



## Behaviour drives morphology: voluntary emersion patterns shape gill structure in genetically identical mangrove rivulus

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The self-fertilizing mangrove rivulus, *Kryptolebias marmoratus*, can produce homozygous 'clonal' offspring and are highly tolerant of severe environmental conditions, including air exposure (emersion) for weeks at a time. We tested the following hypotheses: (1) individual fish that voluntarily emerse more possess gill and skin features better suited for life on land than fish that emerse less often, and (2) individual differences in emersion tendencies cause these morphological changes. We predicted that individuals spending more time in air would have a reduced gill surface area (shorter, thicker gill lamellae and well developed interlamellar cell masses (ILCM)) and a thicker cutaneous epidermis compared to fish preferring to remain in water. These differences were predicted to disappear if fish were prevented from emersing and predicted to reappear if fish were once again allowed to emerse. Fish were videorecorded for 7 days while voluntarily moving between aquatic and terrestrial habitats to determine individual emersion tendencies. We prevented a subset of fish from emersing for 7 days, and then allowed a subset of these fish to emerse for a final 7 days. We found that individual fish spent anywhere from 0 to 78% of the time emersing. Emersion time was positively correlated with gill ILCM height, but not with any other morphological feature. There was no relationship between ILCM height and emersion time after fish were prevented from emersing for 7 days, but this relationship reappeared when fish were once again able to emerse. These results indicate that genetically identical *K. marmoratus* show highly variable behavioural phenotypes that influence gill remodelling. Fish that voluntarily spend more time emersed reduce gill surface area, a modification that may limit branchial water loss and provide support for gill lamellae. This is the first report of respiratory morphologies linked to variation in behavioural phenotype.

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Individuals of a given species possess variable morphological and behavioural phenotypes (Darwin 1859). Genetic and environmental factors interact to generate this variation, although developmental stochasticity may also play a role (e.g. Vogt et al. 2008). Phenotypic plasticity, the result of genotype by environment interactions, can occur in response to acute or chronic environmental conditions, and produce reversible or irreversible changes in phenotype (Scheiner 1993; West-Eberhard 2003; DeWitt & Scheiner 2004). Plasticity can occur at any level of the phenotype (e.g. behaviour, physiology, morphology), and expressions of plasticity across these scales are often correlated (Bolnick et al. 2003). The range of plasticity expressed among taxa varies widely, possibly because of the many costs associated with the expression

of alternative phenotypes (DeWitt et al. 1998; Auld et al. 2010). For this reason it has been hypothesized that organisms that inhabit heterogeneous or fluctuating environments tend to have more variable phenotypes than organisms inhabiting relatively stable environments (West-Eberhard 2003).

Different environmental conditions can result in a variety of plastic responses of behaviour and morphology in fishes. Experiments in stickleback (*Gasterosteus aculeatus*), zebrafish (*Danio rerio*) and guppies (*Poecilia reticulata*) have shown that the developmental environment has irreversible effects on adult behaviour, morphology and physiology (Tulley & Huntingford 1987; Marks et al. 2005; Schaefer & Ryan 2006; Widmer et al. 2006; Chapman et al. 2008). Environmental conditions can also cause reversible phenotypic change in juvenile and adult fishes (Ghalambor et al. 2010). In three freshwater carp species (*Carassius carassius*, *Carassius auratus* and *Gymnocypris przewalskii*), reversible gill remodelling has been reported in response to changes in water temperature or oxygen levels (Sollid et al. 2003, 2005; Nilsson 2007; Matey et al. 2008;

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Mitrovic & Perry 2009). Reversible gill remodelling has also been linked to air exposure and fluctuating salinity in the mangrove rivulus, *Kryptolebias marmoratus* (Ong et al. 2007; LeBlanc et al. 2010). Under these environmental conditions, a cell mass between the gill lamellae (interlamellar cell mass, ILCM) enlarges, which decreases gill surface area; this may be a strategy that prevents lamellae from collapsing and permanently coalescing, which reduces dehydration or ion loss when individuals are on land or exposed to freshwater (Graham 1997; Ong et al. 2007; LeBlanc et al. 2010). It is clear that differences in environmental conditions result in morphological variation in fishes, but an important question that has yet to be elucidated is whether individual differences in behaviour also lead to morphological variation. This is not an easy question to address, as genetic variation can introduce numerous factors that obscure the link between behaviour and morphology. Behaviour and morphology can be directly, and perhaps differentially, influenced by genetic variation. Genetic variation also can control plasticity itself and thereby change the degree of correlation between traits (Ghalambor et al. 2010). Therefore, experiments addressing these phenomena should ideally use genetically identical individuals in order to pinpoint the contribution of environmental variance to the phenotype.

To understand the link between behaviour and gill morphology, we studied the mangrove rivulus, *Kryptolebias* (formerly *Rivulus*) *marmoratus*, one of two known self-fertilizing hermaphroditic vertebrates (Harrington 1961; Tatarenkov et al. 2009; Costa et al. 2010). Hermaphrodites held individually in the laboratory will routinely release fertilized embryos, formed internally from gametes produced in the ovotestis (Harrington 1963; Sakakura et al. 2006). Although males have been discovered in the wild and extensive outcrossing occurs in some populations in Belize, high rates of inbreeding are common in locales where males are rare or absent, and the majority of field-caught animals are homozygous (Turner et al. 1992; Mackiewicz et al. 2006b). Homozygous hermaphrodites produce genetically identical offspring (Vrijenhoek 1985), and isogenic strains are stable for at least three generations (Turner et al. 1990; Laughlin et al. 1995). However, despite this genetic stability, genetically identical offspring can vary in size and growth rate as a result of phenotypic plasticity (Lin & Dunson 1999).

