The Journal of Experimental Biology 209, 1159-1168
Published by The Company of Biologists 2006
doi:10.1242/jeb.02101

Review

Tribute to R. G. Boutilier: The role for skeletal muscle in the hypoxia-induced hypometabolic responses of submerged frogs

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Accepted 17 January 2006

Summary

Much of Bob Boutilier's research characterised the subcellular, organ-level and *in vivo* behavioural responses of frogs to environmental hypoxia. His entirely integrative approach helped to reveal the diversity of tissue-level responses to O_2 lack and to advance our understanding of the ecological relevance of hypoxia tolerance in frogs. Work from Bob's lab mainly focused on the role for skeletal muscle in the hypoxic energetics of overwintering frogs. Muscle energy demand affects whole-body metabolism, not only because of its capacity for rapid increases in ATP usage, but also because hypometabolism of the large skeletal muscle mass in inactive animals impacts so greatly on *in vivo* energetics. The oxyconformance and typical hypoxia-tolerance characteristics (e.g. suppressed heat flux and preserved membrane ion gradients during O_2 lack) of skeletal muscle *in vitro* suggest that muscle hypoperfusion *in vivo* is possibly a key mechanism for (i) downregulating muscle and whole-body metabolic rates and (ii) redistributing O_2 supply to hypoxia-sensitive tissues. The gradual onset of a low-level aerobic metabolic state in the muscle of hypoxic, cold-submerged frogs is indeed important for slowing depletion of on-board fuels and extending overwintering survival time. However, it has long been known that overwintering frogs cannot survive anoxia or even severe hypoxia. Recent work shows that they remain sensitive to ambient O_2 and that they emerge rapidly from quiescence in order to actively avoid environmental hypoxia. Hence, overwintering frogs experience periods of hypometabolic quiescence interspersed with episodes of costly hypoxia avoidance behaviour and exercise recovery.

In keeping with this flexible physiology and behaviour, muscle mechanical properties in frogs do not deteriorate during periods of overwintering quiescence. On-going studies inspired by Bob Boutilier's integrative mindset continue to illuminate the cost–benefit(s) of intermittent locomotion in overwintering frogs, the constraints on muscle function during hypoxia, the mechanisms of tissue-level hypometabolism, and the details of possible muscle atrophy resistance in quiescent frogs.

Key words: *Rana* spp., hypoxia, skeletal muscle, resting metabolism, contraction.

Introduction

One of Bob Boutilier's outstanding contributions to the field of comparative biology is his body of research into amphibian physiology, encompassing areas as diverse as subcellular metabolic regulation to the effects of environmental change on amphibian behaviour. Bob's integrative mindset was a major influence on his research group investigating how frogs (*Rana temporaria* L.) cope with acute and chronic environmental O_2 lack. Research from the Zoology lab at Cambridge University during the period 1993–2003 addressed (i) cellular, molecular and mitochondrial responses to hypoxia/anoxia in skeletal muscle, cardio-myocytes and hepatocytes (St Pierre et al., 2000; Currie and Boutilier, 2001; Court and Boutilier, 2005), (ii) patterns of O_2-dependent heat and ion fluxes in isolated intact skeletal muscles (West and Boutilier, 1998; Donohoe et al., 2000), (iii) *in vivo* metabolism in quiescent overwintering frogs (Donohoe and Boutilier, 1998; Donohoe et al., 1998) and (iv) activity patterns of free-moving frogs submerged in temperature and O_2 gradients (Tattersall and Boutilier, 1997, 1999). Studies that characterise the integrative responses to environmental O_2 lack are important for guiding researchers to relevant (e.g. tissue- and species-specific) mechanistic hypotheses of cellular

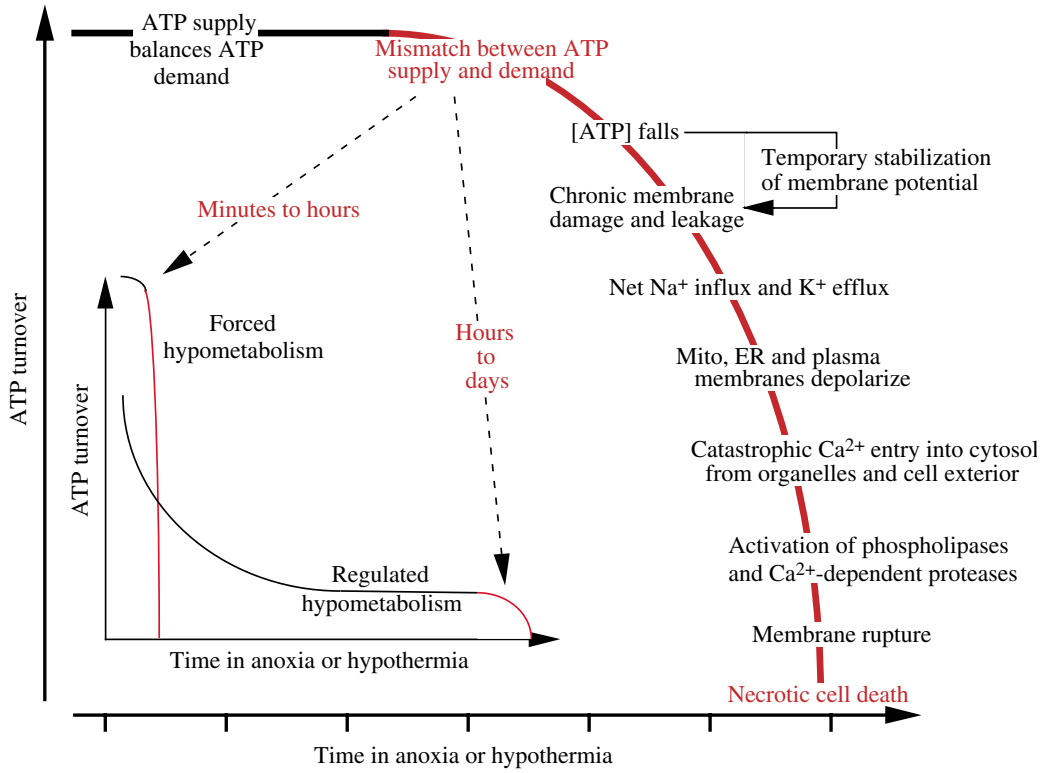

Fig. 1. Boutilier's hypothetical time course of cellular ATP-turnover during anoxia or hypothermia includes the events that promote energy imbalance and cause cell death (red line; Boutilier, 2001a). Neither hypoxia-tolerant nor hypoxia-sensitive cells can survive prolonged energy imbalance, but cells of hypoxia-tolerant animals are adapted to avoid energy imbalance through the coordinated suppression of energy demand and energy supply (inset). Reversible metabolic suppression thus greatly extends hypoxic survival time. (From Boutilier, 2001a.)

hypoxia-tolerance and for increasing our understanding of the energetics and physiological ecology of hypoxia-tolerant species.

