MOLECULAR RESPONSES TO ENVIRONMENTAL STRESS IN TEMPERATE AND POLAR FLIES

DISSERTATION

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ABSTRACT

Understanding the mechanisms used by insects to survive stress is critical for understanding adaptations to the environment and may suggest future tools for pest management. To this end, two very different flies from different parts of the world were examined to determine how they deal with environmental stressors in their habitat. The insects examined include the fruit fly *Rhagoletis pomonella* and the Antarctic midge, *Belgica antarctica*, two species that spend most of their immature period in the soil.

The apple maggot, *Rhagoletis pomonella*, is a major pest of domesticated apples. It can spend most of the year (~11 months) in the soil in a deep diapause. During this time, the pupae are vulnerable to the low temperatures of winter as well as the warm temperatures of early summer. In late summer, adults emerge and oviposit into apples, where the larvae spend several weeks. The larvae then drop to the soil and pupariate. Most pupae enter a deep diapause (300 d), some enter a shallow diapause (100 d), while others directly develop. We cloned and monitored expression of the genes encoding heat shock proteins (Hsps) 70 and 90, molecular chaperones involved in a wide variety of stress responses in insects. Larvae reared at 28°C for 2 weeks did not express Hsp70, but Hsp70 was highly upregulated in larvae reared at 35°C. Hsp90 was expressed in larvae reared at both temperatures. Infested apples were also placed in the field for a
24 hr period during August, and the Hsps were monitored. During this time, the core temperature of the apples exceeded 40°C for at least 2 hrs per day. Hsp70 was mildly upregulated in the field at 9:30 am and upregulation was maximal at 5:30 pm. By 11 pm Hsp expression again declined and by 6 am the next day there was no signal. Hsp90 was again constitutively expressed during the entire day, but a strong upregulation was also noted at 5:30 pm. Hsp expression was also noted during diapause. Diapausing pupae were sampled at 30, 60, 90, 120, 150 and 250 days post pupariation. Hsp70 was expressed throughout diapause but was further upregulated at 120 d (deep diapause). This further upregulation correlates with the onset of winter and lower temperatures, suggesting a possible cold tolerance function for Hsp70 in this pupal diapause as documented for the pupal diapause of another fly, Sarcophaga crassipalpis. Hsp90 was constitutively expressed throughout diapause. We thus provide evidence that certain Hsps cycle daily during larval development under field conditions, and their expression is developmentally upregulated during deep diapause.

The desiccation tolerance of the apple maggot was evaluated for the overwintering diapausing pupae. These pupae have water loss rates similar to that of another desiccation-tolerant fly pupae, Sarcophaga crassipalpis. To investigate the involvement of stress genes, we monitored expression of Hsp70 and Hsp90 during dehydration. The antioxidant enzymes, superoxide dismutase (SOD) and catalase, were cloned and also monitored during dehydration, heat shock and diapause. We found that Hsp70, SOD and catalase were strongly upregulated during dehydration, while Hsp90 was downregulated compared to the controls. SOD was downregulated but expressed at a constant low level.
throughout diapause. Catalase was also downregulated during diapause. SOD and catalase were upregulated in response to heat shock. These results suggest that Hsp70 is crucial in minimizing protein misfoldings and aggregation during periods of water loss, while SOD and catalase scavenge reactive oxygen species that are produced as a result of dehydration.

We also collected several thousand pupae from the field (Grant, MI) and placed them into laboratory conditions to generate direct developers, shallow diapausers and deep diapausers. We monitored weight, size and eclosion time to see if we could identify determinants of these three developmental programs. We found no correlation between weight and/or size with eclosion time or diapause incidence.

Larvae of the Antarctic midge, *Belgica antarctica*, spend nearly 11 months each year encased in a matrix of substrate and ice. During that time the larvae are exposed to the dehydrating conditions of being surrounded by ice, but they also experience numerous freeze and thaw cycles. Those conditions can lead to the generation of oxygen radicals, and thus we monitored the expression of antioxidant enzymes and heat shock proteins. We subjected the larvae to heat shock, freezing, anoxia, direct sunlight and ultraviolet radiation, five of the main stressors that the larvae normally confront. We also evaluated the antioxidant capacity of the larvae and the adults, and monitored markers of oxidative damage. We found that SOD was constitutively expressed by larvae, but not adults, and this expression was not further upregulated by any of the treatments, suggesting maximal threshold levels of expression for this gene (i.e. overexpression). Catalase was expressed in both the larvae and the adults, but larval
expression was far stronger. Catalase, a small heat shock protein and Hsp70 were strongly upregulated in response to sunlight, as well as to ultraviolet radiation A (UVA). The total antioxidant capacity of the adults was higher than that of the larvae, suggesting an antioxidant protection system for sperm and eggs as seen in the mosquito, *Aedes aegypti*. The fact that markers of oxidative damage (lipid peroxidation and carbonyl proteins) showed no changes between the control and the treatments demonstrated that the Antarctic midge is highly resistant to oxidative stress produced by heat shock, freezing and anoxia.

Previous work showed that larvae of the Antarctic midge are prone to desiccation and thus use changes in metabolic rates leading to lower water loss rates, increased cryoprotectants and clustering to manage water loss. To study the genes involved in desiccation tolerance of *B. antarcica* suppression subtractive hybridization (SSH) was carried out by subtracting cDNA from dehydrated larvae from cDNA from hydrated ones. This resulted in 93 cDNA clones, of which 28 were found to be responsive to dehydration. Fifteen candidate genes (*a small Hsp, Hsp70, Hsp90, superoxide dismutase, catalase, metallothionein, P450, fatty acid desaturase, phospholipase A2 activating protein, fatty acyl CoA desaturase, actin, muscle-specific actin, a zinc-finger protein, pacifastin and VATPase*) were chosen from that group and their expression was monitored during fast and slow dehydration, cryoprotective dehydration, fast and slow rehydration and overhydration. The upregulation of all fifteen genes in response to dehydration and the constant expression of some of them during rehydration and overhydration suggests that the larvae of *B. antarctica* upregulate a suite of genes during
periods of water loss and gain that assist in protein folding, scavenging of oxygen radicals, cell membrane and cytoskeletal rearrangement, as well as cellular homeostasis.

This work demonstrates how insects respond to certain environmental stressors (heat shock, freezing, anoxia, sunlight, ultraviolet radiation, dehydration, rehydration, overhydration) and how that response may be widely distributed amongst insects, whether they are from the temperate zone or the polar region. These data show that the insect stress response is not limited to Hsps, and highlights the first examples of SOD and catalase being upregulated in response to dehydration in insects. Thus, to understand how insects survive stressful and life-threatening conditions, we must look at a bigger picture of all the changes that occur at the cellular and tissue levels, as well as synergism in gene expression.
To my parents
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CHAPTER 1

INTRODUCTION

Insects are found in nearly every habitat on the planet, living, thriving and reproducing in some of the most inhospitable environments. However, due to their small size, insects are quite vulnerable to environmental stress and conditions that are barely harmful to larger animals may be harmful to insects. I refer to insect stress as any condition naturally-occurring or artificially-created that threatens normal insect life and can lead to death. One of the main stresses that insects face is temperature. High temperature leads to protein denaturing and inactivation of the ribosomal machinery (Pardue et al. 1987), and death if the heat shock is severe enough (Denlinger et al. 1991), while low temperature causes immobilization and damage to the cell membrane by altering the phase of its lipids (Lee 1991). However, dehydration may be one of the most important stresses for an insect to overcome due to their great amount of surface area to volume ratio, which promotes desiccation. Not only does water loss directly threaten normal cellular function but the increase in production of oxygen radicals during the dehydration process is believed to be responsible for death by desiccation (Pereira et al. 2003). Insects must also deal with changes in oxygen concentration, toxic plant chemicals consumed during feeding, ultraviolet radiation and pathogens, among others.
Many insects enter a hibernation-like state called diapause during the winter months. Although diapause is obviously important for circumventing periods of environmental stress, diapause itself may be stressful because it involves cell cycle arrest and extreme metabolic depression. To counter such stress, insects use common defense mechanisms to cope with environmental conditions and undue stress. Among the most prominent of these defense mechanisms are the heat shock proteins and the antioxidant enzymes. The use of these genes by insects is the focus of my work.

**Heat shock proteins (Hsps)**

One of the most problematic conditions for any organism is maintaining normal cellular function during periods of stress. Small changes in temperature can affect cellular functions such as protein folding, degradation and accumulation of denatured proteins. A common stress countering system used universally by living organisms involves the stress proteins known as heat shock proteins (Hsps). Hsps are among the highest conserved proteins known, and homologous genes have been found in both prokaryotes and eukaryotes (Linquist 1993). These proteins are molecular chaperones that are involved in numerous processes in the cell under normal conditions but can be upregulated as needed under stressful conditions. Mostly they are involved in maintaining proper protein function by preventing misfolding and repairing misfolded proteins. They also prevent protein aggregation and promote degradation of unrepairable misfolded and denatured proteins (Parsell and Lindquist 1993). In extreme cases, Hsps can reverse protein aggregation (Glover and Lindquist 1998). Hsps have been found to

There are five families of Hsps: smHsps, Hsp60, Hsp70, Hsp90 and Hsp100. The small Hsp family includes members ranging in size from 12 to 40 kd (Parsell and Lindquist 1993). These are the least conserved of the Hsps, as well as the least understood. They are true molecular chaperones involved in countless cellular processes under normal conditions, but they are also inducible during stress. In insects, smHsps have been found to be involved in diapause (Yocum et al. 1998), dehydration (Tammaro et al. 1999), heat and cold (Yocum et al. 1991). In addition, small Hsps have been found to be responsive to oxidative stress and ultraviolet radiation (Feder and Hofmann 1999).

Members of the Hsp60 family are known as GroEL and Chaperonin-60 and are one of the most abundant proteins inside the cell under normal temperature conditions (Lindquist 1993), but during stress conditions their abundance can be as high as 15% of the total cellular protein (Parsell and Lundquist 1993). Hsp60 has greater affinity for completely denatured proteins, which other Hsps do not target. In insects, work detailing the involvement of Hsp60 is lacking but recently Hsp60 was found to be upregulated during the diapause of the flesh fly, Sarcophaga crassipalpis (Rinehart et al. 2007).
Hsp70 is the most studied and greatest conserved Hsp. Members of this family can be divided into two groups, the inducible form (Hsp70) and the cognate form (Hsc70). This group was originally found to be upregulated in response to heat in *Drosophila melanogaster*, and that response occurs in as little as 15 minutes (Lindquist 1980). Hsp70 is ATP-dependent and calmodulin-independent. After induction, it has been found to increase up to 1000 times in protein concentration compared to pre-stress concentration (Denlinger et al. 2001). Hsp70 is involved in insect diapause and it is developmentally upregulated during this time in a number of organisms where presumably it promotes cold and heat tolerance for winter and summer diapause, respectively. Hsp70 has been is involved in response to oxidative stress (Williams et al. 2006, Hosoi et al. 1997), apoptosis (Gupta et al. 2007, Beere et al. 2000), dehydration (Tammariello et al. 1999, Hayward et al. 2004, Jonsson and Schill 2007, Schill et al. 2004), ultraviolet radiation (Bonaventura et al. 2005, Matranga et al. 2006, Niu et al. 2006), even hymenopteran venom (Rinehart et al. 2002).

Hsp90 is essential in eukaryotic organisms under normal temperature conditions (Borkovich et al. 1989). Members of this family include Hsp82 and Hsp83, which get their name from being slightly smaller than most of the members in this family. A true molecular chaperone under normal conditions, Hsp90 interacts with other cellular proteins like kinases, hormone receptors, calmodulin, actin and tubulin (Parsell and Lindquist 1993). Those interactions are highly specific, long-lived, prevent the aggregation of those proteins, and promote the disaggregation of aggregated proteins. Hsp90 is ATP-independent but calmodulin dependent. To function properly, Hsp90 must
have calcium attached to one of its receptors. In the absence of calcium, the protein binds to itself, folds and loses confirmation and function (Minami et al. 1993). Under stress conditions, Hsp90 helps in maintaining regular cellular processes and growth at high temperatures. In addition to heat, Hsp90 is involved in certain insect diapauses (Chen et al. 2005), and rehydration (Hayward et al. 2004).

Hsp100s are also highly conserved, but little is known about their function in insects. Hsp100 is heat inducible and is expressed during periods of high temperature (Parsell and Lindquist 1993). However overexpression of Hsp70 can compensate during periods of high temperature if Hsp100 is non-functional (Sanchez et al. 1993). Hsp100s are unique among heat shock proteins in that they are the only Hsp that can reverse protein aggregation (Glover and Lindquist 1998). This was a very controversial issue when it was first proposed in 1996, but since then there has been quite a bit of work detailing the mechanism by which Hsp100s do this. It involves a combination of Hsp100 with Hsp70 and Hsp40 (a small Hsp), where Hsp100 cleaves individual polypeptide chains from the aggregated protein and transfers them through an internal channel to be collected at the other end by another chaperone (Bösl et al. 2006).

Even though these Hsps, as a group, are required for normal cellular processes while still being responsive to a wide variety of stress, they can have deleterious effects. High concentrations of Hsps can be toxic to cells by interfering with ongoing processes or altering function in the cell (Feder and Hofmann 1999). In addition to that, the production of Hsps is a costly process and maintaining strong expression for long periods of time can consume most needed nutrients (Calow 1991, Hoffmann 1995). In the case
of diapausing insects this is particularly crucial as they must survive the winter with the energy stores produced prior to diapause initiation. Therefore the expression of these proteins during diapause must be delicately balance in order to have enough energy left to complete diapause.

**Antioxidant enzymes (AOEs)**

All living creatures have to deal with oxygen radicals created through the process of oxygen metabolism. In addition, highly reactive oxygen based compounds like hydrogen peroxide can have the same deleterious effects as free radicals, and combined they are known as reactive oxygen species (ROS). These ROS will rapidly react with a numbers of molecules and lead to problems with proteins, cell membrane and DNA. In proteins, ROS can lead to amino acid modifications, fragmentation of the peptide chain, and even total enzyme inactivation (Stadtman 1986). Cell membranes are more vulnerable when they are more saturated because ROS targets double bonds, thus disrupting membrane fluidity (Halliwell and Gutteridge 1999). This kind of damage is called lipid peroxidation and its degradation products can initiate apoptosis (Green and Reed 1998). DNA damage ranges from deletions and mutations to base degradation and single-strand breakage (Imlay 2003). The main antioxidant enzymes are superoxide dismutase (SOD) and catalase. However, there are other enzymes that also scavenge ROS like peroxidases (ascorbate peroxide, glutathione S-transferase peroxidases) and glutathione reductases (Felton and Summers 1995). Insects lack glutathione peroxidase,
a common enzyme that breakdown hydrogen peroxide in other organisms (Smith and Shrift 1979).

Superoxide dismutase (SOD) catalyzes the breakdown of the most abundant oxygen radical, the superoxide anion ($O_2^-$), into hydrogen peroxide and water (Fridovich 1995). There are two distinct forms of SOD in insects, the mitochondrial form (SOD2 or SOD-MN) and the cytoplasmic one (SOD1 or SOD Cu-Zn). During periods of oxidative stress, both SODs are upregulated at different concentrations, but if either one is knocked out or is absent, the remaining SOD is upregulated even further to counteract the loss and prevent oxidative damage (Pereira et al 2001). In *Drosophila melanogaster*, SOD overexpression was found to extend the life of larvae (Orr and Sohal 1994, Sun and Tower 1999). An even stronger effect on lifespan was recorded for adults of *Drosophila* and *Caenorhabditis elegans* when a combination of SOD and catalase was used (Orr and Sohal 1994, Melov et al. 2000, Sampayo et al. 2003). This suggests an important synergistic effect of these AOE{s} on reducing oxidative damage and prolonging life span.

Catalase is the enzyme that breaks down hydrogen peroxide ($H_2O_2$) into oxygen and free water. There are several enzymes that do this and they are commonly called peroxidases. But, many of the peroxidases are restricted to specific body tissues, while catalase is a universal scavenger of hydrogen peroxide. Catalase is an interesting enzyme as its mRNA has a very short poly-A tail, and this likely leads to the fast degradation of its signal in the presence of light (Lesser 2006). In *Drosophila melanogaster* and *Musca domestica*, catalase was found to extend life span of the adult flies, either with SOD (Orr and Sohal 1994) or by itself (Bayne and Sohal 2002). Catalase is also responsive to
desiccation, and it is upregulated in yeast during in response to desiccation (Franca et al. 2005). The expression of catalase during dehydration is not fully understood, but there are two mains reasons that have been proposed. Catalase breaks down hydrogen peroxide, which is not an oxygen radical but rather a ROS with great oxidizing ability. Catalyzing hydrogen peroxide not only protects the cell membrane from lipid peroxidation, but that break down yields free water that may also play a role in stabilizing cells during desiccation. Recently a massive EST project in the arctic collembolan, *Onychiurus arcticus*, found both SOD and catalase in desiccated libraries (Clark et al. 2007). This suggests that both antioxidant enzymes are crucial during periods of water loss as several clones of each gene, mostly catalase, were recovered.

**Temperate zone fly**

The apple maggot, *Rhagoletis pomonella*, is a fruit fly in the family Tephritidae. Members of this family are called true fruit flies because the larvae feed within the confines of a fruit to complete their development. These flies are specialists that normally feed and mate within a single host. Ancestrally this native fly fed on hawthorns (*Crataegus* spp.), a native host. Following European colonization of North America and the introduction of apples, a host shift was observed for this species when the maggots were noticed to emerge from apples (Walsh 1867). The host shift occurred while both plants coexisted in geographic proximity without any barriers preventing the flies from reaching the other plant. When a host shift or a new species arises in such a manner, it is said to have occurred in sympatry (i.e. sympatric speciation). This host shift lead to two
distinct populations of *R. pomonella*, one that still feeds on hawthorns and the new population that feeds on domesticated apples (*Malus pumila*). Even though there is debate as to whether these two distinct populations are different species, it is clear that they are distinct host races with different hosts and reproductive strategies. Because of this, the apple maggot has become the classic example for sympatric speciation in animals in North America and has attracted much work because of this important evolutionary example.

