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In vivo dynamics of an immune response in the bumble bee *Bombus terrestris*

P. Korner*, P. Schmid-Hempel

Ecology and Evolution, ETH-Zentrum NW, 8092 Zurich, Switzerland

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Abstract

Concepts from evolutionary ecology have recently been applied to questions of immune defences. However, an important but often neglected aspect is the temporal dynamics of the simple immune measures used in ecological studies. Here, we present observations for workers of the bumble bee *Bombus terrestris* on the dynamics of the phenoloxidase (PO) system, antibacterial activity, and the total number of haemocytes following a challenge with immune elicitors (LPS, Laminarin), over a time-span ranging from 1 min to 14 days. The dynamics of the PO measurement showed a complex pattern and was correlated with haemocyte counts. Antibacterial activity, on the other hand, increased sharply between 2 and 24 h post-challenge followed by a slow decrease. Surprisingly, the effects of a challenge lasted up to 14 days.

Keywords: Immune dynamics; *Bombus terrestris*; Phenoloxidase activity; Antibacterial activity; Haemocytes

1. Introduction

The analysis of the invertebrate immune system, both in terms of molecular mechanisms and the analysis of costs and adaptive strategies, has become a dynamic field of research over the past years (for reviews, see, e.g., Brey and Hultmark, 1998; Hoffmann and Reichhart, 1997; Karp, 1990; Pathak, 1993; Schmid-Hempel, 2003; Söderhäll et al., 1996). In particular, concepts from population biology and evolutionary ecology have been increasingly used to understand the design and adaptive functions of the immune system (Sheldon and Verhulst, 1996). Such studies are greatly facilitated by the availability of techniques to measure specific parameters of the immune response, e.g., antibacterial activity and the enzymatic activity of the phenoloxidase system (Schmid-Hempel, 2003). To understand how an immune response unfolds over time is a particularly relevant but often neglected aspect. On one hand, this temporal dynamics determines the swiftness of a reaction and thus the potential to keep an infection at bay. On the other hand, the temporal dynamics sets the frame when best to measure the strength of a response (e.g., Schmid-Hempel and Ebert, 2003). The existing physiological literature provides some insight into these dynamics (Ashida and Söderhäll, 1984; Ashida et al., 1983; Ratcliffe and Walters, 1983; Ratcliffe et al., 1991). However, these in vitro techniques are typically intended for a different kind of questions and thus may often not allow an extrapolation to studies done with live animals.

The invertebrate immune response rests on a number of pathways. For example, antimicrobial peptides, most of which are synthesized de novo upon an infection, are produced and are effective against bacteria and fungi (Boman, 1995; Bulet et al., 1999; Engström, 1999; Faye and Hultmark, 1993; Hetru et al., 1998; Hoffmann et al., 1996; Tzou et al., 2002). Small particles may become phagocytosed by haemocytes (Bayne, 1990) or entrapped in cell aggregates called nodules (Brookman

* Corresponding author. Fax: +41 1 6321271.
E-mail address: pius.korner@bluewin.ch (P. Korner).

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et al., 1989b; Ratcliffe et al., 1991). Larger invaders are covered with layers of haemocytes, producing a capsule that may become melanized (Dunn, 1986; Gillespie et al., 1997; Karp, 1990). A key enzyme of the melanization process is phenoloxidase (PO), which is activated via a cascade of serine proteinases (Sugumaran and Kanost, 1993). In naïve animals, PO is present in its inactive form, proPO (pPO), contained in the haemocytes and/or in the plasma (Brookman et al., 1989a). The haemocytes and the PO system are always present or very rapidly activated and can thus be thought of as the standing level and first line of defence behind the cuticle (a "constitutive response"; Schmid-Hempel and Ebert, 2003). The antimicrobial peptide titers, by contrast, are typically very low in unchallenged animals. Typically, this kind of defence is only induced when an actual infection has been recognized (for exceptions, see Hetru et al., 1998; Lamberty et al., 2001; Manetti et al., 1998).

