



Behavior of adult and juvenile Japanese sea cucumber *Apostichopus japonicus* on an artificial reef during the breeding season

Peter M. Zhadan¹, Marina A. Vaschenko^{2,*}

¹V. I. Il'ichev Pacific Oceanological Institute, Far Eastern Branch, Russian Academy of Sciences, 43 Baltiyskaya Street, 690041 Vladivostok, Russia

²A. V. Zhirmunsky National Scientific Center of Marine Biology, Far Eastern Branch, Russian Academy of Sciences, 17 Palchevsky Street, 690041 Vladivostok, Russia

ABSTRACT: Despite considerable research interest in various aspects of the biology of the Japanese sea cucumber *Apostichopus japonicus*, a species of high economic value, its diurnal and seasonal behavior in the field is not fully understood. Using high-resolution (1 min) round-the-clock video recording in parallel with measurements of environmental variables (sea level, temperature, salinity), we studied the behavior of *A. japonicus* on an artificial reef populated with the bivalves *Crenomytilus grayanus* and *Crassostrea gigas* during the breeding season (June–September). A total of 12 adult *A. japonicus* were placed on the reef and this number did not change during the study period. A significant number (approximately 23 ind. m⁻²) of 1 yr old juvenile *A. japonicus* were found on the reef. The reef was also colonized by other invertebrates such as sea urchins and brittle stars. Adult *A. japonicus* were predominantly nocturnal, spending 5 times more time outside shelters at night than during daytime. Juveniles were exclusively nocturnal. Extremely high temperatures (up to 28.2°C) and strong temperature variation over approximately 20 d reduced the time spent by adult *A. japonicus* outside shelters by approximately 30% but did not affect the activity of juveniles, which increased the time spent outside shelters with growth and with seasonal lengthening of the night. Spawning in *A. japonicus* occurred exclusively at dusk and night and coincided with the spawning of sea urchins, brittle stars and bivalves. Our results may have implications for the development of co-culture with bivalve mollusks and restocking technologies for *A. japonicus*.

KEY WORDS: Diel rhythms · Holothurian · Light · Seawater temperature · Sheltering behavior · Spawning behavior · Spawning timing · Video recording

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1. INTRODUCTION

The Japanese sea cucumber *Apostichopus japonicus* (Selenka, 1867) is one of the best-known species of the stichopodid holothurians (Echinodermata: Stichopodidae), almost all of which are edible. This species is widespread in the coastal waters of the North Pacific, from Alaska (USA) in the north to Tanegashima Island (Japan) in the south, including the coastal waters of Russia, China and Korea (Zhao 2015).

For many centuries, *A. japonicus* has been used in Asian countries both in dried form (bêche-de-mer or trepang) and fresh as a delicacy, as well as in dietary food and medicine, contributing to the economic value of this species (Chen 2003, Purcell et al. 2014). China has been the most successful in *A. japonicus* aquaculture: production of this holothurian reached 15.8 × 10⁴ t in 2021 (Chen et al. 2022). This is possible because the modern technology of *A. japonicus* aquaculture is based on advances in the study of the bio-

*Corresponding author: mvaschenko@mail.ru

logy, particularly the ecology, of this species in addition to farming techniques. In discussing related strategies that could contribute to the sustainable development of the *A. japonicus* industry, Ru et al. (2019) highlighted advances in behavioral studies. Studying the behavior of *A. japonicus* in relation to feeding, reproduction and predation avoidance provides a theoretical basis for optimizing the techniques and facilities used to farm this species, and for protecting its populations in their natural environment (Ru et al. 2019, Zhang & Lai 2024).

A. japonicus prefers hard and sand-mud substrates, is predominantly nocturnal and spends the daylight hours in shelters (Levin 2000, Dong et al. 2011, Gavrilova & Sukhin 2011, Sun et al. 2015, Zhang et al. 2015, Zhang & Lai 2024). These traits make it difficult to study the behavior of *A. japonicus* under natural conditions. To date, the vast majority of information on the behavioral responses of *A. japonicus* to exogenous (environmental variables, presence of food and/or predators) and endogenous (reproductive and neuro-hormonal status) signals has been obtained in laboratory experiments. The responses of *A. japonicus* to changes in light and temperature have long attracted the attention of researchers. The locomotor activity of *A. japonicus*, which is related to feeding behavior and the need to avoid predators, is subject to a diurnal rhythm that differs between juvenile and adult sea cucumbers (Sun et al. 2015, Yamaguchi et al. 2016). Juveniles about 5 cm long (small-size group, 1 yr old) are maximally active at night and prefer to be sheltered during the day, so the distribution of active juveniles is unimodal with a pronounced peak at night (Dong et al. 2010, 2011, Sun et al. 2015, 2018). Larger juveniles (medium- and large-size groups; 8.4–13.4 cm) have a second peak of activity during the day (Sun et al. 2015). However, in some experiments, *A. japonicus* juveniles were only in shelters during the day in the presence of predators and did not show a diurnal rhythm of activity in their absence (Yamaguchi et al. 2016). The behavior of large adults (300–500 g) of *A. japonicus* was also subject to a diurnal rhythm and dependent on temperature: the distribution of active individuals was bimodal at 12 to 17°C and unimodal at 18 to 25°C (Kato & Hirata 1990).

At the same time, there are currently no data available on the temporal and quantitative characteristics of the daily rhythm of *A. japonicus* activity in the natural environment. Studies on the spatial distribution and size–age structure of populations of this species have been carried out exclusively during the day, and authors have noted the difficulty of finding both

adults and, in particular, juveniles (Hamano et al. 1989, Goshima et al. 1994, Tanaka 2000, Yamana et al. 2006, 2010, Uekusa et al. 2012, Minami et al. 2019).

A. japonicus also exhibits seasonal changes in activity, mainly driven by seasonal changes in seawater temperature, which affect the physiological functions of holothurians (Tanaka 1958a, Kashio et al. 2016, Minami et al. 2019). Laboratory studies have reported that the optimal temperature range for feeding and growth of *A. japonicus* is between 15 and 18°C (Yang et al. 2005, Dong & Dong 2006, Chen et al. 2007). An increase in temperature above 20 to 22°C leads to a state of aestivation, in which sea cucumbers leave the seabed to seek shelter, stop feeding and growing and lose weight due to degeneration of the digestive organs and changes in metabolism (Liu et al. 1996, Yang et al. 2005, 2006, Chen et al. 2007, Yuan et al. 2007, Ji et al. 2008, Yamana et al. 2009a).

Field studies on the relationship between water temperature and *A. japonicus* activity are scarce. Yamana et al. (2008) used individual tracking of several adult *A. japonicus* to find their aestivation sites during the summer season at temperatures of around 23 to 30°C in Yoshimi Bay (Honshu Island). From a field experiment, Nakahara et al. (2018) reported that aestivation of adult *A. japonicus* occurred intermittently from late July to mid-November in Funka Bay (Hokkaido Island) at temperatures ranging from 12 to 24°C. The percentage of aestivating individuals varied from 0 to 24.5% and was positively correlated with seawater temperature. Minami et al. (2019) used SCUBA to study the seasonal and interannual variation in the density of visible (not sheltered) *A. japonicus* in Maizuru Bay (Honshu Island) and found that the density became 0 at an average seawater temperature of $24.7 \pm 3.2^\circ\text{C}$. In that study, individuals with a body length of >10 cm were recorded, whereas the smaller juveniles were not found.

Information on the spawning behavior of adult *A. japonicus* is also very scarce. Data from laboratory studies are limited to observations of the behavior of sea cucumbers in aquaria after stimulation of spawning by temperature shock, desiccation and running seawater (Liu et al. 2015) or injection of substances that induce egg maturation, such as neuronal peptides NGIWyamide and NGLWamide (Kato et al. 2009), and cubifrin-I and its derivative cubifrin-L (Fujiwara et al. 2010). Observations of *A. japonicus* spawning in nature are limited by information received from divers that the sea cucumbers spawn with the anterior part of the body extended vertically (Levin 2000). There are no data on the daily dynamics of *A. japonicus* spawning activity under natural conditions.

To investigate in detail the daily and spawning behavior of *A. japonicus*, we constructed an artificial reef matching the natural habitat of this species. The reef was designed as a truncated pyramid of concrete slabs and stones populated by mussels *Crenomytilus grayanus* and oysters *Crassostrea gigas*. The idea for such a design was based on the observations that (1) in nature, colonies of bivalve mollusks *C. gigas*, *C. grayanus* and *Modiolus* sp. are common habitats for *A. japonicus* (Goshima et al. 1994, Selin 2001, Gavrilova & Sukhin 2011) and (2) the spawning behavior of *A. japonicus* and other closely related holothurian species involves climbing elevated sites to reach the highest point (Fujiwara et al. 2010, Balogh et al. 2019). We used round-the-clock time-lapse photography in parallel with automatic data logging of environmental variables during the summer season and video analysis to (1) clarify *A. japonicus* behavior associated with feeding, sheltering and spawning *in situ* and (2) understand the association of *A. japonicus* activity with changes in environmental variables, primarily daylight and seawater temperature.

2. MATERIALS AND METHODS

2.1. Experimental design and observations

The studies were carried out on an artificial reef constructed in 2017 in Alekseev Bay (Peter the Great Bay, Sea of Japan; 42.981° N, 131.730° E) at a depth of approximately 2.0 m (2.3 and 1.7 m at maximum high and low tide, respectively). The base of the reef was a piece of the bow of a concrete floating pier in the shape of a rectangular parallelepiped (70 × 70 × 60 cm) with a hawse hole in the center, found on the bottom of the bay. It was installed so that the axis of the hawse hole was directed parallel to the bottom. On one side of this piece of concrete, from the bottom to the top, stones populated by the mussel *Crenomytilus grayanus* were placed at an angle of about 40°. The stones were taken from natural settlements of this species in Alekseev Bay. On the opposite side, 2 concrete slabs of 60 × 40 × 5 cm were placed on which *C. grayanus* mussels had previously settled. The slabs with mussels were formed as follows. First, the slabs were laid horizontally, and 70 specimens of *C. grayanus* with a shell height of 7 to 8 cm and with the byssus removed were placed on each of them. Then, after approximately 1 mo, when the mussels had attached themselves to the slabs, they were placed on the reef at an angle of about 45°. The artificial reef was thus a truncated pyramid of about 0.8 m in height with a hole in

the middle. Every year in May from 2018 to 2021, the shells of dead mussels on the concrete slabs were replaced with live mussels. If necessary, some stones with mussel druses on the opposite side of the reef were replaced with new ones.

The reef was located 100 m from the shore. The seabed around the reef was covered with fine gravel and coarse sand with occasional (3–15 m apart) large stones with mussel druses. At 20 m from the reef towards the shore, there was a meadow of *Zostera marina* interrupted by settlements of the Pacific oyster *Crassostrea gigas*.