*Kryptolebias marmoratus* (~20 mm) typically inhabit depressions, drainage ditches or burrows of the land crabs *Cardisoma guanhumi* (Taylor et al. 2004) or *Ucides cordatus* (Davis et al. 1990) within mangrove forests. Water conditions in these habitats fluctuate during both short-term weather and tidal cycles and also as a result of longer-term seasonal changes. *Kryptolebias marmoratus* are able to tolerate salinities of 0–114‰, oxygen concentrations of less than 1 mg/litre, hydrogen sulphide concentrations of 150 parts/billion, and ammonia concentrations of 10 mM (King et al. 1989; Dunson & Dunson 1999; Taylor 2000; Frick & Wright 2002a). Fish leave the water (emerge) to capture prey (Davis et al. 1990) or in response to suboptimal water conditions (Huehner et al. 1985; Abel et al. 1987) or intraspecific aggression (Taylor 1990). In the wild, these emersion periods probably vary in length from minutes to weeks and are usually spent under wet leaves or in moist, rotting logs where the fish respire cutaneously (Taylor 1990; Ong et al. 2007; Taylor et al. 2008).

The combination of its unique reproductive strategy and highly variable habitat makes the mangrove rivulus an ideal model for the study of phenotypic plasticity. In this study, we tested two hypotheses. First, we tested whether fish that spend more time in air (have higher emersion rates) possess anatomical features that differ from fish that emerge less often (experiment 1). We predicted that the cell mass between gill lamellae (ILCM) would be well developed, and lamellae would be shorter and thicker in fish that emerge frequently to prevent the lamellae from collapsing and permanently coalescing. In addition, we predicted that the

cutaneous epidermis would be thicker in fish that spend more time out of water, a feature that would reduce water loss across the skin during air exposure. Second, we tested whether differences in morphology result from behavioural variation (experiment 2). We predicted that differences in morphology related to emersion frequency would disappear if fish were subsequently prevented from emersing, indicating that behavioural variation drives morphological plasticity. Alternatively, results showing the persistence of morphological differences related to emersion frequency after fish were prevented from emersing would be evidence that morphological variation may be driving behavioural differences. To test these hypotheses, we obtained tissue samples from three groups of fish. We observed (videorecorded) the first group (experiment 1 and experiment 2, control) during a period of free access to terrestrial and aquatic environments for 7 days, after which we collected tissue samples. We observed a second group of fish (experiment 2, immersion) as above, but we subsequently limited the group to an aquatic environment for 7 days before collecting tissue samples. We treated the final group (experiment 2, recovery) similarly to the immersion group, followed by a second period of free access to both terrestrial and aquatic environments for 7 days before tissue samples were taken.

## METHODS

### *Experimental Animals*

Mangrove rivulus from two laboratory colonies were used. We conducted an initial experiment (experiment 1) to examine the relationship between voluntary emersion and gill morphology using a single hermaphroditic lineage (*R/W*; originating from Florida, U.S.A.) of rivulus maintained in the Hagen Aqualab, University of Guelph, for over 25 generations. Adult hermaphrodites at least 1 year of age weighing 0.06–0.13 g (wet weight) were used. These fish were recently demonstrated to be homozygous at 36 microsatellite loci (Mackiewicz et al. 2006a; Tatarenkov et al. 2010). Fish were held individually in 100 ml containers (FisherBrand Collection Containers; Fisher Scientific) under identical conditions (25 °C, 16‰, pH 8, 12:12 h light:dark cycle; Frick & Wright 2002a). Water changes were performed weekly using artificial sea water (Crystal Sea® Marinemix; Marine Enterprises International, Inc., Baltimore, MD, U.S.A.) diluted with reverse osmosis freshwater. Fish were fed live *Artemia nauplii* three times per week.

We conducted a second experiment (experiment 2) to test the causal relationship between emersion behaviour and gill structure using a separate lineage (*RHL*; originating from the Bahamas) maintained in the Biology Building at the University of Alabama. This lineage has been in the laboratory for at least 7–12 generations. Adult hermaphrodites at least 1 year of age and weighing 0.13–0.33 g (wet weight) were used. These fish also constitute a homozygous, isogenic lineage (Tatarenkov et al. 2010) and were maintained under similar conditions as the previous group of fish (28 °C, 25‰, pH 8, 11:13 h light:dark cycle). However, these fish were reared in larger (1230 ml) containers (TakeAlongs Deep Squares; Rubbermaid) with established colonies of nitrifying bacteria and algae to maintain water quality. Fish were fed live *Artemia nauplii* seven times per week, and received water changes every 4 months.

### *Experimental Protocol*

#### *Experiment 1: correlation between emersion and gill morphology*

To quantify variation in emersion behaviour, individual fish ( $N = 26$ ) were held for 7 days in 100 ml semitransparent plastic containers (FisherBrand Collection Containers; Fisher Scientific)

with near-vertical ( $83^\circ$ ) sides containing 60 ml of brackish water. Emergence events were defined as fish leaping out of the water and sticking to container sides. Fish were arranged randomly and each container was surrounded by white paper on three sides, ensuring that fish were exposed to the same external stimuli and could not observe one another. Each fish was fed approximately 2 ml of newly hatched *Artemia* nauplii suspension (typical laboratory feeding) immediately prior to experimentation and were not fed or disturbed for the duration of the trial. A digital video camera (Sony DCR-HC32, Tokyo, Japan; or Logitech Quickcam Pro, Fremont, CA, U.S.A.) connected directly to a computer was used to record the fish continuously over the 7 days. Filming at night was accomplished using a combination of the camera's 'Nightshot' or 'RightLight' feature and a red incandescent light (60 W), turned on for the duration of the experiment and located approximately 120 cm from the fish. The time and duration of each emergence event was recorded to the nearest minute; emersions of less than 30 s were considered failed attempts and disregarded. These failed emergence attempts were rare and were typically quickly followed by successful emergence (A. J. Turko, personal observation). At the end of the experiment fish were euthanized (0.2% 2-phenoxy-ethanol), weighed and fixed for histological processing.

