

REVIEW PAPER

Evolution, ecology and physiology of amphibious killifishes (Cyprinodontiformes)

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The order Cyprinodontiformes contains an exceptional diversity of amphibious taxa, including at least 34 species from six families. These cyprinodontiforms often inhabit intertidal or ephemeral habitats characterized by low dissolved oxygen or otherwise poor water quality, conditions that have been hypothesized to drive the evolution of terrestriality. Most of the amphibious species are found in the Rivulidae, Nothobranchiidae and Fundulidae. It is currently unclear whether the pattern of amphibiousness observed in the Cyprinodontiformes is the result of repeated, independent evolutions, or stems from an amphibious common ancestor. Amphibious cyprinodontiforms leave water for a variety of reasons: some species emerge only briefly, to escape predation or capture prey, while others occupy ephemeral habitats by living for months at a time out of water. Fishes able to tolerate months of emersion must maintain respiratory gas exchange, nitrogen excretion and water and salt balance, but to date knowledge of the mechanisms that facilitate homeostasis on land is largely restricted to model species. This review synthesizes the available literature describing amphibious lifestyles in cyprinodontiforms, compares the behavioural and physiological strategies used to exploit the terrestrial environment and suggests directions and ideas for future research.

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Key words: *Fundulus*; *Kryptolebias*; mangrove rivulus; mummichog; terrestriality.

INTRODUCTION

The earliest terrestrial vertebrates were not only able to exploit new ecological opportunities on land, but also faced many challenges (Clack, 2002). Fossils of early tetrapods have provided insight into the morphological adaptations required for the invasion of land, but provide less information regarding the behavioural and physiological adaptations that are necessary. Extant amphibious fishes, despite their relatively distant phylogenetic relationship to modern tetrapods, have probably faced many of the same selection pressures and thus provide another excellent system for understanding the evolution of terrestriality (Graham & Lee, 2004). For the purpose of this review, amphibious fishes are defined as species that are found out of water as part of their normal life history (Gordon *et al.*, 1969; Sayer, 2005). This broad definition includes fishes

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that leave water (emerge) for brief intervals (seconds to minutes) as well as those that remain in terrestrial environments for prolonged periods of days, weeks or even months.

Amphibious fishes are able to exploit a diversity of resources and habitats not available to fully aquatic relatives (Sayer & Davenport, 1991; Graham, 1997; Sayer, 2005). Some species of amphibious cyprinodontiforms are able to escape predation and interspecific competition by moving overland to ephemeral pools of water not available to most fishes, while other species leave the water to snatch terrestrial prey or avoid deteriorating water conditions. These fishes must be able to navigate terrestrial habitats while avoiding water loss and managing gas exchange, osmotic balance and nitrogenous waste excretion. The goals of this review are thus to summarize what is known about amphibious lifestyles in cyprinodontiforms, compare the strategies used by the various amphibious species for exploiting the terrestrial environment and propose ideas and directions for future research. Although the basic biology of many of these fishes is poorly known (Costa *et al.*, 2010), two amphibious cyprinodontiforms, the mummichog *Fundulus heteroclitus* (L. 1766) and mangrove rivulus *Kryptolebias marmoratus* (Poey 1880), have emerged as popular model organisms for research (Burnett *et al.*, 2007; Earley *et al.*, 2012; Wright, 2012). These two species will be highlighted in this review.

DIVERSITY AND EVOLUTION

The order Cyprinodontiformes is large (c. 1000 species, eight families) and includes species from Europe, Africa, Asia and the Americas (Nelson, 2006). Amphibious habits have been described in at least 34 species of cyprinodontiforms, distributed among six families and in both the suborders Aplocheiloidei and Cyprinodontoidei (Fig. 1). Two members of the Cyprinodontidae emerge, *Cyprinodon dearborni* Meek 1909 and *Jordanella floridae* Good & Bean 1879. Anablepidae also contains two amphibious species, *Anableps anableps* (L. 1758) and *Anableps microlepis* Müller & Troschel 1844, as does Poeciliidae, *Gambusia affinis* (Baird & Girard 1853) and an unknown *Poecilia* species. The family Nothobranchiidae contains at least five amphibious species in both the genus *Fundulopanchax* and within the large and much-disputed *Aphyosemion*. These species include *Fundulopanchax gardneri* (Boulenger 1911), *Aphyosemion ahli* Myers 1933, *Aphyosemion cyanostictum* Lambert & Géry 1968, *Aphyosemion franzwerneri* Scheel 1971, *Aphyosemion fulgens* Radda 1975, *Aphyosemion georgiae* Lambert & Géry 1968 and an undescribed *Aphyosemion* species popular in the aquarium hobby and a member of the *Aphyosemion elegans* species group (Collier, 2006).

The family Rivulidae contains at least 13 amphibious species, all formerly considered members of the genus *Rivulus* (Parenti, 1981; Huber, 1992). This is probably an underestimate of the number of amphibious rivulids, however, as numerous species have anecdotally been noted in the aquarium hobbyist literature to be strong jumpers that may emerge (Huber, 1992). In his comprehensive book about the genus *Rivulus*, Huber (1992) characterized the jumping tendencies of 48 species, an amazing 79% of these (38 of 48 species) were considered to be excellent jumpers. In recent years, a great deal of effort has been spent revising the formerly paraphyletic *Rivulus*, and under the most recent classification scheme (Costa, 2011a) amphibious species are now found in four genera split between two subfamilies: the Kryptolebiatinae and

