SEASONAL VARIATIONS IN ANTIFREEZE PROTEIN ACTIVITY AND HAEMOLYMPH OSMOLALITY IN LARVAE OF THE BEETLE *Rhagium mordax* (COLEOPTERA: CERAMBYCIDAE)

C. Wilkens[1] and H. Ramløv*

Department of Science, Systems and Models, Roskilde University, P.O. Box 260, DK-4000 Roskilde, Denmark.
*Corresponding author, e-mail: hr@ruc.dk
[1] Current address: Department of Molecular Biology, University of Copenhagen, Ole Måløevsvej 5, DK-2200 Copenhagen N, Denmark

Abstract

Larvae of *Rhagium mordax* empty their guts in preparation for the winter, which alone may enable the larvae to supercool down to -20°C or below. This should be sufficient for the larvae to over winter in Denmark if they can prevent inoculation. Antifreeze proteins (AFP) prevent inoculation in adult *Rhagium inquisitor* and this is also likely in the larvae of *R. mordax*, as they are in contact with ice in their hibernacula during the winter.
Larvae of *R. mordax* probably produce AFPs in the early autumn, however, in some individuals thermal hysteresis (TH) as high as 5.01 °C was observed in June. Whether or not these individuals have a constant level of TH in their haemolymph all year or if they produce further antifreeze proteins during the autumn is unknown. The lowest measured in January was 7.49°C (the highest during this month was 9.08ºC) so it is likely that the individuals with the highest TH in June also produce AFPs. Haemolymph osmolality in *R. mordax* is relatively low compared to other freeze avoiding insects, samples taken in January peak at 741 mOsm (±127 mOsm). The results of this study are compared with similar data for the closely related *R. inquisitor*.

Key words: Antifreeze proteins; *Rhagium mordax*; thermal hysteresis; Coleoptera; Cerambycidae.

Abbreviations: T_m – melting point; T_h – hysteresis freezing point; AFP – antifreeze protein; T_f – freezing point; TH – thermal hysteresis

INTRODUCTION

Ecothermic animals living in polar, alpine and temperate regions are exposed to temperatures below the melting point (T_m) of their body fluids on a daily or yearly basis. To survive these temperatures, these animals must either avoid freezing of their body fluids or be able to survive ice formation (19).

In the 1960s, Ramsay (18) discovered that when he observed ice crystal formation in fluid from the perinephric space of the larvae of the beetle *Tenebrio molitor* the ice crystals had a "jagged outline and large crystals do not grow on the expense of small ones." Also he

observed that the system was not temperature reversible and that the temperature in this fluid could be lowered as much as 10ºC, in the presence of small ice crystals, before the crystals suddenly grew rapidly. This would later be known as thermal hysteresis (TH). The proteins that cause thermal hysteresis were first identified in the blood serum of Antarctic teleost fishes (2). Since then, these *antifreeze proteins* (AFP) have been observed in several animal taxa, including other insects (5).

AFPs bind to ice crystals and restrict the growth of the ice front to regions between the adsorbed AFPs. These regions grow resulting in an increased local curvature, and therefore, a higher surface free energy. The increased surface area and curvature make it energetically less favourable for water molecules to join the ice crystal (20, 22). This results in a non-colligative lowering of the solution's freezing point (T_f) (21), and the T_m and the T_f of the aqueous solution are separated. The temperature difference between T_m and the "new" T_f is known as thermal hysteresis and the "new" T_f is known as the hysteresis freezing point (T_h) (27).

The physiological role of insect AFPs is still debated (15, 16, 23, 28). It has been claimed that the AFPs stabilize supercooled body fluids by adsorbing to ice embryos (28), however, this if difficult to prove experimentally. It has been shown that AFPs bind to ice nucleating surfaces (15, 16) and this has been suggested as the physiological role of the AFPs because ice embryos are thought to be too small for the AFPs to bind (23). However, the formation of ice embryos in the body fluids is not the only problem faced by animals living in close contact with ice. If an animal is in contact with ice and the body fluids are supercooled, inoculation may occur i.e. body fluids may be seeded through pores in the cuticle, mouth, anus or trachea by the surrounding ice. AFPs have been shown to prevent inoculation in adult *Rhagium inquisitor* (7) and larvae of the beetle *Dendroides canadensis* (17).

TH varies throughout the year with a maximum during the winter in many freeze avoiding organisms (5,6) and this is cued by seasonal photo- and thermoperiodic cycles in the beetle *D. canadensis* (9, 10, 11). In many cold tolerant insects the haemolymph osmolality also varies throughout the year in a similar cycle (27). The elevated haemolymph osmolality in cold tolerant insects during the winter is usually due to the synthesis of low molecular weight cryoprotectants such as glycerol during autumn. The cryoprotectants work in a colligative manner as they suppress the T_f and T_m of water by 1.86°C per kg/mole (4) and the supercooling point is depressed even more (27).

