

# Sex and stress modulate pupal defense response in tobacco hornworm

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## Abstract

In insects, larval and adult defenses against predators have been well studied. However, pupal (also known as resting stage) defenses have been overlooked and not examined thoroughly. Although some pupa possess antipredator strategies such as hairs, spines, cryptic coloration, and exudation of chemicals, few studies have tested these responses and the factors affecting them. Here, we investigated the behavioral responses in tobacco hornworm *Manduca sexta* that pupates in soil by introducing an external stimulus using vibrations from an electric toothbrush to mimic predation. We observed that *M. sexta* made violent wriggling (twitching), followed by pulsating movements in response to the vibrational stimulus. Detailed examination showed that these twitches and pulsating events occurred more frequently and for longer periods of time in male pupa and were dependent on the magnitude of the stress (high and low frequency). However, when we estimated the angular force exerted by pupa using radian and angular momentum of twitches, it was found to be independent of pupal sex. A follow-up experiment on possible cascading effects of stress exposure on eclosion success revealed that low- and high-frequency stress exposure didn't cause any of the common defects in eclosed adults. Our study clearly demonstrates that the so-called defenseless pupal stage uses a wide range of measurable defense behaviors that can actively defend against predators and should be examined further-linking observed behavior with underlying mechanisms.

**Key words:** hornworm, predation, pupal defenses, pupal sex, twitching.

Predator–prey dynamics are a critical component of ecosystem functioning, represented by organisms ranging from bacteria to mammals (Taylor 1990; Toth 2020; Keim et al. 2021). Insecta, the largest class of invertebrates, occupies and possibly dominates most ecosystem functions, and acts as an excellent model to study these dynamics (Ekim et al. 2017). In holometabolous insects, the 4 distinct life stages, including egg, larva, pupa, and adult, all possess different antipredator defensive strategies. Insect larvae, the most studied life stage in predator–prey dynamics, which also represent the immature stage of their life cycle, have the ability to crawl, hang, drop, hide, attack, produce chemical compounds, mimic, or produce aposematic colorations to distract predators (Edmunds 1974; Shackleton et al. 2014; Humphreys and Ruxton 2018; Sugiura 2020). In some species, larvae have been found to have a dense mat of hairs and spines on their body to protect themselves from predator mandibles and parasitoid ovipositors. For example, long and thick hairs on *Lymantria dispar japonica* (Lepidoptera: Erebididae) prevent oviposition by endoparasitoid *Meteorus pulchricornis* (Hymenoptera: Braconidae) (Kageyama and Sugiura 2016). More often observed, some larvae also exhibit cryptic body shapes and colors (Cuthill et al. 2017; Gaitonde et al. 2018), and mimic

twigs or even bird droppings (Skelhorn et al. 2010; Skelhorn 2015) to hide from predators. And, some larvae, while feeding on plants sequester chemical compounds and use them as protection from predators (Weatherstone et al. 1986; Sime 2002; Rayor et al. 2007).

In adults, the ability of flight is touted to be the most important characteristic behind their evolutionary success, and most of the adult insects have been successful in using flight to evade predators (Chai and Srygley 1990; Portman et al. 2015, 2020). Adaptive traits, for example, the presence of special hormones such as adipokinetic hormone (AKH), especially in lepidopteran moths, mobilize glycogen to constantly provide fuel in the contracting flight muscles, enhancing flight ability and performance (Marco et al. 2020). For example, it has been recorded that the female cactus moth *Cactoblastis cactorum* (Lepidoptera: Pyralidae) can fly as far as 24 km to oviposit (Zimmermann et al. 2000). In addition, adults also have compounds sequestered from their host plants at larval stage that protect them against predation (Bowers 1980, 1981; Trigo and Dos Santos 2000). For example, Bowers (1980, 1981) found that *Euphydryas* genus adults are usually not palatable to their predators due to sequestration of iridoid glycosides from host plants (Bowers 1980, 1981).

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However, in between the larval and adult stages, holometabolous insects have a semi-dormant pupal stage that is comparatively immobile and resting (Torres-Muros et al. 2016; Nakahara et al. 2020), making it possibly the most vulnerable stage against potential predators.

Despite being vulnerable, pupa has also been reported to possess behavioral, chemical, and physical defenses (Lindstedt et al. 2019). They include attempts to bypass detection by predators via cryptic coloration or camouflage, which allows the pupa to blend with its external environment (Gaitonde et al. 2018; Payra 2020). For example, the tobacco hornworm *Manduca sexta* (Lepidoptera: Sphingidae) pupates in leaf litter and under the soil surface, turning into a dark brown-colored pupa (Byron and Gillett-Kaufman 2018), seamlessly blending into soil and litter. Pupal defenses in other species also include the building of cases, presence of hairs and spines around pupa, and covering of cocoon with calcium crystals (Ferguson et al. 2018; Lindstedt et al. 2019), to name a few. For instance, the pupa of *Heliconius melpomene* (Lepidoptera: Nymphalidae) is covered in spines that are irritating to predators and can even cause allergic reactions to them (Lindstedt et al. 2019). Some pupa release chemicals that are toxic and unpalatable, making them repulsive to predators (Lindstedt et al. 2019).

