PHILOSOPHICAL
TRANSACTIONS B

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Review

Cite this article: Lindstedt C, Murphy L, Mappes J. 2019 Antipredator strategies of pupae: how to avoid predation in an immobile life stage? *Phil. Trans. R. Soc. B* **374**: 20190069. http://dx.doi.org/10.1098/rstb.2019.0069

Accepted: 21 May 2019

One contribution of 13 to a theme issue 'The evolution of complete metamorphosis'.

Subject Areas:
behaviour, ecology, evolution

Keywords:
pupal defence, protective coloration, chemical defence, physical defence, predator–prey interactions

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Antipredator strategies of pupae: how to avoid predation in an immobile life stage?

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Antipredator strategies of the pupal stage in insects have received little attention in comparison to larval or adult stages. This is despite the fact that predation risk can be high during the pupal stage, making it a critical stage for subsequent fitness. The immobile pupae are not, however, defenceless; a wide range of antipredator strategies have evolved against invertebrate and vertebrate predators. The most common strategy seems to be 'avoiding encounters with predators' by actively hiding in vegetation and soil or via cryptic coloration and masquerade. Pupae have also evolved behavioural and secondary defences such as defensive toxins, physical defences or deimatic movements and sounds. Interestingly, warning coloration used to advertise unprofitability has evolved very rarely, even though the pupal stage often contains defensive toxins in chemically defended species. In some species, pupae gain protection from conspecifics or mimic chemical and auditory signals and thereby manipulate other species to protect them. Our literature survey highlights the importance of studying selection pressures across an individual's life stages to predict how ontogenetic variation in selective environments shapes individual fitness and population dynamics in insects. Finally, we also suggest interesting avenues for future research to pursue.

This article is part of the theme issue 'The evolution of complete metamorphosis'.

1. Introduction

'I spent my time investigating insects. At the beginning, I started with silk worms in my home town of Frankfurt. I realized that other caterpillars produced beautiful butterflies or moths, and that silkworms did the same. This led me to collect all the caterpillars I could find in order to see how they changed'. This is quoted from the foreword by Maria Sibylla Merian in her '*Metamorphosis insectorum Surinamensium*' published in 1705 [1, p. 3]. The idea of the complete metamorphosis was developed by Aristotle 2000 years earlier [2], but Merian was the first entomologist who described insect metamorphosis in detail, including pupation, which makes her one of the most significant contributors to the field of entomology. It is intriguing that more than 300 years after Merian's fundamental observations about insect metamorphosis and the discovery of pupae, this is the life stage, along with the egg stage [3], that we still know almost nothing about compared to the adult and larval stages in insects.

Merian's butterflies, along with the other holometabolous insects, have morphologically distinct immature life stages, larva and pupa, which they need to live through to reach the final reproductive adult stage. Holometabolous insects are thought to have evolved from hemimetabolous insects that have only two life stages: the nymph and the adult [4]. Ancestral forms of immobile 'compact' pupae were most likely mobile nymph-resembling pupae similar to pupa of, for example, the snakeflies [5]. Vulnerability to natural enemies in each of these life stages has resulted in the evolution of an extensive diversity of adaptations protecting individuals against different types of predators and parasitoids [6,7]. These adaptations can vary dramatically in each life stage depending on the individual's lifestyle (e.g. sessile versus mobile life stages), reproductive stage (larval stage versus adult stage) and ontogenetic shifts in resource use [8,9].

Traditionally, the evolution of antipredator defence mechanisms is considered on a scale of one life stage focusing on, for example, larval instars or adult stages [6]. Research has traditionally focused much less on the antipredatory strategies of egg or pupal stages. However, even though the selective environment can change dramatically between each life stage, an individual's fitness (e.g. reproductive success and ability to survive until and beyond a given reproductive stage) is the sum of conditions experienced during the previous life stages [10–12]. To predict how different conditions shape individual fitness or population dynamics of insects, it is therefore not enough to know the factors that contribute to the survival in the larval stage or what affects reproductive success as an adult. We also need to understand what happens between these two stages: what kind of antipredator defences individuals have during the pupal stage, how it is affected by the conditions experienced during earlier life stages or how behaviour during the larval stage affects pupal predation risk. This kind of information helps us to understand the evolution of defensive traits in general, but may also have applied importance in predicting factors that shape population dynamics in potential pest species or in species at risk of extinction. At present, we have surprisingly little data tackling these issues and behavioural and evolutionary research on the topic is scarce.

Here, we first review the literature on predation on the pupal stage to find what attacks insect pupae, how high predation risk is for them, and what different types of antipredator defence mechanisms have evolved for insect pupae as a result of selection by predation. We acknowledge that parasitism is also an important source of mortality in the pupal stage, and it is likely that many defensive mechanisms against predators (e.g. defensive toxins, camouflage, protection gained from other species) can have dual function against both predators and parasitoids [7,13,14]. Therefore, we will take into account parasitism where relevant. However, the main focus of our review will be on antipredator defence strategies. Finally, we discuss the gaps in our knowledge and outline some promising directions for future research to pursue.

