

Research articleSubmitted: February 20th, 2019 - Accepted: October 10th, 2019 - Published: November 15th, 2019**‘Awareness’ in metamorphosing pupae
(Lepidoptera: Pieridae)**

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Abstract

Neurobiological studies of model insect species have established that the nervous system retains some larval innervations, remodels others, and develops other new adult innervations during metamorphosis. Using a simple behavioral response – the ability to ‘kick’ by pupae of the pierid, *Catopsilia pomona* (Fabricius, 1775) - it was possible to assay for the retention of environmental awareness during ontogenetic reorganization. All pupae kicked 24h after ecdysis, 48% of pupae kicked at the pharate adult stage, and 28% of pupae kicked every day of their development (6.52 d ± 0.10). Both the mode and temporal expression of the response indicate that this retained awareness has larval origins. Variability in the response supports the inference that this response is mediated, to some extent, by prior experience rather than purely ‘reflex’. This is consistent with a Darwinian explanation of the behavior (and retained environmental awareness itself) as serving a protective function in pupae.

Keywords: disturbance behavior, ecdysis, metamorphosis, Pieridae.**Introduction**

To what extent do holometabolous pupae retain awareness of their environment as their bodies are re-organized during metamorphosis? One of the reasons this question is so difficult to answer is because many pupae assume postures of immobility as they transform into adults. Thus, it is easy to assume that the insect is either dormant or has gone into a kind of sleep in which it is perceptually closed or insensitive to external stimuli.

Neurobiological studies of model insects have given us a much better understanding of the ways in which the nervous system is re-organized, with some neuromuscular innervations retained, others remodeled and others added (Truman 1992; Consoulas et al. 2000). However, these studies have predominantly sought to discriminate between the larval and adult nervous systems, rather than identifying a pupal neurobiological ‘identity’. This is logically consistent with the view that the pupa is essentially a point of transition between the two and with the apparent predominance of effectively quiescent pupal modes. This emphasis is, of course, not universal, but does generally reflect the tenet of most studies, for whom the incipient adult, with new capabilities like flight, is the logical subject of investigation. However, whilst an entirely legitimate focus, this emphasis has meant that considerations of the pupal stage, as distinct, independent of its role as a transit vehicle for larval and adult characters, have been slightly overshadowed.

A notable exception to this is early work looking at the

gin-trap reflex in the sphingids, *Sphinx ligustri* Linnaeus, 1758 and *Manduca sexta* (Linnaeus, 1763) (Bate 1973a, 1973b, 1973c; Levine & Truman 1983; Levine 1984). These latter studies provide some neurobiological context for the behavioral investigations described here – although this will be further qualified below.

The concept of a pupal neurobiological identity was given early voice by Levine (1984), who noted that: “Even though adult development occurs within the pupal case, there is behavior unique to this transitory stage...”. Preceding these comments, Snodgrass (1954), put the question of the relationship between mobility and metamorphosis into its mechanistic context:

“Of all the reconstructive processes that take place in the pupa the most important is that of the muscular system, which is perhaps the primary reason for the pupa. The dissolution of the larval muscles before the imaginal muscles are formed at least accounts for the immobile condition of the pupa, though, since all the larval muscles are not destroyed at the same time and some may go over intact from the larva to the adult, various pupae retain some degree of activity.” (p. 9)

Pupal movements can probably be considered to be broadly analogous in most derived lineages of lepidopteran pupae in which freedom of movement is retained in a number of abdominal segments: flexion movements are primarily the product of the intersegmental muscles (e.g. Hasenfuss & Kristensen 2003). These muscles have larval origin, enable flexion during metamorphosis, are sub-

sequently involved in emergence, but then die (Truman & Schwartz 1980; Levine 1984). The motor neurons involved in these muscle movements are larval retentions (Levine 1984); however, the elicitor system of sensilla and sensory neurons, responsible for the gin-trap reflex specifically, is modified during the transition from the larval to the pupal stage, and the connection between the sensory and motor components of the reflex is activated only after pupal ecdysis (Levine & Truman 1983; Truman 1992).

