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Air breathing in Magadi tilapia *Alcolapia grahami*, under normoxic and hyperoxic conditions, and the association with sunlight and reactive oxygen species

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Observations of the Magadi tilapia *Alcolapia grahami* in hot, highly alkaline Lake Magadi revealed that they air breathe not only during hypoxia, as described previously, but also during normoxia and hyperoxia. Air breathing under these latter conditions occurred within distinct groupings of fish (pods) and involved only a small proportion of the population. Air breathing properties (duration and frequency) were quantified from video footage. Air breathing within the population followed a diel pattern with the maximum extent of pod formation occurring in early afternoon. High levels of reactive oxygen species (ROS) in the water may be an irritant that encourages the air-breathing behaviour. The diel pattern of air breathing in the field and in experiments followed the diel pattern of ROS concentrations in the water which are amongst the highest reported in the literature (maximum daytime values of 2·53–8·10 μM H_2O_2). Interlamellar cell masses (ILCM) occurred between the gill lamellae of fish from the lagoon with highest ROS and highest oxygen levels, while fish from a normoxic lagoon with one third the ROS had little or no ILCM. This is the first record of air breathing in a facultative air-breathing fish in hyperoxic conditions and the first record of an ILCM in a cichlid species.

Key words: diel patterns; gill remodelling; interlamellar cell mass.

INTRODUCTION

Lake Magadi is a small soda lake situated in the Rift Valley of Africa in southern Kenya, near the Tanzanian border. Most of the lake is covered by a thick layer of

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trona, principally sodium carbonate and bicarbonate (Coe, 1966). Hot springs supply lagoons of open water at points around the edge of the lake. The environment is hostile to life: temperature of 20–43° C, pH *c.* 10, specific density of 1·015 which equates to 60% salinity at 20° C, and oxygen supply of $PO_2 < 2{\cdot}67$ kPa at night to >53·32 kPa during the day, that is, severe hypoxia to hyperoxia (Coe, 1966; Reite *et al.*, 1974; Narahara *et al.*, 1996). A simple but productive foodweb survives, comprising the blue-green cyanobacterium *Arthrospira* sp. (Coe, 1966), on occasion, cyclopoids (Coe, 1966) and immature chironomids (pers. obs.), a small tilapia *Alcolapia grahami* (Boulenger 1912), a number of fish-eating birds (egrets, herons, terns, gulls and the occasional pelican) and flamingos.

Diel hypoxia and hyperoxia are persistent characteristics of at least some of the lagoons (Narahara *et al.*, 1996). The physiological and behavioural responses of fishes to hypoxia and the energetic benefits and ecological costs of these responses have been a major focus of study (Chapman & McKenzie, 2009). In the presence of low oxygen conditions, fishes may use aquatic surface respiration or air breathing to augment their oxygen uptake. Air breathing in *A. grahami* was first reported by Franklin *et al.* (1995) and described by Narahara *et al.* (1996). Both air breathing and aquatic surface respiration were documented as responses to hypoxia in the laboratory. During progressive experimental hypoxia, the species first and predominantly utilized aquatic surface respiration. It resorted to air breathing at the lowest oxygen tensions and after exercise. A role for air breathing is supported by the presence of a highly vascularized, physostomous swimbladder (Maina *et al.*, 1995).

On a reconnaissance trip to Lake Magadi in 2008, *A. grahami* were observed air breathing during the afternoon, when oxygen levels would have been normoxic or hyperoxic in these productive lagoons. Air breathing is ecologically costly and risky (Kramer, 1987). When air breathing, fishes divert time from other behaviours (feeding, mating and social interactions) and expose themselves to avian predation and increased UV radiation. Only some 400 of the 25 000 bony fish species are known to utilize air breathing (Graham, 1997; Chapman & McKenzie, 2009). Some fishes have evolved their air breathing capacity sufficiently that they obtain significant amounts of oxygen from both water and air in normoxic conditions (Lenfant & Johansen, 1972; Randall *et al.*, 1981). Brauner *et al.* (1995) found that the Amazonian armoured catfish *Hoplosternum littorale* (Hancock 1828), a facultative air breather, also utilized air breathing to avoid exposure of its gills to acid waters and hydrogen sulphide.

No previous observations of air breathing by fishes under hyperoxic conditions have been reported. A number of possible reasons for air breathing in *A. grahami* under normoxic or hyperoxic conditions could include the avoidance of an irritant in the water, abnormal behaviour initiated by a parasite to favour predation by birds or the release to or uptake of gases from air for reasons other than aquatic hypoxia.

At Lake Magadi, one possible set of irritants is reactive oxygen species (ROS). During the dry season, the sun shines down fiercely on these shallow productive waters. The high UV light levels, high oxygen saturation, high productivity, shallow depths, high temperature and high pH are all conducive to production of high concentrations of ROS in the water (Scully *et al.*, 1996; Bruskov *et al.*, 2002*a*, *b*). ROS is composed of several components, singlet oxygen ($^1O_2$), superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxide radicals (OH^-) which are all strong oxidizing agents (Skurlatov & Ernestova, 1998). Of these, H_2O_2 can pass through cell membranes into an organism increasing the ROS levels in the body (da Rosa

et al., 2008). The possibility exists that ROS may be stressful to fish in Lake Magadi, particularly to their gills, that are in close contact with the water.

The objectives of this study were: (1) to establish whether air breathing was occurring under normoxic and hyperoxic conditions; (2) to characterize the temporal pattern of air-breathing behaviour of *A. grahami* in relation to changes in the environment including temperature, oxygen levels and ROS (measured as H_2O_2 concentrations); (3) to detail the timing of air breathing actions (breaths s^{-1}, length of time breathing, length of time under water); (4) examine gill structure for evidence of irritation or damage in relation to ROS levels.

MATERIALS AND METHODS

All experiments complied with Kenyan laws and all live fish were returned to their native location at the end of each experiment.

LOCATIONS

The Fish Springs Lagoon complex, located on the east side of Lake Magadi (1·867° S; 36·267° E), comprises four bodies of water (Fig. 1). Fish Springs Lagoon, itself, is bordered by volcanic rock with hot springs and a cement retaining wall. At the time of the study, the water was clear and algae grew on the cement, rock surfaces and sediment. On the other side of the retaining wall is the Pump House Holding Pond which was dark green with algal growth. Bird Lagoon, located north of the road to Fish Springs Lagoon, is connected to the main lake. Its waters were turbid and brown. The extent of Flamingo Lagoon, on the other side of the road, is determined by flooding during the rainy season. At the time of the study, its waters were also turbid and brown. Fish-eating birds were seen over Fish Springs Lagoon, Pump House Holding Pond and along the natural shoreline of Bird and Flamingo Lagoons.

An outdoor laboratory was constructed on the shaded porch of a nearby house(3 km; 15 min drive from the study and collecting site) provided by the Magadi Soda Company (tatachemicals.com/magadi/our_company/profile.html.UrRePvRDsUg). Fish for laboratory observations were held in 20 l, aerated buckets on the porch.