To monitor the behavior of *Apostichopus japonicus* and other invertebrates on the reef, continuous time-lapse video recordings were made using 5 or 6 TLC200 Pro video cameras (Brinno). The cameras were directed at the top of the reef, its lateral surfaces and the area at the base of the reef. Videos were taken at 1 min intervals with a resolution of 1280 × 720 pixels. During the night, the fields of view of the cameras were pulse-illuminated by LED lamps (1 W) with an impulse duration of 1 s. An electronic control module synchronized the lamps and cameras and switched the pulsed illumination on at sunset and off at sunrise when the light level reached 7 units of photosynthetically active radiation (PAR). This PAR level corresponded approximately to the lighting at sunset and sunrise under clear skies.

Video recordings from 2017–2020 served as preliminary data and stimulus for the main experiment in 2021.

On 15 May 2021, 46 d before the start of the main observations, 58 adult oysters *C. gigas* with a shell height of 15 to 22 cm were placed at the base of the reef in an area of 1.1 × 0.8 m near concrete slabs covered with mussels *C. grayanus*. After 3 d, 12 adult sea cucumbers *A. japonicus* were collected from Alekseev Bay and placed on the reef. All sea cucumbers were of the green color variant; according to long-term observations, only this color variant and very rare albino *A. japonicus* inhabit Peter the Great Bay (Levin 2000). The sea cucumbers were transferred to the reef by the diver immediately after capture and were not exposed to air. In addition, juvenile *A. japonicus*, several species of gastropods, small crabs, starfish, brittle stars, sea urchins that colonized the reef themselves, as well as coastal fish species and large planktonic organisms were present on the reef. When analyzing the video recordings, we focused on the behavior of 4 of these animals: juvenile sea cucumbers *A. japonicus*, brittle stars *Ophiura sarsii* and sea urchins *Strongylocentrotus intermedius* and *Mesocentrotus nudus*. Six video cameras were installed around the reef on 19 June

2021 (see Fig. S1 and Video S1 in the Supplement at www.int-res.com/articles/suppl/m752p095_supp/). Two cameras were directed at concrete slabs with mussels, 2 others at the oyster area, 1 camera at the stone side of the reef with natural mussel druses and 1 camera at the top of the reef. Video recording was carried out from 20 June to 6 September, except for short (7–10 h) breaks every 10 to 12 d to collect information from video cameras and to clean the camera boxes and illuminators from fouling. The video-recording period completely overlapped with the spawning season (late July–August) established for *A. japonicus* in Peter the Great Bay (Levin 2000).

2.2. Measurement of environmental variables

Temperature, salinity and sea level were measured using a RBRXRX-620 multi-parameter sonde (Sea and Land Technologies), which was installed 1 m from the base of the reef so that the sensors were 25 cm above the bottom. Parameters were measured at 10 min intervals throughout the study period and averaged hourly for further analysis.

2.3. Video recording analysis

To analyze the video recordings, we used the free video analysis software 'Tracker' (<https://www.compadre.org/osp/items/detail.cfm?ID=7365>).

2.3.1. Measurement of *A. japonicus*

Video fragments suitable for measuring the length of sea cucumbers were exported to the 'Tracker' software. For each sea cucumber, 3 frames were selected in which the animal was moving in a straight line and its body was straightened. The coordinates of the front and back edges of the body were then determined and the length of the individual was calculated. For calibration, markers of known length were placed in the center and along the edges of each camera field of view. Juvenile sea cucumbers were measured twice: once at the beginning and once at the end of the observation period.

2.3.2. Analysis of the daily activity of *A. japonicus*

The total observation period was 79 d. To analyze the daily activity of adult sea cucumbers, the video

records from all 6 video cameras were viewed frame by frame. To analyze the daily activity of *A. japonicus* juveniles, only the video recordings made on the concrete slabs with mussels *C. grayanus* were taken into account. Due to the arrangement of mussels in a monolayer and the light grey flat surface of the slabs, it was much easier to analyze the behavior of the juveniles in these parts of the reef than in others.

The time of the day when sea cucumbers entered and left the shelters as well as when they left and returned to the field of view of the cameras was recorded with an accuracy of 1 min. On the basis of these data, we determined the following parameters: (1) the number of sea cucumbers entering and exiting the shelters each hour of the day for the entire observation period (separately for adults and juveniles); (2) the number of adult sea cucumbers counted inside and outside the shelters per 1 h of observations; (3) the total number of adult sea cucumbers on the reef counted per 1 h of observations; (4) the number of juvenile sea cucumbers on concrete slabs counted per 1 h of observations; (5) average daily numbers of adults and juveniles; (6) average hourly number of adults outside shelters during the day and at night; (7) total time spent out of shelter by all adults and its 24 h distribution; (8) total time spent by juveniles outside shelters on concrete slabs and its 24 h distribution; (9) total time spent by all adult sea cucumbers (both inside and outside shelters) on different parts of the reef; (10) total time spent by all adult sea cucumbers outside shelters during 1 h throughout the observation period; and (11) time spent outside shelters for 1 adult and 1 juvenile per day.

When counting sea cucumbers, we included individuals both outside the shelter (i.e. fully visible) and inside the shelter (but only if we were confident that the individual was there). For example, if any part of the sea cucumber's body was visible while it was inside the shelter, or if its exit from the shelter could not have gone unnoticed, we counted that individual. We also followed this rule when determining the length of time that sea cucumbers were in the shelter.

2.3.3. Analysis of the spawning activity of *A. japonicus*

We started our analysis of the temporal parameters of sea cucumbers' spawning activity by recording the time when they left the shelters (if they were there) and started to climb to the top of the reef, followed by the duration of the spawning posture (vertical stretching and waving of the anterior part of the body) and spawning. The onset and end of spawning was deter-

mined for each individual by the appearance and disappearance of sex products at the anterior end of the body.

2.3.4. Analysis of spawning activity of other invertebrates

Spawning activity was recorded for sea urchins *S. intermedius* and *M. nudus*, mussels *C. grayanus*, oysters *C. gigas* and brittle stars *O. sarsii*. The onset of spawning activity for sea urchins was indicated by increased movement activity and climbing to the top of the reef. For sea urchins, the duration of spawning was determined from the appearance of gametes from gonopores in the first spawning individual to their disappearance in the last spawning individual. This period also included intervals between gamete batches. The duration of spawning activity of brittle stars was determined from their appearance on the reef surface to their leaving for shelters. The main sign of spawning behavior in brittle stars was considered to be the raising of the aboral disc above the reef surface, a characteristic spawning posture of these animals. It was not possible to identify the process of gamete release in brittle stars *O. sarsii* due to their small size. Spawning activity of mussels and oysters was assessed qualitatively by assigning them to 1 of 2 categories: episodic spawning or mass spawning. Spawning was considered episodic if the cloud of gametes covered less than 50% of the field of view of the cameras. The duration of such spawning was determined from the moment the first cloud of gametes appeared until the last cloud disappeared, regardless of its size. Spawning where the cloud of gametes covered more than 50% of the field of view of the video cameras was termed mass spawning.

2.4. Statistical analysis

The quantitative data obtained (length and number of adult and juvenile sea cucumbers, parameters of their behavior, environmental variables) were tested for normality using the D'Agostino-Pearson test. Depending on the test results, parametric or non-parametric methods of statistical analysis were used. Changes in the number of adult and juvenile sea cucumbers during the observation period were analyzed using linear regression. Spearman correlation analysis was used to describe the relationship between environmental variables and between the time when adult and juvenile sea cucumbers left and entered the shelters and the time of sunrise and sunset. Sunrise and sunset

times were obtained from <https://www.timeanddate.com>. Circular statistics using Rayleigh's test for circular uniformity as a criterion of validity (Zar 2010) were performed to determine if there was a relationship between the daily activity of adult and juvenile sea cucumbers (the time of day when sea cucumbers began to enter and exit the shelters and the total time spent outside the shelters) and the time of day. An unpaired *t*-test was used to detect differences between the size of juvenile sea cucumbers at the beginning and end of the observation period.

To analyze the relationship between seawater temperature and activity of adult *A. japonicus*, data on the average hourly time spent by all adult sea cucumbers outside shelters were pooled into 3 data sets: the period between 21 July and 8 August, when water temperature was $\geq 25^{\circ}\text{C}$ (including short periods of temperature decrease to 21.5°C) and the preceding and following periods of temperature below 25°C . The data sets were tested for adherence to a normal distribution using the Kruskal-Wallis test followed by Dunn's multiple comparison test. To compare the effects of temperature on the number and activity of *A. japonicus* adults and juveniles, data on the average daily number of adults and juveniles and the time spent outside shelters per individual per day were pooled into data sets for the same time intervals and tested by 1-way ANOVA followed by Holm-Šidák's multiple comparison test or Kruskal-Wallis test followed by Dunn's multiple comparison test. All statistical analyses were performed using GraphPad Prism v.6.0 for Windows (GraphPad Software). All null hypotheses were tested at a significance level of $\alpha = 0.05$.

3. RESULTS

3.1. Preliminary studies

Preliminary observations showed that the sea cucumbers *Apostichopus japonicus* stayed for several months after being transferred to the new location inhabited by other organisms. Video recordings of the spawning behavior of the sea urchin *Strongylocentrotus intermedius* (Zhadan et al. 2021) showed that 4 *A. japonicus* individuals that had been placed in April 2016 on the feeder with kelp together with sea urchins remained there until the end of June. In the other case, 3 sea cucumbers were placed on an artificial settlement of *Crassostrea gigas* 5 m from the reef in May 2017 and remained there until early September 2019. One *A. japonicus* specimen was placed on the artificial reef in mid-May 2018 and was observed there

until September 2020. These data on *A. japonicus* habitat commitment encouraged us to conduct the main experiment with the placement of a reasonably representative sample of sea cucumbers on the artificial reef. In addition, the preliminary studies allowed us to optimize the conditions for videotaping the behavior of *A. japonicus* juveniles by using the concrete slabs with a monolayer of mussels *Crenomytilus grayanus* and to improve the observation of *A. japonicus* spawning by placing a video camera directly above the reef surface.

