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# Metabolism of Gemmules From the Freshwater Sponge *Eunapius fragilis* During Diapause and Post-Diapause States

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**Abstract.** Post-diapause gemmules of the freshwater sponge *Eunapius fragilis* remained quiescent when maintained at 5°C. Germination occurred within 48 to 72 h following warming to 20°–23°C, culminating with the emergence of a new sponge from the collagenous capsule. Both heat dissipation and oxygen consumption climbed steadily during germination and eventually reached 600% of the starting values. By comparison, energy flow was much lower over the same period of time in diapausing gemmules, clearly demonstrating metabolic depression during diapause. The calorimetric:respirometric (CR) ratio increased significantly from −354 kJ/mol $O_2$ to −541 kJ/mol $O_2$ between hours 3.5 and 56.5 of germination, with an average value across this period of about −495 kJ/mol $O_2$. The low CR ratio at hour 12.5 (−374 ± 21; ± 1 SE, $n$ = 3) was statistically below the oxycaloric equivalent, which suggests that gemmules may have experienced hypoxia during the more than 3 months of storage at 5°C prior to experiments. The increase in metabolism during germination could be blocked by perfusing the gemmules with nitrogen-saturated medium (nominally oxygen free). Developing gemmules were able to survive oxygen limitation for several hours at least; during that time energy flow was depressed to 6% of normoxic values. During germination, the range of values was 3.5 to 4.0 nmol/mg protein for ATP, 0.2 to 0.4 nmol/mg protein for ADP, and 0.5 to 0.8 nmol/mg protein for AMP. Because ATP was high even before gemmules were warmed to room temperature, it is unlikely that levels were severely compromised during the diapause condition.

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## Introduction

Gemmules of the sponge *Eunapius fragilis* are asexually produced reproductive bodies composed of undifferentiated cells surrounded by a complex collagenous capsule. Such bodies serve as the overwintering stage in the life cycle of this sponge. The possession of resting stages in the life cycles of invertebrates is common for organisms inhabiting inconsistent or ephemeral environments and is widespread phylogenetically (Hand, 1991). Newly formed gemmules are in an obligate state of developmental (and potentially metabolic) arrest that is termed diapause. Vernalization in the cold for 2–3 months releases the gemmules from diapause and allows them to resume development when warmed to 20–23°C.

The molecular mechanisms of this diapause breakage are unexplained. *E. fragilis* is well-suited for use in investigating these mechanisms because diapause in its gemmules is easily manipulated and is quite distinct from quiescence (Fell, 1987), a metabolically arrested condition promoted directly by environmental insult. In other sponges, these two states are apparently intertwined (*e.g.*, *Spongilla lacustris*, Simpson and Rodan, 1976), and it can be quite difficult to distinguish whether or not gemmules are in diapause or whether the state has been broken. Other species apparently do not enter diapause at all and exhibit only quiescence (*Ephydatia fluviatilis*, Rasmont, 1963, 1965).

The ecological importance of dormant states has been emphasized by a number of authors (*e.g.*, Clutter, 1978; Crowe and Clegg, 1973; Danks, 1987; Elgmork, 1980; Hand, 1991; Hand and Hardewig, 1996; Hairston, 1987; King, 1980; Lees, 1955; Pourriot and Snell, 1983; Tauber *et al.*, 1986). A recent example of the ecological relevance of resting stages comes from observations on copepods and rotifers, where sediment cores from marine environments have uncovered viable embryos that have apparently withstood severe hypoxia or anoxia for 10–40 years; these forms are now thought to serve as "egg banks" for future generations (Marcus *et al.*, 1994). A report on diapausing eggs from freshwater copepods indicate that even longer survival times are possible (Hairston *et al.*, 1995).