While the antibacterial activity can be measured easily, the PO activity is more difficult to quantify. The activated PO enzyme is known to attach to tissue, and the proenzyme (pPO) is rapidly activated following various treatments, including wounding, mechanical agitation, heat, and various chemicals (Ashida and Brey, 1998). Therefore, it is not clear whether active PO can be taken from the animal, or whether pPO in the haemolymph sample is activated due to haemolymph collection, causing the subsequent increased PO activity to be measured. Subsequently, we will talk of "PO value" to refer to the measured darkening of the test solution (see Section 2), thereby including both the PO activity and the proPO titer. Note, in addition, that our measurements always include proper controls and therefore the appropriate comparisons refer to the differences relative to controls. Looking at differences largely removes the effect of such possible artefacts.

In this article, we present observations on the temporal dynamics of various parameters that characterize the invertebrate immune response (phenoloxidase system, antibacterial activity, total haemocyte count) in live animals following a challenge with an immunogenic elicitor (lipopolysaccharide (LPS) and Laminarin). The use of non-living immune stimuli allows focusing on the unfolding of the immune response as such, excluding effects due to the dynamic behaviour of a real parasite. Note that the purpose of this paper cannot be to unravel the exact physiological mechanisms behind an immune response but to investigate what kind of temporal dynamics is to be expected when the standard methods used for questions of evolutionary ecology are applied. Due to their simplicity and robustness, these methods are extremely valuable for such studies that typically involve large sample sizes and even field conditions. Here, we also mention their shortcomings, which must be scrutinized in future studies, but our immediate goal is to investigate the so-measured temporal dynamics in the immune response.

2. Methods

2.1. Animal rearing

We reared colonies from queens of the bumble bee *Bombus terrestris* caught around Zurich, Switzerland, in the lab under standard conditions (20–25 °C, 60–70% r.H., under red light and food ad libitum). A first experiment was done in the year 2000 with workers of four second-generation colonies that originated from offspring of queens collected in spring 2000. In this experiment, measurements of immune parameters were taken at 2, 4, 8, 12, or 24 h after a challenge by LPS and Ringer (control) ($N = 8$ workers per time and type of challenge). A second series of experiments was done in the year 2002. In this series, we used workers from five colonies (founded by second generation queens, derived from wild queens caught in spring 2001) and tested for the 'long-term dynamics' of the immune parameters (measurements ranging 2–120 h post-challenge). An additional four first-generation colonies from the queens caught in spring 2002 were used for the 'short-term dynamics' (1 min to 2 h post-challenge) experiment. All experiments were done with the same protocol and the following specific modifications. For the long-term dynamics, the workers were age-controlled: callow workers (max 24 h post-hatching) were removed from the colonies and kept in kin groups for 3–5 days before the challenge. For the first experiment of 2000 and the short-term dynamics experiment of 2002, adult workers were randomly selected directly from the colonies and challenged immediately.

2.2. Treatments

In all cases, bees were assigned beforehand to a particular challenge and a designated time for immune measurements and in a randomized fashion so as to balance possible confounding effects of age, colony, treatment or time in the data set. Each individual bee was only used for one treatment and one measurement at the pre-set time point to ensure independence of data.

Workers ready to be challenged were cooled on ice. We used a glass capillary pulled out to a fine tip to inject a volume of 5 μl of sterile Ringer solution containing an elicitor into the abdomen of the worker bees. The capillary tip was washed in methanol between injections. The treatments consisted of injecting either a dose of lipopolysaccharides (LPS) (Sigma L-2755; dose: 0.5 mg/ml dissolved in sterile, i.e., autoclaved, insect Ringer) or Laminarin (Sigma L-9634; dose: 1 mg/ml in autoclaved insect Ringer). LPS is a surface molecule of Gram-neg-

ative bacteria. Laminarin is a β-1,3-glucan found on the surface of fungi and algae. Both substances are known to stimulate the immune system of insects (Engström, 1999; Gillespie et al., 1997).

Because the inevitable wounding of the insect during injection has a known effect on the insect immune system (Yeaton, 1983) we always used a group of workers that were injected with 5 μl of autoclaved insect Ringer solution as a control. In the short-term experiment we also measured the response in naïve animals at each time point as an additional control. These naïve animals were treated identically to bees receiving an injection except that they were not injected or wounded. After the challenge, the animals were kept individually with sugar water (Apinvert, Südzucker, Ochsenfurt, Germany) and pollen ad libitum. For the measurements, 10 μl of haemolymph were taken from each animal at the designated time point. Each animal was bled only once. In all, the time points of measurement were 1, 3, 10, 30 min, 1, 2, 4, 8, 12 h, or 2, 4, 5 days post-challenge. For each combination of time point and type of challenge we assayed a sample of 10–15 workers.

