

Assessing the effect of environmental factors on the spawning activity of the sea urchin *Strongylocentrotus intermedius* through video recording observations

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ABSTRACT: We used video recordings of spawning behaviour of the sea urchin *Strongylocentrotus intermedius* conducted in parallel with an analysis of gonadal state and measurement of environmental variables (tidal level, phytoplankton, temperature, salinity and oxygen concentration) *in situ* to study the role of environmental factors in triggering sea urchin spawning. The observations were performed during 3 reproductive seasons, each of 2 mo duration, in 2 bays with different levels of phytoplankton (measured as chlorophyll *a* [chl *a*]). The only significant positive correlation found was between sea urchin spawning frequency and chl *a* concentration. Despite a high level of gonad maturity, sea urchin spawning was rarely recorded in the bay with low levels of chl *a* whereas mass spawning occurred in the bay with higher chl *a* concentration, with the peak of spawning activity centred around the full moon. Spawning was predominantly nocturnal. Immediately before and during spawning, males actively moved. Females began to spawn only when there were several spawning males nearby. In both sexes, each spawning event included several episodes of gamete release of different duration and with different intervals between them. The same male likely can participate in several spawning events. The following hierarchy of drivers of *S. intermedius* spawning activity was revealed: (1) increasing the phytoplankton concentration induced active motion and spawning in males, (2) both an elevated phytoplankton level and the presence of sperm stimulated spawning in females and (3) the night time and the new and full lunar phases increased the probability of spawning.

KEY WORDS: Broadcast spawning · Echinoderms · Reproductive synchrony · Phytoplankton · Temperature · Circadian rhythm · Lunar cycle

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INTRODUCTION

Many marine invertebrates with external fertilization (broadcast spawners) have feeding (planktotrophic) larvae (Thorson 1950, Jablonski & Lutz 1983, Mercier et al. 2013, Monro & Marshall 2015). It is generally believed that the spawning period of these animals is timed with the seasons, when sets of

most important abiotic (e.g. temperature, salinity) and biotic (phytoplankton production) conditions are favourable for offspring development and survival (Thorson 1950, Giese 1959, Giese & Pearse 1974, Olive 1992, 1995, Himmelman 1999, Reitzel et al. 2004, Mercier & Hamel 2009). Synchronization of spawning between individuals of both sexes is crucial for fertilization success in populations of such spe-

cies, especially when they inhabit shallow subtidal zones under conditions of high hydrodynamic activity that may lead to rapid gamete dispersal upon release (Pennington 1985, Levitan & Petersen 1995, Yund & Meidel 2003, Levitan 2004, Reuter & Levitan 2010).

The concept of seasonality and synchrony of reproduction in marine invertebrates with planktotrophic larvae suggests that animals are capable of perceiving certain environmental cues to determine if conditions are appropriate for spawning. Identification of these cues is necessary to understand the environmental and physiological mechanisms involved in triggering spawning activity in broadcast spawners. Sea urchins are widely used as model organisms to study different aspects of the reproductive biology of broadcast spawners both in laboratory experiments and under field conditions, but despite intensive studies, the environmental factors that trigger and synchronize sea urchin spawning are not yet fully understood (see Mercier & Hamel 2009 for review). Moreover, even results obtained under controlled laboratory conditions sometimes lead to controversial findings regarding the degree of influence and priority of the environmental stimuli that are considered appropriate spawning triggers. For example, Starr et al. (1990, 1992) showed that the addition of various species of microalgae and their extracts to the water stimulated spawning in the sea urchin *Strongylocentrotus droebachiensis* in a dose-dependent manner, and that sea urchin sperm enhanced the phytoplankton stimulus. In contrast, Reuter & Levitan (2010) found that phytoplankton alone did not induce spawning in the sea urchin *Lytechinus variegatus*, but it decreased the reaction time of sea urchins to sperm. In the field experiments of McCarthy & Young (2004) however, no consistent reaction of *L. variegatus* to sperm was observed. Mercier & Hamel (2010) conducted a laboratory mesocosm experiment to study the spawning behaviour of 26 species of marine invertebrates, including the sea urchin *S. droebachiensis*, and suggested that the wave of synchronous spawning was likely triggered by an increased level of phytoplankton and that lunar periodicity may be the external synchronizer for most animals studied.

Field studies of different sea urchin species have also produced uncertain results regarding the role of environmental variables in triggering sea urchin spawning. A coincidence of spawning activity has been observed with an increase in phytoplankton concentration (Himmelman 1975, Starr et al. 1993, Gaudette et al. 2006, González-Irusta et al. 2010, Egea et al. 2011, Bronstein et al. 2016, Zhadan et al. 2016), an increase or decrease in water temperature

(Tsuji et al. 1989, King et al. 1994, Byrne et al. 1998, Guillou & Lumingas 1998, Lamare & Stewart 1998, Himmelman et al. 2008, Egea et al. 2011) and with different phases of the moon cycle (Pearse 1975, Iliffe & Pearse 1982, Lessios 1991, Coppard et al. 2005, Gaudette et al. 2006, Zhadan et al. 2016). A study of the sea urchin *Paracentrotus lividus* from different localities in the Cantabrian Sea (the south portion of the Bay of Biscay in the Atlantic Ocean) concluded that in some cases, sea urchins with completely mature gonads may spawn in the absence of environmental triggers (González-Irusta et al. 2010). On the contrary, the results of our study of the sea urchin *Strongylocentrotus intermedius* from several localities in the northwestern Sea of Japan (Pacific Ocean) showed that in some years, both males and females with fully mature gonads failed to end their reproductive cycle with complete spawning, which strongly suggests the need for external stimulus/stimuli to induce spawning in *S. intermedius* populations (Zhadan et al. 2015).

The multiple and often contradictory inferences regarding the role of environmental cues in the induction of sea urchin spawning may be due to this process being influenced by multiple natural factors (see Mercier & Hamel 2009 for review). However, most field studies provide only indirect evidence of the nature of the stimuli triggering sea urchin spawning due to the low frequency of animal sampling for gonadal analysis (usually once mo⁻¹) and insufficient environmental data. Diving observations of sea urchin spawning events in the field are rare (e.g. Pearse et al. 1988, Levitan 2002, Himmelman et al. 2008), and no one spawning event can be directly linked to a certain environmental cue (Gaudette et al. 2006, Reuter & Levitan 2010). To analyse the relationships between the timing of spawning of *S. intermedius* populations and the associated environmental parameters, we conducted a fine-scale temporal study in which sea urchins were sampled at intervals of 3 to 12 d (4.2 d on average) and environmental variables were directly monitored in sea urchin habitats (Zhadan et al. 2016). This study revealed a significant positive relationship between the probability of spawning events and periods with increased concentration of chlorophyll *a* (chl *a*; an index of phytoplankton biomass), but no relationships were apparent between temperature, salinity, dissolved oxygen or tidal activity and the timing of spawning. Our results also showed that the new and full moon phases may be additional factors that affect the triggering of *S. intermedius* spawning (Zhadan et al. 2016).

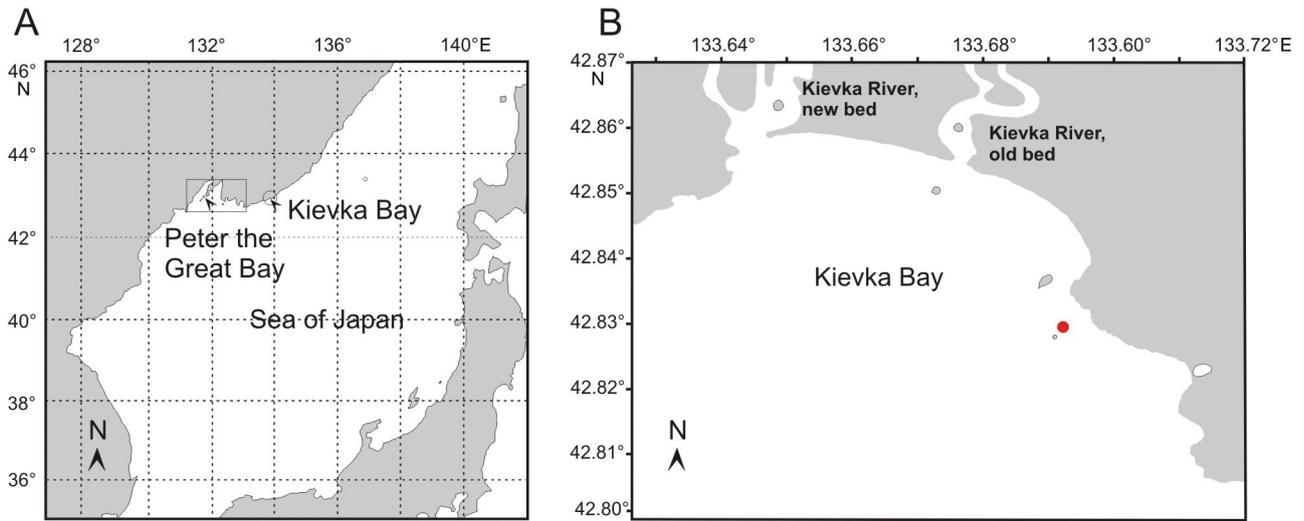


Fig. 1. (A) Study area in the northwestern Sea of Japan. Rectangle: Peter the Great Bay, arrowheads: Popov Island and Kievka Bay. Study sites in (B) Kievka Bay and (C) Alekseev Bay are denoted by red circles

It should be noted that we employed traditional methods in the above-mentioned work to determine the timing of sea urchin spawning based on the analysis of gonadal state, which allows the time interval between 2 sea urchin spawning event(s) (spawning window) to be defined. The accuracy of such a laborious and time-consuming determination greatly depends on sampling frequency, and furthermore, the temporal resolution of this approach does not allow for a correlation between spawning events and the dynamics of environmental variables.

