EFFECTS OF TEMPERATURE ON ESCAPE JETTING IN THE SQUID
LOLIGO OPALESCENS

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Summary

In *Loligo opalescens*, a sudden visual stimulus (flash) elicits a stereotyped, short-latency escape response that is controlled primarily by the giant axon system at 15 °C. We used this startle response as an assay to examine the effects of acute temperature changes down to 6 °C on behavioral and physiological aspects of escape jetting. In free-swimming squid, latency, distance traveled and peak velocity for single escape jets all increased as temperature decreased. In restrained squid, intra-mantle pressure transients during escape jets increased in latency, duration and amplitude at low temperature. Recordings of stellar nerve activity revealed repetitive firing of the giant motor axon accompanied by increased activity in the non-giant motor axons that run in parallel. Selective stimulation of giant and non-giant motor axons in isolated nerve–muscle preparations failed to show the effects seen *in vivo*, i.e. increased peak force and increased neural activity at low temperature. Taken together, these results suggest that *L. opalescens* is able to compensate escape jetting performance for the effects of acute temperature reduction. A major portion of this compensation appears to occur in the central nervous system and involves alterations in the recruitment pattern of both the giant and non-giant axon systems.

Key words: temperature, jet escape, startle response, giant axon, squid, cephalopod, *Loligo opalescens*.

Introduction

The maintenance of essential behavior patterns during sudden changes in temperature presents a major challenge for poikilotherms. Many functional properties of nerve and muscle cells, such as axonal conduction, synaptic transmission and muscle output, are strongly temperature-dependent (e.g. Bennett, 1985, 1990; Lagerspetz, 1974; Prosser and Nelson, 1981). If behavioral performance is to be maintained across a range of temperatures, system-level function must counteract these changes in cellular processes. In fish, such compensation may be due to different effects of temperature on specific peripheral processes, or it may involve control mechanisms in the central nervous system (Macdonald, 1981; Montgomery and Macdonald, 1984, 1990; Montgomery, 1988; Miles, 1992). To our knowledge, comparable studies on cephalopods have not been reported.

Loligo opalescens is a pelagic cephalopod that moves freely between surface waters (14±2 °C) and depths of several hundred meters (≤7 °C; see also O'Dor, 1982), and coordinated swimming and escape behavior have been observed over this range (personal communication from B. Robison, Monterey Bay Aquarium Research Institute; B. Robison and S. Burnett, unpublished data). Squid undoubtedly encounter predators at all depths, but the effects of temperature on escape performance have not been investigated. Previous investigations of the effects of temperature on the squid motor system have focused on the physiological properties of isolated giant axon and giant synapse preparations (e.g. Hodgkin and Katz, 1949; Chapman, 1967; Dexter and Swenberg, 1975; Weight and Erulkar, 1976; Matteson and Armstrong, 1982; Keynes and Meves, 1993).

The giant axon system is important to jet-propelled escape and consists of two first-order giant interneurons that lie in the magnocellular lobe of the brain; each contacts a second-order giant interneuron in the palliovisceral lobe. Each of these second-order cells projects its axon in the pallial nerve and contacts all the third-order giant motoneurons *via* giant synapses in the ipsilateral stellate ganglion (Young, 1939). The giant motor axons exit the ganglion through individual stellar nerves and innervate overlapping fields of circular muscle fibers in the mantle (Young, 1938). A single spike in the giant axons produces an all-or-none mantle twitch, and this generates a hydrostatic pressure transient within the mantle cavity that expels sea water through the funnel and produces a powerful jet (Packard, 1969).

Exclusive control of escape jetting by the giant axon system was originally inferred from isolated nerve–muscle preparations (Prosser and Young, 1937; Young, 1938). However, more recent work in which stellar nerve activity and intra-mantle pressure were recorded *in vivo* suggests an important role for a parallel non-giant axon system in escape jetting (Otis and Gilly, 1990; Gilly et al., 1996). Repetitive activity of multiple non-giant axons produces graded muscle contractions and can result in intra-mantle pressures comparable with those caused by giant axon firing. Moreover, the type of stimulus affects the relative contributions that each system makes to escape jetting. A sudden visual stimulus (e.g. a flash of light) reliably elicits a short-latency (50–60 ms) startle-type escape response in which a giant axon spike is followed by a weak burst of non-giant axon activity. In contrast, other stimuli (e.g. a superficial electrical shock to the arms) elicit a more complex, long-latency (>200 ms) escape response driven by a strong burst of non-giant axon activity that may or may not be followed by giant axon activity. When the giant axon fires at the end of the non-giant axon burst in this manner, it significantly boosts the intra-mantle pressure transient. Repetitive firing of the giant axons is common during escape responses of this latter type, but the additional contribution to the pressure transient appears to be rather small (Otis and Gilly, 1990).

Previous *in vivo* studies on escape responses in *Loligo opalescens* were carried out at temperatures near the upper limit for Monterey Bay, California (15–16 °C). We have extended these studies to the lower thermal limit likely to be encountered by this species at greater depths (5–6 °C). The effects of temperature on flash-stimulated startle-escape responses were investigated at three levels. First, behavioral experiments were carried out with freely swimming animals. Second, intra-mantle pressure transients and corresponding motor activity in stellar nerves were recorded in restrained animals. Third, force transients were recorded during selective electrical stimulation of giant and non-giant axon pathways in isolated nerve–muscle preparations. The results indicate that escape jetting performance in *Loligo opalescens* is compensated at cold temperatures and point to alterations in the pattern of coordinated activity in the giant and non-giant axon systems as an underlying mechanism.

Materials and methods
Animals

Adult squid (*Loligo opalescens* Berry) were collected from Monterey Bay, California, USA, during the spring and autumn of 1997 and were transported to the laboratory in oxygenated sea water. Squid were maintained in circular holding tanks (2.5 m diameter, 1 m height) with flow-through natural sea water at ambient surface temperature (14±2 °C) for up to 2 weeks. All experiments and procedures followed Universities Federation for Animal Welfare guidelines (Boyle, 1991) and Stanford University Institutional Animal Care guidelines.

Behavioral experiments with free-swimming squid

Three squid with dorsal mantle lengths (*ML*) ranging from 95 to 125 mm were placed in a rectangular glass aquarium (120 cm×50 cm×32 cm; 190 l volume) containing aerated, chilled (6 °C) sea water. Water temperature was raised stepwise to 12 °C over 90 min by partially draining some of the cooled sea water and adding an equal amount of sea water at ambient temperature (15 °C). The animals were allowed to acclimate for 10 min before an experimental trial was begun. For each trial, several startle responses were elicited using a strobe-flash unit (Aquaflash 22, Helix Camera and Video, Chicago, IL, USA). Water temperature was monitored using a thermometer.

