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MINIREVIEW

The immune role of the arthropod exoskeleton

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Abstract

The exoskeleton or cuticle of arthropods is an important feature that contributes to their great success in colonising numerous habitats on earth. It has numerous functions among which to provide protection against parasites. Whereas often regarded as a simple physical barrier to the outside world, the immune protection of the cuticle is slightly more complex than that. Here, we provide an overview of the cuticle defensive traits against parasites and examine their variation as a response to parasitism. It appears that the cuticle is an efficient line of defense, which includes physical, biochemical and physiological defensive components that are potentially subject to genetic and plastic variation in response to parasitism. It also appears that the cuticle defense systems are relatively understudied despite it may determine for large part the success of parasitic attacks.

Key Words: invertebrates; immunity; cuticle defense; parasite; ecological immunology

Introduction

Arthropods include insects, spiders, centipedes, shrimp, and crayfish, and make up the most abundant phylum in the animal kingdom. A key feature that contributed to the phenomenal success of this taxon is their cuticle. The cuticle, corresponds to the relatively thin, but tough and flexible, layer of non-cellular material that cover the whole external body surface, as well as respiratory organs, the anterior and posterior portion of the digestive tract, and reproductive ducts (Wigglesworth, 1957). The cuticle is often viewed as the "skin" of arthropods but it has many other functions. It provides physical support and protection of internal organs against chemical and mechanical damages, and serves as skeleton (exoskeleton) to which muscles are attached for locomotion. The cuticle also functions to limit the entry or loss of water, and form an efficient barrier protecting against invasion by eukaryotic parasites and infection by microorganisms. Given the propensity of many arthropods to live in microbial-rich environments, each wounding event is likely to be accompanied by opportunistic infections (Siva-Jothy *et al.*, 2005). Furthermore, even in the most aseptic habitats, the outer surface of arthropod cuticle probably harbours a diverse and abundant community of parasites (Brey *et al.*, 1986).

Since parasites decrease host fitness, arthropods evolved a suite of mechanisms of defense acting at different levels in the sequences of the parasitic infection to reduce the probability and impact of parasite infection (Schmid-Hempel and Ebert, 2003; Siva-Jothy *et al.*, 2005; Duneau *et al*., 2011). These defense components include behavioural, physical and physiological mechanisms and the cuticle is one of these sequential defense mechanisms. The first line of defense involves behavioural mechanisms to avoid or remove parasites (Cade and Wyatt, 1984; Cade, 1991; Bischoff, 2003; and see Kurtz *et al.*, 2002; Thomas and Blandford, 2003). Once an invader has overcome behavioural defenses and breached the protection of the exoskeleton, he is then exposed to the last line of defense that is the innate immune system or haemocoelic internal defenses. From these three lines of defense, boundary defense provided by the cuticle is probably the less known and the most poorly studied mechanism of resistance against parasites in arthropods. Yet, the cuticle contributes greatly to successful defense against most parasites aiming to colonize the haemocoel. The success of the cuticle in this duty is well supported by the increase vulnerability of moulting arthropods to opportunistic parasites entering into the haemocoel with sometime dramatic survival consequences (Le Moullac *et al.*, 1997; Morado *et al.*, 1999). A number of cuticular traits such as the chemical composition, structural

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architecture and thickness reported to be involved in disease resistance, exhibit a great range of variation among and within species. They may also vary within an individual's lifetime, depending on environmental cues directly or indirectly related to the threat of disease. Therefore, it seems that the cuticle of arthropods is subject to modifications, which enhance its ability to act as physical or chemical barrier to penetration by parasites when the threat of disease is high (Wilson *et al.*, 2001).

Whereas moulting is a critical period of vulnerability to parasites, under certain circumstances, it may serve as a defensive mechanism reducing the negative effects of wounding, epibionts or parasites from the shell (Duneau and Ebert, 2012). In this respect, moulting could be included as part of the cuticle defense system, especially if the moulting event is affected by the presence of parasites in the environment (Moret *et al.*, 2010; but see Duneau and Ebert, 2012).

