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High temperature induces downregulation of polydnavirus gene transcription in lepidopteran host and enhances accumulation of host immunity gene transcripts

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ABSTRACT

Endoparasitoids face the challenge of overcoming the immune reaction of their hosts, which typically consists of encapsulation and melanisation of parasitoid eggs or larvae. Some endoparasitic wasps such as the solitary *Tranosema rostrale* (Hymenoptera: Ichneumonidae) that lay their eggs in larvae of the spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae), have evolved a symbiotic relationship with a polydnavirus (PDV), which in turn helps them suppress the host's immune response. We observed an increase in mortality of immature *T. rostrale* with increasing temperature, and we tested two hypotheses about the mechanisms involved: high temperatures (1) hamper the expression of *T. rostrale* PDV genes and (2) enhance the expression of spruce budworm immunity-related genes. Dissections of parasitized spruce budworm larvae reared at 30 °C revealed that most parasitoid eggs or larvae had died as a result of encapsulation and melanisation by the host. A qPCR analysis of *T. rostrale* PDV (TrIV) gene expression showed that the transcription of several TrIV genes in host larvae was downregulated at high temperature. On the other hand, encapsulation, but not melanisation, of foreign bodies in spruce budworm larvae was enhanced at high temperatures, as shown by the injection of Sephadex™ beads into larvae. However, at the molecular level, the transcription of genes related to spruce budworm's melanisation process (prophenoloxidase 1 and 2) was upregulated. Our results support the hypothesis that a temperature-dependent increase of encapsulation response is due to the combined effects of reduced expression of TrIV genes and enhanced expression of host immune genes.

1. Introduction

Encapsulation and melanisation, best studied in the Lepidoptera and the Diptera (Lavine and Strand, 2002), are the two most common immunoreactions that insects mount against invaders such as viruses, spores of fungi and endoparasitoids. Encapsulation is a cellular defense mechanism during which hemocytes (blood cells) spread and adhere to a foreign target (*e.g.*, parasitoid egg), encasing it within a multi-layer cellular shell. As such, encapsulation builds a physical barrier between the foreign organism and the insect hemocoel, thereby contributing to the invader's death (Lavine and Strand, 2002). In contrast, melanisation is a humoral response (*i.e.*, non-cellular) that results in the accumulation of heteropolymer melanin at wounding sites, in plasma or inside hemocytes, sealing off foreign bodies from the host's internal environment (Sugumaran, 2002; Kanost et al., 2004). In addition, melanin promotes a cytotoxic reaction against the intruder (Nappi and Christensen, 2005). The formation of melanin is catalysed by the enzyme phenoloxidase (PO), which is present in the hemolymph or in hemocytes of insects as zymogen prophenoloxidase (PPO) and is brought into its active form by the prophenoloxidase activating enzyme (PPAE) (Cerenius and Söderhäll, 2004). Cellular and humoral immune responses may also work together, as certain types of hemocytes have been found to release PPOs and PPAEs (Jiang et al., 1997; Müller et al., 1999).

To successfully complete their larval development, endoparasitoids must either abrogate or evade the immune reaction of their insect hosts and have evolved various counterstrategies to circumvent the host immune response. One widely studied countermeasure is the use of a viral gene delivery system that enables the transfer of virulence genes from the wasp to the caterpillar, in

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which viral gene expression results in a depression of its immune reaction. These viruses, known as polydnaviruses (PDVs), are obligate symbionts of some endoparasitic wasps. The family *Polydnaviridae* comprises two genera, namely *Bracovirus* (BV), whose members are associated with wasps belonging to a subset of braconid subfamilies, and *Ichnovirus* (IV), whose members are found in two subfamilies of ichneumonid wasps (Strand and Burke, 2014). PDVs are unique in that they constitute the only known virus taxon with a segmented dsDNA circular genome (Webb, 1998; Tanaka et al., 2007). The virus replicates in the ovaries of female wasps, where it accumulates in the lumen of lateral oviducts. Virions are transmitted to the host during oviposition, along with parasitoid eggs. In the host, no viral replication takes place, but virions infect host tissues, express PDV genes, and induce pathologies that include suppression of the host immune response through alterations of hemocyte behaviour or hemocyte production (*e.g.*, Asgari et al., 1996; Doucet and Cusson, 1996a; Pruijssers and Strand, 2007; Suderman et al., 2008), and suppression of PO activity (Doucet and Cusson, 1996a; Shelby et al., 2000). In addition, PDVs can also induce host developmental arrest (Dover et al., 1987; Doucet and Cusson, 1996b; Soller and Lanzrein, 1996; Beckage, 2012) and mobilize host protein reserves for use by the parasitoid (Thompson and Dahlman, 1998; Nakamatsu et al., 2001; Pruijssers et al., 2009).