In addition to these defenses, pupa can also exhibit pronounced movements sensing external stimuli (Umbers et al. 2017). This motion can have a deimatic effect, and potentially scare predators (Umbers et al. 2017). Moreover, these defensive movements (pupa rotates their abdominal region in a fast circular motion) can also make it difficult for the parasitoids to attack (Cole 1959). For example, when parasitoids attempt to lay eggs on the pupa of *M. sexta*, the pupa responds with an intense wriggle, thus prohibiting the parasitoid and parasitoid eggs from settling in or on the pupa (Cole 1959). Behavior-induced defense responses have been well studied in other life stages, specially the larval stage, of insects (Stamp 1986; Bardwell and Averill 1996; Ramirez et al. 2010). For example, Stamp (1986) studied the defensive wriggling body movements of pipevine caterpillars (*Battus hilenor*: Lepidoptera; Papilionidae) against invertebrate predators (Stamp 1986). However, there has been a disproportionate bias in understanding pupal defenses where most studies have focused on either larval or adult defense traits (Kariyat and Portman 2016; Singh et al. 2021). More importantly, few studies have dissected the magnitude of response to different stresses and post-stress effects, if any. To further complicate these defense traits, more often in the case of Lepidoptera, females have been found to be heavier than males, and tend to eat more and accumulate more protein and carbohydrates, clearly suggesting a higher resource investment in females (Telang et al. 2001). Moreover, males also compete for mating rights, which also comes at an additional physiological cost. Therefore, to attract females, they have to be more attractive, produce different chemical compounds, or spend their energy on producing elaborate behavioral modifications to be attractive to females, which can potentially make them less defended than females (Folstad and Karter 1992; McKean and Nunney 2001). Therefore, it is plausible to expect that stress response might also be disproportionately affected by the sex of the pupae.

Inspired by the recent review by Lindstedt, Murphy, and Mappes on pupal defenses (Lindstedt et al. 2019) and the call to empirically test them, we investigated pupal defenses and

possible factors affecting them in male and female pupae of *M. sexta*. Specifically, we hypothesized that *M. sexta* female pupa will elicit a stronger response by producing more twitching and pulsations against any induced stress in comparison to male pupa, and this response will depend on the magnitude of the stress induced. We speculate that the higher the external stimuli, the stronger will be the defensive response, corresponding to intense wriggle. Moreover, we also measured the angular force of pupal defense response, as an additional measure of sex-based differential investment and possible defense trade-offs. We used *M. sexta*, since it is a model herbivore in Lepidoptera extensively used for behavioral (Kariyat et al. 2017, 2019), physiological (Portman et al. 2015), and genetic studies. We used electric toothbrushes to create vibrational (Tayal et al. 2020a; Tayal and Kariyat 2021) as well as mechanical stimuli to mimic predators (similar to Koperski 1997), where electromagnetic vibration excitation was used to create pupal vibrations to evoke response. A total of ~180 *M. sexta* pupae were used to measure abdominal movements/wriggling (twitching), and pupal pulsations were recorded as response variables.

## Materials and Methods

### Model insect

Tobacco hornworm eggs were purchased from Great Lake Hornworm Ltd. Romeo, MI, USA, and placed in a Petri dish on an artificial diet under laboratory conditions at 25 °C and 65% relative humidity (; Tayal et al. 2020c). Newly hatched caterpillars were allowed to feed on an artificial diet until they were ready to pupate (Singh and Kariyat 2020; Tayal et al. 2020a). Pupated caterpillars were sexed based on their genital aperture and anus. Afterward, they were kept in pop-up cages (609.6 × 609.6 × 914.4 mm, Biogentex Laboratories, Inc., TX, USA), and used for the experiments as required. The pupal stage is ~18–21 days in *M. sexta*, after which adults eclose. We used only pupae that had at least 10 more days until eclosion, and were healthy. A total of ~180 pupae were used for the experiments.

