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Source: Zoological Science, 13(2) : 229-234

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.13.229

ZOOLOGICAL SCIENCE 13: 229–234 (1996)

Developmental Changes in the Swimming Behavior and Underlying Motoneuron Activity in the Larval Angelfish, *Pterophyllum scalare*

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ABSTRACT—Developmental changes in the swimming behavior and underlying motoneuron activities of the larval angelfish, *Pterophyllum scalare*, were investigated in the course of post-embryonic development of the fish. For a few days after hatching, angelfish larvae showed spontaneous wriggling movement, which was alternating lateral bending of the tail without locomotion. On the 5th or 6th day after hatching, the larvae started swimming (locomotive activity). Spontaneous ventral root activity was recorded extracellularly from the body surface of the immobilized larvae as a monitor of the spinal motoneuron activity underlying wriggling and swimming. The number of motoneuron impulses and the duration of the burst in each cycle during the spontaneous rhythmic ventral root activity were increased as the larvae developed. In addition, the duration of the motoneuron bursts in older larvae was much more variable compared with that in early larvae, suggesting that the force of tail beat could be regulated flexibly as the larvae developed. In early larvae, two distinguishable patterns of spontaneous ventral root activity were observed: a low frequency bursting and a high frequency bursting. Such bursting patterns were consistent with the behavioral observations of free moving larvae. After the start of swimming, the frequency of the motoneuron bursting converged into a narrow range, corresponding to the stable, continuous swimming rhythm of the free moving larvae. We propose that the early stages of the angelfish larvae provide us with a useful model for investigating the developmental changes in neuronal substrates underlying swimming activity of the teleost fish.

INTRODUCTION

Spinal neuronal circuitry underlying swimming movement has been closely studied in cyclostomes (Cohen *et al.*, 1992; Grillner and Matsushima, 1991; Grillner *et al.*, 1991) and embryonic and larval amphibians (Roberts, 1990; Sillar and Roberts, 1993; Sillar and Simmers, 1994). In amphibians, especially in *Xenopus laevis*, developmental changes of spinal neuronal substrates for swimming have also been investigated (Sillar *et al.*, 1991, 1992; Tunstall and Sillar, 1993).

Fetcho (1992) has pointed out the striking similarities between the fundamental organization of spinal motor networks in lower vertebrates including lamprey, amphibian larvae and teleost fish. In teleost fish, however, the neural basis of swimming motor pattern generation is little known, although the central mechanisms of motor control of startle reflex (C-start) have been well investigated. It has been suggested that the local circuit involved in the startle reflex may well be used in ordinary swimming activity (Faber *et al.*, 1989; Fetcho, 1992).

The development of spinal motoneurons, including the determination of identities of the motoneurons innervating the trunk muscles, has been intensively investigated in zebrafish, *Brachydanio rerio* (Eisen, 1991a, b; Myers, 1985; van Raamsdonk *et al.*, 1983). In this species, development of the reticulospinal neurons that are probably involved in the control of locomotion has also been described in detail (Mendelson, 1986a, b).

In the present paper, as a first step forward understanding of the neural circuitry underlying swimming activity and its development in teleost fish, we describe the development of swimming behavior and underlying spinal motoneuronal activities of larval angelfish, *Pterophyllum scalare*. For 5 days after hatching, angelfish larvae grow on the substrate to which they attach and do not show locomotion, although they show spontaneous wriggling movement, which is alternate lateral bending of the tail without locomotion. Then the larvae detach from the substrate and start to show free swimming. By tracing the developmental changes in the rhythmicity of tail beats and underlying neuronal activities, we aim to reveal how the fish larvae get an ability for swimming in the course of their ontogeny. The basic neuronal circuitry underlying rhythmic movement of the body may well

be developed before hatching, since the animal in late embryonic period shows spontaneous wriggling movement when the egg capsule is artificially removed.