2. Predation risk is often high during the pupal stage

Predation risk during the pupal stage has received the most attention in species that have some economical value such as many forest pest insects. Owing to their outbreak dynamics, pupae of forest pest insects can form an abundant food source during the high-density population peaks for small mammals such as voles, mice and shrews, along with invertebrate predators such as ground beetles, ants and earwigs [15–17]. For example, in gypsy moths (*Lymantria dispar*), small mammal predators such as the white-footed mouse in North America, invertebrate predators such as ground beetles *Carabidae*, and ants have been suggested to be among the most important predators during the pupal stage [15]. Similarly, in ground-dwelling pupae of winter moths (*Operophtera brumata*) and autumnal moths (*Epirrita autumnata*), moles, mice, voles and shrews together with invertebrate predators (larvae of Carabidae, Elateridae and Saphylinidae beetles that prey upon pupae in the soil) have been suggested to be important sources of mortality in Europe [17,18]. Birds have also been reported to feed on pupae, but depending on the insect species, their importance varies from moderate [15] to high [16,19].

Generalist predators of insect cocoons can have a major impact on many insect populations, for example, in stabilizing population cycles [16,18,20]. Based on the mortality rates reported in the literature, the magnitude of predation risk at the pupal stage can be surprisingly high: estimates from studies with gypsy moths report predation rates as high as 90–100% [15]. Studies on winter moths and autumnal moths report pupal predation rates ranging from 20 to 72% [17,18]. In *Neodiprion sertifer* pine sawflies, small mammals caused 70% mortality on the ground and bird predation caused 70–85% mortality [16]. In another forest pest species, the pine processionary caterpillars (*Thaumetopea pityocampa*), predation by Hoopoes (*Upupa epops*) can result in up to 68.3–74.1% mortality in the pupal stage [19]. Much less information from the pupal predation risk exists for the species that do not have cyclic population dynamics and economic value. In two moth species from the family Limacodidae (slug caterpillars), cocoon predation resulted in intermediate mortality ranging from 22 to 29% depending on the moth species [21].

Based on these estimates, predation risk can vary considerably from moderate to very high, making survival through the pupal stage likely to be a critical step for the majority of the insect species. From an evolutionary point of view, the strength of selection on mechanisms enhancing survival at this stage should therefore be extremely strong. It is, however, important to note that these mortality risk estimates are species-specific, perhaps over-estimating the predation risk to some extent. For example, how 'naturally' pupae are located on experimental plots varies among studies and thus, predation risk may sometimes be over-estimated.

The second common feature arising from the literature is that predators that prey on insect pupae are very diverse, including both visual and non-visual predators with, perhaps, the emphasis on latter [15,17,18]. This is different from the larval stage where visual predators such as insectivorous birds are often considered to be the most important predator group [22]. Physical and chemical antipredator strategies play an important role in defence against non-visual predators and could therefore be assumed to be selected for in the pupal stage [6].

3. Avoiding detection by visual predators

The most common strategy to avoid attack is via cryptic coloration that makes prey difficult for a predator to detect (e.g. camouflage). This is also true for insect pupae [8,23]. Cryptic coloration of the pupa is adaptive as it increases survival against visually hunting predators when the colour matches with the visual background [24–26] (figure 1). In some taxonomic groups, variation in the visual background has favoured environmentally plastic variation in pupal coloration such as colour polyphenism. Polyphenism in cryptic pupal coloration has evolved independently at least in Papilionidae, Pierinae, Satyrinae and Nymphalinae [24,25,27,28]. In many of these cases, there are two types of pupae where one form is green–yellow and other brown–black.

Pupal colour variation has been shown to be mainly an environmentally plastic trait, even though individual sensitivity to environmental cues triggering the change in pupal