The room lights were switched off during the experiments, and background red illumination was used for video taping with a Sony SCC-M354 CCD camera, operating at 30 frames s^{-1}, and a 50 mm lens. The camera was mounted on a tripod near the aquarium and provided a lateral view of the animals. A time-code (TG-50, Horita, Mission Viejo, CA, USA) signal was added to the video during filming.

Video data were recorded on Hi-8 tapes for subsequent analysis using a Hi-8 video recorder (EVO-9700, Sony). Video sequences in which animals completed an escape jet without contacting the water surface or tank walls were digitized at 60 Hz using a capture card (LR-3, Scion Corporation, Frederick, MD, USA) and Macintosh computer with NIH image 1.60 software. The distance a squid traveled between successive video fields was measured and converted into multiples of *ML* (i.e. normalized) for each animal. Cumulative distance traveled and instantaneous velocity were calculated from these data.

Experiments with restrained squid

Intra-mantle pressure and *en passant* extracellular stellar nerve activity were recorded in restrained animals as described previously (Otis and Gilly, 1990). Prior to surgery, animals were anesthetized either by topical application of 100 mmol l^{-1} procaine (in sea water) to the intended incision site or by immersion of the animal into a solution of 1.8 % MgCl$_2$ in oxygenated sea water for 15 min. Fully anesthetized animals recovered after 20 min and displayed normal behavior. In some cases, three longitudinal slits (4 cm in length) were cut through the ventral mantle to minimize the intra-mantle pressure developed during an escape jet. This reduced an electrical artifact associated with sea water being forced out of the recording hole during strong jetting and allowed better resolution of non-giant axon activity.

Restrained animals were suspended in an acrylic experimental tank (50 cm×25 cm×20 cm; 25 l volume) supplied with flow-through ambient-temperature sea water. Valves allowed switching to a closed system that circulated cooled, oxygenated sea water from an insulated reservoir (50 cm×30 cm×20 cm; 27 l volume) attached to a cooling unit (Aqualogic, San Diego, CA, USA). In this way, the temperature in the tank was changed from 14 to 6 °C at a rate of 1 °C per 5 min. Temperature was returned to ambient

temperature at a similar rate by reintroducing ambient-temperature sea water. The temperature in the experimental tank was continuously monitored using a thermistor probe and recorded on a chart recorder.

Startle responses were elicited using flash stimuli (see above) triggered by the computer used for data acquisition. Red light was used for illumination and allowed video monitoring of a lateral view in a manner similar to that described above. Changes in mantle diameter were measured in digitized sequences at a point one-third from the anterior end of the mantle (the point of greatest diameter) in successive video fields (see also Preuss et al., 1997). In some cases, incident white-light illumination was employed to monitor chromatophore activity during escape jets. The spatial pattern of chromatophore activation and the degree of expansion of individual chromatophores were analyzed in sequential video fields in the same manner as described for mantle contraction.

Intra-mantle pressure was recorded using a pressure transducer (PX136-015AV; Omega, Stamford, CT, USA) coupled to a hypodermic needle (gauge 20) filled with mineral oil and inserted through the mantle into the mid-posterior mantle cavity. The output of the transducer bridge circuit was amplified using a direct-current-coupled instrumentation amplifier. Force transients were recorded directly onto a laboratory computer disk using either a commercial (Digidata 1200, Axon Instruments, Foster City, CA, USA) or custom-built (D. R. Matteson, University of Maryland, Baltimore, MD, USA) interface and software. Digitized pressure transients were analyzed for latency (time from stimulus to an initial positive pressure increase), peak amplitude and time to peak amplitude (time from the end of the latent period to peak amplitude). All pressure transients in conjunction with Fig. 2 were digitally filtered or 'smoothed' using 30 passes of a binomial (Gaussian) algorithm (Marchand and Marmet, 1983) in IgorPro 9 (Wavemetrics, Eugene, OR, USA), and the maximum rate of pressure rise was estimated by differentiating the smoothed transients.

Conventional extracellular recordings of nerve activity from the hindmost or second-hindmost stellar nerve were made using a polyethylene suction electrode. The electrode was inserted through a hole (5–10 mm in diameter) in the mantle over the left stellate ganglion and attached to a stellar nerve near its emergence from the ganglion (less than 0.5 cm away). Stellar nerve signals were amplified using an alternating-current-coupled preamplifier (Warner Instruments, Hamden, CT, USA) and digitized at 50 kHz on a laboratory computer. Signals were stored on disk and through a PCM-audio input onto the Hi-8 tape used for video recording.

Nerve–muscle preparation

A strip of mantle muscle containing an intact stellate ganglion and several anterior stellar nerves with most, if not all, of their motor fields was removed from a small squid and mounted as described previously (Gilly et al., 1996). The mantle strip (approximately 2 cm long × 0.5 cm wide) was cut parallel to the circular muscle fibers and spanned approximately 75 % of the mantle circumference. This strip was submerged in an experimental chamber (4 cm×6 cm×14 cm) supplied with flow-through sea water. Bath temperature was controlled in the same manner as described for experiments with restrained squid. Bundles of non-giant motor axons were separated from the third-order giant axon by manual dissection under a stereo-microscope. Giant and non-giant axons were selectively stimulated with a small, bipolar electrode (Rhodes Medical Instruments, Woodland Hills, CA, USA) and a polyethylene suction electrode, respectively. Electric shocks were 0.6 ms in duration. During repetitive stimulation, 2–5 shocks were triggered at 50 Hz by the computer used for data acquisition. This is the natural frequency at which the giant axon fires at 15 °C (Otis and Gilly, 1990). Isometric force transients were measured using a force transducer (Load Cell BG-150GM; Kulite Semiconductor Products, Leonia, NJ, USA). These analog data were amplified, sampled and analyzed in a manner analogous to that described above for pressure transients.

In one preparation, the second-order giant axon in the pallial nerve was stimulated with single electrical shocks to excite the third-order giant axons *via* the giant synapse. Motor axon activity was confirmed by recording in the usual manner from a stellar nerve.

