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Maturation of muscle properties and its hormonal control in an adult insect

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Summary

The oviposition of female locusts requires longitudinal muscles to tolerate remarkable lengthening. Whether this ability together with concomitant properties develops during maturation or is present throughout life was investigated. The properties of the locust abdominal muscles involved in oviposition behaviour were investigated with respect to their maturation, segment- and gender-specificity and regulation by juvenile hormone (JH). Muscles from the sixth abdominal segment (an oviposition segment) of mature females (>18 days old) were able to tolerate large extensions (>8 mm). At this length, muscles were still able to generate considerable neurally evoked twitch tension. In contrast, muscle fibres from females less than 5 days old did not tolerate extension of more than 4 mm. At this length, tension generation was negligible. The maximum tension generated at different stimulus frequencies was significantly higher in muscles of females more than 18 days old than in females less than 5 days old. Furthermore, the cross-sectional area of muscle fibres increased significantly during reproductive development. Current-clamp recordings from denervated muscle fibres of females more than 18 days old revealed their ability to generate overshooting action potentials. The potentials were tetrodotoxin (TTX)-insensitive (0.5 μmol l^{-1} TTX), but were blocked by Cd^{2+} (50 μmol l^{-1}) or nifedipine (50 μmol l^{-1}), which suggests the involvement of L-type Ca^{2+} channels. Action potentials recorded from females less than 5 days old differed considerably in amplitude and shape from those recorded from females more than 18 days old, suggesting their maturation during the first 2 weeks of adult life. Inactivation of the corpora allata (CA) by precocene inhibited the maturation of these muscle properties, whereas injection of JH into precocene-treated females reversed this effect. Homologous muscles from the third abdominal segment (a non-oviposition segment, M169) and muscles from males (M214) revealed no comparable changes, although some minor changes occurred during reproductive development. The results suggest a gender- and segment-specific maturation of muscle properties that is related to reproductive behaviour and controlled by JH.

Key words: juvenile hormone, muscle properties, reproductive development, oviposition, insect, *Locusta migratoria*, development.

Introduction

Hormones influence muscle development and morphology in vertebrates and invertebrates. In vertebrates, hormone-induced changes in muscle morphology and function can be related to reproductive development (Venable, 1966; Rand and Breedlove, 1995; Kelly, 1986; Bass, 1986) or to the maintenance of functionality (Everts, 1996; Sacca et al., 1994). In insects, juvenile hormone (JH) and steroids are the major hormones affecting the development, morphology and function of various organs (Weeks and Truman, 1986).

Juvenile hormone, first discovered by Wigglesworth (Wigglesworth, 1934; Wigglesworth, 1936), plays a crucial role in the development and reproduction of insects. During larval and pupal development, JH acts in concert with 20-hydroxyecdysone (20E) to govern metamorphosis by inhibiting the development of adult characters (Riddiford, 1985). However, considerable information has accumulated suggesting that the original role of JH may have been the regulation of reproduction (Sehnal et al., 1996). Many insects use JH to stimulate or depress reproductive development, although the extent and timing of JH action vary considerably (for reviews, see Wyatt and Davey, 1996; Wyatt, 1997).

Reproduction in insects is often accompanied by the expression of specific behaviour that is, in many cases, directly or indirectly regulated by JH. Generally, the effects of JH are diverse, but they predominantly involve courtship behaviour, female sexual receptivity or egg-laying behaviour (Barth and Lester, 1973; Strong and Amerasinghe, 1977; Renucci et al., 1992). One of the most intriguing studies described a direct effect of JH on the neuronal elements responsible for the phonotactic response in female *Acheta domesticus* (Stout et al., 1993). This effect is mediated by JH *via* gene regulation (Stout et al., 1992). In the same species, Cayre et al. (Cayre et al.,

1994) showed that neurogenesis in the mushroom bodies of adult crickets is stimulated by JH, and this might be the basis for hormonal control of oviposition behaviour. Males are less often dependent on the action of JH, although a few studies provide clear evidence for effects of JH in males. Odhiambo (Odhiambo, 1966) reported depressed locomotory activity in male locusts after allatectomy. After re-implantation of the corpora allata (CA), normal activity was restored. In the black cut worm *Agrotis ipsilon*, JH clearly affects male responsiveness to the female sex pheromone (Gadenne et al., 1993).

Analysis of the mechanisms of hormone action requires a detailed knowledge of the developmental changes mediated by hormones. From previous studies, two primary mechanisms of JH action have emerged. First, JH has been shown to bind directly to the membrane and to mediate its effects within minutes without the need for gene transcription (Sevala and Davey, 1989; Yamamoto et al., 1988). The second major mechanism involves gene transcription. Here, JH is thought to modulate gene transcription after it has penetrated the cells and after binding to proteins or nuclei (Jones, 1995; Dubrovsky et al., 2000; Davey, 2000). Several genes have been cloned whose expression is clearly regulated by JH (Wyatt and Davey, 1996). However, the search for a JH receptor has so far been unsuccessful, although the nuclear receptor ultraspiracle, which binds JH with specificity, has been proposed as a good candidate (Jones and Sharp, 1997).

In some insect species, the flight muscles undergo degeneration as a response to high levels of JH (Tanaka, 1994; Davis, 1975; Borden and Slater, 1968; Stegwee et al., 1963). During reproduction, they are no longer needed and now serve to liberate nutrients. In contrast, the Colorado potato beetle (*Leptinotarsa decemlineata*) undergoes a reproductive diapause that results from CA inactivity leading to low levels of JH. During diapause, the flight muscles undergo reversible degeneration (de Kort, 1990; Pener, 1992). However, no direct hormonal regulation of growth and functional maturation of the flight muscles has been demonstrated (Finlayson, 1975).

To determine whether JH has the potential to alter the functional properties of muscle fibres, we investigated locust abdominal longitudinal muscles. These multifunctional muscles are involved in ventilation (Hustert, 1975), flight steering (Baader, 1991) and oviposition (Vincent, 1975; Jorgensen and Rice, 1983a; Rose et al., 2000). During oviposition, females use their abdomen as an ovipositor; they extend their segments in a telescopic manner to up to six times its normal length. During this highly coordinated behaviour (Rose et al., 2000), telescopic extension mainly involves abdominal segments 4–7 in which the longitudinal muscles are required to follow and tolerate lengthening (superextension). In an ultrastructural study, Jorgensen and Rice (Jorgensen and Rice, 1983a) demonstrated pronounced differences between the longitudinal muscles present in abdominal segments 4–7 (oviposition segments) and those in the more anterior segments 1–3 (non-oviposition segments). One of the unique features of muscles from oviposition segments is their ability to fragment their Z-lines into so-called Z-bodies. Since the ability to tolerate extension is closely related to the sexual maturation of female locusts, we investigated whether this ability develops during adult life or is already present at adult emergence. We also explored the possible gender- and/or segment-specificity of muscle properties and whether JH is involved in the regulation of these properties. Our results indicate that, during reproductive development, the properties of the longitudinal muscle of females are subject to change, possibly to adapt them for oviposition behaviour. These properties are segment- and gender-specific and are controlled by JH, as indicated by experiments examining the inhibition of JH release and JH replacement injections.

Materials and methods

Preparations and solutions

Experiments were performed on adult *Locusta migratoria migratorioides* (R&F). Animals were purchased as fifth-instar larvae from a commercial supplier (btbe, Schnürpflingen, Germany) and maintained in laboratory cages at 32±2 °C in a 12 h:12 h dark:light regime. Prior to experiments, cold-anaesthetised (1 h at 4 °C) animals were dissected under physiological saline (in mmol l^{-1}: NaCl, 140; KCl, 5; $CaCl_2$, 5; $MgCl_2$, 1; Tes, 5; $NaHPO_4$, 4;, trehalose, 5; saccharose, 100). The saline for two-electrode current-clamp recordings consisted of (in mmol l^{-1}) NaCl, 140; KCl, 10; $CaCl_2$, 2; $MgCl_2$, 2; Mops, 10; saccharose, 90; and contained 0.5 mmol l^{-1} Tetracaine (Sigma) to reduce spontaneous or stimulus-evoked contractions. Tetracaine strongly reduced Ca^{2+}-induced Ca^{2+} release (CICR; Csernoch et al., 1999) but had no obvious effects on recorded potentials (N=3). All experiments were performed at 22–24 °C. For staging, animals were isolated at the day of emergence. Muscles and nerves were numbered according to the schemes of Snodgrass (Snodgrass, 1935) and Hustert (Hustert, 1974) respectively.

Electrophysiology

Extracellular recordings from peripheral nerves and stimulation of motor axons were obtained using suction or monopolar hook electrodes. Muscle contractions were evoked by stimulating (0.5 ms, 0.5–5 V) the motor axons. To monitor stimulation-evoked action potentials, the terminal branch innervating the muscle was recorded *en passant*.

