

Gill morphology of the mangrove killifish (*Kryptolebias marmoratus*) is plastic and changes in response to terrestrial air exposure

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Summary

Amphibious mangrove killifish, *Kryptolebias marmoratus* (formerly *Rivulus marmoratus*), are frequently exposed to aerial conditions in their natural environment. We tested the hypothesis that gill structure is plastic and that metabolic rate is maintained in response to air exposure. During air exposure, when gills are no longer functional, we predicted that gill surface area would decrease. In the first experiment, *K. marmoratus* were exposed to either water (control) or air for 1 h, 1 day, 1 week, or 1 week followed by a return to water for 1 week (recovery). Scanning electron micrographs (SEM) and light micrographs of gill sections were taken, and morphometric analyses of lamellar width, lamellar length and interlamellar cell mass (ILCM) height were performed. Following 1 week of air exposure, SEM indicated that there was a decrease in lamellar surface area. Morphometric analysis of light micrographs revealed that there were significant changes in the height of the ILCM, but there were no significant differences in lamellae width and length between any of the treatments.

Following 1 week of recovery in water, the ILCM regressed and gill lamellae were similar to control fish, indicating that the morphological changes were reversible. In the second experiment, \dot{V}_{CO_2} was measured in fish continuously over a 5-day period in air and compared with previous measurements of oxygen uptake (\dot{V}_{O_2}) in water. \dot{V}_{CO_2} varied between 6 and 10 $\mu\text{mol g}^{-1} \text{h}^{-1}$ and was significantly higher on days 3, 4 and 5 relative to days 1 and 2. In contrast to \dot{V}_{O_2} in water, \dot{V}_{CO_2} in air showed no diurnal rhythm over a 24 h period. These findings indicate that *K. marmoratus* remodel their gill structures in response to air exposure and that these changes are completely reversible. Furthermore, over a similar time frame, changes in \dot{V}_{CO_2} indicate that metabolic rate is maintained at a rate comparable to that of fish in water, underlying the remarkable ability of *K. marmoratus* to thrive in both aquatic and terrestrial habitats.

Key words: metabolic rate, CO_2 excretion, emersion, gill lamellae, interlamellar cell mass.

Introduction

The mangrove killifish, *Kryptolebias marmoratus*, lives in tropical mangrove forests in Florida, the Caribbean, Central and South America. These fish are the only known self-fertilizing vertebrate hermaphrodite (Harrington, 1961), although there are true males and therefore sexual reproduction occurs in some natural populations (Mackiewicz et al., 2006). They are considered amphibious fish because they survive in both aquatic and terrestrial habitats (Sayer, 2005). *K. marmoratus* have a remarkable tolerance to a wide range of aquatic extremes and can endure over one month of exposure to air (emersion) when among moist detritus or leaf litter (Abel et al., 1987). *K. marmoratus* leave their aquatic environment for varying periods of time in response to aggression between fish (Huehner et al., 1985; Taylor, 1990), as well as in response to environmental stressors, such as high hydrogen sulfide concentrations (Abel et al., 1987; Taylor, 1990), low water temperature (Huehner et al., 1985) or as a result of constant

flux between drought and flooding in the areas they inhabit (Harrington, 1961). Additionally, they may leave the water for short periods of time in order to catch termites on land and then return immediately to eat their prey underwater (Huehner et al., 1985).

In water, most fish rely primarily on gills for gas exchange (Evans et al., 2005). During air exposure, the gills are no longer perfused with water and will collapse if there are no specialized structural modifications. Respiratory adaptations that allow amphibious fishes to live in both terrestrial and aquatic environments include specialized lungs and gas bladders (air breathing organ, ABO), as well as modifications of existing structures, such as the gills and skin (Graham, 1997). The amphibious gourami, *Trichogaster trichopterus*, depends mainly on the labyrinth organs in the suprabranchial chamber for respiration during periods of aerial exposure (Burggren, 1979). Observation of an increased capillary network in the gut of the Chilean clingfish, *Sicyases sanguineus*, after 24 h of

emersion suggests that this fish respire *via* intestinal respiration (Marusic et al., 1981). Cutaneous modifications are present in many different amphibious fish (Park et al., 2003), such as the mudskipper *Periophthalmus magnuspinnatus*, in which an extensive capillary network lies close to the surface of the skin and the middle layer of epidermis contains modified epidermal cells that are thought to facilitate oxygen uptake (Park, 2002).

