terrestrial environments impose a series of constraints on the functional morphology and cardiorespiratory regulation that must have been solved for the complete aquatic-to-terrestrial transition to occur. The specific evolutionary changes in anatomy and physiology associated with the origin of air-breathing in basal bony fishes that occurred hundreds of millions of years ago cannot be directly observed. While the fossil record provides some insight to transitions between aquatic and terrestrial lifestyle, the soft tissue structures that reveal physiological function rarely preserves in the fossil record. Consequently, insight into terrestrial vertebrates' physiological ancestry depends to a large degree on research on extant fishes that differ in aquatic/terrestrial lifestyle and aquatic/aerial breathing mode.

A scientific session of air-breathing fish physiology was organised by Professor Tobias Wang at the Oxygen Symposium in Sandbjerg (Denmark) in May 2018 to honour the scientific contributions of comparative respiratory physiologist Roy E. Weber. At the symposium, the current status and recent advances in research on the physiology of air-breathing - and amphibious fishes were discussed to facilitate future collaborative efforts within the field. While some of the physiological changes associated with air-breathing were well described in recent reviews including specific changes in acid/base status³⁻⁵, ventilatory regulation^{3,6}, blood gas transport⁴, and cardiovascular anatomy^{3,7}, this review aims at taking

a step back to discuss five central themes that are essential for our understanding of the evolutionary physiology of air-breathing - and amphibious fishes: First, we discuss the distinct physiological challenges between aquatic air-breathing fishes and amphibious fishes that emerge from water (Section 2). Next, we review the current state of knowledge on the cardioventilatory regulation of air-breathing that serves as the core physiological mechanisms required for efficient aerial gas-exchange (Section 3). Since intraspecific variation and phenotypic plasticity likely served as the raw material for air-breathing evolution, we discuss how intraspecific variation in personality in air-breathing fishes affects their air-breathing behaviour and respiratory physiology (Section 4) as well as the current state of knowledge on developmental phenotypic plasticity in air-breathing fishes (Section 5). We then summarise the current understanding of the evolution of air-breathing and provide a complete re-evaluation of the evolution of air-breathing and amphibiousness within fishes (Section 6). Finally, we discuss how this model can be used to bridge studies on developmental programming, phenotypic plasticity, and behaviour to gain a detailed understanding of the physiological mechanisms associated with the evolution of air-breathing across time scales (Section 7).

2 | RESPIRATORY CHALLENGES ASSOCIATED WITH LIFE OUT OF WATER

Fishes that can supplement aquatic with aerial gas-exchange are defined as “air-breathing fishes”^{1,2}. There is a massive diversity of air-breathing fishes, with some 656 extant species within 129 genera, 41 families and 22 orders (based on Data Set 1 and the current fish taxonomy⁸). Aerial gas-exchange is achieved by virtue of several distinct respiratory structures, such as respiratory gas-bladders, structures within the buccal cavity, the skin and various sites within the gastrointestinal tract².

Amphibious fishes are a sub-category of air-breathing fishes that are defined as fishes that naturally spend part of their life out of the water, and so considered “amphibious”^{4,9-11}.

There are >200 species of extant amphibious fishes across 93 genera, 41 families, and 19 orders^{4,12}. This diversity matches the wide variation in life histories, phenotypes, and geographic distribution. Some species are emerged (out of water) only in the embryo stage (*e.g.*, California grunion, *Leuresthes tenuis*¹³), while others may transition as adults between water and air multiple times a day (*e.g.*, giant mudskipper, *Periophthalmodon schlosseri*,¹⁴). Still, other amphibious fishes may not actively leave the water, but as the water disappears during the dry season, they are left without water for weeks or months (*e.g.*, African lungfish, *Protopterus aethiopicus*). Thus, it is not easy to generalise about the physiological adaptations that amphibious fishes use to cope with life on land because of their diverse life histories.

What drives fish to leave water? Poor water quality (*e.g.* hypoxia, hypercapnia, elevated H₂S) stimulates many amphibious fishes to emerge¹⁵. Biotic factors, such as competition, predation, terrestrial feeding, and reproduction can also be important^{12,15}. Abiotic and biotic factors can interact to alter emersion behaviour in the mangrove rivulus, *Kryptolebias marmoratus*. This amphibious fish delays emersion as the water temperature rises if they detect a conspecific nearby compared to fish on their own¹⁶. These findings raise several interesting questions about how amphibious fishes process sensory information about their external environment and how the stimulus to leave water can be overridden by intrinsic factors (see section 4 on behavioural regulation of air-breathing).

Once amphibious fishes emerge, whether, for a few hours or months, they face several distinct challenges that may impact respiratory function, which we will now discuss (Fig. 1).

2.1 | CO₂ accumulation

Many amphibious fishes retain CO₂ when they air-breathe out of water because of the absence of water flow over the gills and lower rates of gas convection through lungs or other air-breathing organs¹⁷⁻¹⁹. The rise in blood PCO₂ is similar to aquatic air-breathing fishes – particularly in obligate air-breathers with reduced gill area²⁰. Elevated blood CO₂ results in an initial acidification followed by extracellular pH compensation in some species via a rise in plasma [HCO₃⁻]^{3,5}. The initial reduction in extracellular pH causes both a decrease in blood oxygen-carrying capacity (Root effect) and a decrease in haemoglobin-oxygen affinity (Bohr effect). In some species that are active out of the water, metabolic production of H⁺ via lactate synthesis may further contribute to an acid-base disturbance^{21,22} and affect blood oxygen transport. Do amphibious fishes constitutively express pH/CO₂ insensitive haemoglobins or isoforms with high affinities for oxygen? Or do fish acclimate to air by altering the red blood cell environment (*e.g.*, via regulation of adenylates²³) and/or express different haemoglobin isoforms? The data are sparse and piecemeal. Recent work on blood's functional properties shows that water- and air-breathing fishes have Bohr effects of similar magnitude^{3,24,25}, but air-breathers appear to express haemoglobin with higher buffer capacity, which may serve to alleviate the respiratory acidosis associated with air-breathing²⁶. The same pattern of haemoglobin evolution is likely to have occurred during the evolution of amphibious fishes, but it remains to be tested. Indeed, even very closely related air-breathing fishes can show strikingly different blood transport properties²⁷, and so few assumptions are safe concerning haemoglobin properties in amphibious fishes.

2.2 | Loss of buoyancy

When fishes emerge onto land, they experience an increase in apparent gravity. Movements on land, including respiratory movements²⁸, require more skeletal support due to the increase in apparent gravity^{29,30} and likely more energy in the absence of buoyancy. The cost of

respiration in bimodal breathing fishes has not, to our knowledge, been assessed. Studies of the mechanics of ventilation in *P. aethiopicus* revealed that during aestivation, abdominal muscles were used to forcibly deflate the lung, a mechanism not used when air-breathing in water^{31,32}. Thus, one might predict that aerial respiration on land may be more energetically demanding than in water, a possibility that could be tested in a few different species that are both air-breathing and amphibious, like the lungfishes.

The loss of buoyancy in the air also impacts gill structures. Gill remodelling in two amphibious species, *K. marmoratus*³³⁻³⁵ and bichir, *Polypterus senegalus*³⁶, involved an increase in the height of the interlamellar mass, which was associated with a reduced gill surface area in air-acclimated fish, possibly to fortify delicate lamellar structures or to reduce water loss. As well, air exposure stiffened the gill skeleton of *K. marmoratus* with increased expression of proteins responsible for bone mineralisation and more densely packed collagen fibrils in gill arches and filaments³⁰. Although gill remodelling in the air is reversible, it takes one week for the interlamellar mass to regress³³ and up to 12 weeks for the gill stiffness to return to control aquatic levels³⁵. Therefore, upon returning to water, there are lag time effects that immediately impact aquatic respiration³⁷ and imply that there are trade-offs to gill remodelling.

2.3 | Water loss

Avoiding evaporative water loss across gills and the air-breathing organ surfaces is a challenge for emerged fishes^{10,38,39}. Many species immediately seek humid refugia in terrestrial environments^{22,40-42} or retreat to burrows to breathe air⁴³. In some of these habitats, the gas composition of the microhabitat may be hypercapnic or hypoxic⁴⁴.

Therefore, routine avoidance of desiccation in the air may lead to respiratory challenges. For

example, *K. marmoratus*⁴⁰ and the Dartfish, *Parioglossus interruptus*⁴², seek tunnels in moist rotting logs where low O₂ and high CO₂ levels may prevail⁴⁵. Such conditions would presumably lead to hypoxia, respiratory acidosis, and impaired blood O₂ transport. Further studies are required to characterise more carefully the impact of aerial microhabitat conditions on aspects of respiration and metabolism in amphibious fishes.

2.4 | Decreased ion availability

The gill is normally the major site of ion balance. Ion regulation involves protein transporters that also transport acid-base equivalents (*e.g.*, Na⁺/H⁺ exchange, HCO₃⁻/Cl⁻ exchange, Na⁺/HCO₃⁻ cotransport). Thus, ion transport and acid-base balance are intimately linked, and in turn, any disruption in ion transport in fishes out of water may impact respiration through changes in blood pH and downstream effects on haemoglobin oxygen binding. Little is known about non-branchial alternative sites of ion and acid-base regulation in amphibious fishes. Cutaneous ionocytes have been identified in a few species^{4,46} and ions and water are transported across the skin. One study on Asian swamp eels, *Monopterus albus*, showed that the kidneys play a major role in acid/base regulation when emerged in hypercapnic air similar to the conditions found in their burrows in the dry season⁴⁷. However, whether the kidney or gut take on a larger role in acid-base regulation in the absence of functional gills in amphibious fishes out of the water, in general, is largely unknown. Air-breathing fishes that remain in water during air-breaths tend to have reduced gill surface area with fewer ionocytes relative to water-breathing fishes⁴⁸. Hence, gill iono- and osmoregulation may be augmented by the skin and digestive tracts, as well as the kidney⁴⁹.

2.5 | Threat of toxic nitrogen end products

Nitrogenous wastes are mainly excreted as NH_3 across the gills when fishes are in water. NH_3 can be considered the third respiratory gas along with O_2 and CO_2 ⁵⁰. NH_3 capacitance in the air is 1/700 of that in the water²⁸ and in the absence of gill water flow, potentially toxic nitrogen wastes (such as NH_3) may accumulate in tissues. Amphibious fishes have adopted several mechanisms for storing or excreting nitrogen wastes in air⁵¹. Some amphibious fishes i) suppress amino acid catabolism, ii) alter amino acid metabolism to store nitrogen as alanine and/or glutamine, iii) detoxify NH_3 to urea via the ornithine-urea cycle and/or iv) release gaseous NH_3 across the skin or digestive tract. NH_3 volatilisation has been documented in a few species (*e.g.*, weather loach, *Misgurnus anguillicaudatus*⁵² and aplocheloid killifishes⁵³) and may be coupled to changes in $\text{HCO}_3^-/\text{H}^+$ excretion⁵⁴ to provide an alkaline microenvironment for NH_3 formation. Cutaneous Rhesus proteins in the skin likely facilitate NH_3 release⁵³, but whether they also facilitate cutaneous CO_2 excretion in amphibious fishes is unknown⁵⁵. There is much more to learn about the regulation of NH_3 release across the skin or gut in air-exposed fishes.

In summary, amphibious fishes are a distinct subgroup of air-breathing fishes. They share with air-breathing fishes the advantages of breathing O_2 -rich air, but they face many unique challenges that may impact respiratory function. For example, many fishes out of water must cope with elevated internal CO_2 , increased effective gravity, terrestrial moist microhabitats with altered gas compositions, decreased ion availability and the threat of toxic nitrogen wastes accumulating in the absence of water flow over the gills. Air-breathing fishes that remain in the water and gulp air from the surface may still retain some gill function even if gill surface area is reduced. By separating the physiological challenges encountered by aquatic and amphibious air-breathers, we can gain a better understanding of the evolutionary processes that shaped their respiratory strategies.

3 | CARDIORESPIRATORY INTERACTIONS GOVERNING AIR-BREATHING

Many of the basal ray-finned fishes are air-breathers, and air-breathing has evolved numerous times within teleosts (see section 6). Facultative air-breathing enables these fishes to maintain aerobic metabolism when the supply of dissolved oxygen is limited, or demand is increased during bouts of activity. However, the introduction of an air-breathing organ in a water-breathing fish requires a series of changes in their cardiovascular and ventilatory regulation to take advantage of the new oxygen-rich respiratory medium. As an example, exposure to hypoxia in water-breathing fishes is associated with a reduction in heart rate and branchial hyperventilation to improve branchial gas-exchange efficiency⁵⁶. However, this physiological response to aquatic hypoxia would be disadvantageous in a facultative air-breathing fish, since the air-breathing organ typically drains oxygenated blood into the central veins^{3,7}, creating a potential for oxygen loss to the environment across the gill respiratory surfaces – a phenomenon that would be increased by hyperventilation and bradycardia¹⁷. Cardiorespiratory regulation thus becomes relatively more complex as cardiac and ventilatory rhythms become regulated in response to air-breathing events. In this section, we review the regulatory pathways controlling air-breathing in bimodal breathing fishes and discuss how the lungfishes provide insight into cardioventilatory mechanisms they share with the tetrapod vertebrates.

