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REVIEW

Physiological strategies during animal diapause: lessons from brine shrimp and annual killifish

Jason E. Podrabsky^{1,*} and Steven C. Hand^{2,*}

ABSTRACT

Diapause is a programmed state of developmental arrest that typically occurs as part of the natural developmental progression of organisms that inhabit seasonal environments. The brine shrimp *Artemia franciscana* and annual killifish *Austrofundulus limnaeus* share strikingly similar life histories that include embryonic diapause as a means to synchronize the growth and reproduction phases of their life history to favorable environmental conditions. In both species, respiration rate is severely depressed during diapause and thus alterations in mitochondrial physiology are a key component of the suite of characters associated with cessation of development. Here, we use these two species to illustrate the basic principles of metabolic depression at the physiological and biochemical levels. It is clear that these two species use divergent molecular mechanisms to achieve the same physiological and ecological outcomes. This pattern of convergent physiological strategies supports the importance of biochemical and physiological adaptations to cope with extreme environmental stress and suggests that inferring mechanism from transcriptomics or proteomics or metabolomics alone, without rigorous follow-up at the biochemical and physiological levels, could lead to erroneous conclusions.

KEY WORDS: Diapause, Metabolic Depression, Mitochondria

Introduction: animal diapause

Diapause is a programmed arrest of development that is controlled by endogenous physiological factors and may or may not involve depression of metabolism (Lees, 1955; Tauber and Tauber, 1976; Podrabsky and Hand, 1999; Denlinger, 2002; Reynolds and Hand, 2009; MacRae, 2010; Clegg, 2011; Hahn and Denlinger, 2011; Hand et al., 2011; Patil et al., 2013). Diapause typically precedes the onset of adverse environmental conditions, and production of diapausing embryos is often induced by environmental factors that offer reliable cues for predicting future conditions, such as photoperiod or food quality/availability. Depending on the developmental stage, diapause may be hormonally regulated and occur in response to signaling factors that come before the adverse period. The phenomenon is widespread across animal phyla and critically important for surviving in extreme environmental conditions, synchronizing favorable environmental conditions with the actively feeding stage of the animal, and transport and dissemination of the species in the case of resting eggs.

Interestingly, the intense selection pressures imposed by extreme seasonality appear to have driven the evolution of highly similar life history patterns that include embryonic diapause in very distantly related organisms. In this paper, we use the brine shrimp, *Artemia*

franciscana and the annual killifish *Austrofundulus limnaeus* as exemplar species for the study of dormancy and metabolic depression associated with embryonic diapause. They share strikingly similar life histories and physiological patterns associated with diapause as well as similar responses to environmental stress. These ‘hallmarks of dormancy’ include: (1) a coordinated depression of ATP-producing and ATP-consuming metabolic pathways that typically results in relatively stable ATP:ADP ratios, and thus preserves a favorable energetic poise within the cells; (2) depression in the rate of protein turnover as a major mechanism for reducing ATP demand; (3) reduction in the cost of ionoregulation at the cellular and organismal levels; and (4) stabilization of macromolecules in the face of reduced biosynthetic activity. However, the molecular mechanisms used to regulate metabolic depression and stress tolerance appear to be unique in the two lineages, suggesting that there are several and perhaps even many ways to alter cellular function to support the basic physiological requirements for entrance into a reversible state of metabolic depression.

Diapause during the life cycle of *Artemia franciscana*

During the summer months, females of the brine shrimp *Artemia franciscana* reproduce ovoviviparously by releasing free-swimming nauplius larvae directly from the brood pouch into the water column (Fig. 1). With the onset of shorter days in the autumn, ovigerous females begin reproducing oviparously. Encapsulated (encysted) embryos are introduced into the brood pouch and then released into the lake. These embryos enter diapause at the gastrula stage (Clegg and Conte, 1980). Although the developmental cessation is complete by the time embryos are released by the female into the water column, the metabolic depression requires several days post release. For habitats such as the Great Salt Lake, these events serve to prevent hatching and preserve nutrient stores while the embryo floats on the lake or after it has washed ashore (Patil et al., 2013). Whereas most of these encysted embryos overwinter on the shoreline, a fraction overwinter as floating cysts in the lake. Shoreline-deposited embryos may be subjected to cycles of dehydration and rehydration. Cold exposure and/or dehydration (Drinkwater and Crowe, 1987; Drinkwater and Clegg, 1991) eventually break diapause in overwintering embryos, whether on the lake or shore. Commonly in the spring, the shoreline-deposited embryos (now post diapause) are washed back into the lake and resume active metabolism and development (Patil et al., 2013). However, it should be noted that some of these embryos may become entrapped in sediments/decaying algal mats along the shoreline and experience hypoxia or anoxia for varying lengths of time (Clegg, 1974). Their tolerance to anoxia in this quiescent state (Fig. 1) is impressive but will not be reviewed here (see Stocco et al., 1972; Busa and Crowe, 1983; Clegg and Jackson, 1989; Hand, 1991; Hand and Hardewig, 1996; Warner and Clegg, 2001; Clegg, 2011; Hand et al., 2011).

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List of symbols and abbreviations	
ANT	adenine nucleotide transporter
dpf	days post-fertilization
DII	diapause II
DIII	diapause III
FAA	free amino acid
HSP	heat shock protein
IF ₁	inhibitor of F ₁
pH _i	intracellular pH
pPDH	phospho-pyruvate dehydrogenase
TPMP	triphenylmethyl phosphonium
Δp	proton motive force
ΔΨ _m	mitochondrial membrane potential
V̇ _{O₂}	metabolic rate

of diapausing embryos that exhibit exceptional tolerance to environmental stress (Fig. 1). The typical adult lifespan of an annual killifish ranges from a few weeks to a few months (the duration of pond inundation), while diapausing embryos may remain embedded in dried mud for several months or perhaps several years. Thus, any given individual may spend most of its life as a diapausing embryo encased in the pond sediment.

There are three distinct stages of development where embryos may enter diapause (Fig. 1; Wourms, 1972a,c). Diapause I may occur prior to gastrulation and formation of the embryonic axis during a unique phase in annual killifish development where the cells that will later constitute the embryos disperse across the surface of the yolk (Wourms, 1972b). Diapause II may occur about midway through development after initiation of neurulation and segmentation, but prior to the major phases of organogenesis. Diapause II embryos typically have a near-complete complement of somites, the foundations of the central nervous system and sensory systems, and a functional tubular heart (Podrabsky and Hand, 1999). Importantly, entrance into diapause II appears to be an alternative developmental pathway that may be controlled by maternal provisioning, as well as the developmental environment experienced by the embryo (Podrabsky et al., 2010). Diapause III can occur in the late pre-hatching embryo after development is essentially complete.