For two-electrode current-clamp recordings, muscles were isolated and mounted with the internal side up in a recording chamber. The internal side of longitudinal muscles is directed towards the gut, whereas the external side is adjacent to the body wall. The preparation was continuously perfused (1 ml min^{-1}) with saline (see above). Current and voltage electrodes were made from thin-walled borosilicate glass (Clark Electromedical Instruments, UK) and filled with 2 mol l^{-1} potassium acetate containing 20 mmol l^{-1} potassium chloride (resistance 10–20 MΩ). The current electrode was inserted in the middle of the fibre, whereas the voltage electrode was placed half-way between the end of the fibre and

the current electrode. After impalement of muscle fibres, electrodes were allowed to seal for 15 min. A Ag/AgCl bath electrode served as potential reference. Only fibres from the internal layer were recorded. The signals were controlled and amplified by a two-electrode clamp amplifier (Turbo TEC01C, NPI Electronics, Tamm, Germany) connected to a personal computer. Current injection and acquisition of signals were controlled by a computer program (cell works, NPI electronics, Tamm, Germany). Acquired signals were digitised (sampling rate 10 kHz) and stored on hard disk for subsequent evaluation (Cell Works Reader, NPI Electronics, Tamm, Germany; PlotIt, Scientific Programming Enterprises).

Hormonal treatment

To inactivate the CA chemically, female locusts were treated once at the time of adult emergence by topical administration of precocene I (7-methoxy-2,2-dimethyl-3-chromene;, Sigma, 500 μg dissolved in 15 μl of acetone) onto the dorsal neck fold. Control animals were treated with acetone only (N=15). Approximately 95 % of precocene-treated animals survived the treatment and, at maturation, showed clear signs of CA degeneration (undeveloped oocytes, the cuticle remained light-coloured, no hypertrophy of the abdominal muscles was apparent). Some of the precocene-treated animals were also treated with JH [7.5 μg JH III (Sigma, 75 %) in 5 μl 70 % ethanol] or ethanol alone (N=10, control). JH III has been identified as the prevailing hormone during the gonotrophic cycle of locusts (Rembold, 1981). JH was first injected into the abdomen on day 5. Subsequent injections were made on days 8 and 11 to ensure that a sufficent titre of JH was present. This pattern of injection was chosen after preliminary experiments with a single injection or a smaller amount of JH did not induce signs of normal maturation (development of oocytes, cuticle darkly coloured). Although we have not determined the resulting level of juvenile hormone in the haemolymph, the amount injected was within or below the range of concentrations of JH or its analogue methoprene used in other studies [JH (Tawfik et al., 1997); methoprene (Dhadialla and Wyatt, 1983)]. The survival rate of JH-treated animals was approximately 70 %.

Tension recordings

Tension recordings were made from homologous dorsal longitudinal muscles of the sixth abdominal segment (see Fig. 1A, M214, Fig. 1B) or the third segment (M169). At its anterior insertion, the muscle was fixed to the Petri dish with insect pins. The posterior side was attached to the lever arm of a force transducer (Fort-10, World Precisions Instruments) mounted on a micromanipulator. This was achieved by clamping the piece of cuticle on which the muscle fibres insert to a small clamp made of stainless steel. This arrangement allowed precise lengthening of the muscle during the experiments without influencing muscle contractions. After each series of contractions, the preparation was repeatedly superfused with aerated saline. Between a series of contractions, the muscle was left unexcited for at least 5 min to recover from contractions and to adapt to length changes. Tension recordings were approximately isometric (deflection of the transducer tongue: 6 μm per 1 mN). The response of the transducer was linear over the range used in the experiments and was calibrated after each experiment.

To determine the length/tension relationship, single twitch tension, which minimised fatigue of muscle fibres during the course of an experiment, or passive tension was measured. At each pre-set length (0.5 mm increments), we sampled the mean of five twitches. Experiments were terminated when the fibres started to break.

Cross sections of muscle fibres and measurement of cross-sectional area

In preliminary experiments, we noted considerable hypertrophy of muscle fibres in female locusts during the first 2 weeks of adult life. To examine and quantify this observation, we measured the cross-sectional area of muscle fibres from females in abdominal segments 3 (M169) and 6 (M214). Freshly isolated muscles were pinned in a Petri dish lined with Sylgard and stretched to their resting length (3.5 mm for untreated females more than 18 days old; 2 mm for all other groups). The muscles were than fixed in 2 % glutaraldehyde, post-fixed with 2 % osmium tetroxide and subsequently dehydrated and embedded in Epon 812 (Fluka). Cross sections (0.5 and 1 μm) from the middle region of the muscle were cut on an ultramicrotome, mounted on slides and stained with Methylene Blue. The sections were then examined under a bright-field microscope. From each section, a digital picture was taken with the aid of a CCD camera (Sony ICX038AK, resolution 752×582 pixels). The mean cross-sectional area of a single fibre was determined by calculating the cross-sectional area of the entire muscle (Sigmascan Pro 5.0) and dividing by the number of fibres.

Statistical evaluation

Data are expressed as means and their standard errors (S.E.M.). Statistical significance was determined using non-parametric (Mann–Whitney U-test, Kruskal–Wallis analysis of variance, ANOVA, on ranks) or, when criteria were met, parametric (one-way ANOVA) analysis. *Post-hoc* tests were employed for multiple comparisons (Dunn's method, Tukey test). The significance level was set to P<0.05.

Results

Muscle properties during female reproductive development

After the fifth-instar larvae have moulted to become adults, individuals underwent growth and maturation. In our colony, growth was accompanied by changes in cuticle coloration. The majority of animals attained maturity by days 10–15 of adult life. To determine whether changes in muscle properties occurred during maturation, we measured and compared the muscle properties of immature (<5 days after adult emergence) and mature (>18 days after adult emergence) locusts. We were also interested in comparing the properties of homologous

muscles from segments involved in oviposition (M214, Fig. 1) and from non-oviposition segments (M169).

The length/tension relationship of longitudinal muscle 214 from female locusts less than 5 days old (Fig. 2A, *N*=10) revealed a peak at approximately 2 mm. Further lengthening resulted in a rapid decline in tension and eventually in breakage of muscles fibres (Fig. 2A, arrow). In all experiments, muscles from immature females did not tolerate lengths exceeding 4 mm. In contrast, muscles of females more than 18 days old exerted their maximum tension between 2.5 and 3.5 mm and tolerated stretching of more than 8 mm. At this length, twitch tension was still 35 % of the maximum tension (Fig. 2A, *N*=9). In four experiments, we stretched muscles up to 12 mm. In these experiments, the muscles showed no signs of damage and still generated considerable twitch tension (approximately 10 % of maximum). Our own observations suggest that the normal physiological range of longitudinal muscles is between 1 and 4 mm, and this is supported by the work of Jorgensen and Rice (Jorgensen and Rice, 1983b).

The passive mechanical properties of muscle fibres are determined by their series and parallel elastic elements. These elements are responsible for passive retraction forces exerted by the muscle at different length. The length/passive tension relationship of muscles from females less than 5 days old (*N*=10) showed a steep increase in passive tension, starting at 1 mm, whereas in females more than 18 days old (*N*=9), passive tension developed more slowly and reached a plateau at 5 mm (Fig. 2B). Further lengthening to more than 8 mm resulted in only minor increases in passive tension.

A comparison of the maximum tension exerted by the longitudinal muscles of females less than 5 days old and females more than 18 days old showed significant differences. Both twitch tension and tetanic tension (5, 10, 20 and 50 Hz) were approximately twice as high in muscles of females more than 18 days old (*N*=9) compared with females less than 5 days old (Fig. 2C; $P<0.05$, *N*=11). It was also evident that, during a single twitch, both the contraction time (the time from the beginning to the peak of tension) and the half-relaxation time were significantly increased in mature females (Table 1; $P<0.05$, *N*=6), and these increases were probably responsible for the decrease in the stimulation frequency required to elicit a smooth tetanus (Fig. 2D).

Juvenile hormone and the maturation of muscle properties

JH has been reported to have a variety of functions during sexual maturation of adult insects (Wyatt, 1997; Wyatt and Davey, 1996). To examine the importance of JH for the maturation of longitudinal muscle properties during reproductive development, we chemically inactivated the natural source of JH, the CA, by the application of precocene (Bowers et al., 1976; Pener et al., 1978). Some precocene-treated animals were also injected with JH as a control.

