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PHYSIOLOGY OF INSECT ECDYSIS: NEURAL AND HORMONAL FACTORS INVOLVED IN WING-SPREADING BEHAVIOUR OF MOTHS

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SUMMARY

1. In the tobacco hornworm, wing spreading involves a stereotyped series of movements which take 74 min to complete and which include two components, wing folding and wing inflation.

2. Moths decapitated at the moment of eclosion show neither wing inflation nor wing folding. If decapitation is delayed until 5 sec after emergence, then the full wing-spreading behaviour is displayed.

3. Newly emerged moths which are confined show intense digging-behaviour and delay the onset of wing spreading until after their release. Decapitated moths attempt to spread their wings immediately, regardless of whether or not they are confined.

4. Surgical experiments showed that the brain was not required in a neural capacity in order for wing spreading to occur; it was needed only as a source of the eclosion hormone. The neural influence of the suboesophageal ganglion was required until immediately after eclosion.

5. Newly emerged moths whose abdomens had been removed showed wing-folding behaviour. No inflation occurred and the duration of wing folding was much longer than normal. It was concluded that wing-folding behaviour was centrally programmed but that the abdomen could modify the length of the programme.

6. Injections of bursicon into abdomenless moths reduced the duration of wing-folding behaviour to almost normal levels. Therefore, in some manner, bursicon has an important role in determining when wing-folding behaviour will come to an end.

INTRODUCTION

The escape of the moth from the pupal skin is accomplished through a succession of behaviours which has been termed the 'emergence sequence' (Truman, 1971). This includes the pre-eclosion behaviour, eclosion, post-ecdysis activity, and wing spreading. The progression of the moth through this sequence occurs through a complex series of neural and endocrine events. The first two behaviours are generated by a pre-patterned motor programme built into the abdominal ganglia (Truman and Sokolove, 1972). They are triggered in response to the 'eclosion hormone' which is released from the brain of the pharate moth (Truman, 1971, 1973*a*). Besides the

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eclosion hormone there is a second hormone, bursicon, which is released from the abdominal ganglia during the last part of the emergence sequence (Truman, 1973*b*). This hormone is homologous to the tanning hormone of Diptera (Cottrell, 1962*a*, *b*; Fraenkel and Hsiao, 1962, 1965) since it is responsible for the tanning of the newly spread wings of the moth.

The present paper describes the neural basis of the last portions of the emergence sequence and examines the respective roles of the eclosion hormone and of bursicon in the performance of these behaviours.

MATERIALS AND METHODS

1. *Experimental animals*

The tobacco hornworms, *Manduca sexta*, used in these experiments were derived from a stock obtained from Dr R. A. Bell, A.R.S., U.S.D.A., Fargo, N.D. The rearing of the animals was as described in Truman (1972). Two species of saturniid moths, *Hyalophora cecropia* and *Antheraea pernyi*, were also used for certain experiments. Diapausing pupae of these species were purchased from dealers.

2. *Preparation and injection of homogenates which have bursicon activity*

The abdominal nerve-cord of the pharate adult hornworm is a potent source of bursicon activity (Truman, 1973*b*). Nerve cords were removed from pharate moths, rinsed in saline (Ephrussi & Beadle, 1936), and stored at −5 °C. A few crystals of phenylthiourea were added to prevent blackening (Williams, 1952). When needed, the nerve cords were rapidly thawed and homogenized in saline (20 μl per nerve cord). Routinely, 20 μl of homogenate was injected through the thoracic tergum of each moth by means of a 100 μl syringe.

3. *Surgical methods*

Brain removal and implantation were performed using the techniques described in Williams (1952). The removal of the suboesophageal ganglion was according to the same general procedure. The methods of decapitation are described at the appropriate places in the Results.

