

# Chapter 10

## Water Management by Dormant Insects: Comparisons Between Dehydration Resistance During Summer Aestivation and Winter Diapause

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**Abstract** During summer in temperate regions and tropical dry seasons insects are exposed to extended periods with little available water. To counter this dehydration stress, insects have two options. They can either remain active by utilizing mechanisms to function under severe water stress and high temperatures, or they can escape from the stressful environment by exploiting an aestivation mechanism. During aestivation, insects undergo a variety of molecular and biochemical changes to arrest development, reduce metabolism, tolerate high temperatures, and increase their ability to maintain

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water balance. In this review, I provide a synopsis of known and possible mechanisms utilized by insects to reduce the stress of dehydration during aestivation. Comparative observations of aestivating and diapausing insects are also discussed to assess similarities and differences in the methods used by insects to increase dehydration resistance between these two types of dormancies. Adaptations that alter moisture requirements during diapause (low metabolic rate, increases in osmolytes, shifts in cuticular hydrocarbons, cell membrane restructuring) are likely similar to those utilized at the induction and during the maintenance phase of aestivation. Few studies have been conducted on the physiology, particularly the biochemistry and molecular regulation, of aestivating insects, indicating that much more research is needed to fully assess water balance characteristics of insects during aestivation. Whether an insect is in diapause or aestivation, behavioral, biochemical, and physiological adaptations are essential for suppressing water loss and enhancing survival in a desiccated state.

## 10.1 Introduction

Most insects are capable of developing and reproducing during short periods each year when conditions are appropriate for their growth (Denlinger 1986, 2002). When outside of these optimal conditions, insects have an amazing ability to resist and adapt to fluctuations in their environment for short periods, but when these periods of stress are longer, large scale biological changes are needed (Wolda 1988; Denlinger 2002; Danks 2006). The two most stressful times for most insects are winter (freezing temperatures, lack of water due to its presence as ice, and the absence of food resources) and during the peak of summer or during tropical dry seasons (high temperatures, lack of water due to periods of drought, and inadequate quantities of food). A few insects avoid such periods by migrating to favorable locations, but most species are too small, too slow, or too specialized to engage in long distance flights to new habitats. Thus, most species have mechanisms to tolerate extremely long resting periods under stressful conditions, responses characterized as changes in behavior, reductions in metabolism, prevention of temperature stress, and the maintenance of water balance.

In this chapter, I provide a comparative synopsis of water balance characteristics that occur when insects undergo summer aestivation to tolerate heat and drought and winter diapause to survive low temperature and lack of water. Particularly, I will address behavioral, biochemical, and molecular responses that are involved in water management. I discuss four categories of response: (1) suppressing water loss, including cuticle changes, reductions in metabolic rates and loss of water through regulation of spiracular valves, reducing moisture loss in waste products, and behaviorally by aggregating in favorable microhabitats; (2) increasing the internal water pool by liquid ingestion, water vapor absorption, and/or metabolic water production; (3) reducing the stress of dehydration by the upregulation of stress-related proteins (e.g., heat shock proteins (HSPs)), increasing protective solutes (e.g., trehalose), and partitioning water unevenly into metabolically active tissues;

and (4) exploiting water loss to become anhydrobiotic. Additionally, I provide a brief discussion for the role of water as a cue to initiate and break dormancy.

## 10.2 Maintenance of Water Balance

Development, distribution, and survival are dependent on the insect's ability to maintain water balance. Simply stated, water balance can be summarized by Wharton's (1985) general equation:

$$m = m_s - m_T$$

Where water mass ( $m$ ) is controlled by the movement of water into the insect ( $m_s$ ) and movement of water out of the insect ( $m_T$ ). Thus, if  $m_s > m_T$ , the water pool within the insect increases and if  $m_T > m_s$ , the water pool decreases. The net transpiration rate or water loss rate ( $m_T$ ) usually relates to where the insect resides, those living in arid environments or environments that lack water for extended periods tend to have low water loss rates (Hadley 1994; Gibbs 2002a). Few insects can tolerate either dehydration or overhydration for extended periods. To use chironomids as examples, the Antarctic midge, *Belgica antarctica*, when held at hydrating conditions ( $m_s > m_T$ ) for extended periods eventually succumbs to severe overhydration (Lopez-Martinez et al. 2009). At the opposite extreme, the sleeping midge, *Polypedilum vanderplanki*, when dehydrated slowly ( $m_T > m_s$ , Kikawada et al. 2005; Watanabe 2006) can reduce its water pool to exceptionally low levels, but even in this extreme example a small percent (2–3%) of body water is essential to allow the midge to recover following anhydrobiosis. For an insect to survive and develop within a particular environment, water balance ( $m_s = m_T$ ) must be achieved.

