The Journal of Experimental Biology 204, 457–469 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
JEB3271

DEFENSIVE RESPONSES OF LARVAL *MANDUCA SEXTA* AND THEIR SENSITIZATION BY NOXIOUS STIMULI IN THE LABORATORY AND FIELD

EDGAR T. WALTERS[1,*], PAUL A. ILLICH[1,‡], JANIS C. WEEKS[2] AND MATTHEW R. LEWIN[1]

[1]*Department of Integrative Biology and Pharmacology, University of Texas – Houston Medical School, 6431 Fannin Street, Houston, TX 77030, USA* and [2]*Institute of Neuroscience, University of Oregon, Eugene, OR 97403, USA*

*e-mail: Edgar.T.Walters@uth.tmc.edu

‡Present address: McLennan Community College, Department of Institutional Research, 1400 College Drive, Waco, TX 76708, USA

Accepted 13 November 2000; published on WWW 15 January 2001

Summary

Sensitization of defensive responses following noxious stimulation occurs in diverse species, but no demonstration of nociceptive sensitization in insects has been reported. A set of defensive behavior patterns in larval *Manduca sexta* is described and shown to undergo sensitization following noxious mechanical stimulation. The striking response is a rapid bending that accurately propels the head towards sharply poking or pinching stimuli applied to most abdominal segments. The strike is accompanied by opening of the mandibles and, sometimes, regurgitation. The strike may function to dislodge small attackers and startle larger predators. When the same stimuli are applied to anterior segments, the head is pulled away in a withdrawal response. Noxious stimuli to anterior or posterior segments can evoke a transient withdrawal (cocking) that precedes a strike towards the source of stimulation and may function to maximize the velocity of the strike. More intense noxious stimuli evoke faster, larger strikes and may also elicit thrashing, which consists of large, cyclic, side-to-side movements that are not directed at any target. These are sometimes also associated with low-amplitude quivering cycles. Striking and thrashing sequences elicited by obvious wounding are sometimes followed by grooming-like behavior. Very young larvae also show locomotor responses to noxious stimuli. Observations in the field of attacks on *M. sexta* larvae by *Cardinalis cardinalis*, an avian predator, suggest that thrashing decreases the success of a bird in biting a larva. In the laboratory, noxious stimulation was found to produce two forms of sensitization. Repeated pinching of prolegs produces incremental sensitization, with later pinches evoking more strikes than the first pinch. Brisk pinching or poking of prolegs also produces conventional sensitization, in which weak test stimuli delivered to another site evoke more strikes following noxious stimulation. The degree and duration of sensitization increase with more intense noxious stimulation. The most intense stimulus sequences were found to enhance strike frequency for approximately 60 min. Nociceptive sensitization generalizes to sites distant from sites of noxious stimulation, suggesting that it involves a general, but transient, arousal of defensive responses.

Movies available on-line:
http://www.biologists.com/JEB/movies/jeb3271.html

Key words: injury, learning, memory, arousal, hyperalgaesia, escape, thrashing, avian predator, tobacco hornworm, *Manduca sexta*.

Introduction

Nociceptive sensitization, an enhancement of defensive responses following noxious stimulation, has provided a simple paradigm for studying basic mechanisms of short-term and long-term memory, as well as mechanisms assumed to contribute in humans to hyperalgesia (enhanced pain sensitivity) (Walters, 1994). Nociceptive sensitization has been investigated in models of memory from a few invertebrates, notably *Aplysia californica* and the medicinal leech (Krasne and Glanzman, 1995; Sahley, 1995; Byrne and Kandel, 1996), and extensively in models of hyperalgesia in mammals (Millan, 1999). Its occurrence in at least three different phyla and its potentially general adaptive value suggest that nociceptive sensitization may be a very common form of behavioral plasticity that involves primitive mechanisms (Walters, 1992; Walters et al., 1994). However, the apparent generality of nociceptive sensitization might be questioned by the rarity of published reports of nociceptive sensitization in the largest animal phylum, the Arthropoda. Indeed, we know of no descriptions of nociceptive sensitization in the most abundant class of arthropods, the insects. It would be surprising, however, if sensitization of defensive behavior by noxious stimulation did not occur in some insects, given that insects have highly developed defensive responses (Matthews and Matthews, 1978; Evans and Schmidt, 1990) and can exhibit other forms of behavioral sensitization (Dethier et al., 1965; Duerr and Quinn, 1982; Hammer et al., 1994).

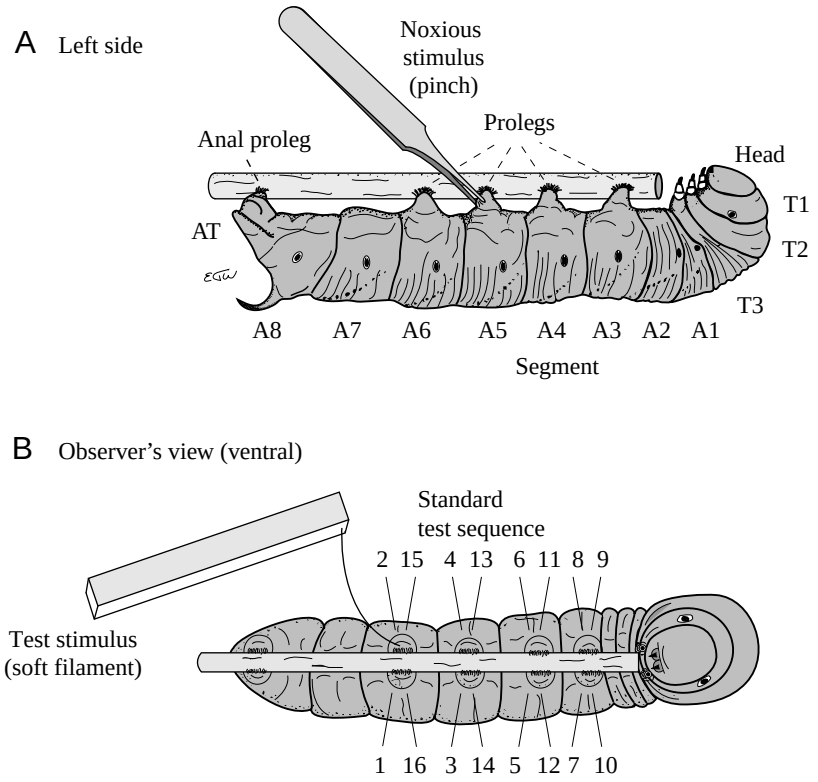

Fig. 1. Diagram of *Manduca sexta* larva and experimental stimuli. (A) Segmental numbering and noxious stimulation. A1–A8 are unfused abdominal segments, whereas the terminal abdominal segment (AT) represents the fusion of segments A9–A11. T1–T3 are thoracic segments. In the text and some figures, L indicates the left side and R the right of a given segment (e.g. RA6 is the right side of abdominal segment 6). In all experiments performed in the laboratory, the wooden rod was horizontal, with the larva hanging underneath. One noxious stimulus, a pinch, was delivered by forceps to the side of a single proleg in segment A5, unilaterally to four abdominal prolegs on one side (excluding the anal proleg) or bilaterally to the four prolegs in segments A5 and A6. In other experiments, poking with a stiff nylon filament to the side of one or more prolegs was used instead of a pinch. Multiple noxious stimuli were separated by 10 s intervals. (B) View of the larva seen by the experimenter, with the standard test sequence indicated. This sequence consisted of 16 pokes to the eight prolegs on segments A6–A3 at 10 s intervals in the order indicated using a soft nylon filament. Each proleg was poked twice in each sequence, with the left proleg on segment A6 receiving the first (1) and last (16) of the soft test pokes.