RESULTS

A. *The emergence sequence of the tobacco hornworm*

At the onset of eclosion the moth ruptures the pupal cuticle along the ecdysial lines by means of violent 'shrugging' of the wing bases. During the next few seconds, it pushes off the mask of pupal cuticle which covers its head and legs and sheds the remainder of the pupal skin through peristaltic contractions of the abdomen. It then coils up its mouthparts (proboscis) and searches for a proper wing-spreading site. Under natural conditions pupation and subsequent adult development take place in the soil. The newly emerged moth must then dig to the surface before wing spreading is possible. Consequently, hours may elapse between eclosion and the beginning of wing-spreading behaviour. In this study, unless otherwise stated, moths were placed in paper bags immediately after eclosion. Wing spreading typically began within a matter of minutes thereafter.

Table 1. *The effects of the time of decapitation on wing spreading of* M. sexta *adults*

Time of decapitation (seconds after eclosion)	Number	% folding wings	% inflating wings	Av. time to end of wing folding (min ± S.D.) With inflation	Without inflation
Eclosion (0)	20	5	0	—	104
Before proboscis coiling (1–5)	12	100	33	78 ± 6	131 ± 25
After proboscis coiling	12	100	100	82 ± 10	—
After coming to rest (> 15)	5	100	100	73 ± 5	—

Wing-spreading behaviour can be divided into two separate components: wing inflation and wing folding. Wing inflation consists of the expansion of the wings to adult size and is accomplished through a tonic contraction of the abdomen which forces blood into the wings. Wing folding comprises a stereotyped series of wing movements. These are produced by the thoracic muscles and occur during the inflation process.

Wing spreading always began within 15 min after the newly emerged moth was placed in the bag. At this time the wings, which had been held close along the sides of the body, were rotated dorso-laterally away from the body (Fig. 1 *a*, Plate 1). Shortly thereafter wing inflation began, and by 30 min after eclosion the fully expanded wings were held straight behind the body in the 'butterfly' position (Fig. 1 *b*, Plate 1). Approximately 45 min later the wings were rapidly rotated at their bases by approximately 130° and brought to rest in a tent-like fashion over the abdomen (the 'moth' position) (Fig. 1 *c*, Plate 1). Wing-spreading behaviour was stereotyped. In 20 moths, the total time between eclosion and folding the wings into the moth position was 74 minutes (S.D. ± 6).

B. *Effects of surgical manipulations on wing spreading*

1. *Decapitation of emerging moths*

The role of the head in the wing-spreading sequence was determined by decapitating adult *Manduca* at various times during and after eclosion. A haemostat was clamped on the neck of the moth, the head was cut off, and the animal was suspended from the haemostat. Twenty eclosing moths were decapitated just before they pushed off the pupal 'mask'; the remainder of the pupal exuviae was then manually removed. As seen in Table 1, only one moth displayed wing-folding behaviour and none showed any sign of wing inflation.

When moths were decapitated a few seconds later, between shedding of the exuviae and proboscis coiling, the results were quite different. All showed wing folding, but only a few (33%) also inflated their wings. When decapitation was delayed until 5 sec after eclosion (after proboscis coiling had occurred) then all moths showed both folding and inflation (Table 1; Fig. 1 *d–f*, Plate 1).

Table 1 also shows that the duration of the wing-folding behaviour was markedly influenced by wing inflation. Those decapitated moths which inflated their wings took usual amount of time for the wing-folding sequence. By contrast, in the moths

Table 2. *The effect of various operations on the wing-spreading behaviour of silkmoth adults*

Operation	Species	Number	% spreading wings
Brain removed	*A. pernyi*	17	0
	H. cecropia	18	0
Brain removed, implanted into abdomen	*A. pernyi*	15	33
	H. cecropia	18	55
Suboesophageal ganglion removed	*A. pernyi*	10	0
	H. cecropia	20	0
Suboesophageal ganglion removed, implanted into abdomen	*H. cecropia*	5	0
Ventral nerve-cord transected behind suboesophageal ganglion	*H. cecropia*	10	0

which did not show wing inflation, the time required for the completion of wing folding was much longer than normal.

