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Development of swimming behaviour in the larva of the ascidian *Ciona intestinalis*

Giuliana Zega[1,2,*], Michael C. Thorndyke[2] and Euan R. Brown[1]

[1]*Neurobiology Laboratory Stazione Zoologica 'Anton Dohrn', Villa Comunale I-80121 Naples, Italy and* [2]*Royal Swedish Academy of Sciences Kristineberg Marine Research Station, SE-45034 Fiskebäckskil, Sweden*

*Author for correspondence at present address: Dipartimento di Biologia 'L. Gorini', Università degli Studi di Milano, Via Celoria 26, I-20133 Milano, Italy (e-mail: giuliana.zega@unimi.it)

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Summary

The aim of this study was to characterize the swimming behaviour of *C. intestinalis* larvae during the first 6 h after hatching by measuring tail muscle field potentials. This recording method allowed a quantitative description of the responses of the larva under light and dark conditions. Three different larval movements were distinguished by their specific frequencies: tail flicks, 'spontaneous' swimming, and shadow response, or dark induced activity, with respective mean frequencies of about 10, 22 and 32 Hz. The shadow response develops at about 1.5 h post hatching (h.p.h.). The frequency of muscle potentials associated with this behaviour became higher than those of spontaneous swimming activity, shifting from 20 to 30 Hz, but only from about 2 h.p.h. onwards. Swimming rate was influenced positively for about 25 s after the beginning of the shadow response. Comparison of swimming activity at three different larval ages (0–2, 2–4 and 4–6 h.p.h.) showed that *Ciona* larvae swim for longer periods and more frequently during the first hours after hatching. Our results provide a starting point for future studies that aim to characterize the nervous control of ascidian locomotion, in wild-type or mutant larvae.

Key words: shadow response, electrophysiology, muscle field potentials, nervous system, locomotion, *Ciona intestinalis*.

Introduction

Ascidians belong to the phylum Chordata, subphylum Urochordata, which is the sister group of vertebrates and thus possess many basal chordate features. The larvae of ascidians have a dorsal tubular nervous system and a tadpole like body, with a tail supported by an axial notochord (Fig. 1A). The notochord is flanked by rows of muscle cells that are responsible for tail movements such as swimming (Fig. 1B) (Kowalewsky, 1866; Mast, 1921; Kats, 1983; Bone, 1992). Ascidian tadpoles actively swim by bending their tail with alternating symmetrical contractions. They also produce asymmetrical contractions, or 'tail flicks', which help the larvae escape from the chorion membrane and, later, to change direction (Mast, 1921).

Tail movements are under the control of the larval nervous system, which comprises around 100 neurons, divided into four regions: the anterior sensory vesicle, the neck, the visceral ganglion and the nerve cord (Nicol and Meinertzhagen, 1991; Meinertzhagen and Okamura, 2001; Meinertzhagen et al., 2004). Muscle contractions are driven by five pairs of motor neurons, found in the visceral ganglion, that project axons to the muscle fibres of the tail (Fig. 1B) (Bone, 1992; Cole and Meinertzhagen, 2004; Brown et al., 2005). The control of swimming has some similarities to vertebrate spinal networks suggesting the existence of some 'universal' chordate features. For example, larval motor neurons innervating the tail express cholinergic promoters and genes (Takamura et al., 2002; Yoshida et al., 2004), neuromuscular junctions are cholinergic (Ohmori and Sasaki, 1977), while GABA is present in the visceral ganglion and, as in vertebrates, has been shown to modulate swimming (Brown et al., 2005).

Impinging on the 'lower' motor network of the visceral ganglion are fibres emanating from the 'higher' part of the nervous system, which includes the sensory vesicle. The sensory vesicle contains the two main sensory organs that allow the larva to detect light and gravity: the ocellus and the otolith (Fig. 1A,B). The ocellus is a pigmented cell associated with 17–30 photoreceptor cells and a lens (Eakin and Kuda, 1971; Nicol and Meinertzhagen, 1991; Horie et al., 2005). The otolith is a single pigmented cell, connected to neurones *via* the floor of the sensory vesicle (Tsuda et al., 2003a; Nagakawa et al., 2002; Sakurai et al., 2004). These two organs are mainly involved in the perception of environmental cues that drive ascidian tadpole behaviour (Tsuda et al., 2003a; Sakurai et al., 2004; Di Jiang et al., 2005).