3.2. Main experiment

3.2.1. Environmental variables

Due to seasonal warming, the temperature in the bottom water layer gradually increased from 14.26 to 24.45°C during the period from 20 June to 20 July (Interval 1; mean: 18.47°C) (Fig. 1). From 21 July to

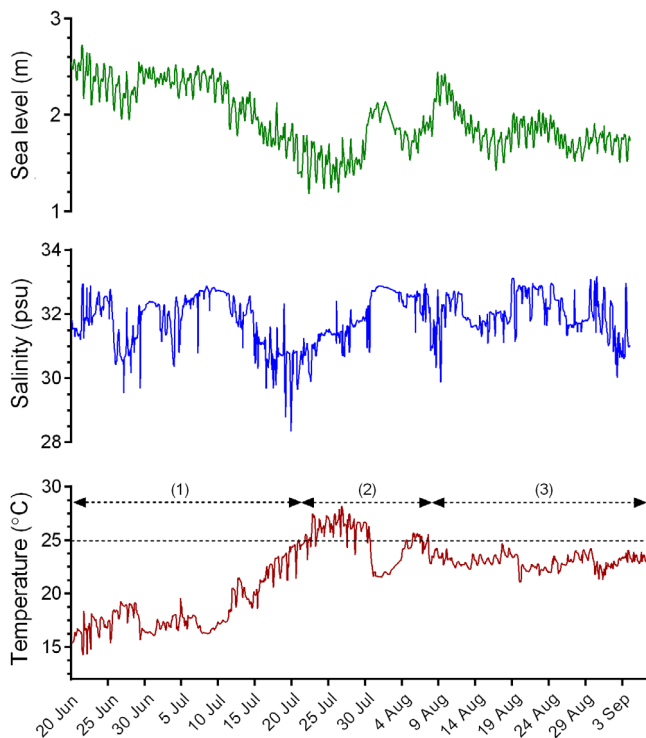


Fig. 1. Seasonal dynamics of environmental variables on the artificial reef in June–September 2021. Data are presented as average hourly values. Horizontal dashed line: 25°C temperature threshold of aestivation in *Apostichopus japonicus* (Yang et al. 2005, 2006). Numbers in parentheses between arrowhead lines delineate time intervals: Interval 1: 21 June–20 July (the period of gradual warming); Interval 2: 21 July–8 August (the period of strong temperature changes); Interval 3: 9 August–6 September (the period of relatively stable temperature)

30 July, there was a period of extremely high temperature (from 24.7 to 28.2°C, mean: 26.12°C), followed by a period of sharp changes when the temperature decreased to 21.51°C, then increased up to 25.72°C and decreased again to 24.7°C (Interval 2; from 21 July to 8 August, mean: 25.04°C). These phenomena were largely determined by the tidal activity, which was highest during the warmest period, probably due to upwelling and downwelling events; maximum sea level changes were approximately 110 cm (Fig. 1). At the same time, the maximum amplitude of an irregular semidiurnal tide was approximately 50 cm. From 9 August to 6 September, seawater temperature varied from 21.06 to 24.67°C (Interval 3; mean: 22.98°C). The salinity in the bottom water layer varied from 28.3 to 33.2 psu during the study period (Fig. 1). A short period when the salinity was sometimes lower than 30 psu (from 18 to 22 July) did not coincide with the period of extremely high temperature. Correlation analysis revealed significant relationships between temperature and sea level (Spearman $r = -0.880$, $p < 0.0001$), temperature and salinity (Spearman $r = -0.308$, $p < 0.0001$) and salinity and sea level (Spearman $r = 0.307$, $p < 0.0001$).

3.2.2. Size of *A. japonicus*

The mean (\pm SD) length of adult sea cucumbers varied from 10.7 ± 0.29 to 22.48 ± 0.29 cm ($n = 3$) (Table S1). Over 79 d of the observation period, the mean length of juveniles increased from 4.28 ± 0.27 cm ($n = 8$) to 5.66 ± 0.16 cm ($n = 11$) (unpaired t -test, $t = 4.652$, $df = 17$, $p = 0.0002$; Table S2).

3.2.3. Dynamics of the number of *A. japonicus*

The number of adult sea cucumbers counted inside and outside shelters per 1 h of observation varied from 0 to 9 and from 0 to 11 individuals, respectively. The total number of adults on the reef per 1 h of observation varied in approximately the same range, from 0 to 12 individuals (Fig. 2A). There was no significant trend in the total number of adult sea cucumbers on the reef throughout the observation period (regression analysis, $y = -0.0001557x + 3.948$, $n = 1767$, $R^2 = 0.0015$, $p = 0.1009$).

The number of juvenile sea cucumbers on the surface of the concrete slabs of the reef varied from 0 to 11 ind. h^{-1} of observation (Fig. 2B). There was a significant increase in the number of juveniles towards the end of the observation period (regression

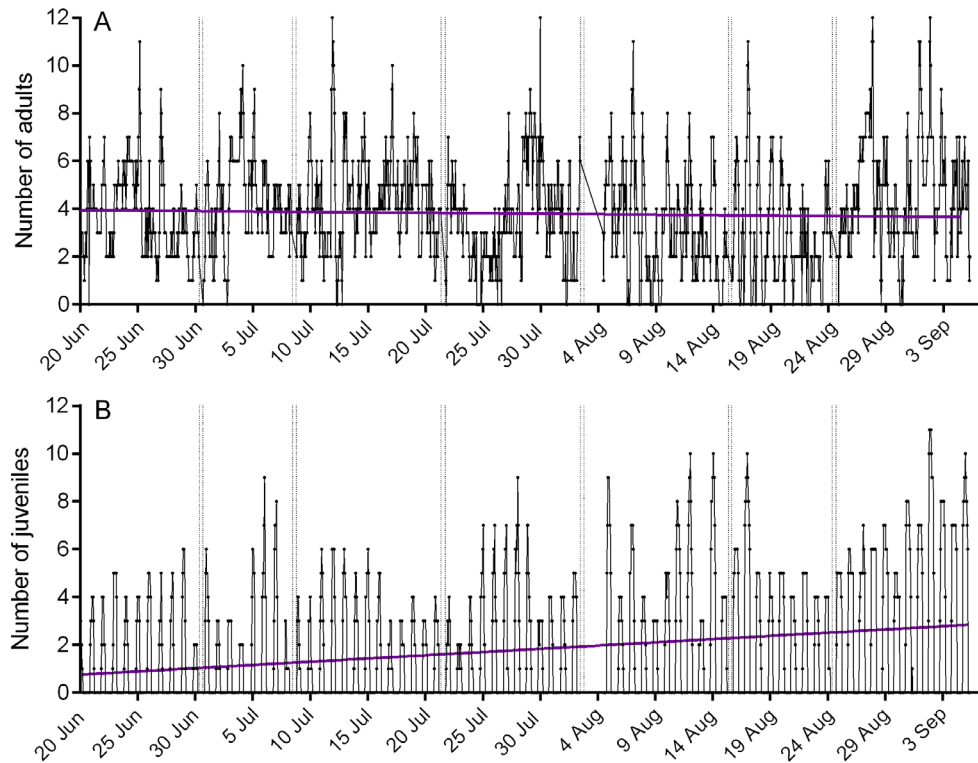


Fig. 2. Dynamics of the number of sea cucumbers *Apostichopus japonicus* on the artificial reef in June–September 2021. (A) Total number of adult sea cucumbers counted per hour of observation. Solid purple line corresponds to the linear regression equation $y = -0.0001557x + 3.948$, $n = 1767$, $R^2 = 0.0015$, $p = 0.1009$. (B) Number of juvenile *A. japonicus* on concrete slabs with mussels *Crenomytilus grayanus* counted per hour of observation. Solid purple line corresponds to the linear regression equation $y = 0.0011x + 0.769$, $n = 1775$, $R^2 = 0.057$, $p < 0.0001$. Paired vertical dotted lines: start and end of periodic 7–10 h breaks in data collection, to allow for equipment maintenance and data retrieval

analysis, $y = 0.0011x + 0.769$, $n = 1775$, $R^2 = 0.057$, $p < 0.0001$) (Fig. 2B). Video recordings did not show juvenile exits and entrances on concrete slabs, so the increase in their number can be explained by the juveniles becoming less secretive as they grew. This increased the likelihood of their being videotaped. Thus, during the first 20 d of observation, juveniles moved 1–5 cm away from the shelters and usually returned to the same shelter. By the end of the observation period, this distance increased to 20–40 cm. At the same time, during the first half of the observation period, the movement of the juveniles was limited by the boundaries of each concrete slab. Juveniles were not observed on the stones inhabited by mussels and on the oyster settlement until 8 August; then, between 1 and 9 individuals were recorded here.

3.2.4. Daily activity of *A. japonicus*

Adults of *A. japonicus* showed a distinct diurnal periodicity in the timing of entering and exiting shelters (Rayleigh's test for circular uniformity, $z =$

23.9, $p < 0.001$ and $z = 43.4$, $p < 0.001$, respectively). Most of the sea cucumbers hid near sunrise (between 05:00 and 06:00 h; Fig. 3A) and emerged at sunset (between 20:00 and 21:00 h; Fig. 3B). Such diurnal activity was recorded throughout the observation period (Fig. 3C). Mean times of the day for entering and exiting the shelters were 05:01 h ($\pm 5:42$ h) (circular statistics, $n = 235$) and 21:10 h ($\pm 5:06$ h) ($n = 252$), respectively.

The mean (\pm SD) hourly number of adults outside shelters during the night (3.60 ± 1.65) was significantly higher than that during the day (0.91 ± 0.65) (Mann-Whitney test, $U = 348$, $p < 0.0001$). The time spent outside shelters by all adults (3580 h in total) was also not uniformly distributed over a 24 h period (Rayleigh's test for circular uniformity, $z = 253.4$, $p < 0.001$). Sea cucumbers were mainly recorded outside shelters between 21:30 and 06:10 h, and the mean of this interval occurred at night at 01:47 ($\pm 4:19$ h) (circular statistics) (Fig. S2A). During the night and day, the mean (\pm SD) hourly time spent outside shelters by all adults was 3.81 ± 1.93 h ($n = 702$) and 0.78 ± 0.93 h ($n = 1162$), respectively. It should be noted that the time

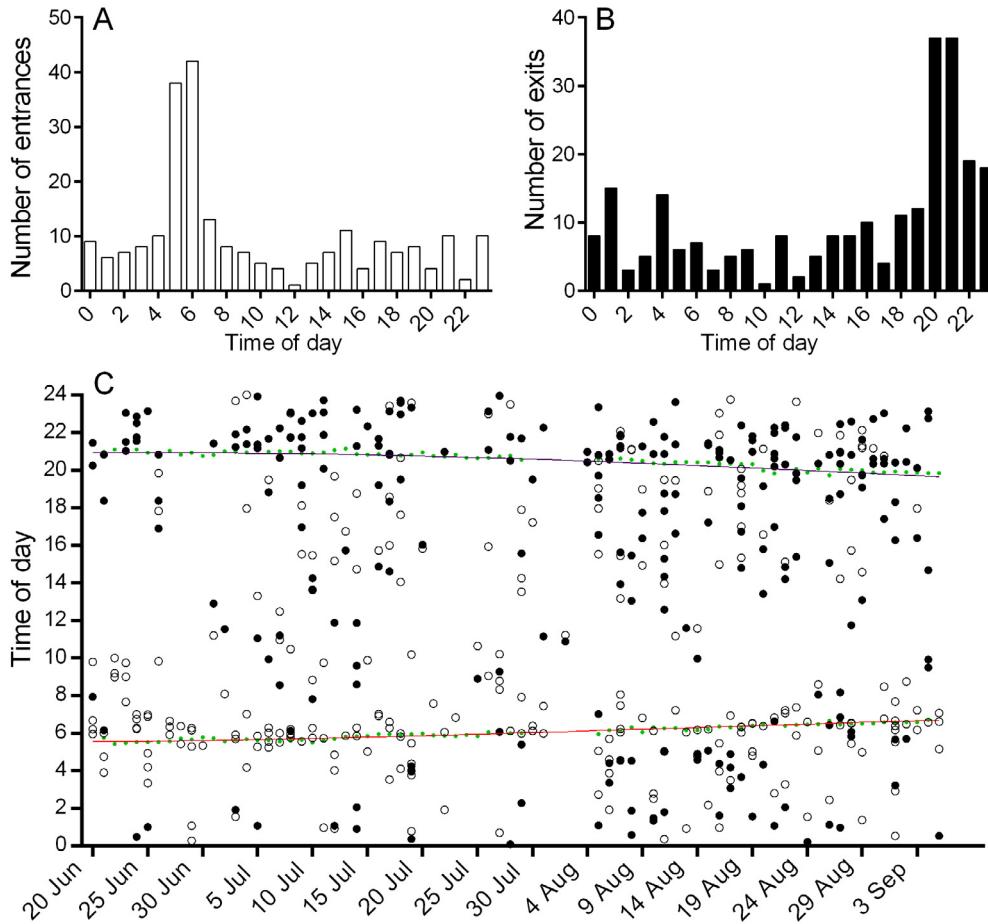


Fig. 3. Daily and seasonal activity of *Apostichopus japonicus* adults on the artificial reef in June–September 2021. Diurnal distribution of sea cucumbers' (A) entrances and (B) exits from the shelters, counted for the whole observation period. (C) Seasonal distribution of the time of day when sea cucumbers entered or left the shelters. Open circles: time entering the shelter; black circles: time leaving the shelter; small green circles: start time of the pulsed illumination; red (lower) and purple (upper) solid lines: astronomical times of sunrise and sunset