Diapause during the life cycle of the annual killifish *Austrofundulus limnaeus*

Annual killifish inhabit ephemeral ponds in regions of Central and South America and Africa that experience distinct dry and rainy seasons (Polacik and Podrabsky, 2015). *Austrofundulus limnaeus* is found in coastal desert and savanna regions on the eastern shore of Lake Maracaibo in Venezuela (Hrbek et al., 2005). Populations of *A. limnaeus* persist in ephemeral habitats through the production

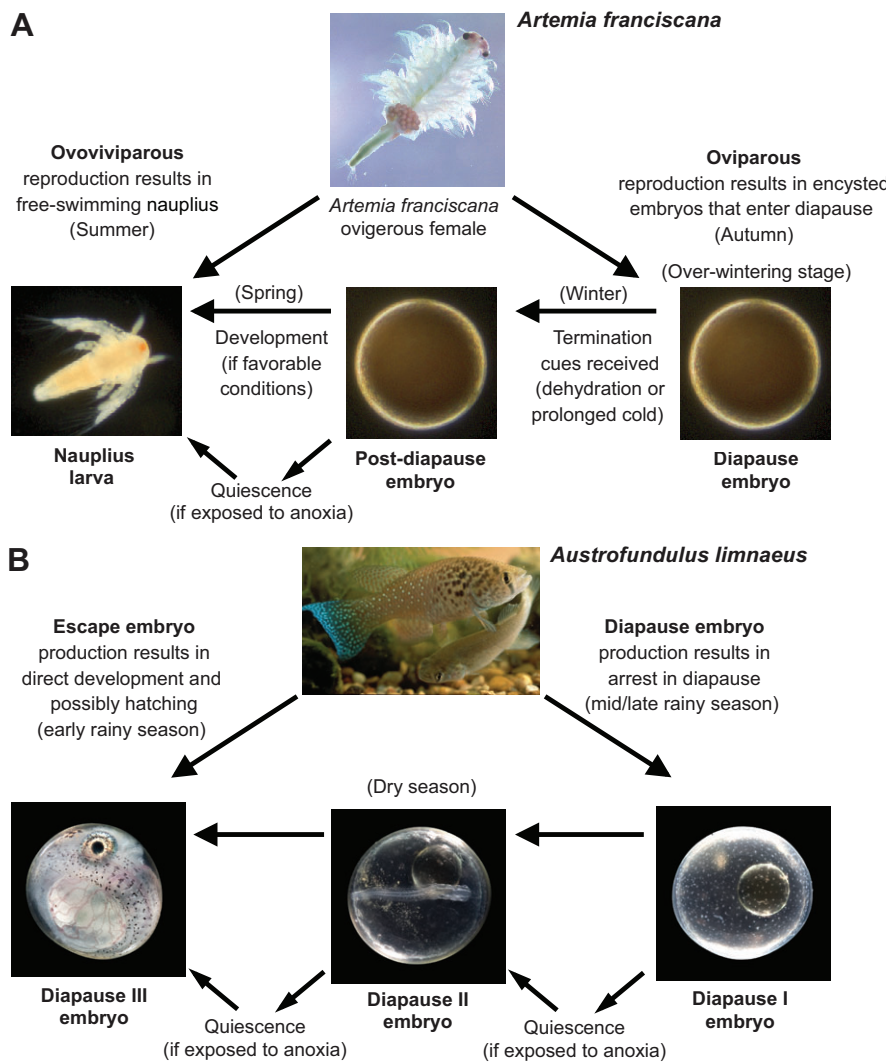


Fig. 1. Diagrammatic representation of the life cycles of the brine shrimp *Artemia franciscana* and the annual killifish *Austrofundulus limnaeus* with emphasis on the entry points into diapause and quiescence.

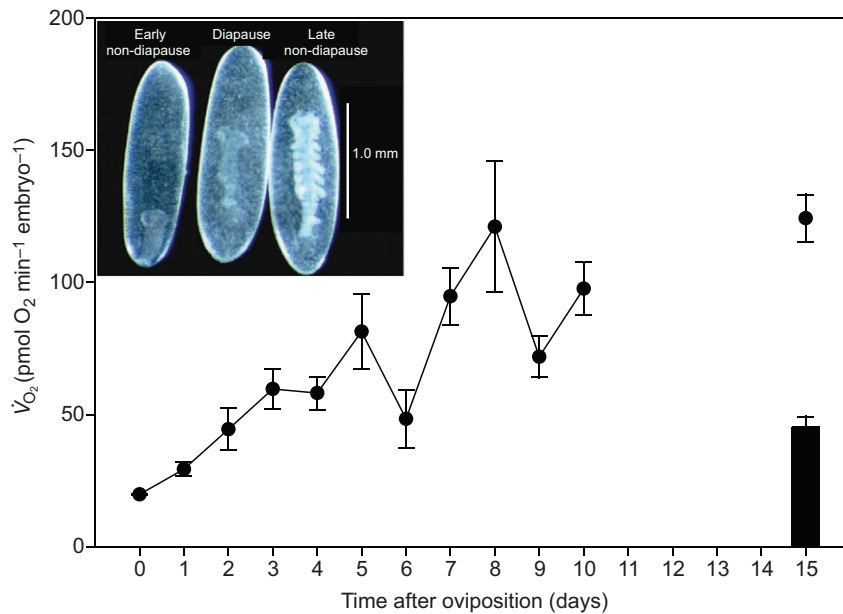


Fig. 2. Respiration rate of ground cricket embryos (*Allonemobius socius*) as a function of time after oviposition. Data for developing, non-diapausing embryos are depicted with filled circles (means \pm s.e.m.; $N=3$ –12 samples of 100 embryos for each time point). The histogram indicates oxygen consumption rate for diapausing embryos 15 days post oviposition (mean \pm s.e.m., $N=22$). Inset shows morphology of diapausing and non-diapausing embryos of *A. socius*. Modified from Reynolds and Hand (2009).