The behaviour of larvae changes during the free swimming

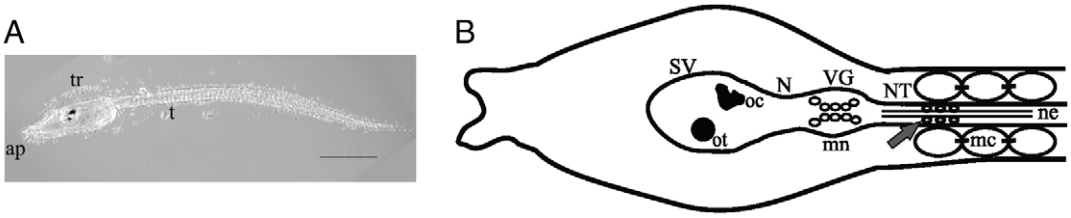

Fig. 1. (A) Swimming larva of *Ciona intestinalis*. In the trunk the two pigmented organs are visible within the sensory vesicle. Scale bar, 100 μm. (B) Diagram of motor neurons of the visceral ganglion and of the innervation pattern of muscle cells in the tail, dorsal view (modified from Bone, 1992; Cole and Meinertzhagen, 2004; Brown et al., 2005). Neurites connecting motor neurons to the muscle cells in the tail present some varicosities (arrow). ap, adhesive papillae; mc, muscle cell; mn, motor neurons; ne, neurites; N, neck; NT, neural tube; oc, ocellus; ot, otolith; SV, sensory vesicle; tr, trunk; t, tail; VG, visceral ganglion).

phase. For example, larvae have been reported to switch their behaviour from photopositive to photonegative during the pre-settlement period (Grave, 1920; Millar, 1971). Resting larvae are stimulated to swim when passing from light to dark conditions and this reaction is known as the shadow response (Mast, 1921; Kajiwara and Yoshida, 1985; Young and Chia, 1985; Bone, 1992). *Ciona savignyi* larvae were found to develop the shadow response 1.5 h after hatching (Kajiwara and Yoshida, 1985), while *C. intestinalis* larvae became sensitive to a reduction in light around 4 h after hatching and during the dark period they swim faster (Kawakami et al., 2002; Tsuda et al., 2003b).

The objective of this work was to record the muscular activity underlying larval swimming during the course of larval life and to determine the time of onset of the shadow response and its influence on larval behaviour. The recording method did not seem to alter significantly the development of swimming behaviour, as restrained larvae demonstrated similar behaviour to that observed with video or by direct observation. This work focuses on the first 6 h period post-hatching since during this phase larval structures complete their development and competence for metamorphic change is acquired (Degnan et al., 1997; Eri et al., 1999; Davidson and Swalla, 2001; Horie et al., 2005; Nakayama et al., 2005). We used a high level of precision because video analysis of swimming larvae does not reflect linearly the output of the nervous system at all stages of larval development; because some larval muscle activity is not concerned with swimming (e.g. changes in direction), and because swimming occurs at intermediate Reynolds numbers in seawater, initial tail movements do not produce instantaneous velocities (McHenry et al., 2003).

Materials and methods

Animals

Ciona intestinalis Linnaeus 1767 adults were collected in the bay of Naples by the fishing service of the Stazione Zoologica and kept in tanks with running seawater. Animals were dissected to remove male and female gametes from the gonoducts for *in vitro* fertilization. Fertilized eggs were allowed to develop in Petri dishes with filtered seawater (FSW, 0.2 μm), at 18°C in an incubator. Embryos were transferred in the lab at 20°C 1–2 h before hatching.

Electrophysiological recordings

For electrophysiological recording, glass micropipettes were drawn from borosilicate glass of 1.5 o.d. on a microelectrode puller (Model P87 Sutter Instrument Company, Novato, CA, USA). The electrodes were mounted on a micromanipulator and their tips broken under microscopic control, so that the internal diameter was about four-fifths the diameter of the larval tail. Using coarse manipulation of the microscope stage and micromanipulation of the electrode, the larval tail was placed in close contact with the tip of the electrode. Then, negative suction was rapidly applied and the larval tail was drawn into the pipette to about two thirds of its length. Muscle action potentials were recorded differentially between the inside of the pipette and the seawater of the bath, and amplified (WPI model DAM 80 World Precision Instruments Ltd, Aston, UK) 10 000× with reference to a silver chloride pellet placed in the bath. Signals were AC-coupled and passed between 0.1 Hz and 10 000 Hz. They were then digitised and stored, using a Digidata 1200 data acquisition system, and analysed using Clampfit software (version 9.0) (Axon Instruments Inc, Molecular Devices Corporation, Sunnyvale, CA, USA). A custom-built shutter was controlled by 5 V control pulses delivered from the Digidata board, allowing a step-down in the light intensity to be applied for 5 s. In order to determine the exact age of larvae used in the recordings, a pool of newly hatched larvae was sampled and transferred to a new Petri dish. Then some larvae were put into a 5 cm Petri dish in FSW and placed under the microscope. When drawn into the pipette, larvae showed some inhibition of swimming activity that lasted around 15 min. Therefore all the experimental runs were started 20 min after the suction electrode was attached to the tail. All experiments were carried out at 20°C and the Petri dish was perfused with FSW (8 ml min^{-1}). Larval activity was recorded in a series of 1 min sweeps, every 5 min, under constant light conditions or with 5 s light-off. The light-off stimulation was always given within the first 30 s of the sweep so that the after-effects of the response could be studied.