3.1 | Autonomic Cardioventilatory Regulation Governing Air-Breathing

3.1.1. Control of ventilation

Our current knowledge of the basis of respiratory rhythm generation in fishes was recently reviewed⁵⁷. In elasmobranchs and bony fishes, the neurons acting as a respiratory rhythm generator are located within the medulla, but their precise location has yet to be determined.

Removal of the fore- and midbrain, spinal cord and even posterior regions of the medulla does not stop breathing⁵⁸. Many early studies suggested there is a longitudinal strip of nervous tissue containing respiration-related neurons extending on each side of the midline over the whole medulla oblongata with the source of rhythmic discharge generated by higher order interneurons, possibly including the reticular formation⁵⁹. Motor neurons showing respiration-related activity are widely distributed within the medulla and the pattern of respiratory activity comprises of a series of coupled oscillators arising from areas associated with the motor nuclei of the trigeminal (Vth), facial (VIIth), glossopharyngeal (IXth), and vagal (Xth) cranial nerves, with that associated with the Vth, which innervates the jaws⁶⁰. These relationships are illustrated schematically in Fig. 2.

Much is still to be known about the central control of respiration and central interactions between respiratory neurons and cardiac vagal preganglionic neurons in the brainstem in the integration of cardiorespiratory control during air-breathing in fishes. In all air-breathing fishes, the gills are ventilated by the same muscles, inserted around the jaws and gill arches, as in water-breathing fishes, while gulping of air to inflate the air-breathing organ is achieved through the action of the jaw musculature together with hypobranchial muscles, used in suction feeding by water-breathing fishes^{61,62}.

The location of neurons associated with cardiorespiratory control was investigated in bowfin, *Amia calva*, by labelling the branchial branches of the vagus nerve and the nerve supplying its air-breathing organ. This showed that cell bodies innervating respiratory muscles in the branchial arches are vagal motor neurons located chiefly in the dorsal motor nucleus of the vagus. However, a small population of vagal motor neurons was in lateral locations in an area termed the reticular formation, where the central respiratory pattern generator in fishes is thought to be located⁶³. The *A. calva* study further showed that the nerve supplying the glottis and air-breathing organ had most of its cell bodies in a ventro-

lateral location in the brainstem and the ventral horn of the anterior spinal cord. The cell bodies of neurons supplying the hypobranchial nerve are located in the ventro-lateral spino-occipital motor nucleus that supply series of ventral muscles inserted from the pectoral girdle of elasmobranch fishes ⁶¹. These are supplied by axons in the XIIth, hypobranchial nerve, innervating musculature associated in water-breathing fishes with the suction movements associated with feeding ⁶³. Recruitment of the hypaxial muscles into the respiratory cycle during periods of increased oxygen demand results in increased ventilation volume. Central connections are possible between vagal respiratory neurons in the dorsal motor nucleus of the vagus and occipital neurons as they have an overlapping distribution in the brain stem ^{61,62}. This could enable a rostrocaudal spread of excitation, from the vagal to occipital then on to the anterior spinal motor nuclei, that together innervate the hypaxial muscles, similar to that observed in the series of cranial nerves supplying the respiratory muscles in elasmobranch fishes ⁶⁴. These results suggest that the neural action of air-breathing would require little change in the pattern of neural control already established for suction feeding, except for control over glottal opening ⁶⁵, which would ensure effective ventilation of the air-breathing organ ⁶⁶. An additional small population of neuron cell bodies innervating the air-breathing organ were located in the Xth, vagal motor nucleus of *A. calva*, suggesting some functional aspects of the organ such as smooth muscle fibres in the tissue lining were under vagal control ⁶³.

3.1.2 Control of the heart

The anatomical and physiological adaptations for air-breathing vary between species, but they share several common characteristics that seemed fundamental to their function ^{3,67}. One characteristic is that heart rate increases during air-breathing, known as surfacing tachycardia ⁶⁶, which may serve to enhance gas-exchange efficiency by matching perfusion and

ventilation of the air-breathing organ ¹⁷. In some species each air-breath is associated with increased perfusion of the air-breathing organ, due to a reduction in peripheral resistance in the pulmonary circuit. This requires tight synchronous regulation of ventilation of the air-breathing organ and associated cardiovascular changes during bouts of air engulfment, which is well described in tetrapods, but much less is known about regulation in air-breathing fishes. The Xth cranial nerve, the vagus plays a dominant role in cardiorespiratory regulation in all vertebrates. In air-breathing fishes it provides both afferent fibres to reflexogenic areas monitoring changes in respiratory gases at the gills, plus in some species volume changes in the air breathing organ and efferent fibres innervating intrinsic respiratory muscles. The vagus also provides preganglionic innervation that modulates heart rate and, in some species, peripheral resistance in the blood supply to the air-breathing organ. These functional connections, plus innervation of respiratory muscles by other cranial and spinal nerves are illustrated diagrammatically in Fig. 2.

In Jeju, *Hoplerythrinus unitaeniatus*, the heart rate increased drastically during each air-breath, which was primarily regulated by the withdrawal of an inhibitory cholinergic tone exerted by the parasympathetic innervation via the vagus nerve ⁶⁸. However, complete inhibition of cholinergic regulation of heart rate did not affect oxygen uptake in this species ⁶⁸. This regulation merits further investigation as peripheral resistance in the pulmonary artery as well as heart rate is under vagal control in vertebrates having an undivided circulation, including air-breathing fishes, so that lung function would seem highly dependent on cholinergic control ⁶⁹.

The marbled swamp eel, *Synbranchus marmoratus*, is a facultative air-breather. When exposed to hypoxia, the submerged fish showed a marked increase in cardiac output related to an increase in both heart rate and stroke volume. Expansion of the buccopharyngeal cavity during a normal breathing cycle also led to a marked rise in heart rate ⁷⁰, suggesting a role for

mechanoreceptor stimulation in this response ². The heart rate then decreased progressively during the breath-hold, implying an additional chemoreceptor stimulation of the response though this could also be a response to the decreasing volume. Similar evidence of a role for peripheral receptors in cardiorespiratory control is available for several species of air-breathing fishes ². This increase in heart rate accompanying both the response to aquatic hypoxia and an air breath in *S. marmoratus* was accompanied by significant increases in central venous blood pressure, mean circulatory filling pressure, and dorsal aortic blood pressures, suggesting that the venous system plays a vital role in determining cardiac filling and consequent stroke volume in this species. These responses of the venous system were shown to be under adrenergic control ⁷⁰.

In water-breathing fishes, most cardiac vagal preganglionic neurons are located in the dorsal motor nucleus of the vagus amongst respiratory neurons. In elasmobranch fishes these cardiac vagal preganglionic neurons in the dorsal motor nucleus of the vagus shown respiration-related activity that can recruit the heart generating cardiorespiratory synchrony ^{64,71}. As this activity was recorded from decerebrated, curarised fish it identifies a role for vagal motor neurons and cardiac vagal preganglionic neurons in interactive central, feed-forward control of the circulatory and respiratory systems. In elasmobranchs about 40% of cardiac vagal preganglionic neurons are located ventrolaterally in the brainstem outside of the dorsal motor nucleus of the vagus, do not show respiration-related activity and may control reflex responses to factors such as hypoxia. Interestingly, this distribution is similar to that of tetrapods ^{62,69}, and though there are important differences in function, this suggests that the neural networks associated with respiratory rhythmogenesis and cardioventilatory integration may be well conserved during vertebrate evolution ⁷². However, we know less about these distributions and their functional properties in all other groups of fishes, and knowledge of these interactions is particularly scarce for air-breathing species.

3.2 | Using lungfishes as model species to illuminate conserved cardioventilatory

mechanisms

Lungfishes arose in the Devonian period around 400 million years ago, together with early tetrapods, with which they share many plesiomorphic features characteristics of the Sarcopterygii such as limb and jaw structure, paired lungs and features of the heart and circulation ^{2,73}.

African and South American lungfishes, *Protopterus* sp. and *Lepidosiren paradoxa*, respectively, are obligate air breathers, rising to the surface at regular intervals to ventilate its lung-like air-breathing organ ², and can survive for long periods in burrows during drought. They fill their lungs, opening off the gut, by means of a buccal force pump. Air is drawn into the buccal cavity by lowering of the floor of the open mouth. This air is then forced into the lungs by raising the floor of the closed mouth, using a set of muscles inserted on the pectoral girdle and innervated by the hypobranchial nerves (Fig. 2). Expiration is brought about by the elasticity of the lung tissue. A similar mechanism operates in amphibians so it can be postulated that lungfishes retain features that reflect the origins of tetrapod evolution, including their mechanisms for air-breathing and for cardiorespiratory control ⁶². The circulatory system is similarly specialised for air-breathing. Blood flowing into the paired pulmonary arteries in lungfishes originates from the efferent arteries of branchial arches 3 and 4 while pulmonary veins conduct blood directly into the heart which is almost completely divided into right and left halves. Because they need to sustain the simultaneous function of both gill- and lung exchange surfaces, *Protopterus* and *Lepidosiren* achieve levels of pulmonary systemic separation surpassing that in most amphibians ⁷³.

In *L. paradoxa* each air-breathing episode was accompanied by an immediate and marked increase in heart rate, due solely to withdrawal of a cholinergic parasympathetic

tonus⁷⁴. The immediate increase in heart rate on surfacing implies an instantaneous change in autonomic tone. This was reflected in the measured conduction velocity of cardiac vagal fibres, which matched mammalian B-fibres and, like them, were shown to be myelinated⁷⁴. Its presence in the *L. paradoxa* as well as in tetrapods suggests an ancient origin of myelination of the vagal efferents, which may be functionally important for allowing instantaneous beat-to-beat control of heart rate. Similar tight control of heart rate could be highly efficient in air-breathing ray-finned fishes, but this has not been investigated and merits further attention.

Following the injection of atropine, *L. paradoxa*, increased air-breathing frequency⁷⁴. This increase was accompanied by an increase in total oxygen consumption, suggesting that the effectiveness of gas exchange over the lung was maintained⁷⁴. Subsequent injection of propranolol (an inhibitor of beta-adrenergic receptors) increased tidal volume and oxygen uptake per breath by 25%⁷⁴. Both acetylcholine and adrenaline/noradrenaline increase pulmonary vascular resistance in lungfish⁷⁵. Consequently, the injection of cholinergic and adrenergic antagonists is likely to have reduced vascular resistance in the pulmonary artery, increasing lung perfusion. Together with the increased heart rate, increased pulmonary perfusion would imply that oxygen uptake over the lung could be enhanced. Clearly, this was not the case as oxygen uptake per breath was markedly reduced, inviting the conclusion that control of patterns of blood and airflow over the lung surface are generally under tight tonic neural control, rather than responding to bulk changes in flow⁷⁴.

The precise nature of this control is unknown, but the recent study on *L. paradoxa* revealed that cardiac vagal preganglionic neurons, responsible for controlling heart rate via the parasympathetic vagus nerve, and possibly also innervating the pulmonary arterial supply, have multiple locations in the brainstem⁷⁴. These locations may be both within the dorsal vagal motor nucleus and ventral to it, as described for the bowfin. It has been suggested that

P. aethiopicus possesses two separate central rhythm generators, one for gill ventilation and the other for air-breathing⁷⁶. These are likely to constitute the central respiratory pattern generator supplying the jaws and branchial innervation via the Vth, VIIth, IXth and Xth cranial nerves, together with a central rhythm generator supplying the hypobranchial complex via occipital and anterior spinal nerves⁶¹. These separate locations and possible interactions are suggested in Fig. 2, but their functional roles and central integration remain to be demonstrated as much of this figure is based on detailed studies of elasmobranch fishes^{62,64,71}.

African and South American lungfishes have pulmonary stretch receptors⁷⁷ as well as both central and peripheral chemoreceptors⁷⁸, making it likely that feedback from these receptors is also involved in cardiorespiratory integration. Since these traits are also present in many tetrapods, the morphological and physiological mechanisms enabling respiratory gas exchange from the air may have been fully evolved in early lobe-finned fishes. This further suggests that these traits may have been secondarily lost (or reduced) in Australian lungfish and coelacanths that are facultative air-breathers and obligate water-breathers, respectively.