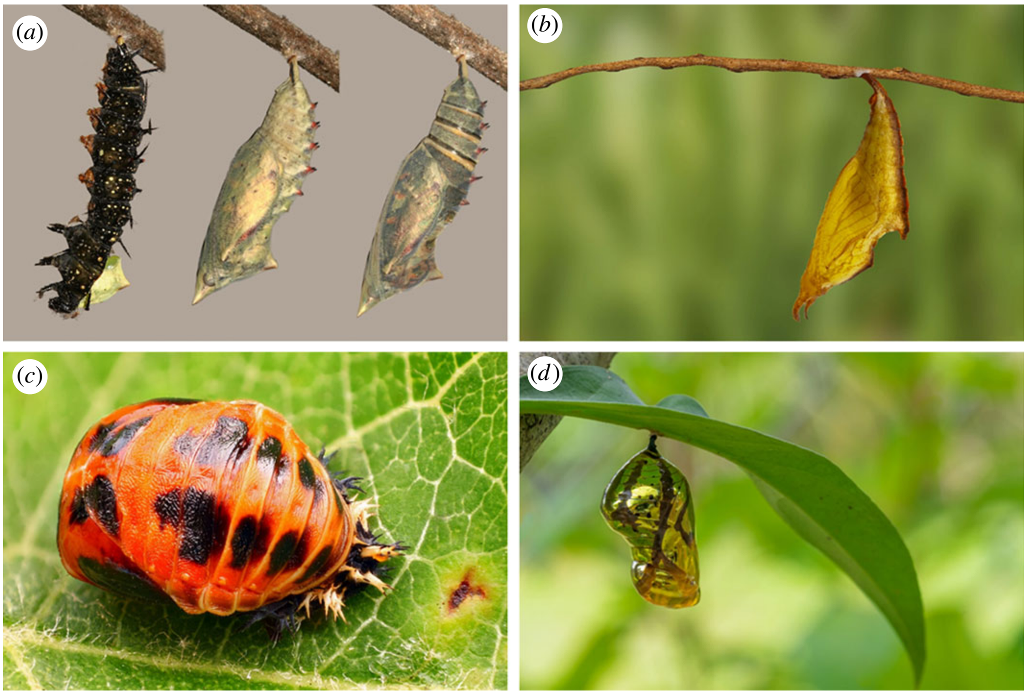

Figure 1. Insects have evolved an extensive diversity of protective coloration strategies in the pupal stage, although their function and mechanisms have largely remained unstudied. (*a*) Pupae of the Peacock butterfly (*Aglais io*) are well camouflaged. (*b*) Pupae can also masquerade themselves to resemble something uninteresting to potential predators such as leaf-mimicking chrysalises of the Common Maplet butterfly (*Chersoneria risa*). (*c*) In some species, such as Harlequin ladybird (*Harmonia axyridis*), pupae may advertise their defensive toxins with conspicuous warning colours. (*d*) Pupae can also have shiny coloration such as chrysalides of the Common Crow butterflies (*Euploea core*), which could both function as a warning signal or conceal the pupa from the predator's eye. (Photos: *a*–*d* Adobe Stock.)

colour can also vary genetically [25,28]. For example, tactile signals such as roughness of the background can impact on whether the pupae develop green (smooth surfaces) or brown (rough textures) in *Papilio xuthus* [29]. In addition to substrate texture, several environmental conditions have been shown to affect pupal colour (reviewed in [30]) such as background colour, relative humidity [30], photoperiod length, temperature, wavelength of light and diet quality [26]. Pupal colour polyphenism can also be influenced by the developmental pathway of the individual. In bivoltine or multivoltine species whose pupal stage length varies considerably depending whether the individual overwinters in a pupal diapause (pupal stage takes several months) or develops directly (pupal stage is only a week or two long), the hormonal state of the pupae has a strong effect on its coloration. For example, directly developing summer pupae have a higher tendency to become green, whereas the overwintering pupae have a higher tendency to become brown [31]. Often, several of the factors listed above can affect the pupal coloration simultaneously [32,33].

Interestingly, the adaptive function of cryptic pupal coloration can also be linked to life-history traits in adult and larval stages [28,34]. In the speckled wood butterfly *Pararge aegeria*, individuals eclosing from the green coloured pupae are larger as adults and have a larger thorax mass, which is relevant to flight behaviour, in comparison to individuals eclosing from the brown pupae. This suggests that there is a trade-off between protection against predators during the pupal stage and adult life stages, maintaining variation both in pupal and adult traits [28].

Attack risk can also decrease if organisms deceive visual predators by mimicking something uninteresting for the potential predator such as sticks or bird droppings [35]. Masquerade is a relatively common defence mechanism in the larval stage in many insect groups, but has sometimes evolved in the pupal stage as well [36] (figure 1). For example, Neochlamisus leaf beetles' larvae build a 'faecal case' under which they hide during the larval and pupal stage. In addition to faeces, they also add trichomes from their host plant on the surface of the case and closer to pupation they build a trichome-filled chamber under the outer layer of faeces. Survival against invertebrate predators is significantly improved owing to these structures. These faecal cases covered with plant-derived thricomes could help to masquerade the pupae on the plants. Cases with trichomes can also offer physical protection against predators as, for example, solder bugs were not able to penetrate the case with their mouth parts [36]. Faeces and plant trichomes can also contain deterrent compounds and therefore have an additional function as a secondary chemical defence. Bagworm moths (Psychidae) build cases that protect them during the larval and pupal stages [37]. These cases often contain material from their environment (sand, twigs, rocks) that potentially makes them uninteresting objects or cryptic for avian and insect predators.

