Short Note

Trade-off between cellular immunity and life span in mealworm beetles *Tenebrio molitor*

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Abstract Encapsulation is a nonspecific, cellular response through which insects defend themselves against multicellular pathogens. During this immune reaction, haemocytes recognize an object as foreign and cause other haemocytes to aggregate and form a capsule around the object, often consisting of melanized cells. The process of melanisation is accompanied by the formation of potentially toxic reactive oxygen species, which can kill not only pathogens but also host cells. In this study we tested whether the encapsulation response is costly in mealworm beetles *Tenebrio molitor*. We found a negative relationship between the duration of implantation via a nylon monofilament and remaining life span. We also found a negative relationship between the strength of immune response and remaining life span, suggesting that cellular immunity is costly in *T. molitor*, and that there is a trade-off between immune response and remaining life span. However, this relationship disappeared at 31-32 hours of implantation at 25 ± 2°C. As the disappearance of a relationship between duration of implantation and lifespan coincided with the highest values of encapsulation response, we concluded that the beetles stopped investment in the production of melanotic cells, as the implant, a synthetic parasite, was fully isolated from the host’s tissues [Current Zoology 59 (3): 340–346, 2013].

Keywords Cost of immunity, Cellular immunity, Encapsulation, Lifespan, *Tenebrio molitor*

Parasite resistance is diverse and range from behavioural adaptations to physiological barriers (Sadd and Schmid-Hempel, 2009; Schmid-Hempel, 2011). The immune defence of insects essentially consists of a cellular and a humoral component (Gupta, 1986; Schmid-Hempel, 2011). In the cellular response, haemocytes phagocytose small foreign particles, or attach themselves to large foreign objects, as an encapsulation response against intruders (Gupta, 1986; Kanost, 2009). The foreign object may become completely encapsulated and isolated from other host tissue as haemocytes attach to its surface and ultimately enclose it (Grimstone et al., 1967; Lavine and Strand, 2002). This cellular response is aided by a humoral response, which consists of proteins able to interfere with a parasitic intruder and regulate coagulation and melanization of hemolymph (Hancock et al., 2006; Lavine and Strand, 2002; Schmid-Hempel, 2011). The melanotic capsule consisting of haemocytes can block absorption of nutrients by parasites and thus contribute to their killing by starvation (Strand, 2008) or by the toxic effects of melanin production, causing necrosis of the intruder (Boucias and Pendland, 2008).

The process of melanization in insects is accompanied by the formation of potentially toxic reactive oxygen species (ROS), including semiquinone radicals (Slepnev et al., 2003), hydrogen peroxide (Komarov et al., 2006; Nappi and Vass, 1998), and superoxide anions (Nappi et al., 1995; Whitten and Ratcliffe, 1999). As such, the formation of the capsule should be fast enough to avoid the cytotoxic ROS action affecting not only entomopathogens, but also the host cells and tissues. The encapsulation response begins as soon as the cuticle of an insect is pierced, and the response usually lasts for hours (Dubovskii et al., 2010) or perhaps days (Eggenberger et al., 1990; Schmit and Ratcliffe, 1977). The formation of such a capsule is considered to be a self-limiting process, and usually involves adherence of
multiple layers of melanotic haemocytes on the surface of the invader (Irving et al., 2005).

Microscopy to measure thickness of the capsule, estimate the volume of the encapsulating cells, and count cell numbers is an important method to study the encapsulation response in insects and nematodes (Schmit and Ratcliffe, 1977; Siva-Jothy et al., 2001). However, this approach requires skilled researchers, sophisticated equipment, and is time-consuming. Hence, recent research has often employed the measurement of an encapsulation response, based on quantifying the optical density of haemocytes attached on the surface of synthetic implants used to activate the immune system of insects (Allander and Schmid-Hempel, 2000). This approach represents a useful way to assess the condition of individual insects (e.g. Kivleniece et al., 2010) since such implants seem to match the activity of real parasites (Rantala and Roff, 2007). Previous research has shown that immune challenge by nylon implants significantly decreased the life span of T. molitor males (Daukste et al. 2012, Krams et al., 2012). However, the relationship between the duration of encapsulation and remaining life span of the host is not well understood.