FIELD OBSERVATIONS

Over the period 21 July to 8 August 2010, measurements of temperature and oxygen were recorded from the lagoons and pond whenever they were visited [YSI Model 54 oxygen metre and probes (Yellow Springs Instruments Company; www.ysi.com) and the Digimed Oxygen metre (Digimed, Model DMO-2, www.digimed.ind.br), both corrected for a salinity of 60% sea water at 20° C based on a specific density of 1·015 (Coe, 1966)]. The % saturation of oxygen was calculated taking into account the altitude of Lake Magadi (605·6 m a.s.l.; Coe, 1966), and temperature using the equations described by http://www.waterontheweb.org/under/waterquality/oxygen.html/ Relevant oxygen levels were converted to kPa using oxygen solubility coefficients described in the study of Boutilier *et al.* (1984).

When *A. grahami* were air breathing, they came to the surface in groups, known as pods, that had a physical cohesion (Fig. S1, Supporting Information). The state of air breathing in the *A. grahami* population in Fish Springs Lagoon, namely the presence or absence of air breathing, location and extent of air breathing pods, and behaviour of individual fish were recorded throughout the study period. The daily pattern of prevalence of air breathing pods within Fish Springs Lagoon was determined from video clips, field notes and photographs. Video clips were used to analyse the air-breathing behaviour itself.

On 7 days between 20 July and 2 August, video clips of air breathing pods were collected using a Sanyo VPCWH1 Exacti camera (www.sanyo.com). Clips covered different periods of the day, in particular early morning and mid-afternoon. These clips were analysed in

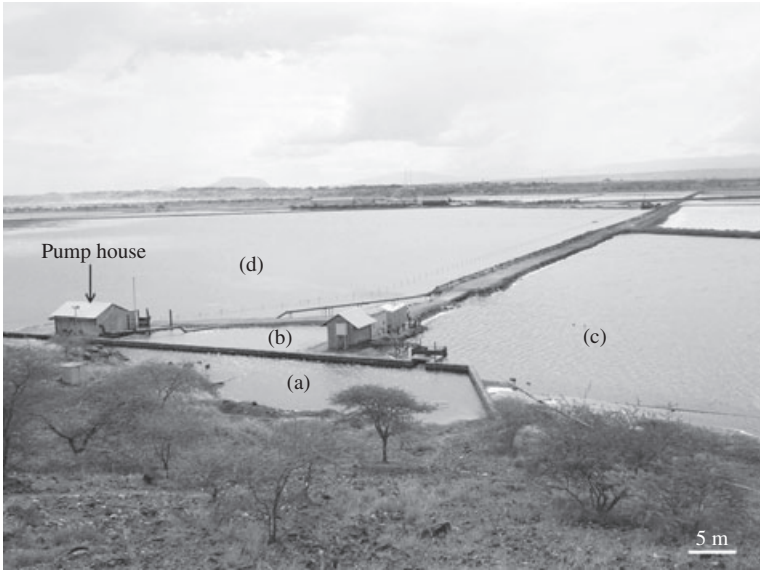

FIG. 1. A west-facing photograph of the Fish Springs Lagoon complex adjacent to the salt flats of the Magadi Soda Company on the mid-eastern shore of Lake Magadi, Kenya (1·867° S; 36·267° E). Fish Springs Lagoon (a), Pump House Holding Pond (b), Bird Lagoon (c) and Flamingo Lagoon (d). Pump House Holding Pond is 30 m on its longest axis.

TotalMediaExtreme for Sanyo (Arcsoft; www.arcsoft.com) recording the length of time individual fish were air breathing, the length of time they remained under water before returning to air breathe and the number of breaths s^{-1}. The fish were sorted into five equal length categories estimated to be between 25 and 65 mm total body length (L_T). Pods were examined to determine the number of fish that were air breathing and the number of fish that were not air breathing, but swimming through the pod. The proportion of fish in a pod that were air breathing was calculated from these data.

STUDY OF DAYTIME TEMPORAL PATTERNS

Daytime patterns in temperature, oxygen, ROS and air breathing were measured from pre-dawn and dawn to dusk and dark: pre-dawn and dawn, 0555–0620 hours; mid-morning, 0955 to 1015 hours; mid-afternoon, 1415 to 1440 hours; dusk and dark, 1805 to 1830 hours on 2 August. Video clips were taken and notes on air breathing were collected at each sampling period from Fish Springs Lagoon only. Temperature, oxygen and ROS samples were collected from the four study sites: Fish Springs Lagoon, Bird Lagoon, Pump House Holding Pond and Flamingo Lagoon. Temperature and oxygen were measured 3–5 cm below the water surface, then three replicate water samples for ROS were collected in the same region at the same depth using syringes fitted with Acrodisc syringe tip filters (0·45 μm) (Pall Corporation; www.pall.com). ROS samples were placed in the dark on ice and analysed within an hour.

BUCKET OBSERVATIONS

Observations of freshly caught *A. grahami* noted that some fish were air breathing when first brought in from the field (28° C, 2·3–4·1 mg O_2 l^{-1}, 6·93–12·26 kPa). If oxygen levels were allowed to decline, surface skimming was observed at 1·3–2·4 mg O_2 l^{-1} (3·87–7·20 kPa, 29–54 Torr), the same PO_2 range in Torr as reported by Narahara *et al.* (1996) for fish held at 31·0–37·5° C. Air breathing commenced again at O_2 levels of 0·71 mg l^{-1} (28·0° C)

in one bucket and 0·46 mg l^{-1} (26·8° C) in the other; that was 2·13 and 1·33 kPa, respectively. A PO_2 of 2·13 kPa (16 Torr) at 30–33° C is the minimal oxygen tension at which fish could survive when denied access to air (Narahara *et al.*, 1996). In aerated buckets, air breathing was not observed.

Bucket observations were employed to answer two questions: (1) Could field observations of afternoon air breathing be replicated? Newly caught fish were placed in two continuously aerated buckets of fresh Fish Spring Lagoon water, 10 fish per bucket. The buckets were first held in the shade and the fish were observed, and then placed in the sun in mid-morning. Temperature and oxygen were monitored throughout the day. Triplicate ROS samples were collected, as above, mid-morning, mid-afternoon and at dusk. Air breathing was monitored at three times during the afternoon while the buckets remained in bright sunlight. The air stones were removed and the fish were observed for 5 min. Fish did not come to the surface in the presence of air stones. (2) Would fish continue to air breath if hyperoxic conditions were created? Two buckets with six fish each were moved into the sun at noon and aeration was turned off after 45 min so that air breathing could be monitored more easily. Oxygen levels had fallen from 6·0 to 4·2 or 5·2 mg l^{-1} (15·40 to 12·14 or 15·03 kPa) and temperatures had risen from 27 to 35° C when aeration with pure oxygen was started at 1600 hours. Within 15 min, oxygen concentrations reached levels of 14·1 and 16·7 mg l^{-1} at 34–35° C (45·59–54·22 kPa or roughly 250% saturation) where they remained until the end of the experiment. The number of fish air breathing was monitored *c*. every 30 min, as above, until 1730 hours.

