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Review

Immune priming in arthropods: an update focusing on the red flour beetle☆

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ABSTRACT

Immune priming has now been demonstrated in a wide range of invertebrate species. Studies testing this phenomenon largely differ in terms of experimental design, host–parasite combinations, agents used for priming, and in particular the degree of demonstrated specificity of the primed response. This review provides an overview of known and putative mechanisms underlying broad-spectrum and specific immune priming in arthropods. We focus on insects and particularly the red flour beetle *Tribolium castaneum*, where priming has been demonstrated within and across generations. We will also draw attention to the relevance of routes of priming and infection, which can occur septically and orally, with largely differing physiology. For oral priming, an involvement of gut microbiota was demonstrated in mosquitoes and flour beetles. Generally, a primed state could result from long-lasting immune activation or a form of memory that does not entail lingering immune components. Moreover, the primed state could also be of a qualitatively different kind than the challenge response. Finally, we will consider that there should be natural variation in priming capability, and therefore a possibility to study this trait with experimental evolution approaches.

Contents

1. Introduction

Interactions between hosts and parasites are particularly strong driving forces in evolution, entailing severe effects on the fitness of both interacting partners (Anderson and May, 1982; Woolhouse et al., 2002). Host–parasite coevolution has thus given rise to a multitude of traits with which parasites (here defined in a broad sense, including viruses, bacteria and fungi) invade and exploit their hosts (Schmid-Hempel, 2011). On the other hand, hosts have evolved numerous countermeasures to defend themselves against parasites, or to limit their deleterious effects on host fitness. These structures and processes are collectively called immune systems, and have classically been separated into innate and adaptive immune systems (Frank, 2002; Murphy et al., 2012). Adaptive (or acquired) immune systems are defined by their ability to create specific immune memory. This form of phenotypic adaptation within the lifetime of an organism allows it to evade the other-

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wise often faster evolutionary adaptation of parasites to their hosts. While adaptive immunity seemed originally restricted to vertebrates that possess the machinery for the production of specific antibodies and memory B and T cells, more recent research has blurred the strict boundary between innate and adaptive immunity (Kurtz, 2005; Criscitiello and de Figueiredo, 2013).

Invertebrates do not possess the underlying molecular machinery for the type of adaptive immunity that we find in vertebrates, but are nevertheless capable of an improved immune response upon a secondary exposure to a parasite (for review, see Kurtz, 2004, 2005; Little et al., 2005; Chambers and Schneider, 2012). These observations in an increasing number of invertebrate taxa gave reason to hypothesise that alternative molecular mechanisms based on components of what is normally called the innate immune system might provide forms of immune memory, which are mostly denoted as "immune priming" (Little and Kraaijeveld, 2004; Little et al., 2005; Kurtz and Armitage, 2006). It is noteworthy (since it might lead to confusion) that other terms have been used for similar phenomena (e.g., "responsive mode prophylaxis" by Moret and Siva-Jothy, 2003; "tolerance" by Rahman et al., 2004 and Kutzer and Armitage, 2016) and that the term priming itself is used for both the (experimental or natural) procedure of "priming", and for the "primed" state of the immune system that is achieved thereby. Moreover, recent discoveries in vertebrates show that their innate immune system is also capable of achieving a primed immune state that has been called "trained immunity" (Netea et al., 2011).

Invertebrate immune priming has now been demonstrated in a wide range of species (e.g., Kurtz and Franz, 2003; Little et al., 2003; Moret and Siva-Jothy, 2003; Witteveldt et al., 2004; Sadd and Schmid-Hempel, 2006; Roth et al., 2009; Pope et al., 2011; Tidbury et al., 2011 Pope et al., 2011; Tidbury et al., 2011). Importantly, these studies differ enormously in terms of experimental design, host–parasite combination, timing of priming and infection, agents that were used for priming, etc. Most importantly, the primed immune responses vary strongly in terms of specificity (Schmid-Hempel and Ebert, 2003). Specificity here defines the degree to which a primed immune response is able to discriminate among different parasites (Frank, 2002; Kurtz, 2005). For example, priming with the bacterial component LPS led to enhanced resistance against infection with the entomopathogenic fungus *Metarhizium anisopliae* in the mealworm beetle *Tenebrio molitor* (Moret and Siva-Jothy, 2003), which indicates that a rather general, unspecific reaction is involved. On the other hand, in a study in the red flour beetle *Tribolium castaneum* protection against subsequent infection with a certain strain of the entomopathogen *Bacillus thuringiensis* was only achieved when exactly the same strain was used for previous priming (Roth et al., 2009). By contrast, within the same study, protection against other bacteria such as *Bacillus subtilis* was also achieved when different bacteria or even Ringer insect saline solution were used for priming (Roth et al., 2009). This indicates that different degrees of specificity can be achieved with different parasites used for priming and challenge. A general state of priming could additionally be attained by even more unspecific stressors, such as a cold or heat shock (Altincicek et al., 2008; Le Bourg et al., 2009) or after mild physical stress (e.g. shaking, Browne et al., 2014). These studies imply that the degree and specificity of priming may strongly depend on the hosts and parasites involved and the specific conditions under which it occurs. On that note, the different forms of priming are likely based on a diversity of mechanisms.

