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Impact of rearing temperature on encapsulation and the accumulation of transcripts putatively involved in capsule formation in a parasitized lepidopteran host

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ABSTRACT

Encapsulation and melanisation are innate immune reactions of insects against foreign intruders such as parasitoids. In an earlier study, we observed that immature life stages of the endoparasitoid *Tranosema rostrale* (Hymenoptera: Ichneumonidae) parasitizing *Choristoneura fumiferana* (Lepidoptera: Tortricidae) larvae experienced higher mortality due to encapsulation and melanisation when reared at high (30 °C) than at lower (10 °C, 20 °C) temperatures. Downregulation of *T. rostrale* polydnavirus genes in parasitized hosts and upregulation of two genes involved in the spruce budworm's melanisation process were identified as likely contributors to parasitoid mortality at high temperature. However, levels of transcripts of genes involved in the spruce budworm's cellular encapsulation process were not measured inasmuch as candidate genes, in the spruce budworm, had not yet been identified. In addition, our assessment of temperature-dependent encapsulation and melanisation of foreign objects in spruce budworm larvae was only partial. To fill these knowledge gaps, we injected Sephadex™ beads into unparasitized spruce budworm larvae and assessed their encapsulation/melanisation after the insects had been held at three different temperatures (10, 20, and 30 °C), and we identified spruce budworm genes putatively involved in the encapsulation process and quantified their transcripts at the same three temperatures, using a qPCR approach. As expected, both encapsulation and melanisation of Sephadex™ beads increased as a function of temperature. At the molecular level, three of the five genes examined (*Integrin β1*, *Hopscotch*, *Stat92E*) clearly displayed temperature-dependent upregulation. The results of this study further support the hypothesis that a temperature-dependent increase in the encapsulation response of *C. fumiferana* against *T. rostrale* is due to the combined effects of reduced expression of polydnavirus genes and enhanced expression of host immune genes.

1. Introduction

Extreme temperatures can result in increased mortality of immature parasitoids, leading to the unimodal relationship typically observed between temperature and parasitoid survival (Spanoudis and Andreadis, 2012; Chen et al., 2015). Successful parasitism may decrease with increasing temperature due to an improvement in the host's immune defense against the intruder. Insect immune defense mechanisms against macroscopic invaders can be divided into two reactions, encapsulation and melanisation. Encapsulation is a cellular defense mechanism that leads to the development of a physical barrier between the foreign organism (e.g., parasitoid egg) and the insect hemocoel, by means of hemocytes (blood cells) that spread and adhere to the foreign target. The resulting multi-layer cellular sheath then contributes to the invader's death (Lavine and Strand, 2002). Melanisation, on the other hand, is an immune reaction that helps seal off foreign bodies from the host's internal environment through the accumulation of heteropolymer melanin at the interface between the foreign body and the capsule (Dubovskiy et al., 2016). Because it has cytotoxic properties, melanin also contributes to the killing of the intruder within the capsule (Nappi and Christensen, 2005). The efficiency of encapsulation of parasitoid eggs by the host generally increases as a function of temperature (Blumberg, 1991; Fellowes et al., 1999). The activity of the enzyme phenoloxidase (PO), which is involved in the formation of

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melanin sealing off foreign bodies from the host's internal environment, is also known to be affected by temperature, with a positive correlation between enzyme activity and temperature, up to an upper threshold varying between 20 and 50 °C, depending on the species (Lockey and Ourth, 1992; Hara et al., 1993; Cherqui et al., 1996; Zufelato et al., 2004).

Increasing temperature was found to be positively related to mortality in the parasitoid *Tranosema rostrale* (Hymenoptera: Ichneumonidae) developing in spruce budworm (*Choristoneura fumiferana*; Lepidoptera: Tortricidae) larvae (Seehausen et al., 2017a). Dissection of parasitized larvae that were reared at high temperature (30 °C) indicated that parasitoid eggs and larvae had died as a result of the host's immune reaction against them: almost 90% of parasitoid eggs and larvae were encapsulated and melanised (Seehausen et al., 2017b). Endoparasitoids like *T. rostrale* have developed countermeasures to evade or abrogate these immune reactions. Along with its egg, this parasitoid injects a polydnavirus (PDV; the *T. rostrale* ichnovirus or "TrIV") into its host, and this virus has been shown to depress hemocyte counts and the formation of melanin in spruce budworm larvae (Doucet and Cusson, 1996a). Because of the presumed protective effect of TrIV against the spruce budworm immune response, enhanced encapsulation and melanisation of *T. rostrale* immatures at high temperatures was somewhat unexpected. Results of a transcriptomic analysis revealed that transcript levels of several TrIV genes in host larvae were downregulated and that the transcription of genes related to spruce budworm's melanisation process [prophenoloxidase (PPO) 1 and 2] was upregulated at high temperature (Seehausen et al., 2017b). However, encapsulation, but not melanisation, of foreign bodies in larvae was enhanced at 30 °C, when compared to 20 °C (Seehausen et al., 2017b).

For the present study, we further characterized the temperature-dependent response of encapsulation and melanisation of foreign bodies in host larvae. In addition, we explored the possibility that genes involved in cellular encapsulation may be downregulated by high temperature, in a way similar to that shown for genes involved in melanisation (Seehausen et al., 2017b). To this end, we selected genes that are shown or believed to be involved in the encapsulation process in other insects (Sorrentino et al., 2004; Chevignon et al., 2015; Cao et al., 2015) and identified their homologs in the spruce budworm genome (Cusson et al., unpublished). Transcript abundance for five such genes was then measured in larvae reared at 10, 20 and 30 °C. We hypothesized that with increasing temperature, (i) encapsulation and melanisation of foreign bodies in spruce budworm larvae would increase linearly, and (ii) that genes putatively involved in spruce budworm's encapsulation process would be upregulated.