R. mordax is a common inhabitant in Danish forests (14). It is a cerambycid beetle whose larvae feed on the phloem of dead stubs and logs of hardwood tree, after at least two years in the egg, larval and pupal stage the adults appear in the late summer or during the autumn. The adults stay in their pupal champer until the spring (8).
In this study we investigated seasonal variations of haemolymph antifreeze protein activity and osmolality in the larvae of the longhorn beetle *R. mordax*.

MATERIALS AND METHODS

Collection of larvae: *R. mordax* larvae were collected from under the bark of hardwood stumps in the forest Heide Overdrev near Osted in Denmark between March 2003 and January 2004. They were kept in darkness at 4°C for up to 48 hours before the haemolymph was sampled.

Haemolymph was sampled by puncturing the cuticula of the larvae. The haemolymph was then drawn into a glass capillary tube. Liquid paraffin was drawn into the tube, and the opposite end of the capillary was sealed by melting in a flame. The capillary tube was then centrifuged, leaving the haemolymph in the closed end of the tube, isolated from air by a column of paraffin oil (for further description of this method see Zachariassen et al. (26)). The samples were stored at -80°C for later use.

Osmolality/melting point: Haemolymph osmolality was measured on a nanolitre osmometer (Clifton Technical Physics, Hartford, NY USA).

Antifreeze protein activity: AFP activity of the haemolymph was measured on the nanolitre osmometer, where fluid samples of approximately 20nl submerged in liquid paraffin were observed through a Zeiss STEMI SV11 APO microscope and a Sony SVT-S 3050 P S-VHS video system while the temperature was regulated to within 0.001 °C. The samples were frozen by rapid cooling to –40 °C and warmed to a temperature just below the expected T_m. The samples were then slowly warmed, and the temperature at which the last ice crystal disappeared was taken as T_m. The sample was then refrozen, and warmed to a pre-set temperature (T_i) below the T_m. The crystal was allowed to stabilise for 1 min. The temperature was then lowered slowly (at approximately 1°C/min.) until rapid ice growth occurred, indicating that the hysteresis freezing point (T_h) had been reached. Measurements of T_m and T_h were given in mOsm by the instrument and were converted to temperature (°C) by multiplying by 0.00186 °C and the hysteresis activity was measured as (T_h-T_m).
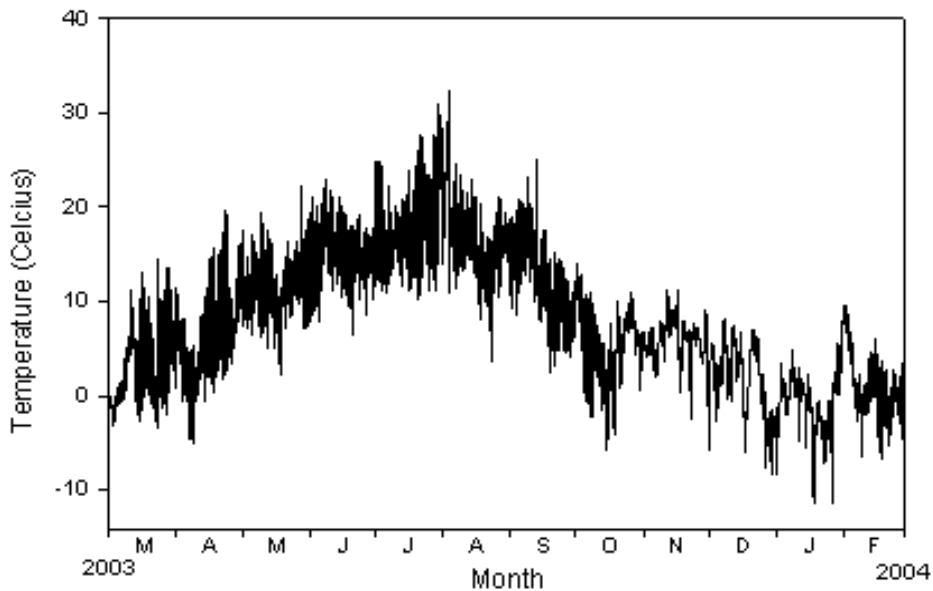

Figure 1: Variation in the temperature at 2 m height at Roskilde University between February 2003 and March 2004.