Plots of instantaneous frequencies of potentials *vs* time and mean frequency of potentials were obtained from raw traces.

The duration of each interval of larval swimming activity, also termed 'burst', and the quantity of activity for each sweep (sum of all burst durations), both with light-off stimulation and in constant light, was obtained. Recordings were made from newly hatched larvae and from larvae up to 6 h post-hatching (h.p.h.). The activity of each larva was recorded for a maximum period of 3 h.

Photographs

To establish the time of onset of the shadow response independently of the suction electrode method, larvae were placed in a square tank (3 cm), and were photographed from above in light conditions. Photographs were taken before and 5, 30 and 60 s after shading (see Kajiwara and Yoshida, 1985). The ambient temperature was 20°C.

Data analysis and statistics

Analysis of variance (ANOVA) was performed to test whether the mean values of muscle potential frequencies associated with different larval activities were significantly different. Mean frequencies of muscle field potentials of different larval activities were calculated from 2 s of sampled traces, with the exception of tail flick values, since these often lasted for shorter periods. For evaluation of the after-effects of the light-off stimulus, average values were obtained from 2.5 s of the sampled trace. The effect of larval age, hours post hatching, and presence or absence of dark stimulation on quantity of activity per sweep (s), were also evaluated. Linear regression analysis was used to examine if trends observed in the after-effects of the light-off stimulus were significant. Values are means ± s.d.

Results

Time course of swimming activity

Tail suction electrode recordings showed the characteristic field potential changes associated with tail twitches and swimming movements (see Brown et al., 2005), i.e. potentials occurred singly or in trains on a flat baseline (Fig. 2A). To examine possible time- or activity-dependent changes in swimming rate, the instantaneous frequency of each tail contraction was plotted *vs* time (Fig. 2B). Significantly different frequencies of potentials were found for three distinctive larval behaviours: tail flicks, spontaneous swimming bursts and shadow responses (dark-induced activities). Tail flicks occurred at 9.8±2.2 Hz (N=7), spontaneous swimming bursts at 22.8±2.4 Hz (N=24) and shadow responses at 32.1±3.9 Hz (N=24) (ANOVA test: F=148.9; $P\leqslant0.00001$) (data from Fig. 2C). For both tail flicks and spontaneous swimming, the frequencies did not alter significantly with time (Fig. 2C). Trains of tail flicks were often recorded in larvae up to 3 h.p.h., both before and after swimming bursts (Fig. 2A,B). Later they occurred rarely.

Development of the shadow response

From 1.5 h.p.h. onwards, larvae started swimming when the light was switched off (shadow response). However the frequency of potentials was similar to that of spontaneous swimming. Over the following hours the frequency of swimming during the shadow response gradually increased, becoming significantly higher than spontaneous swimming frequency (Fig. 2C, Table 1). Occasionally, tail flicks were recorded at the beginning of the shadow response (Fig. 2A,B). Both plots and traces show that resting larvae started swimming immediately when the light went off. Moreover, larvae were stimulated by the light-off signal even if they were already swimming, that is they were able to change frequency during swimming (Fig. 2A,B). The time of onset of the shadow response was also verified by taking photographs of the distribution patterns of swarms of larvae, under light conditions before, and at regular intervals after shading. When illuminated from the side, 1.5 h.p.h. larvae tended to form a 'swarm' in the centre of the tank (Fig. 3A). When a shadow was passed across the light path, larvae started to swim in different directions causing dispersal of the 'swarm'. This indicated that larvae were already sensitive to the switch from light to dark conditions at 1.5 h (Fig. 3B–D).

After-effects of the shadow response.