spent by adults outside shelters during the day would have been less had they not expended an inordinate amount of time in a futile effort to get inside. We repeatedly observed some sea cucumbers spending a long time after sunrise trying to hide in shelters that were no longer large enough. On one occasion, a 20 cm long sea cucumber trying to hide under a 15 cm diameter rock moved the rock by 2 cm and finally moved to a more accessible shelter after 2 h and 43 min.

A. japonicus juveniles showed a much stronger diurnal periodicity in the timing of shelter entry and emergence than adults (Rayleigh's test for circular uniformity, $z = 371.6$, $p < 0.001$ and $z = 324.9$, $p < 0.001$, respectively). The vast majority of juveniles sheltered between 04:00 and 06:00 h (Fig. 4A) and emerged between 20:00 and 22:00 h (Fig. 4B), and the mean times of the day for these events were 04:30 h ($\pm 1:30$ h) (circular statistics, $n = 434$) and 21:30 h ($\pm 1:40$ h) ($n = 394$), respectively. Juveniles showed a

high sensitivity to changes in illumination. The times at which the last individual entered the shelter at sunrise and the first individual left at sunset were positively correlated with seasonal changes in sunrise and sunset times (Spearman $r = 0.82$, $p < 0.0001$ and $r = 0.71$, $p < 0.0001$, respectively; Fig. 4C). We also noticed that *A. japonicus* juveniles entered the shelters later and left them earlier during cloudy weather than during clear weather.

The time spent by all *A. japonicus* juveniles outside the shelters (2734 h in total) was not uniformly distributed over a 24 h period (Rayleigh's test for circular uniformity, $z = 1579.8$, $p < 0.001$). Juveniles showed the highest movement activity between 22:00 and 03:30 h, and the mean of this interval was 00:41 h ($\pm 2:49$ h) (see Fig. S2B). After sunrise and before sunset, *A. japonicus* juveniles appeared extremely rarely on the surface of the concrete slabs, spending a total of about 8 h ($1\text{--}57$ min d^{-1}) there (Fig. S2B).

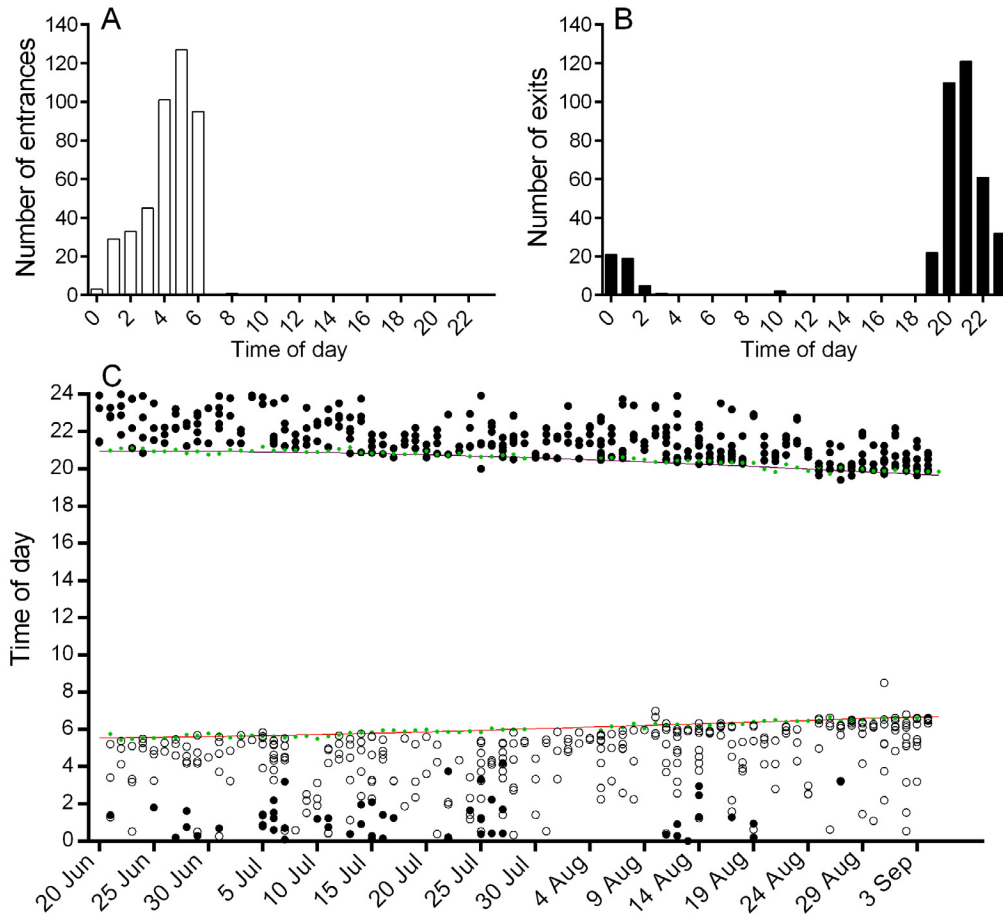


Fig. 4. Same as Fig. 3, but for *Apostichopus japonicus* juveniles

The activity of sea cucumbers, measured as the time spent outside the shelter per individual per day, was significantly higher in adults than in juveniles (mean \pm SD = 4.63 \pm 1.47 h vs. 3.32 \pm 1.55 h, $n = 76$, Mann-Whitney test, $U = 1496$, $p < 0.0001$). The activity of juveniles increased significantly with an increase in the duration of the dark part of the day (non-linear regression, $y = 2.351 - 0.0159x + 0.0007658x^2$, $n = 76$, $R^2 = 0.49$) (Fig. S3). Adult sea cucumbers showed no such relationship with the duration of darkness (Spearman correlation, $r = 0.64$, $p < 0.0001$ and $r = 0.15$, $p = 0.21$ for juveniles and adults, respectively). During the first and last 15 d of the observation period, juveniles spent 2.17 \pm 0.6 and 4.87 \pm 1.44 h (means \pm SD, $n = 15$), respectively, outside the shelters (unpaired t -test, $t = 6.73$, $df = 28$, $p < 0.0001$).

3.2.5. Distribution of *A. japonicus* adults on the reef

Although the number of *A. japonicus* adults on the reef remained constant during the observation period, a redistribution of the total time spent on different

parts of the reef (both inside and outside the shelters) was observed. During the first month (from 20 June to 20 July), the presence of adults at the top of the reef was recorded (Fig. 5A). This was clearly associated with the spawning activity of *A. japonicus*, which was observed from 26 June to 14 July. The maximum daily presence of sea cucumbers at the top of the reef (70 h) was recorded on 12 July (Fig. 5A), when the most intensive spawning activity of *A. japonicus* occurred, with up to 9 ind. h⁻¹ of observation.

On the mussel-covered sides of the reef, the activity of sea cucumbers did not change significantly throughout the observation period (regression analysis, $y = -0.03494x + 78.68x$, $n = 76$, $R^2 = 0.0008$, $p = 0.81$) (Fig. 5B). The total time spent by sea cucumbers at the base of the reef and on the adjacent *C. gigas* settlement increased significantly by the end of the observation period ($y = 0.6030x + 29.73$, $n = 76$, $R^2 = 0.27$, $p < 0.0001$) (Fig. 5C). On the bottom areas adjacent to the reef, the total time spent by *A. japonicus* also increased significantly towards the end of the observation period ($y = 0.1593x - 0.07110$, $n = 76$, $R^2 = 0.42$, $p < 0.0001$) (Fig. 5D).