In summary, the actions of ventilation and perfusion of the air-breathing organ in air-breathing fishes are well described for several species, but the neural control and coordination of their mechanisms are less well understood. Although many recorded responses evidence a role for peripheral receptors in coordination of air-breathing responses in lungfishes, the primary role of the central nervous system in both generating and coordinating the physiological responses during air-breathing seems established² though the details of these interactions remain to be investigated (Fig. 2). There are three outstanding areas for further investigation:

1) Little is still known about the coordination of the actions of primarily respiratory muscles, innervated by cranial nerves, with those innervated by the hypobranchial nerves during gulping of air at the surface ^{61,62}. A proper understanding of the central connections and interactions leading to control in air-breathing fishes may uncover the pathway for the evolution of the control and integration of lung ventilation in tetrapod vertebrates.

2) The typical increase in heart rate at each air-breath needs to be linked to changes in the venous system and patterns of blood flow through the air-breathing organ, including the role of intrinsic factors and the autonomic nervous system in the regulation of these changes.

3) We have also described multiple locations for vagal preganglionic neurons, including specifically those innervating the heart, in the brainstem of the lungfish but have yet to record from these areas in order to apportion separate functional roles to the different groups.

4 | LINKING PHYSIOLOGY AND BEHAVIOUR IN AIR-BREATHING FISHES

Air-breathing by fishes serves, of course, to obtain oxygen to create ATP by oxidative phosphorylation in tissue mitochondria, to sustain the energy demands of metabolism.

Metabolic rate is the rate of energy use for living and, in aerobic animals such as fishes, this requires continuous delivery of oxygen. In fact, metabolic rate is typically measured indirectly in fishes, as their rate of oxygen consumption ⁷⁹. Air-breathing can assure oxygen supply when availability declines in the water (hypoxia) but can also meet the increased oxygen demands associated with ecologically important aerobic activities, such as foraging, digestion, and growth ⁸⁰.

4.1 | Behavioural modulation of air-breathing reflexes

The physiological basis of air-breathing is relatively well understood as a chemoreflex^{81,82}.

The sensory arm of the reflex is provided by oxygen-sensitive chemoreceptors located in the gills and orobranchial cavities, innervated by cranial nerves. Receptors sensitive to water oxygen levels assure the vigorous increase in air-breathing that most species show in aquatic hypoxia. There are also receptors sensitive to blood (or tissue) oxygen levels that are presumably responsible for the fact that air-breathing is sensitive to metabolic rate and the associated oxygen demand^{6,83}. The motor output of the reflex is very complex in air-breathing fishes, as they must approach the water surface and break it to gulp air (see Section 3). Thus, certainly for aquatic air-breathers (as opposed to amphibious species), there is a substantial behavioural component to air-breathing⁸⁴. For example, air-breathing movements can place fishes at risk of predation, both from above and below the water surface. This is presumably why the reflexes can be inhibited if the fish perceive a risk of predation associated with surfacing, and why they may preferentially choose to do so under cover, for example under vegetation in nature^{84,85}. It is also thought to explain the phenomenon of synchronous air-breathing, where of groups of air-breathing fishes may offset the risks associated with surfacing by doing so simultaneously or within a short period^{86,87}. This social surfacing may reduce individual risk of predation⁸⁷, similar to group foraging in fish schools or other animal groups⁸⁸. Although higher-order modulation of air-breathing behaviour, such as the effects of predator avoidance, has been studied much less than the physiological chemoreceptor drive, there is convincing evidence for it^{84,89}. Another striking example is pairs of Siamese fighting fish, *Betta splendens*, which cease fighting to air-breathe in unison, before resuming their battle⁹⁰. This behaviour presumably means that one of the individuals is not necessarily responding to a chemoreceptor drive, but cues from the other fish.

4.2 | Metabolic rate as a driver of air-breathing behaviour

The significant behavioural component to air-breathing reflexes makes bimodal breathing fishes useful models to investigate relationships between respiratory metabolism and behaviour. In particular, understanding the ecological significance of metabolic rate in animals is an active area of research. There has been much focus on why individuals within species vary so widely in their metabolic rate and oxygen requirements. Although the mechanistic basis for such variation is poorly understood, the variation itself is believed to have ecological significance. It has been proposed that the variation persists because it shapes life-history trade-offs between production (growth, maturation, reproduction) and mortality⁹¹⁻⁹³. Individuals with high metabolic rates would have a ‘fast’ life-history traits where they were able to acquire and process resources at high rates, thus maintaining high growth rates and achieving sexual maturity more rapidly than conspecifics with lower metabolic rates. The ‘fast’ phenotypes would, however, incur higher risks of mortality, for example by predation because foraging increases their encounter rate with predators, or by starvation when resources were scarce. This allows the coexistence of ‘slow’ phenotypes, which mature more slowly but, because they are less sensitive to predation and starvation, can nonetheless survive to reproduce⁹⁴. It has, by extension, been demonstrated that individual variation in metabolic rate can be a driver of behaviours linked to resource acquisition, such as taking risks to forage⁹⁵. It has also been proposed that metabolic rate will be intimately linked to variation in behavioural tendencies that comprise elements of ‘personality’, such as boldness, aggression, activity level, and the tendency for exploration^{96,97}. Personality is the persistent expression of such tendencies, over time, and across contexts. Metabolic rate has been invoked as a potential physiological driver for the expression of personalities^{88,97}. For example, animals with high metabolic rates would be predicted to be consistently bolder, as this could enhance the success of a fast high-risk lifestyle^{95,98}.

4.3 | Air-breathing fishes as models for the links between physiology and behaviour

Air-breathing fishes would seem to be excellent models to investigate these questions because aerial oxygen is a resource that is risky to obtain but which is explicitly linked to metabolic rate. They allow us to explore the generality of propositions regarding how the metabolic rate should drive risk-taking to acquire resources in animals, and whether this is associated with differences in behavioural tendencies such as boldness and aggression. The African sharptooth catfish, *Clarias gariepinus*, has been the focus of some studies to investigate associations among metabolic rate, risk-taking to breathe air, and behavioural tendencies^{99,100}. Despite the limited number of studies, the results reveal interesting patterns that will, hopefully, stimulate further research in this field.

C. gariepinus breathes air using a suprabranchial structure termed the arborescent organ. In the wild it is a nocturnally active predator, seeking cover during the day¹⁰¹. It is a facultative air-breather that can meet all of its routine oxygen requirements by gill ventilation in well-aerated water. Thus, air-breathing is not obligatory, but when left undisturbed in respirometer chambers, juvenile catfish show spontaneous diurnal cycles in metabolic rate and air-breathing activity, both being much higher at night than during the day¹⁰⁰.

Individuals show up to an eight-fold variation in their standard metabolic rate (the basal metabolic rate of an ectotherm at their acclimation temperature), but even greater variation in rates of oxygen uptake from air (over 50 fold) and in the proportion of their metabolic rate that they obtained from air (over 20 fold)¹⁰⁰. Individual standard metabolic rate in *C. gariepinus* was strongly correlated with rates of oxygen uptake from the air, especially when surfacing might be perceived as inherently risky (daylight) and also when water oxygen levels drop (aquatic hypoxia)¹⁰⁰. Thus, metabolic rate may be a driver of overall rates of risk-taking, although this seems self-evident since we know that air-breathing responses are driven by chemoreflexes⁸³. Chemoreceptors may assure the link to metabolic rate in areas of

the venous vasculature where blood oxygen levels reflect rates of oxygen removal by respiring tissues and stimulate surfacing reflexes more frequently in animals with high tissue oxygen demand^{80,86,87,100}. However, individual standard metabolic rate was also positively correlated with boldness measured in two contexts, namely the time-lag to resume air-breathing after a startle stimulus in their respirometer (T-res) and the time-lag to enter the centre of a novel environment measured in an open field test¹⁰⁰. These latter two measures of boldness were very highly correlated between themselves¹⁰⁰. Thus, this supports predictions that individuals with higher metabolic rates, which take more risks to acquire resources, should also be intrinsically more bold^{91,97}.

There is also good evidence, however, that *C. gariepinus* is much more complex than such a straightforward paradigm of ‘physiology drives behaviour’. In particular, individual behavioural tendencies such as boldness and aggression have effects on air-breathing that are apparently independent of standard metabolic rate. For example, there was consistent evidence across two studies that individual standard metabolic rate was not correlated with the proportion of metabolic rate that is met by air-breathing under routine conditions in aquatic normoxia¹⁰⁰. The proportion of oxygen obtained from air might be considered a particularly good indicator of boldness because it reveals the predisposition to take risks by choosing to air-breathe. In both studies, the percentage of oxygen uptake derived from air-breathing during daylight hours was, however, correlated with individual boldness, measured as T-res¹⁰⁰. Other evidence comes from a study investigating synchronous air-breathing in *C. gariepinus*, which is gregarious as a juvenile. Groups of air-breathing fishes may offset the risks associated with surfacing by doing so simultaneously or within a short period^{102,103}. Killen et al. (2018) investigated whether the frequency of group surfacing might be driven by individuals with high standard metabolic rate, especially in hypoxia when their oxygen demand would stimulate them to breathe air⁹⁹. There was no evidence for this, in fact, the

frequency of air-breathing was driven by aggression amongst members of the group, with aggressive individuals chasing others until they breathed air, at which point the aggressor would follow them to the surface to breathe also. The fish all breathed much more frequently than they did when in isolation, and all of these effects became more pronounced in aquatic hypoxia.

In summary, the effects of boldness¹⁰⁰ and aggression⁹⁹ are further evidence that higher-order cues can modulate air-breathing behaviours in fishes⁹⁰. At present, the findings are puzzling to interpret because they do not seem to have a simple link to chemo-receptor drive, apparently being either partially (boldness¹⁰⁰) or completely (aggression⁹⁹) independent of any drive from standard metabolic rate and intrinsic oxygen demand. More research is therefore required to understand the underlying drivers for these responses, and how they relate to the accepted physiological role of air-breathing, which is to assure or optimise oxygen supply for metabolism. It is conceivable that, once the neural circuits for air-breathing have evolved, the activity itself may become part of a repertoire of responses that are linked to ecological factors, such as life history strategy or intra-specific competition. It is possible that, although the drivers may be higher-order than chemoreceptor reflexes, the objective may still be 'physiological' if the air-breathing ensures high individual performance or provides a competitive edge, by maintaining plentiful oxygen stores⁸⁹.

5 | DEVELOPMENTAL PHENOTYPIC PLASTICITY IN AIR-BREATHING FISHES

The evolution of air-breathing in fishes allowed for the invasions of aquatic ecological niches with larger variation in environmental abiotic factors such as oxygen and carbon dioxide levels. Transits between habitats with such extreme conditions during their life histories

require both rapid physiological and behavioural responses initially (see sections 3 and 4) and may further stimulate plastic and long-lasting change in the phenotype. Phenotypic plasticity in response to altered environmental conditions has been thoroughly treated in a recent review on fishes including air-breathing fishes ⁴, and this section of our review addresses how early exposure to altered environmental conditions affects the adult phenotype in air-breathing fishes.

5.1 | Phenotypic Plasticity – The Developmental Context

Phenotypic plasticity – the potential for environmental signals to produce multiple phenotypes from a single genotype – is a venerable concept in biology and has been reviewed numerous times ¹⁰⁴⁻¹⁰⁷. Typically, such plasticity is viewed from the perspective of the mature adult animal. Less frequently are the effects of environmental stressors considered during the *development* of that animal. Yet, we know that developing animals pass through so-called ‘critical windows’ when their development can be significantly altered (indeed, sometimes fatally so) by an environmental stressor ^{4,107-114}. Phenotypic modification during development can affect multiple facets of phenotype – morphological, physiological, molecular, behavioural – as we will now explore for air-breathing fishes.

5.2 | Developmental Phenotypic Plasticity in Air-breathing Fishes

Investigating the effects of the environment, specifically in air-breathing fishes, has long been of interest to physiologists ^{1,2}. Yet, most studies, as for other taxa, have considered the effects and responses in adult air-breathing fishes. Study of the embryos and larvae of air-breathing fishes (indeed, almost all fishes) presents challenges because of their small size ¹¹⁵, but several studies have described developmental phenotypic plasticity in especially the

Anabantoidei. So, what *do* we know of developmental plasticity in embryos and larvae of air-breathing fishes?