Here, we provide an overview of the cuticle defensive traits against parasites and examine their variation as a result of parasitism selection pressure. In addition, we further discuss the role of moulting as part of the cuticle defense system based on its interaction with parasite infection success.

Structure and formation of the cuticle of arthropods

A typical arthropod integument is a layered structure mainly composed of three main regions (Fig. 1). The innermost region is the basal membrane (or basal lamina), a thin connective tissue layer attached to the above epidermis and separating the integument from the haemocoel. The epidermis is a more or less continuous monolayer of epidermal cells responsible for the production and secretion of various materials for the above cuticle, which is the extracellular outer layer of the integument that gives most of its rigidity and colour (Nation, 2002).

The cuticle further stratifies into subsequent layers (Fig. 1), typically with a thin waxy epicuticle covering a thicker procuticle consisting of protein, lipid, and chitin cross-linked to a varying degree to provide elasticity and hardness. In crustaceans and myriapods (and in few insects see Leschen and Cutler, 1994), the above cuticular regions are calcified by the addition of a substantial amount of calcium carbonate, leading to great mechanical strength. The epicuticle is the main water-proof barrier. This thin layer is unlikely to provide mechanical protection but may harbour antibacterial and/or cytolytic activity (Harrington *et al.*, 2008). In contrast, the procuticle is the bulk of the cuticle, which provides resistance to mechanical loads and is the next barrier to infection, mainly because of its thickness and architecture. In some part of the body the procuticle differentiates into a hard, outer exocuticle and a soft, inner endocuticle. Differentiation of the exocuticle results from the sclerotisation of proteins that occurs shortly after each moult (see below).

The formation of new cuticle comprises a succession of events that occurs during moulting. The rigidity of the cuticle restricts growth, so arthropods replace it periodically by moulting or shedding the old cuticle after growing a new one (Fig. 2). Moulting cycles occur nearly continuously until the animal reaches full size and are controlled by hormones (Wigglesworth, 1957). The initial phase of moulting, or pre-ecdysis, is induced by an increased level of ecdysone, the arthropod-moulting hormone. Ecdysone stimulates the growth of the epidermis and the release of moulting fluid containing enzymes that digests the endocuticle and thus detaches the old cuticle. This is also the phase when the new deposition of the new cuticle is initiated. When this stage is complete, it is followed by ecdysis during which the remnant of the old cuticle is shed through the uptake of air or water from the environment, causing the exoskeleton to rupture. At this point, the soft new cuticle is wrinkled and the new endocuticle has not yet formed. During postecdysis, the animal continues to pump itself up to expand the new cuticle, then hardens the new exocuticle and eliminates the excess air or water. By the end of this phase the new endocuticle has formed. Hardening of the new exocuticle results from a biochemical process known as sclerotization during which the proteins of the outer procuticle are covalently bound to each other. Sclerotisation is allowed by the presence, beneath the epicuticle, of enzymes including phenoloxidases such as laccase and tyrosinase. These enzymes catalyse the synthesis of quinones that polymerize to form melanin deposits in interaction with the cuticular proteins and chitin, to crosslink and harden them (Andersen *et al.*, 1996; Terwilliger, 1999; Andersen, 2010). In crustaceans and some other arthropods (see above), hardening of the cuticle also results from the process of mineralization, which involves the deposits of calcium salts in all layers of the cuticle except the outer layer of the epicuticle that calcify after ecdysis (Stevenson, 1985).

