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Metabolic Dormancy in Aquatic Invertebrates

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Contents

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> We sat beside the little pool and watched the tree-frogs and the horsehair worms and the water-skaters, and had wondered how they got there, so far from water. It seemed to us that life in every form is incipiently everywhere waiting for a chance to take root and start reproducing; eggs, spores, seeds, bacilli – everywhere. Let a raindrop fall and it is crowded with the waiting life .... And we, seeing the desert country, the hot waterless expanse, and knowing how far away the nearest water must be, say with a kind of disbelief, "How did they get clear here, these little animals?" And until we can attack with our poor blunt weapon of reason that causal process and reduce it, we do not quite believe in the horsehair worms.....
>
> From *Sea of Cortez*. John Steinbeck and Edward Ricketts, 1941

1 Introduction

One major approach used by invertebrates for coping with extreme environmental conditions is to enter states of metabolic and developmental dormancy. The possession of a latent or resting stage is a common occurrence in the life cycle of organisms inhabiting inconsistent or ephemeral environments, and this phenomenon has been a source of long-standing speculation and fascination (cf. van Leeuwenhoek 1702). Considering the remarkable habitat diversity that exists among aquatic invertebrates, it is not surprising that the distribution of dormancy is broadly scattered phylogenetically. Resting stages have now been reported in virtually every major phylum of invertebrates, with the notable exception of Echinodermata. In some taxa, mature adult forms have the ability to enter states of rest, while in others, specialized resting forms are common only during earlier stages of life cycles.

Considerable effort on the part of numerous invertebrate zoologists has been directed toward describing the environmental conditions that induce dormancy, recording both the length and the seasonal timing of these bouts, and relating this information to the life history strategy of the organism. Only more recently have investigators begun tackling the physiological and biochemical mechanisms by which metabolic processes of invertebrates are suppressed and then later reactivated in response to environmental change. It is becoming clear that multiple solutions are employed by different species, and even by different tissues within a species. The purpose of this review is to relate the environmental and life history observations to the growing body of information about the mechanisms governing reversible bouts of dormancy. For the most part I will focus on aquatic invertebrates, the exception being pulmonate snails, where information about regulatory mechanisms will come from both aquatic and terrestrial forms. The voluminous data base on insect dormancy has been reviewed thoroughly in recent volumes (e.g., Tauber et al. 1986; Danks 1987) and will not be treated here.

1.1 Environmentally Induced Quiescence Versus Endogenous Diapause

As pointed out previously by other authors, the very term "dormancy" invites controversy (Bushnell 1974). Undoubtedly, a portion of the confusion arises from the fact that, in nature or in laboratory settings, the same organism may show a

gradation from a state of high metabolic and developmental activity, to states of torpor characterized by suppressed metabolism, to cryptobiosis where energy flow is essentially undetectable by conventional measures (cf. Keilin 1959). So the point at which one assigns the term dormant to describe a state of reduced metabolism involves a degree of subjectivity. In the cases for which data exist, energy flow during invertebrate dormancy is suppressed well below 20% of active values, and quite commonly below 5%. Still, a transient suppression of energy flow to this level is not, in and of itself, sufficient to define a state of dormancy. The potential must also exist for an extended duration of the hypometabolic state, i.e., on the order of weeks, months or years. Normally, arrest of developmental processes accompanies this protracted metabolic suppression, particularly when embryonic or immature stages are involved.

Consistent with the terminology of Keilin (1959) and Mansingh (1971), *dormancy* is a general term that includes virtually all resting states. It is important to distinguish two major categories of dormancy. The first is *quiescence*, which is a metabolic and/or developmental arrest imposed by an unfavorable environmental condition – desiccation, anoxia, temperature extremes, etc. Included here are states like estivation, hibernation, anaerobic dormancy, and anhydrobiosis. Note that this form of dormancy (variously described as environmental, facultative, or exogenous) is controlled simply by application or removal of the relevant physical insult. The second major category of dormancy is *diapause*, a state which also results in metabolic and/or developmental arrest. However, the key difference here is that diapause (obligate, endogenous, constitutive dormancy) is controlled by some type of endogenous physiological factor. In other words, diapausing invertebrates will remain dormant even when exposed to physical conditions that would otherwise promote normal metabolism and development. The release from diapause (activation, diapause breakage) requires exposure to a specific stimulus or cue, or combination of cues. Only after such exposure will development and active metabolism return. It is not unusual for the same organism to be capable of entering both quiescent and diapausing states.

1.2 Biological Rationale

In the broadest sense, arrested states in the life cycles of aquatic invertebrates provide an important means to survive deleterious environmental conditions. Livdahl (1979) presented a mathematical model that predicted an increased selection for life cycle delays in opportunistic species living in ephemeral environments. His model considered a hypothetical population that underwent larval development in an environment which occasionally became totally unsuitable for normal development, such as a temporary pool or water-filled tree hole. The more catastrophic the environment, the stronger the predicted selection for embryos with prolonged durations of dormancy.

The biological advantages of "environmental escape through dormancy" (Pourriot and Snell 1983) include the recolonization of an environment at a later time and, particularly in the case of resting eggs, transport and dissemination of the species to new locations (King 1980; Elgmork 1980). Thus, such resting stages could

serve to synchronize favorable environmental conditions with the actively feeding or growing stage of the organism (Crowe and Clegg 1973). In parasitic species, a cyst or similar propagule could represent an obligate stage for the transmission to a specific host organism. For other organisms, production of encysted forms may represent a process that allows a change between asexual and sexual reproduction (see below), or for the avoidance of predators (Hairston 1987).

2 Phylogenetic Survey of Dormancy and Associated Environmental Cues

2.1 Unicellular Organisms

Before considering multicellular invertebrates, it is appropriate to briefly note that numerous unicellular species exhibit quiescent stages in their life cycles. The occurrence of dormancy in protozoa and freshwater algae has been reviewed by Corliss and Esser (1974) and Schlichting (1974), respectively. More recently, Kirk and Kirk (1986) have examined the mechanism which regulates the change in *Volvox carteri* from asexual reproduction in temporary ponds in the spring to the sexual reproduction of dormant, overwintering zygotes as the ponds dry up in the summer heat. Heat shock of this chromoplast-containing, colonial mastigophoran by exposure to temperatures above 40 °C elicited the production of a sexual inducer. This 30 kDa glycoprotein is one of the most potent biological effectors known. It is effective at 6×10^{-17} M, and one individual can release enough inducer to convert all related males and females in a volume of 1000 liters from asexual to sexual reproduction (Gilles et al. 1984).

Female spheroids of this species normally reproduce by asexual reproduction. However, heat shock causes somatic cells of both asexual males and asexual females to make and release the glycoprotein inducer. The glycoprotein then promotes the production of egg-bearing sexual daughters, leading to the eventual formation of dormant zygotes. Importantly, washing away the inducer from heat-shocked individuals markedly reduced the numbers of sexual progeny, as did the addition of concanavalin A (a lectin which binds to glycoproteins) and the addition of anti-inducer antibody to the medium. These results clearly indicated that the production of sexual progeny was attributable to autoinduction by the released inducer and was not related to activation of inducer-independent sexual development by the heat shock treatment. Thus, for these organisms which inhabit vernal pools in nature, the heat shock caused by warming of soon-to-be-dry shallow ponds would lead to the production of drought-resistant zygospores – an effective adaptation for surviving an ephemeral environment.

2.2 Sponges

Both freshwater and marine members of the phylum Porifera can produce dormant structures called gemmules in response to stressful environmental conditions (cold

temperatures, drought). Gemmules are asexual reproductive bodies composed of aggregations of nutrient-rich, undifferentiated cells (thesocytes) surrounded by a complex collagenous capsule. Under favorable conditions, these structures germinate and hatch, giving rise to an active sponge. Thorough studies have been published describing gemmule morphology and the changes in structure that occur during gemmule formation, germination, and subsequent development (for earlier studies see review by Simpson and Fell 1974; Rozenfeld and Rasmont 1976; Hohr 1977; Langenbruch 1981, 1982; Harrison and Davis 1982; Harrison and Cowden 1983; Jetton et al. 1987). Depending upon the species, thesocytes can be either mononucleate or binucleate. The cells have the normal complement of cellular organelles and additionally have numerous vitelline platelets. The platelets have membranous-like, concentric laminae surrounding them (e.g. Simpson and Fell 1974) as well as internal structures identified as mitochondria (DeVos 1971). Both of these characteristics of gemmule platelets are quite similar to those described for yolk platelets in embryos of the brine shrimp *Artemia* (Vallejo et al. 1979; Marco et al. 1980; Hofmann and Hand 1989). Another morphological feature of some species, which most likely influences the functional properties of the thesocytes, is the presence of intracellular zoochlorellae (Bronsted and Lovtrup 1953; Tessenow 1969; Gilbert and Allen 1973; Simpson and Gilbert 1973). This observation may not be particularly surprising, since mature freshwater sponges are known to contain these algal symbionts as well (e.g., Muscatine et al. 1967). The algal-containing gemmules have higher respiration rates in the light than do gemmules without algae (Bronsted and Lovtrup 1953). However, even the respiration of gemmules lacking symbionts seems to be directly influenced by light (Rasmont and Schmidt 1967).

Both quiescence and diapause have been described for sponge gemmules, and considerable effort has been directed at determining the environmental cues that elicit gemmule formation and initiate the resumption of development (Simpson and Fell 1974; Fell 1974, 1975; Benfey and Reiswig 1982; Bazer and Fell 1986; Fell 1987a,b). Large variation within and among species has been noted in the conditions conducive to gemmule production. For example, in the marine species *Haliclona loosanoffi*, adult sponges in southern New England degenerate in the fall, and only the gemmules survive winter temperatures which sometimes can drop below 0 °C. In contrast, gemmule formation by this same species in North Carolina is observed only in the warm summer months (Fell 1974 and references therein). Variation is also seen in resistance to dehydration. Gemmules of marine sponges are not particularly tolerant of desiccation (Fell 1975), but those of certain freshwater sponges are produced specifically in response to receding water levels in Louisiana swamps (Poirrier 1969). Temperature, photoperiod, and pH of the incubation medium have all been noted as important variable in promoting gemmule hatching, and marked variation even at the interpopulational level has been observed in response to all three variables (e.g., in the freshwater species *Ephydatia mulleri*; Benfey and Reiswig 1982).

A few reports exist regarding the metabolism of gemmules, and information is slowly accumulating about the potential mechanisms that regulate gemmule formation and their release from dormancy. Respiration rates of diapausing and nondiapausing gemmules were compared by Rasmont (1962), who found that oxygen consumption increased threefold upon breakage of dormancy (promoted by

cold treatment of diapausing gemmules). Similarly, the catalytic activity of an acid phosphatase showed a pronounced increase upon cold treatment and germination (Tessenow 1969). Thus, the limited evidence available suggests that there is a metabolic activation in addition to the resumption of development upon diapause breakage.

Some years ago Rasmont (1962, 1963, 1965) suggested the possibility that during quiescence in *Ephydatia fluviatilis* (which do not exhibit true diapause), germination is inhibited by a substance produced by the parent sponge which was termed gemmulostasin. Several observations, including some made by Rozenfeld (1970, 1971), supported the hypothesis. Briefly, when gemmules resided within the adult tissues, they were prevented from hatching presumably by gemmulostasin produced by the adult. Artificially removing the gemmules from the adult tissue promoted hatching unless the gemmules were subsequently maintained at low temperature. Maintaining gemmules in a medium which had been previously used for high-density rearing of adults also prevented the isolated gemmules from hatching. Cytological studies showed that gemmulostasin reversibly inhibited the development of gemmules at an early stage, prior to the first mitoses (Rozenfeld 1970). The agent also inhibited the incorporation of tritiated thymidine into DNA (Rozenfeld 1974). Even after 25 years, gemmulostasin still has not been isolated and chemically characterized, but some additional interpretations and information have been published that may be of relevance.

Building on the original observation of Zeuthen (1939), which indicated that osmotic pressure inside the gemmule decreased immediately prior to germination, Simpson et al. (1973) demonstrated that elevating the concentration of sodium chloride in the medium (to 23 mOsmolar) reversibly prevented the hatching of gemmules. Consequently, Simpson and Fell (1974) suggested that the "gemmulostasin effect" could be explained simply as an increase in ionic strength of the rearing medium rather than by a specific physiological effector molecule. Their proposal is difficult to evaluate, however, in the absence of more detailed information about the ionic permeability of gemmules. Further, the manner in which the relatively low ionic strengths could influence marine gemmules is not clear.

More recently, Simpson and Rodan (1976) reported that the concentration of 3′5′-cyclic AMP (cAMP) decreased during the first 2 h of germination in gemmules of *Spongilla lacustris* (promoted by raising the temperature of these cold, presumably quiescent embryos), and that inhibition of gemmule phosphodiesterase by aminophylline prevented both the decline in cAMP and the gemmule germination. Importantly, the germination was blocked prior to nuclear separation and cell division of the binucleate thesocytes (the same point at which gemmulostasin arrested germination). It would be interesting to see if the inhibitory influence of cAMP on mitosis and gemmule hatching extended to metabolic events in quiescent embryos. It also would be helpful to perform this same series of experiments with gemmules from a species in which there is a more clear cut delineation between quiescence and diapause (e.g., with *Eunapius fragilis* which undergoes true diapause, Fell 1987a; or with *E. fluviatilis* which shows only quiescence, Rasmont 1963, 1965).

Ostrom and Simpson (1978) concluded that calcium ion may also have a role in the germination of quiescent embryos of *S. lacustris*, based on the indirect obser-

vation that calcium can reverse the inhibition of germination at 15 °C caused by other divalent cations. The calcium chelator ethylene glycol bis(beta-aminoethyl ether)N,N-tetraacetic acid (EGTA) actually stimulated germination of the gemmules at 4 °C after 20 days, which is rather perplexing. It is also disconcerting that these presumably quiescent embryos hatched without any EGTA treatment at 4 °C if given a 34-day incubation.

Finally, gemmules from several species of sponge have clearly been identified as undergoing true diapause (e.g., *E. mulleri*, Rasmont 1962; and *E. fragilis*, as stated above). Recent work by Fell (1987a,b) demonstrated a very clear synergistic effect of cold and desiccation in diapause breakage. To my knowledge, however, there is currently no information available regarding the biochemical mechanisms involved in release from this dormant state.

2.3 Cnidarians

The occurrence of dormant stages among the cnidaria is not widespread. The most studied example is the production of podocysts by the scyphistomae (polyps) of scyphozoan jellyfish. Podocysts are basal buds that can remain in a dormant state for up to several years (Herouard 1911; Black and Proud 1976). The cellular mass is composed of peripheral cells and amoebocytes, the latter of which are heavily laden with yolk (Chapman 1968). Mitochondria are primarily restricted to the peripheral cells (Chapman 1968). These cells are enclosed in an outer cuticle that is primarily composed of protein and chitin (Blanquet 1972; Chapman 1968) at a ratio of 10:1 (Black 1981). The environmental conditions that promote the formation or the hatching of podocysts has not been well characterized. In the laboratory, the physical removal of the cuticle stimulates the incorporation of amino acids by cysts and the resumption of development leading to the formation of a new polyp (Black and Proud 1976).