The tobacco hornworm *Manduca sexta* offers well-known advantages for investigations of neural and behavioral plasticity related to development (Weeks et al., 1997; Levine and Weeks, 1990; Hesterlee and Morton, 1996). More recently, learning has been demonstrated in larval *M. sexta* in the form of short-term habituation and dishabituation of the proleg withdrawal reflex (Wiel and Weeks, 1996; Wood et al., 1997; Wiel et al., 2000). These studies yielded indirect evidence that a moderately noxious dishabituating stimulus, body wall pinch, might also produce sensitization, but this possibility was not tested directly. To our knowledge, there have been no reports of the effects of intense noxious stimulation on the behavior of *M. sexta*. However, the larvae of other Lepidoptera respond to noxious stimuli with a rapid bending that has been examined most extensively by Frings (Frings, 1945). Apparently similar responses in various Lepidoptera have been described as 'lashing' (Edmunds, 1975), 'striking' or 'thrashing' of the body (Matthews and Matthews, 1978). In the present study, we use video analysis to characterize directed striking and undirected thrashing responses of larval *M. sexta* to mechanical stimulation both in the laboratory and in the field, and we show that the striking responses are sensitized by noxious stimulation. Some of these results have been reported previously in abstract form (Walters et al., 1996).

Materials and methods

Larvae of the tobacco hornworm, *Manduca sexta* (L.), from a colony at the University of Oregon, were reared and tested in isolation at 24–27 °C, and maintained on an artificial diet (Bell and Joachim, 1976). Laboratory-reared larvae of both sexes were used for experiments during the fourth or fifth instar (usually fifth-instar larvae approximately 1 day after ecdysis). Some behavioral observations were made on first-instar hatchling larvae. A few observations were made on wild *Manduca* larvae (whose markings suggested *M. sexta* rather than other indigenous hornworm species) found on plants in the first author's garden in Houston, Texas, USA: either *Nicotiana tabacum* (tobacco) or *Agnus castus* (chaste tree).

During laboratory studies, each larva was allowed to clasp a horizontal wooden rod. The larvae crawled underneath the rod to its tip, where they usually remained immobile in a 'sphinx-like' posture, with the thoracic legs free and the thorax and head curled (Fig. 1). Observations were made from above the rod, with the larva viewed as in Fig. 1B. Noxious stimuli in some experiments consisted of one or more pokes with a stiff nylon filament (Stoelting), having a calibrated bending force of approximately 40 mN, exerting a pressure of $60\,g\,mm^{-2}$ or approximately 5.5×10^5 Pa. In other experiments, firm pinches were delivered with stainless-steel forceps (tip thickness 0.4 mm). The pressure from each pinch, estimated from measurements of similar pinches using a force transducer, was 5–10 times the pressure exerted by the stiff poke. The duration of each poke and pinch was approximately 0.5 s. In most experiments, noxious stimuli were delivered to one or more prolegs on abdominal segments A3–A6 (Fig. 1A), as described in the Results section. Each larva was used in only one experiment.

Test stimuli used in sensitization experiments were weak pokes delivered with a soft nylon filament (bending force

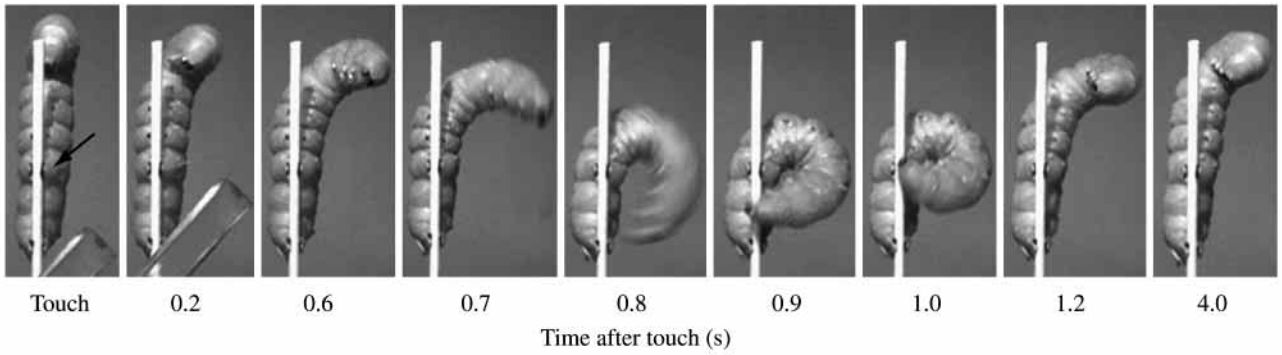

Fig. 2. Strike sequence evoked by poking the left proleg in segment A6 with a soft filament. 'Touch' indicates the video frame in which bending of the filament began during the poke. The point of contact is indicated by the arrow. The complete length of the bent filament is difficult to see because of its translucence and the limited resolution of the video image. The numbers below each frame indicate the time elapsed from the touch (in s). Close inspection of the video recording revealed that the mandibles opened between 0.2 and 0.7 s and had closed by the 1.2 s. Five minutes before this test, the animal had been sensitized by a single strong pinch to the ipsilateral proleg in segment A5 (see video).

of approximately 1.5 mN, exerting a pressure of 15 g mm^{-2} or approximately 1.5×10^5 Pa). The standard test sequence consisted of 16, approximately 0.5 s pokes, at 10 s intervals, to the side of eight prolegs (two pokes per leg; Fig. 1B). Although these 'von Frey hairs' deliver relatively constant maximal forces during each poke, and the tester was instructed to apply the filament with a constant velocity at 90 ° to the surface of the proleg, we wondered whether a tester might unconsciously alter the intensity of the test stimulus by inadvertently changing the angle or velocity of the poke. Several observations indicated that such changes could not account for the modifications we observed in striking responses. In preliminary experiments (N=3 larvae), we were unable to evoke any strikes with 1.5 and 3 mN filaments when we deliberately varied the angle and velocity of the poke well beyond the range used in our formal studies, even though the same filaments often evoked strikes following a pinch. Furthermore, in three animals used in our conventional sensitization studies (one control animal and two pinched animals), the testing was performed 'blind' by an experimenter unaware of the training history of the larvae. The pattern of results of the blind tests was indistinguishable from that in the other tests. In addition, most of the tests in the sensitization studies were performed by technicians who were blind to the guiding hypotheses.

All the behavior of *M. sexta* in the field, and some in the laboratory, was videotaped using a color Hi-8 camcorder (Sony CCD-T3930). Most animals in the laboratory were videotaped on a JVC BR5378U SVHS video recorder with a monochrome CCD camera (COHU). All video sequences were digitized at 29.97 frames s^{-1} using a Media100 video capture card on a Macintosh G3 computer with Adobe Premiere 5.1 software. Illustrations containing video images were prepared with Adobe Photoshop 5.0 and Adobe Illustrator 8.0. The effects of noxious stimulation were assessed statistically using two-way analysis of variance (ANOVA) with repeated measures, followed by Dunnett's tests (for comparison with a single baseline trial) or Newman–Keuls tests. In one study (see Fig. 7), an *a priori* hypothesis, based on pilot data, was assessed with a planned comparison using a paired *t*-test.

Results

Defensive striking response

Various caterpillars exhibit a brisk bending response that propels the head towards a site of intense stimulation on the abdomen (Frings, 1945), but to our knowledge such a response has not been described systematically using stop-motion video or film methods in any insect larva, nor has such a response been reported in *Manduca* spp. We found that *M. sexta* larvae reliably displayed a rapid bending response towards sharp poking or pinching stimuli applied to any of the posterior abdominal segments (from the terminal abdominal segment, AT, to abdominal segment 4, A4, Fig. 1A). Furthermore, larvae could display these responses to much weaker stimuli, provided that the animal had been sensitized beforehand by noxious stimulation. We have termed this response a 'defensive strike' because its function appears to be to strike a source of threatening stimulation (see Discussion). Fig. 2 illustrates a typical strike, which in this case was elicited by poking the left proleg in segment A6 with a soft nylon filament (1 mN bending force) 5 min after sensitization by a noxious pinch delivered to the ipsilateral proleg in segment A5. The latency from the dimpling of the integument by the filament until the first detectable motion of the head was approximately 0.1 s (not shown). The 0.2 s panel in Fig. 2 shows an initial small movement to the animal's left (to the right in Fig. 2, which shows the ventral surface of the animal). The head paused for approximately 0.3 s and then accelerated rapidly (from 0.6 to 0.8 s), passing directly above the site of stimulation (0.9 s) 0.2–0.3 s after beginning the rapid acceleration. The head continued in its arc beyond the site of stimulation (1.0 s) and then returned in a curving trajectory (1.2 s), with the major movement ending approximately 1.4 s after the poke (not shown). Over the next few seconds, there was a very slow recovery towards the original position (4.0 s).