The cutaneous surface is probably a site of respiration in *K. marmoratus* because the epidermis is relatively thin and there is a high density of capillaries near the surface (Grizzle and Thiyagarajah, 1987). During 11 days of air exposure, a significant amount (>40%) of ammonia is released by NH_3 volatilization (Frick and Wright, 2002a). The site of gaseous excretion is likely the skin because both NH_4^+ concentration and pH on the cutaneous surface increase significantly after air exposure (Litwiller et al., 2006). Furthermore, the number of cutaneous vessels perfused on certain areas of the dorsal surface of *K. marmoratus* increases significantly after 30 min of air exposure (S. Litwiller, P.A.W. and C. Murrant, manuscript in preparation). Taken together, these studies suggest that in the absence of functional gills and an ABO, *K. marmoratus* rely on the skin as the major respiratory surface.

Changes in gill morphology have been observed in other teleost fish in response to developmental changes or environmental stressors. For example, in the obligate air breather *Arapaima gigas*, the defined lamellae of the water-breathing juveniles regress and the filaments become smooth columns as they mature and become obligate air breathers (Brauner et al., 2004). The changes in *A. gigas* gills are long term and not reversible. By contrast, the secondary lamellae of the crucian carp, *Carassius carassius*, become much more defined in response to hypoxic conditions (Sollid et al., 2003), and similar changes have been observed in both *C. carassius* and in *C. auratus* in response to warmer water temperatures (Sollid et al., 2005). Exposure to hypoxia induced apoptosis of *C. carassius* gills in between the lamellae (the interlamellar cell mass, or ILCM), thus causing the lamellae to protrude and increase the surface area for gas exchange (Sollid et al., 2003). These changes were completely reversible when *C. carassius* were returned to normoxic water. Hence, gill morphology is plastic in two *Carassius* species in response to temperature and water oxygenation. Do similar changes occur in other fish species in response to a variety of environmental perturbations? In particular, is gill morphology plastic in *K. marmoratus*, a species that tolerates prolonged air exposure?

When *K. marmoratus* are exposed to air they do not appear to aestivate; they remain responsive (K.J.O., personal observation) and there is little change in aerobic enzyme activities (Frick and Wright, 2002a), suggesting that metabolic rate is not depressed as in prolonged emersion in lungfish (Smith, 1930). Graham reviewed the effects of air-exposure on oxygen uptake in air-breathing fish and concluded that the active amphibious species generally maintain oxygen uptake when exposed to air (Graham, 1997). Many of these species

have specialized structures for air breathing, whereas *K. marmoratus* appear to be solely dependent on the passive exchange of gases across the cutaneous surface.

We tested two hypotheses. First, we hypothesized that mangrove killifish gills are plastic and will undergo reversible change when exposed to air. We predicted that the gill surface area would decrease in air and that a return to water would reverse any changes. Scanning electron microscopy and light microscopy techniques were used to document morphological changes in the gills of *K. marmoratus* associated with air exposure for 1 h, 1 day, 1 week and following 1 week of recovery in water. Second, we hypothesized that metabolic rate is maintained during air exposure. If true, we predicted that carbon dioxide excretion (\dot{V}_{CO_2}) would remain unchanged over time and be similar to previously measured values in control killifish in water. *K. marmoratus* were exposed to air for 5 days and \dot{V}_{CO_2} was measured continuously.

Materials and methods

Experimental animals

Kryptolebias marmoratus Poey (hermaphrodites) were held in individual containers at the Hagen Aqualab, University of Guelph under conditions simulating their natural environment (25°C, 16‰, pH 8, 12 h:12 h light:dark cycle) (Frick and Wright, 2002b). Adult killifish used in metabolic rate experiments weighed between 0.07 and 0.16 g, and in microscopy experiments weighed between 0.07 and 0.12 g. Feeding and cleaning regimes were as described by Litwiller et al. (Litwiller et al., 2006).