5.2.1. Morphological Developmental Plasticity

The developmental morphology, especially of the respiratory organs, has been examined in only a few species of air-breathing fishes^{112,116-118}. While branchial morphology is quite plastic in aquatic larval fishes¹¹⁹, it has not been extensively studied in larval air-breathing fishes. Branchial developmental plasticity, in particular, has been probed primarily using aquatic or combined aquatic and aerial hypoxia (sometimes with only nocturnal hypoxia) as an environmental stressor, which is an ecologically relevant approach given that ambient hypoxia was likely one of the environmental drivers for the evolution of air-breathing^{1,2,4,113}.

In the larvae of *B. splendens*, there is a biphasic hypoxic response in body mass, with moderate hypoxic increasing, but severe hypoxia decreasing body mass. However, the closely related blue gourami *Trichopodus trichopterus* shows no significant body mass changes under these conditions¹¹⁶.

Up to the onset of air-breathing (day 35-38), *B. splendens* shows little to no increase in total lamellar surface area in moderate hypoxia, and an actual decrease in severe hypoxia (Fig. 3)¹¹⁶. The surface area of the emerging labyrinth is elevated in moderate hypoxia but is reduced back to control levels when rearing occurs in severe hypoxia. Again, a different suite of responses occurs in the closely related *T. trichopterus*, where neither moderate nor severe hypoxia leads to any changes in branchial or labyrinth surface area up to the onset of air-breathing. These differences in responses between these two anabantoid fishes likely result from differences in habitats and lifestyles - *T. trichopterus* is more active in a better oxygenated habitat, whereas *B. splendens* is more sedentary while inhabiting more hypoxic habitat. Collectively, the data for these two anabantoid species suggest that the environment

has a greater influence on developmental plasticity than the genetic closeness of the two species in these early larval stages. Interestingly, however, after a full 120 days of rearing of *T. trichopterus* in moderate hypoxia, the total lamellar surface area is 16% higher, and labyrinth area is 30% higher in juveniles compared to control populations¹¹⁷, suggesting species-specific differences in the timing of the first appearance of developmental plasticity.

In one of the more intriguing of studies on developmental, morphological plasticity, *P. senegalus* were raised in either aquatic or terrestrial environments, and the influence of terrestriality (the ‘stressor’) on swimming and walking behaviour as influenced by morphological changes in the pectoral girdles was assessed to probe how developmental plasticity might inform on the origin of tetrapods²⁹. Terrestrial upbringing resulted in significantly different shapes of the pectoral girdle and associated changes in terrestrial locomotion (briefer ground contact by fins and shorter stride durations), demonstrating plasticity in even the basic process of skeletal formation (see also section 2.2 on branchial remodelling in amphibious fishes).

5.2.2. Physiological Developmental Plasticity

The gills of air-breathing fishes undergo profound developmental changes associated with the rapid growth of the larvae¹²⁰. As for the morphological features discussed above, there is also evidence for developmental plasticity in physiological performance in larval air-breathing fishes. Whereas neither rates of heartbeat nor opercular pumping were affected by moderate or severe hypoxic rearing in *T. trichopterus* or *B. splendens*, there was an elevation of routine oxygen consumption by hypoxic rearing in the former species¹²¹. Additionally, the ambient partial pressure at which oxygen consumption is no longer maintained, P_{crit} , was decreased by hypoxic rearing in *B. splendens*, but increased in *T. trichopterus*, suggesting marked differences in hypoxia tolerance between the two closely related species.

The developmental transitions in ionoregulatory functions of air-breathing fishes have been documented, as has developmental plasticity in these features. The gills of aquatic larval fishes show changes in ion-transporter expression and density of mitochondrial-rich cells with changes in ambient ion concentrations ¹¹⁹. Presumably, similar plasticity occurs in larval air-breathing fishes, but this has yet to be quantified.

5.2.3 | Behavioural Developmental Plasticity

The air-breathing behaviour of larval *B. splendens* and *T. trichopterus* shows considerable developmental plasticity. The onset of air-breathing in *B. splendens* is modestly accelerated by 2-4 days by rearing in a PO₂ of 14 and 17 kPa compared with normoxia (20 kPa). In contrast, rearing in a PO₂ of 14 and 17 kPa *delays* the onset of air-breathing by 2-4 days in *T. trichopterus* ¹²² (Fig. 4).

As already described under morphological responses, the walking and swimming behaviour of juvenile *P. senegalus* is also quite plastic, being modified when reared in a more terrestrial setting.

5.2.4 | Biochemical/Molecular Developmental Plasticity

Relatively few studies have considered biochemical and molecular developmental plasticity in developing air-breathing fishes. In one of the few such studies, environmentally-induced adjustments in lactate dehydrogenase (LDH) isozyme expression were measured in larval snakeheads, *Channa punctatus* ¹²³ that use a suprabranchial organ for aerial gas-exchange. During the normal transition to bimodal breathing in snakehead larvae (typically 15 days after hatching) LDH-A is induced while LDH-B is down-regulated. If denied access to air, strictly water breathing larvae showed a transient increase in protein abundance of LDH-B over LDH-A in whole animal homogenates, a reversal of the normal developmental sequence

of events. LDH-A levels were correlated with air-deprivation tolerance and scaling of muscle mass during growth ¹²³. However, the functional (*physiological*) implications of these changes in LDH isozyme levels have still to be determined.

The nutritional state was used to explore developmental plasticity in enzymes involved in the urea-generating pathways during early development in larvae of *C. gariepinus* ¹²⁴. Starvation increased the activity of the enzyme uricase, but decreased activities of CPSase III and ornithine carbamoyltransferase, interfering with the normal developmental transition from urea to ammonia excretion.

Exposure of 15-day-old larval walking catfish, *Clarias batrachus*, to ambient ammonium chloride created a significant increase in activity of glutamine synthetase and arginosuccinate lyase, as well as a significantly increased concentration of glutamine synthetase and CPSase III ¹²⁵. These data suggest that ureogenesis can be stimulated early in the life cycle of this species.

Larval fishes appear to be quite plastic in their development, especially when changes occur during their critical windows for development ¹¹⁹. Larval air-breathing fishes are similarly plastic, showing modifications in morphology, physiology, behaviour, and biochemistry/molecular biology when challenged by pervasive environmental stressors. Yet, our knowledge of developmental plasticity in larval air-breathing fishes remains enigmatic. Moreover, the findings to date are dominated by only a few, quite specialised species. Yet, air-breathing has independently evolved multiple times (section 6), employing a wide range of sometimes highly modified organs to breathe air. Thus, there is likely to be an equally wide range of plasticity between species, as hinted at by the differences in morphological and physiological plasticity evident between two closely related anabantoids, *T. trichopterus* and

B. splendens. Future studies comparing the degree of plasticity between uni- and bi-modally breathing larval fishes will be particularly interesting, and possibly provide additional insights into the evolution of air-breathing in vertebrates.

6 | EVOLUTION OF AIR-BREATHING AND AMPHIBIOUS FISHES

Much is known about the physiological mechanisms associated with the water to air transition, such as modifications in the cardiovascular anatomy, gill morphology, ventilatory regulation, and blood's respiratory properties^{2-4,6,7}. As evident from the text above, the vast majority of studies on air-breathing fish physiology have been performed on individual species, which has illuminated the possible physiological mechanisms associated with the evolution of air-breathing and terrestriality in early vertebrates. However, next to nothing is known about the evolutionary dynamics in air-breathing and amphibious lifestyles within vertebrates, which may provide additional insight into how gas-exchange mechanisms evolved over time and inform about selective pressures that have shaped the physiology of extant animals. The most recent analysis on the evolution of air-breathing² used a fish phylogeny from 1983 and suggested that air-breathing had evolved between 34 and 67 times independently. Similarly, an amphibious lifestyle is thought to have evolved multiple times independently within the bony fishes⁴ (Osteichthyes including ray-finned fishes and lobe-finned fishes, where the latter includes tetrapods, lungfishes and coelacanths), both in the lineage leading to tetrapods but also multiple times within the ray-finned fishes (see Section 2). Within the last decade, well-resolved, time-calibrated phylogenies have been developed that allow for re-evaluating the number of independent origins of air-breathing and amphibious lifestyle⁸. Further, we can test in which geological eras these traits evolved, and whether they were secondarily lost. These are questions that have not been addressed

previously, and which may provide novel insight into evolutionary plasticity in the physiological traits associated with air-breathing and terrestriality.

This section re-evaluates the evolution of air-breathing and amphibious lifestyles.

This was done to reassess the number of independent origins (and losses) of these traits, and the timing of those events. We accomplished this by updating the list of air-breathing fish species (Data Set 1), assigned all other genera as water-breathers by default, and applied modern phylogenetic comparative methods to the data set^{126,127} using a complete, fully resolved, time-calibrated fish phylogeny, which included all bony fish genera, except the tetrapods, and using eight species of elasmobranchs and jaw-less vertebrates as outgroup^{8,128} (see Supporting Material for a detailed description of the evolutionary analysis and its limitations). The model is deposited online, so a future updated list of air-breathing and amphibious fish can be analysed automatically. We discuss how this renewed model on air-breathing evolution may inform new detailed studies in evolutionary and cardiorespiratory physiology.

6.1 | Origin of air-breathing

The first evolutionary origin of air-breathing has puzzled zoologists for centuries. It has been suggested that air-breathing was present in early vertebrates by virtue of a lung, which was lost in the branch leading to cartilaginous fishes, and hence, air-breathing may have represented the ancestral condition in the most basal bony fishes^{1,2,113,129}. The single origin of the lung is supported by the presence of conserved elements in the molecular pathways regulating the development of the lung of bichirs (the most basal clade of ray-finned fishes) and lungfishes¹¹². Yet, other aspects of the pulmonary ontogeny suggest independent origins of the lung in bichirs and lungfishes¹¹³.

To model the evolution of air-breathing in fishes, we used an evolutionary model that simulated the evolution of air-breathing 100 times on 100 versions of the fish phylogeny (see supplementary information for full details). Given the diversity of extant air-breathing fish species and the topology of the fish tree of life, we inferred the probability of specific respiratory structures (such as distinct air-breathing organs in air-breathing fishes or gills in water-breathing fishes) being present or absent in all internal branches in the bony fish phylogeny (excluding tetrapods; Figs. 5, 6, S1, Interactive Fig. S1). Here, we used five categories for air-breathing organ (*lung*, *swimbladder*, *mouth* (for species that use any part of the buccal cavity for aerial gas-exchange), *skin*, and *gastro-intestinal tract* (for species that use any part from the esophagus to the anus for aerial gas-exchange)). This analysis predicts that the most recent common ancestor of bony fishes was a water-breather (probability = 99.8%) and predicts convergent evolution of the lung in the bichirs and sarcopterygian lineages (Fig. 7).

This model further supports that species in the line of descent connecting the last recent ancestors of bony fishes and teleosts retained water-breathing, and that air-breathing evolved independently in the radiation leading to extant bowfin and gars (Holostei) and multiple times within the teleosts using numerous respiratory structures (Fig. 7). Our analysis of extant vertebrates predicts that air-breathing evolved from a water-breathing ancestor 82 times, using a lung two times, a swimbladder 19 times, the mouth 13 times, the skin 25 times, and the gastrointestinal tract 23 times (see Figs. 6A, S2 for confidence intervals of these estimates). This total number (82) is significantly higher than the previous estimate of 34-67 *de novo* origins² and it is a result of a longer list of air-breathing fish species, and of air-breathing species previously thought to be monophyletic that have evolved air-breathing in multiple separate events.

While considerable effort has been devoted to identifying the time of origin of the tetrapod lung^{2,113}, nothing is known about when the air-breathing within the ray-finned fishes evolved. Our incorporation of a time-calibrated phylogeny allows for estimating when in geological time air-breathing evolved. The model shows that extant air-breathing lineages are relatively recently and with 89.6% of these originated within the last 65 million years. This period was characterised by a relatively stable atmospheric PO₂¹³⁰, and declining atmospheric carbon dioxide content and temperatures, suggesting that it is unlikely that global hypercapnia, hypoxia or high temperature explain the timing of air-breathing evolution. Hence, it is more likely that other factors may have been key for air-breathing evolution, such as local environmental hypoxia and hypercapnia^{2,131}, advantages of improved oxygenation of the heart¹³², improved resistance to environmental toxicants¹³³⁻¹³⁵ etc. and have probably differed among groups of fishes with different life history and physiological background.

6.2 | Secondary losses of air-breathing

While it is accepted that air-breathing evolved multiple times independently, it has never been tested if air-breathing was *lost* in ray-finned fishes since complete phylogenetic relationships were not developed. Our implementation of a phylogeny including all genera of fishes - both water- and air-breathers - suggest that air-breathing has been secondarily lost 4 times within bony fishes from an aquatic air-breathing ancestor (Fig. 6A, S2). We were able to identify the phylogenetic position of several of these losses, including the loss of a respiratory swim bladder in a common ancestor of Mormyridae (Osteoglossiformes), loss of a respiratory gastro-intestinal tract within siluriform fishes, and loss of a respiratory buccopharyngeal cavity in Indostomidae (Synbranchiformes) (Figs. 5, S1, Interactive Fig. S1).