Results

Behavioral experiments with free-swimming squid

Escape performance in response to a flash stimulus showed strong temperature-dependence. Although squid displayed a slightly longer latency at 6 °C than at 12 °C, the total distance (normalized to *ML*) traveled with one escape jet was much greater at the lower temperature (Fig. 1A): after 600 ms, the animals at 6 °C had traveled approximately 1.5*ML* further than they had at 12 °C. At intermediate temperatures, latencies and total distances traveled were between those obtained at 6 °C and 12 °C.

Peak velocity (calculated from the data in Fig. 1A) was also higher at the colder temperature (Fig. 1B). At 12 °C, maximum velocity was reached 175 ms after the flash, followed by a rapid deceleration. At 6 °C, a greater maximum velocity was reached, and the elevated peak level remained for approximately 200 ms before subsiding. In addition, an inflection was consistently seen in the rise of the velocity curve (Fig. 1B, arrow).

Experiments with restrained squid

To examine the role of the giant and non-giant motor systems during escape jets at different temperatures, extracellular stellar nerve activity and intra-mantle pressure were recorded simultaneously *in vivo*.

Effects of temperature on intra-mantle pressure and mantle kinematics

Intra-mantle pressure provides a useful measure of jetting performance. Assuming that the funnel aperture is constant
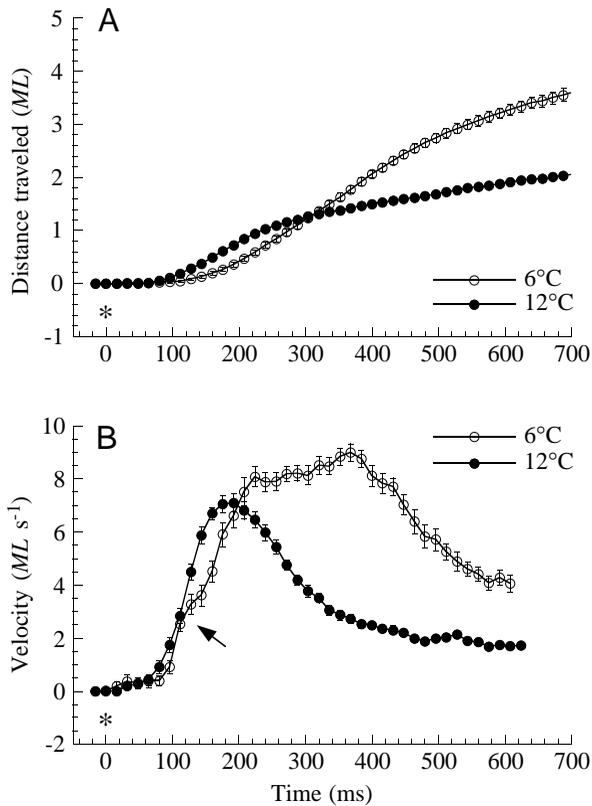
Fig. 1. Comparison of escape jetting performance in free-swimming squid at different temperatures. (A) Distance (in multiples of mantle length, *ML*) traveled during a single escape jet in response to a flash stimulus given at time zero (*) is greater at 6 °C (three animals; *N*=22 trials) than at 12 °C (three animals; *N*=33 trials). (B) Instantaneous velocity calculated from the data in A is greater and sustained for longer at 6 °C. Note the onset of the secondary, slower rise in amplitude at 6 °C (arrow; see also Fig. 2A). Values are means ± S.E.M. Mean *ML*, 10.5 cm.
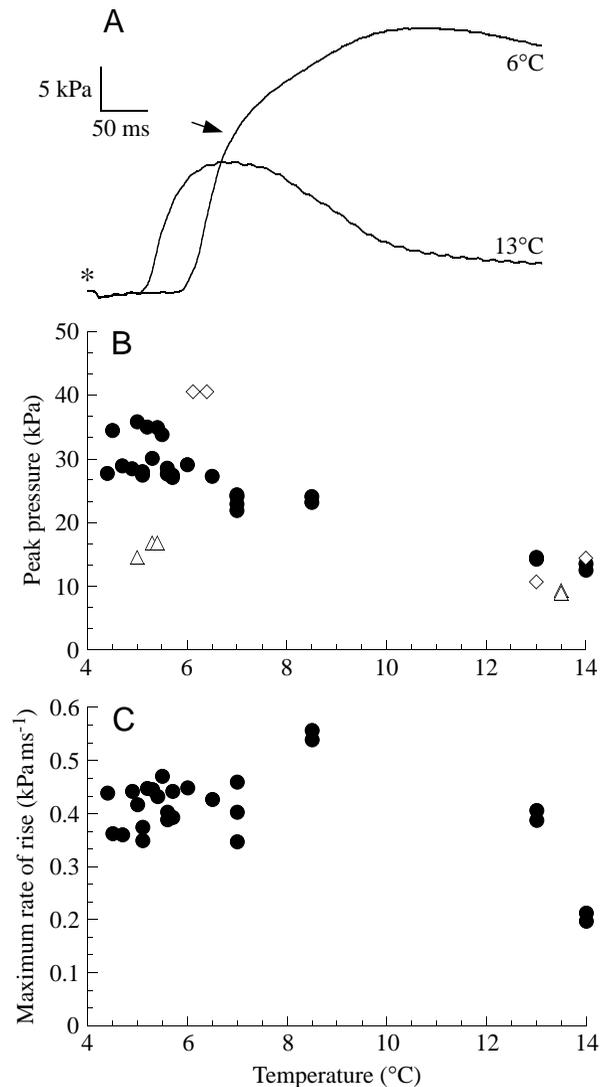

Fig. 2. Comparison of intra-mantle pressures in restrained squid during single escape jets in response to a flash stimulus (*) at different temperatures. (A) The pressure transient at 6 °C is longer in latency and duration and greater in amplitude than that at 13 °C. Note the onset of the secondary, slower rise in amplitude at 6 °C (arrow; see also Fig. 1B). Data are from one animal. (B) Peak pressures are higher at low temperature. Data are from three animals (see text). (C) Maximum rate of pressure rise is well maintained between 4 and 13 °C. Data are from the same animal as in A.

throughout a jet, the pressure (*p*) is related to the velocity of the expelled water (u_j) by the equation:

$$u_j = (2p/d_w)^{1/2}, \qquad (1)$$

where d_w is the density of sea water (O'Dor, 1988a,b).