Metabolic rate

Two days prior to experimentation, fish were placed in the respirometry chambers in water, and food was withheld to conform with previous measurements of oxygen uptake in water (Rodela and Wright, 2006). Fourteen fish were exposed to air for 3 days ($N=14$); for seven of these fish, the exposure continued for 5 days ($N=7$). Fish were not fed in the chamber, but the chamber was opened once or twice each day to moisten the filter paper with 16‰ seawater. Lights were turned on at 08.00 h and off at 20.00 h daily. Temperature was maintained at $25\pm 0.5^\circ\text{C}$ by placing the metabolic chambers on an aluminum water jacket connected to a water bath. Temperature was measured with a thermocouple in the blank chamber.

The air source was filtered external air that was scrubbed to remove CO_2 and humidified to prevent desiccation of the fish. The scrubbed and humidified air was pumped through flow control valves and then into all four chambers (three containing fish, one serving as a blank). The chambers were multiplexed so that the outflow of one went through an ice bath and Drierite® column to remove any water and then to an infrared CO_2 meter (Qubit S151; Qubit Systems, Kingston, ON, Canada). A gas switcher (Qubit G243) switched flow between a fish chamber and the blank chamber every 30 min. Flow was

set to approximately $25 \text{ ml air min}^{-1}$, but the exact flow rate was recorded continuously in all chambers with high-accuracy low flow meters (Qubit G249). The analyzer was calibrated daily using scrubbed gas as zero, and a single high point using a calibrated gas containing a known concentration of CO_2 (1500 p.p.m.), balanced with nitrogen.

Metabolic rate was calculated using the Fick equation:

$$\dot{V}_{\text{CO}_2} = \text{flow} \cdot (\text{CO}_2 \text{ out} - \text{CO}_2 \text{ in}) \cdot (60) / (22.4 \cdot 1000 \cdot m)$$

where \dot{V}_{CO_2} is calculated in $\mu\text{mol CO}_2 \text{ g}^{-1} \text{ h}^{-1}$, flow (ml air min^{-1}) was calculated as the integral of the flow rate through the chamber for the 15 min period when CO_2 levels reached steady-state, $\text{CO}_2 \text{ out}$ = plateau value of CO_2 leaving the chamber containing a fish (p.p.m.), $\text{CO}_2 \text{ in}$ = plateau value of CO_2 leaving the blank chamber (p.p.m.) taken as the average of the value before and after the measurement for each fish, 60 to convert min to h, 22.4 to convert μl to μmol , 1000 to convert ml to l, m is body mass in g.

Experimental protocol for gill structure

Five groups of fish were exposed to either control (immersed) or experimental (emersed) conditions. Control fish (time 0 h) were directly removed from 100 ml plastic chambers (containing 60 ml of 16‰ water), immediately euthanized by spinal cord transection and placed into fixative (see below). Fish exposed to air were placed in 100 ml chambers. Three cotton balls were placed at the bottom of each chamber and a piece of filter paper, cut to fit snugly into the bottom of the chamber, was placed on top of the cotton. Water (10 ml, 16‰) was pipetted onto the filter paper and allowed to soak in evenly. This provided some moisture but did not allow immersion of gills in water. After experimental treatments of 1 h, 1 day and 1 week of air exposure, fish were euthanized and immediately fixed. An additional group of fish, a recovery group, was exposed to air for one week, returned to water (60 ml, 16‰) for a further week, euthanized and fixed.

Scanning electron microscopy

Fish heads were fixed in 1% glutaraldehyde, 1% paraformaldehyde with 16‰ salt water. The left gill arch of fixed fish heads was excised 48 h later. Gills were post fixed in 1% OsO_4 , dehydrated in a series of graded ethanols (50%, 70%, 80%, 90% and three rounds of 100%) and then dried with a critical point drier (custom made at the Physics Workshop, University of Guelph). Samples were then mounted on carbon tape and sputter coated in 30 nm gold with an Emitech K550 Sputter Coater (Ashford, Kent, UK). A Hitachi S-570 Scanning Electron Microscope (Tokyo, Japan) was used to capture micrographs of the gills.

Light microscopy

After 24 h of immersion in 10% phosphate-buffered formalin fixative, the left operculum was cut away and the second gill arch was extracted and then routinely processed for paraffin embedding. The gill arches were serially sectioned in

$4 \mu\text{m}$ increments and then stained with hematoxylin and eosin. The slides were viewed using an Olympus BX60 light microscope (Tokyo, Japan), and images were recorded using Image Pro Plus 5.1 (Media Cybernetics Inc., Silver Spring, MD, USA).