## 10.3 Mechanisms Utilized by Insects to Suppress Transpiration

### 10.3.1 Cuticular Changes

Readers not familiar with the structure of the insect cuticle can refer to comprehensive descriptions by Hepburn (1985) and Hadley (1994). Briefly, the epicuticle, a thin layer at the external surface of the insect, serves as the interface between the insect and the environment, and underneath is the thick procuticle which represents nearly 95% of the cuticle. The procuticle is divided into three sections, the exocuticle, mesocuticle, and endocuticle, all consisting predominantly of a chitin–protein complex. The exocuticle hardens through sclerotization to provide support to the cuticle, the endocuticle remains soft and highly flexible, and the mesocuticle represents a transition between the endocuticle and exocuticle. Though the epicuticle is significantly thinner than the procuticle, it is responsible for regulating most of the

water flux through the cuticle. The cuticulin layer (or outer epicuticle) and the inner epicuticle layer (or dense layer) account for most of the epicuticle, and both are impregnated with lipids secreted by dermal glands. These lipids, which are mainly hydrocarbons, serve to waterproof the cuticle. The outer epicuticle is coated with a wax layer that is secreted through channels from cells below the procuticle. Lastly, the wax layer for some insect species is protected from the environment by an external cement layer composed of lipoproteins. Slight variations in the organization, structure, and composition of the cuticle can significantly alter the waterproofing capacity of the insect cuticle.

Water lost through the cuticle accounts for a significant portion of the total water lost by insects (Hadley 1994; Chown and Nicolson 2004). Commonly, species that reside in moist habitats have highly permeable cuticles and those that live in more arid areas have cuticles resistant to water flux (Edney 1977; Hadley 1994; Gibbs 1998; Bradley et al. 1999; Gibbs and Matzkin 2001). Three possible changes in the cuticle occur to alter water loss: (1) procuticle restructuring, (2) reorganization or changes in the epicuticle, particularly the lipids; and/or (3) the presence of a chorion or pupal case.

Changes in the procuticle have been well documented, but the involvement for this portion of the cuticle in reducing water loss has not been thoroughly studied (Hadley 1994). Some studies have suggested that the structure of the endocuticle may significantly alter the water movement across the cuticle (Machin and Lampert 1987; Hadley 1994). Increases in sclerotization of the cuticle could increase the density of the procuticle, thus improving water retention (Benoit et al. 2005). There is also recent speculation that cuticular proteins are involved in water regulation, but this has not yet been documented conclusively. Currently, no studies have documented changes in the procuticle during aestivation or diapause.

The best-known suppressor of water loss is cuticular lipids located on the epicuticle, particularly the wax layer (Blomquist et al. 1987; Hadley 1981; Lockey 1988; de Renobales et al. 1991; Hadley 1994; Gibbs 1998; Gibbs 2002b). The composition of the epicuticular lipids varies significantly between species, but the dominant constituents are nonpolar, hydrophobic compounds such as hydrocarbons and their derivatives (Blomquist et al. 1987; Hadley 1994; Gibbs 1998; Gibbs 2002b). It has been well-documented that disturbing the epicuticular lipids alters water loss rates of insects and other terrestrial arthropods (Noble-Nesbitt 1991; Hadley 1994), thus their importance as a hydro-insulator is well established. Increases in the amount of cuticular hydrocarbons results in lower cuticular water loss rates (Hood and Tschinkel 1990; Yoder and Denlinger 1991; Hadley 1994; Benoit and Denlinger 2007; Benoit et al. 2008a), and such increases in cuticular lipids have been well documented during diapause for many insects (Yoder and Denlinger 1991; Benoit and Denlinger 2007). In addition to changes in the amount of cuticular lipids, the composition can also change with long-chained, saturated lipids with few methyl side chains acting as a more effective water barrier (Hadley 1994; Gibbs 1998; Benoit et al. 2007a). In most cases, the differences in the cuticular hydrocarbons are the consequence of rearing or storing insects under different conditions. For example, summer-acclimated beetles have more long-chain hydrocarbons than winter-acclimated

beetles, and cuticular lipids change during thermal acclimation in grasshoppers (Hadley 1977, 1994; Gibbs and Mousseau 1994). Thus, it is likely that aestivating insects have modified profiles of epicuticular lipids, but I am not aware of any experiments that have explicitly tested this hypothesis.

Other characteristics that involve the relationship between lipids and water loss are activation energy ( $E_a$ ) and critical transition temperature (CTT) (Hadley 1994). These factors define how temperature influences water loss and cuticle changes (Toolson 1978; Rourke and Gibbs 1999; Gibbs 2002b; Yoder et al. 2005a,b). For many years it was assumed that the CTT involved a transition or melting of lipids that increased water loss rates (Wharton 1985; Gibbs 1998; Hadley 1994). Recent observations on CTT indicate that it does not correlate with increased water loss for many insects, is far above the lethal temperature and may be influenced by factors in addition to lipids; in combination these factors indicate that the CTTs may be an artifact that has no ecological relevance (Gibbs 2002b, Yoder et al. 2005a,b). Thus, the relevance of the CTT and  $E_a$  are in limbo, but trends have been observed. Insects with a low  $E_a$  are more resistant to water loss with increasing temperatures, and those with a CTT are actively suppressing water loss until a particular temperature is reached (Yoder et al. 2005b; Benoit et al. 2008a).