In continuation of our previous work, the present study was undertaken to identify the role of internal (the degree of gonadal maturity) and external (environmental variables) factors in triggering spawning in *S. intermedius* populations. To achieve this goal, we used an integrated methodological approach that included both traditional methods of sea urchin gonad examination (with a sampling frequency of 3 to 7 d) and video recording of sea urchin spawning behaviour (at 1 min intervals) in combination with automatic datalogging of environmental variables in the habitats of the study animals (at 10 min intervals). This approach allowed us to (1) register sea urchin spawning events and the associated environmental variables (temperature, salinity, chl *a* and oxygen concentrations) at the finest temporal resolution for this type of study to date; (2) perform a correlation analysis between the data sets for these parameters and sea urchin spawning activity; (3) perform de-

tailed quantitative analysis of video records of sea urchin behaviour, with the aim of elucidating the features of prespawning and spawning behaviour in males and females, including individual temporal characteristics; (4) assess the distribution of spawning events throughout the day; and (5) compare the data on sea urchin spawning activity assessed by video recording and data on gonadal maturity.

MATERIALS AND METHODS

Study areas and populations

The study was conducted from 2014 to 2016 in 2 bays in the northwestern Sea of Japan (Fig. 1A): Kievka Bay (42.830°N, 133.691°E) (Fig. 1B) and Alekseev Bay (42.981°N, 131.730°E) (Fig. 1C). Three separate sets of observations were performed; 2 in

Kievka Bay (August and September of 2014 and 2015) and one in Alekseev Bay (May and June of 2016). The timing of the studies was chosen to coincide with *Strongylocentrotus intermedius* spawning seasons, which occur in August and September in Kievka Bay (Zhadan et al. 2015), and May and June in Alekseev Bay (Zaslavskaya et al. 2012). Kievka Bay (Fig. 1B) is an open bay with a width of 8.3 km and a length of 3.3 km, and is typical of the south-eastern coast of the Primorye region of Russia; the bay is open to the prevailing winds from the south-east to the southwest. This bay is located in a sparsely populated area adjoining the forest reserve and therefore is not subjected to any important human impact. Primary productivity in this bay is influenced by the wind-induced upwelling associated with the monsoon, which is most pronounced in summer and autumn seasons (Zvalinskii et al. 2006). Alekseev Bay (Fig. 1C) is one of the bays of Popov Island, in Amursky Bay (Peter the Great Bay, Sea of Japan). Amursky Bay is subject to a chronic influence of industrial and municipal wastes of Vladivostok, the largest sea port in the Russian Far East, and has been recognized as being the most eutrophicated area of Peter the Great Bay (Aleksanin et al. 2012). Our previous studies in 2011 and 2012 (Zhadan et al. 2016) and a preliminary experiment in 2015 (P. Zhadan unpubl. data) showed that the concentration of chl *a* in Alekseev Bay is higher than that in Kievka Bay in *S. intermedius* spawning seasons, which provides an opportunity to compare the spawning of sea urchins in water bodies with different phytoplankton levels. Alekseev Bay has a width of 0.8 km and a length of 1.3 km, and is thus more closed than Kievka Bay, and subjected to winds from the north.

Video recording experimental setup and analysis

The video observation of sea urchins was performed with TLC200 Pro (Brinno Incorporated) time-lapse video cameras; time-lapse videos were taken in 1 min intervals at a resolution of 1280 × 720 pixels. During the night, the fields of view of the cameras (approximately 1.0 × 1.5 m) were illuminated by lanterns (1 W) with an impulse duration of 1 s. The camera flash LED indicator was used to synchronize the cameras and lanterns, which were mounted on stanchions approximately 1 m above the bottom. Flat mesh containers, 1.1 × 0.75 m in size, were filled with approximately 30 kg of the kelp *Saccharina japonica* (Laminariales, Phaeophyta; hereafter simply laminaria) and placed near each camera to attract the sea

urchins into the field of view. During each study season (2 mo), the contents of the containers were changed 3 times. In Kievka Bay, 2 and 4 cameras were used in 2014 and 2015, respectively, and they were placed at a depth of 6 m on relatively flat bedrock with an area of 10 × 15 m surrounded by large stones. In Alekseev Bay, the cameras were located at a depth of 2.5 m near the base of a concrete pier. Two cameras were directed at the containers filled with laminaria, which were located near the base of the pier, and 2 additional cameras were aimed at the pier wall above the containers.

The recorded videos were viewed frame by frame to determine the number of sea urchins in the field of view of each camera, the numbers of spawning males and females, the exact time and duration of the release of each gamete batch, the time intervals between the gamete batches, and the individual spawning duration, which included both the duration of gamete release and the intervals between the gamete batches. During spawning, *S. intermedius* sex is easily distinguishable due to different colors of gametes (white in males and orange in females; see Video S1 in the Supplement at www.int-res.com/articles/suppl/m588p101_supp/). The beginning and end of gamete releases were determined with an accuracy of 1 min. In the cases when spawning of the specimen was recorded on 1 frame only, the individual spawning duration was considered to be of 1 min. Short periods of time (a few minutes) when several males spawned simultaneously were not included in this analysis because of high turbidity of the water.

Sea urchin motion activity was analysed using the free software 'Tracker' for video analysis (www.open-sourcephysics.org/items/detail.cfm?ID=7365). The cell size (2 × 2 cm) of the mesh container was used as a scale to measure the distance moved by each sea urchin. Videos showing the presence of 10 or more spawning sea urchins during 60 to 90 min were used for tracking purposes. The position of each individual was tracked over a period of 200 min (total 200 points). The distance moved by each spawning sea urchin during a period which started 10 min before the first gamete release and finished 5 min after the end of spawning was determined and compared with the distance for a period of equal duration prior to the beginning of spawning. The distances moved by each nonspawning sea urchin for 1 h from the beginning of the tracking (in the absence of spawning sea urchins) and for a period of 1 h in the presence of spawning sea urchins were also compared. Overall, 33 spawning males, 11 spawning females and 96 nonspawning sea urchins were tracked.

Measurement of environmental variables

The video camera stanchions were placed 2 m apart from each other, so the distance between the containers with laminaria was approximately 1 to 2 m. Midway between the video camera stanchions, a multi-parameter RBRXR-620 sonde (Sea and Land Technologies) was installed 50 cm above the bottom, and the water depth, temperature, and salinity as well as photosynthetically active radiation (PAR) and the concentrations of chl *a* and dissolved oxygen were measured at 10 min intervals. The video camera stanchions and the data logger were connected by an imaginary line parallel to the direction of the tidal currents. This arrangement of devices ensured that the environmental parameters at locations of the video cameras and data logger were the same.

The chl *a* sensors were calibrated as previously described (Zhadan et al. 2016). Briefly, 1.0 to 1.5 l of sea water was filtered through Whatman glass-fibre filters GF/F (0.7 µm nominal pore size) and frozen (−20°C) until subsequent analysis. Pigments were extracted with 90% acetone and measured by spectrophotometry, and chl *a* concentrations were calculated using the SCORE UNESCO equations (UNESCO 1966). To compensate for the dependence of chl *a* sensor data on illumination intensity, PAR data were used to correct them.

Sea urchin sampling and processing

A rough estimate showed that there were approximately 200 specimens of *S. intermedius* within a radius of 20 m from the cameras before the experiment in Kievka Bay in both survey seasons, so the sea urchin population in this location was replenished with 450 and 650 individuals in 2014 and 2015, respectively. For this purpose, sea urchins were collected within a 100 to 150 m radius of the cameras and carefully transplanted within a 5 to 10 m radius. In Alekseev Bay, there were initially approximately 60 individuals within a radius of 10 m from the cameras, so an additional 450 individuals were transplanted. At the beginning of each experiment, 35 sea urchins were placed on the containers with laminaria, directly in the cameras' field of view.

To analyse the gonadal state, between 20 and 30 sea urchins (5 to 10 individuals from the field of view of each camera) were sampled from 10:00 to 11:00 h every 3 to 4 d in Kievka Bay and every 7 d in Alekseev Bay. After sampling, the number of sea urchins in the field of view of the cameras was replenished

from the 'stock' sea urchin populations near the installations. Thus, for the duration of the experiment, no less than 568, 521 and 422 sea urchins in the field of view of the cameras were replaced with other individuals in 2014, 2015 and 2016, respectively.

The gonadal state was examined as previously described (Zhadan et al. 2016). Briefly, all specimens were dissected after being measured and weighed; the gonads were weighed, and the gonad index (GI) was calculated using the following formula: $GI (\%) = [(\text{gonad wet weight} / \text{total wet weight}) \times 100]$. Individuals of each sex that released gametes from their gonopores after dissection were categorized as ready for spawning. To determine the state of maturation, small pieces of female gonads were used to prepare smears for examination under a compound light microscope, and based on the micro- and macroscopic characteristics of the gonads, the following stages of ovarian maturation were determined: (1) immature, (2) premature, (3) partially mature, (4) mature, (5) spent and (6) over-mature (Zhadan et al. 2016).