Relative simplicity of anatomy of the larval neuromuscular system, e.g., compared with the adult, the larvae have relatively small number of spinal motoneurons and thin axial muscles (Yoshida *et al.*, unpublished observations), may facilitate the physiological and histological manipulations. In addition, angelfish larvae spend a considerably longer period before the onset of free swimming, compared with zebrafish (Kimmel *et al.*, 1995), which has been widely used for developmental neurological research. This characteristic of angelfish is helpful to examine the developmental changes in swimming behavior. The angelfish larvae, therefore, seem to provide a useful model to investigate the development of neural mechanisms involved in the swimming behavior of teleost fishes. Furthermore, it is important from a comparative point of view to reveal the features of the development of neural circuits underlying teleost locomotion, since some of the mechanisms involved in development of locomotion are likely to be shared by many vertebrates (Sillar *et al.*, 1993).

MATERIALS AND METHODS

Larvae of the angelfish, *Pterophyllum scalare*, used in the present experiments were obtained from two pairs of laboratory-reared adult *P. scalare* that bred spontaneously at 26.5°C. Spawned eggs, together with the substrate (plastic tube having 18 mm diameter and 150 mm length) on which the eggs were laid, were transferred to a 1 litter bottle filled with aerated water kept at 26.5°C. Larvae of the angelfish were fed on newly-hatched brine shrimp larvae after the start of feeding. Behavior of the fish was observed visually and recorded on video tape (30 frames per sec). The video-taped records were used for analysing the beating pattern of larvae.

To record the ventral root activity (VRA) of the larvae, animals were first anesthetized in saline containing 100-200 ppm MS-222 and 10^{-3} M curare. In the solution, small incisions in the trunk skin of the larvae were made to facilitate the penetration of curare. After the complete immobilization, small parts of the trunk skin at the level of the anus on both sides of the larvae were removed with fine forceps. The preparation was then transferred to a recording chamber that was perfused continuously with saline containing 10^{-4} M curare. Spontaneous VRA, which mainly consisted of action potentials in motoneuronal axons, was recorded using glass suction electrodes placed on the intermyotomal clefts, at the level of the anus, on both sides of the trunk. The amplified signals were monitored on an oscilloscope (Nihon Kohden, VC-11). The data were stored on FM tape using a data recorder (Sony, NFR-3000) for later analyses, and the digitized data were stored on magnetooptical disk through the MacLab system (AD Instruments) connected with the Apple Macintosh computer. Permanent records were made using a thermal array recorder (Nihon Kohden, RTA-1100).

To estimate the distribution of frequency of cyclic motoneuron bursting, i.e. power spectrum of the bursting frequency, relative occurrence time of the activities with each frequency (increment factor of the frequency=1) was calculated, since the absolute values of the number of occurrence lead to an underestimate of the occurrence probability of lower-frequency bursting. In the calculation, total time of occurrence of the most frequently observed bursting frequency was set at 1. According to this method, 1 cycle of bursting with 5 Hz has equal occurrence time to 2 cycles of bursting with 10 Hz, for example.

The composition of physiological saline used in the experiments was as follows (in mM): NaCl, 118.5; KCl, 4.74; $CaCl_2$, 3.0; KH_2PO_4, 1.18; $MgSO_4$, 1.18; $NaHCO_3$, 24.9; glucose, 5.0. The saline was bubbled with 95% O_2, 5% CO_2. The pH of the solution was 7.4. All physiological experiments were performed at 25–27°C.

RESULTS

Development of swimming behavior

The eggs of the angelfish were laid on a substrate provided by plastic tubing in the present experiment. After about 60 hr of pre-hatching development at 26.5°C, the embryo hatched out of the egg capsule. On the day of hatching (day 1), the eyes of the larvae are not pigmented and the mouth is not open yet.