GILL MORPHOLOGY

Alcolapia grahami from Fish Springs Lagoon (lowest ROS levels) and Bird Lagoon (highest ROS levels) were collected between 0700 and 0800 hours and transported to the laboratory. Six fish from each pond were individually blotted dried, weighed to 0·01 g, euthanized and dissected as per Laurent & Hebibi, (1990). Dorsal, middle and ventral pieces of the second gill arch were preserved in 5% glutaraldehyde buffered with 0·15 M sodium cacodylate (pH 7·4) at 4° C. Pieces of anterior and posterior filaments from each piece were removed and embedded in Araldite blocks (www.go-araldite.com). The blocks were trimmed under the stereomicroscope and then sectioned (1 μm) using an automatic ultramicrotome (Ultracut; www.labequip.com). Every 10th section was mounted on a slide and stained with toluidine blue. The lamellae were examined for signs of irritation or change and for the presence or absence of an interlamellar cell mass (ILCM) (Nilsson, 2007).

ROS ANALYSIS

ROS was measured as H_2O_2 equivalents using the p-iodophenol, horse radish peroxidase (HRP), enhanced chemoluminescence method of Bruskov *et al.* (2002*a*). Prepared reagents were stored at 4° C and kept on ice packs in the field. Reagents consisted of 4-iodophenyl (5×10^{-5} M), luminol (5×10^{-5} M), HRP (10 U l^{-1} of final solution), Tris-HCl buffer at pH 8·5 (10^{-2} M) and H_2O_2. Small stock solutions of 4-iodophenol and luminol were created by dissolving 10 mg of each in 100 μl of dimethylsulphoxide (DMSO) in separate, foil-wrapped bullet tubes. The other solutions were made up in distilled water and stored in dark bottles. H_2O_2 standards were made just prior to collecting the samples.

The decay time of ROS in Fish Springs Lagoon water was measured so that ROS-free Fish Springs Lagoon water could be used in the standards as a control for any other chemoluminescence present in the water. Fish Springs Lagoon water collected on 19 July was held for over 24 h at outside temperatures in dark conditions in a sealed container. This should allow for the bacterial degradation of any ROS present (Cooper & Lean, 1989). New Fish Springs Lagoon water was collected at 1345 hours on 20 July and taken to the laboratory. ROS concentrations in the old and new waters were measured at 1510 and 1915 hours as luminescence. Decay of ROS follows first-order kinetics (Price *et al.*, 1998). The decay rate of ROS in Fish Springs Lagoon water was determined from the rate of change in the ratio, expressed in natural logarithms, of the luminescence of new to old water. Thereafter, Fish Springs Lagoon control water was held in the dark in a sealed container until >99% of ROS should have decayed (12·3 h).

Luminescence was determined by counting photons for 30 s in a Triathler luminometer [Triathler 425-004 Multilabel Tester (Triathler; www.hidex.com)]. All measurements were carried out in dim light and all reagents were kept on ice. Samples consisted of 0·3 ml of lagoon water plus distilled water to construct a 1/30 dilution. Six standards were prepared to cover the range from 0·00 to 0·31 μM H_2O_2 and were composed of 0·3 ml of the previously prepared control Fish Springs Lagoon water, distilled water and H_2O_2. Three replicates of each sample and standard were measured. The standard curves were best represented by a polynomial regression ($y = a + b_1x + b_2x^2$), where y is luminosity at the end of the 30 s measurement, x is the H_2O_2 concentration and a and b are constants (calculated in Origin Software; www.originlab.com). In order to determine the sample H_2O_2 concentration, the equation was solved for $x = 0$ to 0·25 μM H_2O_2 in 0·005 μM H_2O_2 increments and the x values were compared with field results.

STATISTICS

The effect of L_T and time of day on air breathing characteristics were analysed using two-way analysis of variance (ANOVA). Differences in ROS levels on 2 August amongst the four lagoons and pond and time of day were assessed using Freidman's two-way ANOVA based on ranks. Kruskall–Wallis tests were run to evaluate the significance of the time of day and location independently. If a variable was significant, a two-sample Kruskall–Wallis test was employed to determine which pairs were significantly different. The data are presented as means ± S.E. (n). Differences in gill structure between Fish Springs Lagoon and Bird Lagoon fish were assessed using the Fisher exact probability test. All statistical analyses were performed in Systat 11.0 (Systat Software Inc.; www.systat.com). A probability $\leq 0{\cdot}05$ was considered significant.

RESULTS

FIELD OBSERVATIONS

Diurnal patterns were observed across the study period in oxygen concentrations, temperature and incidence of air breathing. No rainfall was observed during the study period. It was generally sunny with occasional periods of cloud cover. Water temperatures were highest and more stable at Fish Springs Lagoon than at the other sites, with only a shallow diurnal pattern ranging from 32·0 to 35·1° C (Fig. 2). At the other sites, lowest temperatures ranged from 19·9 to 24·0° C. Maximum temperatures of 29·2–31·8° C were reached by 1600 hours. With respect to oxygen, all sites were hypoxic at dawn (<2·3 mg l^{-1} before 0630 hours, with Flamingo Lagoon ranging from 0·80 to 1·87 kPa, Pump House Holding Pond 1·33 to 6·27 kPa, Bird Lagoon 0·53 to 4·67 kPa and Fish Springs Lagoon from 3·87 to 4·80 kPa), with one exception: Flamingo Lagoon had an oxygen concentration of 6·1 mg l^{-1} (14·00 kPa) on 2 August. Oxygen concentrations increased to maximum levels of 5·0 ± 0·5 mg l^{-1} ($n = 9$) (16·66 kPa) at Fish Springs Lagoon, representing 75·2 ± 5·8% saturation, range 48–124% saturation. Hyperoxic conditions occurred by mid-afternoon at the other sites with oxygen concentrations exceeding 20 mg l^{-1} (59·72 kPa) and 250% saturation (Fig. 2).

Air breathing was studied systematically only at Fish Springs Lagoon. Aquatic surface respiration and some air breathing, however, were observed at dawn at Pump House Holding Pond. Detailed observations were not possible at Bird and Flamingo Lagoons as these could not be entered safely to observe fish. The daily pattern of prevalence of air breathing pods within Fish Springs Lagoon was determined from