The idea that the immune system is costly and must be traded off against other important physiological systems is a cornerstone of the rapidly developing field of ecological immunity (Sheldon and Verhulst, 1996; Schmid-Hempel, 2011). It is known that encapsulation is a costly response of the immune system and a response that should be stopped as soon as the foreign object is isolated from the host tissue by the completed capsule (Gillespie et al., 1997; Lawniczak et al., 2007). Resource allocation to immune function may be subject to a trade-off between other life history traits such as reproduction and remaining life span (Sheldon and Verhulst, 1996; Schmid-Hempel, 2011). Some studies have found a negative relationship between the duration of implantation and the longevity of affected insects (Armitage et al., 2003; Jactot et al., 2004; Vainikka et al., 2007; Maciel-de-Freitas et al., 2011). However, this earlier work did not distinguish between the effect of duration of implantation or duration of encapsulation response on longevity. In this study we investigated the relationship between duration of implantation and the remaining life span of mealworm beetles Tenebrio molitor to test whether there is any maximum value of encapsulation response against a nylon monofilament and whether this maximum value of encapsulation is related to the remaining life span of the insects. We predicted the reduction of lifespan before reaching the maximum value of encapsulation and the disappearance of any relationship between duration of implantation and lifespan after reaching the highest values of encapsulation response. The life span of T. molitor usually reaches four months in our laboratory, which suggests excellent condition (Daukste et al., 2012).

1 Material and Methods

1.1 Experimental insects

The beetles used in the experiment were collected from natural populations in several barns in southeastern Latvia. The stock culture was maintained at 25 ± 2°C. We removed pupae from the culture on the day of pupation, weighed them, and determined their sex (Bhattacharya et al., 1970). The pupae and newly emerged adults were kept individually in 200 ml plastic containers filled with a mixture of bran and wheat flour and with fresh carrot pieces offered *ad libitum*.

1.2 Activation of immune system and survival of beetles

To eliminate the potential confounding variable of sex and body size (Rantala and Roff, 2005; Krams et al., 2011a), we tested only males. We weighed 10-day-old males (*n* = 920; mean body weight = 0.1437 ± 0.450 g) and randomly divided them into 39 experimental groups (number of individuals = 20–42 in each group). At day 8 after imaginal eclosion we immobilized males on ice and inserted a piece of sterile nylon monofilament (2 mm length, 0.18 mm diameter, knotted at one end, Grand Rider 30/018 fishing line by Salmo SIA Latvia), through its pleural membrane between the third and fourth abdominal sternite (Kivleniece et al., 2010; Krams et al., 2011a,b; Rantala et al., 2002) for 1–45 hours at 25 ± 2°C. We did not perform implantations for 14, 16, 18, 29, 36, and 37 hours. The surface of the monofilament was scratched with sandpaper P600 before use to enhance the likelihood that haemocytes would stick to the implant. To ensure sterility the scratched implants were then stored in ethanol (96%) prior to use in experimental animals. After the specified number of hours for the experimental group, we carefully grasped the knots and removed the nylon monofilaments from the males. The removed implants were dried and stored in the freezer (−40°C) for later analysis. Most of the experimental males (*n* = 872) were included in the survival group to see whether survival rates of each group males are dependent on the duration of implantation. The males were kept at constant temperature in 200 ml
plastic containers filled with a mixture of bran and wheat flour and with fresh carrot pieces offered *ad libitum* at least twice a week.