Morphometry

Measurements of lamellar width, lamellar length and height of ILCM were performed for each fish (Fig. 1). Width of lamellae was measured parallel to the filament at the base of the lamellae from one edge to the other. Lamellar length was measured from the edge adjacent to the filament to the most distal point of the lamellae from the filament. Height of ILCM was measured parallel to the total lamellar length, starting from the edge of the ILCM bordering the filament to the most distal edge of the ILCM from the filament.

Statistical analysis

Changes in mean metabolic rate over the period of air exposure were analyzed using analysis of covariance (ANCOVA) (with body mass as the covariate) and Tukey's tests. For gill morphometrics, a one-way ANOVA was used to compare differences between treatments with a significance level of $P < 0.05$. If significance was found, a Tukey's test was used to estimate where the significant differences occurred. A $P < 0.05$ was deemed significant.

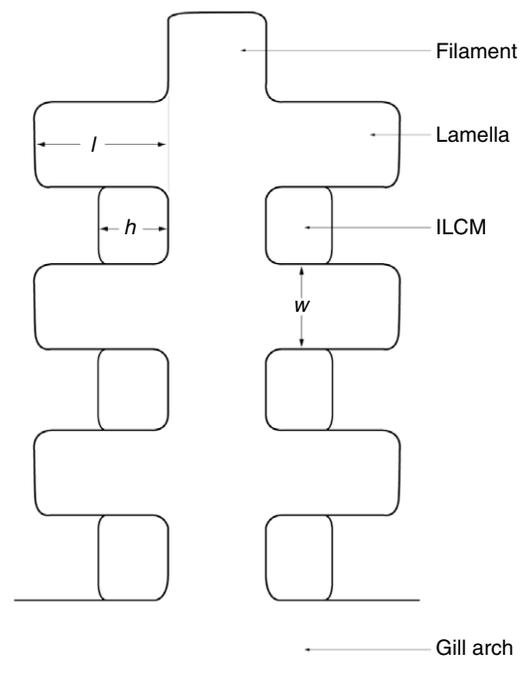


Fig. 1. Schematic diagram of one filament from *K. marmoratus* indicating the filament, secondary lamellae, interlamellar cell mass (ILCM) and gill arch. Measures (μm) of total lamellar length (l), ILCM height (h) and lamellar width (w) are indicated.

Results

Metabolic rate

\dot{V}_{CO_2} values varied between 6 and 10 $\mu\text{mol CO}_2 \text{ g}^{-1} \text{ h}^{-1}$ in fish exposed to air for 5 days (Fig. 2A). Mean metabolic rate of the 14 fish was $8.02 \pm 0.75 \mu\text{mol CO}_2 \text{ g}^{-1} \text{ h}^{-1}$ and decreased with an increase in body mass (ANCOVA, $F_{1,237}=47.92$, $P<0.001$).

\dot{V}_{CO_2} increased with air-exposure time over a five-day period (ANCOVA, $F_{4,237}=13.16$, $P=0.000$) (Fig. 2A). Tukey's tests revealed that there were no significant differences between metabolic rates on days 1 and 2 or between days 3, 4 and 5. However, metabolic rate on both days 1 and 2 was significantly less than on days 3, 4 or 5 ($P<0.05$). When hourly rates were averaged from different days, metabolic rate did not change significantly with time of day (Fig. 2B) (ANCOVA, $F_{22,237}=0.28$, $P=1.00$).

Gill structure

Scanning electron micrographs revealed marked differences in morphological appearance between control gills and gills emersed for 1 week (Fig. 3). The lamellae of the control fish