In addition to morphological and physical traits, insects have different types of behavioural adaptations that decrease their detectability to predators and parasites during the pupal stage. For example, many species burrow into the ground to pupate, which offers a refuge from the predators preying above the ground such as birds [17]. Before individuals enter the pre-pupal stage and start to spin their cocoon, they often disperse from their larval host plant or food source [9,24,38,39]. Staying close to the host plant can increase the risk of being detected by natural enemies that often use the host plant as a cue to locate their potential prey [13,40]. These behavioural adaptations before entering the pupal stage can also vary within species depending on the life-history strategy of an individual. For example, most

Lepidopteran larvae have a wandering phase prior to pupation when larvae move away from their host plant while seeking a suitable substrate for pupation [38]. In the European swallowtail butterfly, *Papilio machaon*, this wandering phase is longer in the overwintering generation, which has a longer pupal stage, in comparison to larvae that are under direct development (non-overwintering larvae), which have a pupal stage of only one to two weeks. Owing to increased mobility and distances moved, the wandering phase is risky for a larva [9,38] and this may suggest that individuals trade-off host-related predation risk by wandering less when the pupal stage is shorter [38].

4. Avoiding detection by non-visual predators

Individuals do not always need to mimic visual components in their environment. Instead, if the most important predators are non-visual, it can be more effective for a prey to use chemical 'camouflage' [41]. Most of the literature on the use of chemical compounds in confounding the detection of prey by predators comes from species that live in a close association with ants [41]. For example, myrmecophilous *Lycaeides argyrognomon* butterflies larvae have organs that they use to produce nectar for attending ants. In return, the ants protect them against different types of natural enemies. Individuals lose this organ during the pupal stage, but instead start to produce cuticular compounds that supress attacks from the ants and make the pupae less prone to ant predation [42]. In this way, *L. argyrognomon* butterflies can pupate in ant nests where they are protected from natural enemies. Since non-visual predators are an import predator group during the pupal stage, these kind of tactics relying on chemical communication for protection against predators could be more common than previously thought.

5. High detectability cost as a constraint for the evolution of warning coloration in the pupal stage?

The opposite strategy for hiding from the predators' senses is aposematism. Aposematic individuals can advertise their unprofitability as prey (e.g. toxicity) to predators with conspicuous signals (i.e. aposematism), which can include colours, sounds or odours. Predators learn to associate the signal with the unprofitability of the prey and avoid attacking individuals sharing a similar appearance in future encounters [6,43]. Intriguingly, the aposematic defence strategy seems to be rare during the pupal stage [8,23] (figure 1). For example, in swallowtail butterflies (family Papilionidae), all species have a cryptically coloured pupal stage even though their larval and adult defence strategies vary from cryptic and masquerade to aposematism [23].

The evolution of aposematic defence strategies may be constrained in the pupal stage owing to some physiological or developmental characteristics. However, developmental constraints at least are unlikely; studies on the pupal coloration of Papilio species have shown that brown or green pupal coloration is based on a blend of brightly coloured pigments (red, yellow, black, blue) [44], which excludes the possibility that lack of bright pigments would constrain the development of colourful pupae. However, background matching in the pupal stage combined with chemical defences can be favoured under conditions where the detectability risk for conspicuously coloured aposematic prey is high. In their mathematical model, Endler & Mappes [45] showed that low conspicuousness can evolve for a chemically defended prey if there is variation in the cognitive and perceptual capacity among predators or in their sensitivity to the secondary defences of prey. This increases the detectability costs for a prey, favouring lower conspicuousness. This could also be true for insect pupae that are vulnerable for long periods of time to a diverse predator community that contains both visual and non-visual predators. In addition, a conspicuous appearance can also invite attacks from specialist parasitoids that have evolved to tolerate, or even benefit, from the defensive toxins of the pupal stage [40].

Cryptic coloration or masquerade can also guarantee highest survival during an immobile life stage [46,47]: aposematism can be favoured if it facilitates the mobility of the organisms among different visual environments and backgrounds [48]. This is because individuals with camouflage are always dependent on their visual background to conceal them from the predator's eyes, but aposematism is expected to function irrespective of the background. This enables aposematic individuals to acquire resources more effectively. For example, in *Acronicta alni*, the larval mobility increases towards the later instars when larvae grow larger and need to move more to feed [49]. This dual benefit in terms of predator avoidance and resource collection can be one factor that explains why many Lepidopteran larvae switch strategy from crypsis or masquerade to aposematism when they grow larger or why immobile pupae rely on cryptic coloration [8,47,50].

6. Chemical defence

Chemical defences are one of the most widespread defensive mechanisms against predators [51]. Among insects, chemical defences can be defensive secretions that are actively released in the presence of a predator, decreasing the likelihood that the predator needs to taste (and potentially kill) the prey before it finds it unpalatable. Chemical defences can also be stored in body parts such as wings in many toxic butterflies [6,51]. In both of these cases, the efficacy of chemical defence is based on shared predator education costs where predators learn to avoid the chemically defended prey sharing a similar appearance [52]. If prey species offer conspicuous cues (aposematism) associated with a chemical defence, this avoidance learning rate becomes even more effective [52].

At present, the focus of research has been on chemical defence in larval and adult stages and much less is known about the role of chemical defences in protection against predators during the pupal stage. In species whose larvae and adults contain defensive compounds, pupal stages are also often chemically defended [53–55]. For example, both larvae and pupae in *Delphastus catalinae* ladybirds have minute secretory hairs that produce defensive secretions deterrent to their predators [56]. Eclosion fluid in pupae can also be bitter and play role in chemical defence (J.M. 2017, personal observation) even though its function in chemical defence has not been tested.