#### *Experiment 2: causal relationship between emergence and gill morphology*

To determine the causal relationship between emergence behaviour and gill morphology, a three-stage follow up study was conducted on a different clonal lineage (*RHL*). An initial group of fish ( $N = 40$ ) was monitored with a video camera (Logitech Quickcam Pro) for 7 days as described above. In experiment 1, there was no difference in the time spent emerged during the day versus night during the 7-day trial (paired  $t$  test:  $t_{20} = 2.09$ ,  $P = 0.25$ ); therefore, to simplify the set-up, fish were filmed only during the day for this second experiment. Emergence behaviour was continuously quantified during the trial to divide the sample population into three subgroups (control, immersion, recovery) on day 7. To ensure that each subgroup contained individuals representing the complete emergence spectrum, the three fish that showed the highest levels of emergence were randomly divided between the subgroups, and then the next three highest-emerging individuals were divided in the same manner until the three complete subgroups were created.

All animals of the control subgroup ( $N = 18$ ) were immediately euthanized (6 g/litre buffered tricaine methanesulfonate), weighed and fixed for histology after the 7-day observation period. Because no relationship was found between cutaneous epithelial thickness and emergence time in experiment 1, only the gills were collected for histology in experiment 2. The remaining fish (immersion and recovery subgroups, each  $N = 11$ ) received a water change and were prevented from emerging by securing a piece of mesh at the air–water interface. These fish were fed *Artemia* nauplii every other day to minimize the potential effects of hunger on future emergence behaviour. After 7 days the immersion subgroup was euthanized for histology as described for the control subgroup. The remaining fish (recovery subgroup) received a water change, were fed *Artemia* and were videorecorded for 7 days with free access to both aquatic and terrestrial conditions. Tissue samples were then immediately collected for histology as described previously.

#### *Histology*

Euthanized fish were fixed in 10% neutral buffered formalin at  $4^\circ\text{C}$  for 24 h, placed in a decalcification solution (Surgipath Decalcifier II, Winnipeg, MB, Canada) for 1 h at  $20^\circ\text{C}$ , and then transferred to 70% ethanol at  $4^\circ\text{C}$  until dissection. Gill arches were obtained from the left side. Dissected tissues were routinely

processed for paraffin embedding, serially sectioned in  $4\ \mu\text{m}$  increments and stained with haematoxylin and eosin. Slides were viewed using a Nikon Eclipse 90i epifluorescent microscope and measurements were taken using NIS Elements software (Nikon, Melville, NY, U.S.A.). Five gill lamellae from each gill arch were measured for morphometric analyses. These were randomly selected by numbering all intact gill lamellae in a section and using randomly generated numbers in Microsoft Excel to decide which lamellae to measure (LeBlanc et al. 2010). The fourth gill arch was sometimes distorted or sectioned along the wrong axis on the prepared slides, and in these cases only measurements from the first three gill arches were used. The height of the proximal interlamellar cell mass (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 (Fig. 1; see also Ong et al. 2007). The 20 measurements of each trait were averaged to provide an overall value for each individual. We measured cutaneous epidermal thickness of fish from experiment 1 on the dorsal, ventral and lateral surfaces relative to the spinal column. Random labelling of the slides served to blind the single observer (A.T.) and reduce observational bias.

#### *Ethical Note*

The experiments in this study were approved by the University of Guelph Animal Care Committee (Animal Utilization Protocol 10G008) and the University of Alabama Institutional Animal Care and Use Committee (Protocol 08-309-2). Behavioural experiments were conducted under conditions similar to standard laboratory rearing conditions, and at no time did fish appear to be distressed.