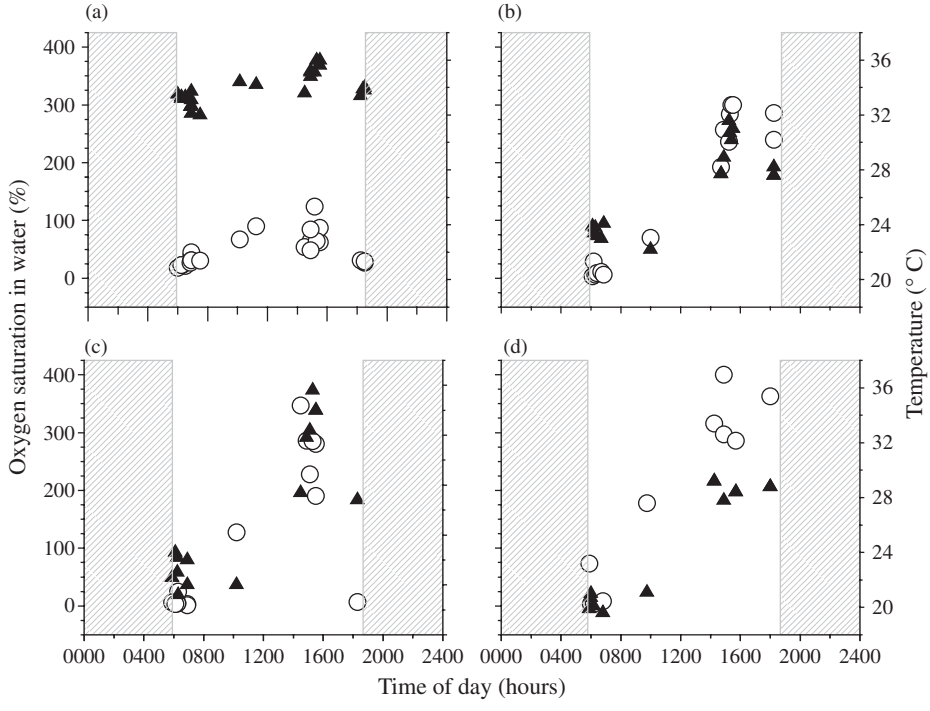

FIG. 2. Daytime patterns in temperature (▲) and oxygen saturation (○) observed between mid-July and mid-August 2010 at the four water bodies comprising the Fish Springs Lagoon complex, Lake Magadi, Kenya. Periods of darkness are indicated (▨). (a) Fish Spring Lagoon $n = 25$, (b) Pump House Holding Pond $n = 15$, (c) Bird Lagoon $n = 15$ and (d) Flamingo Lagoon $n = 11$.

video clips, field notes and photographs and was based on the spatial extent of air breathing pods. A distinct pattern emerged that was classified into three categories of increased intensity of air breathing within the lagoon [Fig. 3(a)]. (1) Few ($\leq$3) pods existed. This included the condition at dawn (0600 hours) when only two pods of *A. grahami* were observed air breathing, both pods in areas protected from avian predation (Movie S1, Supporting Information). Although different in pattern, the extent of air breathing at dusk (1800–1830 hours) was similar, a couple of pods were observed either near the wall or more diffusely in the open water (Figs S1 and S2, Supporting Information). On at least one day none of the fish were air breathing (2 August). (2) The period of time when more pods were forming. The time at which other pods started to form was variable. It could be as early as 0630 hours and as late as 0830 hours; however, most records showed some additional air breathing by 0700 hours. During this period, air-breathing fish started to gather into small groups near the wall which provided protection from avian predation. In the beginning, the groups were a mix of air breathing and non-air-breathing fish; however, by 0800 hours, the pods of air-breathing fish were generally well formed and >80% of fish within the pod were air breathing [Fig. 3(b)]. (3) The period of the day when maximum pod development could occur was between mid-morning and mid-afternoon. During this time, air-breathing fish were observed along long stretches of the wall and in the shallows along the eastern shore. The occurrence

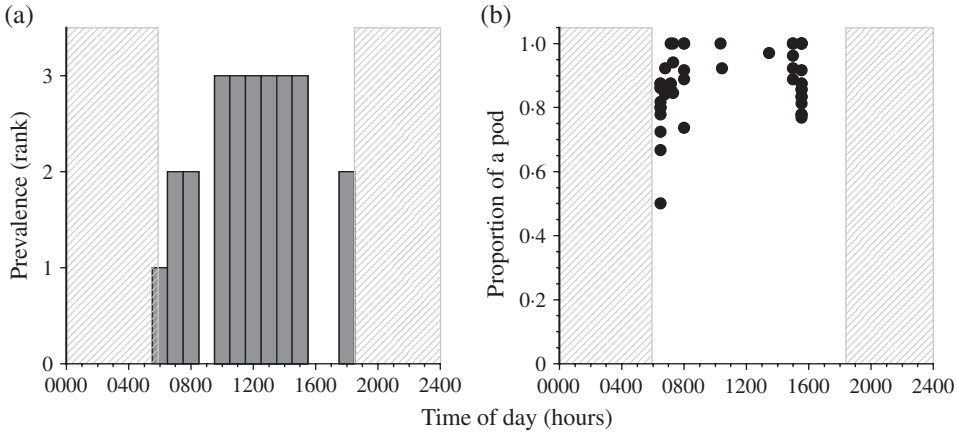

FIG. 3. Diurnal patterns in air breathing in *Alcolapia grahami* observed at Fish Springs Lagoon between 20 July and 8 August 2010. (a) A semi-quantitative measure of prevalence of air breathing in Fish Springs Lagoon based on field notes, videos and photographs: (1) ≤3 pods as seen near dawn each morning or at dusk, (2) period of pod development along the wall and (3) period of major extent of pods along the wall and in shallower waters. (b) The proportion of *A. grahami* in a pod that were air breathing.

and extent of pod development appeared to be related to sunlight: when the sky was cloudy, fewer fish were air breathing and fewer pods were observed. On 2 August, the peak occurred in mid-afternoon (1400–1500 hours).

Pods occupied specific, although mobile, locations (Movie S2, Supporting Information). *Alcolapia grahami* entered the pods before commencing air breathing. Several fish were observed specifically swimming into a pod and air breathing immediately, suggesting a strong drive to air breathe. Non-air-breathing fish tended to stay out of the pods, and fish that were air breathing outside the pod could be attacked by the large fish nearby [Fig 4(a) and Movie S3, Supporting Information], which would emerge from the water at high speed and land on or ram the target individual. These attacks were observed both in buckets at the laboratory and in video clips from the field. These behaviours are in stark contrast to the synchronous and surreptitious movement of a population of *A. grahami* coming to the surface to perform aquatic surface respiration (skimming) as seen under extreme hypoxia in the Pump House Holding Pond: 0·5 mg l^{-1} oxygen (1·20 kPa) [Movie S4(a), (b), Supporting Information].

Air breathing consisted of very rapid movement of air in and out of the mouth of the fish [Fig. 4(b) and Movie S5(a), Supporting Information]. A close-up video of individual fish in the laboratory also showed a reduction in opercular amplitude with air breathing [Movie S5(a), (b), (c) Supporting Information]. Air-breathing behaviour could be characterized by the length of time fish air breathed, the length of time during which the fish stayed under water before the next bout of air breathing, and the number of breaths they took per second when air breathing. In Fish Springs Lagoon, the mean ± S.E. length of a bout of air breathing was 9·8 ± 0·8 s ($n = 24$): data were only taken from fish where the complete air breathing bout was observed. A fish remained under water for mean ± S.E. 2·6 ± 0·3 s ($n = 29$) between air breathing bouts. During this time, they often released bubbles from the mouth and were observed shaking them away [Fig. 4(c) and Movie S5(c), Supporting Information].