Marked differences in amplitude and time course are evident in intra-mantle pressure transients recorded during startle responses at 6 °C compared with 13 °C (Fig. 2A). Although the initial rate of rise is similar, a slower, secondary rise in amplitude occurs at 6 °C (arrow in Fig. 2A). This secondary rise in pressure was consistently observed at low temperature, and its onset appears to coincide with the inflection in the velocity waveform mentioned above (arrow in Fig. 1B).

Although peak pressure amplitude always increased as temperature was lowered (Fig. 2A), the extent of this increase varied in the six animals studied. Filled circles in Fig. 2B indicate peak pressures from a representative animal in which the most extensive testing was carried out (same animal as in Fig. 2A). Open symbols represent data from two other animals and indicate the total range of the extent of increase at low

temperature. The maximal rate of rise in mantle pressure was well mantained across the temperature range studied up to 13 °C (Fig. 2C), and this effect was consistently observed in all six animals. Latency and duration also increased monotonically with decreasing temperature. All these effects of temperature on mantle pressures during escape jetting were reversible.

Changes in mantle diameter were measured during startle responses at different temperatures in one squid from eight video sequences at 6 °C and at 12 °C. The greatest mantle contractions were observed at 6 °C, and mantle diameter
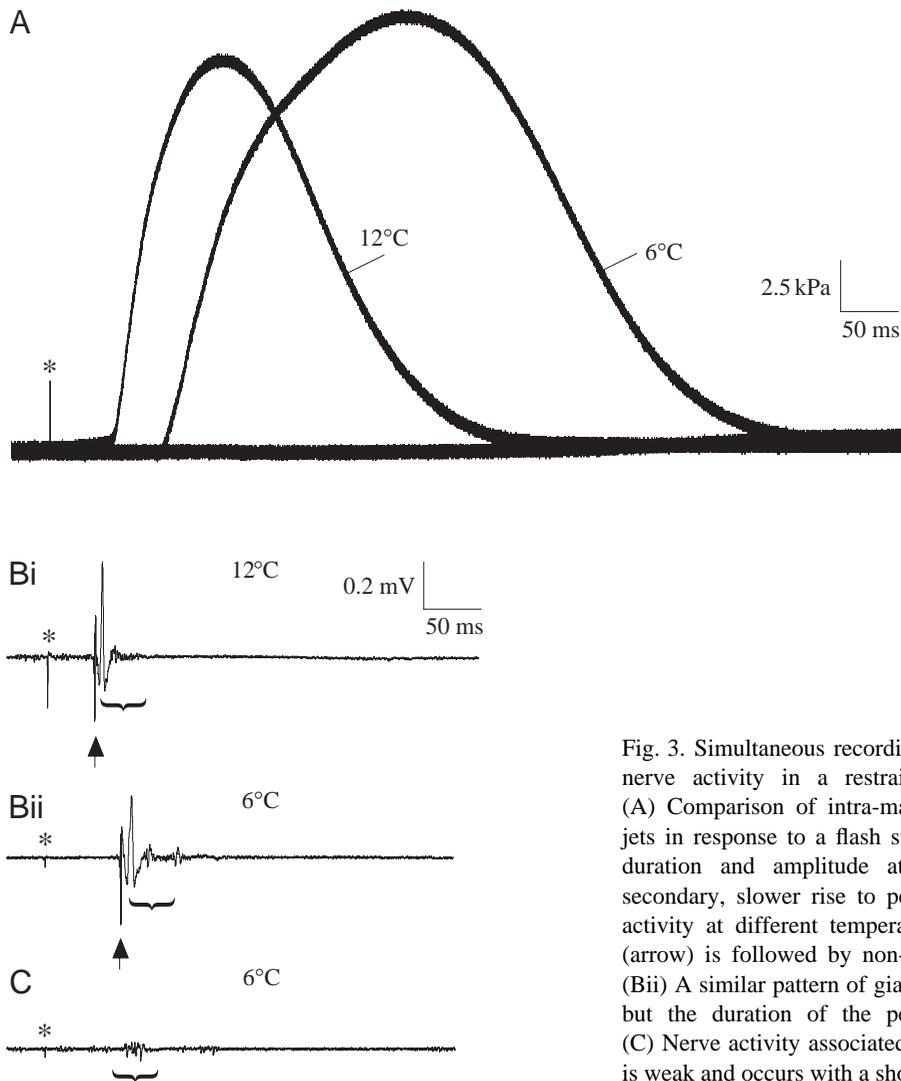
A

Bi

Bii

C

Fig. 3. Simultaneous recordings of intra-mantle pressure and stellar nerve activity in a restrained squid at different temperatures. (A) Comparison of intra-mantle pressure transients during escape jets in response to a flash stimulus (*) indicates increased latency, duration and amplitude at 6 °C compared with 12 °C and a secondary, slower rise to peak pressure. (B) Corresponding nerve activity at different temperatures. (Bi) A single giant axon spike (arrow) is followed by non-giant axon activity (bracket) at 12 °C. (Bii) A similar pattern of giant and non-giant activity occurs at 6 °C, but the duration of the period of non-giant activity is longer. (C) Nerve activity associated with chromatophore expansion at 6 °C is weak and occurs with a short latency (see text for further details).

during contraction was 85±1.4 % of that before the flash stimulus (mean ± S.E.M.). During maximal contraction at 12 °C, mantle diameter were 89±0.9 % of that before the stimulus (mean ± S.E.M.). The latency and the duration of diameter change increased at 6 °C. These data are therefore consistent with the results of the pressure measurements described above.

Effects of temperature on non-giant axon activity

Fig. 3 illustrates intra-mantle pressure transients (Fig. 3A) and corresponding stellar nerve activity (Fig. 3B) for startle responses at warm compared with cold temperature. At 12 °C, a single giant axon spike (arrow) was typically followed by a burst of non-giant axon spikes (bracket, Fig. 3Bi). A similar pattern was evident at 6 °C (Fig. 3Bii), but non-giant axon activity persisted for longer at this temperature. Non-gaint axon activity gradually increased in duration as temperature decreased (data not shown) and was accompanied by the changes in pressure transients described above.

Two prominent groups of non-giant motor axons exist in a stellar nerve. One group innervates a subset of circular mantle muscle fibers (Wilson, 1960), whereas the other innervates chromatophore muscle fibers in the dermal layers (Ferguson et al., 1988). In the experiments described above, the onset of chromatophore expansion following flash stimulation (as recorded on video) closely matched that of mantle contraction throughout the temperature range 6–12 °C, and expansion of the chromatophores reached its maximum extent at approximately the same time as did mantle contraction. However, no difference in the number or size of expanded chromatophores was evident between 6 and 12 °C. This suggests that increased activity in chromatophore axons is not responsible for the increased non-giant axon activity shown in Fig. 3Bii at 6 °C.