Skeletal muscle function was really the focal point for much of our work because shifts in muscle energy demand, either upward or downward from the resting state, of the large skeletal muscle mass (40% of total body mass) impact so greatly on whole-animal metabolism and on the circulation of substrates and O_2. We hypothesised that quiescence and hypometabolism in skeletal muscle was a basic attribute of the O_2 management and metabolic strategy in submerged frogs. Metabolic suppression is certainly a key aspect of energy conservation in frogs during acute anoxia at room temperature (Schultz et al., 1991) and during chronic overwintering submergence in normoxic and hypoxic water (Donohoe and Boutilier, 1998; Donohoe et al., 1998). For example, in normoxic cold-submerged frogs, oxygen uptake across the skin was found to be 50% of air-access controls, while the rate during hypoxic cold-submergence was suppressed to 25% of normal. Quiescence and muscle hypometabolism are indeed important components of whole-body O_2 and metabolite handling in such conditions of uniform normoxia or mild hypoxia (Donohoe and Boutilier, 1998). However, laboratory behavioural studies (Tattersall and Boutilier, 1997; Tattersall and Boutilier, 1999) further indicate that cold-submerged frogs remain relatively active when placed in O_2 and thermal gradients; they explore their environment and eventually settle in areas of optimal ambient O_2 content and temperature. This behaviour in non-uniform environments is consistent with the inability of frogs to survive prolonged and severe O_2 lack (Bradford, 1983; Boutilier et al., 1997). Submerged frogs exploit thermal and O_2 gradients in natural waterways to seek out cold water with high levels of dissolved O_2 and thereby achieve a state of slow, aerobic metabolism (Tattersall and Boutilier, 1997; Tattersall and Boutilier, 1999). Throughout the winter, frogs probably experience periods of stable hypometabolic quiescence that are interrupted by episodes of high-cost muscle contraction and post-exercise recovery.

Integrative studies of energy partitioning are essential for assessing the cost–benefit of intermittent locomotion in heterogeneous environments, especially in overwintering conditions where air access may be cut off for months. Improved understanding of energy supply/demand relationships in cold-submerged frogs will also help to predict the impact of water quality and environmental change on survival and on the capacity for strenuous reproductive activities upon emergence from hibernation. The aim of this tribute paper is to review studies that have helped to characterise the hypoxia/anoxia tolerance capacity of skeletal muscle in *R. temporaria* and to highlight some of the on-going work into the ionic homeostasis and contractile performance of frog muscle during acute and chronic O_2 lack.

A model of anoxic cell death

Much has been written about the causes of anoxia induced energy imbalances in vertebrate cells and about the proposed mechanisms needed to avoid the catastrophic events that lead

to cell death (see Hochachka et al., 1996; Boutilier, 2001a; Boutilier, 2001b; Hochachka and Lutz, 2001). Fig. 1 is a hypothetical time course for anoxia-induced cell death, as presented originally by Boutilier (Boutilier, 2001a) in a timely review of the hypoxia–hypothermia field. One feature of this model is that the danger period for all cells begins as anaerobic ATP supply starts to fall short of the ATP demanded by various cellular ATPases. Irreversible damage begins essentially as ion gradients across cellular and subcellular membranes fail; the ensuing osmosis and proteolysis cause membrane rupture and necrotic cell death. A second feature of the model is that anoxia-tolerant animals and cells are not any less susceptible to mismatches in ATP supply/demand than are anoxia-sensitive systems. However, anoxia-tolerant animals are able to avoid energy imbalances by entering into 'regulated hypometabolic states' (see inset, Fig. 1) when environmental O_2 is limiting. Energetic homeostasis is achieved through the coordinated downregulation of ATP supply and demand. Hypometabolism, together with the large on-board reserves of tissue glycogen in anoxia-tolerant animals, provide the capacity for the organism to endure natural cycles of environmental O_2 availability and thus prevent the onset of necrotic cell death.

The shoulder of the anoxic time course in Fig. 1, where energy imbalance leads directly to failure of membrane ion gradients, highlights the critical role for membrane–metabolic coupling in cell survival of O_2 lack. Mechanisms that promote hypoxia-induced hypometabolism and membrane–metabolic coupling in different vertebrate systems are discussed in detail elsewhere (Hochachka et al., 1996; Boutilier, 2001a; Boutilier, 2001b; Hochachka and Lutz, 2001; Lutz and Nilsson, 2004). The general events that have been identified and/or proposed are; (i) reallocation of ATP supply away from 'dispensable' energy consumers such as protein and RNA/DNA synthesis (Land and Hochachka, 1994; Buttgereit and Brand, 1995) and toward the ATP-dependent ion pumps; (ii) reduction in the rates of ion pumping; (iii) reduction of membrane ion-leak; (iv) reduction of glycolytic ATP production to match overall anaerobic energy demand; and (v) the capacity for rapid return to normoxic rates of energy supply/demand when O_2 availability increases. Ion pumping is a dominant energy sink during anoxia because pump activities are not inhibited as completely as other processes (e.g. see Buck and Hochachka, 1993). In cells where anoxia does not affect transmembrane ion gradients there must be a reduction in passive channel-mediated ion fluxes to match the suppression of ATP-dependent pump rates (Hochachka et al., 1996; Hochachka and Lutz, 2001). This so-called channel- and pump-arrest may be mediated by reversible inhibition and/or changes in density of membrane channel proteins (Perez-Pinzon et al., 1992). There are important species and tissue-type variations on this theme, for example, the discussion on anoxia-tolerance in vertebrate brains (Lutz and Nilsson, 2004), but it is generally true that hypometabolic homeostasis requires coordinated stabilization of cytosolic ATP concentration/turnover and transmembrane ion gradients.