Statistical analysis

The distributions of all data sets were checked for normality (D'Agostino and Pearson normality tests, $p < 0.05$) and homogeneity of variance (Bartlett's test and Fisher's *t*-test, $p < 0.05$), and normally distributed data were analysed by parametric tests (unpaired *t*-tests, 1-sample *t*-tests, 1-way ANOVA). The GI values were transformed using the equation $p' = \arcsin\sqrt{p}$ to normalize their distribution, and were then analysed by 1-way ANOVA followed by Tukey's post hoc test. For skewed distributions, non-parametric tests (Spearman's rank-order correlation, Fisher's exact test, Wilcoxon test, Mann-Whitney test) were applied. All statistical analyses were run using GraphPad Prism v.6.0 for Windows (GraphPad Software). The number of females and males ready for spawning and the number of spent females in each sample were analysed using binomial statistics (Zar 2010), and the data obtained by the RBRXR-620 data logger were edited to remove spikes and then averaged per hour.

Circular statistics for axially distributed data (Zar 2010) were performed to determine if there was a relationship between spawning timing and time of day.

To determine whether there were relationships between sea urchin seasonal spawning activity and environmental variables (temperature, chl *a*, oxygen concentrations and salinity), 2 statistical methods

were used. First, Spearman's rank-order correlation was applied to examine the relationship between the hourly averaged values of each parameter and the hourly frequency of spawning events determined as the ratio of sea urchins that spawned during the corresponding hour to the total number of sea urchins recorded in the camera field of view. This analysis was performed separately for each data set (Kievka Bay 2014, Kievka Bay 2015 and Alekseev Bay 2016), which allowed the effects of environmental variables on the seasonal spawning activity of sea urchins to be assessed for different study areas and years. For calculation of the threshold values of environmental variables responsible for spawning initiation, linear regression analysis for non-uniformly distributed data was performed to fit a straight-line model. These analyses were run using GraphPad Prism v.6.0. Second, generalized linear models for binomial data (logistic regression) were applied to assess the relationships between the interday variation in sea urchin spawning activity and environmental variables; this analysis was performed on pooled data from Kievka Bay in 2014 and 2015. The observation of a spawning event was used as a binary dependent variable; i.e. a value of 1 was assigned to each time interval between 16:00 and 02:00 h over 2 reproductive seasons when at least 1 spawning individual was found, and a value of 0 was assigned to each such time interval when spawning did not

occur. The hourly averaged values of each parameter were automatically arranged into 3 groups (low, middle and high) and used as independent factors (predictors), and both the separate and combined influences of these predictors were analysed. The significance of the relationships between the spawning events and predictors was evaluated by dispersion analysis (1-way ANOVA) using a chi-square test. Logistic regression procedures were implemented using the free and open-source R software (R Development Core Team 2010). However, we were unable to analyse a data set obtained in Alekseev Bay in the same way because of the short period of intensive sea urchin spawning recorded in May 2016; R software failed to properly arrange the environmental data set to obtain the predictors.

RESULTS

Environmental characteristics of the study areas

Sea level and weather

In both bays, an irregular semidiurnal tide with a maximum amplitude of approximately 50 cm was detected (Figs. 2–4), and the sea urchins in the more open Kievka Bay were subjected to storms to a greater extent than those in Alekseev Bay (the

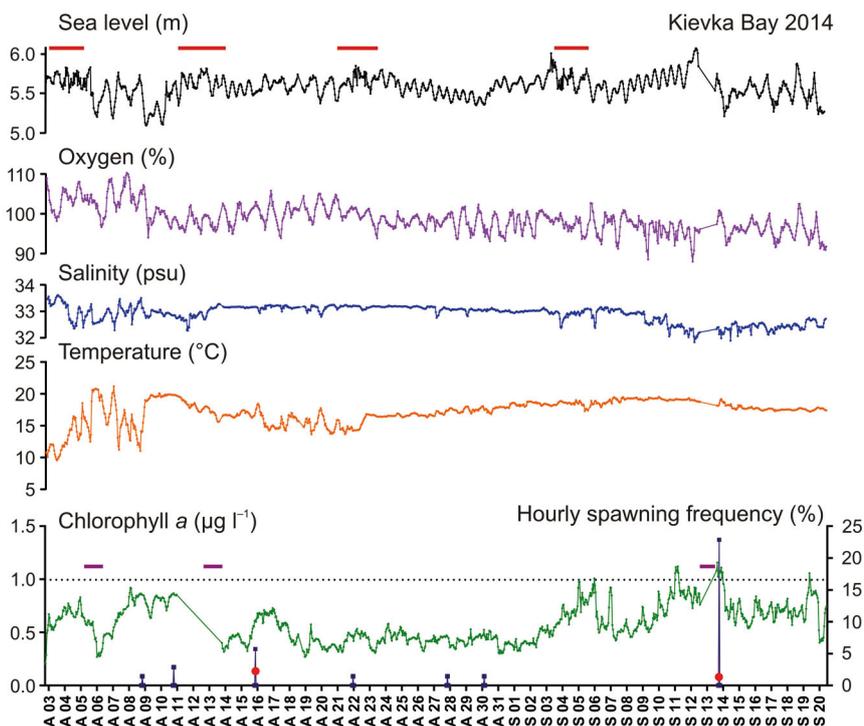


Fig. 2. Timing of spawning of the sea urchin *Strongylocentrotus intermedius* assessed through video recording and associated environmental conditions in August and September 2014 in Kievka Bay (northwestern Sea of Japan). Data for environmental variables are presented as average hourly values. Horizontal dotted line: concentration of chl *a* corresponding to $1 \mu\text{g l}^{-1}$; blue squares and red circles in the lower graph: hourly spawning frequency of males and females, respectively; horizontal solid red lines in the upper graph: storm periods; horizontal solid violet lines in the lower graph: periods when the video cameras did not work because of a full memory card, high turbidity, etc. x-axis: month and date

Fig. 3. Timing of spawning of the sea urchin *Strongylocentrotus intermedius* assessed through video recording and associated environmental conditions in August and September 2015 in Kievka Bay (northwestern Sea of Japan). Details as in Fig. 2. Detection of oxygen was stopped on 27 August because of destruction of the oxygen sensor

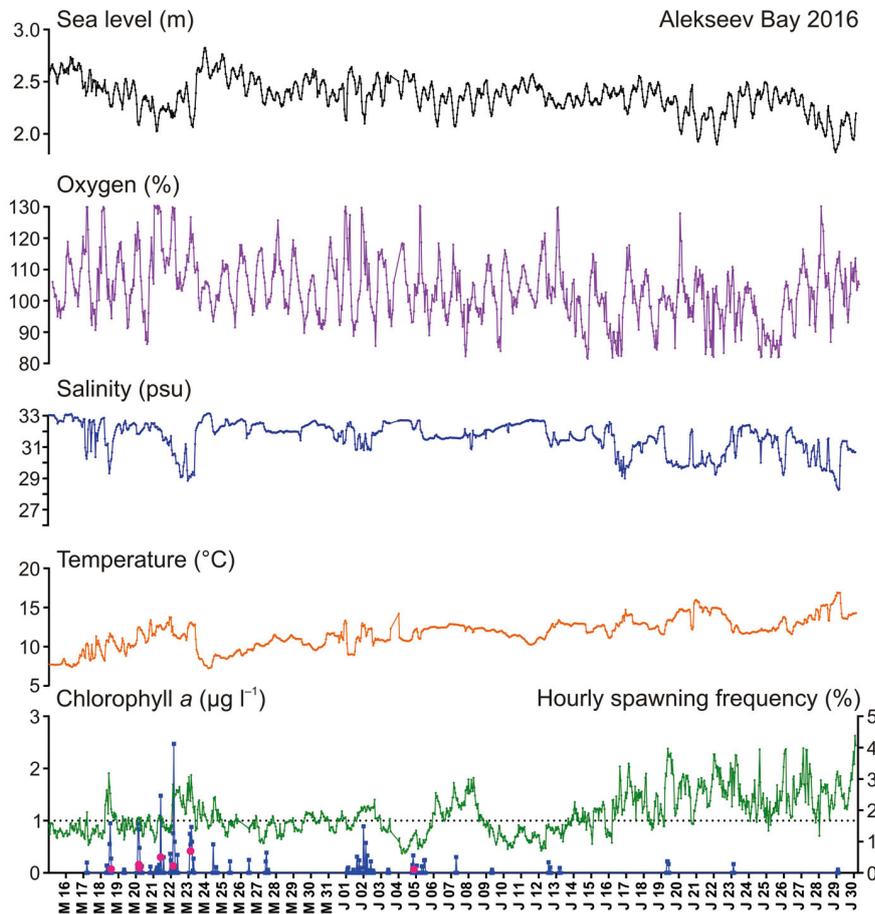
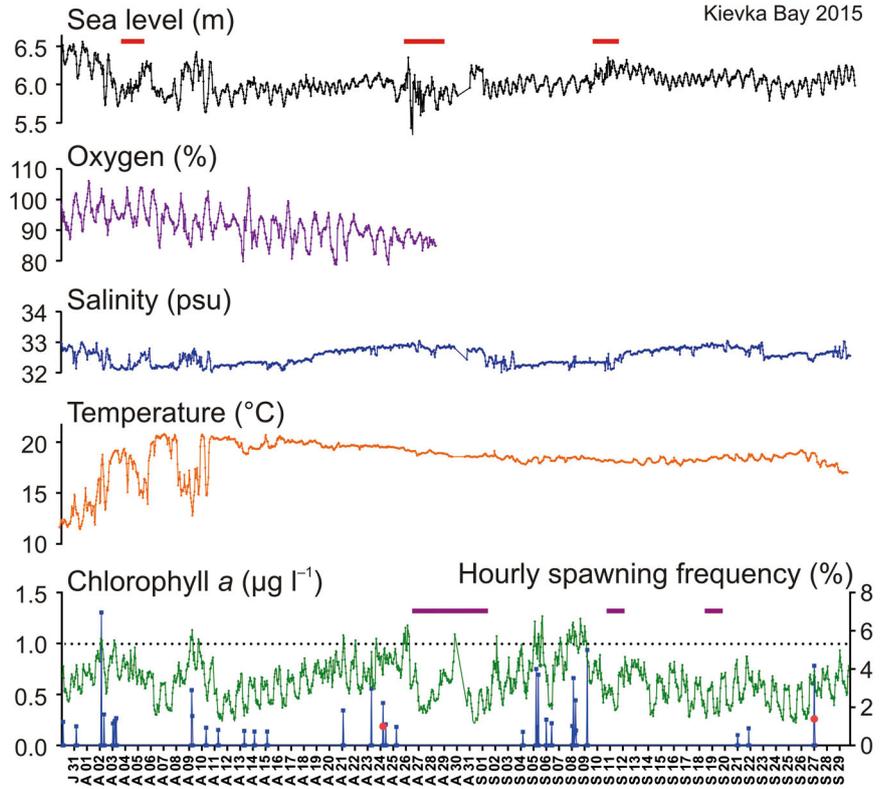