The subject of this study, the pierid, *Catopsilia pomona*, is a different species, from a completely different Family, and even utilizes a different pupation mode to *M. sexta*. Thus, unlike *M. sexta*, *C. pomona* is not a model species, and lacks a pre-existing body of behavioral or neurobiological investigations. Nonetheless, it and other pierids common to South and SE Asia, might well serve as useful models, both generally and regionally. (Regional origins have some significance to environmental context; see below). This is not to delimit the taxonomic basis of this type of pupal response: *C. pomona*, is, on the basis of observations, an excellent demonstrator of the abdominal response, and by virtue of identical or similar modes of pupation, other closely related pierids are probably also; however, the distantly related sphingids, *S. ligustri* and *M. sexta*, with a different phylogenetic history, demonstrate analogous defensive movements (Bate 1973a, b). Indeed, the only requirements for the possession of this behavior (in some analogical form) may be the possession of some degree of freedom of movement in the latter abdominal segments. The dual-attachment system of pupation (i.e. cremaster + silk girdle) is not employed by Sphingidae, but it is characteristic of the Pieridae and some other groups. This attachment system may, however, be responsible for conferring some of the particular vigor observed in the kicks of *C. pomona* by providing sites of ground reaction force. Overall, these observations are therefore completely novel, although, it is emphasized, they have comparative value also.

The term ‘gin-trap’ was formulated by Hinton (1946, 1955) to describe a much more localized response, which he primarily detailed in beetles, in which the modified margins of movable abdominal segments are used to pinch micro-intrusions between two adjacent segments of the pupa. Hinton (1955) noted but did not elaborate extensively on lepidopteran responses. However, studies of lepidopteran gin-traps, using Sphingidae as models, were subsequently made by Bate (1973a, b, c) and Waldrop & Levine (1989, 1992). Interestingly, Bate (1973b) noted, but did not explore, the employment by a typical sphingid pupa, of more generalized movements to ‘repel predators and parasites and to clear debris which obstructs its cell and deforms its integument’. The extent to which the responses are anatomically analogous in these and other species, requires further examination. Given that the gin-trap, as originally conceived, operates as a ‘jawed’ intersegmental trap initiated by localized sensilla, it may well be considered

a supernumerary adjunct to more prevalent preservations of freedom of movement in holometabolous pupae. However, its referencing and inclusion within this study context are important because its operations are the only ones examined in any detail to date; and are clearly reliant on identical or analogous principles of abdominal movement and musculature.

It is emphasized however, that the response of interest here is the generalized one: the flexion of the abdomen afforded by the retention of freedom of movement in a number of medial abdominal segments. This response has far greater ecological significance: intersegmental intrusions delimit disturbance to individuals either of a certain size or with some degree of specialized anatomy; but a generalized model is appropriate to realistic ecological contexts in which disturbance might originate from a vast array of sources and in which the response, whether considered as ‘signal’ or ‘physical defense’, is conveyed across the entire form of the pupa(e). The source pool of biotic disturbances is further accentuated in *C. pomona* and its regional congenics as a result of a tropical situation in which biodiversity is more extreme. The term ‘kicking’ is therefore adopted here from Hawes (2018), which also provides further discussion and context, as a continuation of this conceptualization of a generalized response.

The term ‘kicking’ specifically simultaneously evokes the generalized (whole-body) form of the response and its caudal origins in the medial abdominal segments. ‘Kicking’ fits the visual appearance of the movement(s) and is also appropriate to the vigor of the response in its archetypal form in fresh pupae. The figures of body contortions during kicking (Hawes 2018) illustrate this well. Although both Hinton (1946, 1955) and subsequent investigators (e.g. Eisner & Eisner 1992) have demonstrated the defensive functions of the gin-trap, these have all followed the conceptualization of the abdominal contractions as specific responses to individualized disturbances. ‘Kicking’ allows for a much more inclusive (and ecologically realistic) definition and conceptualization of defense. A specific example of its employment against coordinated ant attacks is documented by Hawes (2018), however the response allows for defensive options that are both communicatory and mechanical and against a range of disturbances from individual to multiple agents, and from incidental enquiries by wandering arthropods to sustained attack by predators.

This defensive function is important for establishing the connection between a pupa-specific neurobiological identity and its adaptive significance – i.e. evolutionary context. The general ‘need’ for a defensive mode necessitated by extremely limited mobility and exposure to unpredictable environments argue for the importance of seeing the pupa’s neurobiological identity as something more than a transition point. In nature, pupae develop in ecological context. The retention of awareness provides a Darwinian advantage similar to the way other pupal adaptations like camouflage or spiky protuberances contribute to

defense. Metamorphosis is in some ways the most vulnerable life stage and the ability to respond to environmental disturbance gives it a selective advantage over an entirely passive metamorphic passage. As with other defense systems, of course, kicking must be said to only mitigate, rather than remove, environmental vulnerability.