2. *Decapitation and confinement of moths*

In *M. sexta*, bursicon release and wing spreading can be delayed for a number of hours when the newly emerged moth is confined in a glass vial and forced to dig (Truman, 1973*b*). It was therefore of interest to determine whether a decapitated moth would respond to confinement in a similar fashion. Twenty moths were decapitated within 5–10 sec after eclosion, the haemostats removed, and the moths placed in vials. All animals bled considerably. Three individuals showed sporadic digging-movements and never spread their wings, but the other 17 moths showed clear wing-spreading behaviour. These animals folded their partially spread and crumpled wings to the moth position at 65 min (S.D. $\pm$ 5) after eclosion. Therefore, in the confined moth the head is necessary to promote digging behaviour and to inhibit the behaviour involved in wing spreading.

3. *Removal of the brain and suboesophageal ganglion*

In our experience *Manduca* which have had their brain or suboesophageal ganglion removed as pupae are usually unable to shed the pupal skin. Consequently, we turned to the giant silkmoths for this portion of the study. Ganglia were removed from each insect early in adult development and the ability of the resulting moth to spread its wings was then determined. As seen in Table 2, none of the debrained *H. cecropia* or *A. pernyi* moths showed wing-spreading behaviour.

The effect of brain extirpation could be due to the removal of neural circuits which are necessary for wing spreading, the absence of the eclosion hormone, or to both. In order to distinguish between these possibilities, brains were removed from pupae and a brain was then implanted into the abdomen of each. Approximately 40 percent of the resulting 'loose-brain' moths showed complete wing-spreading (Table 2).

The effect of the removal of the suboesophageal ganglion was also examined. In no case did a moth which lacked this ganglion spread its wings (Table 2). Implantation of a suboesophageal ganglion into the abdominal haemocoel of an operated animal was without effect. As seen in Table 2, transection of the ventral nerve-cord posterior to the suboesophageal ganglion also served to abolish wing-spreading behaviour. Th

Table 3. *Effect of bursicon injections on the wing-spreading behaviour of* M. sexta *which were confined in glass vials immediately after emergence*

Treatment (in addition to confinement for 90 min)	Number	% folding wings while confined	% inflating wings while confined	Av. duration of wing folding after release (min ± S.D.)
None	5	0	0	71 ± 3
Injected with saline	5	0	0	73 ± 4
Injected with bursicon	11	0	82	75 ± 13

Table 4. *The effect of bursicon injections on the wing-spreading behaviour of decapitated* M. sexta

Treatment	Number	% folding wings	% inflating wings	Av. time to end of wing folding (min ± S.D.)
Decapitated at eclosion; injected with bursicon	4	0	0	—
Decapitated before proboscis coiling	12	100	33	113 ± 32
Decapitated before proboscis coiling; injected with bursicon	8	100	88	83 ± 17

results indicate that neural influences from the suboesophageal ganglion are also involved in wing-spreading behaviour.

4. *Removal of the abdomen*

The abdomen was severed from each of eleven *Manduca* within 10 to 15 sec after eclosion. Each animal was then supplied with a suitable wing-spreading site. Ten of these moths subsequently performed the wing-folding sequence, but the duration of the sequence was much longer (145 min S.D. ± 15) than that seen in intact moths (see Table 5). Obviously, wing inflation was not observed in these animals.

C. *Injection of bursicon*

1. *Effects on 'digging' moths*

As indicated above, confinement of the newly emerged hornworm promotes digging behaviour and delays both bursicon release and the onset of wing spreading (Truman, 1973*b*). In order to investigate a possible role of bursicon in ending digging behaviour and in initiating wing spreading, newly emerged moths were injected with bursicon and placed in glass vials. These moths displayed intense digging-behaviour throughout their 1·5 h confinement, and none showed wing-folding behaviour (Table 3). When finally released, all performed the wing-folding sequence in a time which was only slightly greater than that seen in the controls.

It was of interest that 9 out of 11 of these animals showed partially inflated wings at the time of release from the vial. This result was probably due to the action of bursicon to increase cuticle elasticity (Cottrell, 1962*d*; see Discussion), coupled with the increased haemolymph pressure generated by the digging movements of the abdomen.

