Physiological Studies on the Heart Photoresponses in Crustaceans

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Hiroshi MIYAMOTO

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ABSTRACT

The heart of animals is regulated through the central nervous system in response to external sensory stimuli. No direct response of the heart to external sensory stimuli has been reported. I found, however, that the adult neurogenic heart of the isopod crustacean *Ligia exotica* has photosensitivity. The beat frequency of the isolated heart decreased in response to a light stimulus. Magnitude of the response was stimulus intensity dependent and the heartbeat frequency decreased to less than 80% of the dark value during illumination of the white light with an intensity of 6.0 mW cm^{-2}. The spectral sensitivity curve of the heart photoresponse peaked at a wavelength around 520 nm. In response to 530 nm monochromatic light, the relationship between light intensity and response magnitude was linear and the threshold intensity was 7.26×10^{12} quanta cm^{-2} s^{-1}. Bursting activity of the cardiac ganglion, which is located in the heart and acts as the cardiac pacemaker deceased in frequency in response to illumination by white light. These observations suggest that the heart photoresponse of *L. exotica* results from the photosensitivity of the cardiac ganglion neurons.

The cardiac pacemaker of *L. exotica* is transferred from the myocardium to the cardiac ganglion of the neurogenic heart during juvenile

development. To elucidate developmental changes in the photosensitivity of the juvenile *Ligia* heart, I examined the effect of a light stimulus on the semi-isolated heart of juveniles at various developmental stages by recording membrane potential of the myocardium. The myogenic heartbeat of early juveniles did not change in response to light, meanwhile the neurogenic heartbeat of mid and late juveniles decreased in frequency in response to light. The proportion of juveniles exhibiting a heart photoresponse increased gradually up to 100% during the period between 3 and 10 days after hatching. The results suggest that the heart photoresponse of *L. exotica* appears in association with transfer of the cardiac pacemaker from the myocardium to the cardiac ganglion during juvenile development.

To explore the diversity of heart photosensitivity in crustaceans, I examined the heart photoresponses in several crustacean species. The neurogenic hearts of the ostracod *Vargula hilgendorfii* and the isopod *Porcellio scaber* respond to light by an increase in beat frequency. But in the other animals tested, the heart photoresponse was not observed.

1 INTRODUCTION

The heart of animals is a pump of haemolymph circulation and changes in the heartbeat affect directly all metabolic activities of organs and tissues. Therefore, the heartbeat is controlled neurally and hormonally by the central nervous system and regulation of the heartbeat in response to changes in animal's internal and external conditions has been reported in various kinds of invertebrates (reviewed by Maynard, 1960; Prosser, 1973; McMahon *et al*., 1997). For changes in environmental light conditions, cardiac responses to visual sensory inputs via neural and neurohormonal pathways of the central nervous system have been reported in crustaceans (Hara, 1952; Miyazaki *et al*., 1985; Li *et al*., 2000) and insects (Campan, 1972; Thon, 1982). Moreover, cardiac regulation by photosensitive neurons in the central nervous system is suggested in the chelicerate *Limulus polyphemus* (Mori & Kuramoto, 2004; Mori *et al*., 2004).

Thus the cardiac responses to external sensory stimuli via the central nervous system have been used as a useful model for sensory reception of the animal (cf. Larimer & Tindel, 1966; Angioy *et al*., 1998). However, no direct response of the heart to external sensory stimuli has been reported. I found here that the adult neurogenic heart of the isopod crustacean *Ligia*

exotica responds directly to light stimulus by a decrease in beat frequency. There are some early reports suggesting the presence of photosensitivity in the myocardium (in the branchiopod crustacean *Daphnia magna*, Schultz, 1928, cited by Maynard, 1960; in the mollusc *Helix pomatia*, Arvanitaki & Chalazonitis, 1947). Recently, in the cultured heart of zebrafish, photosensitivity of the myocardial cells was shown by photoentrainment of rhythmic expression of circadian clock genes (Whitmore *et al*., 2000). To my knowledge, however, there have been no reports that show physiological evidence for the presence of a photosensitive heart.

The presence of the photosensitive heart in *Ligia exotica* implies that external light affects directly all metabolic activities of organs and tissues of the animal by changing the cardiac output aside from any possible regulation through the central nervous system. In order to understand the physiological role of the heart photosensitivity, a detailed analysis of the heart photoresponse is first required. I therefore elucidated detailed characteristics of the heart photoresponse, including determination of the spectral sensitivity and the threshold stimulus intensity of the response. I also aimed to determine the site of photoreception in the heart.

The heart photoresponse of a decrease in heartbeat frequency suggests

the effect of a light stimulation on the cardiac pacemaker. In *L. exotica*, the heart of embryos and early juveniles is myogenic, with the myocardium acting as a dominant pacemaker (Yamagishi, 1996). During juvenile development, the cardiac pacemaker is transferred to the cardiac ganglion, which becomes the primary pacemaker, leaving the myocardium with latent pacemaker properties (Yamagishi & Hirose, 1997). This finding of the transfer of the cardiac pacemaker during ontogeny predicts developmental changes in the cardioregulatory mechanisms in accordance with changes in the target tissue for regulation. Indeed, the mechanisms underlying nervous regulation and neurohormonal modulation of the heart change in association with the cardiac pacemaker transfer from the myocardium to the cardiac ganglion during juvenile development (Sakurai *et al*., 1999; Yamagishi *et al*., 2001, 2004). These facts predict that the photosensitivity of the heart changes in association with the cardiac pacemaker transfer during juvenile development. I therefore examined the heart photoresponses in juveniles at various developmental stages.

In an early report, Schultz (1928) described that local illumination of light to the heart of the intact branchiopod crustacean *Daphnia magna* increases the heartbeat frequency. Since the heartbeat of branchiopods has

been supposed to be myogenic, his observations were interpreted to suggest the photosensitivity of the myocardium (Maynard, 1960), but no direct evidence for the heart photoresponse of *D. magna* has been shown yet. Recently, it was reported that the heart of the branchiopod *Triops longicaudatus* is myogenic with having no neuron in the heart (Yamagishi *et al.*, 1997). On the other hand, the heartbeat of many crustaceans is neurogenic; the myocardium has no inherent automaticity, and the cardiac ganglion acts as a dominant pacemaker, producing periodic bursts of impulses to drive the myocardium (reviewed by Maynard, 1960; Prosser, 1973; McMahon, 1997). Moreover, recent investigations showed that the heartbeat pacemaker mechanism in crustaceans is more diverse than previously thought (Yamagishi, 1999). To explore diversity of the heart photosensitivity among crustaceans, I examined photoresponses of the heart in several crustacean species, including the myogenic heart of the branchiopod *T. longicaudatus*.

2 MATERIALS AND METHODS

2.1 Animals and preparations

2.1.1 *Ligia exotica*

Adult males and females of the littoral isopod crustacean *L. exotica*, 25–35 mm in body length, were collected at Pacific seashores Kamogawa and Shimoda, Japan. They were kept in a plastic tank containing a small amount of artificial seawater (Matsuda) in the laboratory, at room temperature (22–26˚C). Females that held eggs were kept individually in small plastic containers to obtain specimens of known developmental stages. Developmental stages of juveniles were determined and expressed as the number of days after hatching (Yamagishi & Hirose, 1997).

The heart of *L. exotica* is located dorsally, extending over the posterior half of the body (Fig. 1A). The heart is tubular and the wall consists of a single layer of striated muscle cells. The cardiac ganglion composed of six neurons runs longitudinally along the midline of the ventral surface of the dorsal heart wall and gives off nerve branches to the myocardial cells (Fig. 1B, C; Alexandrowicz, 1952; Yamagishi & Ebara, 1985).

After the ventral carapace was cut off, all the viscera and central nervous system were removed with the head. The heart was kept intact in

the pericardial cavity and isolated together with the dorsal carapace. I mainly used heart preparations of this semi-isolated type. I also used heart preparations that were completely isolated from the body. The preparation was pinned, ventral side up, in the experimental chamber, which was continuously perfused with aerated physiological saline solution of the following composition (mM): NaCl 557, KCl 14, $CaCl_2$ 25, $MgCl_2$ 21, Na_2SO_4 4.5 and Tris 5 (Yamagishi & Ebara, 1985). The pH was adjusted to 7.4 using HCl. In some experiments, tetrodotoxin (TTX; Wako) was added to the saline.

2.1.2 *Triops longicaudatus*

Adult specimens of the freshwater branchiopod crustacean *T. longicaudatus*, 20–30 mm in body length, were collected from rice fields in Saitama, Japan. They were kept in a freshwater aquarium in the laboratory, at room temperature.

The heart is tubular, situated at the dorsal side of the thorax, and lacks the cardiac ganglion (Yamagishi *et al*., 1997). The heart was kept intact in the pericardial cavity and isolated together with the dorsal carapace. The preparation was pinned, ventral side up, in the experimental chamber,

which was continuously perfused with aerated physiological saline solution of the following composition (mM): NaCl 75, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1 and Tris 5 (Yamagishi *et al*., 1997). The pH was adjusted to 7.4 using HCl.

2.1.3 *Vargula hilgendorfii*

Adult specimens of the marine ostracod crustacean *V. hilgendorfii*, 2.8–3.5 mm in body length, were collected at Pacific seashore Tateyama, Japan. They were kept in an artificial seawater aquarium in the laboratory, at room temperature.

The heart is globular, composed of a single layer of myocardial cells, and located dorsally at the central position of the body. A single cardiac neuron located outer surface of the heart wall gives off branches to the myocardial cells (Ando *et al*., 2001). One of the valves of the specimen was fixed to a small sheet of Parafilm with an adhesive. Then, the specimen was fixed to the bottom of the chamber by pins through the Parafilm sheet. The heart was exposed by breaking the upper side valve and isolated by removing the tissues around the heart, taking care to keep the suspensory ligaments between the heart and the lower side valve. The chamber was continuously perfused with aerated modified Pntin’s saline solution of the

following composition (mM) (Matsui, 1955); NaCl 530, KCl 10.7, $CaCl_2$ 18.0, $MgCl_2$ 24.6, $NaHCO_3$ 2.3. The pH was adjusted to 7.4 using HCl.

2.1.4 *Porcellio scaber*

Adult specimens of the terrestrial isopod crustacean *P. scaber*, 6–12 mm in body length, were collected from forests in Tsukuba, Japan. They were kept in a plastic container containing soil and dead leaves in the laboratory, at room temperature.

The heart is located dorsally, extending over the posterior half of the body. The heart is tubular and the wall consists of a single layer of striated muscle cells (Holley & Delareu, 1972). After the ventral carapace was cut off, all the viscera and central nervous system were removed with the head. The heart was kept intact in the pericardial cavity and isolated together with the dorsal carapace. The preparation was pinned, ventral side up, in the experimental chamber, which was continuously perfused with aerated physiological saline solution of the following composition (mM): NaCl 306.6, KCl 6, $CaCl_2$ 13.5 and Tris 5 (Holley & Delareu, 1972). The pH was adjusted to 7.4 using HCl.