Many key life-history traits in insects are influenced by ambient temperature because they are poikilothermic organisms, and this affects parasitoids and hosts alike (*e.g.*, Hance et al., 2007). Temperature also affects the immune system of insects; for example, efficiency of encapsulation of parasitoid eggs is generally enhanced by a rise in temperature (Blumberg, 1997; Fellowes et al., 1999). In addition, PO activity in insect hemolymph is positively correlated with 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). It has been hypothesised that high temperatures negatively influence PDV gene expression (Khafagi and Hegazi, 2004), however, to our knowledge, no study has confirmed this hypothesis to date.

In recent work we showed that survival of the larval endoparasitoid *Tranosema rostrale* (Brischke) (Hymenoptera: Ichneumonidae) within its host, the spruce budworm *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae), is negatively correlated with rearing temperatures above 20 °C (Seehausen et al., 2017). During oviposition, this parasitoid wasp transmits a PDV (the *T. rostrale* ichnovirus or "TrIV") to its host (Cusson et al., 1998), for which both viral gene expression (Béliveau et al., 2000, 2003; Rasoolizadeh et al., 2009a, 2009b; Djoumad et al., 2013) and function (Doucet and Cusson, 1996a, 1996b; Cusson et al., 2000; Djoumad et al., 2013; Doucet et al., 2008) have been characterized. As such, this host-parasitoid association provides an excellent model to study the effect of temperature on PDV gene expression and on the performance of the host immune system. Given our earlier results on temperature-dependent survival of *T. rostrale*, we hypothesised that high temperature; (1) reduces the performance of TrIV through a depression of viral gene expression that limits TrIV's effectiveness in abrogating the host immune response, and (2) enhances the spruce budworm's immune system, enabling more effective encapsulation and melanisation reactions as a result of a rise in the expression of immunity-related genes.

2. Material and methods

2.1. Rearing and parasitization of spruce budworm larvae

Parasitoids were obtained from two study sites near Québec, QC, Canada, through implantation of spruce budworm larvae on balsam fir *Abies balsamea* (L.) Miller, Pinaceae, as described in detail by Seehausen et al. (2016, 2017). Overwintering spruce budworm larvae obtained from the insect rearing facility of the Canadian Forest Service (Great Lakes Forest Research Centre, Sault Ste. Marie, ON, Canada) were reared on current-year balsam fir foliage in growth chambers at 20 °C and under a 16 h photoperiod up to the 5th-instar. Larvae were then exposed to *T. rostrale* females for parasitization in plastic containers (Seehausen et al., 2016, 2017). Wasp and larvae were monitored until stinging was observed, and only stung larvae were used for subsequent experiments. Within 30 min after parasitization, spruce budworm larvae were transferred to 237-ml plastic containers featuring a top window screen for ventilation and provided balsam fir foliage for food. The containers were then placed at different temperatures for rearing.

2.2. Dissection of parasitized larvae

To identify the causes of in-host mortality of parasitoids at high temperature, spruce budworm larvae were placed in rearing chambers immediately after parasitization at either 20 °C (n = 10) or 30 °C (n = 10), where they were reared for 5 days. Larvae were then immobilized by exposure to 20 °C for 3–5 min and dissected under a microscope in a buffered saline solution. The developmental stage of the parasitoid (egg or larva) and its status (live, encapsulated or melanised) were determined visually.

2.3. Hemolymph melanisation

As for the dissections, spruce budworm larvae were reared immediately after parasitization for 5 days at 20 (n = 10) or 30 °C (n = 10). Hemolymph was collected from parasitized larvae by puncturing the dorsal cuticle with an insect pin and drawing hemolymph with a capillary tube. Placed on a Parafilm^{TM} sheet, the hemolymph was then monitored at room temperature for a display of melanisation, as described in Doucet and Cusson (1996a). A change of hemolymph colouration from yellow-green to dark-brown was considered normal melanisation, whereas the maintenance of the original colour after 30 min of exposure to air was considered inhibition of melanisation.