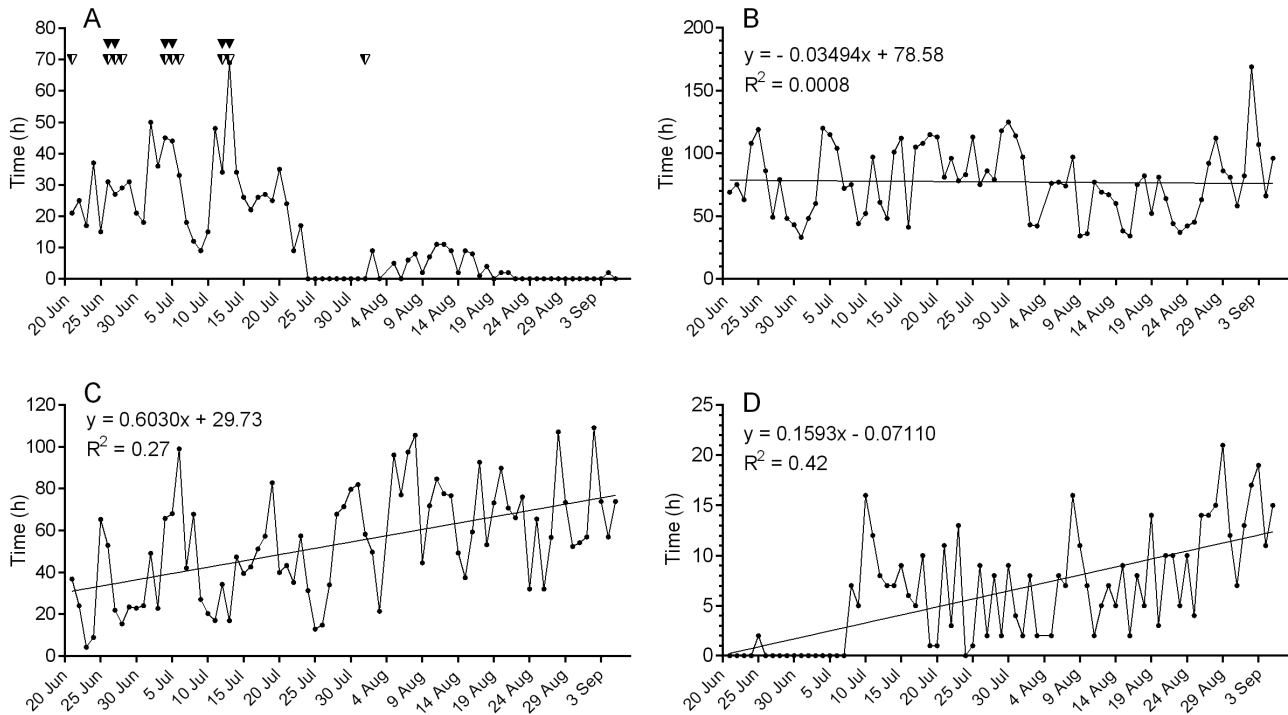


Fig. 5. Distribution of total time spent by all adult *Apostichopus japonicus* (both inside and outside shelters) on different parts of the artificial reef in June–September 2021. (A) Top of the reef. Full and half-filled triangles: dates when *A. japonicus* spawning events and spawning behavior were observed, respectively. (B) Sides of the reef with mussels *Crenomytilus grayanus*. Solid straight line corresponds to the linear regression equation $y = -0.03494x + 78.68x$, $n = 76$, $R^2 = 0.0008$, $p = 0.81$. (C) Base of the reef and adjacent *Crassostrea gigas* settlement. Solid straight line corresponds to the linear regression equation $y = 0.6030x + 29.73$, $n = 76$, $R^2 = 0.27$, $p < 0.0001$. (D) Bottom areas adjacent to the reef. Solid straight line corresponds to the linear regression equation $y = 0.1593x - 0.07110$, $n = 76$, $R^2 = 0.42$, $p < 0.0001$

3.2.6. Abundance and activity of *A. japonicus* in relation to temperature

Temperature fluctuations during the survey season did not significantly affect the numbers of adult and juvenile sea cucumbers. The average daily numbers of adults determined for Intervals 1–3 with different temperature conditions (see Fig. 1) did not differ significantly (see Fig. S4A and Table S3 for statistics). The average daily number of juveniles did not differ significantly between Intervals 1 and 2 (the period of strong temperature changes) and was higher in Interval 3 compared to Intervals 1 and 2 (see Fig. S4B and Table S4 for statistics). This is consistent with the results of the linear regression (Fig. 2B) and non-linear regression (Fig. S4B) and confirms a significant increase in the number of juveniles recorded by the end of the observation period.

Strong temperature fluctuations during Interval 2 reduced the activity of

A. japonicus adults, measured as the total time spent by all adult sea cucumbers outside shelters during 1 h (Fig. 6). It was significantly lower than during Intervals 1 and 3 ($p = 0.0145$ and $p = 0.0018$, respectively; see Table S5 for statistics).

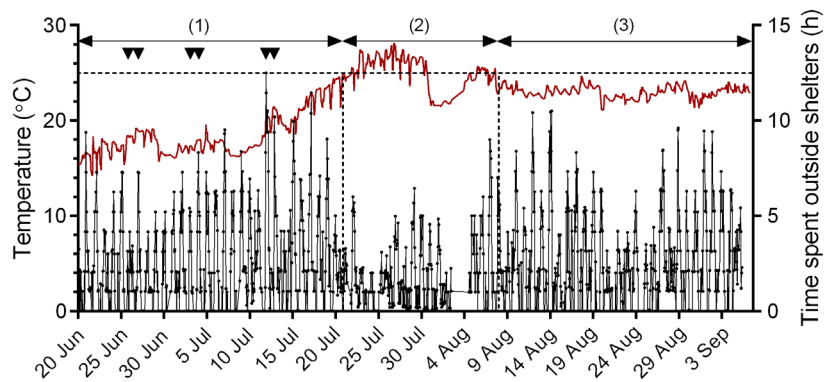


Fig. 6. Seasonal activity of *Apostichopus japonicus* adults relative to seawater temperature on the artificial reef in June–September 2021. Black lines and symbols: total time spent by all adult sea cucumbers outside shelters during 1 h; red line: average hourly seawater temperature. Horizontal dashed line: 25°C. Numbers in parentheses between arrowhead lines delineate Intervals 1, 2 and 3 (see Fig. 1). Black downward-pointing triangles: dates when *A. japonicus* spawning events were observed

A comparison of the activity of adult and juvenile *A. japonicus*, measured as the time spent outside shelters per individual per day, showed that adults were significantly less active during Interval 2 than during Intervals 1 and 3 (Fig. 7A, see Table S6 for statistics), while the activity of juveniles gradually increased during the observation period (non-linear regression, $y = 2.351 - 0.0159x + 0.0007658x^2$, $n = 76$, $R^2 = 0.49$; Fig. 7B). No significant difference in the time spent by juveniles outside shelters was found between Interval 2 (the period of strong temperature changes) and Interval 1. At the same time, the activity of the juveniles was significantly higher during Interval 3 than during Intervals 1 and 2 (Fig. 7B, see Table S7 for statistics).

3.2.7. Spawning activity of *A. japonicus* and other invertebrates

Spawning activity of *A. japonicus* and several invertebrate species inhabiting the artificial reef was observed from 26 June to 14 July (Fig. 8). Spawning events occurred at temperatures ranging from 16.64 to 21.5°C, salinities from 30.52 to 32.65 psu and sea levels from 1.85 to 2.51 m (Table S8). Adult *A. japonicus* showed a clear sequence of behaviors during spawning activity. First, they left the shelters (if present) and climbed to the top of the reef. Second, the sea cucumbers displayed a typical spawning posture, lifting the anterior part of the body and moving it vigorously from side to side. Such postures were recorded 31 times, and in 27 cases, only at the top of the reef. In 4 cases, the sea cucumbers started to show the spawning posture on the side surfaces of the reef but soon stopped and continued climbing. The time difference between the first and last individual reaching the top of the reef was 15 ± 12 min (mean \pm SD, $n = 27$), with a maximum of 39 min. Typically, one individual occupied the highest point: a stone placed at the top of the reef.

All adult sea cucumbers exhibited spawning behavior (climbing to the top of the reef and displaying the spawning posture) but this was not necessarily accompanied by spawning (i.e. gamete release). The third step of spawning activity, spawning, was recorded 8

times in sea cucumbers of 18–22 cm (Fig. 8). Judging by the white color of the gametes, all these sea cucumbers were males.

The time between reaching the reef top and displaying the spawning posture was 14 ± 8 min (mean \pm SD, $n = 27$). When spawning did occur, it started 18 ± 13 min ($n = 8$) after the onset of the spawning posture, and the duration of spawning varied from 60 to 288 min (140 ± 89 min). In non-spawning sea cucumbers, the display of the spawning posture lasted from 7 to 247 min (103 ± 85 min, $n = 19$). After the end of spawning activity, the sea cucumbers left the top of the reef. The maximum total number of sea cucumbers displaying spawning behavior and spawning on the upper and lateral surfaces of the reef was recorded on 12 July and amounted to 9 ind. h^{-1} of observation (75% of the total number of adult sea cucumbers on the reef) (see Fig. S5).

The spawning activity of sea cucumbers occurred mainly at night, after sunset and before sunrise (Fig. 8). The exception was 2 sea cucumbers that started to show spawning behavior 1 h 44 min and 1 h 20 min

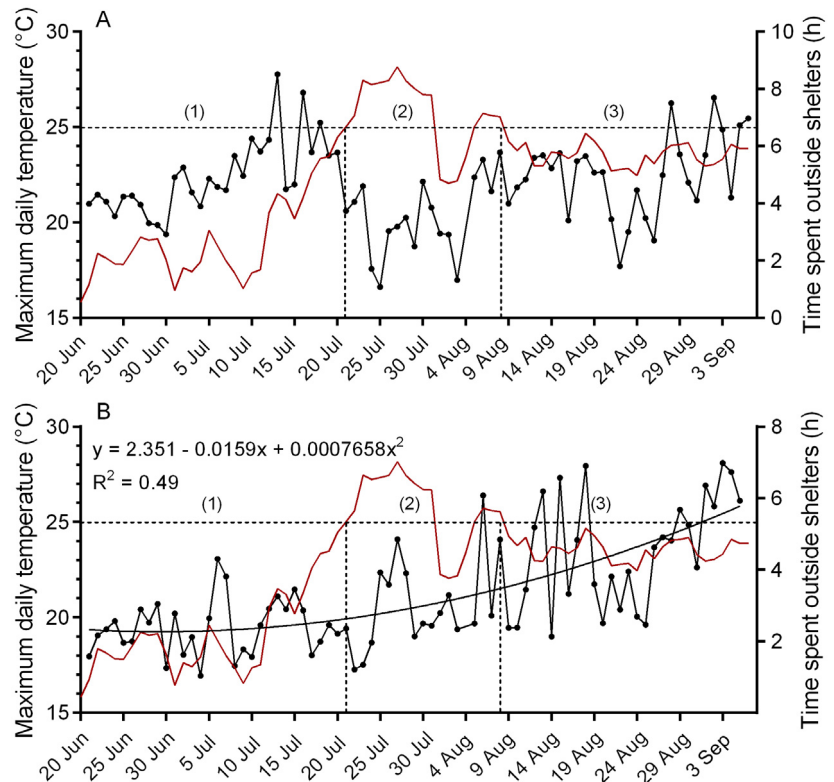


Fig. 7. Seasonal dynamics of the activity of *Apostichopus japonicus* (A) adults and (B) juveniles (represented as time spent outside shelters, counted per individual per day) and maximum daily temperature (denoted by red solid line without symbols) on the artificial reef in June–September 2021. Horizontal dashed line: 25°C. Vertical dashed lines indicate Intervals 1, 2 and 3 (see Fig. 1). The curve line corresponds to the non-linear regression equation $y = 2.351 - 0.0159x + 0.0007658x^2$, $n = 76$, $R^2 = 0.49$

before sunset on 26 June and started to spawn 57 and 37 min before sunset.

Spawning activity of sea urchins *Mesocentrotus nudus* and *S. intermedius*, mussels *C. grayanus* and oysters *C. gigas* was observed at dusk and at night, from 18:20 to 07:20 h (Fig. 8). According to our data, during the period from 25 June to 13 July, the PAR level at 19:00 h was approximately 10 times lower than at 12:00 h. Sea urchins showed spawning behavior by significantly increasing their movement activity and climbing the reef top. During periods of spawning activity, the number of sea urchins *M. nudus* on the reef top was 2.7 times higher than before these periods (paired *t*-test, $t = 7.486$, $df = 38$, $p < 0.0001$, $n = 39$). However, only 45% of *M. nudus* (19 out of 42 individuals) and 75% of *S. intermedius* (3 out of 4 individuals) spawned (Fig. 8). A total of 9 spawning episodes were recorded for both species, 6 of which coincided with the intervals of *A. japonicus* spawning activity (Fig. 8). Spawning behavior of the brittle star *Ophiura sarsii* was observed from 18:20 to 02:20 h (Fig. 8). A total of 5 episodes were recorded, all coinciding with the spawning activity of *A. japonicus* and other invertebrate species. The largest mass spawning events, involving 6 species, were recorded on 26–28 June (near the full moon) and 12–13 July (near the new moon); 3 other multispecies spawning events occurred on 3–6 July when the moon was in waning phase (third quarter) (Fig. 8).