Frogs and their muscles as models of hypoxia tolerance

The common frog (*Rana temporaria*) possesses only modest hypoxia/anoxia tolerance capacity in comparison to better known anoxia-tolerant models, like some freshwater turtles (e.g. *Chrysemys picta*) and the crucian carp *Carassius carassius* (Bradford, 1983; Wegener and Krause, 1993; Boutilier et al., 1997; Lutz and Nilsson, 2004). Frogs survive severe hypoxia for hours to days depending on ambient temperature, whereas turtles and carp tolerate total anoxia for several weeks or even months. Nevertheless, interest in the frog model has grown, largely from the desire to understand the physiological and behavioural mechanisms that frogs without air-access must exploit in order to survive in waterways that are naturally heterogenous with respect to temperature and dissolved O_2.

It has been argued previously (e.g. Boutilier et al., 1997) that the modest hypoxia-tolerance capacity of frogs may mean that they express a wide range of tissue-level hypoxia tolerances; from true anoxia-tolerance to O_2-dependence. We hypothesised that the skeletal muscle of *R. temporaria* displays the typical characteristics of cellular anoxia-tolerance, whereas the core organs like liver, brain and heart require a minimal level of continuous O_2 delivery. Periods of muscular quiescence are certainly important for energy conservation in overwintering frogs, not only because of the shut-down of ATP demand for contraction, but also because of the overall savings brought about by hypometabolism of the large skeletal muscle mass (making up 40% of total body mass). Similarly, non-hibernating frogs at summer temperatures will need to conserve energy if they have to evade predators by remaining submerged in hypoxic water. It may be that hypoperfusion of skeletal muscle accounts for the bulk of the whole-body metabolic adjustment to hypoxia by lowering muscle energy demand and sparing blood O_2 and substrates for tissues that tolerate hypoxia to a lesser degree.

Room temperature anoxic responses

It has been known for more than 70 years (Feng, 1932) that the anoxic heat flux of frog sartorius is stable at 20–30% of the normoxic rate at 20°C. Hypometabolism in sartorius was reversible and repeatable (Feng, 1932), and the excess recovery heat after re-oxygenation was exquisitely responsive to the level of metabolic load imposed on the muscle during anoxia. Recent experiments have further demonstrated that (i) step changes in O_2 availability cause step changes in the stable metabolic heat production of isolated skeletal muscle (West and Boutilier, 1998; Boutilier, 2001b) and of the whole animal (Schulz et al., 1991); (ii) the extent of suppression of muscle heat flux (to 20–30% of normal; Fig. 2) matches the reduction in muscle ATP synthesis as well as the anoxia-induced change in whole-animal metabolism (Schulz et al., 1991; West and Boutilier, 1998; Vezzoli et al., 2003; Vezzoli et al., 2004); and (iii) muscle ATP concentration is protected during anoxia. Anaerobic lactate accumulation is relatively low in anoxic sartorius (West and Boutilier, 1998) and gastrocnemius (Vezzoli et al., 2004) at rest. This may partly reflect the fact

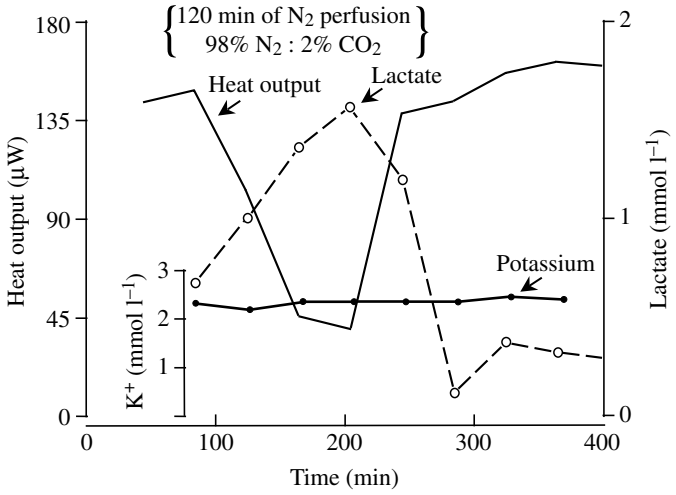

Fig. 2. The effect of a cycle of normoxia, anoxia and re-oxgenation on heat output and interstitial lactate and K^+ concentrations (means ± s.e.m.) in frog sartorius muscle at 20°C. Interstitial lactate and K^+ levels were determined from microdialysis effluents of muscle interstitial space during continuous micro-calorimetric monitoring of heat flux. The lack of change in interstitial [K^+] suggests that anoxia and re-oxygenation did not increase K^+ loss from skeletal muscle. (From West and Boutilier, 1998.)

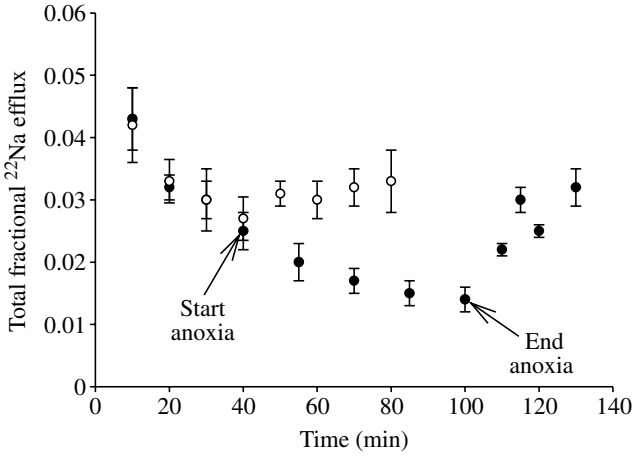

Fig. 3. Time courses for fractional efflux of $^{22}Na^+$ (mean ± s.e.m.) in isolated frog sartorius (T. G. West and R. G. Boutilier, unpublished observations) at 20°C during normoxia (98% O_2:2% CO_2; open symbols) and anoxia (98% N_2:2% CO_2; closed symbols). Fractional efflux was determined using methods modified from Overgaard et al. (Overgaard et al., 1997). Briefly, whole sartorius muscles were first preloaded with $^{22}Na^+$ (2 μCi ml^{-1} for 30 min at 20°C in oxygenated Ringer solution). Then the muscles were washed (4× 10 min) in ice-cold Na-free Ringer; a process expected to remove extracellular $^{22}Na^+$ (see Overgaard et al., 1997). Finally, the muscles were transferred through 2 ml volumes of normal Ringer at 20°C with the treatments and time courses shown. Fractional efflux was calculated as the amount of $^{22}Na^+$ released to the bathing medium during each time interval divided by the total amount of $^{22}Na^+$ loaded into the muscle. The total $^{22}Na^+$ load was the cumulative sum of $^{22}Na^+$ c.p.m. released plus the c.p.m. remaining in the muscle at the end of the experiment (measured in trichloroacetic acid extracts). Fractional efflux after 30 min anoxia was significantly lower (*t*-test; t=4.16, P=0.005) than that of normoxic controls at the same time point (i.e. 70 min into the time courses). The ouabain-sensitive component of total fractional efflux (i.e. giving the portion of efflux attributed specifically to pump activity) could not be assessed in these studies because uncontrolled muscle twitches were always induced within 10 min by the addition of 1 mmol l^{-1} ouabain, possibly owing to rising cytosolic Ca^{2+} mediated by increased Na^+/Ca^{2+} exchange after Na^+-pump blockade. (From T. G. West and R. G. Boutilier, unpublished observations.)