In the following, we will review the current state of research on immune priming in arthropods, and specifically in the red flour beetle *T. castaneum*. We will pay particular attention to different routes of priming and potential underlying mechanisms. Finally, we will discuss the suitability of *T. castaneum* as a promising model organism for evolutionary experiments and give an outlook on currently on-going experiments addressing the question whether priming and its specificity are in itself traits that could undergo evolutionary change.

2. Routes of infection and priming in the red flour beetle, *T. castaneum*

Only recently, the infection route of a parasite has been recognised as a relevant aspect for the evolution of host defences (Martins et al., 2013). The point of entry of a parasite into its host can strongly vary in its underlying physiology; different immune parameters are relevant in the insect cuticle, haemolymph and gut. Consequently, depending on the infection route, both host and parasite will need to adjust their defence and counter defence mechanisms.

For the red flour beetle, cuticular, septic and oral routes of infection are experimentally used to investigate host–parasite interactions. The entomopathogenic fungus *Beauveria bassiana*, which is also used to control *T. castaneum*, infects via penetration of the cuticle (Pedrini et al., 2015). For septic infections, pathogens such as vegetative bacterial cells are directly introduced into the insect's body cavity by pricking the intersegmental membrane with a contaminated needle (Roth et al., 2009; Tate and Graham, 2015 Tate and Graham, 2015). Alternatively, injection methods serve to better control the amount of bacteria introduced (Ferro et al., unpublished data). In contrast, infections via the oral route are performed by exposing the insects to parasite-contaminated diet (Blaser and Schmid-Hempel, 2005; Milutinović et al., 2013; Rafaluk et al., 2015). The benefit of oral exposure is that it avoids the wounding that is intrinsic to septic infection techniques, which in itself induces a strong immune response (Johnston and Rolff, 2013; Behrens et al., 2014). Making use of both septic and oral routes of infection in the same host species, specifically when using the same parasite strain as well, allows studying a wide range of immunological, physiological and behavioural responses (Behrens et al., 2014; Peuß et al., 2016). For example, septic and oral infections by *B. thuringiensis* were shown to activate substantially different physiological and immunological reactions in *T. castaneum* (Behrens et al., 2014). Moreover, transcriptomic responses to infection also differed between the two insect populations that were studied and that had previously been shown to differ in their susceptibility to *B. thuringiensis* (Milutinović et al., 2013). Oral uptake of spores is generally considered the natural route of infection for *B. thuringiensis* (Raymond et al., 2010) and indeed, *T. castaneum* larvae can experimentally get re-infected by cannibalising on *B. thuringiensis* infected cadavers (Milutinović et al., 2015). However, septic infections are also likely to happen in nature when animals are wounded, which might regularly occur in *T. castaneum* (B.M., personal observation upon collection of wild *T. castaneum* populations, cf. Milutinović et al., 2013). Hosts therefore also need to be able to shape their defence responses depending on the route by which parasites enter.

Consistent with the same argument, the phenomenon of immune priming has also been demonstrated for pricking and for oral infections in *T. castaneum*, with potentially very different underlying mechanisms (discussed in Section 3). Priming induced via the septic route in *T. castaneum* is achieved by introducing the heat-killed bacteria into the insects' body cavity, followed by administration of a lethal dose of live bacteria several days later (Roth et al., 2009). Enhanced resistance can be transferred to offspring by mothers and even fathers (Roth et al., 2010; Eggert et al., 2014; Tate and Graham, 2015). Interestingly, such transgenerational immune priming was also found to be triggered by a pathogen-unrelated stressor; offspring of parents experiencing a short cold shock survived a bacterial challenge better than the off-