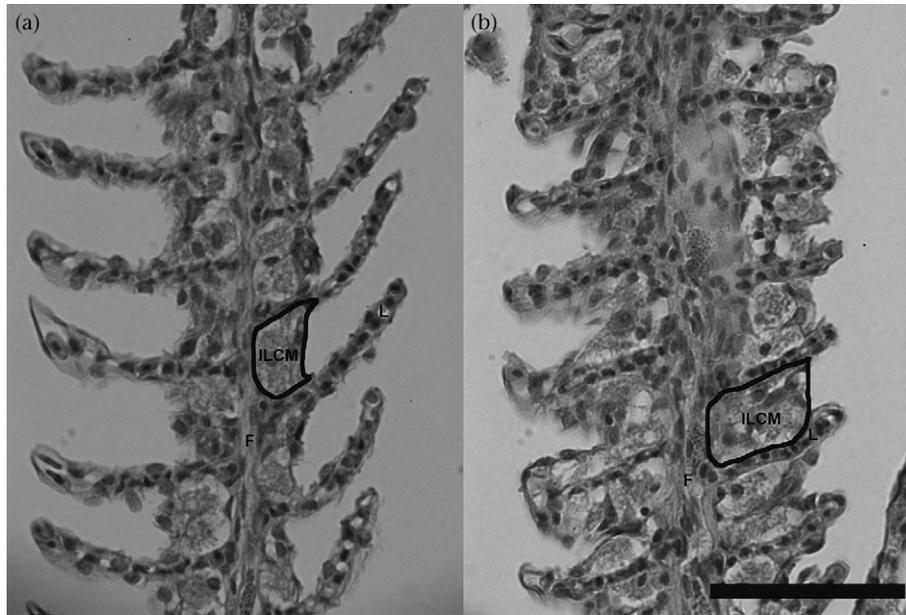
#### *Statistical Analysis*

We used simple linear regressions to examine the relations between emergence time and interlamellar cell mass height, lamellar length, lamellar width and cutaneous epithelial thickness (where applicable). To reduce the chance of committing a type I error resulting from the multiple comparisons of linear regressions, we used Bonferroni-corrected  $\alpha$  levels of 0.008 (experiment 1:  $0.05/6$ ) and 0.017 (experiment 2:  $0.05/3$ ). For all other tests, we used an  $\alpha$  level of 0.05. We used one-way ANOVA followed by post hoc Tukey's tests to test for differences in mean ILCM height between the three subgroups in experiment 2. Multiple linear regressions were used to test whether variation in total emergence time was the result of differences in emergence frequency or duration of emergence events. Despite collecting diurnal and nocturnal emergence data in experiment 1, only diurnal values were used for analysis to be consistent with experiment 2; this had no effect on the significance of our results (data not shown). SigmaStat 3.5 (Systat Software, San Jose, CA, U.S.A.) was used for all analyses. Throughout the text means are given as mean  $\pm$  SE.

## **RESULTS**

### *Emergence and Morphology*

In experiment 1, individual fish spent 0–64% of the diurnal recording period out of water. Emergence durations ranged from 1 to 403 min (mean  $\pm$  SE =  $61.0 \pm 3.0$  min; Fig. 2a), and individual fish emerged between 0 and 73 times over the 7-day period. Total emergence time was significantly positively correlated with both emergence frequency and average emergence duration (multiple linear regression:  $R^2 = 0.84$ ,  $F_{2,23} = 60.55$ ,  $N = 26$ ; frequency:  $P < 0.001$ ; duration:  $P = 0.029$ ). Emergence time was not correlated



**Figure 1.** Representative light micrographs of *Kryptolebias marmoratus* gill filaments (F) and lamellae (L) showing (a) interlamellar cell mass (ILCM) development in an individual that rarely left the water and (b) high ILCM development in an individual that frequently emerged. Adobe Photoshop (Adobe Photoshop 7, Toronto, Canada) 'auto contrast' was used to improve image clarity. Scale bar = 50  $\mu$ m.

with body size (linear regression:  $R^2 = 0.0015$ ,  $F_{1,24} = 0.037$ ,  $N = 26$ ,  $P = 0.85$ ).

Total emersion time was positively correlated with ILCM height (linear regression:  $R^2 = 0.34$ ,  $F_{1,24} = 12.46$ ,  $N = 26$ ,  $P = 0.002$ ; Fig. 2b), but not with lamellar length or width (linear regression: length:  $R^2 = 0.016$ ,  $F_{1,24} = 0.39$ ,  $N = 26$ ,  $P = 0.54$ ; width:  $R^2 = 0.0037$ ,  $F_{1,24} = 0.09$ ,  $N = 26$ ,  $P = 0.77$ ). In those fish that spent most of their time in water, a reduced ILCM resulted in lamellae that were clearly separated and well defined (Fig. 1a). Conversely, in fish that spent much of their time in air, the spaces between gill lamellae were mostly filled by the ILCM (Fig. 1b). Total emersion time was not correlated with cutaneous epithelial thickness in any region (linear regression: dorsal  $R^2 = 0.055$ ,  $F_{1,24} = 1.42$ ,  $N = 26$ ,  $P = 0.25$ ; ventral:  $R^2 = 0.0016$ ,  $F_{1,24} = 0.039$ ,  $N = 26$ ,  $P = 0.85$ ; lateral:  $R^2 = 0.041$ ,  $F_{1,24} = 1.02$ ,  $N = 26$ ,  $P = 0.32$ ).

#### Causal Relationships

During the control period of experiment 2, individual fish spent 0–22% of the time emersed. Emersion durations ranged from 1 to 157 min (mean  $\pm$  SE =  $18.8 \pm 0.7$  min; Fig. 3a), and individual fish emersed 0–67 times over the 7-day period. Total emersion time was significantly positively correlated with both emersion frequency and average emersion duration (multiple linear regression:  $R^2 = 0.86$ ,  $F_{2,37} = 116.91$ ,  $N = 40$ ; frequency:  $P < 0.001$ ; duration:  $P < 0.001$ ). Emersion time was not significantly related to body size or any gill parameter (linear regression: mass:  $R^2 = 0.011$ ,  $F_{1,38} = 0.41$ ,  $N = 40$ ,  $P = 0.52$ ; ILCM:  $R^2 = 0.12$ ,  $F_{1,16} = 2.10$ ,  $N = 18$ ,  $P = 0.17$ ; Fig. 3b; lamellar length:  $R^2 = 0.16$ ,  $F_{1,16} = 2.79$ ,  $N = 18$ ,  $P = 0.12$ ; lamellar width:  $R^2 = 0.064$ ,  $F_{1,16} = 1.03$ ,  $N = 18$ ,  $P = 0.33$ ).

Fish in the immersion subgroup were prevented from emersing for 7 days after emersion tendencies were measured during the control phase. There were no relationships in the immersion group between emersion time during the control period and any gill parameter after the immersion period (linear regression: ILCM:  $R^2 = 0.11$ ,  $F_{1,9} = 1.16$ ,  $N = 11$ ,  $P = 0.31$ ; Fig. 3c; lamellar length:  $R^2 = 0.03$ ,  $F_{1,9} = 0.33$ ,  $N = 11$ ,  $P = 0.58$ ; lamellar width:  $R^2 = 0.11$ ,  $F_{1,9} = 1.12$ ,  $N = 11$ ,  $P = 0.32$ ).