### 1.3 Immune assays

The degree of encapsulation was analyzed as grey values of reflecting light from implants (0–100%). To quantify lightness of nylon monofilaments, each removed implant was photographed from two different directions under consistent light conditions using a Zeiss Lumar V12 Stereo microscope and Axio Cam MRc5 digital recorder. We then analysed the digital images using image analysis software (Image J, http://rsbweb.nih.gov/ij/), which calculated the lightness value. The lightness of implants was analyzed blind with regard to experimental group. Data were transformed so that grey values of the darkest areas of melanized implants correspond to the highest encapsulation rate. This transformation was done by subtracting observed grey values from the control grey value (clear implant), after (Rantala and Roff, 2005).

### 1.4 Statistics

Plotting the mean lightness of the melanotic capsule and the mean residual life of the beetles together with standard errors (Fig. 1) suggests that both (as functions of implantation time) reach an asymptote. The increase of the mean values of encapsulation response and the decrease in life span values seem to stop at time values less than 35 hours. Since it cannot be properly tested without assuming that the functions are eventually constant *a priori*, we asked how long the functions remain roughly constant when starting form the largest argument values by employing Helmert contrasts, as discussed in (Venables and Ripley, 2002) in a linear model. We reversed the order of levels (descending form 45) and tested stepwise whether the value (i.e. mean) of the function can be distinguished from the mean of all previous levels, thus enabling us to identify the highest value of implantation time at which the function is definitely not yet constant. We used R (R Development Core Team 2011) to estimate the light reflectance of melanized implants. Because we expected just a decrease or an increase of encapsulation response, a one-tailed test is appropriate.

### 2 Results

We found a significant positive correlation between duration of implantation and the strength of encapsulation response ($r = 0.901, n = 39, P = 0.001$, Fig. 1), which increased from $4.35 \pm 1.81 \%$ after the first hour to $71.50 \pm 6.39 \%$ up to hour 34. In contrast, we found a negative correlation between duration of implantation and the remaining life span of *T. molitor* males ($r = -0.918, n = 39, P < 0.001$, Fig. 1), which decreased from $5.81 \pm 0.48$ months to its minimum of $1.19 \pm 0.23$ months.

![Relationship between duration of implantation and encapsulation response (lightness of implant, %) and duration of implantation and residual life span in mealworm beetles](image_url)
Table 1  Test of time intervals (hours of implantation) to determine when the increase in encapsulation response (31 hours) and decrease in residual life span are no longer constant (32 hours)

| Encapsulation response (lightness of implant) | Time (hours) | Estimate | Standard error | t-value | p-value |
|------------------------------------------------|-------------|----------|----------------|---------|---------|
| 33                                             | -0.004      | 0.087    | -0.050         | 0.480   |
| 32                                             | -0.018      | 0.084    | -0.213         | 0.416   |
| 31                                             | -0.181      | 0.077    | -2.345         | 0.010   |
| 30                                             | -0.211      | 0.070    | -3.015         | 0.001   |

| Residual life (days) | Time (hours) | Estimate | Standard error | t-value | p-value |
|----------------------|-------------|----------|----------------|---------|---------|
| 33                   | -0.109      | 0.296    | -0.369         | 0.644   |
| 32                   | 0.201       | 0.271    | 0.741          | 0.229   |
| 31                   | 0.298       | 0.249    | 1.197          | 0.116   |
| 30                   | 0.456       | 0.225    | 2.032          | 0.021   |

months at hour 33. This suggests that the full encapsulation of an intruder brings substantial costs. However, further analysis revealed that relationships between duration of implantation and remaining life span, and between duration of implantation and the strength of immune response, disappear between 31-33 hours of implantation (Table 1, Fig. 1). Table 1 shows only the relevant part of the analysis, revealing that the mean value of encapsulation of the melanotic capsule at the time of 30-31 hours is less than it is for all the higher time values combined. P-values of Table 1 show the probability of obtaining a value of the t-statistic that is as small/large or smaller/larger under the null hypothesis that the function value at the tested time value is equal to the average function value for larger times. The times at which the respective functions cannot be considered constant were identified as 31 hours for the encapsulation response and 32 hours for residual life span (Table 1, Fig. 1). This indicates that the encapsulation did not increase after 31 hours, while remaining life span remained unaffected after 32 hours of the onset of implantation.