2. Material and methods

2.1. Injection of Sephadex™ beads into larvae

To measure the immune reaction of spruce budworm larvae towards foreign bodies at different temperatures, we injected Sephadex™ G25 beads into larvae. To this end, overwintered 2nd-instar spruce budworm larvae (obtained from the Insect Production Services of the Canadian Forest Service, Great Lakes Forest Research Centre, Sault Ste. Marie, ON, Canada) were reared on artificial diet (McMorran, 1965) in 37 mL plastic cups (5 larvae per cup) at 20 °C until the 6th instar. Following the protocol described in detail by Seehausen et al. (2017b; section 2.5), injection of three beads with a measured diameter between 140 and 180 μm was conducted three days after the molt to the 6th instar, using a 5 μL syringe (Model 7105 KHWG SYR, Knurled Hub NDL) and a 0.31 mm-gage needle. Immediately after injection, larvae were transferred individually to 37 mL plastic cups containing artificial diet and placed into a growth chamber at either 10, 20, or 30 °C. Two h after injection, a total of 50 larvae (14, 18, and 18 at 10, 20, and 30 °C, respectively) were dissected in a buffer solution to measure the encapsulation surface around beads, as well as the degree of melanisation of beads, as described in detail by Seehausen et al. (2017b; section 2.5). Encapsulation surface was calculated by taking a photo of all three encapsulated beads per larva using a digital camera (Dino-Lite AM7023B) mounted on a microscope and measuring the area within the boundaries of the capsule using an image analysis software (Dino-Capture). Subsequently, a mean encapsulation surface (S) per larva was calculated using $S = (\sum S_E - \sum S_B)/3$, where S_E is the surface of the encapsulated bead and S_B is the surface of the bead prior to injection. Extent of melanisation was scored on a scale of 0–5, where 0 denotes no change in colour and 5 denotes a dark brown colouration of the entire bead. An average melanisation index for each larva was then calculated by taking the mean of the values from all three beads.

2.2. RNA isolation and qPCR

Fifth-instar spruce budworm larvae were parasitized by *T. rostrale* [obtained from two study sites in Québec, near Armagh (46°46′ N, 70°39′ W) and Petit lac à l'Épaule (47°18′ N, 71°120′ W)] in the laboratory and immediately thereafter randomly assigned to three rearing temperatures, 10, 20, or 30 °C, where they were reared for 6, 24, 72, and 120 h. A total of 120 parasitized larvae were processed, with 10 larvae at each temperature and time. Larvae were homogenized in 500 μL TRIzol reagent (Invitrogen Life Technologies) to isolate RNA, and larval cuticle debris were removed by centrifugation at 17,000*g* for 5 min. RNA purification was performed according to the Direct-zol™ RNA Mini-Prep Instruction Manual (Zymo Research Corp.), including an in-column DNase I digestion for 15 min at room temperature. Total RNA was quantified using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific Inc.). Based on the RNA concentration (160–900 ng μL^{-1}) and its ratio of absorbance at 260 nm and 280 nm (~2.0), three samples from each temperature and holding time treatment were chosen for the following steps. Reverse transcription was performed according to the protocol described in the QuantiTect Reverse Transcription Handbook (Qiagen®). This included elimination of genomic DNA in a 14 μL reaction volume containing 2 μL gDNA Wipeout Buffer, which was incubated for 2 min at 42 °C. The reaction was carried out in a 20 μL reaction volume containing 1 μL Quantiscript Reverse Transcriptase, 4 μL Quantiscript RT Buffer, 1 μL RT Primer Mix and 14 μL template RNA from the previous step. The master mix was held for 30 min at 42 °C, followed by 3 min at 95 °C to inactivate the reverse transcriptase. The cDNA reaction was then diluted in 180 μL 10 mM TRIS/HCl.

Quantitative Real Time PCR (qPCR) was performed using an Applied Biosystems 7500 Fast Real Time PCR machine, 96-well Bright/White real-time PCR plates (Cat. BW-FAST, Primer Design, UK) with Applied Biosystems MicroAmp Optical Adhesive Film, and the Quantitect SYBR Green PCR Kit (Qiagen®). Expression levels were measured for five immunity related genes (*HAIP*, *GBP*, *Integrin β1*, *Hopscotch*, and *Stat92E*) and two housekeeping genes (GAPDH and gTubulin). The immunity-related genes were chosen based on their presumed role in the encapsulation response of other insects (Sorrentino et al., 2004; Chevignon et al., 2015; Cao et al., 2015). *Choristoneura fumiferana* homologs of the five genes examined here were then obtained by searching a *C. fumiferana* transcriptome (Cusson et al., unpublished) using *Bombyx mori* (Lepidoptera: Bombycidae) amino acid sequences as queries. Primers were designed using OligoExplorer software (Gene Link; Table 1). No-RT samples were also included to confirm the absence of genomic DNA in the samples. PCR analyses were performed on the same RNA samples used in a previous study (Seehausen et al., 2017b), with three technical replicates for each sample containing 2 μL of cDNA (10 ng of converted RNA) and 50 cycles of 95 °C for 15 s, 60 °C for 30 s, and 65 °C for 90 s. To determine absolute quantities of target molecules, linear regression of efficiency (LRE) analysis developed for modelling qPCR amplification (Rutledge, 2011) was used with lambda genomic DNA as a quantitative standard. Copy number results were normalized using the GeNorm algorithm