Data for measurements done 14 days post-challenge under the LPS treatment became available from another experiment (with an identical experimental protocol) (P. Korner and P. Schmid-Hempel, in prep.) and were added here for comparison ($N = 14$ workers per time and challenge).

2.3. Haemolymph collection and immune measures

The haemolymph (10 μl) was collected from ice-cooled bees into an EDTA-moistened glass capillary and immediately blown into 50 μl ice-cold buffer (sodium cacodilate: 0.01 M Na-Cac, 0.005 M $CaCl_2$). A part of this solution was pipetted onto an improved Neubaur haemocytometer for the count of the total haemocyte numbers (the haemocytes were allowed to settle for 5–15 min before counting). The rest was snap-frozen in liquid nitrogen and stored at −80 °C for later measurements of antibacterial and phenoloxidase values. In the first experiment from 2000, instead of bleeding, we homogenized the bees' thorax in 300 ml cacodilate buffer. The homogenate was shock-frozen in liquid nitrogen and stored at −80 °C for later analysis. We used the supernatant (after centrifugation for 10 min at 3700*g* and 4 °C) for PO measurements (see below).

Besides the haemocyte count, further immune parameters were measured as described in Moret (2001), with a few minor modifications and as follows. For the antibacterial activity, we placed 2 μl of the haemolymph solution into holes (diameter 2 mm) punched into a thin layer of agar (6 ml in Petri dishes, diameter 9 cm; agar recipe: 10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl, 1000 ml distilled water, pH 7.5) mixed with the test bacteria *Arthrobacter globiformis* (Institut Pasteur Paris, nr. 81.84 T) adjusted to 10^5 bacteria/ml. After 24 h of incubation at 37 °C we measured the diameter of the clear zone of inhibition around the haemolymph. The diameter was used as a measure of antibacterial activity in the haemolymph. The PO system was quantified photospectrometrically with a SpectraMAX-340PC together with SOFTmax PRO 3.1 software. For this purpose, haemolymph was defrosted in an ice-water bath and centrifuged for 7 min (3700*g*, 4 °C). Of each probe, 20 μl of supernatant was added to 140 μl ice-cold deionized water and 20 μl buffer (PBS: 8.74 g NaCl, 1.78 g NaH_2PO_4, 1000 ml H_2O, pH 6.5) in a well of a flat bottom microtiter plate. Then, 20 μl of L-DOPA (Sigma D-9628; 4 mg/ml deionized water), the substrate for the enzymatic reaction, was added. Immediately afterwards, the plate was put into the photospectrometer set to a reaction temperature of 30 °C allowing the conversion of L-DOPA to melanin, the key reaction catalyzed by PO. Light absorbance at 480 nm was measured every 10 s between 10 and 20 min after the start of the reaction. The accumulating reaction product is reflected in the increasing absorbance. From this, the PO activity of the reaction mixture can be expressed as the slope of the reaction curve during the steady-state reaction, which emerges as a straight line describing the change in optical density with time (as the substrate is used up and the product of the reaction catalyzed by PO appears at a steady rate; defining the so-called V_{max} value, i.e., the rate of change of the optical density per minute).

2.4. Statistical analysis

For each of the immune parameters measured we performed a univariate general linear model with 'time' (i.e., time period elapsed since the challenge) and 'challenge' (Ringer, LPS, or Laminarin) as fixed factors (including their interaction) and 'colony' (identifies the colony the bee came from) as random factor. Total haemocyte count was square root-transformed and the PO values were ln-transformed to normalize variances in the general linear model (GLM) and as inspected with cumulative probability plots. No effort was made to correct p-values for multiple correlations because the pattern rather than the individual values of the correlations is of interest. We used SPSS 10 for Macintosh.