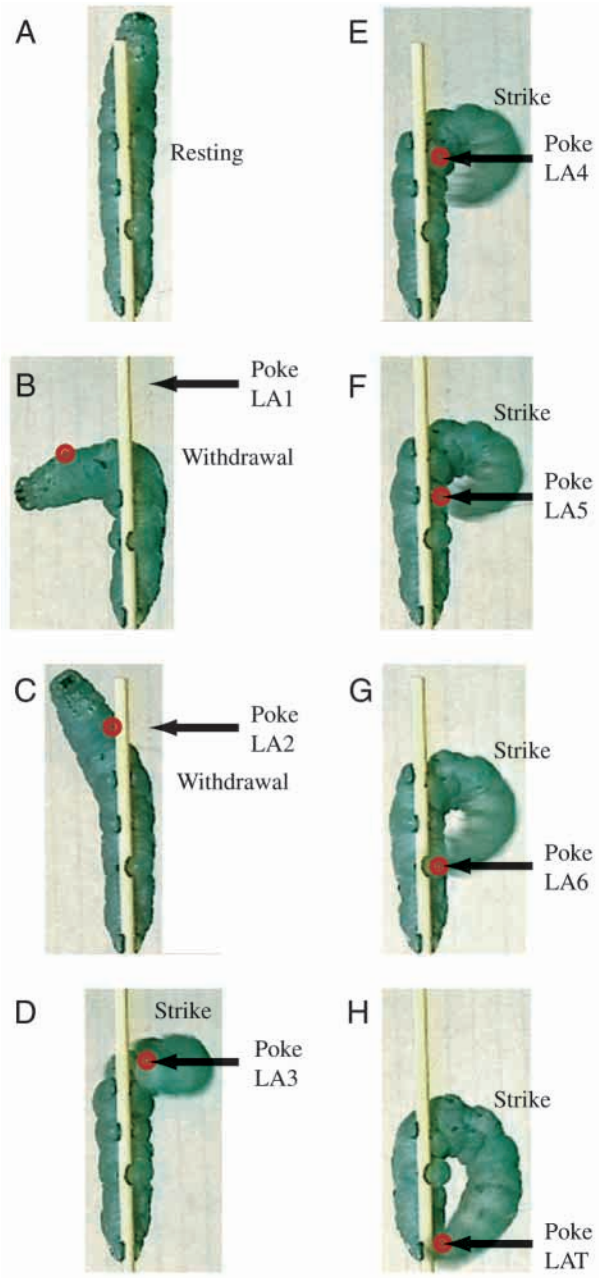

Fig. 3. Directed responses to a noxious poke delivered to different segments. (A) A larva at rest. (B–H) Video frames taken at the point of maximal movement shortly after a single poke to the location indicated (e.g. 'LA1' indicates the left side of segment A1) with a stiff nylon filament. The position in space where a poke was delivered is indicated by the tip of the arrow. The spot on the body that had been poked is indicated by a red circle. Separation of the red circle from the arrow tip shows withdrawal from the poke (B,C). A poke to more posterior segments evoked a strike that came very close to the site of the poke (C–H).

Site-specific striking, withdrawal and cocking responses

The site-specificity of responses to a stiff nylon filament (40 mN bending force) is illustrated in Fig. 3. Identical results were found in three other animals whose responses were analyzed with stop-motion video methods. Pokes to the head, to the thoracic segments or to the first two abdominal segments (Fig. 3B,C) failed to evoke immediate strikes, instead evoking a withdrawal of the anterior body from the site of stimulation (see also Fig. 4, 0.2 s). In contrast, pokes to any of the more posterior abdominal segments evoked immediate strikes (Fig. 3D–H). Strikes evoked by strong stimuli such as this stiff poke were identical to the strike shown in Fig. 2, except that the pause between the initial movement of the head and the peak of the strike was typically absent. In this and most other animals tested, the maximum excursion of the head and its peak velocity occurred close to the abdominal segment that received the poke (Fig. 3D–H). However, in some cases (e.g. Fig. 2), the trajectory of the head went beyond the site of stimulation, passing directly over the stimulated site during both the acceleration (0.8 s) and deceleration (1.0 s) phases of the strike. In this and most animals, the strikes came within 1–2 mm of, and often grazed, the site of stimulation, but rarely impacted the integument. Close inspection of the recordings revealed that the mandibles invariably opened at the beginning of an immediate strike and closed at the end of the strike. Opening of the mandibles and the rapid velocity and precise aim of the strike are consistent with a function of producing maximal impact on a threatening target. The impact of the strike on a human finger was forceful but did not cause tissue damage or pain. Fluid was sometimes regurgitated from the open mouth at the peak of the strike, especially in the few cases in which an eliciting pinch was sharp enough to cause a visible wound in the integument. In some of these cases, the animal later brought its head slowly up to the wound and displayed grooming-like behavior for several seconds, with its open mouthparts repeatedly contacting the wound.

Videotaped responses in more than 50 larvae (most analyzed without painstaking stop-motion methods) confirmed that rapid strikes were directed at the stimulated site in posterior segments. However, the same strong stimuli that elicited immediate strikes in posterior segments evoked immediate withdrawals when the stimuli were applied to any segment anterior to A3 (Figs 3, 4). Stimulation of segment A3 itself produced mixed effects, sometimes evoking strikes (e.g. Fig. 3D) and sometimes withdrawals (not shown). In other larvae, we found that the most intense stimuli evoked the most vigorous withdrawal responses. Although the withdrawals were clearly graded with the intensity of the stimulus, we did not systematically examine relationships between stimulus intensity and response strength. After some withdrawal responses to anterior stimulation, the body remained in the resulting curved posture for tens of seconds without further movement. In other cases, and especially if the animal had received several noxious stimuli, the withdrawal away from the stimulus was followed quickly by a vigorous swing towards the stimulus (Fig. 4, 1.4 s). Rapid swings (directed towards an anterior stimulus) that followed a withdrawal response appeared identical to immediate strikes, except that the mandibles often failed to open. A very brief (approximately 0.2 s) withdrawal of the anterior body away from the side of stimulation was