Following on from previous work describing the ‘kicking’ behavior of *Catopsilia pomona* (Hawes 2018), this study sought to examine the time course of this behavior during metamorphosis. These previous observations had indicated a decay in the response, but this was not sufficiently temporally discriminated, nor was it clear whether this decay represented individual variability or whether the response was itself ultimately confined to the early stages of metamorphosis. The primary aim of the study was to further develop the previous description of kicking in *C. pomona* by Hawes (2018), however the question is also of some interest to framing the response within a neurobiological context, particularly with regard to the temporal availability of sensitivity. In addition, some informal observations made of one of the Jezebel pierids (*Delias* sp.) in which a pupa was reared to imago stage and demonstrated kicking directly prior to eclosion, prompted a suspicion that such movements may span the pupal ontogenetic timeframe.

Material and methods

Individual pupae of *C. pomona* were followed through metamorphosis and tested for their kicking response. The hypothesis that pupae are capable of being environmentally aware throughout their metamorphosis was tested. (Awareness is defined here as referring to an ability to perceive perceptual stimuli, whereas environmental awareness specifically designates an ability to perceive perceptual stimuli that are external to the organism). A number of factors argue for the occurrence of some form of mediation of the response, such that the traditional conceptualization of pupal movements as a ‘reflex’ (Truman 1992), may need reconsideration. A ‘reflex’ response may well still be a reasonable descriptor of the gin-trap mechanism. But, while sharing some commonalities with the gin-trap response, the responses described here show some aspects which deviate from the concept of a ‘reflex’ as a strictly stereotyped response in which both the eliciting cues and the response are basically invariant in form.

Butterflies were identified as *Catopsilia pomona* (Fabricius, 1775) by reference to Ek-Amnuay (2012) using caterpillars previously reared out to adult imagos. The form of *C. pomona* used here was identified as *C. pomona* f. *jugurtha*, as first described by Cramer (1779: 138, Pl. 187, Figs E, F) (see Hawes 2018). Final instar caterpillars were collected from the walls of an avenue lined with *Cassia fistula* L. trees in the city of Khon Kaen, northeastern Thailand in June 2018. *Cassia fistula* leaves are a preferred food

plant of the caterpillars of *C. pomona* (Ek-Amnuay 2012; van der Poorten & van der Poorten 2013) and a population of this pierid is present at this sampling locality. During their pre-pupation wandering phase, large numbers of caterpillars leave the trees and climb up the nearby white-washed walls to attach themselves and pupate. Caterpillars were transferred to individual chambers with a ball of moistened tissue. Caterpillars that did not pupate were excluded from the experiment. A high incidence of parasitism meant that nearly 40% of the original test population had to be excluded ($n = 40$). Kicking behavior was only tabulated for individuals that completed development and successfully eclosed out of the chrysalis ($n = 25$). Emerged imagos were released back into the wild.

Kicking behavior was described by Hawes (2018). To test for the occurrence and maintenance of this disturbance response behavior, the chamber of each individual pupae was opened in the morning of each development day and the pupa was assayed for a response. Total developmental time was calculated as the mean number of days of pupation before emergence. The assay differentiated between 3 categories of response with a hierarchy of sensitivity: 1) kicking prior to tactile stimulation (i.e. from the disturbance of the container being opened); 2) kicking in response to 2 light strokes of the middle abdominal segments with a paintbrush; 3) kicking not initiated by #2 but in response to further light stroking of the entire body three times. Responses were categorized on a scale of 0-3, where 0 = no kick, 1 = kick prior to direct contact, 2 = kick from first direct contact, and 3 = kick after more sustained repeated contact. Tests were only carried out once a day as it was felt that excessive testing could increase the stress and/or familiarity of the tactile assays. The relative ‘vigor’ of the responses was not measured but qualitative differences in the response were noted.

One-way and multi-way chi square tests were carried out to identify significant differences in the proportions of responses by pupae. Expected values for one-way tests were calculated based on the assumption of equal distribution of values between compared groups. For comparisons of different developmental days as regards to the number of pupae which showed behavioral categories 0-3, low counts for some of the categories led to expected values < 5 , so Fisher’s exact test was used instead of chi-squared tests. In the Fisher’s exact tests, contingency tables of 4×2 were used to compare the responses of pupae between each pair of developmental days. For all tests, statistical significance (p) was calculated with a two-tailed significance level of $p < 0.05$. Tests were calculated using online calculators at in-silico.online (accessed July 2019).