The apple and hawthorn races display a very high degree of host fidelity. Even when the host trees occur in such sympatry that their branches cross each other, only about 6% of the population will go to the other’s host plant (Feder et al. 1994). Because hawthorns mature in September and apples mature in August, the host shift probably resulted in the altered diapause behavior that these host races display. The adults emerge days before fruit maturity occurs in their respective host, and undergo gonad maturation in under 10 days (Prokopy et al. 1971). At this point, the flies mate on the surface of their host plant, and the females oviposit into the fruit. It takes two to three weeks, depending on temperature, for the larvae to develop within the fruit and then they fall to the ground, burrow and pupariate. It is at this point that the pupae can go into one of three different developmental pathways. Some pupae will directly develop into adults in about 30 days when host fruit may not be available. The remaining pupae will go into diapause. The first group of diapausing pupae will emerge between 60 and 100 d after going into diapause (Dambroski and Feder 2007). This group emerges in the middle of winter, during a time when no fruit is available and thus there is no fitness advantage to
this strategy. However, most of the diapausing pupae will go into a deep diapause that lasts almost 11 months and requires experiencing a period of low temperature in order to break diapause (Feder et al. 1997). This long diapause can also last as much as 22 months but this only happens in less than 10% of the deep diapausing population (personal observation).

**Polar fly**

The Antarctic midge, *Belgica antarctica*, is the southernmost hotometabolous insect in the world, and it is endemic to the maritime Antarctic (Convey and Block 1996). It is a wingless chironomid that has a patchy distribution in the Antarctic Peninsula (Gressit 1967). At 5 mm, *B. antarcticca* is the largest free-living, year round terrestrial resident of Antarctica. The larvae hatch in the austral summer and must undergo two winters frozen before pupating and emerging as adults on the third summer (Sugg et al. 1983). Larvae can overwinter in any of the four instars; all instars are freeze-tolerant. The adults live for an average of 10 days during the austral summer. Given that the adults are short-lived and are freeze-intolerant, they do not overwinter. The larvae feed on animal detritus, algae, fungi and bacteria (Baust and Edwards 1979), and are normally associated with the alga *Prasiola crispa* (Sugg et al 1983). However, dense aggregations of larvae have also been recorded in association with animal detritus, grass (*Deschampsia antarctica*), moss (*Drepanocladius* sp.), mats of cyanobacteria (*Phormidium* sp.) and nutrient-rich soils in the vicinity of seal wallows and bird nests (Peckham 1971, Richard et al. 1994).
The larvae spend a large portion of their life over-wintering while encased in their substrate and ice. During this time multiple cycles of freezing and thawing occur (Block 1997), which can lead to devastating cellular damage. Being in the presence of ice during the winter leads to dehydration, and the larvae have several strategies in place to cope with this, like reduced metabolic rates that lead to reduced water loss rates, production of cryoprotectants, clustering, and cryoprotective dehydration (Benoit et al. 2007, Elnitsky et al. 2007).

My goals

Comparative physiology studies can yield great amounts of knowledge and understanding. My goal was to compare the molecular insect stress response of a temperate zone insect with a polar one. To do this I decided to focus on several specific stresses: heat, desiccation and their overwintering state. The apple maggot, Rhagoletis pomonella, is an insect that spends as much as 11 months burrowed in the soil in an inactive pupal diapause. During this time, the pupae are vulnerable to the freezing temperatures of winter, the dryness of the soil and the high temperatures of early to mid summer. A very different insect is the Antarctic midge, Belgica antarctica, a soil-dwelling insect that spends nearly 11 months encased in ice in Antarctica, dealing with freezing temperatures and numerous freezing and thawing events. I tested the heat shock response and the antioxidant protection of these insects by looking at heat shock protein (Hsp) and antioxidant enzyme (AOE) expression.
I cloned heat shock proteins 70 and 90 from the apple maggot and used northern blot hybridizations to monitor their expression during larval feeding inside the apple under laboratory conditions and in the field. I also looked at Hsp and AOE expression during diapause, dehydration and rehydration.

A suppressive subtractive hybridization (SSH) was performed for the Antarctic midge, comparing control and dehydrated larvae. Because periods of no oxygen normally accompany periods of freezing, anoxia was also tested in the Antarctic midge, in addition to heat shock and freezing. I also monitored the expression of Hsps and AOE in response to direct Antarctic sunlight, UVA and UVC radiations. The expression of Hsps, AOE and several other genes was tracked during fast and slow dehydration, fast and slow rehydration and overhydration.
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CHAPTER 2

Heat shock proteins are upregulated in larvae of the apple maggot, *Rhagoletis pomonella*, during hot summer days and in pupae during their overwintering diapause.

ABSTRACT

Developing larvae of the apple maggot, *Rhagoletis pomonella*, are frequently exposed to summertime apple temperatures that exceed 40°C, and during their overwintering diapause pupae are exposed to sub-zero soil temperatures for prolonged periods. To investigate the potential involvement of heat shock proteins (Hsps) in response to these environmental extremes, we cloned the genes encoding Hsp70 and Hsp90 in *R. pomonella* and monitored expression during larval feeding within the apple and during its overwintering pupal diapause. Larvae reared in the laboratory at constant temperatures of 25, 28 or 35°C expressed *Hsp90* but very little *Hsp70*. Larvae did not survive rearing at 40°C. The temperature cycles to which larvae were exposed inside apples in the field, ranging from 16 to 46.9°C in a 24 h period, elicited strong *Hsp70* and *Hsp90* expression that began at mid-day and reached a peak in late afternoon, coinciding with peak air and apple temperatures. *Hsps* were also expressed strongly by pupae during their overwintering diapause. *Hsp70* was not expressed in non-diapausing pupae.
but was highly expressed throughout diapause. *Hsp90* was constitutively expressed in both diapausing and nondiapausing pupae. *R. pomonella* thus strongly expresses its *Hsps* during pupal diapause, presumably as a protection against low temperature injury, and during larval development to cope with natural temperature cycles prevailing in late summer.

**INTRODUCTION**

The apple maggot, *Rhagoletis pomonella*, is a North American species that fed exclusively on hawthorns (*Crataegus* spp.) until the introduction of domesticated apples (*Malus pumila*) following European colonization (Walsh 1867). The introduction of apples allowed a host shift resulting in a new population that quickly became a major apple pest (Illingworth 1912, Bush 1969). Though the hawthorn and apple populations occur in close proximity, they are separated in time due to host phenology (Bush 1966). Apples mature in August and hawthorns in September, and a high degree of host fidelity suggests that these two variants should be considered different host races (Feder *et al.* 1994). Adults mate on the fruit surface, oviposit into the fruit, and the larvae feed for approximately two weeks, depending on temperature. After completion of feeding, the larvae leave the fruit, drop to the soil and burrow underground to pupariate and enter a facultative overwintering pupal diapause (Prokopy 1968).

In this study, we examine expression of the genes encoding heat shock proteins (*Hsps*) in both the feeding larvae as well as the overwintering pupae in diapause. We hypothesize that the *Hsps* may be quite important for larval survival due to the high
temperatures that likely prevail in apples during late summer. Previous work with *Drosophila melanogaster* suggests that fruit temperatures can sometimes be high enough to generate the production of Hsps (Feder 1997). We cloned the genes encoding two of the Hsps, *Hsp70* and *Hsp90*, monitored their expression in response to different rearing temperatures in the laboratory and under field conditions within an apple orchard. The fact that Hsps are also known to be upregulated during diapause in numerous species (Rinehart *et al.* 2007) prompted us to also monitor expression of these two Hsps during different phases of diapause in *R. pomonella*.

**MATERIALS AND METHODS**

*Insect rearing*

The stock colony of *Rhagoletis pomonella* (Walsh), derived from a laboratory colony maintained at the New York State Agricultural Experiment Station in Geneva, was reared on ‘delicious’ apples at 25°C under long day (15L:9D) conditions. Apples with oviposition punctures were placed in environmental chambers (Percival-Scientific, Perry, IA) at 28, 35, or 40°C under long day (17L:7D) conditions. Feeding larvae were dissected from the apples, and wandering larvae were collected from cups containing infested apples. Diapausing pupae were generated by rearing larvae at 18°C under short day (8L:16D) conditions. Diapausing pupae continued to be held at 18°C (8L:16D) and were sampled 30, 60, 90, 120, 150 and 230 d post pupariation.
**Field observations**

Apples containing oviposition scars were transferred from the laboratory to an apple orchard (Waterman Farm, Ohio State University, Franklin County), where they were suspended with string to branches of ‘delicious’ apple trees bearing fruit of approximately the same size as the laboratory apples. Transfers were made a few days before the larvae were expected to exit the apples; under our rearing conditions larvae exit the apple approximately 15 d after oviposition. Temperature probes (Onset Corp, Bourne, MA), approximately 3cm in length, were inserted into the top surface of each apple, halfway into the fruit, and into the core. Differences among the temperature probes within any given apple did not exceed 0.25°C, thus only temperatures of the probes nearest the larvae are shown. An additional probe was placed in the air several centimeters from the apple to record air temperature. Apples were removed from the trees the day after they were transferred at 7:00, 9:30, 13:30, 17:30 and 23:00, and carefully cut open to remove the larvae. Groups of larvae (10 to 14) were then immediately subjected to RNA extraction. Preliminary temperature data and RNA samples were collected in the summers of 2005, 2006, and 2007 with nearly identical results. Experiments reported here were conducted on August 22-24, 2007.

**Clones**

Clones of *Hsp70* and *Hsp90* were obtained using previously available primers (Rinehart *et al.* 2006a, b). The initial 200 bp sequence of *Hsp70* was extended to the full
coding sequence using 5’ and 3’ Rapid amplification of cDNA ends (Race) kits (Invitrogen, Carlsbad, CA). The clone of *Hsp90* consisted of 710 bp. A 210 bp fragment of 28S was cloned from homology primers designed for dipterans (forward primer 5’-CTGTGGATGAACCAAACGTG-3’ and reverse primer 5’-TGTACGCCAGCGGTGTAATGTA-3’).

*Northern Blot Hybridization*

RNA was extracted from groups of 10-14 larvae or pupae (~70 mg) using Trizol reagent (Invitrogen) in accordance with the manufacturer’s protocol. 4 µg of RNA was run on a 1.4% agarose, 0.41 M formaldehyde gel, and the RNA was transferred to a positively-charged nylon membrane (Hybond-N+, Amersham Biosciences) using the rapid downward transfer system from Schleicher & Schuell. DNA clones were labeled using Roche Diagnostics’ DIG-High Prime labeling kit, and hybridization was done with their DNA Labeling and Detection Starter Kit II. Membranes were exposed to Blue Lite Autorad Film (ISC BioExpress) for 5 to 30 min, depending on probe strength. All membranes were stripped in accordance with the DIG kit guidelines and re-hybridization was done using 28S as the control gene. All northern blots were replicated five times.

**RESULTS**

*Hsp70, Hsp90 and 28S sequences*

Identity of the full length clone of *Hsp70* (GenBank accession no. EF103584) was confirmed by nucleotide and amino acid comparisons. The sequence consists of two
regions, the ATP domain (amino terminus) and the substrate binding region (carboxyl terminus), that are characteristic of the Hsp70 family. The clone shares 84% and 89% amino acid identity with Hsp70 from *Ceratitis capitata* (Mediterranean fruit fly) and *Delia antiqua* (onion maggot), respectively (Fig. 1).

The partial clone of *Hsp90* (GenBank accession no. EF397426) was confirmed by nucleotide and amino acid comparisons. The 710 bp region that was cloned is highly conserved among Hsp90s and shares 97 and 89% amino acid identity with Hsp83 from *C. capitata* and Hsp90 from *D. antiqua* (Fig. 2).

The 210 nucleotide clone of *28S* (GenBank accession no. EU559628) was confirmed by nucleotide comparisons and shares 98% identity with three other members of the family Tephritidae: *Trypeta tortilis*, *Euleia fratria* and *Ceratitis capitata*.

**Hsp expression in larvae reared at constant temperatures**

Larvae reared in the laboratory at a constant temperature of 25°C did not express *Hsp70* while feeding nor during the post-feeding wandering stage, and *Hsp90* was only modestly upregulated (Fig 3a). When the larvae were reared at 28°C, *Hsp70* was still not upregulated but *Hsp90* was highly expressed. When larvae were reared at 35°C, *Hsp70* was expressed at a very low level, and once again *Hsp90* was highly expressed. Rearing larvae at a constant temperature of 40°C was lethal, thus no RNA extractions were made at that temperature. Expression patterns did not differ between actively feeding and wandering 3rd instar larvae, thus only data from the actively feeding larvae are presented to allow a direct comparison with the field data.
Hsp upregulation in the apple orchard

Air temperatures in the orchard averaged 26°C, with extremes of 16 and 34°C during the 48 h experimental period on August 22-24, 2007 (Fig. 4a). Within the apples, temperatures averaged 28°C, with extremes of 16 and 46.9°C (Fig. 4b). Both air and apple temperatures peaked around 17:30 on the two test days. The warming effect of sunlight on the apples, even those in partial shade, caused the apples to warm faster and cool slower than the surrounding air. Both infested apples from laboratory cultures and apples already growing on the trees were monitored, and no differences in temperatures were noted. All apples monitored were exposed to a mixture of sun and shade throughout the day. No mortality was recorded.

Hsp70 expression was not evident in larvae at our earliest time point, 7:00 (Fig. 3b), at which time ambient temperature (T_E) was 20°C and the apple temperature (T_A) was 19°C. Mild upregulation of Hsp70 was noted at 9:30 (T_E=28.5°C; T_A=30°C) and persisted until 13:30 (T_E=30°C; T_A=35°C). Temperature reached its peak at 17:30 when T_E=35.5°C and T_A=46.9°C, at which time upregulation of Hsp70 was very strong. Hsp70 continued to be expressed at 23:00, although the intensity of expression had declined by this time (T_E=23°C; T_A=27°C).

Hsp90 was expressed at all time points measured, but expression increased with increases in temperatures (Fig. 3b). At 7:00 expression was modest, expression increased by 9:30, reached its peak at 17:30, and again declined by 23:00, a daily pattern much akin to the cycle of Hsp70 expression.
**Hsp expression during diapause**

*Hsp70* expression was not evident in nondiapausing pupae but was highly upregulated throughout diapause (Fig. 3c). Interestingly, expression was conspicuously further elevated at 120 d, an elevation that was noted in all five replicates of the experiment. *Hsp70* was still highly expressed at 150 and 230 d but was not as high as noted at 120 d. The level of *Hsp90* expression was high in nondiapausing pupae, somewhat lower throughout diapause but constant and still present at 234 d.

**DISCUSSION**

The sequences of *Hsp70* and *Hsp90* from *R. pomonella* are quite similar to corresponding genes identified in other Diptera. Likewise, our experiments show that *Hsp70* is heat-inducible as it is in other tephritid species (Thanaphum & Haymer 1998, Rinehart et al. 2000a, Teixeira & Polavarapu 2005). By contrast, *Hsp90* is constitutively expressed but also responds to high temperature with an elevation in expression. This expression pattern was also observed for *Hsp90* in the flesh fly (Rinehart & Denlinger 2000b) and the onion maggot (Chen et al. 2005).

When larvae were reared in the laboratory at constant temperatures ranging from 25 to 35°C, *Hsp70* was expressed only at 35°C, and at that temperature expression was very modest. *Hsp90*, by contrast, was expressed at all three rearing temperatures but at varying intensities. Expression was most intense at 28°C. Larvae were unable to survive at a constant rearing temperature of 40°C, although it was clear from our orchard experiments that they can survive brief exposures to temperatures exceeding 40°C.
Temperatures in the field, of course, are not constant, and summer air temperatures in our experimental orchard in late August ranged from 16 to 34°C (Fig 4a). Apple temperatures were notably higher: temperatures were higher than 35°C on both of our experimental days and spiked briefly to 46.9°C on day 2 of our experiment (Fig 4b). The daily temperature cycle was reflected in a corresponding but slightly delayed cycle of Hsp70 and Hsp90 expression in R. pomonella. Lowest expression for both Hsps was noted in our early morning samples (7:00) and peak expression was observed at 17:30. Peak Hsp expression occurred after the temperature peaked, and expression remained elevated at 23:00, long after air and apple temperatures dropped.

Peak apple temperatures far exceeded temperatures normally required for induction of the heat shock response (Velazquez et al. 1983), but it is interesting to note that both Hsp70 and Hsp90 were already moderately upregulated at 9:30, at which time temperatures were around 30°C, a temperature we would not have predicted to elicit Hsp expression. Possibly the gradual increase in temperature during early morning triggers a preventive heat shock response, which is then supplemented with strong upregulation in response to protein damage at the higher temperatures prevailing at midday. Such a scenario was postulated for larvae of Drosophila melanogaster feeding in necrotic fruit (Feder et al. 1997), but in D. melanogaster the evidence did not support this hypothesis because Hsp70 elevation was modest and mortality increased, even with a gradual increase in temperature.