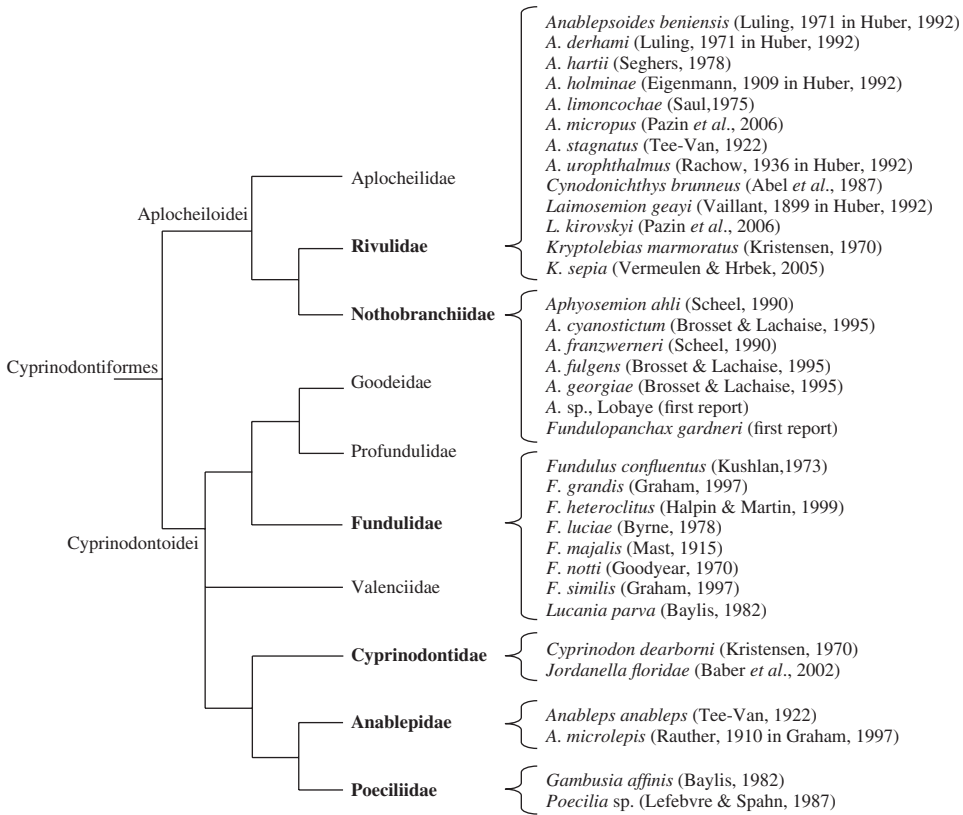


FIG. 1. Phylogenetic placement of cyprinodontiform families containing amphibious species (after Costa, 2012). Families including amphibious species are in bold; the amphibious species are listed to the right along with the first report of emersion in that species.

the Rivulinae [Fig. 2(a)]. *Kryptolebias* contains two amphibious species, *K. marmoratus* and *Kryptolebias sepia* Vermeulen & Hrbek 2005. Two more amphibious species are found within *Laimosemion*, *Laimosemion geayi* (Vaillant 1899) and *Laimosemion kirovskiy* (Costa 2004). The remaining amphibious rivulids are found in the sister genera *Cynodonichthys* and *Anablepsoides*. These species include *Cynodonichthys brunneus* (Meek & Hildebrand 1913), *Anablepsoides beniensis* (Myers 1927), *Anablepsoides derhami* (Fels & Huber 1985), *Anablepsoides hartii* (Boulenger 1890), *Anablepsoides holminae* (Eigenmann 1909), *Anablepsoides limoncochae* (Hoedeman 1962), *Anablepsoides micropus* (Steindachner 1863), *Anablepsoides stagnatus* (Eigenmann 1909) and *Anablepsoides urophthalmus* (Günther 1866).

Amphibious behaviour has been reported in eight species of the family Fundulidae. These North American species are widely distributed phylogenetically and occur in two deeply divergent genera, *Lucania* and *Fundulus* [Fig. 2(b)]. Only one species of *Lucania*, *Lucania parva* (Baird & Girard 1855) has been recorded to leave the water (Baylis, 1982). Within *Fundulus*, seven amphibious species have been described in two subgenera (Ghedotti & Davis, 2013). In the subgenus *Fundulus*, amphibious species include *Fundulus confluentus* Goode & Bean 1879, *Fundulus grandis* Baird & Girard

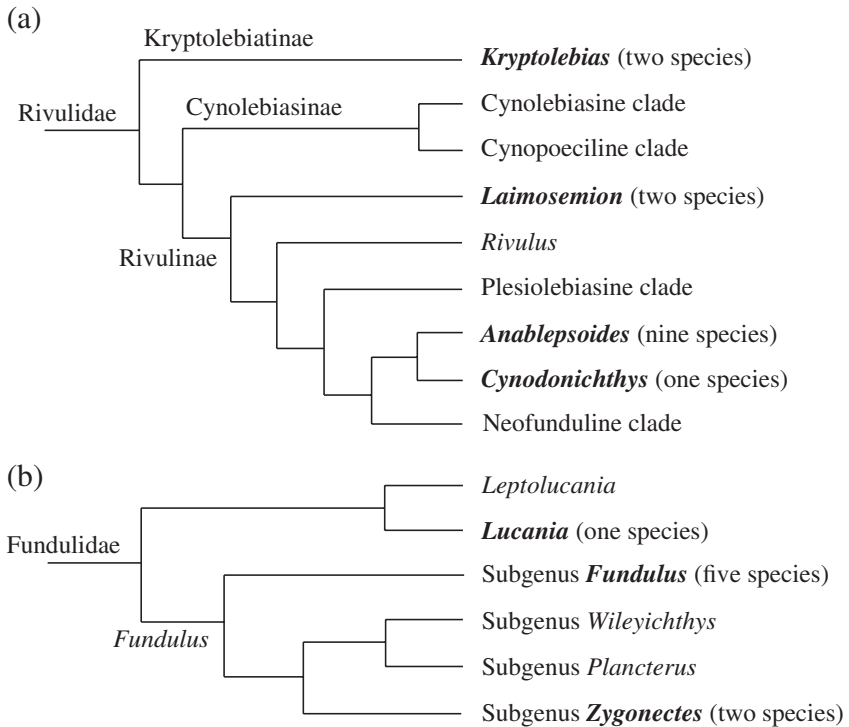


FIG. 2. Phylogenetic distribution of amphibious fishes within the cyprinodontiform families (a) Rivulidae (after Costa, 2011a, 2013) and (b) Fundulidae (after Ghedotti & Davis, 2013). Terminal clades containing amphibious species are in bold.

1853, *F. heteroclitus*, *Fundulus majalis* (Walbaum 1792) and *Fundulus similis* (Baird & Girard 1853). Within *Zygonectes*, *Fundulus notti* (Agassiz 1854) and *Fundulus luciae* (Baird 1855) are known to emerge.

The large phylogenetic diversity of amphibious cyprinodontiforms raises the question whether this behaviour is ancestral or has evolved repeatedly. To date, there is no clear pattern. The six families containing amphibious species are found on either side of the most basal division in the order (between the Aplocheiloidei and Cyprinodontoidei; Fig. 1), suggesting the most parsimonious explanation may be that the common cyprinodontiform ancestor was able to leave water. This hypothesis requires amphibiousness to have been repeatedly lost in many cyprinodontiform families, genera and species (Fig. 1). Furthermore, the relative rarity of amphibious taxa (only about 3% of cyprinodontiforms demonstrate the trait) suggests that the ability to emerge may not be the basal condition. Instead, the tendency of cyprinodontiforms to inhabit shallow, murky or ephemeral habitats may have exposed many species to the environmental conditions thought to drive the evolution of amphibious habits (Parenti, 1981; Sayer & Davenport, 1991). Annual life cycles in cyprinodontiforms are thought to have evolved independently six times, probably as an alternative strategy for occupying the same habitat types that favour amphibiousness (Furness *et al.*, 2015). Until more is known about the emersion tolerances and adaptive specializations of many more cyprinodontiform species, no firm conclusions are possible.