Since the selective environment and lifestyle are likely to change during the larval, pupal and adult stages, we can expect qualitative and quantitative changes in defensive

Figure 2. Pupae can defend against predators with different kinds of morphological and chemical defences or even rely on protection from other individuals. (*a*) Aposematic *Heliconius melpomene* butterflies' pupae are covered with spines. (*b*) Many moth species produce a silk cocoon or loose silk nets to protect the pupa (white satin moth, *Leucoma salicis*). (*c*) Larval, pupal and adult stage in the common buckeye (*J. coenia*) contain iridoid glycosides that give them chemical protection predators. (*d*) *Maculinea rebeli* butterfly pupae use vocal communication to recruit ants to guard them. (Photos: *a*–*c* Adobe Stock, D. Marco Gherlenda.)

chemistry during development [57,58]. In leaf beetles (*Oreina gloriosa*), individuals contain defensive compounds (cardenolides) during all the life stages [57]. However, the composition of the chemical cocktail changes from larval to pupal and pupal to adult stage. The authors suggest that these differences can partly be explained by changes in the lifestyle and respective predator and parasite community structures. Studying this hypothesis experimentally would offer a promising route to test how changes in predator community structure shape and maintain diversity in chemical protection [51].

The quality and quantity of defensive compounds in the pupal stage can also depend on the developmental environment during the larval stage. For example, *Junonia coenia* (Nymphalidae) larvae sequester iridoid glycosides from their host plant (*Plantago lanceolata*) [54]. These compounds have been shown to be deterrent against different types of invertebrate predators such as ants [59]. Both host plant genotype and the predator type present have been shown to affect the quality and quantity of defensive chemical content in the pupal stage [54]. Catalpol content was higher during the pupal stage in individuals that grew in the presence of stinkbugs in comparison to individuals that had suffered predation by wasps. Thus, *J. coenia* individuals can alter their chemical protection in the pupal stage to match the predation risk from the most important predator species (target-specific defence; see also [60]).

Sometimes possessing defensive compounds in the pupal stage can increase predation risk from conspecifics. Pyrrolizidine alkaloids are plant-derived compounds that are widely used in chemical defence and communication, especially among Arctiid species [53,61]. *Utetheisa ornatrix* larvae derive pyrrolizidine alkaloids (PAs) from their host plants *Crotalaria* spp. and retain alkaloids through life stages and metamorphosis. These compounds also occur during the pupal stage, offering potential protection against natural enemies. However, these same compounds can make the pupal stage vulnerable to cannibalism. Before pupation, larvae tend to wander further from the host plant as *U. ornatrix* larvae cannibalize pupae with high PA content to acquire PAs for themselves [62]. In addition to protection against predators such as spiders or birds, PAs play an important role in sexual selection in *U. ornatrix*, offering one explanation for the cannibalistic behaviour in the larval stage [61,62].

7. Physical and behavioural defences

Pupae can also offer physical defence against potential predators (figure 2). Hairs and spines have been shown to be especially effective defence mechanisms against invertebrate predators [63–65] during the larval stage and may play a potentially important role in pupal defence. For example, pine processionary caterpillars are covered by urticating hairs that can cause strong allergic reactions in humans. Thus, it is possible that they are unprofitable to other predators too. In addition, their cocoons contain exuvia of the last larval moult, bearing urticating hairs twined into the cocoon walls [19]. Gonometa-moth species such as African wild silk moth (*Gonometa postica*) larvae have urticating hairs that they incorporate into the pupal cocoon walls [66]. Cocoon walls can also be enforced with other structures that make them less likely to break when attacked: Veldtman *et al.* [66] suggest that in *G. postica*, cocoons appear conspicuous against their background. They also suffer much lower bird predation risk (2%) in comparison to *Gonometa rufobrunnea* (50%), whose cocoons are otherwise similar but appear more cryptic for the human eye [66]. Rather than aposematic defence, authors suggest that lower predation risk in *G. postica* is likely to be owing to the difference in the structure of the cocoon. *Gonometa postica* cocoons are covered by small calcium crystals that make the cocoon more difficult to break, in addition to differences in cocoon colour. The function and origin of these different types of physical protective mechanisms in defence against predators in the pupal stage have not, however, been tested experimentally. For example, larvae may need to get rid of the hairs before successful pupation. Incorporating hairs into the cocoon walls may therefore be a physiological necessity offering additional benefits in defence against predators or parasitoids.