3. Results

3.1. Immune dynamics

3.1.1. Measurement of PO-activity

Fig. 1 shows the temporal dynamics of the PO values. Over the time period measured, we found significant differences among the various treatments and a significant time × treatment interaction indicating that the activity

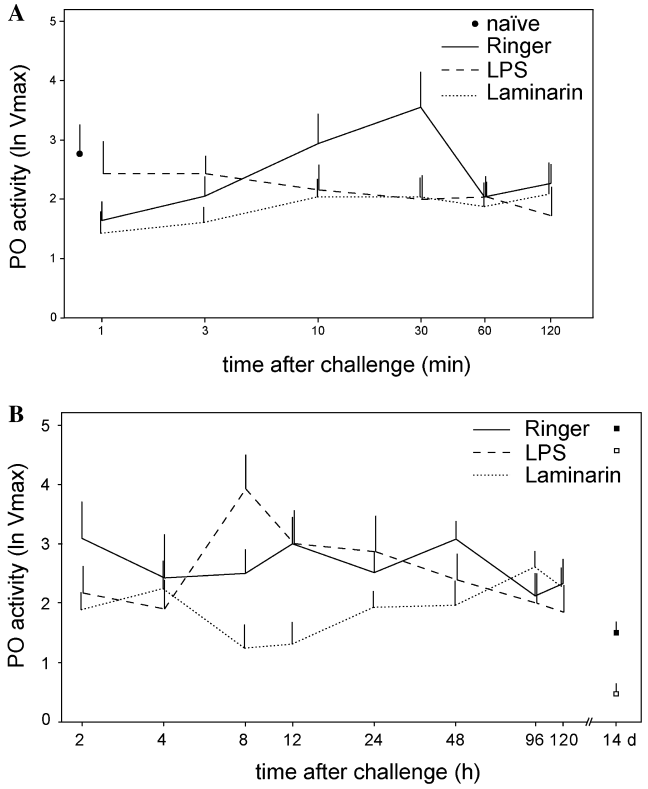

Fig. 1. Dynamics of the PO values (ln V_{max}, i.e., rate of change in optical density per minute, see Section 2) measured after injection of LPS (dashed line, open square) or Laminarin (dotted line) into the abdomen of bumble bee workers. Control bees were injected with physiological saline (Ringer, solid line, solid square). Naïve bees (solid dot) were sham-manipulated but not injected or wounded. Time points on the horizontal axis indicate the times of haemolymph collection and measurement post-challenge (note the logarithmic scale). Sample size was 10–15 bees per time point and treatment. Means + SE are depicted (for clarity only one error bar shown; some nudged sideways for clarity). (A) Naïve bees and short-term dynamics (1 min to 2 h post-challenge). (B) Long-term dynamics (2 h to 5 days post-challenge) and one additional measurement at 14 days.

changed over time but according to treatment (Table 1). By contrast, the mean PO value of naïve bees measured at the different time points, 1 min to 2 h post-challenge, varied between 2.28 and 3.25, with no pattern or trend detectable (overall mean thus depicted in Fig. 1A).

The PO values of Ringer-injected bees (i.e., the control for wounding and injection) showed a transient increase during the first hour, although the peak was based on two data points only (i.e., measurement at 10 min: $\ln(V_{max}) = 2.93$, at 30 min: $\ln(V_{max}) = 3.54$) and was thus not really higher than the mean found in naïve bees ($\ln(V_{max}) = 2.75$).

However, PO values for Ringer-injected bees were higher than the values for LPS- and Laminarin-challenged bees during the first hours after the treatment. Between 8 and 24 h, PO values of LPS bees were higher than from Ringer-injected bees (most clearly at 8 h), while Laminarin-challenged bees remained at low PO-values. Interestingly, PO-values 14 days after an LPS challenge were lower than after a Ringer injection (see dots at right in Fig. 1B, t test $t = 3.89$; $P = 0.001$). Overall therefore, the dynamics of the PO values seems to be rather complex. The pattern seen after an LPS challenge in the experimental series of 2002 is similar to that found in the first experiment in 2000, where low values after a LPS challenge at 2 and 4 h and a peak at 8–24 h post-injection were observed (Fig. 2).