that lactate efflux is favoured in isolated muscle preparations that are perifused with lactate-free solution (Boutilier et al., 1986). However, the chief reason seems to be that glycolytic activation is minimal in non-contracting anoxic muscle (Hsu and Dawson, 2003). Interestingly, Vezzoli et al. (Vezzoli et al., 2004) observed that the time course of suppression of ATP synthesis rate was essentially paralleled by changes in the phosphocreatine (PCr) breakdown rate, perhaps indicating that anaerobic energy turnover in resting and/or hypometabolic muscle is controlled to a large extent by ATP supply from PCr hydrolysis. Glycolysis clearly has a role in energy provision, particularly at higher temperatures (≥15°C) and when PCr is exhausted (Vizzoli et al., 2004), but the low level of glycolytic activation overall is consistent with the so-called reversed Pasteur effect that is characteristic of so many other anoxia-tolerant tissues (Hochachka et al., 1996; Hochachka and Lutz, 2001). The apparent dependence of anoxic muscle on the limited on-board PCr reserves emphasises the fundamental importance of hypometabolism for prolonging energetic homeostasis.

There is also evidence of tight membrane–metabolic coupling during acute room temperature anoxia in frog muscle. Interstitial K^+ concentration is stable during anoxia and reoxygenation (Fig. 2), indicating that maintenance of membrane integrity does not depend on O_2 availability (West and Boutilier, 1998). Total (i.e. not pump-specific) fractional efflux of $^{22}Na^+$ is also suppressed during anoxia at 20°C (Fig. 3; T. G. West and R. G. Boutilier, unpublished observations). The pattern of fractional efflux loosely parallels that of total metabolic heat flux (Fig. 2) during anoxia and reoxygenation, possibly indicating that Na^+ homeostasis is closely coupled to muscle energy economy. Preliminary data on a small number of sartorius preparations (N=2; T. G. West and R. G. Boutilier, unpublished observations) suggests that Na^+ uptake may also be suppressed during anoxia. Reduced leakiness of sartorius membranes could directly account for reduced effluxes, but further studies are needed to quantify (i) the significance of any reduced Na^+ channel leak and (ii) Na^+-pump specific (i.e. ouabain-dependent) changes in ion fluxes in cycles of normoxia–anoxia–reoxygenation.

Another proposed mechanism of pump-arrest is the anoxia-induced switch from Na^+/H^+ exchange to Na^+-dependent $Cl^-/HCO_3^-/H^+$ exchange (Vezzoli et al., 2004). With increased dependence on the multi-ion exchanger there is reduced uptake of Na^+ needed for regulating intracellular pH (pH_i). Thus the

amount of ATP used by the Na^+K^+-ATPase mol^{-1} H^+ removed from muscle is suppressed (Reipschläger and Pörtner, 1996; Vezzoli et al., 2004). This mechanism could be important *in vivo* because anoxia-induced reductions in extracellular pH (pH_e) are thought to inhibit Na^+/H^+ exchange. However, the low anaerobic heat flux and stable ionic characteristics of isolated muscle during continuous-flow anoxic perifusions, in the absence of direct manipulation of pH_e (Fig. 2; West and Boutilier, 1998), suggest that frog muscle has other intrinsic mechanisms for anoxic ion homeostasis and reversible metabolic suppression.

Regulated hypoperfusion of skeletal muscle is possibly an important mechanism of *in vivo* metabolic suppression and whole-body O_2 conservation. However, it should be emphasized that there is currently only indirect evidence that this mechanism operates in hypoxic frogs. Skeletal muscle clearly displays typical anoxia tolerance characteristics, but a key point is that the room-temperature oxyconformation response in isolated muscle (West and Boutilier, 1998) was observed in perifused, not cannulated or perfused, muscle. The onset of a true (i.e. physiological) oxyconformance response is likely to be overestimated because the gradient of O_2 into the core of a perifused muscle will initiate hypometabolic responses in some fibres at an apparently high P_{O_2}. The sensitivity of resting muscle to changes in blood or O_2 flow, and the possibility that O_2 acts directly to trigger suppression of channel and pump activities, needs to be assessed in more physiological circumstances. Similarly, there needs to be further study of *in vivo* blood flow patterns to strengthen the idea that muscle hypoperfusion is linked directly to muscle quiescence and whole-body oxyconformance at room temperature and to the hypometabolic rescue response seen in cold-submerged frogs (discussed in the next section).

Hypoxia responses during cold-submergence

Frog skeletal muscle expresses the typical characteristics of hypoxia-tolerance and metabolic suppression. This capacity is almost certainly exploited in frogs that overwinter without air-access and gradually suppress their whole-body metabolic rate by up to 75% of normal (Donohoe et al., 1998). Greater understanding of the precise role of skeletal muscle in overwintering frogs has come from laboratory studies into how the metabolic and ionic characteristics of muscle change as whole-animal hypometabolism progresses during normoxic and hypoxic cold-submergence (Donohoe and Boutilier, 1998; Donohoe et al., 1998; Donohoe et al., 2000).

Stable normoxic conditions impose no apparent anaerobic stress on quiescent overwintering animals; body glycogen stores remain relatively high (Donohoe and Boutilier, 1998) and there is no change in muscle and plasma lactate levels throughout 16 weeks of continuous cold-submergence (Fig. 4). Muscle ATP and PCr concentrations are also unchanged during normoxic submergence.