Additional water-proofing barriers can also occur, particularly during diapause and aestivation. The two most common examples are found in insects that are dormant as eggs or pupae. Insects that undergo dormancy as pharate first-instar larvae within the chorion of the egg are extremely resistant to dehydration. This is likely due to the presence of two water proofing barriers: the egg chorion and the larval integument. This high resistance to water loss has been demonstrated in many insect eggs (Krysan et al. 1977; Yoder et al. 2004; Roberts 2004; Benoit et al. 2007b) and is particularly striking in mosquito eggs that undergo diapause (Sota and Mogi 1992). For fly pupae, the puparium serves a similar function as the egg chorion; it provides an additional layer that helps to suppress water loss. Also, it is important to note that the inside of the puparium or chorion may be coated with additional lipids, providing even more resistance to water loss (Yoder et al. 1992).

### ***10.3.2 Respiratory Water Loss and Metabolism Reduction***

Along with water lost through the cuticle, respiration represents a secondary route usually accounting for 5–20% of the total water loss for most insect (Hadley 1994; Chown 2002). In comparison to hydrophilic species, insects residing in arid regions usually lose a higher proportion of water through respiration, up to 70%, than through their highly water-proofed cuticle (Hadley 1994; Chown 2002). During respiration, water is lost rapidly when the spiracles are open due to the humidity gradient within the tracheal system, which is very high, and the external environment, which is very low, thus causing an outward water flux (Hadley 1994). Water loss through the spiracles can be suppressed by opening within an internal cavity (Ahearn 1970; Benoit et al. 2005); such is the case for beetles with fused-elytra or

elytra that rarely open, such as in the tenebrionid, *Eleodes armata* (Ahearn 1970; Cloudsley-Thompson 2001) and the spider beetle, *Mezium affine* (Benoit et al. 2005). Another mechanism is to locate the spiracles closely together. This method increases the local humidity around the spiracle cluster, which in turn suppresses water loss through interference (Pugh et al. 1988). Both of these structural arrangements retard water flux out of the spiracles.

The most prominent mechanism to reduce respiratory water loss is the most simple, closing the spiracles. By closing or even partially blocking the spiracles, water loss, particularly at low relative humidities, is reduced (Bursell 1957; Hadley 1994; Chown 2002). Discontinuous gas exchange (DSC) can also reduce water loss through the spiracles by limiting gaseous diffusion to short periods when carbon dioxide accumulates to high levels (Lighton et al. 1993; Hadley 1994). Recent research has questioned the ability of DSC to reduce water loss, indicating that more studies are needed to determine exactly how DSC relates to water loss suppression (Sláma 1999; Chown 2002). Some of the arguments against DSC are that it is abandoned during periods of water stress, respiratory water loss only accounts for a small portion of water loss and that ability of DSC to limit water loss is dependent on increasing the  $PCO_2$  to the highest levels, which does not occur naturally (Chown 2002). Arguments for DSC are other factors such as hemolymph pH and oxygen demand may be more critical under dehydrating conditions than water retention due to changes associated with declining hemolymph volume, thus respiration increases during dehydration (Chown 2002). Even so, without spiracle control water loss rates would be higher (Bursell 1957; Lighton 1996; Sláma 1999; Chown 2002). One of the hallmarks of dormancy is the suppression of metabolic rate, which allow spiracles to remain closed for extended periods (Hadley 1994; Storey 2002; Denlinger 2002). In particular, flight is a period when water is lost rapidly through respiration (Lehmann 2001; Chown and Nicolson 2004), and flight is usually reduced during adult diapause (Denlinger 1986, 2002).

### 10.3.3 Excretory System

The elimination of waste is another conduit for the loss of water. The alimentary canal is responsible for the internal regulation of salt and water levels. The regions of the alimentary canal that regulate a majority of the fluid levels are the Malpighian tubules and the hindgut, divided into the ileum and rectum. Many studies have focused on the role of the alimentary canal on water and osmotic regulation, as reviewed by Bradley (1985) and Chown and Nicolson (2004). Briefly, water, along with organic molecules, particularly urine and ions, are absorbed from the hemolymph into the upper portion of the Malpighian tubules, and the urine is then actively concentrated in the lower portion of the tubules. The hindgut, particularly the rectum, acts as the primary site for the reabsorption of water and select solutes. Insects that are dehydrated or reside in dry regions generate extremely hyperosmotic rectal products as a result of efficient water reabsorption, while aquatic species, those

living in moist regions, or those feeding on water-diluted fluid have hyposmotic rectal products. In some larval Coleoptera, Diptera, and Lepidoptera, Malpighian tubules are not free floating, but instead the distal ends of the tubules are physically attached to the rectum; this arrangement known as the cryptonephridial system allows the feces to be dried extremely efficiently and in some species is associated with rectal water vapor uptake (Hadley 1994).