Within the Rivulidae, the evolutionary picture appears clearer. Amphibious habits are documented in four genera and in two subfamilies, including the basal Kryptolebiatinae [Fig. 2(a)], and anecdotes of emersion in *Rivulus* species are widespread (Scheel, 1990; Huber, 1992). Thus, it appears probable that amphibiousness is a trait that existed in the earliest rivulids. Considering that Nothobranchiidae, the sister family to Rivulidae, also contains many amphibious species, this origin of amphibiousness might even predate the nothobranchiid–rivulid split. Why then are there so many fully aquatic species in these families? One possibility is that jack-of-all-trades species able to survive in and out of water may be competitively disadvantaged in aquatic habitats. For example, both locomotion (Gibb *et al.*, 2013) and respiration (Turko *et al.*, 2012) in amphibious fishes appear to require morphological trade-offs that may result in these species being outperformed in water by fully aquatic fishes. Thus, the potential cost of being amphibious in environments where emersion is unnecessary may have driven the reversion to aquatic habits in many rivulids. Annual fishes within these groups may represent an extreme end of this continuum, in which all energy is allocated to rapid growth and reproduction. Although further work is required to clarify the evolution of amphibious and annual natural histories in the Nothobranchiidae and Rivulidae, this system appears to have great potential for studying the life-history trade-offs required to colonize variable and ephemeral habitats (Edward & Chapman, 2011).

In their report demonstrating air-breathing ability by emersed *F. heteroclitus*, Halpin & Martin (1999) suggested that the common ancestor of all *Fundulus* species was amphibious. This conclusion was based on the identification of three amphibious species, *F. heteroclitus*, *F. majalis* and *F. nottii*, which are divided by the deepest fork in the phylogeny of *Fundulus* [Fig. 2(b); Ghedotti & Davis, 2013]. Five additional fundulid species have also been reported to emerse (Kushlan, 1973; Baylis, 1982; Graham, 1997), one in the sister genus to *Fundulus* (*L. parva*), which further supports this idea and suggests that emersion may be a basal trait in the family Fundulidae, rather than only present in *Fundulus*. As is seen in the Rivulidae, however, there are many fully aquatic species in the Fundulidae. Therefore, an alternative hypothesis is that amphibious habits have evolved repeatedly in this family. The common ancestor of the subgenus *Fundulus* (which contains five amphibious species) may have evolved the ability to emerse, while the more distantly related amphibious species *F. nottii*, *F. luciae* and *L. parva* may represent two or three additional independent origins of emersion ability [Fig. 2(b)]. Overall, it appears probable that emersion tolerance is a trait ancestral to the family Fundulidae (Halpin & Martin, 1999) because it is probably more difficult to evolve a complex ability, such as amphibiousness, than to lose it (Murphy & Collier, 1997).

The majority of amphibious cyprinodontiforms are found in the Rivulidae and Fundulidae, but members of the Nothobranchiidae, Cyprinodontidae, Anablepidae and Poeciliidae will also emerse. There are probably many additional cyprinodontiform species that emerse as part of their natural history, but there are as yet no formal reports of these behaviours. The evolutionary origins of amphibious habits within this diverse group of fishes are unclear and merit additional study. Phylogenetically broad studies of both the environmental factors that cause amphibious cyprinodontiforms to emerse and the physiological mechanisms they use will be valuable for discovering common themes between species.

BEHAVIOUR AND ECOLOGY

Amphibious fishes emerge for many reasons and exhibit a variety of behaviours while out of water. Sayer & Davenport (1991) reviewed the factors driving amphibious fishes to emerge and divided them broadly into abiotic factors (*e.g.* habitat drying and poor water quality) and biotic factors (*e.g.* competition, predation and feeding). Almost all of these factors can trigger emersion in cyprinodontiforms. One recurring reason for emersion in many amphibious fishes is reproduction (Sayer & Davenport, 1991; Graham, 1997). Terrestrial egg laying is common in both the Rivulidae and Fundulidae (Huber, 1992). Depositing eggs in air has many advantages for fishes, including increasing the availability of oxygen and offering protection from aquatic predators, but these developing embryos must overcome other challenges such as preventing desiccation. Recent reviews (Podrabsky *et al.*, 2010; Martin & Carter, 2013; Polacik & Podrabsky, 2015) do an excellent job summarizing the current state of knowledge in the field.

EMERSION STIMULI

Habitat drying is perhaps the most common environmental factor that drives amphibious cyprinodontiforms to emerge. When the water evaporates from salt marshes or inland lagoons, *C. dearborni* is able to survive in thin films of water between pebbles or in algae for several days (Kristensen, 1970). Similarly, *J. floridae* is capable of surviving in damp mud if water levels are low (Baber *et al.*, 2002), and *F. confluentus* can tolerate at least 24 h out of water during dry periods in Floridian cypress and willow swamps (Kushlan, 1973). Instead of remaining during low tide, groups of *F. majalis* stranded in tide pools will travel several metres overland to return to the ocean (Mast, 1915). At the beginning of the dry season in the Amazon, the rivulid *A. derhami* will move overland to congregate in the deepest pools of water (Lüling, 1971). The closely related *A. micropus* uses a different strategy to tolerate the dry season, retreating to mud and leaf litter at the bottom of ponds where it can survive for at least 3 months (Pazin *et al.*, 2006). Leaf litter is also a common terrestrial refuge for *K. marmoratus*, and the amount of leaf litter in a habitat may be a significant predictor of abundances of this species in Florida (Huehner *et al.*, 1985; Richards *et al.*, 2011). During emersion in the field, *K. marmoratus* will also hide in damp mud, under coconuts, in discarded beer cans and even within termite tunnels in rotting logs [Fig. 3(a); Taylor *et al.*, 2008]. This log packing behaviour has been documented in both U.S.A. and Belize populations. Many fish are able to occupy a single log; more than 100 individuals were recorded from a 1.5 m long log (9 cm in diameter) at Peter Douglas Cay, Belize, and 57 fish were collected from a similarly sized log at Big Pine Key, Florida, U.S.A. (Taylor *et al.*, 2008). The interrupted dartfish *Parioglossus interruptus* Suzuki & Senou 1994, a perciform from Indonesia, has independently evolved a similar habit of hiding in teredinid tunnels in rotten mangrove logs during emersion (Hendy *et al.*, 2013). The logs inhabited by *P. interruptus* were up to 6.5° C cooler than nearby tide pools due to evaporative cooling and may thus provide a thermal refuge for emersed fish; mangrove logs in the New World may play a similar role for *K. marmoratus*.