2.4. RNA isolation and qPCR

Immediately after parasitization, spruce budworm larvae were randomly assigned to three rearing temperatures, 10, 20, or 30 °C, where they were reared for 6, 24, 72, and 120 h. At each temperature and time, 10 parasitized larvae were processed, for a total of 120 larvae in the experiment. These larvae were homogenized in 500 μL TRIzol reagent (Invitrogen Life Technologies) to isolate RNA. Larval cuticle debris were removed by centrifugation at 17,000*g* for 5 min, and the supernatant was transferred to a new tube. 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.). Three samples from each temperature and time treatment were chosen for the following steps based on the RNA concentration (160–900 ng μL^{-1}) and its ratio of absorbance at 260 nm and 280 nm (~2.0). Reverse transcription was performed according to the protocol described in the QuantiTect Reverse Transcription Handbook (Qiagen®), including 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 reverse-transcription 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. We used 96-well BrightWhite real-time PCR plates (Cat. BW-FAST, Primer Design, UK), with Applied Biosystems MicroAmp Optical Adhesive Film. Expression levels were measured for four TrIV genes (*TrV1*, *TrFrep1*, *Ank2*, *Cys*), Prophenoloxidases 1 and 2 (PPO1, PPO2), PPO1 activating enzyme (PPAE1) and two housekeeping genes (GAPDH and gTubulin). The immunity-related genes, PPO1, PPO2 and PPAE1, were chosen based on the availability of their DNA sequences and their documented roles in melanisation (Dubovskiy et al., 2016). No-RT samples were also included to confirm the absence of genomic DNA in the samples. Primer pairs for TrIV genes were obtained from Rasoolizadeh et al. (2009a); for all other genes, primers were designed using OligoExplorer software (Gene Link). Two technical replicates were run for each sample containing 2 µL of cDNA (10 ng of converted RNA). PCRs were performed using the Quantitect SYBR Green PCR Kit (Qiagen) with 50 cycles of 95 °C for 15 s, 60 °C for 30 s, and 65 °C for 90 s. Linear regression of efficiency (LRE) analysis developed for modelling qPCR amplification (Rutledge, 2011) was used to determine absolute quantities of target molecules. Lambda genomic DNA was used as a quantitative standard. Copy number results were normalized using the GeNorm algorithm (Vandesompele et al., 2002).

Samples with no polydnavirus-related RNA detected (4 samples, 11.1%) were replaced with new ones, assuming that these larvae had not been effectively parasitized.

2.5. Injection of Sephadex™ beads into larvae

To measure the immunoreaction (encapsulation and melanisation) 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 were reared on artificial diet (McMorran, 1965) in 21 mL plastic cups (6 larvae per cup) at 20 and 30 °C until the 6th instar. Injections of beads took place 3 and 2 days after the molt to the 6th instar at 20 and 30 °C, respectively, so that measurements were taken at approximately the same physiological age in the two groups of larvae. Prior to injection, larvae were anesthetised with CO_2 for 1–2 min and fixed with metal clips to a wax pad. Three beads with a measured diameter between 100 and 200 µm were injected in 1 µL PBS into each larva using a 5 µL syringe (Model 7105 KHWG SYR, Knurled Hub NDL) and a 0.31 mm-gage needle. The needle was inserted between two segments of the larva's thorax and beads were injected right under the cuticle to avoid injury of the inner organs. After injection, a liquid bandage (New-Skin®, Prestige Brands Holdings Inc.) was applied to the wound to avoid bleeding and ejection of the beads. At both temperatures, dissections of larvae were carried out at 2, 4, and 6 h post-injection. To this end, larvae were immobilized by exposure to −20 °C for 3–4 min, after which they were dissected in PBS under a microscope. Once isolated, pictures of encapsulated beads were taken under standardized light conditions with a digital camera (Dino-Lite AM7023B) mounted onto a microscope (Optiphot, Nikon). Using an image analysis software (DinoCapture), the surface of each encapsulated bead was measured by tracing a line around the capsule containing the bead and calculating the area within the line. The surface of encapsulation S was measured for the three beads and averaged 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. Encapsulation was measured in a total of 79 larvae. In addition to encapsulation, the degree of melanisation of beads was measured by grading the colour and coverage of melanisation after injection, using a melanisation scale of 0 to 5 (Fig. 1), 0 and 5 denoting no change in colour and a dark brown colouration of the entire bead, respectively. An average melanisation index for each larva was then calculated by taking the mean of the values from all three beads. Melanisation was measured in a total of 59 larvae.

2.6. Statistical analysis

We used Fisher's Exact Test to compare parasitoid mortality and hemolymph melanisation at 20 and 30 °C (PROC FREQ; SAS Inc., 2015). Backward model selection using the Corrected Akaike Information Criterion (AICc) for selecting the best fitting model was used to analyse the influence of temperature (T) over time (t) on the expression of polydnavirus genes and genes related to spruce budworm immunity, as well as encapsulation and melanisation of Sephadex™ beads (PROC GLMSELECT; SAS Inc., 2015). The full model included the two independent variables (T and t), their squares, and all two-way interactions. The best fitting model (Table 1) was then submitted to a multiple regression analysis (PROC GLM; SAS Inc., 2015). To meet the assumptions of normality and variance stability, all dependent variables related to gene expression were log-transformed.