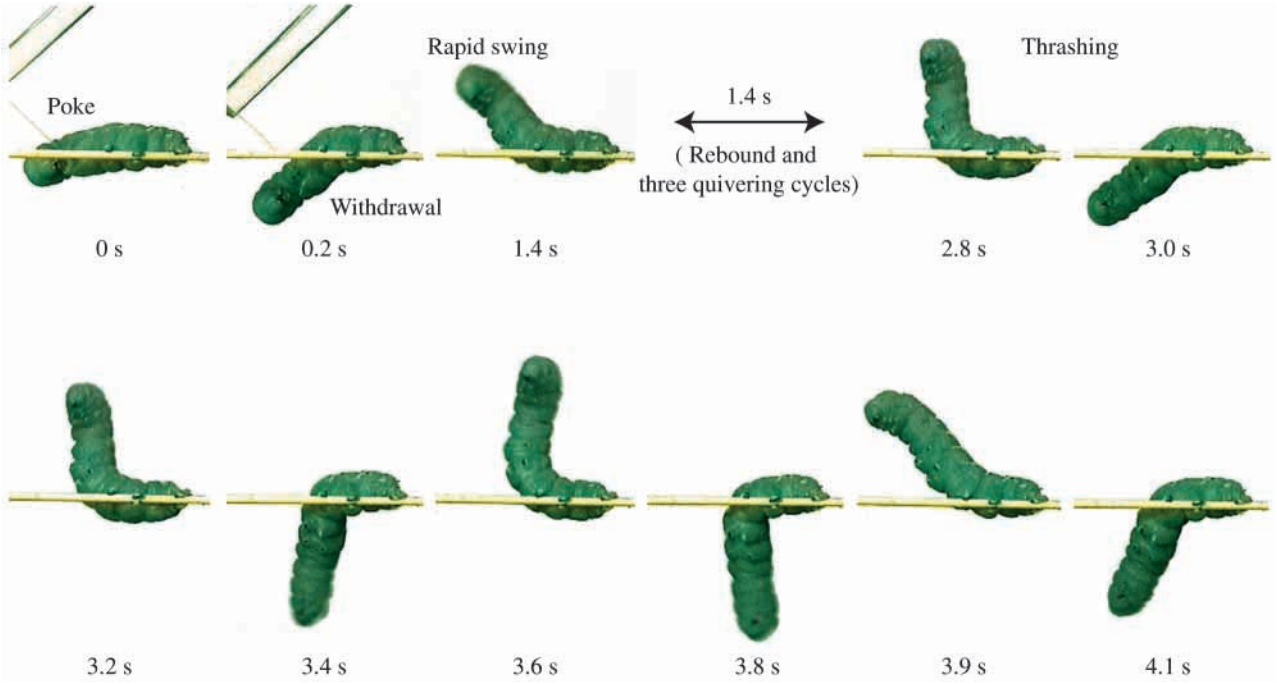

Fig. 4. Withdrawal and thrashing evoked by a relatively soft poke shortly after noxious stimulation (see text). The numbers below each frame indicate the time elapsed (in s) from the poke. Following the poke, the larva withdrew and then rapidly swung its head in the direction of the stimulus (without opening the mandibles). A rebound to the contralateral side then occurred (not shown), which was smaller than the preceding swing. There followed three very small, symmetrical, side-to-side 'quivering' cycles (not shown). Clear thrashing began 2.8 s after the poke, with four complete side-to-side cycles shown here between 2.8 s and 4.1 s. The mandibles did not open (see video).

sometimes also observed after an immediate strike evoked by posterior stimulation (not shown). This withdrawal began after recovery from the initial strike and was usually followed by a second strike. Both strikes were characterized by targeting to the site of stimulation and opening of the mandibles. The second strike displayed a greater amplitude and peak velocity, suggesting that the brief withdrawal of the head between strikes serves a 'cocking' function, increasing the distance of the head from the target to maximize the velocity of the ensuing strike.

Site-specific striking and withdrawal/cocking responses were also recorded in wild *Manduca* spp. (*N*=2) found in the first author's garden (see video). These responses were qualitatively indistinguishable from the responses observed within the laboratory by laboratory-reared *Manduca sexta*, although the responses in the field were faster. This could have been a consequence of the higher temperatures (>30 °C) compared with the laboratory (approximately 25 °C) or possibly a result of physiological differences between wild and laboratory-reared animals.

Both in the laboratory and in the field, our studies focused on larger larvae in the fourth and fifth instars. We observed similar defensive responses in very small first-instar hatchlings reared in the laboratory and tested while hanging from a tiny rod (*N*=5), while on their food (their typical substratum in the laboratory; *N*=6) or on paper (KimWipe; *N*=4). In each group, a pinch with fine forceps evoked withdrawal responses to stimulation of the anterior segments and well-directed strikes in response to stimulation of the posterior segments. Two larvae regurgitated during strikes. One difference from larger larvae was that the hatchlings exhibited locomotor responses to pinches, rapidly crawling away. Strikes and withdrawals could also be elicited while the larvae were crawling.

Thrashing and quivering responses in the laboratory

Mechanical stimulation of a larva occasionally evoked relatively symmetrical, side-to-side bending movements that bore no fixed relationship to the site of stimulation. The likelihood of observing these dramatic 'thrashing' responses appeared to increase after prior noxious stimulation (see below), especially if noxious stimuli were delivered to anterior segments. However, under all our conditions, thrashing was uncommon, and we have not yet systematically examined its stimulus control. An example of a brief thrashing sequence evoked in the laboratory by a relatively soft nylon filament (6 mN bending force) is shown in Fig. 4. Two minutes prior to the illustrated response, this animal had received two strong, noxious pinches to the left side of the first thoracic segment (LT1), and the second pinch had evoked a 3 s thrashing sequence (not shown). The subsequent test poke to segment LA3 (which had failed to evoke strikes or thrashing prior to pinching LT1) then elicited an immediate withdrawal of the anterior body (Fig. 4, 0.2 s) which lasted 0.5 s, followed by a rapid swing towards the stimulated side (1.4 s). Although this quick, unilateral swing resembled a strike, its amplitude was smaller, the moving head did not approach the body of the larva, and the mandibles did not open. The anterior body then

Fig. 5. Prolonged thrashing of a larva in the field during attack by an avian predator. (A) Early, unsuccessful attempt of a female cardinal to seize the larva by flying up from underneath the tobacco leaf, the stem of which is clasped by the larva. At this time, the leaf obscures the larva from the camera, but thrashing is revealed by the rhythmic swaying of the leaf. (B) The cardinal tears away parts of the leaf, exposing the thrashing larva (red circle) from above. (C,D) Unsuccessful attempts to bite the thrashing larva from above. Note the opposite positions of the head of the larva near the left (C) and right (D) peaks of each swing. (E) The cardinal directs its bites to the stem of the leaf near the larva. (F) After cutting off the end of the leaf, the cardinal picks up the leaf fragment with the attached larva, which is now unable to evade subsequent bites (see text and video).

rebounded in a smaller bend to the opposite side, and there followed a series of three symmetrical, side-to-side swing cycles of very small amplitude (approximately 5° in each direction; not shown). These weak 'quivering' movements of the anterior body occurred with a regular period of 0.3 s and were also seen in other larvae that displayed thrashing both in the laboratory and in the field.

In this example, the quivering cycles were then followed by five thrashing cycles (period 0.4 s), the first four cycles of which are shown in between 2.8 and 4.1 s in Fig. 4. Thrashing is distinguished from quivering by the much larger amplitude of the swings, but thrashing and quivering may represent two different states of the same underlying motor program. The maximum amplitude of each swing in these thrash cycles was approximately 90°, with the total cycle amplitude being approximately 180° (Fig. 4; 3.6 and 3.8 s). The thrashing cycles were followed by four more quivering cycles and then seven more thrashing cycles. Although most thrashing cycles had symmetrical swings to each side, it is notable that the first swing (2.8 s and possibly 1.4 s, which may also represent a thrashing movement) is towards the stimulated side and is greater in amplitude and velocity than the contralateral swing in the cycle. During the entire sequence of thrashing and quivering, the prolegs on segments A5 and A6 (as well as AT) remained attached to the rod. In some animals, however, the prolegs on segment A5 were released, so that attachment was only through the prolegs on segments A6 and AT. In these cases, the thrash amplitude was much greater (up to 360° for each cycle).

Prolonged thrashing responses to predatory attack in the field

Following some of the laboratory experiments, we placed *M. sexta* larvae onto tobacco plants growing in the garden of the first author. Three of these larvae were attacked by a natural avian predator, the northern cardinal *Cardinalis cardinalis*. In one case, the first author was able to record much of an attack sequence using a camcorder from a distance of approximately 6 m (Fig. 5). The responses of the hornworm to this attack appeared similar to those observed in an unrecorded attack by another female cardinal. In both cases, the attacked larva was clasping the main stem on the underside of a tobacco leaf. In the third case, a male cardinal had already seized the larva when first observed, and it flew away soon afterwards. In the recorded sequence, the female cardinal was first observed standing in the grass underneath the tobacco leaf. It repeatedly flew up and tried to bite or seize the larva (Fig. 5A). Inspection of the video recording indicated that the larva was not moving