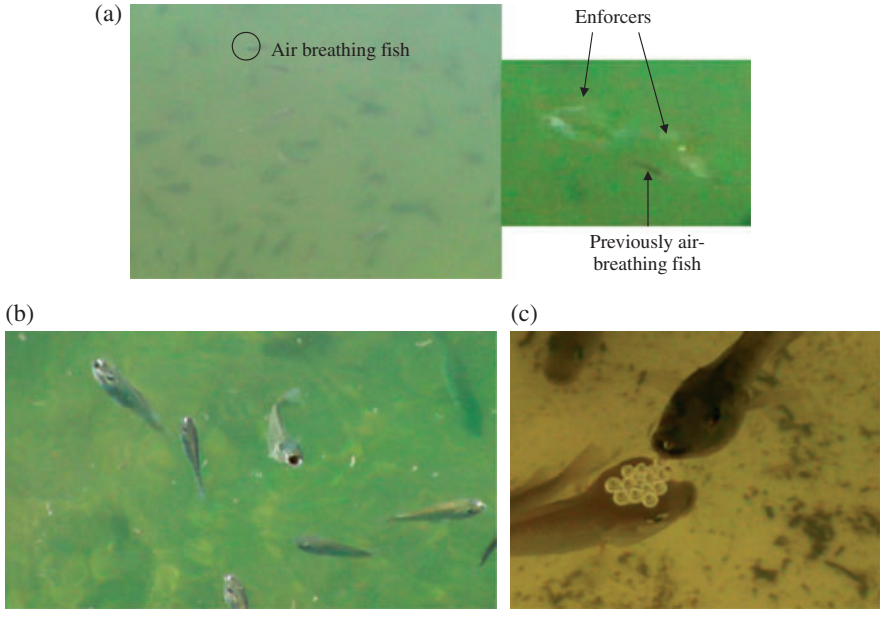

FIG. 4. Photographs extracted from the videos of *Alcolapia grahami* taken from Fish Springs Lagoon, Lake Magadi, Kenya, between 22 July and 7 August 2010. (a) A fish air breathing outside the pod and the action of larger fish (the enforcers) to stop the air breathing activity. (see also Movie S3, Supporting Information). (b) Pod of air-breathing fish. (c) A fish under water after an air breathing bout, expelling bubbles.

Sometimes, the bubbles were re-inhaled. The mean ± S.E. number of breaths s^{-1} was $3{\cdot}1 \pm 0{\cdot}1$ ($n = 73$). Length of fish did not affect the air breathing frequency or the length of a bout of air breathing (ANOVA: d.f. = 3, both $P > 0{\cdot}05$, $n = 74$). Small fish, however, stayed under water for shorter periods than large fish (ANOVA: $P < 0{\cdot}05$, $n = 28$): progression was observed through length ranges from $1{\cdot}5 \pm 0{\cdot}3$ s (mean ± S.E., $n = 4$) for the smallest fish to $4{\cdot}2 \pm 1{\cdot}0$ s ($n = 5$) for the largest fish (Fig. 5). The number of breaths s^{-1} and lengths of time spent air breathing and under water did not change with the time of day.

STUDY OF DAYTIME TEMPORAL PATTERNS

The study of environmental conditions, ROS concentrations and air-breathing behaviour started at 0555 hours. The temperature patterns were typical while the oxygen levels and per cent saturation were higher than normally recorded at dawn for three sites: 2 mg l^{-1} (4·80 kPa) at Bird Lagoon, 2·3 mg l^{-1} (5·60 kPa) at Pump House Holding Pond and 6·1 mg l^{-1} (14·00 kPa) at Flamingo Lagoon. [Oxygen concentration at Fish Spring Lagoon was 1·5 mg l^{-1} (4·27 kPa) at dawn.] Otherwise, they followed normal patterns (Fig. 6). Measured ROS concentrations covered the range from below detection to 8·1 μM H_2O_2 (Fig. 7). Concentrations were significantly different amongst locations and time of day (Freidman two-way ANOVA, d.f. = 3, 3, $P < 0{\cdot}001$; Kruskall–Wallis one-way ANOVA for location, d.f. = 3, $P < 0{\cdot}01$ and for time of day, d.f. = 3, $P < 0{\cdot}001$). ROS levels at Bird Lagoon were higher than those at Fish Springs Lagoon and Flamingo Lagoon (Kruskall–Wallis

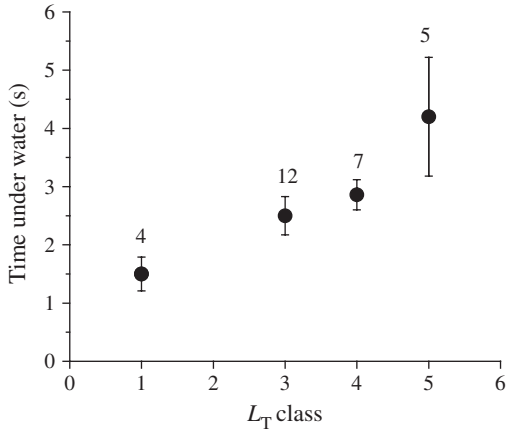

FIG. 5. Relationship between the time spent under water between bouts of air breathing and the total length (L_T) of *Alcolapia grahami*. Five categories were created of roughly equal length. The smallest individuals were in category 1 and the longest in category 5. The number of individual *A. grahami* measured is given. Values are means ± S.E.

paired tests, d.f. = 1, $P < 0{\cdot}05$). No other significant differences occurred amongst sites. Levels were lowest in the morning and climbed slowly to 1000 hours and then more rapidly to 1400 hours (Kruskall–Wallis paired tests, d.f. = 1, $P < 0{\cdot}05$). The levels at 1400 hours were not significantly different from those at 1800 hours, perhaps because the ROS levels at Bird Lagoon went up noticeably while those at Fish Springs Lagoon and Flamingo Lagoon decreased between these two periods (Fig. 7). Early morning, ROS levels (0600 hours) were 0·23 μM at Flamingo Lagoon, 2·75 μM at Bird Lagoon and below detection at Fish Springs Lagoon and Pump House Holding Pond. At their peak, ROS levels reached $2{\cdot}63 \pm 0{\cdot}64$ μM (mean ± S.E., $n = 2$) at Fish Springs Lagoon, $3{\cdot}55 \pm 0{\cdot}13$ μM ($n = 3$) at Flamingo Lagoon, $4{\cdot}95 \pm 0{\cdot}00$ μM ($n = 2$) at Pump House Holding Pond and $8{\cdot}10 \pm 0{\cdot}64$ μM ($n = 2$) at Bird Lagoon.

BUCKET OBSERVATIONS

Replication of field observations

Air breathing initiated when the fish were exposed to sunlight. Oxygen and temperature conditions in the two buckets were very similar at 1015 hours just before the buckets were removed from the porch to the bright sun: 27·7° C and 7·7 mg l^{-1} O_2 (23·06 kPa). Oxygen levels were maintained by aeration. Temperature, ROS concentrations and air breathing increased through the day when the buckets were exposed to sunlight (Fig. 8). ROS increased from 0·22 and 1·15 μM in the morning to 6·50 and 7·80 μM by 1600 hours and remained high until dusk. The aerator was removed to observe the fish. They were not air breathing when in the shade or first placed into sunlight. Occasional air breathing was observed at 1320 hours, more at 1440 hours and much greater air breathing at 1600 hours. Fish were also observed trying to escape from the bucket. Temperature rose to 33·5–34·0° C by 1320 hours and remained high during the early afternoon. Oxygen levels remained high: 6·7–7·0 mg l^{-1} O_2 (22·00 kPa).

