

RESEARCH ARTICLE

Frogs seek hypoxic microhabitats that accentuate metabolic depression during dormancy

Giulia S. Rossi^{1,*}, Rebecca L. Cramp², Patricia A. Wright¹ and Craig E. Franklin²

ABSTRACT

Many animals occupy microhabitats during dormancy where they may encounter hypoxic conditions (e.g. subterranean burrows). We used the green-striped burrowing frog (*Cyclorana alboguttata*) to test the hypothesis that animals seek hypoxic microhabitats that accentuate metabolic depression during dormancy. We first measured the partial pressure of oxygen (P_{O_2}) within artificial cavities excavated in wet clay soil, which simulated *C. alboguttata* underground aestivation chambers, and recorded hypoxic conditions (P_{O_2} as low as 8.9 kPa). Using custom-built tunnels that maintained a longitudinal P_{O_2} gradient (hypoxic to normoxic), we then examined the P_{O_2} preference of *C. alboguttata* in response to drying habitat conditions. In support of our hypothesis, we found that *C. alboguttata* chose to spend a greater proportion of time at the hypoxic end of the P_{O_2} gradient compared with the normoxic end. To determine whether hypoxia accentuates metabolic depression in *C. alboguttata*, we exposed frogs to normoxia (21.0 kPa) or hypoxia (10.5 kPa) for 7 weeks during the transition from an active to an aestivating state. We found that hypoxia exposure accelerated the onset of metabolic depression in *C. alboguttata* by 2 weeks. Furthermore, we found that frogs exposed to hypoxia exhibited a 66% reduction in O_2 consumption after 7 weeks compared with active frogs in normoxia, whereas frogs exposed to normoxia reduced O_2 consumption by only 51%. Overall, our findings indicate that some animals may seek microhabitats to maximally depress metabolic rate during dormancy, and that microhabitat O_2 availability can have significant implications for energy metabolism.

KEY WORDS: Dormancy, Aestivation, Hypometabolism, Hypoxia, Oxygen gradient, Amphibians

INTRODUCTION

A number of animals enter a state of dormancy (e.g. aestivation) to tolerate seasonally arid environmental conditions. During aestivation, animals undergo a suite of physiological modifications to cope with the challenges associated with arid environments, including limited food and water availability (Pinder et al., 1992). Among the more striking physiological changes is the strong depression of metabolic rate to conserve limited endogenous energy reserves in the absence of external food sources (for reviews, see Guppy and Withers, 1999; Storey, 2002; Storey and Storey, 2012). Here, we use the term metabolic depression to describe a reduction in metabolic rate below the routine value. The extent to which different animals suppress metabolic rate during

aestivation falls along a continuum, but a considerable metabolic depression is necessary for animals that remain dormant for many months (Guppy and Withers, 1999). The premature exhaustion of endogenous reserves can result in the atrophy of critical muscles and tissues (Hudson and Franklin, 2002; Mantle et al., 2009; Secor and Lignot, 2010) and the impairment of reproduction when animals resume activity (Pusey, 1990), and may be ultimately fatal (Horne, 1979; Etheridge, 1990). In the extreme, aestivating populations may be threatened with extirpation if all individuals completely exhaust endogenous reserves before the return of environmental conditions favourable for active life (van Beurden, 1980).

The extent of metabolic depression in aestivating ectotherms can be influenced by the microhabitat. For example, microhabitats with elevated temperatures can increase metabolic rate and, consequently, expedite substrate utilisation (Young et al., 2011), whereas microhabitat conditions that constrain aerobic metabolism may promote metabolic depression. Hypoxic conditions, for example, have been reported to depress metabolism in a number of ectothermic animals (e.g. Boutilier et al., 1997; Hicks and Wang, 1999; St Pierre et al., 2000). Hypoxic hypometabolism is accomplished by downregulating physiological processes involved in ATP turnover (Hochachka et al., 1996; Boutilier, 2001), thereby conserving energy and limiting the accumulation of toxic metabolic end-products (e.g. lactate) in animals during conditions of limited oxygen availability (Hochachka and Somero, 1984). For animals that aestivate for many months, hypoxic hypometabolism may be beneficial if it accentuates metabolic depression and considerably slows the rate of substrate utilisation.

Several animals aestivate within underground burrows, where they may encounter hypoxic conditions. Burrowing has been reported in annelids (Bayley et al., 2010), molluscs (Kotsakiozi et al., 2012; Osborne and Wright, 2018), fishes (Smith, 1931; Chew et al., 2004), amphibians (Ruibal and Hillman, 1981; Etheridge, 1990; Booth, 2006) and reptiles (Kennett and Christian, 1994) as a strategy to avoid desiccation, elevated temperatures and predation while in an aestivating state. Although burrows dug in sandy soils are typically well ventilated (Hanks and Thorp, 1956), heavy clay soils pose a significant barrier for gas exchange, and thus hypoxic conditions within the burrow can develop (Chew et al., 2004; Shams et al., 2005). The influence of hypoxic microhabitats on the metabolic rate of aestivating amphibians has not been thoroughly investigated. However, we have recently demonstrated that amphibious fish (*Kryptolebias marmoratus*) seek hypoxic microhabitats during prolonged air exposure that accentuate metabolic depression (Rossi and Wright, 2020). Do other animals also seek hypoxic microhabitats to enhance hypometabolism during dormancy?