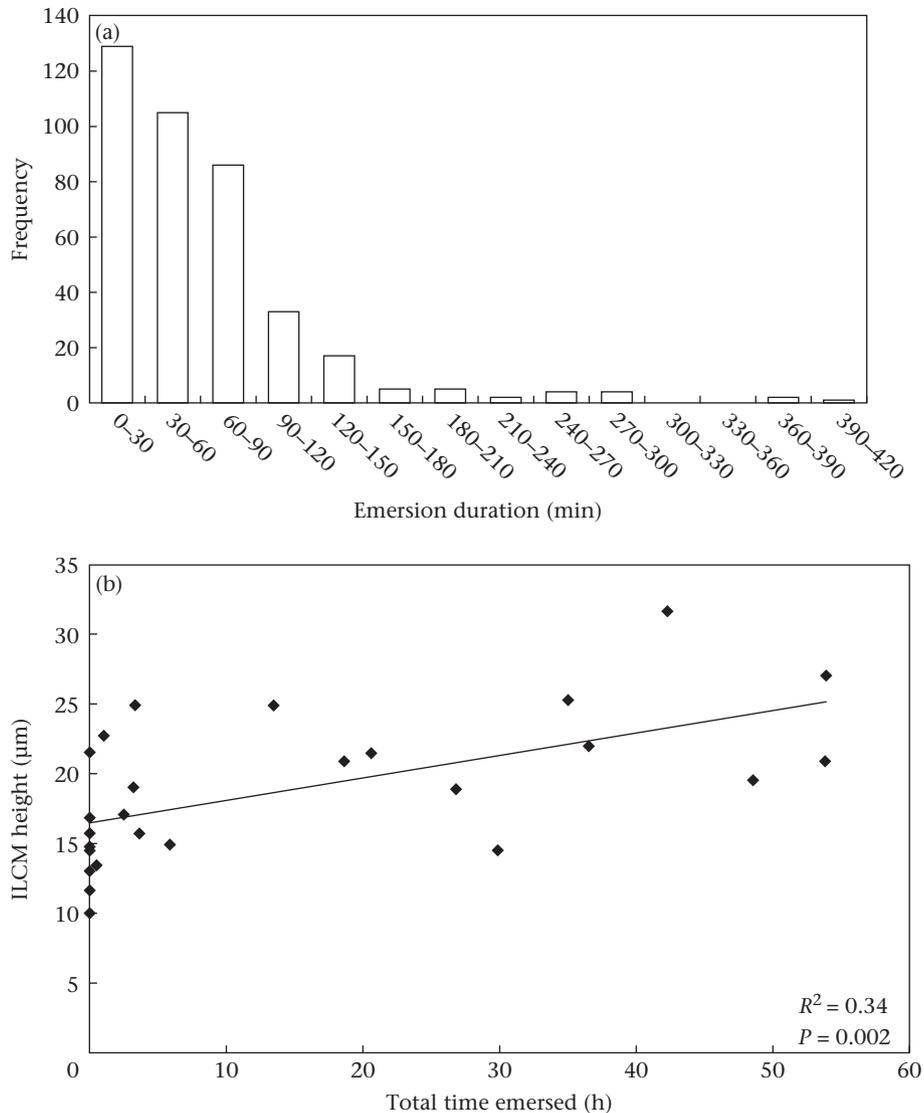
Emersion rates in the recovery subgroup increased relative to the other subgroups and fish spent 3–78% of the time out of water. Emersion durations ranged from 1 to 167 min (mean  $\pm$  SE =  $21.9 \pm 0.5$ ; Fig. 4a), and individual fish emersed 9–204 times over the 7-day period. Total emersion time was significantly positively correlated with both emersion frequency and average emersion duration (multiple linear regression:  $R^2 = 0.94$ ,  $F_{2,8} = 66.72$ ,  $N = 11$ ; frequency:  $P < 0.001$ ; duration:  $P = 0.015$ ). Emersion time was not correlated with body size (linear regression:  $R^2 = 0.076$ ,  $F_{1,9} = 0.74$ ,  $N = 11$ ,  $P = 0.41$ ). In these recovery fish, emersion time was strongly related to ILCM height (linear regression:  $R^2 = 0.53$ ,  $F_{1,9} = 10.32$ ,  $N = 11$ ,  $P = 0.01$ ; Fig. 4b). No other gill measurements showed a relationship with emersion time (lamellar length:  $R^2 = 0.072$ ,  $F_{1,9} = 0.69$ ,  $N = 11$ ,  $P = 0.43$ ; lamellar width:  $R^2 = 0.03$ ,  $F_{1,9} = 0.28$ ,  $N = 11$ ,  $P = 0.61$ ).

Mean ILCM height differed significantly between the three subgroups (one-way ANOVA:  $F_{2,38} = 8.26$ ,  $P = 0.001$ ; Fig. 5). The ILCM was significantly enlarged in the recovery subgroup relative to both the control subgroup (Tukey's test:  $P = 0.04$ ) and immersion subgroup (Tukey's test:  $P < 0.001$ ). There was no difference in ILCM height between the control and immersion subgroups, however (Tukey's test:  $P = 0.14$ ).