2.1.5 *Ligidium japonicum*

Adult specimens of the terrestrial isopod crustacean *L. japonicum*, 4–10 mm in body length, were collected from forests in Hamamatsu, Japan. They were kept in a plastic container containing soil and dead leaves in the laboratory, at room temperature.

The heart is tubular and is located dorsally extending over the posterior half of the body. After the ventral carapace was cut off, all the viscera and central nervous system were removed with the head. The heart was kept intact and isolated together with the dorsal carapace. The preparation was pinned, ventral side up, in the experimental chamber, which was continuously perfused with aerated physiological saline solution of *P. scaber* (see §2.1.4). On this saline solution, the heart preparation continued a stable beat.

2.1.6 *Orchestia platensis*

Adult specimens of littoral amphipod crustacean *O. platensis*, 8–15 mm in body length, were collected at Pacific seashores Kamogawa, Japan. They were kept in a plastic tank containing a small amount of artificial seawater in the laboratory, at room temperature.

The heart is tubular and situated at the dorsal side of the thorax. After the ventral carapace was cut off, all the viscera and central nervous system were removed with the head. The heart was kept intact and isolated together with the dorsal carapace. The preparation was pinned in the experimental chamber, which was continuously perfused with aerated modified Pntin's saline solution (see §2.1.3). On this saline solution, the heart preparation continued a stable beat.

2.1.7 *Hemigrapsus sangnineus*

Adult specimens of littoral decapod crustacean *H. sangnineus* were collected at Pacific seashore Kamogawa and Nakaminato, Japan. They were kept in an artificial seawater aquarium in the laboratory, at room temperature.

The heart is cubical and situated at the dorsal side of the thorax. After the ventral carapace was cut off, all the viscera and central nervous system were removed. The heart was kept intact and isolated together with the dorsal carapace. The preparation was pinned in the experimental chamber, which was continuously perfused with aerated modified Pntin's saline solution (see §2.1.3). On this saline solution, the heart preparation

continued a stable beat.

2.2 Electrical recordings

Contraction of the heart (mechanogram of the heartbeat) was recorded in the semi-isolated preparation pinned dorsal side up in the experimental chamber. Part of the dorsal carapace was removed over the middle region of the heart. The dorsal suspensory ligament, which was left attached to the heart, was tied using fine thread and was connected to the mechanoelectric transducer (TB-611T, Nihon Kohden). Impulse activity of the cardiac ganglion was extracellulary recorded from the ganglionic nerve branches. The anterior or posterior nerve branch of the cardiac ganglion was cut at the peripheral side and the proximal cut end of the nerve was sucked into the suction electrode. The membrane potential of the myocardial cells was recorded with a conventional glass microelectrode filled with 3 M KCl (electric resistance, 10–30 MΩ). For current injection, a second microelectrode was inserted into the myocardium at a distance from the recording electrode. The signals were amplified with a microelectrode amplifier (MEZ-7200, Nihon Kohden). The electrocardiogram was recorded with the suction electrode sucking the heart wall. The signal was

amplified with a bioelectric amplifier (AB-651J, Nihon Kohden).

In all experiments, the signals were displayed on a cathode ray oscilloscope (SS-7810, Iwatsu), stored on magnetic tape with a data recorder (PC204Ax, Sony), digitalized with an analog-digital converter (PowerLab/4SP, AD Instruments) and analysed with CHART software versions 4.2 and 5.0.2 (AD Instruments).

2.3 Light stimulation

White or monochromatic light from a 500 W xenon arc lamp (UXL-500D-O, Ushio) and white light from a 1 kW halogen lamp (Hilux-Zoom 210, Master) were used for illumination. A collimated beam of light produced with quartz lenses was passed through a heat-absorbing filter (IRA-25S, Toshiba). Monochromatic light was produced by passing the light beam through one of a set of nine narrow-band interference color filters (MIF-S, Optical Coatings Japan; half-band width less than 13 nm). The light-emitting end of the light-guide (diameter 5 mm) was positioned approximately 3 cm above the preparation. The light intensity was altered with quartz neutral density filters (Optical Coatings Japan), with which the monochromatic light was adjusted to contain an equal number of photons.

The intensity of white light was measured with a power meter (TPM-310, Gentec Electro-Optics, Inc; LI-250, LI-COR) and that of monochromatic light was measured with a silicon photodiode calibrated with a photoelectric tube (S1227-1010BR, Hamamatsu Photonics). The maximum intensity of white light was 6.0 mW cm^{-2} and the intensity range of monochromatic light was between 5.87×10^{12} and 7.03×10^{14} quanta cm^{-2} s^{-1} at the surface of the preparation. The timing and duration of light stimulation were determined by an electro-magnetic shutter (LS6 and LS3T2, Uniblitz) controlled by an electronic stimulator (SEN-7103 and SEN-3301, Nihon Kohden).

3 RESULTS

3.1 Photoresponse of the *Ligia* heart

I examined the effect of light on the semi-isolated heart by recording a mechanogram of the heartbeat. In constant darkness, the heart beat regularly at a frequency of 148.0 ± 19.6 beats min^{-1} (mean ± s.d., n = 65). When white light illuminated the heart, the heartbeat frequency decreased immediately and remained low during illumination (Fig. 2). In this case, illumination of 6.0 mW cm^{-2} white light for 20 s caused a decrease in the heartbeat frequency from 169 to 129 beats min^{-1}. When illumination ended, the heartbeat frequency recovered to the control dark value quickly. Sometimes, the heartbeat frequency became a little faster transiently than the control value just after the end of strong light illumination (cf. Fig. 2). There was little change in the amplitude of the heartbeat in response to light stimulus.

I also measured beat frequency changes in response to light stimulus in the heart isolated completely from the body by visual observations or electrical recordings of the myocardial activity. I found no noticeable differences in the light response of the heart (decrease in beat frequency) between semi-isolated and completely isolated heart preparations. In

addition, I examined the effects of indirect light illumination to estimate the magnitude of the heart photoresponse in living animals. When white light was applied through the dorsal carapace, the magnitude of the heart photoresponse decreased to approximately 80% of the response to direct illumination (not shown).

To examine characteristics of the heart photoresponse, I applied white light of various intensities to the heart. The magnitude of the response to white light increased with the light intensity (Fig. 3). Under weak light, the beat frequency decreased monotonically to a steady-state level (Fig. 3A(i),(ii)). As the light intensity was increased, the response became biphasic with an initial trough phase and a subsequent steady phase (Fig. 3A(iii),(iv)). To determine the relationships between light intensity and response magnitude (% change in the heartbeat frequency) in the trough and steady phases, the control heartbeat frequency was defined as the mean frequency during 5 s period just before the onset of light stimulus. The heartbeat frequency of the steady phase was defined as the mean frequency during 5 s period between 12 and 17 s after the onset of light stimulus, because the duration of the trough phase was almost constant (cf. Fig. 3C). The response magnitude in the trough phase increased as the light intensity

was gradually increased to 6.0 mW cm^{-2}, at which point the heartbeat frequency had decreased to approximately 80% of the dark value (Fig. 3B). The response magnitude in the steady phase also increased depending on the light intensity, but it began to saturate to strong light stimuli. I further examined the heart photoresponse to strong white light with various durations. Regardless of the duration of illumination, the trough phase of the response always lasted 7–8 s in duration at the beginning of the following steady phase (Fig. 3C). In contrast, the steady phase of the response prolonged with increasing the duration of illumination and persisted during illumination for more than 1 h (Fig. 3C, bottom trace).

3.2 Spectral sensitivity of the heart photoresponse

To obtain a spectral sensitivity curve of the heart photoresponse, nine monochromatic lights each of which has a wavelength between 430 and 590 nm were tested on the heart. These monochromatic lights were applied at various intensities in the range between 5.87×10^{12} and 7.03×10^{14} quanta cm^{-2} s^{-1}. To all the monochromatic lights of this intensity range, the heart photoresponse was monophasic and no trough phase appeared. For the five monochromatic lights tested (450, 470, 530, 570 and 590 nm), each

response magnitude was plotted against each light intensity (logarithmic unit). The relationships between response magnitude and light intensity of these monochromatic lights were almost linear and parallel with each other (Fig. 4A). Similar results were obtained for the other four monochromatic lights tested (430, 490, 510 and 550 nm; not shown).

Based on the above results, I determined a spectral sensitivity curve of the heart photoresponse. The spectral sensitivity was defined as a reciprocal of the light intensity at which each of the nine monochromatic lights evokes a response with equal magnitude. Then sensitivity at each monochromatic light was normalized against that at 530 nm monochromatic light and plotted as a function of wavelength. The spectral sensitivity curve obtained peaked at a wavelength around 520 nm (510–530 nm; Fig. 4B). The minimum intensity of 530 nm monochromatic light to induce the heart photoresponse, the threshold light intensity, was 7.26×10^{12} quanta cm^{-2} s^{-1}. In addition, selective light adaptation experiments in which 380 or 610 nm monochromatic light was applied for 10 min showed that magnitude of the responses decreased but no spectral response shift occurred (Fig. 4C).

3.3 Photosensitive site in the heart

The heart of adult *L. exotica* is basically neurogenic; the cardiac ganglion acts as the primary pacemaker of the heartbeat with the myocardium having a latent pacemaker property (Fig. 5A). A burst of impulses generated spontaneously in the cardiac ganglion neurons produces an excitatory neuromuscular junctional potential and induces an action potential of the myocardium which is followed by a heartbeat (Fig. 5B; Yamagishi & Ebara, 1985; Yamagishi & Hirose, 1997). To determine a photoreceptive site in the heart, I examined the effect of strong white light on cardiac ganglion activity. The frequency of periodic bursting activity of the cardiac ganglion decreased in response to the light stimulus (Fig. 6A). The photoresponse of the cardiac ganglion was similar to that of the heartbeat with trough and steady phases. Moreover, simultaneous recording of the heartbeat and the cardiac ganglion activity showed that the heartbeat frequency decreased in association with the frequency decrease of the cardiac ganglion activity (Fig. 6B). These results suggest that the heart photoresponse results from the photosensitivity of the cardiac ganglion neurons.

In the heart of adult L. exotica, the myocardium also acts as a

secondary pacemaker (Fig. 5A) and the heartbeat changes reversibly from neurogenic to myogenic upon application of TTX which suppresses the cardiac ganglion activity (Yamagishi & Hirose, 1997). To confirm the neural origin of the heart photoresponse, I examined the effect of white light on the myogenic heartbeat induced by TTX. The heart photoresponse observed in the neurogenic heartbeat abolished in the myogenic heartbeat induced by 10 mM TTX and recovered after washout of TTX (Fig. 7). This result supports the idea that the heart photoresponse results from the photosensitivity of the cardiac ganglion neurons.