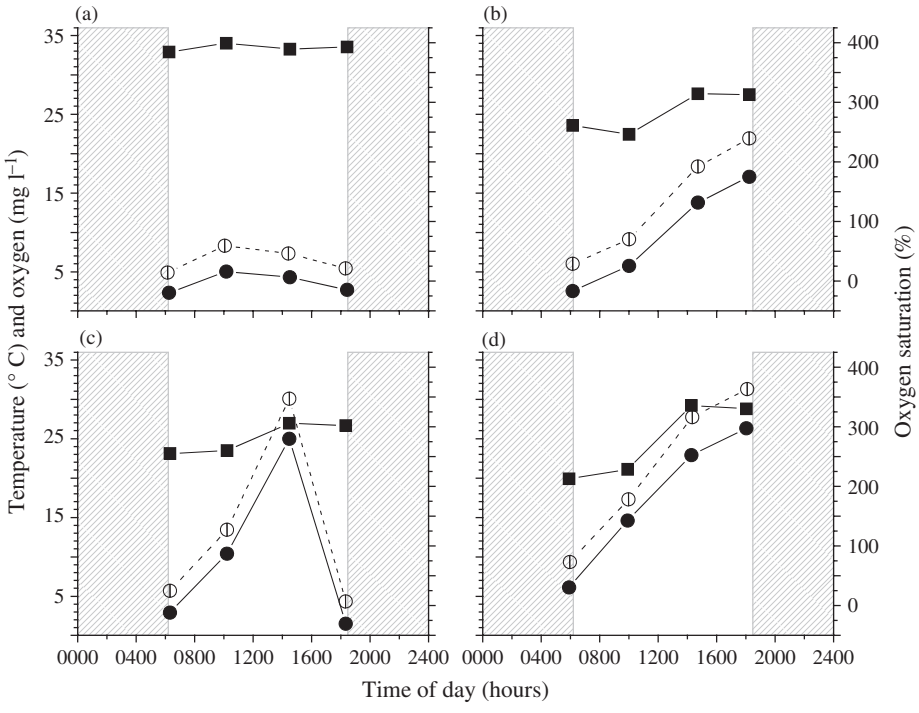

FIG. 6. Temperature (■), oxygen concentration (●) and oxygen saturation (⌽) changes throughout the day (2 August) in the four water bodies comprising the Fish Springs Lagoon complex, Lake Magadi, Kenya: (a) Fish Springs Lagoon, (b) Pump House Holding Pond, (c) Bird Lagoon, (d) Flamingo Lagoon. Periods of darkness are indicated (▨).

Air breathing in hyperoxia

Air breathing was observed in both normoxia and hyperoxia. Seven out of 12 fish were air breathing by mid-afternoon under normoxia and similar levels of air breathing were still observed after 75 min in hyperoxic conditions (14·1–16·7 mg l^{-1}, 45·59–54·12 kPa). ROS could not be measured due to equipment failure.

COMPARISON OF GILL MORPHOLOGY

Mass of the fish from Fish Springs Lagoon ranged from 2·24 to 2·65 g and those from Bird Lagoon ranged from 2·24 to 3·37 g. The fish in Fish Springs Lagoon and Bird Lagoon had significantly different gill structure (Fisher exact probability = 0·002, d.f. = 6, 6), *i.e.* the gills of all six fish from Fish Springs Lagoon were normal and showed little or no signs of ILCM [Fig. 9(a)]. In the gills of all six fish from Bird Lagoon, ILCMs had completely filled the interlamellar spaces [Fig. 9(b)].

DISCUSSION

This study established that *A. grahami* air breathe under normoxic and hyperoxic conditions in Lake Magadi. Air breathing under hyperoxic conditions has not been reported previously for any facultative air-breathing fishes. Within Fish

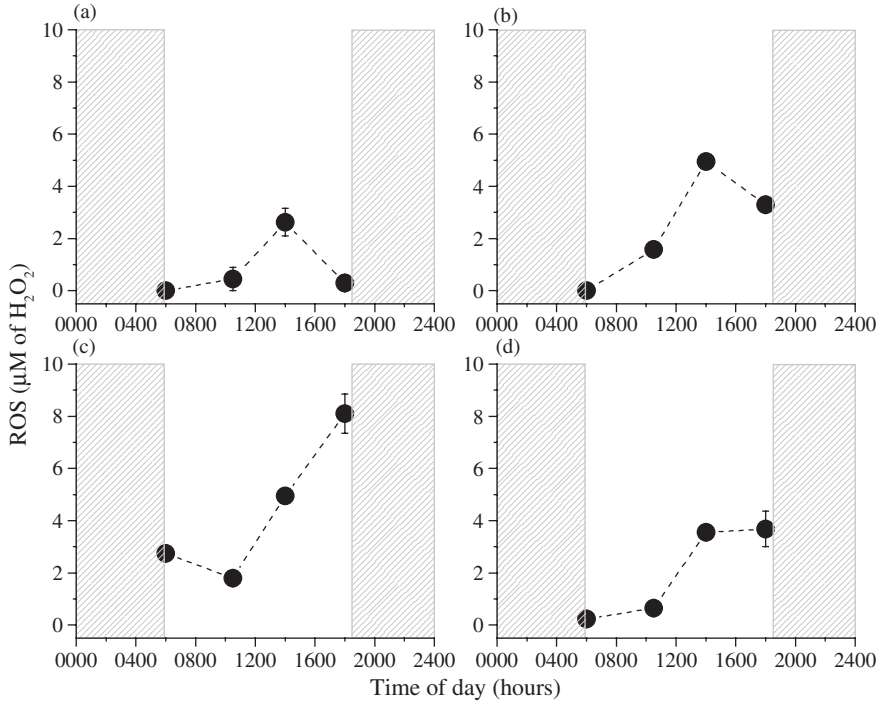

FIG. 7. Diel reactive oxygen species (ROS) concentrations (2 August) in the four water bodies comprising the Fish Springs Lagoon complex, Lake Magadi, Kenya: (a) Fish Springs Lagoon, (b) Pump House Holding Pond, (c) Bird Lagoon, (d) Flamingo Lagoon. Periods of darkness are indicated (▨). Values are means ± S.E. (n = 2–4).

Springs Lagoon, air breathing under normoxic and hyperoxic conditions was performed within a social context in that it occurred within certain regions of the pond and air-breathing fish aggregated into pods that were separated from the remainder of the population. Negative social interactions between air breathing and non-air-breathing fish outside the pods helped to maintain their integrity. These behaviours may reduce the risk of avian predation for both the air breathing and non-air-breathing fish. Although the fish were in a cohesive pod, there was no obvious synchrony to their movements. This behaviour was in stark contrast to the synchronous and surreptitious movement of a population of *A. grahami* coming to the surface to perform aquatic surface respiration.

In *A. grahami*, air breathing is performed in bouts of limited duration, interrupted by short spells under water during which time the fish often expels mucous-coated bubbles. The air breathing frequency was not influenced by fish length or the time of day. ROS, oxygen and temperature levels changed throughout the day; therefore, within these ranges, ROS (<0·12–2·63 μM H_2O_2), oxygen (1·4–8·1 mg l^{-1}, 4·00–23·46 kPa) and temperature (32·0–35·1° C) did not affect the air breathing frequency of *A. grahami*.