Development of behavior of the angelfish larvae in relation to the swimming activity is summarized in Fig. 1. For 4 or 5 days after hatching, angelfish larvae adhered to the substrate with sticky mucus secreted from the cement glands on the head. During this early post-embryonic period, the larvae intermittently showed spontaneous wriggling movements but did not show locomotor activity (Fig. 2A). The wriggling movements of the young larvae did not seem to be for swimming but, at least in part, for exposing the body surface to fresh water for effective gas exchange. The young larvae also showed occasional tail beats at a higher frequency than that of periodical wriggling as described above (Fig. 2A). These high-frequency tail beats occurred spontaneously as well as in response to sudden dimming of the illumination or tactile stimuli. From the video analysis, the average frequency of low-frequency pattern (wriggling) was found to be 5.33±0.70 (cycles/sec±SD, n=48 from 4 animals). The frequency of high-frequency pattern appeared to be more than 12 cycles/sec (c/s), although, with the limita-

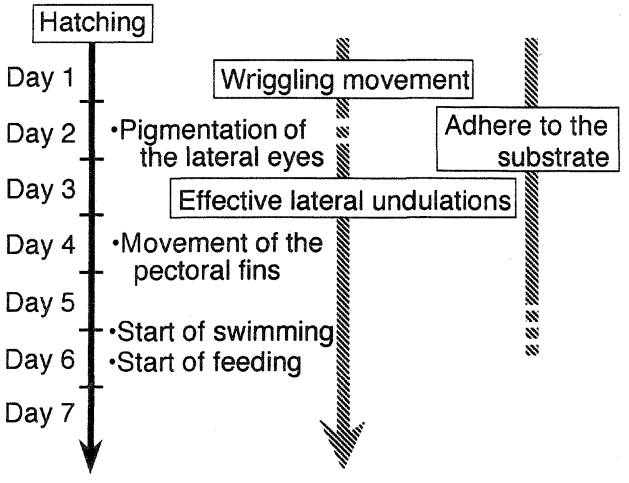

Fig. 1. Development of behavior of angelfish larvae in relation to the swimming activity. Day 1 is the day of hatching. Simple alternating bending movement of the tail (wriggling movement) gradually develops into effective lateral undulations, which can produce driving force for locomotion, then, at late day 5 or day 6, the larvae start free swimming.

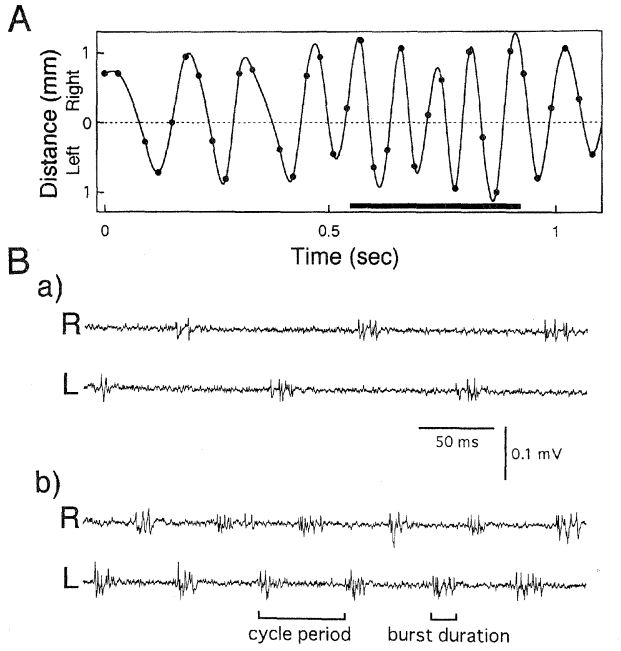

Fig. 2. Beating pattern of spontaneous tail beat and the recording of ventral root activities in angelfish larvae. (A) A trace of the beating pattern of the larvae at day 2. Dashed line indicates the axis that runs through the center of the head and the center of the rostral part of the trunk. Distances between the tip of the tail fin and the axis are plotted every 30 ms. Note that the larva shows the tail beat at higher frequency during the priod indicated by a thick bar. (B) Spontaneous ventral root activities recorded extracellularly from the right side (R) and left side (L) of the body surface of immobilized skinned larvae at day 2. (Ba) Low-frequency rhythmic bursts. (Bb) High-frequency rhythmic bursts. Traces shown in (Ba) and (Bb) were obtained from the same preparation.

tion of sampling rate of video tape recorder, an average value could not be calculated. Transition from wriggling to the high-frequency tail beat appeared to be abrupt (Fig. 2A).