Moreover, it was found that chromatophore expansion could be triggered in the absence of an escape response by positioning the flash unit further from the squid. Stellar nerve activity recorded under these conditions is likely to represent primarily activity in chromatophore motor axons (Fig. 3C), but may also include some weak activity in non-giant motor axons
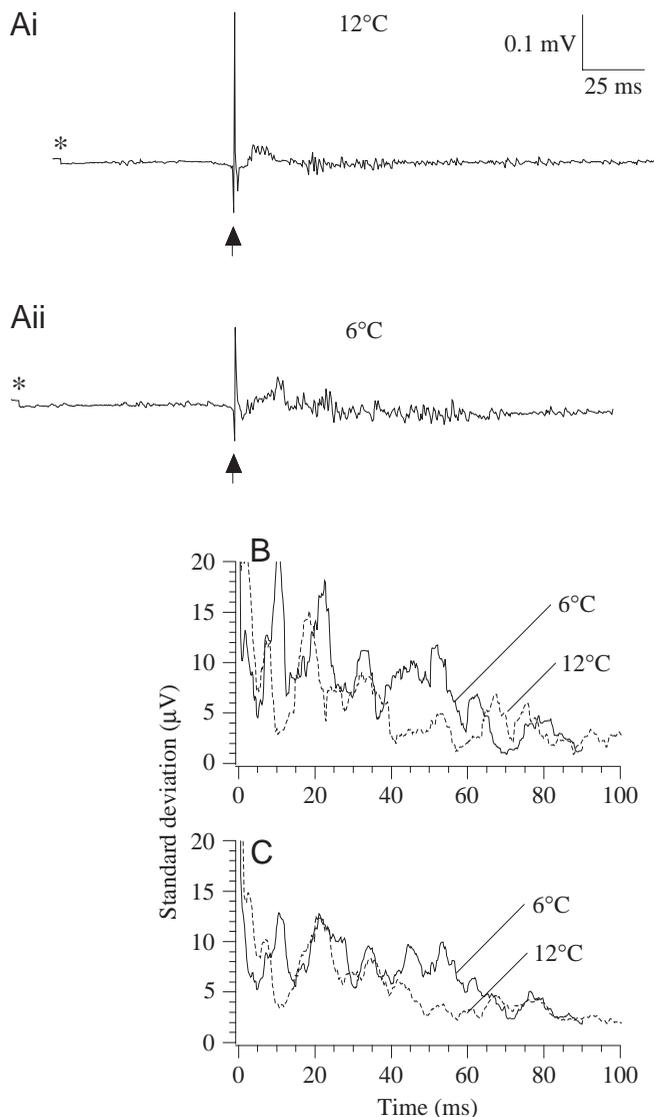
Fig. 4. Analysis of non-giant axon activity during single escape jets at different temperatures. (A) Recordings of stellar nerve activity during flash-stimulated (*) escape responses at 12 °C (Ai) and at 6 °C (Aii). The recordings have been aligned so that the giant axon spikes (arrows) coincide. The non-giant burst at 6 °C is visibly more intense and long-lasting. (B) A standard deviation for nerve activity was calculated for each time point in an individual trace starting at the peak of the giant axon spike (time zero) using a 20-point window extending to the right. These values are plotted as the *x*-coordinate at the time of the first point of each window. Increased non-giant activity at 6 °C is evident during much of the response. (C) Averaged standard deviation traces from four trials at each temperature in the same animal confirm increased non-giant axon activity at 6 °C.

that is insufficient to produce muscular force. In these trials, chromatophore expansion was similar in time course and extent to that accompanying an escape jet, and the main burst of associated stellar nerve activity was relatively weak and persisted for only 20 or 30 ms after the stimulus. The characteristics of this chromatophore-related discharge do not match those described in conjunction with the activity in

Fig. 3B, which we associate with the non-giant motor axons that innervate circular mantle muscle fibers.

A more quantitative way of demonstrating the stronger burst of non-giant axon activity at low temperature is shown in Fig. 4. Examples of the motor discharge accompanying a startle response at 12 °C and 6 °C are shown in Fig. 4A. An overall index of non-giant spike activity following firing of the giant axon (arrow; defined as time zero) was generated by computing a running standard deviation as described in the legend to Fig. 4, and these traces are illustrated in Fig. 4B. An increase in non-giant axon activity throughout most of the response is evident at 6 °C (solid trace). This result was reproducible, as demonstrated in Fig. 4C by the averaged standard deviation traces from four trials at both 6 °C (solid trace) and 12 °C (broken trace).

Effects of temperature on giant motor axon activity

Repetitive firing of the giant axon was often observed during startle responses at low temperature. Fig. 5A illustrates intra-mantle pressure transients at 6 °C, together with two corresponding traces of stellar nerve activity (Fig. 5B). Double firing of the giant axon (Fig. 5Bii) was associated with pressure transients of increased amplitude and duration primarily because of a steeper rate of rise in pressure for the second phase and a longer-lasting plateau (Fig. 5A). A response showing double firing of the giant axon was always accompanied by a more intense burst of non-giant axon activity (Fig. 5Bii; bracket). These phenomena were observed in all six animals tested, although individual animals varied with respect to both the fraction of responses with double firing at a given low temperature (30–100 %) and the temperature at which double firing first appeared (9–6 °C). Triple firing was observed in one animal, producing an additional increase in pressure.

Effects of temperature on nerve–muscle preparations

To investigate how the recruitment of giant and non-giant motor axons might individually influence escape jetting at different temperatures, the two systems were stimulated independently in nerve–muscle preparations. Electric shocks to either system caused contraction of the mantle muscle strip, exerting a measurable force on the transducer. In an intact squid, the force (f) exerted by a strip of mantle muscle is linearly related to intra-mantle pressure (p) by the equation:

$$2pr = f/l, \qquad (2)$$

where r is the radius of the mantle cavity and l is the width of the muscle strip. Therefore, the time course of force transients can be directly related to intra-mantle pressure (personal communication from M. Denny, Hopkins Marine Station, Stanford University, USA).