Air-breathing appears to offer a variety of physiological advantages compared to water-breathing species, particularly for the exploitation of hypoxia/hypercapnic aquatic water bodies and terrestrial habitats, as well as tolerance of periods of drought, so the secondary losses of air-breathing could be viewed as strikingly maladaptive. One possibility for the loss of air-breathing may be due to drift after the invasion of more oxygenated / deeper waters, such that water-breathing subsequently became evolutionarily fixed. It may even be that there has been negative selective pressure on air-breathing capabilities, such as through functional trade-offs between gas-exchange and nutrient absorption in fishes that use parts of their gastrointestinal tract for air-breathing, trade-offs between gas-exchange and protective function of the skin in fishes with cutaneous aerial respiration, spatial constraints between air-breathing organ volume and egg fecundity, aerial predation ⁸⁹, increased metabolic costs associated with air-breathing ⁸⁰, or through cardiac constraints imposed by the very high haematocrit typical of most air-breathing fishes ³.

To test if losses of air-breathing was a general phenomenon across air-breathing fishes, we estimated within the same model the rates that air-breathing has originated and has been lost, as well as the speciation and extinction rates among water- and air-breathing fishes (Fig. 8; see supplementary material for details). This model showed that the speciation rate in air-breathing fishes is 4.5 times lower than in water-breathing fishes ($P < 0.001$) and that the rate by which air-breathing was lost is 5.2 times higher than the rates at which air-breathing evolved ($P < 0.001$). These findings further support the notion that air-breathing brings physiological trade-offs that may select against this trait in species where they are not strictly required.

6.3 | Evolutionary dynamics of air-breathing fishes

Our phylogenetic analyses show that the evolution of air-breathing has been much more dynamic compared to previous assessments². This evolutionary plasticity is reflected in a higher number of independent origins of air-breathing organs, but especially by the identification of secondary transitions into water-breathing. The line of descent connecting the last common ancestor of bony fishes and elephantfish, *Brienomyrus niger*, even provides an example of secondary origin of air-breathing among fishes (Fig. 6C): The last common ancestor of bony fishes was most likely water-breathing, whereas air-breathing in a swimbladder evolved in the early branches of osteoglossiformes around 100 million years ago. This respiratory swimbladder was secondarily lost in the radiation leading to elephantfishes (Mormyridae) around 70 million years ago, and then air-breathing recently reappeared in the branch leading to *B. niger* using the mouth as an air-breathing organ (Fig. 6C). This secondary origin of air-breathing is supported by the lack of air-breathing in other mormyrids¹³⁶ and by the adoption of two distinct respiratory structures for air-breathing organs in this evolutionary trajectory.

Most of the origins of air-breathing among the extant fishes appear to have been very recent. This temporal heterogeneity shows that extant air-breathing lineages are young but, since the extant species only represent a fraction of the pre-existing biodiversity of fishes, air-breathing has most likely been gained and lost continuously throughout vertebrate evolution. Hence, the number of gains and losses of air-breathing among all extinct and extant species is expected to greatly exceed our estimation based upon extant species alone.

This model shows that air-breathing evolution in ray-finned fishes is in contrast to that of lobe-finned fishes; air-breathing in lobe-finned fishes occurred via a single origin of the lung around 325 million years ago, whereas air-breathing within the ray-finned fishes occurred very recently on a series of occasions (Fig. 6B). The reason for these temporal and numerical differences likely stem from the lung evolving early in lobe-finned fish evolution,

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followed by terrestrial invasion and loss of the gill in the tetrapods. Subsequent back-transitions to sole water-breathing within this group must have been impossible, as the gills were absent, and hence no respiratory structure was available to support aquatic gas-exchange, and air-breathing became evolutionarily fixed within the tetrapods. In the ray-finned fishes, air-breathing evolved much later after the huge diversification and invasions of a range of habitats, so there was a much higher number of species from which air-breathing could evolve.

This model is based solely on the current state of knowledge on air-breathing fish diversity and, hence, the outcome may change with the further identification of new species of air-breathing fishes. For this reason, we have deposited the source code for the analysis on GitHub allowing for automatically repeating the entire analysis and re-answering all questions raised in this section if new air-breathing fishes are identified.

6.4 | Evolutionary dynamics of amphibiousness in fishes

Amphibious behaviour has been suggested to have evolved multiple times independently within vertebrates⁴, which is strongly supported by applying the evolutionary modelling framework from above to a previously published list of amphibious fishes. This analysis showed that amphibious behaviour is indeed a trait that has evolved convergently multiple times within the bony fishes (Fig. 9). Among the extant species of bony fishes, amphibiousness appears to have originated 87 times independently, and most of these transitions appear to have occurred within the last 65 million year. Hence, the extraordinary evolutionary plasticity observed in breathing-mode (water- versus air-breathing) was also observed for an amphibious lifestyle.

7 | TOWARD AN UNDERSTANDING OF AIR-BREATHING EVOLUTION ACROSS TIMESCALES

The evolution of air-breathing and subsequent invasion of land is one of the most iconic transitions in animal evolution and has inspired research within comparative physiology and evolutionary biology for over a century. We suggest that an additional in-depth understanding of the general physiological shifts associated with the evolution of air-breathing and terrestrial invasions can be gained by combining model-organism and model-clade approaches. A classical model-organism approach is essential for illuminating specific physiological mechanisms and for investigating how physiological, morphological, and behavioural plasticity in air-breathing are affected by biotic and abiotic factors on time scales of the individual. These can include developmental studies to illuminate the physiological, morphological, and behavioural changes associated with the transition from a water-breathing larva to an air-breathing adult. Likewise, they can include acclimation of adult air-breathing fishes to altered environmental conditions to characterise physiological and behavioural responses during short exposures and their phenotypic plasticity during longer exposures. Such model-species approaches are essential for understanding the physiological basis for air-breathing and for understanding how phenotypic plasticity in response to environmental conditions may facilitate the evolution of fixed traits associated air-breathing.

In combination with model-species studies, a “model-clade” approach may provide an additional macroevolutionary dimension to understanding the physiological changes associated with air-breathing evolution. Here, individual monophyletic groups containing both uni- and bimodally breathing fishes can be systematically compared, which allows for detailed evolutionary reconstructions of how physiological, morphological and behavioural traits have evolved over time in the lines of descent connecting water-breathing ancestors to extant air-breathers (Fig. 10). Biochemical and soft tissue structures are rarely preserved in

the fossil record, and hence phylogenetic reconstruction provides the safest strategy available to infer the physiological transitions associated with air-breathing by systematically investigating closely related water- and air-breathing species and analysing data in a phylogenetic comparative framework. Such reconstructions can now use time-calibrated phylogenies, which may further inform about the time spans required for the evolution of adaptive physiological mechanisms associated with air-breathing. Combining the multispecies comparisons with phenotypic responses to environmental change in model-species provides an opportunity to test how phenotypic responses within an individual can resemble macroevolutionary patterns of physiological, morphological, and behavioural change.

The vast majority of studies on air-breathing evolution has been performed using model-species approaches similar to those described above, while very few studies have adopted a model-clade approach to reconstruct the evolutionary changes in respiratory function during water-to-air transitions in fishes, because the phylogenetic tools have not been available. Hence, we suggest that future studies should combine model-organism with model clade approaches to gain a more comprehensive understanding of air-breathing evolution across temporal regimes. Further, we emphasise the importance of using groups of air-breathing ray-finned fishes as model organisms/clades for air-breathing evolution, since these groups have all evolved air-breathing very recently compared to the evolution of the lung in lobe-finned fishes several hundred million years ago. Hence, ray-finned fishes can provide a better understanding of the initial adaptations associated with air-breathing evolution and they allow to test whether different clades of fishes used distinct physiological and morphological adaptations to solve the same physiological challenges associated with air-breathing and terrestriality. Lastly, ray-finned fishes that have secondarily lost air-breathing offer an opportunity to test the physiological basis for air-to-water transition within

vertebrates, and to test further if physiological traits associated with air-breathing are reversible during evolutionary back-transitions.

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CONFLICT OF INTEREST

There is no conflict of interest in this work.

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LEGENDS TO FIGURES

Figure 1 Phenotypic modifications associated with life out of water. During terrestrial excursions, the shift in respiratory medium from water to air affects the exchange of gasses with the environment, including internal accumulation of carbon dioxide (CO₂), cutaneous water loss, and cutaneous angiogenesis facilitating aerial gas-exchange. Further the increase in effective gravity in air collapses the gills affecting gill function and ammonia excretion, which is associated with changes in the cutaneous ionocyte distribution, stiffening of the gills, and volatilisation of ammonia (NH₃) across the skin. Fish illustration by Ian Smith (University of Guelph, Canada).

Figure 2 Diagrammatic representation of the possible mechanisms controlling breathing and the associated changes in heart rate in the lungfish (the size of the brain is exaggerated, and the mechanisms illustrated are derived from work on a range of species and could be applied to a similar range). The reduced gills are ventilated by jaw muscles and intrinsic muscles in the gill arches (not illustrated) innervated by the Vth, VIIth, IXth and Xth cranial nerves (only the Vth and Xth are illustrated). Surface air-breathing utilises both jaw and hypobranchial muscles that are separately innervated (the latter by the green - occipital and spinal nuclei). Central co-ordination of their activity may be achieved by independent inputs from a respiratory rhythm generator in the brainstem, attributed here to the reticular formation and the nucleus of the Vth cranial nerve. The lungs and heart are supplied by the Xth cranial nerve with both sensory (blue fibres - from the afferent nucleus) and motor (red fibres - from the efferent nuclei) innervation. The heart receives efferent innervation from cardiac vagal preganglionic neurons located in separate nuclei (labelled medial and lateral) within the brainstem that may subserve separate control functions. The schematic transverse section through the brainstem indicates possible interactions between nuclei responsible for

generating and controlling breathing and cardio-respiratory interactions. More details of these interactions are given in the text. *Lepidosiren* silhouette was modified from an original silhouette made by Roberto Díaz Sibaja.

Figure 3 Respiratory surface areas after rearing in control, moderate or severe nocturnal hypoxia until the onset of air-breathing (38 days in *Betta splendens*, 35 days in *Trichopodus trichopterus*). Hypoxic rearing reveals developmental plasticity in *B. splendens* but not in *T. trichogaster*, in which hypoxia-induced developmental changes occur in juveniles. From Mendez-Sanchez and Burggren (2019) ¹¹⁶.

Figure 4 Changes in the onset of air-breathing in larval *Trichopodus trichopterus* and *Betta splendens* as affected by rearing oxygen levels. Larval *T. trichopterus* and *B. splendens* reared in total normoxia (aquatic and aerial) began air-breathing at 34- and 38-days post-fertilisation, respectively. Total hypoxic rearing delayed the onset of air-breathing in *T. trichopterus*, to 39 days post-fertilisation ($PO_2 = 17$ kPa) and 40 days post-fertilisation ($PO_2 = 14$ kPa). However, hypoxic rearing actually accelerated the onset of air-breathing in *B. splendens* to 33 days post-fertilisation at the lowest rearing oxygen level ($PO_2 = 14$ kPa). The day of air-breathing onset was determined as the day when 50% of the population was air-breathing (*i.e.*, no error bars). After Mendez-Sanchez and Burggren (2014) ¹²².

Figure 5 Evolution of breathing mode traced across the bony fish tree of life. Ancestral state reconstruction of air-breathing, where branch colours depict the probabilities of water-breathing (bluer) or air-breathing (redder). Circles indicate branches in which air-breathing evolved (red) and was lost (blue). The phylogeny included all genera of bony fishes excluding tetrapods and using eight genera of agnathans and elasmobranchs as outgroup. The time from the centre to periphery is 615 million years with dashed circles for each 100-million-year period. See Supporting Material for an annotated high-resolution version of the

figure with genus, family and order names (Fig. S1 + Interactive Fig. S1), detailed model description, and computer-code to run the model.

Figure 6 Evolution of air-breathing in bony fishes. Air-breathing evolution was simulated 100 times on 100 versions of the phylogeny, and the number of gains and losses of air-breathing in those 10,000 simulations were summarised. The phylogeny included all genera of bony fishes excluding tetrapods and using eight genera of agnathans and elasmobranchs as outgroup. This analysis shows that air-breathing (red) evolved 82 times independently and was secondarily lost (blue) 4 times (A). On each of the 100 trees, the timing of transitions to water- and air-breathing (blue and red, respectively) were identified and the time of transition from all 100 trees was summarised in a density plot showing that most origins and losses of air-breathing occurred within the last 100 million years (B). Evolution of air-breathing in the line of descent connecting the last common ancestor of bony fishes and the elephant fish, *Brienomyrus niger*, illustrating an example of a secondary origin of air-breathing (C). The graph depicts the Bayesian posterior probability of air-breathing in all the internal branches in the phylogeny connecting the last common ancestor of bony fishes and elephant fish.