#### DISCUSSION

These findings demonstrate that differences in emersion behaviour exist within isogenic lineages of *K. marmoratus*. Some fish generally remained in water, venturing out only rarely, whereas other fish consistently attempted to emerse and spent up to 78% of their time out of water. In support of our first hypothesis, we found that the size of the cell mass between gill lamellae (ILCM height) was positively correlated with the amount of time emersed. Fish that voluntarily spend more time out of water showed reduced gill surface area, a modification that may limit branchial water loss and provide support for delicate lamellar structures.

There was no difference in the thickness of the cutaneous epidermis related to emersion time within the one lineage studied. Although preventing desiccation is probably the greatest challenge



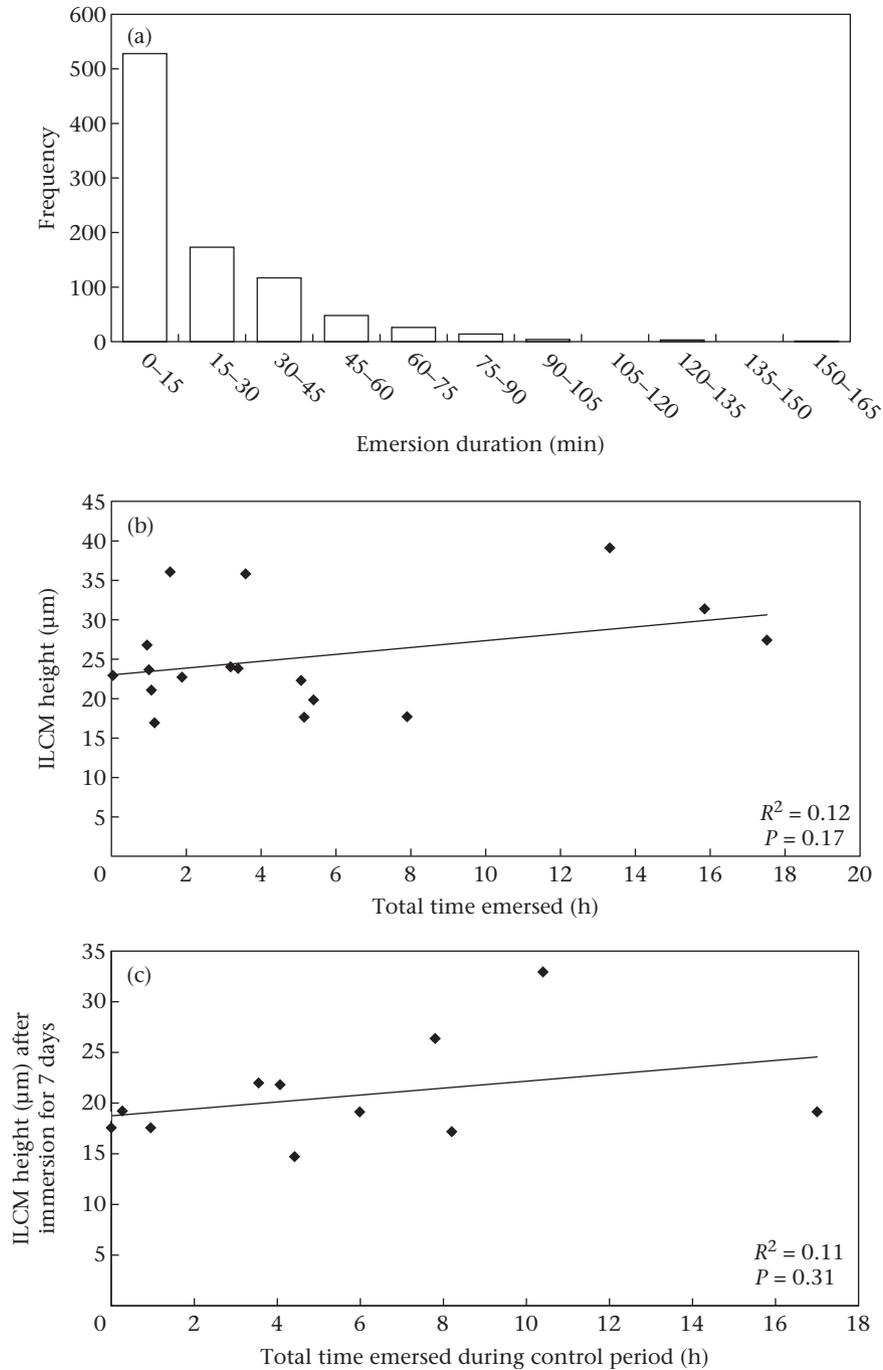
**Figure 2.** (a) Frequency of diurnal emersion events ( $N = 393$ ) of different duration (range 1–403 min) recorded in experiment 1. (b) Relation between height of the interlamellar cell mass (ILCM) and total emersion time (linear regression:  $R^2 = 0.34$ ,  $F_{1,24} = 12.46$ ,  $N = 26$ ,  $P = 0.002$ ).

facing amphibious fish during emersion, mangrove rivulus face a trade-off between reducing water loss across the skin while maintaining functionality for respiration, ionoregulation, osmoregulation and ammonia excretion (Frick & Wright 2002b; Sayer 2005; Litwiller et al. 2006; Ong et al. 2007; LeBlanc et al. 2010). Mangrove rivulus do not undergo metabolic depression during emersion, but the gills are remodelled and opercular movements cease (Ong et al. 2007), suggesting that the skin is especially important during air exposure. Additionally, mangrove rivulus are one of only a few amphibious fishes that possess epidermal rather than dermal capillary networks to allow for cutaneous gas exchange (Grizzle & Thiyagarajah 1987; Graham 1997). Placement of capillaries close to the surface of the skin may negate any possible advantage of a thicker epithelium. Changes in the location and density of the epidermal capillaries may also be important in gas exchange and water conservation during emersion, and this should be examined in future studies.