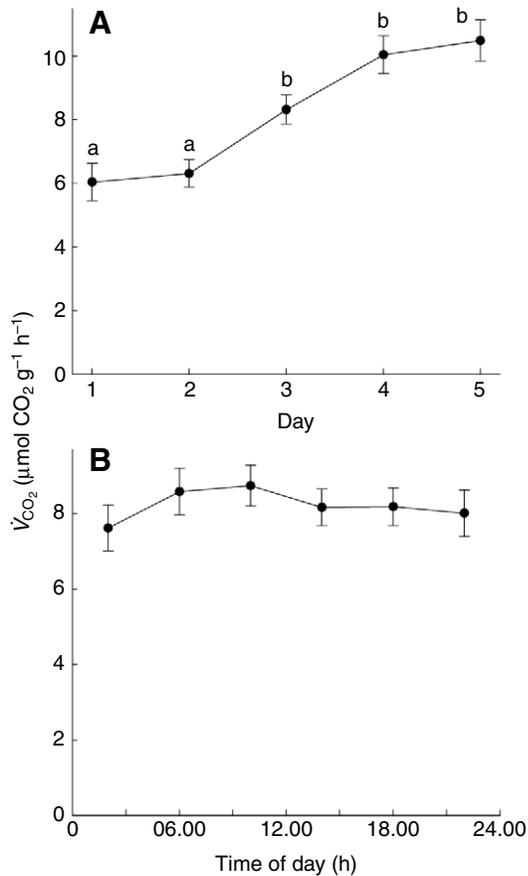


Fig. 2. Metabolic rate ($\mu\text{mol CO}_2 \text{ g}^{-1} \text{ h}^{-1}$) of *K. marmoratus* exposed to air: (A) over a 5-day period and (B) over 24 h. In B, hourly rates were averaged from different days. Values represent means \pm s.e.m. (days 1, 2, 3 $N=14$; days 4, 5 $N=7$). Values with different letters are significantly different ($P<0.05$).

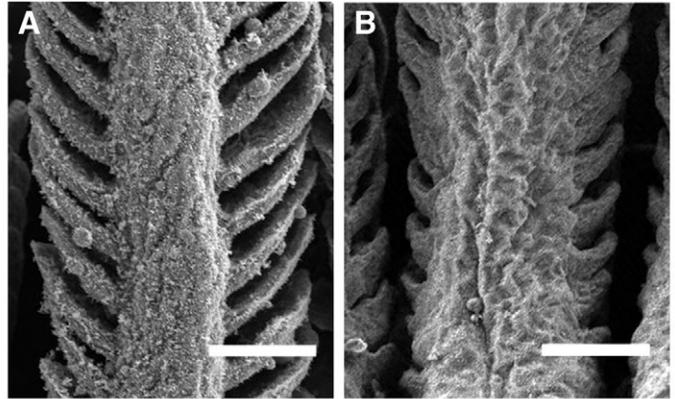


Fig. 3. Scanning electron micrographs of gill filaments from *K. marmoratus* in (A) control conditions in 16‰ seawater and (B) after 1 week of air exposure. Scale bars, 40 μm .

were defined, not fused together, and had a relatively large surface area for exchange with water (Fig. 3A). Conversely, the lamellae of the fish exposed to air for one week appeared to be shorter with a decreased surface area (Fig. 3B). Similarly, light micrographs indicated that the lamellae became more embedded (i.e. less surface area was exposed to the air as a result of ILCM growth) with an increase in the time exposed to air (Fig. 4). Most samples exhibited an intermediate pattern where lamellae were partially embedded after 1 week of air exposure (Fig. 4D), but in one of the fish sampled the lamellae were completely embedded (Fig. 4E). After 1 week of recovery in water, the gill lamellae appeared very similar to control fish (Fig. 4F).

Mean lamellar widths from each treatment were not significantly different ($P>0.05$) and ranged from 4.5 to 5.1 μm (Fig. 5A). Analysis of the lamellar length from light micrographs revealed no significant differences ($P>0.05$) between fish in any treatment (Fig. 5B). Short-term exposure to air (1 h and 1 day) did not yield significant changes in the height of the ILCM ($P>0.05$), but after 1 week of air exposure there was a significant increase ($P<0.05$, $N=6$) (Fig. 5C). After 1 week of recovery in water, there was a significant decrease ($P<0.05$) in ILCM height compared with air-exposed fish (1 week), and the ILCM height was not significantly different ($P<0.05$) from control values (Fig. 5C).