3.1.2. Measurement of haemocytes

The dynamics of the total haemocyte count is shown in Fig. 3, and the results of the statistical analysis are given in Table 1. Naïve bees (overall mean given in Fig. 3A) and Ringer-injected bees both showed no trend across time in haemocyte counts and were similar in their counts. LPS- and Laminarin-challenged bees, by contrast, showed an increase in the haemocyte count relative to the Ringer controls a few minutes after the challenge (Fig. 3A) and which lasted (with fluctuations in the Laminarin group) for about 12 h (Fig. 3B). After 12–24 h post-challenge the challenged bees had consistently lower hemocyte counts than the Ringer controls (Fig. 3B). Haemocyte counts 14 days after an LPS challenge appeared lower than control bees, but the difference

Table 1
General linear model for the dynamics of immune parameters

Immune measure[a]	Time[b]	Treatment[c]	Time × treatment	Colony[d]
PO value	$F_{12,493} = 0.66$ $P = 0.79$	$F_{2,493} = 8.45$ **$P < 0.001$**	$F_{26,493} = 1.67$ **$P = 0.021$**	$F_{7,493} = 1.35$ $P = 0.22$
Total haemocyte count	$F_{12,488} = 5.73$ **$P < 0.001$**	$F_{2,488} = 5.09$ **$P = 0.006$**	$F_{26,488} = 2.34$ **$P < 0.001$**	$F_{7,488} = 3.56$ **$P = 0.001$**
Antibacterial activity[e]	$F_{7,297} = 52.51$ **$P < 0.001$**	$F_{2,297} = 75.10$ **$P < 0.001$**	$F_{14,297} = 7.74$ **$P < 0.001$**	$F_{4,297} = 0.83$ $P = 0.51$

Entries are F and P values (significant values in bold; df's in subscript) for each factor in the univariate models for each immune measure.

[a] See text for details of measurement.

[b] Time interval post-challenge.

[c] Treatments were Ringer (control), LPS, and Laminarin.

[d] The colony where the tested worker bee came from.

[e] Only data from the 'long-term experiment' (2–120 h) included, since antibacterial activity takes about 2 h to develop.

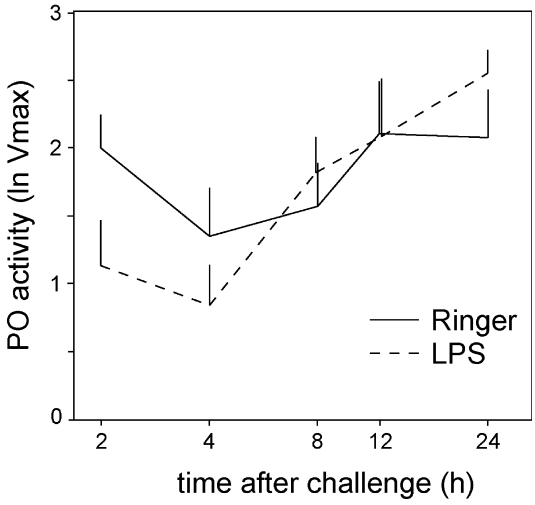

Fig. 2. Dynamics of PO values from the first experiment of 2000 (see also Fig. 1). Sample size was 10–15 bees per time point and treatment. Labelling as in Fig. 1.