Stimulation of non-giant axons at different temperatures

A muscle force transient produced in response to a single (supramaximal) electrical stimulation of non-giant axons at 13 °C is illustrated in Fig. 6Ai (arrow), and its amplitude is

A   6°C

2.5 kPa

25 ms

Two giant axon spikes

One giant axon spike

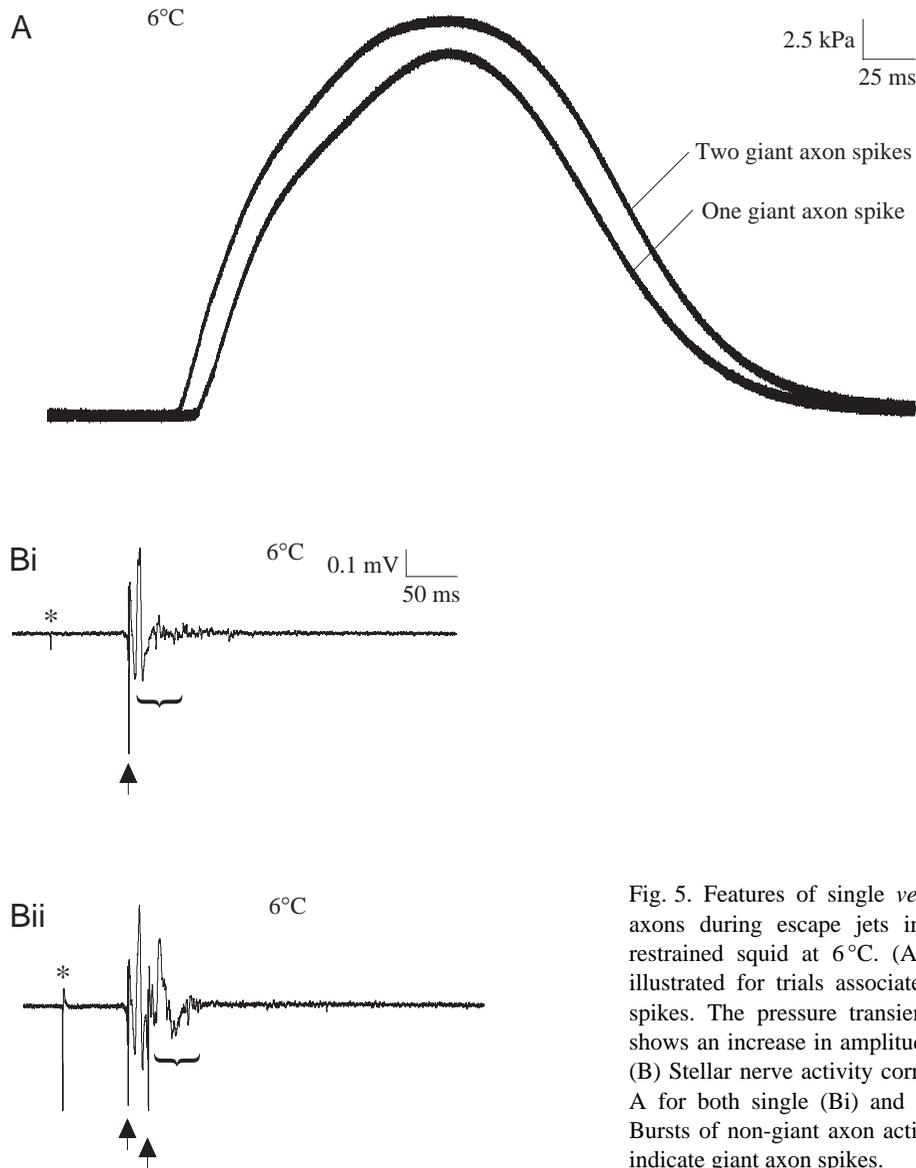Bi   6°C   0.1 mV

50 ms

*

Bii   6°C

*

Fig. 5. Features of single *versus* double firing of the giant motor axons during escape jets in response to flash stimuli (*) in a restrained squid at 6 °C. (A) Intra-mantle pressure transients are illustrated for trials associated with either one or two giant axon spikes. The pressure transient involving double giant axon firing shows an increase in amplitude, secondary rate of rise and duration. (B) Stellar nerve activity corresponding to the pressure transients in A for both single (Bi) and double (Bii) firing of the giant axon. Bursts of non-giant axon activity are indicated by brackets. Arrows indicate giant axon spikes.

comparable with that at 6 °C (×1). Peak force and maximum rate of rise for a single shock both reach a maximum at approximately 10 °C (Fig. 6Aii,iii; triangles); this feature becomes more obvious if repetitive stimuli are used (see below). Latency and time to peak force increased monotonically as temperature was reduced.

Repetitive stimulation of non-giant axons produced force transients with amplitudes that were highly dependent on the number of stimuli delivered at 50 Hz. Examples of muscle responses to one, three and five shocks at 6 °C are shown in Fig. 6Ai. Such potentiation with repetitive stimulation was maximal at approximately five shocks, and the chosen stimulation rate appeared to be about optimal for producing this effect. Although we do not know the natural firing frequency of individual non-giant axons, 50 Hz is comparable with the frequency observed for repetitive firing in the giant axon (see Fig. 5Bii). These results confirm that the muscular

output of the non-giant motor system depends strongly on the number of stimuli (Wilson, 1960). Output could also be graded with stimulus strength, but all the results in the present study were obtained using a stimulus strength optimal for force production with a single shock.

Thus, temporal summation exerts a strong influence on the output of the non-giant system at all temperatures. The data in Fig. 6Aii indicate that the peak amplitude of force transients at any stimulation frequency is comparable at the temperature extremes studied (5–6 and 13 °C), but the system appears to be optimally effective in the intermediate temperature range (7–10 °C). Results similar to those in Fig. 6Aii,iii were obtained in two other animals.