In Fig. 2B, the scatter plot of instantaneous frequency of muscle potentials in a 3.5 h.p.h. larva, shows a gradual decrease after the 5 s dark period. We observed that the frequency of potentials of the shadow response activity remained higher than the spontaneous swimming frequency for a period after the light was switched on again. A linear regression model explained about the 31% of the frequency decrease in time (N=15, R^2=0.310; F=116.257; $P\leqslant0.00001$) (Fig. 4). The mean frequency of tail contractions, for each time interval, was significantly higher than the frequency of spontaneous swimming (recorded before the light off stimulation) for 25 s after the beginning of shadow response (Table 2).

Quantity of swimming activity

To examine if there were changes in the quantity of swimming with time, the shadow response duration, the duration of spontaneous swimming, mean burst duration and quantity of total swimming activity during larval aging was calculated. The duration of the shadow response stimulated by 5 s light-off was very variable and no significant trend was found during larval aging (data not shown). The mean duration of the shadow response was 20±10.4 s and the minimum duration was 5 s, corresponding to the duration of the imposed dark period. The mean duration of spontaneous swimming bursts changed significantly during the three periods considered. In larvae from 0–2 h.p.h., bursts lasted for 10.2±14.2 s, while in older larvae, from 2–4 h.p.h. and from 4–6 h.p.h., mean burst duration was 4.1±5.1 s and 5.7±6.0 s, respectively (ANOVA: F=11.304, $P\leqslant0.0001$; Tukey's *Post Hoc*: 0–2 h.p.h. *vs* 2–4 h.p.h. $P\leqslant0.0001$; 0–2 h.p.h. *vs* 4–6 h.p.h.: $P\leqslant0.002$; 2–4 h.p.h. *vs* 4–6 h.p.h.: no significant difference) (N=20) (Fig. 5).

Comparison between mean total activity per sweep with and without the light-off stimulation during these three periods of