3.2.8. Potential predators of *A. japonicus*

The Japanese cormorant *Phalacrocorax capillatus* (Temminck & Schlegel, 1849) is probably a threat to the sea cucumber *A. japonicus*. During the study

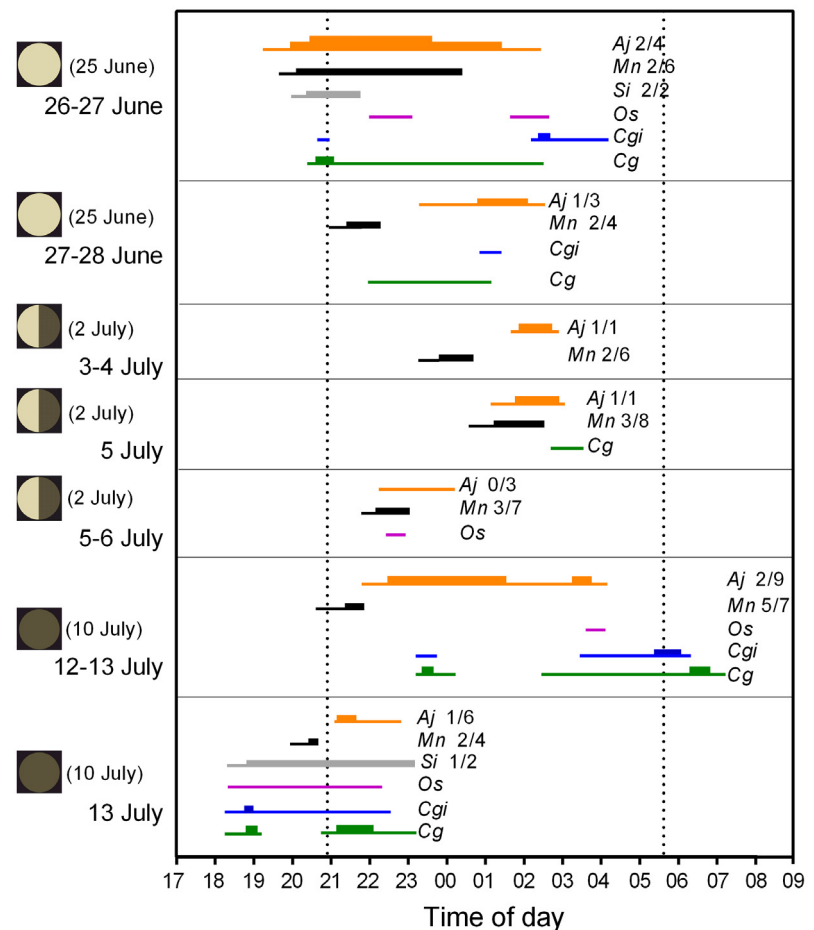


Fig. 8. Seasonal and daily intervals of spawning activity of several invertebrate species on the artificial reef in 2021. Horizontal solid lines and different letters indicate spawning activity intervals for *Apostichopus japonicus* (Aj), *Crenomytilus grayanus* (Cg), *Crassostrea gigas* (Cgi), *Mesocentrotus nudus* (Mn), *Strongylocentrotus intermedius* (Si) and *Ophiura sarsii* (Os). For *A. japonicus*, *M. nudus* and *S. intermedius*, thin and thick parts of the lines correspond to spawning behavior and spawning, respectively. For *C. grayanus* and *C. gigas*, thin and thick parts of the lines correspond to episodic and mass spawning, respectively. For brittle stars, thin lines indicate spawning behavior. Numbers indicate the number of spawning individuals (numerator) and total number of individuals showing spawning behavior (denominator). Dotted lines correspond to the sunset (on the left) and sunrise (on the right) at the beginning and end of the observation period. Observation dates and moon phases (symbols, with exact date of occurrence in parenthesis) are given to the left of the figure

period, 5 observations of *P. capillatus* were recorded on the reef. We also believe that some fish species may prey on the gametes released by the sea cucumbers. On one occasion, 10 min after the mussels began to spawn, we observed the appearance of 7 individuals of the so-iuy mullet *Planiliza haematocheilus* (Temminck & Schlegel, 1845), also known as the redlip mullet, with a length of 19–22 cm. They positioned themselves close to the mussels and, judging by the movement of their lips and gill covers, swallowed the water with suspended gametes. After

the cloud of gametes disappeared, *P. haematocheilus* left the reef.

4. DISCUSSION

Round-the-clock time-lapse video recording of the number and activity (time spent outside shelters and spawning activity) of the Japanese sea cucumber *Apostichopus japonicus* on the multispecies artificial reef *in situ* during the breeding season allowed us to clarify the association of these parameters with environmental variables, primarily daylight and seawater temperature.

4.1. Population dynamics of *A. japonicus* adults on the reef

The number of *A. japonicus* adults transferred to the artificial reef remained the same during the summer period over a wide range of seawater temperatures (14.3–28.2°C). Furthermore, no significant differences were found in the average number of adults between the periods with different temperature regimes (Table S3). From studies on the seasonal distribution of *A. japonicus* adults in the coastal zone of Japan, it was reported that during the warmest season, when the temperature was higher than 24.5–25°C (July–October), no sea cucumbers were observed on the seabed because they took shelter in rock crevices, oyster shells or under the harbor pier (Yamana et al. 2009b, Minami et al. 2019). This apparent discrepancy may be explained by the fact that these studies were conducted during the day, whereas we counted sea cucumbers around the clock, both visible and in shelters when we were sure they were inside, and showed that the average number of *A. japonicus* adults outside shelters at night was about 4 times higher than during the day.

The fact that the number of adult *A. japonicus* transferred to the artificial reef did not decrease during the summer season indicates the attractiveness of this habitat for sea cucumbers, which may be related to their physiological needs for feeding, sheltering and reproduction. First, filter-feeding bivalves produce a large amount of organic and inorganic material in the form of feces and pseudofeces (biodeposits), which serve as a food source for the deposit-feeding *A. japonicus* (Zhou et al. 2006, Yokoyama 2015). Furthermore, the abundance of bivalves on the reef is a factor that attracts other species of benthic invertebrates. The translocation of druses of the mussel *Modiolus*

modiolus to an artificial reef led to a significant increase in faunal diversity and species abundance in a relatively short time (12 mo) (Fariñas-Franco et al. 2013). In our study, several species including sea urchins *Strongylocentrotus intermedius* and *Mesocentrotus nudus*, brittle stars *Ophiura sarsii* and small crabs self-populated the artificial reef within 1.5 mo after *Crassostrea gigas* and *Crenomytilus grayanus* were translocated onto it. Biodeposits from these species may also provide food for sea cucumbers (Yu et al. 2023). Indeed, our observations showed that non-sheltered sea cucumbers on the reef and adjacent bottom areas exhibited feeding behavior typical of deposit-feeding holothurians (characteristic 'sweeping' movements with the anterior end of the body where fine oral papillae are located).

Second, the crevices between the shells of the mollusks and between the rocks and concrete slabs that form the base of the reef can serve as shelters to spend the day and survive under unfavorable ecological conditions, such as extremely high temperatures, wave activity, etc. The presence of shelters is a prerequisite for the normal vital activity of *A. japonicus* (Levin 2000). Laboratory experiments have shown that artificial reefs with crevices increase survival and improve fitness-related behavior of small sea cucumbers at high temperatures (Wang et al. 2023a,b).

Third, our artificial reef in the form of a truncated pyramid provides an attractive spawning site for *A. japonicus*. Holothurians and other echinoderms use elevated areas of the bottom and natural reefs for spawning (Friedman 2008, Himmelman et al. 2008, Balogh et al. 2019, Webb et al. 2021, Zhadan et al. 2021).

4.2. Population dynamics of *A. japonicus* juveniles on the reef

In addition to *A. japonicus* adults which were placed on the artificial reef, a significant number (up to 23 ind. m⁻²) of juvenile sea cucumbers were found on the concrete slabs of the reef populated by mussels *C. grayanus*. This population density was higher than the density of *A. japonicus* juveniles of different ages (up to 7 ind. m⁻²) found in one of the bays of Peter the Great Bay (Sea of Japan) (Levin 2000) and at one of 8 stations in the intertidal zone of Hirao Bay (Seto Inland Sea, Japan) (Yamana et al. 2006) but was close to the density (22 ind. m⁻²) found in a wave-protected area off the southern part of Hokkaido (Sea of Japan) at 1.5 m depth (Yamana et al. 2014). The highest known density of *A. japonicus* juveniles (50–200 ind. m⁻²)

was recorded in the intertidal zone of the Stark Strait in Peter the Great Bay (Levin 2000).

The age of *A. japonicus* juveniles on the reef corresponded to approximately 1 yr (12 mo) in terms of size (body length) (Hamano et al. 1989, Ito & Kitamura 1997, Yamana et al. 2010, Sun et al. 2015, Sakurai et al. 2017). This can be explained by the fact that the concrete slabs with mussels were installed on the reef before the start of the previous spawning season of *A. japonicus*. Given the specific biological preferences of holothurian larvae with respect to the substrate on which they settle (Young & Chia 1982, Hamel & Mercier 1996, Ito & Kitamura 1997, Mercier et al. 2000, Qiu et al. 2015), the appearance of *A. japonicus* juveniles of the same age at high density on the artificial reef seems to be the result of habitat selection by settling larvae of this species. Recently, Ru et al. (2022) tested different substrates to evaluate their effect on the settlement of *A. japonicus* larvae and found that the substrates made of oyster and scallop shells were optimal.

The natural habitat characteristics of *A. japonicus* juveniles are poorly understood. Several studies on the distribution of *A. japonicus* in coastal waters of Japan and Russia have reported that the highest densities of young-of-the-year and 1–3 yr old individuals were found at shallow depths (0–1.5 m) and that juveniles preferred moderately eutrophicated rocky bottom with clusters of bivalve mollusks (mussels *C. grayanus*, *Modiolus kurilensis* and oysters) and macroalgae (*Sargassum* spp., *Ulva* spp.) (Hamano et al. 1989, Selin 2001, Yamana et al. 2006, 2014, Minami et al. 2018). These and our results suggest that *A. japonicus* juveniles require habitats with food availability for detritivores (i.e. algal detritus and bivalve feces) as well as hard substrate with shelters (boulders and bivalve shells). Studies of the distribution patterns of juveniles of the other stichopodid holothurian, *Australostichopus mollis*, also showed that relatively high densities of juveniles were associated with the enrichment of sediments with organic matter from algal and terrigenous detrital inputs and with the presence of large shell fragments of the horse mussel *Atrina zelandica* (Slater et al. 2010).