Table 5. *The effect of injections of bursicon and the removal of the abdomen on the wing-spreading behaviour of* M. sexta

Treatment	Number	% folding wings	% inflating wings	Av. time to end of wing folding (min ± S.D.)
None	20	100	100	74 ± 6
Abdomen removed	11	91	0	145 ± 15
Abdomen removed; moth injected with saline	5	100	0	154 ± 17
Abdomen removed; moth injected with bursicon	15	100	0	97 ± 16

2. *Effects on decapitated moths*

In a further investigation of the role of bursicon in wing spreading, we decapitated moths which were in the process of eclosion and then immediately injected them with bursicon. The injection caused neither wing inflation nor wing folding (Table 4).

In another experiment moths were decapitated within the few seconds between eclosion and proboscis coiling and then injected with bursicon. Seven out of eight of these moths showed at least partial wing-inflation. Additionally, the duration of the wing-folding behaviour in this group was significantly shorter ($0{\cdot}05 > P > 0{\cdot}02$; *t*-test) than that seen in decapitated controls (Table 4).

3. *Effects on abdomenless moths*

The experiments on abdomenless moths showed that some form of feedback from the abdomen was required for the normal timing of wing folding. The data above (Table 4) indicate that bursicon might contribute to this feedback. This possibility was tested by injecting abdomenless moths with bursicon. As seen in Table 5, the time required for wing folding was shorter in the bursicon-injected individuals than in moths which had been injected with only saline ($P < 0{\cdot}01$; *t*-test).

DISCUSSION

1. *External and internal factors controlling the expression of wing-spreading behaviour*

In the higher Diptera expansion of the cuticle of the newly emerged fly is accomplished by swallowing air into the gut and by muscular contractions of the abdomen (Cottrell, 1962*c*). If expansion is prevented either by deflating the gut (Fraenkel, 1935) or by blocking the proboscis (Cottrell, 1962*c*), then the pharyngeal and abdominal pumping still occur, and they follow a time-course which is very similar to that seen in the intact fly. Thus, the muscular contractions which bring about expansion appear to be caused by a central neural programme which is largely independent of sensory-feedback stimuli. However, sensory information can produce some minor modifications in this programme (Cottrell, 1964).

As described above, in *M. sexta* wing-spreading behaviour has two separate components: wing folding and wing inflation. The former involves the thoracic movements which progressively shift the position of the wings. The latter consists of the expansion of the wings with haemolymph and is caused by a tonic contraction of the

abdomen. In this species swallowing of air appears to be of little importance for the wing-spreading process. The two components are intimately associated in the intact animal, but the decapitation experiments show that they are triggered separately around the time of proboscis coiling. This latter event was originally selected by us because in the newly emerged moth it was the only early behaviour which could be readily distinguished. Its occurrence between the times of initiation of wing inflation and of wing folding is probably fortuitous.

The wing-folding behaviour of *Manduca* appears to be centrally patterned into the thoracic ganglia. This was indicated because it occurred in decapitated moths and by the fact that abdomenless moths performed wing-folding behaviour even though their wings did not inflate. But since these animals required twice as long as did intact moths, the duration of the wing-folding programme can be substantially influenced by the abdomen. It is of interest that this influence is not mediated centrally through the ventral nerve-cord. Peripheral stimuli due to the stretching of the wings are also ruled out because the bursicon-injected moths in Table 5 showed no wing inflation. Rather, the duration of the wing-folding behaviour is shortened primarily through the action of the bursicon which is liberated into the blood from the abdominal nerve-cord. The manner by which bursicon exerts this effect is unknown. The hormone could act directly on the neural centres in the thoracic ganglia, or it could cause peripheral changes in the cuticle which are then transmitted neurally to the ganglia. Since intact moths rotate their wings to the moth position at a time when bursicon has caused hardening of the wings, we think the latter mode-of-action is more likely.