3.4 Developmental changes in the heart photosensitivity

After copulation, the female of *L. exotica* molts and holds approximately 80 to 120 fertilized eggs in the brood pouch on the ventral surface of the abdomen. The embryo develops in the egg for approximately 3 weeks before hatching. Several days after hatching, the juveniles are released from the mother's brood pouch. The juvenile stage lasts for approximately 3 weeks, during which the juvenile molts twice to become an immature adult (Fig. 8A). The heart of early juveniles is myogenic with the myocardium acting as a pacemaker. But, during juvenile development,

the cardiac ganglion begins to produce periodic burst discharges and becomes a primary pacemaker (Fig. 8B; Yamagishi & Hirose, 1997). This change of heartbeat from myogenic to neurogenic implies developmental changes in the heart photoresponse which is likely derived from the photosensitivity of the cardiac ganglion.

I therefore examined the effect of illumination of white light on the beat frequency of semi-isolated hearts of juveniles at various developmental stages by recording the membrane potential of the myocardial cells. I also examined the effect of injection of a hyperpolarizing current into the myocardium, because it decreases the beat frequency of the myogenic heart but not of the neurogenic one (Yamagishi & Hirose, 1997). In constant darkness, the heartbeat frequency of the juveniles ranged between 404 and 165 beats min^{-1}, tending to decrease with age (Yamagishi & Hirose, 1997). The heartbeat of *L. exotica* follows a myocardial action potential composed of a plateau potential with spike potentials superimposed on it (Fig. 5B; Yamagishi & Hirose, 1997). In juveniles 1 to 2 days after hatching, injection of a hyperpolarizing current (15 nA) into the myocardium caused an approximately 10% decrease in heartbeat frequency (Fig. 9A, left), but illumination with white light (4.0 mW cm^{-2}) caused no changes (Fig. 9A,

right). In contrast, in juveniles more than 10 days after hatching, injection of a hyperpolarizing current caused no changes in heartbeat frequency (Fig. 9B, left), but illumination caused an approximately 10% decrease (Fig. 9B, right). Regardless of the developmental stage, the heart photoresponse was observed only in juveniles whose beat frequency did not change upon injection of a hyperpolarizing current into the myocardium.

In adult *L. exotica*, the heartbeat can be reversibly changed from neurogenic to myogenic by application of TTX (Yamagishi & Hirose, 1997; see the previous section). To confirm the neural origin of the heart photoresponse in juveniles, I examined the effects of TTX on the heart photoresponse. The heart photoresponse observed in a juvenile 13 days after hatching (Fig. 10A) was eliminated by application of 1 μM TTX (Fig. 10B) and recovered after washout of TTX (Fig. 10C).

To determine the time course of the appearance of the heart photoresponse during juvenile development, I examined the effect of white light on the heartbeat frequency in juveniles of successive developmental stages (n = 20 for each day after hatching). Juveniles earlier than 3 days after hatching exhibited no photoresponses, then the proportion exhibiting a definite photoresponse (a 3% to 19% decrease in beat frequency) increased

gradually from 3 days to 100% at 10 days after hatching (Fig. 11).

3.5 Diversity of the heart photoresponse

I examined the effects of white light on the semi-isolated heart of several crustacean animals by recording the action potentials the membrane potential of the myocardial cells or the electrocardiogram. The heartbeat frequency of the ostracod *V. hilgendorfii* is unstable both in the intact animal and in the semi-isolated preparation (cf. Fig. 12A; Ishii & Yamagishi, 2002). When white light of 4.0 mW cm^{-2} illuminated the heart, the frequency of the myocardial action potential increased (Fig. 12A). The mean of the heartbeat frequency during illumination was 141 ± 1.9% of that before illumination (3 trials from every 5 specimens, Fig. 12B). Moreover, the heartbeat frequency of the isopod *P. scaber* also increased during illumination (Fig. 13). In this case, heartbeat frequency increased to 3.5% of the dark value during illumination of 4.0 mW cm^{-2} white light. On the other hand, in the branchiopod *T. longicaudatus*, the isopod *L. japonicum*, the amphipod *O. platensis*, and the decapod *H. sangnineus*, the hearts exhibited no response to white light (Table 1).

4 DISCUSSION

4.1 Photosensitivity of the heart

The results of the present study show that the isolated adult heart of the isopod crustacean *L. exotica* responds to illumination by white light by decreasing its beat frequency (Fig. 2). Moreover, the magnitude of the heart photoresponse depended on the intensity (Fig. 3) and wavelength (Fig. 4) of the light stimulus. These results suggest that the adult heart of *L. exotica* is photosensitive.

The heart of adult *L. exotica* is basically neurogenic; the cardiac ganglion acts as the primary pacemaker with the myocardium acting as a secondary pacemaker (Fig. 5A; Yamagishi & Hirose, 1997). Periodic bursts of the cardiac ganglion, each of which consists of two or three impulses, induce the action potentials of the myocardium through excitatory junctional potentials (Fig. 5B; Yamagishi & Ebara, 1985; Sakurai *et al*., 1998). The frequency of bursting activity of the cardiac ganglion decreased in response to light (Fig. 6A) and a heartbeat always followed each ganglionic burst discharge (Fig. 6B). Moreover, the heart photoresponse vanished reversibly when the heartbeat was reversibly changed from neurogenic to myogenic by application of TTX (Fig. 7).

These results suggest that the cardiac ganglion is responsible for the heart photoresponse in adult *L. exotica* (Fig. 14).

4.2 Photoresponse characteristics of the cardiac ganglion

The *Ligia* cardiac ganglion is composed of six neurons that lie longitudinally along the midline of the inner surface of the dorsal heart wall (Alexandrowicz, 1952; Yamagishi & Ebara, 1985). All the cardiac ganglion neurons are glutamatergic motoneurons with pacemaker property and discharge synchronously periodic bursts of impulses via electrical connections among the neurons (Fig. 1B, C; Yamagishi & Ebara, 1985; Sakurai *et al*., 1998). Partial illumination by white light to the anterior or posterior half of the heart resulted in a smaller decrease in the heartbeat frequency than that by the illumination to the whole heart (not shown). These results suggest that all the six ganglion neurons are photosensitive and their summed response is reflected in the frequency change of the heartbeat.

The pacemaker bursting activity of the *Ligia* cardiac ganglion decreased in frequency by injection of a hyperpolarizing current into a cardiac ganglion neuron (Yamagishi & Ebara, 1985). A frequency decrease

of the cardiac ganglion activity in response to light (Fig. 6A) predicts generation of a hyperpolarizing photoreceptor potential in the cardiac ganglion neurons. Extraocular photosensitive neurons have been found in the central nervous system of many invertebrates, but most of which produces a depolarizing receptor potential (reviewed by Musio, 1997). Photosensitive neurons producing a hyperpolarizing receptor potential are found in the central nervous system of the molluscs, *Onchidium verruculatum* (Hisano *et al*., 1972; Gotow & Nishi, 2002) and *Aplysia californica* (Brown & Brown, 1973; Andresen & Brown, 1979). These hyperpolarizing receptor potentials, however, are monophasic and no biphasic receptor potentials predicted from the heart response are found yet. It is interesting to examine the membrane potential responses of the cardiac ganglion neurons to light and these investigations are now in progress.

The spectral sensitivity curve of the heart photoresponse peaked at a wavelength around 520 nm. Maximum responses in green light have been reported in extraocular photoreceptors of various invertebrates (Felisberti *et al*., 1997; Nishi & Gotow, 1998). Moreover, the spectral sensitivity curve of the Ligia heart photoresponse well resembles those reported in crayfish extraocular photosensitive neurons (Sandeman *et al*., 1990) and *Ligia* green

photoreceptor cells, one of the three types photoreceptor cells found in the compound eye (Hariyama *et al*., 1993). On the other hand, the spectral sensitivity curve of the heart photoresponse seems to fit well to the vitamin A1-based visual pigment absorption curve (dashed line in Fig. 4B) obtained from the template by Govardovskii *et al*. (2000). In addition, selective light adaptation experiments resulted in no spectral shift in the heart photoresponse (Fig. 4C). These results suggest that the spectral response of the cardiac ganglion is caused by one type of visual pigment. The cardiac ganglion neurons of *L. exotica* may have a visual pigment similar to that of the green photoreceptor neurons in the compound eye.

When the monochromatic light of the most effective wavelength of 530 nm was applied, the threshold light intensity to induce the heart photoresponse was 7.26×10^{12} quanta cm^{-2} s^{-1} (Fig. 4A). This threshold value is comparable with those obtained in extraocular photosensitive neurons of other invertebrates (Larimer, 1967; Andresen & Brown, 1979; Nishi & Gotow, 1998).

4.3 Developmental change in the heart photosensitivity

The results show clearly that the heart photoresponse of *L. exotica*

appears during juvenile development (Figs. 9 and 11). The cardiac pacemaker of *L. exotica* is transferred from the myocardium to the cardiac ganglion during juvenile development (Fig. 8B; Yamagishi & Hirose, 1997). Ordinarily, no detectable differences are found in the spontaneous membrane potential changes of the myocardium between myogenic and neurogenic hearts, but the effects of current injection into the myocardium differ between them: a hyperpolarizing current causes a decrease in beat frequency in the myogenic heart but no change in the neurogenic heart (Yamagishi & Hirose, 1997). The heart photoresponse was recorded only from juveniles whose beat frequency remained unchanged upon injection of hyperpolarizing current into the myocardium (Fig. 9). Moreover, as observed in adult heart (Fig. 7), the heart photoresponse of juveniles was reversibly eliminated by TTX (Fig. 10), which reversibly changes the heartbeat from neurogenic to myogenic by suppressing cardiac ganglion activity (Yamagishi & Hirose, 1997). These results suggest that the heart photoresponse of juveniles originates in the cardiac ganglion, as it does in the adult neurogenic heart.

The heart photoresponse in juveniles appeared gradually from 3 to 10 days after hatching (Fig. 11). Transfer of the cardiac pacemaker in *L.*

exotica does not occur synchronously among juveniles released from the same mother, but occurs individually during the early to mid phase of juvenile development, over approximately 3 weeks (Yamagishi & Hirose, 1997). Dopamine decreases the heartbeat frequency of the myogenic heart but increases that of the neurogenic heart, and the proportion of juveniles exhibiting the reversed response increases gradually during the period of pacemaker transfer (Yamagishi *et al.*, 2004). The time course of the appearance of the heart photoresponse obtained in this study (Fig. 11) is similar to that of the cardiac pacemaker transfer but seems to be somewhat earlier and rapider than it. The period of embryonic development of *L. exotica* is 18 days under conditions of a constant temperature at 25°C but changes depending on the temperature (Mori & Yamagishi 1996, unpublished observations). The discrepancy of the time courses might result from differences in collecting and rearing conditions of animals. These results lead to the conclusion that the heart photoresponse of *L. exotica* results from the photosensitivity of the cardiac ganglion and not of the myocardium (Fig. 14), and appears after the transfer of the cardiac pacemaker from the myocardium to the cardiac ganglion during juvenile development.