Although both Hsp70 and Hsp90 display similar cyclic patterns of expression in the field, expression extremes were more pronounced for Hsp70. While Hsp90 was
present all the time, \emph{Hsp70} was absent at 7:00 and then reached a higher level of peak expression than observed for \emph{Hsp90} at 17:30. This observation is consistent with the known role of Hsp70 in preventing thermal damage and repairing misfoldings of proteins (Parsell & Lindquist 1993), while Hsp90 appears to be less involved in preventing thermal damage and more involved in regulating the rate at which heat-damaged proteins are reactivated (Nathan \textit{et al.} 1997).

Hsps also play an integral role in the pupal diapauses of flies including the flesh fly, \emph{Sarcophaga crassipalpis} (Rinehart \textit{et al.} 2000a), the blueberry maggot, \emph{Rhagoletis mendax} (Teixeira & Polavarapu 2005), and the onion maggot, \emph{Delia antiqua} (Chen \textit{et al.} 2005, 2006). During diapause, Hsps provide enhanced tolerance to both low and high temperatures (Teixeira & Polavarapu 2005, Chen \textit{et al.} 2006, Rinehart \textit{et al.} 2007). In \emph{R. pomonella} \emph{Hsp70} is developmentally upregulated at the onset of diapause and high expression persists throughout diapause.

Interestingly, all replicates of our experiment showed higher \emph{Hsp70} expression at 120 d. There was no introduced stress or any measurable change in diapause conditions at this time, however this time point coincides with the end of diapause for individuals that have entered a “shallow diapause”. Only pupae that have entered a “deep diapause” are still in diapause at this time. These two categories of pupal diapause are well known for \emph{R. pomonella} (Feder \textit{et al.} 1997, Dambroski & Feder 2007), and our observations may reflect different intensities of \emph{Hsp} expression associated with these two types of diapause. Currently there are no molecular or morphological traits that can distinguish the two types of diapause \textit{a priori}. The diapause of the apple maggot is a bit peculiar for a
univoltine species because it is facultative. After pupariation, development can proceed without diapause, be halted by a shallow diapause lasting 50-90 d, or pupae can enter a deep diapause that may last over 300 d and require a period of low temperature for termination (Feder et al. 1997, Dambroski & Feder 2007). Given that shallow diapause and deep diapause pupae cannot be separated on the basis of size or weight (unpublished data), RNA extracted from the pupae at 30, 60 and 90 d would include both phenotypes. With this scenario samples collected after 100 d would likely include only “deep diapause” pupae. The strong expression we note at 120 d may thus suggest higher expression of Hsp70 in “deep diapause” pupae. This would be consistent with an important role for Hsp70 in protection against cold injury as seen in the flesh fly (Rinehart et al. 2007).

Hsp90 is expressed throughout diapause in R. pomonella, but expression is lower than in nondiapausing pupae. Hsp90 is also downregulated during diapause in S. crassipalpis (Rinehart et al. 2000b). Thus, in both cases Hsp70 is highly upregulated during diapause, while Hsp90 is downregulated. This is in contrast to most stress responses where all Hsps synchronously are elevated (Denlinger et al. 2001). Clearly the diapause pattern of Hsp expression is quite different than the classic stress response. It is also evident that downregulation of Hsp90 does not occur in all cases of diapause. For example, in the diapuses of the onion maggot, D. antiqua (Chen et al. 2005), and the rice stem borer, Chilo suppressalis (Sonoda et al. 2006), Hsp90 is upregulated. The precise role for Hsp90 during diapause is unclear, but this Hsp is known to be involved in certain signal transduction pathways and in many cases tends to form long-lived
interactions with its targets (Parsell & Lindquist 1993). Hsp90 is also involved in the ecdysone receptor/ultraspiracle complex (Arbeitman & Hogness 2000) and the inactivity of that complex during diapause correlates with a downregulation of Hsp90 (Rinehart et al. 2000b).

It is evident from this study that the daily thermal cycle in summer evokes a corresponding cycle of Hsp expression in larvae of R. pomonella. And, during the winter, Hsp70 is highly elevated throughout the duration of pupal diapause. The Hsps are thus utilized not just in rare periods of extreme stress but on a possible daily basis in summer and all of the time during winter.

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Rhagoletis 1 RIGINMVAIGIDLTGTY occasional substitutions
Delia 1 RIGINMYAIGIDLTY occasional substitutions
Ceratitis 1 RIGINMVAIGIDLTGTY occasional substitutions

Rhagoletis 51 RLIGDAAKNQVAMNPKNTVFDAKRLIGRKFDDQKIQEDMKHWPFKVINDC
Delia 51 RLIGDAAKNQVAMNPQNTVFDAKRLIGRKFDDQKIQEDMKHWPFKVINDC
Ceratitis 51 RLIGDAAKNQVAMNPKNTVFDAKRLIGRKFDDQKIQEDMKHWPFKVINDC

Rhagoletis 101 GKPKICVEYKGENKRFAPEEISSMVLTKMKETAEAYLGTTVTDAVITVPA
Delia 101 GKPKISVEFKGEQRKFAPEEISSMVLTKMKETAEAFLGTTVRDAVVTVPA
Ceratitis 101 GKPKISVEYKEENKQFAPEEISSMVLTKMKETAEVILGTTVTDAVITVPA

Rhagoletis 151 YFNDSQRQATKDAGRIAGLNVLRIINEPTAAALAYGLDKNLKGERNVLIF
Delia 151 YFNDSQRQATKDAGRIAGLNVLRIINEPTAAALAYGLDKNLKGERNVLIF
Ceratitis 151 YFNDSQRQATKDAGRIAGLNVLRIINEPTAAALAYGLDKNLKGERNVLIF

Rhagoletis 201 DLGGGTFDVSILTIDEGSLFEVRATAGDTHLGGEDFDNRLVNHFAEEFKR
Delia 201 DLGGGTFDVSILTIDEGSLFEVRATAGDTHLGGEDFDNRLVTHLAEEFKR
Ceratitis 201 DLGGGTFDVSILTIDEGSLFEVRATAGDTHLG-EDFDNRLVSHLAEEFKR

Rhagoletis 251 KYKKDLRTNPRALRRLRSAAERAKRTLSSSTEATIEIDALYEGVDFYTKV
Delia 251 KYKKDLRSNPRALRRLRSAAERAKRTLSSSTEATIEIDALYEGVDFYTKV
Ceratitis 250 KYKKDLRSNPRALRRLRTAAERAKRTLSSSTEATIEIDALYEGVDFYTKV

Rhagoletis 301 SRARFEELCADLFRNTLQPVEKALNDAKMDKNQIHVYVLVGGSTRIPKVQ
Delia 301 SRARFEELCADLFRNTLQPVEKALNDAKMDKNQIHVYVLVGGSTRIPKVQ
Ceratitis 300 SRARFEELCADLFRQTLEPVEKALNDAKMDKNQIHVYVLVGGSTRIPKVQ

Rhagoletis 351 NLLQQFFCGKSLNLSINPDEAVAYGAAVQAAILSGDKSTEIQDVLLVDVA
Delia 351 NLLQQFFCGKSLNLSINPDEAVAYGAAVQAAILSGDKSTEIQDVLLVDVA
Ceratitis 350 NLLQQFFCGKSLNLSINPDEAVAYGAAIQAAILSGDKSTEIQDVLLVDVA

Rhagoletis 401 PLSLGIETAGGVMTKLIERNSRIPCKQTQTFSTYSDNQPGVNIQVYEGER
Delia 401 PLSLGIETAGGVMTKLIERNSRIPCKQTQTFSTYSDNQPGVNIQVYEGER
Ceratitis 400 PLSLGIETAGGVMTKLIERNSRIPCKQTQTFSTYSDNQPGVNIQVYEGER

Rhagoletis 451 AMTKDNNRLGTFDLSGIPPAPRGVPQIEVTFDVDANGNNLNVSAKEMSSG
Delia 451 AMTKDNNRLGTFDLSGIPPAPRGVPQIEVTFDVDANGNNLNVSAKEMSSG
Ceratitis 450 AMTKDNNRLGTFDLSGIPPAPRGVPQIEVTFDVDANGNNLNVSAKEMSSG

Rhagoletis 500 NAKNIVIKNDKGRLSQAEIDRMVNEAGRYAEEDERQRNKIAARNNLESYV
Delia 500 NAKNIVIKNDKGRLSQAEIDRMVNEAGRYAEEDERQRNKIAARNNLESYV
Ceratitis 500 NAKNIVIKNDKGRLSQAEIDRMVNEAGRYAEEDERQRNKIAARNNLESYV

Figure 2.1. The translated full length clone of *Rhagoletis pomonella*’s Hsp70 shares 84% and 89% amino acid identity with the Mediterranean fruit fly, *Ceratitis capitata*, and the onion maggot, *Delia antiqua*, respectively.
Figure 2.2. Amino acid sequence for the partial clone of Hsp90 from Rhagoletis pomonella, which shares an amino acid identity of 97 and 89% with the Mediterranean fruit fly, Ceratitis capitata, and the onion maggot, Delia antiqua, respectively.
Figure 2.3. Northern blot hybridizations showing the expression of *Hsp70* and *Hsp90* in larvae reared at 25°C, 28°C and 35°C under laboratory conditions (A), during the field test experiencing a natural fluctuation of temperature (B), and during the apple maggot’s pupal diapause at a constant 18°C (C). 28S was used as the control gene. ND = nondiapause.
Figure 2.4. Temperature readings from outside (A) and inside (B) red delicious apples used for the field study. A daily fluctuating pattern can be seen over the nearly three day interval that was monitored, August 22-24, 2007.
CHAPTER 3
Heat shock protein and antioxidant enzyme expression in response to dehydration in the apple maggot, *Rhagoletis pomonella*.

ABSTRACT

The apple maggot, *Rhagoletis pomonella*, spends up to 11 months in a deep diapause in which it must cope with the low temperatures of winter. Another challenge that the pupae face is the desiccating condition in the frozen soil during the winter months. To investigate the involvement of stress genes during desiccation in the apple maggot, we monitored expression of heat shock proteins (Hsps), Hsp70 and Hsp90, two genes that had been previously cloned. We cloned the antioxidant enzymes, superoxide dismutase (SOD) and catalase, and monitored their expression during dehydration, heat shock and diapause. We found that SOD and catalase were strongly upregulated during dehydration while Hsp90 was unaffected and constitutively expressed. Hsp70 was not expressed in response to dehydration. SOD and catalase were upregulated in response to heat shock. SOD was downregulated during diapause but expressed at a constant low level throughout diapause. Catalase was also downregulated during diapause. These results suggest that SOD and catalase scavenge reactive oxygen species that are produced as a result of dehydration while Hsp90 serves its role as a molecular chaperone.
INTRODUCTION

The apple maggot, *Rhagoletis pomonella*, is a major pest of domesticated apples. The flies emerge in mid to late summer, mate on the surface of the apples and the females oviposit into the fruit. After a 2-3 week period of larval feeding inside the fruit, the larvae drop to the soil and pupariate. The pupae can undergo three developmental strategies (Dambroski & Feder 2007). Some directly develop into adults (~30 d), other enter a shallow diapause (up to 100 d), but most enter a deep diapause that can last as much as 11 months. During this time the pupae are vulnerable to environmental extremes in temperature, experiencing both the low temperatures of winter and the warm temperatures of spring and early summer. Dehydration is also a challenge for the apple maggot as the soil can be dry in the fall and frozen in the winter.

Dehydration is one of the biggest challenges that any insect can face. Due to their small surface area to volume ratio, insects are prone to desiccation (Hadley 1994). The damage incurred during water loss is not simply repaired by water gain. Protein denaturing, nucleic acid damage and lipid peroxidation are among three of the harshest effects that dehydration elicits at the cellular level (Hansen et al. 2006). Some of this damage is caused by a 10-fold increase in oxygen radicals that occurs during water loss (Pereira et al. 2003), the origin of which is still unknown (Franca et al. 2007). Oxygen radicals and non-radicals, like hydrogen peroxide, are collectively called reactive oxygen species (ROS). ROS are compounds with strong oxidizing capacity that originate by the partial reduction of oxygen during aerobic respiration and oxygen metabolism. Because
of this increase in ROS during dehydration, a strong antioxidant defense is necessary. Superoxide dismutase (SOD) is one of the main antioxidant enzymes; it catalyzes the breakdown of the superoxide anion into hydrogen peroxide and oxygen. Catalase in turn breaks down hydrogen peroxide into oxygen and water.

Given the many ways that cellular function can be impaired by dehydration, it would seem that the best strategy to deal with damage to the cell membrane and proteins is preventively rather than curatively (Franca et al. 2007). To investigate the involvement of these two antioxidant enzymes (SOD and catalase) in the apple maggot, we cloned both genes and monitored their expression during dehydration, heat shock and diapause. We also monitored the expression of Hsp70 and Hsp90 in response to dehydration; both genes were previously cloned (Lopez-Martinez and Denlinger 2008).

MATERIALS AND METHODS

Insects

A colony of *Rhagoletis pomonella* (Walsh) was maintained at 25°C under long day (15L:9D) conditions. This colony originated from a laboratory colony maintained at the New York State Agricultural Experiment Station in Geneva. Adults were fed on a 50:50 slurry of brown sugar and enzymatic yeast, while the larvae were reared on ‘delicious’ apples. Larvae were reared at a constant 25, 28 and 35°C under long day (17L:7D) conditions in incubators (Percival-Scientific, Perry, IA). Third instar larvae were carefully removed from the apples, and exposed to a 1 h heat shock 40°C. Diapausing pupae were reared by maintaining maggot-infested apples at 18°C under
short day (8L:16D) conditions. Pupae from those apples were sampled 30 days post-pupariation. Non-diapausing pupae were maintained at 25°C under long day (15L:9D) conditions and used as controls. To achieve a 10% loss of water content, pupae were desiccated at 0% relative humidity (CaSO₄) for 15 days.

Clones

The 312 bp clone of SOD was obtained by designing homology primers using the SOD sequence from the walnut husk fly, *Rhagoletis suavis* (forward primer 5’-GGTCTCTCCAAGGGTCAACA-3’ and reverse primer 5’-CAATAGTGCCTCCGACAATG-3’). The 109 bp clone of catalase was obtained using previously available primers (Lopez-Martinez et al. 2008a). The heat shock proteins (Hsp70 and Hsp90), as well as the control gene, 28S, were previously isolated from the apple maggot (Lopez-Martinez and Denlinger 2008).

Northern Blot Hybridization

RNA extractions were performed using the Trizol reagent (Invitrogen). Groups of 12 larvae or pupae (~75mg) were homogenized following the manufacturer’s protocol. 4 µg of RNA was run on a 1.4% agarose, 0.41 M formaldehyde gel. Using the rapid downward transfer system from Schleicher & Schuell, the RNA was transferred to a positively-charged nylon membrane (Hybond-N+, Amersham Biosciences). The Roche Diagnostics’ DIG-High Prime labeling kit was used to label the DNA clones, and hybridization was done with Roche’s DNA Labeling and Detection Starter Kit II.
Membranes were exposed to Blue Lite Autorad Film (ISC BioExpress) for 5 to 30 min, depending on probe strength. All membranes were stripped in accordance with the DIG kit guidelines and re-hybridization was done using 28S as the control gene. All northern blots were replicated three times.

RESULTS

Clones

The superoxide dismutase (SOD) clone (Genbank accession number EU******) was identified as SOD Cu-Zn (SOD 1) and shares 99 and 84% amino acid with SOD1 from the walnut husk fly, *Rhagoletis suavis* and the house fly, *Musca domestica*, respectively (Fig. 1).

The catalase clones (Genbank accession number EU******) contains the heme binding domain that is characteristic of catalases. The clone shares a 100 and 94% amino acid identity with the sand fly, *Lutzomyia longipalpis* and the fly *Drosophila melanogaster*, respectively (Fig. 2).

Antioxidant expression in the larvae and during pupal diapause

*SOD* was expressed in larvae reared at 25°C and a mild upregulation was noted for larvae reared at 28°C but expression decreased in larvae reared at 35°C (Fig 3b).
SOD was upregulated in larvae heat shocked at 40°C. Catalase was expressed in larvae reared at 25, 28 and 35°C, but the level of expression increased in response to a 40°C heat shock (Fig 3b).

Both SOD and catalase were downregulated in diapause compared to nondiapausing controls (Fig. 3b). However, the signal for both genes was still present during diapause but it was at a constant low level of expression that was still present 200 d into diapause (data not shown). Non-diapausing pupae had very strong upregulation of both genes.

Expression during dehydration

SOD and catalase were strongly upregulated in response to a 10% water loss in pupae (Fig. 4b). Hsp70 was not expressed at all in the controls or the desiccated pupae. Hsp90 expression did not change from what we saw in the controls.

DISCUSSION

The real challenge of overcoming ROS damage to the membrane is maintaining membrane integrity. As the cells lose water, the membrane becomes perturbed and fluidized which makes it more susceptible to ROS attack (Crowe et al. 1989). The effects of these attacks are lipid peroxidation and de-esterification. The damage of such effects is noted when water is gained and the membrane becomes leaky due to an increase in the phase transition temperature as a result of packing density of the phospholipids head groups (Franca et al. 2007).
The involvement of antioxidant enzymes during dehydration has been studied in other systems. In yeast, the model system for desiccation, both SOD and catalase are upregulated in response to desiccation (Pereira et al. 2001). In addition these investigators monitored the expression of both of superoxide dismutases, SOD1 (cytoplasmic) and SOD2 (mitochondrial) and found that expression of either SOD, in the absence of the other, is enough to acquire tolerance against oxidative damage (Pereira et al. 2003). This is of interest to note as we monitored the expression of SOD1, which was very responsive to dehydration. SOD expression during dehydration has not been investigated in many insects. In fact it has only been shown to be upregulated in polar insects. An EST project from the Arctic collembolan, *Onychiurus arcticus*, revealed SOD clones in desiccated libraries (Clark et al. 2007), thus indicating expression in response to water loss. A recent study on the Antarctic midge, *Belgica antarctica*, has also revealed a strong upregulation in response to dehydration (Lopez-Martinez et al. 2008b). SOD is also upregulated in moss where it is considered to play a vital role in dehydration tolerance (Mayaba and Beckett 2003).