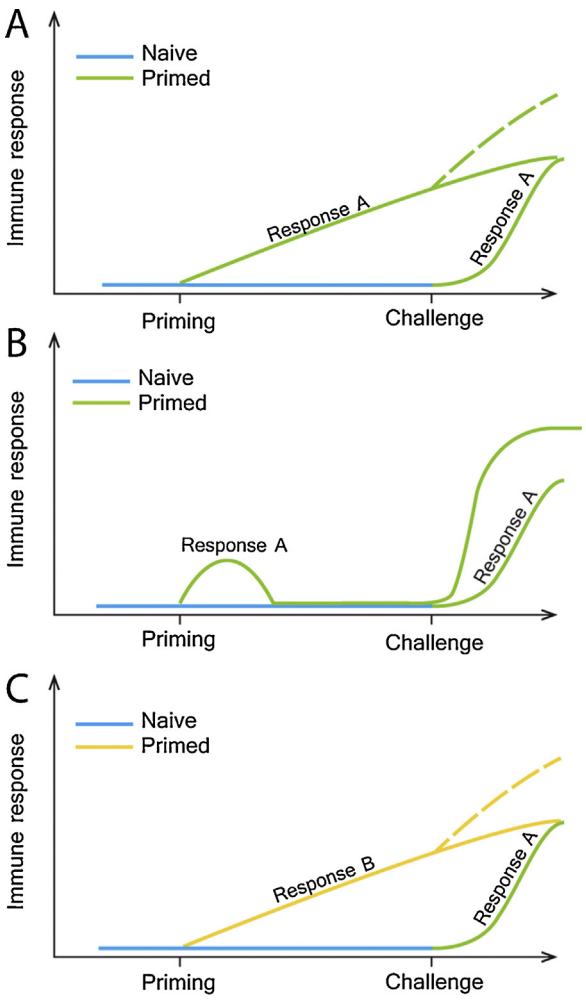

Fig. 1. Hypothetical scenarios for immune priming. (A) Survival benefit upon secondary challenge with a parasite could result from long-lasting defences induced upon priming. Additionally, priming-activated defences may be combined with challenge-activated responses (dashed line), resulting in an overall stronger reaction. (B) After priming, the initial immune response could fade with time, with the secondary exposure eliciting a stronger and/or faster reaction. This scenario implies the existence of a form of memory, where the information on a previously encountered pathogen is stored and rapidly accessed upon demand. (C) In contrast to scenarios (A) and (B), priming might alternatively mount a qualitatively different immune response from the one following a simple challenge. (Fig. 1A and B after Schmid-Hempel 2011, p. 181).

spring of non-treated parents (Eggert et al., 2015), suggesting an intimate connection between immunity and stress in general.

Priming studies via the oral route are still scarce for insect models and were only recently described in *T. castaneum* (Milutinović et al., 2014). In contrast to septic priming where heat-killed bacterial cells are used, oral priming was achieved using medium supernatants from bacterial spore cultures that were centrifuged and filter-sterilised before mixing with the insect flour diet (Milutinović et al., 2014), i.e. the observed priming effect could not be attributed to latent infection, potentially occurring when live bacteria are fed to the host (Freitak et al., 2009). Moreover, oral priming in *T. castaneum* was induced only by spore culture supernatants derived from a *B. thuringiensis* strain that is pathogenic to *T. castaneum*, which might indicate specificity, however, this needs to be studied in more detail, using several bacterial strains and/or species for challenge.

Consistent with the life-history theory, which predicts trade-offs between activation of a costly immune response and other biological processes (Sheldon and Verhulst, 1996), paternal priming reduced offspring fecundity in *T. castaneum* (Roth et al., 2010) and pupal mass in the related mealworm beetle, *Tenebrio molitor* (Zanchi et al., 2011). Interestingly, effects of priming on development time seem more complex: while maternal septic priming prolonged larval development in *T. molitor* (Zanchi et al., 2011), septic priming accelerated larval development in *T. castaneum*, both in a trans-generational setting (Tate and Graham, 2015) and within a generation (Roth and Kurtz, 2008). In contrast, oral priming delayed larval development within a generation (Milutinović et al., 2014) pointing again to very distinct physiological differences between the two infection routes.

3. The immunological and molecular underpinnings of priming

3.1. The key components of insect immune systems

The core elements of the insect immune system are well described today. Most of our knowledge is based on the immune system of *Drosophila melanogaster* (for review, see Lemaitre and Hoffmann, 2007). However, it should be noted that in addition to the generic set of immune components defined by work on *D. melanogaster*, additional taxon-specific immune factors have been identified in other species (Loker et al., 2004; Altincicek et al., 2008; Gerardo et al., 2010; Vilcinskas et al., 2012). The insect immune response can be broadly divided into cellular and humoral components (Lemaitre and Hoffmann, 2007), with the latter term being used in analogy to the vertebrate immune system, but here mainly referring to any type of non-cellular immune component (typically antimicrobial peptides, AMPs), mostly produced by the insect fat body. AMPs are small cationic peptides that integrate into and disrupt the bacterial cell wall and thereby kill the microbes (Zasloff, 2002; Bulet and Stöcklin, 2005). The cellular immune response is based on haemocytes, which act similar to vertebrate macrophages. Specialised haemocytes phagocytose pathogens or encapsulate larger pathogens like parasitoid eggs. In addition (and somewhere in between cellular and humoral), coagulation and melanisation responses are mediated by the enzyme phenoloxidase (PO). Its inactive state (prophenoloxidase, proPO) is generally produced by haemocytes and released into the haemolymph (Cerenius et al., 2008). The activation of PO is mediated by pathogen-recognising host receptors, which activate a complex cascade of serine proteases, tightly regulated by serine protease inhibitors (serpins) (Cerenius et al., 2008). Active PO catalyses the production of melanin and cytotoxic products and enables the encapsulation of pathogens (Cerenius et al., 2008).