4.4 Diversity of the heart photosensitivity

The heartbeat of branchiopods has been supposed to be myogenic (Maynard, 1960). In the branchiopod *D. magna,* Schultz (1928) reported that local illumination of light to the heart of the intact body increases the heartbeat frequency. His observations were interpreted to suggest the photosensitivity of the myocardium (Maynard, 1960). I demonstrated, however, that photosensitivity of the *Ligia* heart does not reside in the myocardium. Moreover, I showed that the heartbeat of the branchiopod *T. longicaudatus*, which has been demonstrated to be myogenic (Yamagishi *et al*., 1997), exhibits no responses to light (see §3.4). It seems unlikely that the crustacean myocardium has photosensitivity.

The heartbeat of crustaceans except branchiopods are neurogenic with the cardiac ganglion acting as cardiac pacemaker (reviewed by Maynard, 1960; Prosser, 1973; McMahon *et al*., 1997). The beat frequency of the neurogenic hearts of the ostracod *V. hilgendorfii* and the isopod *P. scaber* increased during illumination of white light (Figs. 12 and 13) while that of *L. exotica* decreases in response to light (Fig. 2). These results suggest that there are two types of heart photoresponses in beat frequency change; increasing and decreasing types. On the other hand, the other four

crustaceans tested exhibited no change in heartbeat frequency in response to light stimulation (see Table 1). These results suggest the presence of some diversity of heart photosensitivity in crustaceans. Moreover, direction of beat frequency change in a heart photoresponse was different even among close species of the isopod. The type of photoresponse appears to vary in a species specific manner and investigations on the heart photoresponse of more diverse species are required.

4.5 Physiological role of the heart photosensitivity

Most of the extraocular photosensitive neurons found in the invertebrate central nervous systems are inter-neurons making synaptic connections with many other neurons; so, their functions are still uncertain. In contrast, the photosensitive cardiac ganglion neurons of *L. exotica* are motoneurons of the myocardium with a pacemaker function. Therefore, changes in the pacemaker activity of the cardiac ganglion neurons by light stimulus affect directly the cardiac outflow in the hemolymph circulation.

L. exotica is a largely diurnally active seashore animal, whose terrestrial habitat is well lit by sunlight (Hariyama *et al*., 1986). In addition, white light whose intensity is much weaker than sunlight (approx. 100 mW

cm^{-2}) could induce the heart photoresponse, even when it was applied passing through the dorsal carapace (see §3.1). These facts suggest that, in living animals, sunlight passing through the carapace directly affects metabolic activities of all tissues and organs by changing the cardiac output, aside from any possible regulation through the central nervous system. But the physiological role of the heart photosensitivity is uncertain yet. Investigations on its function in the diurnal activity and circadian rhythm of living animals are required.

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7 TABLE

(1 table)

Table 1. Heart photoresponses in seven crustaceans. Photosensitivity of the heart was not found in all the species tested. There are two types of heart photoresponses; decreasing of beat frequency and increasing of beat frequency.

Specific name	Classification	Heartbeat	Photoresponse in heartbeat frequency
Triops longicaudatus	Branchiopod	Myogenic	No response
Vargula hilgendorfii	Ostracod	Neurogenic	Increase
Ligia exotica	Isopod	Neurogenic	Decrease
Porcellio scaber	Isopod	Neurogenic	Increase
Ligidium japonicum	Isopod	Neurogenic	No response
Orchestia platensis	Amphiopod	Neurogenic	No response
Hemigrapsus sangnineus	Decapod	Neurogenic	No response

8 FIGRUES AND LEGENDS

(14 figures)

Fig. 1. Schematic drawings of the heart of *L. exotica*. (A) Dorsal view of the heart with removing the dorsal carapace. (B) Ventral view of the heart with removing the ventral heart wall. (C) Circuitry in the cardiac ganglion. Circles, cardiac ganglion neurons; waved lines, pacemaker properties; zigzagged lines, electrical connections; triangles, synapses.

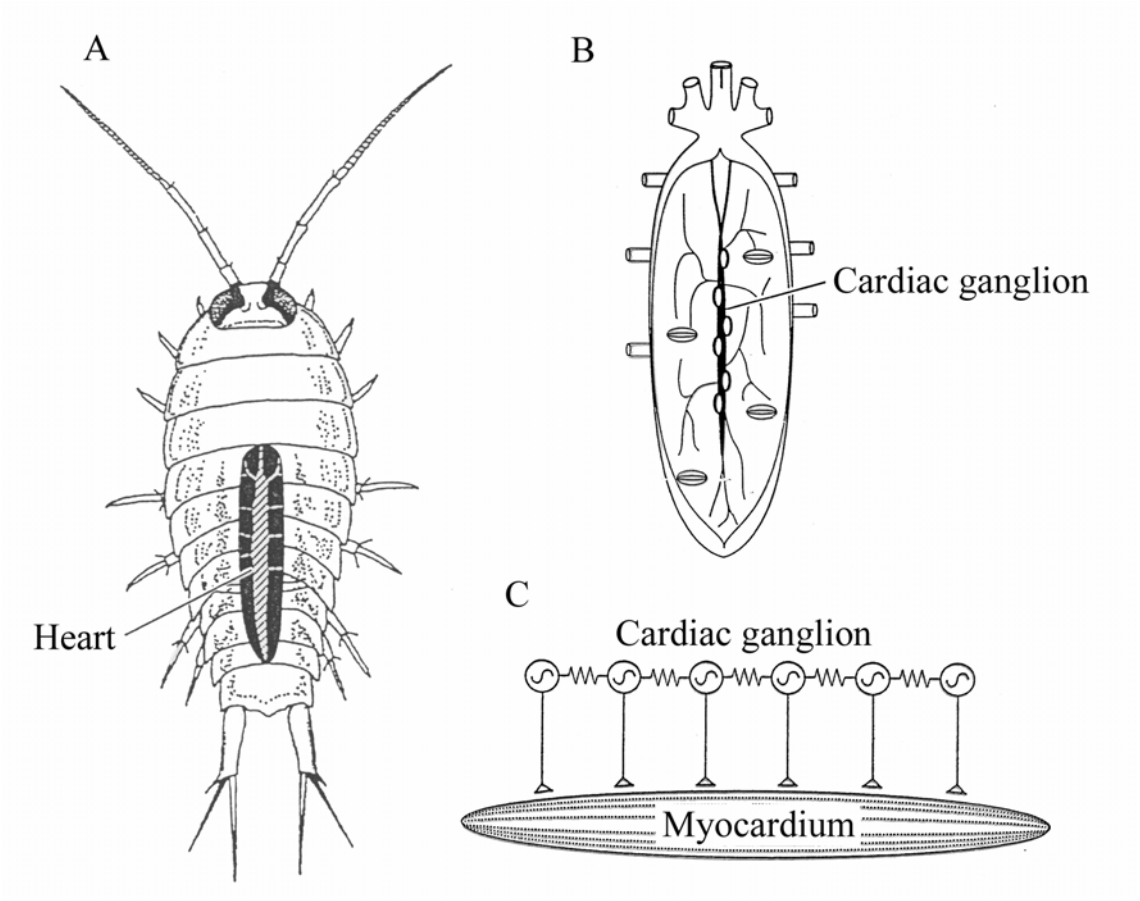

A
Heart
B
Cardiac ganglion
C
Cardiac ganglion
Myocardium

Fig. 2. An example of a photoresponse of a semi-isolated heart. Mechanogram of heartbeat (upper trace) and heartbeat frequency (beats min^{-1}, lower trace) are shown. Black and white parts of the horizontal bar indicate the periods of constant darkness and illumination, respectively. White light of 6.0 mW cm^{-2} was applied during for 20 s. Note the different time-scale in the left portion separated by the vertical dashed line.

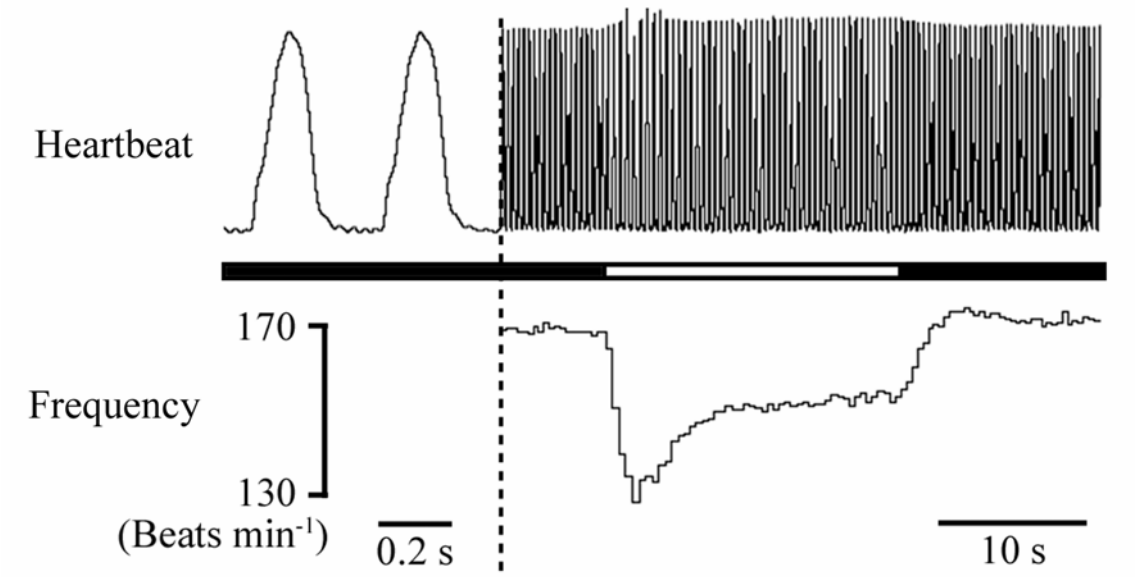

Heartbeat
170
Frequency
130
(Beats min-1)
0.2 s
10 s

Fig. 3. Characteristics of the heart photoresponse. (A) Photoresponses to white light of four different intensities are superimposed. Light intensities in trace i, ii, iii and iv are 9.5×10^{-3}, 9.5×10^{-2}, 9.5×10^{-1} and 3.8 mW cm^{-2}, respectively. Black and white parts of the horizontal bar indicate the periods of constant darkness and illumination, respectively. White light was applied for 20 s. (B) Relationships between light intensity and magnitude of photoresponse in the steady phase (closed circle) and in the trough phase (open circle). White light was applied at eighteen different intensities and the maximum intensity (log 0) was 6.0 mW cm^{-2}. Each data point shows mean ± s.e. (n = 4). (C) Photoresponses to white light of three different durations. Light intensity was 6.0 mW cm^{-2}. Black and white parts of the horizontal bar indicate the periods of constant darkness and illumination, respectively. Durations of illumination were 30 s (upper trace), 5 min (middle trace) and 63 min (bottom trace).