Fig. 4. Timing of spawning of the sea urchin *Strongylocentrotus intermedius* assessed through video recording and associated environmental conditions in May and June 2016 in Alekseev Bay (northwestern Sea of Japan). Data for environmental variables are presented as average hourly values. Horizontal dotted line: concentration of chl a corresponding to $1 \mu\text{g l}^{-1}$; blue squares and red circles in the lower graph: hourly spawning frequency of males and females, respectively. x-axis: month and date

periods of stormy weather are denoted by solid red lines in the upper pictures in Figs. 2 & 3). An especially heavy storm was associated with Typhoon Goni in late August 2015, and based on the loss of spikes, approximately 5% of sea urchins were damaged during this storm.

Oxygen

In both bays, the median oxygen saturation value of the bottom water was close to 100%. The level of oxygen saturation increased in the daytime and decreased at night, with a significant positive correlation between time of day and oxygen saturation (Spearman's $r = 0.39$, $p < 0.001$). The diurnal oscillation of this parameter was higher in Alekseev Bay than in Kievka Bay (Figs. 2–4).

Salinity

In Kievka Bay, salinity was relatively stable during both study periods (Figs. 2 & 3), varying from 31.03 to 33.04 psu with a median value of 32.47 psu. In Alekseev Bay, the variation in salinity was greater (Fig. 4), from 28.17 to 33.08 (median = 31.72 psu), probably due to the site's proximity to the coast and shallower depth.

Temperature

The temperature dynamics in Kievka Bay were similar in 2014 and 2015. In the first half of August, there were sharp temperature fluctuations of approximately 10 to 21°C, after which there was a period of relatively stable temperature until the end of September (Figs. 2 & 3). In Alekseev Bay, a gradual seasonal heating of seawater occurred during the study period, and the temperature increased from approximately 7 to 17°C (Fig. 4). A sharp decrease in temperature was observed on 23 and 24 May, followed by warming from 25 to 28 May (Fig. 4).

Chl *a*

In Kievka Bay, the chl *a* concentration rarely exceeded 1 $\mu\text{g l}^{-1}$ (Figs. 2 & 3). In 2015, the median chl *a* concentration was 0.64 $\mu\text{g l}^{-1}$ (min. = 0.23; max. = 1.27 $\mu\text{g l}^{-1}$), which was significantly higher than in 2014 (Mann-Whitney test, $p < 0.0001$; median = 0.52,

min. = 0.26, max. = 1.13 $\mu\text{g l}^{-1}$). In Alekseev Bay, chl *a* concentrations often exceeded 1 $\mu\text{g l}^{-1}$ (Fig. 4), and the median value of 0.83 $\mu\text{g l}^{-1}$ (min. = 0.35, max. = 2.62 $\mu\text{g l}^{-1}$) was significantly higher than that of Kievka Bay (Mann-Whitney test, $p < 0.0001$).

Dynamics of gonadal state

Sex ratio and gonad index

In both bays, the overall mean male:female ratio in all samples did not deviate significantly from 1 (1-sample *t*-test, $t_{38} = 1.465$, $p = 0.151$ and $t_{18} = 1.549$, $p = 0.139$ for Kievka Bay and Alekseev Bay, respectively). As no significant difference in the GI was detected between sexes (unpaired *t*-test, all $p > 0.05$), the GI data from both sexes were pooled (Figs. 5A, 6A & 7A). In both study seasons in Kievka Bay, no significant differences in the GI were observed between each 2 successive sea urchin samples (ANOVA and Tukey's post hoc test, all $p > 0.05$) (Figs. 5A & 6A). However, ANOVA revealed a significant decrease in the GI during both reproductive seasons ($F_{14,593} = 2.916$, $p < 0.01$ and $F_{19,501} = 2.141$, $p < 0.01$ for 2014 and 2015, respectively). In Alekseev Bay in 2016, the only significant decrease in the GI occurred between 22 and 29 May (ANOVA and Tukey's post hoc test, $q_{211} = 5.394$, $p < 0.01$), and there were no significant changes thereafter (ANOVA, $F_{5,131} = 0.483$, $p = 0.79$) (Fig. 7A). However, ANOVA revealed a significant decrease in the GI over the study season (ANOVA, $F_{8,211} = 6.92$, $p < 0.0001$).

Readiness for spawning

In both bays, the proportions of male sea urchins ready for spawning were higher than those of the females (blue and red lines in Figs. 5A, 6A & 7A). In Kievka Bay, this parameter ranged from 80 to 100% in both study years. In 2014, the proportion of females ready for spawning increased from 40 to 80% in August and decreased to 70% (including the individuals with over-mature fragmented eggs) by the end of September (Fig. 5A), while in August and September 2015, this parameter ranged from 50 to 100% (Fig. 6A). However, there were no significant differences between successive samples in both years (binomial statistics, $p > 0.05$). In Alekseev Bay during 8 to 22 May 2016, the proportions of males and females ready for spawning were 60 and 40%, respectively (Fig. 7A), and this parameter signi-

Fig. 5. (A) Temporal dynamics of the gonad index (brown bars: mean \pm SD), percentages of males (blue lines with squares) and females (red lines with circles) ready for spawning and (B) percentages of ovarian maturity stages in sea urchins *Strongylocentrotus intermedius* from Kievka Bay (northwestern Sea of Japan) in 2014. Shaded areas: storm periods. x-axis: month and date

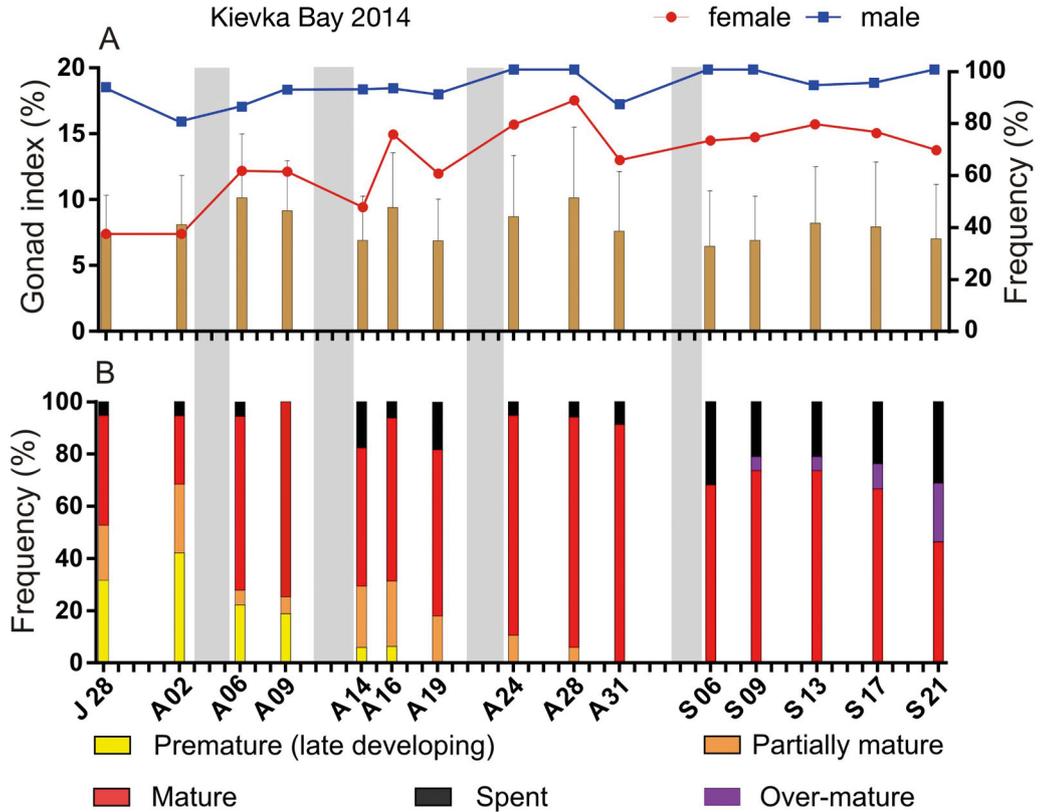
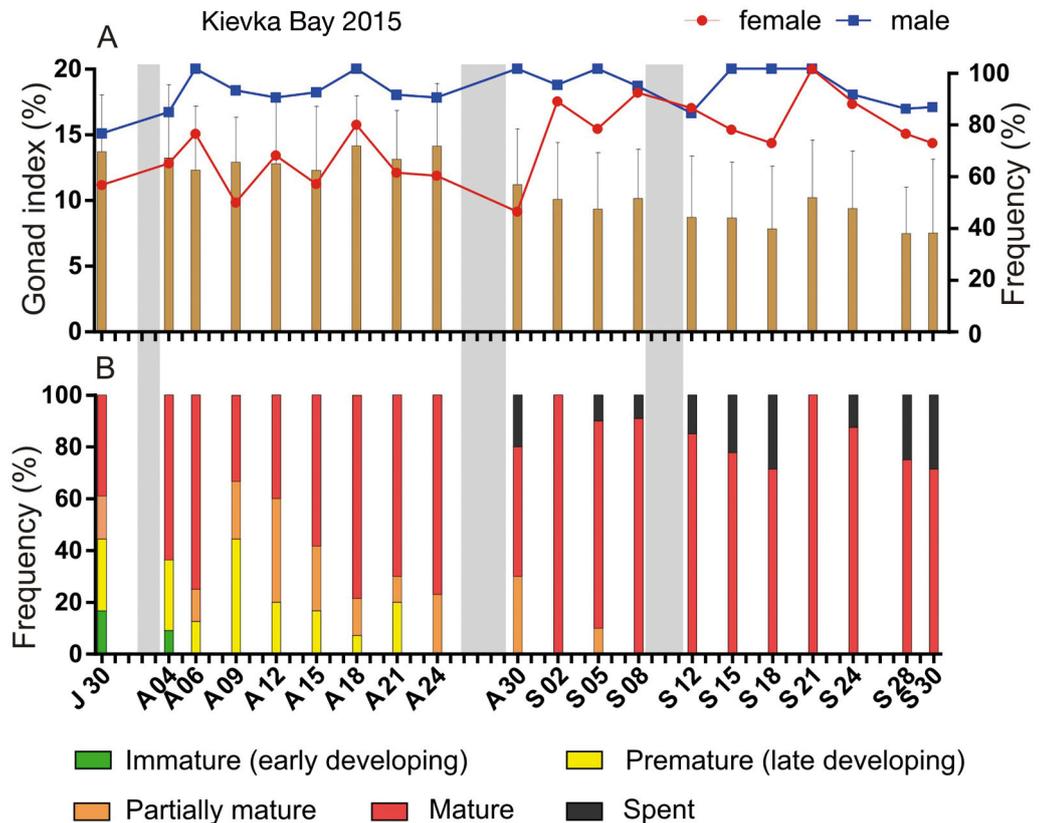