Each stage of diapause is physiologically distinct, and responds to environmental stress and cues in a unique manner. For example, diapause I is rare in *Austrofundulus*, and may only occur in embryos that are incubated at low temperatures (Wourms, 1972c). However, the dispersion and reaggregation phases of development that are associated with diapause I may act as a buffer against otherwise teratogenic levels of cellular damage (Wagner and Podrabsky, 2015). Entrance into diapause II is favored at temperatures of 25°C or below in the lab, and lower temperatures are associated with the dry season in their native habitat. Diapause II embryos have the highest tolerance to a number of environmental stresses, and in fact are able to withstand anoxia longer than any other vertebrate at 25°C (Podrabsky et al., 2001, 2007; Machado and Podrabsky, 2007; Wagner and Podrabsky, 2015). An important parallel between the *Artemia* and *Austrofundulus* life histories is the presence of a post-diapause period of active development that retains the high tolerance to environmental stress observed in diapausing embryos. For embryos of *A. limnaeus*, extreme tolerance of anoxia is retained for 4–6 days of post-diapause II development. Embryos in diapause III remain resistant to a number of environmental stresses compared with other species of fish, but have lost the extreme tolerance associated with diapause II (see references above).

Metabolic depression during diapause

In many cases depression of metabolism is not observed during diapause entry. Whether or not downregulation of energy flow occurs depends upon the species and the stage of development in question. For example, in the southern ground cricket, *Allonemobius socius*, embryonic diapause is not accompanied by a statistically significant change in metabolism (Reynolds and Hand, 2009) when one compares non-diapausing and diapausing embryos at the same developmental stage (Fig. 2). Specifically, diapausing embryos at 15 days post oviposition have the same metabolic rate as non-diapausing embryos at days 4–5, which are at equivalent morphological stages. Thus, there is not a detectable metabolic depression upon diapause entry. However, as development proceeds, the metabolism of non-diapause embryos continues to rise along with the increase in cell number per embryo. Accordingly, the aerobic metabolism in 15-day diapausing embryos

is only 36% of the rate measured for 15-day non-diapausing embryos, which emphasizes that the ontogenetic increase in metabolism observed in actively developing embryos is blocked during diapause (Reynolds and Hand, 2009).

In marked contrast to this example is the extreme metabolic depression seen in gastrula-stage embryos of *A. franciscana* during entry into diapause, which is arguably one of the deepest arrests ever reported for a diapause state. The metabolic depression was first quantified by Clegg et al. (1996). The embryos in the study by Clegg et al. were collected from the San Francisco Bay population and synchronized to within 24 h of the time of release from ovigerous females; across the intervening 20 days after release, the respiration rate drops to approximately 2% of the initial rate (Fig. 3; closed symbols). A very similar

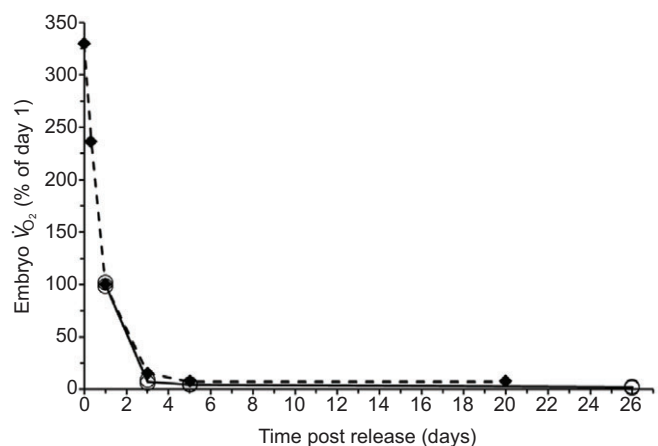


Fig. 3. Respiration rate for diapausing embryos of *Artemia franciscana* from the Great Salt Lake (solid line) as a function of time after release from the ovigerous female. Embryos were synchronized to within 4 h of their release. Independent replicates are displayed with superimposed open circles. Data from Clegg et al. (1996) for diapause embryos from San Francisco Bay (dashed line, diamonds) are expressed relative to day 1 values to allow comparison. Metabolism is depressed in diapausing embryos to <1% of that for the active embryo. Modified from Patil et al. (2013).

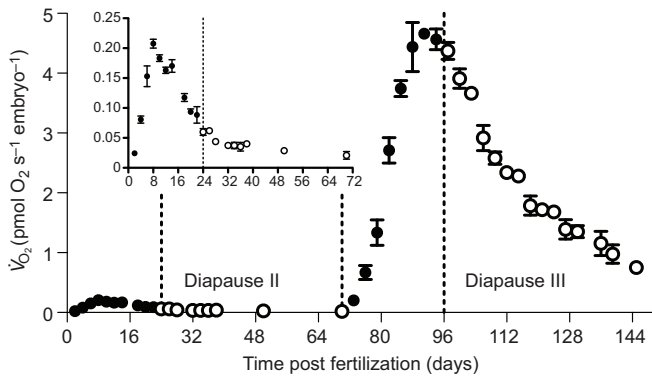


Fig. 4. Respiration rate is depressed by 80–90% in both diapause II and diapause III compared with actively developing pre-diapause embryos of *Austrofundulus limnaeus*. Note that respiration rate is extremely low for all of early development through diapause II compared with post-diapause II development. Inset shows expanded axis for early development and diapause II. Filled symbols are means for actively developing embryos whereas open circles are from embryos in diapause. Data are means±s.e.m. (N=4–8). Data are from Podrabsky and Hand (1999).

pattern is seen for embryos from the Great Salt Lake, Utah population (Patil et al., 2013). In this study embryos were synchronized to within 4 h of release from the female by collecting adults on site at the lake, incubating the adults on board the research vessel in large well-aerated holding tanks for 4 h, and then separating the newly-released embryos from adults by filtering through sieves. In this case respiration rate decreases to less than 1% of the day 0 value across the subsequent 26-day time course. As in the study by Clegg et al. (1996), the vast majority of the metabolic depression occurs during the first 3–5 days after release from the ovigerous females. The physiological ramifications of this deep arrest, which to re-emphasize occurs under conditions of normoxia, full hydration, and at euthermic temperatures, are substantial and will be discussed below.

Diapause in annual killifish is a profound state of developmental and metabolic dormancy (Levels et al., 1986; Podrabsky and Hand, 1999). Each stage of diapause exhibits a unique pattern of

metabolic depression (Fig. 4). For example, respiration rate begins to decline several days prior to the cessation of development in diapause II and is already depressed by 70% compared with peak pre-diapause II rates upon entry into diapause. Respiration rates continue to decline during the first week of diapause, finally reaching rates that are depressed by over 90% compared with peak pre-diapause II rates (Podrabsky and Hand, 1999). In contrast, embryos enter diapause III at peak rates of respiration for development and exhibit a slow and steady decline in respiration rates over many weeks. After almost 50 days in diapause III respiration rates were depressed by over 80% compared with rates at diapause entry (Fig. 4; Podrabsky and Hand, 1999). As noted above for *A. franciscana*, these profound reductions in respiration associated with diapause in *A. limnaeus* occur under normoxic conditions, in fully hydrated embryos exposed to near optimal temperatures for development.