Using the green-striped burrowing frog (*Cyclorana alboguttata*), we tested the hypothesis that aestivating animals select hypoxic microhabitats that accentuate metabolic depression during aestivation. During periods of prolonged drought, *C. alboguttata* burrow underground and aestivate within a cocoon of shed skin and

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mucus (Lee and Mercer, 1967). As *C. alboguttata* dig down into wet clay, they form a subterranean chamber, effectively becoming completely encased/entombed in the mud with no direct connection with the surface. Consequently, hypoxic conditions may develop in the underground cavities occupied by *C. alboguttata* during aestivation (Booth, 2006). The hypoxic habitat hypothesis predicts that frogs will preferentially occupy hypoxic rather than normoxic microhabitats in response to habitat drying. We placed frogs in custom-built experimental choice chambers that maintained an oxygen (O_2) gradient (hypoxic to normoxic) to determine the preferred partial pressure of O_2 (P_{O_2}) of frogs in response to drying habitat conditions. The hypoxic habitat hypothesis also predicts that hypoxia will accentuate metabolic depression in aestivating *C. alboguttata*. Thus, we compared the rate of O_2 consumption in frogs maintained in either normoxia (control) or hypoxia during the transition from an active to an aestivating state.

MATERIALS AND METHODS

Experimental animals

Adult *Cyclorana alboguttata* (Günther 1867) (mean \pm s.e.m. body mass=20.8 \pm 0.7 g, mixed sex) were collected from wet roads in non-protected areas near Lake Broadwater in Dalby, Queensland, Australia, in December 2018 ($n=21$). Frogs were individually placed into large Ziploc plastic bags for transport to The University of Queensland, where they were maintained for several months prior to experimentation. Frogs were housed individually in either small (235 \times 170 \times 120 mm) or large (265 \times 235 \times 12 mm) well-ventilated clear plastic containers. Each housing container was lined with paper towels saturated with chemically aged water (dilution 1:4000; VitaPet, NSW, Australia), and contained a half PVC pipe for shelter. Frogs were fed vitamin-dusted crickets (*Acheta domesticus*) once per week, and the housing containers were cleaned weekly. The photoperiod was maintained on a 12 h:12 h light:dark cycle and room temperature was kept constant at 23°C. All animals were collected with approval of the Queensland Department of Environment and Heritage Protection (SPP WA0011256), and all experiments were carried out with the approval of The University of Queensland Animal Welfare Committee (SBS/502/18).

Microhabitat O_2 levels

The P_{O_2} within natural *C. alboguttata* aestivation cavities has never been measured. The underground chambers are extremely difficult to locate from above ground because an air tunnel conduit is not maintained. Thus, we collected clay soil from the *C. alboguttata* collection site to create artificial chambers in which P_{O_2} could be measured. We buried three hollow, perforated rubber balls (95 mm diameter) at approximately 10 cm depth in individual soil-filled buckets (6 litres) to simulate the underground cavity in which frogs reside during aestivation (Fig. S1). We maintained access to the aestivation cavity through a plastic tube (27 mm diameter) that protruded from the soil. The soil in each container was saturated with water to promote microbial activity as would be present when *C. alboguttata* excavate their burrows. We monitored P_{O_2} within the artificial aestivation cavity over a 2 week period (between 10:00 and 14:00 h) using an O_2 -sensing optode and Presens Optical Oxygen Sensor (Precision Sensing, Regensburg, Germany) inserted down the plastic tube and into the perforated ball. The plastic tube was closed to the external environment when O_2 measurements were not being taken. The artificial burrows remained outdoors during the 2 week period, where temperatures ranged from 14°C (night) to 27°C (day), and were typical of temperatures that *C. alboguttata* would experience during aestivation.

Experimental protocol

Environmental O_2 preference

To determine whether *C. alboguttata* select hypoxic microhabitats in response to drying habitat conditions, we built custom experimental choice chambers (750 \times 240 \times 120 mm) through which frogs could move freely (Fig. 1A). The choice chambers were lined with paper towels saturated with aged water. We individually placed frogs in the centre of an experimental choice chamber and then generated an O_2 gradient (10.9 \pm 0.1 to 18.8 \pm 0.1 kPa; Fig. 1) by introducing a gentle stream of air at one end of the chamber, and a gentle stream of N_2 at the other. We periodically monitored the O_2 levels along the gradient during each behavioural trial using four evenly spaced O_2 -sensing optodes glued to the choice chamber, and a Presens Optical Oxygen Sensor. The O_2 gradient remained stable over time (Fig. S2). The gradient direction (low to high O_2) was reversed (high to low O_2) between trials. Frogs remained in the experimental choice chambers for 24 h, during which time the paper towel dried gradually. We continuously video-recorded frogs during the 24 h experimental period using infrared surveillance cameras (Eonboom Electronics Limited, and K Guard Security, New Taipei City, Taiwan), and a 16-channel H.264 Digital Video Recorder (DVR) system. We chose a 24 h experimental period because preliminary behavioural trials lasting 48 h revealed that frogs were relatively immobile after 24 h. We conducted all O_2 choice trials in the dark to simulate the burrowing conditions of *C. alboguttata* in the wild. All monitoring of the O_2 gradient was performed under red light to minimise disturbance to the frogs. All frogs were fasted for at least 48 h prior to choice experiments.