Results and Discussion

The mean development time of pupae was $6.52 \text{ d} \pm 0.10$. Table 1 shows the frequency of kicking responses across

development by individual pupae. Responses of the sample population are summarized in Figure 1 and Table 2. Fisher’s exact test found significant differences between the proportions of pupal responses between days: 1 and 2 ($p = 0.008$), 1 and 3 ($p < 0.003$), 1 and 4 ($p = 0.015$), 1 and 5 ($p = 0.004$), 1 and 6 ($p < 0.001$), 3 and 6 ($p = 0.040$); and 4 and 6 ($p = 0.049$). When categories were collapsed into a binary comparison of the frequency of kicking (1+2+3) versus non-kicking (0) pupae, a chi-square test found significant differences between the proportions of pupal responses ($df = 5$; $\chi^2 = 21.429$; $p = 0.0007$). Table 2 summarizes the main characteristics of kicking and non-kicking behavior in the sample population of pupae. One-way chi-squared tests found significant variability in the number of response types of individual pupae ($df = 3$; $\chi^2 = 16.76$; $p = 0.0008$). However, no significant differences were found between the numbers of days with non-kicking responses

Table 1 – Kicking responses by individual pupae across the developmental timeframe (0 = no kick, 1 = kick prior to direct contact, 2 = kick after first direct contact, and 3 = kick after sustained repeated contact; E = eclosion of imago).

# Pupa	Pupal developmental day (d)							
	1	2	3	4	5	6	7	8
1	2	2	2	1	2	0	E	
2	2	2	2	2	2	2	2	E
3	2	3	2	2	0	0	E	
4	2	2	3	3	2	0	2	E
5	2	2	2	2	2	1	E	
6	1	2	3	2	2	0	E	
7	1	2	2	2	2	2	E	
8	1	2	3	2	0	2	E	
9	1	2	2	3	2	2	E	
10	2	2	2	2	2	2	E	
11	2	1	2	2	2	0	E	
12	2	0	0	2	2	0	E	
13	2	0	0	0	0	0	E	
14	2	2	2	2	2	0	E	
15	2	0	2	1	1	0	E	
16	2	2	2	0	0	2	E	
17	2	0	0	2	0	0	E	
18	2	0	0	2	2	0	E	
19	2	2	0	0	2	2	E	
20	2	2	2	0	0	0	E	
21	2	2	0	0	0	0	E	
22	2	2	2	2	2	0	E	
23	2	0	2	2	2	2	E	
24	2	2	2	2	2	2	E	
25	2	2	3	3	3	3	E	

in individual pupae, nor were significant differences found in which day non-kicking behavior was initiated by individual pupae.

These assays of the pupal disturbance behavior of *C. pomona* demonstrate that the pupae of this species retain some form of environmental awareness across metamorphosis. Up until the latter half of the last century it was not even certain whether the insect nervous system functioned at all during metamorphosis. Bate (1973b) noted that:

“...this view is based on the assumption that the delicacy of the metamorphosing nervous system and the large-scale rearrangements which it undergoes prevent it from functioning. However, the interesting point is not that the pupal nervous system ceases to function but that it continues to do so throughout the period of reconstruction, until the onset of the adult moult.”

These observations on *C. pomona* extend this knowledge further by demonstrating that the nervous system is not only operant, but that it is sensitive to the external environment across metamorphosis in some species.

This modified and enlarged perspective of pupal neuro-activity is already present to some extent latent in the gin-trap literature, although without a systematic examination of the time course of pupal behaviors it has lacked any definite basis for explicit articulation. Of course, without further comparative work it is best not to extrapolate too far, but certainly for this species, the conceptualization of metamorphosis as either a form of dormancy or sleep in which the insect is perceptually closed to its surroundings is inappropriate. Indeed, some pupae – those that kicked in response to their pupation chambers being opened without any direct contact disturbance – might even be considered hypersensitive to their environment. It should be emphasized however, that response #1 (kicking prior to direct contact) should not be taken to indicate any kind of extrasensory perception: it just means that the pupae were so sensitive to environmental change that they responded to the mechanical disturbances inherent in the opening of their chambers (communicated to them via their girdle and cremaster attachments to the container walls), without the need for direct contact stimuli.