In contrast, hypoxic cold-submergence does mobilise liver glycogen and activate muscle glycolysis (Donohoe and Boutilier, 1998). The time courses of liver glycogen depletion

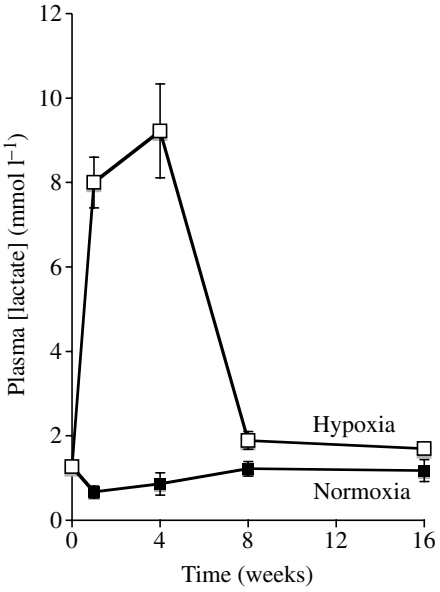

Fig. 4. Plasma lactate changes (means ± s.e.m.) in normoxic (air-equilibrated water) and hypoxic (60 mmHg O_2) cold-submerged (16 weeks, 4°C) frogs. (From Donohoe and Boutilier, 1998.)

and the onset of muscle glycogenolysis are essentially mirrored by the rise in plasma lactate levels in the first few weeks of hypoxic submergence (Fig. 4). In isolated frog muscle, energy turnover during acute anoxia at 4°C is apparently supported entirely by PCr hydrolysis; glycolytic activation seems tightly coupled to the onset of increased contractions (Hsu and Dawson, 2003). The clear activation of muscle glycogenolysis *in vivo* may mean that some low level of muscular activity occurs continuously during the initial stages of hypoxic cold-submergence and/or that circulatory factors are important for mobilising muscle glycogen. Muscle lactate concentration rises only moderately during enforced hypoxia, and muscle pHi remains stable at 7.2–7.3 at the same time that glycogen levels drop by more than 60% (Donohoe and Boutilier, 1998). This suggests that glycolytic end-products are removed from the muscle and are a major contributor to changes in blood chemistry. Lactate released from muscle is probably oxidised in other tissues and used for liver gluconeogenesis.

Remarkably, whole-body O_2 consumption in hypoxic cold-submerged frogs approaches a phase of deep suppression before muscle glycogen stores become depleted. Donohoe and Boutilier (Donohoe and Boutilier, 1998) refer to this response as ‘aerobic hypometabolic rescue’ because the lower metabolic demands of the animal can at this point be supported entirely aerobically. Continued muscle quiescence is of course essential for minimising perturbations in whole-animal metabolism. Indeed, the time course of plasma lactate concentration (Fig. 4) supports the view that frogs switch from an initial hypoxic, high-activity state to a phase of prolonged aerobic hypometabolic quiescence. Long-term energy supply to skeletal muscle during chronic environmental hypoxia seems to be largely shared between anaerobic glycolysis and the

aerobic oxidation of mixed fuel. It is perhaps expected that muscle PCr should participate in the early phases of these gradual metabolic transitions, but the metabolite time courses presented by Donohoe and Boutilier (Donohoe and Boutilier, 1998) indicate that PCr concentration remained essentially stable in hypoxic cold-submerged frogs. Future studies that relate more detailed metabolite time courses specifically to episodes of muscle contraction and/or ischemia (i.e. when PCr hydrolysis is expected to be important) may possibly reveal acute fluctuations in PCr level. Clearly, having flexibility in energy supply is important for frogs that overwinter in environments where ambient O_2 is heterogeneous and when periods of quiescence are interspersed with costly hypoxia avoidance behaviour. Moreover, hypometabolic rescue is critical for extending the time that on-board fuel reserves can support all of the complex behaviours of overwintering frogs (Boutilier et al., 1997).

Two characteristics of ion balance processes in cold-submerged frogs support the hypothesis that channel- and pump-arrest help to lower muscle energy demands. Firstly, membrane permeability of Na^+ and K^+ is gradually reduced in sartorius from both normoxic and hypoxic cold-submerged frogs, and the ouabain-dependent $^{22}Na^+$ efflux from sartorius muscle is also significantly suppressed (Donohoe et al., 2000). The time courses for the reduction of Na^+ and K^+ permeability are similar in normoxic and hypoxic frogs, but there is greater reduction of Na^+K^+-ATPase activity in the hypoxic frogs. The time courses of changes in muscle ion transport properties in cold-submerged frogs are in good accord with the onset of aerobic hypometabolic rescue. Reduced costs for muscle ion pumping will help to rescue the tissue by ensuring that energy imbalance due to any O_2 limitation is not prolonged (Boutilier, 2001b), but it remains unclear whether controlled hypoperfusion of muscle triggers the downregulation of channel and pump densities/activities.

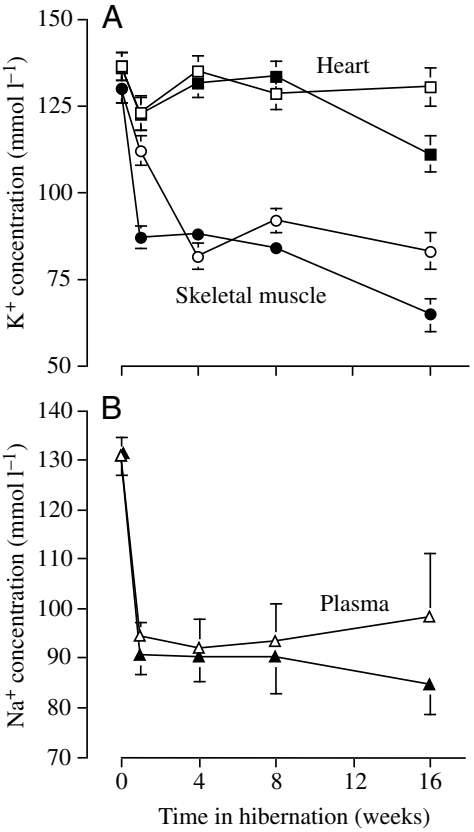

Fig. 5. Changes in intracellular [K^+] (means ± s.e.m.) in muscle and heart (A) and extracellular [Na^+] (B) in frogs during 16 weeks of cold-submerged (4°C) normoxia (air equilibrated water; open symbols) and hypoxia (60 mmHg; closed symbols). (From Donohoe et al., 2000.)