3. Results

3.1. Effect of temperature on in-host mortality of T. rostrale and melanisation

Tranosema rostrale eggs or larvae were recovered in 19 out of 20 dissected larvae. The spruce budworm larva that had not been truly parasitized was excluded from the experiment. Significantly more parasitoids were found dead at 30 °C than at 20 °C ($P = 0.0011$): 89% ($n = 9$) of larvae were found dead (encapsulated and/or melanised as eggs or larvae) at 30 °C compared to 10% ($n = 10$) at 20 °C. No hemolymph samples drawn from parasitized spruce budworm larvae reared at 20 °C melanised at ambient air ($n = 10$); however, 33% ($n = 9$) of the samples from insects reared at 30 °C melanised (Fisher's Exact Test: $P = 0.0867$).

3.2. Effect of temperature on TrIV gene expression

Among the four TrIV genes whose transcript levels were quantified in this study, *TrV1* was by far the most highly expressed, followed by *TrFrep1*, *Ank2*, and *Cys* (Fig. 2). The time-dependent pattern of *TrV1*, *TrFep1* and *Cys* expression was generally parabolic (first increasing, reaching maximum expression after 72 h, and decreasing thereafter), and varied as a function of temperature (relatively low at 10 °C, highest at 20 °C, and again lower at 30 °C) (Table 1A, Fig. 2). However, the expression of *TrV1* and *Cys* deviated slightly from this general pattern, as indicated by the significant interaction terms (Table 1A). *Ank2* transcript levels increased in a more linear fashion over time, a trend that was particularly apparent at 20 °C (Fig. 2), which resulted in a significant $t \times T^2$ interaction (Table 1A).

3.3. Effect of temperature on the spruce budworm immune response

There was a significant $t^2 \times T$ interaction with respect to the encapsulation surface of Sephadex™ beads (Table 1B). The extent of encapsulation increased at both temperatures over time; however, the increase was faster at 30 °C, reaching 13,000 µm^2 4 h after injection, as compared to ~10,000 µm^2 at 20 °C (Fig. 3A). Melanisation intensity increased significantly over time, but was not

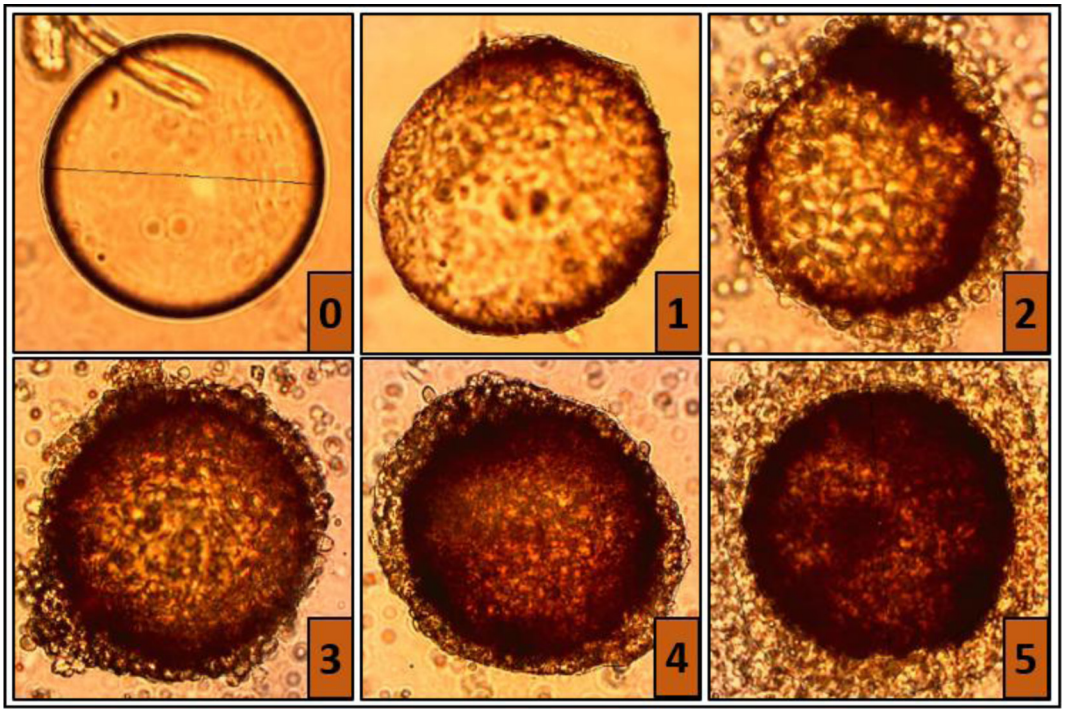

Fig. 1. Scale for ranking melanisation intensity of Sephadex™ beads injected into spruce budworm larvae. This scale was used to assess speed of melanisation at two different rearing temperatures (20 and 30 °C). Index values (0–5) provide an estimate of the degree of melanisation, which is based on colour (light to dark) and coverage level of bead melanisation after injection and ranges between 0 (no change in colour) and 5 (dark brown colouration of the entire bead).