Poor water quality is another common trigger for emersion. Under conditions of aquatic hypoxia, many fishes use aquatic surface respiration to obtain oxygen from the thin and relatively oxygen-rich layer of water at the surface (Kramer & McClure, 1982). Some amphibious cyprinodontiforms go a step further and emerge in response

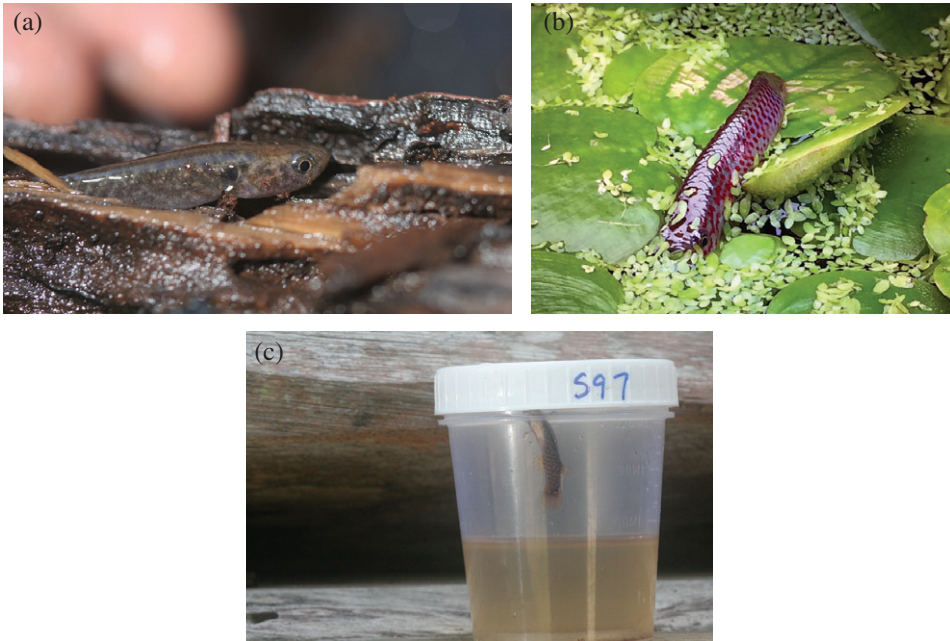


FIG. 3. (a) In Long Caye, Belize, *Kryptolebias marmoratus* were found inside rotting logs, as first described by Taylor *et al.* (2008). (b) The undescribed *Aphyosemion* sp. Lobaye often leaves water, apparently voluntarily, and basks on top of floating vegetation in aquaria (photo credit: Dominique Taylor). (c) An individual *K. marmoratus* voluntarily emerged in a cup above water taken from its crab burrow in Belize.

to aquatic stressors. For example, *F. heteroclitus* may emerge under hypoxic conditions and then switch to aerial respiration (Halpin & Martin, 1999). Abel *et al.* (1987) were the first to test whether hypoxia caused emersion in *K. marmoratus*, but used a moderate level of dissolved oxygen (*c.* $2 \text{ mg O}_2 \text{ l}^{-1}$) and concluded that the fish were unresponsive. Extreme hypoxia ($<0.4 \text{ mg O}_2 \text{ l}^{-1}$), however, stimulated emersion in *K. marmoratus* (Regan *et al.*, 2011). High levels of hydrogen sulphide, common in the mangrove swamps typically inhabited by *K. marmoratus* (Taylor, 2012), also cause emersion (Abel *et al.*, 1987). Receding water levels led to increased concentrations of hydrogen sulphide, and Abel *et al.* (1987) speculated that this is the cue used by *K. marmoratus* to leave mangrove pools and find new bodies of water. Other acute abiotic changes, such as fluctuations in temperature, salinity or pH have also been hypothesized to elicit emersion (Sayer & Davenport, 1991). *K. marmoratus* will emerge when water temperatures decrease from 25 to 20° C (Huehner *et al.*, 1985). Increased water temperatures (*c.* 36° C) have also recently been found to induce emersion by *K. marmoratus*, which may then be able to benefit from evaporative cooling in air (D. Gibson, E. Sylvester, A. Turko, G. Tattersall & P. Wright, unpubl. data). Additional data indicates that aquatic acidosis resulting from either hypercapnia or the addition of dilute HCl also stimulates emersion by *K. marmoratus* (Robertson *et al.* online). In mangrove forest pools and crab burrows inhabited by *K. marmoratus*, many water quality variables (*e.g.* oxygen, carbon dioxide, hydrogen sulphide, temperature, ammonia and pH) fluctuate on a daily basis (Ellison *et al.*, 2012). A systematic study of the behavioural responses of fishes to the isolated effects of each of these potential

stimuli, as well as their interactive effects on behaviour, is warranted. In addition, nothing is known about the potential interactions between abiotic and biotic factors. For example, would emersion in response to competition or aggression occur more readily in hypoxic or hydrogen sulphide-rich habitats? Finally, only the acute emersion responses of amphibious cyprinodontiforms have been investigated thus far. Future experiments should investigate if chronic but less severely degraded water quality also leads to increased rates of emersion.

Emersion can be used to escape competition, aggression and predation by other fishes. Intraspecific aggression has been shown to cause emersion in *K. marmoratus* under laboratory conditions (Taylor, 1990), and these fish will also emerge in response to a human disturbance (submerging a hand underwater) in crab burrows in the field (Taylor, 1988). The closely related *K. sepia* will also emerge in response to disturbance by humans (Vermeulen & Hrbek, 2005), as will the more distantly related *A. ahli* (Scheel, 1990). African killifish in the *Aphyosemion* subgenus *Diapteron* (*A. fulgens*, *A. cyanostictum* and *A. georgiae*) have been seen leaping from water after losing an intraspecific aggressive contest; this behaviour occurs both in the wild and in aquaria (Brosset & Lachaise, 1995). Emersion in response to the threat of predation is commonly reported. *Cynodonichthys brunneus* escapes predation from species of *Hoplias* by jumping out of the water (Abel *et al.*, 1987), and *A. hartii* also emerge in response to a predator threat (Seghers, 1978). Goodyear (1970) reported that *F. nottii* will leap out of water and remain on the bank of a pond for several minutes to escape predation by largemouth bass *Micropterus salmoides* (Lacépède 1802). Similarly, *G. affinis* and *L. parva* will jump onto water lilies to escape predatory bluegill *Lepomis macrochirus* Rafinesque 1819 (Baylis, 1982). Emersion is, however, not a foolproof strategy. Baylis (1982) noted that small fish flipping across a lily pad are sometimes spotted from below by the predatory *L. macrochirus* and consumed lily and all.