As Zachariassen and Husby (25) reported for *R. inquisitor*, the hysteresis activity in the haemolymph of *R. mordax* also shows a log linear negative relationship with the ice crystal size, therefore the crystal size has to be taken into consideration when the hysteresis activity is quantified. Accordingly, TH was determined several times for each sample with different ice crystal fractions. The ice crystal fraction was not measured directly but was calculated from the formula: $1 – (T_m/T_i)$ (25). The hysteresis activity in each sample was determined by drawing the best fitting line for a series of corresponding values of hysteresis and ice crystal fraction. The hysteresis activity was defined as zero at ice crystal fraction 1.0, and the best fitting line was drawn between this point and the median value of the observed values. To standardise the hysteresis activity in the different samples, the hysteresis was expressed

throughout as the ordinate value at an ice crystal fraction of 0.001 (a further description of this technique can be found in Zachariassen et al. - 28).

Temperature data: Temperature was measured at 2 m height every 20 minute at Roskilde University which is located ~20 km northeast of the collection site (Fig 1).

RESULTS

Haemolymph osmolality was lowest in spring/summer and highest in winter (approximately 420 mOsm and 741 mOsm respectively) (Fig. 2).
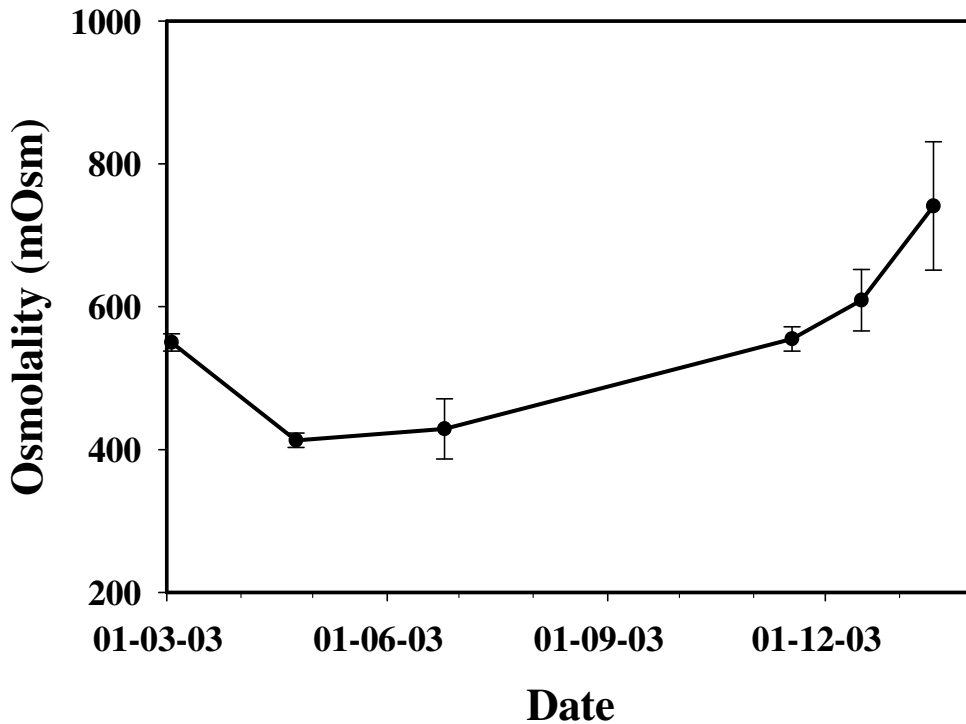

Figure 2. Seasonal variations in haemolymph osmolality in larvae of *Rhagium mordax*. Data are presented as mean ± SD, (n = 5-6 with one exception on January 15, 2004: n = 3).

The TH in the haemolymph of the larvae varied considerably both on a seasonal basis and between individual animals (Fig. 3). However, larvae of *R. mordax* showed strong hysteresis especially in winter (Fig. 4).

The average TH was highest during winter and early spring (Fig. 4) and the yearly variation between individuals peaked during the summer (25th of June) and showed a minimum in the winter (15th of January) (Fig. 3). On the 25th of June two individuals had a TH as high as 5.01°C and 4.23°C while others showed no appreciable TH. The average TH rose throughout the autumn and winter, reaching a maximum in January.