Figure 7 The evolution of air-breathing in early fishes. Pie charts at the most recent common ancestors (MRCA) of major fish radiations represents the probability for water- (blue) and air-breathing (red) summarised from Fig. 5. This analysis shows that water-breathing was most likely the ancestral state for bony fishes, and that air-breathing evolved independently in lungfishes, Polypteriformes, holostei, and within later within the teleosts.

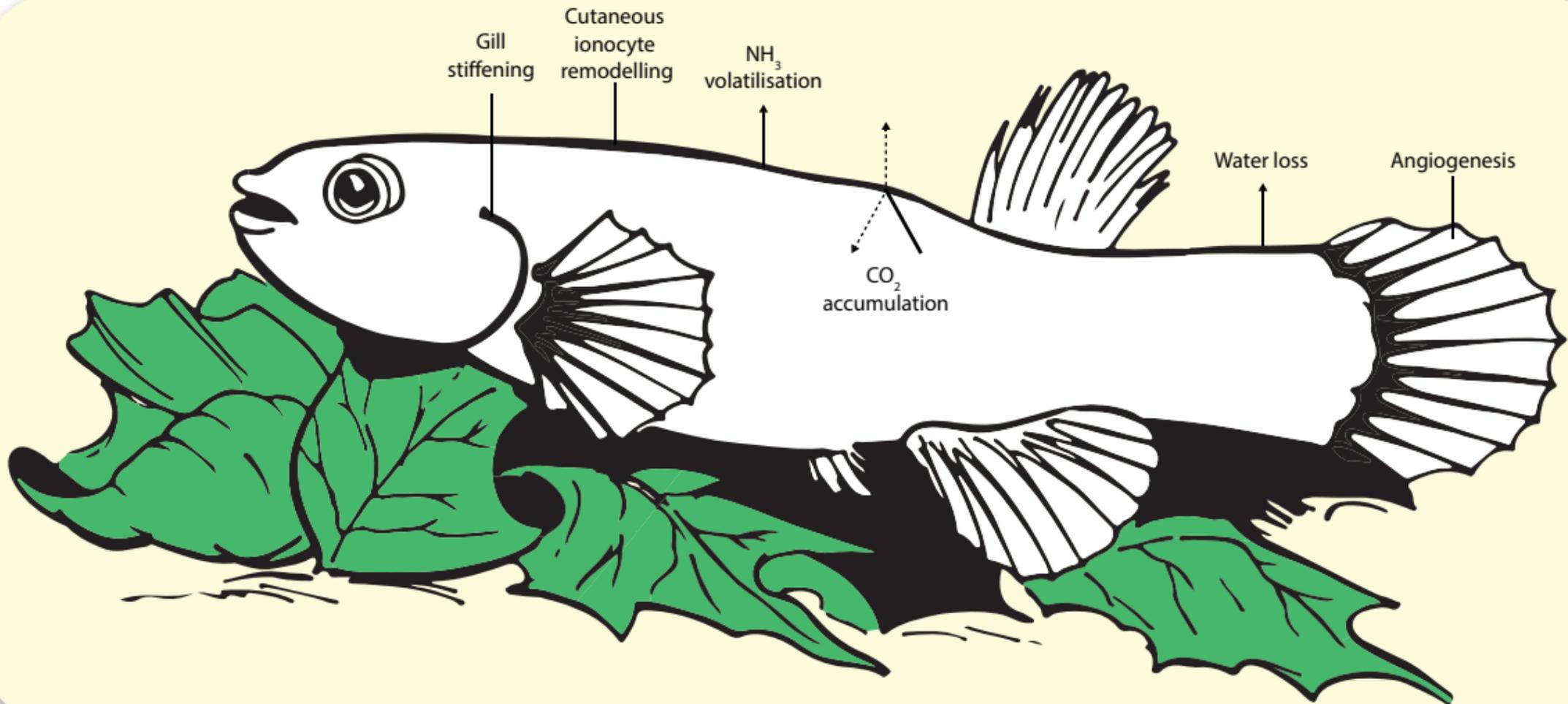
Figure 8 Evolution and diversification of air- and water-breathing fishes. The rates of transitions between water- and air-breathing, as well as speciation and extinction rates of water- and air-breathing fishes was found using a Binary State Speciation and Extinction

model and maximum likelihood. The figure shows that speciation rate was 4.5 times higher for water- compared to air-breathers ($P < 0.001$ and indicated by thicker lines), extinction rates were identical, and the rate of loss of air-breathing was 5.2 times higher than the rate of air-breathing origin ($P < 0.001$ and indicated by thicker lines).

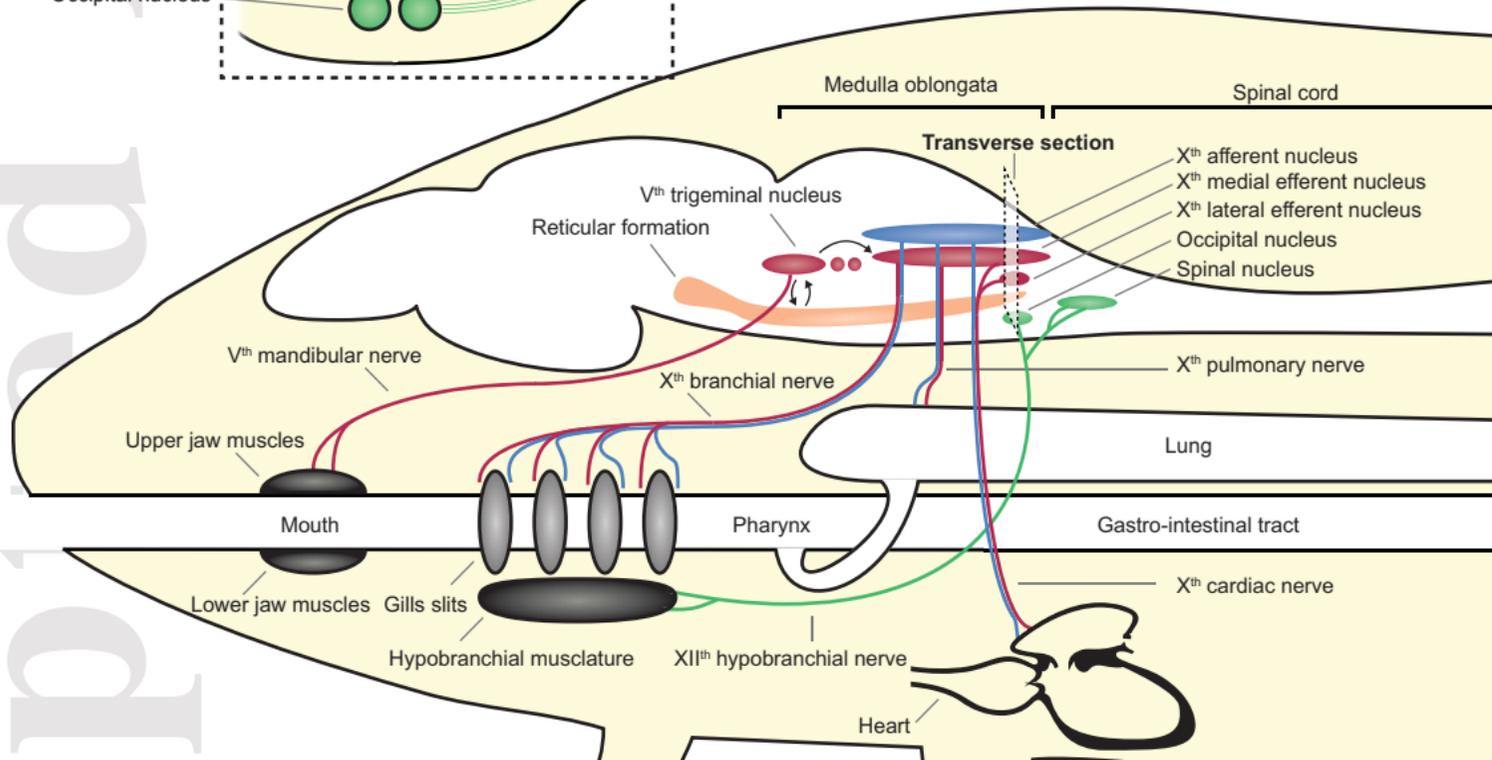
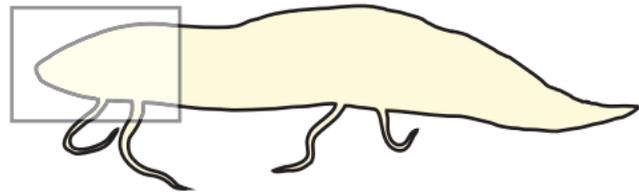
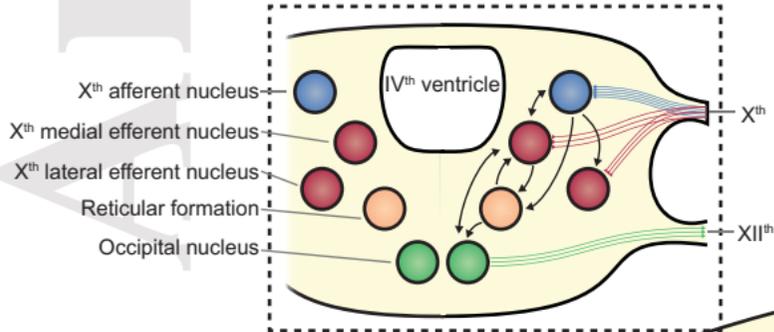
Figure 9 The evolution of amphibious lifestyle traced across the bony fish tree of life.

