Environmental Physiology of the Mangrove Rivulus, *Kryptolebias marmoratus*, A Cutaneously Breathing Fish That Survives for Weeks Out of Water

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Synopsis

The mangrove rivulus (*Kryptolebias marmoratus*) is an excellent model species for understanding the physiological mechanisms that fish use in coping with extreme environmental conditions, particularly cutaneous exchange during prolonged exposure to air. Their ability to self-fertilize and produce highly homozygous lineages provides the potential for examining environmental influences on structures and related functions without the complications of genetic variation. Over the past 10 years or so, we have gained a broader understanding of the mechanisms *K. marmoratus* use to maintain homeostasis when out of water for days to weeks. Gaseous exchange occurs across the skin, as dramatic remodeling of the gill reduces its effective surface area for exchange. Ionoregulation and osmoregulation are maintained in air by exchanging Na⁺, Cl⁻, and H₂O across skin that contains a rich population of ionocytes. Ammonia excretion occurs in part by cutaneous NH₃ volatilization facilitated by ammonia transporters on the surface of the epidermis. Finally, new evidence indicates that cutaneous angiogenesis occurs when *K. marmoratus* are emersed for a week, suggesting a higher rate of blood flow to surface vessels. Taken together, these and other findings demonstrate that the skin of *K. marmoratus* takes on all the major functions attributed to fish gills, allowing them to move between aquatic and terrestrial environments with ease. Future studies should focus on variation in response to environmental changes between homozygous lineages to identify the genetic underpinnings of physiological responses.

Introduction

The environment, whether aquatic or terrestrial, has had a large impact on the evolution of structure and function in animals. If one considers respiratory structures, for example, breathing water is challenging because it is 800 times more dense, 60 times more viscous, and contains 30 times less oxygen relative to air (Dejours 1988). Consequently, gills in extant fishes are highly efficient at extracting oxygen. Air breathers can breathe fewer breaths relative to water breathers to take up the same amount of oxygen (Dejours 1974). However, the greatest challenge for air breathers is dehydration. Over evolutionary time, the benefits of breathing air must have outweighed the costs because more than 370 species of air-breathing fish are known (Graham 1997). So what’s so special about *Kryptolebias marmoratus*—is it just one of many or is it a remarkable species that can provide us with novel insights into the physiology of breathing air? I will argue the latter for three key reasons: (1) these fish are self-fertilizing, (2) tolerant of extreme environments, and (3) they completely rely on cutaneous respiration when out of water.

Self-fertilization

*K. marmoratus* are self-fertilizing hermaphrodites (Harrington 1961; Tatarenkov et al. 2009) and produce highly homozygous lineages when isolated in the laboratory for many generations (Vrijenhoek 1985; Turner et al. 1990). Wild populations are androdioecious; males occur at low, but variable, rates and outcrossing between males and females is rare.
hormaphrodites is thought to be the cause of heterozygosity (see Tatarenkov this issue). For physiologists interested in the effects of environment on the phenotype (e.g. structure, function), the ability to study isogenic laboratory strains eliminates the confounding factor of genotype. Further, if differences in physiological responses to environmental change are detected between isogenic strains and between wild populations, then a more detailed differential genetic screening may provide new insights into regulatory pathways. For example, acclimation to changing environmental salinity is variable among lineages of K. marmoratus. We have discovered that in hypersaline water, some lineages remodel their gills and decrease the effective surface area for exchange, presumably limiting excessive ion uptake (discussed below), whereas other lineages show no response (A. Turko and P. Wright, unpublished data). If the gene and/or protein expression profiles of the gills from lineages showing a differential response to salinity are compared, then the genes and proteins responsible for regulating remodeling of the gills could be identified. In addition, comparing plasma hormone profiles among those fish that do undergo changes in the gills and those that do not would be one step toward understanding whether gill remodeling in response to salinity is under endocrine control. A comparative genomics approach has been used in euryhaline Fundulus sp. to identify the importance of particular genes in acclimation to salinity and in physiological systems that may be linked to osmotic tolerance and niche segregation (Whitehead 2012).