Physical component of the cuticle defense

Intuitively, the protective function of the cuticle of arthropods relies on boundary defense, which consists of a tough and flexible integument covering the animal surface. This protection even extends to the digestive system, where a protective cuticular membrane called the peritrophic membrane, covers the midgut. (Peters, 1992). Despite this physical barrier, parasites, can invade directly through the exoskeleton. Parasites that penetrate the cuticle are mainly bacteria, fungi and parasitoids. For instance, the entomopathogenic fungi such as *Metarhizium anisopliae* and *Beauvaria bassiana*, or bacteria responsible for the shell disease syndrome (*e.g.*, *Vibrio sp*., which induces characteristic black-spot lesions on the exoskeleton of marine crustaceans) use a combination of physical and enzymatic processes, such as chitinase and protease, to breach the cuticle (Charnley and St. Leger, 1991; Vogan *et al.*, 2001; Freimoser *et al.*, 2003, 2005; Cho *et al.*, 2006). Resistance to these microbial infections resides mainly in cuticular thickness, the degree of cross-linking within the cuticular laminae

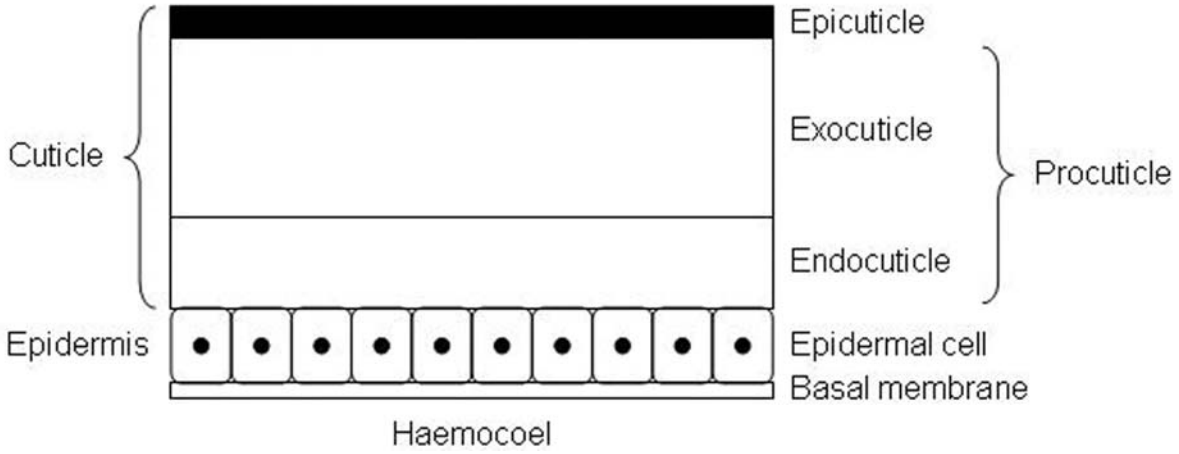

Fig. 1 A schematic figure of the arthropod integument, showing the different structural layers of cuticle.

and the degree of sclerotisation in the cuticle. In this respect, melanin deposits in the exocuticle and the epicuticle are likely to have an important role to increase the immune protection of the cuticle. As a polymer, melanin may strengthen the cuticle and so improves its ability to act as a physical barrier to the penetration of parasites (St. Leger *et al.*, 1988; Hajek and St. Leger, 1994). Furthermore, melanin is toxic to microorganisms and has potent antimicrobial activity (e.g. Söderhäll and Ajaxon, 1982; Montefiori and Zhou, 1991; Ourth and Renis, 1993; Sidibe *et al.*, 1996; Ishikawa *et al.*, 2000), perhaps by binding a range of proteins (Doering *et al.*, 1999) and inhibiting lytic enzymes produced by microorganisms, including proteases and chitinases (Kuo and Alexander, 1967; Bull, 1970).