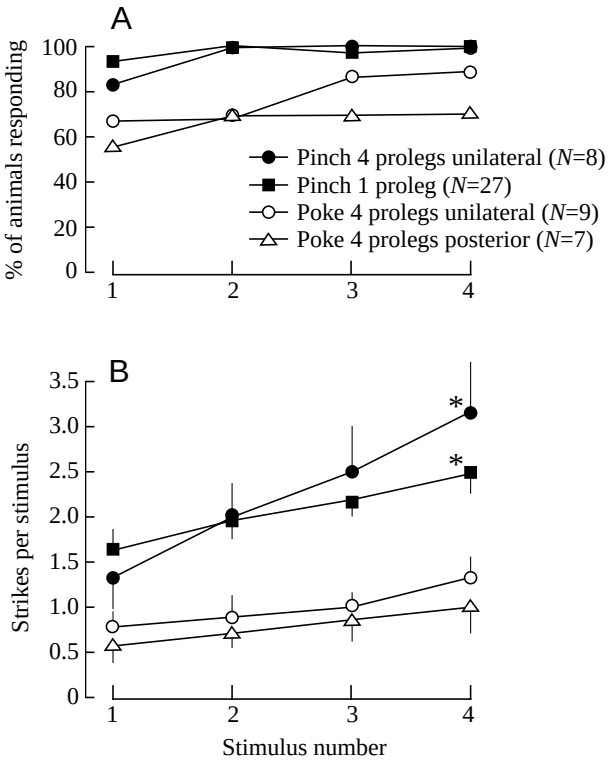

Fig. 6. Incremental sensitization of the striking response to noxious stimuli in the laboratory. (A) The mean percentage of animals that exhibited a striking response to each of the first four stimuli (pinches or stiff pokes, as indicated; see text for details) during the noxious stimulation sequence. In some animals, eight stimuli were given (see Fig. 7), but the last four responses are omitted here. (B) The mean (±S.E.M.) number of strikes evoked per stimulus in each animal during the first four applications of the indicated stimuli. An asterisk indicates a significant difference from the first response in the sequence (P<0.05).

when the recording began, but that it soon responded to the attack with a 30 s episode of thrashing. The early thrashing was revealed primarily by large, rhythmic movements of the overlying tobacco leaf. After several unsuccessful attempts to seize the thrashing larva with its beak, the cardinal landed on top of the tobacco leaf (Fig. 5B) and spent approximately 30 s tearing strips off the leaf with its beak. This partially exposed the larva, and the cardinal then lunged (not shown) and apparently hit its target because the larva responded with a lengthy thrashing sequence (Fig. 5C–F). The thrashing movements are seen as bending of the body to both sides in Fig. 5B–E, which occurred in regular cycles with a period of 0.6–0.8 s. This second sequence of thrashing continued without interruption for more than 1 min with more than 90 cycles. A similarly long sequence of thrashing was seen in the unrecorded attack sequence. In contrast to these observations, thrashing sequences evoked in the laboratory were rare and much shorter (e.g. Fig. 4), perhaps because the stimuli used in the laboratory were briefer and less intense than the attacks by a bird, because the temperature in the laboratory was cooler, or because the larvae also responded to other cues from the bird.

Although thrashing was generally undirected with respect to the eliciting stimulus, it did appear to help larvae evade a bird's bites. In the attack shown in Fig. 5 and in the unrecorded attack, we observed five bites by the cardinals on immobile (but responsive) larvae suspended from leaves, and in all five cases the bite struck its target. However, when the larva was thrashing, the success rate dropped from 100 to 29 % (four of 14 bite attempts observed in two attacks). In the attack shown in Fig. 5, the cardinal was unable to seize the larva while it continued to thrash under the swaying tobacco leaf.

Following its unsuccessful attempts to bite the thrashing larva (Fig. 5C,D), the strategy of the cardinal changed. Instead of targeting the larva, the cardinal resumed biting and tearing the leaf (Fig. 5E). It severed the main stem, tore the end of the leaf off with the larva still attached (Fig. 5F), and flew down to the grass holding the leaf tip and attached larva. The cardinal then bit the larva twice, carried it to a nearby log, and continued to bite the now unresponsive larva. The bird repeatedly swung the limp larva against the log, causing drops of fluid to fly with each impact (not shown). The cardinal then wiped its beak against the log several times, and flew away with the larva in its beak. In each observed attack, the bird departed with its prey before we could see whether the larva was eaten.

Incremental sensitization of striking during repeated noxious stimulation

To begin to explore properties of nociceptive sensitization in *M. sexta*, we used protocols in which sensitization was quantified as an increase in the frequency of strikes evoked by mechanical stimuli delivered to the lateral surface of abdominal prolegs. In this section, we describe an incremental increase in striking responses evoked during successive applications of a noxious stimulus. A progressive increase in responsiveness to a repeated stimulus has been termed 'incremental sensitization' (also 'warm-up' or 'wind-up'; see Walters, 1994).

In two groups of larvae, the repeated stimulus was a poke to a proleg delivered with a stiff nylon filament (40 mN bending force). This stimulus was considered moderately noxious because prolonged poking to the same spot would eventually damage the integument. In one group of larvae (Fig. 6, 'poke 4 prolegs unilateral'), a single stiff poke was delivered at 10 s intervals successively to each proleg on one side of the animal, going from segment A6 to A3 (i.e. in the sequence RA6, RA5, RA4, RA3, or the corresponding sequence on the left side). In a second group ('poke 4 prolegs posterior'), four pokes were delivered at 10 s intervals only to the prolegs on segments A6 and A5, but on both sides of the animal in the sequence LA6, RA6, LA5, RA5. In most cases, the pokes evoked proleg withdrawal (not shown) and often elicited strikes. The percentage of animals displaying strike responses (Fig. 6A) and the number of strikes evoked per poke in each animal (Fig. 6B) appeared to increase across the poke sequence, but neither of these apparent increases was statistically significant (ANOVA and Fisher's exact test comparing the first and fourth pokes, N=9 for unilateral poke and N=7 for posterior poke).

A third group ('pinch 4 prolegs unilateral') received a series of four strong pinches (one per proleg) delivered to one side of the body at 10 s intervals from segment A6 to A3 (the same pattern as delivered to the unilateral poke group above). Every pinch after the first pinch evoked strikes in nearly all the animals tested (Fig. 6A), a response rate that was higher than for poke stimuli. Individual pinches in the series often evoked withdrawal of the pinched proleg (not shown) and evoked more strikes per stimulus than did pokes (Fig. 6B). Two-way ANOVA with repeated trials followed by *post-hoc* comparisons with Dunnett's tests revealed that the number of strikes after the fourth pinch (but not the second or third pinches) was significantly greater than that after the first pinch (N=8; P<0.01, Fig. 6B). A fourth group ('pinch 1 proleg') received four pinches to a single proleg (either the right or left proleg in segment A5). The same statistical tests again revealed that the number of strikes after the fourth pinch (but not the second or third pinches) was significantly greater than that seen after the first pinch (N=27; P<0.01, Fig. 6B). Although not analyzed quantitatively, the strikes evoked by pinches appeared to be faster and of larger amplitude than the strikes evoked by stiff pokes. Repeated pinches to the same site clearly damaged the integument and occasionally resulted in drops of hemolymph appearing at the wound. The few cases of grooming-like behavior observed in the laboratory were usually associated with evidence of perforation.

Taken together, the data in Fig. 6 show that both the likelihood of evoking a strike and the number of strikes evoked increase with more noxious stimuli (pinch *versus* stiff poke). Moreover, incremental sensitization of the striking response occurs during repeated application of a noxious pinch.