To date, physiologists have not capitalized on this opportunity in K. marmoratus. Significant progress has been made in understanding the genetics of K. marmoratus (see Tararenkov this issue) and the annotated genome of multiple lineages is soon to be available (see Kelly this issue). In addition, identifying phenotypic differences in growth and behavior between isogenic lineages (see Earley this issue) is valuable information when assessing which lineages are most suited for specific environmental manipulations. Thus, in the next few years, there is potential for great gains and novel insights to be made in determining the factors controlling physiological responses to environmental change using K. marmoratus as a model species.

**Extremophilic**

The second reason why K. marmoratus are of particular physiological interest is that they thrive under relatively extreme conditions. Physiologists interested in understanding the underlying mechanisms of a response to the environment may gain more information from an animal that is surviving at the limits of tolerance rather than an organism that tolerates only moderate conditions. Textbooks of comparative animal physiology routinely present this tenet in their opening pages as the August Krogh Principle “For every well-defined physiological problem there is an animal optimally suited to yield an answer” (Randall et al. 2002). For example, if one is interested in the physiological consequences of air-breathing in fish, more insight may be gained from studying K. marmoratus that survives for ~2 months out of water (emersed) compared with a species that gulps air occasionally at the surface of the water.

The extreme nature of the habitat of K. marmoratus is readily apparent by taking a walk through the mangrove forests of the western Atlantic region (Taylor et al. 1995; see also Taylor this issue). Progress is slow as you climb over a tangle of aerial roots from the red mangrove trees and sink deep into the thick mud where microbial activity releases malodorous hydrogen sulphide from deep layers of decaying vegetation. K. marmoratus typically reside in crab burrows (8.0 ± 0.5 cm [diameter] × 33.9 ± 2.0 cm [long], n = 13) (P. Wright and D.S. Taylor, unpublished data) on the forest floor. Water quality for aquatic respiration in these crab burrows could not be worse—the water is extremely hypoxic (Fig. 1), has elevated levels of H₂S (Abel et al. 1987), and is warm (25–30°C) (Davis et al. 1990; Taylor 2000; Frick and Wright 2002a). We measured dissolved oxygen (DO) levels in crab burrows where K. marmoratus were found on Calabash Caye in December 2009 over a 24-h period (Fig. 1; see also Ellison et al. 2012). Normally, fully air-saturated water at these temperatures and salinity would contain ~7.5 mg L⁻¹ DO; however, mean DO in the crab burrows was <1 mg L⁻¹, a level of oxygen few aquatic species tolerate for long. Earlier studies on K. marmoratus reported that the fish were insensitive to hypoxia alone but emersed when exposed to a combination of elevated H₂S (0.003 mg L⁻¹) and mild hypoxia (2 mg L⁻¹ DO) (Abel et al. 1987). Obviously K. marmoratus are tolerant of extreme water conditions, but emersion may be a convenient escape when environmental conditions are beyond the range of tolerance.

Using a remote video camera, we recorded activity at the surface of a crab burrow in Calabash Caye, Belize (see Supplementary Video). Several mangrove rivulus were observed resting on a small branch at the air–water interface. At the same time, the crab Cardisoma guanhumi rose to the surface from deep
within the burrow and aerated its gills. No doubt aerial respiration for both animals is a routine and possibly necessary daily behavior due to the severely hypoxic water in which they reside. To our knowledge, there have been no studies quantifying the amount of time *K. marmoratus* remains immersed in crab burrows under these conditions. This information would be valuable to assess their tolerance to hypoxia (and H2S) in the field.