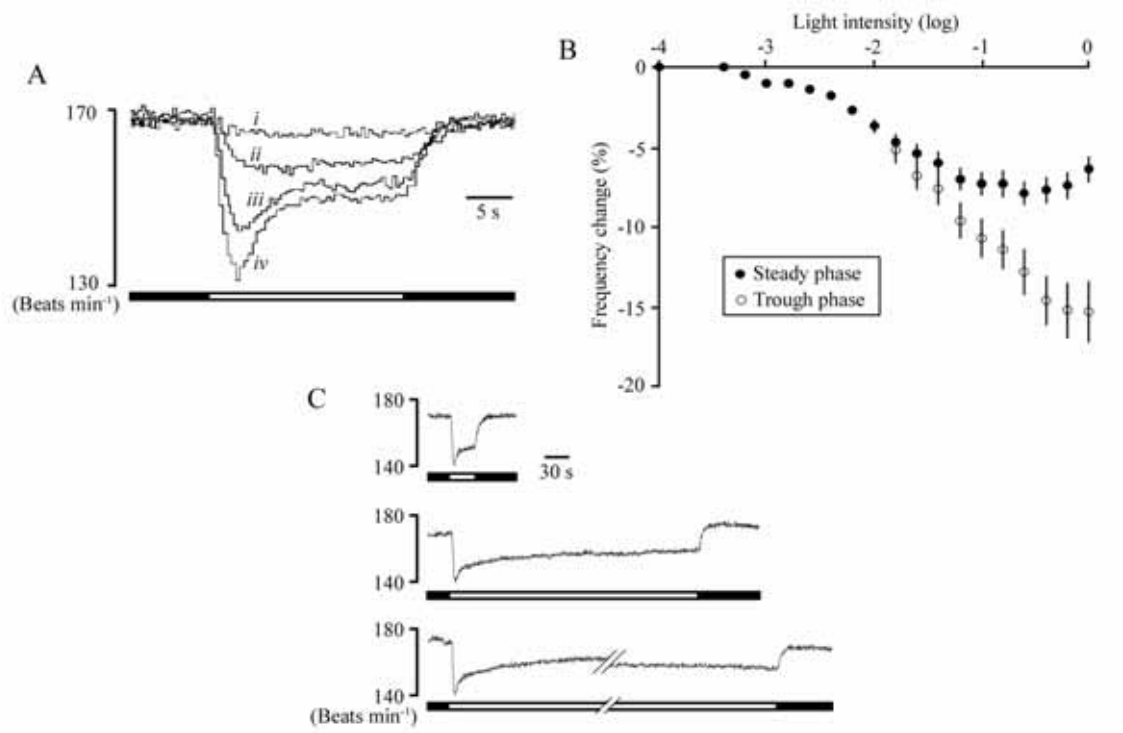

A
170
130
(Beats min⁻¹)
i
ii
iii
iv
5 s
B
Light intensity (log)
-4
-3
-2
-1
0
0
-5
-10
-15
-20
Frequency change (%)
Steady phase
Trough phase
C
180
140
30 s
180
140
180
140
(Beats min⁻¹)

Fig. 4. Heart photoresponse to various monochromatic lights. (A) Relationship between intensity of monochromatic light and magnitude of photoresponse. Five monochromatic lights of various intensities were successively applied. The maximum intensity (log 0) was 7.03×10^{14} quanta cm^{-2} s^{-1}. Each data point shows the mean of six specimens. Closed circle, 450 nm; open circle, 470 nm; closed square, 530 nm; open square, 570 nm; closed triangle, 590 nm. (B) Spectral sensitivity curve of the heart photoresponse. Each data point shows the mean of six specimens. The dashed line shows the vitamin A1-based visual pigment absorption curve obtained from the template by Govardovskii *et al*. (2000). (C) Spectral sensitivity of the heart photoresponse (black) and that in 380 nm (blue) and 600 nm (red) monochromatic light adaptation. (i) Each data point was normalized against the value of 530 nm in control. Black and red dashed lines are handwritten spectral sensitivity curves in control and in 600 nm monochromatic light adaptation, respectively. (ii) Each data point was normalized against the maximum value of each experiment (530 nm in control and in 380 nm monochromatic light adaptation; 510 nm in 600 nm monochromatic light adaptation). Dashed line is handwritten spectral sensitivity curve in control.

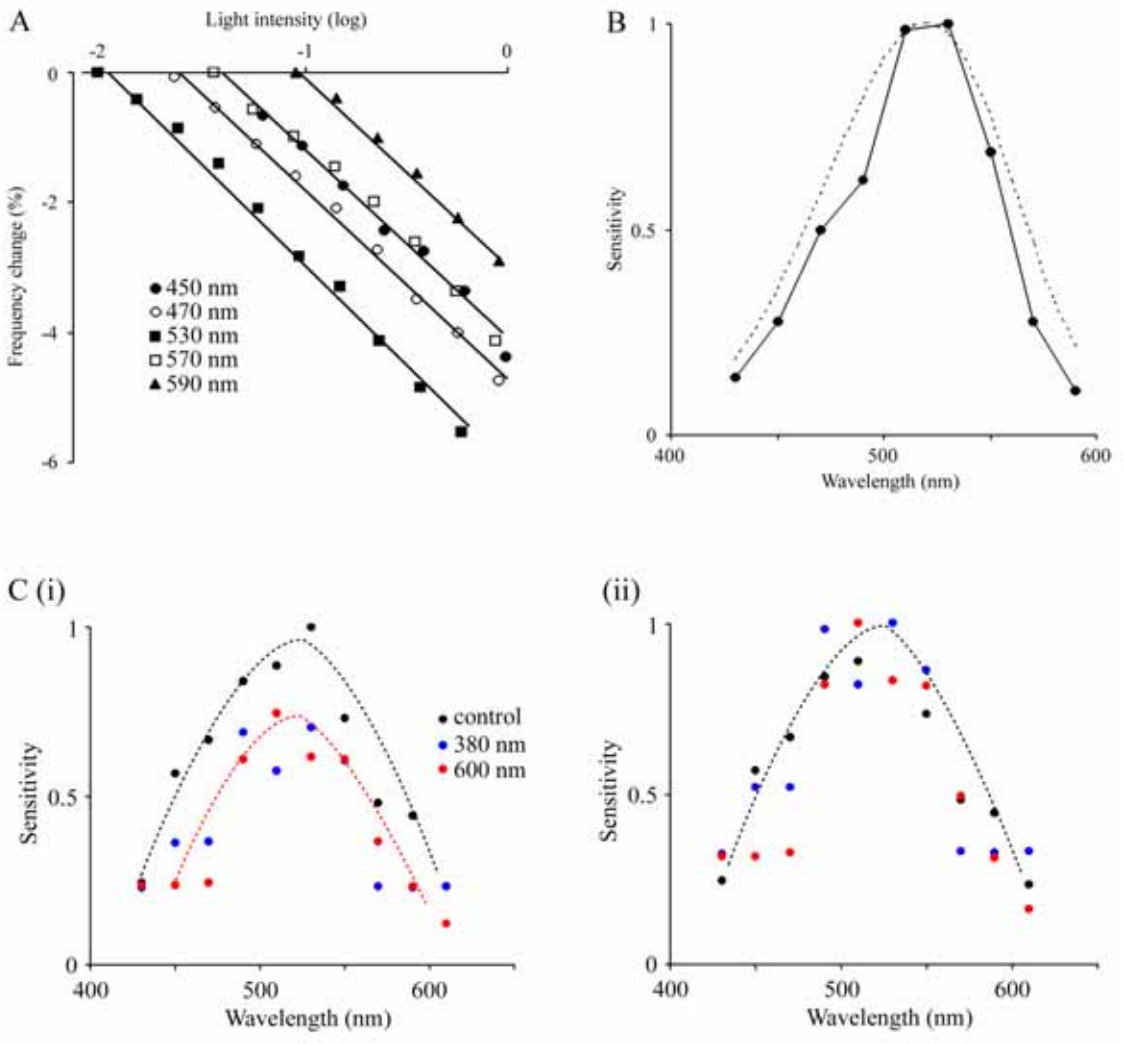

A
Light intensity (log)
-2
-1
0
0
-2
-4
-6
Frequency change (%)
450 nm
470 nm
530 nm
570 nm
590 nm
B
1
0.5
0
Sensitivity
400
500
600
Wavelength (nm)
C (i)
1
0.5
0
Sensitivity
control
380 nm
600 nm
400
500
600
Wavelength (nm)
(ii)
1
0.5
0
Sensitivity
400
500
600
Wavelength (nm)

Fig. 5. Schematic drawings of the cardiac pacemaker mechanism of adult *L. exotica*. (A) The heart of adult *L. exotica* is basically neurogenic. A waved line and a dotted waved line indicate the primary pacemaker and the latent pacemaker, respectively. (B) From the top, membrane potential change of the cardiac ganglion neurons, bursts of impulses on nerve branches from the cardiac ganglion to the myocardium, membrane potential change of the myocardium (dashed line indicates neuromuscular junctional potentials), and mechanogram of the heartbeat are shown.

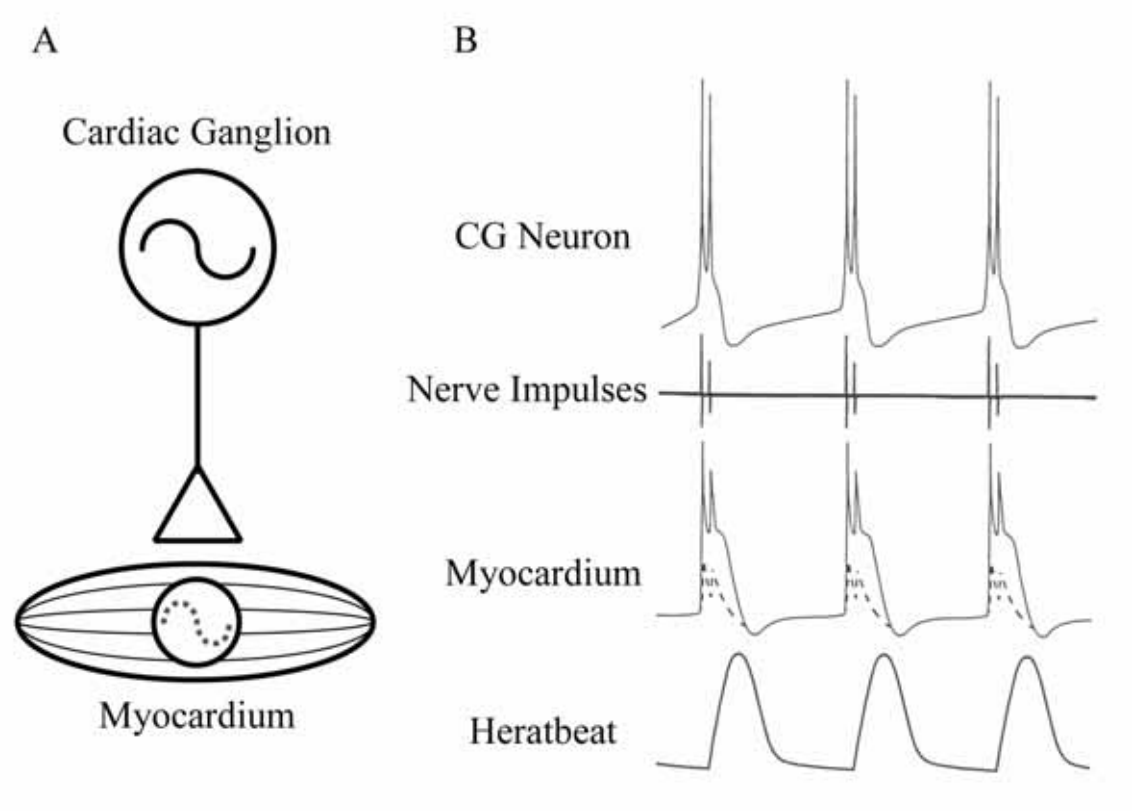

A
Cardiac Ganglion
Myocardium
B
CG Neuron
Nerve Impulses
Myocardium
Heratbeat

Fig. 6. Photoresponses of the cardiac ganglion. (A) Spontaneous burst discharge of the cardiac ganglion (upper trace) and frequency of the burst discharge (lower trace) are shown. Black and white parts of the horizontal bar indicate the periods of constant darkness and illumination, respectively. White light of 6.0 mW cm^{-2} was applied for 20 s. Note the different time-scale in the left portion separated by the vertical dashed line. (B) Burst discharge of the cardiac ganglion (upper trace) and mechanogram of the heartbeat (lower trace) recorded simultaneously in (i) constant darkness and (ii) during illumination with white light at intensity of 6.0 mW cm^{-2}. The timing of the fifth burst discharge was delayed during illumination (dashed lines and an arrow).