### Experiment methodology

We used an electric toothbrush (Tayal et al. 2020a; Tayal and Kariyat 2021) to stimulate predation by using 2 different vibration frequencies to gauge the intensity of the *M. sexta* pupal defense response. We used the electric toothbrush to simulate the stress conditions and mimic the stress induced by the predators or due to environmental conditions. Before the experiment, each pupa (separated based on sex) was subjected to mass, length, and width (to calculate pupal volume— $\pi \times r^2 \times l$ ; where  $r$  is the radius of the pupa and  $l$  is the length of the pupa assuming pupal shape as a cylinder) measurements. Utmost care was taken to allow only minimal disturbance to the pupa while conducting these measurements.

To perform the experiments, each pupa was laid flat on an 21.59" × 27.94" sheet of white paper on a lab bench with the proboscis facing up and was gently taped using scotch tape to keep them in place to record the data. Fifteen male and 15 female pupae were then exposed to a high-frequency stimulus of 333 Hz using an electric toothbrush (Colgate 360 powered toothbrush, Colgate Co. Pvt. Ltd; Tayal et al. 2020a). To induce the stimulus, the toothbrush was turned on and the bristle head was allowed to gently touch the pupal abdomen for 3 s. After 3 s, the pupae's horizontal movements (twitches)

were recorded for 1 min. Data on the number of horizontal twitches as well as the length of the shortest and longest horizontal twitches was recorded (Supplementary Video 1). In addition, the time taken by the pupae (in seconds) to complete the first twitch, and the number of in-and-out pulsing movements (Supplementary Video 2) post-stimuli were also recorded. The pupae were then allowed to rest for 24 h before testing them again; this time using the low-frequency stimulus (15 male and 15 female pupae; 233 Hz; Oral-B 3d White Action Power Toothbrush; Tayal et al. 2020a), and similar recordings were carried out. Control pupae (not exposed to the stress treatments) were separately maintained by gently taping them on paper to record the same measurements as in stressed pupa; 22 males and 18 females.

In addition to these parameters, we also estimated the angular force exerted by the pupal twitch right after the stimulus (force of the first twitch). To estimate this, the pupae (21 male and 20 female pupae) were fastened in a similar fashion as in previous experiments, and different parameters were used to measure the force (angle of the first twitch, time to complete the first twitch, and length of twitch) were recorded. As pupal twitch is similar to the movement of a pendulum, we used the following equations to measure radian, angular acceleration, and, finally, the force. Similarly, angular force was measured for control pupa by taping them on a paper to measure the parameters used for calculating angular force as mentioned above.

$F = ma$ , where  $m$  = Pupal mass and  $a$  (angular acceleration)  
 $= \omega/t$ , where  $t$  = time,  $\omega$  = radian/ $d$ , where  
 $d$  = distance and radian  $= \theta/180$ .

$$m = \text{Pupal mass}; a = \text{angular acceleration}; \text{radian} \\ = \frac{\theta}{180}; \omega = \frac{\text{radian}}{d}; a = \frac{\omega}{t};$$

$$F = ma.$$

Following the experiments, all stressed pupae were placed back in pop-up cages separately based on sex and treatments induced, and allowed to successfully eclose. After eclosion, we examined whether these stimuli (stress) affected their eclosion success, a common concern in *M. sexta* adults. Similarly, control pupae (which were not exposed to stress treatment) were also placed in separate cages on basis of sex (male/female) to record their eclosion success.

### Statistical analysis

Pupal length, mass, and volume data were normally distributed and were analyzed using unpaired *t*-tests. Data for time to start twitching, number of twitches, twitch length, time of pulsations post-stress, and number of pulses based on the sex (male and female) and frequency (high and low) were not normally distributed. These datasets were analyzed using generalized regression with Poisson distribution for a number of pulses and twitches; gamma for pupal response time, and time to start twitching, and multiple pairwise comparisons were carried out using Tukey. Data of pupal force parameters (length and time of 1 complete twitch and radian and angular force of pupae) were normally distributed and analyzed using unpaired *t*-tests. Data of the proportion of pupae successfully eclosed were analyzed with generalized regression and binomial distribution. All data analyses were carried out using

SAS JMP (SAS Inc, NC, USA) version 13.0, and plots were made using GraphPad prism version 8.0 (La Jolla, CA, USA).

## Results

### *M. sexta* pupal length, mass, and volume

We found that female pupae were significantly longer than male pupae (Unpaired *t*-test;  $t = 3.597$ ;  $P = 0.0004$ ; Figure 1A). Moreover, female pupae were also significantly heavier than male pupae (*t*-test;  $t = 4.442$ ;  $P < 0.0001$ ; Figure 1B), but there was no significant difference in the volume of male and female pupae (unpaired *t*-test;  $t = 1.367$ ;  $P = 0.1732$ ; Figure 1C). Clearly, the female pupae were larger/had increased allocation toward body size when compared to males from the same population.