Stimulation of the giant motor axon at different temperatures

Fig. 6Bi compares force transients during selective stimulation of the giant axon resulting from a single electrical
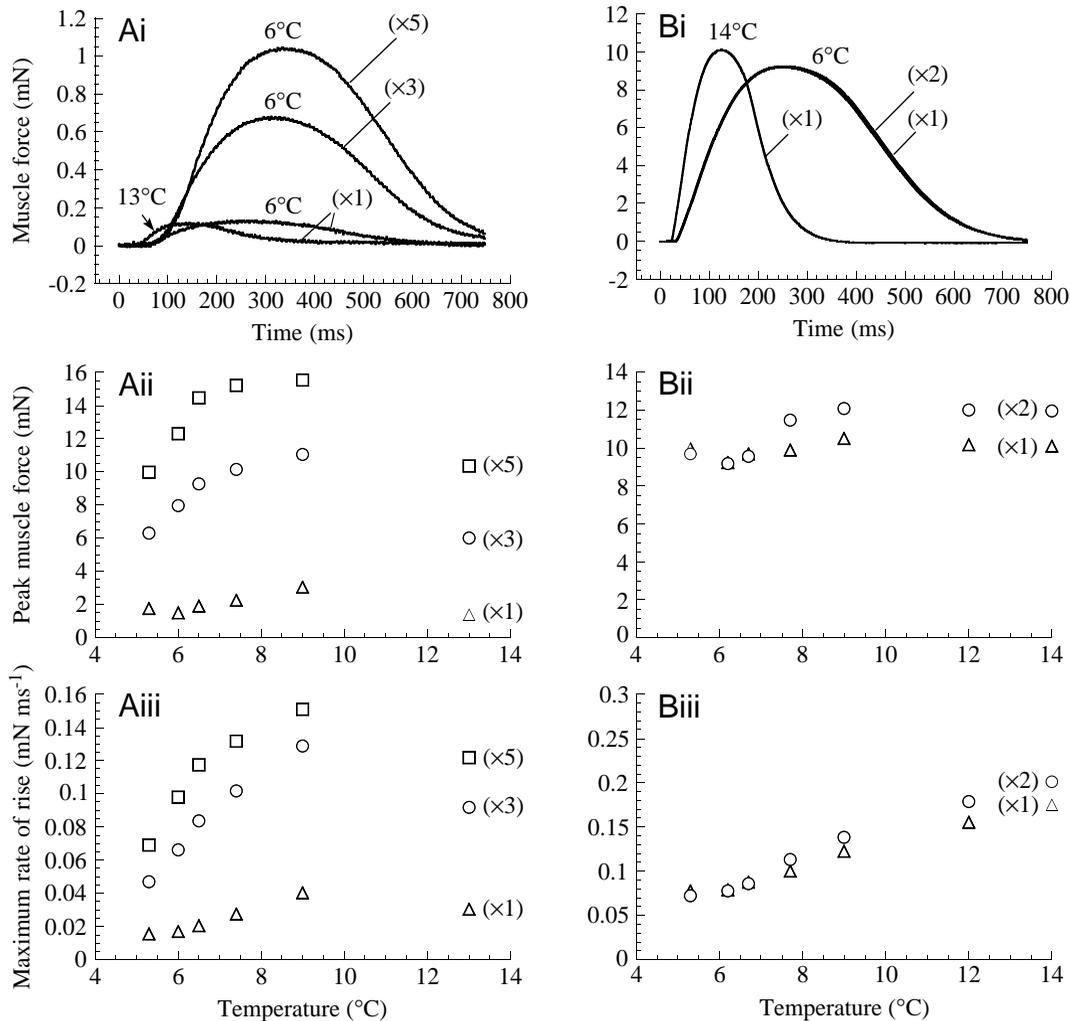
Fig. 6. Temperature-dependence of force production in isolated nerve–muscle preparations in response to selective electrical stimulation of giant and non-giant axons. (A) Properties of the non-giant axon system. (Ai) Single stimuli (×1) at both 13 °C and 6 °C produce responses with comparable force amplitudes, although the response at 6 °C is substantially slower. Repetitive stimulation at 6 °C with either three (×3) or five (×5) shocks at 50 Hz produces strong summation. Summation at 13 °C was similar (not illustrated). (Aii) Peak muscle force in response to a single stimulus (×1) is steady across the temperature range studied. Repetitive stimulation produces similar-sized responses at 5 °C and 13 °C, but forces are larger in the intermediate temperature range. (Aiii) Analysis of the maximum rate of rise of force from the experiments illustrated in Aii. The rate of force increase shows a temperature-dependence similar to that in Aii. (B) Properties of the giant axon system. (Bi) Force transients in response to single stimuli (×1) at 6 °C and 14 °C have a similar amplitude, but the response at 6 °C has a longer duration and latency. Double stimulation (×2) at 6 °C does not increase the response. (Bii) Peak amplitude shows very little temperature-dependence between 5 and 14 °C for either single (×1) or double (×2) stimulation. (Biii) The maximum rate of rise in force increases steadily with temperature for both single and double stimulation. The rate of rise in force was estimated by differentiating digitally filtered (smoothed) force transients as described in conjunction with the analysis of pressure transients in Materials and methods.

shock (×1) at 6 °C and 14 °C. Although the rate of rise of force transients was significantly reduced at 6 °C, peak amplitude was not greatly affected. Peak amplitude did not vary much across the temperature range studied (Fig. 6Bii, triangles), but the rate of rise was clearly temperature-dependent (Fig. 6Biii, triangles). Latency and duration both increased as temperature decreased. Similar results were obtained with two other animals.

An analogous series of measurements was also made in which giant motor axons were stimulated with the doublet pattern described above (20 ms interval). At 6 °C, the force

transients for single (×1) and double (×2) stimulation were virtually identical (Fig. 6Bi). However, at temperatures above 8 °C, the doublet firing pattern yielded a small increase in both peak twitch amplitude (Fig. 6Bii) and rate of rise (Fig. 6Biii). At the temperatures at which repetitive firing of the giant motor axon typically occurs *in vivo* (i.e. below 8 °C), there is no detectable augmentation of the twitch that can be directly ascribed to summation in the giant axon system. Similar results were obtained in experiments with two other animals.

In addition, giant motor axons were stimulated *via* the giant

synapse by stimulating the second-order giant axon in the pallial nerve. Single shocks never produced a doublet pattern of firing in the giant motor axon regardless of temperature.

Discussion

Compensation of jet escape performance at low temperature

Reducing the temperature slows the reaction rates underlying biological processes. The results presented here show that some temporal features of the startle-escape response in *Loligo opalescens*, such as response latency and duration, are affected in this manner over the temperature range studied (6–14 °C). Similar results were obtained with isolated nerve–muscle preparations. These observations undoubtedly reflect direct effects of temperature on basic functional properties such as axonal conduction velocity (Dexter and Swenberg, 1975), the strength of synaptic transmission (Weight and Erulklar, 1976) and the speed of muscle contraction and relaxation (Prosser and Nelson, 1981).