Variability in the sensitivity of the pupae supports a number of inferences about the behavior as well as raising further questions. Non-kicking occurs throughout the developmental timeframe, so it is difficult to attribute it to a temporary loss of sensitivity as a result of physiological reorganization. For example, if pupal sensitivity went ‘offline’ temporarily due to internal changes, then it would be predicted to occur at similar points in the metamorphosis of individual pupae. However, the chi-square test comparing the numbers of pupae which initiated non-kicking behavior on different developmental days provided no significant evidence in favour of this hypothesis. This result, together with the fact that 28% of the pupae kicked every

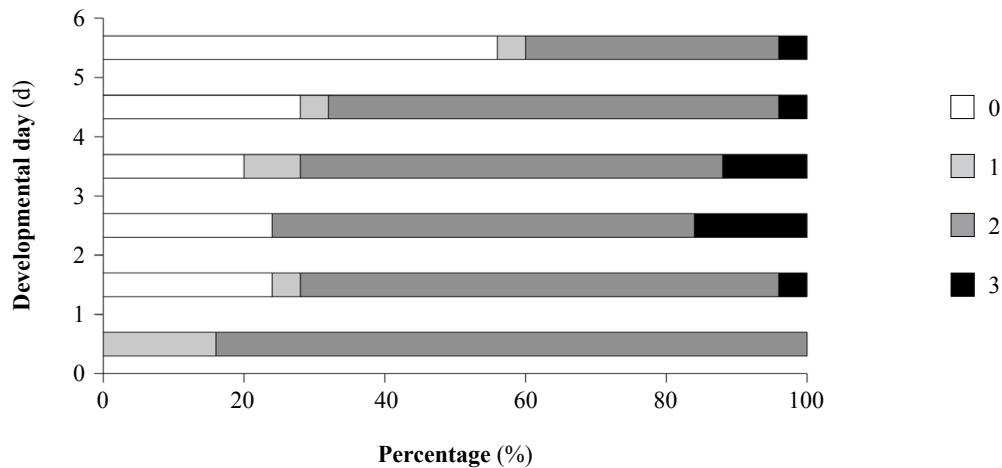


Fig. 1 – Abdominal disturbance responses of *Catopsilia pomona* pupae assayed daily through metamorphosis (0 = no kick, 1 = kick prior to direct contact, 2 = kick after first direct contact, and 3 = kick after sustained repeated contact).

day, and that almost half of the pupae (48%) demonstrated the behavior in their final developmental day, suggests that sensitivity and associated response behaviors may be retained throughout development in the majority of pupae. On the other hand, there are two developmental days in which the frequency of kicking (day 1) or of non-kicking (day 6) is strikingly above the expected values if there were no differences between the developmental days, and this accounts for the positive results of the chi-square test when all developmental days were simultaneously compared with each other, and of some of the Fisher’s exact tests comparing couples of days. Thus, at this stage it is not possible to determine the effects of metamorphic stage on behavioural expression (e.g. in the pharate adult stage the pupal sensilla are not directly connected to the nervous system of the adult, due to the space between the pupal and adult cuticles). Nor is it possible to properly determine whether non-kicking is the result, not of a loss of sensitivity, but rather due to factors typical to the inhibition of behavioral expression generally (e.g. familiarization, modified threat assessment). With regard to this latter point, there is clearly some scope for further examining these behaviors within hypothetical models designed to test for familiarization through comparisons of responses to repetitive and novel stimuli.

What if the temporal synchronization of response tests used here missed ‘offline’ pupae? Both the responses observed and the fact that the nerve connections involved must form a small part of the overall reorganization of the metamorphosing pupae, suggest that even if kicking is not entirely a larval retention, its availability for use and any mediation of this use by environmental assessment are probably distributed across the greater portion of metamorphosis. If remodeling does occur and/or if other changes in the organism cause external sensitivity to indirectly ‘unplug’ from the nervous system, such disruptions can reasonably be inferred to be temporary. In familiarization

tests, such gaps, if they exist, might be detected by using a wide temporal bandwidth of test conditions or be indicated by a layer of general artefactual ‘noise’ spread across response measures. Regardless, the presence of environmental awareness in the pupae would only be redefined slightly, from across the entirety of metamorphosis, to across most of it. Here, it is sufficient to note that environmental awareness is potentially available every developmental day, but not necessarily 24h of every developmental day.