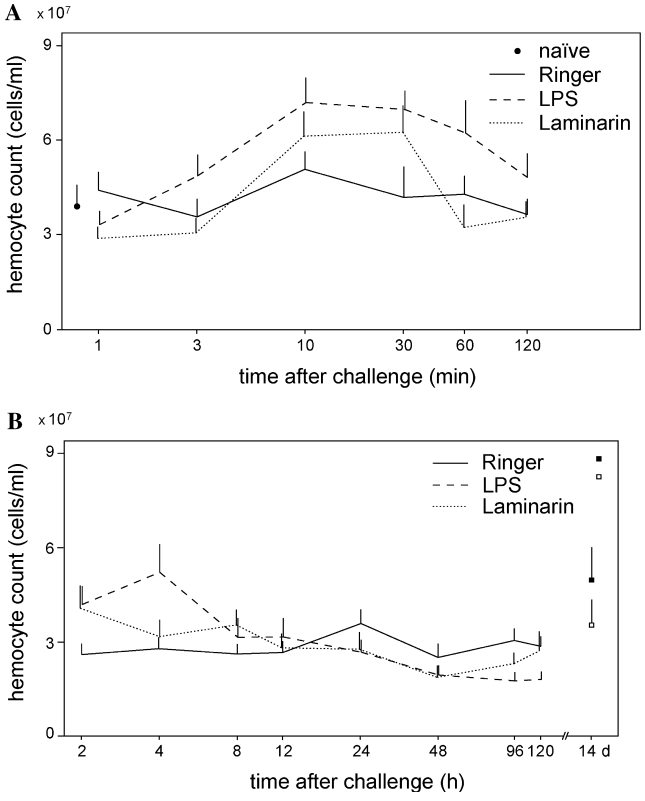

Fig. 3. Dynamics of the total haemocyte count, given as cells per ml haemolymph. (A) Naïve bees and short-term dynamics (1 min to 2 h post-challenge). (B) Long-term dynamics (2 h to 5 days post-challenge) and 14 days. Labelling as in Fig. 1.

was not significant (see dots at right in Fig. 3B, $t = 1.51$, $P = 0.14$).

3.1.3. Measurement of antibacterial activity

Antibacterial activity, by contrast, showed a clear and simple dynamics (Fig. 4, Table 1). Virtually no

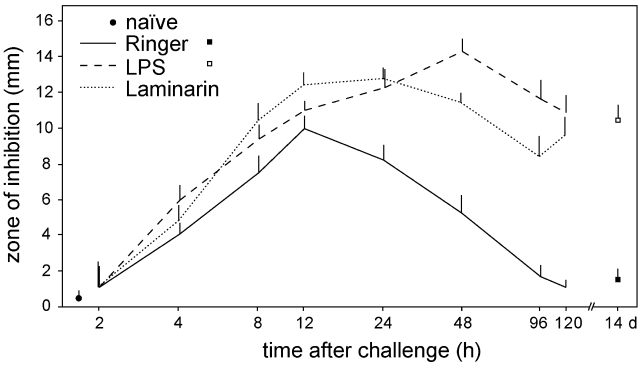

Fig. 4. Dynamics of antibacterial activity, expressed as the zone of inhibition around a drop of haemolymph on a bacterial test plate coated with *A. globiformis*. Labelling as in Fig. 1.

activity was observed during the first 2 h (data not shown). Thereafter, antibacterial activity increased in all bees, independent of the treatment (Fig. 4). However, after about 12 h, the activity of Ringer-injected bees decreased continuously to reach naïve levels within 120 h (Fig. 4). Bees challenged with either LPS or Laminarin maintained high antibacterial activity until the end of the experimental period (120 h). Even 14 days after LPS-challenge, the antibacterial activity of these animals was still considerably higher than in the Ringer controls (see dots at right in Fig. 4, $t = 8.25$, $P < 0.001$).

3.2. Correlations between immune parameters

The dynamics of the correlation between PO values and haemocyte count over time may help to better understand the course of an immune response. We found that early after the injection, there was no correlation between haemocyte numbers and PO-activity in any of the treatments (Table 2). However, negative correlations appeared as the immune response unfolded with time after the challenge. This was most pronounced in LPS-challenged animals. The correlations vanished towards the end of the time interval investigated here so that no correlation was found 14 days after the challenge. If anything, at that time, we found a trend towards a positive correlation (pcc = 0.348, $P = 0.075$, $N = 25$). By contrast, all significant correlations were of negative value.

4. Discussion

We found that PO-values showed complex dynamics after an experimental immune challenge in the living animal. However, one can identify three different periods on the time scale examined here: 10 min to 4 h, when Ringer bees showed highest values; 8–24 h, when LPS- and Laminarin-challenged bees behaved differently;

Table 2
Dynamics of the correlation between total haemocyte count and PO value after injection of immune elicitors or Ringer

Time post-challenge[a]	Treatment[b]			
	Ringer	LPS	Laminarin	Pooled
1 min	0.11	−0.44	0.03	−0.09
3 min	−0.10	0.06	0.28	0.15
10 min	−0.51	0.02	0.51	−0.11
30 min	−0.71*	−0.15	0.33	−0.51**
1 h	0.21	−0.04	0.01	0.14
2 h	−0.44	0.18	−0.58*	−0.27
2 h	−0.27	−0.72**	−0.36	−0.47**
4 h	−0.37	−0.44	−0.51*	−0.41**
8 h	−0.06	−0.62*	−0.35	−0.42**
12 h	−0.28	−0.34	0.38	−0.11
1 day	−0.49*	−0.60*	−0.37	−0.44**
2 days	−0.46	−0.22	−0.38	−0.22
4 days	0.33	−0.69*	0.42	−0.05
5 days	−0.23	0.19	0.12	0.06

Entries are the partial correlation coefficients controlling for the colony effect. Naïve bees are not included.