The second key observation by Donohoe et al. (Donohoe et al., 2000) was that changes in extracellular and intramuscular concentrations of Na^+ and K^+ occur in cold-submerged frogs (see Fig. 5). Lower Na^+- and K^+-gradient set-points in skeletal muscle will suppress membrane ion leak and rates of ion-pumping. Hence, energy demand by muscle Na^+K^+-ATPase seems to be influenced by intrinsic changes in membrane channel/pump activities and by the resetting of transmembrane ion gradients. Suppressed muscle excitability, as occurs after acute contraction-induced fatigue in frog muscles (Balog and Fitts, 1996), could also be an important component of chronic muscle quiescence. It is noteworthy that in a previous study of ionic status during cold-acclimation (with air-access) *R. temporaria* displayed no changes in plasma Na^+, significant reductions in plasma Ca^{2+}, and significant increases in urine K^+ and Mg^{2+} levels (Sinsch, 1991). Differences between the two studies (Sinsch, 1991; Donohoe et al. 2000) could possibly be related to the degree of air-access provided to frogs and to the extent of quiescence observed during the hibernation periods. Clearly, further work is needed with overwintering frogs to confirm how concentrations of intramuscular, plasma and urine ions change, and to determine the significance of any chronic shifts in muscle ion gradients on metabolism and muscle function.

Muscle function during hypoxia/anoxia

While it is undoubtedly true that muscle quiescence is an important energy-saving feature of submerged frogs, it is nevertheless clear that skeletal muscle must remain continuously poised for rapid and perhaps even strenuous contractions. Energy provision for skeletal muscle must be flexible in order to support the range of behaviours characteristic of submerged frogs: from the slow exploratory behaviour of natural O_2 and thermal gradients to the burst behaviour needed for predator avoidance. How might O_2-dependent changes of muscle metabolic/ionic properties constrain muscle function in submerged frogs?

Metabolite changes during hypoxia/anoxia

Frogs in continuous normoxic cold-submergence show low accumulation of lactate together with stable levels of muscle pH_i, adenylates, PCr, creatine (Donohoe and Boutilier, 1998)

and presumably inorganic phosphate (P_i). Hence, during continuous normoxic cold-submergence there seems to be no substantial changes in metabolite levels that might limit muscle contraction. In frogs confined to uniformly hypoxic cold-submerged conditions (Fig. 4), the time course of lactate changes suggests that muscle tissue recruits glycolysis to defend energetic homeostasis in the early stages of O_2 lack. It is possible that the initial phase of glycolysis protects PCr and ATP levels, and that the end products of anaerobic glycolysis are removed from the muscle for disposal in other tissues (as discussed previously). Hence, even before the phase of aerobic hypometabolic rescue, there appear to be no drastic changes in muscle metabolite levels. Key aspects of the aerobic 'hypometabolic rescue' response therefore seem to be the preservation of muscle fuel reserves and, possibly, the inhibition of muscle atrophy (see next section), both of which will be important for powering the animals' movements if ambient water quality deteriorates.

As discussed above, energy provision during acute anoxia/ischaemia in quiescent isolated skeletal muscle (over the temperature range 4–25°C) is highly dependent on PCr hydrolysis (Hsu and Dawson, 2003; Vezzoli et al., 2004). Complete and sustained muscle anoxia or ischemia is likely to be a rare occurrence *in vivo* because of the frogs' need to avoid severe environmental hypoxia. Even so, skeletal muscles of frogs do express typical anoxia-tolerant characteristics. In natural waterways, quiescent muscle anoxia/ischaemia could conceivably arise in situations where overwintering frogs are not able to retreat from progressive environmental hypoxia. One might expect that the accumulation of P_i, which generally mirrors the depletion of PCr, could limit the mechanical function of skeletal muscle. A growing view, however, is that there are few direct metabolic consequences of P_i build-up at physiological temperature. For example, glycolytic activation is associated more with contractions than with increases in P-metabolites (Hsu and Dawson, 2004). Moreover, at physiological temperatures elevated P_i seems to have minimal effect on the capacity for skinned muscle fibres to produce isometric force (Coupland et al., 2001; Debold et al., 2004). Recent work on intact dogfish fibres at physiological/acclimation temperature (12°C) indicates that P_i build up after 3.5 s of isometric contraction reduces ATP usage by actomyosin ATPase to 20% of initial values, while only diminishing plateau force to 97.5% (West et al., 2004; West et al., 2005). Hence, there is a significant effect of P_i accumulation on energy release of intact fibres at physiological temperature, but not on the generation and maintenance of isometric force. It would be of interest to examine these relationships at physiological temperature in the skeletal muscle of overwintering frogs to determine, for example, whether anoxia- or ischemia-induced increases in P_i concentration affect subsequent contraction mechanics and energetics. Effects of muscle pH_i are also important to consider because it believed that there are only minor effects of increased H^+ concentration on muscle function at physiological temperatures (Westerblad et al., 2000). Further studies are needed to determine the effects of specific metabolites on shortening velocity, Ca^{2+} kinetics and work output in frog skeletal muscle at overwintering tempeatures. Given that submerged frogs would normally exploit ambient thermal gradients and risk temporary 'hypothermia' in order to avoid anaerobiosis, close attention to the thermal and O_2 histories and to the activity levels of overwintering frogs will be important for assessing the physiological/ecological significance of any interactive effects of temperature and metabolite levels on muscle contractile properties.