As the larvae developed, their wriggling movement gradually changed into effective lateral undulations of the trunk and tail that could produce a driving force. At day 5, some larvae detached from the substrate and occasionally showed locomotion on the bottom of the aquarium. At day 6, almost all larvae showed free swimming and feeding. They swam above the bottom continuously and oriented to the food, such as brine-shrimp larvae, apparently visually. Around the start of swimming, coordinated movements of a pair of pectoral fins appeared to begin to take part in postural control of the larvae.

Development of ventral root activity

Ventral root activity (VRA), consisting of action potentials in motoneuron axons innervating the trunk muscles, was recorded by using suction electrodes placed on the intermyotomal clefts of immobilized larvae. Alternating bursts of motoneuron spikes were extracellularly recorded from the right and left sides of the body surface (Fig. 2B). Since rhythmic bursts of spinal motoneurons that were probably underlying tail beating could be observed in the immobilized animal, the bursting pattern must be produced by a central pattern generator that is intrinsic to the central nervous system and that does not require sensory feedback for the pattern generation. The cyclic activity of spinal neurons observed in the present experiment is "fictive swimming" that is not accompanied by actual locomotion.

From behavioral observations, as described above, the young larvae at day 1 and day 2 were found to show two distinguishable patterns of body movement, a low frequency wriggling and a higher frequency tail beat (Fig. 2A). Two patterns of cyclic motoneuronal activities, corresponding to the behavioral observations, were seen in typical spontaneous VRAs recorded from immobilized day 2 larvae (Fig. 2B). Figure 3Aa, b shows the instantaneous frequency of each cycle of rhythmic bursts in two spontaneous episodes recorded from the immobilized day 2 larvae. The low frequency of cyclic rhythm was found to shift rapidly to a higher one and then recovered relatively gradually to the initial level (Fig. 3Aa). Continuous low frequency rhythm with moderate fluctuation was also observed (Fig. 3Ab). Maintained cyclic bursts having intermediate frequency were rarely observed. Figure 3Ac shows frequency of cyclic rhythm versus relative period of occurrence of spontaneous rhythmic ventral root activities in immobilized animals at day 2. Two components of spontaneous rhythm, having peaks of occurrence at 5-7 c/s and 18-20 c/s each, were observed in day 2 larvae (Fig. 3Ac). The above results were consistent with the observations in free moving animals at day 2 or earlier.

Frequency of cyclic burst activity in spontaneous episodes changed as larvae developed. At day 3 or 4, the low frequency component, which was seen in day 1 or day 2 larva, had almost disappeared, while the higher one was preserved to some extent (Fig. 3Bc). In addition, a component having a peak at about 10 c/s emerged (Fig. 3Bc). Figure 3Ba, b shows the frequency of cyclic bursts in two episodes in day 4 larva. Although the transition from cyclic bursts at about 10 c/s to higher-frequency cyclic bursts was rapid, the latter, unlike those in younger larvae, were variable in frequency (Fig. 3Ba). This tendency was also represented in the histogram shown in Figure 3Bc as an unclear peak of higher frequency bursts.

The frequency of the bursts in spontaneous episodes appeared to converge at 8-10 c/s in the larvae at the later developmental periods (Fig. 3C, D). In addition, the spontaneously occurring episodes in older (day 8 or later) larvae were much more stable in frequency than those in younger (day 1 to day 4) larvae. At day 8 or later, distinct lower frequency cyclic bursts were again observed (at the end of the episode shown in Fig. 3Ca; Fig. 3Cc, Dc). However, the lower frequency cyclic bursts observed in the youngest and oldest larvae did not appear to have the same function in controlling fish movements. While a burst during the low frequency rhythm in older larvae consisted of markedly larger numbers of spikes and had a longer duration than in the