We decided to examine the emersion response in more detail in the laboratory to carefully quantify the threshold for breathing air in response to hypoxia. When the oxygen content of water was acutely lowered from normoxia to hypoxia, 50% of *K. marmoratus* emersed at 0.23 mg L\(^{-1}\) DO (Fig. 2) (Regan et al. 2011), an O\(_2\) level similar to that in water in burrows (Fig. 1). These results demonstrate that hypoxia alone induces emersion, in contrast to the findings of Abel et al. (1987). The level of hypoxia used by Abel et al. (2 mg L\(^{-1}\) DO) was well above the threshold for emersion (<0.4 mg L\(^{-1}\) DO) found in the study by Regan et al. (2011). In most air-breathing fish, hypoxia induces air-breathing at higher levels of DO relative to *K. marmoratus* and full emersion is typically unnecessary (Graham 1997; Chapman and McKenzie 2009). Before emersion, *K. marmoratus* appear to spend more time at the surface, but a careful study to characterize aquatic surface respiration (ASR) has not been conducted for this species. ASR is a common behavior in hypoxia-tolerant fish when oxygen is depleted from deeper waters (Chapman and McKenzie 2009). What mechanisms enable *K. marmoratus* to tolerate extreme hypoxia? The answer is unknown. In fish, the short-term critical adjustment to lower levels of oxygen in the water that limit the mismatch between demand and supply of oxygen is the hypoxia ventilatory response (HVR) (Perry et al. 2009). The HVR involves an increase in the rate of ventilation and/or the volume of water pumped over the gills per breath. Mangrove rivulus have a robust HVR, increasing mostly the rate of ventilation rather than the ventilatory amplitude when water DO is lowered to 3–5 mg L\(^{-1}\) (A. Turko et al., submitted for publication).

![Fig. 1](https://example.com/fig1.png) DO levels in three neighboring crab burrows (site 1) on Calabash Caye, Belize, at the end of the wet season (December 2009) over a 24-h period. Note that fully oxygenated water (28°C, 38%) would have a DO level of ~7.5 mg L\(^{-1}\) (P. Wright et al., unpublished data). Means ± S.E. (n = 3).

![Fig. 2](https://example.com/fig2.png) Proportion of *K. marmoratus* immersed in water at low levels of DO (DO; mg L\(^{-1}\)). At lower DO levels (<0.4 mg L\(^{-1}\)), fish emersed and adhered to the side of the experimental chamber (N = 21, EC\(_{50}\) = 0.23 mg L\(^{-1}\) DO). From Regan et al. (2011).
It is also adaptive if fish can increase the rate of perfusion through cardiovascular adjustments during exposure to hypoxia, but there are no data in this regard with respect to K. marmoratus. Longer term resistance to hypoxia in animals has been linked to the hypoxia inducible factor (HIF-1α), a transcription factor that controls the expression of numerous genes controlling angiogenesis, formation of red blood cells, glycolysis, and other pathways (reviewed by Nikinmaa and Rees 2005). Increased hemoglobin levels or changes in the expression of hemoglobin isoforms toward higher-affinity isoforms have been reported (Frey et al. 1998; Rutjes et al. 2007; Campo et al. 2008; Wells 2009), but, again, whether similar adjustments occur in K. marmoratus is unknown. Investigations of HIF-1α expression and downstream effects in response to hypoxia in K. marmoratus are warranted.

**Respiration in air**

The third advantage of studying K. marmoratus is that the mechanisms of cutaneous exchange in air-breathing fishes are not completely understood. Cutaneous respiration is not uncommon in amphibious fishes (Graham 1997; Sayer 2005), but few depend on the skin as the sole site of exchange like K. marmoratus does without accessory air-breathing organs (ABOs). To maintain homeostasis in air, fish must continue to transport O₂ and CO₂, balance ions and water, and prevent the accumulation of potentially toxic nitrogenous wastes. There is a vast body of literature on the structure of ABOs and the respiratory/cardiovascular changes that occur when air-breathing fishes switch from aquatic to aerial respiration (see Hughes 1976; Graham 1997). In addition, detailed work has been carried out more recently on various strategies for nitrogen excretion in air-breathing fish (reviewed by Ip et al. 2001; Sayer 2005; Chew et al. 2006). However, many questions remain. If cutaneous respiration dominates during emersion, does the structure and function of the skin and gills reversibly remodel to accommodate changing roles on land? Over the past decade, researchers in my laboratory have focused on mechanisms responsible for cutaneous exchange and tissue remodeling when K. marmoratus emerse. Highlights from this and other work are reviewed below.