Without metabolic energy conservation, survivorship during dormancy would be greatly reduced. General principles governing metabolic arrest are beginning to emerge (for reviews see Guppy *et al.*, 1994; Hand, 1991, 1993a, b; Hand and Hardewig, 1996; Hochachka and Guppy, 1987; Storey and Storey, 1990). Recent physiological studies have investigated cues associated with the termination of diapause (Drinkwater and Clegg, 1991; Drinkwater and Crowe, 1987, 1991; Lavens and Sorgeloos, 1987; van der Linden *et al.*, 1988) and have documented the metabolic transitions associated with this state (Clegg *et al.*, 1996). In this study, we wanted to establish a quantitative metabolic framework from which to ultimately investigate molecular mechanisms of diapause breakage. We employed microcalorimetry (Gnaiger, 1983a; Hand and Gnaiger, 1988) to assess overall energy flow in diapausing and post-diapause gemmules. Oxygen consumption was measured both simultaneously and independently of the calorimetric measurements. Finally, we measured ATP, ADP, and AMP in post-diapause gemmules to gain insight into the adenylate status during development.

In the accompanying paper (Loomis *et al.*, 1996), we address changes in carbohydrate levels during germination and potential enzymatic mechanisms for modulating these changes. Results of these studies could have general implications for organisms or tissues in which acute modulation of development and energy metabolism are known to occur. From an ecological perspective, the ongoing work may eventually provide a better understanding of how environmental stimuli, ones promoting entry into and exit from dormancy, are linked to the requisite physiological responses of invertebrates.

## Materials and Methods

Diapausing gemmules were collected from Stony Brook in South Hadley, Massachusetts, in late September of 1993 and maintained at 4°C until early October when calorimetry was performed (storage for 2 weeks at 4°C is insufficient time to break diapause). Post-diapause gemmules were collected from the same location in February of 1993 and maintained for an additional 3 months in the laboratory at 4°C prior to metabolic experiments. The hatching percentage of post-diapause gemmules was always greater than 95%.

### *Closed-system respirometry*

Experiments were initiated by filtering vernalized gemmules from the 4°C storage water and placing them into 20°C stream water that was continuously bubbled with air. Respiratory measurements were made at the indicated time intervals by transferring samples of gemmules to a sealed respiration vessel containing air-saturated stream water. Partial pressure of oxygen was measured using a model 5300 Yellow Springs Instruments biological oxygen monitor. Gemmules were continuously stirred through the 15-min measurement interval. The rate of oxygen consumption was linear down to 40% saturation, and during these experiments the oxygen level never dropped below 60% saturation.

### *Flow-through calorimetry and respirometry*

Heat dissipation and oxygen consumption of gemmules were measured simultaneously with an LKB 2277 thermal activity monitor coupled *via* stainless steel tubing to a twin-flow respirometer (Cyclobios, Innsbruck, Austria) (Gnaiger, 1983a). All experiments were performed at 21°C. At time zero, gemmules were filtered from the 4°C stream water, transferred to the calorimeter, and perfused at 15 ml/h with 21°C air-saturated stream water (<10 mOsm/kg $H_2O$; 0.22 $\mu$m filtered). The partial pressure of oxygen was above 60% air saturation on average in the excurrent flow after passage by the gemmule sample. One data point was sampled every 60 s and recorded with Datgraf acquisition software (Cyclobios; Innsbruck, Austria). After each run, the gemmules were removed and dried at 60°C to constant weight. Baselines were measured before and after each run, without gemmules in the perfusion chamber. For the experiment involving a bout of oxygen limitation, post-diapause gemmules were given aerobic perfusion for 21 h, and then the perfusion was switched to nitrogen-saturated stream water for 7.5 h. After the bout of oxygen limitation, gemmules were returned to aerobic perfusion to initiate a recovery period. One advantage of flow-through calorimetry for such experiments is the ease and speed with which oxygen availability can be manipulated. Although considerable effort is devoted to main-

taining a nominally oxygen-free status in the flow-through system (for methodological precautions employed, see Gnaiger 1983a; Hand and Gnaiger, 1988; Hand, 1990), evidence indicates that a sealed ampoule system, while experimentally cumbersome in terms of reversibility, may be slightly more effective in providing an anoxic environment (Hand, 1995).

