Widespread use of emersion and cutaneous ammonia excretion in Aplocheiloid killifishes (2018). Livingston MD, Bhargav VV, Turko AJ, Wilson JM. and Wright PA. Proceedings of the Royal Society B.

Supplemental Material Contains:

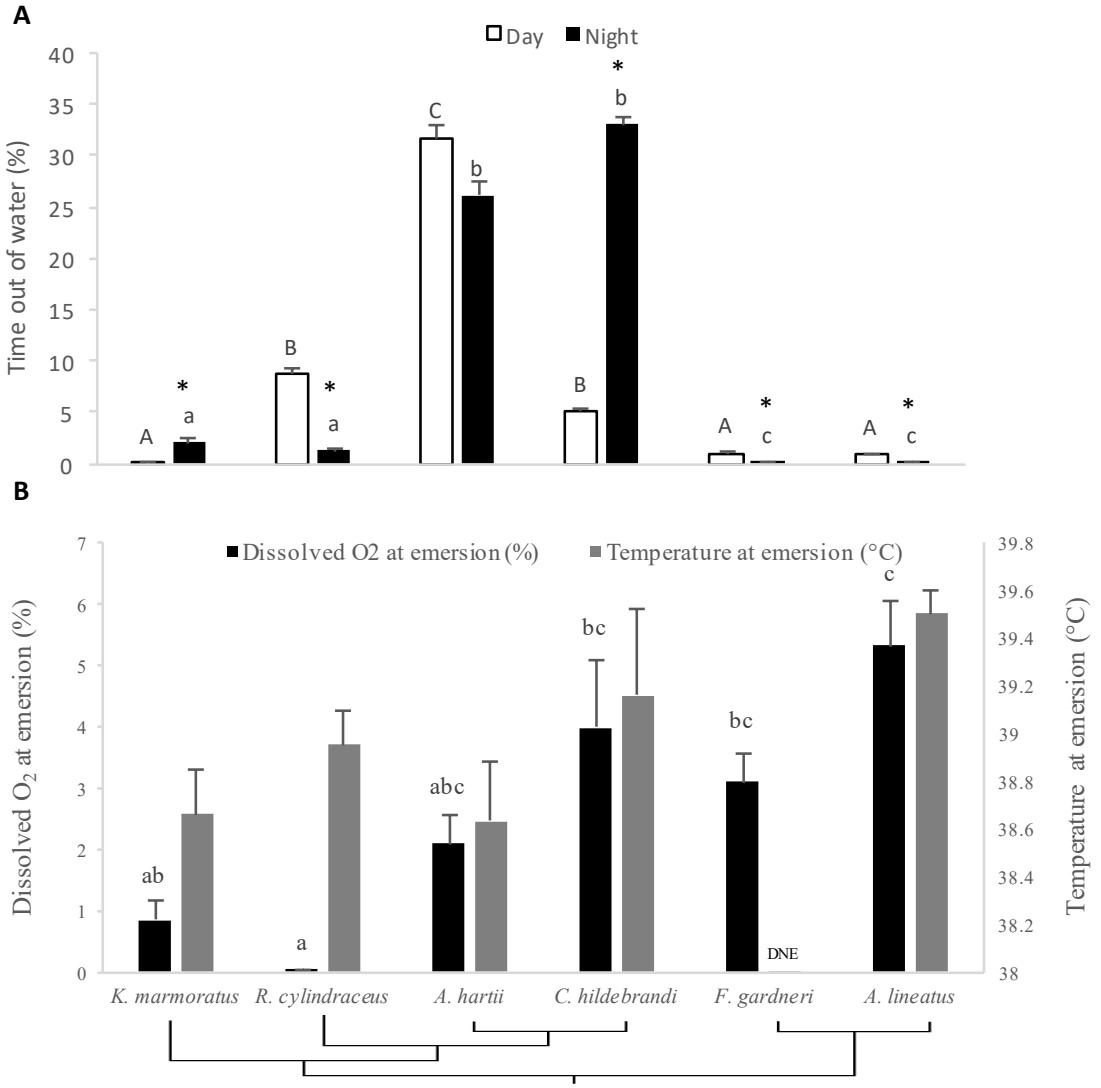

Figure S1. Voluntary emersion (A) and threshold for emersion in response to an acute increase in water temperature or decrease in water oxygen (B) for six species of Aplocheiloid killifishes (*Kryptolebias marmoratus, Rivulus cylindraceus*, *Anablepsoides hartii, Cynodonichthys hildebrandi, Fundulopanchax gardneri*, *Aplocheilus lineatus*). Phylogenetic relationships are indicated by the tree below the x-axis. Values are expressed as means ± s.e.m. In panel A, different capital letters denote significant inter-specific differences in emersion rate during the day, and lowercase levels indicate significant