Conventional sensitization of striking: intensity-dependence and time course

Sensitization is conventionally demonstrated by showing that a strong stimulus enhances the response to a separate, weak test stimulus. We first observed conventional sensitization both in the laboratory (N=6) and in the field (N=4) when we poked *Manduca* spp. with a finger tip. In the absence of any noxious stimulation, even hard pokes with a finger failed to evoke strikes. However, following a pinch or pokes with sharp forceps to another site on the body, even gentle pokes with a finger tip evoked strikes. To study the time course and possible site specificity (see next section) of conventional sensitization, we tested the larvae with a soft filament (1 mN bending pressure). This was briskly poked against all eight prolegs in the sequence shown in Fig. 1B, with 10 s between each poke. Each test sequence consisted of 16 soft pokes applied over a period of 150 s, and each proleg was poked twice during the sequence. The 16-poke test sequence was repeated at intervals of 5 min or longer. In the absence of separate noxious stimulation, repetition of the soft-poke test sequence at 5 min intervals evoked very few strikes (N=5 animals, Fig. 7A), and the few strikes that did occur showed no increase in frequency during the course of testing. Moreover, these few strikes were very weak, exhibiting much lower velocity and smaller maximal excursions (<45 °) than were displayed in response to noxious stimuli (which usually produced excursions of approximately 180 °). This response pattern changed when a brief series of eight moderately noxious, stiff pokes was delivered after the first soft-poke test sequence (Fig. 7B). The stiff pokes were applied in a single sequence to all four prolegs on one side of the animal at 10 s intervals, beginning with A6 and ending with A3. The stiff poke sequence began 5 min after the beginning of the first (baseline) test sequence and 5 min before the beginning of the second test sequence. The mean numbers of strikes directly evoked by the first four stiff pokes in these animals are presented in Fig. 6 ('poke 4 legs unilateral'). During the remaining four stiff pokes (not shown in Fig. 6), the strike frequency showed no further change. Although significant incremental sensitization of striking responses to the stiff pokes was not found during repetition of the pokes (Fig. 6), the stiff pokes did sensitize responses to the soft pokes delivered to either side of the animal 5 min later (Fig. 7B; two-way ANOVA with repeated trials, P<0.01). *Post-hoc* comparisons with baseline in each group using Dunnett's tests showed that stimulation of both sides evoked more strikes 5 min after the poke (P<0.05 in each case, N=9 animals), but not during tests performed 10–30 min after the stiff poke sequence. There was no significant difference between the sides ipsilateral and contralateral to the poke. Many of the strikes evoked by soft pokes after the stiff poke sequence had large amplitudes (approximately 180 °). However, neither multiple striking nor thrashing was evoked by the soft pokes after the stiff poke sequence.

Application of stronger noxious stimuli increased the degree and duration of sensitization of the defensive striking response. Delivering a single strong pinch to one proleg (LA5 or RA5) significantly increased the number of strikes elicited during the soft-poke test sequence (Fig. 7C; two-way ANOVA with repeated trials, P<0.001). Dunnett's tests revealed significant sensitization of striking 5 min and 10 min after the pinch (P<0.05 in each case, N=8). Again, there was no significant difference between the sides ipsilateral and contralateral to the pinch. Most strikes in response to the soft poke were rapid and of large amplitude, and in a few cases double strikes were evoked. No thrashing was elicited.

Delivery of a brief series of eight sharp pinches at 10 s intervals to a single proleg in segment A5 significantly enhanced strike number at every time point between 5 and 30 min when the test sequences were given at 5 min intervals (P<0.05 at each point, N=11, data not shown). We repeated this study with longer intervals between tests to estimate the duration of sensitization following intense noxious stimulation (Fig. 7D). Significant increases in strike number were observed in response to test stimulation of both the ipsilateral and contralateral sides 15 and 30 min after the pinch sequence (two-way ANOVA with repeated trials, P<0.001, and Dunnett's tests, P<0.01 for ipsilateral stimulation and P<0.05 contralateral stimulation, N=8 animals). No significant sensitization was found 1 h after pinch or during two test sequences separated by 15 min that were given 24 h after the pinches.

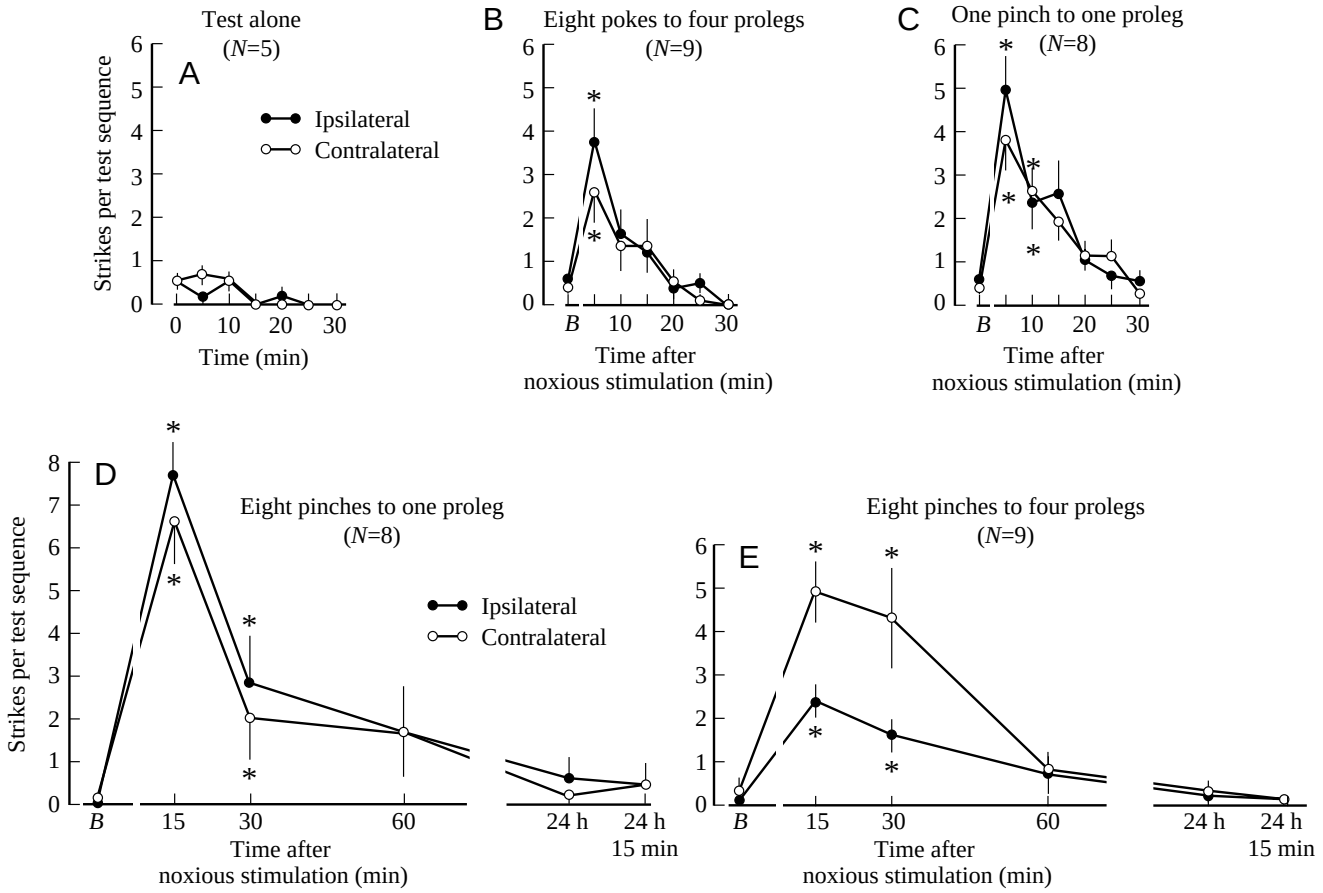

Fig. 7. Conventional sensitization, by noxious stimulation, of the striking response evoked by weak test stimuli in the laboratory. (A) Few strike responses were evoked by soft-poke test sequences delivered at 5 min intervals in the absence of noxious stimulation. Each point represents the mean (±S.E.M.) number of strikes evoked during a test sequence of 16 pokes with a soft filament at 10 s intervals to eight abdominal prolegs as shown in Fig. 1B. In this part, 'ipsilateral' refers to responses evoked by test stimuli to the same side as the first test stimulus and 'contralateral' to responses evoked on the opposite side. In B–E, the same terms refer to the side of noxious stimulation. (B–E) Enhancement of strike number following the noxious stimulation protocols indicated. Note the differences in test intervals between A–C and D,E. In B and C, noxious stimuli were delivered during the breaks in the lines (strikes evoked by the noxious stimuli are not included) 5 min after the baseline test (*B*) and 5 min before the next soft-poke test. In D and E, the noxious stimuli were delivered 15 min after the baseline test and 15 min before the next soft-poke test. Asterisks indicate significant differences ($P<0.01$ or $P<0.05$, see text) from the baseline test.

