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Detecting seasonal variation of antifreeze protein distribution in *Rhagium mordax* using immunofluorescence and high resolution microscopy

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ABSTRACT

Larvae of the blackspotted pliers support beetle, *Rhagium mordax*, were collected monthly, for the duration of 2012 and fixed. The larvae were embedded in paraffin wax and sectioned. Using fluorophore-coupled antibodies specific to the *R. mordax* antifreeze protein, RmAFP1, sections were visualised with UV reflected light microscopy. An automated software analysis method was developed in order to discard autofluorescence, and quantify fluorescence from bound antibodies. The results show that *R. mordax* cuticle and gut exhibit a higher degree of fluorophore-bound fluorescence during summer, than in the cold months. It is hypothesised that *R. mordax* stores RmAFP1 in, or near, the fat body during times when freeze avoidance is not needed.

1. Introduction

A fundamental aspect of the temperate climate is its seasons. Terrestrial animals inhabiting this environmental zone, must therefore be able to cope with large seasonal variations in temperature, light, humidity and snow cover. During winter, the northern regions of the temperate zone may reach temperatures well below −10 °C [22] [24], while summers may be above 20 °C [14] [18].

Organisms vary in their response to cold and freezing conditions. Some are tolerant to freezing of their body fluids, while others must avoid such freezing. Insects may have a variety of behavioural responses, to initially avoid the cold [16] [14], but evolution has in many cases favoured the adaptation of a physiological cold response [19] [13]. In overwintering insects the external environment can produce conditions that, put the organism in danger of inoculative freezing [12]. As the environment cools, environmental ice may start to form from condensed water, and contact with such ice could be fatal for a freeze avoiding organism. Also, as insect larvae often have partly digested plant remains in their gut lumen, this internal environment may freeze, making the whole organism susceptible to inoculative freezing from the inside. As such, an emptying of the gut is often observed in larvae preparing to overwinter [18].

Freeze avoiding insects often employ a certain group of biological antifreeze agents called antifreeze proteins (AFP),[1] as well as colligative antifreeze compounds such as polyols [25], free amino acids [14] and inorganic salts p [18].

When water freezes, solutes are excluded from the growing crystal, leading to a sharp concentration gradient near the crystal surface. This process is believed to be one of the main causes of tissue damage in freezing organisms [7] [1]. By inhibiting inoculation and growth of ice crystals in the body fluids, AFP's can essentially extend the period of time before permanent damage occurs from freezing of the tissues.

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole (blue, 461 nm); FITC, Fluorescein isothiocyanate (green, 518 nm); TRITC, Rhodamine (red, 580 nm).

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[1] As more intricate knowledge about the mechanism of these proteins have become available in recent years, they are often referred to as Ice Binding Proteins or Ice Structuring Proteins.

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A study by Olsen et al., (1998) [12], showed that AFP distribution varied in tissue sections from *Dendroids canadensis* larvae as a function of season. In their immunofluorescence study [12] they observed that during winter the integument was bright from AFP-bound fluorescence, while the integument from summer larvae showed a much smaller degree of fluorescence from AFP binding. This indicated that the amount of tissue-bound AFP varies with season, and that it is strongest during winter. *R. mordax* is a freeze avoiding coleopteran, whose larvae can be found overwintering beneath the bark of rotting beech tree trunks and stumps [18]. This micro environment provides some protection from convective cooling, as well as ice crystal inoculation from surface snow or ice.

However, a previous study on the seasonal variation of AFP in *R. mordax*, by Wilkens and Ramløv (2008) [18], observed that larvae hemolymph showed thermal hysteresis activity (THA) all year, but that TH was highest during the coldest months. It was hypothesised that *R. mordax* primarily avoids freezing through AFP production, and that the synthesis of osmolytes was of secondary importance. The sister species *Rhagium inquisitor* accumulates large amounts of osmolytes, such as glycerol, during winter [23], but its distribution in general is higher north, and thus colder during winter. *R. mordax* is adapted to a warmer climate, and accumulates colligative cryoprotectants to a much smaller degree [18]. *R. mordax* can thus spend less energy on the maintenance of high hemolymph osmolality [17], and extend its growing season compared to its sister species *R. inquisitor*. A study of the thermodynamic stability of RmAFP1, the primary AFP found in *R. mordax* [5], showed that RmAFP1 has a melting point of 28.5 °C, but that it had exceptionally strong refolding capabilities. This lends credibility to the hypothesis that RmAFP1 has a long half-life in *R. mordax*, and that antifreeze activity can be detected year round because of this.