All these insect immune reactions (and more generally, most innate immune reactions) seem to share a basic, similar architecture: recognition of pathogens via pattern recognition receptors (PRRs) that bind to evolutionarily conserved pathogen associated molecular patterns (PAMPs). Such binding stimulates regulatory networks, which in turn activate effector mechanisms. The most important immune signalling pathways are the well-known Toll and Imd pathways that can already grant a broad specificity, e.g. to discriminate between different bacterial Gram types (i.e., bacteria carrying Lys- and DAP-type peptidoglycans (Lemaitre and Hoffmann, 2007)). However, the underpinnings of higher degrees of specificity are not yet well understood. Specificity needs to entail all parts of the process, since specific recognition will not lead to a gross specific response if the involved signalling or effectors act non-specifically, and vice versa. In this context, it is noteworthy that vertebrate antibodies are at the same time highly specific antigen receptors and effectors, since they can recognise and directly combat a pathogen. Moreover, an initially unspecific immune reaction, such as phagocytosis or the release of cytotoxic effectors,

can be harnessed with specificity when a specific opsonin directs these reactions towards a specific target (Murphy et al., 2012). In the following, we will highlight a few (non-exhaustive) hypotheses for potential mechanisms mediating immune priming and its specificity in arthropods, and then again focus on the red flour beetle.

3.2. Molecular basics of immune priming in arthropods

In general, the phenomenon of immune priming in invertebrates could follow two main hypothetical scenarios of immune induction (Schmid-Hempel, 2011). In the first scenario, priming antigens induce long-lasting defences, leaving circulating immune molecules in the host body until the secondary challenge occurs. In this case, the survival benefit upon secondary exposure is a consequence of such previously activated defences. Additionally, previously activated defences may be combined with new, challenge-activated responses, resulting in an overall stronger reaction (Fig. 1A). In a second scenario, the initial priming response fades with time and the secondary exposure elicits stronger and/or faster defences, facilitating rapid removal of the parasite (Fig. 1B). Hence, the mechanism by which the two priming scenarios could take place are essentially different; whereas the first relies on lastingly induced defences, the second implies the existence of a form of memory, where the information on a previously encountered pathogen is stored and rapidly accessed upon demand (Kurtz, 2005; Schmid-Hempel, 2011). In reality, priming could be based on a combination of both scenarios. Moreover, the two priming scenarios could differ in the degree of specificity. While the first could also be triggered by more general stressors such as a temperature shock, the second might be tailored to a specific pathogen (e.g., different strains of the same parasite genus).

A graphical account of some of the currently discussed molecular underpinnings of septic and oral immune priming and effects on the following challenge is presented in Fig. 2. Antimicrobial peptides (AMPs) are without doubt relevant for immune priming in insects (Dubuffet et al., 2015), potentially playing a role as lastingly induced defences (cf. Fig. 1A). For example, AMPs are strongly induced upon septic wounding and infection and remain up-regulated for long periods of time. In *D. melanogaster*, mass spectrometric analysis of haemolymph samples from adult flies challenged with a mixture of *E. coli* and *M. luteus* revealed that some AMPs are even active in the haemolymph of the fly for up to three weeks after the initial exposure (Uttenweiler-Joseph et al., 1998). Moret and Siva-Jothy (2003) demonstrated that the haemolymph of *T. molitor* showed induced antimicrobial activity 7 days post priming. Haine et al. (2008) showed that haemolymph antimicrobial activity remains active long (28 days) after infection in *T. molitor* and likely protects the insect against the bacteria that persist within its body. However, given that most AMPs show limited effector specificity (Lemaitre and Hoffmann, 2007) it is as yet unclear to what extent such antimicrobial activity could also confer highly specific protection. The combination of AMPs, though, could potentially mediate higher specificity than single AMPs (Schulenburg et al., 2007; Johnston and Rolff, 2013; Rahnamaeian et al., 2015).