Approximately 90 % of the females treated with precocene on the day after adult emergence showed clear signs of impaired maturation (undeveloped oocytes, cuticle remained light coloured, no hypertrophy of abdominal muscles). Muscle

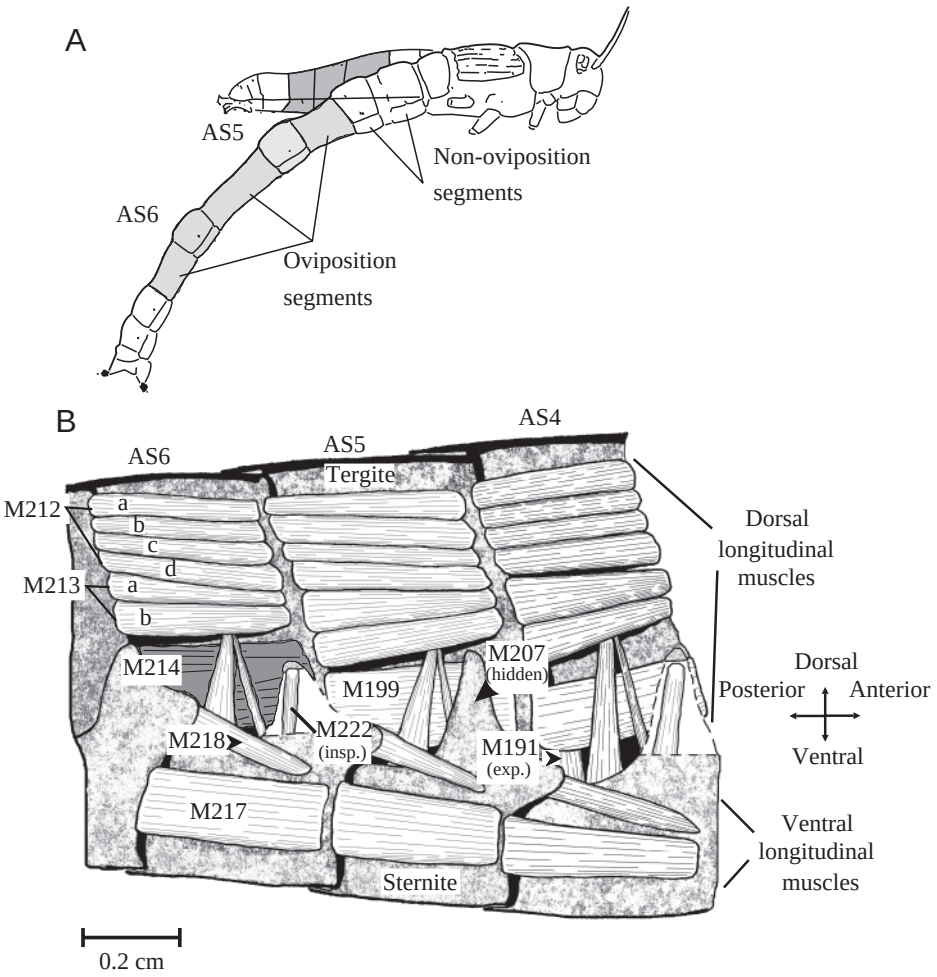

Fig. 1. (A) Sketch of an ovipositing locust. Extension of the abdomen is achieved by unfolding the intersegmental membranes between the fourth and seventh segments (oviposition segments, shaded). (B) The internal anatomy of the fourth, fifth and sixth abdominal segment (AS4–AS6; the internal layer of muscle fibres is at the top of the figure). Longitudinal muscles are referred to as ventral (sternal) or dorsal (tergal) longitudinal muscles. During oviposition, these muscles must follow and tolerate the extension. A and B were adapted from Rose et al. (Rose et al., 2000). a–d and a,b designate part of the muscles M212 and M213, respectively; exp., expiratory; insp., inspiratory.

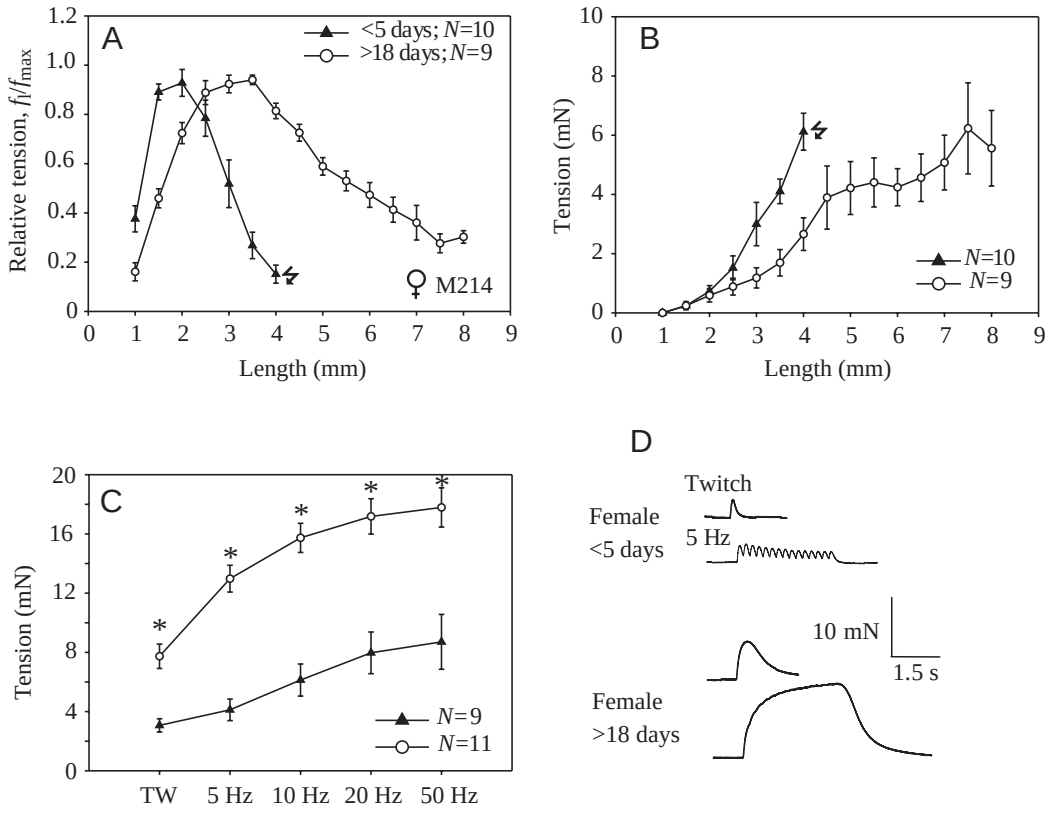

Fig. 2. Contraction properties of longitudinal muscle M214 measured in females less than 5 days old and females more than 18 days old. (A) The length/twitch tension relationship revealed that only muscles from mature females (>18 days old) were able to tolerate extensive lengthening without breakage of fibres. At 8 mm muscle length, the fibres still generated approximately 30 % of maximum tension. The tension generation of muscle fibres from females less than 5 days old declined rapidly after reaching a maximum, and the fibres broke at 4 mm (indicated by an arrow). f/f_{max}, muscle tension at a particular length normalized to maximum tension. (B) Passive tension rose more steeply in muscles from females less than 5 days old than in females more than 18 days old. Above 4.5 mm, passive tension reached a plateau in muscles of females more than 18 days old. (C) A comparison of maximum forces generated by different stimulation frequencies revealed significantly higher tension in muscles from females more than 18 days old compared with muscles from females less than 5 days old (P<0.05). TW, twitch. An asterisk indicates statistical significance (Mann–Whitney rank sum test, P<0.05). Values are means ± S.E.M. (D) Examples of twitch and 5 Hz contractions. Muscles from females less than 5 days old had shorter twitch durations (see also Table 1) and higher tetanus fusion frequencies than muscles from females more than 18 days old.

Table 1. *Twitch contraction and half-relaxation times of muscles 169 and 214*

	Male		Female					
	M214 <5 days	M214 >18 days	M169 <5 days	M169 >18 days	M214 <5 days	M214 >18 days	M214 >18 days Precocene	M214 >18 days Precocene+JH
Contraction time (ms)	80.0±7.9	104±8.1	76.3±2.9	73.6±5.5	138.5±12.5	273.6±17.2	159.6±19.5	280.7±23.4
50 % relaxation time (ms)	123±17.7	157.0±25.3	105.4±7.8	111.0±11	158.4±18.9	374.4±43.5	243.7±12.5	332.2±42.3
N	5	5	6	5	6	6	6	6

Contraction time (time from the onset to peak contraction) and 50 % relaxation time (time measured from peak contraction to 50 % relaxation) did not change significantly during maturation of male M214 or female M169 (P>0.05). In contrast, twitch kinetics of M214 from untreated females changed significantly during reproductive development (<5 days old *versus* >18 days old). Changes were suppressed in females treated with precocene (M214, >18 days old, precocene), but re-appeared after application of juvenile hormone (JH). Examples of single twitches are given in Fig. 2, Fig. 3, Fig. 7 and Fig. 8.

For males (M214) and females (M169), statistical differences were tested by Mann–Whitney rank sum test, whereas differences between female M214 were determined by one-way ANOVA with a subsequent test for multiple comparisons (Tukey test).

Values are means ± S.E.M.