The majority of available information about podocyst metabolism comes from a study by Black (1981), who measured the respiration rate, utilization of energy stores, and anoxia tolerance of podocysts formed by polyps of the brackish water medusa *Chrysaora quinquecirrha*. Respiration rate (microliters O_2 h^{-1} per 1000 cysts) decreased from 1.6 ± 0.3 (SD) after 1–3 months dormancy to 1.1 ± 0.3 after 11–13 months. Protein specific oxygen consumption of podocysts was one-fourth that of recently fed scyphistomae. One hour after opening the cuticle (which reinitiates development), respiration rate increased approximately threefold. Viability of the podocysts was not compromised by a 2-day exposure to anoxia at 25 °C, but no cysts (out of 300) survived a 1-month bout of anoxia. Polysaccharide content of the cysts was low and judged not to be of importance as a metabolic fuel during dormancy. In contrast, one-third of cyst protein and one-fifth of the lipid was depleted in 11–13-month cysts compared to 1–3-month cysts. The most unusual biochemical observation was a 50% drop in total DNA content over this period. Ultrastructural observations by Black (1981) led him to conclude that the outer peripheral cells were utilizing the yolk-rich amoebocytes located in the center of the cyst for nutrition. The apparent depletion of nuclei in the central region of the cyst was consistent with the DNA measurements. These conclusions were similar to those of Chapman (1968),

who concluded that peripheral cells were phagocytizing the yolk-rich amoebocytes during dormancy. Black suggested that this strategy for long-term survival during dormancy may be unique among aquatic metazoans. I am unaware of any information to the contrary.

2.4 Turbellarians

Freshwater flatworms often produce two types of eggs (embryos), depending on the season and environmental factors, primarily temperature (Heitkamp 1977). Subitaneous eggs are produced in the warm summer months, develop within the parental uteri, and hatch within a short period of time. Autoradiographic studies indicate that subitaneous eggs receive nutrients from the parent during their development (Gremigni and Domenici 1977). Dormant or resting eggs are produced in the autumn and are usually released into the water at the time of death of the parent (Domenici and Gremigni 1977). The resting egg can tolerate desiccation and cold temperatures over the winter and then hatches at the next warm season. The capsules surrounding these two types of turbellarian eggs differ markedly in thickness, biochemical composition, and ultrastructural features (Domenici and Gremigni 1977). In the case of *Mesostoma ehrenbergii* a single adult can produce about 10–20 subitaneous eggs and about 30–60 resting eggs in a given season (Heitkamp 1977). In addition to the free-living species above, dormant eggs are also produced by symbiotic species inhabiting the intestine of sea urchins (Shinn 1983).

Based on the work of Heitkamp (1977), resting eggs from *M. ehrenbergii* apparently exist in a state of diapause. An obligate period of dormancy is observed, which can be broken by exposure to low temperatures for an extended period (optimally, 90 days at 3.5 °C). Mechanisms governing the entry and exit from this dormant state have not been investigated.

2.5 Aschelminths

One of the most striking examples of metabolic dormancy – anhydrobiosis, or life without water – is a commonly exhibited feature in both embryonic and adults stages of this group of invertebrates. While desiccation-tolerant resting eggs are known to occur in freshwater gastrotrichs, the majority of existing information on aschelminth dormancy comes from rotifers and nematodes. Tardigrades share several diagnostic features with the aschelminths and eventually may be included formally in this assemblage, but the phylogenetic status of this group is far from resolved; the decision here to discuss "water bears" along with the aschelminths is primarily a matter of convenience to facilitate comparisons.

Both major groups of rotifers, the Bdelloidea and the Monogononta, exhibit dormant states. As emphasized in the useful review by Gilbert (1974), dormancy is predominantly a feature of adult stages in bdelloid rotifers, but pertains to resting eggs in monogonont species. Due to the absence of males among bdelloid species, these rotifers reproduce only parthenogenetically, and resting eggs are uncommon. Considering the absence of overwintering eggs, one might suspect bdelloid rotifers

to preferentially inhabit temporally stable bodies of water located in warmer climates. In contrast, these rotifers are commonly found in habitats subjected to desiccation and freezing on a frequent basis – lakes and ponds in polar regions, and mosses lichens and soils of different types (Gilbert 1974). Survival under these conditions is a result of the remarkable ability of adult females to withstand desiccation and freezing temperatures. The available literature on this topic was reviewed by Gilbert (1974), and in the intervening 15 years, there still exists a scarcity of controlled physiological and biochemical studies addressing anhydrobiosis and freeze tolerance in rotifers. For example, as related to dehydration, the quantitative limits on cellular water removal and the nature of the internal osmolyte systems have not been identified. Whether or not the tolerance to subfreezing temperatures is a result of supercooling due to antifreeze compounds, extracellular ice formation promoted by ice-nucleating agents, or other (as yet unidentified) protective mechanisms is not known. One could speculate that parallels might exist with mechanisms operative in tardigrades and nematodes, but at present there is no experimental basis from which to judge.

Adults stages of most monogonont rotifers apparently are unable to tolerate desiccation or exposure to freezing temperatures (Gilbert 1974). While diploid parthenogenesis (amictic reproduction) is common, this group also has the capacity for bisexual (mictic) reproduction, a process which results in the formation of encysted embryos. Wurdak et al. (1978) have described the fine structure of the encysted embryos from two monogonont species. It is the mictic (resting) eggs that provide monogonont populations the ability to survive environmental extremes. In addition to endogenous factors like age and genotype of the parental female, environmental conditions which favor the production of mictic females include photoperiod, population density, and diet (see citations in Pourriot and Snell 1983; Pourriot et al. 1986). For some species, ingestion of food like algae and algivorous zooplankton that contains vitamin E (tocopherol) is a potent stimulus for causing amictic females to produce a high proportion of mictic daughters (Gilbert and Thompson 1968; Gilbert and Litton 1978). Ingestion of prey containing as little as 0.02 picogram of vitamin E was sufficient for eliciting this effect in *Asplanchna brightwelli* (Gilbert 1983). The mechanism for the vitamin E effect is not understood, but tocopherol is known to stimulate the formation of various reproductive structures and processes in arthropods (cf. citations in Gilbert and Thompson 1968).

Available evidence indicates that resting eggs of rotifers exist in a true state of diapause, although the minimum length of obligate dormancy varies widely among species (3 to 90 days) (Pourriot and Snell 1983). Activation can be accomplished by exposing eggs to various combinations of light, low temperature, and salinity (e.g., Minkoff et al. 1983; Pourriot et al. 1981). Like adult stages of bdelloid rotifers, monogonont resting eggs can withstand desiccation, and to a lesser degree, exposure to freezing conditions (Bogoslavsky 1963; Ito 1960, as interpreted from the Russian and Japanese by Gilbert 1974). Drying for up to 12 months is tolerated by some species of *Brachionus*. Sharp declines in hatching were noted for embryos exposed for a few days to –5 °C.

Members of the phylum Tardigrada can be viewed as primarily aquatic, but the exceptional abilities of adult water bears to withstand desiccation have allowed habitation of increasingly xeric environments (Wright 1989a). Thus, in addition to

freshwater and marine species, tardigrades also live in the surface water films of terrestrial mosses, lichens, and forest litter. Some freshwater species produce thin-shelled summer eggs and thick-shelled, presumably dormant, eggs (Barnes 1980). Little information is available on these resting eggs, and consequently our attention will focus on anhydrobiosis in adult tardigrades.

In order to appreciate the temporal interplay between progressive dehydration on the one hand, and the requisite biochemical and physiological events permitting successful anhydrobiosis on the other, it is appropriate to consider first the mechanisms governing rates of water loss in tardigrades as well as nematodes. From a qualitative standpoint, it has been known for some time that many tardigrades require slow drying in order to survive dehydration (Broca 1860). Crowe and colleagues assessed this issue quantitatively by measuring the water content and survivorship of the tardigrade *Macrobiotus areolatus* under various regimes of fast and slow drying (Crowe 1972, 1975; Crowe and Madin 1974). When animals were dried in relative humidities between 70 and 95%, evaporative water loss resulted in the lowering of tardigrade water content from 80–90% to 50–60% in the first hour, and to 10–25% over the next 4 days. Returning these animals to water promoted revival of over 90% of the individuals. In contrast, dehydrating the tardigrades at humidities below 60% caused a rapid rate of water loss; for example, at 0% humidity water content reached 2–3% in 15 min. No animals survived this latter treatment. A key observation from these studies of Crowe was that tardigrades apparently had the ability to control their own rates of water loss if dehydration occurred in humidities above 70%. Further, the rate of water loss from tardigrades under these conditions was biphasic – there was an initial rapid loss followed by a much slower rate of transpiration.

One factor that influenced the rate of water loss, particularly in the early phase of tardigrade desiccation, was a change in the morphology of the animal (Crowe 1972). Anterior-posterior contraction resulted in the formation of a "tun" (Tönnchenstadien, Baumann 1922; Crowe et al. 1971; Walz 1979). If animals are anesthetized prior to desiccation so that tun formation was prevented, water loss was two to three times faster during the first hour at 80% relative humidity. Crowe attributed the slower rate of water loss observed in the tun conformation to the fact that intersegmental areas of high permeability were withdrawn into the body and removed from contact with the air. The degree to which the tun is effective in slowing the rate of water loss differs between the Eutardigrada and the Heterotardigrada, due to the relative degree of contraction experienced during tun formation (Wright 1989a).

In addition to tun formation in the early stage of desiccation, Crowe (1975) postulated that there might be changes occurring in cuticular lipids that could result in the formation of a permeability barrier – a barrier which could be important in explaining the reduction in water loss in the latter stages of dehydration. Until recently, this hypothesis had not been tested experimentally. In an illuminating series of papers, Wright explored the contribution of cuticular lipids in reducing tardigrade permeability during the latter stage of dehydration – a period referred to by Wright as the "permeability slump" (Wright 1988a,b; 1989a,b). The permeability slump is not a metabolic phenomenon or a result of tun formation because this pronounced decline in water permeability was seen in both live and dead specimens

(Wright 1989a). The permeability slump began after several minutes of dehydration and resulted in a 100-fold decrease in the rate of water loss (Wright 1989a). Wright (1988a, 1989b) extended the existing information on the tardigrade cuticle (e.g., Crowe et al. 1970, 1971; Baccetti and Rosati 1971; Greven 1983; Greven and Peters 1986) to show that cuticular lipids underwent distinct morphological changes during dehydration and that these changes were functionally related to the permeability slump. Identification and localization of the cuticular lipids was accomplished by osmicating tardigrade cuticles before and after lipid extraction and then viewing with transmission electron microscopy (Wright 1988a). Observing dehydrated tuns with cryo-SEM revealed the presence of dense aggregations in the surface of the cuticle. These surface masses were osmiophilic under SEM and could be removed by chloroform treatment. Thus Wright (1988a) suggested that the extruded masses represented lipid and that these lipid plugs could block pores in the tardigrade cuticle. These hypothetical pores could be sites of extensive water loss and their occlusion could substantially reduce cuticular permeability.

Of particular importance was the observation that treating tardigrades with hot chloroform prior to desiccation markedly diminished the permeability slump when compared to either untreated, heat-killed or hot-water-extracted tardigrades (Fig. 1) (Wright 1989b). GC-MS analyses of the chloroform wash revealed substantial quantities of lipid, predominantly straight-chain fatty acids. These data in combination with lanthanum tracer studies (Wright 1989b) were taken as strong support that changes in intracuticular lipids were responsible for the permeability slump. Across various tardigrade species, the positive correlation between the thickness of the intracuticular lipid layer and the rapidity of induction of the permeability slump was taken as further evidence for this conclusion (Wright 1988b).

Water relations during the induction of anhydrobiosis in nematodes also have received considerable investigation (see reviews by Ellenby 1969; Simons 1973; Crowe and Madin 1974; Womersley 1981, 1987). As in the case of tardigrades, dormant states have been documented for both immature and mature forms. For example, diapausing eggs of the nematode *Nematodirus battus* withstand wide

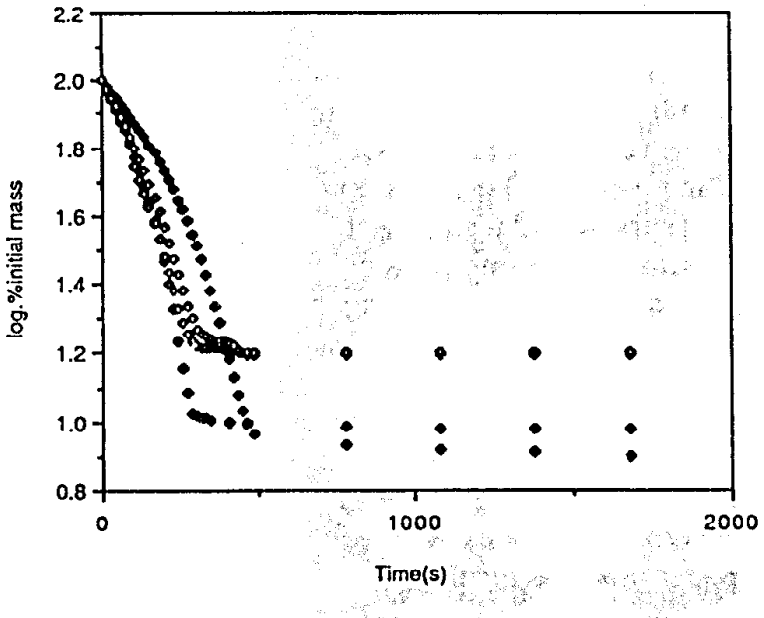

Fig. 1. A comparison of desiccation curves for 25-h chloroform-treated specimens (*lower pair of traces*) and dead specimens (*upper pair of traces*) of the tardigrade *Macrobiotus richtersi*. Note the much greater drop in water content (mass) for the chloroform-extracted organisms, i.e., the "permeability slump" has been eliminated. Dead (control) animals were briefly heated, and the results obtained with this treatment were very similar to those obtained with living tardigrades. (Wright 1989b)

fluctuations in temperature and moisture and remain dormant until they are exposed to low temperatures (Ash and Atkinson 1983). Most investigative efforts into anhydrobiosis have centered on larval, preadult, and adult stages.

As can be inferred from the above discussion regarding tardigrades, the conditions under which desiccation occurs, and the resulting water loss rates, are critical to the survival of anhydrobiotes. This premise has been strongly emphasized for nematodes by Womersley (1981, 1987), who pointed out that successful induction of anhydrobiosis in the laboratory may require careful simulation of conditions existing in the organism's natural habitat. For the slow-drying strategist, *Rotylenchulus reniformis*, Womersley and Ching (1989) have shown that the larval and preadult stages of this nematode could not survive even short-term exposure to 97% relative humidity. Rather, successful anhydrobiosis required dehydration in model substrates that mimicked the natural rate of soil moisture loss. Maximum coiling of *R. reniformis* required 10–12 days. Coiling of nematodes has been identified previously as an important behavioral change that can retard rates of water loss (e.g., Crowe and Madin 1974; Demeure et al. 1979). Similar to the changes in cuticular permeability observed for tardigrades, dehydration-dependent decreases in cuticular transpiration in nematodes have been documented by Ellenby (1968) and Perry (1977). However, Crowe and Madin (1974) concluded that nematodes were not as proficient at regulating their rate of evaporative water loss as the tardigrades, which may partly explain why it appears to be so crucial that natural conditions be simulated during dehydration of nematodes.

It is appropriate to note, however, that all nematodes do not require slow drying, and the adaptations allowing rapid drying during induction could be of interest (see discussion in Womersley 1987). Since there is a substantial body of information on the physiological and biochemical events accompanying anhydrobiosis in nematodes, discussion of these topics will be addressed separately in Section 5.