Many holometabolous insects spin their cocoon before metamorphosis. The silk cocoon can protect them against predators and parasitoids, but also protect against desiccation or microparasites [67,68]. The silk that is the primary constituent of Lepidopteran cocoons is also thought to play a defensive role for pupae. Although the majority of silk is made up of proteins that combine to maintain the silk's structural properties, there are a number of other proteins present in different species' silk [69]. These alternative roles are thought to include contributing to unpalatability of the cocoons and defending their contents against microbes [70,71]. The majority of studies on these non-structural proteins have focused on the silk of the silkmoth, *Bombyx mori*; however, the silk components of moths from different families have been shown to be very similar to one another [69]. The silk of the silkmoth cocoons contains protease inhibitors that could act against a range of bacterial- and fungal-derived proteases. Yet, Kaur *et al.* [72] have argued that many of the purported antibiotic properties of silk were owing to experiments using contaminated silk. These contaminants originated from the processes of breaking apart components of the silk prior to the experiments they were used in. So, the extent to which the silk of cocoons defends the pupae against bacterial or fungal infections or makes them unpalatable for predators is far from clear cut and requires further experimental studies.

Pupae can also display defensive movements that can potentially have a deimatic function against predators [73] or, they can make attacks by predators and parasitoids physically difficult [74]. For example, pupae of *Tenebrio molitor* and *Zophobas atratus* rotate their abdominal segments in response to tactile stimulation and this behaviour has been shown to decrease the risk of cannibalism by the larvae of the same species [75]. Pupae of the small tortoiseshell (*Aglais urticae*) start to wriggle very intensively when the parasitoid tries to land on it, often preventing the parasitoid from depositing its egg into the pupa [74]. In the same paper, Cole [74] reported higher oviposition success of parasitoids with Ledidopteran species *P. aegeria* and *Pieris brassicae*, whose pupae are not able to wriggle as intensively as *A. urticae* pupae.

8. Intraspecific interactions and survival throughout the pupal stage

Predation risk is often quoted as a major selective force favouring sociality [76], including cooperative protection of offspring during the pupal stage. In many socially behaving insects, such as eusocial hymenoptera, different types of cooperative breeding strategies have evolved where adults take care of the immature stages, including pupae, and defend them against different types of natural enemies [77]. However, in eusocial species such as ants it is also the case that pupae are not safe from predators: for example, Asiatic black bears are more likely to forage on ant nests that have plentiful pupae [78] (but see [79]).

In some species, individuals can form aggregations in the pupal stage that decrease mortality risk. These aggregations can be passive: for example, owing to features in the landscape (if suitable habitats for pupation are scattered) that lead to the clumping of individuals. Alternatively, aggregations can be active when individuals actively maintain contact with each other. Aggregating in the pupal stage (or in any life stage) can function in three ways: first, it minimizes encounters with random search predators, but may increase vulnerability to, for example, visual predators that are good at detecting groups of prey [16,80]. Second, it dilutes the effect of the predator after it has been encountered (safety in numbers owing to predator satiation and handling times) [80–82]. For example, in a stream-dwelling trichopteran, *Rhyacophila vao*, pupal aggregations were disadvantageous in terms of encounter risk with the predators. However, grouping was beneficial in terms of dilution of predation risk. When evaluated together (attack-abatement effect [80]), grouping in the pupal stage offered higher net benefits against predation risk [83].

The third way in which aggregation can lower predation risk is by enhancing the avoidance learning of predators if the prey is unprofitable [81]. Predators have been shown to learn to avoid chemically defended cryptic (immobile) artificial and real prey items more effectively when these prey items are in groups compared to solitary prey [81,84]. This mechanism has not been experimentally tested at the pupal stage, but it could play an important role in species whose pupae possess chemical defences and are often aggregated spatially and temporally. For example, in the gregarious chemically defended pine sawfly species, pupae are often aggregated in the vicinity of the host trees where larvae feed gregariously in actively maintained groups. In Neodiprion pine sawflies [39], individuals switch from gregarious behaviour to solitary in non-feeding final instar where larvae disperse to spin their cocoons on the ground. Yet, cocoons are often clumped in the space under host trees and can form attractive feeding sites for mammalian predators such as shrews and voles [20] or birds [16]. Pine sawfly pupa include defensive chemicals as the defensive glands of larvae are disposed into the pupa during metamorphosis [85]. However, during the pre-adult stage, individuals actively turn inside the cocoon if the cocoon wall is pierced and move the disposed defensive gland sacs towards the potential attacker [85]. Whether this chemical defence mechanism of pupa has any effect on the predators' behaviour has not been directly tested [16,20] and its role might be more important in defence against macroparasites [85].

9. Protection from other species and survival throughout the pupal stage

Individuals can gain protection against predators from other species during the pupal stage [9,14,86]. These interactions can vary from mutualistic (both partners benefit from it) to parasitic (costly for the other partner without any gained benefits). Sometimes, the relationship can also be commensalistic: for example, *Platyperpia virginalis* (Arctiidae) caterpillars shift host plant and habitat during pupation. Individuals prefer to pupate within a spiny plant species, which are in a different habitat and not the host plant they feed upon during the larval stage. Furthermore, individual survival during the pupal stage was higher in these physically defended plants. This was confirmed experimentally: in plants where the physical defence structures were removed, survival of pupae decreased [9]. Similarly, in papilionidae *Battus polydamas archidamas*, pupae have a higher probability of survival when they are on cacti in comparison to shrubs, rocks or the ground [26].