Discussion

K. marmoratus is a fish that constantly undergoes changes of habitat (Davis et al., 1990). The most dramatic change is no doubt the move from an aquatic to a terrestrial habitat. In normal aquatic conditions, the gills of *K. marmoratus* did not exhibit any general external features unique to many air-breathing and amphibious fish (see below). The ILCM did not fill the areas between the secondary lamellae in aquatic conditions. *K. marmoratus* gills in water were organized in a fashion typical of aquatic teleosts with relatively long, thin secondary lamellae projecting from the filament. The move to

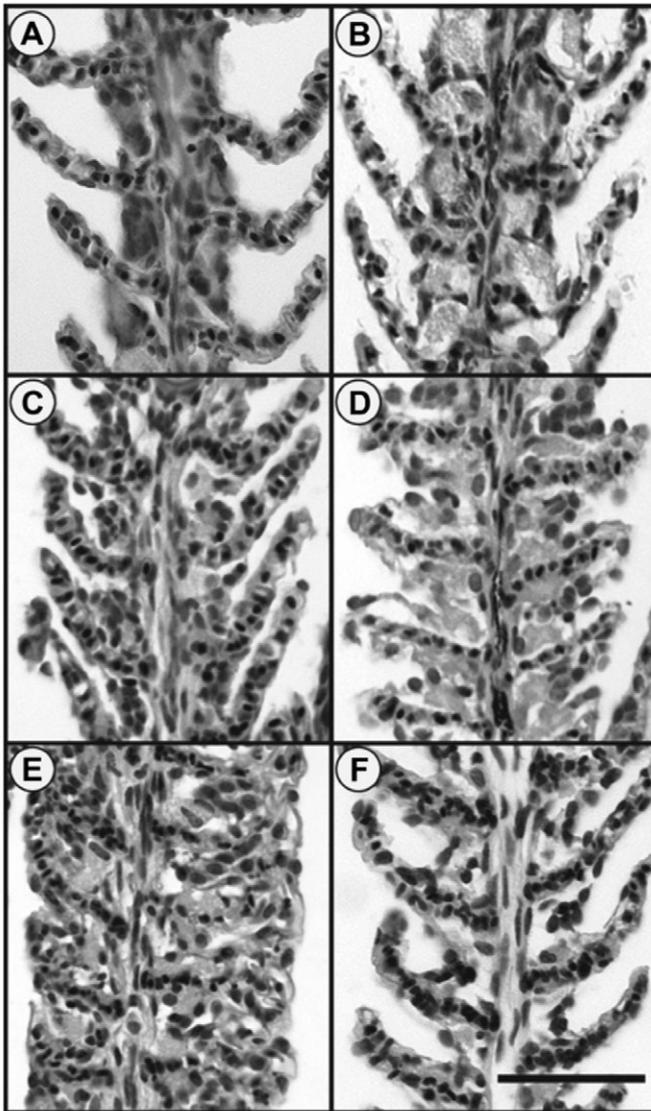


Fig. 4. Light micrographs of gill filaments of *K. marmoratus* from (A) control conditions in water, (B) 1 h of air exposure, (C) 1 day of air exposure, (D,E) 1 week of air exposure and (F) 1 week of air exposure followed by 1 week of recovery in water. Experimental treatments for the two fish shown in D and E are identical, but in D the interlamellar cell mass (ILCM) partially fills the channel between secondary lamellae, and in E the ILCM completely fills this same space. Scale bar, 40 μm .

a terrestrial habitat induced morphological changes in the gills that were reversible. The embedding of the lamellae *via* ILCM growth during terrestrial exposure may serve to protect lamellae from collapse, aid the fish in aerial respiration or possibly prevent desiccation.

Many amphibious fish have developed structural modifications in their gills to prevent lamellar collapse when emersed. Tamura et al. reported that the mudskipper *Boleophthalmus chinensis* has short, widely spaced lamellae in order to reduce coalescence during emersion (Tamura et al., 1976). The gills of *Mnierpes macrocephalus* are enlarged, thick