2.6 Molluscs

Particularly among the freshwater and terrestrial taxa, the capacity for estivation and hibernation has figured prominently in the expansion of geographical ranges and in the dynamics of evolution among the Mollusca (Boss 1974). Literally hundreds of examples exist that document quiescence in bivalves and gastropods (for an extensive review, see Boss 1974), and some of the cases are quite spectacular. The report by Dance (1958) described an African freshwater bivalve (*Aspatharia*) that survived 12 months out of water and had been collected protruding from dried stream beds among grass tufts. The pulmonate snail *Oxystyla pulchella*, the alleged Rip van Winkle of estivating snails, reportedly survived 23 years of dormancy (Baker 1934). From a physiological standpoint, however, it is frustrating that so few species have received detailed analysis and documentation. What is known about quiescence in molluscs comes from a trivial percentage of the total cases.

Anecdotal information has existed for some time suggesting that the tiny fingernail clam, which often inhabits vernal pools, lakes, and temporary streams, can survive period of extreme cold, drought, and excessive heat. A series of informative papers are now available that address the life history tactics, desiccation resistance,

and respiratory adaptations of these freshwater pisidiid (sphaeriid) clams (Way et al. 1980, 1981; McKee and Mackie 1980, 1981, 1983). Work with the species *Sphaerium occidentale*, *Musculium securis*, and *M. partumeium* indicates that growth and reproduction occur primarily in the early spring when snowmelt fills the temporary ponds, and sometimes in the fall due to water accumulation from rainfall. For much of the summer the ponds are dry, and in the winter the clams typically survive under ice.

These species are ovoviviparous and brood their young. A stochastic theory has been offered to explain the life-history traits of these clams relative to the ephemeral, variable environment in which they live (Way et al. 1980; McKee and Mackie 1981; Stearns 1977). Remarkably, the embryos and larvae continue to develop inside the tightly adducted valves of the adults even through prolonged bouts of parental estivation in the summer (McKee and Mackie 1981). Marine intertidal bivalves are known to gape during aerial exposure (e.g., Widdows et al. 1979; Shick et al. 1986), but the desiccation brought about by such behavior presumably could be lethal to estivating fingernail clams, due to the much longer periods without water (McKee and Mackie 1983). Thus, the question of oxygen availability during estivation is of interest. Fingernail clams are noted for having small perforations through the calcareous layers of their shells, resulting in a peppered appearance due to the density of punctae on the surface (Collins 1967; Mackie 1977). One obvious possibility suggested by McKee and Mackie (1983) was that oxygen could diffuse through these passages when valves were closed during estivation, thereby supporting some degree of aerobic metabolism. The oxygen and water permeability characteristics of these shell perforations have not been studied.

In the laboratory, estivating specimens of *Sphaerium occidentale* could survive aerial exposure in 100% relative humidity for at least 48 days at 20 °C (McKee and Mackie 1980). Survival was extended by incubation in moist soils; no mortality was observed over 80 days when clams were kept in soil with a 20–30% moisture content (Fig. 2). Estivating clams were at least ten times more tolerant to these conditions than nonestivating clams, which suggested that metabolic adjustments most likely occurred in estivating specimens. Clams that were allowed to enter estivation in the field were brought into the laboratory and their respiration rates measured at field temperature (McKee and Mackie 1983). Even though clams were typically placed in water for 15 h prior to measuring oxygen consumption (which compromised the metabolic quiescence to some degree), a clear depression in respiration rate was documented during estivation. In one experiment where measurements were taken on estivating clams immediately after submergence in water, respiration rate at 25 °C was 0.132 ± 0.140 microliters O_2 mg^{-1} dry mass h^{-1} compared to 0.733 ± 0.441 (SD) for nonestivating clams. The relative contributions of aerobic and anaerobic metabolism during estivation in these clams was far from being conclusively resolved. McKee and Mackie (1983) reported that estivating specimens exposed to 1 week of anoxia in moist soil exhibited 60 to 100% survival, depending on the species.

Both freshwater and marine prosobranch gastropods have been observed to enter estivation and hibernation (cf. Boss 1974), and the amphibious Indian apple snail *Pila globosa* is the species from this group that has received the most study. Ramanan (1903) reported that this snail burrowed up to 4 feet into dried lake beds

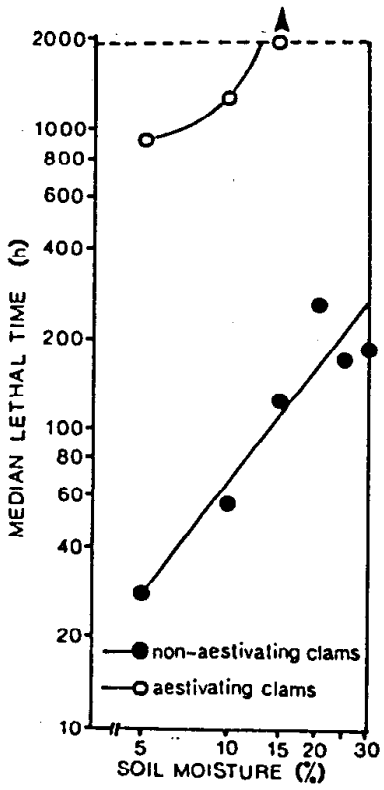

Fig. 2. Mortality curves for active specimens of the fingernail clam *Sphaerium occidentale* collected from a flooded pond and for estivating *S. occidentale* collected from a dry pond. Clams were placed in soils of varying moisture content at 20 °C. (McKee and Mackie 1980)

in India during the warmest months. However, a more recent and complete study by Haniffa (1978) documented that the majority of *P. globosa* buried themselves between 15 and 30 cm depending on the month in which estivation occurred. Based on Haniffa's data, the maximum number of snails estivating during any given month was 26% of the total population. As would be expected, the percentage of snails estivating increased as the pond slowly dried. The pond always retained at least some water year round, but the decreasing dissolved oxygen in the water during the warmer months apparently encouraged snails to leave the pond and burrow into the exposed mud flats. Survivorship of dormant snails depended upon the length of estivation as well as their depth in the sediment (Fig. 3). The increase in soil moisture and the decrease in soil temperature with increasing depth were clearly important in survival.

It is studies of the pulmonate snails, including freshwater species (Basommatophora) and terrestrial forms (Stylommatophora), that provide the most information about the potential mechanisms regulating gastropod dormancy. Environments inhabited by estivating pulmonates range from temperate freshwater ponds in the case of the limpet *Ferrissia wautieri* (Richardot 1977a,b) to the Sinai desert, where the helicid snail *Sphincterochila prophetarum* can remain dormant for 97% of the year (Steinberger et al. 1981). Biochemical and physiological insights about the regulation of dormancy in helicid snails will be considered below (Sect. 6).

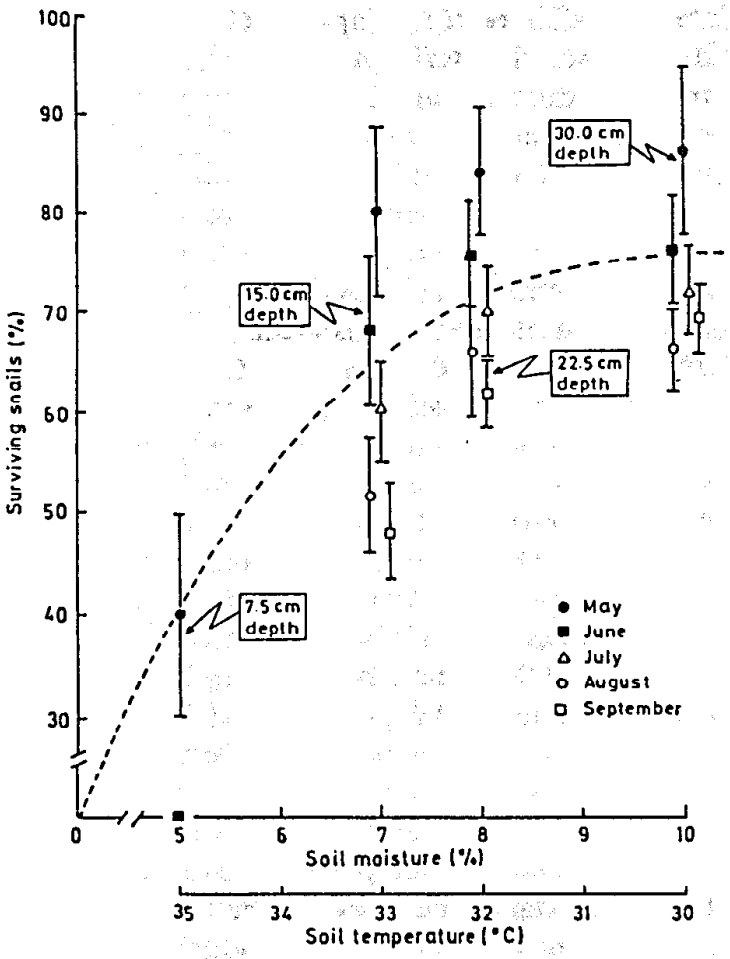

Fig. 3. Survivorship of the aquatic snail *Pila globosa* during estivation as a function of soil temperature and moisture. *Each value* represents 12–20 animals, and the *vertical lines* are one SD. (Haniffa 1978)

2.7 Annelids

A few aquatic oligochaetes are capable of burrowing into sediments, secreting a mucous cyst wall, and entering dormancy as an avoidance mechanism against desiccation or winter temperatures (Barnes 1980). Physiologically, the phenomenon is not well studied. The analogous behavior in terrestrial annelids, most notably the earthworm *Allolobophora*, results in a profound state of dormancy that can last up to 2 months (Gerard 1967; Cloudsley-Thompson 1970). During this period the posterior end of the worm actually degenerates and the clitellum regresses. Body mass may decrease by as much as 50%. A collagenase-type enzyme has been reported from *Allolobophora* which may play a role in this curious tissue degeneration (Kaloustian 1981).

2.8 Crustaceans

Marine calanoid copepods are noted for their abilities to produce resting eggs in the state of diapause (Sazhina 1968; Zillioux and Gonzalez 1972; Kasahara et al. 1974, 1975a,b; Grice and Gibson 1975; Uye and Fleminger 1976; Marcus 1979, 1980, 1982). Genotype and the conditions prevailing during oocyte formation influence

the duration of this diapause (Marcus 1987); temperature, photoperiod, and deoxygenated water have all been implicated in the maintenance and/or release from diapause of marine calanoid eggs (see references above). A least one species of marine harpacticoid copepod is capable of encystment as a sexually immature adult (Coull and Grant 1981).

The majority of limnic zooplankton produce a resting stage at some point during their life cycle, a process which has been particularly well studied among the copepods and branchiopods. Freshwater calanoid copepods alternate between production of subitaneous (immediately hatching) and diapausing eggs (Cooley 1978; Hairston and Olds 1984, 1987 and references therein), and one genus of freshwater harpacticoid copepod is known to encyst as an adult (Sarvala 1979a). Diapause in freshwater cyclopoid copepods is quite interesting in that the arrest seems to be limited exclusively to the copepodite instars (Elgmork 1955; George 1973; Elgmork and Nilssen 1978, and references therein; Sarvala 1979b; Lacroix and Lescher-Moutoue 1984). Apparently cyclopoid eggs are all of the subitaneous type (Cooley 1978). Available evidence indicates that the diapause in these fourth and fifth stage instars is analogous physiologically with insect diapause (Einsle 1967; Watson and Smallman 1971; Elgmork and Nilssen 1978). Working with the fourth copepodite instar of *Diacyclops navus*, Watson and Smallman (1971) characterized an initial refractory period in the diapause where oxygen consumption was 0.01 μl O_2 h^{-1} $individual^{-1}$ and tolerance to cyanide and anoxia exposure was high. The diapause was promoted by exposing first copepodite instars to specific combinations of photoperiod and temperature. In the later competent phase of diapause, where the fourth-stage instars were capable of resuming development, respiration rate increased to 0.06 μl O_2/h/individual. Survival under anoxia was reduced, but cyanide tolerance was about the same.

Among branchiopods, the cladocerans (water fleas) possess many life-cycle features in common with the monogonont rotifers: parthenogenetic eggs are produced for multiple generations that hatch into females, and under certain conditions, males appear in the population leading to the production of sexual, resting eggs (cf. Frey 1982). In genera like *Daphnia*, pairs of these eggs (ephippia) are encased in a protective capsule derived from the walls of the adult brood chamber, and they can withstand drying, temperature extremes, and passage through vertebrate digestive tracts (Mellors 1975). Exposure to light and CO_2 at receptive points during dormancy is important for activation of these diapausing eggs (Stross 1971). Other cladoceran genera, like *Leptodora* and *Bythotrephes*, produce nearly spherical resting eggs that are structurally distinct from the ephippial type (Andrew and Herzig 1984).

While cytological aspects of cladoceran eggs have been investigated (Makrushin 1980), the metabolism of this resting stage has been ignored, with the exception of the respirometric study by Andrew and Herzig (1984). These workers laboriously hand-sorted numerous resting eggs (gastrula stage) of *L. kindti* and *B. longimanus* from core samples taken from an Austrian lake and measured oxygen consumption at environmentally encountered temperatures. Diapause had presumably been broken in these eggs, since development was clearly underway. Still the metabolic rates were low – about 0.5 μg O_2 h^{-1} mg^{-1} dry mass (2–4 °C) for embryos at the earliest stages of post-diapause development. This value was far lower than those obtained

for hatched planktonic juveniles (3–4 μg O_2 h^{-1} mg^{-1} dry mass; Moshiri and Cummins 1969), but Andrew and Herzig (1984) pointed out that such comparisons can be misleading when one considers that metabolically active embryo tissue comprises only a portion of the total egg weight.

It is fair to say that more is known about the mechanisms governing metabolic and developmental arrest in embryos of the brine shrimp *Artemia* (Branchiopoda: Anostraca) than for all other crustacean species combined, due in part to the commercial availability of large quantities of encysted, anhydrobiotic embryos. Information dealing with the quiescent states of anaerobic dormancy and anhydrobiosis in *Artemia* will be reviewed in Sections 3, 4, and 5. Less work has been performed on the topic of *Artemia* diapause because of the difficulty in collecting and maintaining embryos in this state. Cysts must be collected and stored fully hydrated, since dehydration is one stimulus that serves to break diapause, at least for the Great Salt Lake and San Francisco Bay populations. During late summer in the Great Salt Lake (Utah), ovigerous females release encysted embryos into hypersaline lakes, where these eggs float. While in the lake, the encysted embryos are in a true state of diapause; conditions normally known to promote the development and hatching of post-diapause embryos (ample oxygenation, hydration, and warm temperature) are ineffective at this point (reviewed by Clegg 1974b). The developmentally arrested cysts eventually wash to shore and accumulate in thick windrows, where they are then subjected to prolonged desiccation. Cellular dehydration releases the embryo from diapause, so that when the dry embryos are rehydrated and washed into the lake again by spring rains, development is reinitiated. This scenario differs among *Artemia* populations. For example, cysts in Mono Lake sink to the lake bottom when released by females (Lenz 1980; Hand, pers. observ.), and consequently never experience dehydration.