Our results showed that *A. japonicus* juveniles exhibited seasonal population dynamics that were distinctly different from those of adults. The total number of juveniles recorded on the reef increased significantly towards the end of the survey season. In parallel, the time spent by juveniles outside the shelters also increased. It is unlikely that the increase in the number of juveniles was due to an influx of juveniles from outside. Such an influx would have

been easy to detect due to the high temporal resolution of the video recording, the low movement rate of the juveniles, and the light grey background color of the concrete slabs. Therefore, we suggest that the increase in the number and activity of juveniles during the summer season is due to a decrease in the time spent in the shelters and an increase in feeding activity. This conclusion is consistent with the results of laboratory experiments showing that the rate of oral tentacle locomotion and food consumption in *A. japonicus* increased with increasing body weight and size (Yang et al. 2005, Sun et al. 2015).

4.3. Daily rhythm in *A. japonicus* activity

The most striking feature of the behavior of adult and juvenile *A. japonicus* on the artificial reef was the pronounced nocturnal activity. It has long been known that *A. japonicus*, like many other holothurian species, is predominantly nocturnal (Levin 2000). However, to our knowledge, the present study is the first to provide a detailed analysis of the daily behavior of *A. japonicus* under field conditions. First, we recorded the timing of *A. japonicus* sheltering behavior and showed that both adults and juveniles sheltered at sunrise and emerged at sunset. Such a rhythm was most pronounced in juveniles: they sheltered before sunrise and emerged after sunset, and these times correlated positively with seasonal changes in sunrise and sunset times. Adults were able to perform these actions during both day and night, but most of them also moved into shelters around sunrise and emerged from shelters around sunset. Second, we showed that *A. japonicus* adults spent 4.4 times more time outside the shelters at night than during the day and that juveniles were outside the shelters only at night. Such a strict relationship between the activity of *A. japonicus* juveniles and the darkness of the day has not been previously reported. In laboratory experiments, *A. japonicus* juveniles of about 5 cm in length (1 yr old) were maximally active at night but they also showed marked diurnal activity (Dong et al. 2010, 2011, Sun et al. 2015, 2018). Furthermore, Yamaguchi et al. (2016) reported that there was no difference between nighttime and daytime activity of juvenile *A. japonicus*. However, in the presence of a predator (the filefish *Stephanolepis cirrhifer*), the juveniles were more active at night.

In general, our results are consistent with those of laboratory studies on the diurnal periodicity in *A. japonicus* behavior (Kato & Hirata 1990, Yamana et al. 2009a, Dong et al. 2010, 2011, Sun et al. 2015). These

results suggest that the diurnal rhythm of activity in *A. japonicus* is under the control of both an endogenous mechanism (circadian clock) and an exogenous factor (change in illumination). Dong et al. (2011) reported that *A. japonicus* juveniles retain the nocturnal activity pattern after rearing under conditions of different light/dark ratios, even under constant illumination or constant darkness. The other stichopodid holothurian, *Stichopus* cf. *horrens*, exhibited nocturnal activity 48–57 d after larval settlement, even when larvae were not exposed to light during development (Rioja et al. 2020). This suggests the existence of an endogenous mechanism responsible for the regulation of daily rhythms in *A. japonicus*. Our finding that juvenile *A. japonicus* on the reef sheltered before sunrise and emerged only after sunset also suggests the involvement of an endogenous factor in regulating the daily activity of young sea cucumbers. On the other hand, the fact that the time of sheltering and emergence of juveniles was strongly linked to seasonal changes in the time of sunrise and sunset suggests that the exogenous factor (illumination) also influences the behavior of *A. japonicus*. Recently, Ye et al. (2024) reported that the expression of clock genes (*AjClock*, *AjArnt1*, *AjCry1* and *AjTimeless*) in different tissues of adult *A. japonicus* showed clear circadian rhythmicity and was influenced by environmental light fluctuations.

4.4. Activity of *A. japonicus* in relation to environmental variables

Our results showed that seasonal temperature changes affected the activity of adult *A. japonicus* (time spent outside shelters, i.e. the time that sea cucumbers could use for feeding). In our study, when assessing the effect of elevated temperature on the behavior of *A. japonicus*, we considered the temperature of 25°C as the temperature threshold of aestivation, which was determined in laboratory experiments on the effects of temperature on the feeding intensity and metabolism of *A. japonicus* (Yang et al. 2005, 2006). During most of the study period, the seawater temperature was below 25°C (see Fig. 1). The activity of *A. japonicus* adults between 21 July and 8 August — when water temperature first reached or exceeded 25°C, including short periods of temperature decrease (average: 25.04°C) — was decreased by approximately 30% compared to the preceding period of gradual warming (average: 18.5°C) and the following period of relatively stable temperature (average: 23.0°C). Experimental research shows that

daily water temperature fluctuations of 4–6°C amplitude around average temperatures of 15 and 18°C significantly affect the growth and metabolism of *A. japonicus* (Dong & Dong 2006, Dong et al. 2006). We suspected that such temperature fluctuations might also cause the changes in the behavior of *A. japonicus*, so we consider the whole period of strong temperature fluctuations — an increase above 25°C on 21 July followed by a decrease of 4°C (from 25.6 to 21.6°C) during 12 h from 31 July to 1 August, an increase from 22.2 to 25.0°C (i.e. of approximately 3°C), during 2 d (from 3 August to 5 August) and a decrease of approximately 3°C (from 25.6 to 22.7°C) during several hours on 8 August — as the time interval during which the activity of *A. japonicus* was inhibited (see Fig. 6).

Our results indicate that in the study area, under conditions of maximum summer temperature and its severe fluctuations, the potential decrease in feeding activity of *A. japonicus* adults is temporary and reversible. The maximum recorded duration of *A. japonicus* individuals in the shelter was 4.5 d. Our results are consistent with the study conducted by Nakahara et al. (2018), who found that in the relatively cold waters of Funka Bay, Hokkaido Island, the duration of the aestivation period of *A. japonicus* was 3 d on average (maximum: 12 d). They also found that not all individuals aestivated. At the same time, in the warmer waters of Yoshimi Bay, Honshu Island, where the period of temperature above 25°C was long (from July to October), *A. japonicus* aestivation lasted 84–104 d (Yamana et al. 2008). Thus, in a cool temperate zone, *A. japonicus* is able to feed for a much longer time than in warmer waters.

It should be noted that 2 other environmental variables (sea level and salinity) measured in parallel with temperature in our study were also quite variable during the study period, and their variations correlated with those of temperature. The Japanese sea cucumber *A. japonicus* inhabits a wide range of depths, from the intertidal zone down to depths of more than 100 m (Levin 2000). On the artificial reef, maximum and minimum depths of 2.74 and 1.18 m, respectively, were recorded. The lowest sea level coincided with the highest temperature in the bottom water layer (Fig. S7), probably due to downwelling as the warm surface water sinks. Water flow may be one of the factors influencing sea cucumber activity. Lin et al. (2020) reported from laboratory experiments that *A. japonicus* slowed down or even stopped moving at a water velocity of approximately 15 cm s⁻¹. Unfortunately, we did not measure the water velocity. However, Minami et al. (2019) found

that depth changes associated with tidal activity were a less important factor influencing the density of visible *A. japonicus* on the seafloor than temperature changes.

The normal salinity of seawater in the natural habitats of *A. japonicus* is usually between 29 and 34 psu (Gavrilova 1995, Kashenko 2000, Levin 2000, Liu et al. 2015). At the artificial reef, the maximum and minimum salinities were 33.2 and 28.3 psu, respectively. During the whole study period, the average hourly salinity was below 29 psu on only 3 occasions (28.3, 28.7 and 28.8 psu). This decrease in water salinity occurred on 20–21 July and coincided with the beginning of the period of extremely high temperatures (see Fig. S7). During this period, the salinity gradually increased to 32 psu. As the period of reduced salinity was very short, we believe that it was unlikely to be responsible for the decline in *A. japonicus* activity. Adult *A. japonicus* can survive a short-term decrease in salinity to 20–18 psu (Kashenko 2000).

A. japonicus juveniles showed no significant response to extremely high temperatures. This is consistent with the results of laboratory experiments showing that *A. japonicus* juveniles have a higher tolerance to elevated temperatures than adults. Based on the analysis of food consumption rate, Yang et al. (2005) concluded that the threshold temperature for aestivation of juvenile sea cucumbers was between 25.5 and 30.5°C. According to other laboratory reports, under experimental conditions, juvenile sea cucumbers did not enter aestivation even when exposed to 26°C for 20 d (Liu et al. 1996, Gao et al. 2018, 2019). Mature adults and immature juveniles of *A. japonicus* exhibited different metabolic physiological responses to heating, such as the rates of oxygen consumption (OCR) and ammonia-nitrogen excretion (AER) (Yang et al. 2006). The OCR of adults peaked at 20°C and then decreased significantly at higher temperatures, whereas the OCR of juveniles continued to increase slightly, even after 25°C. The AER of adults peaked at 20°C, while that of juveniles peaked at 25°C. A combination of lipid and protein was the main energy source in adults while protein was the main energy source in juveniles (Yang et al. 2006). Yuan et al. (2007) found that aestivation differently affected energy allocation in growth, respiration, feces discharge and ammonia excretion in *A. japonicus* with different body weights. Molecular studies revealed differences between adult and juvenile individuals of *A. japonicus* in the expression of genes encoding the heat shock proteins SHSP, HSP60/10, HSP90, HSP70/110 and DNAJ proteins (co-chaperones of

HSP70), which promote cell survival in response to elevated temperatures (Gao et al. 2018, 2019, 2022). Thus, an extremely high level of inducible expression of some *HSP70/110* was found in adult *A. japonicus* after entry into aestivation, whereas a relatively high level of constitutive expression of these genes was maintained in juveniles in the absence of aestivation. These results suggest that adult and juvenile individuals of *A. japonicus* use different strategies to adapt to heat stress.

Our results showed that *A. japonicus* juveniles increased significantly in size by the end of the observation period. This indicates that the habitat conditions on the artificial reef were suitable for their growth. Seasonal variations in the main environmental parameters, temperature and salinity, were not outside the optimal range for juvenile growth identified in laboratory experiments (Dong et al. 2006, 2008), and the feces of bivalve mollusks can serve as a food source for sea cucumbers, as shown in other laboratory experiments (Zhou et al. 2006, Yokoyama 2015).

4.5. Behavior of *A. japonicus* adults on the reef in relation to reproduction

The behavior of holothurians is driven by physiological requirements such as feeding, reproduction and the avoidance of stressors (Pierrat et al. 2022). We studied the behavior of *A. japonicus* adults, specifically their responses to the changes in illumination and temperature, during the spawning season, which coincides with the seasonal increase in seawater temperature and occurs during the summer (Tanaka 1958b, Gavrilova 1995, Zhou et al. 2001, Park et al. 2007, Wang et al. 2015, Kang et al. 2017). We recorded several *A. japonicus* spawning events, which took place between 26 June and 13 July, when temperature and salinity levels were within the optimal range for larval development of this species (reviewed by Qiu et al. 2015).