Fig. 6. (A) Temporal dynamics of the gonad index (brown bars: mean \pm SD), percentages of males (blue lines with squares) and females (red lines with circles) ready for spawning and (B) percentages of ovarian maturity stages in sea urchins *Strongylocentrotus intermedius* from Kievka Bay (northwestern Sea of Japan) in 2015. Shaded areas: storm periods. x-axis: month and date



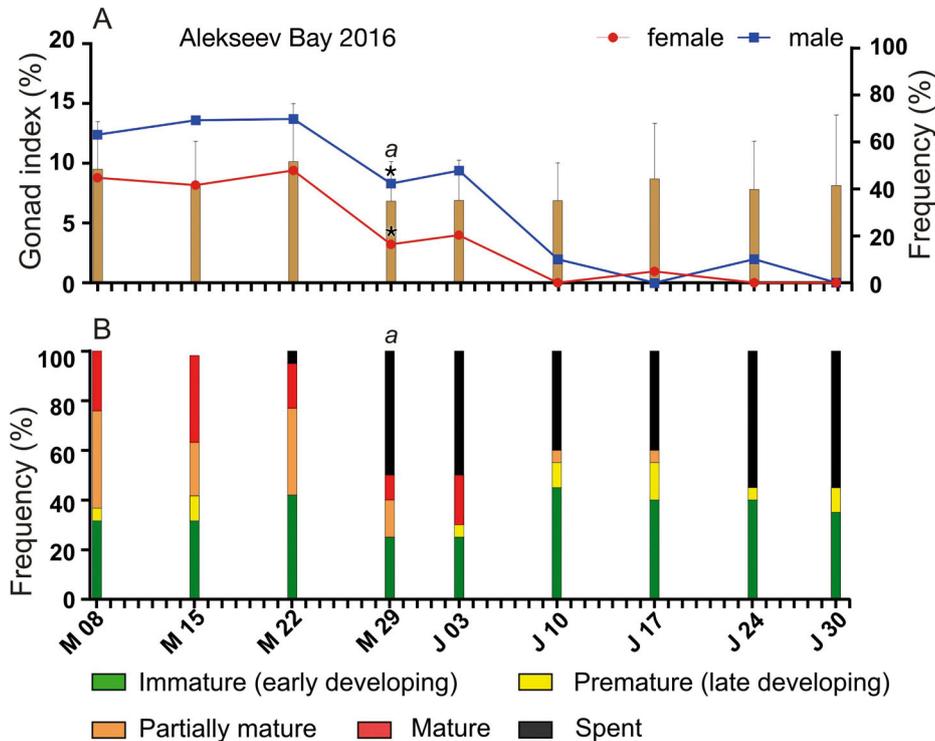


Fig. 7. (A) Temporal dynamics of the gonad index (brown bars: mean \pm SD), percentages of males (blue lines with squares) and females (red lines with circles) ready for spawning and (B) percentages of ovarian maturity stages in sea urchins *Strongylocentrotus intermedius* from Alekseev Bay (northwestern Sea of Japan) in 2016. Lowercase 'a' above the column in (A) denotes a significant decrease in the gonad index ($p < 0.01$, 1-way ANOVA, Tukey's multiple comparison test); asterisks indicate a significant decrease in the proportions of males and females ready for spawning ($p < 0.05$, binomial statistics). Lowercase 'a' above the column in (B) denotes a significant increase in the proportion of spent females ($p < 0.05$, binomial statistics). x-axis: month and date

significantly decreased in both sexes between 22 and 29 May (binomial statistics, $p < 0.05$) and was close to zero between 10 and 30 June (Fig. 7A).

Ovarian maturity

In Kievka Bay in August 2014 and 2015, most of the sampled females were at the premature (2), partially mature (3) and mature (4) stages of maturation (Figs. 5B & 6B), but in 2015, ovarian development was slightly retarded compared with that in 2014, based on the presence of immature females (less than 20%) in the 30 July and 4 August samples. Fully spent females (stage 5 of ovarian maturation) were observed from late July 2014 and late August 2015 to the end of September, but their proportion was not higher than 31%. There were no significant differences in this parameter between successive samples (binomial statistics, $p > 0.05$). In the samples from September 2014, from 5 to 23% of females had large gonads that released both morphologically normal and over-mature fragmented eggs (stage 6 of ovarian maturity) (Fig. 5B).

In Alekseev Bay over the period of 8 May through 30 June 2016, 30 to 40% of females were in the immature (1) stage of maturation (Fig. 7B), which is likely because the *Strongylocentrotus intermedius* population in this bay has 2 spawning peaks, one in

May–June and another in August–September (Zaslavskaya et al. 2012). Between 8 and 22 May, approximately 60% of females were in the partially mature (3) and mature (4) maturation stages, and by 29 May, that proportion decreased to 25% (Fig. 7B). At the same time, the number of spent females sharply increased (Fig. 7B; binomial statistics, $p < 0.05$), as to by the end of June, no females with mature or partially mature gonads were found.

Spawning behaviour

The mean (\pm SD) number of sea urchins in the field of view of the cameras was relatively stable at 32 ± 9 individuals. During storms, the number of sea urchins on the containers with laminaria significantly decreased (unpaired t -test, $t_{428} = 10$, $p < 0.0001$); the animals stopped feeding and grouped around the bases of the large stones.

Just before and during spawning, the males actively moved. The distance moved by spawning males for a period beginning approximately 10 min before and ending approximately 5 min after spawning was 5.5 times longer than the distance for a period of equal duration preceding spawning (t -test, $t_{64} = 5.007$, $p < 0.0001$) (Fig. 8). The distances moved by spawning females before and during spawning did not differ significantly (t -test, $t_{20} = 1.139$, $p =$

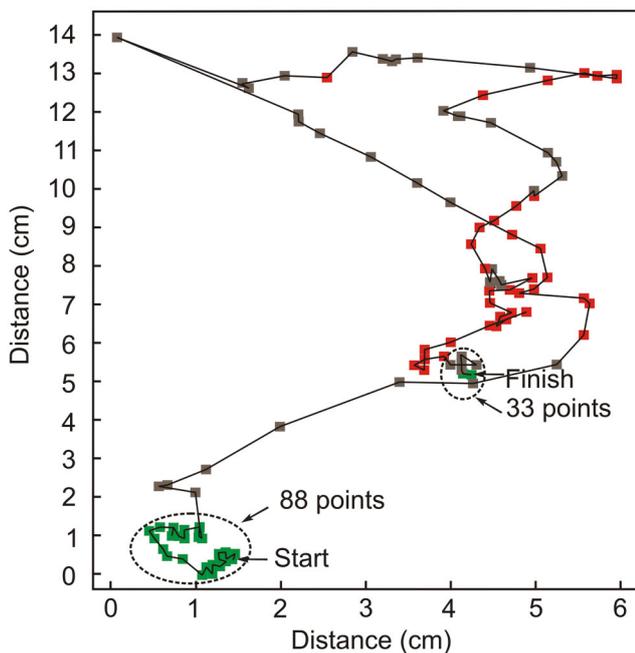


Fig. 8. Representative movement trajectory of an individual male sea urchin *Strongylocentrotus intermedius* on a horizontal surface. Distance measurements were made at 1 min intervals. Green squares bounded by dashed lines (start and finish): trajectory of the sea urchin before and after spawning and include numerous overlapped points; brown and red squares: the distance moved by spawning males for a period beginning approximately 10 min before and ending approximately 5 min after spawning; red squares: periods of sperm release

0.268). The distances that nonspawning sea urchins moved for 1 h periods in the absence and presence of spawning sea urchins did not differ significantly (t -test, $t_{94} = 0.628$, $p = 0.541$). On a horizontal surface, sea urchin movements were multidirectional (Fig. 8, Video S1 in the Supplement). On a vertical surface (the wall of a concrete pier in Alekseev Bay), sea urchins moved strongly upward before and during spawning, and their motion activity was distinctly visible (see Video S2 in the Supplement). Downward movement of sea urchins started 6 to 8 h after the beginning of their upward movement and lasted approximately 1.5 h.