For analysis, we binned the choice chambers into three O_2 zones of equal size: the 'low O_2 zone' (10.9 \pm 0.1 to 15.1 \pm 0.1 kPa), the 'medium O_2 zone' (15.1 \pm 0.1 to 17.4 \pm 0.1 kPa) and the 'high O_2 zone' (17.4 \pm 0.1 to 18.8 \pm 0.1 kPa). We calculated the proportion of time frogs spent in each O_2 zone after they had sampled every zone. We also recorded the first O_2 zone in which frogs remained immobile for at least 1 h and assumed a water conserving posture (Fig. S3) as a proxy for a suitable burrowing location. The 1 h time period was chosen because it takes *C. alboguttata* approximately 1 h to burrow in wet clay substrates (Booth, 2006). One frog did not sample all O_2 zones, and was consequently excluded from all behavioural analyses. All animals were weighed before and after each experimental trial to determine whether any dehydration occurred owing to habitat drying.

O_2 consumption in normoxia and hypoxia

Following the O_2 choice experiments, we randomly assigned frogs to one of two 7 week aestivation treatments: normoxia (control; $n=9$) or hypoxia ($n=10$). All frogs were fasted for 72 h in their housing containers, then individually placed in a 500 ml respirometry chamber lined with two 15 cm² pieces of paper towel saturated with aged water. The normoxia (21.0 kPa) and hypoxia (10.5 kPa) exposures were accomplished by continuously flushing the respirometry chambers with a gentle stream of humidified air or air- N_2 mixture. We verified the O_2 levels within the respirometry chambers periodically throughout the 7 week experimental period using O_2 -sensing optodes glued to the inside of each respirometry chamber, and a Presens Optical Oxygen Sensor. Aestivation was induced by allowing the paper towel to dry gradually, as well as maintaining frogs in the dark throughout the experimental period (24°C) (Flanigan et al., 1991).

The mass-specific rate of O_2 consumption in *C. alboguttata* was measured weekly using closed-system respirometry. Frogs were given 24 h to acclimate to their respirometry chamber and respective

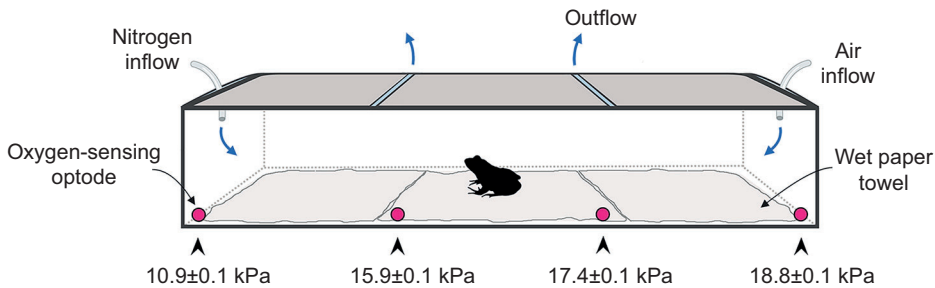


Fig. 1. Schematic representation of a custom-built experimental choice chamber. The partial pressure of O_2 (P_{O_2}) range within the low O_2 zone (left), medium O_2 zone (centre) and high O_2 zone (right) was determined using O_2 -sensing optodes and a Presens Optical Oxygen Sensor. The choice chambers used for *Cyclorana alboguttata* were 750×240×120 mm and made from clear acrylic.

O_2 exposure before the week 0 measurements. The O_2 consumption measurements were made by sealing respirometry chambers for a 2–8 h period and measuring the decline in P_{O_2} (longer measurement periods were required as the rate of O_2 consumption declined throughout the experimental period). Week 0 measurements consisted of three trials (repeated measures) to establish a mean 'resting' O_2 consumption rate for each frog. In order to minimise disturbance during the onset of aestivation, only one O_2 consumption measurement was made per animal in the remaining weeks as previously described (Young et al., 2011). All O_2 consumption measurements were performed in the dark under red light to minimise disturbance to the frogs. When necessary, 1 ml of aged water was discreetly added to the respirometry chambers to prevent complete desiccation. Two control frogs died during the 7 week experimental period and were thus excluded from the analysis. One hypoxia-exposed frog was also excluded because it did not assume the water conserving posture by the end of the experimental period. Frogs were weighed before and after the experimental period to assess hydration status.