In addition to leaving the water to avoid predation, some cyprinodontiforms emerge to capture prey. Huehner *et al.* (1985) reported that *K. marmoratus* emerge to capture termites on land. According to Davis *et al.* (1990), *K. marmoratus* are capable of pursuing and capturing prey on land by flipping or leaping, but always return to water to consume their meal. In captivity, *K. marmoratus* leap 11 cm in the air to catch terrestrial prey, an impressive feat for a fish that is typically 2–4 cm in length (Taylor, 1990). Impressive jumping ability is also exhibited by *A. hartii*, which can jump 14 cm vertically to capture ants or other insects (Seghers, 1978). Pronko *et al.* (2013) recently reported that *K. marmoratus* will employ three distinct locomotory behaviours, ‘pounces’, ‘squiggles’ and ‘launches’, to leave water and capture prey presented in a laboratory environment. Launches are driven by a swimming burst that propels the fish out of water, while pounces are a shorter-distance movement in which the fish curls into a C-shape and rapidly extends, sliding its ventral aspect over the substratum. Squiggles are oscillatory movements used to travel over land without breaking contact with the substratum. A similar behaviour to the launch of *K. marmoratus* is used by *A. anableps* in the laboratory to capture terrestrial insects near the water’s edge (Kushner *et al.*, 2009). In the wild, *A. anableps* are often found emersed for several hours at low tide during which time they feed on small invertebrates (Tee-Van, 1922; Zahl *et al.*, 1977; Brenner & Krumme, 2007).

The ability to leave water and travel over land allows amphibious cyprinodontiforms to colonize new habitats unavailable to fully aquatic species. Several species, including

F. majalis, *A. micropus* and *L. kirovskiyi*, have been reported to move over land into new aquatic habitats (Mast, 1915; Espírito-Santo *et al.*, 2013). These terrestrial sojourns can be brief, as in the case of an unidentified species of *Poecilia* observed briefly ‘flipping’ across the top of a sandbar to reach an upstream segment of a creek (Lefebvre & Spahn, 1987) or *F. luciae*, which moves over mud between shallow pools (Byrne, 1978). At the other end of the spectrum, *A. hartii* can cover 53 cm in a single jump and move 363 cm in 5 min, allowing these fish to disperse widely (Seghers, 1978). *Anablepsoides hartii* frequently move between pools of water after rainfall, thus rapidly occupying open habitats. In Trinidad, *A. hartii* colonized a newly dug pit within 2 months, even though the pit was at least 20 m from the nearest temporary water body and 500 m from the nearest permanent stream (Jordan, 1923). To determine the ability of *K. marmoratus* to colonize new habitats, Taylor (1990) dug artificial holes that simulated burrows of the land crab *Cardisoma guanhumii*, the typical habitat of this species. When the burrows were sampled 6 months later, *K. marmoratus* were found within them. These fish have also been observed in puddles that form in the middle of roadways after rainfall, suggesting that new or ephemeral habitats can be rapidly colonized (Davis *et al.*, 1990). In a laboratory mesocosm, *K. marmoratus* moved over land between artificial burrows up to six times per day, providing further evidence for the ability of these fish to exploit new habitats (Taylor, 1990). Little is known about the movement patterns of individual fish in the wild, in large part, because performing mark–recapture studies of such small fish in the mangrove habitat is quite difficult.

While many cyprinodontiforms emerge to reproduce, disperse or avoid deteriorating environmental conditions, some species emerge even when there is no discernable stimulus. Sayer & Davenport (1991) urge caution before concluding that emersions are voluntary, as subtle biological or abiotic factors may influence behavioural decisions. Therefore, while the emersion behaviours in the following cases may appear voluntary, the cues for emersion are best summarized as unknown. Tee-Van (1922) described apparently voluntary emersion behaviour in the rivulid *A. stagnatus*, which would often jump out of the water in holding aquariums and adhere to leaves or stones. One individual, lying alert, remained emersed for 9 days before returning to water. Similarly, *A. beniensis* is frequently inexplicably found emersed and on blades of grass or algae filaments above well-oxygenated water (Lüling, 1971). *Aphyosemion franzwernerii* remain emersed among tree roots overhanging small pools by day (Scheel, 1990). *Fundulopanchax gardnerii* has been observed emersed in aquaria on top of floating plants or if provided with a cradle of filter floss that sits just above the water (M. Davies, pers. comm.). The undescribed *Aphyosemion* sp. Lobaye, a close relative of *A. elegans* (Collier, 2006), will similarly emerge on top of floating vegetation [Fig. 3(b); D. Taylor, pers. comm.]. *Kryptolebias marmoratus* voluntarily leave water under both laboratory and field conditions. Turko *et al.* (2011) video-recorded fish for 7 days in clean brackish water in the laboratory to minimize abiotic cues for emersion and analysed the time each spent voluntarily out of water [Fig. 3(c)]. Some individuals never emersed, but others spent up to 78% of the 7 day period out of water. In Belize, a remotely operated video camera was placed inside a crab burrow to record emersion patterns of *K. marmoratus* under natural conditions [Video S1 (Supporting Information)]. Emersion by an unknown number of fish was common in this habitat, in 30 min of filming 11 instances of emersion were recorded, ranging in duration from 6 s to 7 min (mean \pm S.E. duration of 2.8 ± 0.7 min). These emersions were shorter than those recorded in the laboratory by Turko *et al.* (2011), which averaged 61 min in one population and 20 min in another.

At several points in the video, the movement of predatory mangrove tree crabs *Goniopsis cruentata* appear to startle emersed *K. marmoratus* and triggered their return to the water, which may explain why the emersion durations were shorter in the field relative to undisturbed laboratory conditions. Interestingly, movements by another, larger species of detritivorous crab (*C. guanhumi*, the burrow-excavating species) did not startle emersed *K. marmoratus* in the same way. These emersions occurred without any clear biotic stimulus (*e.g.* no observable chasing by conspecifics, risk of predation or terrestrial feeding opportunities), although abiotic factors such as hypoxia or elevated hydrogen sulphide probably play a role. Perhaps voluntary emersion allows some species to exploit oxygen-rich air and minimize energy expenditure by not swimming and reducing ventilation of the gills.