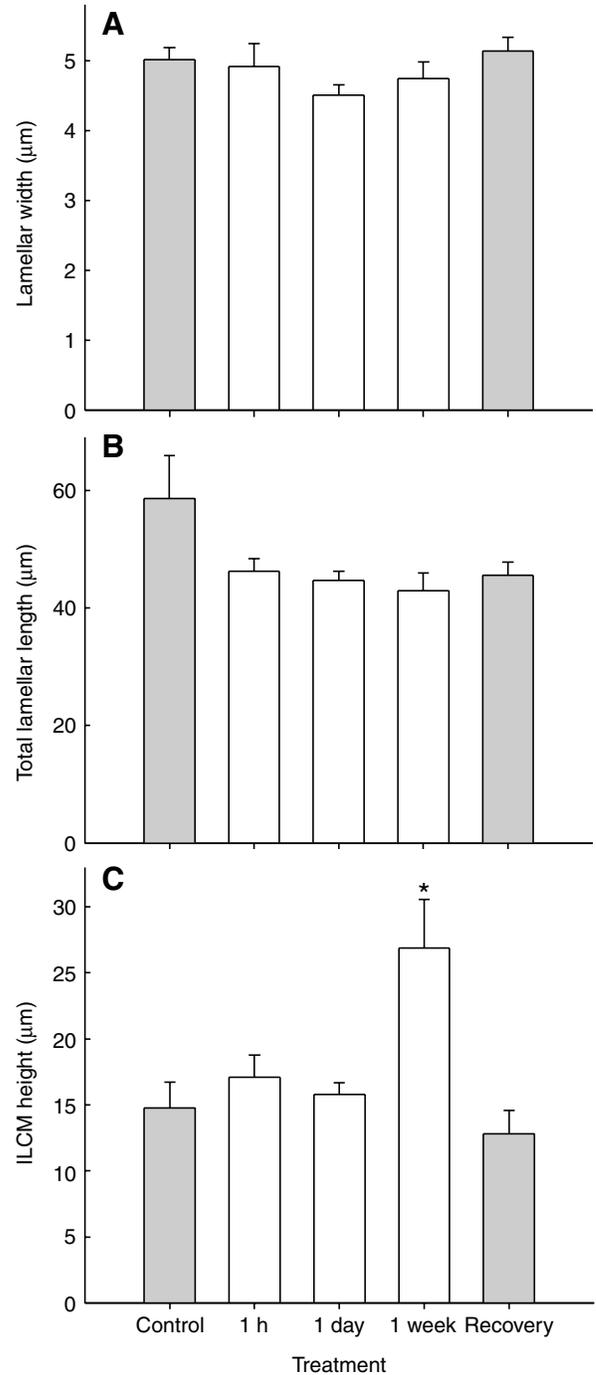


Fig. 5. Effect of water and air exposure treatments on: (A) lamellar width, (B) total lamellar length and (C) interlamellar cell mass (ILCM) height. Values are mean \pm s.e.m. ($N=6$). Dark bars indicate immersion, light bars indicate emersion. Significantly different ($P<0.05$) values are indicated with an asterisk (*).

and long, which prevents their collapse in air (Graham, 1970). The gills of the mudskipper *Periophthalmodon schlosseri* have permanent fusions between the lamellae in order to prevent collapse, and their gills have been found to be better adapted for air breathing than for water breathing (Kok et al., 1998;

Wilson et al., 1999). In *K. marmoratus* exposed to air, there was no change in lamellar width, indicating that thickened lamellae are not a strategy adopted to prevent lamellar collapse. However, we did observe growth of the ILCM in air-exposed killifish, which may serve to provide structural support. Although significant structural changes were not detected until 1 week of emersion, more subtle changes in the ILCM may have helped prevent collapse and coalescing of the secondary lamellae in the first few hours to days of air exposure. Alternatively, the ILCM growth during air exposure may have helped to prevent water loss across the gills. Water conservation is of prime importance to *K. marmoratus* because death occurs after only a few hours in air in the lab if the substratum is dry (P.A.W., personal observation) and in the field emersed fish aggregate, which is thought to be a mechanism to reduce water loss (Taylor, 2000).

The length of time exposed to air varies in nature depending on circumstance. Periods of drought can leave fish stranded on land for over a month, whereas terrestrial forays in search of food can last mere minutes. Although there is some evidence that cutaneous respiration may be the primary mode of respiration in air (see Introduction), we cannot rule out the possibility that the gills may be involved in aerial respiration. Much like *P. schlosseri*, *K. marmoratus* may partially use their gills for respiration when in air; the growth of the ILCM between the lamellae may serve to separate the lamellae so that they can function as respiratory structures (Sayer, 2005). The use of both skin and gills in respiration occurs in some amphibious fish, for example *Periophthalmus cantonensis* and *Boleophthalmus chinensis* (Tamura et al., 1976). Careful observations of buccal and opercular movements in air are necessary to establish if, indeed, branchial respiration occurs.