The influence of antibiotics (50 mg/l each of penicillin and streptomycin) on the heat dissipation was evaluated by exposing gemmules for 5 h to perfusion with nitrogen-saturated medium and then switching to the same nitrogen-saturated medium containing antibiotics. The slope of the $\mu$W-*versus*-time curve before the switch to antibiotics was compared to the slope 4 h after the switch. The slopes were not significantly different ($P$ = 0.343, $n$ = 3, paired, two-tailed $t$ test). Similarly, the absolute $\mu$W values before and after the switch were not significantly different ($P$ = 0.258, $n$ = 3, paired, two-tailed $t$ test). Thus, antibiotics were not included in the water used for calorespirometry experiments.

*Adenylate measurements*

Post-diapause gemmules were incubated aerobically in stream water at 20°–23°C. At the indicated time points, triplicate samples were extracted with perchloric acid (Rees and Hand, 1991). Perchloric acid precipitates were resuspended in 0.5 $N$ NaOH and assayed for total protein with a modified Lowry assay (Peterson, 1977). The neutralized supernatants were analyzed for adenylates by high-performance liquid chromatography using weak anion exchange as previously described (Rees and Hand, 1991). Briefly, the separation column of amino propyl silica (250 × 4.6 mm, 5 $\mu$m particle size; Phenomenex, Torrance, CA) was eluted isocratically with a 2:1 mixture of acetonitrile and 60 m$M$ potassium phosphate buffer (pH 6.5). The potassium phosphate replaced the ammonium bicarbonate used by Rees and Hand (1991) in the mobile phase, which avoided the changes in composition caused by slow outgassing of $CO_2$ from the mixture. Adenylates were monitored at 254 nm and identified by co-elution with bona fide standards.

## Results

*Heat dissipation and oxygen consumption of gemmules*

A pronounced increase in heat dissipation of 5.8-fold is observed upon incubating post-diapause gemmules at 21°C (Fig. 1). In comparison, two distinct differences are seen with diapausing gemmules (Fig. 1). First, the absolute level of heat dissipation after 3.5 h of incubation at 21°C is 50% of that measured for the post-diapause gemmules at the same temperature. Thus, breakage of diapause apparently results in an increased heat flow. Second, the large increase in heat dissipation observed over time for the post-diapause condition is absent with the diapausing embryos. Even though the diapausing embryos are presented with optimal conditions for development and germination, they remain dormant (no morphological signs of germination were observed). These quantitative data illustrate the developmental and metabolic differences between these two states of diapause and post-diapause.

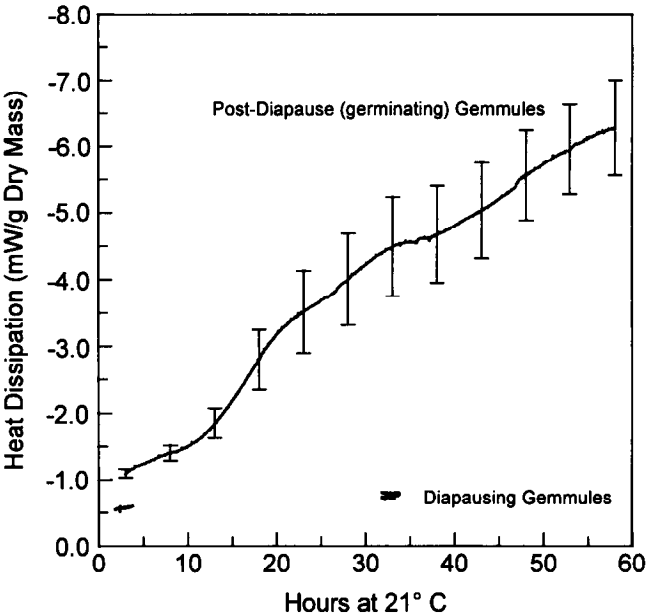


**Figure 1.** Calorimetric measurements of energy flow in germinating and diapausing gemmules of *Eunapius fragilis* at 21°C. Data for germinating gemmules are expressed as means of three independent experiments; bars represent one standard error of the mean at the time points indicated. Values for diapausing embryos are presented for two independent experiments at the time intervals shown.