In earlier studies it was determined that the fat bodies of larvae from *D. Canadensis* [20] [3] and *Tenebrio molitor* [21] [4] were the primary production site of AFP. The fat body of *T. molitor* is filled with protein-rich granules, first observed by Easton & Horwath in 1994 [4]. These granules are immunologically active for Tm [12].[86], a *T. molitor* specific AFP. In 1996, Horwath et al. [8], observed an accumulation of Tm 12.86 in these granules, during summer. During winter, the granules had been emptied for Tm 12.86, presumably because of release of cryoprotectants to the hemolymph. The fat body in insects is closely connected to both the integument and the gut. Due to its role in energy metabolism and AFP synthesis in *T. molitor* [21] and *D. canadensis* [20], the tissues connected to the fat body are therefore of great interest, when seeking to determine seasonal variations in AFP levels.

In two previously unpublished studies of AFP localisation in *Zoarces viviparus* [9] and *R. mordax* [10] it was confirmed that the immunofluorescence method used in this paper was functional, but needed to be expanded upon. It was further determined that, AFP distribution in the cuticle and gut epithelium of *R. mordax* did not differ significantly between winter (Fig. 2a) and summer (Fig. 2b) months, which is in stark contrast to the clear seasonal variation seen in 1998 by Olsen et al. in larvae of *D. canadensis* [12]. If anything, the amount of fluorescence during summer seemed higher than in winter in Fig. 2. This discrepancy was the primary motivation, for the present study. Previous immunofluorescence micrographs of *R. mordax* larvae (Fig. 2) were all non-adjacent images, taken at various points of interest. This meant that limited structural information about the cross section could be extracted. The field of view was simply too narrow. This study expands upon the technique by using several hundred micrographs, and stitching them together, forming large composites of whole larva cross sections. The goal is a greater perspective on the possible seasonal variation in tissue distribution of AFP.

Based on the information presented here, the working hypothesis for this study is that, the amount of RmAFP1 associated fluorescence in *R. mordax* larvae will decrease as ambient temperature increases, as seen in *D. canadensis*. This is especially expected to be the case in the cuticle, and gut epithelium, as these two tissue types are involved in prevention of inoculative freezing.

2. Materials and methods

2.1. Capture and fixation of specimens

Larvae of *R. mordax* were collected once a month from February 2012 until January 2013 in Boserup Forest near Roskilde, Denmark. By collecting larvae for a whole year, every season is represented in the data set. A temperature logger was set up at the forest location, logging both the tree stump temperature beneath the bark and the air temperature. See Fig. 1 for temperatures throughout 2012. Larvae were located by removing decomposing bark from stumps of beech trees that had been cut down a few years earlier. Larvae were transported back to the laboratory in 2 ml Eppendorf tubes covered with perforated parafilm to ensure airflow, inside an insulating styrofoam box. Larvae were placed in embedding cassettes (Klinipath 2020) for easier storage. Larvae were fixed using FineFix (HD Scientific ML 70147) and kept at 4°C until further processing. See Table 1 for an overview of the experiments.

2.2. Tissue sectioning

The fixed larvae were cut with a scalpel to allow better fluid penetration and dehydrated in a series of increasing concentrations of ethanol and finally xylene. The dehydrated larvae were then infiltrated by molten paraffin wax (Thermo Scientific 6774006) at 60°C. Hardened blocks of paraffin wax and larvae were mounted on a Biocut 2030 microtome, and cut at 5 Âµm thickness. Sections were transferred to a floatation bath (Agar Scientific, L4136) filled with distilled water kept at 45–50 °C, and allowed to expand and stretch out for a few seconds before being collected onto a polysine coated glass slide (Agar Scientific L4345). Polysine slides were air dried and gently wiped around the cross sections. They were then allowed to anneal in a 40°C drying chamber for at least two hours. See Sup1 and Fig. S1 in the supplementary materials for a detailed protocol.

2.3. Tissue staining

Paraffin wax was removed by xylene submersion, and the tissue sections were rehydrated by submerging in decreasing concentrations of ethanol in water. For each batch of immunofluorescence stained tissue sections, one or two sections were also prepared for staining with hematoxylin and eosin. The main purpose of these HE stains was to help identify distinct internal anatomical structures, that may not have been visible using only UV-reflected light. Using a light polarizer, the cuticle and gut were identified in HE stains. Their unique light pattern was used as identification of tissue in the transparent immunofluorescence stains. The two staining procedures share the same 4-step protocol used for dehydration, embedding, sectioning and rehydration. See Sup2 in the supplementary materials for a detailed immunofluorescence protocol. See Sup3 for a detailed HE protocol.

2.4. Antibody production

Primary antibodies against the antifreeze proteins found in *R. mordax* were raised in rabbits against *R. mordax* AFP isoform 1 [5] and purified from serum by BioGenes GMBH. The primary antibodies were polyclonal, but this was regarded an advantage rather