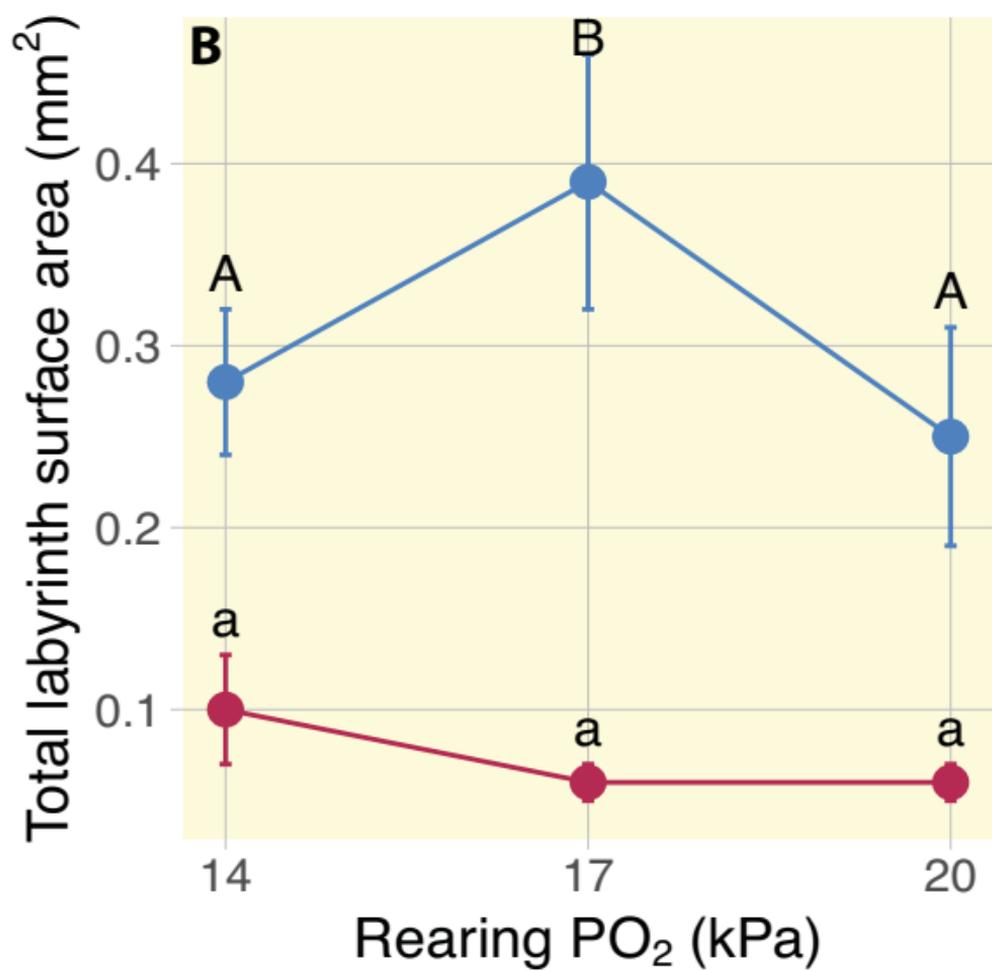
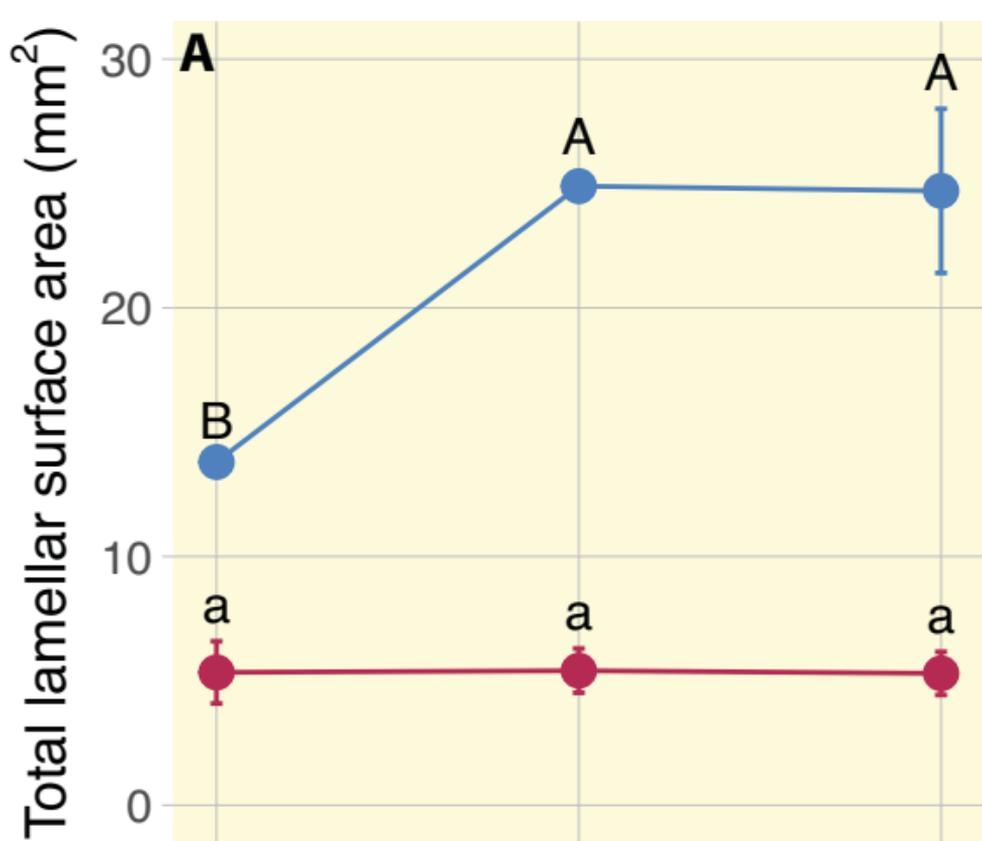
Lifestyle (aquatic or amphibious) was simulated across a complete genus-level phylogeny of the bony fishes (excluding the tetrapods) as in Fig. 5 using the list of amphibious fishes from ⁴. The probability for specific lifestyles in internal branches of the tree is colour coded with bluer (or redder) branches indicating a higher probability for aquatic (or amphibious) lifestyle. Red circles indicate branches where amphibious lifestyle had evolved. The time from the centre to periphery is 615 million years with dashed circles for each 100-million-year period. See Supporting Material for an annotated high-resolution version of the figure with genus, family, and order names (Fig. S3).

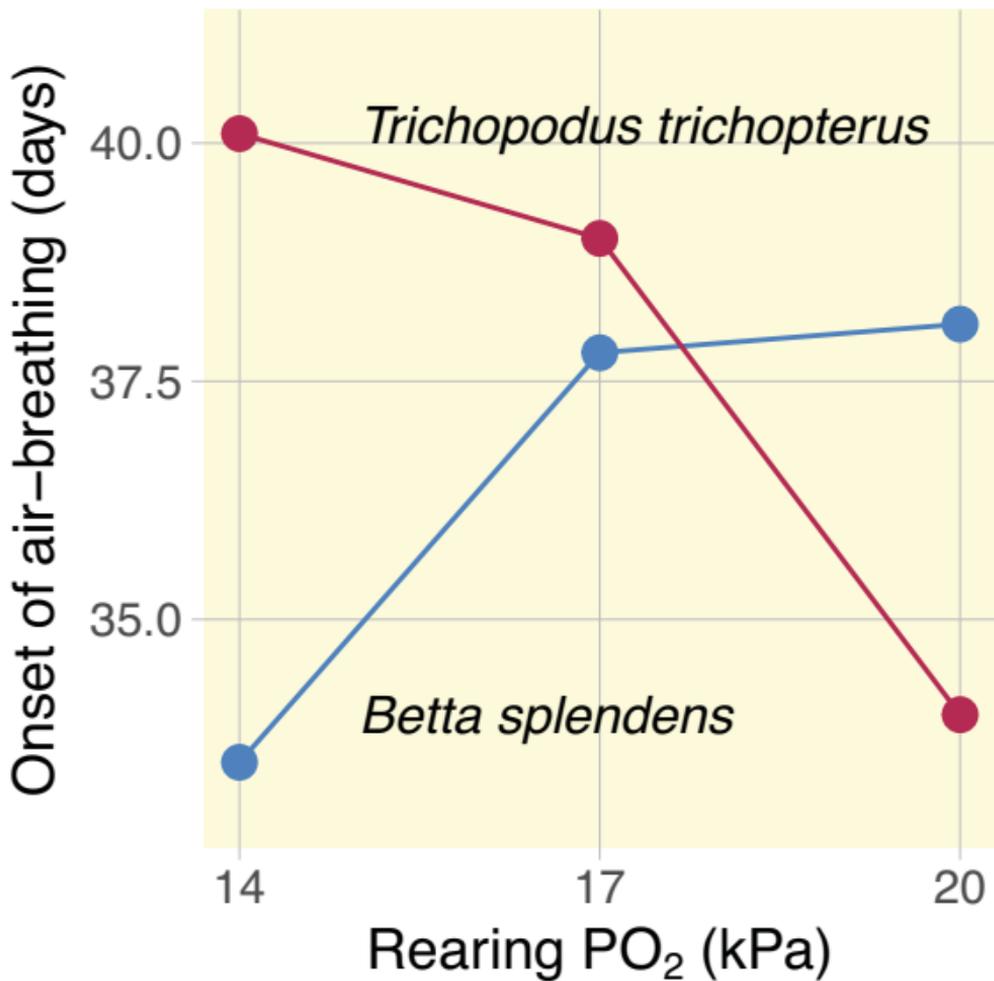
Figure 10 Model-clade approach to understanding functional transitions during evolutionary origins of air-breathing. Measuring functional traits in closely related water- (blue) and air-breathing (red) species allows reconstruction of the evolutionary changes in physiology and anatomy associated with air-breathing evolution.