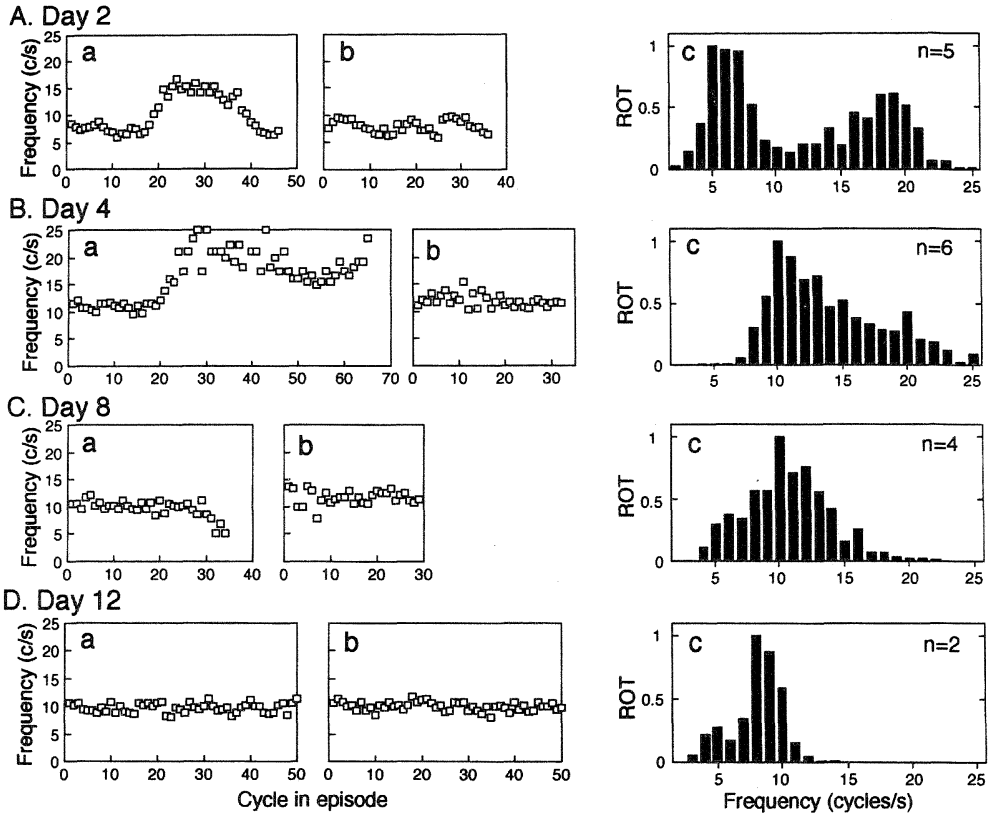

Fig. 3. Changes of patterns of spontaneous cyclic ventral root activities in the course of the development of the larvae. (Aab, Bab, Cab, Dab) Frequency of each burst cycle in spontaneously occurring episodes recorded from immobilized day 2 (Aab), day 4 (Bab), day 8 (Cab) and day 12 (Dab) larvae. Two typical examples at each age are shown (a, b). (Ac, Bc, Cc, Dc) Relationships between the relative occurrence time (ROT) and the frequency of burst cycles, i.e. the power spectrum of frequency of cyclic rhythm, in spontaneously occurring ventral root activities in day 2 (Ac), day 4 (Bc), day 8 (Cc) and day 12 (Dc) larvae. Total time of occurrence of the most frequently observed bursting frequency was set al 1. At least 540 cycles in spontaneous episodes were measured in each animal.