### *M. sexta* pupal defense parameters

#### Time to start twitching

We then examined whether there are any differences in time to first twitch based on the sex (male and female) of pupae and stress frequency (low/high). Our results show that there is no significant difference in time taken to the first twitch between male and female pupae (generalized regression; gamma distribution; Wald Chi-square = 2.81;  $P = 0.0935$ ; Figure 2A). However, the time taken by pupae for the first twitch was significantly longer on lower frequency than higher frequency stress conditions (generalized regression; gamma distribution; Wald Chi-square = 9.97;  $P = 0.0016$ ; Figure 2B). In other words, the pupae responded faster to stronger stress, in this case, a higher frequency.

#### Number of twitches

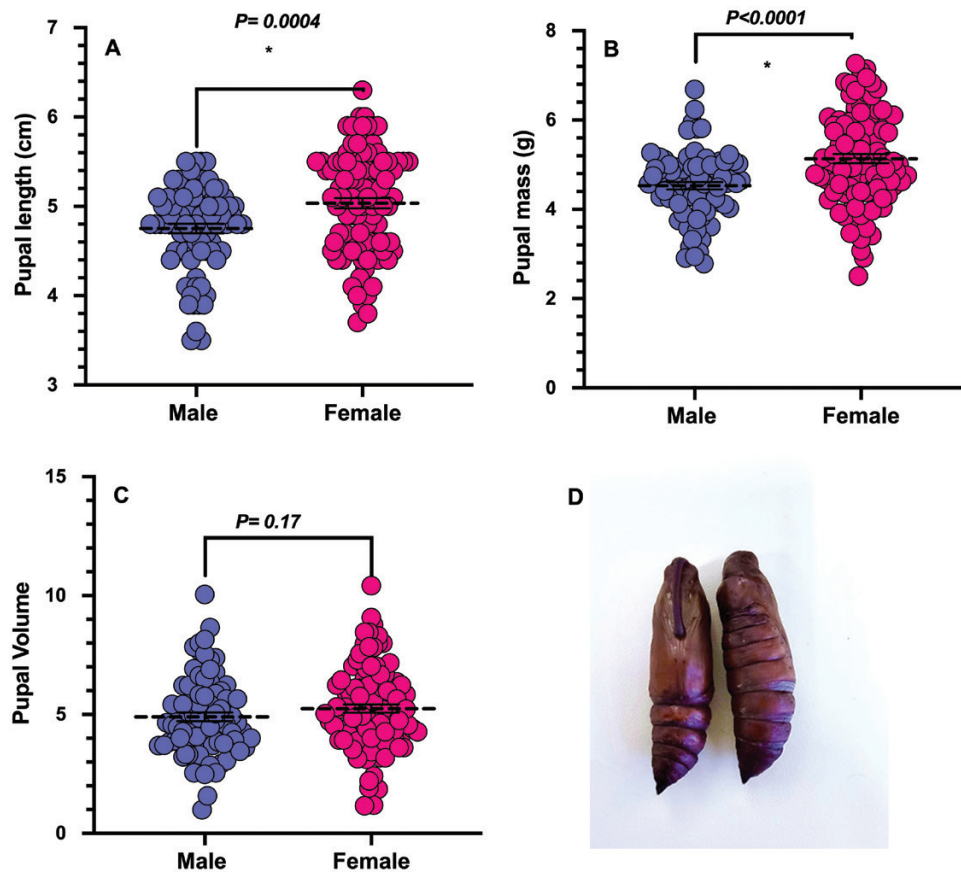
Our results show that post-stress, the number of twitches was significantly higher in male pupae in comparison to female pupae (generalized regression; Poisson distribution; Wald Chi-square = 13.27;  $P = 0.0003$ ; Figure 2C). Moreover, the number of twitches in pupae exposed to lower frequency was significantly higher than in pupae exposed to higher frequency stress (generalized regression; Poisson distribution; Wald Chi-square = 6.21;  $P = 0.0127$ ; Figure 2D).

#### Pupal twitch length

We found that twitch length (movement of pupae from one end point to another, i.e. similar to the movement of pendulum; Supplementary Figure 2) of male pupae was significantly longer than female pupae (generalized regression; Poisson distribution; Wald Chi-square = 4.49;  $P = 0.0341$ ; Figure 2E), but there was no significant difference in the twitch length of pupae under lower or higher frequency stress conditions (generalized regression; Poisson distribution; Wald Chi-square = 0.77;  $P = 0.3779$ ; Figure 2F).