214 of mature precocene-treated animals had a length/tension curve that showed a steep increase to a maximum tension at approximately 2 mm (N=10). Additional extension resulted in a rapid decline in tension and eventual rupture of muscles fibres at 4.5 mm (Fig. 3A). The curve therefore resembled the length/tension relationship obtained from untreated females less than 5 days old (compare with Fig. 2A). Control animals (only acetone applied) showed no signs of impaired maturation (15 out of 15 locusts). Females (>18 days old, N=6) injected with both precocene and JH had well-developed oocytes and a

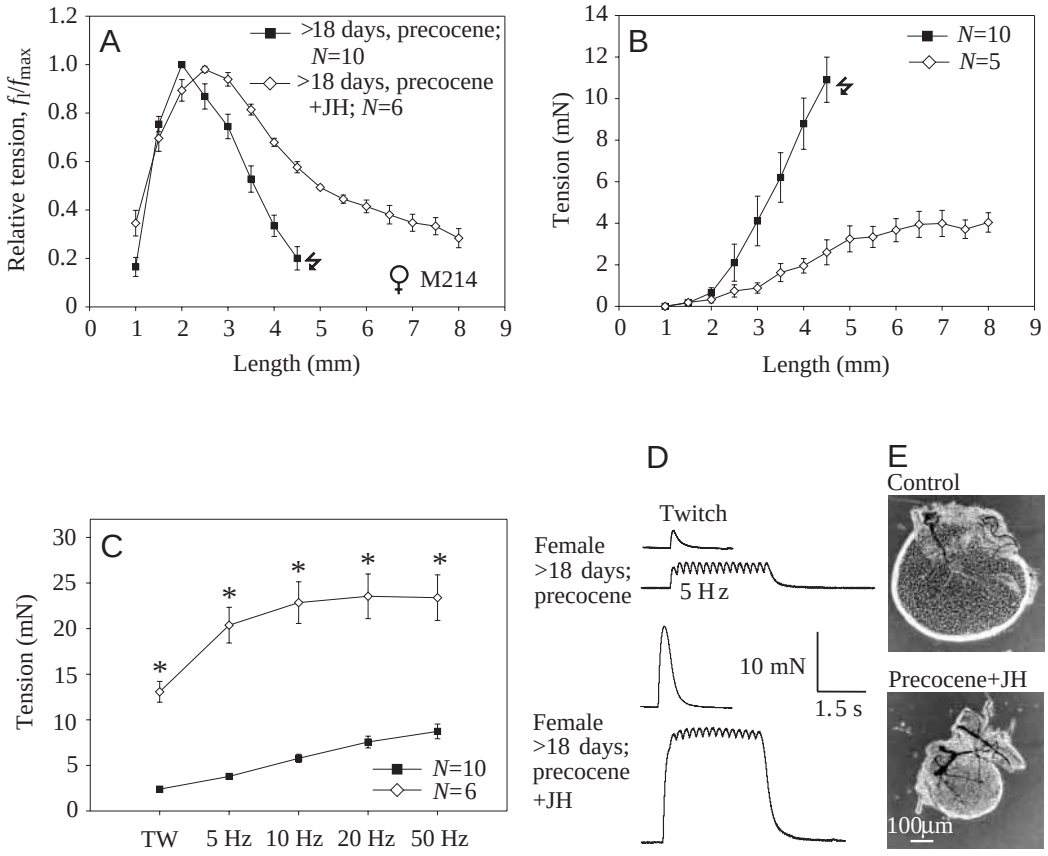

Fig. 3. A comparison of the properties of muscle fibres (M214) from mature females (>18 days old) treated with precocene or precocene plus juvenile hormone (JH). (A) The length/twitch tension relationship, showing that longitudinal muscles from precocene-treated females were not able to tolerate a length exceeding 4.5 mm without breaking (arrow). At this length, approximately 20 % of maximum tension was generated. In contrast, muscles from females treated with precocene plus JH tolerated extensions up to 8 mm and above and still generated more than 30 % of maximum tension. f/f_{max}, muscle tension at a particular length normalized to maximum tension. (B) The passive length/tension relationship revealed pronounced differences. Passive tension increased steeply in muscles from precocene-treated females, whereas muscles from females treated with precocene plus JH generally exerted a lower tension that increased more slowly. (C) The maximum tension generated by the two groups differed significantly (P<0.05). The tension generated by muscles from precocene-treated animals was approximately one-third of the tension generated by muscles from animals treated with precocene plus JH. TW, twitch. An asterisk indicates statistical significance (Mann–Whitney rank sum test, P<0.05). Values are means ± S.E.M. (D) A comparison of tetanus fusion frequencies revealed no consistent differences comparable with those in Fig. 2D. (E) To confirm that the corpora allata (CA) from animals injected with precocene plus JH were indeed affected by precocene, the CA from these females were compared with those of untreated females (Control). The diameter of the CA from treated females was approximately 30 % of that of control females.

darkly coloured cuticle that was most obvious at the site of JH injection. Muscle 214 of these animals tolerated extensions up to a length of 8 mm and above (some were extended up to 10 mm with no signs of muscle fibre damage). At approximately 8 mm, the muscles generated approximately 30 % of their maximum tension (Fig. 3A, precocene+JH). Control animals (precocene+ethanol) did not differ from animals treated with precocene alone with respect to their maturational status (10 out of 10 locusts, data not shown).

The passive tension exerted by the muscles increased rapidly in precocene-treated females (N=10), whereas muscles from animals injected with JH showed pronounced viscoelastic properties, as indicated by a slow increase in passive tension (Fig. 3B, N=5).

The measurement of maximum tension revealed a pronounced difference between the two groups. Muscles from females treated with precocene+JH (N=6) exerted tension that was 2.5–3.0 times greater than that of females treated with precocene alone (Fig. 3C, N=10). A comparison of the twitch kinetics revealed significantly increased twitch contraction times in precocene+JH-treated animals (Table 1, Fig. 3D; P<0.05, N=6). Although consistent differences were also apparent for the half-relaxation time, the values were not statistically different (P>0.05, N=6).

Because the effects of CA inactivation were obvious in females treated with precocene alone (cuticle coloration, undeveloped oocytes, massive fat body), but not in females treated with precocene+ JH (precocene might have failed to inactivate the CA, which would have been indistinguishable from the case in which precocene inactivated the CA and JH reversed this effect), we compared the morphology of the CA with that of non-treated females more than 18 days old (control, Fig. 3E, N=2). Females treated with precocene+JH were expected to have CA showing a noticeable atrophy (Pener et al., 1978). Freshly dissected CA from females more than 18 days old (precocene+JH) had atrophied dramatically compared with those of control animals (Fig. 3E), providing further evidence that precocene was indeed effective in females treated with precocene+JH.

Comparing the cross-sectional area of muscle fibres from females less than 5 days old with that of females more than 18 days old, we observed a pronounced hypertrophy (Fig. 4A,B,