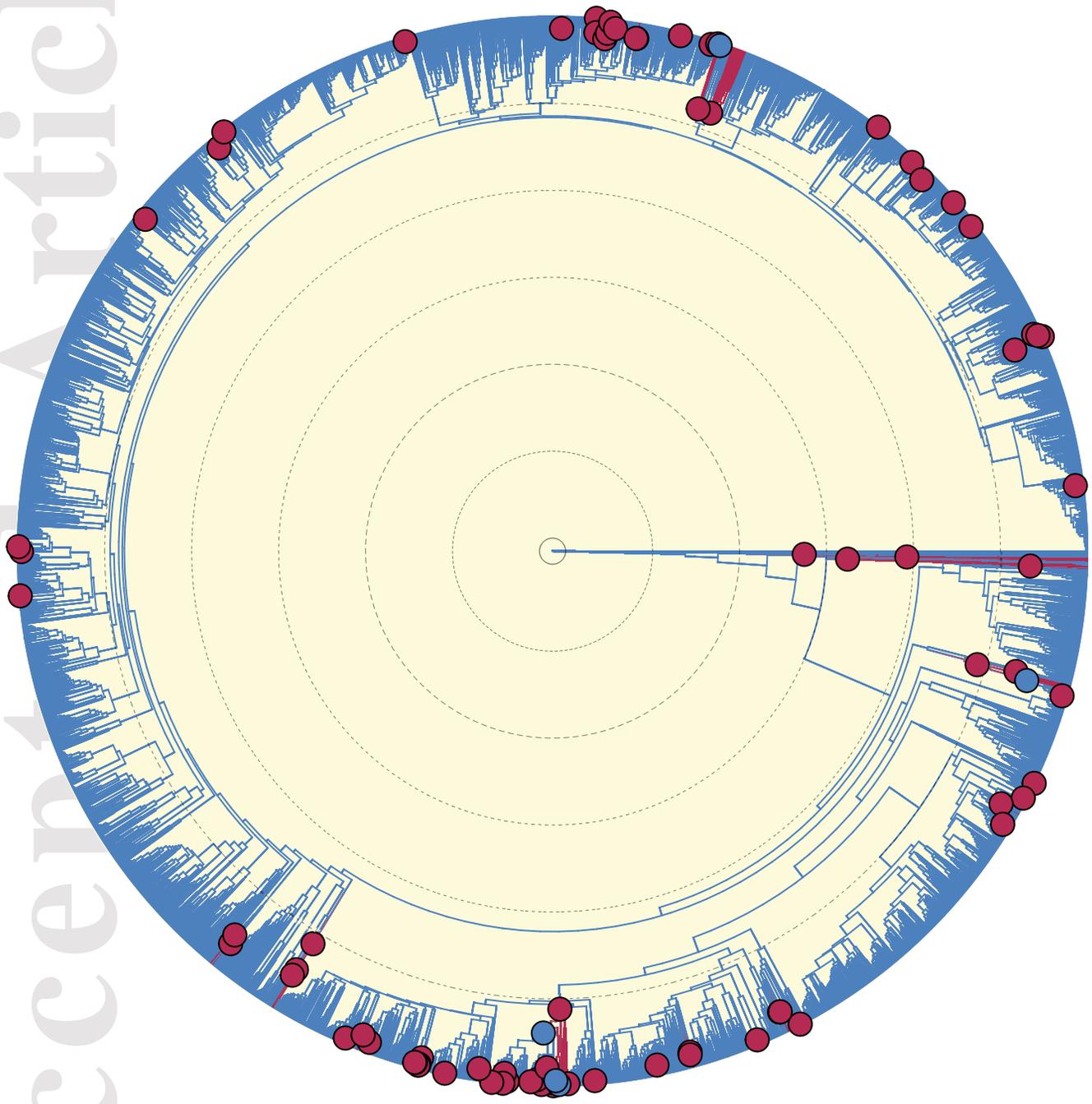


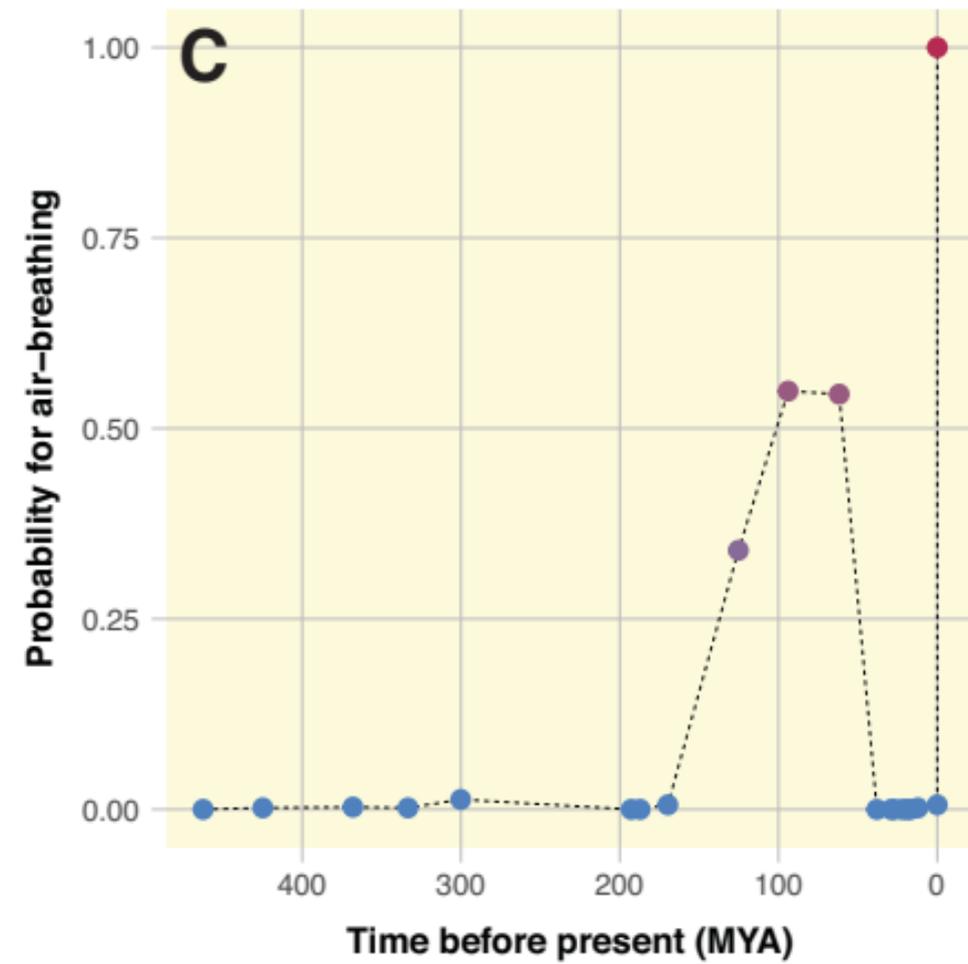
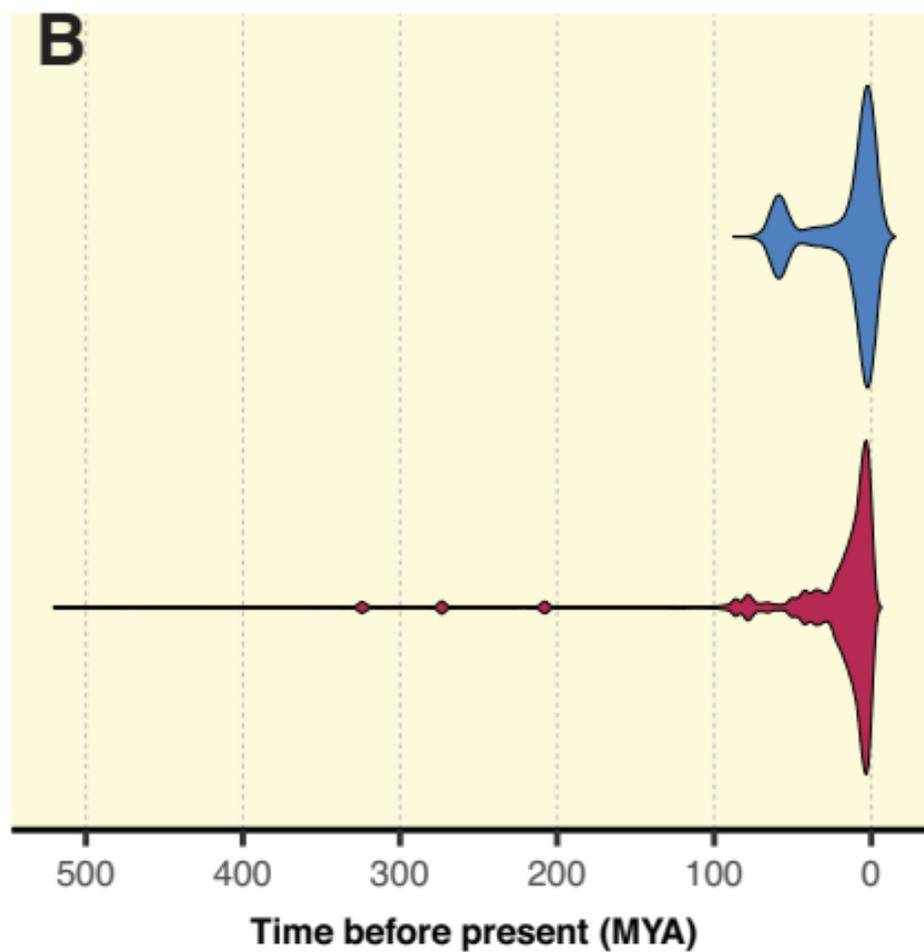
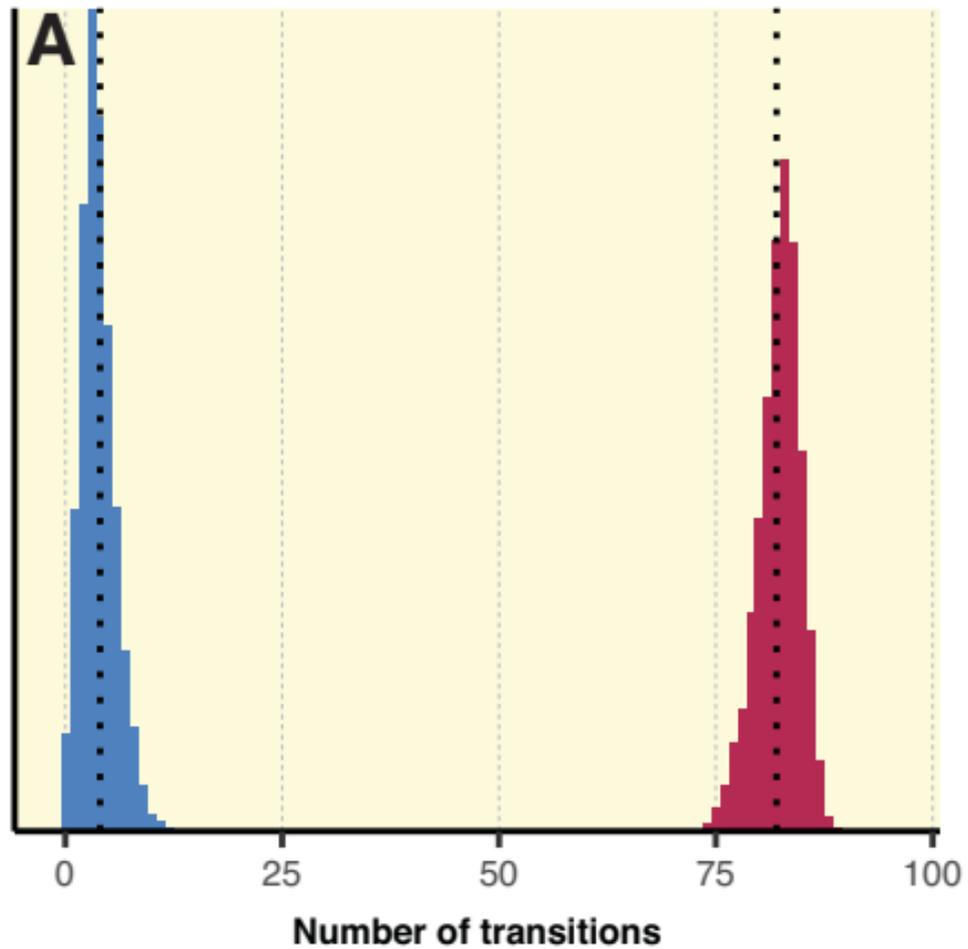
Transverse section

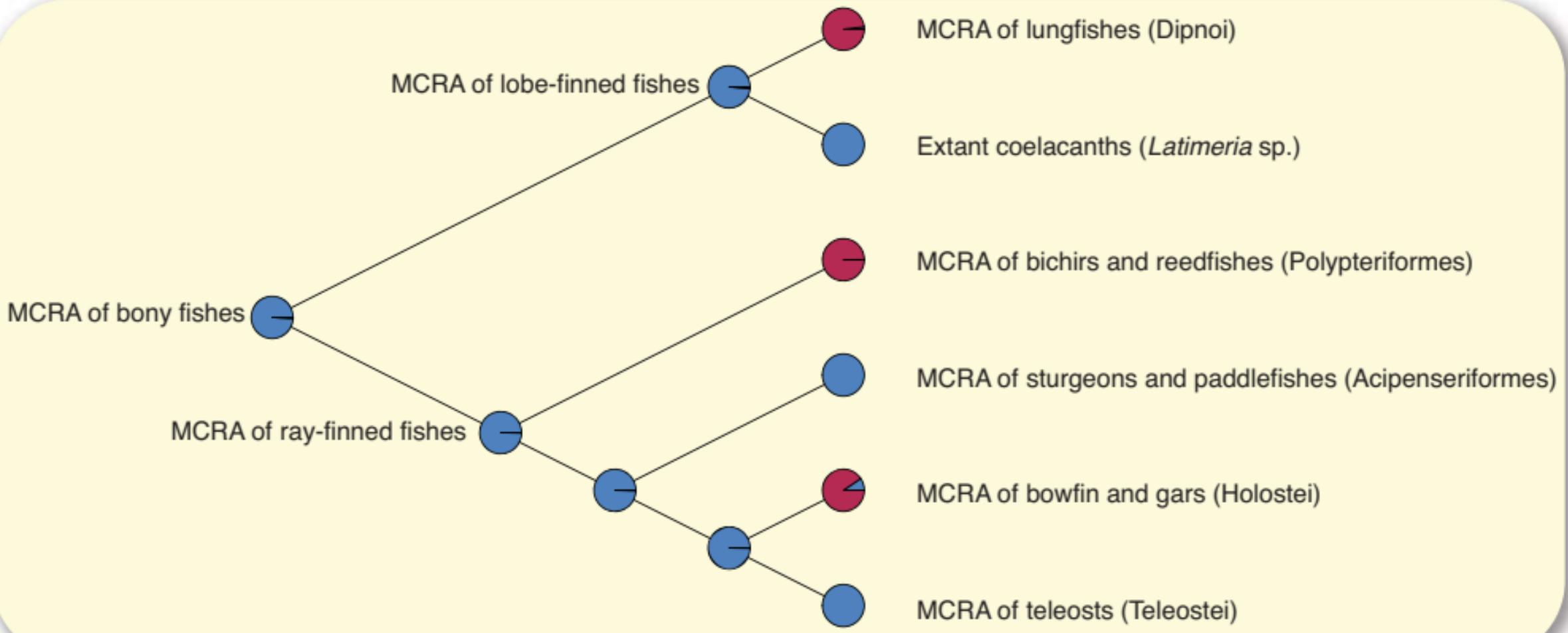












MCRA of lungfishes (Dipnoi)

MCRA of lobe-finned fishes

Extant coelacanths (*Latimeria* sp.)

MCRA of bony fishes

MCRA of bichirs and reedfishes (Polypteriformes)

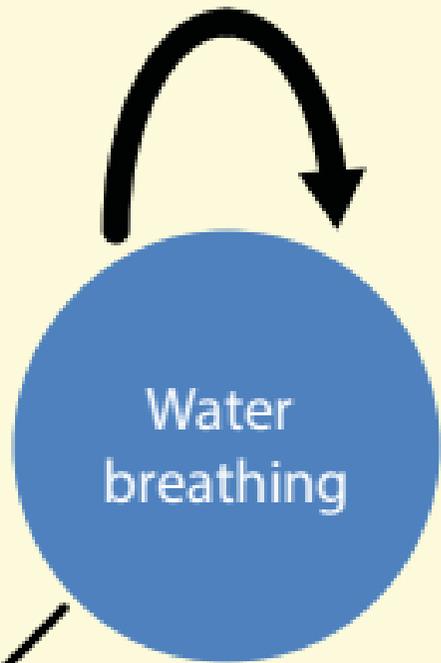
MCRA of sturgeons and paddlefishes (Acipenseriformes)

MCRA of ray-finned fishes

MCRA of bowfin and gars (Holostei)

MCRA of teleosts (Teleostei)

speciation



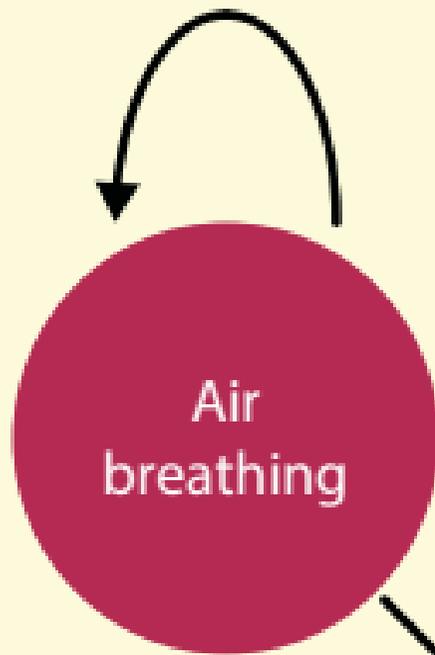
extinction

loss of air-breathing



origin of air-breathing

speciation



extinction