Secretion and absorption by the Malpighian tubules and hindgut reabsorption are regulated by neuropeptide hormones: diuretic hormones cause the secretion of water into the alimentary canal, thus increasing the net water loss, while antidiuretic hormones act in the opposite direction to retain water. Many of the neuropeptides involved in water balance are summarized in papers by Coast et al. (2002), Riehle et al. (2002), Gäde (2004), and Coast (2006). Some key examples are calcitonin-like peptides, corticotropin-releasing factor related peptides, insects kinins, and cardioacceleratory peptides, which all function as diuretic hormones, and chloride transport-stimulating hormone and an ion-transport process peptide that serve as antidiuretics (Coast 2006). The presence and absence of these hormones regulate urine production and subsequent reabsorption of the lost water. Studies involving aestivation and diuretic/antidiuretic hormones are currently lacking, but I suspect such hormones, particularly antidiuretics, may be involved in regulating water loss during dormancy.

### 10.3.4 Nitrogenous Waste

Uric acid is the dominant nitrogenous waste product produced by terrestrial arthropods, but guanine and other closely-related nitrogenous products are used by ticks and spiders (Hadley 1994; Benoit et al. 2008b). Why use uric acid rather than urea or ammonia? Ammonia is highly toxic and highly soluble, thus requiring insects to immediately expel this waste product, a response that would require large quantities of water; this metabolic end product is thus usually restricted to aquatic insects. Although urea is significantly less toxic than ammonia, it still has to be eliminated in solution. Increases in urea have been linked to increased temperature resistance (Storey 2004), thus accumulation of this molecule may be useful under certain environmental situations. Uric acid is the least toxic of the potential waste products for insects, and due to its low solubility, excretion of a nearly dry waste product is possible. Also, due to its low toxicity, uric acid can be accumulated within the body of the insect (storage excretion), a situation that completely prevents a loss of water by defecation. This is especially important for insects that aestivate as eggs or pupae since defecation does not normally occur during these stages. Uric acid accumulation occurs in adult insects as well, and has been documented in a few types of bugs, including the shield bug, *Parastrachia japonensis*, during diapause (Kashima et al. 2006). Some insects contain microbes that generate uricase, an enzyme that breaks down uric acid, thus allowing uric acid to be recycled (Sasaki et al. 1996; Kashima et al. 2006). The reduced metabolism associated with

dormancy also means that less nitrogenous waste is produced, thus insects in dormancy generally defecate less or not at all and if they do defecate they utilize uric acid, two features that maximize water conservation.

### ***10.3.5 Behavioral Changes***

Since aestivation is characterized by heat and water deprivation, changes in insect behavior are frequently involved in the response. The simplest response is to move into a more favorable region or to hide in protective harborages until favorable conditions return (Denlinger 1986). Long-distance migrations occurs in only a few insect species (e.g., Monarch butterflies), thus retreating to a near-by protected refugia is the most common response. Within these protective sites the relative humidity is usually higher and the temperature lower, both of which reduce water loss. The protective capacity of these sites can be enhanced by construction of a nest or by lining their microhabitat with silk or wax (Roubik and Michener 1980; Denlinger 1986; Kikawada et al. 2005). In many cases, plants can be altered by insect (e.g., galls) to generate small protective harborages (Chown and Nicolson 2004). Aggregation is also a frequent behavior response associated with dormancy. Formation of an aggregation increases the local humidity, suppressing water loss for members of the group (Yoder et al. 1993; Benoit et al. 2005, 2007b, 2007c). For example, adults of the tropical beetle, *Stenotarsus rotundus*, form aggregations of up to 70,000 individuals at the base of a palm tree in Panama (Wolda and Denlinger 1984; Tanaka 2000). As the group size increases, metabolic rate drops and water conservation is enhanced (Yoder et al. 1993). These benefits gained by forming an aggregation are likely adaptive advantages behind the frequent occurrences of aggregations during periods of aestivation.

## **10.4 Increases in the Water Pool during Aestivation and Diapause**

### ***10.4.1 Ingestion of Water***

Ingestion of water represents the main route used by terrestrial insect to replenish their water content (Hadley 1994). Many insects simply drink free standing water to rehydrate, and this is usually regulated by hemolymph volume (Chown and Nicolson 2004). For fluid-feeding insects, as with those that live in saline environments, presence of excess ions ( $K^+$  in plant phloem and  $Na^+$  in vertebrate blood and salt water) ingested needs to be removed (Wharton 1985; Hadley 1994). For blood feeders such as the bed bug, *Cimex lectularius*, blood represents the only source of water between long periods of quiescence (Benoit et al. 2007c). Moisture contained within solid food is sufficient to replace water stores for certain



insects. For example, food is the only water source for the spider beetle, *M. affine*, a species that survives on the water present in dry stored grain throughout its entire development (Benoit et al. 2005). Some insects retain access to free water during diapause if their habitat temperature remains above freezing and their dormant stage is capable of movement; e.g., females of the northern house mosquito, *Culex pipiens* (Benoit and Denlinger 2007). But, for many other overwintering species or aestivating species, water may be inaccessible and certain stages such as eggs and pupae do not have the capacity to imbibe water. Thus dormancy in such stages eliminates drinking as an option. Other insects, particularly tenebrionid beetles, ingest massive amounts of water that may push their water content over 70% before entering extremely dry periods (Hadley 1994; Zachariassen and Pedersen 2002).