Metabolic support during diapause

When considering the proximal mechanisms for metabolic downregulation in *A. franciscana*, it is first appropriate to note that the sole source of metabolic fuel for the embryo prior to hatching is the endogenous store of the disaccharide trehalose, a conclusion based upon respiratory quotients and biochemical measurements of stored fuels (Dutrieu, 1960; Muramatsu, 1960; Emerson, 1963; Clegg, 1964; Carpenter and Hand, 1986). Thus, unraveling the mechanisms by which trehalose catabolism is brought to a halt during diapause is central to an understanding of the overall energetic arrest (Patil et al., 2013).

Based on an extensive analysis of trehalose plus all metabolites in the glycolytic pathway involved in flux (i.e. ‘pathway intermediates’), the product-to-substrate ratios show there are four enzymatic steps at which inhibition occurs during entry into diapause: trehalase, hexokinase, pyruvate kinase and pyruvate dehydrogenase (Fig. 5). The orchestrated interplay at these points in the metabolic pathway results in blockage in the delivery of carbon fuel to the mitochondrion, thereby explaining a large portion of the metabolic depression during diapause. Previously, our lab has reported that the respiratory capacities of mitochondria isolated from diapausing versus post-diapausing embryos are quite similar,

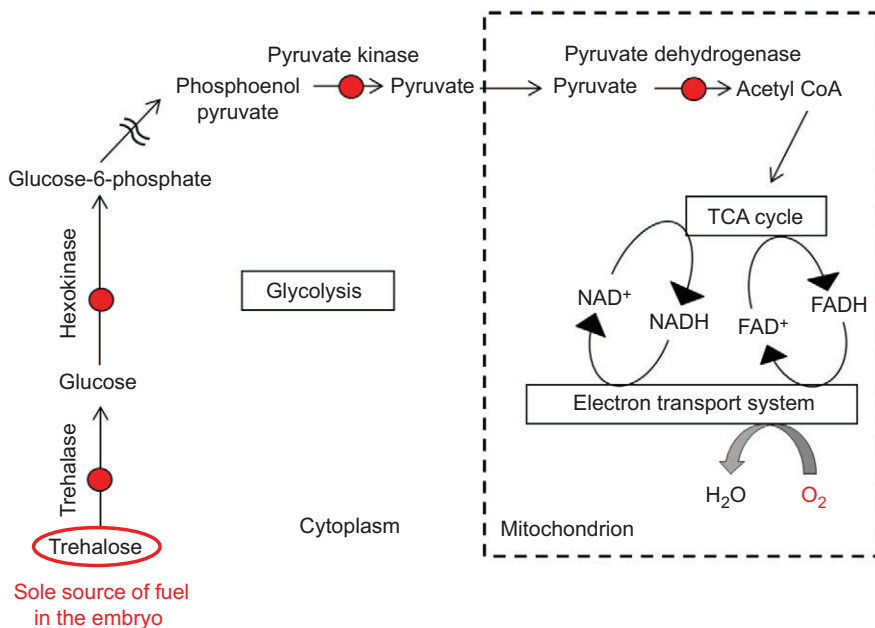


Fig. 5. Schematic depiction of metabolic arrest in *Artemia franciscana* embryos during diapause that shows the enzymatic sites of inhibition based on metabolite analyses. Modified from Patil et al. (2013).

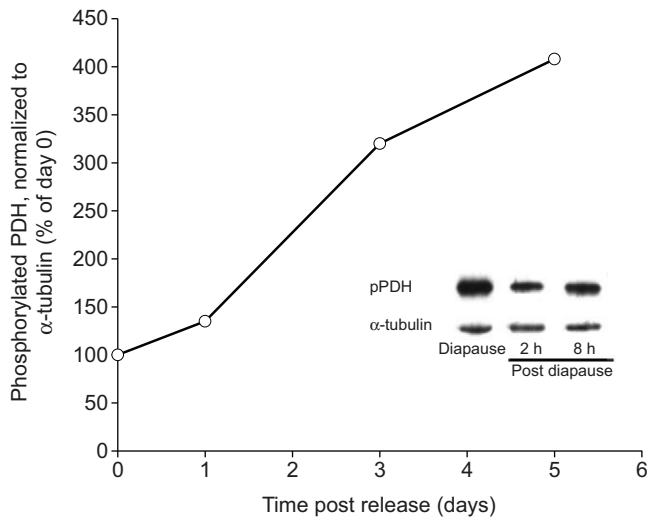


Fig. 6. The phosphorylation status of pyruvate dehydrogenase (PDH) in diapausing embryos of *Artemia franciscana* as determined by western blots is shown for 4-h-synchronized embryos as a function of days after release from females. Intensity was quantified by densitometry. Inset shows western blot for phospho-PDH (pPDH) from bulk (nonsynchronized) diapausing embryos compared with post-diapausing embryos incubated for 2 and 8 h at room temperature. α -tubulin is shown as the loading control. Antibody against pPDH site 1 (serine 293, subunit E1 α) was used. Modified from Patil et al. (2013).

as are the quantities and structures of the respective mitochondria *in situ* (Reynolds and Hand, 2004). Recent data (Y. Patil, E. Gnaiger and S.C.H., unpublished results) indicate that diminished respiration with substrates for complex I and II can be detected in crude lysates of diapausing embryos compared with those that are post diapause. The result is consistent with the concept that various types of covalent modification of mitochondrial proteins are present during diapause that are not preserved during the mitochondrial isolation steps, and/or a soluble inhibitor is possibly removed during the purification.

Recently, we have documented that phosphorylation of pyruvate dehydrogenase (serine 293, E1 α ; 'site 1') increases substantially during diapause (Patil et al., 2013), as measured by extracting whole embryos in SDS buffer, followed by western blot analysis with an antibody against phospho-PDH site 1. Phosphorylation at site 1 is well established to strongly inhibit PDH activity (Kolobova et al., 2001; Patel and Korotchikina, 2001). The increase in phosphorylation can be seen with non-synchronized diapausing embryos (Fig. 6, inset) as well as during the time course for entry into diapause with embryos that have been synchronized to within 4 h of release from the female (Fig. 6). The time course for the increase in phosphorylation matches the time course for the metabolic depression (Fig. 3) quite well.