TERRESTRIAL LOCOMOTION

Locomotion in terrestrial environments is challenging for most fishes, which have body forms, support structures and sensory systems that have evolved to facilitate life in water (Sayer, 2005). Many fully aquatic fishes, such as *M. salmoides*, are only capable of producing a chaotic thrashing movement that results in vertical but not in horizontal displacement (the kinematics of terrestrial locomotion in fishes were recently reviewed by Gibb *et al.*, 2013). Amphibious cyprinodontiforms, however, are able to navigate out of water in a controlled and directional manner despite their lack of obvious morphological specializations for terrestrial locomotion (Ashley-Ross *et al.*, 2014). Body orientation on a slope can be sensed by emersed *G. affinis*, which always roll to return to water if they are positioned perpendicularly to the incline, but often jump or flip back to water if they are pointed up or downhill (Boumis *et al.*, 2014). At low tide, *F. majalis* emerge from tide pools and jump in the direction of the ocean, even if this requires moving uphill or into a breeze (Mast, 1915). The navigational cues used by *F. majalis* are unknown, but Goodyear (1970) has demonstrated that *F. nottii* use the sun to navigate while emersed. In these experiments, fish were captured from four different locations around a pond, at the four cardinal points of the compass. The fish were then placed, without water, on a flat table and their direction of travel was recorded. Goodyear (1970) found that, on sunny days, *F. nottii* would overwhelmingly travel in the direction that would have led them to the water's edge based on their location of capture from the pond, *e.g.* fish captured from the north shore travelled south, which would have returned them to the water had they been emersed on the northern bank. On overcast days, however, fish moved randomly. These results suggest that emersed *F. nottii* use the sun to navigate, and also that these fish may emerge frequently enough prior to being captured for experimentation to learn the local environment and optimal direction of travel to return to water. *Kryptolebias marmoratus* also appear capable of learning and navigating terrestrial landscapes. In the field, emersed *K. marmoratus* tend to jump in the direction of crab burrows when disturbed or accidentally dropped by a collector (Huehner *et al.*, 1985). Local landscapes can also be learned under laboratory conditions (Taylor, 1990; Pronko *et al.*, 2013). Taylor (1990) built a simulated mangrove swamp aquarium that contained two replica crab burrows separated by 8 cm of emersed substratum in order to film a fish moving between the burrows. When a barrier was installed to force the fish to take a longer (23 cm) and indirect path to switch between burrows, the new route was learned within 24 h. A new barrier was

then inserted which completely separated the two burrows except for a small hole (1 cm diameter) drilled at ground level; the fish learned the location of this hole within 5 days and used it to move between burrows. When the small hole was subsequently plugged, the *K. marmoratus* would repeatedly orient towards the location of the former hole and try to cross to the other burrow.

Despite their ability to move overland to inhabit unoccupied pools of water, many amphibious rivulids are endemic to only small regions of South America (Huber, 1992). In contrast, *K. marmoratus* are found in mangrove habitats throughout the tropical west Atlantic, from southern U.S.A. to northern Brazil, although the species designation in Brazil is uncertain (Costa, 2011b; Taylor, 2012). Why are *K. marmoratus* so widespread when other rivulid species are not? One possibility is that logs invaded by fish during periods of low water are spread throughout the western Atlantic Ocean by storms and ocean currents (Taylor *et al.*, 2008; Taylor, 2012). Another possible scenario is the dispersal of embryos, which are highly adhesive and likely candidates for transport on floating mangrove leaves or detritus. While this type of waif dispersal is probably rare, as suggested by strong population structure among Belizean, Bahamian and American populations, a few specimens of *K. marmoratus* genetically similar to Belizean populations have been discovered in Florida (Tatarenkov *et al.*, 2007). Log or debris transport aside, two other derived traits in *K. marmoratus* are also required for their observed wide distribution. Unlike most other rivulids, *K. marmoratus* are euryhaline (Costa, 1998) and capable of tolerating fresh water and salinities as high as 114 (King *et al.*, 1989; LeBlanc *et al.*, 2010). This salinity tolerance not only allowed the colonization of brackish mangrove swamps, but also would enable *K. marmoratus* to survive exposure to seawater during dispersal voyages (Costa *et al.*, 2010; Taylor, 2012). Second, *K. marmoratus* is one of the only two (the other being the sister species *Kryptolebias hermaphroditus* Costa 2011) known self-fertilizing hermaphroditic vertebrates (Harrington, 1961; Tatarenkov *et al.*, 2009; Costa *et al.*, 2010; Costa, 2011b). The ability to self-fertilize would allow a single *K. marmoratus* to found a new population after long-range dispersal, and would also be advantageous even after shorter movements over land to unoccupied crab burrows or pools (Tatarenkov *et al.*, 2009; Avise & Tatarenkov, 2012).

Overall, amphibious cyprinodontiforms emerge to reproduce, escape poor or deteriorating habitats or to exploit new niches, and some also appear to emerge voluntarily. Emersions range in duration from seconds to months, and may involve actively moving about on land or simply retreating to a damp crevice to wait for water to return. A suite of morphological and physiological adaptations permit these terrestrial sojourns, and these are reviewed in the next section.

PHYSIOLOGY

To survive both in and out of water, amphibious fishes require adaptations that facilitate the maintenance of water balance, gas exchange, nitrogenous waste excretion and ionoregulation (Sayer, 2005). In fully aquatic fishes, the gills are the main site of these physiological processes, but maintaining gill functionality during emersion is problematic. Gill tissues must remain moist, but without the buoyant support of water the secondary lamellae of the gills may collapse and coalesce thus reducing their effective surface area. Many amphibious fishes have evolved specialized gill morphologies or

air-breathing organs, but these strategies do not appear to be used by cyprinodontiforms. Instead, during emersion, the cutaneous epithelium appears to take on many of the roles typically assigned to the gills (Wright, 2012; Glover *et al.*, 2013). Unfortunately, reports of amphibious cyprinodontiform physiology are limited to studies of *K. marmoratus* and one investigation of *F. heteroclitus*, so while other amphibious species conceivably also use their skin during emersion, the role of the cutaneous surface in these fishes is unknown.

The skin may be especially important to *K. marmoratus* during emersion, as the gills are remodelled during terrestrial acclimation and surface area is reduced. Ong *et al.* (2007) demonstrated that 1 week of emersion induces the proliferation of a group of cells in the spaces between the secondary lamellae in *K. marmoratus* but recedes within 7 days of returning to water. This group of cells, the inter-lamellar cell mass, was also enlarged in *K. marmoratus* that voluntarily emersed frequently over a 7 day recording period (Turko *et al.*, 2011). Aquatic respiratory function was impaired in *K. marmoratus* with reduced gill surface area after they returned to water but before the inter-lamellar cell mass regressed (Turko *et al.*, 2012). The cost imposed by an enlarged inter-lamellar cell mass in water suggests that gill remodelling provides some kind of benefit during emersion, although the nature of the benefit is not yet clear. The inter-lamellar cell mass may protect and support the delicate secondary lamellae from collapsing and coalescing on land when they are not supported by water, or the reduced gill surface area resulting from an enlarged cell mass may minimize water loss (Ong *et al.*, 2007). Gill remodelling may also reduce the surface area available for parasite attachment (Nilsson *et al.*, 2012). Gill surface area is correlated with parasite loads in the gills of *Carassius carassius* (L. 1758) (Nilsson *et al.*, 2012) and *Liza ramada* (Risso 1826) (Caltran & Silan, 1996), and the gills of wild *K. marmoratus* sometimes contain bacterial cysts (Ellison *et al.*, 2011).