differences at night (two-way RM ANOVA, interaction $P<0.05$). Asterisks indicate significant differences in time out of water between day and night within a species. In panel B, different letters above bars indicate significant inter-specific differences (ANOVA on ranks, $P<0.05$). DNE, "did not emerse".

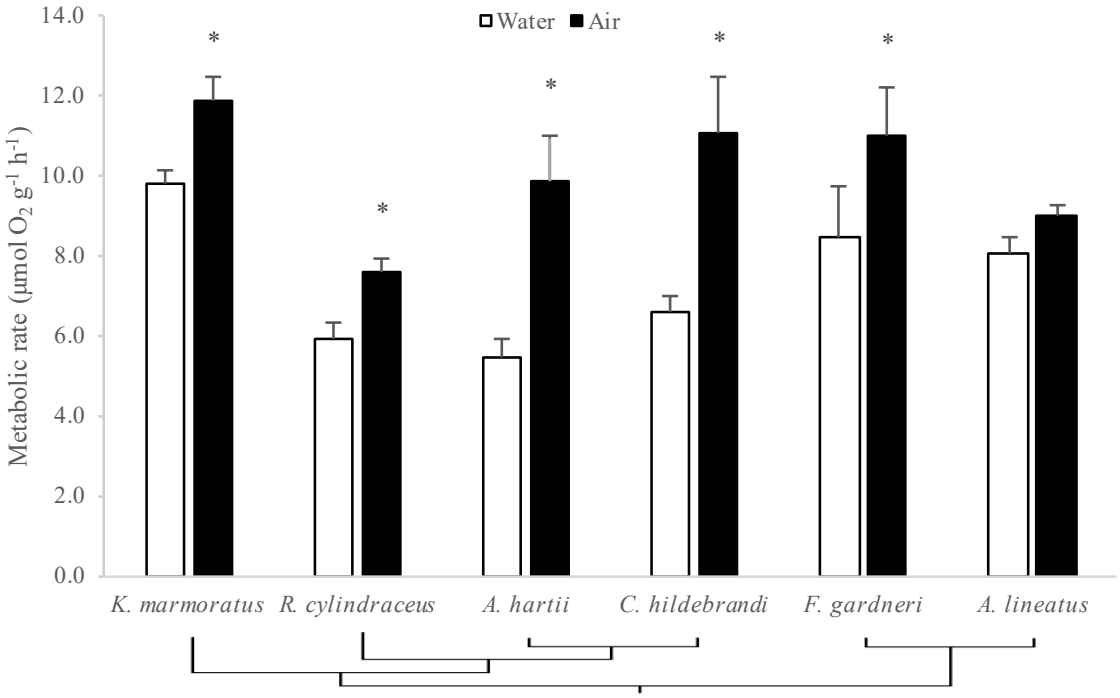

Figure S2. Rates of O_2 consumption in water (white bars) and air (6 h exposure, black bars) for six species of Aplocheiloid killifish (*Kryptolebias marmoratus, Rivulus cylindraceus*, *Anablepsoides hartii, Cynodonichthys hildebrandi, Fundulopanchax gardneri*, *Aplocheilus lineatus*). Phylogenetic relationships are indicated by the tree below the x-axis. Values are expressed as means ± s.e.m. The presence of an asterisk denotes a significant difference in O_2 consumption between water and air-breathing (N=6, paired t-test, $p<0.05$).