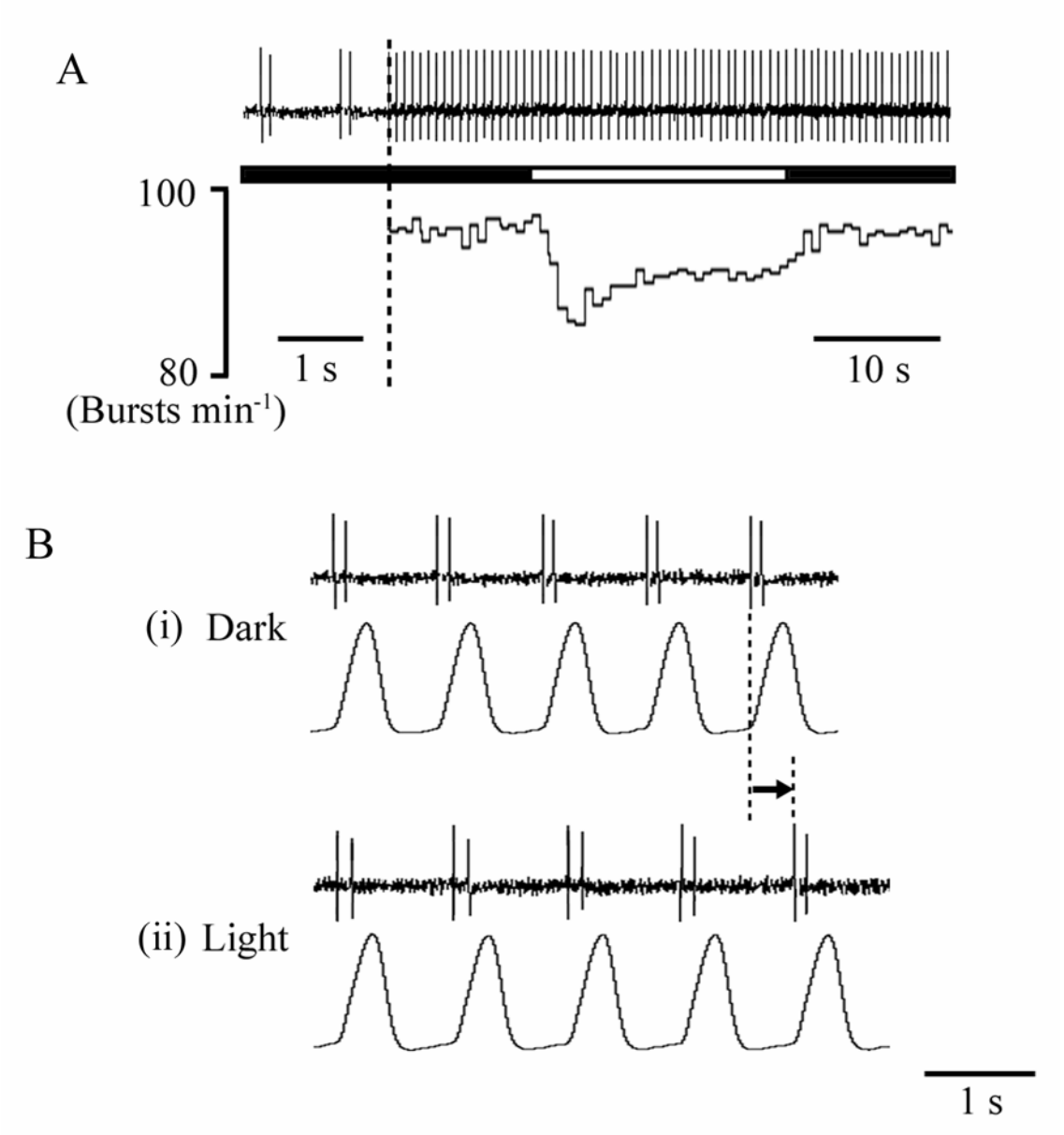

A
100
80
(Bursts min-1)
1 s
10 s
B
(i) Dark
(ii) Light
1 s

Fig. 7. Effects of tetrodotoxin (TTX) on heart photoresponse. To change reversibly the heartbeat from neurogenic to myogenic, 10 μM TTX was applied. Photoresponses (A) before application, (B) during application and (C) after washout of TTX are shown. Black and white parts of the horizontal bar indicate the periods of constant darkness and illumination, respectively. White light of 6.0 mW cm^{-2} was applied for 20 s.

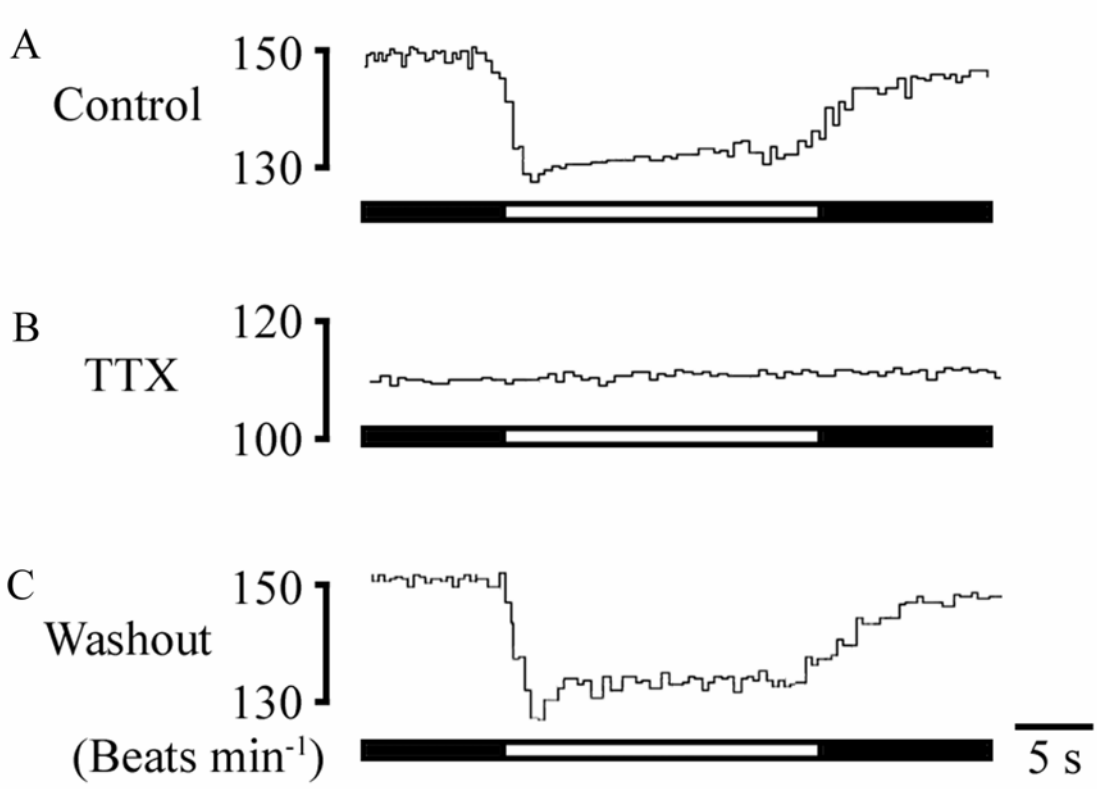
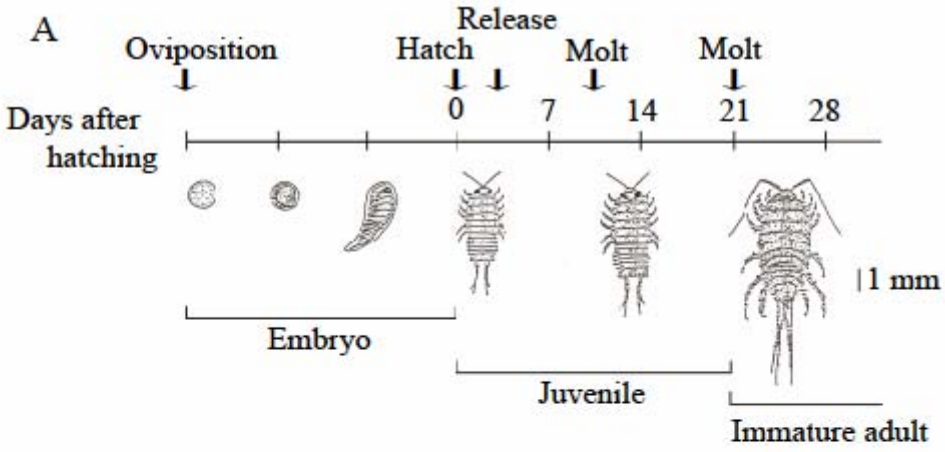

A
Control
150
130
B
TTX
120
100
C
Washout
150
130
(Beats min-1)
5 s

Fig. 8. Cardiac pacemaker transfer during development in *L. exotica*. (A) Schematic drawing of the time course of the embryonic and juvenile development of *L. exotica*. (B) During juvenile development, heartbeat changes from myogenic to neurogenic in association with the pacemaker transfer from the myocardium to the cardiac ganglion.