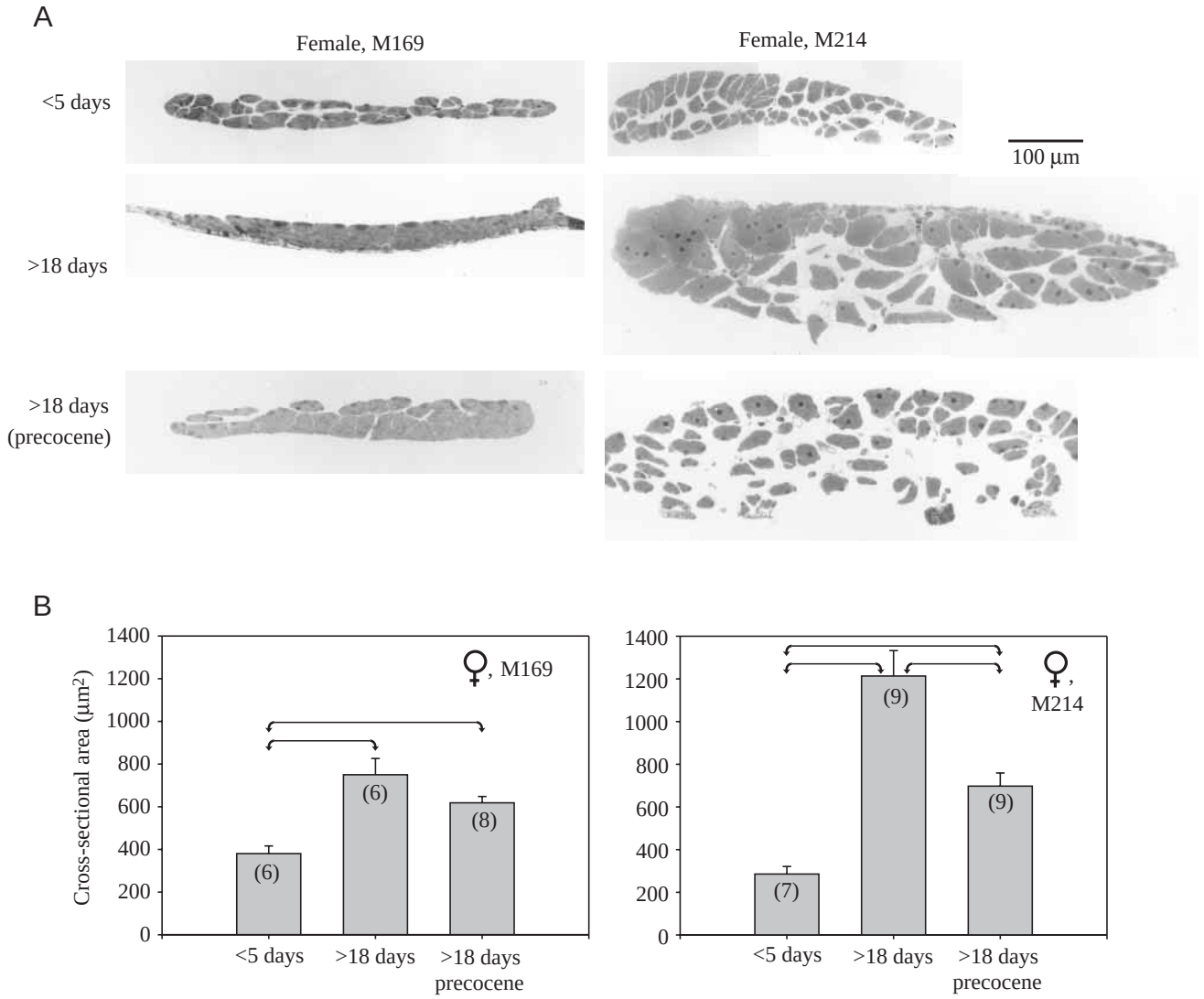

Fig. 4. A comparison of the cross-sectional area of longitudinal muscles 169 and 214 from female locusts at different stages of development. (A,B) Cross sections revealed a significant, but not dramatic, hypertrophy of M169 during reproductive development (left-hand panel, <5 days old *versus* >18 days old). To examine a possible role for juvenile hormone in the hypertrophy, muscles from precocene-treated animals were also compared. For M169, there was no significant reduction in cross-sectional area evident for females treated with precocene (A,B; >18 days old *versus* >18 days old, precocene-treated). Muscle 214 (A,B, right-hand panel) showed marked hypertrophy during maturation. The degree of hypertrophy was significantly decreased in females treated with precocene (B, right-hand panel >18 days old *versus* >18 days old, precocene-treated), although values were still considerably higher than those of females less than 5 days old. The lines in B indicate significant differences (P<0.05, ANOVA with Tukey test). The number of experiments is given in parentheses. Values are means + S.E.M.

right-hand panel). The mean cross-sectional area of muscle fibres from females more than 18 days old was considerably greater (1213±119.3 μm^2, N=9) than that of muscles of females less than 5 days old (285±35.3 μm^2, N=7; P<0.05). The muscles of females treated with precocene hypertrophied to a lesser extent. These muscle fibres had a mean cross-sectional area of 697.2±61.8 μm^2 (N=9), which was still significantly different from that of untreated females (P<0.05). Mean fibre numbers were not significant different for M214 (<5 days old, 68±5; >18 days old, 73±2; >18 days old, precocene-treated, 71±3) and M169 (<5 days old, 48±2; >18 days old, 42±3; 18 days old, precocene-treated, 42±2).

In most of the preparations of muscle 214 from females more than 18 days old, we observed spontaneous contractions of muscle fibres even when the muscle was denervated. The frequency of contractions initially increased when the muscle was stimulated mechanically (stretch or release). To investigate muscle fibre excitability, we made current-clamp recordings from single denervated muscle fibres. Current injection into muscle fibres of females less than 5 days old and more than 18 days old evoked action potentials (Fig. 5A). The threshold for action potentials was consistently more depolarised in females less than 5 days old (–35 to –40 mV, N=3) than in females more than 18 days old (–45 to –50 mV;

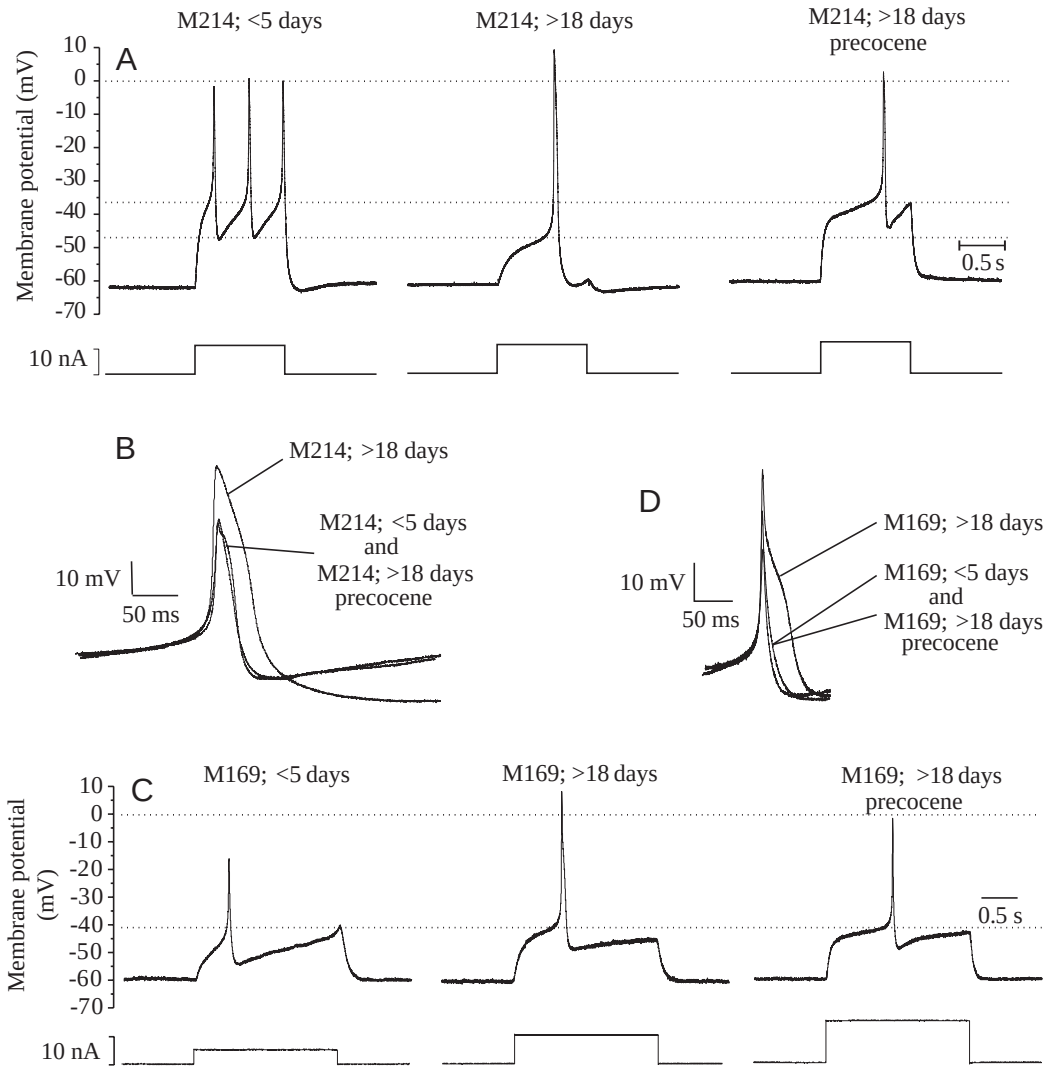

Fig. 5. Stimulus-evoked action potentials revealed by current-clamp recordings from denervated muscle fibres. The amount of current injected was adjusted for each preparation to just reach the threshold at which action potentials were generated. (A) In females less than 5 days old, action potentials were non-overshooting with a moderate afterhyperpolarisation (left-hand panel). Muscles from females more than 18 days old generated overshooting action potentials with a pronounced afterhyperpolarisation (centre panel, see also B). In contrast, action potentials generated by fibres from females more than 18 days old treated with precocene resembled those from females less than 5 days old. (B) Action potentials from A shown on an expanded time scale for comparison. The action potential width for muscles of females more than 18 days old was increased. This increase was consistently absent from females treated with precocene. (C) Current injection evoked action potentials in fibres of muscle 169. Potentials from females less than 5 days old were non-overshooting and were followed by a small afterhyperpolarisation (C, left-hand panel). When females attained maturity, action potentials were overshooting but had still a small afterhyperpolarisation (C, centre panel). The action potentials of females treated with precocene were larger in amplitude than those of females less than 5 days old but were still non-overshooting. The afterhyperpolarisation of all groups was comparable (C, right-hand panel). (D) Potentials from C shown on an expanded time scale. The action potential width of mature female (untreated) muscle was increased.

N=5). The peak amplitude of the action potential was higher (>18 days old, +10 to +25 mV; <5 days old, –5 to+10 mV, Fig. 5B) and the spike width was greater in fibres of females more than 18 days old compared with females less than 5 days old. The spike afterhyperpolarisation was more pronounced in females more than 18 days old (Fig. 5B). To test whether Na^+ is the predominant inward charge carrier of action potentials, we applied TTX to muscle fibres of females more than 18 days old. In all experiments, TTX (0.5 μmol l^{-1}, *N*=3) was ineffective in blocking the generation or even in changing the shape of the action potential (Fig. 6A). In contrast, in five out of five experiments, Cd^{2+} (50 μmol l^{-1}, Fig. 6B), but not Ni^{2+} (data not shown), completely blocked the action potentials. The effect of Cd^{2+} was readily reversible by washing with saline.