[a] Values for 1 min to 2 h from "short-dynamics" experiment; values for 2 h to 5 days from "long-term" dynamics experiment.

[b] $P < 0.1$.

* $P < 0.05$.

** $P < 0.01$.

and between 2 and 5 days (14 days) the appearance of the long-term consequences of a challenge.

Over a short time span, i.e., up to 4 h, but especially between 10 min and 1 h, we measured lower PO-values for immune-stimulated animals. This observation seems to be at odds with previous work, where the activation of pPO by LPS and Laminarin has been found in vitro, although this is actually not always the case (discussed in Ashida and Brey, 1998; Brookman et al., 1989a). Our results show that in *B. terrestris*, both substances do have an effect on the PO value, but immediately after a challenge we seem to observe a deactivation rather than an activation. Perhaps, the following scenario may explain this observation. In the naïve insect, phenoloxidase is mainly present as its pro-enzyme, i.e., pPO (Brookman et al., 1989a). The injection of LPS and Laminarin causes the rapid conversion of pPO to PO and correspondingly reduces the pPO-titer in the insect. Much of the newly produced PO sticks to body tissue and to glassware upon haemolymph collection (Ashida and Brey, 1998), but not yet converted pPO is collected with the haemolymph. Subsequently, when this haemolymph sample is measured, some or all of the remaining pPO converts to PO, because pPO is easily activated already by mechanical agitation and heat (Ashida and Brey, 1998). The produced PO then catalyzes the production of melanin in the test solution and, therefore, its darkening, which is measured by the photospectrometer. Therefore, we think that our measure is a reasonably good estimate of the pPO titre in the insect. This would explain the observed reduction in the initial phase of the time series measured. In addition, the time course of low PO values occurring shortly after injection of the immune stimulant is in good agreement with physiological studies (Saul and Sugumaran, 1987). Note also that our interpretation refers to the differences between treatment(s) and control, which balances these effects. Furthermore, the interest is in gaining a general understanding of immune dynamics and, therefore, our measures even if these contained a systematic bias will nevertheless describe the relevant changes over time.

Kato et al. (1994) reported clearance of LPS in *Bombyx mori* larvae within 8–12 h. Around this time, pPO in the haemolymph may no longer be converted to PO. At about this time (8–24 h), PO values after an LPS challenge were higher than the control in our experiment. The effect was pronounced at 8 h, but weak at 12 h and 24 h. However, the same pattern was found in a previous experiment (Fig. 2). Possibly, the bees managed to replenish their pPO stocks by that time. In this context, it is interesting to find significant negative correlations between total haemocyte count and PO value 2–8 h post-LPS challenge (Table 2). At this time, haemocytes may have burst and released pPO into the haemolymph in order to replenish pPO levels.

As opposed to the LPS treatment, 8–12 h after a Laminarin challenge, PO values were very low. This difference may represent alternative strategies of the host to combat different types of infection (bacteria, mimicked by LPS, versus fungi, mimicked by Laminarin). The difference is also observed in the dynamics of the negative correlation between PO values and total haemocyte count, which is stronger in LPS treated bees (Table 2).

After 24 h, the effect of Laminarin on the PO values faded away. The long-term effect of LPS on *B. terrestris* workers seems to down-regulate the PO system. Moret (2001) found PO values to be only half compared to the Ringer treatment 4 days after challenge. We found clearly reduced PO values 14 days after LPS injection compared to Ringer (Fig. 1B). At that time, antibacterial activity is still high, and the low PO values may indicate a trade-off between the two pathways. The trade-off could, for example, be based on limited energy, crucial nutrition resources, or on negative side-effects associated with immune activation (Nappi and Vass, 1993). One alternative explanation is based on the fact that PO is involved in several physiological processes, e.g., also in the hardening of the cuticle (Marmaras et al., 1996). A large PO activity after a challenge may temporarily accelerate such other tasks of the PO enzyme, and a subsequently reduced overall need of PO activity could ensue (C. Yourth, pers. comm.).