### 10.4.2 Metabolic Water Production

Metabolism of food resources generates water and this water source is immediately transferred to the water pool of the insect (Edney 1977; Hadley 1994). The amount of water released in this manner is dependent on the biomolecules utilized: fat releases the most water (1.07 ml g<sup>-1</sup>), followed by carbohydrates (0.56 ml g<sup>-1</sup>) and proteins (0.40 ml g<sup>-1</sup>). Even though fat metabolism releases the most water, water is also utilized in the metabolism of fat, thus metabolism of sugar, particularly when stored as glycogen, is the most efficient form of metabolism for releasing water (Loveridge and Bursell 1975; Hadley 1994; Chown and Nicolson 2004). The contribution of metabolic water is rather small for most insects, but for species with extremely low water loss rates and those in flight the amount is proportionally higher. This proportional increase has been noted in a few species of desert-dwelling and dry-adapted beetles. For two tenebrionids (*E. armata* and *Cryptoglossa verrucosa*) and a spider beetle (*M. affine*), water generated by metabolism contributed substantially to their water pool during their prolonged exposure to dehydrating conditions (Cooper 1985; Benoit et al. 2005). Metabolic water production can be increased only if ATP accumulation can be reduced (Hadley 1994). This can be achieved by decoupling within the mitochondria to prevent the conversion of ADP to ATP, but this has been demonstrated only in wax moth larvae (*Galleria mellonella*, Jinda and Sehnal 1990) and is not considered by Hadley (1994) to be true for most insects. During periods of aestivation, water is unavailable for extended periods, thus I assume utilization of metabolic water is especially important at this time, yet there are actually few examples documenting the use of metabolic water by terrestrial arthropods during dormancy periods (Dautel 1999; Hadley 1994; Danks 2000). Most likely the paucity of examples reflects the fact that this question has not been adequately addressed. Also, since it is more efficient to retain water than generate water metabolically, increasing metabolism solely to generate water would deplete food reserves that are critically needed to survive extended dormant periods (Danks 2000). Thus, I suspect that water generated during aestivation is likely a small but vital byproduct of metabolism that contributes to the water pool during dry periods.

### 10.4.3 Water Vapor Absorption

Quite a few arthropods, including acarines, beetles, collembolans, fleas, grasshoppers, walking sticks, flies, lice and isopods, are able to absorb water from subsaturated air (<99% RH, Hadley 1994). A relatively complete list of arthropods capable of absorbing water vapor from the air is presented by Edney (1977), with later examples listed by Hadley (1994). Water vapor absorption is particularly important for dormant stages such as eggs, pupae and other stages that lack the ability to ingest free water (Danks 2000). Examples include diapausing flesh fly (*S. crassipalpis*) pupae (Yoder and Denlinger 1991), walking stick (*Extatosoma tiaratum*) eggs (Yoder and Denlinger 1992) and larvae of the ectoparasitoid, *Nasonia vitripennis* (Yoder et al. 1994). Although *S. crassipalpis* and *N. vitripennis* are both examples of species with temperate zone diapause, the example of the walking stick diapause is a subtropical example with an aestivation diapause, and indeed this species is able to absorb water vapor at relative humidities as low as 30% RH, the lowest reported critical equilibrium humidity (CEH, lowest threshold where water movement into and out of the insect is equal). Water vapor absorption is either active (requiring energy input) or passive (Hadley 1994; Bayley and Holmstrup 1999; Danks 2000). It is important to note that passive absorption occurs at all relative humidities and requires no energy, but it cannot counter water loss unless the relative humidity is at saturation (100% RH, Hadley 1994; Chown and Nicolson 2004). Potential sites utilized by insects for active water vapor absorption are the mouth and anus, and rarely water may be absorbed directly through the cuticle (Hadley 1994; Bayley and Holmstrup 1999). Oral and anal sites utilize hyperosmotic or hygroscopic secretions to absorb water (Knülle 1984; Hadley 1994). In the case of Collembola, absorption through the cuticle involves the accumulation of osmolytes (Bayley and Holmstrup 1999). Thus far, the mechanisms of water vapor absorption have been most extensively studied for nondormant insects, but it is likely that similar mechanisms observed will be evident in aestivating or diapausing insects. Larvae, particularly beetle and lepidopteron larvae, utilize rectal uptake mechanisms. (Edney 1977; Hadley 1994). Adult insects predominantly use oral mechanisms (Hadley 1994). Exactly how eggs and pupae absorb water vapor is currently unknown. Possibly pharate larvae and adults ingest or absorb water that has condensed on the inside of the chorion or puparium, but this remains speculation. Thus, the only aestivating insect known to absorb water from subsaturated air is the Australian walking stick, *E. tiaratum*, which aestivates in the egg stage but quite likely many more examples will be found as the study of insect aestivation expands.