Our results also support the second hypothesis that differences in behaviour are responsible for the observed differences in gill morphology. We found a strong positive correlation between emersion time and ILCM height in the experiment 2 recovery fish

that were allowed to emerge after being held in water for 7 days. Experiment 2 showed no relationship between emersion time and gill morphology in either control individuals or in immersion individuals. However, the control individuals in experiment 2 did not display the same breadth of variation in emersion behaviour as the fish in experiment 1 (see below). The observation that a correlation between emersion time and ILCM height only occurs when some fish spend a relatively large amount of their time emersed may indicate that a threshold level of air exposure is required before gill remodelling begins. The increased emersion rates of recovery fish in experiment 2 may have crossed the threshold amount of time spent in air responsible for initiating gill remodelling. Whatever the reason for increased rates of emersion in some fish after 7 days of forced immersion, these results strongly suggest that high levels of emersion caused ILCM enlargement in these individuals.

The difference in emersion frequency observed between the *R/W* fish used for experiment 1 and the *RHL* fish used for the control phase of experiment 2 either resulted from genetic differences between the lineages, or were due to differences in husbandry protocols between laboratories. Differences in growth rate and

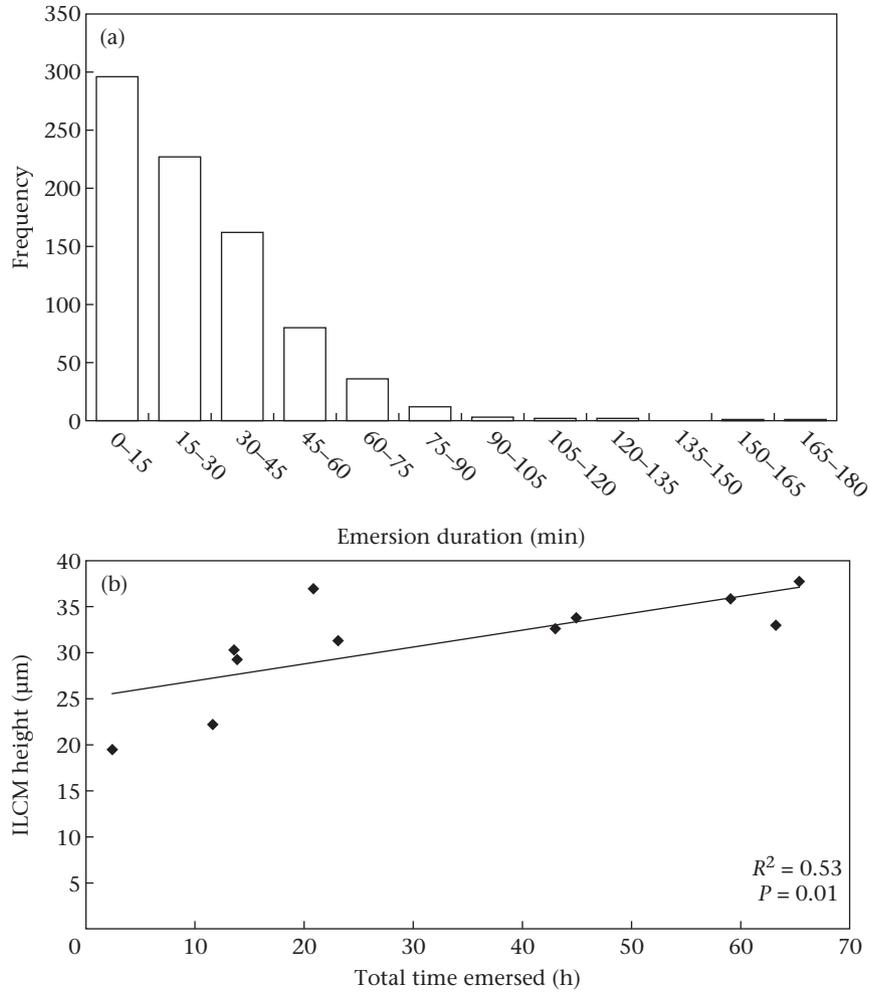


**Figure 3.** (a) Frequency of diurnal emersion events ( $N = 914$ ) of different duration (range 1–157 min) recorded in experiment 2. Relation between height of the interlamellar cell mass (ILCM) and total emersion time immediately after the control phase (b:  $R^2 = 0.12$ ,  $F_{1,16} = 2.10$ ,  $N = 18$ ,  $P = 0.17$ ) and after the immersion phase (c:  $R^2 = 0.11$ ,  $F_{1,9} = 1.16$ ,  $N = 11$ ,  $P = 0.31$ ).

reproductive investment have been reported between isogenic strains of mangrove rivulus (Grageda et al. 2005), and there are strain differences in aggressiveness (R. L. Earley, unpublished data). It is possible that emersion behaviour also varies among mangrove rivulus lineages, and this may explain the observed differences in emersion rates between experiment 1 and the control phase of experiment 2. These behavioural differences between experiments may also have resulted from differences in rearing conditions at each laboratory. Fish used in experiment 1 were housed in smaller containers, maintained at a slightly lower temperature and salinity, and were fed less often than fish used in experiment 2. Despite these

rearing differences, however, when fish from experiment 2 emersed at similar rates to fish from experiment 1 (during the recovery phase of experiment 2), a similar ILCM response was observed in both groups. This suggests that, while genetic or environmental factors may have differentially influenced initial emersion rates in the present experiments, high emersion rates triggered the same gill remodelling response despite these differences between populations and/or laboratories.