Statistical analysis

We determined the preferred P_{O_2} of *C. alboguttata* using methods for compositional data, because the proportion of time frogs spend in any one O_2 zone is dependent upon the time spent in other O_2 zones. All proportions were isometric log-ratio (ILR) transformed and then analysed using an ordinary least squares (OLS) regression. We subsequently back-transformed the coefficients for meaningful interpretation of the results (van den Boogaart and Tolosana-Delgado, 2013). We performed a chi-squared goodness-of-fit test to determine which O_2 zone *C. alboguttata* selected as the first suitable 'burrowing' location in response to habitat drying. A two-way repeated-measures ANOVA with a Greenhouse–Geisser correction was used to compare the rate of O_2 consumption in normoxia- and hypoxia-exposed frogs over time. We performed a Dunnett's many-to-one comparison test to determine when the rate of O_2 consumption within each P_{O_2} treatment group differed from that of active frogs (all reported P -values are adjusted for multiple comparisons). Paired t -tests were used to compare the body mass of *C. alboguttata* before and after use in the behavioural and O_2 consumption experiments. Prior to analysis, we assessed all data for normality of residuals and homogeneity of variance. All results were considered significant at $P < 0.05$.

RESULTS

Microhabitat O_2 levels

The P_{O_2} in the artificial aestivation cavities varied considerably across our three replicates, and over time. We found that the P_{O_2} within the aestivation chambers became hypoxic (P_{O_2} as low as 8.9 kPa) while the clay soil was saturated with water (Fig. 2). As the soil began to dry and crack several days later, the P_{O_2} within the aestivation cavities returned to relatively normoxic levels.

Environmental O_2 preference

The proportion of time *C. alboguttata* spent in each O_2 zone was not equally distributed. We found that frogs spent $57.4 \pm 6.9\%$ of the time in the low O_2 zone, and only $23.9 \pm 5.6\%$ and $18.7 \pm 5.2\%$ in the medium and high O_2 zones, respectively (OLS: $P < 0.001$; Fig. 3A). *Cyclorana alboguttata* did not seek any one zone as their preferred 'burrowing' location (chi-squared: $P = 0.12$), although 55% of frogs selected the low O_2 zone as their first suitable 'burrowing' location, and only 20% and 25% selected the medium and high O_2 zones, respectively. The body mass of *C. alboguttata* after the O_2 choice experiments was 7% lower than that before (t -test: $P < 0.001$).

O_2 consumption in normoxia and hypoxia

The mass-specific rate of O_2 consumption in *C. alboguttata* was significantly influenced by P_{O_2} exposure and time (two-way ANOVA: $P = 0.002$, $P < 0.001$; Fig. 4). On average, the O_2 consumption rate in hypoxia-exposed frogs was $27.7 \pm 0.1\%$ lower than that of normoxic (control) frogs over the 7 weeks. The rate of O_2 consumption in hypoxia-exposed frogs during weeks 2–7 was significantly different from that of week 0 (Dunnett's: $P < 0.01$). In contrast, it took longer for metabolic depression to occur in normoxic frogs (weeks 4–7 were different from week 0; Dunnett's: $P < 0.05$). By the end of the experimental period (week 7), hypoxia-exposed frogs exhibited a 59% reduction in the rate of O_2 consumption from active (week 0) counterparts (from 41.4 ± 4.6 to $21.1 \pm 1.8 \mu l O_2 g^{-1} h^{-1}$; Dunnett's: $P < 0.001$), whereas control frogs exhibited a 51% reduction (from 34.2 ± 1.8 to $14.2 \pm 1.1 \mu l O_2 g^{-1} h^{-1}$; Dunnett's: $P > 0.001$). We found no change in the body mass of *C. alboguttata*

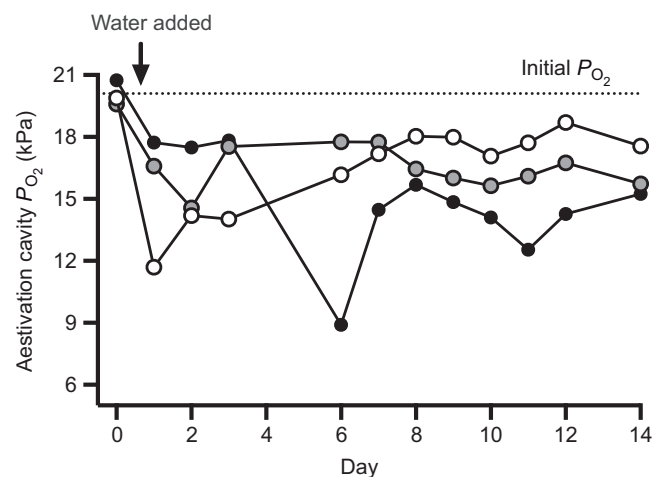


Fig. 2. The P_{O_2} profile in three artificial aestivation cavities excavated in clay soil over a 2 week period. Saturating the clay soil with water resulted in a considerable decline in P_{O_2} within the aestivation cavities. The P_{O_2} in the aestivation cavities did not return to the initial normoxic levels (20.1 ± 0.3 kPa; dashed line) during the 2 week experimental period.