Only a few studies have investigated the morphological specializations that permit cutaneous respiration in cyprinodontiforms. Grizzle & Thiyagarajah (1987) described capillaries in *K. marmoratus* that reach within 1 μm of the epidermal surface. These capillaries are densest anteriorly and dorsally, but blood vessels are also present in the fins. Recent use of immunohistochemical labelling of the protein Cd31, a common marker for angiogenesis, has revealed that these surfaces receive increased blood flow during emersion (Fig. 4; Turko *et al.*, 2014). The fins, which comprise *c.* 40% of the total cutaneous surface area of *K. marmoratus*, also demonstrated significant angiogenesis in terrestrially acclimated fish (Cooper *et al.*, 2012). Unexpectedly, Turko *et al.* (2014) noticed that increased Cd31 fluorescence was not limited to the cutaneous surface, blood flow to the epithelium lining the mouth and opercula also increased during emersion. Further experiments demonstrated that *K. marmoratus* gulp air approximately five times per hour over several days after emersion, suggesting that the bucco-opercular chamber and possibly the gills may supplement cutaneous respiration. The use of cutaneous capillaries for gas exchange has never been directly investigated in *F. heteroclitus*, but Connolly (1925) demonstrated that the head and caudal fin of these fish are well supplied with blood. Vasodilation of these cutaneous blood vessels occurred when aquatic *F. heteroclitus* were placed on a red background, turning the fish slightly pink and improving camouflage. Whether similar changes in blood flow occur in response to emersion remains an open question.

Aerial gas exchange rates in both *K. marmoratus* and *F. heteroclitus* have been measured in several studies. Halpin & Martin (1999) measured oxygen consumption by *F.*

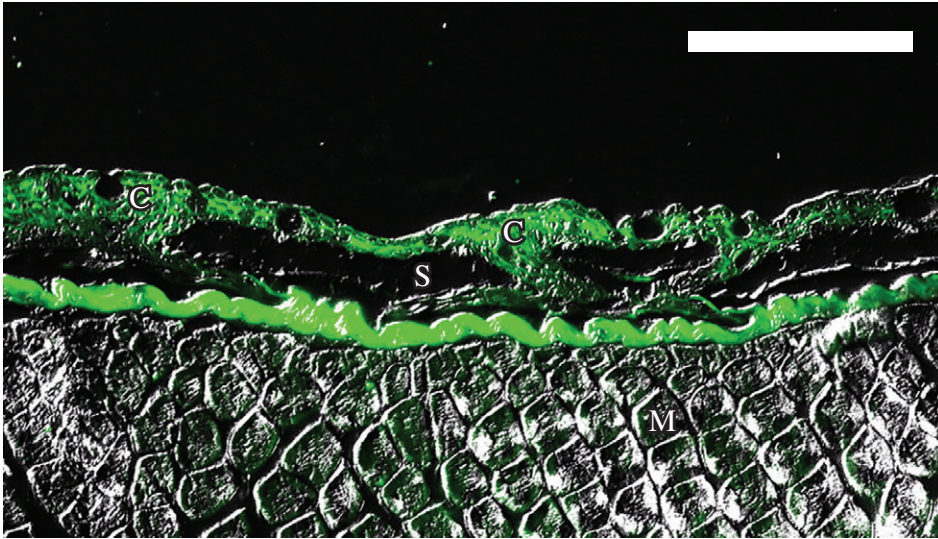


FIG. 4. Capillaries (●) extend between the scales of *Kryptolebias marmoratus* into the epidermis. This photograph of a sagittal section was taken through the dorsal surface of the head, but these capillaries are seen across the cutaneous epithelium. An antibody against the endothelial protein Cd31 was used to label capillaries; for methods see Turko *et al.* (2014). C, capillaries; S, scales; M, muscle. Scale bar = 100 μ m.

heteroclitus in and out of water. These studies found that emersed fish reduced oxygen consumption by *c.* 75% relative to their rate of consumption in water, but despite these low rates of oxygen consumption no oxygen debt was evident upon returning to water after 1 h of emersion. Excretion of carbon dioxide was probably not problematic for emersed *F. heteroclitus* either, as respiratory exchange ratios (carbon dioxide released:oxygen consumed) were determined to be between 0.7 and 1.0 depending on temperature. To date, the only published report of aerial oxygen consumption by *K. marmoratus* suggests that fish decrease oxygen uptake after 1 day of emersion (Abel *et al.*, 1987). Carbon dioxide excretion by emersed *K. marmoratus*, however, occurs at rates similar to those in water immediately after emersion and at significantly higher rates (*c.* 40%) after 3 days of terrestrial acclimation (Ong *et al.*, 2007). Similarly, the activities of several enzymes involved in amino-acid and mitochondrial oxidative metabolism are unchanged or increased during emersion (Frick & Wright, 2002). How are these contradictory results explained? Measuring oxygen consumption by very small animals, <100 mg in the case of *K. marmoratus*, in relatively oxygen-rich atmospheric air (compared with water) is notoriously difficult (Ong *et al.*, 2007). With the recent availability of optode technology that allows precise sensing of oxygen concentrations, it should now be possible to directly measure oxygen consumption in and out of water in *K. marmoratus* and other minute amphibious fishes.

While there is some information on gas exchange in emersed *F. heteroclitus* (Halpin & Martin, 1999) and *K. marmoratus* (Ong *et al.*, 2007), there are no data available on partial pressures within the blood. Carbon dioxide is relatively insoluble in air compared with water, and thus tends to accumulate in the blood of air-breathing animals (Rahn, 1966; Ultsch, 1987). An increased partial pressure of carbon dioxide in the blood could potentially impair oxygen uptake by reducing both the oxygen

binding affinity (*via* the Bohr effect) and carrying capacity (*via* the Root effect) of haemoglobin, thus resulting in hypoxemia (Graham, 1997; Shartau & Brauner, 2014). Considering that cyprinodontiforms have probably evolved under conditions of aquatic hypoxia, Turko *et al.* (2014) tested the hypothesis that *K. marmoratus* use a co-opted hypoxia response to maintain oxygen transport in the face of carbon dioxide accumulation during emersion. This hypothesis was soundly rejected, terrestrially acclimated fish increased haemoglobin-oxygen binding affinity, while fish acclimated to hypoxia in water increased the number of circulating red blood cells. Increased haemoglobin-oxygen binding affinity in terrestrially acclimated *K. marmoratus* probably offsets the decrease in affinity resulting from the Bohr effect, maintaining the balance between oxygen uptake and oxygen delivery. If not hypoxia, what cue initiates a change in haemoglobin affinity in *K. marmoratus*? One possibility is that emersed fish respond to increased partial pressures of carbon dioxide in the blood.