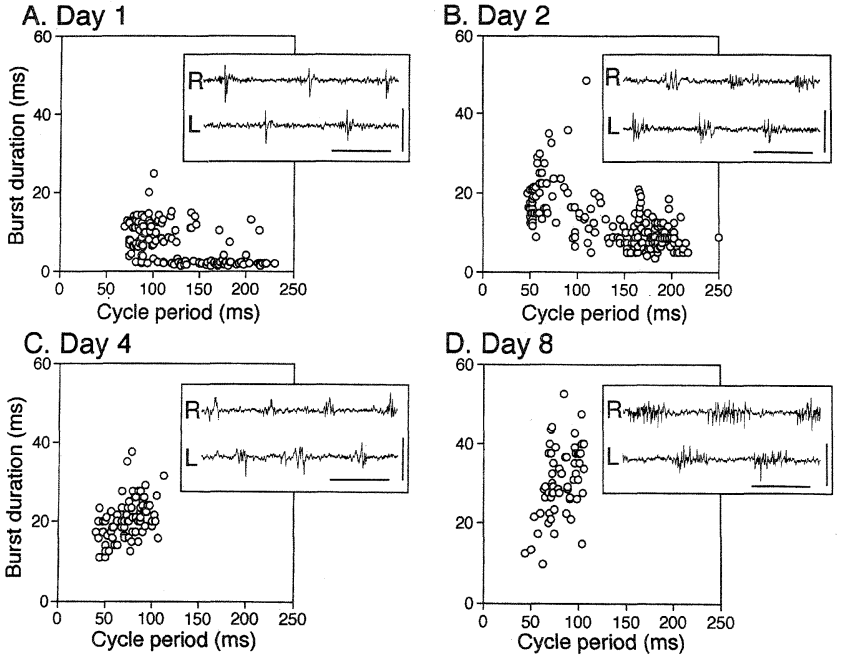

Fig. 4. Relationships between burst duration and cylce period in spontaneous ventral root activities in larvae at day 1 (A), day 2 (B), day 4 (C) and day 8 (D). Insets show the examples of recording of spontaneously occurring ventral root activities. Calibrations in insets: horizontal bars, 50 ms; vertical bars, 0.1 mV. R, right side; L, left side.

higher frequency rhythm, bursts in the low frequency rhythm in younger larvae consisted of fewer spikes and had shorter durations than in the higher-frequency rhythm (not shown).

Figure 4 shows the relationships between burst duration and cycle period in spontaneous episodes of day 1, 2, 4, and 8 larvae. In day 1 larvae, each burst in spontaneous episodes included only a single or a few spikes (see inset of Fig. 4A). As the larvae developed, the number of motoneuronal spikes included in each cyclic burst was increased (Fig. 4 insets) and variability of the burst duration was also increased (Fig. 4). Furthermore, in early larvae (day 1 and day 2), durations of bursts in shorter cycle periods, which means the frequency of the burst cycle was higher, appeared to be longer than those in longer cycle periods (lower frequency rhythm). The burst duration and the cycle period, therefore, had negative correlation ($r=0.517$ in day 1 ($n=137$) and $r=0.688$ in day 2 ($n=128$), $P<0.05$) in the early larva. After day 4, however, the relationships between burst duration and cycle period reversed, and the burst duration was significantly increased as the cycle period was increased ($r=0.42$ in day 4 ($n=81$) and $r=0.465$ in day 8 ($n=60$), $P<0.05$) (Fig. 4C,D). In addition, in the larvae after the start of swimming (day 6 or later), cycle periods in the spontaneous fictive swimming appeared to converge at a certain narrow range (Fig. 4D), and this observation is consistent with the result shown in Figure 3. In contrast, burst durations in the fictive swimming were distributed over a wide range in the older larvae (Fig. 4D), suggesting that the intensity of tail beats can be controlled independently of the beating frequency.

DISCUSSION

In the angelfish, *Pterophyllum scalare*, the basic neural circuit generating the alternating right and left bending of the tail appears to be developed before hatching. However, it takes 4 or 5 days after hatching for larvae to develop adult-like lateral undulations of the trunk and tail that can produce enough driving force. It is interesting to note that the angelfish larvae get an ability for swimming during the post-embryonic period. The early post-embryonic stage of larval angelfish, therefore, should provide us with a good model to investigate the development of the neural circuitry controlling the swimming behavior of the teleost fish. This is facilitated by the accessibility of the larva, with the anatomical simplicity of the neuromuscular system (see INTRODUCTION), for example, for physiological and morphological examinations.