A
Release
Oviposition
Hatch
Molt
Molt
Days after hatching
0
7
14
21
28
1 mm
Embryo
Juvenile
Immature adult

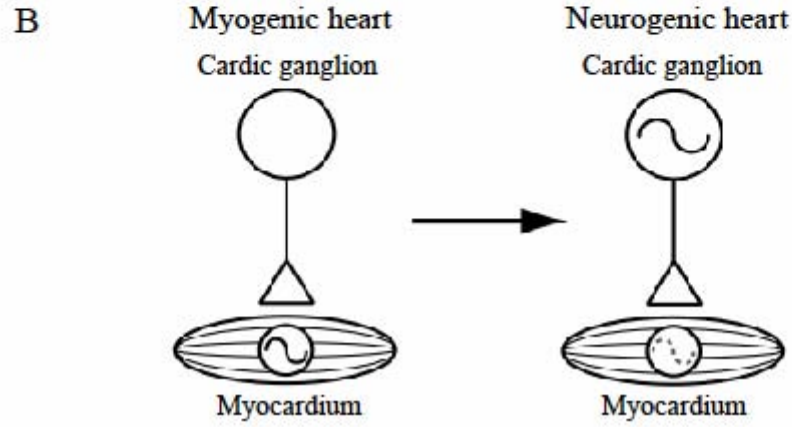

B
Myogenic heart
Cardic ganglion
Myocardium
Neurogenic heart
Cardic ganglion
Myocardium

Fig. 9. Effects of current injection into the myocardium and illumination of white light on the heart of a juvenile 2 (A) and 12 days (B) after hatching. Each record shows membrane potential of the myocardial cell (upper trace), instantaneous frequency (min^{-1}) of the myocardial action potential (middle trace), and stimulus (bottom trace). Left: a hyperpolarizing current of 15 nA was injected into the myocardium for 8 s. Note the different time scale in the left portion of each upper trace. Right: white light of 4.0 mW cm^{-2} was applied for 20 s. Black and white indicate periods of darkness and illumination, respectively.

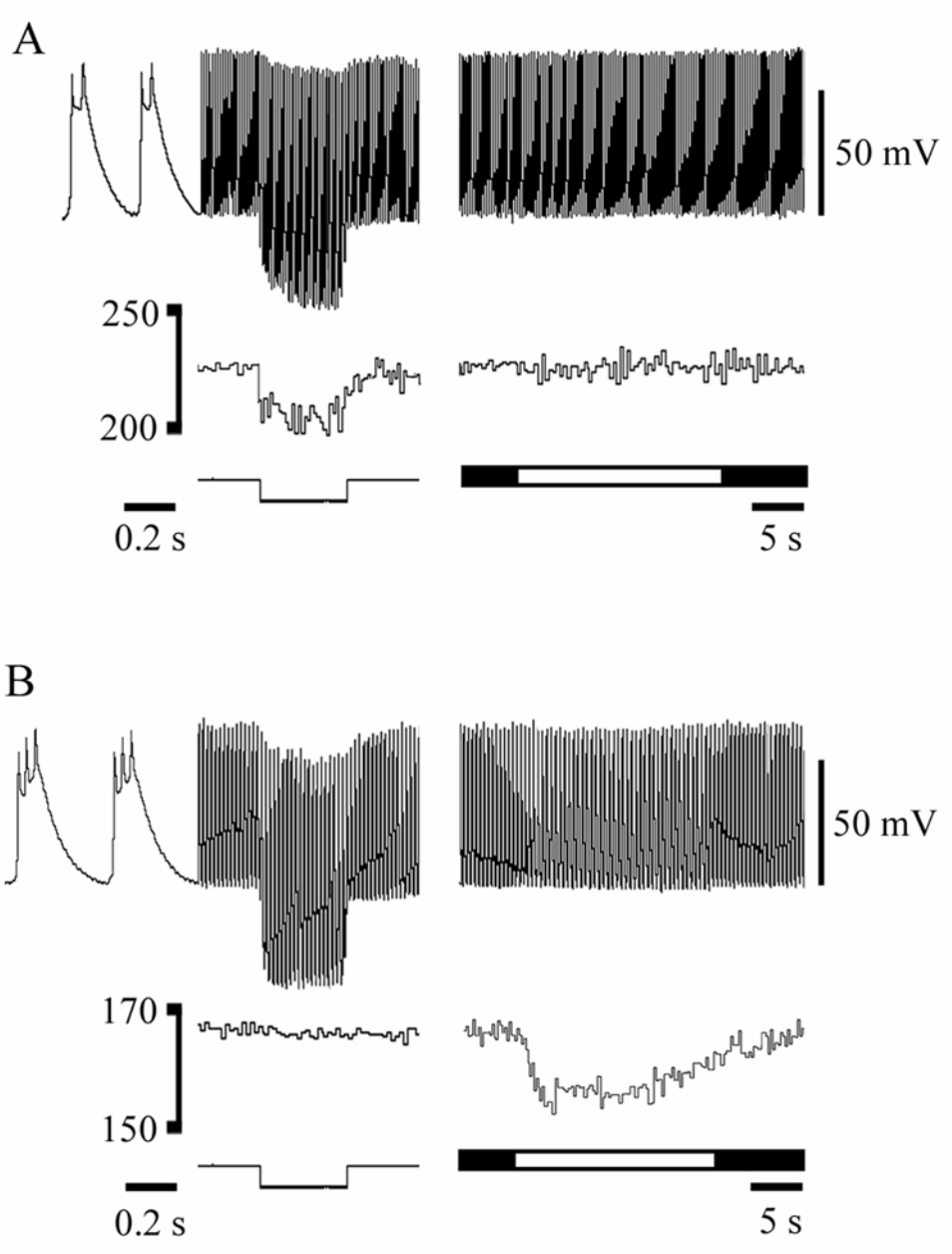

A
50 mV
250
200
0.2 s
5 s
B
50 mV
170
150
0.2 s
5 s

Fig. 10. Effects of TTX on the heart photoresponse of juveniles before (A), during (B), and after (C) application of 1 μM TTX. Each record shows membrane potential of the myocardial cell (upper trace), instantaneous frequency (min^{-1}) of the myocardial action potential (middle trace), and stimulus (bottom trace). Note the different time scale in the left portion of each upper trace. White light of 4.0 mW cm^{-2} was shone for 20 s. Black and white indicate darkness and illumination, respectively. From a juvenile 13 days after hatching.

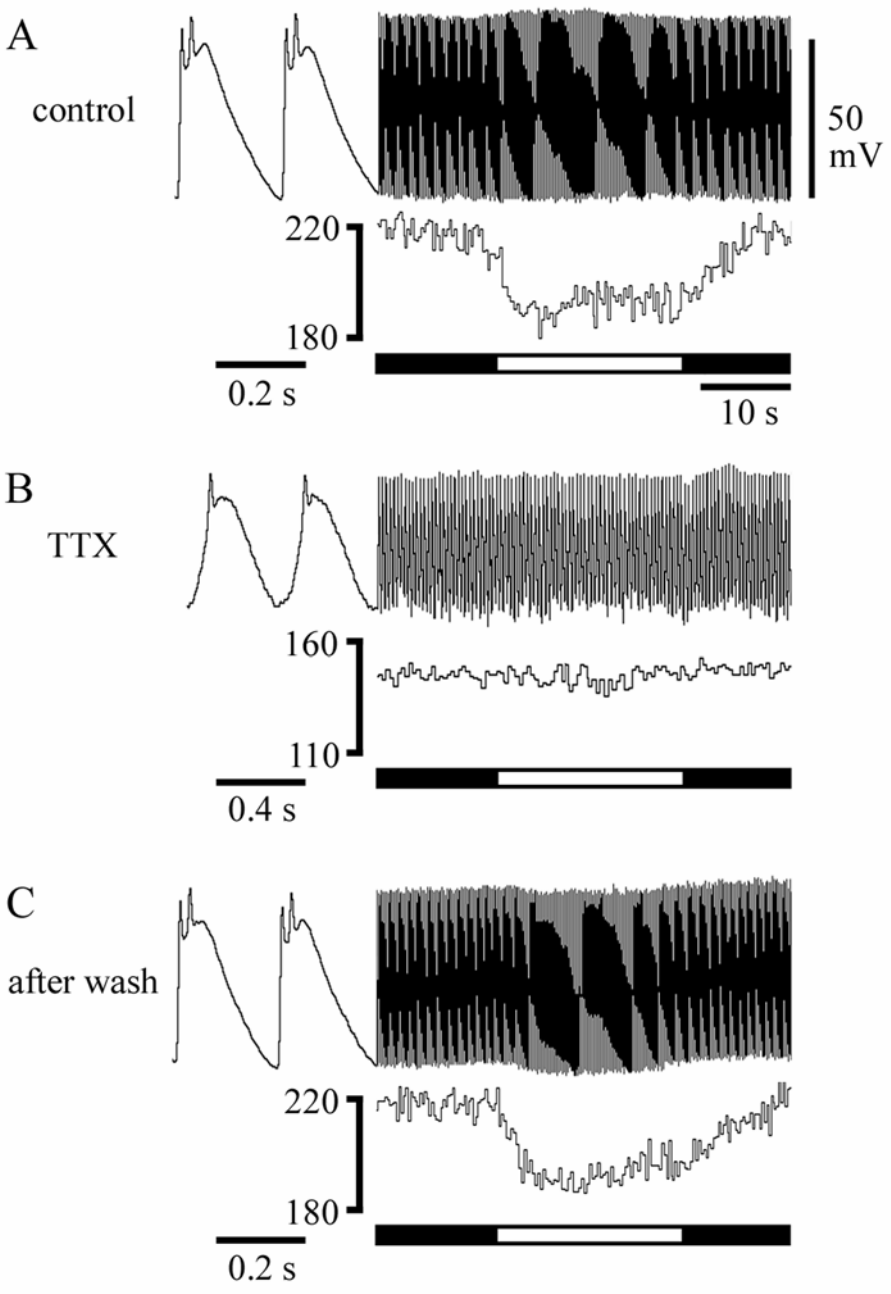

A
control
50
mV
220
180
0.2 s
10 s
B
TTX
160
110
0.4 s
C
after wash
220
180
0.2 s

Fig. 11. Time course of the appearance of heart photoresponse during juvenile development. The relationship between the proportion of juveniles exhibiting a heart photoresponse and the developmental stage of the juveniles is shown. Heart photoresponses to white light of 4.0 mW cm^{-2} were obtained from 20 different juveniles at each developmental stage.