While mechanisms governing the production of diapausing embryos are not clear (Clegg and Conte 1980), a recent study by Drinkwater and Crowe (1987) has elucidated many physiological and environmental features involved with release of *Artemia* embryos from diapause. Two species of diapausing embryos were used in their studies – *A. franciscana* from the salterns adjacent to San Francisco Bay, California, and *A. monica* from Mono Lake, California. *A. franciscana* was activated by pretreatment with various combinations of temperature and dehydration in saline. Saline solutions can be used to precisely control hydration state in *Artemia* embryos due to the impermeability of the outer cuticular membrane to essentially everything except low molecular dissolved gasses and water (Clegg 1966; Conte et al. 1977; Busa et al. 1982; Clegg and Conte 1980). Overall, dehydration was the more effective cue for diapause breakage in *A. franciscana*. The highest hatch was obtained by pretreating cysts with 3.5 M NaCl at 23 °C (Fig. 4). In contrast, *A. monica* cysts were not activated by dehydration, but rather, low temperature was the key factor. Considering that these cysts from Mono Lake are not exposed to dehydration in the field, the latter observation would seem to be ecologically important (see discussion in Drinkwater and Crowe 1987). The presence or absence of oxygen had no influence on diapause breakage in either population. Based on ^{31}P nuclear magnetic resonance, diapause embryos had alkaline intracellular pH (pH_i) values similar to those reported for developing (post-diapause) cysts (Drinkwater and Crowe 1987). Hence, diapause was not imposed by low pH_i. Interestingly, however,

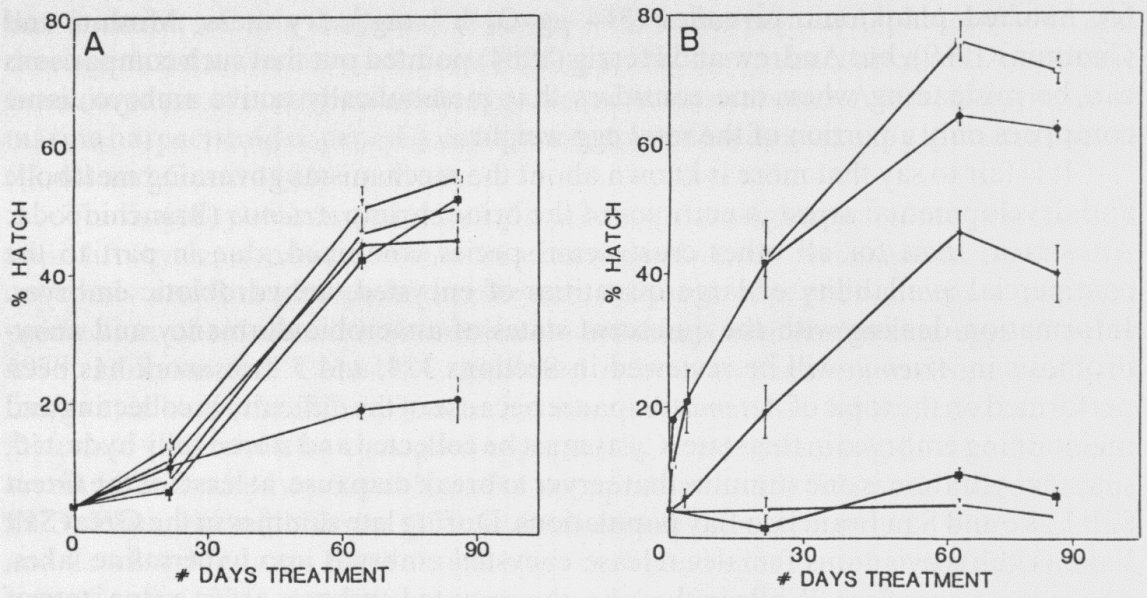

Fig. 4A,B. The influence of dehydration and temperature on diapause breakage for the encysted embryos of *Artemia franciscana* (San Francisco Bay). Percent hatch is plotted after preincubation at 7.5 °C (*A*) and 23 °C (*B*) in NaCl solutions of 1.25 M, N_2 (*closed squares*), 1.25 M (*open circles*), 2.0 M (*closed stars*), 3.5 M (*open squares*), 5.0 M (*closed circles*). Unless otherwise specified, flasks were equilibrated with atmospheric air prior to being stoppered. After the indicated period of time, all cysts were hatched at 23 °C in 0.25 M NaCl and counted at 48 h. *Points* are means ± SD, n = 3. (Drinkwater and Crowe 1987)

exposure of *A. franciscana* to 20% CO_2 for 20–60 days activated these cysts. Such treatment acidifies pH_i to approximately 7.2 (Busa and Crowe 1983; Drinkwater and Crowe 1987).

Van der Linden et al. (1985, 1986, 1988) have investigated the influence of light on diapause breakage in *A. franciscana* from the Great Salt Lake population. Depending on the batch, these workers found that a variable percentage of these anhydrobiotic cysts (about 27%) would not hatch when incubated in low ionic strength medium (1% seawater, 10 mM Hepes buffer, pH 8.0) at 25 °C in the dark. Apparently, previous dehydration was by itself insufficient to promote diapause breakage. However, exposing this 27% of unhatched cysts to a short 30-min burst of illumination (3.5 mW cm^{-2}) during incubation triggered activation and eventual hatching. Cysts activated in this manner showed the typical increases in respiration and trehalose catabolism expected for developing embryos (Van der Linden et al. 1988). Van der Linden and colleagues have suggested that there could be a photorefractory and photosensitive phase in the diapause of *Artemia* cysts, analogous to the case mentioned above for *Daphnia* (Stross 1971). Such a scenario would seemingly lend some environmental relevance to the light-induced activation. Clearly from the work of Drinkwater and Crowe (1987) and Van der Linden et al. (1988), considerable variation exists among cysts in the ease of diapause breakage. Versichele and Sorgeloos (1980) have suggested that abiotic conditions at the time of cyst production by the ovigerous female have a significant influence on the subsequent environmental requirements for diapause breakage in *Artemia*.

Scattered reports of dormancy exist for adult freshwater decapods (Bovbjerg 1952; Priutt 1988). Certain species of crayfish are known to overwinter in a quiescent

state or to burrow deeply into mud during dry periods. Pruitt (1988) showed differences in membrane lipid composition in thermally acclimated crayfish depending on the type of overwintering strategy employed. *Cambarus bartoni* exhibited a significant change in head-group composition of membrane phospholipids during acclimation to low temperature; relative amounts of phosphatidylethanolamine, sphingomyelin, and phosphatidylinositol were increased at the expense of phosphatidylcholine. In contrast, *Orconectes propinquus*, which overwinters in a quiescent state, showed no significant alteration in headgroup composition during cold acclimation. While other factors in addition to head group composition influence lipid fluidity, the changes in head groups seen in *C. bartoni* could potentially help maintain lipid fluidity in membranes at low temperature (cf. Hazel 1988). This feature could be important for the winter-active species.

2.9 Bryozoans

Among the ectoprocts, a minor percentage of the total species produce asexual structures that can remain dormant for considerable periods (Bushnell and Rao 1974). The best known of these resting stages is the statoblast produced by the freshwater Phylactolaemata (Fig. 5). Functionally similar structures termed hibernacula (winter buds) have been described in the Gymnolaemata (freshwater, brackish, and marine species) (Bushnell and Rao 1974). While both structures are heavily sclerotized, they are fundamentally different in that statoblasts are double valve structures produced within the body cavity of individual zooids (cf. Mukai and Oda 1980), whereas hibernacula are formed as outgrowths of stolons or erect

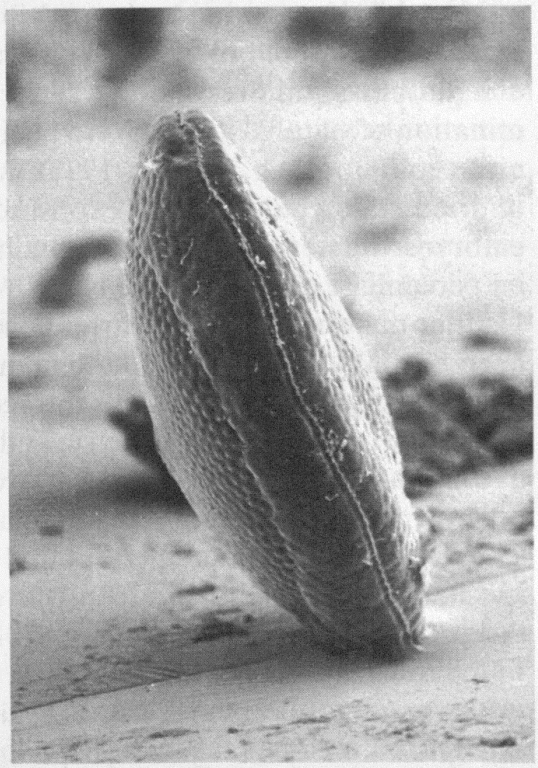

Fig. 5. Scanning electron micrograph of a statoblast (floatoblast) of the freshwater bryozoan *Plumatella casmiana*. The suture zone between the two valves is visible in this lateral view. (Bushnell and Rao 1974)

branches of the colony (Bushnell and Rao 1974). Statoblasts are often divided into two groups based on the presence of an air sac (sessoblasts versus floatoblasts). For a recent morphological and developmental study of these two forms of statoblasts see Mukai and Kobayashi (1988). Two types of cells constitute the germinal mass of statoblasts, an epithelial layer and an inner mass of yolk cells that contain large quantities of yolk granules, lipid droplets, and glycogen (Terakado and Mukai 1978).

Quiescence and diapause have been reported for statoblasts from the same species, but unfortunately, physiological and biochemical information addressing the regulation of dormancy in these structures is not extensive. Mukai (1974) reported that the aqueous medium in which statoblasts had been incubated contained a dialyzable and relatively heat-stable factor, termed blastostasin, which could inhibit the germination of statoblasts. This substance may be functionally similar to gemmulostasin described for freshwater sponges (see Sect. 2.2 above). Black and Proud (1976) showed that respiration rate and the levels of RNA and DNA were much lower in diapausing statoblasts from *Pectinatella magnifica* compared to the values measured upon germination. In their study, diapause was broken by incubating statoblasts for several months at 4 °C. After this cold treatment, returning the cysts to 25 °C led to essentially 100% germination in 48–72 h. Respiration rate of diapausing cysts released from the parent tissue was as low as 0.02 ± 0.01 μl O_2 h^{-1} per 100 cysts. Within 6 h after germination of post-diapause cysts, the respiration rate increased to over 6 μl O_2 h^{-1} per 100 cysts. A sevenfold increase in polysomes was measured after diapause breakage, and total levels of RNA and DNA in cysts increased about threefold during the first 70 h after germination. Black and Proud (1976) found that diapausing cysts were impermeable to ^{14}C uridine and phenylalanine. Similarly, Mukai (1977) observed that exposure of statoblasts from *P. gelatinosa* to high salt, acid, and alkali concentrations caused intense dehydration but had no inhibitory effects on germination, which suggested that the cysts were impermeable to the solutes.

Statoblasts are resistant to desiccation and cold temperatures. Successful germination of statoblasts that have been maintained in a dried state for over a year is quite common (e.g., Rogick 1938). Mukai (1974) reported that statoblasts in the undried state could survive exposure to –20 °C for 60 days. Work with *Artemia* embryos and nematodes has shown that the ability to survive freezing is dependent on percentage of water in the bound "unfreezable" state (Crowe et al. 1981, 1983). Neither the worms nor the shrimp embryos could survive freezing unless they had been partially dehydrated to the point that all bulk water was removed from their tissues. Under these conditions intracellular ice formation did not occur, as judged by thermal analyses, calorimetry, and ion leakage studies. It is noteworthy that the hydration level at which bulk water was removed differed twofold between these organisms (0.3 g H_2O g^{-1} dry nematode; 0.6 g H_2O g^{-1} dry *Artemia* embryo). Consequently, the amount of bound water in a tissue is to some degree species-specific. If the statoblasts were fully hydrated in the freezing studies of Mukai (1974), then one would predict that they have an exceptionally high percentage of bound water and virtually no freezable, bulk water. Unfortunately, quantitative hydration values were not determined in Mukai's study.

3 Metabolic Arrest During Anaerobic Dormancy

During the life cycle of the brine shrimp *Artemia*, the development of postdiapause embryos (cysts) is sometimes interrupted by anaerobic dormancy, an extreme state of quiescence under environmental anoxia (Busa and Crowe 1983; Hand and Gnaiger 1989). Anoxia is common in the hypersaline and often high-temperature lakes into which these embryos are released, and the condition is often prevalent within the thick windrows of cysts (and decaying algal mats) that accumulate along shorelines (Clegg 1974b). Survival of these embryos under anoxia is rather remarkable. In the laboratory cysts can survive up to six months of anoxia in aqueous medium (Dutrieu and Chrestia-Blanchine 1966; Clegg and Jackson 1989). Thus, the physiological and biochemical features governing this profound state of dormancy are apt to be considerably different from those seen in short-term facultatively anaerobic invertebrates (cf. Storey 1988). We are unaware of any other euryoxic invertebrate with the capacity to reduce cellular energy flow to the levels seen for *Artemia* (less than 0.5% of aerobic values, see below) or to tolerate extended anoxia to these lengths. These features, plus the easily manipulated and reversible shifts from aerobic development to anaerobic dormancy in post-diapause embryos, make *Artemia* embryos an excellent system for the study of quiescent states.

It is clear from measurements of respiratory quotients that carbohydrate is the primary fuel used by *Artemia* cysts during aerobic incubation (Muramatsu 1960; Emerson 1963; Clegg 1964). Trehalose, glycogen, and glycerol comprise 98% of the total embryo carbohydrates, but only trehalose is degraded during this time while glycogen and glycerol are synthesized (Ewing and Clegg 1969). Trehalose is thought to be the source of glucose equivalents for these syntheses, and evidence suggests that catabolism of this disaccharide provides the majority of cellular energy required for preemergence development (Clegg 1964). When cysts are placed under 100% nitrogen, the degradation of trehalose is acutely suppressed (Ewing and Clegg 1969). Upon restoration of oxygen, a population of cysts will hatch at the same rate and to the same percentage as cysts not previously incubated anaerobically (Ewing and Clegg 1969; Stocco et al. 1972; Busa et al. 1982). Accompanying the entry of cysts into anaerobic dormancy is the largest acidification ever reported for a biological tissue – a change in intracellular pH (pH_i) from $\geq$7.9 to as low as 6.3 (Busa et al. 1982). The extent of acidification during short-term oxygen deprivation in bivalves, for example, is small relative to the transition in *Artemia* (e.g., Ellington 1983a,b). This observation prompted several investigations into the potential importance of pH in metabolic arrest in these embryos.

3.1 A Role for Intracellular pH

The involvement of intracellular pH in the suppression and activation of cellular metabolism has become well documented for several unicellular and multicellular organisms (Busa and Nuccitelli 1984; Busa 1986). As Busa and Nuccitelli emphasized in their review, a physiological pH_i change can only be considered of regulatory significance if (1) it is shown to occur under conditions relevant to the organism in question, (2) artificial induction of a pH_i change of similar timing and

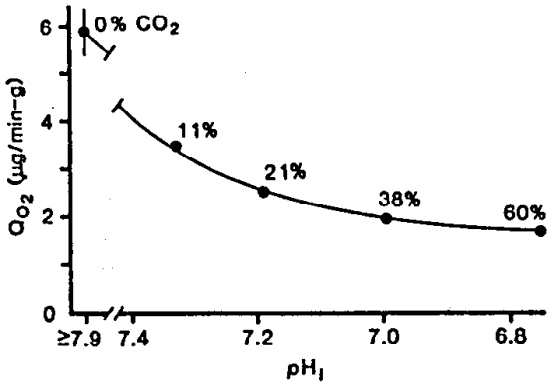

Fig. 6. Dependence of respiratory rate (Q_{O2}; µg oxygen consumed/min/mg dry mass) on the intracellular pH of aerobic *Artemia franciscana* embryos (Great Salt Lake). The pH_i was manipulated by increasing the CO_2 partial pressure in the superfusion medium (at constant partial pressure of oxygen). *Points* represent the mean of two ^{31}P NMR experiments. *Bar* depicts two standard errors of the mean, where this was larger than the graphical presentation. (Busa and Crowe 1983)

magnitude results in the same metabolic response, and (3) reversal or inhibition of the pH_i shift results in modification or reversal of the putative pH-dependent response. All three of these criteria have been met in the case of anaerobic dormancy in *Artemia*.