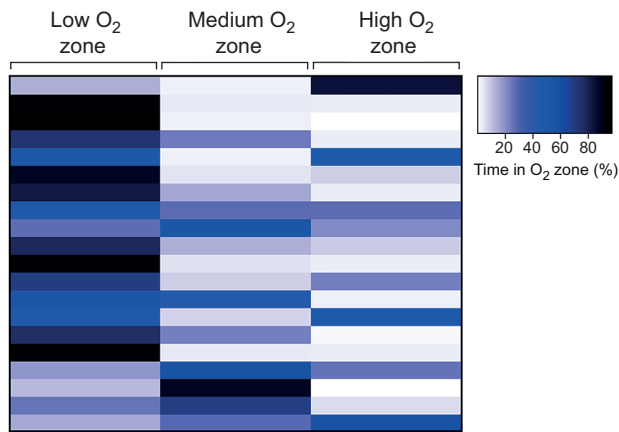


Fig. 3. Heat map representing the percentage of time spent in the low, medium and high O_2 zones by *C. alboguttata* ($n=20$). Each row represents an individual and each column represents an O_2 zone.

before (22.0 ± 0.8 g) and after (22.2 ± 1.0 g) the 7 week experimental period (t -test; $P=0.71$).

DISCUSSION

We used *C. alboguttata* to test the hypothesis that animals seek hypoxic microhabitats that accentuate metabolic depression during dormancy. Indeed, we found that *C. alboguttata* spent a significantly greater proportion of time in the low O_2 zone compared with the medium and high O_2 zones in response to drying habitat conditions. We also found that hypoxic microhabitats had a significant influence on the rate of O_2 consumption in *C. alboguttata* during the transition from an active to an aestivating state. Hypoxia exposure accelerated the onset of metabolic depression in *C. alboguttata* by 2 weeks, and resulted in a more profound metabolic depression than could be achieved under normoxic conditions. Taken together, our findings suggest that some animals may seek microhabitats that maximally depress metabolic rate during aestivation, and that microhabitat O_2 availability can have significant implications for energy metabolism.

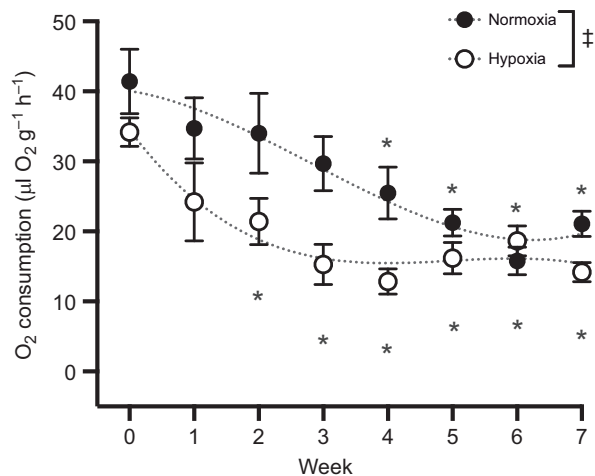


Fig. 4. The mass-specific rate of O_2 consumption in *C. alboguttata* exposed to normoxia (21.0 kPa) and hypoxia (10.5 kPa) during the transition from an active to an aestivating state. The rate of O_2 consumption in *C. alboguttata* was significantly influenced by the P_{O_2} exposure and by the week measurements were taken (two-way ANOVA: $P=0.002$, $P<0.001$). The double dagger denotes a significant difference in the rate of O_2 consumption between P_{O_2} treatments. The asterisks denote significant differences in the rate of O_2 consumption from that of active (week 0) frogs exposed to the same P_{O_2} treatment. The dashed lines indicate the third-order polynomial fit for each P_{O_2} exposure.

Microhabitat selection

Cyclorana alboguttata chose to spend more time in hypoxia compared with normoxia in response to drying habitat conditions, and one possible benefit is metabolic depression. Our laboratory findings are consistent with the observed habitat preference of *C. alboguttata* in the wild, as frogs have only been found burrowed in heavy clay soils despite the presence of sandy soils within their distribution range (Lee and Mercer, 1967; Booth, 2006). Interestingly, a previous laboratory study reported that *C. alboguttata* preferentially burrowed in wet sand rather than wet clay, likely because of the reduced energetic cost of burrowing in friable substrates (Booth, 2006). However, in the Booth (2006) study, frogs were not provided with hypoxic soils, which may affect their burrowing behaviour. In the wild, burrowing in clay soils may also provide aestivating frogs with benefits beyond the presence of environmental hypoxia. For example, clay soils dry much slower than sandy soils because of the smaller spaces between soil particles (Hubble, 1984), which would delay the onset of desiccating conditions during aestivation. Similarly, the soils from which *C. alboguttata* were collected smell of ‘rotten eggs’ (C. E. Franklin, R. L. Cramp, personal observations), suggesting that hydrogen sulphide (H_2S) may be present. Because H_2S is a potent inhibitor of aerobic metabolism (Smith et al., 1977), it may also promote metabolic depression in aestivating frogs. Numerous aestivating animals spend the vast majority of their life in subterranean burrows (Shoemaker, 1988; Abe, 1995), yet the factors that contribute to microhabitat selection remain poorly understood.