Table 1
Independent variables chosen by AICc-based backward model selection and their corresponding P-values explaining variation in levels of (A) TrIV gene-specific mRNA, (B) Sephadex™ bead encapsulation surface and intensity of melanisation, and (C) spruce budworm gene-specific mRNA.

Dependent variable	Model	R^2	Independent variables							
			Time	Time^2	Temperature	Temperature^2	Time x Temperature	Time^2 x Temperature	Time x Temp^2	Time^2 x Temperature^2
A) TrIV gene-specific mRNA										
TrV1	<0.0001	0.64	0.0003	0.0192	<0.0001	0.0004	–	–	–	0.0059
Rep1	<0.0001	0.55	0.0064	0.0421	0.0008	0.0023	–	–	–	–
Cys	0.0001	0.59	0.0006	0.0022	0.0001	0.0003	0.0455	–	0.0957	–
Ank2	<0.0001	0.75	0.0064	–	0.0128	–	<0.0001	0.0559	<0.0001	–
B) Sephadex™ bead encapsulation and melanisation										
Encapsulation	<0.0001	0.66	<0.0001	–	–	–	<0.0001	0.0002	–	–
Melanisation	0.0059	0.17	0.0242	0.0681	–	–	–	–	–	–
C) Spruce budworm gene-specific mRNA										
PPO1	0.0037	0.46	–	0.0339	–	0.0783	0.0044	0.0020	0.0016	0.0009
PPO2	0.0081	0.39	–	0.0783	–	0.0362	–	0.0124	0.0101	0.0063
PPAE1	0.1518	0.15	–	0.0711	–	–	0.0971	0.0686	–	–

affected by temperature (Table 1B). It reached a plateau just over 3 on the melanisation intensity index at both temperatures (Fig. 3B).

There was a significant $t^2 \times T^2$ interaction in the expression of PPO1 and PPO2 (Table 1C). PPO1 transcript abundance increased fastest at 30 °C, up to the 72 h sampling point, after which it dropped slightly. In contrast, at 20 °C PPO1 transcript levels first declined, reaching their lowest point at the 24 h sampling point, but increased thereafter. At 10 °C, no significant change in PPO1 over time was observed (Fig. 4). The time-dependent pattern of PPO2 transcript abundance was very similar to that of PPO1, with the exception that its highest transcript levels were observed at the 120 h sampling point at 30 °C (Fig. 4). Transcript abundance for the prophenoloxidase-activating enzyme, PPAE1, was neither influenced by time nor by temperature (Table 1C, Fig. 4).

4. Discussion

The data presented here provide support for the hypotheses we set out to test: enhanced encapsulation and melanisation of *T. rostrale* eggs and larvae in parasitized *C. fumiferana* hosts exposed to high temperature (30 °C) are associated with reduced TrIV gene transcription and elevated expression of two spruce budworm immunity-related genes, as compared with hosts held at 20 °C (Table 1; Figs. 2 and 4). However, the causal link between these two types of gene expression alterations and enhanced encapsulation/melanisation at high temperature is not yet firmly established, and the results presented here need to be interpreted in the light of prior observations on *T. rostrale-C. fumiferana* interactions.

Although TrIV has been shown to have a strong inhibitory effect on melanisation and PPO activity in *C. fumiferana*, its effect on the host cellular immune response appears weaker than that observed