In adult vertebrates, circuitry underlying locomotor rhythm generation has been considered to share many common mechanisms (Sillar *et al.*, 1993). Investigating neuronal properties of the swimming behavior in the course of development of lower vertebrates, such as teleost fish and amphibian larvae, should provide us with insight into the rhythmic locomotory behavior in higher vertebrates (Sillar *et al.*, 1991). Rhythmic motor output underlying most vertebrate locomotor patterns includes multiple motoneuron impulses per cycle, whereas the spinal motoneurons in the angelfish larva in the early post-embryonic period show single or a few impulses in each cycle. Motoneuron impulses in each rhythmic burst increased in number as the larvae developed, and hence the burst duration in each cycle was prolonged. A similar phenomenon has also been observed in *Xenopus laevis* tadpoles (Sillar *et al.*, 1991). In *Xenopus* tadpoles, the increase in motoneuron impulses per cycle has been shown to be a result of increased complexity of synaptic drive on the motoneuron and acquisition of the ability of motoneurons themselves to produce multiple spikes per cycle (Siller *et al.*, 1992). Although the cause of the increment in motoneuron impulse number in each cyclic burst during the course of development of the angelfish larvae is yet to be determined, the mechanisms may be similar to those seen in the *Xenopus* tadpole system. However, the increase in the impulses recorded extracellularly from the ventral root in angelfish larvae appears to be partly due to the increase in the number of spinal motoneurons innervating the trunk muscle (Yoshida *et al.*, unpublished observations).

Angelfish larvae in the early post-embryonic period show spontaneous wriggling movements on the substrate, to which they adhere by means of sticky mucus secreted from the cement gland in the head. Immediately after detaching from the substrate, around day 6, they start swimming and feeding. The spontaneous cyclic motoneuron activity recorded from the ventral roots in immobilized larvae thus can be considered to underlie the wriggling movements of larvae in the early post-embryonic period and swimming movements of the larvae after the start of swimming, respectively. Observation of the occurrence of such spontaneous activities in paralyzed animals suggests the existence of an endogenous mechanism that activates the neural circuit underlying swimming activity in the absence of exogenously applied stimuli. Clarifying whether the activating mechanism is intrinsic to the spinal neuronal circuit or to any other region in the higher brain will be important in understanding the central developmental mechanism of swimming activity in teleost fish.

Furthermore, in the early peirod of the larval stage of angelfish, two apparently distinguishable patterns of rhythmic movements were observed in freely moving animals. Tentatively corresponding patterns of cyclic spinal motoneuronal activities were also observed in paralyzed animals. Intermittently occurring low frequency wriggling movement is possibly at least in part used for effective gas exchange on the body surface, since, in this period, the gill is not yet well developed. In contrast, however, the meaning of occasionally occurring high frequency beating is not clear. Since both patterns were frequently observed in single episodes and the transition from one to the other appeared to be quick, there may be a switching mechanism that is activated depending on the internal circumstances of the larvae.

In addition to the changes in burst duration and the number of motoneuron impulses in single bursts, we also found the change in frequency of spontaneous cyclic rhythm is one of the major characteristics of the development of the

neuronal circuitry underling swimming activity in angelfish. As the larvae developed, after day 3 or day 4, an intermediate frequency rhythm (8-10 c/s) emerged and became a major component after the start of swimming. This observation suggests that the appearance of the two distinct patterns of spontaneous cyclic rhythm is specific to the early larval stage. In addition, after the start of swimming, day 6 or later, the observed episodes in paralyzed larvae were found to be more stable with respect to their burst frequency. This observation is consistent with the steady swimming activity seen in freely moving animals after the start of swimming. The development of the pectoral fins may help to stabilize the body in the water. In contrast, the motoneuron burst duration was considerably more variable in the larvae at later developmental periods (day 8 or later). The expansion of the range of burst duration after the start of swimming of larvae suggests that the beating force can be regulated in a wider range. This is probably involved in the development of the flexibility of swimming behavior of the larvae.

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(Received October 6, 1995 / Accepted January 31, 1996)