Once in Kievka Bay and thrice in Alekseev Bay, aggregations of 10 to 15 spawning sea urchins crawling over one another were observed (see Video S1); there were always spawning females in these aggregations.

The sea urchins released their gametes in batches, and the release duration of one gamete portion (the time over which a gamete clot was present in the area near the gonopores) varied from 1 to 17 min (3.9

± 3.8 min, mean \pm SD). The intervals between the batches ranged from 1 to 27 min (6.8 ± 7.2 min), and the length of such intermittent spawning by an individual sea urchin varied from 1 to 46 min (21.4 ± 12.4 min). The maximum number of gamete batches was 3 and 5 for females and males, respectively.

Males spawned both alone (only males that spawned alone during a 1 h interval were classified as alone-spawning males) and in groups of 2 or more individuals. In Kievka Bay, the portion of alone-spawning males (55%) was significantly higher than in Alekseev Bay (31%; Fisher's exact test, $p = 0.0297$). Females began to spawn only when there were several spawning males nearby (see Video S1), so the numbers of spawning males and females were positively correlated (Spearman's $r = 0.637$, $p < 0.0001$).

Relationships between spawning activity and environmental variables

The significantly higher chl *a* concentration in Alekseev Bay corresponded to a higher average daily frequency of *S. intermedius* spawning events compared to that in Kievka Bay (Fig. 9).

Seasonal *S. intermedius* spawning activity as assessed by video recording varied between the different bays. In Kievka Bay, sea urchin spawning was rarely recorded; during August and September 2014, 23 spawning males and 3 spawning females were

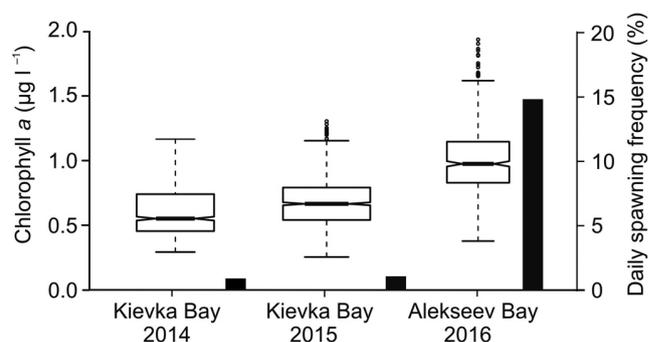


Fig. 9. Chl *a* concentrations (box plot) determined for the spawning seasons of the sea urchin *Strongylocentrotus intermedius* in Kievka and Alekseev bays (northwestern Sea of Japan) and average daily spawning frequency (black columns). Boxes indicate 25th and 75th percentiles; solid line in the box: the median (50th percentile); whiskers: minimum and maximum values; circles: outliers. The width of each box is proportional to the sample size; the notch indicates the 95% confidence interval of the median. The median chl *a* concentrations are all significantly different from each other ($p < 0.001$, Wilcoxon rank sum test with continuity correction)

recorded, and in 2015, 83 spawning males and 3 spawning females were observed. The time intervals between spawning events varied from 1 to 14 d (Figs. 2 & 3). In Alekseev Bay in 2016, spawning intensity was much higher: 388 spawning males and 16 spawning females were recorded. The peak of spawning activity was centred around the full moon between 20 and 24 May, when 55% of males and 88% of females spawned (Fig. 4). Most spawning events (95%) occurred between 17 May and 13 June, and after that, only solitary spawning males were found. Therefore, this period was used for the correlation analysis between environmental variables and sea urchin spawning activity in Alekseev Bay, whereas the data from both study periods in Kievka Bay were used.

Spearman's correlation analysis revealed no significant relationships between the hourly frequency of spawning events in *S. intermedius* populations from both study areas and temperature, salinity or oxygen saturation. At the same time, there was a significant positive relationship between the hourly frequency of spawning events and chl *a* concentration. This correlation was characterized by relatively low Spearman's correlation coefficients (0.112, 0.167 and 0.305 for 2014, 2015 and 2016, respectively) and a high level of significance ($p < 0.001$), which was due to the presence of a large number of structural zeros in the contingency tables because the number of en-

vironmental variable measurements greatly exceeded the number of recorded spawning events.

The fact that the significantly higher chl *a* concentration in Alekseev Bay compared to that in Kievka Bay corresponded to a higher average daily frequency of spawning events (Fig. 9) also indicates a cause-and-effect relationship between phytoplankton concentration and sea urchin spawning activity. Linear regression analysis revealed that sea urchin spawning was initiated when a certain chl *a* concentration (the threshold value) was attained. For Alekseev Bay, this threshold value was $0.79 \mu\text{g l}^{-1}$ (95% CI = $0.68\text{--}0.87 \mu\text{g l}^{-1}$) whereas for Kievka Bay it was lower: $0.47 \mu\text{g l}^{-1}$ ($0.29\text{--}0.56 \mu\text{g l}^{-1}$) and $0.49 \mu\text{g l}^{-1}$ ($0.34\text{--}0.57 \mu\text{g l}^{-1}$) in 2014 and 2015, respectively.

In both bays, *S. intermedius* spawning events were not uniformly distributed over a 24 h period (Rayleigh's test for circular uniformity, $p < 0.005$ and $p < 0.002$ for Kievka Bay and Alekseev Bay, respectively). In Kievka Bay, all spawning events were recorded between 16:00 and 02:00 h (Fig. 10A), and the mean of this interval of increased spawning activity occurred at sunset, $20:58 \pm 03:20$ h (mean \pm SD, circular statistics), when PAR levels at the bottom in August and September were $6.7 \pm 7.9 \mu\text{M m}^{-2} \text{s}^{-1}$ and close to zero, respectively. For comparison, PAR during the period from 12:00 to 13:00 h was $379 \pm 162 \mu\text{M m}^{-2} \text{s}^{-1}$. In Alekseev Bay, most spawning events (74.8%) were recorded at night, and the

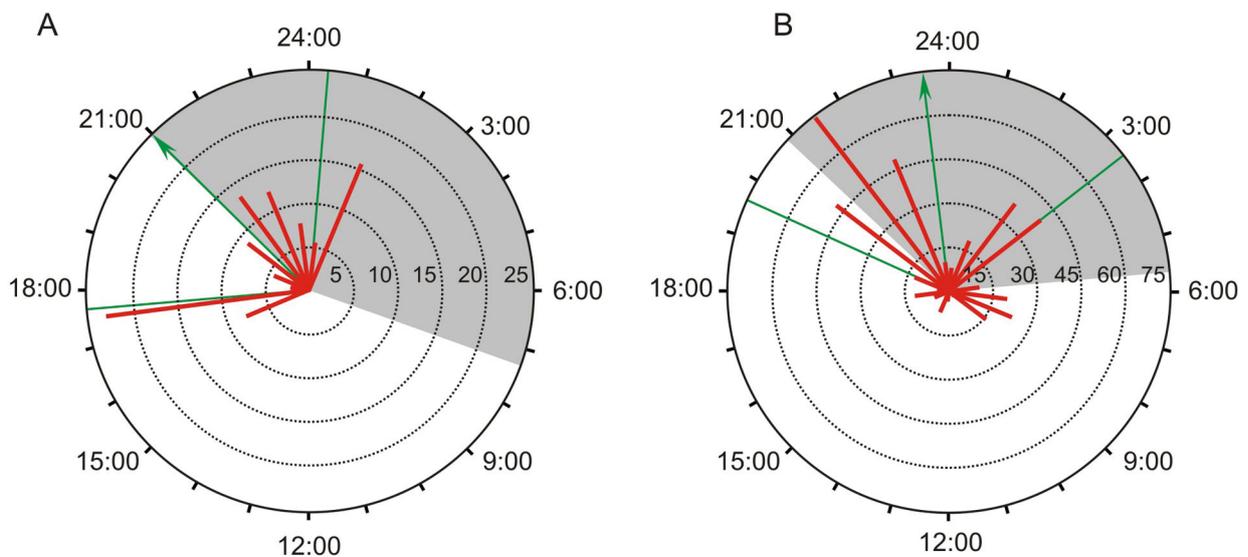


Fig. 10. Distribution of spawning events of the sea urchin *Strongylocentrotus intermedius* over a 24 h period: (A) Kievka Bay, pooled data from 2014 and 2015; (B) Alekseev Bay, data from 2016. Shaded areas: time of day when photosynthetically active radiation at the bottom level was less than $6.7 \mu\text{M m}^{-2} \text{s}^{-1}$. Solid red lines and figures inside the circles denote the numbers of spawning events. Green arrows and solid lines indicate the means of distributions of spawning events and standard deviations, respectively

Table 1. Medians of environmental variables for 2 periods: the period of increased diurnal spawning activity of the sea urchin *Strongylocentrotus intermedius* and the rest of the day (separated by a slash) and significance ($p < 0.05$, in **bold**) of their differences (Mann-Whitney test)

Study area and year	Chlorophyll <i>a</i> ($\mu\text{g l}^{-1}$)	<i>p</i>	Temperature ($^{\circ}\text{C}$)	<i>p</i>	Salinity (psu)	<i>p</i>	Oxygen (%)	<i>p</i>
Kievka Bay (16:00–02:00 h / 02:00–16:00 h)								
2014	0.51 / 0.54	0.79	17.4 / 17.5	0.29	33.0 / 33.1	0.09	100.5 / 99.5	<0.0001
2015	0.65 / 0.63	0.85	18.8 / 18.5	<0.0001	32.6 / 32.5	0.29	102.3 / 100.5	0.002
Alekseev Bay (20:50–05:40 h / 05:40–20:50 h)								
2016	0.78 / 0.88	0.26	10.1 / 10.3	0.83	32.2 / 32.1	0.92	96.5 / 101.0	0.002

mean of the increased spawning activity interval corresponded to $23:33 \pm 04:35$ h (mean \pm SD, circular statistics) (Fig. 10B). The value of this parameter for the pooled sample of sea urchins from both bays was $22:03 \pm 04:35$ (mean \pm SD, circular statistics).