**Morphological plasticity of gills**

For most fish, the gills are the primary site of exchange between the external environment and the blood (internal environment). Gaseous exchange, excretion of nitrogenous wastes, acid–base regulation, and osmoregulation and ionoregulation occur at the gills (Evans et al. 2005), with the cutaneous surface and kidneys playing a minor role in most cases. In the absence of water, gill lamellae (also called secondary lamellae) collapse due to high surface tension, coalesce, and the fused structures reduce the effective surface area for exchange, with irreversible loss of function in most fish (Lam et al. 2006). K. marmoratus may spend minutes to weeks out of water, but gross morphology of the gill when fish are in water (immersed) is typical of other fully aquatic teleosts (Ong et al. 2007). There is no evidence of gill rods, fused lamellae, or widely spaced lamellae typical of some air-breathing fish, such as the mudskipper that partially uses the gills for gaseous exchange in air (e.g. Low et al. 1988; Lam et al. 2006). Also, K. marmoratus do not gulp air or ventilate the opercular chamber when out of water (LeBlanc et al. 2010). Instead, K. marmoratus reversibly remodel their gills in response to exposure to air. A cell mass appears between the lamellae (interlamellar cell mass [ILCM]) which effectively reduces the gill’s surface area after a week in air; after recovery in water, the ILCM degenerates (Fig. 3; Ong et al. 2007; Turko et al. 2011). Reversible changes in morphology of the gills were first reported in the fully aquatic crucian carp (Carassius carassius) and closely related goldfish (C. auratus) with changes in temperature and in oxygen content of the water, a response that is thought to be effective in balancing oxygen uptake with loss of ions in these freshwater species (Sollid et al. 2003, 2005; Nilsson 2007). The physiological value of reduced surface area of the gills in emersed K. marmoratus is unknown. Remodeling of the gills in K. marmoratus may be a mechanism that decreases the risk of desiccation and/or preserves lamellar structure when K. marmoratus are out of water for extended periods (Ong et al. 2007).

**Gaseous exchange**

Metabolic rate does not typically decline when amphibious fish emerse (e.g. Gordon et al. 1969; Gordon et al. 1978; Steeger and Bridges 1995; Kok et al. 1998; Takeda et al. 1999), although in cases when the habitat dries aestivalion is critical for survival (e.g. African lungfish; Janssen 1964). On land, K. marmoratus requires a moist habitat and metabolic rate (as measured by excretion of CO₂) is maintained, or even enhanced, over the first 5 days of exposure to air (Ong et al. 2007). Moreover, mitochondrial oxidative enzymes (plus seven enzymes involved in amino acid metabolism) were unchanged or slightly increased in K. marmoratus held for 10
days in air (Frick and Wright 2002b). These data suggest that cutaneous respiration adequately meets the metabolic needs of *K. marmoratus* at least over the first week or so.

Gaseous exchange may be facilitated during aerial episodes by an increase in cutaneous blood flow. Grizzle and Thiyagarajah (1987) reported that the dorsal epidermis of *K. marmoratus* contains capillaries within 1 μm of the skin’s surface providing a very short diffusion distance between air and blood. In other amphibious species, estimated diffusion distances are slightly greater and vary considerably (2–340 μm) (e.g. Yokoya and Tamura 1992; Graham 1997; Zhang et al. 2000; Park 2002; Park et al. 2003). In *K. marmoratus*, arteriole diameter narrows in vessels of the caudal fin within the first 20 seconds of exposure to air (Cooper et al. 2011), a change that would diminish, not enhance, blood flow. The fact that these changes were reversed upon application of the α-adrenoreceptor blocker phentolamine indicates that this initial vasoconstriction was probably a catecholamine-induced response to stress. Long-term acclimation to a terrestrial environment (10 days) induced cutaneous angiogenesis (Cooper et al. 2011). Using immunohistochemistry and the antibody for the endothelial protein CD31 (Baluk and McDonald 2008), we showed an increased CD31 signal in the caudal vessels of air-exposed *K. marmoratus* (Cooper et al. 2011). These results indicate that the turnover of endothelial cells or the overall number of endothelial cells was increased in air. The fins of *K. marmoratus* constitute ~40% of the body’s surface area (Cooper et al. 2011), and therefore if angiogenesis is restricted to the fins only (but may also be occurring across the total surface of the body), then these changes would potentially have a significant impact on the exchange of gas during prolonged terrestrial episodes.