A similar overall trend is observed when respiration rate is plotted as a function of developmental time (Fig. 2). As seen in the inset to Figure 2, closed-system respirometry provides results comparable to those obtained with the open-flow system. Figure 3 depicts the changes in the CR ratio during development of post-diapause embryos. The value increases significantly ($P$ = 0.023, unpaired, two-tailed $t$ test) from −354 ± 46 kJ/mol (±1 SE, $n$ = 3) to −541 ± 36 kJ/mol (±1 SE; $n$ = 4) between hours 3.5 and 56.5 of germination, with an average value of about −495 kJ/mol across this period. For comparison, the oxycaloric equivalent for carbohydrate catabolism under aerobic conditions is −478 kJ/mol $O_2$ (Gnaiger, 1983b, c). The CR value of −374 ± 21 (±1 SE;

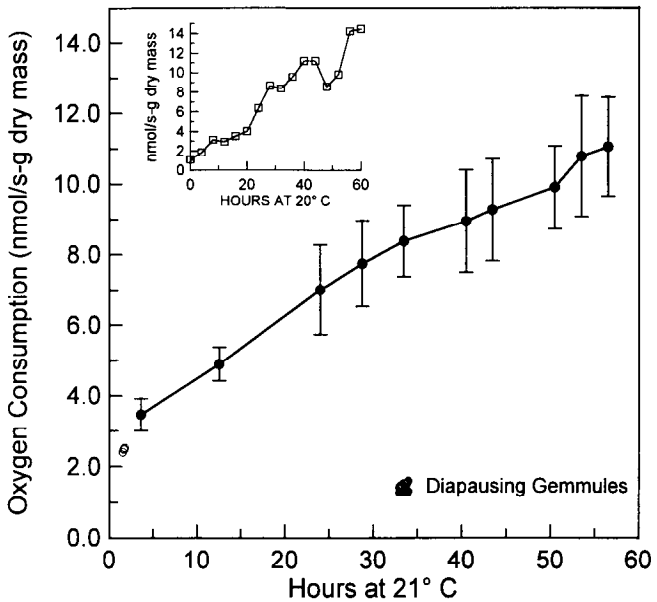


**Figure 2.** Oxygen consumption rates at 21°C, measured using the flow-through respiration system, for post-diapause and diapausing gemmules of *Eunapius fragilis* during germination. Values for post-diapause gemmules are means for 3-h intervals (except for a 1.25-h interval at the first time point), with the symbol placed at the mid-point of each time interval. Error bars represent ±1 SE for three independent experiments, except at hours 50.5 and 56.5, where $n = 4$. Values for diapausing embryos are presented for two independent batches of embryos at the time intervals shown. Inset: For comparison, respiration rates measured using closed-system respirometry at 20°C are presented for post-diapause gemmules; $n = 1$.

$n = 3$) determined at hour 12.5 is statistically different from the oxycaloric equivalent ($t_s = 4.95$). This lower C:R ratio near the beginning of the incubation could be attributed to gemmules that are undergoing recovery from hypoxia or anoxia. Because they were stored for months at 4°C without aeration, it is likely that these quiescent gemmules could have experienced oxygen limitation.

### *Tolerance of gemmules to oxygen limitation*

Figure 4 illustrates the effect of interrupting the aerobic germination of post-diapause gemmules with a 7.5-h bout of oxygen limitation. Heat flow is depressed to 6% of the aerobic rate. Upon reperfusion with air-saturated medium (at hour 28), heat flow increases, and in due course, new sponge tissue emerges from the collagenous capsule. Emergence is delayed by an increment of time equal to the bout of nitrogen perfusion (data not shown). Thus, oxygen limitation appears to halt development in addition to depressing heat dissipation. These observations are similar to those reported for embryos of the brine shrimp *Artemia franciscana* (Hand and Gnaiger, 1988; Hand, 1990). Even the biphasic profile seen with the gemmules during recovery (hours 28–30) is virtually identical to that observed with *A. franciscana* embryos.