The difficulty of distinguishing nuclei in the light micrographs made it impossible to tell whether the growth of the ILCM was due to hypertrophy or hyperplasia. Hypertrophy is a more energy-efficient method of increasing size than hyperplasia because it does not involve cell duplication (Cheek and Hill, 1970; Overgaard et al., 2002). A reduced energy intake, as in the case of the mangrove killifish during air exposure, compromises the nuclear division necessary for hyperplasia, but not necessarily for hypertrophy (Cheek and Hill, 1970). We do not know what type of cells comprise the ILCM, nor whether hyperplasia or hypertrophy is involved in the ILCM growth. However, there was an increase in \dot{V}_{CO_2} after several days in air, which may or may not be linked partly to the gill remodeling (see below).

In air-exposed *K. marmoratus*, CO_2 excretion was measured instead of O_2 uptake because it is a more precise measure. The respiratory exchange ratio (CO_2 released per O_2 consumed) usually varies between 0.7 and 0.9 in amphibious air-breathing fish (Bridges, 1988; Martin, 1993; Graham, 1997). Using a respiratory exchange ratio of 0.8 and our \dot{V}_{CO_2} values of *K. marmoratus* in air (6–10 $\mu\text{mol g}^{-1} \text{h}^{-1}$), the oxygen uptake in air is estimated to be between 7.5 and 12.5 $\mu\text{mol g}^{-1} \text{h}^{-1}$. Rodela and Wright reported that \dot{V}_{O_2} in

water ranged from 8 $\mu\text{mol g}^{-1} \text{h}^{-1}$ (nighttime, inactive period) to 22 $\mu\text{mol g}^{-1} \text{h}^{-1}$ (daytime, active period) in *K. marmoratus* (Rodela and Wright, 2006), values similar to or slightly higher than our estimated oxygen uptake in air. The fact that our values in air correspond to the previously measured nighttime values in water is most likely a result of the observed quiescence when the fish were exposed to air, as well as their unfed state.

Other amphibious marine fish, such as *Oligocottus snyderi*, *Clinocottus globiceps* and *Anoplarchus pupurescens*, have equivalent oxygen uptake rates in both air and water (Bridges, 1988), whereas *Ascelichthys rhodorus* and *Oligocottus maculosus* have a decreased oxygen uptake when exposed to air (Yoshiyama and Cech, 1994). The salt marsh killifish *Fundulus heteroclitus* also undergoes a significant decrease in oxygen uptake upon aerial emergence (Halpin and Martin, 1999).

Over a 24 h period of air exposure, there were no variations in \dot{V}_{CO_2} in *K. marmoratus*. This finding contrasts with the results of our previous study of \dot{V}_{O_2} in control *K. marmoratus* in water (Rodela and Wright, 2006). Over a 3-day period, \dot{V}_{O_2} consistently peaked at midday and decreased to the lowest rate midway through the night. The lack of a diurnal pattern in air-exposed *K. marmoratus* in the present study is likely due to inactivity during emersion. Despite no fluctuations of daily \dot{V}_{O_2} when emersed, there was an increase in \dot{V}_{CO_2} after two days of emersion in *K. marmoratus*. Gordon et al. observed a similar increase in small Chilean clingfish, *Sicyases sanguineus*, over 13 h but could not provide an explanation for this rise in oxygen uptake (Gordon et al., 1970). We speculate that the increase of metabolic rate over time is due to a complex series of changes, possibly including repaying an oxygen debt, alterations in biochemical pathways, cutaneous structures and/or gill morphology.

Our study is the first to show that the gills of *K. marmoratus* are plastic and are capable of undergoing reversible changes when emersed and then returned to water. We suggest that the growth of the ILCM may prevent the lamellae from coalescing (which could render the gills non-functional when returned to water), facilitate branchial aerial respiration or have another function such as resistance against desiccation. Over the period of time of gill remodeling in air, metabolic rate is maintained at a rate similar to that of fish in aquatic conditions. Hence, *K. marmoratus* are supremely adapted to the challenges of respiring in air, explaining in part why they tolerate weeks of air exposure.

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