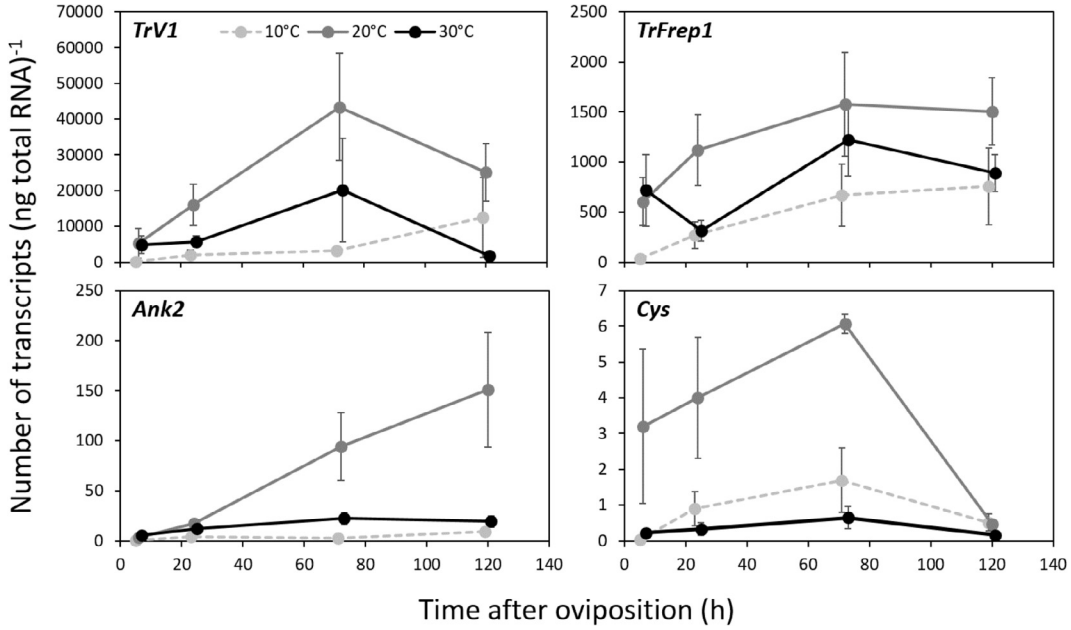

Fig. 2. qPCR assessment of transcript abundance (mean ± SEM) for four TrIV genes (*TrV1*, *TrFrep1*, *Ank2*, and *Cys*) in spruce budworm larvae parasitized by *Tranosema rostrale* and reared at three different temperatures (10, 20, and 30 °C). Each data point is the mean number of transcripts, as measured from three larvae, with two technical replicate run for each RNA extract.

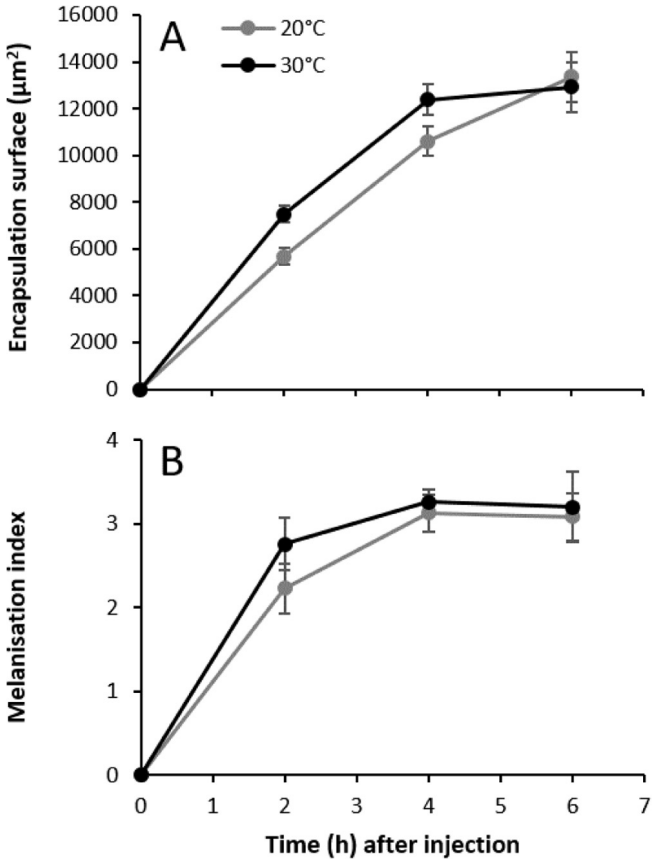

Fig. 3. Effect of temperature on temporal pattern of (A) encapsulation surface (mean ± SEM; n = 79) and (B) melanisation intensity (mean ± SEM; n = 59) of Sephadex™ beads injected into spruce budworm larvae.