Some evidence points to phagocytosis playing an important role in the specificity of immune priming (Pham et al., 2007; Roth et al., 2009; Wu et al., 2015 Wu et al., 2015). Pham et al. (2007) reported that specific immune priming in *D. melanogaster* was mediated by phagocytosis. There, priming with heat-killed *Staphylococcus pneumoniae* protected the fly against a subsequent infection with a sub-lethal dose, but not against an infection with another bacterium or fungus (Pham et al., 2007). When the authors blocked phagocytosis by injecting beads, the specific immune response was also lost (Pham et al., 2007). Further evidence that phagocytosis might be a mediator of specific priming comes from the woodlouse *Porcellio scaber* which, when primed with homologous bacteria, had a higher phagocytic activity against the same bacterium two weeks later (Roth and Kurtz, 2009). As in Pham et al. (2007), a specific reaction was only observed against certain bacterial species (i.e., a subset of the pathogens used).

A hotly debated candidate for a specific immune receptor in arthropods is the *Down syndrome cell adhesion molecule* (*Dscam*) (for review see Armitage et al., 2014a). For this gene, intra-individual mRNA diversity is achieved by mutually exclusive alternative splicing, creating thousands of different isoforms (Schmucker et al., 2000; Graveley, 2005). *Dscam* was initially described as a vital receptor of neurons in *D. melanogaster* (Schmucker et al., 2000), giving a single neuron the possibility to discriminate between self and non-self axons. Later on, Watson et al. (2005) reported *Dscam* expression in haemocytes and fat body, two main immune tissues in insects. The authors showed that a reduction in *Dscam* expression in haemocytes leads to a reduction in phagocytosis and that different *Dscam* isoforms bind to a different degree to *E. coli,* indicating that secreted *Dscam* might play a role as a specific opsonin. Moreover, Dong et al. (2006) showed that infection of *Anopheles gambiae* mosquitoes with different parasites induced the expression of different splice form repertoires (Dong et al., 2006). Dong et al. (2012) were later on able to identify possible mechanisms for parasite-induced expression shifts in *Dscam* isoforms by showing that knockdown of downstream factors of the *Imd* and *Toll* pathways has a direct impact on the alternative splicing in *A. gambiae*. Hence, such isoform diversity of *Dscam* makes it a candidate receptor, opsonin or effector, potentially recognising diverse parasite antigens and reacting with a high degree of specificity (Watson et al., 2005; Dong et al., 2006, 2012; Kurtz and Armitage, 2006; Boehm, 2007; Watthanasurorot et al., 2011; Ng et al., 2014). However, using long-read RNA sequencing, Armitage et al. (2014b) could not find any changes in the isoform repertoire after exposure of *D. melanogaster* or S2 cells to *E. coli*. To summarise, while *Dscam* certainly plays a role in arthropod immunity, its specific function for priming and specificity still needs to be clarified.

There is yet another way by which a primed immune response can be elicited in insects. When ookinetes of the *Plasmodium* parasite invade the gut of its intermediate host *Anopheles*, they disrupt the gut barriers, allowing in turn commensal bacteria to migrate into the body cavity of the mosquito (Rodrigues et al., 2010). Rodrigues et al. (2010) were able to show that the “infection” of the body cavity with commensal bacteria from the gut of *A. gambiae* after *Plasmodium* infection triggers a life-long increase in granulocyte (a distinct haemocyte population) cell numbers by an increased differentiation rate of pro-haemocytes, mediated by a lipoxin/lipocalin complex serving as a haemocyte differentiation factor (Ramirez et al., 2015). This led to immune priming and protected the mosquito against a second infection with *Plasmodium* ookinetes, whereas the removal of the gut microbiota prior to priming abolished protection against subsequent challenges (Rodrigues et al., 2010). It is noteworthy that another study with a different *Anopheles* species demonstrated that immune priming can also be achieved irrespective of the presence or absence of the gut microbiota (Contreras-Garduño et al., 2015).

3.3. Molecular processes involved in immune priming in T. castaneum

Septic immune priming in *T. castaneum* seems to be associated to some degree with the rather unspecific reaction to wounding. A general state of priming can be achieved via up-regulation of phenoloxidase (PO) (see Fig. 2). In *T. castaneum*, wounding of an adult beetle leads to an increase of PO activity in the haemolymph (Peuß