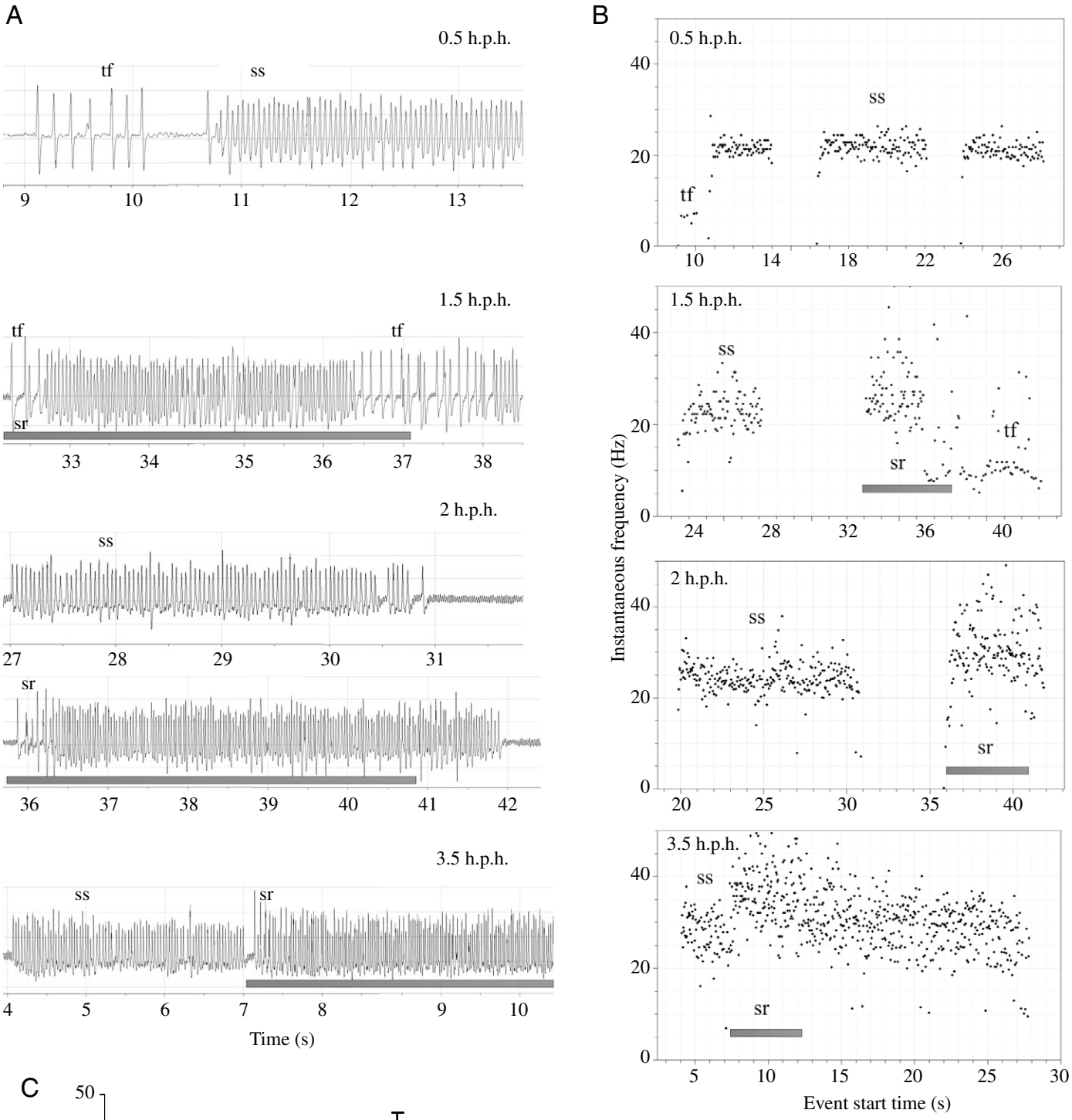

Fig. 2. See next page for legend.

Fig. 2. Muscle field potential recordings, instantaneous frequencies and mean frequencies. (A) Examples of larval activity at different ages (h.p.h.). Solid bars indicate dark periods (of 5 s). (B) Plots of instantaneous frequency of tail contractions (Hz) *vs* time (s) from the traces shown in A. Every tail contraction is represented by one dot in the chart. Solid bars under plots and traces indicate the 5s dark period, imposed by the automatic shutter. There was an exact correspondence between the beginning of the light off-period and the beginning of swimming activity. Series of tail flicks, with a mean frequency of about 10 Hz, can be seen to precede or follow swimming bursts. Tail flicks are of larger amplitude than the potentials during swimming periods. Tail flicks can also be seen at the beginning of the shadow response that developed at 1.5 h.p.h. The frequency of potentials during the shadow response increased during larval aging and from 2 h.p.h. it was always higher than spontaneous swimming frequency. The 3.5 h.p.h. plot is also an example of how an active larva could change frequency of swimming if stimulated by a step-down in light. tf, tail ficks; ss, spontaneous swimming; sr, shadow response. (C) Mean frequency of muscle field potentials generated by tail flicks, spontaneous swimming and shadow response at different larval ages (h.p.h.). Frequency values (Hz) are means ± s.d.. Open triangles, tail flicks; grey circles, spontaneous swimming; black circles, shadow response.

larval life revealed significant differences in the quantity of larval swimming (Fig. 6). We found significant interactions between the mean total activity per sweep both with respect to larval age, hours post hatching, and presence or absence of dark stimulation (effect of larval age: h.p.h. *vs* total activity F=12.760, $P \leqslant 0.00001$; effect of presence or absence of light-off stimulation *vs* total activity F=18.495, $P \leqslant 0.00001$; interaction: larval age × presence or absence of light-off stimulation F=22.803, $P<0.00001$). From 0 to 2 h.p.h., larvae under constant light swam for 40.1±15.1 s per sweep, while larvae stimulated by the 5 s light-off swam for 31.0±17.8 s. In the next two periods, larvae under constant light conditions swam for shorter times per sweep when compared to larvae stimulated by the light-off (2–4 h.p.h. and 4–6 h.p.h. without light-off: 15.2±11.2 s and 20.3±11.1 s; 2–4 h.p.h. and 4–6 h.p.h. with light-off: 33.1±14.4 s and 36.2±12.5 s) (ANOVA: 2–4 h.p.h. F=27.242, $P \leqslant 0.00001$; 4–6: F=35.269, $P \leqslant 0.00001$) (N=40) (Fig. 6). These results showed that there was a significant decrease in the quantity of activity during aging if larvae were not 'stimulated' by light-off. On the other hand, in the three different time intervals considered, there was an increase in the total activity of the larvae during sweeps with light off stimulation. Moreover in each of the three periods, there was a significant difference between mean total activity in sweeps without light-off compared to that in sweeps with light-off. Under our experimental conditions, larvae from 0 to 2 h.p.h. swam for longer in the absence of dark stimulation while later on (2–4 and 4–6 h.p.h.) they swam for longer times only if stimulated by a light step down, when compared to 0–2 h.p.h. larvae.