The metabolic fuels used to support metabolism during diapause remain unverified in embryos of annual killifish. Elevated calorimetric to respirometric ratios (C:R ratios) during diapause II indicate that anaerobic pathways contribute significantly to the overall metabolism of aerobic diapause II embryos (Podrabsky and Hand, 1999). Carbohydrate metabolism is not likely to play a key role because glycogen content does not change significantly during even extended sojourns in diapause II and extensive metabolomics screens have not identified accumulation of any common glycolytic end-products (Podrabsky and Hand, 1999; Podrabsky et al., 2007). It is worth noting, however, that fish embryos are highly dependent on protein catabolism to support development. Most of the yolk

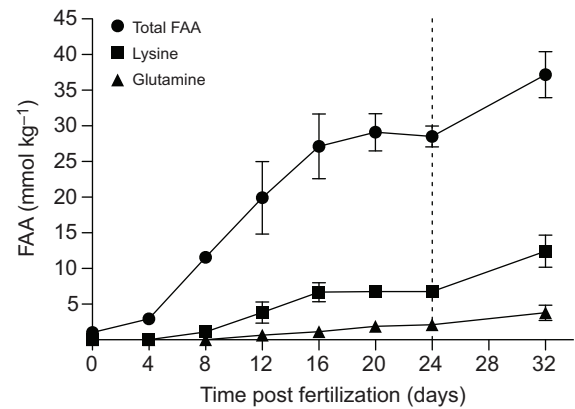


Fig. 7. Free amino acids (FAAs) accumulate during diapause II in embryos of *Austrofundulus limnaeus*. Note that during the first week of diapause total FAAs continue to increase, despite the cessation of development and cell proliferation. Most of this increase is accounted for by lysine and glutamine. Symbols are means \pm s.e.m. ($N=3$). Data are from Podrabsky et al. (2007).

energy stores, the sole source of energy and building blocks for the completion of development in fish embryos, are invested in phospho- and lipoproteins (LaFleur et al., 2005; Sawaguchi et al., 2005; Kristoffersen et al., 2009). In fact, free amino acid (FAA) levels do increase during diapause II, with lysine and glutamine accounting for most of the almost 8 mmol l⁻¹ increase in FAAs observed during the first week of diapause II (Fig. 7; Podrabsky et al., 2007). Total protein levels remain relatively constant during diapause II (Podrabsky and Hand, 1999; Machado and Podrabsky, 2007), and thus it is not clear whether this increase in FAA represents differential use of FAAs released from a very limited amount of protein degradation, or whether some biosynthetic pathways are also at play. Ammonia accumulation is a possible negative consequence of amino acid catabolism, especially when diapausing embryos are encased in dried mud. It is interesting that the two amino acids that account for most of the increase in FAAs during diapause have side chains containing nitrogen. It is possible that biosynthesis of these amino acids could serve as a sink for ammonia produced by the catabolism of other amino acids. Additional studies will be needed to identify the possible role of protein catabolism in supporting the metabolic requirements for diapause in *A. limnaeus*.

Mitochondrial membrane potential and proton leak

Because the downregulation of respiration in diapausing embryos is so severe, this observation brings into question whether the proton motive force (Δp) across the inner mitochondrial membrane is defended during diapause. At less than 1%, embryo respiration in *A. franciscana* is depressed far lower than that required to even compensate for mitochondrial proton leak under basal conditions. Consequently, one mechanism to prevent dissipation of Δp during diapause would be to dramatically lower the proton conductance across the inner membrane. Thus, we measured proton leak respiration as a function of the membrane potential ($\Delta\Psi_m$) in mitochondria isolated from diapausing and post-diapausing embryos. Because $\Delta\Psi_m$ is the driving force for the leak, it is essential to compare proton conductances between different states only at the same driving force (Affourtit et al., 2012). When the F₁F₀-ATP synthase (complex V) is inhibited, mitochondrial respiration is then proportional to proton leak rate through the inner membrane. Accordingly, the kinetic response of the proton

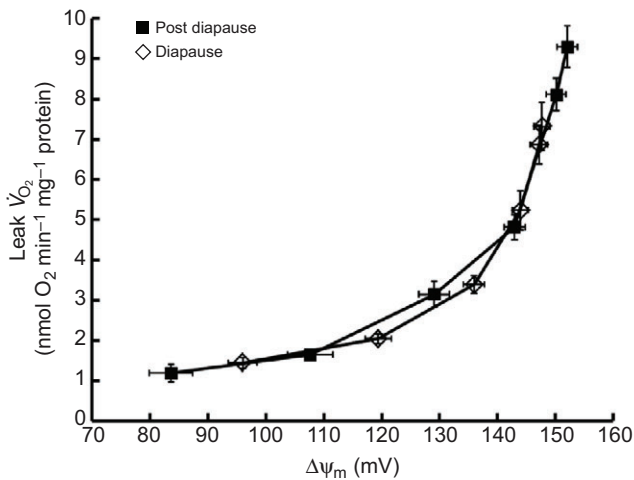


Fig. 8. Kinetic response of proton leak at 25°C (measured as respiration rate) as a function of the driving force (mitochondrial membrane potential, $\Delta\Psi_m$). Mitochondria were isolated from diapausing and post-diapausing embryos of *Artemia franciscana*. Succinate plus rotenone was used as the substrate to support leak respiration (state 4) in the presence of oligomycin (inhibitor of the F_1F_0 -ATP synthase) and nigericin (K^+/H^+ exchanger), the latter of which served to convert the ΔpH to $\Delta\Psi_m$. Stepwise addition of malonate (complex II inhibitor) was employed to progressively inhibit the electron transport system and thus lower overall $\Delta\Psi_m$. Triphenylmethyl phosphonium (TPMP)-sensitive electrodes were used to measure the distribution of this lipophilic cation across the inner membrane, from which the $\Delta\Psi_m$ was calculated with the Nernst equation. Values are means \pm s.e.m. for $N=6$ independent determinations. Modified from Patil et al. (2013).

conductance pathway to its driving force can be measured as the relationship between respiration rate and $\Delta\Psi_m$ when the latter is varied by titrating the electron transport system (ETS) with inhibitors (Cadenas and Brand, 2000; Affourt et al., 2012; Patil et al., 2013). Our results indicate clearly that proton conductance, as judged by leak respiration, does not differ between mitochondria isolated from diapausing and post-diapausing embryos (Fig. 8). Consequently, because proton conductance across the inner mitochondrial membrane is not restricted during diapause, $\Delta\Psi_m$ is

undoubtedly compromised. One biological advantage for not defending Δp is that it reduces energy expenditure considerably during diapause. Some of the potential disadvantages have been treated elsewhere (Hand and Menze, 2008; Menze et al., 2010) and include an increased probability of opening of the mitochondrial permeability transition pore and the attendant signaling for cell death. However, all inducers for the mitochondrial permeability transition that have been tried so far are ineffective in promoting the transition in *A. franciscana* mitochondria (Menze et al., 2005).