## 10.5 Mechanisms to Reduce Water Stress

### 10.5.1 Protein and Molecular Changes

Protein and molecular responses common during dehydration are likely essential to insects during aestivation, but few insect studies have focused on this topic. In terrestrial snails, several stress-related proteins (HSPs, etc.) change in abundance as a result of

aestivation and appear to prevent dehydration-induced stress and mortality (Reuner et al. 2008). Insects may have similar responses during aestivation, but little is known. It is likely more efficient to upregulate proteins to prevent damage rather than repair damage after it has already occur (França et al. 2007; Lopez-Martinez et al. 2008, 2009).

Hsps are among the most studied proteins in relation to water balance (Tammariello et al. 1999; Bayley et al. 2001; Hayward et al. 2004; Sinclair et al. 2007; Lopez-Martinez et al. 2008, 2009). Three types of Hsps (smHsp, Hsp70, and Hsp90) have been linked to dehydration and diapause in insects, but no studies have been conducted on aestivating insects thus far. Many insects produce Hsps continually during diapause (Rinehart et al. 2007), but whether this is a component of aestivation is unknown. Nondiapausing insects also express one suite of Hsps during dehydration and a different suite during rehydration (Hayward et al. 2004; Lopez-Martinez et al. 2009). The increase of Hsps either prevents stress damage by acting to prevent unwanted biochemical interactions, by repairing protein damage or prompts the disassembly of proteins damaged by dehydration (Parsell and Lindquist 1993; Feder and Hofmann 1999; Rinehart et al. 2007). Recently, we have shown that Hsps are essential for insects to reach their normal dehydration tolerance by blocking their expression with RNA interference (Benoit et al. 2009b). Late embryogenesis abundant proteins (LEAs) seem to act by stabilizing the structure of proteins as the water content within the insect declines (Kikawada et al. 2008). Antioxidant enzymes, such as catalase and superoxide dismutase (SOD), are elevated during dehydration, presumably to reduce damage from oxygen radicals formed from desiccation-induced stress (França et al. 2007; Lopez-Martinez et al. 2008, 2009). Changes in proteins involved with the membrane and cytoskeleton may be fairly common in relation to dehydration (Li et al. 2009). One of the main functions of proteins involved with the cell membrane is to restructure the membrane to reduce water movement out of the cells as the hemolymph osmolality changes. Cytoskeletal protein changes serve to stabilize the cells during the pressure and size changes caused by the osmotic stress of dehydration (Li et al. 2009).

### ***10.5.2 Osmolality, Solutes and Regulation of Size and Volume***

Insect hemolymph osmolality, measured in  $\text{mOsm kg}^{-1}$ , ranges between 100 to 1,400  $\text{mOsm kg}^{-1}$  and for most insects is around 400–500  $\text{mOsm kg}^{-1}$  (Hadley 1994). It is important to note that increasing osmolality, even 2–3 $\times$ , reduces water loss only slightly, and the net water flux out of the insect persists unless the individuals are in an environment near saturation or above their CEH (Willmer 1980; Chown and Nicolson, 2004). The alimentary canal efficiently regulates ion content, and usually maintains osmolality within 200–300  $\text{mOsm kg}^{-1}$  range for most insects that reside in mesic and xeric regions, without causing internal damage. In some cases, insects sequester ions as insoluble forms, usually in the fat body, during dehydration and these ions can be release when the hemolymph volume increases during rehydration, effectively buffering the osmolality changes within the insect (Chown and Nicolson 2004). Additionally, combining free fatty acids and amino acids into large insoluble molecules can be used to regulate osmolality.

Poor osmoregulators usually have osmolalities that vary nearly 1000 mOsm kg<sup>-1</sup> (Hadley 1994; Elnitsky et al. 2008), and such insects usually reside in moist microhabitats. Osmolality fluctuations are influenced by a variety of molecules, including, but not limited to, salts (NaCl, KCl), polyols (glycerol), sugars (trehalose), free amino acids (proline, etc.), and free fatty acids.

Some of these molecules that increase during dehydration have protective qualities (Goyal et al. 2005; Benoit et al. 2009a). Trehalose and glycerol are two of the most common molecules that can suppress water loss and reduce stress (Yoder et al. 2006; Watanabe 2006). Trehalose is especially important during severe dehydration by preventing unwanted protein interactions, decreasing metabolism by altering fluid dynamics and protecting proteins and cellular membranes (Crowe et al. 1992; Suemoto et al. 2004; Goyal et al. 2005; Yoder et al. 2006; Benoit et al. 2009a). Additionally, it is important to note that glycerol and trehalose act to prevent heat and cold stress, resulting in cross-tolerance between temperature and dehydration stress (Yoder et al. 2006; Benoit et al. 2009a). Proline, as a free amino acid, may have similar effects (Yancey 2005; Ignatova and Gierasch 2006). This amino acid has been documented to increase during stress in a few insects (Michaud and Denlinger 2007; Michaud et al. 2008), but future studies are needed to determine the exact function of proline within insects during dehydration. Dehydration-induced changes have also been documented for glucose and sorbitol (Hadley 1994; Elnitsky et al. 2008). Currently, only two studies have looked for changes in protective osmolytes during aestivation. In the case of the blossom weevil, *Anthonomus pomorum*, trehalose was increased (Košťál and Šimek 1996), but no changes in osmolytes were noted in Mediterranean tiger moth, *Cymbalophora pudica* (Košťál et al. 1997).