Melanisation of the cuticle largely occurs shortly after moult. Thus moult represents a critical period during which melanisation of the cuticle might be plastically altered from one intermoult period to the other, depending on the perceived risk of infection by the animal. Examples of such plastic changes in the degree of melanisation of the cuticle are illustrated by the literature on density dependent polyphenism of the cuticular colour in insects (Wilson, 2005). Parasite transmission is assumed to be positively density-dependent (Anderson and May, 1979), and the increased density of conspecific may represent a reliable cue for increased threat of diseases (Wilson and Reeson, 1998; McCallum *et al.*, 2001). In response to this cue, individual insects exhibit a stronger melanisation of the cuticle during the next moult, which is associated with increased parasite resistance and immune function (Kunimi and Yamada, 1990; Reeson *et al*., 1998; Barnes and Siva-Jothy, 2000; Wilson *et al*., 2001; Cotter *et al*., 2004a, b). For instance, melanic caterpillars of two lepidopteran species, *Spodoptera exempta* and *Spodoptera littoralis*, were respectively found more resistant to the ectoparasitoid wasp, *Euplectrus laphygmae*, and the entomopathogenic fungus, *B. bassiana*, than non-melanic caterpillars (Wilson *et al*., 2001). Similarly, the mealworm beetle, *Tenebrio molitor*, shows density dependent polyphenism of adult cuticular colour. In this insect, the degree of cuticular melanization is a strong indicator of resistance to the entomopathogenic fungus, *M. anisopliae*, with darker beetles being more resistant than lighter ones (Barnes and Siva-Jothy, 2000). Thus, cuticular melanisation could be considered as an immune parameter in its own right (Wilson *et al.*, 2001).

Biochemical component of the cuticle defense

If cuticular melanisation occurs mainly during the process of moulting, it could also be induced in response to a mechanical scratch or by microbial invasion. This defense response results from the presence of phenoloxidase zymogens, which are produced by haemocytes and transported to the cuticle across the epidermis (Ashida and Brey, 1995). This process is often used for wound healing and results in the formation of dark melanised plugs around the damage zone of the cuticle (Plaistow *et al.*, 2003; Fig. 3). Yet, whether phenoloxidase enzymes are present in the cuticle for the purpose of improving the physical structure of the integument or to afford defense against infection is unclear (Siva-Jothy *et al.*, 2005). Nevertheless, the activity of these enzymes is directed towards parasites in the cuticle since fungal germ tubes are melanised as they pass through the cuticle before they entered the haemocoel (Golkar *et al.*, 1993). Inducible biochemical defense also involves the production of antibacterial peptides by epidermal cells, such as cecropins, which are transported in the vicinity of the microbial challenge to abraded cuticle (Brey *et al.*, 1993). Hence, in addition to its obvious physical characteristics, the cuticle of arthropods also provides an active biochemical barrier.

Moulting as a component of cuticle defense

Growth and development of the arthropods involve a series of moults during which the old cuticle is digested while a new cuticle is formed and the remnant is discarded (Fig. 2; see above). In addition to allow growth of the animal, moulting may

Fig. 2 *Gammarus pulex* (Crustacea: Amphipoda) shortly after ecdysis on the right side with its old cuticle on the left side. Photo by F Vogelweith.

serve as a defensive mechanism reducing the negative effects of wounding or parasites from the shell. In nature, wounds can be extremely prevalent and form the main points of entry for parasites (Plaistow *et al.*, 2003). Although wounds are rapidly healed by melanotic plugs (Fig. 3), they may be less protective than a new cuticle. Wounds favour the development of shell disease in crustaceans, resulting from the spread of chynolytic micro-organisms in the cuticle (Vogan *et al.*, 2008). Recent data show that moulting soon after parasite exposure helps to remove parasites attached to the cuticle and reduces the likelihood of successful infection. For instance, in the cotton aphid, *Aphis gossypii*, exposed to the entomopathogen fungus, *Lecanicillium attenuatum*, moulting removes conidia attached on the cuticle before their germ tubes penetrated the insect haemolymph (Kim and Robert, 2012). Similarly, in the crustacean *Daphnia magna*, moulting helps to remove up to 30 % of the spores of the castrating bacterium, *Pasteuria ramosa*, attached on the oesophageal cuticle and reduces greatly the infection success of the parasite when it occurs within 12 h after exposure (Duneau and Ebert, 2012). These results show that moulting is an effective mechanism of resistance against parasites attached on the cuticle. However, they also stress the point that moulting, in order to be really beneficial, has to occur rapidly after the parasite is attached to the cuticle (before the parasite penetrates the host). It seems then that the benefit of moulting could be improved if it was combined with the ability for the host to perform precocious moulting in response to wounding or parasite attachment. Unfortunately, available data on this matter are scarce and not conclusive. The only study that examined specifically whether hosts exposed to parasite could shorten time to ecdysis is the one by Duneau and Ebert (2012) who found no significant difference in moult interval between experimentally exposed and unexposed *D. magna* to spores of *P. ramosa*. However, there are indirect evidences that suggest different outcomes in other systems. Indeed, Laufer *et al.* (2005) found that levels of the moulting hormone (ecdysone, which initiate the process of moulting) are much higher in shell diseased lobsters than in unaffected ones. Unfortunately, moult cycling was not measured in this study. Maybe another indirect evidence is the one provided by the study of Roth and Kurtz (2008) on the red flour beetle, *Tribolium castaneum*. The authors found that larvae wounded with a needle, either sterile or mucked with heat-killed bacteria, reduce larval development time compare to unmanipulated larvae. Rapid growth allows moulting events to occur more frequently. In addition, in species such as *T. castenum*, the number of larval moults could be highly variable ranging from 8 to 20. One could hypothesise that in a parasite rich environment, larvae should perform more moults than in a parasite poor environment. However, such a response to parasitism should be costly because it