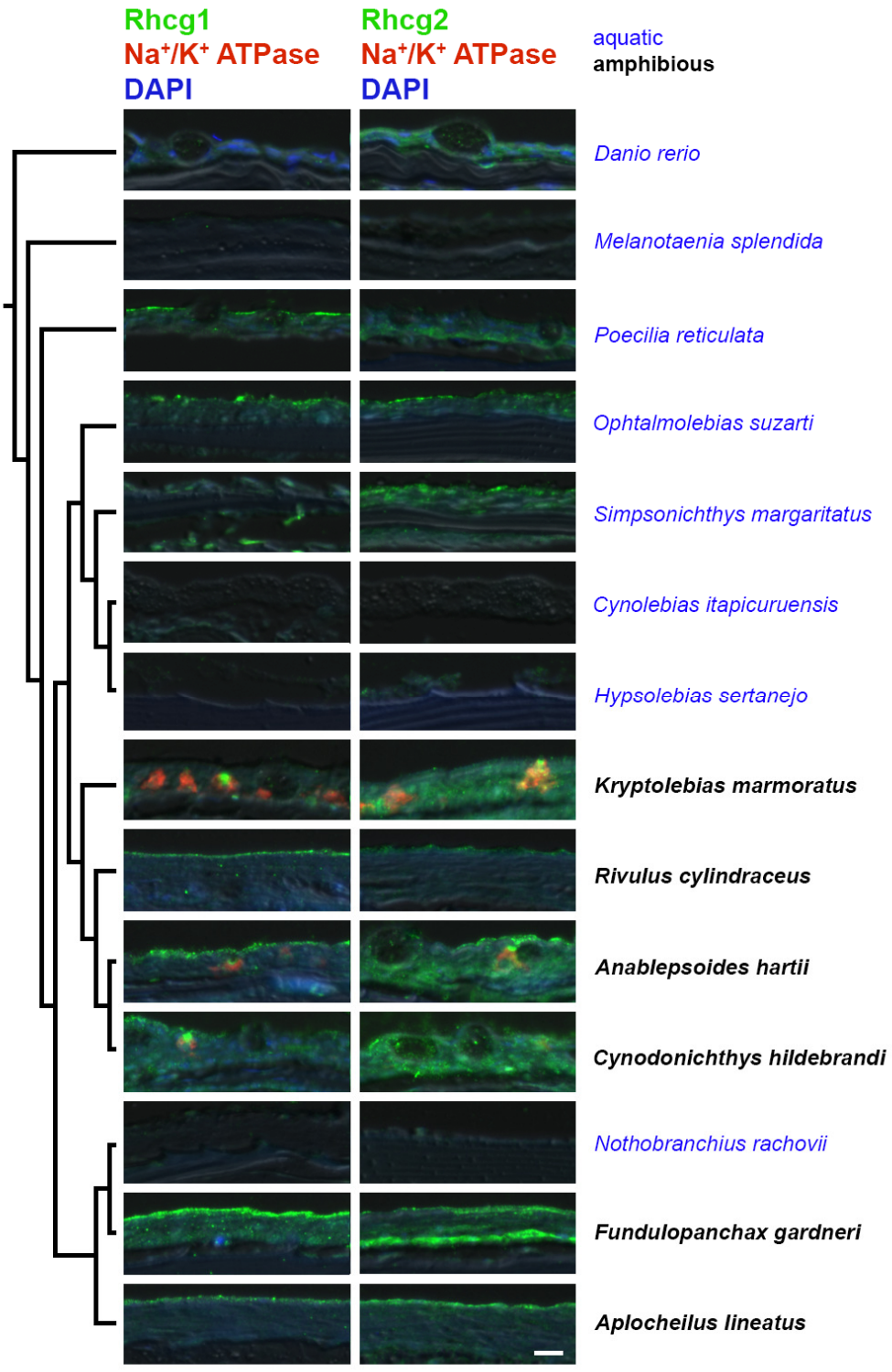

Figure S3. Skin cross-sections of aquatic and amphibious fishes labelled by immunofluorescence for nuclei (DAPI; blue) NKA (red) and either Rhcg1 (left column, green) or Rhcg2 (right column, green). Aquatic fishes are labelled in blue, amphibious species in black. Scale bar = 10 µm.

Table S1. Contribution of NH_3 volatilization, washout ammonia, and urea-N synthesis to the ammonia deficit of six amphibious killifish species during 6 h of air exposure. Remaining ammonia deficits were calculated to include contributions from the three nitrogen excretion mechanisms we tested (volatilization, accumulation and washout, or urea-N synthesis). Urea-N synthesis includes only the increase in urea-N levels throughout emersion (emersion – control), while washout ammonia includes only the increase in ammonia excretion upon returning to water (re-immersion – control). NS represents a non-significant difference between ammonia (during re-immersion) or urea (during emersion) excretion values as compared to the control. Values are means ± S.E.M. (N=6).