Some species can even manipulate other species to gain protection from them [87]. Lycaenidae and Riodinidae butterfly families are famous for their relationships with ants, which vary from mutualistic to parasitic (reviewed in [86,88]). In general, larvae of several species of this group get protection from ants against parasites and predators. Ants in return receive a nutritious secretion from the larvae. How dependent these species are on ants varies among species. Some of the species are obligatory myrmecophiles such as *Jalmenus evagoras*, which are practically unable to survive without ants. Some other species are facultative myrmechophiles.

Interestingly, not only larvae but also pupae of this group of butterflies have evolved mechanisms to manipulate the ants' behaviour. All Lycaenid pupae produce sound via stridulation (sound-producing organs) regardless whether they are ant-associated or not [86]. These pupal sounds can be considered to have a deimatic function [73], as these sounds are produced when pupae are disturbed [89]. However, in some species, pupal sounds are involved in ant recruitment. For example, in *J. evagoras* pupae, sound production can attract ants and maintain ant guard. These examples show that the pupal stage is not 'a passive and inactive' life stage but pupae can actively communicate with their environment. Perhaps the most fascinating example of this comes from the parasitic *Maculinea rebeli* butterflies (*Lycaenidae*). Both their larvae and pupae produce sound that accurately mimics the sound of its host, the queen ants of *Myrmica schenki* [90]. Sound produced by pupae is actually more accurate than the sound produced by larvae. This pupal sound elicits a similar response to sounds made by queens, giving *M. rebeli* the ability to manipulate its host and gain protection from ants.

10. Conclusion and future questions

Our literature survey shows that an extensive diversity of defensive mechanisms have evolved to increase protection against predators through the pupal stage in insects. These strategies include different types of protective coloration strategies, defensive toxins, protection gained from conspecifics and manipulation of host species' sensitivity to specific chemical or auditory cues to gain their protection. Thus, the pupal stage is not an 'inactive' stage but can protect against predators with an extensive diversity of morphological, chemical and behavioural mechanisms. However, considering the literature available from the larval or adult stage, understanding of the defensive strategies at the pupal stage is still in its early stages. Therefore, predation risk, and how to protect from it in the pupal stage will evidently offer an interesting and important topic for future research. Determining these selection pressures across life stages is critical to forming a realistic view of the evolution of life-history strategies in species with complex lifecycles and to predict factors that shape their population dynamics. This information can even provide insights to mechanisms behind dramatic declines in insect populations [91,92] and be beneficial for effective conservation planning and management. With these aspects in mind, we have outlined several potentially interesting and important routes for future research to follow.

(a) How does variation in predator community composition shape the evolution of prey defences throughout an individual's life cycle?

Variation in the predator community structure can be an important selective agent that shapes the evolution of prey defences. At present, these effects have been mainly considered within the individual life stage (e.g. larval or adult) [60,93]. However, based on our literature review, predator community structure is also likely to change across different life stages [15,17]. How it shapes the function and diversity of defensive mechanisms between life stages, including the pupal stage, is not known [8]. For example, variation in the composition of predator and parasitoid community structure could explain why we sometimes observe variation in the quantity and quality of defensive compounds in larval, pupal and adult stages. To study those unstudied aspects, we can benefit from representative model systems for prey species [54,55,61], where we already have accumulating information on the multiple factors that shape individual defensive strategies in different life stages. We also need to focus on relevant predator species as a recent study suggest that chemical defences can be target-specific [60], addressing the importance of choosing the correct focal predator species when the efficacy of chemical defences is studied. With the accumulating empirical research information from different species, we can then perform systematic analyses to evaluate how much defences across different life stages are linked or whether they evolve independently.

(b) Function of chemical defences during the pupal stage: can non-visual predators learn to avoid cryptically coloured pupae based on chemical, tactical or auditory signals?

Aposematism has repeatedly evolved in larval and adult stages, but very rarely in the pupal stage [8,23]. However, a general assumption is that defensive compounds also occur in the pupal stage when larval and adult stages are chemically defended. This is also supported by empirical data in species where defensive chemical content in the pupal stage has been analysed [54,61]. As conspicuous signals should enhance the predator's avoidance learning efficiency, the occurrence of these defended prey species with low signal intensity is puzzling [45]; this is especially true if chemical defences are costly to produce and maintain and the individual is already well protected by camouflage [94]. If a predator always needs to bite the pupa to find it unpalatable (i.e. predators do not learn to avoid chemically defended pupae), defensive chemicals in the pupal stage do not benefit the individual, which will die anyway after the cocoon shell is pierced. For example, Wiklund & Sillén-Tullberg [23] suggested that pupae might even be innately more vulnerable to handling by predators because the hard cuticle of pupae is more likely to crack broken in comparison with more flexible larval and adult cuticles.