Discussion

We have characterised the swimming behaviour of *C. intestinalis* larvae in terms of the frequency of muscle field potentials and the quantity of activity during larval aging. The simplest of larval movements were tail flicks or asymmetrical tail contractions that occurred at a frequency of about 10 Hz. Tail flicks are the first movements of the tail and may help hatching larvae to escape from the chorionic membrane. Tail flicks are not confined to pre-hatching and newly hatched larvae, however, since trains of tail flicks were often recorded from larvae for up to 3 h.p.h. It has been suggested that in the first hours after hatching, tail flick contractions could represent 'warm-up' movements, in turn activating symmetrical swimming (Bone, 1992). In agreement with this view, we often observed tail flicks in the period immediately preceding a 'bout' of spontaneous symmetrical swimming and also in the first movements of the shadow response. However, tail flicks were not an absolute prerequisite for both types of symmetrical swimming and it has been observed that they could determine a change in direction in free-swimming larvae (Mast, 1921; Bone, 1992).

Table 1. *Values* of frequency of muscle field potentials of larval swimming activity at different times after hatching*

	Frequency (Hz)				
Larval age (h.p.h.)	Tail flicks	Spontaneous swimming	Shadow response	*F*	*P*
0.5	6.3±0.9	21.1±1.7	–	–	
1.5	12.5±5.2	23.2±3.2	25.5±4.5		NS
2	–	23.6±2.0	31.4±5.2	110.5	<0.00001
2.5	10.0±1.7	24.7±3.7	31.2±5.0	18.3	<0.00001
3	12.1±6.2	27.0±3.4	36.0±6.2	121.6	<0.00001
3.5	–	21.3±7.4	33.0±7.9	101.8	<0.00001
4	–	21.4±2.4	35.1±7.4	131.0	<0.00001
5	–	23.4±1.0	30.5±7.3	290.1	<0.00001
6	9.0±2.8	29.3±3.6	39.7±6.4	124.7	<0.00001

*Values (means ± s.d.; N=9) were chosen from among those represented in Fig. 2.

h.p.h., hours post hatching.

F and *P* values (ANOVA) indicate significant differences between spontaneous swimming and shadow response frequency (NS, not significant).

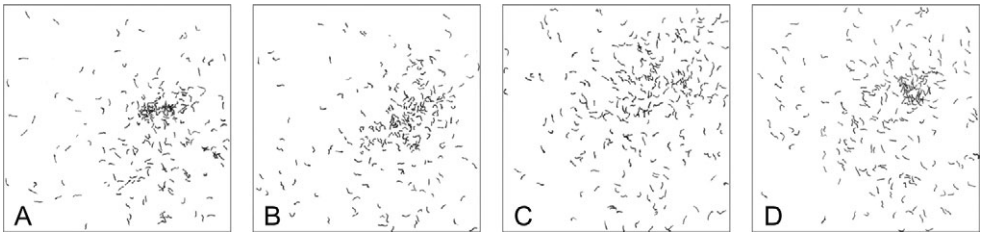

Fig. 3. Larval distribution pattern photographed from above. (A) Larvae are grouped in a swarm when the light was on. (B) 5 s after the shading the swarm was dispersed. (C) 30 s after the shading, larvae were still swimming in different directions. (D) After 1 min larvae tended to group again.

Symmetrical swimming activity, or spontaneous activity, was characterized by rates of around 20 Hz, which is about double the frequency of the single tail twitches. The fact that both tail flicks and symmetrical swimming have such tightly controlled frequencies would seem to suggest that both are subject to control by impulses coming from the main sensory organs in the trunk, which act to coordinate firing rate and, in consequence, muscle contractions. In terms of control, it should be noted that there was no superposition of tail flicks, spontaneous swimming or shadow response. In other words, there is a final common pathway for nervous system output, and cross-inhibition ensures temporal separation of the different behaviours.

The onset of the shadow response in *C. intestinalis* larvae occurred at 1.5 h.p.h., and this was confirmed by photographs of swarms of larvae taken before and after shading. This showed that even when restrained in the suction electrode, larvae develop shadow responses at the same time as freely moving specimens. The frequency of potentials of the shadow response activity was initially no different from the frequency of spontaneous swimming. About half an hour after its appearance, the frequency of the potentials during the shadow response increased to 30–35 Hz. Under our experimental conditions, even actively swimming specimens reacted to light-off by increasing the frequency of muscle contractions (tail beating).