Busa and Crowe (1983) showed that *Artemia* embryos incubated under fully aerobic conditions with increasing partial pressures of CO_2 did not hatch, and that pH_i, as measured by ^{31}P nuclear magnetic resonance, dropped from its normal values of ≥ 7.9 to as low as 6.8. Under these conditions of aerobic acidosis, respiration was inhibited up to 70% in a pH-dependent manner (Fig. 6). The effect of pH_i depression was completely reversed by removal of CO_2. This method for increasing the proton concentration would also elevate bicarbonate levels in the embryo. However, active compensatory alterations in the strong ion difference involving active ion extrusion (Stewart 1978), as typically displayed by other cellular systems in response to CO_2 loading, is improbable due to the striking impermeability of the embryo's outer cuticular membrane (even to protons, Busa et al. 1982). Thus, the above observations, coupled with the known influences of pH on enzyme kinetics and structural equilibria, implicated pH_i as a principle regulator of these modulations in energy metabolism.

3.2 Suppression of Energy Flow Quantified with Microcalorimetry

By using open-flow microcalorimetry, we were able to perform a direct test of the proposed role of pH_i in metabolic switching in brine shrimp embryos (Hand and Gnaiger 1988). Uninterrupted measurements of metabolic heat dissipation during transitions from aerobic to anaerobic states allowed us to quantify the actual depression of energy flow under anaerobic dormancy. These calorimetric studies were designed to answer three questions regarding transitions of *Artemia* into anaerobic dormancy. First, what influence did experimental modulation of embryo pH_i have on energy flow during anaerobic dormancy? If the shutdown of energy metabolism under anoxia was indeed due to intracellular acidification, then artificial *alkalization* in the absence of oxygen would be expected to cause an elevation of metabolism. To what degree was total metabolic rate of *Artemia* embryos suppressed during short-term and long-term quiescence? This point was of particular interest considering the extended periods that the organism can survive total anoxia, and the

answer could improve our understanding of the exceptional tolerance of *Artemia* to long-term oxygen deprivation. Finally, what was the identity of the substrate(s) supporting the energy metabolism observed during quiescence? The application of microcalorimetry (Gnaiger 1983a,c), coupled with respirometric and biochemical measurements, afforded an opportunity to address these points.

Figure 7 shows the continuous record of heat dissipation during a cycle of anaerobic dormancy in *Artemia* embryos (Hand and Gnaiger 1988). When the intracellular pH of cysts was alkalinized with ammonia under anoxia, the energy flow increased fivefold. This elevated energy flow was accompanied by a reinitiation of trehalose catabolism and an elevation of ATP/ADP ratio. Thus, elevating the pH_i under strict anoxia promoted a marked deinhibition of metabolic rate. Since oxygen was not present for mitochondrial-based metabolism during this treatment, an increase in heat dissipation to control (aerobic) values was not expected. Also evident from Fig. 7 was that total energy flow was rapidly suppressed under anaerobic dormancy to 2.4% of control values within the first few hours of anoxic exposure, which was the lowest percentage reported for euryoxic animals (Gnaiger 1983b).

However, since embryos were not ametabolic during this period of anoxia, it was of interest to know what substrates were utilized to fuel this metabolism. Stocco et al. (1972) showed that an unusual nucleotide stored in *Artemia* embryos, diguanosine tetraphosphate (Gp_4G), was catabolized under anoxia to some extent. These workers proposed that this compound could fuel maintenance level metabolism during anoxia. However, during the first 9 h of anoxia, thermochemical calculations indicate that the rate of Gp_4G catabolism could explain less that 2% of the heat dissipation (Hand and Gnaiger 1988). This observation led us to propose that another fuel, possibly carbohydrate, was being slowly degraded across this period. However, to accurately detect this process, it was clear that the anoxic exposure would need to be extended.

Thus, a calorimetric experiment was conducted in which calorimetric measurements were extended to span a period of 6 days (Hand 1990). Results showed that heat dissipation continued to decline over 6 days, reaching 0.4% of control rates. Trehalose and glycogen stores declined for at least 72h during this period. Ther-

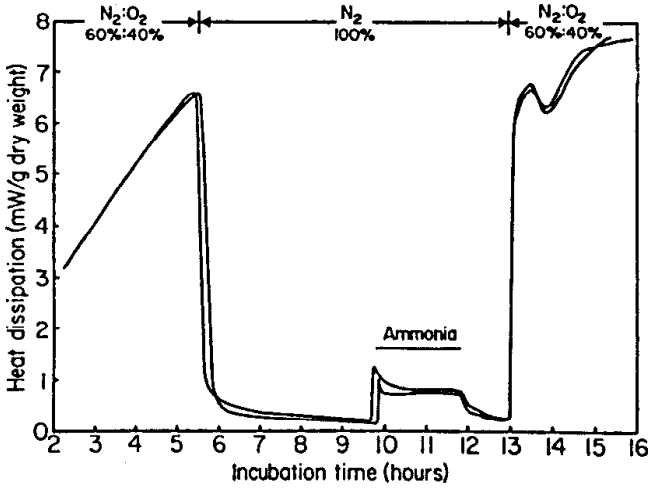

Fig. 7. Superimposed traces of two independent experiments showing heat dissipation of *Artemia franciscana* embryos (Great Salt Lake) during aerobic development (hour 0 to 5.5) and anoxia (hour 5.5 to 13). During the continuous period of anoxia, the acidotic state of anaerobic dormancy was interrupted with the addition of ammonia, which serves to alkalinize pH_i. This period of activated anoxibiosis (hour 9.5 to 12) was reversed by removal of ammonia. The recovery response (hour 13–16) showed a reproducible biphasic pattern. Experiments were performed at 25 °C. (Hand and Gnaiger 1988)

mochemical calculations showed that carbohydrate catabolism accounted for 84% of the total heat dissipation measured over the 6 day anoxic bout; only 3% of the heat could be explained by the catabolism of diguanosine tetraphosphate (Gp_4G). The results suggested that a perceptible energy flow in *Artemia* embryos still remained after 6 days of anoxia. While an ametabolic state might be reached with time, the length of this prolonged transition into anaerobic dormancy had never been appreciated.

It is important to note that Clegg and Jackson (1989) have recently reported constancy of the trehalose pool during 9 weeks of anoxia exposure in *Artemia* embryos from the San Francisco Bay. Their experiments differed from those of Hand (1990) in at least one important way – hydrated embryos were not given a preincubation under aerobic conditions prior to anoxic exposure. Since dry embryos were introduced directly into anoxic medium, a major metabolic transition (aerobic to anaerobic) was not simulated in their studies, and consequently, it is understandable that early declines in carbohydrate stores were not detected under anoxia. Interestingly, the work of Clegg and Jackson (1989) does suggest that the existence of an ametabolic state is indeed possible for hydrated embryos under anoxia, an observation which may explain the exceptional tolerance of *Artemia* to long-term oxygen deprivation.

The recovery phase from long-term anoxia in *Artemia* embryos was biphasic when monitored by heat dissipation (Hand 1990), as previously reported for short-term anaerobic dormancy (Hand and Gnaiger 1988). The calorimetric-respirometric ratios across the first 2 hours of the recovery period were very low – -226 kJ mol^{-1} O_2 after 45 min, and increasing to -346 by 120 min. Similarly low CR ratios have been reported during recovery from anoxia in the marine mussel *Mytilus edulis* (Shick et al. 1986, 1988). Within the first hour of recovery in *Mytilus*, the CR ratio can be as low as -210 kJ mol^{-1} O_2. While a small portion of the excess oxygen consumed relative to heat liberated can be attributed to reoxygenation of tissues, the predominant feature causing the low CR ratios apparently is the occurrence of anabolic, heat-conserving metabolism (Shick et al. 1986, 1988). For example, the theoretical oxycaloric equivalent for gluconeogenic succinate clearance is estimated to be about -200 kJ mol^{-1} O_2, and the oxycaloric equivalent for the partial oxidation of succinate to malate or aspartate is projected to be quite low as well (Gnaiger et al. in prep., as reviewed by Shick et al. 1988). Thus, the low CR ratios during recovery in *Artemia* embryos suggest that processing of organic acids may be one metabolic activity involved in the early stages. Reinitiation of trehalose catabolism, which may be delayed due to hysteretic properties of *Artemia* trehalase (Hand and Carpenter 1986), may contribute more prominently to the overall energy flow as recovery continues.

3.3 Biochemical Coupling of Acidosis to the Arrest of Trehalose Catabolism

All of the above results suggested that pH_i has an inhibitory influence on the pathway of trehalose catabolism during anaerobic dormancy. Consequently, we initiated a study to identify the regulatory steps involved in this arrest in *Artemia* embryos (Carpenter and Hand 1986a). We measured the changes in concentration

of trehalose, glycogen, glycerol, glycolytic intermediates and adenylate nucleotides that occurred during aerobic development, anaerobic dormancy, and aerobic acidosis. During aerobic development, trehalose levels declined while glycogen and glycerol were synthesized. These changes were blocked during both anaerobic dormancy and aerobic acidosis, but were resumed upon return of embryos to aerobic incubation. Similarly, elevated CO_2 also suppressed trehalose catabolism in the tardigrade *Adorybiotus coronifer* (Westh and Ramlov 1990). Evaluation of glycolytic intermediates with crossover point analysis (Crabtree and Newsholme 1985) supported the conclusion that the conversion of trehalose to glucose (catalyzed by trehalase) was the first nonequilibrium reaction reversibly inhibited by pH_i during both aerobic acidosis and anaerobic dormancy (Carpenter and Hand 1986a). Inhibition of the hexokinase and phosphofructokinase reactions was also indicated. If one assumes that all mobilized trehalose that is not converted to glycogen or glycerol is completely oxidized under aerobic conditions, then the 95% shutdown of trehalose catabolism during aerobic acidosis (Carpenter and Hand 1986a) quantitatively accounts for the large suppression of oxygen consumption seen under these conditions (Busa and Crowe 1983). These results provided clear evidence that pH_i was a primary regulator of carbohydrate catabolism in *Artemia* cysts during aerobic/anaerobic transitions, a conclusion further supported by the observation that a change in energy charge was not necessary to achieve this effect (Carpenter and Hand 1986a).

Any mechanism proposed for the pH-induced metabolic transitions in *Artemia* first needed to explain the proton modulation of the trehalase reaction. We provided evidence that the shutdown of trehalose mobilization resulted from a shift in the assembly equilibrium of trehalase (Hand and Carpenter 1986). Isolated trehalase from *Artemia* embryos existed in two active forms that interconvert when exposed to physiological transitions in pH. This interconversion was reversible, required on the order of minutes for completion, and involved a change in enzyme polymerization. The two states differed twofold in molecular weight and were distinguishable electrophoretically. Compared to the smaller species, the polymerized form was strongly inhibited by acidic pH, ATP, and the substrate trehalose. No evidence suggested that phosphorylation was involved in this process. Thus, the shift in assembly equilibrium toward the aggregated enzyme caused by pH values less than or equal to 7.4 apparently is one step involved in mediating the arrest of trehalose-fueled metabolism during anaerobic dormancy.

As indicated earlier, the reaction catalyzed by hexokinase was also severely inhibited during arrest of carbohydrate catabolism in *Artemia* embryos under anoxia. Thus, the kinetic features of hexokinase purified from *Artemia* embryos were studied in order to explain the molecular basis for this inhibition (Rees et al. 1989). We found that pH-dependent changes in the kinetic constants of the enzyme could explain only a modest inhibition of the enzyme under intracellular conditions prevalent during dormancy. However, we discovered that aluminum ion has an acute inhibitory effect on the *Artemia* enzyme at pH values below 7.2. Other workers have seen this aluminum effect, but viewed the inhibition as an unfortunate technical annoyance due to metal contamination of commercial ATP preparations (Womack and Colowick 1979). We measured total aluminum concentration in *Artemia* embryos and found the value to be 72 μM. While no detectable inhibition

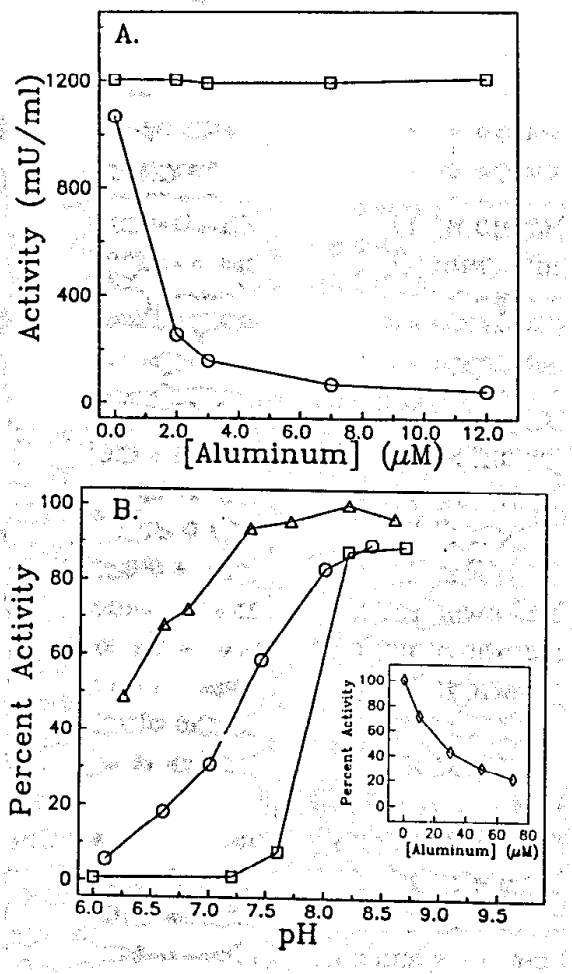

Fig. 8A,B. pH-dependent aluminum inhibition of hexokinase purified from *Artemia franciscana* embryos (Great Salt Lake). In frame *A*, the assay pH was either 8.2 (*open squares*) or 7.0 (*open circles*). Frame *B* depicts the pH dependency of inhibition by aluminum: control conditions, no aluminum (*open triangles*); aluminum at the total concentration measured in embryos, 72 μm (*open squares*); 72 μm aluminum in the presence of physiological levels of metal chelators (0.5 mM citrate, 12.5 mM sodium phosphate) (*open circles*). Reaction rate was expressed as a percentage of the activity at pH 8.0. Inset, the effect of aluminum concentration on hexokinase activity at pH 6.8 in the presence of 0.5 mM citrate and 12.5 mM phosphate. (Rees et al. 1989)

of *Artemia* hexokinase is measurable at pH 8.0, over 90% inhibition occurred at pH 7.0 in the presence of 3 μM aluminum (Fig. 8.).