Another potential source of variability in behavior is individual differences. This possibility merits further examination. Individual differences in the magnitude of response, perception and assessment of disturbance would readily fit the patterns of variability observed. This variability is not inconsistent with different responses by individuals. The minority of pupae showed non-varying responses, most showed some degree of variability, with the majority of individual pupae altering response type a few, rather than the maximum number of times. Again, this would need further examination but, for example, a scenario in which pupae kick in their first few days, then stop kicking, then resume kicking, readily fits a model of behavior based on changing information and ongoing assessment (where assessments might change over time or be different between pupae). Thus, a ‘on-off-on’ response pattern might be produced by re-assessment of a persistent disturbance across the metamorphic timeline: repeated disturbance (without injurious outcome) causes a disturbance to become familiar (leading to inhibition of the response), but its persistence over an extended timeframe, accompanied by the finalization of metamorphic development, makes it appear more like a threat again (leading to re-expression of the response).

Considerations of kicking behavior within a combined ontogenetic and ecological context suggest that the behavior may be both anatomically and perceptually informed by larval history. The movements of the abdominal segments

Table 2 – Summary of kicking and non-kicking responses in individual pupae of *C. pomona* across metamorphosis.

	% pupae
Kick at first development day (12h after ecdysis)	100
Kick at final development day (pharate adult stage)	48
Kicking every day	28
Variability in responses (# per pupa ¹) across developmental days	
- 1	16
- 2	60
- 3	16
- 4	8
Number of days (#) in which pupae did not kick across development	
- 0	24
- 1	36
- 2	16
- 3	12
- 4	8
- 5	4
First occurrence of non-kicking among pupae by developmental day (d) ²	
- 1	0
- 2	35
- 3	12
- 4	12
- 5	12
- 6	29

¹ Number of disturbance responses types defined on a scale of 0-3. See Methods.

² Percentages calculated in terms of pupae that did not kick at least once (n = 17) as pupae that kicked are automatically excluded from the comparison of days.

involved are undoubtedly retained from the intersegmental muscle innervations of the larvae (Levine 1984; Truman 1992), although to an unknown extent. Indeed, the origins of the kicking behavior may be quite specifically connected to larval defense: many caterpillars (and other similarly shaped organisms like millipedes, worms, etc.) exhibit a flexing, twisting form of movement when they are picked up or grabbed by pincers. Specifically, *C. pomona* larvae produce a kind of sinuous jerky escape movement by looping and rapidly unlooping their bodies when picked up. Likewise, in addition to these anatomical connections to larval defensive behaviors, perceptual assessment of threat or disturbance is most efficiently achieved by the retention of some form of larval memory. This is also consistent with the preservation of the behavior across metamorphosis. However, kicking in response to disturbance does not necessarily require a means of threat assessment inherited from a previous ontogenetic stage (contact during such a vulnerable point in development may be sufficient signal of threat in and of itself or), but, again, it would certainly be advantageous for such inheritance to occur.

Memory is the most logical referential basis for a capa-

bility to assess threat or disturbance. Certainly, these observations are some way from establishing the existence of this capability, but this inference is not inconsistent with the behaviors observed. Likewise, ‘instinct’ might offer an equally plausible alternative hypothesis. Then again, ‘instinctive’ responses may find a better fit with conceptualizations of stereotyped reflex responses. Although traditionally gin-trap behaviors have been considered to be ‘reflex’ actions (Truman 1992), aspects of the behavior observed here appear to distinguish pupal kicking from such wholly reflex movements. Pupae exhibited demonstrably differentiated sensitivity: some pupal responses were elicited without even direct contact; others had to be coaxed out of pupae by repeating and increasing direct contact. Likewise, the sequence in which behaviors were modified followed a pattern consistent with habituation (behaviors mediated by previous experience). Thus, both the dampened types of response, in which kicking was either only elicited after more extensive contact, or not elicited at all (non-kicking response), were only expressed after prior expression of a more immediate kicking response, respectively: the first occurrence of behavior #3 was always im-

mediately preceded by behavior #2; and the first occurrence of behavior #0 was always immediately preceded by either behavior #1 or #2.

A mediated response is also probably more consistent within a Darwinian perspective of this kicking behavior as a defensive adaptation. This defensive role has been directly observed and documented (Hawes 2018) and under conditions of real threat, the response was actively maintained over a sustained period of time with alterations of intensity and frequency that served to delay, deter and shake off external attack. Although the behavior here can be considered as just an isolated adaptation of a single species, it occurs at a point in ontogeny in which it would seem beneficial for many holometabolous insect species to possess similar environmental ‘awareness’. If such a wider context were demonstrated, the Darwinian argument would be elevated from that of an isolated adaptation to a more prevalent adaptive component of many metamorphic lifestyles. This tallies with informal observations, but obviously a lot more comparative work is needed to develop this hypothesis.

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