We also delivered eight pinches to four prolegs on one side (Fig. 7E). The pinches were delivered at 10 s intervals in the sequence A6, A5, A4, A3, A6, A5, A4, A3 on either the left or right side. Sensitization of strike number was found 15 and 30 min after the pinch sequence (two-way ANOVA with repeated trials, $P<0.001$, and Dunnett's tests, $P<0.01$ on both tests for contralateral prolegs; $P<0.01$ for the 15 min test and $P<0.05$ for the 30 min test for ipsilateral prolegs, $N=9$). Again, no sensitization was observed 24 h later. Although sensitization was not significant at 60 min in this experiment (Fig. 7E), in both this and the experiment in which eight pinches were delivered to a single proleg (Fig. 7D), more strikes were evoked in the 60 min test than the baseline test. Therefore, we asked how long eight pinches to either one or four prolegs sensitized the strike response. When we pooled the data from the two experiments and repeated the analysis, significant sensitization was also found at 60 min ($P<0.05$ on each side, Dunnett's tests), but not in the 24 h tests. In both protocols, multiple strikes per poke sometimes occurred during testing. In two animals, multiple strikes during the 15 min test were followed by 2–4 cycles of undirected thrashing. Neither multiple striking nor thrashing continued for more than 5 s, and therefore neither interfered with the next test stimulus.

Taken together, these results show that noxious stimuli sensitize the strike response (and perhaps thrashing). The sensitization can last approximately 1 h and is graded with the intensity of the noxious stimulus. The duration of sensitization is similar whether the noxious stimulation is restricted to a single proleg or distributed across four prolegs.

Generalization of sensitization across prolegs

The results shown in Fig. 7 demonstrate that sensitization of the strike response by noxious stimulation of one or more prolegs is not specific to the side receiving noxious

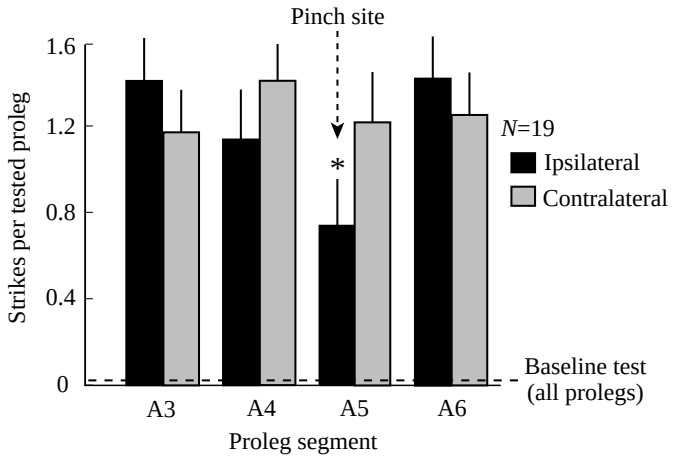

Fig. 8. Generalization of sensitization across prolegs and reduction of sensitization at the site of noxious stimulation. The data represent the mean (+S.E.M.) number of strikes evoked per proleg during each soft-poke test (16 pokes per test) 15 min after the delivery of eight pinches to a single proleg in segment A5 ('pinch site'). The mean number of strikes for all prolegs during all the baseline tests is indicated by the dashed line; this value ranged between 0 and 0.06 strikes per proleg for different prolegs (data not shown). The difference in strike number between the corresponding baseline test and the 15 min test was statistically significant (P<0.001) for all prolegs, both ipsilateral and contralateral to the pinch site. The single asterisk indicates that the soft poke evoked significantly fewer strikes when delivered to the pinched proleg than to the contralateral proleg (P<0.05).

stimulation. To investigate the generality of sensitization in more detail, we asked whether the pattern of responsiveness across all the tested prolegs was altered by intense noxious stimulation to a single proleg. Fig. 8 shows the pattern observed before and 15 min after eight pinches delivered at 10 s intervals to a single proleg (either the right or left proleg in segment A5). Data were pooled from experiments in which the animals were tested at 5 min intervals (N=11) and at longer intervals (N=8; the same animals as in Fig. 7D). Fifteen minutes after the pinch sequence, soft test pokes of every proleg (both ipsilateral and contralateral to the pinches) evoked significantly more strikes per animal (Fig. 8), and every proleg was associated with a greater frequency of animals showing strikes (not shown) compared with baseline (paired t-tests and Fisher exact tests, P<0.001 in each case, N=19). An *a posteriori* analysis with two-way ANOVA indicated that there was no overall significant difference among the eight prolegs in the number of strikes evoked. However, we also tested an *a priori* hypothesis, that proleg damage from the strong pinches would produce a local sensory deficit that would reduce the responsiveness of the pinched proleg compared to its contralateral homolog. This hypothesis was supported by a paired t-test that revealed a significant reduction of strike number from the pinched proleg compared with the contralateral proleg in segment A5 (P<0.05, N=19). These data indicate that pinch-induced sensitization is expressed generally across all the prolegs, although its expression is somewhat weaker in the pinched prolegs.

Discussion

Striking, withdrawal, cocking, thrashing and quivering

Although striking, withdrawal and thrashing responses had been described in various lepidopteran larvae (Frings, 1945), they had not been reported in *Manduca* spp., and in no species had these responses been investigated with stop-frame motion analysis. In *M. sexta*, we have distinguished five different responses to noxious stimulation that involve swinging movements of the anterior body: striking, withdrawal, cocking, thrashing and quivering. A strike was defined as a rapid, large-amplitude swing of the anterior body to one side, directed at the source of stimulation. As noted in other caterpillars (Frings, 1945), it appeared that strikes in *M. sexta* larvae were directed in the dorsoventral plane as well as laterally, although we did not investigate this systematically. When multiple strikes occurred, each swing went to just one side. The high velocity and precise targeting of the strike suggest that it functions to produce maximal impact on a source of noxious stimulation, perhaps dislodging small attackers (see below) or startling some predators. Supporting this conclusion is the hardness of the heavily sclerotized head and the opening of the sclerotized mandibles during the strike. We do not know whether the regurgitation that sometimes occurred during a strike has a defensive function. However, one of us (J.C.W.) found that the regurgitant is highly irritating to the human eye. Striking responses normally have a high threshold; in the absence of prior noxious stimulation, a strike was never evoked by touch with a finger tip and was rarely evoked by pokes with a soft nylon filament. In contrast, a firm pinch with sharp forceps always evoked a strike in species of *Manduca* larvae tested in the field and nearly always in the laboratory (Fig. 6A). The few strikes that were evoked by soft pokes in unsensitized animals were slower and smaller in amplitude than those evoked by sharp pokes or pinches, indicating that this high-threshold response is graded with the intensity of a suprathreshold stimulus.

Withdrawal of the anterior body was defined as a bending away from a source of noxious stimulation. It occurred with short latency and, like other withdrawal responses (Weeks and Jacobs, 1987; Walters, 1994), was graded with stimulus intensity. A novel finding was that a similar bending of the anterior body away from a stimulus sometimes preceded rapid swings evoked by either anterior (Figs 3, 4) or posterior (see video) stimuli. Rather than serving to move the head away from a threat, this cocking movement appears to enhance a subsequent strike towards the threat by increasing the distance through which the head accelerates to reach maximal velocity.

Thrashing was defined as large swinging movements to both sides in a largely symmetrical fashion. Thrashing occurred in regular cycles with periods of 0.3–1 s, and the movements bore no fixed relationship to the site of stimulation, except that the first swing in a thrashing sequence was usually towards the

stimulated side (Fig. 4) and was often larger than the rebound swing to the opposite side. It is possible that the first swing in a thrashing sequence blends the motor outputs for a thrash and strike. Thrashing sequences observed in the field sometimes lasted more than 1 min and consisted of more than 90 cycles. We sometimes observed multiple strikes with interstrike intervals similar to thrashing periods, but the strikes only went to one side of the body, and never numbered more than four in episodes that lasted less than 2 s. Thrashing amplitudes in different animals varied, with individual swing amplitudes ranging from approximately 30 to 180 ° (60 to 360 ° for a complete thrash cycle). Sometimes preceding or following the thrashing sequences were much weaker quivering sequences. These movements had cycle periods (0.2–0.4 s) similar to those of thrashing, but the swing amplitudes were only 2–10 °. We do not know whether quivering has a distinct function (e.g. mimicking a shaking leaf); it may simply represent a very weak state of thrashing. In the field, thrashing seemed to be unaffected by the position or proximity of the predator. Indeed, it seems unlikely that the visual system of the hornworm could recognize or localize a predator (Blum, 1985). Our observation that the cardinal had a lower success rate biting the larva when it was thrashing than when it was immobile suggests that one function of thrashing is to evade the bite of a predator. A defense that slows the attack of a skittish predator (such as a cardinal) could be adaptive by increasing the likelihood that the attack would be interrupted by external events.