Recent evidence suggests that the skin of *K. marmoratus* may be used for oxygen sensing as well as gas exchange. In fishes, innervated chemosensory cells called neuroepithelial cells are responsible for sensing oxygen concentrations and initiating cardiorespiratory responses to hypoxia (Zachar & Jonz, 2012; Jonz *et al.*, 2015). Neuroepithelial cells are typically not only found in the gills of adult fishes, but are also present on the cutaneous surface of larval fishes before the gills are developed. The skin of adult *K. marmoratus* has recently been reported to contain an abundance of neuroepithelial cells (45% more cells in the skin than the gills) and these cells respond to aquatic hypoxia (Regan *et al.*, 2011). Whether these cutaneous oxygen sensors are also used during emersion has yet to be investigated. The situation is paradoxical, as aerial environments are rich in oxygen and these conditions are thought to reduce the need for oxygen sensing. Acclimation to hyperoxia, for example, reduced the densities of neuroepithelial cells in zebrafish *Danio rerio* (Hamilton 1822) (Vulesevic *et al.*, 2006). Future studies are required to determine the functions and benefits of cutaneous neuroepithelial cells during emersion.

In addition to being the main location for respiration, the cutaneous epithelium of *K. marmoratus* is used for nitrogenous waste excretion during emersion. Fishes typically excrete waste nitrogen as ammonia by diffusion across the gills, but this is difficult in the absence of water. Uniquely among amphibious fishes, *K. marmoratus* excrete a relatively large proportion (40%) of nitrogenous waste as ammonia gas *via* volatilization (Frick & Wright, 2002; Wright, 2012). Emersed fish volatilize ammonia gas within 24 h of leaving water, and continue to use this strategy over at least 11 days of terrestrial acclimation (Litwiller *et al.*, 2006). This strategy depends on the presence of ammonia-transporting Rhesus glycoproteins in the skin, as well as the ability for fish to finely regulate the pH of the skin surface (Hung *et al.*, 2007; Wright & Wood, 2009; Cooper *et al.*, 2013). It is unknown whether other amphibious cyprinodontiforms also possess these specializations to permit ammonia volatilization during emersion, or whether alternative strategies are used, such as suppressing amino acid catabolism, detoxifying ammonia *via* urea or glutamine synthesis or enhancing cellular tolerance to high ammonia concentrations (Ip *et al.*, 2004).

Maintaining water and ion balance during air exposure is one of the greatest challenges for amphibious fishes (Sayer, 2005; Takei, 2015). Many species rely on a thick skin or mucous coat during emersion, but for fish such as *K. marmoratus* the multi-functional nature of the cutaneous epithelium may preclude these methods of retaining water (LeBlanc *et al.*, 2010; Turko *et al.*, 2011). Nonetheless, emersed *K.*

marmoratus are able to maintain water balance. Terrestrially acclimated fish, which are thought to be incapable of feeding while out of water, lose no more mass than fasted fish in water and the proportional body water content may actually increase as fatty energy stores are utilized (Frick & Wright, 2002; Litwiller *et al.*, 2006; LeBlanc *et al.*, 2010). Embryos of *F. heteroclitus* that develop terrestrially downregulate aquaporin expression, possibly to conserve water (Tingaud-Sequeira *et al.*, 2009). The ability of adult cyprinodontiforms to similarly regulate cutaneous water permeability at the protein level has yet to be investigated.

Ion balance is largely maintained during emersion in *K. marmoratus* by a large cutaneous population of ionocytes (LeBlanc *et al.*, 2010). These cells are typically found in the gills of adult fishes, but *K. marmoratus* possess approximately equal numbers in the skin and gills. Cutaneous ionocytes are also known in some other cyprinodontiforms. Ionocytes are present in the opercular epithelium of *F. heteroclitus* and in the skin of *Poecilia reticulata* (Schwerdtfeger & Bereiter-Hahn, 1978; Hiroi & McCormick, 2012; Glover *et al.*, 2013). Given the diversity of amphibious cyprinodontiforms, it would not be surprising if cutaneous ionocytes are found in other species as well.

While many advances have been made in the understanding of the physiological adaptations used by amphibious cyprinodontiforms to tolerate life out of water, many questions remain unanswered. One avenue of research that appears particularly promising is to utilize phylogenetic and inter-population comparisons to understand the particular traits that influence emersion tolerance. For example, why can *K. marmoratus* and *A. micropus* survive for many months out of water, while congeners do not emerge at all? Or perhaps most species within the genera *Kryptolebias* and *Anablepsoides* are in fact amphibious, but natural history knowledge is lacking. Intraspecific comparisons across populations are also worthwhile. Life-history traits are known to vary among populations of *A. hartii* (Walsh & Reznick, 2010; Oufiero *et al.*, 2011; Walter *et al.*, 2011), *F. heteroclitus* (Burnett *et al.*, 2007; Whitehead *et al.*, 2011) and *K. marmoratus* (Grageda *et al.*, 2005; Earley *et al.*, 2012). Determining if there is similar variation in amphibious ability and addressing the physiological mechanisms that underlie this variation are promising future directions for research.

In conclusion, the order Cyprinodontiformes contains a diversity of amphibious species and more are sure to be discovered. For example, 97 valid species of *Rivulus* were described 20 years ago by Huber (1992). Since then, at least 54 new species have been described within the various genera that formerly comprised *Rivulus* (Costa, 2011a). Behavioural observations and natural history data are lacking for many of these species, but some will almost certainly prove to be amphibious. Is there some basal cyprinodontiform trait that has allowed so many species to invade land? Perhaps amphibiousness is a key evolutionary innovation that has enabled cyprinodontiforms to invade new ecological niches, as in the cichlids where the evolution of pharyngeal jaws is thought to underlie their dramatic radiation in Africa (Liem, 1973). The ability to leave water has certainly allowed cyprinodontiforms to take advantage of several ecological niches not available to fully aquatic fishes. Emersion can be used to exploit ephemeral bodies of water, reproduce in oxygen-rich terrestrial environments safe from aquatic predators, tolerate deteriorating water conditions, feed on terrestrial prey and avoid aggressive or predatory interactions in water. Understanding the various environmental factors that drive emersion in cyprinodontiforms may even provide insight into why the earliest tetrapods made the transition to a terrestrial existence.

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Supporting Information

Supporting Information may be found in the online version of this paper:

Video S1 A video camera placed inside a crab burrow captured several emersions by *Kryptolebias marmoratus* in Long Caye, Belize (17° 13-08' N; 087° 35-66' W). Several attempts were often required before fish would remain emersed. Movements by the partially submerged blue land crab *Cardisoma guanhumi*, a detritivore, did not readily startle the emersed *K. marmoratus*. Sudden movements by emersed mangrove tree crabs *Goniopsis cruentata*, a predatory species, caused emersed *K. marmoratus* to immediately flip back into the water. This video is also available online at <http://youtu.be/0nBaE5rTwIM/>

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