### *Adenylate levels during germination*

Considerable variance was noted in the ATP levels of gemmules during the early stages of the germination process, as manifested in the large error bars over the first 20 h (Fig. 5). Overall, however, the mean values are relatively consistent across the entire period. There is a small decrease in ADP at hour 42 (Fig. 5) that results in a significant change ($P = 0.008$) in the ATP:ADP ratio when compared to hour 0 (Table I). AMP levels fluctuate between 0.5 and 0.8 nmol/mg protein (Fig. 5). The calculated adenylate energy charge was statistically unchanged over the 54 h of aerobic incubation (Table I). Even at the hour 0 time point (*i.e.*, immediately before transferring the post-diapause gemmules to room temperature), the adenylate pool is already well charged. The

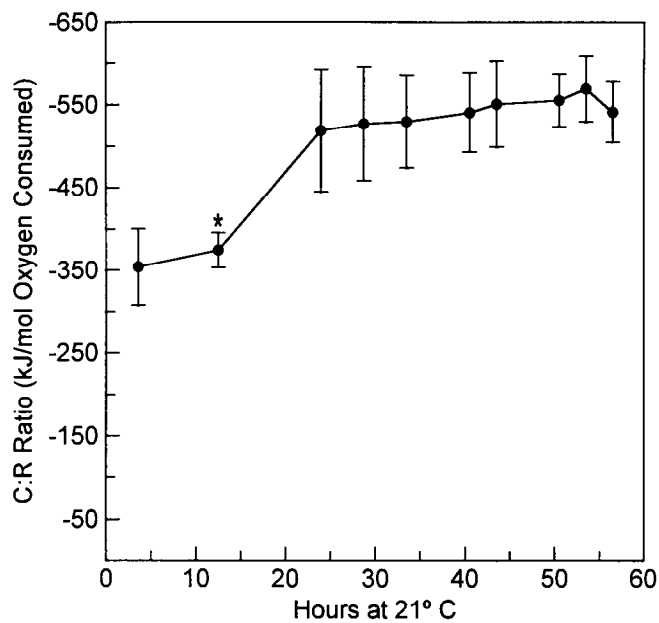


**Figure 3.** Calorimetric:respirometric (CR) ratios for post-diapause gemmules of *Eunapius fragilis* during germination at 21°C. Values are means for 3-h intervals (except for a 1.25-h interval at the first time point), with the symbol placed at the mid-point of each time interval. Error bars represent ±1 SE for three independent experiments, except at hours 50.5 and 56.5, where $n = 4$. Analysis of variance shows a significant increase in CR ratio between the first and last time points ($P = 0.023$; unpaired, two-tailed $t$ test). The asterisk denotes the only value significantly different ($t_s = 4.95$) from the theoretical oxycaloric equivalent for carbohydrate (−478 kJ/mol $O_2$), based on comparison of the experimental means and associated variances to the parametric value.

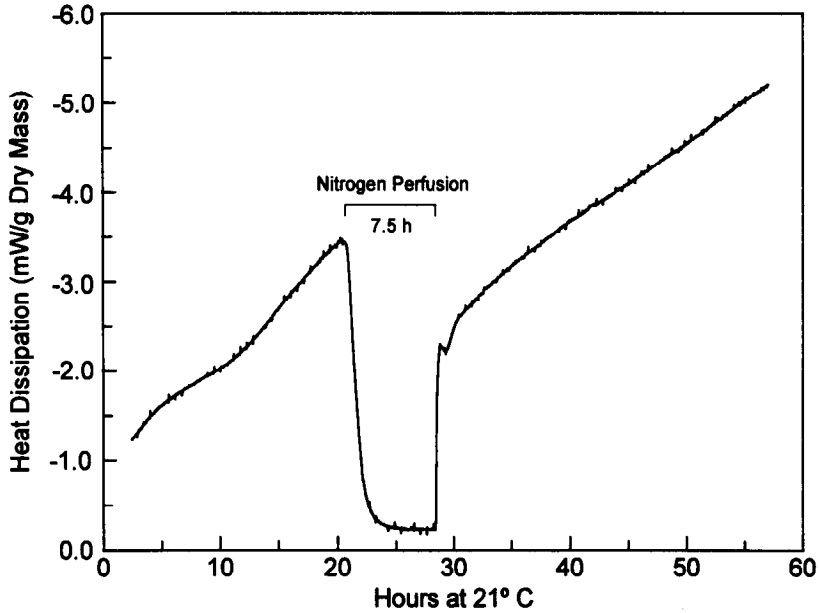


**Figure 4.** Interruption of heat dissipation during germination of *Eunapius fragilis* gemmules by perfusion with nitrogen-saturated medium (nominally oxygen free). The period of oxygen deprivation is indicated on the figure.