Metabolic depression in hypoxia

We showed that hypoxic microhabitats can have a significant influence on the metabolic rate of *C. alboguttata* entering aestivation. Hypoxia exposure accelerated the onset of metabolic depression in *C. alboguttata* by 2 weeks. Although *C. alboguttata* may aestivate for several months of the year (Withers and Richards, 1995), any reduction in metabolic rate during the first weeks of aestivation – when O_2 consumption rates are highest – may result in considerable energy savings. Environmental hypoxia has previously been shown to accelerate metabolic depression. For example, partially submerged freshwater turtles (*Pseudemys scripta*) with access to hypoxic air depressed metabolic rate five times faster than those with access to normoxic air (Jackson and Schmidt-Nielsen, 1966). Surprisingly, very few studies have examined how the rate at which metabolic depression is achieved impacts the endogenous fuel stores of dormant animals, but this is a fascinating area for future investigation.

We also showed that frogs in hypoxia exhibited a 59% reduction in O_2 consumption after 7 weeks, whereas frogs in normoxia only reduced O_2 consumption by 51%. The more ecologically relevant comparison, however, is between active frogs in normoxia (above ground) and aestivating frogs in hypoxia (underground). Under these circumstances, the rate of O_2 consumption in *C. alboguttata* is depressed by 66% after 7 weeks. The extent to which animals depress metabolic rate during dormancy gives a proportional extension to the time they can survive using endogenous reserves (Storey and Storey, 1990). For example, the predicted survival time of *Rana temporaria* overwintering in ice-covered lakes is extended from 129 to 254 days for a given quantity of lipid stores (1% of total body mass) when metabolic depression is accentuated by ~50% owing to hypoxia exposure (Boutilier et al., 1997). If we perform similar calculations using the average O_2 consumption rate of *C. alboguttata* in hypoxia and normoxia during week 7, and assume that 4.5% of the total body mass is lipid (as reported for aestivating *Cyclorana platycephalus* of similar size; van Beurden, 1980), we find that hypoxia exposure would extend survival time from 180 to

267 days using the following equation:

$$\text{Survival time} = \frac{(2.02L \times 1000)/[(M \times \dot{V}_{O_2})/1000]}{24}, \quad (1)$$

where L is the mass of the lipid body (g), M is the total mass of the frog (g) and \dot{V}_{O_2} is the O_2 consumption rate of the frog ($\mu\text{l } O_2 \text{ g}^{-1} \text{ h}^{-1}$). Based on the assumption of Klieber (1961), the only substrate utilized was lipid, and 2.02 l of oxygen is required to oxidise 1 g of lipid. Remarkably, *C. alboguttata* aestivating in normoxia can depress metabolic rate to less than 20% of the normal resting value after 10 weeks, thereby extending survival time to several years (Kayes et al., 2009). It is unknown whether there is a limit to the extent frogs can depress metabolic rate during aestivation. Regardless, hypoxia exposure significantly accelerated and accentuated metabolic depression in *C. alboguttata* and may therefore delay the exhaustion of critical endogenous energy reserves during periods of prolonged drought.

Body mass

The body mass of *C. alboguttata* was differentially affected by our experimental procedures. On the one hand, frogs exhibited a 7% reduction in body mass after the 24 h behavioural trials, probably owing to loss of body water in the rapidly drying choice chambers. Although many frogs can maintain water balance during aestivation, even in desiccating soils (e.g. *Neobatrachus aquilonius*; Cartledge et al., 2006), complete substrate drying is unlikely to occur in natural aestivation cavities over a 24 h period. On the other hand, these same frogs exhibited no further change in body mass following the 7 week O_2 consumption experiment. Although the respirometry chambers dried gradually over several weeks, we prevented complete desiccation by periodically adding 1 ml of water to each chamber, which may have helped frogs to maintain hydration. Furthermore, *C. alboguttata* can absorb and store up to 20% of their body mass in water as dilute urine in preparation for aestivation (Booth, 2006), and the closely related water-holding frog (*Cyclorana platycephalus*) can similarly store more than 50% of their body mass in water (van Beurden, 1984). The gradual drying in the respirometry chambers may have allowed for water uptake and storage that was not possible in the choice chambers, which dried far more rapidly.