Thus, we felt it was appropriate to pursue this observation more rigorously. The concentrations of all known effectors of hexokinase activity were measured in these embryos under aerobic development, anaerobic dormancy, and aerobic acidosis. Similarly, the known chelators of aluminum ion (citrate and phosphate) were also measured. In assays simulating the intracellular concentrations of aluminum, its known chelators, hexokinase substrates and effectors, and intracellular pH, a 90% inhibition of the enzyme was observed under anoxia relative to control (aerobic) conditions (Rees et al. 1989). To our knowledge, this is the first report to investigate and support a regulatory role for aluminum inhibition of hexokinase under simulated in vivo conditions. The applied, biomedical implications of this observation could be substantial: Alzheimer's disease (Crapper et al. 1976), dialysis encephalopathy syndrome (Alfrey et al. 1976), and Parkinsonism (Garruto et al. 1984) all represent metabolic dysfunctions which are correlated with elevated levels of aluminum in brain tissues. In this context, Lai and Blass (1984) have noted that micromolar amounts of aluminum inhibit glycolysis in mammalian brain extracts.

A final observation, which has implications for the evolution of hexokinases, is the glucose-6-phosphate inhibition pattern that we measured for the 40 kDa hexokinase of *Artemia* embryos. G-6-P inhibition occurs by competition with ATP

at the active site. Mammalian hexokinases are thought to have arisen by the process of gene duplication and fusion (White and Wilson 1987; Schwab and Wilson 1988). Briefly, the argument holds that the ancestral hexokinase was a yeast-like enzyme (on the order of 50 kDa with no G-6-P inhibition). Duplication of the gene coding for this protein, followed by fusion of the gene copies, resulted in a 100 kDa molecule. One active site remained catalytically competent while an allosteric site arose from modification of the alternative active site. Since the *Artemia* enzyme is expected to be homologous with the original C-terminal active site domain of the mammalian enzyme, it seemed that the G-6-P inhibition in mammalian HK might actually be a property inherent to the active site (as opposed to the allosteric domain). Recently, White and Wilson (1990) used limited tryptic cleavage of the mammalian enzyme to yield separate catalytic and allosteric fragments. The 40 kDa catalytic peptide, just like *Artemia* hexokinase, clearly showed G-6-P inhibition. Thus, it now looks as if the mammalian enzyme arose from an invertebrate-like enzyme, rather that from the yeast-like enzyme as originally proposed (cf. White and Wilson 1990).

Investigations centering on the inhibition of *Artemia* phosphofructokinase (PFK) during acidosis were designed to answer two questions (Carpenter and Hand 1986b). First, what physical and kinetic properties of the enzyme were responsible for the inhibition of fructose-6-phosphate phosphorylation during intracellular acidification? Kinetic measurements with the purified enzyme showed severe suppression of catalytic activity at acidic pH even when assayed at the adenylate nucleotide concentrations existing in anaerobic embryos. Based on measurements of 90° light scattering, this observed inhibition was not due to pH-induced dissociation of tetrameric *Artemia* PFK into inactive subunits, as is observed with certain other PFK homologs (reviewed in Somero and Hand 1990). The possible contribution of enzyme phosphorylation to the observed pH-dependent inhibition has not been investigated.

The second part of our studies with brine shrimp PFK (Carpenter and Hand 1986b) was initiated in response to our observation that this PFK homolog displayed allosteric kinetics at pH 8.0, a feature that is functionally significant since the pH_i of the developing brine shrimp is ≥ 7.9. However, the result was unusual in that allosteric properties of PFK from vertebrate muscle were seen only near neutrality; binding of allosteric modifiers (e.g., ATP) is dependent on the presence of protonated histidyl residues (reviewed in Somero and Hand 1990). Thus, we compared the kinetics of native *Artemia* PFK to those of the enzyme modified with diethylpyrocarbonate, a reagent shown to react with histidyl residues in PFK. Our findings showed that histidyl residues were indeed involved in allosteric kinetics of this enzyme at pH 8.0. Assuming that these residues in brine shrimp PFK must be protonated for binding of modifiers, it is likely that evolutionary changes in the protein microenvironment surrounding the histidyl residues have occurred which elevated the pK_a. Such a shift in pK_a would mirror the more alkaline pH_i of *Artemia* embryos relative to vertebrate muscle.

4 Coordinated Arrest of Development Under Anoxia

One major aspect concerning animals in quiescent states that has received little attention is the status of biosynthetic events (e.g., protein synthesis, membrane biogenesis, nucleic acid synthesis). The use of metabolic depression as a response to extreme physical environments would seem to require the coordinated suppression of both ATP-producing pathways and the anabolic, ATP-utilizing processes (cf. Hochachka and Guppy 1987; Hofmann and Hand 1990b). Relatively few examples of this coupled relationship have been demonstrated. Rather, the vast majority of studies concerning quiescence have focused only on the arrest of catabolic events. Are these energy-utilizing processes arrested simultaneously with ATP-yielding metabolic pathways? Anabolic processes could simply run down during dormancy due to reduced availability of energy-rich adenylate nucleotides. However, it could also be argued that anabolic processes must be closely regulated upon entrance into a dormant state in order to insure that cellular energy levels do not reach critically low levels from which the animal might never recover (Hofmann and Hand 1990b).

4.1 Subcellular Differentiation During Preemergence Development in *Artemia* and its Reversible Blockage

Across preemergence development in *Artemia* embryos, the features most indicative of differentiation are for the most part subcellular and include mitochondrial morphogenesis, elaboration of glycogen storage sites, and changes in yolk platelet structure (Hofmann and Hand 1990a). Preemergence development (PED) encompasses all active developmental periods of the encysted embryo from the gastrula to the emergence stage (rupture of the cyst wall and extension of embryo tissue). Mitochondrial maturation involves two populations of mitochondria that are spatially segregated in embryonic cells – one in the cytoplasm and the other located inside of yolk platelets. The latter population was identified by histochemical staining of DNA and supports the original suggestion of such a mitochondrial location by Marco et al. (1980) and Vallejo et al. (1979). While both mitochondrial populations matured structurally during PED, our enzymatic evidence indicated that only the cytoplasmic population was functional across this period.

Busa and Crowe (1983) demonstrated that embryos under anoxia or aerobic acidosis had suppressed hatching rates. We have extended their results to show that these two treatments block subcellular differentiation (Hofmann and Hand 1990a; Utterback and Hand 1987). Lipoprotein storage granules (yolk platelets) in cysts incubated for 10 h under aerobic development showed a significant decrease in numbers and in total platelet protein; these changes did not occur when embryos were incubated under aerobic acidosis (Utterback and Hand 1987). Furthermore, the release of protein and lipid from isolated yolk platelets was shown to be dependent on solution pH. The extent of release increased markedly as pH was raised in increments from 6.3 to 8.0 (Fig. 9). Protein release could be suppressed, but not reversed, by reacidification. Electron microscopy revealed that embryonic cells exposed to either anoxia or aerobic acidosis for 12 h were essentially identical to cells of the undifferentiated, hour 0 embryo (Hofmann and Hand 1990a). Similarly,

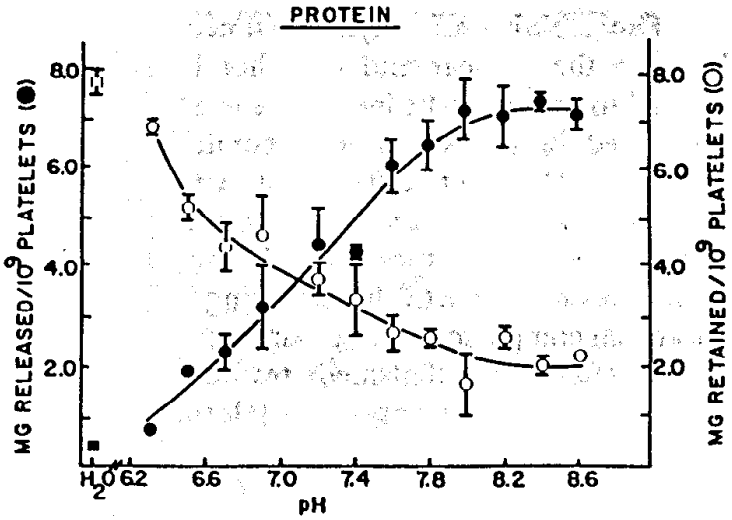

Fig. 9. Mobilization of protein from isolated yolk platelets of *Artemia franciscana* (Great Salt Lake) as a function of pH. Values for platelets incubated in deionized water (pH 5.7) are represented by *open* and *closed squares*. All symbols represent means ± SE (n = 3). Where *vertical bars* are absent, SE is less than the size of symbol. (Utterback and Hand 1987)

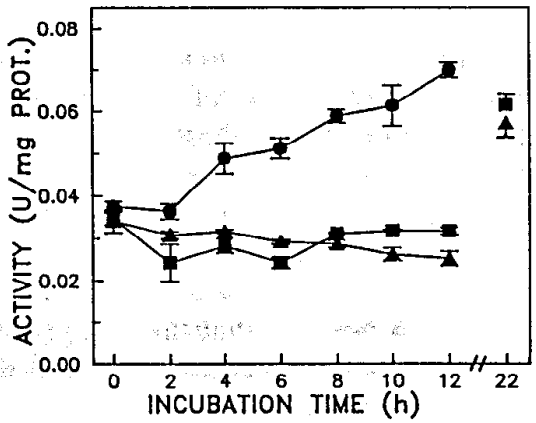

Fig. 10. Cytochrome oxidase activity in *Artemia franciscana* embryos (Great Salt Lake) during aerobic development (*closed circles*), anaerobic dormancy (*closed triangles*), and aerobic acidosis (*closed squares*). Each *point* represents the mean ± SD for three independent samples. After 12 h, embryos were returned to control, aerobic conditions for an additional 10 h. (Hofmann and Hand 1990a)

cytochrome c oxidase (COX) activity increased 230% over 12 h of aerobic development, but this ontogenetic increase was arrested during 12 h of anoxia or aerobic acidosis (Fig. 10). Alkalinizing the pH_i of embryos under anoxia by adding ammonia to the medium resulted in a 37% increase in COX levels relative to those under anoxia. These data support a pH_i-dependent regulation of development in preemergence *Artemia* embryos.

4.2 Disruption of Protein Synthesis: Cytochrome c Oxidase

The above changes in cytochrome c oxidase activity during preemergence development suggested that this enzyme could be a useful specific marker for protein synthesis in these embryos. Consequently, we have addressed whether or not protein synthesis is inhibited during anaerobic dormancy and whether such regulation might involve transitions in pH_i (Hofmann and Hand 1990b). First we developed a method for purifying COX from *Artemia*. Then, by following the incorporation of radiolabeled amino acids into COX, we showed that substantial biosynthesis of the enzyme occurred during aerobic development, and that this process was blocked under anaerobic dormancy and aerobic acidosis.

The assembly of catalytically functional COX is dependent on subunits encoded by both the nuclear and mitochondrial genomes (Poyton 1980). Thus, if pH_i acidification were to be ineffective in arresting mitochondrial protein synthesis, the continued biogenesis of mitochondrially translated subunits (I–III) would be predicted. However, differential synthesis of mitochondrially versus cytoplasmically translated subunits was not evident under aerobic acidosis. This observation suggests that depression of pH_i may be accompanied by intramitochondrial acidification – effectively inhibiting protein synthesis in cytoplasmic *and* mitochondrial compartments. To our knowledge, this study is one of few to identify an intracellular signal that mediates both catalytic and anabolic arrest during dormancy in a eucaryotic organism (Hofmann and Hand 1990b).

5 Anhydrobiosis: Dehydration-Induced Quiescence

Examples from relevant taxa illustrating patterns of water loss during anhydrobiotic bouts, as well as some of the morphological, physiological and behavioral phenomena responsible for these patterns, were addressed in Section 2. As will be seen below, animals able to tolerate the removal of most or all of their cell-associated water without irreversible damage exhibit a reduction in metabolic rate during the dehydrated condition. In some cases the metabolic arrest is so severe that it becomes virtually impossible with available methodologies to measure energy flow at all. Thus, it is possible that these organisms are actually ametabolic (Clegg 1973). In these instances, the quiescent state induced by desiccation is commonly referred to as cryptobiosis.

5.1 Patterns of Metabolic Suppression: Respirometric and Calorimetric Measurements

Crowe et al. (1977) showed that the respiration rate of anhydrobiotic nematodes (*Aphelenchus avenae*), which were transferred from dry air to 97% relative humidity for 24 h and then to water for 10 min, was about 25 nl O_2 mg^{-1} dry mass min^{-1} at 25 °C. Oxygen consumption of fully hydrated nematodes was about 150 nl mg^{-1} dry mass min^{-1}. These values compare well to others obtained for anhydrobiotic (Bhatt and Rohde 1970) and fully hydrated (Cooper and Van Gundy 1970) nematodes.

Early manometric measurements of oxygen consumption of dry *Artemia* embryos (San Francisco Bay population) yielded values that were considered to be negligible compared to those for hydrated embryos (Muramatsu 1960). Sometime later Morris (1971) studied respiration of dried embryos undergoing hydration in 0.5 M NaCl and detected significant oxygen consumption with polarographic oxygen electrodes after 10 min of equilibration. Clegg (1976b) related the precise water content of San Francisco Bay embryos to the onset of respiration. His results showed that oxygen consumption, as measured polarographically at 25 °C, was not detectable below about 0.5–0.6 g water g^{-1} dry mass embryo, but respiration increased rapidly as hydration climbed during incubation of embryos in 0.5 M NaCl (Fig. 11).

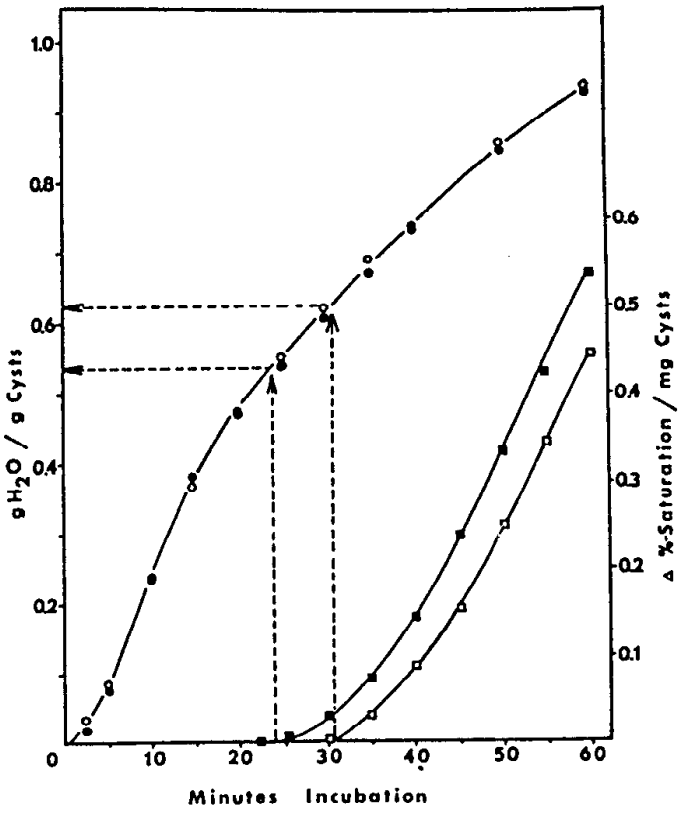

Fig. 11. Hydration and polarographic measurements of respiration of dried cysts (*Artemia franciscana*, San Francisco Bay) incubated in 0.5 M NaCl. Hydration (*open* and *closed circles*) and respiration (*open* and *closed squares*) values represent maximum variation noted in five replicate experiments. The *dashed lines* indicate the estimated hydration level at which respiration begins. (Clegg 1976b)

A similar "critical hydration level" for initiation of respiration was determined in the same study by pre-equilibrating embryos in solutions of increasing ionic strength. The rapid onset of respiration was confirmed with manometric measurements, which indicated that respiration rate of embryos increased from undetectable levels to 1.0 μg O_2 mg^{-1} dry mass h^{-1} within the first 45 min of hydration in the vapor phase of 0.5 M NaCl (Clegg 1976b).