Catalase has been shown to be crucial during periods of dehydration in yeast (Pereira et al. 2001, 2003). More recent work has shown catalase mutants that have a 70% increase in intracellular oxidation (Franca et al. 2005). Thus, the upregulation of catalase that we saw in the apple maggot supports the role of scavenging ROS during water loss. Catalase is also very efficient at rapidly breaking down high concentrations of hydrogen peroxide (Kranner and Birtić 2005). Much like SOD, work showing the involvement of catalase in insects during dehydration is lacking. SOD/catalase research
in insects has focused on the synergistic effect that expression of these antioxidants has on life span extension (Orr and Sohal 1994), rather than stress responses. However, when wide gene capturing techniques like ESTs and SSHs were performed in polar insects, both SOD and catalase were found in desiccated samples of an arctic collembolan (Clark et al. 2007) and the Antarctic midge (Lopez-Martinez et al. 2008b). Even though polar insects are more prone to desiccation due to the xeric environments they live in because of their limited access to free water during winter, there is no reason to doubt the involvement of such a strong antioxidant defense in temperate zone insects that are also limited in available water during winter, and we, in fact, show that this is the case in the apple maggot.

Heat shock proteins are regulated in response to dehydration and rehydration in insects. A common duality exists where Hsp70 has been found upregulated in the flesh fly, *Sarcophaga crassipalpis* (Tammariello et al. 1999, Hayward et al. 2004), a soil collembolan, *Folsomia candida* (Bayley et al. 2001) and the eutardigrade, *Richtersius coronifer* (Schill et al. 2004, Jönsson and Schill 2007), while Hsp90 has been found to be involved in rehydration in the flesh fly (Hayward et al. 2004). The inducible form of Hsp70 is the most conserved Hsp, and it is upregulated in response to a wide array of stressors (Feder and Hofmann 1997). Hsp70 was found to be upregulated during deep diapause in the apple maggot, and that upregulation persisted for at least 250 days into diapause (Lopez-Martinez and Denlinger 2008). The fact that Hsp70 is not expressed in response to desiccation in the apple maggot suggests that the long period of time (15 d) needed to result in 10% water content loss does not challenge normal cellular function.
enough to require upregulation of this gene. On the other hand, even though Hsp90 is still inducible during stress, it plays a more regulatory role as a molecular chaperone. Under normal conditions, Hsp90 is involved in regulatory pathways where it interacts with kinases, hormones receptors calmodulin, actin and tubulin (Parsell and Lindquist 1993). We also reported a low level of expression of Hsp90 during diapause in this species. Thus, the lack of responsiveness from Hsp90 during dehydration is expected given that the main function of Hsp90 seems to be maintenance of normal cellular processes and growth at elevated temperatures.

In summary, the apple maggot pupae are able to deal with water loss during their deep diapause by upregulating SOD and catalase to reduce oxidative damage created by dehydration. Hsp70 is not expressed during dehydration, most likely because a 10% water loss in a period of 15 days does not challenge normal cellular function in the apple maggot, whereas Hsp90 remains expressed at a constant level. The low level expression of SOD and catalase as a consequence of diapause may be an indication of the lack of a need for these enzymes during periods of low respiration activity.

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REFERENCES


Figure 3.1. Amino acid homology comparison of superoxide dismutase 1 from *R. pomonella* with *Rhagoletis suavis* and *Musca domestica*. The percentage identity shared is 99% and 84%, respectively.
Figure 3.2. Amino acid homology comparison of catalase from *R. pomonella* with catalase from *Lutzomyia longipalpis* and *Drosophila melanogaster*. The percent identity shared is 100 and 94%, respectively (Fig. 2).
Figure 3.3. Northern blot hybridizations showing mRNA signals for SOD and catalase during larval rearing at different temperatures (A) and during pupal diapause (B). 28S was used as the control gene.
Figure 3.4. Northern blot hybridizations showing mRNA signals for *Hsp70* and *Hsp90* (A) and *SOD* and *catalase* (B) in response to 10% water content loss in pupae of *R. pomonella*. 28S was used as the control gene.
CHAPTER 4

High resistance to oxidative damage in the Antarctic midge *Belgica antarctica*, and developmentally-linked expression of genes encoding superoxide dismutase, catalase and heat shock proteins.

ABSTRACT

Intense ultraviolet radiation, coupled with frequent bouts of freezing-thawing and anoxia, have the potential to generate high levels of oxidative stress in Antarctic organisms. In this study we examined mechanisms used by the Antarctic midge, *Belgica antarctica*, to counter oxidative stress. We cloned genes encoding two key antioxidant enzymes, superoxide dismutase (SOD) and catalase (Cat), and showed that SOD mRNA was expressed continuously and at very high levels in larvae, but not in adults, while Cat mRNA was expressed in both larvae and adults but at a somewhat reduced level. SOD mRNA was present in greater amounts in larvae that were exposed to direct sunlight. Catalase, a small heat shock protein, and Hsp70 mRNAs were also strongly upregulated in response to sunlight. Total antioxidant capacity of the adults was higher than that of the larvae, but levels in both stages of the midge were much greater than observed in a freeze-tolerant, temperate zone insect, the gall fly *Eurosta solidaginis*. Assays to
measure oxidative damage (lipid peroxidation TBARS and carbonyl proteins) demonstrated that the Antarctic midge is highly resistant to oxidative stress.

**INTRODUCTION**

In addition to the conspicuous challenges of low temperature and desiccation, organisms living in Antarctica are bombarded with especially large amounts of ultraviolet radiation during the summer, an effect that has been exacerbated recently due to openings in the ozone layer (Solomon 1990; Liao and Frederick 2005, Weatherhead and Andersen 2006). Adding to the oxidative stress caused by ultraviolet radiation is the potential for oxygen radical generation by frequent freeze-thaw and anoxia cycles (Joanisse and Storey 1998; Hermes-Lima and Zenteno-Savin 2002).

Oxygen radicals and non-radicals, like hydrogen peroxide, are known collectively as reactive oxygen species (ROS). ROS can cause lipid peroxidation which disrupts membrane fluidity, and the degradation products can initiate apoptosis in the mitochondria (Halliwell and Gutteridge 1999; Green and Reed 1998). Oxidative damage to proteins can range from specific amino acid modifications and fragmentation of the peptide chain to total enzyme inactivation by superoxide anions (Stadtman 1986). ROS can also lead to DNA deletions, mutations, base degradation, single-strand breakage and cross-linkage of proteins (Imlay and Linn 1988; Imlay 2003). Superoxide radicals generated by oxidative stress act as oxidants or reductants that lead to the production of hydroxyl radicals (Fridovich 1995). The hydroxyl radicals, though short-lived, are highly reactive and readily damage DNA by denaturing nucleic acids (Lesser 2006). Two of the
enzymes most crucial for inactivating these potentially damaging oxygen agents are superoxide dismutase (SOD) and catalase (Orr and Sohal 1994). SOD converts superoxide into oxygen and hydrogen peroxide, while catalase then converts the highly reactive hydrogen peroxide into oxygen and free water.

In this study we examine the response of an Antarctic insect, the midge Belgica antarctica Jacobs (Diptera, Chironomidae), to oxidative stress. This insect, which is endemic to maritime Antarctic, has a patchy distribution on the Antarctic Peninsula and its nearby islands (Gressitt 1967; Convey and Block 1996). The midge is freeze tolerant and spends much of its two-year life cycle as a larva encased in a matrix of ice and substrate, but during the brief (approximately 2 month) austral summer the larvae feed on algae and bacteria located in the substrate near penguin rookeries (Sugg et al. 1983). The wingless adults emerge late December to early January. They can be found in aggregations on the surfaces of rocks located near the larvae, but they never stray far from the moist habitats that are essential for their survival (Benoit et al. 2007a).

A previous study (Grubor-Lajsic et al. 1996) reported elevated antioxidant enzyme activity in the midge larvae. We extend that work by cloning the genes that encode SOD and catalase in B. antarctica and by examining the expression patterns of those genes in the larvae and adults. Because Antarctic organisms are particularly vulnerable to ultraviolet radiation (Weiler and Penhale 1994, Weatherhead and Andersen 2006), we monitored the expression of the genes encoding SOD and catalase, as well as the genes encoding three heat shock proteins in response to ultraviolet radiation. We also assessed total soluble antioxidant capacity, trehalose concentrations, and measures of
oxidative damage, lipid peroxidation (TBARS) and protein damage (carbonyls). For a point of reference, we compared the antioxidant capacity of *B. antarctica* to that of a temperate zone species, *Eurosta solidaginis*, a gall fly that is also freeze tolerant (Baust and Lee 1982).

**MATERIALS AND METHODS**

*Insects*

*B. antarctica* Jacobs larvae and adults were collected in January and February, 2006 and 2007, on Norsel Point, and Torgersen and Cormorant Islands near the United States Antarctic Program’s research station, Palmer Station (Anvers Island, Antarctica). Larvae were collected with the substrate, while the adults were collected using aspirators. Prior to use in our experiments, the larvae were sorted from the substrate and placed, as groups of ten, in 1.5 ml microcentrifuge tubes containing 0.5 ml of water and held at 4°C until used. Adults were kept in Petri dishes in groups of 50 at 4°C until used.

*Clones*

The clones were obtained by two methods. The clone of *SOD* was obtained by suppressive subtractive hybridization (SSH), in which an RNA pool from hydrated larvae held at 4°C was subtracted from an RNA pool of larvae held at 4°C that had been desiccated for 12 h at 98.2% RH to remove roughly 10% of their body water. The cDNAs were synthesized using the BD SMART™ PCR cDNA Synthesis kit and
subtracted using a PCR-Select™ cDNA Subtraction Kit (Clontech, Mountain View, CA). cDNAs encoding SOD, a small heat shock protein and a control gene, 28S, were among the cDNAs obtained by this method, although none of the cDNAs proved to be unique to either of the original RNA pools. Full length sequence for SOD was obtained by rapid amplification of cDNA ends using a 3’ Race kit (Invitrogen, Carlsbad, CA).

*Catalase* was obtained by designing primers using sequences from *Anopheles gambiae*, *Aedes aegypti* and *Drosophila melanogaster* (forward primer 5’-CCGTCAGTTCTACACTGAGGA-3’ and reverse primer 5’-CAGAACATGTCCGGATCCTT-3’). The full coding sequence of catalase was obtained using the 3’Race kit (Invitrogen) and the Genome Walker Universal kit (Clontech).

The heat shock protein clones (*hsp70 and hsp90*) were previously isolated from *B. antarctica* by our group with designed primers based on homology (Rinehart et al. 2006).

*Northern blot hybridization*

RNA used for northern blot hybridizations was extracted from the treatment groups of 75 larvae or 50 adults described below, using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. Northern blot hybridization was performed by running 4 µg of RNA in a 1.4% agarose, 0.41 M formaldehyde gel. Based on signal strength, 1 µg of RNA was used for the 28S control to verify equal loading. The RNA was transferred to a positively charged nylon membrane (Hybond-N+, Amersham Biosciences, Piscataway, NJ) using the Turboblotter rapid downward transfer system.
(Schleicher & Schuell Inc.). DNA clones for SOD, catalase, a small heat shock protein (smHsp), Hsp70, Hsp90 and 28S were labeled using the DIG-High Prime labeling kit and hybridization was performed using the DNA Labeling and Detection Starter Kit II (both from Roche Diagnostics, Switzerland). The membranes were exposed using Blue Lite Autorad Film (ISC BioExpress) for 5 to 30 min depending on DIG probe strength. The membranes were stripped using the DIG kit guidelines and re-hybridized with the control gene (28S). All northern blots were performed in triplicate.

**Stress Treatments**

To evaluate gene expression and to assess total antioxidant capacity and damage due to oxidative stress, larvae were subjected to three stresses that commonly occur in their habitat: heat shock, freezing and anoxia. To administer heat shock and freezing stresses, groups of 10 larvae were placed in 1.5 ml microcentrifuge tubes with 100 µl of distilled water and exposed to 1 h heat shock at 30°C or freezing at -5°C for 2 d, with and without a 2 h recovery. For exposure to anoxia, groups of 10 larvae were placed in 15 ml glass vials with distilled water, and a stream of nitrogen was bubbled into the container for 2 min; larvae were then maintained in the sealed vials at 4°C for 2 d. Even though the adults are not likely to experience anoxic conditions in their short life, we wanted to record any possible SOD and catalase upregulation following anoxia therefore we exposed adults to anoxic conditions in the same manner but without water. Controls consisted of larvae and adults that were maintained at their normal summer habitat.
temperature, 4°C. RNA was extracted, as described above, immediately after the stresses were administered.

Larvae were also stressed by exposure to unfiltered ultraviolet radiation from direct Antarctic sunlight. For this experiment, larvae were held in water (0.5 cm in depth) in 100 mm i.d. glass Petri dishes that were maintained at 4-5°C and monitored with a thermometer hourly. Groups of larvae (N = 75) were exposed to direct sunlight for 3, 6, and 9 h on January 25, 2007. During the entire period, the test site remained in the sunlight, with the exception of two 3-5 min periods of cloud cover. Immediately after exposure, RNA was extracted from the samples. The experiment was independently replicated three times.

Total antioxidant capacity

Larvae were collected and held in distilled water overnight (12 –16 h) to allow clearance of the gut prior to assessment. For comparison, the antioxidant capacity of the freeze-tolerant, over-wintering larvae of a temperate zone species, the goldenrod gall fly was also assessed. Eurosta solidaginis (Fitch)(Diptera, Tephritidae) larvae were collected near Miami University in Oxford, OH in late November, 2006 and maintained within the intact galls at 4°C for 4 months prior to measurement of antioxidant capacity.

Groups of 25 larvae or 40 adults of B. antarctica were homogenized in ice-cold Coast’s solution (Coast 1988), and sonicated for 30 s to disrupt cells. A 25 µl aliquot of the crude homogenate was stored at -80°C and reserved for the TBARS assay. The remainder of the homogenate was then centrifuged at 4°C for 10 min at 2000 g to
separate cellular fragments. The supernatant was collected and stored at -80°C until assayed. Individual larvae of *E. solidaginis* were similarly prepared and stored.

The trolox equivalent antioxidant capacity (TEAC) was measured using an ABTS [2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation decolorization assay (Re et al. 1999). The ABTS radical cation (ABTS⁺) was produced by reacting an ABTS stock solution (7.0 mM) with potassium persulfate (2.45 mM final concentration) and allowing the mixture to stand in the dark at room temperature for 12-16 h. The radical cation was stable in the dark at room temperature for 48 h. For use in the assay, the ABTS⁺ was diluted with phosphate buffered saline (PBS) to an absorbance of 0.7 at 734 nm. The antioxidant capacity was assessed by monitoring the decrease in absorbance at 734 nm exactly 10 min after the addition of 1 ml of diluted ABTS⁺ to 10 µl of biological sample. Samples were compared to Trolox standards (0 – 15 µmol/ml; final concentration) and expressed as Trolox equivalents.

**Lipid Peroxidation**

The concentration of thiobarbituric acid reactive substances (TBARS) in larvae of *B. antarctica* was determined using a commercially available assay kit (OXItek; ZeptoMetrix Corp., Buffalo, NY). The assay was conducted on crude tissue homogenates by combining 25 µl of homogenate with 25 µl of the supplied SDS solution and 625 µl of TBA reagent in a 2 ml microcentrifuge tube. The mixture was then incubated at 95°C for 1 h. After cooling to room temperature, 675 µl of butanol was added to each sample and the tubes were then centrifuged 5 min at 1,000 g. Two 220 µl
aliquots of the butanol layer were transferred to a 96-well plate and the absorbance (\( \lambda = 532 \) nm) read on a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA). The TBARS concentration (nmol/ml) was determined by averaging duplicate samples calculated from a standard curve prepared from various concentrations (0 to 50 nmol/ml) of malondialdehyde. TBARS concentration was expressed per mg of soluble protein in the crude homogenate. Protein concentrations in the prepared tissue homogenates were determined by the Bradford method (Bio-Rad, Hercules, and CA) using bovine serum albumin (0 to 1 mg/ml) as a standard.

**Carbonyl proteins**

Carbonyl proteins were measured from whole body homogenates of larvae by reacting samples with 2, 4-dinitrophenylhydrazine (DNPH) to generate dinitrophenyl-hydrazone (Reznick and Packer 1994). For the assay, a 200 \( \mu \)l aliquot of sample supernatant was added to each of two 2 ml microcentrifuge tubes; 800 \( \mu \)l of a DNPH solution (10mmol DNPH in 2M HCl) was added to one tube, and 800 \( \mu \)l of 2 M HCl to the other. The sample mixtures were then incubated at room temperature in darkness for 1 h. Proteins were precipitated with the addition of 1 ml 20% TCA and centrifuged at 10,000 g for 10 min. The resulting pellet was washed 3x with 1 ml volumes of an ethanol:ethyl acetate (1:1) solution and similarly centrifuged between washes to remove excess DNPH. The final pellet was solubilized in 500 \( \mu \)l of 6 M guanidine hydrochloride. Carbonyl content was determined from the difference between the average absorbance (\( \lambda = 370 \) nm) of duplicate samples of DNPH-reacted and unreacted
HCl samples. Carbonyl proteins were expressed per milligram of protein in the sample as described above.