Another factor that contributes to regulation of osmolality and water content is volume regulation and/or compartmentalization (Zachariassen and Einarson 1993; Hadley 1994; Zachariassen and Pedersen 2002). For example, a significant portion of water may be lost from one water pool (i.e., the hemolymph), but water in another region of the body (e.g., salivary glands and midgut) may remain relatively constant. Typically, water in the tissues is conserved at the expense of the hemolymph. This is particularly intriguing since the osmolality of the hemolymph is usually higher than within the cells during dehydration. Even though the exact mechanisms for retaining water in a tissue at the expense of the hemolymph are not known it is extremely important in order to retain the integrity of biologically active tissue in the body. Overall, much of the stress induced by dehydration can be reduced by regulating osmolality and water pools of the insects.

Body size change represents a mechanism that can increase dehydration tolerance of insects. A key example is provided by the experiments of Gibbs and colleagues on adaptations of *Drosophila* for dehydration resistance. In these studies, dehydration was used as a selective mechanism for *D. melanogaster*, and it was determined that increased water content (over 30%), along with respiratory changes, were the factors that increased dehydration resistance (Gibbs et al. 1997; Folk et al. 2001). Of interest, is the fact that size changes are not present in cactophilic, desert-adapted *Drosophila* when compared to mesic species, thus raising the question whether size changes are ecologically relevant or can only be used for interspecies comparisons

(Gibbs and Matzkin 2001). Size changes have also been noted during dormancy in insects. The size of the northern house mosquito, *C. pipiens*, increases substantially in preparation for the winter (Benoit and Denlinger 2007). Interestingly, the water pool does not increase; instead this represents an increase in the dry mass, but even so, the decrease in the surface area to volume ratio reduces water loss (Benoit and Denlinger 2007). Increasing body size would be a fairly simple mechanism for increasing dehydration resistance in aestivating insects.

### 10.5.3 Membrane Restructuring

As mentioned in Sect. 5.1, membrane changes can result in response to dehydration or in preparation for dormancy (Holmstrup et al. 2002; Tomala et al. 2006). Only a few such studies have been conducted in general and even fewer on insects. Of interest, dehydration-induced changes are minor in comparison to membrane restructuring that results from the induction of dormancy (Michaud and Denlinger 2006; Tomčala et al. 2006). Nearly all of these studies have focused on cold tolerance or the diapause syndrome (Košťál et al. 2003; Michaud and Denlinger 2006; Tomčala et al. 2006), but I expect that such changes also occur during aestivation. Most responses during diapause involved changes in glycerophospholipids that improve membrane fluidity and improve cold tolerance.

## 10.6 Extreme Dehydration Tolerance (Anhydrobiosis)

Anhydrobiosis as a mechanism to survive dry seasons deserves special attention. A loss 30–40% of body water content causes death in most insect (Hadley 1994). However, a few species can survive even after losing over 70% of their water content, and anhydrobiotic species can lose over 95% of their water content and still survive (Crowe et al. 1992; Danks 2000; Watanabe 2006). Most organisms capable of anhydrobiosis are relatively small, such as tardigrades, springtails and chironomid larvae (Watanabe 2006). Nearly all insects capable of losing over 70% of their water content are larvae within the midge family, Chironomidae (Suemoto et al. 2004; Watanabe 2006; Benoit et al. 2007a). Thus, the remaining discussion in this section focuses predominately on the mechanisms used by chironomid larvae to tolerate extreme dehydration.

For midge larvae to tolerate high levels of dehydration, a particular sequence of changes has been noted. First, water loss has to occur at a relatively controlled rate, which is done so by decreasing cuticular permeability, aggregating, or building structures to reduce water loss (Watanabe 2006). After dehydration reaches a certain point, high concentrations of certain ions trigger the synthesis of trehalose and other protective molecules such as glycerol and free amino acids (Watanabe 2006). Slow dehydration yields more osmolytes than rapid dehydration, which is the result of more time available to respond to dehydration stress (Kikawada et al. 2005;

Benoit et al. 2007a, 2009a). Within the insects, aquaporins regulate water movement between compartments (Kikawada et al. 2008). These channel proteins seem to be particularly important in tandem with cells accumulating trehalose, ensuring that vitrification and water-replacement occurs properly. Additionally, LEA and Hsp genes are upregulated during this time to avoid protein denaturation and aggregation (Watanabe 2006; Kikawada et al. 2008). Throughout this entire process, metabolism is suppressed to prevent oxidative stress and respiratory water loss (Benoit et al. 2007a, Lopez-Martinez et al. 2008). Oxidative stress proteins such as catalase and SOD may be increased to reduce dehydration-induced oxidative stress. Normal biological activity is resumed once larvae become rehydrated due to the presence of liquid water.