What is the stimulus for emersion in *K. marmoratus*? There is limited quantitative information on this topic. Abel et al. (1987) reported that 50% of *K. marmoratus* emersed at a hydrogen sulphide



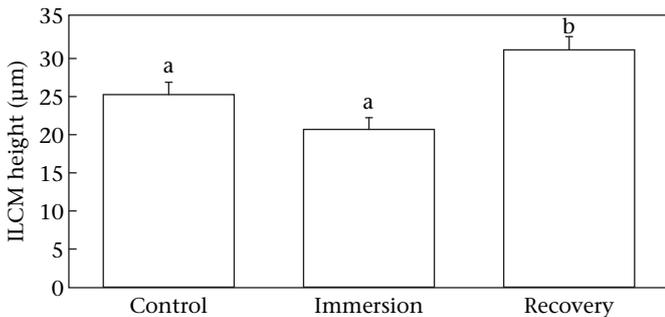
**Figure 4.** (a) Frequency of diurnal emersion events ( $N = 822$ ) of different duration (range 1–167 min) in the recovery phase of experiment 2. (b) Relation between height of the interlamellar cell mass (ILCM) and total emersion time ( $R^2 = 0.53$ ,  $F_{1,9} = 10.32$ ,  $N = 11$ ,  $P = 0.01$ ).

concentration of about 150 ppb in the laboratory. Although no hydrogen sulphide accumulated in the present experiment, water quality undoubtedly changed over the 7-day experiment due to the normal exchange of gases and ions between fish and chamber water. For example, water ammonia concentrations increased from 0.0 to 0.4 mmol/litre by the end of the experiment 1 (A. J. Turko, unpublished data). No mortalities or behavioural changes were reported in *K. marmoratus* exposed to 5 mmol/litre (7 days) or 10 mmol/litre

(48 h)  $\text{NH}_4\text{Cl}$  (Frick & Wright 2002a), so it is unlikely that ammonia levels at least 10 times lower would have caused undue stress. Furthermore, differences in emersion rates were observed between the control and recovery subgroups of experiment 2 despite the fact that ammonia would have probably accumulated similarly in both experiments.

An alternative possibility is that hunger influences emersion behaviour in the laboratory. Hunger is known to increase exploratory behaviour and activity levels in fish (Stoner 2003; Vehanen 2003; Petrie & Ryer 2006), and we found that emersion rates increased over the course of each 7-day filming period (data not shown). In our experiments fish were fed only on the first day of each 7-day recording period and presumably food availability would have quickly declined thereafter. Although these captive-bred fish were only fed live *Artemia*, which were added to the water, some individuals might have left the water to seek prey once *Artemia* numbers had decreased. Mangrove rivulus are known to emerse to feed on terrestrial insects in the laboratory, and in a survey of 45 wild fish with food in their guts, 41% contained terrestrial prey (Huehner et al. 1985; Taylor 1992).

Since the *K. marmoratus* hermaphrodites used here were drawn from two isogenic (self-fertilizing and homozygous) lineages, the observed behavioural variation within each experiment was not a result of genetic differences. Lin & Dunson (1999) were the first to report phenotypic plasticity within clonal lines of this species. In



**Figure 5.** Mean  $\pm$  SE interlamellar cell mass (ILCM) height in fish from the control, immersion and recovery subgroups of experiment 2. Significant differences ( $P < 0.05$ ) between groups are indicated by different letters.

their studies, the condition of parent *K. marmoratus* was a key factor in phenotypic differences between clonal offspring (Lin & Dunson 1995, 1999). Offspring of well-fed parents grow and mature more slowly than do offspring of adults provided smaller food rations (Lin & Dunson 1999). These developmental differences occur despite the fact that parents' food availability does not influence the size of eggs or newly hatched larvae (Lin & Dunson 1995). While all reasonable efforts were taken to rear the fish used in the present experiments under standardized conditions, it is likely that there were small variations in the care of each individual. Small differences in parents' age or condition or food availability may have affected the behavioural phenotype of offspring. Other environmental factors experienced by the developing embryos may have varied slightly, including systemic disturbance due to placement within the laboratory or proximity to the ceiling lighting or the climate control system. Additionally, water conditions and food availability may have differed slightly between individuals due to the standardization of feeding and water change schedules. It is possible that exposure to slightly different environments during a critical period of development would cause persistent phenotypic differences to arise between genetically identical individuals, especially because these critical periods are often only a few days long (Browman 1989).

This is the first study to report a causal link between behaviour and gill morphology in any fish. We show that frequent voluntary emersion increased ILCM height to a level similar to that of fish maintained in air for 1 week (Ong et al. 2007), while fish that rarely emersed had reduced ILCMs. This finding indicates that short but frequent exposure to air can have the same effect on gill morphology as continuous emersion (Ong et al. 2007). It should be noted that the naked carp, *G. przewalskii*, alters the size and shape of lamellae in addition to the development of the ILCM in response to environmental hypoxia (Matey et al. 2008), but in *K. marmoratus* there were no changes in lamellae length or thickness. There is a great diversity of respiratory structures (e.g. lungs, buccal cavities) that have evolved for breathing air in amphibious fishes. However, there is no information in the literature on whether these respiratory structures are reversibly remodelled in other amphibious fishes.

In summary, our results show that a spectrum of emersion tendencies exists within isogenic lineages of mangrove rivulus, indicating that subtle developmental and/or environmental factors alone can give rise to distinct behaviours in this fish. These behavioural differences, in turn, induce dramatic plastic changes in gill morphology that may provide protection against dehydration or gill damage when fish are out of water. The high degree of phenotypic plasticity expressed by this species may be a key to its successful exploitation of highly variable mangrove habitats.

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