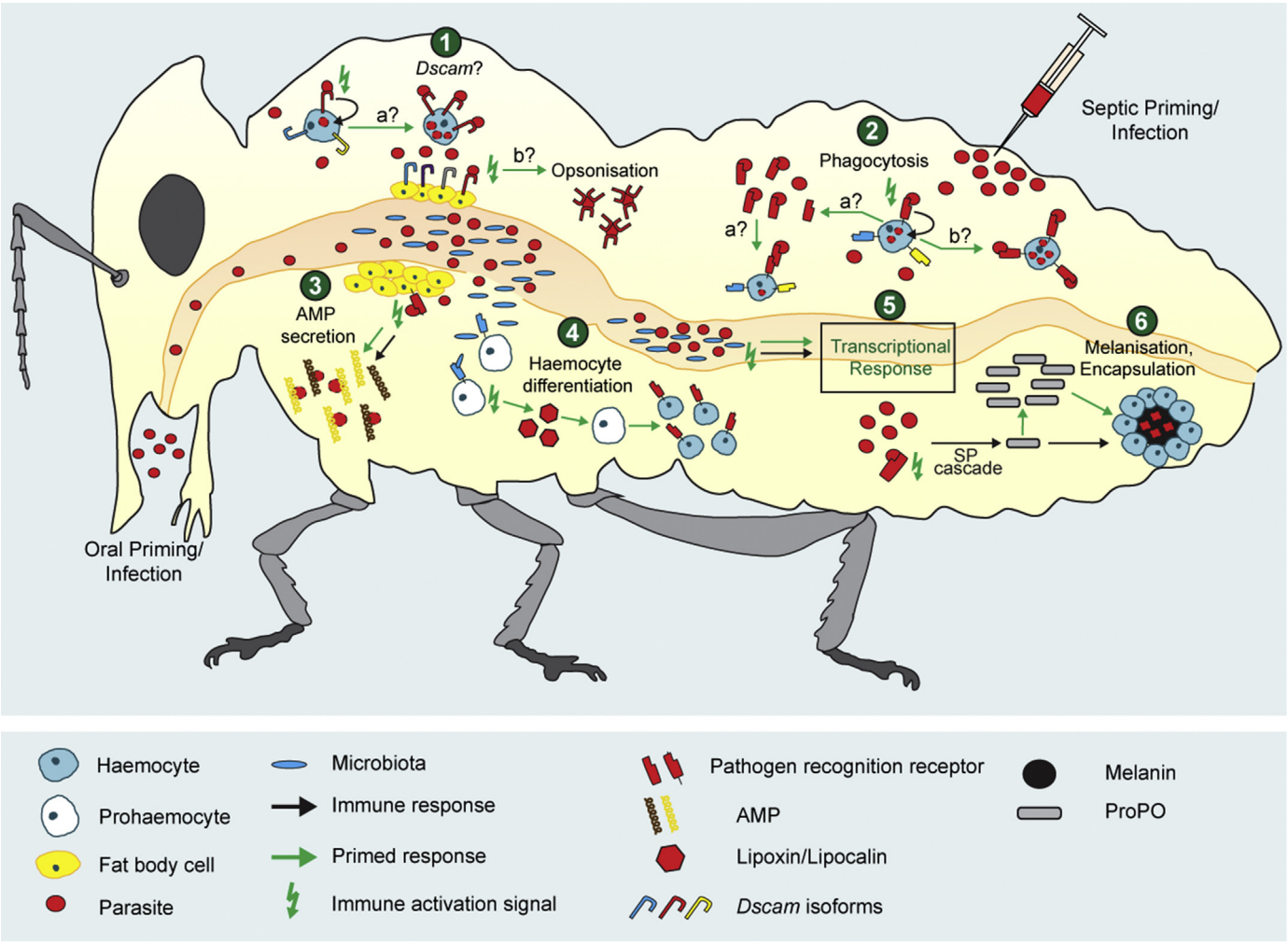

Fig. 2. Potential mechanisms of septic and oral immune priming in arthropods. The figure combines knowledge from different arthropods to show some of the mechanisms by which priming could mediate increased resistance upon secondary challenge. (1) Hypothetical function of *Dscam* in the arthropod immune system. (a) *Dscam* could act as a mediator of phagocytosis (Watson et al., 2005), where upon first exposure to a parasite, isoform expression changes towards a parasite-specific isoform (Dong et al., 2006). (b) *Dscam* could also act as an opsonin, as first suggested by Watson et al. (2005). ? indicates the lack of studies specifically testing these functions of *Dscam* in immune priming (e.g. priming/challenge experiments). (2) Immune specificity mediated by phagocytosis upon priming through septic infection (Pham et al., 2007). (a) Specific PRRs could be secreted by haemocytes, which recognise the parasite upon secondary exposure and mediate phagocytosis; or (b) immune specificity could also potentially be mediated by a specific PRR, which is induced after priming in phagocytosing haemocytes. (3) Antimicrobial peptides (AMPs) could mediate immune priming (Moret and Siva-Jothy, 2003; Schulenburg et al., 2007; Haine et al., 2008; Johnston and Rolff, 2013; Rahnamaeian et al., 2015). Fat body cells release AMPs after recognition of a parasite through PRRs. (4) Induced priming response after oral infection based upon haemocyte proliferation dependent on the gut microbiota (Rodrigues et al., 2010). Invasion of gut microbiota into the hemocoel induces expression of a haemocyte proliferation factor (lipoxin/lipocalin, see Ramirez et al., 2015), which promotes haemocyte proliferation and differentiation. (5) A recent study using oral priming/challenge experiments in *T. castaneum* and *B. thuringiensis* bacteria showed dependance of priming induction on resident microbiota (Futo et al., 2015), with potential candidate genes mediating such immune priming revealed by a transcriptome study (Greenwood et al., unpublished data). (6) Immune priming could be based on activation and overexpression of proPO after recognition of a parasite by PRR and serin protease (SP) cascades, mediating a general state of priming resulting in encapsulation and melanisation of parasites (see Roth et al., 2010; Eggert et al., 2014; Peuß et al., 2015). Insect drawing based on an illustration by Piotr Jaworski (Creative Commons license).