The skin of *K. marmoratus* appears to play a role in sensing oxygen levels in the environment. In most fish, hypoxia is detected by chemoreceptive neuroepithelial cells (NECs) in the gills (Dunel-Erb et al. 1982; Jonz and Nurse 2003; Saltys et al. 2006). Gill NECs are positioned externally (where they are in contact with the incident flow of water and may detect aquatic hypoxia) or internally (where they respond to changes in oxygen levels of the blood) (Perry et al. 2009). In some air-breathing fish, gill NECs mediate hypoxia-induced air-breathing at the surface (Smatresk 1986; Shingles et al. 2005; Lopes et al. 2010). In *K. marmoratus*, NECs were found in the gill and over the entire cutaneous surface, occupying the uppermost epithelial layer (Regan et al. 2011). NECs of both the skin and gills increase

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**Fig. 3** Representative light micrographs of gill filaments and lamellae of a control *K. marmoratus* in water (A), a fish exposed to air for 1 week (B), and a fish recovered in water for 1 week after a week in air (C). Scale bar = 50 μm. From Ong et al. (2007).
cell area in response to chronic hypoxia, and pharmacological studies suggest that they may be involved in regulating the response to emersion in *K. marmoratus* (Regan et al. 2011). Further studies are required to determine whether cutaneous sensing of oxygen is ubiquitous in air-breathing fishes.

**Ionoregulation and osmoregulation**

As mangrove rivulus move from water onto land, they must maintain homeostasis of water and ions without the use of branchial epithelium and possibly, under some circumstances, in an environment with <100% humidity. Variations in salinity commonly occur in mangroves, both spatially (freshwater or brackish streams flowing over mudflats) and temporally (heavy rainfalls versus dry season) (Gordon et al. 1985). Even when fish leave water, the salinity of the moist substratum would presumably vary depending on these same factors. Given these challenges, *K. marmoratus* managed to maintain perfect whole-body homeostasis of Cl⁻ and water but not Na⁺ balance over a 9-day period of exposure to air in the laboratory (LeBlanc et al. 2010).

Ion-transporting cells (ionocytes) in the skin have been reported in some amphibious fish but not in others (Schwerdtfeger and Bereiter-Hahn 1978; King et al. 1989; Yokoya and Tamura 1992). In *K. marmoratus*, a rich population of ionocytes are present on the cutaneous surface (Fig. 4A) as well as on the gills (Fig. 4B). Skin ionocytes form clusters of 20–30 cells and the physiological significance of this pattern is unknown. Is the clustering synchronous with the discontinuous subepidermal scales and/or with the coordinated signaling pathway Delta/Jagged-Notch that regulates cell clustering in zebrafish embryos? (Hsiao et al. 2007; Jänicke et al. 2007) Further studies are required to understand the functional role of these clusters in *K. marmoratus*.

Cutaneous ionocytes are probably the key site of ion transport when *K. marmoratus* are emersed. The surface area of cutaneous ionocytes was larger in air-exposed rivulus in contact with a moist hypersaline (45%) compared with a freshwater (1%) surface, suggesting that a larger surface area of cells would be beneficial for ionoregulation at the higher salinity (LeBlanc et al. 2010). The number of mucous cells declined significantly in air-exposed rivulus (45% substrate), indicating that increased mucus production in *K. marmoratus* is probably not a strategy that protects body tissues from dehydration in air, unlike the case for other air-breathing fish (Lam et al. 2006). Do other structural changes occur in the skin that retain body water but exchange critical molecules during prolonged exposure to air? There are many avenues for further investigation.