observation implies that adenylate status is probably not compromised by the diapause period prior to reinitiation of differentiation. Otherwise, one would expect to see a substantial rise in ATP levels during the early stages of germination.

## Discussion

Energy flows in gemmules of the freshwater sponge *Eunapius fragilis* differ between the states of diapause and post-diapause (germination). Diapausing gemmules exhibit an energy flow only 50% that of post-diapause gemmules at the beginning of germination, and this rate of heat flow is only 11% of that seen in post-diapausing gemmules at the point of cell emergence from the collagenous capsules. Thus, diapause is characterized by a substantial metabolic and developmental suppression. Nevertheless, there is still energy metabolism in this state of obligate (constitutive) dormancy when measured at 21°C. Presumably, the energy flow during diapause would be depressed further by the low temperatures naturally experienced during the overwintering state. Gas-

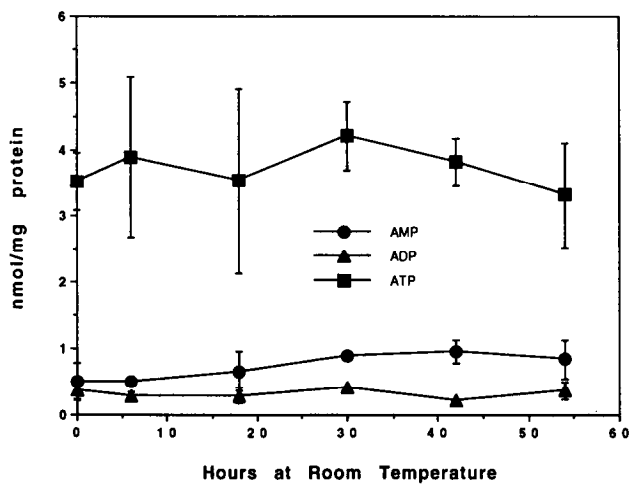


**Figure 5.** Chemical measurements of ATP, ADP, and AMP extracted from gemmules of *Eunapius fragilis* at various points during germination. Values are expressed as means ±1 SE ($n$ = 3).

**Table I**

*ATP:ADP ratios and adenylate energy charge in gemmules of* Eunapius fragilis *during germination*

| Time at room temperature (h) | ATP:ADP ratio[a] | Energy charge[a] |
|---|---|---|
| 0 | 6.91 ± 0.96 | 0.81 ± 0.02 |
| 6 | 15.12 ± 1.31 | 0.86 ± 0.01 |
| 18 | 12.15 ± 1.89 | 0.83 ± 0.01 |
| 30 | 10.28 ± 0.05 | 0.80 ± 0.01 |
| 42 | 17.55 ± 0.92* | 0.79 ± 0.01 |
| 54 | 9.86 ± 2.57 | 0.77 ± 0.05 |

[a] Values represent means ± 1 SE ($n$ = 3).

* Significantly different from hour 0 value ($P$ = 0.008), based on ANOVA with Bonferroni adjustments for multiple comparisons. No significant differences were found for the energy charge values.

trula-stage embryos of the brine shrimp *Artemia franciscana* exhibit an exponential decline in respiration rate as a function of time in diapause; after 5 days of diapause, respiration reached "barely detectable" levels (Clegg *et al.*, 1996). Considering that gemmules in this study had been in diapause for 2 weeks before the metabolic measurements, it seems that gemmules of *Eunapius fragilis* do not undergo as severe a metabolic depression as that reported for brine shrimp embryos.