should lead to the development of smaller adults that usually exhibit a low investment to reproduction (Thornhill and Alcock, 1983). This hypothesis remains to be tested. Whether parasite may speed up or not its occurrence, moulting, by preventing infection, might select parasite for higher parasite penetration speed (Duneau and Ebert, 2012) or to develop means that prevent or slowdown moulting (Kamimura *et al.*, 2012). If this is true, there is therefore room for an arm-race between moulting defence and parasite infectivity.

On the other hand, when shedding the old cuticle, the animal exposes a new, soft and untanned cuticle to the outside world. This may represent a critical period of vulnerability to parasites entering the host through the cuticle until the properties of the integument are fully re-established (Le Moullac *et al*., 1997; Morado *et al*., 1999). However, recent findings show that antimicrobial peptides can be prophylactically produced during this period of vulnerability (An *et al*., 2012). The presence of parasites in the environment is temporally and spatially variable, resulting in periods or sites of variable risk of infection. Therefore, arthropods may gain a survival advantage by adjusting moult timing to correspond to the lowest risks of infection. Consistent with this hypothesis, the crustacean, *Gammarus pulex*, was found to exhibit temporal adjustments of its moult cycling in response to elevated risks of infection by postponing ecdysis by several days when the individuals are exposed to 'micro-organism-enriched' water (Moret *et al*., 2010).

Concluding remarks

This minireview intended to provide the reader with an overview of the defensive traits of the cuticle of arthropods against parasites in order to highlight its important role in the immune protection of these animals. The defense line that confers the cuticle of arthropods is probably less complex but also fairly understudied compare to haemocoelic defense systems (see Lemaître and Hoffmann, 2007 for a review). Consequently, its involvement in resistance to parasitic infections is probably often underestimated. Yet, the outcome of parasitic attacks depends to a large extent on cuticular defenses. Furthermore, beyond the acknowledgement of its great protective efficacy, the immune role of the exoskeleton of arthropods cannot be simply regarded as an inert and invariable physical barrier to the outside world. Indeed, defense components of the exoskeleton also comprise inducible biochemical processes and ecdysis in addition of the physical structure of the cuticle. All these traits are potentially subject to variation depending on level of the parasitic selective pressure. Furthermore, variation could also occur plastically within an individual lifetime in response to environmental cues presaging changes in the threat of disease. From a functional as well as ecological point of view, investigation about the causes and consequences of variation of cuticular defenses should provide significant insight into the evolution of immune defence in a larger picture.

Fig. 3 Larvae of the European grape berry moth, *Eupoecilia ambiguella* (Lepidoptera: Tortricidae) exhibiting melanic plugs on sites of wounding of the cuticle. Photo by F Vogelweith.

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