**Volatilization of NH₃**

Animals catabolize proteins and amino acids for fuel and maintenance/turnover of body proteins, resulting in the synthesis of ammonia (Wright 1995). Ammonia is a neurotoxin and elevated concentrations of ammonia in the tissues are harmful to fish (reviewed by Ip et al. 2001). Ammonia exists in solution as both the dissolved gas NH₃ and the ion NH₄⁺, although at physiological pH the equilibrium is shifted toward NH₄⁺ (pKₐm = 9). Normally, fish excrete ammonia as NH₃ down the gill’s blood-to-water NH₃ partial pressure gradient by
way of ammonia gas channels, the Rhesus (Rh) glycoproteins (for reviews see Weihrauch et al. 2009; Wright and Wood 2009). Diffusion of ammonia across the gills and its dilution in the aquatic environment prevents its accumulation in the tissues, but elevated environmental levels or exposure to air may prevent efficient elimination of waste nitrogen.

When *K. marmoratus* are out of water, accumulation of ammonia is avoided by its excretion through alternative routes (see below) or its conversion to less toxic compounds (e.g. urea, glutamine). Urea is a byproduct of arginine catabolism, uric acid degradation, or, in a few unusual cases in fish, the end product of the ornithine urea cycle (OUC) (Anderson 2001). The percent of nitrogen excreted as urea in tropical amphibious fishes varies between 3 and 58% (Graham 1997), with mangrove rivulus excreting 10–40% as urea (Frick and Wright 2002a; Rodela and Wright, 2006a,b). *K. marmoratus* do not appear to synthesize urea via the OUC because activities of OUC enzymes were relatively low and only modestly increased in response to exposure to air (Frick and Wright 2002b). Thus, urea production in *K. marmoratus* is likely the result of a combination of arginolysis and uricolyis.

Our work shows that in air, *K. marmoratus* are one of the rare teleosts that volatilize a significant amount of NH₃ from the cutaneous surface (Tsui et al. 2002; Frick and Wright 2002b; Litwiller et al. 2006). An 18-fold increase in NH₄⁺ concentration and a small elevation of pH at the skin’s surface result in a substantial rise in cutaneous partial pressure of NH₃ (Litwiller et al. 2006). These changes in the ion composition of the surface of the skin may be related partly to changes in the rate of transport of NH₄⁺/NH₃ and H⁺ (C. Cooper et al., submitted for publication). An induction of Rh glycoproteins Rhcg1 and Rhcg2 mRNA in air may translate into an increase in apical Rhcg proteins in cutaneous ionocytes (Hung et al. 2007; Wright and Wood 2009), facilitating transfer of NH₃ to the skin’s surface. Overall, *K. marmoratus* are highly efficient in eliminating ammonia when emersed because over a 10-day period there was no significant accumulation of whole-body ammonia (Frick and Wright 2002b).

**Perspectives and Conclusions**

Mangrove rivulus are extremophilic fish that breathe cutaneously when emersed. They are an ideal model species for examining the specific mechanisms of cutaneous exchange that air-breathing fish use to maintain homeostasis over long periods of exposure to air. Our studies have demonstrated that the skin of *K. marmoratus* shares responsibility for gaseous exchange (Ong et al. 2007; Regan et al. 2011), ionoregulation (LeBlanc et al. 2010), and excretion of nitrogenous wastes (Frick and Wright 2002a; Litwiller et al. 2006; Hung et al. 2007; Wright and Wood 2009) with the gills when immersed but plays a more dominant role when fish emerse. Angiogenesis in the fins could potentially increase blood flow and enhance the capacity for cutaneous exchange in air (Cooper et al. 2011). Moreover, there is a strong potential for further insights into regulatory pathways by identifying differences among strains in responses to environmental perturbations using isotopic lineages of laboratory-reared *K. marmoratus*.

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**Supplementary Data**

Supplementary Data are available at *ICB* online.

**References**