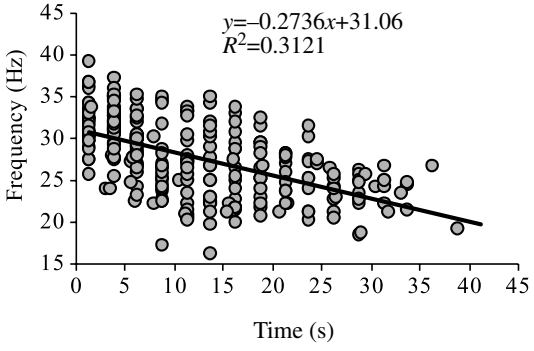

Fig. 4. After–effect of the shadow response. Time 0 s represents the beginning of the shadow response: all frequency values of swimming activity during the dark period are shown with respect to this time. The regression line shows the negative trend of the frequency of muscle field potentials *vs* time.

Our results on the timing of the onset of the shadow response are in accordance with observations made on *C. savignyi* (Kajiwara and Yoshida, 1985). Kajiwara and Yoshida described how different larval behaviour, from the onset of the shadow response to the beginning of the photonegative period, was related to the different developmental stages of the ocellus, which becomes fully differentiated 3.5 h after hatching. In particular, the authors described how pigment granules are gradually accumulated while the sensory structure of the photoreceptor is folded into its definitive form. It is possible that changes in the ability to respond to dark stimulation are determined by a particular course of development of the light sensing organ. In *C. intestinalis*, Horie et al. described that in 1 h.p.h. *C. intestinalis* larvae all the photoreceptor cells are already present, while their nervous connections expanded remarkably only later, at around 3 h.p.h. (Horie et al., 2005). Therefore, the observed increase in frequency of the shadow response activity could be related to the completion of the development of the light sensory organ and of the neural

Table 2. *Lasting effect of the shadow response*

Time interval (s)	Mean frequency (Hz)	*P*
0–2.5	31.9±2.9	<0.00001
2.5–5	31.8±3.6	<0.00001
5–7.5	29.1±3.1	<0.00001
7.5–10	26.7±4.4	<0.0011
10–12.5	26.2±4.5	<0.01
12.5–15	26.2±5.0	<0.01
15–17.5	25.9±4.3	<0.01
17.5–20	26.4±3.6	<0.01
20–22.5	25.3±2.6	<0.01
22.5–25	25.7±3.5	<0.01
25–27.5	23.7±2.1	NS
27.5–30	23.3±2.6	NS

Values are means ± s.d. (*N*=15).

The list of mean frequency values of activity potentials during 30 s from the beginning of light-off shows the decrease following the 5 s dark period. *P* values indicate significant differences of these values when compared to the mean frequency of spontaneous swimming potentials, determined by ANOVA (NS, not significant).

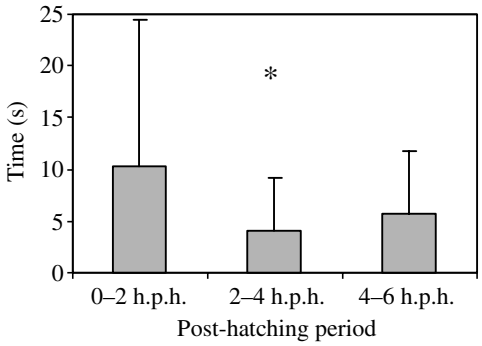

Fig. 5. Mean duration of bursts of spontaneous swimming during three different post-hatching periods. Data are means ± s.d. For statistical significance, see Results. *$P \leq 0.0001$.

network connecting the sensory organs to motor area in the visceral ganglion.

It has been reported that in *C. intestinalis* larvae the shadow response did not develop until 4 h.p.h. (Kawakami et al., 2002; Tsuda et al., 2003b). Such different observations could be explained by the different temperatures at which the experiments were carried out (20°C *vs* 18°C). Temperature strongly influences the timing of development in *Ciona* embryos and it is possible that larvae kept at a lower temperature develop the shadow response later. Perhaps the major difference, however, was that the determination of the onset of this response was obtained by carrying out video recording and producing a mean linear speed for a large number of swimming tadpoles. As larval swimming speed does not deviate significantly from that of spontaneous swimming until 2 h.p.h., it would be difficult to determine the onset of this response using the video recording method. In any case, and in accordance with our results, it was established that linear speed of swimming increased to a maximum value during the dark period and then decreased when the light was on again (Tsuda et al., 2003b).