Thrashing and striking may also be important for countering other threats to *M. sexta*, such as parasitic wasps. Interestingly, vigorous striking and thrashing responses in *M. sexta* larvae appear to be common during attacks by braconid wasps and other wasp species (N. Beckage, personal communication). One of us (M.R.L.) has observed violent thrashing lasting minutes by unidentified lepidopteran larvae in the west Texas desert during prolonged attempts by braconid wasps to deposit eggs in the larvae. These observations suggest that striking and thrashing responses to parasitic wasps are common in lepidopteran larvae. An interesting question is whether the grooming-like actions of the mouthparts at a perforation of the integument have biological functions. To our knowledge, neither tending nor guarding a wound has been demonstrated in any insect (Eisemann et al., 1984).

Nociceptive sensitization in an insect

Nociceptive sensitization is defined as an enhancement of defensive responses following stimulation that either causes tissue damage or activates sensory neurons tuned to damaging stimuli (see Walters, 1994). Its occurrence in chordates, annelids and molluscs suggested that nociceptive sensitization is a common form of behavioral plasticity, perhaps involving primitive mechanisms (Walters, 1992; Walters et al., 1994). However, evidence for nociceptive sensitization in the Arthropoda is meager. No enhancement of defensive responses has been reported after tissue damage or strong mechanical stimulation, although the defensive responses of crustaceans are reported to show enhancement following electric shock (Krasne and Glanzman, 1986; Rakitin et al., 1991). Shock has also been used with insects in associative learning paradigms to modify limb position (Horridge, 1962; Eisenstein and Carlson, 1994), appetitive behavior and olfactory choice (Dudai, 1988; Davis, 1996; Menzel et al., 1996; Tully et al., 1996), but nonassociative sensitization by shock of defensive behavior in insects has not been reported. Shock directly elicits defensive responses in arthropods, and some species will learn new responses to avoid shock (Horridge, 1962; Punzo, 1983; Eisenstein and Carlson, 1994). These observations raise the possibility that shock activates nociceptive systems designed to recognize bodily injury and trigger adaptive responses. However, the apparent disregard shown by some insects to severe bodily trauma supports the view that adaptive responses to injury may be lacking in this group whose evolutionary success is assumed to depend more upon short generation times and exuberant reproduction than upon reparative and mnemonic capabilities (Guthrie, 1975; Eisemann et al., 1984).

Our results demonstrate that an insect, *M. sexta*, can display nociceptive sensitization. One form is incremental sensitization, in which sensitization is both induced and revealed by repeated application of a noxious stimulus (Walters, 1994). In the present study, successive pinches elicited progressively larger numbers of strikes (Fig. 6). ‘Warm-up’ or ‘wind-up’ of defensive responses during a series of noxious stimuli has been described in rats (Woolf, 1984; Illich et al., 1995), frogs (Franzisket, 1963), spinal cats (Thompson and Spencer, 1966), *Aplysia californica* (Walters et al., 1983; Walters, 1987a; Walters, 1987b), leeches (Lockery and Kristan, 1991) and crabs (Rakitin et al., 1991). Incremental sensitization to innocuous stimuli has been reported in the leech (Burrell and Sahley, 1998) and may also occur in *Manduca sexta* (Wiel and Weeks, 1996). The present results are among the first in any species to show incremental sensitization of a defensive behavior pattern in response to repetition of a noxious mechanical stimulus.

We also demonstrated sensitization in *M. sexta* using the conventional procedure in which noxious stimulation of one pathway enhances responses evoked by test stimuli to another pathway. The degree and duration of conventional sensitization were graded with the intensity and duration of noxious stimulation (Fig. 7). Following multiple pinch protocols, sensitization lasted approximately 1 h, whereas with a single pinch the sensitization lasted only 5–10 min. No significant nociceptive sensitization was found 1 day after multiple pinches. This modest duration following intense noxious stimulation contrasts with nociceptive sensitization in mammals (Woolf, 1984) and in *Aplysia californica* (Walters, 1987a; Walters, 1987b), which can persist for weeks. It is possible that other noxious stimuli may induce more persistent sensitization in hornworms. Nevertheless, differences in properties of nociceptive sensitization (such as duration and site specificity) are likely to exist between *Manduca sexta* and *Aplysia californica*, and such differences may provide clues about both the functions and evolution of this widespread form of behavioral plasticity.

In principle, incremental and conventional sensitization could be different reflections of the same underlying mechanisms. Consistent with this possibility is that neither incremental (Fig. 6) nor conventional (Figs 7, 8) sensitization is specific to either the side or site of noxious stimulation; all prolegs on both sides expressed sensitization whether the sensitization was monitored by strikes evoked by the noxious stimulus itself or by a separate test stimulus. The only difference seen among different prolegs was that, when a single side or single proleg received all the pinches, then that side or that proleg became somewhat less likely to evoke strikes than the other side or other prolegs. This weaker sensitization could involve either damage to afferents in the pinched proleg(s) or site-specific neural inhibition (functionally similar to some forms of analgesia in mammals; see Illich et al., 1994; Illich et al., 1995). In contrast to effects reported in mammals and *Aplysia californica*, there was no site-specific enhancement of sensitization at the site of noxious stimulation (see Walters, 1987a; Walters, 1987b; Walters, 1994). Thus, a major component of nociceptive sensitization in *Manduca sexta* may be a general defensive arousal, perhaps a counterpart of the arousal of appetitive responses, such as feeding, which has been attributed to a 'central excitatory state' in insects (Dethier et al., 1965; Duerr and Quinn, 1982; Hammer et al., 1994).

Neural implications

Our behavioral findings raise interesting questions about underlying neural mechanisms. For example, how does an insect distinguish noxious from innocuous mechanical stimulation? There has long been speculation about whether insects are capable of feeling 'pain' (Norman, 1900; Wigglesworth, 1980; Eisemann et al., 1984), but little is known about the effects of noxious stimulation in this group. The relatively high threshold for eliciting striking and thrashing responses (before sensitization), the selective ability of noxious stimuli to sensitize defensive responses and the existence of multidendritic sensory neurons within the subepidermal plexus (Grueber and Truman, 1999) that are activated preferentially by noxious stimuli (W. Grueber, personal communication) suggest, but do not prove, that nociceptors exist in insects and contribute to the selective triggering and sensitization of defensive reflexes by noxious stimuli.

How is targeting of the striking response achieved, and what are the roles of the subepidermal plexus of multidendritic neurons (Grueber and Truman, 1999) and the low-threshold mechanoreceptors in bristle sensilla (Levine et al., 1985; Peterson and Weeks, 1988)? At least some of these sensory neurons project somatotopically within sensory neuropil and might provide positional information required for targeting. How is accurate targeting maintained while larvae undergo massive allometric growth? To what extent do head withdrawal, cocking, striking, thrashing and quivering utilize common neural networks, including motor neurons known to mediate larval bending (Waldrop and Levine, 1989)? How does the animal choose between withdrawal, striking and other responses? In this regard, segments A3 and A2 are particularly interesting because they form a transition zone where the primary response to a noxious stimulus inverts, changing from an immediate strike to withdrawal. Is this choice influenced by prior sensitizing stimulation? How does sensitization-related plasticity interact with developmental plasticity? These questions can be directly tested in the larvae of *M. sexta.*

The authors thank Stephanie Bell and Phi-Nga Le for expert technical assistance, and Wesley Grueber, Richard Levine and Nathan Tublitz for helpful comments. The authors are grateful to the Neural Systems and Behavior course at the Marine Biological Laboratory, Woods Hole, MA, USA, for facilitating the interactions that led to this study. Supported by NIH grants MH38726 and NS35882 to E.T.W and NS23208 to J.C.W.

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