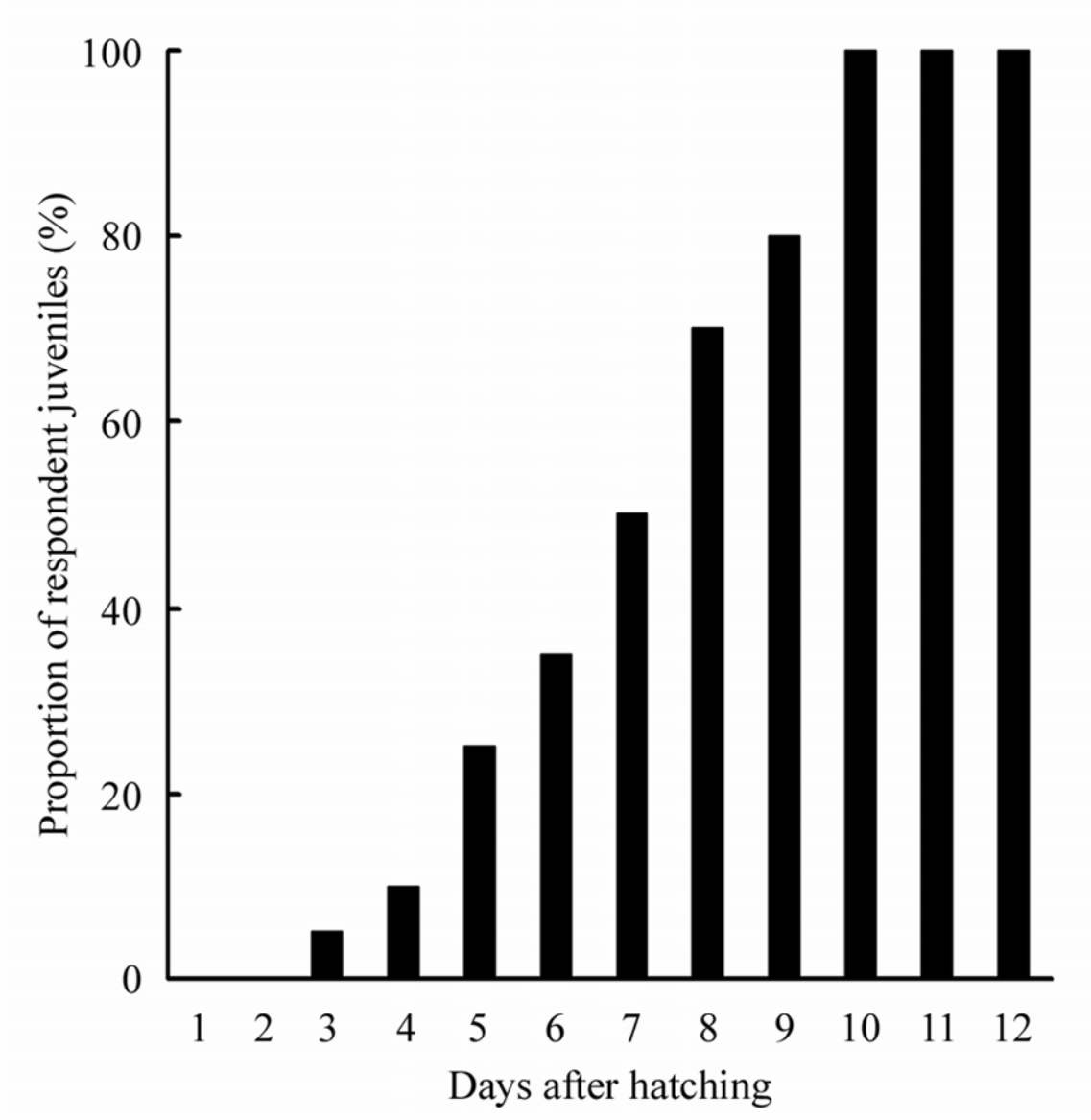

Proportion of respondent juveniles (%)
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
12
Days after hatching

Fig. 12. Heart photoresponse of *V. hilgendorfii*. (A) An example of a photoresponse of a semi-isolated heart. Membrane potential changes of the myocardium is shown. Black and white parts of the horizontal bar indicate the periods of constant darkness and illumination, respectively. White light of 4.0 mW cm^{-2} was applied for 20 s. (B) Each data shows the mean ± s.e. (15 trials from 5 species) of heartbeat frequency for 20 s before, during and after application of white light normalized against the value of before application (relative heart rate). *, $p<0.01$.

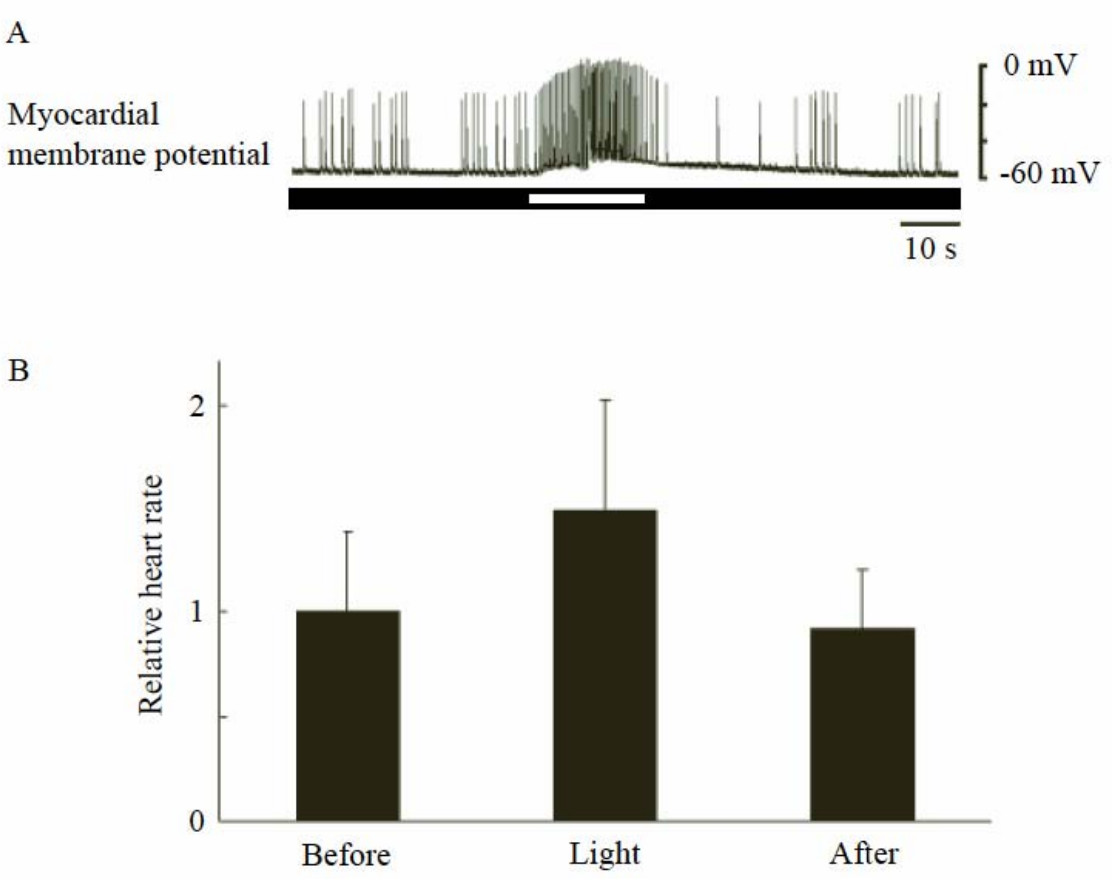

A
Myocardial
membrane potential
0 mV
-60 mV
10 s
B
Relative heart rate
2
1
0
Before
Light
After

Fig. 13. An example of a photoresponse of a semi-isolated heart of *P. scaber*. Electrocardiogram (ECG, upper trace) and heartbeat frequency obtained from ECG (lower trace) are shown. Black and white parts of the horizontal bar indicate the periods of constant darkness and illumination, respectively. White light of 4.0 mW cm^{-2} was applied for 20 s. Note the different time-scale in the left portion separated by the vertical dashed line.

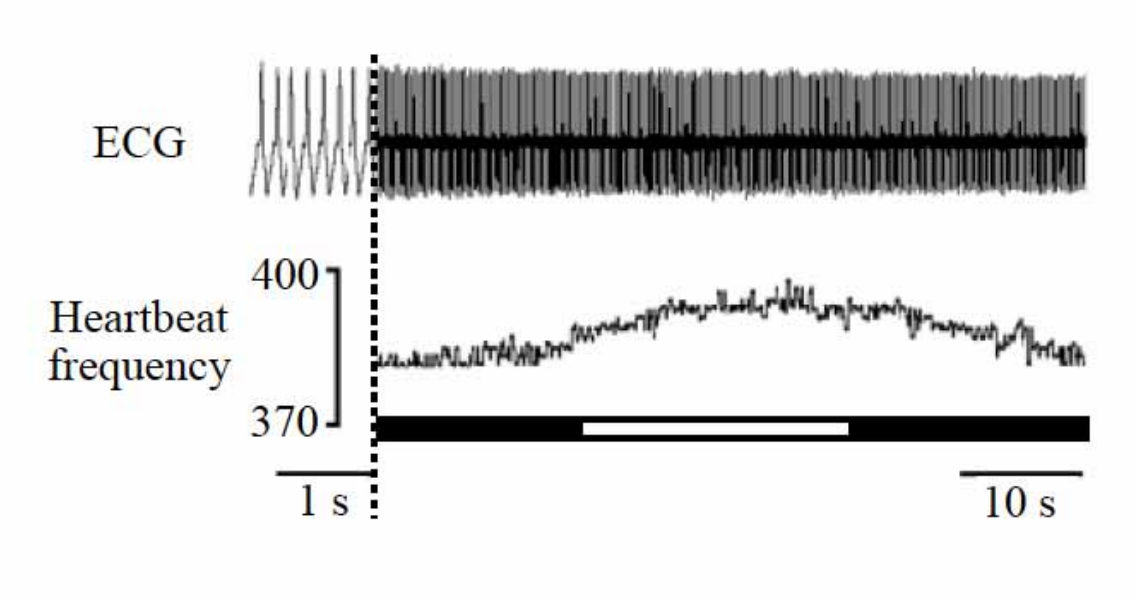
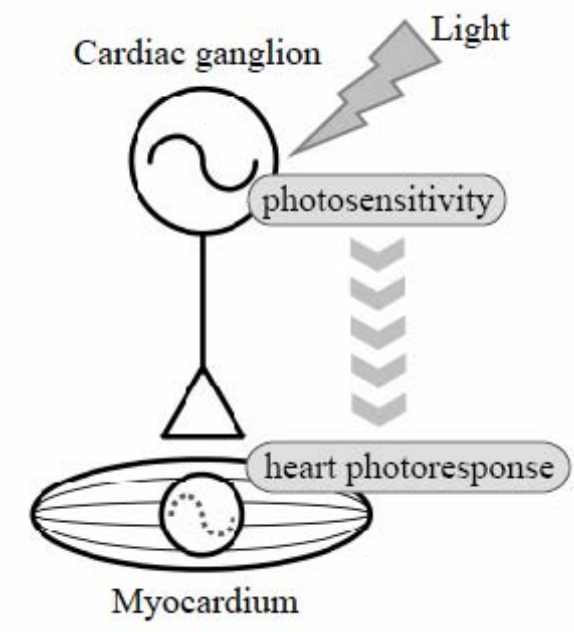

ECG
400
Heartbeat
frequency
370
1 s
10 s

Fig. 14. Schematic drawing of photosensitive heart of *L. exotica*. The heartbeat changes in response to light because the cardiac ganglion neurons are photosensitive.


Cardiac ganglion
Light
photosensitivity
heart photoresponse
Myocardium