More recently, Glasheen and Hand (1989) used open-flow microcalorimetry to follow changes in energy flow in *Artemia* embryos (Great Salt Lake, Utah) undergoing cycles of dehydration-rehydration in NaCl solutions. As illustrated above, metabolic heat dissipation can be a sensitive measure of total energy flow in the embryos. Calorimetry avoids the technical difficulties that arise when oxygen consumption is chosen as a means to estimate aerobic metabolism in solutions of high ionic strength; electrolyte solutions within polarographic electrodes dehydrate under such conditions (Hale 1983). Heat dissipation from developing embryos rose steadily during the first 4 h in control 0.25 M NaCl (Fig. 12). When the perfusion medium was switched to 1.0 M NaCl, energy flow continued to rise, reaching 137% of control (hour 4) values. However, when control medium was switched to 2.0 M NaCl, energy flow fell exponentially to 21% of control values after approximately 18 h. In experiments with 3 M NaCl, heat dissipation fell sharply to 6%. At higher ionic concentrations, heat dissipation declined to as low as 3%, or an absolute rate of 0.14 milliwatt g^{-1} dry mass. Thus metabolism in developing embryos was disrupted at a critical hydration level promoted by an external NaCl concentration between 2.0 and 3.0 M. When embryos were returned to control perfusion after dehydration, recovery of energy flow was rapid, showing the reversibility of the metabolic arrest.

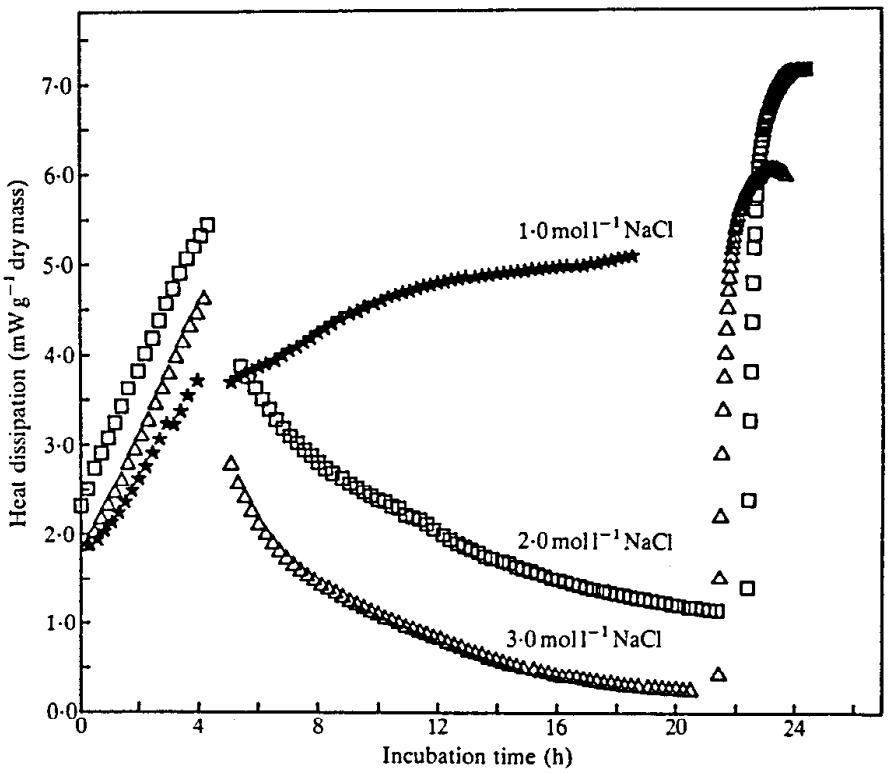

Fig. 12. Heat dissipation of *Artemia franciscana* embryos (Great Salt Lake) during incubations in various concentrations of NaCl. For all experiments, embryos were initially incubated for 4 h in 0.25 M NaCl, after which the medium was changed to either 1.0 M NaCl (*closed stars*), 2.0 M (*open squares*), or 3.0 M (*open triangles*). After the 2.0 and 3.0 M treatments, the incubation medium was switched to 0.25 M NaCl, and the recovery period monitored. (Glasheen and Hand 1989)

Based on hydration values determined for Great Salt Lake embryos (Glasheen and Hand 1989) and Clegg's values for San Francisco Bay embryos (Clegg 1974a), the critical hydration necessary for the onset of major metabolic events was also similar between the two studies. If the hydration state is expressed as water content of metabolically active embryo tissue, a value of 0.7 g water g^{-1} dry mass was needed for respiration in the San Francisco Bay embryos, while 0.7–0.85 g water g^{-1} dry mass was required for substantial heat dissipation in Great Salt Lake embryos.

5.2 Biochemical Correlates of Cellular Dehydration State

5.2.1 Nematodes and Tardigrades

Biochemical changes during the early stages of dehydration in nematodes are important for survival, particularly in species which are often referred to as "slow dehydration strategists" (Womersley 1987; Womersley and Ching 1989). A key finding made by Madin and Crowe (1975), which stimulated several productive avenues of research still underway today, was that the accumulation of the non-reducing sugar trehalose and glycerol during dehydration was tightly correlated with survivorship in *Aphelenchus avenae*. A reciprocal relation existed between ac-

cumulation of trehalose (and glycerol) and the decline in glycogen and lipid content as water loss occurred (Madin and Crowe 1975); the pattern was reversed upon rehydration (Crowe et al. 1977) (Fig. 13). Recently, the tardigrade *Adorybiotus coronifer* has also been shown to accumulate trehalose during slow dehydration (Westh and Ramlov 1990). Calculations suggested that the declines in lipid and glycogen in nematodes could, even after energy metabolism was considered, fully account for the trehalose and glycerol synthesized (Madin and Crowe 1975). A similar pattern of carbohydrate synthesis in the face of lipid depletion was reported in the early stages of dormancy in nematode eggs (Ash and Atkinson 1983). Subsequent work addressed the synthesis of trehalose from its immediate precursors (Loomis et al. 1980a,b) and showed that the majority of the carbon skeletons for carbohydrate synthesis were generated from lipid via the glyoxylate cycle (Madin et al. 1985).

The survival advantage offered by trehalose accumulation during anhydrobiosis is most likely multifaceted. A compelling series of studies by Crowe and colleagues suggests that trehalose serves as a natural stabilizer of biological membranes (for review see Crowe et al. 1987). Dehydration of biological membranes can

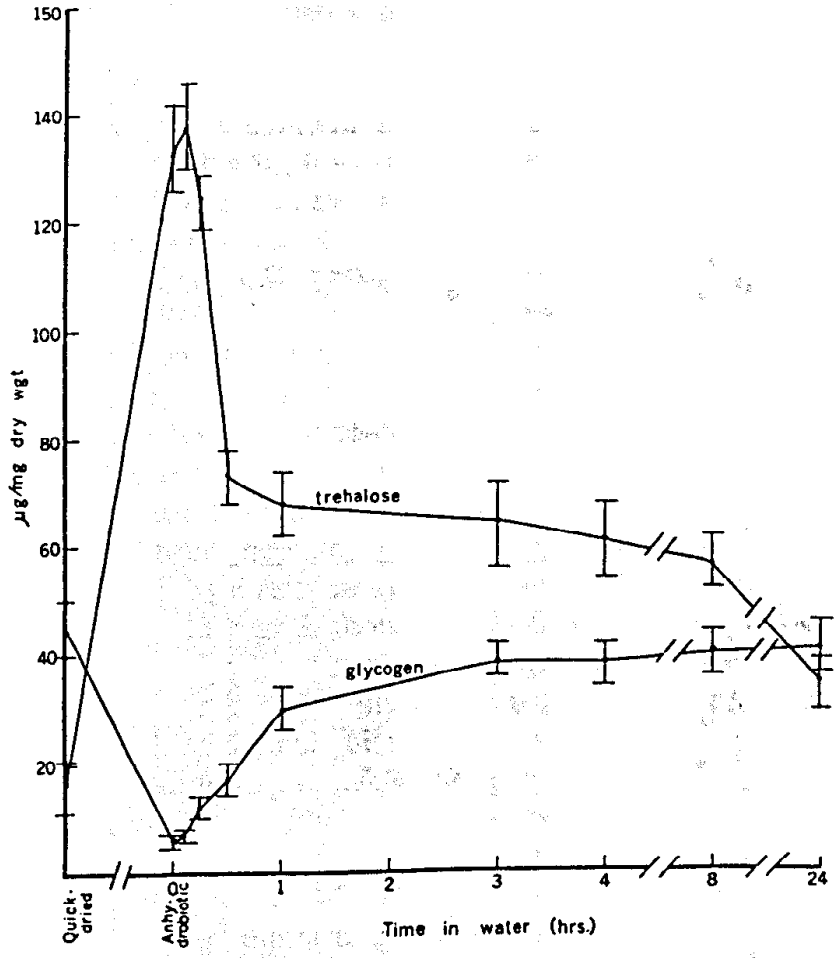

Fig. 13. Trehalose and glycogen contents of quick-dried nematode worms (*Aphelenchus avenae*) and of anhydrobiotic *A. avenae* in dry air and at intervals following transfer of the anhydrobiotic worms to water. (Crowe et al. 1977)

result in several deleterious changes in bilayer configuration – for example, phase transitions from the hydrated liquid crystalline phase to the gel phase, and formation of an inverted hexagonal phase lipid (a nonbilayer configuration). These transitions can be disruptive to normal membrane functioning, but in the presence of trehalose their formation during dehydration is markedly reduced. Trehalose seems to be superior to most other naturally occurring carbohydrates in its membrane-protective role. Yet the beneficial effect of trehalose may not be limited to this single class of macromolecule.

Loomis et al. (1979) showed that the reduction of glycogen coupled with the increase in trehalose could serve to suppress "browning reactions" in proteins, i.e., at high concentrations, reducing sugars can interact with free amine groups of

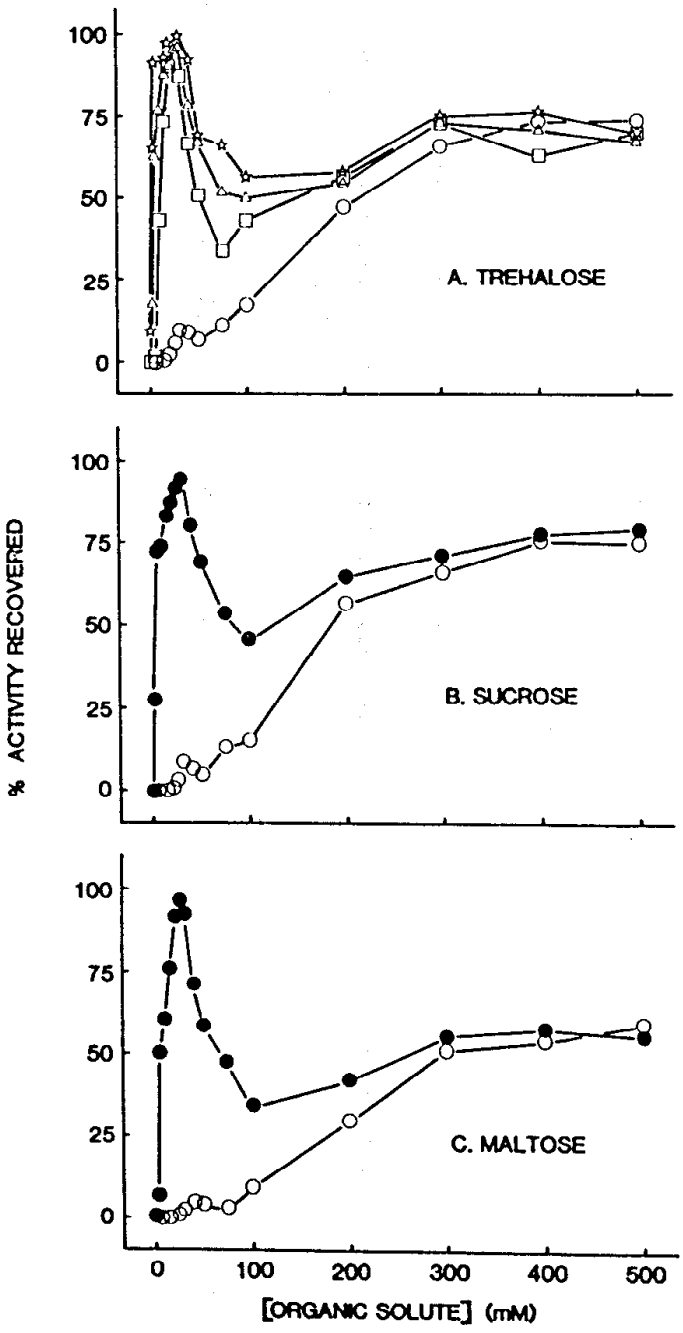

Fig. 14A-C. Protection of purified phosphofructokinase against freeze-thaw damage by the addition of sugars and zinc ion. *A* Percentage of activity recovered after freeze-thawing with trehalose alone (*open circles*) and in combination with the 0.3 mM (*open squares*), 0.6 mM (*open triangles*) and 0.9 mM (*open stars*) $ZnSO_4$. *B* and *C*, percentage of activity recovered in the presence of sucrose and maltose alone (*open circles*) and in combination with 0.6 mM $ZnSO_4$ (*closed circles*). (Carpenter et al. 1986)

proteins causing irreversible protein denaturation. Thus simultaneously lowering the concentration of glycogen (with its reducing termini) and increasing the concentration of nonreducing trehalose could be a protective feature against protein perturbation. Furthermore, trehalose and other carbohydrates, particularly in combination with transition metal ions, have a profound ability to stabilize proteins against damage incurred during freeze-thawing, freeze-drying and air-drying (Fig. 14) (Carpenter et al. 1986, 1987a,b). It has been known for some time that polyols, including sugars like trehalose, could serve as nonperturbing, compatible osmolytes in cells (see review by Yancey et al. 1982).

Undoubtedly, there are other biochemical adaptations to anhydrobiosis in nematodes of which we are currently unaware. For example, a definitive role for the increased glycerol concentrations during dehydration has yet to be identified. In fact, at least as it relates to membrane stabilization, glycerol has a negative, fusion-promoting influence on lipid bilayers. Further, as pointed out by Womersley (1987; Womersley and Ching 1989) the protective mechanisms that may be operative in nematodes which do not require slow dehydration deserve attention, since these species would seem to be "preadapted" for dehydration stress.

5.2.2 Artemia Embryos

The protective influences of trehalose discussed above are surely relevant to anhydrobiotic *Artemia* embryos, considering that up to 17% of the cyst's dry weight is composed of this disaccharide (Clegg 1964). However, for purposes of this analysis, the existing information on biochemical and physiological events in *Artemia* embryos is particularly useful from a different standpoint – that of improving our understanding of the mechanisms controlling energy metabolism during anhydrobiotic bouts.