*Trehalose concentration*

Trehalose content was determined as described by Chen et al. (2002). Five groups of 25 larvae were weighed and immediately frozen at -80°C. Larvae were homogenized in 0.25 ml of 0.25 M Na₂CO₃ and incubated at 95°C for 2 h to denature proteins. Following the denaturing of proteins, 0.15 ml of 1 M acetic acid and 0.6 ml of 0.2 M sodium acetate were added, and the mixture was centrifuged at room temperature for 10 min at 12,000 g. Aliquots (200 µL) of the supernatant were placed into two tubes; one was used as background, and the other was digested by trehalase (0.05 units/ml) overnight at 37°C. Glucose concentration was then measured using the glucose (GO) assay kit (Sigma, St. Louis, MO, USA).

*Statistical analyses*

Means were compared with analysis of variance (ANOVA) and Bonferroni-Dunn tests (Statview from SAS Institute, Cary, NC, USA). Data are presented as mean ± SEM. Statistical significance was set at $P<0.05$. 
RESULTS

*Superoxide dismutase*

The full length clone of *SOD* that was produced was identified, by nucleotide and amino acid sequence comparisons, as SOD2 (Fig. 1). The identification was confirmed by the presence of two highly conserved domains for iron/manganese superoxide dismutases. The N-terminus contains the long alpha antiparallel hairpin and the C-terminal ends in the characteristic mixed alpha/beta fold. An amino acid comparison of the 217 amino acid clone shows that *SOD2* (*SOD-Mn*) from the Antarctic midge (GenBank accession no. DQ507228) shares a 67% and 68% amino acid identity to *Drosophila melanogaster* and *Aedes aegypti*, respectively.

Strong upregulation of transcripts encoding SOD2 was found in all samples from the midge larvae, *i.e.* the untreated controls as well as those exposed to heat shock, freezing or anoxia (Fig. 2). By contrast, the gene was not expressed in control or anoxia-treated adults. Thus, in the larvae, *SOD2* was strongly and continuously expressed, and expression levels were not further elevated by any of the environmental stresses that we measured. By contrast northern blots did not reveal the transcript in the adults, regardless of the stress applied. The transcript of the control gene, 28S (GenBank accession no. DQ459549), was present in all treatments and replications, including the adults.

*SOD2* was also continuously expressed in larvae exposed to direct sunlight (Fig. 3). A modest upregulation occurred after a 6 and 9 h exposure to direct sunlight.
Catalase

The catalase gene clone (GenBank accession no. EU344974) shares an amino acid identity of 93% and 89% with Drosophila melanogaster and Aedes aegypti, respectively (Fig. 4). The 506 amino acid coding sequence obtained contains the heme-binding pocket characteristic of heme-catalases (Fig. 4).

Although the transcript encoding catalase was not as strongly expressed as the SOD transcript, the catalase transcript was present in both larvae and adults. Expression at 4°C was higher in larvae than in adults (Fig. 2). Freezing and anoxia both lowered the level of expression in larvae, but expression was elevated by heat shock. In the adults, anoxia elevated expression.

Catalase was upregulated in larvae in response to natural sunlight (Fig. 3). An elevated upregulation was detected when the larvae were exposed to direct sunlight for 3h but declined somewhat after 6 and 9 h.

Small Hsp

A small heat shock protein (GenBank accession no. DQ459548) was equally expressed in both control larvae and those that were heat shocked (Fig. 2). Expression in response to freezing and anoxia was not as great as observed following heat shock. The adults did not express this gene under control conditions, but a mild upregulation was evident following anoxia (Fig. 2). Larval expression of this gene was reduced compared to that of the controls, after 3 h of direct sunlight exposure (Fig. 3). However, strong upregulation occurred by 6 h and persisted to 9 h.
Hsp70

Heat shock protein 70 (GenBank accession no. DQ459546) was constitutively expressed in larvae, but a decline in expression was evident in response to freezing and anoxia (Fig. 2). Hsp70 was not expressed in control adults but was upregulated in adults exposed to anoxia. Larvae exposed to direct sunlight for 3 h (Fig. 3) expressed the gene at a level similar to the controls, but a 6 h exposure to direct sunlight elicited strong upregulation that persisted for at least 9 h.

Hsp90

Heat shock protein 90 (GenBank accession no. DQ459547) was constantly expressed in all larval treatments (Fig. 2). HSP90 was also present in the adult controls and following anoxia. A mild upregulation was seen in larvae exposed to direct sunlight for 6 or 9 h (Fig. 3).

Total antioxidant capacity

B. antarctica appears to be well equipped to defend against ROS, as they possessed relatively high antioxidant capacity (Fig. 5). Relative to another freeze-tolerant insect, the larvae of the goldenrod gall fly E. solidaginis, the antioxidant capacity of B. antarctica larvae was nearly 5 times higher. Surprisingly, the short-lived midge adults possessed higher (>1.5x) antioxidant defense than the larvae (Fig. 5).

Relative to control larvae (maintained at 4°C), no significant differences in total antioxidant capacity were observed following larval freezing, anoxia or heat shock (Fig.
6). All measurements were taken after a 2-h recovery at 4°C; however, these values did not differ significantly from measurements taken 30 min following removal from the stresses (data not shown).

**Trehalose content**

Compared to the control (23.21 ± 0.41 µg/mg DM; n = 5) no significant increase in trehalose concentration of the larvae was observed following exposure to anoxia or heat shock (Fig. 7). Trehalose concentration was greater (~1.3x) following freezing (P<0.05); however, this increase did not correspond to an elevation of the total antioxidant capacity of larvae following freezing.

**Oxidative damage**

Markers of oxidative damage were assessed following exposure to the aforementioned environmental stressors. Relative to controls, no significant differences in either TBARS or carbonyl proteins were observed following freezing, anoxia, or heat shock (Fig. 8A and B). These results suggest that the antioxidant capacity of the larvae is sufficient to prevent oxidative damage induced by ROS, and no further elevation of these markers for oxidative damage occurs when the larvae are subjected to stress.
DISCUSSION

The continuous and large amount of the mRNA encoding superoxide dismutase, in the absence of any measurable stress, indicates that the midge larvae have evolved a mechanism to prevent oxidative stress by the massive production of antioxidant agents. Rather than responding to stress by initiating transcription of the SOD gene, the midge larvae are already maximally expressing this gene, and none of the environmental stressors we examined caused the gene to be further upregulated. This is much akin to the continuous upregulation of heat shock proteins observed in these larvae (Rinehart et al. 2006). The continuous expression of this gene suggests that the larvae have adapted to produce the enzyme in anticipation of the wide range of environmental stressors that they confront in the Antarctic environment, and the lack of increased oxidative damage we report is consistent with that. None of the three stresses we tested (heat shock, freezing, or anoxia) generated an increase in ROS damage to lipids or proteins beyond what was observed in the control. This result is consistent with a previous report showing significantly greater SOD activity in B. antarctica compared to temperate zone insects (Grubor-Lajsic et al. 1996).

In addition to the freeze-thaw cycles that invertebrates experience in Antarctica, up to 140/yr (Block 1997), ultraviolet radiation (UVR) may be a driving force contributing to the persistent SOD expression pattern we observed. The active, summer larvae of B. antarctica are normally found within the substrate, in close association with algae, grass, moss, soil and animal detritus (Peckham 1971). This habitat is likely to provide considerable protection against UVR, yet periods of melting snow, driving
summer rainstorms and ocean spray can readily expose the larvae, and it is in these situations that the larvae are particularly vulnerable to UVR. Ultraviolet radiation is classified into three major forms based on wavelength (UVA, UVB, UVC), but UVA and UVB present a greater challenge than UVC, which is absorbed in the upper atmosphere (Karentz 1994, Cockell 2001). UVA radiation in the 320 – 400 nm range, is not attenuated by the ozone layer and leads to the production of ROS. This is due to the fact that nucleic acid bases absorb UV radiation weakly above 320nm (Cadet et al. 2005). UVB, radiation in the 280 – 320 nm range, damages DNA by the formation of cyclobutane pyrimidine dimers at adjacent thymine residues and also generates ROS (Tevini 1993).

In the austral summer the surface temperature of the midge larval habitat can reach 25-27°C, an extremely high temperature for this insect (Rinehart et al. 2006). The larvae may avoid these high temperatures by moving further down into the substrate to find cooler locations (Peckham 1971). However, melting snow and summer rain storms commonly displace larvae from their microhabitats, exposing them directly to ultraviolet radiation.

Even though the hole in the ozone layer allows UVB radiation to reach the surface of the Antarctic Peninsula, peak UVB activity in this region occurs in November (Karentz and Bosch 2001), and during this time the larvae are still well protected from UV radiation by a layer of snow. The hole in the ozone layer appears to have formed only within the past 25 yr (Solomon 1990), thus it is not likely that this midge, with its two-year life cycle, has already adapted to that type of ultraviolet radiation. UVA
radiation, however, is at its highest in the months of December and January (Liao and Frederick 2005), a period when the larvae are actively feeding and are most likely to be vulnerable to exposure. It is during this late December/early January period that adults are present and can be seen crawling over the rocks surfaces, where they are clearly vulnerable to UVA radiation.

We thus suspect that the constant upregulation of $SOD$ in the larvae serves as a preventive measure against UVA exposure. The lack of further upregulation $SOD$ in response to Antarctic sunlight supports this conjecture. We also suspect that the numerous freeze-thaw cycles in Antarctica render invertebrates from this continent especially susceptible to the production of free radicals. The larvae become anoxic during freezing and are subjected to oxygen reperfusion and free radical generation upon thawing (Joanisse and Storey 1998). We thus anticipate that SOD provides protection against the oxygen reperfusion following such bouts of anoxia.

The lack of $SOD$ expression in adults suggests that the short-lived adults (7-10 days) do not degrade superoxide anions. This is surprising given that the adults spend most of their time walking on the surfaces of rocks or plant material where they are vulnerable to sunlight and the heated substrate. Even though adults are less likely than larvae to experience bouts of freezing or anoxia, the lack of $SOD$ expression after an anoxic period is surprising and suggests the possibility that adults may lack the ability to produce this antioxidant enzyme. Due to a shortage of adults available during our field seasons in Antarctica, we were unable to directly test this hypothesis.
We suspect the difference in SOD expression between larvae and adults of B. antarctica reflects the contrasting durations of these two life stages. The brief period of adult life would not likely necessitate an elaborate protection mechanism against oxidative stress, while the larvae are much more susceptible to such injury during the onslaught of environmental stresses they encounter during their two-year developmental period. Such a dichotomy is also evident in the heat shock response (Rinehart et al. 2006): the larvae continuously mount a heat shock response, i.e. express their heat shock proteins, while the adults do not. But, unlike the heat shock response, the adults did not upregulate SOD following exposure to anoxia. Likewise, larvae are extremely drought tolerant (Hayward et al. 2007; Benoit et al. 2007b), while adults are not (Benoit et al. 2007a).

Unlike SOD mRNA, catalase mRNA was present in both larvae and adults. Catalase reduces the amount of hydrogen peroxide, and its expression is known to extend the life span of Drosophila melanogaster (Orr and Sohal 1994), c.f. the house fly Musca domestica (Bayne and Sohal 2002). A synergistic effect between SOD and catalase further extends the life span of D. melanogaster (Orr and Sohal 1994). Catalase mimetics also protect against oxidative stress and increase life span in Caenorhabditis elegans (Melov et al. 2000; Sampayo et al. 2003). Catalase is an unusual antioxidant enzyme in that it is sensitive to light and has a rapid turnover (Lesser 2006). The expression levels we observed for catalase were less strong than those observed for SOD, suggesting the possibility that catalase may be present at lower levels than SOD. The low expression level may also reflect the possibility that alternate enzymes, such as peroxidases, that also
catalyze the breakdown of hydrogen peroxide, may be upregulated at this time. However, strong upregulation of *catalase* was seen in response to sunlight, suggesting a possible increase of ROS production due to UVA radiation and/or rapid turnover of the protein in the presence of light.

The *smHsp* and *Hsp70* were continually expressed in larvae, but not adults, as previously reported (Rinehart et al. 2006). It is interesting to note that *Hsp70* was upregulated by anoxia to a greater extent in adults than in larvae, while the *smHsp* was barely expressed. Certainly, larvae experience anoxia more frequently than adults, and quite possibly the adults never experience it. The larvae appear capable of handling anoxia without further upregulation of the *Hsps* while adults possibly rely on the *Hsps*. Expression of *Hsp90* persisted at a constant level in both larvae and adults, and no responsiveness to stress was apparent, but we cannot discount a role for *Hsp90* in other stresses, *e.g.* desiccation.

In response to Antarctic sunlight, all three *Hsps* were strongly upregulated in larvae within 6 h, and that upregulation persisted for at least 9 h. We carefully controlled temperature during this time, thus we do not think this upregulation can be attributed to heat shock. In addition, the *Hsp* upregulation in response to sunlight was stronger than expression of these three genes following 1 h of heat shock. It is evident that these expression patterns exceed that of the control, suggesting that something else is involved, possibly UVB. UVB elicits *Hsp70* upregulation in sea urchin embryos (Bonaventura et al. 2005; Matranga et al. 2006). UVC also prompts the upregulation of *Hsp70* in certain tissues (Niu et al. 2006), but so little UVC reaches the Antarctic surface that we think this
is an unlikely cause of Hsp70 upregulation. The involvement of Hsps in various ultraviolet radiation treatments suggests that these chaperones are critical for adequate cell function during UV exposure.

The total antioxidant capacity of *B. antarctica* larvae was quite high when compared to another freeze-tolerant insect originating from the temperate zone, *E. solidaginis*. With a capacity of up to five times that of the gall fly *E. solidaginis*, the Antarctic midge appears exceedingly capable of dealing with oxidative stress and free radical formation. Our results from lipid and protein oxidation also confirm that this is indeed the case. The larvae apparently do not increase their antioxidant defense in response to environmental stress as their antioxidant defense is already quite high. Total antioxidant capacity of adults was even higher than that of larvae. This result was surprising because adults do not express SOD, and even catalase levels were much lower than those observed in larvae. Still, adults are more susceptible to ROS formation due to UV radiation given the fact that they are surface dwellers and are exposed to over 20 h of daylight daily during the austral summer. In addition, certain antioxidant enzymes and compounds with antioxidant capacity are upregulated during reproduction to protect both the ovaries and eggs from ROS formation. Catalase is one such enzyme that has been found in the mosquito *Anopheles gambiae* to not only be upregulated during reproduction but also to have a rescue effect against the decline of egg laying in aging females (DeJong et al. 2007). Chorion peroxidases have been found in *Aedes aegypti* and presumably play an important role in ROS defense during egg formation (Li and Li 2006). Therefore it seems reasonable that the antioxidant defenses that the adults use to
protect sperm and eggs result in a higher total antioxidant capacity. But, our experiments have not identified the factors contributing to the ROS defense present in the adult midge.

Sugar-based cryoprotectants are well known to reduce osmotic outflow from the cell and thus reduce injury due to cell dehydration (Storey and Storey 1988). Trehalose has been implicated in anoxia tolerance by reducing protein aggregation and maintaining proteins in a partially folded position aiding chaperone refolding, enhancing recovery from anoxia exposure and possibly as a carbon source under extraordinary conditions (Chen et al. 2002). Trehalose has also been demonstrated to prevent damage caused by oxygen radicals when combined with heat shock (Benaroudj et al. 2001, Pereira et al. 2001). Our data suggest that larvae do not rely upon stress-induced increases in trehalose concentration as a means to prevent or limit potential oxidative damage. Other sugar-based compounds such as mannitol and glycerol can reduce damage to proteins caused by ROS (Benaroudj et al. 2001), and we cannot eliminate the possibility that the midge larvae use such compounds. We recorded a strong increase in glycerol levels during desiccation (Benoit et al. 2007b), but we did not measure glycerol changes in response to the present stressors.

In summary, the strong and constant upregulation of SOD and the rapid upregulation of catalase in response to sunlight in the larvae are a likely defense against oxidative damage due to the accumulation of ROS. This protection was noted under field conditions, as well as in response to experimentally-induced heat shock, freezing and anoxia. In contrast, the short-lived adults also have a strong antioxidant defense but mount this defense in the absence of SOD expression. Possibly the adults are protected
from ROS by peroxidases involved in the protection of sperm and eggs. Clearly the total antioxidant capacity of adults exceeds that of larvae, which in turn is much higher than observed in *E. solidaginis*, a freezing-tolerant insect from the temperate zone. A *smHsp* and *Hsp70* are upregulated when the adults are exposed to anoxia but not under normal conditions, suggesting that the adults are capable of mobilizing Hsps under stressful conditions. For the three stresses (heat shock, freezing and anoxia) tested, the larvae did not further upregulate the *Hsp70s* beyond their normal constitutive levels. However, all three *Hsp70s* were strongly upregulated in the defense that the larvae mounted against prolonged exposure to ultraviolet radiation. This indicates that UVR possibly is more threatening to larval well-being than other naturally occurring stresses on the continent. Thus, the larvae and adults have distinctly different protection strategies against ROS, a distinction that most likely reflects their different microhabitats (underground vs rock surfaces), stage duration (2 yr vs 1-2 wk), and functional differences (non-reproductive vs reproductive).