Only 3 larvae from the 15th of January 2004 are shown because we where unable to freeze the other samples in the nanolitre osmometer, which could indicate a very high concentration of AFPs; the highest TH measured was 9.08°C on this date.
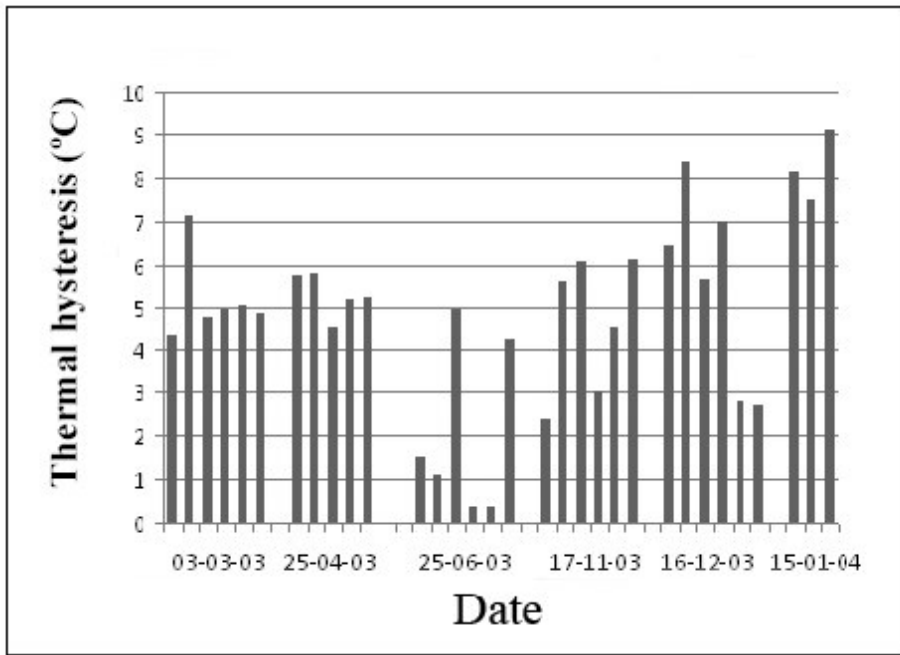
Figure 3. Histogram showing variation in antifreeze activity in individual *R. mordax* larvae at various dates throughout the year.
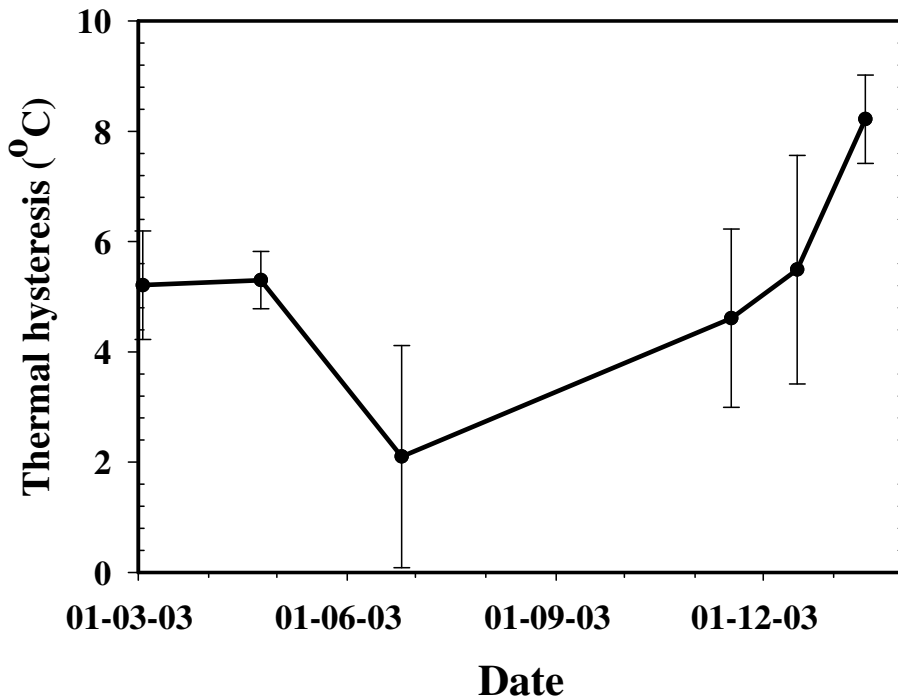

Figure 4. Seasonal variations in haemolymph thermal hysteresis in larvae of *Rhagium mordax*. Data are presented as mean ± SD, (n = 5-6 with one exception on January 15, 2004: n = 3)

DISCUSSION

Larvae of *R. mordax* emptied their guts during autumn and they did not have any contents in their guts until spring (personal observations C. Wilkens). Emptying their digestive system and thereby removing any ice nucleators (INA) may alone enable the larvae to supercool down to -20°C or below (12), which should be sufficient for the larvae to overwinter in Denmark if inoculation can be prevented. However, as long as a larva is supercooled there is a chance that it may freeze. Freezing may be prevented by the AFPs either by masking INAs (15, 23), by preventing inoculation (7) or by inhibiting the growth of ice embryos (25).