#### Pupal response time and post-stress pulsation behavior

Post-stress, we also examined the total time for which pupae responded by twitching before becoming inert again, and the number of pulses (see Supplementary Video 2) based on their sex and frequency of stress. We found that males responded significantly longer than female pupae (generalized regression; gamma distribution; Wald Chi-square = 4.98;  $P = 0.0256$ ; Figure 2G), and the number of pulses was also significantly higher in male pupae than female pupae (generalized



**Figure 1** The results of the  $t$ -test analysis (unpaired) of tobacco hornworm (*Manduca sexta*) male and female (A) pupal mass ( $P = 0.0004$ ), (B) length ( $P < 0.0001$ ), and (C) volume ( $P = 0.17$ ). Asterisk shows significant results at  $P < 0.05$ .

regression; Poisson distribution; Wald Chi-square = 47.86;  $P < 0.0001$ ; [Figure 2I](#)). Also, pupae responded significantly longer when exposed to lower frequency than higher frequency stress (generalized regression; gamma distribution; Wald Chi-square = 8.45;  $P = 0.0037$ ; [Figure 2H](#)), and the number of pulses was also significantly more on lower frequency than on higher frequency stress (generalized regression; Poisson distribution; Wald Chi-square = 203.1;  $P < 0.0001$ ; [Figure 2J](#)).

### *M. sexta* pupal force parameters

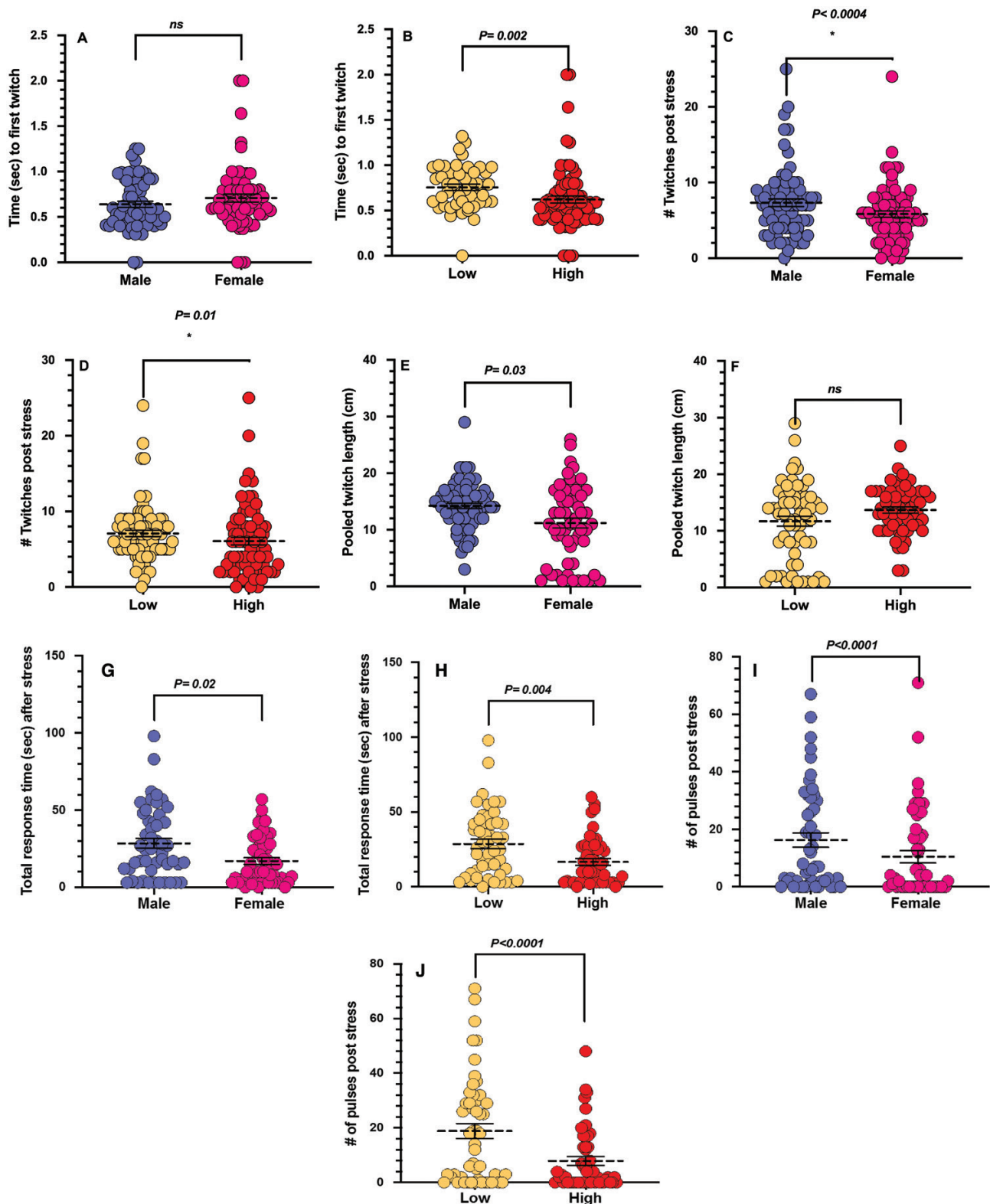
We measured the mass and length of male and female pupae to study force parameters and our results show that female pupal length (unpaired  $t$ -test;  $t = 2.291$ ;  $P = 0.0279$ ; [Supplementary Figure 1A](#)) and female pupal mass (unpaired  $t$ -test;  $t = 4.008$ ;  $P = 0.0003$ ; [Supplementary Figure 1B](#)) were significantly higher than male pupae. Additionally, we analyzed pupal force parameters of twitch for both sexes. We found that the twitch length of female pupae was higher than the twitch length of male pupae (unpaired  $t$ -test;  $t = 2.18$ ;  $P = 0.0353$ ; [Figure 3A](#)). However, there was no significant effect of stress on male and female pupae for the time to complete a twitch post-stress (unpaired  $t$ -test;  $t = 1.193$ ;  $P = 0.0638$ ; [Figure 3B](#)), or the radian (angle) of twitch (unpaired  $t$ -test;  $t = 1.159$ ;  $P = 0.2541$ ; [Figure 3C](#)), and consequently, the angular force (unpaired  $t$ -test;  $t = 0.3743$ ;  $P = 0.7104$ ; [Figure 3D](#)) produced by the twitch. Collectively, we didn't detect any significant sex differences in the force exerted by a twitch, although male pupae had a significantly longer response and more pulsations post-stress.