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Figure 4.1. Amino acid homology comparison of superoxide dismutase 2 (Mn) from *B. antarctica*, *A. aegypti* and *D. melanogaster*. The percentage identity shared is 68% and 67%, respectively. The solid line (___) shows the conserved N-terminal long alpha antiparallel hairpin. The dashed line (---) shows the conserved C-terminal mixed alpha/beta fold. Consensus matches are shown in black, partial matches in grey and no matches in white.
Figure 4.2. Northern blot hybridizations showing mRNA expression of SOD, catalase, a smHsp, Hsp70 and Hsp90 in response to a variety of environmental stresses. 28S was used as a control. The treatments were: larvae at 4°C (summer habitat temperature), adults at 4°C, larvae heat shocked at 30°C for 1 h, larvae frozen at -5°C for 2 d, larvae under anoxic conditions for 2 d, and adults under anoxic conditions for 2 d.
Figure 4.3. Northern blot hybridizations showing the mRNA expression patterns of SOD, catalase, a smHsp, Hsp70 and Hsp90 in response to direct Antarctic sunlight. 28S was used as a control. The larvae were exposed to sunlight for 0, 3, 6 or 9 h. Water temperature within the Petri dishes was maintained at 4°C (summer habitat temperature).
Figure 4.4. Amino acid homology comparison of catalase from *B. antarctica*, *A. aegypti* and *D. melanogaster*. The percentage identity shared for the cloned gene fragment is 71% and 69% respectively. The dashed line (---) area marks the heme-binding pocket characteristically found in heme-catalases. Consensus matches are shown in black, partial matches in grey and no matches in white.
Antioxidant capacity (umol Trolox equiv. mg⁻¹ DM)

Figure 4.5. Total soluble antioxidant capacity of *B. antarctica* adults and larvae, and freeze-tolerant larvae of the goldenrod gall fly, *E. solidaginis*. Values are mean ± SEM (n = 5). Different letters represent significant differences (P<0.05) between groups.
Figure 4.6. Total soluble antioxidant capacity of larvae of *B. antarctica* following exposure to a variety of environmental stressors. Larvae were frozen (-5°C/ 24 h), held under anoxia (48 h at 4°C), or heat shocked (30°C/ 1 h) and allowed to recover 2 h at 4°C prior to assessment of antioxidant capacity. Control larvae were maintained in water at 4°C. Values are mean ± SEM (n = 5).
Figure 4.7. Trehalose concentration in *B. antarctica* larvae following exposure to a variety of environmental stressors. Larvae were frozen (-5°C/24 h), held under anoxia (48 h at 4°C), or heat shocked (30°C/1 h) and allowed to recover 2 h at 4°C prior to measurement of trehalose concentration. Control larvae were maintained in water at 4°C. Values are mean ± SEM (n = 5). Different letters represent significant differences (P<0.05) between treatments.
Figure 4.8. Indices of oxidative damage, TBARS (A) and carbonyl proteins (B), in *B. antarctica* larvae following exposure to several environmental stressors that may be expected to result in production of reactive oxygen species. Larvae were frozen (-5°C/ 24 h), held under anoxia (48 h at 4°C), or heat shocked (30°C/ 1 h) and allowed to recover 2 h at 4°C prior to measurement of oxidative damage. Control larvae were maintained in water at 4°C. Values are mean ± SEM (n = 5).
CHAPTER 5

Dehydration, rehydration and overhydration alter patterns of gene expression in the Antarctic midge, Belgica antarctica.

ABSTRACT

We investigated molecular responses elicited by three types of dehydration (fast, slow and cryoprotective), rehydration and overhydration in larvae of the Antarctic midge, Belgica antarctica. The larvae spend most the year encased in ice but during the austral summer are vulnerable to summer storms, ocean spray and drying conditions due to intense sunlight. Using suppressive subtractive hybridization (SSH), we obtained clones that were potentially responsive to dehydration and then used northern blots to evaluate the gene’s responsiveness to different dehydration rates and hydration states. Genes encoding three heat shock proteins (smHsp, Hsp70, Hsp90), superoxide dismutase (SOD) catalase, metallothionein (Mtn), cytochrome p450, fatty acid desaturase (FAD), phospholipase A2 activating protein (PLAP), fatty acyl CoA desaturase, actin, muscle-specific actin (MSA), a zinc-finger protein (ZFP), pacifastin and VATPase were upregulated during fast dehydration. A smaller subset of these genes was upregulated in response to slow dehydration: Hsp70, catalase, p450, fatty acyl CoA desaturase, actin, MSA and pacifastin. Cryoprotective dehydration elicited increased expression of all three
Hsps, catalase, Mtn, FAD, PLAP, fatty acyl CoA desaturase, actin, MSA, ZFP and VATPase. Following fast dehydration, most genes continued to be expressed during rehydration, but SOD, Mtn, PLAP and ZFP were downregulated by rehydration. FAD was the only gene to be upregulated in response rehydration. All genes examined, except VATPase, were upregulated in response to overhydration. We suggest that these changes in gene expression during water loss and gain in the midge contribute to the maintenance of proper protein function and overall cell homeostasis during times of osmotic flux, a challenge that is particularly acute in this Antarctic midge.

INTRODUCTION

The Antarctic midge, Belgica antarctica, is endemic to the maritime Antarctic, and in this habitat it is confronted with a wide range of environmental stresses. The larvae spend up to 10 months each year frozen within the icy substrate, but during the austral summer when the ice melts, they thaw, feed and reinitiate their growth and development. Larval development is completed after two years, and then the wingless adults emerge, reproduce and die within a 7-10 day window in late December-early January (Sugg et al. 1983). During the austral summer the larvae are exposed to rain storms, pools of melting snow and ice, ocean spray and periods of dry habitat due to intense sunlight. One of the major problems that the midge larvae encounter in this environment is management of their water content.
Our previous work on water balance of this insect demonstrated that these hydrophilic larvae employ several mechanisms to both conserve water (Benoit et al. 2007) as well as lose water to enhance their freeze tolerance (Hayward et al. 2007). Water conserving mechanisms include increasing cryoprotectants, decreasing their metabolic rates and thereby decreasing their water loss rates, and clustering (Benoit et al. 2007). In addition, the larvae are able to tolerate the loss of up to 70% of their body water and thereby enhance their freeze tolerance (Hayward et al. 2007). Larvae may experience as many as 140 freeze-thaws cycles throughout the year (Block 1997), thus providing yet another challenge for maintenance of water balance.

Under natural conditions, dehydration can occur rapidly or slowly. During winter, when the larvae are directly exposed to ice, dehydration likely occurs slowly, until the vapor pressure of the midge larva matches that of the ice. This slow form of dehydration, known as cryoprotective dehydration, is likely to be the major water management strategy used during the winter (Elnitsky et al. 2008). Thus far, little is known about the molecular changes associated with these changes in hydration state.

In this study, we performed suppressive subtractive hybridization (SHH) using hydrated and dehydrated individuals, and from this we identified 70 genes that were putatively altered in expression. Northern blot hybridizations were used to confirm expression of those genes during dehydration. We then chose 15 candidate genes (smHsp, Hsp70, Hsp90, SOD, catalase, metallothionein, p450, FAD, PLAP, fatty acyl CoA desaturase, actin, MSA, a zinc-finger protein, pacifastin, and VATPase) to monitor
expression changes during fast and slow dehydration, cryoprotective dehydration, fast and slow rehydration, and overhydration.

MATERIALS AND METHODS

Insects

*Belgica antarctica* larvae were collected in January, February, and March 2006 and 2007, on Humble, Norsel Point, Torgersen and Cormorant Islands near Palmer Station, Anvers Island, Antarctica (64°46’S, 64°04’W). Larvae were collected with the substrate. The larvae and substrate were frozen and transported to Ohio State University, where the genes were cloned and their expression patterns monitored. In our laboratory, the larvae were maintained in a cold room at 4°C. Before the experimental larvae were subjected to the various treatments, they were sorted from the substrate in iced water and then transferred to glass beakers containing moist paper towels at 4°C.

Stress Treatments

Control larvae were sorted directly from the substrate and stored at high humidity conditions (moist paper towels in sealed glass beakers) to avoid the prolonged ice water sorting and water storage protocol we used in previous experiments (Rinehart et al. 2006, Hayward et al. 2007, Lopez-Martinez et al. 2008). This control thus represents conditions that preclude the possibility of hydration or overhydration due to storage of the larvae in water, and yet still allows for gut clearance and synchronization of the larval hydration state. Thus, none of the control larvae were stored in water, but instead were
held at a high relative humidity, simulating substrate conditions during the austral summer.

Several dehydration series were evaluated. Fast dehydration was achieved by exposing larvae to 75% RH (saturated NaCl) for 36 hr; this resulted in a loss of approximately 50% of their total water content. A shorter exposure (12h) to 75% RH, resulting in a 30% water loss was also tested to mimic what occurs on a summer day in the Antarctic. Slow dehydration was achieved at 98% RH (saturated K₂SO₄) for 5 d, a treatment that resulted in approximately a 50% water loss. Larvae were also subjected to cryoprotective dehydration (Elnitsky et al. 2008), a slow dehydration over a 14 d period in the presence of ice.

Fast and slow rehydration series were also carried out following both fast (75% RH for 36 h) and slow dehydration (98% RH for 5 d). Fast rehydration was done by submerging larvae in water for 2 hrs, and slow rehydration was achieved by exposing larvae to 100% RH for 12 hr.

To evaluate the effect of holding larvae in water, larval survival was determined after different durations of exposure to water. Ten larvae were placed into 1.7 ml microcentrifuge tubes containing 1 ml distilled water and held at 4°C for up to 30 d. Each day, 0.75 ml water was replaced with fresh water to prevent anoxia and build up of waste products. Every 5 days, the larvae were poured into Petri dishes to monitor survival. The midges were observed under a dissection microscope, and individuals were considered living if movement was noted. After assessing survival, dead larvae were removed and stored in separate tubes for re-evaluation of mortality 5 d later. Controls
consisted of groups of ten larvae held in mesh-covered tubes (N =10) that were switched between 98% and 100% RH every 12 hrs, to maintain proper hydration levels (Benoit et al. 2007).

Source of clones

Most clones used in this study were obtained by a suppressive subtractive hybridization (SSH) procedure in which hydrated control larvae were subtracted from a group of larvae desiccated for 12h at 98.4% RH. The BD SMART™ PCR cDNA Synthesis kit (Clontech Laboratories) was used to prepare the clones and a PCR-Select™ cDNA Subtraction Kit (Clontech Laboratories) was used for the subtraction. Clones of Hsp70, Hsp90 and catalase, that were also evaluated in this study, were isolated previously (Rinehart et al. 2006, Lopez-Martinez et al. 2008).

Northern blot hybridization

Trizol reagent (Invitrogen) was used to extract RNA from groups of 75 larvae, following the manufacturer’s protocol. Four micrograms of RNA per treatment were run on a 1.4% agarose, 0.41 M formaldehyde gel. The RNA was then transferred to a nylon membrane (Hybond-N+, Amersham Biosciences, Piscataway, NJ) using a Schleicher & Schuell’s Turboblotter transfer system. Roche Diagnostics’s DIG-High Prime labeling kit was used to label the DNA clones for hybridization, which was done at 42°C overnight. The northern blots were treated following the protocol from the DNA Labeling and Detection Starter Kit II, also from Roche, and exposed using Blue Lite
Autorad Film (ISC BioExpress) for 30 minutes. All membranes were stripped and re-
hybridized with 28S (our control gene). All northerns were done in triplicate.

RESULTS

Suppressive subtractive hybridization (SSH)

The SSH produced 70 unique clones whose putative identity could be deduced by
blasting their amino acid translations using GenBank (http://www.ncbi.nlm.nih.gov/
blast/Blast.cgi). Accession numbers are registered in GenBank as numbers DQ507279
thru DQ507371. Northern blot hybridizations were used to confirm expression of each
gene. Among the 70 clones, 42 were unaffected by fast, slow or cryoprotective
dehydration and thus their expression was not monitored for rehydration or
overhydration. The 15 genes that were most responsive to dehydration were also
monitored by northern blot hybridization for responses to rehydration and overhydration.

Fast and slow dehydration series

The identity, size and match of clones that were upregulated in response to fast
dehydration are shown in Table 1. Table 2 shows the same information for the 8 genes
that were downregulated during fast dehydration. Three heat shock proteins (a smHsp,
Hsp70, Hsp90), superoxide dismutase, catalase, metallothionein, p450, fatty acid
desaturase, phospholipase A2 activating protein, fatty acyl CoA desaturase, actin,
muscle-specific actin, a zinc-finger protein, pacifastin, and Vacuolar (H+) ATPase were
upregulated in response to 12 h at 75% RH (fast dehydration) (Fig 1). When the duration
of fast dehydration was increased to 36 hrs, most of the genes were still upregulated (Fig. 2), but expression of $Mtn$ and $ZFP$ decreased with the longer exposure time.

Slow dehydration (5 d at 98% RH) led to the upregulation of $Hsp70$, $catalase$, $p450$, fatty acyl CoA desaturase, actin, $MSA$, and $pacifastin$ (Fig 2). Thus, slow dehydration increased expression of some of the same genes upregulated by fast dehydration ($Hsp70$, catalase, $p450$, fatty acyl CoA desaturase, actin, $MSA$, and $pacifastin$) but there was no difference from the controls in expression levels for the $s\text{m}Hsp$, $Hsp90$, $FAD$ and $PLAP$ (Fig 2). $SOD$, $Mtn$, $ZFP$ and $VATPase$ were downregulated in response to slow dehydration when compared to hydrated controls (Fig 2).

Cryoprotective dehydration

The three $Hsps$, catalase, $Mtn$, $FAD$, $PLAP$, fatty acyl CoA desaturase, actin, $MSA$, the $ZFP$, and $VATPase$ were strongly upregulated in response to cryoprotective dehydration, dehydration in the presence of ice that lasted 14 d (Fig 1). Expression of the $s\text{m}Hsp$, fatty acyl CoA desaturase and $VATPase$ was greater for cryoprotective dehydration than for fast dehydration. But, expression of $Hsp90$, $Mtn$, $FAD$, $PLAP$ and actin was less strong than elicited by fast dehydration. Expression of $SOD$, $p450$ and $pacifastin$ did not differ from that of the controls (Fig 1). Cryoprotective dehydration elicited a response that was very similar to the response to slow dehydration (98% RH for 5 d) (Fig 2).
**Fast and slow rehydration**

Most differences in gene expression that occurred during rehydration were noted in larvae that were first subjected to fast dehydration (36 h at 75% RH). The only notable change in expression during rehydration following slow dehydration was the upregulation of *Hps90*, while the expression of the other genes remained mostly unchanged (data not shown). Thus, the following rehydration results focus on rehydration following fast dehydration.

Following dehydration, *smHsp, Hsp70, fatty acyl CoA desaturase* and *actin* did not change in response to either fast or slow rehydration (Fig 2). Expression was elevated by fast dehydration and it remained high during both types of rehydration. Expression of *SOD, Mtn, PLAP* and *ZFP* decreased following both types of rehydration (Fig 2). *Hsp90, catalase, MSA, pacifastin* and *VATPase* were downregulated as a result of fast rehydration, while *P450* and *FAD* expression remained unchanged (Fig 2). *FAD* was the only gene that was uniquely upregulated by slow rehydration (Fig 2d).

Expression of the three *Hsps, catalase, fatty acyl CoA desaturase, actin, MSA, pacifastin* and *VATPase* was not changed by slow rehydration, beyond the strong response noted during the initial fast dehydration regime (Fig 2), whereas *SOD, Mtn, p450, PLAP* and the *ZFP* were downregulated in response to slow rehydration (Fig 2).

**Overhydration**

The *smHsp, Hsp70, Hsp90, SOD, catalase, Mtn, p450, FAD, PLAP, fatty acyl CoA desaturase, actin, MSA, ZFP* and *pacifastin* were upregulated when larvae were
transferred from moist filter paper to water and held there for 10 days (Fig 3). Among the genes evaluated, VATPase was the only gene that was not upregulated by overhydration (Fig 3f).

*Survival of overhydration*

When larvae were submerged in water, internal water content increased over the first 10 days by approximately 10% (Fig 4); thereafter water content remained stable. After 1 week in water, the larvae became less active and were clearly more turgid. This coincided with a decrease in survival from approximately 80% at the end of week 1 to nearly 50% by the end of week 2 (Fig. 4). By the end of week 4, only 30% of the larvae were still alive, and nearly all larvae died by week 5 (data not shown).

**DISCUSSION**

One of the biggest challenges that an insect faces is water loss. Dehydration leads to significant physiological injury including protein denaturation, nucleic acid damage and lipid peroxidation, among others (Hansen et al. 2006) and can ultimately result in death. The severe damage that dehydration generates is better managed preventively than by attempting to repair massive cellular damage, thus it is presumably advantageous to mount an early response to dehydration (Franca et al. 2007). In this study, we have identified several genes that respond to changes in the hydration state of the Antarctic midge, *Belgica antarctica*. 
Heat shock proteins

The heat shock proteins are constitutively upregulated in the larvae of this midge (Rinehart et al. 2006), and our group previously found no further upregulation in response to dehydration (Hayward et al. 2007). We suspect that the discrepancy between our earlier results and the current dehydration response we note is due to the different methodologies used in our different studies. In our previous experiments, the larvae were held in water, a condition that our current experiments suggest results in overhydration. Although we still observed constitutive expression of Hsps in our current study when larvae were assayed immediately after collection from the substrate or held experimentally in moist paper towels, we now see that Hsps can be further upregulated when their hydration state is either increased (overhydration) or decreased (dehydration).