The blocking effect of Cd^{2+} suggests the involvement of Ca^{2+} as the predominant charge carrier. This was supported by experiments in which we omitted Ca^{2+} from the saline, preventing the generation of action potentials (*N*=3, data not

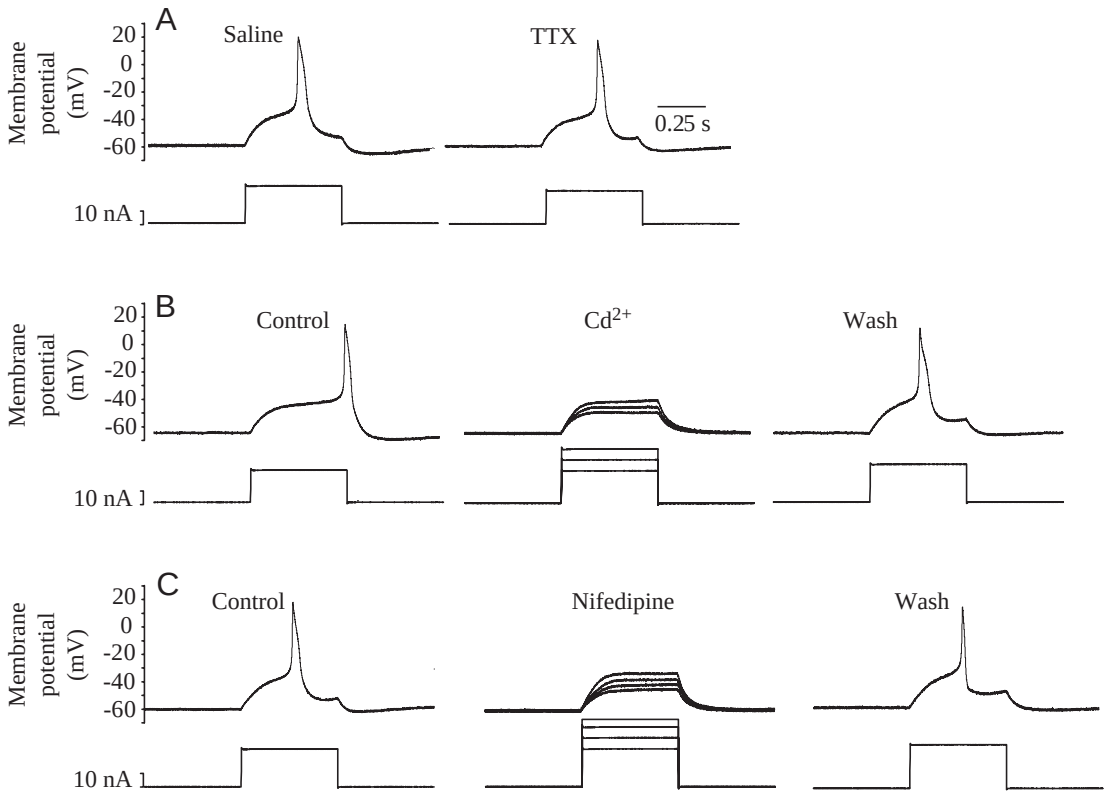

Fig. 6. Characterisation of action potentials recorded from muscle 214 from mature females. (A) The application of tetrodotoxin (TTX) (0.5 μmol l^{-1}) had no effect on the generation or the shape of the action potential. (B) Application of Cd^{2+} (50 μmol l^{-1}) completely blocked action potentials. Even increasing the injected current (from 24 to 40 nA) was not effective in generating potentials when Cd^{2+} was present in the bath. After washing in normal saline (10 min), action potentials reappeared. (C) Nifedipine (50 μmol l^{-1}), which is known to modulate L-type Ca^{2+} channels, blocked the generation of action potentials even when the amount of current was increased (from 27.5 to 49 nA). Washing with normal saline reversed the blocking effect of nifedipine. The examples shown in A, B, C are from two different preparations.

shown). To determine the type of channel involved, we tested the specific L-type Ca^{2+} channel modulator nifedipine. Nifedipine (50 μmol l^{-1}) effectively blocked action potential in four out of four experiments (Fig. 6C). Even the injection of considerable current (up to 49 nA, Fig. 6C, middle trace) was not sufficient to evoke action potentials. The effect of nifedipine was fully reversible after 5 min of washing with normal saline.

Segment-specificity of muscle properties

The toleration of extensive lengthening and the concomitant muscle properties were measured as described above for muscle 214 of the sixth abdominal segment, which is involved in telescopic extension during oviposition. To investigate whether similar properties and their changes can be found in homologous muscles of non-oviposition segments, we measured the properties of muscle 169 in the third abdominal segment (M169, non-oviposition segment) before and after locusts underwent reproductive development.

The length/tension relationship of muscle 169 was similar in females less than 5 days old (N=6) and in females more than 18 days old (Fig. 7A, N=9). Muscles from neither developmental stage tolerated extension of more than 4–4.5 mm length (Fig. 7A, arrows). At this length, twitch tension was almost negligible. The passive tension exerted by the muscles was consistently higher in females more than 18 days old (N=5) than in females less than 5 days old (Fig. 7B, N=6). A similar relationship was obtained for the maximum tension. Mean values were higher in females more than 18 days old (N=5) than in females less than 5 days old (Fig. 7C, N=6), although the difference was not significant (P>0.05).

The time required for a twitch to reach peak tension and the half-relaxation time was similar in females less than 5 day old and females more than 18 days old (Table 1). This was consistent with the finding that muscles from both stages had a comparable tetanus fusion frequency of approximately 20 Hz (Fig. 7D; >18 days old, N=5; <5 days old, N=6). Because of the similarities in contraction properties of muscle M169 from females less than 5 days old and females more than 18 days old, we decided not to measure contraction properties from precocene-treated females.

The cross-sectional area of muscle 169 was found to increase in females more than 18 days old (Fig. 4A,B, left-hand panel). The mean cross-sectional area of muscles from females less than 5 days old was 380.3±35.7 μm^2 (N=6), but increased to 750±76.5 μm^2 (N=6) in females more than 18 days

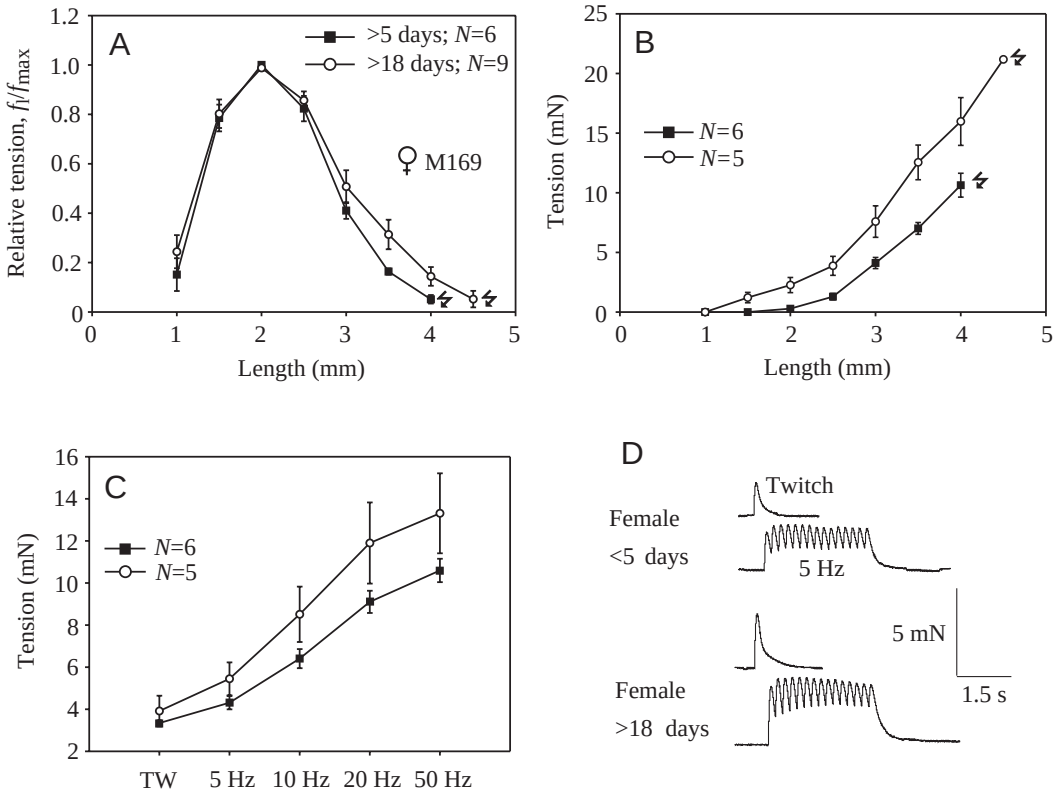

Fig. 7. Contraction properties of longitudinal muscle 169 (from non-oviposition segments) from females less than 5 days old and females more than 18 days old. (A,B) The length/tension curves revealed no age-dependent differences. Muscle fibres were not able to tolerate extensions of more than 4–4.5 mm (arrows indicate breakage of muscle fibres). Only the passive tension (B) was slightly increased in muscles of females more than 18 days old. f/f_{max}, muscle tension at a particular length normalized to maximum tension. (C) The maximum tension exerted by muscle 169 of females more than 18 days old was consistently higher than that of females less than 5 days old, although the difference was not significant (P>0.05; Mann–Whitney rank sum test). TW, twitch. Values are means + S.E.M. (D) No consistent difference was found with respect to contraction kinetics (see also Table 1).

old. These values were significantly different (P<0.05). Females treated with precocene had cross-sectional areas that were similar to those of untreated females (618±30 μm^2, N=8, Fig. 4A,B; P>0.05).