Besides its influence on wing-folding behaviour, bursicon is important for wing inflation through its effects on cuticle elasticity. In *Calliphora*, Cottrell (1962*d*) clearly demonstrated that bursicon increased the elasticity of the cuticle of the newly emerged fly. A consideration of the moths which were decapitated after eclosion but before proboscis coiling indicates that the same effect also occurs in *Manduca*. In the headless animals which were injected with bursicon, the amount of inflation observed was often asymmetrical. Moths which were injected to the left of midline showed a longer extension of the left wing over that of the right. Similarly, injection to the right of the midline often produced a greater inflation of the right wing. No cases were observed in which the contralateral wing was longer. The effect of bursicon on wing inflation appears to be a local effect on cuticle extensibility, and, thereby, on the length to which the wing can be inflated. Consequently, the abdomen supplies both the haemolymph pressure needed to expand the wings and also the hormone which allows the wings to respond to this pressure.

2. *The triggering of the wing-spreading behaviour*

In the higher flies the release of bursicon from the thoracic ganglionic-mass is reported to be triggered by neural commands from the brain (Fraenkel & Hsiao, 1965). In the moth the secretion of bursicon and wing spreading are closely associated and are under a two-step control which involves both the brain and the suboesophageal ganglion. The first step depends on the activity of the brain. In brainless moths wing spreading is never observed, but 'loose-brain' moths often spread their wings. Thus, the brain is required for the performance of this behaviour, but only in its endocrine capacity as a source of the eclosion hormone (Truman, 1973*a*). This need for eclosion

hormone is further demonstrated by the fact that injection of the hormone into normal pharate moths, or those from which the pupal cuticle has been removed, provokes eclosion followed by the precocious spreading of the wings (Truman, 1971).

The second step requires the neural influence of the suboesophageal ganglion. This conclusion is based on the fact that moths which lack this ganglion, or which have had their ventral nerve-cord severed posterior to it, do not spread their wings. Additionally, the decapitation experiments indicate that the head is required for wing spreading even after the brain has released the eclosion hormone. This continuing need for the head up to the time of eclosion is presumably related to the neural function of the suboesophageal ganglion. At eclosion this ganglion somehow triggers wing-spreading behaviour, but then it is not required for the subsequent performance of the behaviour.

Although the wing-spreading pattern is triggered within seconds of eclosion, confinement of the moth can subsequently delay its onset for hours. The results of placing decapitated moths in glass vials showed that this inhibition of wing spreading was mediated through the head. Whether this mediation involves the brain or the suboesophageal ganglion has not been determined.

3. *The organization of the emergence sequence*

The emergence sequence of moths (Truman, 1971) appears to be made up of two, long, centrally programmed pieces of behaviour: the pre-eclosion behaviour (which includes eclosion) and wing-spreading behaviour. The eclosion hormone initiates the emergence sequence by directly releasing the pre-eclosion behaviour (Truman & Sokolove, 1972). This behaviour involves only abdominal movements during its first part and both abdominal contractions and wing 'shrugging' during the eclosion portion. At the end of eclosion the suboesophageal ganglion triggers the post-ecdysial portion of the emergence sequence. Depending upon environmental conditions the moth then has two options. If confined it shows digging behaviour and wing spreading is inhibited. In *Manduca* wing-spreading can be delayed for over a day; in some of the silkmoths the inhibition is not as strong and some individuals will eventually attempt to spread their wings even though still confined (Truman, 1971). With the release from confinement the moth shows normal walking-behaviour and climbing-behaviour until a suitable site is reached. The wing-spreading programme is then begun. At its end, full, adult behaviour is attained.