In contrast to *A. franciscana*, respiration rates during diapause in *A. limnaeus* are more than sufficient to support rates of proton leak associated with actively developing embryos and appear to be supported in large part by mechanisms intrinsic to mitochondria (Duerr and Podrabsky, 2010). Multiple lines of evidence support this statement. First, respiration rates of isolated mitochondria can account for much of the observed decreases in respiration rate observed *in vivo* (Duerr and Podrabsky, 2010). Second, the activity of respiratory complexes II, IV and V are significantly reduced or inhibited in mitochondria isolated from diapausing embryos compared with developing embryos (Fig. 9). The reduction in the activity of complex V, the F_1F_0 -ATP synthase, is quite dramatic, with almost no measurable activity in mitochondria isolated from diapause II embryos. This inhibition of activity for all the complexes is rather quickly reversed (within 24 h) when a diapause III embryo is prompted to hatch (compare DIII and larval means) and thus is either due to a dramatic activation of gene expression for mitochondrial enzymes of key importance to supporting oxidative phosphorylation, or perhaps more likely, to covalent modification of existing proteins. Interestingly, the small amount of respiration that is supported during diapause II can be accounted for almost exclusively by an increased proton leak across the inner mitochondrial membrane. In fact, the maximum $\Delta\Psi_m$ achievable under non-phosphorylating conditions is lower for mitochondria isolated from diapausing compared with non-diapausing embryos (100 vs 140 mV), but the proton leak respiration at a common $\Delta\Psi_m$ is much (over 4-fold) higher in mitochondria isolated from diapausing compared with non-diapausing embryos (J. M. Duerr and J.E.P., unpublished results). We hypothesize that this seemingly paradoxical increase in proton leak during diapause may be a

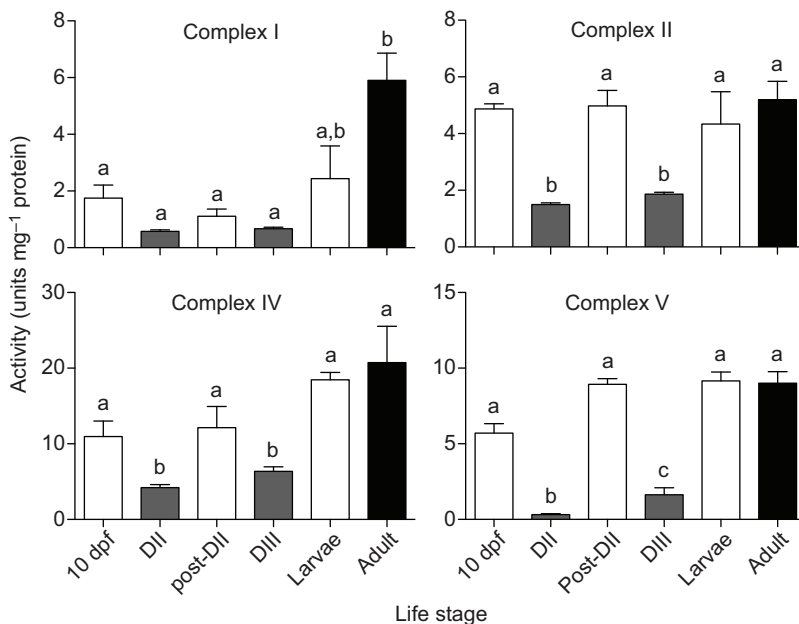


Fig. 9. The activity of respiratory complexes II, IV and V are decreased in isolated mitochondria from diapausing embryos of *Austrofundulus limnaeus*. DII, diapause II; DIII, diapause III; post-DII embryos are Wourms' stage 39 (Wourms, 1972a); larvae are 1–2 days post hatch; adult data are for isolated liver mitochondria. Bars represent means \pm s.e.m. ($N=5$). Bars with different letters are statistically different as determined by ANOVA with Tukey's *post hoc* tests, $P<0.05$. Data are from Duerr and Podrabsky (2010).

Table 1. Adenylate status in diapausing and active (post-diapause) embryos of *Artemia franciscana* and *Austrofundulus limnaeus*

Adenylates	Diapause	Active (post diapause)
<i>Artemia</i> ^a		
ATP/ADP	1.31±0.04	7.30±0.28
ADP ^b	0.13±0.01	0.11±0.01
AMP ^b	0.62±0.04	0.03±0.01
<i>Austrofundulus</i> ^c		
ATP/ADP	3.3±0.36	5.5±0.4
ADP ^d	0.067±0.011	0.085±0.009
AMP ^d	0.014±0.007	0.005±0.005

^aValues are means±s.e.m., N=10 (diapause), N=8 (post diapause) (Patil et al., 2013).

^bValues are expressed as $\mu\text{mol g}^{-1}$ hydrated embryo.

^cValues are means±s.e.m., N=3 (Podrabsky et al., 2012).

^dValues are expressed in nmol per embryo. Each embryo has a mass of approximately 2.5 mg.

mechanism to reduce $\Delta\Psi_m$ and thus reduce or prevent the production of reactive oxygen species that might occur if membrane potentials were allowed to increase – as predicted by the severely limited activity of the F_1F_0 -ATP synthase. The mechanism to explain this increased proton leak has not yet been identified and is currently under investigation.

Defending ATP levels during diapause: a role for the inhibitor of F_1 protein (IF₁)?

During diapause in arthropods, it is not surprising that cellular ATP content is lower than in the non-diapause state, but it is noteworthy that ATP is by no means depleted. For embryos of the cricket *A. socius*, the ATP:ADP ratio is approximately 40–50% lower in diapausing versus non-diapausing embryos (Reynolds and Hand, 2009). Likewise, diapausing cysts and embryos from *A. franciscana* and *A. limnaeus* contain considerably more ATP than ADP, although the ratio is substantially lower than that measured for post-diapause embryos (Table 1).