Muscle 169 was also able to generate action potentials, although we never observed spontaneous contractions of single muscle fibres. Potentials recorded from fibres of females less than 5 days old had a low threshold (approximately −45 mV), and the peak amplitude was −20 to −10 mV (Fig. 5C, N=3). After the females had attained maturity, the threshold for action potential generation increased to approximately −40 mV, and the peak amplitude was approximately 10 mV (Fig. 5C, N=3). The width of the action potential was considerably greater in mature females compared with females less than 5 days old (Fig. 5D). Muscle fibres from females treated with precocene were also able to generate action potentials (N=2). Their threshold was comparable with that of untreated females more than 18 days old. The peak amplitude was around 0 mV, and the width of the potentials was within the range of that of females less than 5 days old (Fig. 5D). The powerful afterhyperpolarisation that was characteristic of the fibres of muscle 214 (see Fig. 5A) was not evident in muscle 169 (N=3). The effect of Cd^{2+} on the generation of action potentials in M169 was similar to that on muscle 214: Cd^{2+} (50 μmol l^{-1}) blocked action potentials in all stages (N=2; data not shown).

Gender-specificity of muscle properties

To investigate whether the properties of muscle from males changed during reproductive development, we measured the contraction properties of muscle 214 from immature (<5 days old) and mature (>18 days old) male locusts.

Longitudinal muscles 214 of males were not able to tolerate stretch of more than 4 mm at either stage (Fig. 8A, arrows; <5 days old, N=9; >18 days old, N=7). Furthermore, the length/tension curves were nearly identical, with muscles exerting very little twitch tension at lengths between 3.5 and 4 mm.

Passive tension was consistently higher in muscles from males more than 18 days old (N=6) compared with muscles from males less than 5 days old (N=5), although the variation in individual values was high. Compared with males less than 5 days old (N=4–6), the maximum tension of muscles from males more than 18 days old (N=6–9) was higher at all

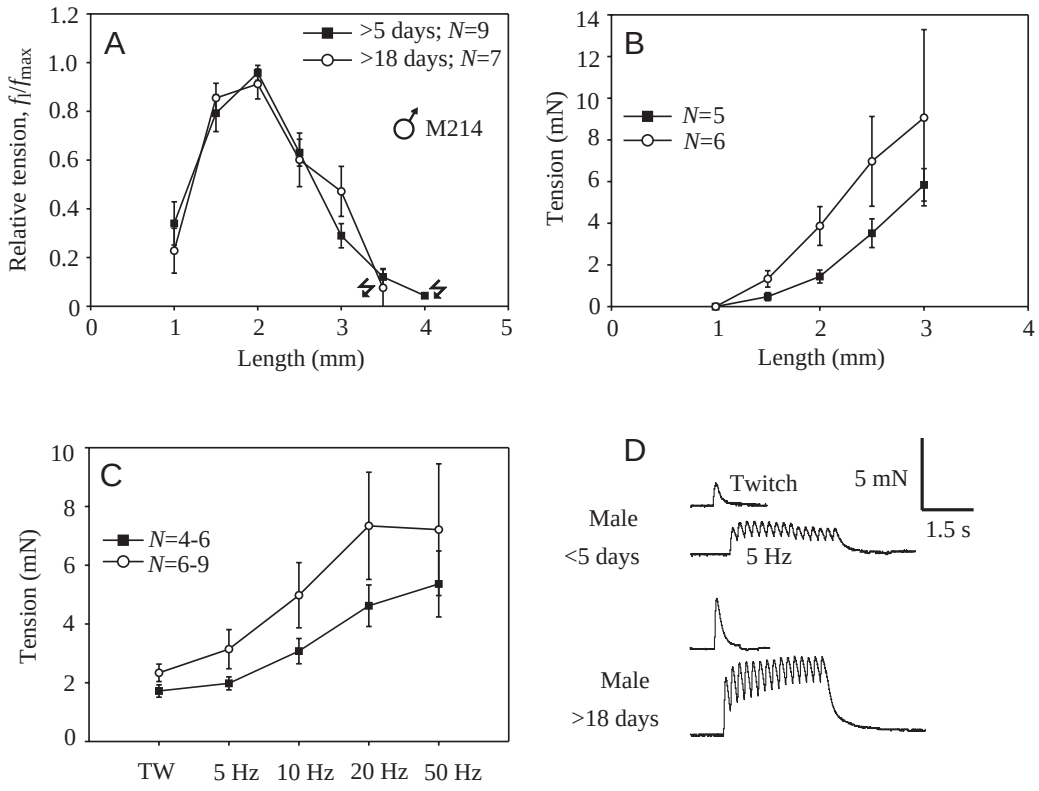

Fig. 8. Contraction properties of longitudinal muscle 214 measured in males less than 5 days old and males more than 18 days old. (A) The length/twitch tension curves of the two groups were comparable. Muscle fibres were not able to tolerate extensions of more than 4 mm (arrows). f/f_{max}, muscle tension at a particular length normalized to maximum tension. (B) Passive tension increased steeply in both groups, starting at 1 mm length, but was consistently higher in mature males (>18 days old), although the difference was not significant (P>0.05; Mann–Whitney rank sum test). (C) The maximum tension generated by muscles from males more than 18 days old exceeded that of muscles of males less than 5 days old. However, the means were not statistically different (P>0.05; Mann–Whitney rank sum test). Values are means ± S.E.M. TW, twitch. (D) No differences were evident with regard to the speed of contraction (see also Table 1) or tetanus fusion frequency.

stimulation frequencies tested (Fig. 8C). However, the values were not significantly different (P>0.05). Furthermore, the time to peak contraction and the half-relaxation time were higher in males more than 18 days old, but the difference was not significant (Table 1, P>0.05, N=5). The tetanus fusion frequency of muscle was approximately 20 Hz for both mature and immature males (N=5). In a few experiments, we current-clamped muscle fibres from male locusts. These fibres generated small action potentials upon current injection (N=3), but these were not further characterised.

Discussion

During the sexual maturation of locusts, the longitudinal muscles of females acquire specific properties probably necessary for appropriate oviposition behaviour. Most evident is their ability to tolerate large extensions, a property unique among invertebrates muscles (Hardie, 1975; Miller, 1974; Ishii and Takahashi, 1982; Josephson and Ellington, 1997). In an ultrastructural study of locust longitudinal muscles, Jorgensen and Rice (Jorgensen and Rice, 1983a) underlined the importance of Z-line fragmentation for their extensibility. In stretched muscles, they found Z-lines that were fragmented into so called ‘Z-bodies’. Z-line fragmentation might also influence the passive properties of muscle fibres. As indicated by the passive length/tension relationship, the longitudinal muscles became more viscoelastic. We can only speculate about the molecular mechanisms of Z-line fragmentation, but it seems likely that specific proteins that determine the elastic properties of invertebrate and vertebrate muscle fibres are involved [for a review, see (Maruyama, 1999)]. In arthropods, connectin, in concert with projectin, links the Z-line to the myosin filament and is responsible for the generation of passive tension upon stretch (Manabe et al., 1993), whereas kettin is regarded as a scaffold of insect muscle Z-lines (Bullard and Leonard, 1996). We therefore assume that, during locust reproductive development, muscle proteins undergo changes in their relative expression and/or structure to enable Z-line fragmentation and low passive tensions upon stretch. As a first step towards characterization of the molecular mechanism of Z-line fragmentation, we plan to compare the expression of selected proteins in immature and mature females.

The development of specific muscle properties during

maturation depends on JH, as indicated by the experiments in which we chemically inactivated the CA with precocene. Despite its name, JH has been shown to have multiple effects in adult insects [for reviews, see (Wyatt, 1997; Wyatt and Davey, 1996)]. Most of these involve degeneration and regeneration of flight muscle (Chudacova and Gutmann, 1978; Stegwee et al., 1963). Our results, however, suggest various changes in contractile and membrane properties. These changes are correlated with maturation and are a prerequisite for successful oviposition behaviour.