Brauner *et al.* (1995) have shown that *H. littorale* air breathes under normoxic conditions in the laboratory when exposed to irritants (acidic waters and hydrogen sulphide), and thus employs air breathing for purposes other than a response to

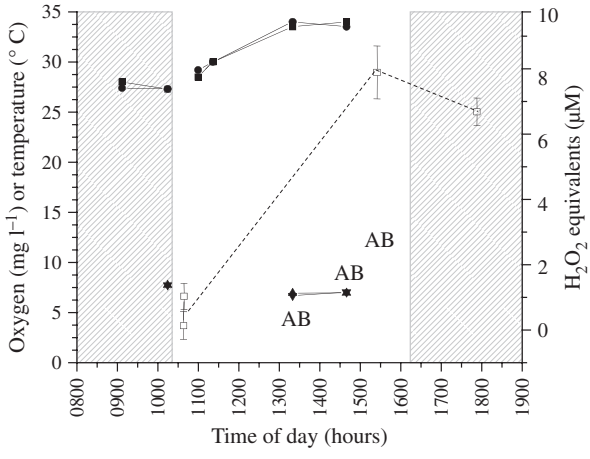

FIG. 8. Daytime patterns in temperature (■, ●), oxygen concentration (▲, ▼), diel reactive oxygen species (□) and air breathing by *Alcolapia grahami* (AB) in two 20 l buckets placed in sunlight at 1015 hours and monitored throughout the day. AB is based on a relative scale. ▨, period when the buckets were in the shade.

hypoxia. Could *A. grahami* do the same? Freyer & Iles (1969) point out that species of the genus *Tilapia*, as part of the extensive radiation of cichlid species in Africa, have adopted a generalist strategy and have often adapted to areas with harsher, more extreme environments. The evolution of *Tilapia* spp. in the Natron-Magadi basin has been very rapid (Tichy & Seegers, 1999). In Lake Magadi, *A. grahami* have altered their nitrogenous waste excretion, using urea instead of ammonia (Randall *et al.*, 1989; Wood *et al.*, 1989) and exhibit extreme adaptations in their strategies for acid-base regulation (Johansen *et al.*, 1975; Wood *et al.*, 1994, 2002) and ionoregulation and osmoregulation (Laurent *et al.*, 1995; Bergman *et al.*, 2003). They have shown considerable evolutionary plasticity. Thus, it is conceivable that they could also adapt air breathing for other purposes.

The actual purpose for air breathing under normoxic and hyperoxic circumstances are unknown. They may include response to an irritant, alteration in behaviour caused

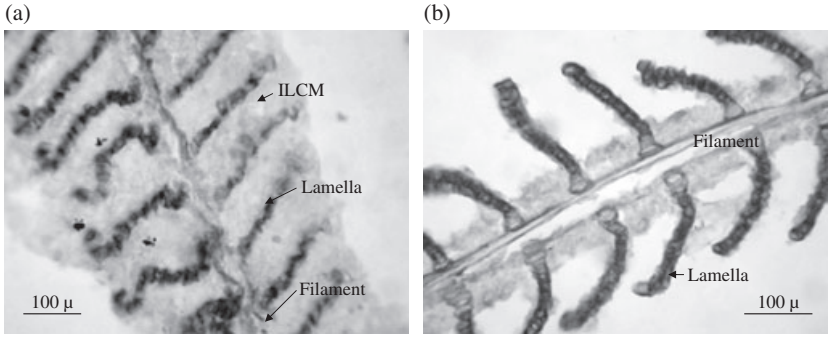

FIG. 9. Photographs of the gills of *Alcolapia grahami* showing (a) the presence of the interlamellar cell mass (ILCM) at Bird Lagoon and (b) the low level or remnants of the ILCM at Fish Springs Lagoon. *Alcolapia grahami* were collected between 0700 and 0800 hours.

by a parasite or gas exchange considerations, perhaps the exhalation of excess O_2 taken from the water so as to reduce ROS formation in the body, or preferential retention of CO_2 to maintain acid-base balance in this highly alkaline environment. ROS was the most obvious, general, potential irritant given the environmental conditions at Lake Magadi. High temperature, high pH, high UV light levels, high oxygen and high productivity all promote ROS production during the day (Scully *et al.*, 1996; Bruskov *et al.*, 2002*a*, *b*). The current data cannot prove that air breathing lessens the contact of an irritant with the gills. It does seem probable that if the fish were getting O_2 from the air, they would ventilate less water across the gills. The visible reduction in opercular amplitude during air breathing would suggest that less water is passing across the gills. High ROS, coupled with high water oxygen levels, could also cause very high oxygen blood levels which may be lowered by air breathing. Lake Magadi would be a reasonable place to expect such effects, as the maximum levels of H_2O_2 recorded are close to the highest observed in nature. Most marine and freshwater environments have H_2O_2 concentrations $<$420 nM (Millar & Kester, 1994). High values, comparable to the present observations, have been recorded for agricultural drainage ditch water exposed to sunlight, 6·8 μM (Draper & Crosby, 1983), the maximum for waters in the former Soviet Union, 3·0 μM (Skurlatov & Ernestova, 1998), 10 μM (Draäbkovaä *et al.*, 2007) and for intertidal rock pools, 5 μM (Abele-Oeschger *et al.*, 1997). The high Lake Magadi ROS levels in mid-afternoon and lower concentrations in the morning are probably part of a daily pattern. The presence of a diel cycle in ROS concentration at Lake Magadi is supported by the following: the rapid decline of ROS in water held in the dark at ambient temperature as would occur at night, the low ROS levels observed in morning water collected from Fish Springs Lagoon (bucket and field observations) and high ROS levels observed in the presence of mid-afternoon sunlight (bucket and field observations). Diel patterns in ROS concentrations with a peak in mid-afternoon are common in freshwater and coastal systems (Cooper & Lean, 1989; Wilson *et al.*, 2000; Häkkinen *et al.*, 2004).

At Lake Magadi, samples for ROS were collected 3–5 cm below the water surface, yet the fish live at all depths. Would fish be exposed to similar ROS levels at depth? ROS production in freshwater areas appears to be derived predominantly from photochemical reactions (Yuan & Shiller, 2005). Consequently, UV extinction coefficients (vertical penetration of UV light), vertical mixing, vertical distribution of reactive substances (*e.g.* oxygen) and vertical decay rates of ROS govern the vertical profile of ROS in fresh waters (Cooper & Lean, 1989; Häkkinen *et al.*, 2004). The maximum depth of Fish Springs Lagoon was 1 m; most of the lagoon was much shallower. The water was relatively clear, fish and bottom structure could be readily observed in the deepest areas and temperature and oxygen levels near the bottom were the same as at the surface, indicating that the water was mixed. Thus, ROS concentrations were presumably similar throughout the water column in Fish Springs Lagoon. In the other three water bodies, the water was turbid either with algae (Pump House Holding Pond) or with sediment and algae (Flamingo and Bird Lagoons). It is unlikely that photochemical ROS production occurred far into the water column. Distribution of ROS to deeper reaches would have depended on mixing, but it is not known to what extent that may occur at these sites.