Behavioral results, however, show that other critical aspects of escape performance are remarkably constant over this temperature range. In restrained squid, the maximum rate of rise of intra-mantle pressure transients varied little over the temperature range studied, and peak pressure increased with decreasing temperature. In free-swimming squid, maximum velocity during a single escape jet was higher at 6 °C than at 12 °C, and the total distance traveled was substantially greater. However, *in vitro* experiments with nerve–muscle preparations failed to show a comparable compensation of force production.

Although we cannot completely rule out peripheral temperature- or frequency-dependent effects that *in vivo* might modulate axonal transmission into the fine terminal branches in mantle muscle (Parnas, 1972; Westerfield et al., 1978), our results suggest that mechanisms exist upstream from the neuromuscular junction that can counteract the direct effects of temperature on cellular excitability. Neural recordings *in vivo* indicate that this temperature compensation involves alterations in recruitment pattern of both giant and non-giant motor axons. Temperature-dependent recruitment of different types of motor unit has also been reported to be a compensatory mechanism for locomotion in teleost fish (Rome, 1990) and may represent another example of the striking convergent evolution between cephalopods and fish (Packard, 1972).

Roles of giant and non-giant motor systems in compensation of escape jetting

Escape jets at low temperature were accompanied by increased activity in both the non-giant axon (Figs 3B, 4) and giant axon (Fig. 5) systems. Since both systems can produce strong escape jets, the question arises as to the contribution made by each system to temperature compensation.

Several observations support the idea that the increase in non-giant axon activity is directly associated with compensation of escape jetting. First, the amplitude of pressure transients increased gradually during cooling, and this was associated with an increasingly prominent secondary pressure rise (arrow; Fig. 2) and an increase in the duration of the burst of non-giant axon activity (Figs 3B, 4). These changes occurred independently of double firing of the giant axons. Second, at a given temperature, significant variation in the secondary rise of pressure transients was observed from trial to trial, with no change in giant axon firing. Third, the inflection that identifies the secondary rise of the pressure transient occurs towards the end of the burst of non-giant motor activity (Figs 3B, 5). This coincidence in timing suggests a relationship between these phenomena.

Although the doublet firing pattern for the giant axons occurs regularly at low temperature, it is more difficult to assess the contribution of this phenomenon to compensation of escape jetting. Double firing of giant axons *in vivo* was always associated with an increase in both the secondary rise and the peak amplitude of the pressure transient (Fig. 5), but no such effect was apparent with force transients in the experiments with isolated nerve–muscle preparations (Fig. 6B). Furthermore, because double firing of the giant axons was also accompanied by an increase in the non-giant axon discharge (Fig. 5Bii), it is not clear to what extent the second giant axon spike directly contributes to the compensation observed at low temperature *in vivo*.

An alternative role for the doublet firing pattern could indirectly involve the non-giant axon system, whose muscular output is graded with the number (and frequency) of axonal impulses. If activity somewhere in the giant axon system (including in the brain) served to coordinate a synchronized discharge from non-giant motor axons, then the additional burst of non-giant axon activity might thereby contribute to the increase in the secondary rise of a pressure transient. The recordings in Fig. 5B show a large summed wave shortly after the giant axon spike at 6 °C, and this may represent such a synchronized discharge of non-giant motor axons. Such a synchronizing effect would be consistent with the strong dependence of force production on the number of stimuli delivered to the non-giant axon system in the nerve–muscle experiments (Fig. 6A). Repetitive stimulation of unidentified axons in the pallial nerve that provide synaptic inputs to the non-giant motoneurons also produces strong facilitation of synaptic potentials with only a few stimuli (Miledi, 1967).

This synchronization effect would also be likely to occur with only a single giant axon spike, and temperature-dependent modulation of such giant/non-giant axon interactions could constitute an important mechanism for compensation as defined in this study. For example, if the postulated excitatory influences of activity in the giant axon system were more effective at 6 °C than at 12 °C, then a single giant axon spike would be accompanied by increased activity of non-giant motor axons at the lower temperature and produce the secondary rise in pressure during an escape jet.

Role of inhibitory influences on escape jetting and compensation

The ideas developed above are also consistent with the generalization that inhibitory synapses are more temperature-

sensitive than are excitatory ones (Prosser and Nelson, 1981). For example, the behavioral hyperexcitability observed in goldfish (*Carassius auratus*) during cooling has been ascribed to the removal of background inhibition (Friedlander et al., 1976; Prosser and Nelson, 1981). Reduced levels of ongoing inhibition in the non-giant axon system of the squid at low temperature may also be an important factor underlying increased activity in this motoneuron pool during an escape response. This would effectively increase the excitatory influence of the giant axon system, as postulated above. At warmer temperatures, an increased level of inhibition would be consistent with a minimal contribution of the non-giant axon system to flash-stimulated startle responses (Otis and Gilly, 1990). Thus, inhibition-dependent coordination between the giant and non-giant axon systems may underlie the temperature compensation of escape jetting described here.

At present, one can only speculate about the inhibitory pathways involved or the nature of any excitatory influences of the giant axon system on the non-giant axon system. Limited intracellular recordings from stellate ganglion motoneurons of both systems have not revealed inhibitory synaptic potentials (Miledi, 1967), but cholinergic inhibitory control over the giant motor axon has been suggested (Stanley, 1984). Additional physiological studies are clearly required to settle this issue. We are unaware of any published work aimed at revealing an excitatory influence of the giant axon system on the non-giant motoneurons, and future work should be directed to detect such an interaction.

Although the postulated inhibitory or excitatory interactions might take place in the stellate ganglion, it seems more likely that they would occur in the brain. The results of the present study strongly suggest that the site of processing leading to double firing of the giant axons does lie in the brain, because single stimulation of the pallial nerve does not produce double firing even at low temperature in the nerve–muscle preparation. Presumably, the command for double firing is issued at the level of the first- or second-order giant interneurons (Young, 1939), but recordings from these cells during escape jetting have not been reported. Unfortunately, very little is known about the anatomy of the non-giant motor system, and even the location of the cell bodies providing excitatory synaptic inputs to the motoneurons in the stellate ganglion has yet to be established (Mackie, 1990).

In summary, our findings indicate that important characteristics of escape jetting in *Loligo opalescens* are maintained over the entire temperature range likely to be encountered by this species in the wild. An important mechanism in temperature-compensation of this vital behavior appears to be an alteration in the nature of coordination of motor activity in the parallel motor systems consisting of giant and non-giant axons. At least some of the temperature-dependent modulation of this coordination appears to be carried out in the central nervous system.

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