### *M. sexta* pupal eclosion

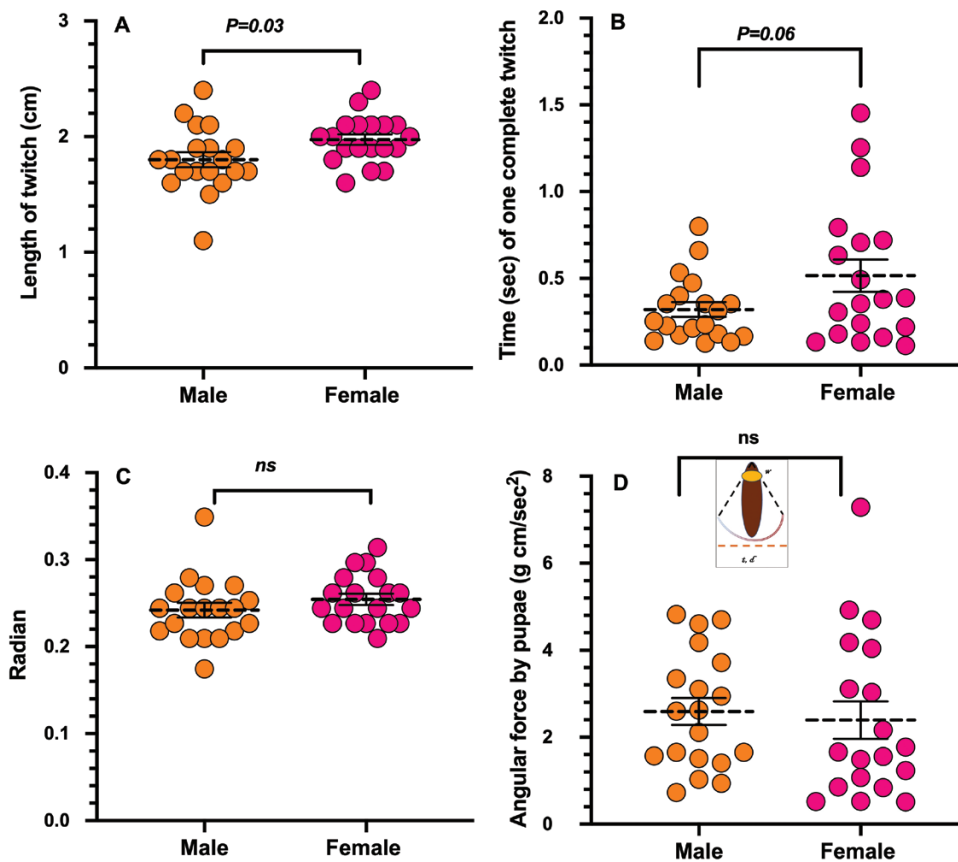
We also examined whether the stress conditions introduced at pupal stages had any effect on pupal eclosion. We analyzed the proportion of pupae eclosed from stressed and control groups and found that there was no significant effect of stress on eclosion success (deformed wings, mortality, or inability to fly and/or feed) of pupae. The proportion of stressed and control, and male and female eclosed were 71.6% and 64.6% and 59.3% and 57.1%, respectively ([Figure 4](#)). Clearly, a short exposure to two different stress regimes did not affect eclosion success.