Microhabitat conditions

The ecological significance of our study is reinforced by the empirical evidence of hypoxia in the aestivation substrates of *C. alboguttata*. Although frogs were not present within the artificial aestivation cavities, we showed that the P_{O_2} declined to hypoxic levels (P_{O_2} as low as 8.9 kPa) in the days following soil saturation. Soils often become hypoxic when saturated with water owing to rapid O_2 consumption by soil microbes (Drew, 1992), and because the presence of water between soil particles creates a significant diffusion barrier for O_2 (Arieli, 1979; Maclean, 1981). The presence of *C. alboguttata* within natural aestivation cavities would likely exacerbate the hypoxia because of their respiratory processes. Consequently, the P_{O_2} levels we measured in the artificial aestivation cavities were likely an overestimate of those experienced by *C. alboguttata* in the wild. Furthermore, the rate of drying in the field would be conceivably much slower than in our artificial aestivation cavities, suggesting that hypoxia could persist for longer than observed in our artificial cavities. A previous study has suggested that the clay soils inhabited by *C. alboguttata* during aestivation in the wild can remain wet for several months following heavy rains

(Booth, 2006). However, as wet clay soils dry and crack, re-oxygenation can occur. We found that the P_{O_2} within some of the aestivation cavities returned to relatively normoxic levels, particularly when cracks started to form in the drying substrate. Overall, we suggest that *C. alboguttata* likely experience several months of hypoxia during aestivation, which ultimately may help frogs to depress metabolism and economise on endogenous reserves.

Perspectives

Hypoxia avoidance behaviour has been reported in virtually all major animal phyla, yet we demonstrated here hypoxia-seeking behaviour in *C. alboguttata*. At the proximate level, seeking hypoxic microhabitats during dormancy may ensure that endogenous reserves do not become limiting to an individual's survival. Although uncommon, there are some reports of hypoxia-seeking behaviour in other animals. For example, the ocean quahog (*Arctica islandica*) burrows into hypoxic substrates during winter months when food is scarce as an energy-saving strategy (Strahl et al., 2011). Is strategic microhabitat selection widespread among aestivating and hibernating taxa? Exploring the microhabitat selection strategies of dormant animals remains an exciting avenue for future investigation.

Moving from proximate to ultimate questions, does the persistence of dormant populations over evolutionary time depend on microhabitat conditions that help animals economise on endogenous reserves? If so, then climatic changes (e.g. chronically elevated temperatures) that result in higher metabolic rates and, consequently, faster rates of substrate utilisation, may necessitate strategic microhabitat selection. Overall, we suggest that microhabitat selection strategies are at least as important as the physiological mechanisms used by dormant animals to tolerate environmental extremes. Moreover, the microhabitats animals occupy during dormancy may modulate, in part, their ecological and evolutionary success.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: G.S.R., R.L.C., P.A.W., C.E.F.; Methodology: G.S.R., R.L.C., P.A.W., C.E.F.; Validation: G.S.R., R.L.C., P.A.W., C.E.F.; Formal analysis: G.S.R., R.L.C.; Investigation: G.S.R., R.L.C.; Resources: C.E.F.; Writing - original draft: G.S.R.; Writing - review & editing: G.S.R., R.L.C., P.A.W., C.E.F.; Visualization: G.S.R.; Supervision: P.A.W., C.E.F.; Funding acquisition: G.S.R., P.A.W., C.E.F.

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Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.218743.supplemental>

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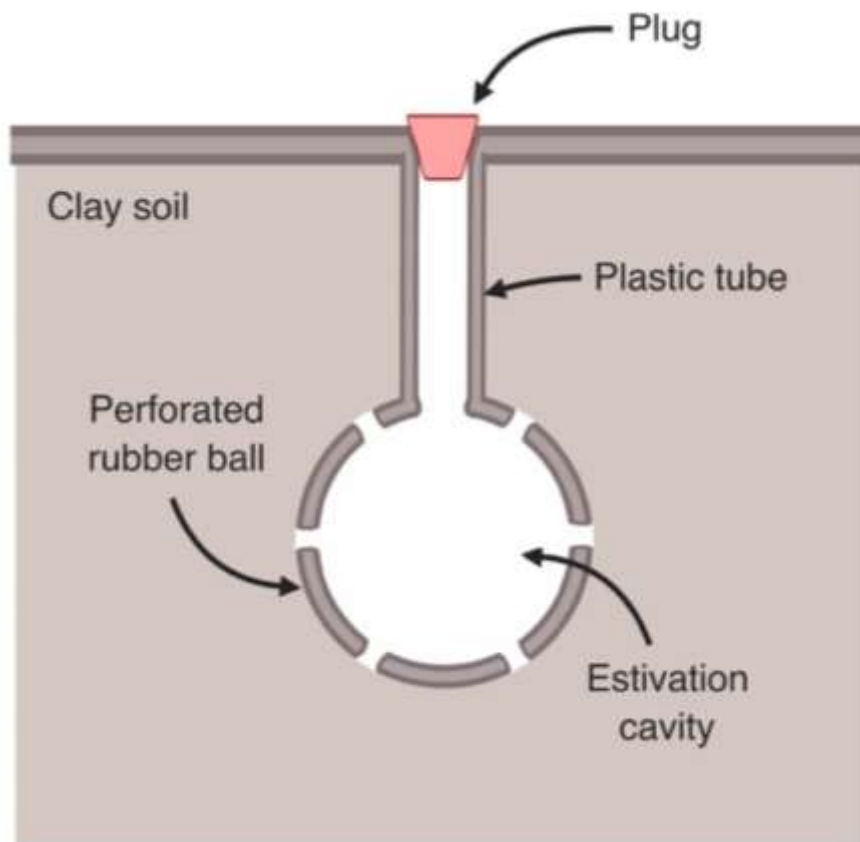