for other PDVs (Doucet and Cusson, 1996a). Indeed, TrIV infection was shown to cause a significant reduction in hemocyte counts, but glass rods introduced into the hemocoel of *C. fumiferana* larvae parasitized by *T. rostrale* were nonetheless encapsulated (while remaining unmelanized). Whether the artificial surface provided by the foreign object was, in part, responsible for its encapsulation is unclear, but *T. rostrale* eggs and larvae always escaped encapsulation (Doucet and Cusson, 1996a). Thus, TrIV may provide protection against parasitoid encapsulation while allowing encapsulation of other foreign bodies. Whether this protection is the result of TrIV gene expression remains unclear as the presence of TrIV virions at the surface of *T. rostrale* eggs has been hypothesised to provide passive protection against encapsulation, presumably through a mechanism where the layer of virions at the egg surface prevents recognition of eggs as foreign (Cusson et al., 1998b). However, such a mechanism would not account for the ability of *T. rostrale* larvae to evade encapsulation. It therefore appears likely that the expression of some TrIV genes is required for the immature parasitoid to escape encapsulation and melanisation. Downregulation of TrIV genes at high temperature may thus have been responsible, at least in part, for the observed encapsulation and melanisation of *T. rostrale* eggs and larvae. However, we cannot rule out, at this point, the possibility that encapsulation of parasitoid larvae may have occurred as a result of, and subsequent to, death of the parasitoid induced directly by high temperature.

Which TrIV genes are responsible for abrogating the encapsulation/melanisation reaction against *T. rostrale* is currently unknown. The two TrIV genes that are most highly expressed in parasitized *C. fumiferana* larvae are *TrV1* and *TrFrep1* (Béliveau et al., 2000; Volkoff et al., 2002; Rasoolizadeh et al., 2009a, 2009b; Djoumad et al., 2013), but their exact function(s) remain uncertain. However, *TrV1* is strongly suspected of being responsible for the important developmental arrest observed in last-instar *C. fumiferana* larvae parasitized by *T. rostrale* (Béliveau et al., 2000, 2003; Djoumad et al., 2013). Although *TrV1* and *TrFrep1* transcript accumulation was depressed at 30 °C relative to levels measured at 20 °C (Fig. 2), the impact of temperature on transcript abundance was greater for the other two genes examined, *Ank2* and *Cys*, for which homologs have been shown in other host-parasitoid-PDV systems, to play a direct role in the inhibition of the encapsula-

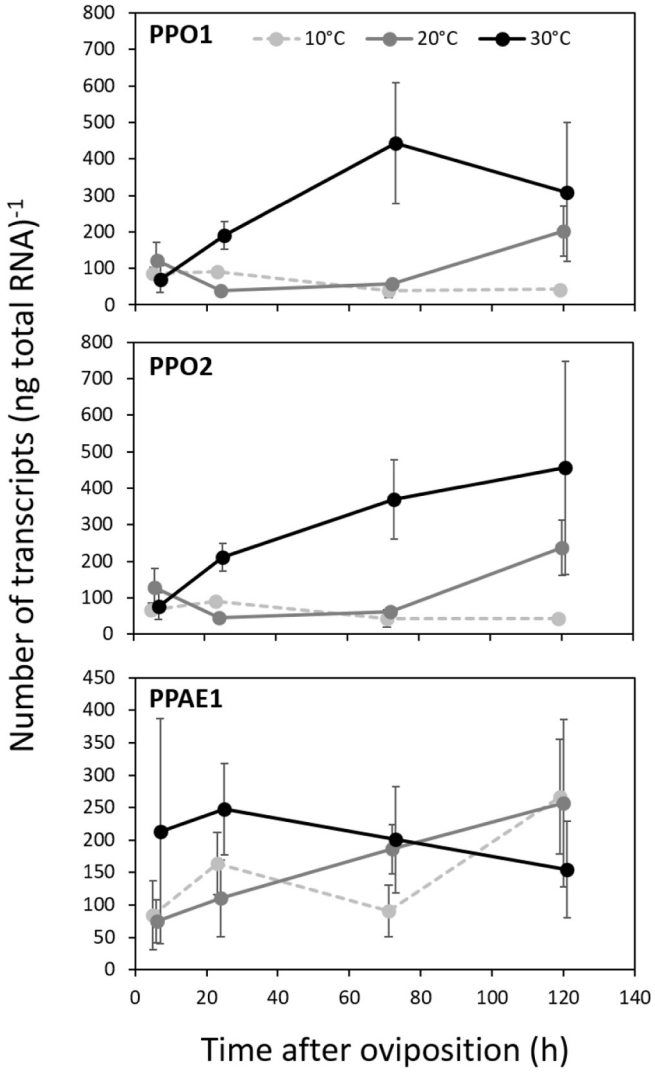

Fig. 4. Temporal mean (±SEM) transcription levels assessed by qPCR of three spruce budworm genes, prophenoloxidase (PPO) 1 and 2, and PPO activating enzyme (PPAE1) in spruce budworm larvae parasitized by *Tranosema rostrale* and reared at three different temperatures (10, 20, and 30 °C). Each point is the mean number of transcriptions from three larvae and two technical replicates carried out on each RNA extract.

tion/melanisation reaction (Li and Webb, 1994; Cui et al., 1997; Kroemer and Webb, 2005; Gueguen et al., 2013) and developmental disturbance (Kroemer and Webb, 2004). Although these genes were expressed at much lower levels than *TrV1* and *TrFrep1*, as shown earlier (Rasoolizadeh et al., 2009b), the strong effect of temperature on the accumulation of their transcripts suggests a role in the interaction between temperature, their expression and the encapsulation/melanisation response observed here. It must be pointed out that, for the present study, we sampled only four representative TrIV genes; the possibility remains that transcript abundance of other genes may have been more strongly affected than that of the four we selected.