Overall, this ability to tolerate complete dehydration allows larvae to reside in seasonal water pools. When the dry season begins, these habitats quickly desiccate and the larvae respond accordingly. These responses by the larvae to dehydration prevent subsequent damage from heat and other stresses (Watanabe 2006; Benoit et al. 2009a). For *P. vanderplanki*, this cross tolerance is extremely important with temperatures in the African dry season pools reaching 60–70°C (Watanabe 2006). Cross tolerance is also evident in *B. antarctica*: dehydrated larvae are more tolerant to high Antarctic temperatures (30°C; Benoit et al. 2009a). Thus, high levels of dehydration allow chironomid larvae to survive a range of environmental stresses.

## 10.7 Water as a Development Cue

Moisture serves as a cue for breaking aestivation in a number of tropical insects after a long period of water scarcity (Masaki 1980; Denlinger 1986; Pires et al. 2000). Although water may be commonly used to break aestivation, it is used less commonly to initiate aestivation. Waiting until the dry season arrives may be too late to program the entrance into dormancy. Thus, other triggers such as temperature or photoperiod serve more commonly as the cues for the onset of aestivation. One key aspect is that a single, short exposure to water or high humidity does not usually cause termination of dormancy; termination more frequently requires a sustained exposure to wet conditions (Denlinger 1986; Watanabe 2006). Brief rains, which are not infrequent during the dry season, can replenish water stores, but using a brief rain to terminate aestivation could be disastrous if the favorable conditions prompted by the onset of rainy season have not been initiated. One way to prevent this accidental break is by suppressing moisture-triggered development until a fixed period of latency has elapsed (Denlinger 1986; Pires et al. 2000).

## 10.8 Conclusions and Future Directions

Since maintaining water balance is such a high priority for insects, it is not surprising that a variety of methods are used to accomplish this task, as shown in Table 1.

**Table 10.1** Factors that influence water balance characteristics of insects during dormant periods. + indicates it has been documented, (+) indicates it has not been demonstrated, but it will likely be established based on related insect physiology studies

	Aestivation	Diapause
<i>Suppression of water loss</i>		
Reduced respiration	+	+
Spiracle control	+	+
Increased cuticular lipids	(+)	+
Cuticle modifications	(+)	+
Presence of 2nd barrier	+	+
Size increases	(+)	+
Dry waste	+	+
<i>Water intake</i>		
Water vapor absorption	(+)	+
Metabolic water production	+	+
Ingestion of free water	+	+
<i>Stress-reduction</i>		
Proteins		
Heat shock proteins	(+)	+
Late embryo genes is abundant	(+)	(+)
Antioxidant	(+)	+
Cytoskeletal	(+)	+
Protective or colligative solutes		
Glucose	+	+
Glycerol	+	+
Proline	(+)	+
Sorbital	(+)	+
Trehalose	+	+
Membrane alterations	(+)	+
Osmolality regulation	+	+

As Table 1 indicates many of the same methods are likely used during aestivation and winter diapause, and I suspect that responses during aestivation and winter diapause will be seen as even more similar when more experiments have been completed. Why are the responses of diapausing and aestivating insects so similar? During winter (diapause) and summer (aestivation), the stresses in respect to moisture requirements are similar: a lack of free water and food resources and low ambient relative humidity. The only major difference is temperature. While temperatures are low during the winter resulting in lower water loss rates, aestivating insects are exposed to high temperatures, which further reduce their ability to retain water. Additionally, only certain biological factors can be altered without expending considerable energy, thus, overlap between the responses of aestivation and diapause is even more likely. Due to higher temperatures, aestivation, particularly during the dry seasons in tropical regions, is likely a more challenging period to maintain water balance than winter diapause even though both types of dormancy use similar methods to increase dehydration resistance.

The paucity of research on insect aestivation indicates a clear need for more focus on this topic if we are to understand the mechanisms involved in water balance of dormant insects. Particularly, studies that focus on environmental cues that trigger aestivation-based changes in behavior and physiology are required. This is not an easy task. To use insect diapause as an example, many groups have focused on triggers, changes and termination of diapause, but considerably more research is still needed to elucidate the fine points of these mechanisms (Denlinger 2002). With the considerable overlap noted in the water requirements of winter diapausing and aestivating insects, it is likely that other physiological similarities exist. Based on this observation, it is reasonable to use research on winter diapause as a baseline for expanding research on aestivation. Previous papers by Denlinger (2002) and Košťál (2006) discuss changes in diapause that could be utilized for comparative work on aestivation.

Responses of other aestivating organisms, such as snails, anurans, and fish (Storey 2002, 2004) have been studied more thoroughly, and offer potential insights that may also be applicable to insect aestivation. To reduce water stress, mechanisms such as reduced respiration and metabolism, increased cuticle barriers, changes in osmolality, and the ability to tolerate losing a high proportion of water content are used by organisms such as snails and anurans (Storey 2002). Many proteins have been studied for noninsect aestivating organisms (Storey 2002), and comparative studies could be beneficial for elucidating protein expression themes for insects.

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