et al., 2015). Moreover, it was shown that PO activity is increased in offspring after paternal priming of *T. castaneum* (Roth et al., 2010; Eggert et al., 2014). However, the different bacteria used to prime the parental generation did not lead to markedly different degrees of PO response in the offspring (Roth et al., 2010). Hence, PO regulation can be associated with a rather unspecific primed response in *T. castaneum*. Here it is noteworthy that not only a vertical transfer of the primed state can be achieved, but also a horizontal transfer, i.e. within a group of beetles (Peuß et al., 2015).

Next to this unspecific component of priming, an additional, more specific immune reaction seems to be involved as well, as survival after challenge depended on the bacterium used for priming within the same individual (Roth et al., 2009) or in mothers (Roth et al., 2010). Maternal priming could be achieved by the direct transmission of bacteria or bacterial components from primed mothers to the eggs, as recently shown in *T. castaneum* (Knorr et al., 2015), which might even enable specificity. Paternal priming seems less specific, but a study by Eggert et al. (2014) could tease apart paternal priming effects. One component, similarly affecting offspring and step-offspring (e.g. mediated by seminal fluid) led to increased PO activity and expression of the pattern recognition receptor PGRP in the offspring. Another component was only transmitted to the offspring and led to differences in challenge survival, with an as of yet unknown molecular underpinning.

Despite these results, the molecular details of septic priming and, in particular, its specificity still remain rather unclear in *T. castaneum*. Importantly, recent studies suggest that the probably "hottest" candidate for specificity in priming, *Dscam*, might not have an essential function for resistance against bacteria in *T. castaneum* (Peuß et al., unpublished data).

Regarding the molecular basis of oral priming in *T. castaneum*, a recent RNAseq experiment provided more detailed insight (Greenwood et al., unpublished data). The study showed that the transcriptomic responses towards oral priming with *B. thuringiensis* are qualitatively different from the immune response that is elicited upon challenge. Whereas challenge without priming induced a broader repertoire of defences by activating both *Imd* and *Toll* cascade pathways, prior priming seems to have directed the immune response towards the expression of different effectors. For example, upon priming, a large number of genes were expressed that were otherwise not induced upon challenge, many of which are involved in gut defence. Interestingly, a substantial number of genes even showed a reversed pattern of expression for challenge and priming; about 18% of genes significantly upregulated upon priming were in fact downregulated upon challenge alone. As in the first scenario described above (Fig. 1A), some immune mechanisms were already active before the secondary exposure; however, in this case they were also to a large extent qualitatively different.

These results are relevant with regard to two main hypothetical scenarios of immune induction, which both assume that the reaction mounted upon secondary exposure does not differ qualitatively from the first one, i.e. that the challenge elicits a stronger or faster reaction than the priming itself, but similar in kind (Fig. 1A and B). We would therefore add a third possible scenario, where the reaction mounted upon priming qualitatively differs from the kind of reaction that follows simple challenge (Fig. 1C). It would be interesting to see how general such a scenario is in the currently studied host–parasite systems. We speculate that it could largely depend on the specific host–parasite combinations and their potential (co)evolutionary history, as the observed switch in the expression patterns, given the likely costs such priming involves (Milutinović et al., 2014), should only be advantageous if a secondary exposure indeed occurs, for example when a population faces an epidemic. The *B. thuringiensis* strain used in this oral priming study was shown to be able to transmit to other *T. castaneum* larvae via cannibalism (Milutinović et al., 2015), in which case such an anticipatory response could be adaptive.