What cues could predators then use to learn to avoid chemically defended pupae? First, conspicuousness may not be as important as long as defended prey are sufficiently distinctive from the palatable prey [95]. Second, most of the research on pupal coloration has focused on conspicuousness to human eyes. However, we know very little about the conspicuousness of pupa to predators with different types of visual systems, such as birds that also perceive UV wavelengths [96]. Some species have metallic golden or silver shiny pupae (figure 1) [97]. This kind of shiny coloration may function either as a warning signal in a similar way to iridescent colours, or as a camouflage reflecting the visual environment [97–100]. Third, our literature review shows that pupae have evolved many other ways to communicate such as movements, sounds and chemical cues. These kind of cues could function as effective warning signals of defensive toxins [101], especially for non-visual predators. Currently, we lack experimental studies that test if these non-visual predators are able to learn to avoid chemically defended pupae based on the cues available. Finally, it is possible that defensive compounds occur in the pupal stage simply because they need to be transported from the larval stage to adult stage without any adaptive function during the pupal stage. Future research could try further experiments with dummy prey, similar to those that are widely used to test predation risk during the larval [22] and adult stages [102]. This would offer a way to test how different phenotypic traits such as size, coloration or pupation site are under selection by predation.

(c) Life-history trade-offs across life stages and how they link with predation risk during the pupal stage

Another less studied aspect is potential life-history trade-offs between pupal and adult stages [28,34]. For example, if allocation to effective camouflage pigmentation during the pupal stage trades-off with the size or flight capacity during the adult stage, selective predation during the pupal stage will shape phenotypic variation in adults indirectly. Similarly, if predation favours either large or small size during the pupal stage [21], it is also likely to reflect traits such as size in adults. Defensive traits, such as chemical defence, can also be costly to produce and maintain during the larval stage [12,103,104] and constrain performance during the pupal and adult stages. For example, Lindstedt *et al.* [103] found that when the costs of chemical defence were high, *Diprion pini* pine sawfly individuals were less likely to reach the pupal stage and grew more slowly. In *P. brassicae* larvae, higher contribution to chemical defence decreased their likelihood of reaching the pupal stage and they were smaller in the pupal stage [12]. Finally, allocating resources to building up the protective cocoon during the pupal stage can also be costly and constrain resources available during the adult stage [105].

How pupal traits are associated with adult or larval traits could be especially interesting questions to test with species that are polymorphic at some life stage. For example, if adult and pupal traits are correlated and predation risk varies accordingly with life-stage, selection during the pupal stage could be an important factor explaining variation in the frequencies of adult morphs. Studies that link phenotypic variation in pupal traits with the variation in larval and adult traits are, however, very scarce, and would therefore be important in understanding the evolution of life-history strategies in insects.

(d) Do defensive traits have multiple functions in defence against multiple enemies?

Even though our main focus in this literature survey has been on predation, we want to address the fact that many of the defensive mechanisms listed above can be equally critical in protection against parasitoids and pathogens [13,85,106]. Currently, there is accumulating evidence that studying these two selection pressures simultaneously in 'multienemy-framework' can help to understand how defensive traits have evolved initially [106–109]. For example, some defensive compounds can have a dual function and serve multiple purposes in protection against predators and parasites [106,107] (but see [40]). Thus, it is possible that the same defensive compounds that play an important role in the protection of the larval stage against predators may play a more important role in protection against fungi and pathogens during the pupation. If defensive toxins have evolved primarily against parasitoids or diseases, it will offer one more evolutionary explanation for the weak visual signals of chemically defended pupae. Similarly, defensive movements during the pupal stage or camouflage can increase survival both against predators and parasitoids [13]. Again, there is a clear need for experimental studies where the importance of visual and chemical cues in protection against parasitoids and predators can be tested.

(e) How often do individuals switch habitats or rely on protection from other species during the pupal stage?

Our review shows that individuals can switch habitats [9], host plants [9,26] or even evolve parasitic or cooperative interactions with other species [14,68,90] to gain enemy-free space during the pupal stage. How often pupae actually rely on protection from other species in their defence [14] or how dependent insect species are from multiple habitats during their lifecycle would offer an interesting topic for future research to consider. This would require natural history data and behavioural observations combined with experimental manipulations and phylogenetic studies [110]. This information could also have applied importance in planning of conservation areas targeted to protect certain species: often efforts in conservation are allocated on protection of areas abundant in larval host plants. However, if insect species are dependent on other species to survive through the pupal stage [14], or even need to change habitats to pupate successfully, effective conservation needs to take these requirements into account.

Data accessibility. This article has no additional data.

Authors' contributions. C.L. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Competing interests. We declare no competing interests.

Funding. This study was funded by the Academy of Finland via Centre of Excellence in Biological Interactions.

Acknowledgements. We thank Paul Johnston, Stuart Reynolds and Jens Rolff for the invitation to the 'Evolution of complete metamorphosis' theme issue. We thank an anonymous reviewer, Dirk Mikolajewski, Jens Rolff, Tapio Mappes and Emily Burdfield-Steel for commenting the earlier versions of the manuscript. Francesca Barbero together with Marco Gherlenda kindly provided the photo of *M. rebeli* pupae.

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