The above values reflect the adenylate content in whole embryos, but it is pertinent to recall that the status of adenylate pools in the cytoplasm versus the mitochondrial matrix differ appreciably in cells. Relative to the cytoplasm, ATP is concentrated in mitochondria and ADP even more so (Tables 2 and 3). In classic studies with digitonin permeabilization of the plasma membrane, cytosolic adenylates can be separated from the pelleted mitochondrial fraction and then quantified separately (Siess et al., 1977; Akerboom et al., 1978). Roughly 60% of total adenine nucleotides are found in the cytoplasm. However, this value depends greatly on the cell volume occupied by mitochondria, which for rodent hepatocytes is 23% (Beauvoit et al., 1994). By

Table 2. ATP and ADP partitioning between cytoplasmic and mitochondrial compartments based on the study by Siess et al. (1977) with rat hepatocytes

Adenylate	Concentration (nmol g ⁻¹ dry wt cells)	Percentage of total cellular content	Concentration	
			(mmol l ⁻¹)	
Cytoplasm	ATP	6020±390 (N=9)	68.2%	4.36
	ADP	1080±70 (N=9)	46.2%	0.78
Mitochondria	ATP	2810±280 (N=9)	31.8%	16.98
	ADP	1260±60 (N=9)	53.8%	7.61

Values are means±s.e.m.

Table 3. ATP and ADP partitioning between cytoplasmic and mitochondrial compartments based on the study by Akerboom et al. (1978) with rat hepatocytes

	ATP (mmol l ⁻¹)	ADP (mmol l ⁻¹)	AMP (mmol l ⁻¹)	ATP:ADP ratio
Cytoplasm	2.76	0.32	0.13	8.76
Mitochondria	10.38	5.86	4.33	1.77

Relative to the cytoplasm, ATP is concentrated in mitochondria and ADP even more so.

comparison, the cellular volume of mitochondria is around 5% in *A. franciscana* embryos (Rees et al., 1989). The ATP:ADP ratio in the cytosolic fraction is much higher than in the mitochondrial fraction (Tables 2 and 3). During diapause, it is remarkable that ATP stores are not depleted in the face of extreme metabolic arrest, especially considering that mitochondrial Δp is compromised and the matrix pH would become more acidic. Under these conditions, the F_1F_0 -ATP synthase can reverse and function in the ATPase direction (Nicholls and Ferguson, 2013). Because the adenine nucleotide transporter (ANT) would also reverse, the ANT would transport cytoplasmic ATP into the matrix and remove matrix ADP, thus tending to equalize the ATP:ADP ratios shown in Tables 2 and 3. Under this scenario, the possibility exists that all cellular ATP could be hydrolyzed in a futile attempt to restore the Δp . Bob Boutilier once referred to this phenomenon as ‘cellular treason’ (St-Pierre et al., 2000).

However, at low matrix pH it is now established that a matrix-resident inhibitor protein, termed inhibitor of F_1 (IF₁), can bind to the ATP synthase and block the reverse reaction from occurring (e.g. Gledhill et al., 2007; Bason et al., 2011, 2014). Bovine monomeric IF₁ comprises 84 amino acids (9.6 kDa) and its N-terminal inhibitory region is intrinsically disordered (Bason et al., 2014). The inhibitor binds at a catalytic interface between the β DP and α DP subunits. IF₁ exists as inactive tetramers at alkaline pH, but these can depolymerize to free dimers at low pH, which bind to the enzyme and inhibit ATP hydrolysis. It is possible that such a mechanism may operate during diapause in *A. franciscana*, and this hypothesis is currently being tested.

Reduction in ionoregulation

Maintenance of ion balance across the plasma membrane can account for 30–70% of basal cellular metabolism (Rolfe and Brown, 1997), and thus severe metabolic depression requires that the rates of ion leak and pumping at the cellular and organismal levels are addressed. For aquatic organisms, this also means that exchange of ions with the external environment must be tightly regulated and reduced during dormancy to avoid rapid depletion of cellular ATP levels in the face of reduced ATP production. Accordingly, the costs for ion homeostasis in diapausing and post-diapausing embryos of *A. franciscana* are exceedingly low because the shell of these encysted embryos is impermeable to ions, including protons (Conte et al., 1977; Busa et al., 1982; Clegg and Conte, 1980). Furthermore, the embryos do not even possess measurable activities of the Na^+/K^+ -ATPase during these stages (Peterson et al., 1978). Consequently, energy requirements for ion balance between the inside and outside of embryos are perhaps non-existent until emergence and hatching. Similarly, diapausing embryos of *A. limnaeus* have extremely low rates of Na^+/K^+ -ATPase and overall ATPase activity during early development and diapause II (Machado and Podrabsky, 2007). In fact, Na^+/K^+ -ATPase activity does not increase significantly following fertilization until several days after diapause II is broken. Associated with this low activity is an extremely low exchange of ions

and water with the environment (Machado and Podrabsky, 2007). Thus, osmoregulation in both organisms is achieved by minimizing environmental exchange, a strategy that is equally effective in both hypo- and hyper-osmotic environments without the restructuring of osmo- and ionoregulatory machineries. This strategy is likely to be especially effective in the extreme and often unpredictable environments to which dormant embryos may be exposed.

Reduction in protein turnover

A great deal of the ATP turnover in animal cells can be attributed to protein turnover (e.g. Rolfe and Brown, 1997). In fact, in many different species, protein synthesis can account for about 30% or more of the total oxygen consumed to support basal cellular metabolism (e.g. Rolfe and Brown, 1997; Podrabsky and Hand, 2000). Not surprisingly, considering the degree of metabolic depression, protein synthesis in embryos of *A. franciscana* is severely depressed in prolonged diapause, perhaps by 99%, as estimated by incorporation of radiolabel into protein of field-collected embryos (Clegg et al., 1996). In this study, Clegg and colleagues observed little evidence of proteolysis in diapausing embryos and assumed that pathways for macromolecular degradation are also brought to a standstill during diapause. Rates of protein synthesis are depressed by over 90% during diapause II in embryos of *A. limnaeus* which results in a reduction in ATP demand equivalent to about 36% of the metabolic depression observed (Podrabsky and Hand, 2000). Reduction in the rate of protein synthesis during diapause presents challenges to a diapausing embryo and may limit the ability of the embryo to mount an effective translational response to environmental challenges. Thus, the production of proteins that support metabolic depression and increased stress tolerance are likely to occur prior to entrance into diapause, when metabolic and protein synthetic capacity are still high. This supposition is supported by the accumulation of stress-inducible forms of heat shock proteins (HSPs) during early development that peak during diapause II in *A. limnaeus* (Podrabsky and Somero, 2007) and by the accumulation of small HSPs in diapausing embryos of *A. franciscana* (Liang and MacRae, 1999). While accumulation of HSPs may help protect embryos from environmental stress during diapause, they are typically not expressed at high levels during embryonic development and overexpression has been shown to impede development in some species (Feder et al., 1992). The trade-offs associated with depression of protein synthesis during dormancy and the possible costs to a developing embryo have yet to be addressed in species that produce diapausing embryos.