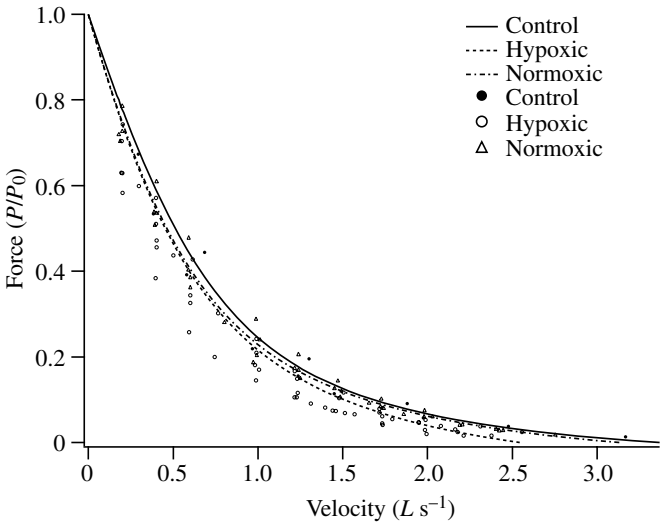

Fig. 6. Force–velocity relationships in frog sartorius muscle taken from (4°C) air-access control frogs, normoxic cold-submerged frogs (12–16 weeks in air equilibrated water) and hypoxic cold-submerged frogs (12–16 weeks in 60 mmHg O_2) determined using isovelocity contractions (see Appendix for experimental details). Muscle force (P) is normalised to maximal isometric force (P_0). Velocity (V) is in muscle lengths (L) s^{-1}. The lines are hyperbolic-linear fits for each treatment of the form $V=B(1–P/P_0)/(A+P/P_0)+C(1–P/P_0)$ (Marsh and Bennett, 1986), calculated using the mean values for the constants A, B and C determined for each muscle for each group. Maximum shortening velocity was significantly different for the three groups (one-way ANOVA: $F_{1,8}$=4.47, P=0.05). The curvature of the force–velocity relationship ($F_{1,8}$=0.745, P=0.51) and maximum power output ($F_{1,8}$=0.832, P=0.47) were not significantly different between the three groups. (G. N. Askew and R. G. Boutilier, unpublished observations.)

Muscle mechanics in overwintering frogs

Overwintering frogs must remain alert to changes in their ambient thermal and O_2 conditions in order to avoid extreme hypoxic conditions. While the time courses of muscle metabolite changes in normoxic and hypoxic submerged frogs would seem to be consistent with continued muscle readiness for locomotion, the mechanical properties may nevertheless be compromised during extended inactivity. Hence, we have begun to assess how the physiological properties of frog locomotory (sartorius) and calling (external oblique) muscles are affected by 3–4 months of normoxic and hypoxic cold-

submergence (i.e. into the phase of quiescent hypometabolic rescue; see Appendix).

Results indicate that the mechanical properties of both locomotory and calling muscles are in fact not greatly influenced by quiescence and hypoxia. Maximum isometric tetanic stress in muscles from cold-submerged hypoxic and normoxic frogs is similar to that of the relatively active air-access control animals; ~205 kN m^{-2} (one-way ANOVA $F_{1,8}$=0.151, P=0.86) for the sartorius muscle and ~160 kN m^{-2} for the external oblique muscles (one-way ANOVA $F_{1,7}$=2.578, P=0.15). The maximum power output measured during isovelocity force–velocity contractions for the sartorius muscle was not significantly different between the three groups of frogs (approximately 50 W kg^{-1}). The degree of curvature of the force–velocity relationship was similar in all groups; however, the maximum shortening velocity from hypoxic submerged frogs was reduced by approximately 20% compared to control and normoxic submerged frogs (Fig. 6; see Appendix for experimental details). The relationship between net power output determined using the work loop technique and cycle frequency was broadly similar for the sartorius and external oblique muscles across all treatment groups (Fig. 7). Peak cyclical power output was approximately 13 W kg^{-1} in the sartorius muscle and ranged from 5.0–6.0 W kg^{-1} in the external oblique muscles. While there are small differences in the maximum unloaded shortening velocity for the sartorius muscle, the data overall indicate that the mechanical properties of frog locomotory and calling muscles are essentially stable throughout overwintering submergence. The general uniformity of the mechanical properties in both muscles shows that major fibre-type remodelling did not occur. The relative sartorius muscle mass was largely preserved and similar in all treatment groups, representing approximately 0.29% of body mass (one-way ANOVA $F_{1,8}$=0.746, P=0.51). Thus body mass-specific power – that which relates to whole animal performance – is also unaffected by submergence.

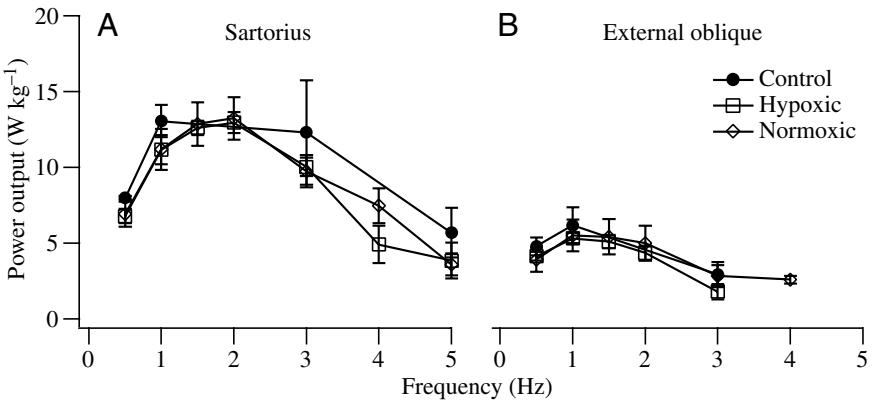

Fig. 7. Power-cycle frequency relationships (means ± s.e.m.) at 4°C for sartorius and external oblique muscles taken from (4°C) air-access control frogs, normoxic cold-submerged frogs (12–16 weeks in air equilibrated water) and hypoxic cold-submerged frogs (12–16 weeks in 60 mmHg O_2). A general linear model was used to test for changes in power with cycle frequency and treatment, considering power as the dependent variable and frequency and treatment as independent variables. Power was significantly affected by cycle frequency but there was no significant effect of treatment (Frequency $F_{6,40}$=7.208, P<0.001; Treatment $F_{2,40}$=1.381, P=0.265). (G. N. Askew and R. G. Boutilier, unpublished observations.)

Preservation of muscle mechanical properties is in keeping with the active hypoxia avoidance strategy of overwintering frogs. Since frogs will engage in strenuous mate-calling and reproductive behaviours soon after emergence from their overwintering hibernaculum (Boutilier et al., 1997), it is likely that their muscles resist any dramatic deterioration or fibre-type remodelling for the entire overwintering season. At present, however, it is difficult to conclude that muscles of overwintering *R. temporaria* express true atrophy-resistance, as is the case with aestivating frogs *Cyclorana alboguttata* (Hudson and Franklin, 2002). On the one hand, *R. temporaria* overwintering in natural waterways are clearly not victims of their changing environments (Boutilier, 2001a; Boutilier, 2001b) since they can, and must, react to dwindling ambient O_2 levels. In contrast, muscular quiescence is sustained for months in *C. alboguttata* without any sign of fibre atrophy (Hudson and Franklin, 2002). On the other hand, constant O_2 levels were provided in our laboratory studies of cold-submerged *R. temporaria* and these stable conditions should promote extended quiescence, particularly during normoxia. The preservation of mechanical properties in these circumstances is indeed remarkable.