Species	Ammonia deficit ($\mu mol\ g^{-1}\ h^{-1}$)	Volatilized NH_3 ($\mu mol\ g^{-1}\ h^{-1}$)	Washout ammonia ($\mu mol\ g^{-1}\ h^{-1}$)	Urea-N synthesis ($\mu mol\ urea\text{-}N\ g^{-1}\ h^{-1}$)	Remaining ammonia deficit (%)
K. marmoratus	1.42 ± 0.26	0.31 ± 0.07	NS	NS	78
R. cylindraceus	0.43 ± 0.10	0.28 ± 0.07	NS	NS	35
A. hartii	1.02 ± 027	0.38 ± 0.03	NS	0.42 ± 0.10	22
C. hildebrandi	0.92 ± 0.22	0.44 ± 0.07	NS	NS	52
F. gardneri	0.67 ± 0.10	0.26 ± 0.02	0.85 ± 0.17	NS	0
A. lineatus	0.63 ± 0.08	0.49 ± 0.12	NS	NS	22

Supplementary Methods

Nitrogen excretion

To determine if bacterial metabolism could account for any changes in water nitrogen levels, fish (N=6) were placed in experimental containers (30 ml of water) for two hours. Fish were then removed and a known amount of stock ammonia (20 uL of 0.1 M) was added. Water samples were collected at 0 h, 2 h, and 16 h. No significant differences were found in ammonia or urea-N levels throughout a 16-hour water control (Paired t-test, p=0.40, N=6), or air control period (just filter paper, Paired t-test, p=0.12, N=4). Additionally, no significant difference (Paired t-test, p=0.13, N=4) in ammonia levels was found throughout a 6 h control period in the acidified distilled water flasks used to measure ammonia volatilization.

The ammonia deficit was calculated by subtracting the amount of ammonia excreted in air from the amount of ammonia excreted while in water. We attempted to account for this deficit by looking at three parameters: i) washout ammonia (excess ammonia excretion upon re-immersion as compared to control) ii) $NH_{3(g)}$ volatilization, and iii) urea synthesis/excretion. Washout ammonia was calculated by subtracting the ammonia excretion rates during recovery (re-immersion) from the control. Urea synthesis values were calculated by subtracting urea excretion values during emersion from control urea excretion values. If washout ammonia or urea excretion values were negative (i.e. lower respective values in air than the water control for any individual, they were treated as zero).

Oxygen uptake

To measure O_2 consumption, optodes (Loligo) were first calibrated using 2 M sodium sulfite (0% O_2 saturation) and 25°C humidified air (100% O_2 saturation). Stir bars were placed in the chambers over a stir plate, separated from the fish by mesh, to ensure that water in the

chambers was well mixed. Prior to experimentation, moist filter paper was placed into the glass chambers to ensure a moist environment during air exposure. Individuals were placed in the chambers for a three-hour acclimation period in well oxygenated (>90% O_2 saturation), flow-through water (25°C, species dependent water type). Water flow was stopped and the change in O_2 levels was recorded over three 10 minute intervals. Water flow was reopened for an additional 10 minutes in between each measurement period, to replenish the chambers with well-oxygenated water. Water was then completely drained from the chambers to initiate a 6-hour air exposure period. To determine O_2 consumption rates, O_2 saturation (%) was converted to O_2 concentration (at 25°C, 100% dissolved O_2 saturation = 8.1 μmol/L of O_2). For each trial, a linear regression was fit to raw data to calculate the slope of O_2 consumption (μmol O_2 g^{-1} h^{-1}); R^2 values were always ≥0.95.

Control trials were performed without fish to ensure measured changes in PO_2 were due to the fish alone. Over the water control period there were no changes in PO_2 in the chambers, however, when water was removed from the chambers measured PO_2 levels initially decreased due to dehydration of the O_2 sensing spots. After 1h, there were no changes in the PO_2 of the air-filled chamber in the absence of a fish. Therefore, O_2 consumption measurements from the first hour of air exposure were excluded from metabolic rate determinations.