Data presented in Fig. 2 also show a strong suppression of TrIV gene transcript accumulation in parasitized *C. fumiferana* larvae held at 10 °C, relative to values measured at 20 °C. Such an effect of low temperature on gene transcription was not unexpected given that most enzyme reactions involved in gene expression will be inhibited by low temperature. Interestingly, of the three temperatures examined here, 10 °C provided the best conditions for *T. rostrale* survival in *C. fumiferana* (Seehausen et al., 2017). Since low temperatures are also expected to considerably slow down enzymatic reactions involved in the host immune response (*e.g.*, see PPO1 and PPO2 transcript accumulation at 10 °C; Fig. 4), we hypothesize that the reduced level of TrIV gene expression observed here at 10 °C may be sufficient to prevent encapsulation and melanisation at this temperature.

To assess the impact of rearing temperature on the expression of host immune genes, we selected three *C. fumiferana* genes for which we had sequence data; all three are involved in the melanisation reaction. The expression of PPO1 and PPO2 was strongly enhanced by exposure of parasitized *C. fumiferana* larvae to 30 °C, as compared to larvae held at 20 °C, while accumulation of PPAE1 appeared little affected by temperature (Fig. 4). These results provide strong evidence that the transcription of some, but not all, immunity-related genes is stimulated by elevated temperatures, which could enhance the immune response mounted against parasitoids. Interestingly, the higher transcript abundance measured for PPO1 and PPO2 at 30 °C, relative to 20 °C, was not accompanied by a significant rise in the melanisation of Sephadex™ beads injected in unparasitized *C. fumiferana* larvae held at 30 °C (Fig. 3B). However, comparisons between these two experiments must be made with caution as budworm larvae differed in their parasitism status (and, as a consequence, in their TrIV infection status), and the foreign body used to assess encapsulation and melanisation (Sephadex™ beads) had an artificial surface, generating strikingly different results from those reported here for immature parasitoids assessed through dissection. In fact, the results obtained for the Sephadex™ bead experiment (Fig. 3) point to a marginal impact of temperature (20 °C versus 30 °C) on the encapsulation and melanisation of such beads in *C. fumiferana*. These results suggest that, in the absence of TrIV, the *C. fumiferana* immune response is not strongly affected by temperature, at least not enough to account for the important temperature-related differences observed in the encapsulation and melanisation of *T. rostrale* immatures in parasitized hosts. Thus, the question of whether the strong accumulation of PPO1 and PPO2 transcripts in parasitized larvae held at 30 °C (Fig. 4) is due to an inhibition of TrIV gene expression needs to be assessed. However, earlier work indicated that TrIV-dependent downregulation of PPO1 and PPO2 expression plays a minor role in the inhibition of melanisation in this host-parasitoid system (Doucet et al., 2008).

In conclusion, the present work points to a role of PDV and host immune gene expression in the success of immature parasitoid encapsulation and melanisation as affected by rearing temperature. Importantly, observed differences may be due to interactions between the virus and host immune genes, as suggested by the marginal impact of temperature on the encapsulation and melanisation of Sephadex™ beads introduced into unparasitized *C. fumiferana* hosts. Efforts to further explore these hypotheses will require the use of gene-silencing approaches such as RNA interference (RNAi) to assess which, if any, TrIV genes are required to abrogate the host immune reaction and, conversely, which host immune genes are required to mount it. The range of temperatures to which *T. rostrale* and spruce budworm larvae were exposed under the laboratory conditions used for our experiments is similar to that encountered in the known habitats of both the parasitoid and the host (e.g., Lethiecq and Régnière, 1988). Given that extreme high temperatures are predicted to be reached more frequently in the future as a result of climate change (Meehl and Tebaldi, 2004; Rummukainen, 2012; Rahmstorf and Coumou, 2011), the temperature-immunity interaction reported here will likely be of significant relevance in the regulation of natural *T. rostrale* populations in the field. It would also be interesting to determine whether the relationship between temperature and encapsulation/melanisation described here applies to other host-parasitoid-PDV systems; among the latter, those where PDVs have

been shown to play a strong role in the inhibition of the host immune response may provide alternative models to test our hypotheses.

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