To understand the non-uniform distribution of *S. intermedius* spawning events throughout the day, we compared environmental variables within each bay over the entire season for 2 periods: (1) the period of increased spawning activity (16:00 to 02:00 h for Kievka Bay, 100% of spawning events; and 20:50 to 05:40 h for Alekseev Bay, 75% of spawning events) and (2) the rest of the day. In Kievka Bay, the first period differed in terms of slightly increased temperature in 2015 (a median difference of 0.3°C) and oxygen saturation (a median difference of 1.0 to 1.8%), whereas in Alekseev Bay, the only difference was 4.5% lower oxygen saturation (Table 1). Salinity and chl *a* concentration did not differ significantly between these 2 periods (Table 1). This indicates that the non-uniform distribution of spawning events over the day was not determined by intraday variability of the parameters studied.

As demonstrated above (Figs. 2 & 3), sea urchin spawning activity in Kievka Bay was characterized by high interday variability during 2 reproductive seasons, so to understand the reasons, we conducted logistic regression analysis. No significant relationships were revealed between the interday variation in the spawning activity of the *S. intermedius* population from Kievka Bay and temperature, salinity or oxygen saturation, while a significant positive relationship was found between the probability of sea urchin spawning and chl *a* concentration (ANOVA, $p = 0.01$). In other words, the probability of sea urchin spawning increased markedly when the favourable time of day (night) and an elevated phytoplankton concentration coincided.

DISCUSSION

Combining of methods of gonadal analysis and video recording of *Strongylocentrotus intermedius* spawning behaviour in parallel with automatic data-logging of environmental variables (a combination that has been applied for the first time for this type of study) allowed us to reveal the relationships between these variables and the spawning process in natural populations of sea urchins. The studies were conducted in 2 bays with different levels of primary production; low (Kievka Bay) and relatively high (Alekseev Bay). This design provided an additional opportunity to clarify the link between sea urchin spawning and phytoplankton abundance, the most likely key environmental spawning trigger in natural sea urchin populations (see Mercier & Hamel 2009 for review).

The results of the present study confirmed our previous findings regarding the temporal patterns of gonad ripening and spawning in *S. intermedius* populations inhabiting these 2 bays and the relationships between sea urchin spawning and environmental conditions (Zaslavskaya et al. 2012, Zhadan et al. 2015, 2016). First, the ripening of sea urchin gonads and spawning in Kievka and Alekseev bays took place in different seasons, namely, August–September and May–June, respectively. This agrees with our previous observations in the northwestern Sea of Japan, where there are 3 types of *S. intermedius* populations that differ from each other in the proportions of individuals with different spawning schedules: (1) almost 100% of individuals exhibit autumn spawning, (2) almost 100% of individuals exhibit early summer spawning, and (3) approximately equal proportions of individuals exhibit autumn and early summer spawning (Zaslavskaya et al. 2012). Sea urchin populations from Kievka and Alekseev bays corresponded to the first and third types,

respectively, but the reasons for such inter- and intra-population variation in spawning timing remain unclear and are beyond the scope of the present discussion.

Secondly, the results of the present study showed that although *S. intermedius* in Kievka Bay has the potential to spawn at any time in August and throughout most of September, its reproductive cycle may not end in complete spawning; in 2014 and 2015, approximately 70% of females failed to spawn by the end of September (Figs. 5B & 6B). In 2008, 2009 and 2011, we found 95, 53 and 30% of *S. intermedius* females, respectively, to be unspawned at this site (Zhadan et al. 2015, 2016).

Thirdly, the most important result of the present study is the demonstration of a significant positive correlation between increased phytoplankton production and *S. intermedius* spawning activity as assessed through video recording. Thus, we have found strong statistical support for the existence of a key link between an environmental cue (phytoplankton) and spawning induction in sea urchin species with planktotrophic larvae *in situ*. This result confirmed our earlier suggestion that increasing phytoplankton concentration might be the proximate stimulus for triggering spawning in *S. intermedius* natural populations (Zhadan et al. 2016). Based on the data obtained from our previous and present studies, we can derive some general conclusions about the patterns of the *S. intermedius* reproductive cycle and spawning under conditions of low and relatively high primary production. When phytoplankton concentration is lower than a certain level (corresponding to a chl *a* concentration of approximately $1 \mu\text{g l}^{-1}$) over the whole summer–autumn period, the spawning season is extended, the reproductive parameters (such as GI and readiness for spawning) exhibit no significant changes, and the average daily frequency of spawning events, as assessed by video recording, is very low. Part of the sea urchin population does not complete the reproductive cycle by spawning, so the unspawned eggs and sperm are resorbed. This phenomenon of spawning failure seems to be common in *S. intermedius* populations in waters with low phytoplankton production during the summer–autumn period; as supported by the findings that the sea urchin spawning season in Kievka Bay can last for more than 2 mo (this study), and spawning failure was observed during 4 reproductive seasons between 2008 and 2015 (Zhadan et al. 2015, 2016, this study). Under conditions of relatively high phytoplankton abundance (chl *a* concentration of approximately $1 \mu\text{g l}^{-1}$ and

higher over the whole study season), the spawning period lasts <1 mo as determined from a sharp decrease in parameters such as GI and readiness for spawning, and a significant increase in the proportion of spent females. Additionally, the average daily frequency of spawning events observed through video recording was much higher.

Another important finding of this study involves insights into the spawning behaviour of *S. intermedius in situ*. The most representative data were obtained from observations in Alekseev Bay, where spawning intensity was higher than in Kievka Bay, and we showed, for the first time, that sea urchin males began to move actively before spawning and retained this activity over the spawning process, and that the vertical movement of both males and females was directed strongly upward. The latter agrees with observations of the spawning behaviour of the sea urchin *Lytechinus variegatus* under experimental conditions (Mercier & Hamel 2010, Reuter & Levitan 2010), and there is evidence in the literature of similar spawning behaviour in other groups of echinoderms in their natural habitat (Fishelson 1968, Hendler & Meyer 1982, Babcock et al. 1992, Gladstone 1992, Himmelman et al. 2008). We suggest that more active movement of sea urchin males as well as the vertical movement behaviour of both sexes during spawning are directed to help disperse the sperm and eggs after their release from the gonopores.

Data on spawning duration and patterns of gamete release in sea urchins *in situ* are scant (see Lotterhos & Levitan 2010 for review). Based on occasional diving observations of natural spawning events in 3 sea urchin species (*Strongylocentrotus franciscanus*, *S. droebachiensis* and *Diadema antillarum*), Lotterhos & Levitan (2010) characterized their spawning process as ‘continuous’ (plume) lasting for 1 to 2 h. In contrast, our results revealed an intermittent (‘pulse’) pattern of spawning of *S. intermedius* males and females. Quantitative analysis of video records showed that each spawning event lasts from 1 to 46 min and includes several episodes of gamete release of different duration, with different intervals between them. This discrepancy indicates that further studies are necessary to clarify the gaps in knowledge about temporal characteristics of sea urchin spawning.

It is generally believed that gregarious behaviour allows for gametes to be concentrated, which can enhance fertilization in broadcast spawners (Pennington 1985, Babcock et al. 1992, Young et al. 1992, Himmelman et al. 2008). During our observations (i.e. over 3 reproductive seasons), only 4 large sea

urchin aggregations (10 to 15 spawning males and females) were observed in the field of view of the cameras. No spawning aggregations of the sea urchin *S. droebachiensis* were found during a study of mass-spawning echinoderms off the Mingan Islands in the northern Gulf of St. Lawrence in eastern Canada (Himmelman et al. 2008), but it should be noted that the sea urchin density in that study (50 ind. m^{-2}) as well as in our studies (60 ind. m^{-2}) seems to be high enough to result in successful fertilization. This density value is one order higher than that in the areas adjacent to the cameras (1 to 2 ind. m^{-2}). However, a SCUBA diving survey of the adjacent area revealed no aggregations of spawning sea urchins (P. Zhadan pers. obs.), but *S. intermedius* aggregations frequently occurred around the base of large stones under storm conditions and in the laminaria thickets. Thus, our observations confirm the suggestion by Pennington (1985), the *in situ* experimental findings by McCarthy & Young (2002, 2004) and the direct field observations by Himmelman et al. (2008) that echinoids do not aggregate in preparation for spawning, although some of the bathyal echinoid species may be an exception (Young et al. 1992).