Hsps previously were shown to be upregulated in response to dehydration in the flesh fly, Sarcophaga crassipalpis (Tammarrello et al. 1999, Hayward et al. 2004), the collembolan Folsomia candida (Bayley et al. 2001), the eutardigrade Richtersius coronifer (Jönsson and Schill 2007) and during rehydration in the flesh fly, Sarcophaga crassipalpis (Hayward et al. 2004). In B. antarctica the smHsp and Hsp70 were expressed more highly in the long cryoprotective dehydration treatment than in either fast or slow dehydration. Even though cryoprotective dehydration represents a slower rate of water loss than our fast 12 hr treatment, it seems to be a more stressful situation for larvae of B. antarctica, as evidenced by Hsp expression. Possibly the slow but constant loss of water during this treatment elicited a need for more chaperones. In addition, the stronger upregulation of the smHsp and Hsp70 when dehydration time increased from 12 to 36 h
suggests the need for these proteins during prolonged dehydration, a result consistent with what we observed for cryoprotective dehydration. As the larvae lose more water, increased protein and membrane damage occurs and hence the upregulation of the \textit{smHsp} and \textit{Hsp70} during prolonged fast dehydration suggests that there is potentially more threat to protein integrity and function that was not as severe during short dehydration. \textit{Hsp90} was most highly upregulated during fast dehydration and slow rehydration. Previously, Hsp90 was implicated in rehydration of the flesh fly (Hayward et al. 2004). Expression of different Hsps following dehydration and rehydration was previously observed in the flesh fly: a \textit{smHsp} and \textit{Hsp70} were upregulated during dehydration, while \textit{Hsp90} and \textit{Hsc70} were upregulated during rehydration (Tammariello et al. 1999, Hayward et al. 2004).

\textit{Antioxidant enzymes}

One of the most deleterious effects of dehydration in the cell is oxidative damage (Franca et al. 2007). Though the origin of the excess oxygen radicals is not fully understood, a 10-fold increase in oxidation as a result of dehydration was recorded in yeast cells (Pereira et al. 2003). It is suspected that greater oxidation may play a major role in death caused by dehydration. Thus, protection against oxidation by enzymatic breakdown of oxygen radicals is crucial during water loss. A universal primary defense against oxygen toxicity is superoxide dismutase (SOD). SOD catalyzes the breakdown of the superoxide anion into hydrogen peroxide and oxygen. Hydrogen peroxide is then broken down to water and oxygen by several enzymes, the main one...
being catalase. Our previous work showed that SOD is highly expressed under normal control conditions, heat shock, freezing and anoxia in the larvae of B. antarctica (Lopez-Martinez et al. 2008). The upregulation of SOD in response to dehydration was stronger than what we observed for freezing and anoxia, which both lead to the production of oxygen radicals as a result of oxygen reperfusion. Possibly the challenge of dehydration is stronger than the challenges of the other stresses previously tested. SOD was upregulated in response to dehydration in moss (Mayaba and Beckett 2003) and yeast (Pereira et al. 2003); in both of those systems SOD is considered to play a vital role in dehydration tolerance, and we suspect that this is the case with B. antarctica as well.

Catalase was also involved in the dehydration response of B. antarctica. Catalase was also linked to dehydration in yeast (Franca et al. 2005), where a 70% increase in intracellular oxidation was observed in catalase mutants. Catalase rapidly breaks down high concentrations of hydrogen peroxide (Kranner and Birtić 2005). Thus, as water is lost and osmolality increases during fast dehydration in B. antarctica (Benoit et al. 2007b), the increase in ROS may trigger a strong signal for catalase upregulation. Recently, catalase clones from the arctic collembolan, Onychiurus arcticus, were isolated in higher numbers from desiccated individuals than from controls in an EST project (Clark et al. 2007). These results suggest that catalase plays an important role during dehydration by eliminating hydrogen peroxide molecules that can lead to lipid peroxidation and that its role during dehydration is more critical than during rehydration.
Detoxification

Metallothioneins (MTs) are a group of cystine-rich small proteins that are involved in heavy metal detoxification and cellular homeostasis. These proteins sequester heavy metals, sustain the balance of essential trace metals and maintain a reservoir for copper and zinc (Sato and Bremner 1993). MTs have also been shown to have antioxidant capabilities by rapidly reacting with hydroxyl radicals with a higher affinity than to the superoxide anion (Thornalley and Vasäk 1985). This is of importance because there is no specific enzymatic defense against the most potent reactive oxygen species, the hydroxyl radicals (\(-\text{OH}\)). The role of MTs as an oxygen radical scavenger has been questioned because, in the presence of hydrogen peroxide, the protein can be oxidized and its functionality compromised; however, readily available catalase can prevent this from occurring (Jiménez et al. 1997). We have recorded an upregulation in Mtn2 by B. antarctica in response to freezing and anoxia (unpublished data), both of which result in the generation of oxygen radicals. Thus, the upregulation of Mtn2 in response to dehydration, but not rehydration, supports a role for Mtn2 in scavenging oxygen radicals during the process of water loss and maintaining the homeostasis of trace metals as the osmolality in the cell increases. The increase we saw for catalase may also serve a protective role for maintaining the integrity of metallothionein as catalase scavenges the hydrogen peroxide that will readily oxidize metallothionein proteins.

Cytochrome P450 monooxygenases are a family of enzymes involved in the metabolism of endogenous compounds and xenobiotics (Scott and Wen 2001). This huge family of enzymes is widely studied, but the complete extent of their function is still not
fully known. The main reactions they catalyze can lead to the reduction of harmful substances, including oxygen radicals, and this is possibly their role in response to fast dehydration.

_Fat metabolism_

Fatty acid desaturases catalyze the synthesis of mono and polyunsaturated fatty acids by introducing double bonds at specific locations in the saturated fatty acid molecules (Macartney et al. 1994). The Δ9 FAD that we cloned from _B. antarctica_ introduces a double bond at the Δ9 position. In insects, changes in fatty acid composition of the membrane have been most frequently observed in response to cold and in relation to homeoviscous adaptation (Koštál et al. 2003, Kayukawa et al. 2007). However, in the soil collembolan _Folsomia candida_, a large increase in the proportion of mono-unsaturated fatty acids was also recorded in response to drought tolerance (Bayley et al. 2001). Thus the strong upregulation we see in response to fast dehydration and the consistent expression during rehydration indicates that membrane re-structuring likely occurs during dehydration. Given that oxygen radicals are increased by dehydration and that they target polyunsaturated fatty acids in the membrane, the activity of FAD, _i.e._ the increase in mono-unsaturated fatty acids in the membrane, may be important during dehydration to maintain membrane integrity.

Phospholipase A2 activating protein is the enzyme that activates phospholipase A2 (PLA$_2$). Once activated this enzyme cleaves fatty acids at the sn2 position of phospholipids (Oliver et al. 1995). Normally it is polyunsaturated fatty acids, such as
arachidonic acid, that are cleaved and then replaced with more saturated fatty acids (Clark et al. 1991). This is beneficial not only because it allows another fatty acid to enter the membrane to change its fluidity but also because it can prevent lipid peroxidation. One of the reactive oxygen species, singlet oxygen, can directly interact with polyunsaturated fatty acid side chains and initiate lipid peroxidation (Halliwell 1987). A membrane that has already been compromised by lipid peroxidation is more likely to undergo further damage by oxygen radicals (Franca et al. 2007). Therefore the cleaving of side chains that will react with oxygen radicals is likely to be a key component of the dehydration survival mechanism used by this insect. Hence the upregulation of phospholipase A2 activating protein (PLAP) in response to dehydration, along with FAD, suggests that some membrane restructuring occurs in response to water loss. In such a scenario, we would not expect the upregulation of PLAP to occur in response to rehydration because the membrane may not need to again change its confirmation until the fully hydrated state is achieved.

Fatty Acyl CoA ∆9 desaturases are genes that are also involved in restructuring membranes, and these enzymes are essential in regulating membrane fluidity in eukaryotes (Eigenheer et al. 2002). Most functional studies of these enzymes suggest they play an important role in maintaining homeoviscous adaptation during cold exposure and acclimation by assisting in the membrane transition from the liquid crystalline phase to the gel phase (Drobnis et al. 1993, Koštál et al. 2003). These desaturases also play a prominent role in pheromone synthesis (Rosenfield et al. 2001). However, their role during dehydration has not previously been explored; the most likely scenario is that the
upregulation of this gene during dehydration is related to homeoviscous adaptation or other forms of membrane reorganization. Membrane restructuring during water loss is crucial because as oxygen radicals increase, the cell needs both a mechanism to prevent lipid peroxidation and the ability to restructure its membrane to prevent rupture. The expression of FAD, PLAP and fatty acyl CoA desaturase in response to dehydration suggests that a suite of enzymes are involved in protecting the cell and its membrane from damage due to water loss.

Cytoskeletal genes

A major component of the cytoskeleton is actin. It is a ubiquitous protein that is involved in filament formation and is crucial for cell motility and locomotion (Lovato et al. 2001). Just like vertebrates, insects have muscle-specific actins (MSAs), and in larvae these MSAs form filaments that are found along the abdominal walls, in the head, and in the alimentary canal (Mounier and Prudhomme 1991). Actin is upregulated in response to dehydration in the nematode Steinernema feltiae, where it maintains the cell skeleton and rapidly forms filaments as the nematodes shrink (Chen et al. 2005). Thus, the high expression of actin and muscle-specific actin that we observe in this study of B. antarctica suggests the restructuring of both the cellular cytoskeleton and the locomotory muscles of the larvae occurs in response to dehydration, rehydration and overhydration.
Other genes

The zinc-finger protein (ZFP) we found in *B. antarctica* may be responsive to stress in a similar fashion as other zinc-finger proteins reported in desiccation-tolerant plants (Sugano et al. 2003, Mukhopadhyay et al. 2004). The high expression of a zinc finger protein in petunias leads to an increase in drought tolerance and thus survival (Sugano et al. 2003). We also found this ZFP to be upregulated in response to heat shock (1 hr at 30°C), freezing (-5°C for 2 d) and anoxia (unpublished data), thus it is possible that this particular ZFP gene is responsive to a wide variety of stresses and that it is involved in maintaining cellular homeostasis.

A relatively new family of serine protease inhibitors, the pacifastin family, has been found in arthropods (Simonet et al. 2003). The function of this family of protease inhibitors is unknown, but they have been implicated in the prophenoloxidase activating system (ProPO-AS), which is part of the immune system of arthropods and is involved in wound healing and melanotic encapsulation. Even though no activation of the ProPO-AS was observed in *Schistocerca gregaria*, the pacifastin peptides appear to contribute to the immune system (Franssens et al. 2008). Pacifastin peptides also have been found in the corpora cardiaca of *Locusta migratoria*, suggesting a possible neurosecretory function (Clynen et al. 2001). The upregulation of this peptide in response to desiccation has not been previously reported.

Vacuolar (H⁺) ATPases are proton pumps with a highly conserved structure (Kane 2006). Their main function is the acidification of certain organelles such as lysosomes, early and late endosomes, and the late Golgi apparatus (Mellman et al. 1986). This
acidification is crucial for a range of cellular processes such as ion homeostasis, protein sorting and degradation (Kane 2006). More recently a link between oxidative stress and V-ATPase has been found. In *vma* (V-ATPase subunits) mutants an increase in the levels of reactive oxygen species was found, accompanied by protein damage (Kane 2007). This dual function of VATPases may be important during dehydration as they not only maintain acidification of the cell but also help counter the mass increase in reactive oxygen species that occurs during dehydration. As water re-enters the cell after dehydration, these pumps would presumably be active to maintain regular cellular function.

We are aware of no other studies examining molecular responses to overhydration in insects. The strong upregulation of many of the genes we evaluated suggests a massive defense strategy against increased water presence in the cells. Many of these genes are likely to be instrumental for maintaining cellular homeostasis after the 10% increase in water content caused by submersion in water. Given that larvae of *B. antarctica* are commonly encountered in pools of rain water and ocean spray, their ability to defend themselves during events of overhydration is likely to be an important component of their survival strategy in the constantly changing environment of the Antarctic peninsula during the austral summer.

In summary the fifteen genes that were monitored during dehydration, rehydration and overhydration define an array of processes that occur during changes in the hydration state and the cellular defenses that are mounted against water loss. As water rushes out of the cell, as it does during dehydration, maintaining normal cellular function is a key to
survival. The ability to sequester molecular chaperones such as the Hsps may be important for maintaining proper functions of other cellular proteins. The cellular membrane likely becomes re-structured and unsaturated by FAD, PLAP and fatty acyl CoA desaturases, while the antioxidant enzymes scavenge oxygen radicals to prevent lipid peroxidation in the membrane. Maintaining cellular integrity requires the fast production of actin filaments. It is important that, during this process, the normal ionic balance of the cell be maintained by controlling cell acidity (VATPase) and metal homeostasis (Mtn). Collectively, these genes we have identified are likely to enable the midge larvae to response to the changes in hydration state that are prevalent during the short austral summer and during the long Antarctic winter when the larvae are encased in an icy matrix.

ACKNOWLEDGEMENTS

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REFERENCES


Kayukawa, T., Chen, B., Hoshizaki, S., and Ishikawa, I. 2007. Upregulation of a desaturase is associated with the enhancement of cold hardiness in the onion maggot, Delia antiqua. Insect Biochemistry and Molecular Biology 37, 1160-1167.


<table>
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<th>Abbrev</th>
<th>% ID, Organism</th>
<th>D</th>
<th>R</th>
<th>O</th>
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<td>Hsp70</td>
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<td>Hsp90</td>
<td>Hsp90</td>
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<td>Catalase</td>
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<td>Mtn</td>
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<td>x</td>
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<td>DQ507342</td>
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<td>x</td>
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</tr>
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<td>FAD</td>
<td>52%, <em>Aedes aegypti</em></td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
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<td>DQ507304</td>
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<td>PLAP</td>
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<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Ba-U117</td>
<td>DQ507336</td>
<td>Fatty Acyl-CoA desaturase (delta-9)</td>
<td>∆9 desat</td>
<td>52%, <em>Culex pipiens quinquefasciatus</em></td>
<td>x</td>
<td>x</td>
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<td><strong>Cytoskeletal</strong></td>
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<td>Ba-U41</td>
<td>DQ507302</td>
<td>Actin</td>
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<td>85%, <em>Aedes aegypti</em></td>
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<td>x</td>
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<td>DQ507341</td>
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<td>MSA</td>
<td>100%, <em>Aedes aegypti</em></td>
<td>x</td>
<td>x</td>
<td>x</td>
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<td></td>
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<td></td>
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<td>Zinc-finger protein</td>
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<td>Pacifastin</td>
<td>32%, <em>Locusta migratoria</em></td>
<td>x</td>
<td>x</td>
<td>x</td>
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<td>DQ507282</td>
<td>Vacuolar (H+) ATPase</td>
<td>VATPase</td>
<td>97%, <em>Aedes aegypti</em></td>
<td>x</td>
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</table>

Table 5.1 - Dehydration/Rehydration/Overhydration upregulated genes from the Antarctic midge, *Belgica antarctica*. Organism matches, percent amino acid identities and putative identities were confirmed using GenBank blast x([http://www.ncbi.nlm.nih.gov/blast/Blast.cgi](http://www.ncbi.nlm.nih.gov/blast/Blast.cgi)). Northern blot expression is shown for dehydration (D), rehydration (R) and overhydration (O).
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<th>Blast</th>
<th>% ID, Organism</th>
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<td>Ba-U50</td>
<td>661</td>
<td>DQ507306</td>
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<td>x</td>
<td>48%, Aedes aegypti</td>
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<td>Ba-D46</td>
<td>331</td>
<td>DQ507366</td>
<td>aliphatic nitrilase</td>
<td>x</td>
<td>89%, Aedes aegypti</td>
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<tr>
<td>Ba-D32</td>
<td>743</td>
<td>DQ507355</td>
<td>cytochrome c oxidase subunit II</td>
<td>x</td>
<td>77%, Anopheles punctulatus</td>
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<td>Ba-U52</td>
<td>189</td>
<td>DQ507313</td>
<td>cytochrome oxidase subunit III</td>
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<td>84%, Drosophila waddingtoni</td>
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<td>Ba-U37</td>
<td>762</td>
<td>DQ507299</td>
<td>juvenile hormone epoxydase hydrolase</td>
<td>x</td>
<td>64%, Aedes aegypti</td>
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<td>Ba-U32</td>
<td>552</td>
<td>DQ507297</td>
<td>L-asparaginase</td>
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<td>80%, Culex pipiens quinquefasciatus</td>
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<td>Ba-D02</td>
<td>555</td>
<td>DQ507338</td>
<td>thymus-specific serine protease</td>
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<td>57%, Culex pipiens quinquefasciatus</td>
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<td>DQ507295</td>
<td>leucine-rich transmembrane protein</td>
<td>x</td>
<td>64%, Aedes aegypti</td>
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Table 5.2 - Dehydration downregulated genes from the Antarcite midge, *Belgica antarctica*. Organism matches, percent and putative identities were confirmed using GenBank blast x(https://www.ncbi.nlm.nih.gov/blast/Blast.cgi).
Figure 5.1. Northern blot hybridizations of the 15 genes that were found to be upregulated in response to dehydration in *B. antarctica*. The treatments are control (C), fast dehydration at 75% RH for 12 hrs and cryoprotective dehydration (CP). 28S was used as the control.
Figure 5.2. Northern blot hybridizations showing the dehydration and rehydration series for *B. antarctica*. The treatments are control (C), prolonged fast dehydration at 75% RH for 36 h, fast rehydration in the presence of water (FR) and slow rehydration at 100% RH (SR). 28S was used as the control.
Figure 5.3. Northern blot hybridizations showing the effects of overhydration in *B. antarctica*. The treatments are control (C) and overhydrated larvae held in water for 19 days (OH). The control gene used was 28S.
Figure 5.4. Survival and water mass of *B. antarctica* during overhydration. The closed cells (■) indicate water mass and the open cells (□) indicate percent survival.
The apple maggot can undergo three developmental pathways at the time of pupariation. Most pupae enter a deep diapause, lasting up to 11 months, which requires a period of low temperature exposure to break. Some pupae will enter a shallow diapause that can last between 30 and 100 days and others will directly develop. I wanted to test whether photoperiod or temperature was more important for the induction of these developmental strategies.