3 Discussion

Our results clearly show that immune activation via nylon monofilament decreased the lifespan of male mealworm beetles, suggesting that encapsulation is costly in *T. molitor*. Since the duration of encapsulation negatively correlated with remaining life span, it reveals a trade-off between immunity and longevity. However, this relationship was direct and monotonic only during the first 30 hours of implantation. As soon as the implantation time exceeded 30 hours we found no relationship between the duration of implantation and lifespan of the males, since all of them lived for about 1.3 months after being implanted by nylon monofilaments for 31–45 hours. As the disappearance of a relationship between duration of implantation and lifespan seemed to coincide with the time of reaching the highest values of encapsulation response, it can be concluded that the males stopped investment in their immune response as the implants were fully isolated from the host’s tissues. This is a likely explanation, since the production of melanotic cells is supposed to be a key element in fighting multicellular pathogens as fungi, nematodes and parasitoids (Yourth et al., 2001; Youth et al., 2002) and even viruses (Washburn et al., 1996).

Existing evidence shows that the increase of thickness of the protective capsule can progress for several days after nylon insertion (Forton et al., 1985; Ryder, 1999; Siva-Jothy and Thompson, 2002), and the maximum values of thickness may be achieved long after reaching the maximum value of melanization (Dubovskii et al., 2010). Our results cannot either support or reject the idea of the increase of the thickness of the protective capsule well beyond the point of the highest melanization of the insert, because we did not measure its thickness. However, we would agree that the final stages of encapsulation response in insects may be relatively slow as it has been shown earlier by Ryder (2007) in his study on the cricket *Acheta domesticus*. In addition, non-melanotic haemocytes may continue to attach to the nylon long after the cessation of melanotic cell production. Since the lifespan of mealworm beetles appeared to be non-dependent on the duration of implantation after 32–34 hours of the implantation procedure, we can conclude that the production of a possible encapsulation consisting of non-melanotic cells has
relatively low costs with no apparent effect to residual life span. Melanotic cells release toxic ROS compounds, which kill the intruder. However, cytotoxic ROS activity also increases lipid peroxidation and damage of DNA and protein molecules of the host organism (Zenkov, 2001). Therefore, the outer layer of the capsule might be needed to protect host tissues against ROS instead of fighting against an already encapsulated foreign body. This is supported by a recent experiment by Dubovskii et al. (2010) that revealed a significant relationship between the production of melanotic hemocytes and the concentration of ROS in a moth. However, other studies have not found a direct relationship between the intensity of melanisation and the release of ROS (Mastore et al., 2005; Nappi & Christensen, 2005; Slepneva et al., 1999).

Overall, our results suggest that the encapsulation response is costly up to a point in time, which results in a negative relationship between the duration of encapsulation and remaining life span in mealworm beetles. We also show that the maximum value of implant melanization can be used as an indicator of completion of isolation of a foreign object from the host’s tissue, as evidenced by the recovery in remaining life span of male beetles. Results of other studies show considerable variation in the completion of the encapsulation response, which may take up to 70 hours (Allander and Schmid-Hempel, 2000; Eggenberger et al., 1990; Grimstone et al., 1967; Siva-Jothy et al., 2001; Takahashi and Enomoto, 1987). The observed discrepancy between our results and other studies may be explained by species-specific immunity, differences in metabolic rates, and ambient temperature, which significantly affect the strength of immune response in insects (Kortet and Vainikka, 2008; Mikkola and Rantala, 2010; Karl et al., 2011; Lalouette et al., 2011; Marshall and Sinclair, 2010; Rantala and Roff, 2006). Finally, our results show that important life history traits such as immunity and longevity can be experimentally affected in *T. molitor*, and this species is therefore an excellent model in the field of ecological immunology and immunogenetics.

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