AFPs prevent inoculation in adult *R. inquisitor* (7) and in larvae of the beetle *D. canadensis* (17). This is also the probable role of AFPs in the larvae of *R. mordax*. In their hibernacula they are in contact with ice during the winter and early spring, but we found no frozen larvae at any time, which must thus be protected against inoculation from surrounding ice crystals. It is possible that the pores in the cuticle are so small that ice crystals are unable to pass through them, however, it was possible for ice to inoculate adult *R. inquisitor* that lacked AFPs (7) and this may well pertain to *R. mordax* larvae as well. Thus we assume that in *R. mordax* the AFPs also protect the insects against inoculation whether through pores in the cuticle or elsewhere.

It has been reported that AFP activity varies during the year in other insects (3, 5) and this is also the case for *R. mordax*. Larvae of *R. mordax* probably produce AFPs in the early autumn, however, in some individuals TH as high as 5.01°C was observed in June. Whether or not these individuals have a constant level of TH in their haemolymph all year or if they produce further antifreeze proteins during the autumn is unknown. But in January the individual with the lowest TH measured had a TH of 7.49°C so it is likely that the individuals with the highest TH in June also produce additional AFPs in winter.

The mean TH measured in June was 2.10± 2.01°C. It is unknown what is the function, if any, of the AFPs during the summer. However, temperatures below the T_f occur in Denmark near the surface of the earth during the summer so they might protect the animals from freezing during such events. Events of this kind are not shown in Fig. 1 as the data shown here are all measured at a height of 2 m with no vegetation nearby. However, it can be seen that even in summer temperatures can fall to approximately 4°C at this height and on clear nights this may give rise to freezing temperatures at ground level. Relatively high TH similar to the ones observed during summer in the present study is also seen in both *R. mordax* (29) and *R. inquisitor* populations in Norway during the summer (Pers. comm. Sindre A. Pedersen).

Overwintering *R. mordax* have an average TH at 8.22 ± 0.80°C on the 15th of January. In a number of cases it was not possible to freeze the samples in the nanolitre osmometer from animals collected in January, thus TH could be even greater than that reported here. To our knowledge the TH in this study is the highest measured in any animal only the sibling species *R. inquisitor* has nearly such a high hysteresis during winter. However, Bennett et al. (1) have showed an antifreeze activity of 12.85°C in 3.2 times concentrated haemolymph of the beetle *Cucujus clavipes*.

In late April the larvae also had a high TH, consistent with sub zero temperatures often occurring at this time of the year.

The haemolymph osmolality was 312 mOsm higher in the larvae collected in January compared to those collected in June. The higher osmolality found in the overwintering larvae of *R. mordax* may be due to loss of water but retention of ions. Lundheim and Zachariassen (13) have proposed that supercooled insects are more prone to water loss in their hibernacula than are freeze tolerant insects, due to the higher water vapour pressure of supercooled water

compared to that of ice. Ødegaard (29) suggests that *R. mordax* in contrast to *R. inquisitor* does not accumulate polyols as part of its cold hardening process. However, a slight increase in polyols during cold exposure during the autumn can not be ruled out, but was not measured. Also the decrease in haemolymph osmolality from the 3rd of March till 24th of April corresponded with the fact that the larvae were eating in late April and not in early March. This could result in a reestablishment of the water content and thus a decrease in osmolyte concentrations.

The haemolymph osmolality in *R. mordax* is relatively low compared to other freeze avoiding insects with a peak at 741 mOsm (±127 mOsm) on the 15th of January. In adult *R. inquisitor* the haemolymph osmolality is as high as 3200 mOsm during January and February (24). This gives *R. inquisitor* an advantage over *R. mordax* in very cold regions and this is seen in Norway where *R. mordax* is not found in the coldest parts. If present in the colder parts of Norway, it prefers hibernacula which are situated below the snow cover during winter (29). In Denmark *R. mordax* hibernacula were observed above the snow cover during winter. Snow cover was light or not present at all. However, it should be noted that the temperatures the Danish animals experience are higher than those which the Norwegian animals experience during the winter.

The high haemolymph osmolality in adult *R. inquisitor* is mainly due to a high concentration of glycerol (24) and this gives *R. inquisitor* an advantage over *R. mordax* in very cold regions. However, this mechanism may be expensive metabolically. Maintenance of high levels of polyols and carbohydrates is disadvantageous due to the metabolic costs (12). The low levels of low molecular weight cryoprotectants may thus allow *R. mordax* to develop in periods where *R. inquisitor* is preparing for the winter.

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