Maggot-infested apples were placed in incubators (Percival-Scientific, Perry, IA) under different combinations of photoperiod and temperature. The treatments were: 28°C and long day conditions (17L:7D), 28°C and short day conditions (8L:16D), 28°C and short day conditions and 18°C and long day conditions (17L:7D). The treatment with high temperature (28°C) and long day conditions is supposed to generate pupae that will directly develop into adults while the treatment at low temperature (18°C) and short day conditions is supposed to generate mostly deep diapausing pupae.

Among the results we gathered, we found that the larval feeding period (time spent inside the apple feeding) at 28°C under long day conditions was 15.1 days on
average while larvae feeding at 18°C took 28.7 days (Table A.1). This is the time required to complete their development (egg to third instar larva), emerge from the apple and pupariate in the soil or substrate. It is important to note that the pupae from both treatments did not differ in size or weight (Figs. A.1 & A.2) but unpublished data suggest that they differ in fat content.

Larvae reared under direct development promoting conditions (28°C and long day) resulted in 45.9% of the pupae directly developing into adults, 53.6% going into shallow diapause and 0.05% undergoing deep diapause (n =272) (Fig. A.3). These data suggest that temperature and photoperiod play an equal role in direct development and shallow diapause and perhaps a third factor is involved in the decision to diapause. Only one deep diapauser was recorded but a lot of mortality was observed and it is possible that deep diapausers died of dehydration as the conditions were not optimal for maintaining deep diapause.

Larvae reared under diapause inducting conditions (18°C and short day) resulted in 62.8% of the pupae going into shallow diapause and 37.1% underwent deep diapause (n = 156) (Fig. A.5). Interestingly enough, 2 pupae were able to directly develop under this treatment and 5 of the deep diapausers (~10%) remained in diapause for an additional year. These results suggest that there may be other important factors in diapause induction.

Larvae reared at 28°C under short day conditions resulted in 46.4% of the pupae directly developing into adults and 53.6% going into shallow diapause (n = 28) (Fig A.5).
Because of the small sample size compared to the other treatments, there is not much that can be said about this treatment.

Larvae reared at 18°C under long day conditions resulted in 1.6% of the pupae directly developing into adults, 88.6% entering a shallow diapause and 9.8% undergoing deep diapause (n = 61) (Fig. A6). This treatment also has a small size, like the previous one because we were unable to finish the study. In spite of that, it is of interest to note that under these rearing conditions 88.5% of all pupae went into shallow diapause while only 9.5% went into deep diapause.

Even though we would have liked to have had 18 and 28°C with equal amounts of light and dark (12L:12:D), we did not have the time to do that. Thus we can only speculate that both temperature and photoperiod are important for diapause induction in _Rhagoletis pomonella_ and variations in those two factors can heavily affect the percentage of pupae that will undertake each developmental pathway.
Table A.1. Mean feeding and larval developmental time from egg to 3rd instar larva in the apple maggot, *Rhagoletis pomonella*, while confined inside the apple. *this mean feeding time has over 10,000 individual observations because it is our normal rearing non-diapause treatment.

<table>
<thead>
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<th>Temperature</th>
<th>Photoperiod</th>
<th>Mean feeding time</th>
<th># of larvae</th>
</tr>
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<tr>
<td>18°C</td>
<td>long day (17L:7D)</td>
<td>28</td>
<td>305</td>
</tr>
<tr>
<td>18°C</td>
<td>short day (8L:16D)</td>
<td>28.7</td>
<td>1542</td>
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<td>28°C</td>
<td>long day (17L:7D)</td>
<td>15.1</td>
<td>1895*</td>
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<tr>
<td>28°C</td>
<td>short day (8L:16D)</td>
<td>16.7</td>
<td>685</td>
</tr>
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</table>
Pupal diapause at 28°C and long day conditions

Figure A.1. Correlation between weight and days until adult emergence for *Rhagoletis pomonella* pupae reared at 28°C and long day conditions (17L:7D).
Pupal diapause at 18°C and short day conditions

Figure A.2. Correlation between weight and days until adult emergence for *Rhagoletis pomonella* pupae reared at 18°C and short day conditions (8L:16D).
Pupal development at 28°C under long day conditions

Figure A.3. Time of adult emergence (eclosion) following larval rearing at 28°C under long day conditions (17L:7D). The pupae were also maintained at that temperature and photoperiod until eclosion.
Figure A.4. Time of adult emergence (eclosion) following larval rearing at 18°C under short day conditions (8L:16D). The pupae were also maintained at that temperature and photoperiod until eclosion.
Figure A.5. Time of adult emergence (eclosion) following larval rearing at 28°C under short day conditions (8L:16D). The pupae were also maintained at that temperature and photoperiod until eclosion.
Pupal development at 18°C under long day conditions

Figure A.6. Time of adult emergence (eclosion) following larval rearing at 18°C under long day conditions (17L:7D). The pupae were also maintained at the temperature and photoperiod until the eclosion occurred.
APPENDIX B

Suppressive subtractive hybridization (SSH) in the Antarctic midge,

Belgica antarctica

A cDNA pool consisting of dehydrated larvae of B. antarctica was subtracted from a cDNA pool of control larvae producing 93 cDNA clones. Of those clones, 58 resulted from the dehydrated pool (Table B.1) and 35 from the control pool (Table B.2). None of the clones was restricted to either pool when confirmed by northern blot hybridization. All sequences have been deposited in Genbank and given accession numbers indicated in the tables.
<table>
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<td>60%, Culex pipiens quinquefasciatus</td>
</tr>
<tr>
<td>Ba-U27</td>
<td>493</td>
<td>DQ507293</td>
<td>Anterior fat body protein</td>
<td>47%, Culex pipiens quinquefasciatus</td>
</tr>
<tr>
<td>Ba-U28</td>
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<td>DQ507294</td>
<td>mRNA sequence</td>
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<td>Ba-U29</td>
<td>588</td>
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<td>Leucine-rich transmembrane protein</td>
<td>64%, Aedes Aegypti</td>
</tr>
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<td>Ba-U31</td>
<td>486</td>
<td>DQ507296</td>
<td>mRNA sequence</td>
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<tr>
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<td>DQ507297</td>
<td>L-asparaginase</td>
<td>80%, Culex pipiens quinquefasciatus</td>
</tr>
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<td>Ba-U34</td>
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<td>Oocyte-testis gene 1</td>
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<tr>
<td>Ba-U37</td>
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<td>DQ507299</td>
<td>Juvenile hormone epoxide hydrolase</td>
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<td>50%, Drosophila melanogaster</td>
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<tr>
<td>Ba-U41</td>
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<td>Actin</td>
<td>85%, Aedes aegypti</td>
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<td>DQ507303</td>
<td>mRNA sequence</td>
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<tr>
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<td>DQ507304</td>
<td>Phospholipase A2 activating protein</td>
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<td>Ba-U49</td>
<td>323</td>
<td>DQ507305</td>
<td>mRNA sequence</td>
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<td>4-nitrophenylphosphatase</td>
<td>48%, Aedes Aegypti</td>
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<tr>
<td>Ba-U53</td>
<td>828</td>
<td>DQ507307</td>
<td>Epsilon-trimethyllysine 2-oxoglutarate dioxygenase</td>
<td>25%, Aedes Aegypti</td>
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<tr>
<td>Ba-U54</td>
<td>104</td>
<td>DQ507308</td>
<td>mRNA sequence</td>
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<td>DQ507309</td>
<td>Oligosaccharyl transferase</td>
<td>73%, Aedes Aegypti</td>
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<tr>
<td>Ba-U56</td>
<td>391</td>
<td>DQ507310</td>
<td>Metallothionein 2</td>
<td>54%, Anopheles gambiæ</td>
</tr>
<tr>
<td>Ba-U59</td>
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<td>Acetyl-coa acetyltransferase</td>
<td>75%, Aedes Aegypti</td>
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<td>D5S ribosomal protein S2</td>
<td>89%, Culex pipiens quinquefasciatus</td>
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<td>DQ507313</td>
<td>Cytochrome c oxidase subunit III</td>
<td>84%, Drosophila nigella</td>
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<td>mRNA sequence</td>
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<td>Delta(9)-desaturase</td>
<td>54%, Culex pipiens quinquefasciatus</td>
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<td>Ba-U66</td>
<td>1168</td>
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<td>Axel_AAE005993</td>
<td>39%, Aedes Aegypti</td>
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<td>Ba-U69</td>
<td>724</td>
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<td>Hemolectin</td>
<td>41%, Apis mellifera</td>
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<td>Coatomer delta subunit</td>
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<td>Axel_AAE003336</td>
<td>89%, Aedes Aegypti</td>
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<td>RE14390q</td>
<td>85%, Drosophila melanogaster</td>
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<tr>
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<td>DQ507322</td>
<td>mRNA sequence</td>
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<td>DQ507323</td>
<td>Transmembrane protein 38A</td>
<td>91%, Culex pipiens quinquefasciatus</td>
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<td>DQ507324</td>
<td>GPI transamidase component PIG-T</td>
<td>45%, Culex pipiens quinquefasciatus</td>
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<td>551</td>
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<td>L-asparaginase</td>
<td>80%, Culex pipiens quinquefasciatus</td>
</tr>
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<td>DQ507326</td>
<td>Phenylalanine hydroxylase</td>
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<td>Ba-U96</td>
<td>627</td>
<td>DQ507327</td>
<td>Serine protease precursor</td>
<td>43%, Bombyx mori</td>
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<tr>
<td>Ba-U97</td>
<td>1231</td>
<td>DQ507328</td>
<td>ATP-binding cassette transporter</td>
<td>37%, Culex pipiens quinquefasciatus</td>
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<tr>
<td>Ba-U102</td>
<td>459</td>
<td>DQ507329</td>
<td>Plasma membrane calcium-transporting ATPase 2</td>
<td>82%, Culex pipiens quinquefasciatus</td>
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<tr>
<td>Ba-U107</td>
<td>554</td>
<td>DQ507330</td>
<td>Zygote-specific protein</td>
<td>61%, Chlamydomonas reinhardtii</td>
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<tr>
<td>Ba-U108</td>
<td>532</td>
<td>DQ507331</td>
<td>mRNA sequence</td>
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<td>DQ507332</td>
<td>mRNA sequence</td>
<td>N/A</td>
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<td>Ba-U112</td>
<td>712</td>
<td>DQ507333</td>
<td>Connexin43-interacting protein of 150 kDa</td>
<td>51%, Culex pipiens quinquefasciatus</td>
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<tr>
<td>Ba-U114</td>
<td>338</td>
<td>DQ507334</td>
<td>mRNA sequence</td>
<td>N/A</td>
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<td>Ba-U115</td>
<td>1275</td>
<td>DQ507335</td>
<td>Axel_AAE00815</td>
<td>61%, Aedes Aegypti</td>
</tr>
<tr>
<td>Ba-U117</td>
<td>1026</td>
<td>DQ507336</td>
<td>Delta(9)-desaturase</td>
<td>52%, Culex pipiens quinquefasciatus</td>
</tr>
</tbody>
</table>

Table B.1 - Putative dehydration upregulated genes resulting from the *Belgica antarctica* suppressive subtractive hybridization (SSH)
<table>
<thead>
<tr>
<th>Clone</th>
<th>Size (bps)</th>
<th>Accession No.</th>
<th>Putative Identity</th>
<th>% ID, Organism</th>
</tr>
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<tr>
<td>Ba-D1</td>
<td>577</td>
<td>DQ507337</td>
<td>Deoxyribonuclease ii</td>
<td>52%, Aedes aegypti</td>
</tr>
<tr>
<td>Ba-D2</td>
<td>555</td>
<td>DQ507338</td>
<td>Thymus-specific serine protease</td>
<td>57%, Culex pipiens quinquefasciatus</td>
</tr>
<tr>
<td>Ba-D3</td>
<td>758</td>
<td>DQ507339</td>
<td>1-acylglycerol-3-phosphate acyltransferase</td>
<td>34%, Aedes aegypti</td>
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<tr>
<td>Ba-D6</td>
<td>1095</td>
<td>DQ507340</td>
<td>Farnesoic acid o-methyltransferase-like</td>
<td>53%, Aedes aegypti</td>
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<tr>
<td>Ba-D10</td>
<td>543</td>
<td>DQ507341</td>
<td>Muscle-specific actin 2</td>
<td>100% Aedes aegypti</td>
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<tr>
<td>Ba-D11</td>
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<td>DQ507342</td>
<td>Cytochrome P450</td>
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</tr>
<tr>
<td>Ba-D13</td>
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<td>DQ507343</td>
<td>Glucose dehydrogenase</td>
<td>48%, Aedes aegypti</td>
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<tr>
<td>Ba-D16</td>
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<td>Alcohol dehydrogenase</td>
<td>57%, Aedes aegypti</td>
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<tr>
<td>Ba-D17</td>
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<td>mRNA sequence</td>
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<td>Ba-D20</td>
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<td>DQ507346</td>
<td>Xanthine dehydrogenase</td>
<td>74%, Culex pipiens quinquefasciatus</td>
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<td>Ba-D23</td>
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<td>W-repeat protein</td>
<td>65%, Aedes aegypti</td>
</tr>
<tr>
<td>Ba-D24</td>
<td>224</td>
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<td>N/A</td>
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<td>Ba-D25</td>
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<td>Purple acid phosphatase</td>
<td>60%, Culex pipiens quinquefasciatus</td>
</tr>
<tr>
<td>Ba-D27</td>
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<td>DEAD box ATP-dependent RNA helicase</td>
<td>80%, Aedes aegypti</td>
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<tr>
<td>Ba-D28</td>
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<td>DQ507351</td>
<td>RhoGTPase</td>
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<td>Ba-D29</td>
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<td>6-phosphogluconate dehydrogenase</td>
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<td>Tyrosyl-tRNA synthetase</td>
<td>88%, Aedes aegypti</td>
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<tr>
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<td>Aael_AAELO12158</td>
<td>50%, Aedes aegypti</td>
</tr>
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<td>Ba-D34</td>
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<td>DQ507357</td>
<td>SLender lobes</td>
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<td>Ba-D35</td>
<td>353</td>
<td>DQ507358</td>
<td>Putative salivary Cys-rich secreted peptide</td>
<td>36%, Aedes albopictus</td>
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<td>Ba-D36</td>
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<td>DQ507359</td>
<td>mRNA sequence</td>
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<td>Ba-D39</td>
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<td>Inosine-5-monophosphate dehydrogenase</td>
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<td>Ba-D41</td>
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<td>Delta (9) desaturase</td>
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<tr>
<td>Ba-D43</td>
<td>813</td>
<td>DQ507364</td>
<td>N-myc downstream regulated</td>
<td>84%, Aedes aegypti</td>
</tr>
<tr>
<td>Ba-D45</td>
<td>771</td>
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<td>mRNA sequence</td>
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<td>Ba-D46</td>
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<td>Mucin-like protein</td>
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<td>mRNA sequence</td>
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Table B.2 - Putative dehydration downregulated genes resulting from the *Belgica antarctica* suppressive subtractive hybridization (SSH).
APPENDIX C

Expression of antioxidant enzymes and heat shock proteins in response to ultraviolet radiation on the Antarctic midge, *Belgica antarctica*

My previous work on the Antarctic midge showed that superoxide dismutase (SOD), catalase (cat), a small heat shock protein (smHsp), Hsp 70 and Hsp90 were upregulated in response to direct Antarctic sunlight (Chapter 4). Thus, we decided to monitor these five genes during prolonged exposure to ultraviolet radiation, given that they have also been shown to be associated with various stressful conditions in this species (Chapters 4 & 5).

Larvae of *B. antarctica* were exposed to ultraviolet radiation A & C under laboratory conditions. The larvae were placed in Petri dishes that had been partially filled with distilled water. They were then subjected to 3, 6 or 9 h of UVA or UVC radiation. Afterwards the larvae were homogenized using Trizol reagent to extract their total RNA and northern blot hybridizations were performed. No larval mortality was recorded for any of the treatments.

*SOD* was the only gene downregulated in response to UVA (Fig. C.1). However, it was still constitutively expressed as *B. antarctica* larvae express *SOD* throughout larval development (Chapters 4 & 5). *Catalase* expression did not change from high expression.
level seen in the controls (Fig C.1). All three Hsps were upregulated in response to UVA (Fig. C.1), but expression of Hsp70 and Hps90 was stronger than that seen for the smHsp. *SOD* was also downregulated in response to UVC, although an upregulation occurred at 9 h. But, that signal was not stronger than the one recorded for the controls (Fig C.2). *Catalase* was downregulated in response to UVC but was still consistently expressed at a low level (Fig C.2). Expression for the *smHsp* mirrored the expression of *SOD* (Fig C.2), with an initial downregulation and a subsequent upregulation at 9 h. *Hsp70* was strongly downregulated and no there was no detectable signal during UVC treatments (Fig C.2). *Hsp90* was continually expressed at a low level (Fig C.2), a feature of Hsp90 that can be seen across taxa (Chapter 2, 3, 4 and 5).

These results suggest that the larvae of *B. antarctica* can upregulated genes responsible for reducing reactive oxygen species (AOEs) and maintaining proper protein function (Hsp) during prolonged exposures to UVA, a stress normally encountered in the Antarctic Peninsula. The response to UVC was not as strong, probably due to the fact that the larvae do not normally encounter UVC radiation.
Figure C.1 Northern blot hybridizations for antioxidant enzymes (AOE) and heat shock proteins (Hsps) in response to ultraviolet radiation A (UVA) in larvae of the Antarctic midge, *Belgica antarctica*. 
Figure C.2  Northern blot hybridizations for antioxidant enzymes (AOE) and heat shock proteins (Hsps) in response to ultraviolet radiation C (UVC) in larvae of the Antarctic midge, *Belgica antarctica*. 
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