To our knowledge, this work is the first description of the reproductive behavior and diurnal spawning timing of *A. japonicus* in the field. The most important finding of our study is the clear dependence of the spawning activity of *A. japonicus* on the diurnal cycle. With rare exceptions, sea cucumbers exhibited spawning behavior and spawned after sunset and before sunrise. This finding is consistent with other results showing a strong dependence of *A. japonicus* activity on light levels, as well as previously reported data on the predominantly nocturnal lifestyle of this

species (reviewed by Zhang et al. 2015, Zhang & Lai 2024). However, to our surprise, data on the relationship between *A. japonicus* spawning activity and time of day are scarce in the available literature. Soliman et al. (2013) studied spontaneous spawning in *A. japonicus* in the laboratory and suggested nocturnal spawning based on the embryonic development of eggs collected in the morning. Previously, we studied the diurnal timing and temporal parameters of spawning in sea urchins *S. intermedius* and *M. nudus* and showed that they spawn mainly at dusk and during the night (Zhadan et al. 2018, 2021). These prior observations are consistent with the present results showing that spawning behavior and spawning of *A. japonicus* and other invertebrates (sea urchins, brittle stars and bivalves) on the artificial reef occurred exclusively in the evening or at night. Simultaneous spawning of several species was also recorded.

It should be emphasized that the multispecies spawning events on the artificial reef involving *A. japonicus* were sequential in nature, i.e. they took place over 2–3 d with breaks during daylight hours. Studies on the diel periodicity of multispecies spawning in marine invertebrates other than corals *in situ* are scarce. It appears that the closest to our study is the field study reported by Babcock et al. (1992), who collected data on spawning time and number of spawners of 69 invertebrate species (echinoderms, sponges, anthozoans, mollusks and polychaetes) during 159 diurnal and 55 nocturnal SCUBA dives on the Great Barrier Reef (GBR). Over the course of 3 summer seasons, 10 multispecies spawning events involving 11 holothurian species (the orders Holothuriida, Apodida, Synallactida and Dendrochirotida) were recorded (see Table 1 in Babcock et al. 1992), 9 of which occurred during the evening and at night (between 19:00 and 23:45 h). Spawning of single holothurian species was recorded 15 times, 11 of which were also nocturnal. Thus, although night observations were limited to the first half of the night, these results showed a predominantly nocturnal spawning pattern for holothurians and other participants in multispecies spawning. Our round-the-clock observations of invertebrate behavior on the artificial reef support the conclusions of Babcock et al. (1992) that (1) multispecies spawning is common for benthic invertebrates, and echinoderms in particular, and (2) multispecies spawning of invertebrates occurs predominantly at night. In addition, our results provide strong evidence that the spawning of sea cucumbers and other invertebrates is serial in nature, continuing over several consecutive nights with a break during daylight hours. There is also some evidence that

spawning of the same invertebrate species occurs on consecutive nights with a daytime break on the GBR (see Table 1 in Babcock et al. 1992).

Our observations have revealed several interesting features in the spawning behavior of *A. japonicus in situ*. The first is the apparent increase in movement speed and the intention to reach the highest point on the reef prior to spawning. Climbing up the side of the tank towards the water surface has previously been observed in this holothurian species following artificial spawn stimulation in the laboratory (Fujiwara et al. 2010, Liu et al. 2015). At the same time, there are surprisingly few reports of similar reproductive behavior in holothurians in the wild. McEuen (1988) observed a wandering behavior of *Cucumaria lubrica* males both in the laboratory and in the field, in which they climbed to the tops of culture dishes, the sides of seawater tanks, rock walls and boulders prior to spawning, while females remained firmly attached to the substrate. Babcock et al. (1992) reported that during multispecies spawning on the GBR, the sea cucumbers *Stichopus variegatus* (now *Stichopus herrmanni* Semper) and *Actinopyga lecanora* were commonly observed climbing up onto coral outcrops before spawning. The rest of the 11 holothurian species did not exhibit this behavior and often spawned on open ground.

The second important feature of *A. japonicus* spawning behavior was that the sea cucumbers gathered at the top of the reef, raised the anterior end of the body in a vertical position and swayed vigorously. Such a characteristic posture appears to be a widespread behavioral trait exhibited by many holothurian species belonging to different holothurian orders (Cameron & Fankboner 1986, McEuen 1988, Pearse et al. 1988, Conand 1993; see Mercier & Hamel 2009 for a review).

In general, such behavior of *A. japonicus* during spawning seems to be aimed at grouping individuals at the elevated site and promoting an increase in the quantity of gametes shed and their dispersal, thus increasing the chances of egg fertilization. The other mobile echinoderms, the sea urchins *S. intermedius* and *M. nudus*, showed similar behavior before and during spawning (highly accelerated upward movement on a vertical surface) (Zhadan et al. 2018, 2021). During mass spawning events with 10 or more individuals, the sea urchins also moved closer to each other by increasing their speed of movement (Zhadan et al. 2021).

An important feature of the spawning process itself (i.e. gamete shedding) was that spawning did not occur in all individuals of *A. japonicus* and sea

urchins that exhibited spawning behavior. Only 8 out of 27 individuals of *A. japonicus* that reached the top of the reef and exhibited the typical spawning posture actually spawned. Similarly, only about 30% of sea urchins *M. nudus* that reached the top of the reef spawned. During previously described mass spawning events, both spawners and non-spawners of sea urchins *S. intermedius* and *M. nudus* also exhibited spawning behavior (Zhadan et al. 2018, 2021). Babcock et al. (1992) also reported several cases of holothurians exhibiting a spawning posture without gamete shedding.

As can be seen from Fig. 8, multispecies spawning events on the reef started with both the accelerated movement of sea cucumbers and sea urchins and the spawning of bivalves. Furthermore, during the largest multispecies spawning events (26–27 June and 13 July), echinoderm spawning behavior and bivalve spawning started synchronously. This suggests that both processes (spawning behavior and spawning) were triggered by the same external stimulus. The fact that the spawning behavior of sea cucumbers and sea urchins does not always result in spawning may be explained by insufficient stimulus intensity and/or immaturity of the animals. According to Thorson (1950), an increase in the phytoplankton level may be the universal trigger of spawning in marine invertebrates with external fertilization (broadcast spawners) that have feeding (planktotrophic) larvae. Many other environmental factors — photoperiod, time of day, drastic changes in light intensity at sunset, temperature, salinity, seawater velocity and lunar phase — have also been proposed as spawning cues for different broadcast spawners, and especially holothurians (see Mercier & Hamel 2009 for a review). In our study, during the 6 h period preceding the spawning of *A. japonicus*, small fluctuations in temperature (within 1°C, except for the spawning event on 12 July when the temperature increased by 2.2°C), salinity (within 1 psu) and sea level (within 0.5 m) were observed (Table S8). The most intensive spawning events started 1 h before sunset on 26 June and 1.5 h after sunset on 12 July, 1 d after the full moon and 2 d after the new moon, respectively (see Fig. 8). These results suggest that none of the above environmental variables can be considered as the main trigger for *A. japonicus* spawning on the artificial reef during our study. Given the planktotrophic mode of development of *A. japonicus*, we suggest that phytoplankton abundance is the most likely environmental cue for spawning in this species. This suggestion is supported by previous studies on the role of phytoplankton in stimulating spawning in natural popula-

tions of another echinoderm species with planktotrophic larva, the sea urchin *S. intermedius*. Under conditions of extremely low phytoplankton concentration, the reproductive cycle of *S. intermedius* populations did not end with complete spawning, and unshed gametes were resorbed (Zhadan et al. 2016, 2018). In addition, we previously found a direct positive correlation between chlorophyll *a* concentrations and spawning intensity in natural populations of *S. intermedius* (Zhadan et al. 2018). It should be noted that in the present study, the sea urchin *S. intermedius* exhibited spawning behavior at the same time as *A. japonicus*. Therefore, it is not unlikely that these 2 species are sensitive to the same spawning trigger. Further studies are needed to test whether there is a relationship between phytoplankton abundance and spawning of *A. japonicus* in nature.

The reproductive state obviously influenced the seasonal distribution of *A. japonicus* adults on the reef. Their presence on the reef top was only recorded during the first month of observations and was clearly related to the spawning events that occurred at that time. The end of the breeding season coincided with the beginning of the period of strong changes in seawater temperature, when the state of reduced activity of *A. japonicus* adults was recorded. It is interesting to note that this state could be associated not only with extremely high temperature but also with the post-spawning rest period. The coincidence of aestivation with the end of the reproductive cycle in *A. japonicus* inhabiting Peter the Great Bay (Sea of Japan) was previously noted by Levin (2000). He also emphasized that the onset and end of sea cucumber aestivation occurred at different times in different areas of the bay and could not always be explained by high temperature alone. In our study, the period of reduced activity of *A. japonicus* adults was rather short (~20 d) and did not significantly affect their total number on the reef. However, the redistribution of sea cucumbers on the reef during the observation period was evident and seemed to be related to the changes in the functional needs of *A. japonicus*. The tendency for sea cucumbers to spend more time off the reef towards the end of the study period could be explained by the need for intensive feeding due to the need to replenish energy resources used for reproduction. This change in the behavior of *A. japonicus* can probably be considered the first stage of a seasonal transition to a less cryptic mode, which underlies the seasonal variation in the visible density of this species in nature (Yamana et al. 2009b, Kashio et al. 2016, Minami et al. 2019). However, it should be noted that throughout the entire period of our obser-

vations, *A. japonicus* adults spent most of their time on the side surfaces of the reef populated by mussels *C. grayanus* and on the settlement of the Pacific oyster *C. gigas* (Fig. 3B,C). This indicates that bivalve settlements on the reef met the feeding and shelter needs of *A. japonicus* during the breeding season.

In conclusion, the results of our field study on the behavior of adult and juvenile individuals of *A. japonicus* on an artificial reef populated by mussels and oysters may have implications for the development of co-culture with bivalve mollusks and restocking technologies for this species. Knowledge of the following behavioral traits associated with the reproduction of *A. japonicus* may be of the most interest: (1) the use of the reef as a shelter during the daytime during the spawning period reduces the risk of predation and helps to maintain the reproductive potential of the species; (2) adult *A. japonicus* group together on the reef top during spawning, apparently ensuring successful fertilization and production of numerous offspring; (3) simultaneous spawning of sea cucumbers and other invertebrates inhabiting the reef (multi-species spawning events) may help to reduce predation pressure on offspring, thereby enhancing the reproductive success of *A. japonicus*; (4) during the period of high temperature fluctuation, adult *A. japonicus* increase the time they use the reef for shelter; and (5) live bivalve mollusks provide a suitable substrate for the settlement of *A. japonicus* larvae and a suitable habitat for *A. japonicus* juveniles, which can use the mollusk shells for shelter and the mollusk feces as a food source.

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