Figure S1: Schematic representation of the artificial estivation burrow used for pO_2 measurements.

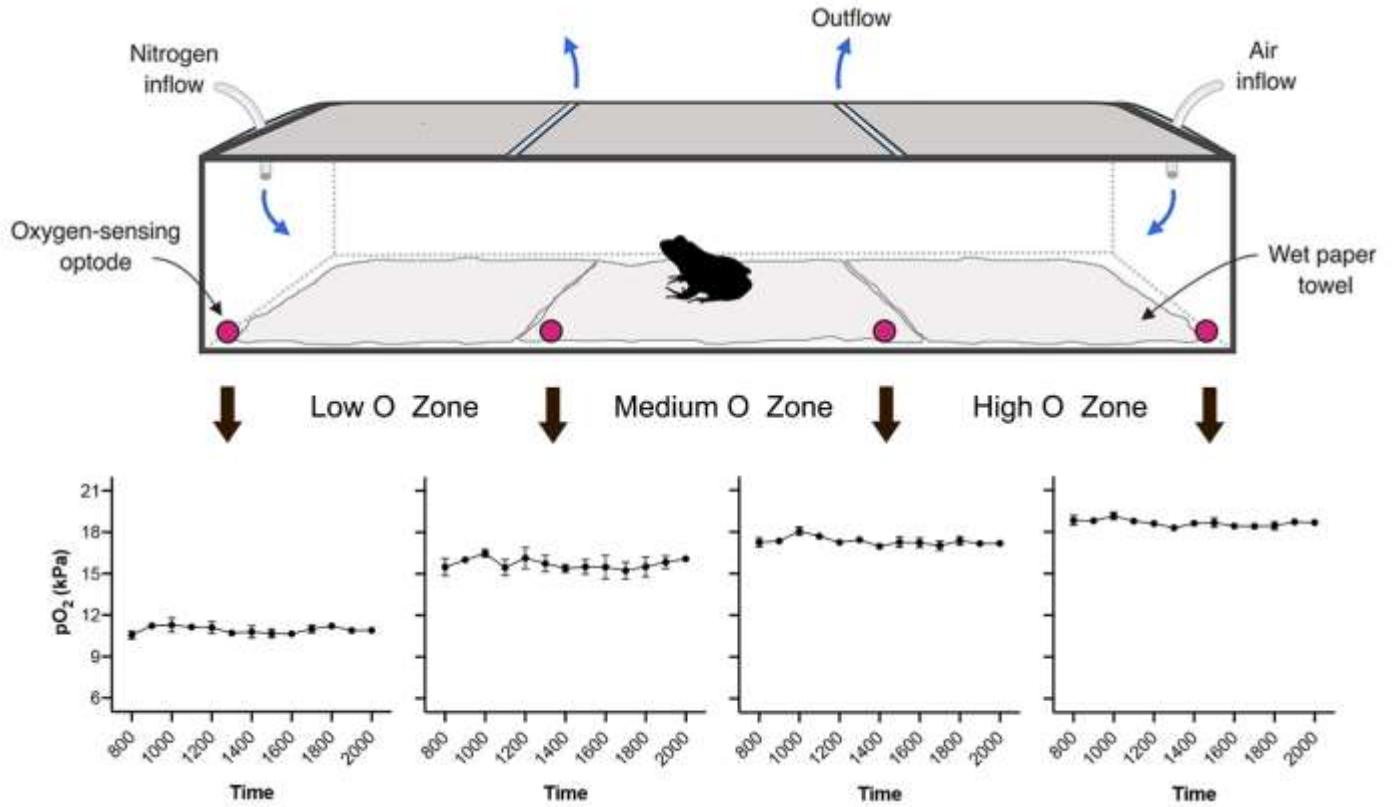


Figure S2: Schematic representation of a custom-built experimental choice chamber. The pO₂ measurements at each O₂-sensing optode from all behavioural trials are shown over a 12 hour period (8:00 AM to 8:00 PM) to demonstrate the stability of the pO₂ gradient over time.



Figure S3: *Cyclorana alboguttata* in the water conserving posture; all limbs are tucked under the body, and the head is lowered to be in contact with the substrate.