Future systematic analysis of muscle fibre-types and fibre dimensions in conjunction with *in vitro* mechanical assays will help determine how cold-submerged *R. temporaria* compare with known atrophy-resistant hibernation and aestivation models. Direct comparison of mechanical and energetic properties of intact and skinned muscle fibres from *R. temporaria* can further help to characterise any changes in actomyosin function and sensitivity to metabolites/ions during time-courses of hypoxic and normoxic submergence. Plasma Ca^{2+} levels were also shown to be reduced by 30–40% in cold-adapted *R. temporaria* (Sinsch, 1991). Mechanical and energetic properties of intact muscle fibres could be affected, particularly if intramuscular Ca^{2+} stores follow the pattern of changes in plasma Ca^{2+} levels. It may be important in future work to incorporate changes in extracellular ion concentrations (Fig. 5) into *in vitro* protocols to determine (i) the impact of reduced ion gradients on membrane excitability and energy demand, (ii) Ca^{2+} sensitivity of mechanical properties and (iii) ionic-strength dependence of mechanical properties. West et al. (West et al., 2005) note that, like the effects of P_i on muscle force, alterations in ionic strength seem to perturb peak isometric force and energy demand minimally when measured

at physiological, or acclimation, temperatures. Even so, it is important to determine how force production, power output and energy demand might be affected by ions and metabolites over an ecologically relevant winter thermal range, in which relatively small shifts of body temperature can have potentially large impacts on rate processes.

Conclusion

Characterisation of frog hypoxia/anoxia responses has begun to verify that frogs express a range of tissue-level modes of hypometabolism (Boutilier et al., 1997); from the true anoxia-tolerance capacity of skeletal muscle, to the intermediate, and probably O_2-dependent, hypometabolic capacity of brain (Lutz and Nilsson, 2004). Skeletal muscle physiology is possibly the focal point of an integrated physiological and behavioural hypoxia defense strategy in frogs. Hypometabolism in the large, possibly oxy-conforming and often quiescent skeletal muscle mass contributes significantly to (i) whole animal hypometabolism and (ii) the conservation of O_2 supply for use by more hypoxia-sensitive core tissues. Metabolic and ionic responses of skeletal muscle are consistent with the channel/pump arrest hypothesis (Hochachka and Lutz, 2001), although a possibly unique feature of membrane energetics during cold-submergence may arise from the alteration of muscle Na^+ and K^+ gradients and plasma Ca^{2+} level to new and stable set-points (Sinsch, 1991; Donohoe et al., 2001). The lack of effect of prolonged normoxic and hypoxic cold-submergence on muscle mechanical properties highlights the readiness of frogs to move away from severe hypoxia in natural waterways. More work on muscle dimensions and energetics/mechanics during overwintering time courses is needed to assess (i) how changes in metabolites and ionic strength affect contraction and (ii) the quiescent cold-submerged frog as a model of muscle atrophy-resistance.

Appendix

Animals

Frogs (*Rana temporaria* L.) collected from wild populations in the British Isles and Ireland were purchased from commercial suppliers (Blades Biological, Cowden, UK). The animals were acclimated in the laboratory for 2–3 weeks in two 3400 l 'Living Streams' (LS900, Frigid Units Inc, Toledo, OH, USA), at a water temperature of 4°C. During this time, the frogs had access to air-breathing and the water was held at air-saturated levels of oxygen (i.e. 'normoxic conditions'). Two randomly selected groups of animals were submerged in water-filled perspex boxes placed below the water surface of both tanks to prevent any further air-breathing and to induce hibernation. The water of one Living Stream was held at air-saturated partial pressures of oxygen (P_{O_2} ~150 mmHg), while the water of the other was adjusted with mixed gases to achieve an ambient P_{O_2} of 60 mmHg (Donohoe and Boutilier, 1998). A control group of animals was held in normoxic conditions with air access.

Muscle physiology

In vitro muscle experiments were made at 4°C after 3–4 months for control and normoxic and hypoxic cold submergence frogs. The animals were killed and a sartorius muscle and a fascicle bundle dissected from the external oblique muscle were removed. One muscle was used immediately, while the other was pinned out at approximately resting length in oxygenated Ringer's solution (composition after Fischmeister and Hartzell, 1987).

For physiological measurements, the muscle was attached using aluminium foil clips between a force transducer (SensoNor AE801, Horten, Norway) and an electrodymanic shaker (Ling Dynamic Systems V201, Royston, UK) and bathed in oxygenated Ringer's solution at 4°C. Custom written software controlled the shaker and a stimulator (model S4, Grass-Telefactor, West Warwick, RI, USA) and was used to impose length changes and phasic stimulation on the muscle. Muscle force and length were recorded onto a personal computer at 5 kHz *via* a data acquisition card (Keithley Instruments model DAS1802AO, Theale, UK).

Following a recovery period of approximately 30 min, a series of isometric twitches was used to set the length of the muscle to that at which twitch force was maximal (L_0). At this length maximum isometric tetanic force was determined using a 500 ms train of stimuli at 50 Hz. Following the initial optimisation of muscle length, the work loop technique (Josephson, 1985) was used to measure muscle power output during sinusoidal length changes. Cycle frequency was varied between 0.5 and 5 Hz using a constant strain of 0.1 L_0, symmetrical around L_0. The net power output of the muscle was calculated from the force and differentiated strain trajectory data (i.e. velocity). The onset and duration of stimulation were optimised to maximise net power output.

Force–velocity characteristics were determined for a number of muscles during isovelocity contractions imposed during the plateau of an isometric tetanus. Tetani were performed at regular intervals to allow for correction for the decline in muscle performance. A hyperbolic linear regression equation (Marsh and Bennett, 1986) was fitted to the data using the non-linear curve fitting routine in the application Igor (version 5.0, Wavemetrics, Portland, OR, USA), and the maximum velocity of shortening estimated by extrapolation to zero force.

The authors dedicate this paper to the memory of Bob Boutilier; a true friend, a talented mentor, and a gifted comparative biologist. This work was supported by the Biotechnology and Biological Sciences Research Council (to R.G.B.), the Natural Environment Research Council (to R.G.B. and G.N.A.), and the Wellcome Trust (to T.G.W.).

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