### Discussion

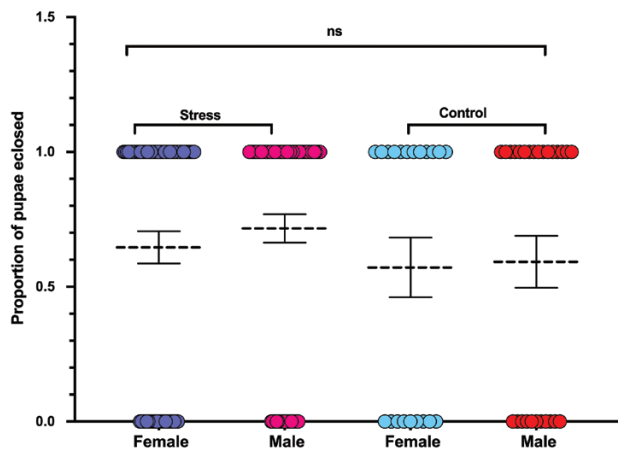
Despite the presence of other defenses including hairs, spines camouflage, and chemical defenses, pupal twitching/wriggling in response to external stimuli is an important pupal defense behavior ([Chandrasegaran et al. 2018](#); [Lindstedt et al. 2019](#); [Atijegbe et al. 2020](#)). In this study, we demonstrate that the response of *M. sexta* pupae varies under differing stress conditions and sex. Altogether, we found that low-frequency stress caused more twitching and pulsating than high-frequency stress, which was delivered by the electric toothbrush. Moreover, stress induced more twitching and pulsating response in males than female pupae. However, male and female pupae produced similar force upon introducing stress regardless of female pupae having more mass than males, thereby effectively nullifying any notion of higher investment in defense traits by female ([Telang et al. 2001](#)).



**Figure 2** (A and B) The results of generalized regression analysis of time (in seconds) taken by tobacco hornworm (*Manduca sexta*) pupae to first twitch after introduction of stress (A) in male and female pupae ( $P = 0.0935$ ) and (B) at low and high frequency ( $P = 0.002$ ). Asterisk shows significant results. (C and D) The results of generalized regression analysis of number of twitches post-stress in tobacco hornworm (*M. sexta*) pupae (C) in male and female pupae ( $P < 0.0004$ ) and (D) at low and high frequency ( $P = 0.01$ ). Asterisk shows significant results at  $P < 0.05$ . (E and F) The results of generalized regression analysis representing pooled twitch length post-stress in tobacco hornworm (*M. sexta*) pupae (E) in male and female pupae ( $P = 0.03$ ) and (F) at low and high frequency. Asterisk shows significant results. (G and H) The results of generalized regression analysis representing total response time (in seconds) post-stress in tobacco hornworm (*M. sexta*) pupae (G) in male and female pupae ( $P = 0.02$ ) and (H) at low and high frequency ( $P = 0.004$ ). Asterisk shows significant results at  $P < 0.05$ . (I and J) The results of generalized regression analysis of number of pulses post-stress in tobacco hornworm (*M. sexta*) pupae (I) in male and female pupae ( $P < 0.0001$ ) and (J) at low and high frequency ( $P < 0.0001$ ). Asterisk shows significant results at  $P < 0.05$ .



**Figure 3** The results of *t*-test analysis (unpaired) of tobacco hornworm (*Manduca sexta*) male and female pupae (A) length of twitch (in cm;  $P = 0.03$ ), (B) time of one complete twitch (in seconds;  $P = 0.06$ ), (C) radian (angle) of twitch ( $P = 0.2541$ ) and (D) time of 1 complete twitch (in g cm/s;  $P = 0.7104$ ) after stress. Asterisk shows significant results.



**Figure 4** *t*-Test analysis (unpaired) of tobacco hornworm (*Manduca sexta*) male and female pupae enclosed in both treatments, that is, stress and control.