Haemocyte counts after LPS and Laminarin challenge increased. Similar observations have been made in *Manduca sexta* (Horohov and Dunn, 1982), and the authors suggested that, possibly, sessile haemocytes are

recruited upon a bacterial insult. However, Ratcliffe and Walters (1983) and Chain and Anderson (1983) observed the opposite in *Galleria mellonella*, namely a decrease in total haemocyte count 30 min to several hours after injection of live bacteria (see Bahadur, 1993; for a discussion on the effect of challenges on total haemocyte count). In our experiment, haemocyte counts between 0.5 and 1–2 h decreased in the LPS and Laminarin treated bees, which may represent a loss of haemocytes through degranulation in the course of the immune reaction (Rowley and Ratcliffe, 1976). Gradually, counts dropped below the level of Ringer treated bees. Two weeks after LPS injection, total haemocyte count was only non-significantly lower than after Ringer injection, suggesting a recovery towards pre-challenge total haemocyte counts.

The dynamics of the antibacterial activity following an immune insult is straightforward. The simplicity can be explained in that we now look at a phenomenon of de novo protein synthesis, while the pPO/PO measurement is based on an enzyme system, which, of course, is likely to respond more rapidly. *Bombus pascuorum* is know to synthesize a number of antibacterial proteins closely related to the ones found in honey bees *Apis mellifera* (Rees et al., 1997), and the same can be expected for *B. terrestris*. In line with the notion that antibacterial activity is only induced upon a challenge, almost no activity was found in naïve animals (Fig. 4). After a challenge with Ringer, we observed antibacterial activity after 2–4 h, peaking at 12 h, which then is fading away over a period of 4–5 days. Both LPS and Laminarin led to a slightly steeper increase of antibacterial activity 2 h post-challenge, the peak was reached after 24 or 48 h, followed by a plateau or gradual, slow decrease. Interestingly, even 14 days post-LPS challenge, a very significant antibacterial activity was still observed (Fig. 4). Usually, antibacterial activities last for several days (Kaaya, 1993), although longer effects are known (e.g., 44 days in dragonflies, Bulet et al., 1992).

It may seem strange that injection of Laminarin, a β-1,3-glucan simulating a fungal infection (Ochiai et al., 1992), induced activity against *A. globiformis*, our test bacterium. Fungi usually enter the insect haemocoel across the cuticle (Roberts and Airst, 1984; Schmid-Hempel, 1998). From an adaptive point of view, this penetration may increase the risk of bacteria entering the body cavity, too, and the induction of antibacterial activity following Laminarin injection may thus be a pre-emptive response of the insect. On the other hand, some antimicrobial proteins are known to be active against fungi as well as bacteria (Casteels, 1998; Hoffmann, 1995). This second argument holds true for the observation that LPS from *E. coli* (Gram-negative) triggers activity against Gram-positive *A. globiformis*. However, it has to be stressed that some degree of specificity of the insect immune response has been demonstrated (Hetru et al., 1998). Indeed, it can be seen from the differences in the dynamics of all three immune parameters measured here that the immune responses to LPS and Laminarin were not identical.

5. Conclusion

Simple measures of immune parameters gained from experiments with live insects are often indispensable for ecological questions. Our findings can help to better interpret such immune measurements. Within the limitations given by our methods, the results suggest the following possible scenario of an infection in *B. terrestris*: upon infection, pPO is activated and haemocytes are released from stocks, representing the first line of defense. At 2–4 h, antibacterial activity starts. Also, haemocytes burst and release pPO. This process, however, seems to be stronger after LPS-challenge than after Laminarin-challenge, indicating some degree of specificity. The effect of an LPS challenge lasts for at least 14 days, when antibacterial activity is still at high levels, while PO value is reduced, possibly due to a trade-off with antibacterial activity, or due to a long-term change in the optima of the PO system.

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