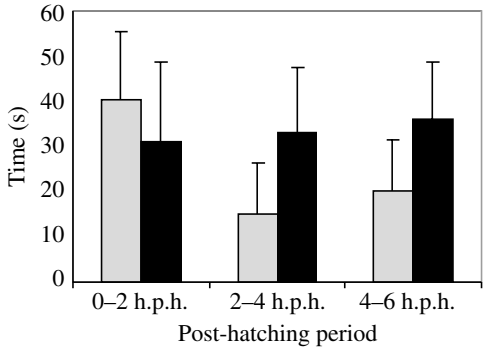

Fig. 6. Mean total activity per sweep for three different post-hatching periods. Grey columns: without light-off stimulation; black columns: with light-off stimulation. Data are the mean (± s.d.) totals of activity per sweep (s). For the analysis of interaction between larval age and presence or absence of light-off stimulation, see Results.

The frequency of potentials of shadow response activity showed after-effects. Maximum values were recorded during the 5 s dark period, while after that, when the light was on, the frequency gradually decreased for 25 s until it was equal to the frequency observed during spontaneous swimming. Ascidian photoreceptors are of the hyperpolarizing type (Gorman, 1971) and darkness should produce a depolarizing response, giving an excitatory stimulus to nearby neurones. This stimulus could determine a change in muscle tail contraction frequency, through excitation of interneurone circuits that drive the firing rate of motor neurones in the visceral ganglion. Such interneurones, located close to the photoreceptors and forming part of a retinal territory that sends 'descending' neuronal process to the visceral ganglion, have been detected morphologically (D'Aniello et al., 2006). When the light is on, hyperpolarization of photoreceptors occurs and the motor response frequency begins to wane, following the trend shown in Fig. 4. Brown et al. localized GABA immunoreactivity in the nervous system of larvae of *C. savigny*, particularly at the level of the sensory vesicle and the visceral ganglion (Brown et al., 2005). Their pharmacological results with *Ciona intestinalis* showed that GABA is released during swimming and could act as a modulator of swimming frequency. Another potential inhibitory transmitter system in ascidian larvae is dopamine. Moret et al. detected tyrosine hydroxylase (dopamine synthesis rate-limiting enzyme) expression in the hypothalamus-related domain of the sensory vesicle of *C. intestinalis* larvae (Moret et al., 2005) and suggested that dopamine could be involved in modulating larval locomotion. These authors showed that dopamine synthesis begins only some hours after hatching. It could be that one of these inhibitory neurotransmitters cause the decrease in frequency of muscle contraction after dark stimulation, when the light is on. Indeed the increase in frequency of the shadow response with time may reflect a gradual disappearance of inhibitory control over the 'normal' (spontaneous) swimming rate.

The duration of the shadow response was very variable during the course of larval life. The fact that such a diffuse reaction among ascidian tadpoles has an unpredictable duration during larval aging, supports the hypothesis that this response is not involved in locating shaded habitats and does not allow larvae to encounter a suitable place for settlement with a higher probability (Young and Chia, 1985; Svane and Young, 1989). Its most probable function is to help orientation of swimming larvae in light, first in photopositive and later in photonegative taxis. A mechanism was described supporting the hypothesis that the shadow response may help the larvae to orientate towards or away from the light direction (Mast, 1921). Mast noted that while swimming, ascidian tadpoles continuously rotate on their longitudinal axis clockwise and they twitched the tail in different directions depending on the orientation of the ocellus to the light source As the unpaired photoreceptor of the ascidian tadpole is situated on the right posterior wall of the sensory vesicle, he suggested that the orientation of larvae to light could be the result of one or more reactions, caused by

the alternate shading and illumination of the optic nerve endings, owing to the rotation of the tadpoles on the longitudinal axis.

Mean burst duration and mean total activity per sweep were higher in larvae up to 2 h.p.h. than in older ones. As a consequence, under our experimental conditions, ascidian larvae swam for longer time intervals and more often during the first hours after hatching, compared to older ones. It is reasonable to suppose that, under natural conditions, newly hatched larvae are more active to improve dispersal, while older larvae swim less and most probably sink for longer, to increase the chance of finding a suitable place to settle (Bone, 1992; McHenry, 2005). Our observation provides additional evidence that, even if restrained in the suction electrode, larvae retained an apparently normal behaviour.

To date the neural networks connecting the sensory vesicle to motor neurons and how they might drive ascidian larvae locomotion are still poorly known. The method used here to characterize ascidian larvae behaviour is an essential first step towards describing in detail how single neurone behaviour and networks work together to produce whole-larva behaviour.

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