We found that the time taken by *M. sexta* pupae for its first twitch was significantly longer at a lower frequency (our proxy for a stress of lower magnitude), possibly due to a higher latency in response (Figure 2B). Interestingly, more twitches as well as more pulsating movements were observed at low stress (Figure 2D, J), and that too were longer at low stress when compared to our high-stress treatment (Figure 2F, H). We speculate that the stronger reaction of pupae at low stress conditions in the form of abdominal movements is akin

to their response against pupal predators, as pupae are mostly encountered by small predators such as ground beetles and ants (Elkinton and Liebhold 1990). This stress was induced for 3 s, so it would be interesting to test if there are any differences in the defense response and its later fitness consequences if they were exposed to stronger and/or longer duration of stimuli. Previously, in *M. sexta*, we have demonstrated that low-quality diet can lead to reduced growth and development in larval stages with such effects cascading to adults and even to the next generation (Lakes-Harlan et al. 1991; Tayal et al. 2020a, 2020b, 2020c). Interestingly, we found no evidence for any such cascading effects on eclosion success, possibly because we only exposed the pupae for a few seconds of stress instead of prolonged and continuous exposure.

Antennae and setae (hairs) present in the abdomen cavity of pupae have been found to function as mechanoreceptors to external stimulus (Lakes-Harlan et al. 1991). This external stimulus can originate from physical presence as well as vibrational signals emitted by enemies which induce anti-predatory behavior (Chandrasegaran et al. 2020), leading to abdominal movements commonly known as sinusoidal wriggling and lateral bending/up-down forth motion (pulsating events). These responses are a direct result of neuronal activation that culminates in the movement of abdominal segments, turning their dorsum away from the enemy (Ichikawa and Sakamoto 2013). In our study, the observed wriggling and pulsating movement in *M. sexta* pupae upon the introduction of vibrational signals to the pupal abdomen for 3 s were also an attempt of pupae to turn themselves (by turning their

dorsum) away in the wake of an enemy or any warning signals. These movements act as an important defense strategy against predators. Previous studies have been documented in the pupae of other species which undergo similar wriggling motion against predators such as Kiwi, Weka, and Rock wren. For instance, Atijebde et al. (2020) observed vibrational stimuli-activated defenses in *Wiseana viette* (Lepidoptera: Hepialidae) pupae; similar behavior was also observed in *Zophobas atratus* (Coleoptera: Tenebrionidae) beetle pupae (Grosman et al. 2008; Ichikawa et al. 2012; Ichikawa and Sakamoto 2013).

In addition, we also imposed the same type of stress on male and female pupae to understand if there are any sex differences in mechanical pupal defenses. The male and female pupae used for our experiment differed significantly in pupal length and mass with female pupae being heavier and longer (Figure 1A, B) but having similar pupal volume. We found that males twitched longer, had a larger twitching length, and were compounded with more twitches post-stress pulses when compared to females. Grossman et al. (2008) observed 15% more violent head swing movement on pupae that were better defended against predators, suggesting that the more twitches, the stronger the pupal defense mechanism is, as observed in this study as well. It is well known that resource allocation among the three broad activities, that is, growth, reproduction, and defense (Neilson et al. 2013), can lead to trade-offs. For instance, green-colored pupae of speckled wood butterfly *Pararge aegeria* (Lepidoptera: Nymphalidae), although more vulnerable when compared to brown pupae, become bigger adults with more thorax mass, compensating for the lower pupal defense (Van Dyck et al. 1998). Similarly, pupal behavioral defenses in mosquito *Aedes aegypti* (Diptera: Culicidae) have a cost of lower energy available for development and metamorphosis (Lakes-Harlan et al. 1991). In our study, we speculate that the stronger response exhibited by males could be because female lepidopteran invests more in reproduction, with a possible trade-off in defense during their pupal stage (Rutowski 1982).

In addition to these responses, we also computed the magnitude of angular force exerted by the pupae to get a sense of how powerful the twitch was, and whether pupal sex affected this force. Since the stress simulated predation, the force exerted by the pupae in response would directly fend off the predator, ultimately pupal survival. We estimated the force of the first twitch using the radian of angular movement along with the velocity of movement. Interestingly, regardless of the stress magnitude, male and female pupae exerted similar force to defend, although females were heavier and longer. Clearly, smaller sized males generated more angular acceleration, thereby compensating for their lower mass. However, we do acknowledge that force measurement could be refined using more precise tools, since the swift movement of pupae made it difficult to calculate angular velocity precisely in our studies.

Taken together, we conclude that *M. sexta* pupae are not helpless but actively respond against stressors. More specifically, their response is both stress magnitude and sex dependent. A short exposure to stress regardless of magnitude has no impact on adult eclosion rate and did not lead to deformities in adults. Future studies should be focused on dissecting the effects of early versus late pupal stages, and testing varying levels of stresses in both quantity and quality, and their effects on traits such as growth and reproduction. Our study also demonstrates that pupal defenses should be examined further

focusing on linking behavioral responses and defense traits with underlying mechanisms, and then expanding to orders beyond Lepidoptera.

## Conflict of Interest Statement

Authors report no conflict of interest.

## Supplementary Material

Supplementary material can be found at <https://academic.oup.com/cz>.

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