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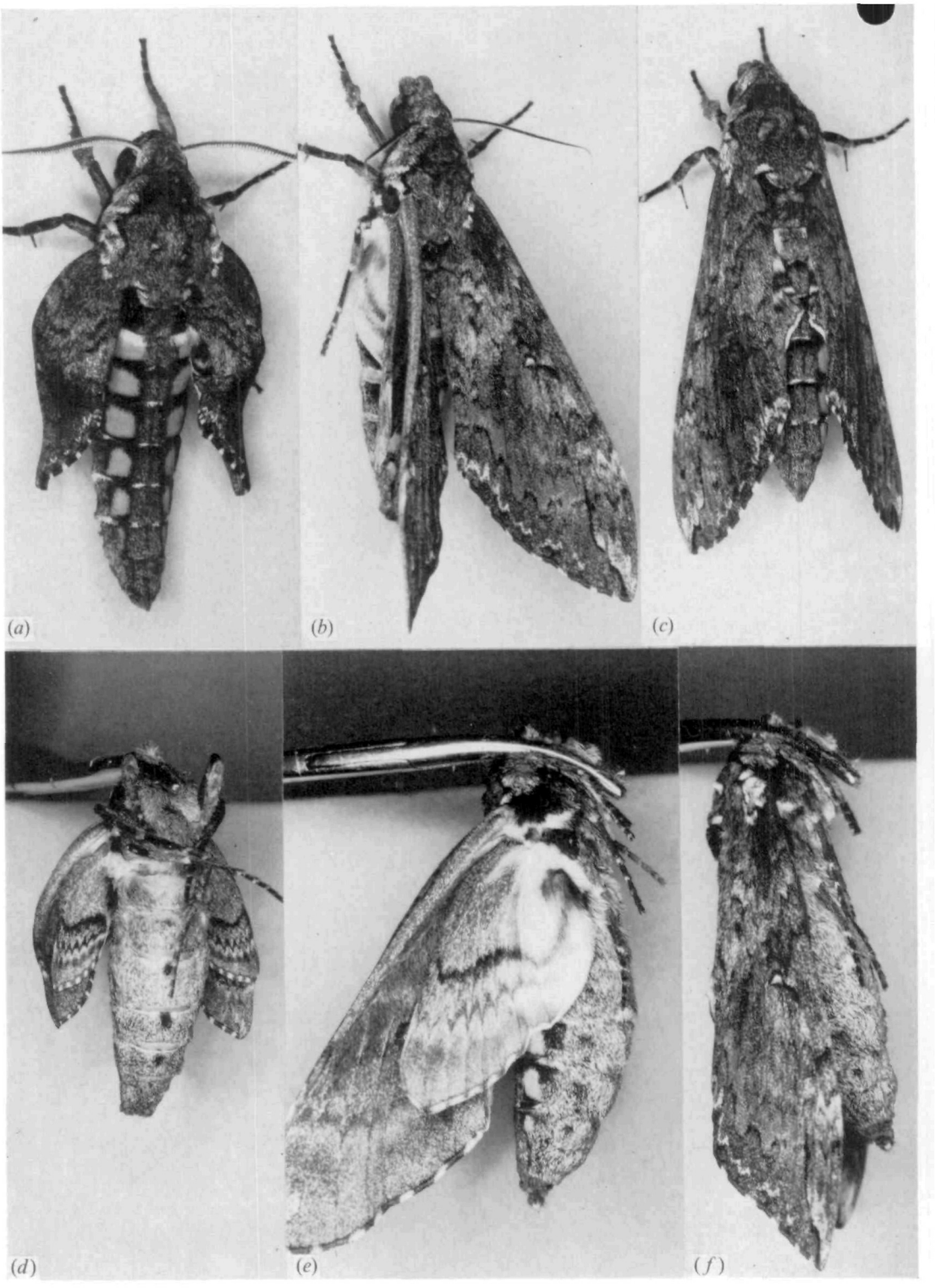
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EXPLANATION OF PLATE

Fig. 1. The postures assumed by *Manduca* moths during various phases of wing spreading. An intact moth (*a*) at the beginning of wing spreading; (*b*) in the butterfly position; and (*c*) in the moth position. A decapitated moth (*d*) at the start of spreading; (*e*) in the butterfly position; and (*g*) in the moth position.