Finally, a recent study in the *T. castaneum* – *B. thuringiensis* system showed that gut microbiota plays a crucial role in oral immune priming (Futo et al., 2015). Larvae depleted of their gut microbiota did not show oral immune priming. It needs to be further investigated how general such a dependence of priming on microbiota is (Rodrigues et al., 2010; Contreras-Garduño et al., 2014), and which molecular processes cause the microbiota dependency of oral priming in *T. castaneum*.

4. Evolution of priming ability – an outlook

A phenotypically plastic, i.e. primed immune response can be expected to evolve when parasite pressure is not constant but varies over time (Shudo and Iwasa, 2001; Schmid-Hempel, 2011). Since immunity is costly, an induced expression should be favoured over a constitutive expression of immune traits. However, the machinery for being phenotypically plastic might in itself be costly, such that it depends on the probability and dynamics of parasite re-exposure whether or not the ability to raise primed responses is maintained or not. Since parasite exposure is highly variable depending on the environment, we can thus expect that there might be genetic variation for the ability to raise primed immune responses, and in consequence the ability to react with phenotypic plasticity (i.e., priming) might in itself be an evolvable trait (Pigliucci, 2005). There are also costs and benefits of being specific; a too narrow (i.e., highly specific) immune response might fail against genetically diverse or rapidly changing parasites, while a too broad (i.e., cross-reactive) response might be less efficient against a particular parasite type (Frank, 2002). Moreover, the more antigenic space is surveyed by a single immune receptor, the more likely this will lead to the detection of self-antigens and autoimmunity as a consequence (Fairlie-Clarke et al., 2009). Many host organisms circumvent this trade-off by genetically and/or somatically increasing the number of available immune receptors, although the extent of this receptor diversification is ultimately limited by its evolutionary and physiological costs (Du Pasquier, 2006). Therefore, we may find that priming ability and its specificity are limited by the evolutionary history of a host organism and optimised for the defence against natural parasites.

To reconstruct the evolutionary history and macro-evolutionary pattern of immune priming, it is of mayor importance to explore the link between immune priming and its genetic variability and heritability. While such studies have been performed in a more general eco-immunological context (Kurtz and Sauer, 1999; Lazzaro, 2004; Feis et al., 2016) and the role of environmental variance for priming ability has been explored as well (Tate and Graham, 2015), more comprehensive studies addressing the fundamental aspect of its genetic variability remain to be done. Experimental evolution approaches might provide possible alternatives to study the adaptive value and micro-evolutionary changes of immune priming ability. Experimental evolution as a means to study host–parasite interactions and more generally rapid adaptations under controlled laboratory conditions have increasingly been used in recent years. In these studies, *T. castaneum* has often been used, e.g. to address the role of genetic architecture, sexual reproduction and resistance and virulence evolution (Wegner et al., 2009; Kerstes et al., 2013; Rafaluk et al., 2015). We are currently using *T. castaneum* in evolution experiments that address the evolution of priming ability. Specifically, we want to know if we can select for an enhanced (or lowered) priming response, connect this phenotypic change to respective differences in genotypes and thereby also identify candidate genes underlying specific immune priming.

It will be interesting to see how such evolution is connected to transgenerational immune priming (TGIP) (Roth et al., 2010; Eggert et al., 2014; Beemelmanns and Roth, 2016) which might be mediated by epigenetic processes and thus enable short-term rapid adaptations of the epigenome (Vilcinskas, 2016). Even more intriguing, *T. castaneum* might be able to release cryptic genetic variation upon recognition of wounding of conspecifics via down-regulation of heat shock protein 90 (Hsp 90) (Peuß et al., 2015). Given that the respective population is not too inbred, this could potentially modulate the evolvability of *T. castaneum* and accelerate the response to selection.

Importantly, *T. castaneum* allows for in-depth functional follow-up analysis of candidate genes for the evolved traits, e.g. using systemic RNAi. With the "iBeetle" project, a comprehensive RNA interference library is available for studying genes identified in evolved populations (Dönitz et al., 2014). Most recently, the powerful Crispr/Cas technology has successfully been used in *T. castaneum* (Gilles et al., 2015) and is offered as a service to the *Tribolium* community ("TriGenES"). While the RNAi database offers phenotypic analysis for a number of genes, exploring gene function is often based on homology approaches, using gene information from other insect model organism. However, such homology-based approaches, especially when considering non-annotated transcripts, are sometimes misleading since the function of a gene might differ and is often context-related. Considering this aspect, it is indeed possible that priming mechanisms are highly taxon-specific (as illustrated in Fig. 2). Nevertheless, functional and comparative genetics using RNAseq datasets remain a state-of-the-art approach (Greenwood et al., 2016, Pinaud et al., 2016) to understand the underlying principles of how immune priming evolves in invertebrates.

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