Clegg and associates have identified relationships between cellular water content, the physical properties of cell water, and various metabolic events in *Artemia* embryos. This extensive body of work has been reviewed on several occasions (e.g., Clegg 1978, 1979, 1981, 1984). In brief, the unifying theme of the work is that cyst metabolism is a strict function of water content. Various physical measurements made by Clegg's group indicate that in a fully hydrated embryo, about 50% of the total water is in a bulk (unrestricted) phase, about 40% is in a vicinal phase (translationally restricted water), and about 10% is tightly bound water (reflecting the primary hydration shells of macromolecular components) (cf. Clegg 1981). These various forms of water are sequentially removed during dehydration, and Clegg has correlated the inhibition of metabolic events with the disappearance of these water classes. From full hydration (about 1.4 g water g^{-1} dry mass embryo) down to about 0.65 g/g^{-1}, metabolism is qualitatively the same, and this range of hydration is referred to as the domain of conventional metabolism. Clegg refers to the hydration range from 0.65 to 0.3 g g^{-1} as the domain of restricted metabolism. Here major metabolic events, including respiration, carbohydrate metabolism, and protein synthesis, have ceased. Finally, the ametabolic domain is the hydration range from 0.3 to 0 g g^{-1}, where Clegg has no evidence for enzymatically catalyzed events. A key feature of this model is that the major metabolic events are not

qualitatively changed until all bulk water has been eliminated from cells, and the vicinal water has begun to be perturbed.

As mentioned earlier, trehalose is the exclusive metabolic fuel used during development of encysted embryos (Clegg 1964); thus, an understanding of mechanisms involved in the arrest of carbohydrate metabolism is central to explaining the suppression of energy flow in these embryos. Consequently, we compared the changes in concentrations of trehalose, glycogen, glycolytic intermediates, and adenylate nucleotides occurring during normal aerobic development of hydrated embryos with those occurring after dehydration of embryos in concentrated salines (Glasheen and Hand 1988). The trehalose utilization and glycogen synthesis that occurred during development of fully hydrated cysts were both blocked during desiccation (Fig. 15) (Glasheen and Hand 1988; cf. Clegg 1976a). Upon return to 0.25 M NaCl solution both processes were resumed. Analysis of glycolytic intermediates suggested that the inhibition was localized at the trehalase, hexokinase and phosphofructokinase reactions. ATP level remained constant during the 6-h period of dehydration, as did the adenylate energy charge.

Because it is clear that pH_i acidification in fully hydrated embryos arrests carbohydrate metabolism (Sect. 3), we felt it would be informative to dehydrate cysts under conditions where acidification (if it were to occur) would be precluded (Glasheen and Hand 1988). Thus an additional dehydration experiment was performed in saline containing a level of ammonia known to maintain an alkaline pH_i in the embryos. The metabolic response to dehydration under these conditions was very similar to the previous dehydration series. Thus, these results were taken as strong evidence that the metabolic suppression observed during dehydration does not require cellular acidification.

While interpretations based on *solvent* status have proved highly useful, the important corollary of intracellular *solute* content has been relatively neglected vis a vis metabolic organization and control in *Artemia* (Glasheen and Hand 1989). Until recently, one deficiency preventing investigators from evaluating the role of intracellular solutes in metabolic arrest during anhydrobiosis was the lack of a comprehensive analysis of the internal osmolyte system for *Artemia* embryos.

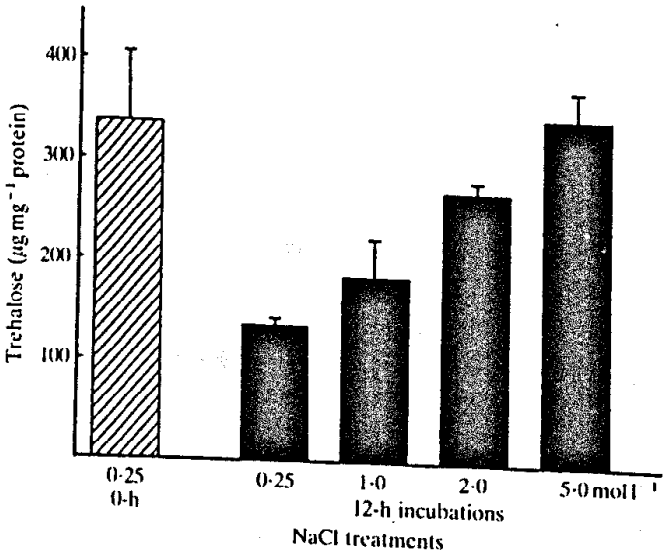

Fig. 15. Trehalose levels after 12 h of aerobic incubations of *Artemia franciscana* embryos at 23 °C. All embryos were prehydrated overnight at 0 °C in the indicated NaCl solutions prior to incubation in those same solutions at 23 °C. Histogram on the *left* represents time 0 controls. Note that increasing concentrations of NaCl, which progressively dehydrate the embryos, serve to suppress the catabolism of trehalose over the 12-h period. *Each histogram* is the mean of three independent samples, and the *vertical line* represents ± one SE. (Glasheen and Hand 1988)

Without such information it has been impossible to estimate the ionic and organic solute concentrations present in the embryo at the onset of metabolic arrest. Thus, we recently undertook and completed a comprehensive analysis of the internal solutes and macromolecular components of *Artemia* embryos (Glasheen and Hand 1989). Taken together, 97.4% of the dry cyst mass was identified. When the cellular hydration values for these embryos were coupled to the intracellular solutes levels, it was possible to estimate the composition of the internal milieu of these embryos at any point along the graded series of dehydration. As a direct consequence of dehydration, we found that the total concentration of inorganic ions rose dramatically. At the cellular hydration level where energy flow was blocked in these embryos, the internal inorganic ion concentration approached 500 mM.

In most species studied, levels of univalent ions above 100–200 mM are severely deleterious to enzyme function, as judged by the lowering of reaction velocities and elevation of K_m values (reviewed by Yancey et al. 1982). In addition to these direct influences on enzyme function, evidence suggests that the binding of enzymes to subcellular components may be disrupted by elevated levels of inorganic ions. Partitioning of enzymes into soluble and particulate fractions (with differing kinetic properties), or altering the cohesiveness of multienzyme associations are attractive models for metabolic regulation (Welch 1977; Srere 1987; Ovadi 1988; Somero and Hand 1990). In mammalian muscle, the adsorption of the glycolytic enzymes aldolase, lactate dehydrogenase, and pyruvate kinase to an F-actin-tropomyosin-troponin complex was significantly reduced in 150 mM KCl (Clarke and Masters 1975). Likewise, 150 mM NaCl resulted in desorption of glyceraldehyde-3-phosphate dehydrogenase from Band 3 protein in erythrocytes (Yu and Steck 1975). It is possible that ionic perturbation of protein-protein or protein-cytoskeletal interactions may account for at least part of the observed dehydration-induced arrest of *Artemia* metabolism. Consequently, we suggest that the role of intracellular osmolytes should be more fully integrated into hypotheses designed to explain such phenomena during anhydrobiosis (Glasheen and Hand 1989).

6 Estivation in Gastropods

As emphasized in Section 2, many aquatic and terrestrial snails respond to desiccating environmental conditions by entering estivation, a behavior which minimizes evaporative water loss (Machin 1975). Some species are capable of remaining in estivation for several years (e.g. Stearns 1877; Machin 1967). During this time there is no intake of foodstuffs, so energy requirements must be met exclusively by stored reserves. Clearly, metabolic suppression during dormancy would help to extend the period of time that energy stores will last.

6.1 Metabolic Patterns

Values for oxygen consumption from a variety of pulmonate snails indicate that the depth of metabolic arrest during estivation is related to environmental water

availability. The slug *Limax flavus*, which occurs in mesic habitats and has a limited tolerance of desiccating conditions, is characterized by a relatively high rate of respiration during estivation, 27 μmol O_2 g^{-1} dry mass h^{-1} (Horne 1979). At the other extreme is *Sphincterocilla boissieri*, a snail from the deserts of the Near East, which exhibits the lowest rate of respiration during estivation (0.9 μmol O_2 g^{-1} dry mass h^{-1}) (Schmidt-Nielsen et al. 1971). Intermediate in terms of environmental water availability are *Bulimulus dealbatus* (12 μmol O_2 g^{-1} h^{-1}; Horne 1973a), *Otala lactea* (4.2 μmol O_2 g^{-1} h^{-1}; Herreid 1977; Barnhart and McMahon 1987), and *Oreohelix strigosa* (6.2 μmol O_2 g^{-1} h^{-1}; Rees and Hand 1990). All of the values above have been adjusted to 25 °C using a Q_{10} values of 2.44 (Schmidt-Nielsen et al. 1971). Therefore among land snails, the capacity to reduce respiration (and consequently water loss) may represent an adaptation to survival in xeric environments.

Another interesting feature of the metabolic suppression is the rate at which it occurs during entry into estivation. In the two species for which the time course has been studied (*Bulimulus* Horne 1973a; and *Oreohelix*; Rees and Hand 1990), the most rapid decline in respiration occurred within the first few days of dormancy. Within 4 days respiration rate was reduced sixfold in both species, and in *Bulimulus* the respiration rate did not decrease any further over 6 months. Once the minimal metabolic rate had been established during extended periods of estivation, the respiration of *Otala lactea* was characterized by periodic bursts of oxygen consumption; rates would increase fivefold at intervals between 20 and 50 h (Barnhart and McMahon 1987). The beginning of each burst in metabolism was associated with a bout of hyperventilation that resulted in a net release of CO_2. The close correlation of oxygen consumption rate and whole-body CO_2 content led these workers to suggest that CO_2 or acid-base balance might influence metabolic rate during dormancy (see Sect. 6.3 below).

6.2 Catabolism of Energy Reserves and Accumulation of Urea

Carbohydrate and protein are the primary energy stores utilized by pulmonate snails during periods of estivation and/or starvation (von Brand et al. 1957; Emerson 1967; Schmidt-Nielsen et al. 1971; Horne et al. 1973a, 1979; Rees and Hand in prep.). Typically, carbohydrate reserves are catabolized early in estivation with protein being utilized in latter stages. Lipid does not appear to be an importat energy source in dormant snails (Horne 1973a). At least during the early phases of estivation, it does not appear that anaerobic metabolism is operative in snails. Churchill and Storey (1989) have recently reported that anaerobic end products do not accumulate in *Otala* during 3 days of estivation. Later in estivation, lactate was measured in *Otala* (Churchill and Storey 1989), and alanine and succinate accumulate in the estivating African snail *Achatina achatina* (Umezurike and Iheanacho 1983). It has been known for some time that helicid snails have moderate anoxia tolerance and accumulate lactate and succinate immediately upon the onset of anoxia (Wieser 1981). Recently we have made simultaneous measurements of heat dissipation and oxygen consumption of estivating and nonestivating *Oreohelix strigosa* (Rees and Hand 1990), and under both conditions, the quantity of heat dissipated per mole of oxygen consumed was indicative of a completely aerobic metabolism. The calo-

rimetric-respirometric (C/R) ratio was –461 ± 12 kJ mol^{-1} O_2 under nonestivating conditions and –464 ± 26 kJ mol^{-1} O_2 during estivation (3–4-day duration). Thus anaerobic pathways of energy production are not recruited during short-term estivation in this snail.

For snails that catabolize significant quantities of protein during estivation, the accumulation of nitrogenous end products can be substantial since there is no elimination of nonvolatile wastes during this period. Horne (1973b) noted that urease activity was present in active snails, offering the possibility of ammonia production. However, during estivation urease activity decreased two orders of magnitude and urea production was enhanced tenfold. The mountains snails *Oreohelix strigosa* and *O. subrudis* both accumulate urea to remarkably high levels in the later months of estivation. Both the rate and extent of accumulation were greater in *O. subrudis*, where the concentration rose from pre-estivation values of 0.4 mM to 289 mM after 7 months (Rees and Hand, in prep.). Because urea at these concentration can perturb macromolecular structure and function (Hand and Somero 1982; Yancey et al. 1982), it was of interest to quantify the methylamine compounds in estivating snails. In elasmobranch fishes, which have similarly high urea concentrations, quaternary ammonium compounds are accumulated with urea in the ratio of 1:2 (methylamine:urea). At this molar ratio, methylamines can offset in vitro the disruptive effects of urea on macromolecules (Yancey et al. 1982). The concentration of quaternary ammonium compounds in *Oreohelix* was only 12 mM after 7 months. Relative to the urea at this point in estivation, this concentration of quaternary ammonium compounds was far below that needed to counteract urea perturbation of enzyme proteins. Thus within limits, it is possible that urea could serve as a reversible inhibitor of metabolism (Yancey et al. 1982). It is also possible that urea accumulation could serve in water conservation in a dehydrating environment, as it does in terrestrial toads (Jones 1980) and elasmobranch fishes (Forster and Goldstein 1976). Since some snails do not accumulate urea (Barnhart 1986a), it would be informative to see if the capacity for urea buildup among land snails correlated with increasingly xeric habitats. Unfortunately, sufficient information is not available at present for such a comparison.

6.3 Potential Mechanisms for Metabolic Arrest

Barnhart (1986a,b) was the first to report the association between hypercapnia, extracellular acidosis, and metabolic depression in land snails. Exposure of active snails to elevated CO_2 levels (65 mm Hg) reduced respiration rate by 50% within 1 h and lowered whole-body intracellular pH by approximately 0.4 pH unit (Barnhart and McMahon 1988). This level of CO_2 was within the range that occurred naturally in the lungs of estivating snails. These results supported a hypothesis that hypercapnia or the resulting respiratory acidosis depressed metabolic rate during estivation.

A similar respiratory acidosis occurs during estivation in *Oreohelix strigosa*, which was not compensated by elevation of hemolymph bicarbonate (Rees and Hand 1990). Hemolymph pH declined from 8.03 to 7.4 within 2 days of estivation. The temporal pattern of this decline was very similar to that seen for the decline in

metabolic rate as judged by oxygen consumption, suggesting a possible linkage between these two variables. Exposure of active snails to artificial hypercapnia (5.75% ambient CO_2; 30 mm Hg in hemolymph) depressed the hemolymph pH to 7.59, and over the same time course (6 h), respiration rate declined 50%. Thus, these data along with those of Barnhart suggest that the influence of acidosis on metabolism may be a common feature of land snails.

7 Directions for Future Study

Hopefully, it is clear from this review that only a small fraction of the cases of metabolic dormancy in aquatic invertebrates has been investigated at the physiological and biochemical levels. Consequently, our current notions about the existence of pleiotropic mechanisms governing metabolic arrest during dormancy in these organisms are limited. Only through a comparative approach can these unifying principles be discovered. Information gained from studies of anhydrobiosis and anaerobic dormancy, particularly with nematodes, tardigrades, and brine shrimp embryos, has begun to provide complete pictures of the types of biochemical mechanisms operative during quiescence. However, our understanding of processes regulating the entry and breakage of diapause is still rudimentary. It seems that selected species of sponge gemmules and *Artemia* embryos may represent valuable systems for diapause study.

Finally, the majority of investigations reviewed above have focused on the arrest of catabolic, energy-producing pathways. The manner in which this phenomenon is coordinated with the suppression of ATP-utilizing (biosynthetic) processes has received virtually no attention at the mechanistic level. An integrated understanding of dormancy will require knowledge of both. Eventually, we may gain a better appreciation of how certain stages in the life cycle of aquatic invertebrates survive environmental conditions that destroy the vast majority of living organisms. The results should have implications for a variety of biological systems that experience stress resulting from temperature extremes, desiccation, and oxygen deprivation.

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