Respiration rates of diapausing and post-diapause gemmules were compared by Rasmont (1962), who found that oxygen consumption increased threefold upon breakage of diapause (promoted by cold treatment of gemmules). These studies were conducted with gemmules of *Ephydatia muelleri,* which apparently do not exhibit as distinct a diapause state as do gemmules of *Eunapius fragilis.* The current evidence suggests that there is a metabolic activation that coincides with the resumption of development after diapause breakage and subsequent exposure to elevated temperatures.

Over the entire period of germination, the CR ratio for gemmules of *Eunapius fragilis* averaged −495 kJ/mol $O_2$, a value indistinguishable from the oxycaloric equivalent for aerobic metabolism of carbohydrate of −478 kJ/mol $O_2$. As noted previously, however, the CR ratio was significantly lower (−374 ± 21 ± 1 SE; $n = 3$) at hour 12.5 of the germination process (similarly low at hour 3.5), an observation that leads us to suspect that the gemmules were hypoxic prior to the aerobic incubation period of germination. Values significantly below the oxycaloric equivalent have been reported before for organisms recovering from anoxia. For the marine mussel *Mytilus edulis,* values as low as −210 kJ/mol $O_2$ have been observed during the first hour of recovery from anoxia (Shick *et al.*, 1986; Shick *et al.*, 1988). A small portion of the excess oxygen consumed (relative to heat liberated) can be attributed to reoxygenation of tissues, but the predominant cause of the low CR ratios is apparently the occurrence of anabolic, heat-conserving metabolism (Shick *et al.*, 1986; Shick *et al.*, 1988). The theoretical oxycaloric equivalent for gluconeogenic succinate clearance is estimated to be about −200 kJ/mol $O_2$, and the oxycaloric equivalent for the partial oxidation of succinate to malate or aspartate is projected to be quite low as well (reviewed by Shick *et al.*, 1988). Further support for this argument comes from the calculated CR ratio soon (2.5 to 3.5 h) after the short anoxic bout in Figure 4. The value was a low −363 kJ/mol $O_2$, compared to −438 kJ/mol $O_2$ immediatedly before the anoxic bout (hour 19.5–20.5). By 3.5–6.5 h post-anoxia, the CR ratio had risen to −457 kJ/mol $O_2$ and to −527 kJ/mol $O_2$ by 10.5–13.5 h post-anoxia.

Adenylate pools appear to be well charged in post-diapause gemmules even before warming to room temperature (hour 0). Preliminary data (not shown) suggest that ATP levels are also quite high in diapausing gemmules, and consequently, severe depletion of ATP is unlikely in either of these two metabolic states. Data are currently not available for anoxic gemmules, but our prediction is that ATP levels could be compromised under anoxia, similar to the situation observed for *Artemia* embryos, where ATP drops rapidly during oxygen limitation (Anchordoguy and Hand, 1994, 1995; Carpenter and Hand, 1986; Hand and Gnaiger, 1988; Rees *et al.*, 1989; Stocco *et al.*, 1972).

Post-diapause gemmules are tolerant of severe hypoxia or anoxia, at least for a period of 7.5 h. During nitrogen perfusion (nominally oxygen free), heat dissipation is reduced to 6% of the aerobic value. In comparison, after 50 h of oxygen deprivation, heat dissipation from post-diapause embryos of *Artemia franciscana* drops at least an order of magnitude and is still declining at that point (Hand, 1990, 1995; compare with Hontoria *et al.*, 1993). It is possible that such low values of heat dissipation in *Artemia* embryos reflect an ametabolic state, as defined by the absence of processes associated with ATP turnover. Upon return of gemmules to aerobic perfusion, heat dissipation climbs rapidly, and gemmules resume development and emerge. Exploration of biochemical and metabolic features underlying this tolerance to oxygen limitation and the capacity for long-term survivorship under anoxia is biologically relevant because the natural habitat of these gemmules during winter can include burial in pond sediments of low oxygen tension (S. H. Loomis and P. E. Fell, unpubl. observations).

The results from this study, in conjunction with those of the companion paper, provide a quantitative framework from which to explore molecular bases for diapause. The data represent the first characterization of the metabolic transitions in sponge gemmules that are capable of entering and exiting diapause.

## Acknowledgments

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