Although the results of this study suggest an important role for JH, we have to be cautious in the interpretation of our results since we have not measured the effective concentration of JH in precocene-treated animals or those additionally injected with JH. In female locusts (*Schistocerca gregaria*, *Locusta migratoria*), the JH titre increased significantly during maturation, starting at the seventh day after adult emergence (Rembold, 1981; Tawfik et al., 2000). In male *Schistocerca gregaria*, JH titres are comparatively low, but increase gradually within the first 30 days after the final moult (Tawfik et al., 2000). It is possible that JH titres, rather than the simple presence or absence of JH, affected our results. For example, some active and passive properties (length/tension curve, time to peak contraction, twitch tetanus fusion frequency) of females treated with precocene+JH (Fig. 3) apparently differ from those of normal control females (Fig. 2). This might be due to inappropriate JH titres leading to impaired synchronisation of maturational events. It has been assumed that JH generally mediates its various effects through different pathways specified by different concentrations (Gäde et al., 1997). However, our experiments strongly suggest that at least the acquisition of specific muscle properties depends on the existence of JH.

During maturation, the muscle fibres apparently increased their cross-sectional area. This hypertrophy also results in the ability of muscle 214 to exert a dramatically increased maximum tension after completion of maturation. Similar processes have been reported during moulting of crustaceans. Here, the growth of the myofibrils from leg muscles may occur by addition of thick and thin filaments and by longitudinal myofibrillar splitting (El Haj et al., 1984). These processes seemed to be under the control of ecdysteroids, as suggested by elevated levels of RNA synthesis in muscles after ecdysteroid administration (Whiteley et al., 1992). Interestingly, polyamines, which are known to play a fundamental role in tissue growth and development [for a review, see (Morgan, 1999)], are involved in JH-dependent oviposition behaviour in crickets (Cayre et al., 1996) and in the mitogenic action of JH on adult insect neuroblasts (Cayre et al., 1997). Thus, polyamines might be involved in the hypertrophy and/or expression of longitudinal muscle properties of locusts, possibly by influencing the transcriptional or translational stages of protein synthesis or by acting as an intracellular messenger (Morgan, 1999).

The kinetics of twitch contraction determined for M214 changed significantly during the reproductive development of females (Table 1). Precocene treatment reduced contraction and half-relaxation times, whereas injection of JH caused an increase. In contrast, contraction and half-relaxation times did not increase in M169. The twitch contraction times determined for the asynchronous dorsoventral flight muscle of the bumblebee *Bombus terrestris* (58 ms) (Josephson and Ellington, 1997) and for the tymbal sound-producing muscle of cicadas (107 ms) (Josephson and Young, 1981) were less than or within the range of values determined in the present study, but we are not aware of quantitative data for twitch kinetics that are directly comparable with those obtained in the present study.

The kinetics of insect muscle contraction are determined mainly by the muscle fibre architecture (type of muscle fibres and their composition). Müller et al. (Müller et al., 1992) demonstrated a direct correlation between fibre type and contraction speed. Rapidly contracting fibres (fast-type) had a high mATPase activity, whereas slow fibre types exhibited a low mATPase activity. Thus, the changes in twitch kinetics in M214 during maturation might be due to changes in fibre type expression. In addition, the modulatory action of proctolin or octopamine might play a role. Octopamine increases the amplitude of neurally evoked contractions and speeds up the relaxation of skeletal muscles (O'Shea and Evans, 1979), and a neurone has been identified, which presumably releases octopamine, that supplies the longitudinal muscles in locusts (Ferber and Pflüger, 1990). Proctolin has also been shown to modulate the contraction of skeletal muscles [for a review, see (Orchard et al., 1989)]. Proctolin appears to be co-localised in motoneurons with the excitatory transmitter glutamate (Usherwood and Cull-Candy, 1975; O'Shea et al., 1985; Worden et al., 1985). The amplitude and kinetics of the contractions measured in this study might therefore be affected by the individual history of modulation or by maturation-dependent differences in the responsiveness of muscle fibres to modulatory substances such as proctolin or octopamine.

With the exception of action potential generation, JH seems to affect predominantly the longitudinal muscles in oviposition segments of females, since the maturation of muscle properties was less pronounced in muscles from non-oviposition segments (M169). However, a slight, but consistent, increase in the maximum force (Fig. 7C) and a significant hypertrophy (Fig. 4A,B) indicated that these muscle were also subject to maturational changes. Similar changes occurred during the maturation of muscle 214 in males. In M214 of precocene-treated females, the properties differed from those of females less than 5 days old (compare Fig. 2 and Fig. 3). These results suggest a developmental process that applies qualitatively to all longitudinal muscles under investigation but is quantitatively increased by JH in muscles involved in oviposition. This hypothesis is further supported by the proposal that the original role of JH may have been the regulation of reproduction (Sehnal et al., 1996; Tobe and Bendena, 1999). Medawar (Medawar, 1953) concludes that 'endocrine evolution is not an evolution of hormones, but an evolution of the uses to which they are put'. Along this line of argument, we assume that the

evolutionary pressure for the protection and survival of offspring led to the ability of specific intersegmental muscle to superextend. Since this ability is not needed before the time of egg-laying, it might have become regulated by JH. The specificity of JH for longitudinal muscles in ovipositional segments might be controlled through differential hormone receptor expression, although no JH receptor has so far been identified. Furthermore, we do not know whether JH acts *via* a direct or an indirect pathway.

The visual observation that muscle fibres from mature females contract spontaneously after mechanical stimulation (stretch or release) led us to investigate the underlying conductances. All muscle fibres investigated generated action potentials upon current injection. The action potentials were dependent on Ca^{2+}, as demonstrated in experiments in which Cd^{2+} was added to or Ca^{2+} was omitted from the saline. There is good evidence that Ca^{2+} is the principal inward charge carrier for action potential generation in insect muscle fibres (Washio, 1972; Yamamoto et al., 1978; Ashcroft and Stanfield, 1982). Blocking of voltage-dependent Na^{+} channels by TTX had almost no effect on the shape of action potentials. Similar TTX-insensitive potentials were found in a tonic muscle fibre bundle of the locust hindleg (Burns and Usherwood, 1978). The sensitivity of action potentials to nifedipine suggests the involvement of an L-type Ca^{2+} channel. L-type Ca^{2+} channels, sensitive to dihydropyridines, have been characterized in vertebrates and invertebrates (Catterall et al., 1988, Triggle, 1990; Erxleben and Rathmayer, 1997; Gielow et al., 1995). However, we cannot exclude the participation of other types of Ca^{2+} channel in the generation of action potentials. Gielow et al. (Gielow et al., 1995) characterised two voltage-dependent Ca^{2+} currents (L- and T-type) in the body wall muscles of larval *Drosophila melanogaster*. These Ca^{2+} channels activate between −40 and −30 mV, which is within the range of activation observed in our experiments.

The observed differences in the amplitude, activation, width and afterhyperpolarisation of action potentials are related to the presence or absence of JH. The differences may be due to a differential expression of ion channels. In the nervous system, JH has been shown to influence the properties of identified neurons in the auditory system of crickets, in which the expression of the nicotinic acetylcholine receptor gene in an auditory neuron was increased in the presence of JH (Stout et al., 1992; Stout et al., 1993). This led to an improved directionality and decreased the threshold for phonotaxis of females more than 18 days old (Koudele et al., 1987; Walikonis et al., 1991). It is also likely that the hypertrophy of muscle fibres influenced the properties of the action potentials. When fibres increase their volume, the surface-to-volume ratio decreases. This slows the kinetics of underlying conductances (Hille, 1992) which should, in turn, reduce the amplitude of the action potential. However, our results suggest the opposite. It is possible that the increase in the surface area of the muscle fibres was independent of the volume, as suggested by the work of Jorgensen and Rice (Jorgensen and Rice, 1983a). They found highly corrugated sarcolemma and basal lamina in abdominal longitudinal muscles of mature female locusts. To gain further insight into the maturation of muscle properties, we have recently started to voltage-clamp longitudinal muscle fibres.

The reduced threshold of action potentials during reproductive development led us to assume a specific role for these potentials during oviposition. This role might be related (i) to a general activation (contraction) of muscle fibres by action potentials or (ii) to the Ca^{2+} influx into the muscle fibres. When fibres were stretched, twitches were visible as a result of the generation of action potentials. This activation of muscle fibres might prevent myosin and actin filaments from sliding apart when the muscle is stretched during oviposition and, thus, direct the mechanical forces to the Z-lines to enable their fragmentation. A similar mechanism was suggested by the work of Jorgensen and Rice (Jorgensen and Rice, 1983a). In addition, the influx of Ca^{2+} into the muscle fibres could activate a second-messenger system that might lead to the activation of enzymes or of additional intracellular events. The Ca^{2+}-activated protease calpain, for example, digests kettin, a Z-line protein, and disrupts the Z-discs of striated muscle in insect flight muscle (Lakey et al., 1993). This process leads eventually to the disassembly of myofibrils. Similar events might be involved during superextension of longitudinal muscles.

The development and specificity of muscle fibre properties make this system well suited for further investigations of the cellular and possibly molecular changes underlying the action of JH. By comparing muscles from oviposition segments with homologous muscles from non-oviposition segments, we hope to gain further insight into how adaptive behaviour is hormonally controlled and achieved.

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