Future questions

In addition to the IF₁ hypothesis above, numerous unexplained issues remain regarding the control of diapause and survival in this arrested state. For example, whether there is a regulatory role for acidification of pH_i during diapause entry (as there is during anoxia; see Busa and Crowe, 1983 and for a review, see Hand et al., 2011) is still an open question. In *A. franciscana*, diapause embryos have an alkaline pH_i shortly after release from the female (Drinkwater and Crowe, 1987), but direct evidence is lacking for an acidification during the time course of metabolic depression for diapause. Similarly, regulation of intracellular pH has never been addressed in diapausing embryos of *A. limnaeus*. Addressing the issue of intracellular pH is particularly important in light of the recent evidence that the mitochondrial permeability transition pore is composed of dimeric units of the F₁F₀-ATP synthase (Giorgio et al., 2013). It would be good to understand why the transition pore in *A. franciscana* is resistant to opening and what structural differences might exist for its ATP synthase. A similar lack of calcium-induced opening has been observed for mitochondria

of the ghost shrimp, *Lepidophthalmus louisianensis* (Holman and Hand, 2009). While no direct evidence exists concerning opening of the transition pore in embryos of *A. limnaeus*, they are highly resistant to anoxia-induced apoptosis (Meller and Podrabsky, 2013), and thus may share similar resistance to mitochondrial-induced apoptosis. Additionally, it will be important to explore the cell-signaling pathways involved in the induction and breakage of diapause. For example, cells of *A. limnaeus* arrest in the G₁ phase of the cell cycle (Meller et al., 2012), while some species of insect arrest in G₁ and others in G₂/M phases (Denlinger, 2002). Where arrest occurs in the cell cycle will have a dramatic affect on the gene expression status of the cells and thus the molecular pathways that must be regulated. In addition, there is increasing evidence that insulin-like signaling pathways, which are known to favor arrest of the cell cycle in G₁, may play a central role in the regulation of diapause in a variety of lineages (e.g. Kimura et al., 1997; Sim and Denlinger, 2013; S. C. Woll and J.E.P., unpublished results), and thus may represent a universal way to regulate cellular physiology in favor of metabolic depression. Finally, the importance of understanding how diapause is broken is critical. Extreme environmental conditions are often associated with diapause breakage (desiccation, long-term cold or freezing), and thus understanding how an organism can increase resistance to environmental stress while still maintaining the ability to sense and respond to these same variables will likely lead to new insights in our understanding of cell sensing and signaling in response to environmental change.

Many routes to similar phenotypes and the importance of mechanistic follow-up

Despite the striking similarities of the life history and very similar physiological characteristics associated with diapause in *A. franciscana* and *A. limnaeus*, evolution has clearly driven different molecular adaptations to support diapause in each lineage. This route to adaptation parallels that observed for individual proteins evolving in response to environmental temperature (see Fields et al., 2015). While this observation may not be surprising, it highlights the importance of physiology as a central regulator of organismal performance that is of paramount importance in the process of natural selection. Perhaps because organisms are such complex systems, there will always be many mechanistic ways to alter physiological function or maintain homeostasis in response to ecological or environmental selection pressures.

However, as powerful as the comparative approach is for scientific discovery, a disturbing trend is becoming prevalent in comparative physiology. The use of hypothesis-generating approaches – such as transcriptomics, proteomics and mass-spectroscopy-based metabolomics – as stand-alone techniques for scientific inquiry has become problematic for the field. As the costs of transcriptomics (e.g. RNA-Seq) and similar tools continue to decline, many investigators are applying these tools as ultimate end points for their studies, instead of as means to an end. The painstaking, time-intensive work of testing the resultant hypotheses with mechanistic analyses is, more often than not, ignored. Yet instead, another round of mRNA or protein profiling for a new combination of environmental variables is performed, the results and hypotheses from which are again not experimentally tested or confirmed. Unfortunately, this practice may be helping to nudge comparative physiology toward the backwaters of science; the comparative section of *Annual Reviews of Physiology* has been terminated, with more emphasis placed on medical physiology, where apparently investigators appreciate the obligation of driving a project forward to proofs of function and mechanistic conclusions.

Without follow-up, ‘omics’ studies are of limited value. Applebaum et al. (2014) have made a compelling case for combining quantitative genetics with omics approaches to provide genotype-to-phenotype mapping in order to narrow the focus for mechanistic physiological studies. Generating and integrating the relevant functional data is important in order to move past correlations (e.g. Furlong, 2011; Nikinmaa and Rytönen, 2012). Accordingly, rigorous enzyme kinetics, macromolecular structural studies, metabolic control analysis, cellular signal transduction studies and other approaches are required to test physiological mechanisms, which make collaborations among laboratories often essential. In the realm of bioenergetics, it seems that physiologists have forgotten that modulating the titer of one or more enzymes within a metabolic pathway (assessed by proteomics and often incorrectly inferred by transcriptomics) is often a trivial aspect in metabolic control. Rather, allosteric regulation, control of pathway flux by substrate availability and covalent modification of proteins (including far more than phosphorylation) are frequently more important physiologically than changes in gene expression (see Chouchani et al., 2013 for a compelling case of metabolic modulation by protein nitrosation). In some cases omics techniques themselves have serious deficiencies. For example, broad-based, mass spectroscopy scans often provide quantitative information only for a subset of intermediates within a given pathway because of a lack of resolution or identification. Without traditional benchwork to obtain data for the remaining pathway metabolites, virtually nothing can be concluded about sites of inhibition/activation or sites inferred regarding metabolic flux.

Thus, as genomic information continues to accumulate, it is imperative that we follow through to elucidate mechanisms in order to appreciate the central importance of physiology as a primary target of natural selection. This mindfulness will ensure that we continue to focus on the attributes that are critical for organismal performance and adaptation.

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Author contributions

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