Of all the pro-oxidants in ROS, H_2O_2 is not ionized and can pass across cell membranes where it interacts with transition metals to form the highly reactive hydroxyl

radical, OH^- (da Rosa *et al.*, 2008). The strongest effects of ROS under natural conditions have been seen in bacterial production and cyanobacteria photosynthesis (Xenopoulos & Bird, 1997; Draäbkovaä *et al.*, 2007). Some invertebrates have responded with symptoms of oxidative stress when exposed to H_2O_2 concentrations near the levels observed in Lake Magadi. Reductions in oxygen uptake have been seen in *Nereis diversicolor* exposed to 5 μM H_2O_2 for 6 h (Buchner *et al.*, 1994) and the Antarctic intertidal limpet *Nacella concinna* exposed to 3 and 5 μM H_2O_2 for 4 h at 4° C (Abele *et al.*, 1998). The latter also suffered enhanced lysosome damage. Studies of sublethal effects of H_2O_2 on fishes are generally lacking except when H_2O_2 was used to treat disease, such as fish lice in Atlantic salmon *Salmo salar* L. 1758 (Kiemer & Black, 1997) and columnar disease in juvenile rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) (Speare & Arsenault, 1997). A few studies, however, reported on the oxidative stress response of *O. mykiss* juveniles, *S. salar* smolts and goldfish *Carassius auratus* (L. 1758) exposed to hyperoxia up to 180% saturation (Lygren *et al.*, 2000; Dabrowskia *et al.*, 2004; Lushchak *et al.*, 2005; Lushchak & Bagnyukova, 2006). These investigations indicate that exposure to hyperoxia, *per se*, is an oxidative stress with enzyme responses either during hyperoxia (*S. salar*) or on return to normoxia (*C. auratus*), but they did not consider the possibility of ROS formation in the external water. Growth rate and ascorbic acid levels in the gills declined in hyperoxic conditions in *O. mykiss*. A morphological study of the effects of hyperoxia (60 kPa) on *O. mykiss* gills described changes in the structure of the epithelium and in the internal structure of filamental chloride cells suggesting increased activity (Laurent & Perry, 1993). Additional ROS created by high temperatures and UV radiation outdoors in the sunshine should make the hyperoxic environment even more stressful to fish.

Taken together, these considerations suggest that cellular responses may be initiated at the concentrations of ROS measured in Lake Magadi, especially under chronic (albeit cyclic) exposure. The effect on Lake Magadi may be even higher than the concentrations would suggest because stressors do not act in isolation. Lake Magadi is a harsh environment with high energy costs to the fish, resulting in the highest routine metabolic rates ever recorded for a teleost of this size (Franklin *et al.*, 1995; Narahara *et al.*, 1996). These high energy expenditures are associated with (1) high metabolic demand due to the costs of acid-base regulation, estimated as 50% of routine O_2 consumption (Wood *et al.*, 2002), (2) the additional physiological costs of conversion of waste nitrogen to urea instead of ammonia for excretion at pH 10 (Randall *et al.*, 1989; Wood *et al.*, 1989, 1994) and for life at high temperature, (3) high feeding demands in response to these elevated metabolic rates, (4) the necessity of using either aquatic surface respiration or air breathing, and added vigilance against avian predators, on exposure to hypoxic conditions which develop overnight and are seen at dawn and (5) energetic costs of more extensive ventilation and loss of time feeding when mouth brooding.

Exposing organisms to H_2O_2 may also underestimate the exposure to ROS because ROS comprises a number of components, some of which are more reactive than H_2O_2 and may cause a response at more exposed surfaces such as the gills. Gill structure can change in response to environmental conditions (Chapman *et al.*, 2000; Sollid & Nilsson, 2006). Depending on the oxygen concentration and temperature, some species of cyprinid can alter the proportion of the gill exposed to the environment by infilling between the lamellae to various degrees producing an ILCM (Nilsson,

2007; Nilsson *et al.*, 2012). Recently, Brauner *et al.* (2011) and Tzaneva *et al.* (2011) have shown that the extent of the ILCM may be related to the oxygen levels and demand within the fish. Similar ILCMs have been observed in response to toxicants in the environment (*e.g.* acid pH and certain metals) and the presence of gill parasites (Nilsson *et al.*, 2012). The *A. grahami* from Fish Springs Lagoon showed distinct lamellae with very little evidence of ILCM. This may mean that for a fish experiencing cyclic hypoxia and low levels of hyperoxia, including species with a high blood affinity for oxygen [$p_{50} = 6$ Torr (0·80 kPa) at 30–32° C in *A. graham*; Narahara *et al.*, 1996], it is preferable to maintain high gill surface area. The interlamellar space of all fish from Bird Lagoon was largely filled with an ILCM. Thus, *A. grahami* may be added to the number of fishes known to be capable of forming an ILCM, the first cichlid. In Bird Lagoon, infilling may be a response to more severe hyperoxia as seen in *C. auratus* by Tzaneva *et al.*(2011) or to ROS or the combination of hyperoxia and high ROS. As Bird Lagoon also experiences hypoxia at dawn, it is possible that protection of the gills under hyperoxic (and high ROS) conditions is more important than adjustments of the gill for daily hypoxic periods, which can be accommodated by aquatic surface respiration and air breathing, as necessary. Nonetheless, the possibility that the ILCM response at Bird Lagoon was a response to another stressor cannot be discounted.

The influence of ROS on daytime air breathing under normoxic or hyperoxic conditions cannot be confirmed at this point. This study does indicate that the role of ROS in the behaviour and changing gill structure of *A. grahami* requires further examination. Relevant observations include (1) the similar daily patterns in ROS production and in the extent of air breathing in Fish Springs Lagoon, (2) the tendency of fish to increase their air breathing and to try to escape from buckets placed in direct sunlight, when ROS levels were high, (3) the response in the extent of air breathing in Fish Springs Lagoon to the level of sunlight (cloudy *v.* clear skies) and (4) the remodelling of the gills of *A. grahami* in Bird Lagoon, but not in Fish Springs Lagoon; the former experienced daily hyperoxia and three times the level of ROS in the water as compared with Fish Springs Lagoon.

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Supporting Information

Supporting Information may be found in the online version of this paper:
FIG. S1. Two pods of air breathing *Alcolapia grahami* present at dusk in Fish Springs Lagoon, 1804 hours. (b) An enlargement of the air breathing pods in photograph (a). The two pods are circled. Note the cohesive structure of each pod.

FIG. S2. Air breathing *Alcolapia grahami* located diffusely over part of Fish Springs Lagoon at dusk, 1815 hours. The regions are circled. The open mouths of the fish appear as black dots.

MOVIE S1. Air breathing *Alcolapia grahami* in the protective corner of the wall at Fish Springs Lagoon half an hour after dawn, 0628 hours.

MOVIE S2. An example of the mobile boundaries and movement of a pod of air breathing *Alcolapia grahami* in Fish Springs Lagoon.

MOVIE S3. An example of an *Alcolapia grahami* air breathing outside of the pod (see top centre of the video) and the ramming action of larger fish which stop the former from air breathing 5·5–6 s into the video. An air breathing pod is towards the bottom left.

MOVIE S4. A comparison of the movement within (a) a pod of air breathing *Alcolapia grahami* in the early morning (Fish Springs Lagoon, 0759 hours) and (b) a group of fish using aquatic surface respiration (ASR) (Pump House Holding Pond, 0733 hours). Note that at Pump House Holding Pond, while the majority of fish are using ASR some fish are air breathing, as noted by the dark open mouths.

MOVIE S5. Close up of individual *Alcolapia grahami* that are air breathing. (a) Air breathing posture, the rapid buccal movements characteristic of air breathing, the reduced amplitude of opercular movement during air breathing and release of air bubbles at the end of a bout of air breathing. (b) Transition of a fish with regular movement of water past the gills with larger opercular movements to air breathing and smaller opercular movements. Speed=one fifth of true time. (c) A fish going between air breathing and not air breathing, as viewed from the side.

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