Judging from *in situ* observations, the males of different classes of echinoderms usually begin to spawn first (see Mercier & Hamel 2009 for review), as exhibited by the sea urchins *S. droebachiensis* and *L. variegatus* under experimental conditions (Starr et al. 1990, Mercier & Hamel 2010, Reuter & Levitan 2010) and *S. intermedius* under field conditions (this study). Our study revealed many instances of males spawning alone over 1 h, and such spawning prevailed in Kievka Bay, where a predominantly low phytoplankton concentration was observed over the 2 survey seasons. This means that even under conditions of high sea urchin density and a high level of gonad maturity in both males and females, sperm release alone cannot trigger spawning in other individuals. This is consistent with the *in situ* experimental observation that the sperm of *L. variegatus* failed to initiate spawning in conspecifics (McCarthy & Young 2004), but it is important to note that environmental parameters were not monitored in that study. In contrast, high spawning frequency prevailed in Alekseev Bay, where a relatively high phytoplankton concentration was recorded, and this agrees with the results of both field and laboratory studies indicating that the presence of phytoplankton can induce spawning in the sea urchins *S. droebachiensis* and *S. intermedius*, and that a combination of phytoplankton and sperm exerts a synergistic effect on spawning activity (Starr et al. 1990, 1992, Zhadan et al. 2016). At the same

time, our data contradict the results of a laboratory study that showed that sperm alone but not phytoplankton alone could stimulate spawning in *L. variegatus* (Reuter & Levitan 2010). Our finding that spawning behaviour of sea urchins begins from an increase in movement activity of males which are able to spawn alone clearly indicates that sperm cannot be considered as the primary inducer of *S. intermedius* spawning. This discrepancy may be explained by species-specific reactions to spawning triggers.

The next important finding is that *S. intermedius* males may spawn repeatedly during the spawning season, which is evident from the data obtained from Alekseev Bay. There were approximately 400 individuals in the camera field of view (see 'Materials and methods'), and 388 spawning males were recorded. Considering that the female:male ratio in *S. intermedius* is close to 1 and, as mentioned above, only approximately half of the sea urchin population in this bay spawns in May–June, one can conclude that a single spawning event could be recorded approximately 100 times. The actual observation of 388 spawning events suggests that the males partially spawn 3 to 4 times during the season.

It should be also noted that the female:male ratio during the spawning events in our study was 1:17, which is one order lower than the corresponding value (approximately 1:1.7 to 1:1.5) obtained for the sea urchins *S. droebachiensis* and *L. variegatus* in laboratory experiments (Starr et al. 1992, Reuter & Levitan 2010). At the same time, the results of a SCUBA diving survey conducted in the northern Gulf of St. Lawrence showed that the ratio of spawning *S. droebachiensis* females and males was 20% to 80% (i.e. 1:4; Himmelman et al. 2008). Field observations of spawning events in several groups of echinoderms (Holothuroidea, Asteroidea, Crinoidea and Ophiuroidea) conducted by SCUBA divers at Davies Reef (Great Barrier Reef) found an average female:male ratio for all of the study species to be approximately 1:7 (Babcock et al. 1992). Possible explanations for these quantitative differences lie in variations between field and laboratory conditions, features of the methodological approaches as well as species-specific characteristics.

One additional quantitative discrepancy that should be discussed is the difference between the proportion of spawning females recorded by the video cameras and the proportion of spent females determined by gonadal analysis, which was most obvious from the results obtained from Kievka Bay. In 2014 and 2015, 3 spawning females were recorded each year, which constitutes approximately 9.6 and 4.8% of the

total number of females (as estimated from the average number of sea urchins in the field of view of the cameras, assuming a 1:1 female:male ratio). At the same time, the proportions of spent females by the end of September were 31 and 28% in 2014 and 2015, respectively; such a deficit could be due to underestimating the actual number of spawning females because of several breaks in the video recording caused by storms (see Figs. 2 & 3). Thus, after Typhoon Goni (25 to 30 August 2015), 20% of spent females appeared (see Fig. 6), but this could not be video-recorded because of damage to the installation. The study season in Alekseev Bay was characterized by calm weather and the absence of installation faults, so the proportion of spawning females was approximately 90% of the total number of females calculated.

Taken together, our results revealed the following link between spawning events in natural *S. intermedius* populations and spawning triggers: under low phytoplankton concentrations, the spawning of solitary males prevails, which cannot stimulate mass spawning in other males and females. An increase in phytoplankton concentration promotes the spawning frequency of males, and an elevated concentration of phytoplankton combined with a high sperm concentration stimulates spawning in female sea urchins. In addition, there is an evident link between spawning events in *S. intermedius* populations and the phases of the new and full moons (Zhadan et al. 2016, this study). However, given the fact that complete spawning of *S. intermedius* populations might or might not take place despite the high level of gonadal maturity and the same moon cycle, lunar phases cannot be regarded to be the proximate environmental cue triggering *S. intermedius* spawning.

Another interesting finding of our study is the clear dependence of *S. intermedius* spawning activity on the time of the day, which was more evident under conditions of low phytoplankton concentration in Kievka Bay, where spawning was only recorded between 16:00 and 02:00 h. The absence of evident differences in environmental variables between the period of increased spawning activity and the rest of the day suggests the influence of a circadian rhythm on sea urchin spawning activity. Because throughout the reproductive season, nocturnal spawning predominantly occurred on days with elevated phytoplankton concentration, we can suggest a synergistic effect of phytoplankton and circadian rhythm on *S. intermedius* spawning. Such a synergistic effect may be realized through a decrease in the threshold of sea urchin reaction to phytoplankton at night. We did

not find any data in the available literature on the relationship between sea urchin spawning activity and the time of day, but there are a number of studies showing that the movement and migration activity of sea urchins changes throughout the day. Field observations have revealed mostly nocturnal activity patterns in sea urchins (Dance 1987, Crook et al. 2000, Rosenberg & Lundberg 2004, Hereu 2005, Yeo et al. 2013, Hasegawa 2014), which concurs with our finding of predominantly nocturnal *S. intermedius* spawning activity. There is an interesting study by Babcock et al. (1992), in which data on the spawning time and number of spawning echinoderms belonging to different classes (Holothuroidea, Asteroidea, Crinoidea and Ophiuroidea) were collected by SCUBA divers over the summers (November to January) of 1990–1991 and 1991–1992 at Davies Reef in the central Great Barrier Reef. We previously analysed the data from this work using Rayleigh's test for circular uniformity (Zar 2010) and revealed that the spawning events of the studied echinoderms were also non-uniformly distributed throughout the day; spawning episodes were concentrated around 19:00 ± 02:00 h (mean ± SD). It should be mentioned, however, that the number of night SCUBA dives in Babcock et al. (1992) was almost 3 times lower than the number of daytime dives, which may explain the shift in the centre of the distribution of echinoderm spawning events to the light time of the day compared to our data.

Thus, a non-uniform distribution of spawning activity throughout the day seems to be a general feature of echinoderms. Considering that we did not find any significant difference in the values of the environmental variables (except for the light level) that could be responsible for the difference in *S. intermedius* spawning activity between 2 diurnal periods (i.e. the period of increased spawning activity and the rest of the day), the non-uniform distribution of spawning events over 24 h (the day/night cycle or the circadian rhythm) may instead be explained by the participation of some endogenous factor(s) in their regulation. A possible molecular basis for this phenomenon may be the genes belonging to the cryptochrome/photolyase family (*Cpf*), the products of which are involved in DNA repair, light perception and circadian clock regulation; *Cpf* genes have been found in the genomes of different aquatic organisms, including sea urchins (Oliveri et al. 2014). Spawning during dusk and at night seems to be a useful evolutionary adaptation enhancing the likelihood of offspring survival due to decreased predation pressure. Based on the results of the present study and the findings from our previous study, which explored the

relationships between spawning windows (the time intervals between 2 sea urchin samples when spawning was recorded based on gonadal analysis) in several *S. intermedius* populations and environmental variables (Zhadan et al. 2016), we have shown the following hierarchy of drivers of *S. intermedius* spawning activity: (1) phytoplankton is the most important factor because under conditions of low phytoplankton abundance, spawning failure occurs in natural *S. intermedius* populations (Zhadan et al. 2015, 2016, this study). (2) Increasing the concentration of phytoplankton induces moving activity and spawning in male sea urchins, whereas both an elevated phytoplankton level and sperm are necessary to stimulate spawning in females (this study). Furthermore, (3) night-time (this study) and the new and full lunar phases (Zhadan et al. 2016, this study) increase the probability of *S. intermedius* spawning, probably by enhancing the sensitivity of sea urchins to phytoplankton.

A similar, but not exactly the same, hierarchy of the drivers regulating spawning process in sea urchins with planktotrophic larvae has been previously proposed based on both laboratory and field studies: phytoplankton (Himmelman 1975, Starr et al. 1993); phytoplankton and sperm (Starr et al. 1990, 1992); sperm and phytoplankton, and the lunar cycle as a modulating factor (Reuter & Levitan 2010); a common external spawning signal (most likely, the spring phytoplankton bloom), sperm and the lunar cycle (Gaudette et al. 2006); and a sharp increase in phytoplankton level and the lunar cycle (Mercier & Hamel 2010, Zhadan et al. 2016). It is important to stress, however, that despite the fact that phytoplankton has long been considered to be the most likely proximate cue for triggering spawning in sea urchins (see Mercier & Hamel 2009 for review), no previous field study has reported a direct correlation between this environmental variable and sea urchin spawning events; therefore, the investigators could only suggest/hypothesize, based on the observed coincidence between increased chl *a*/phytoplankton concentration and sea urchin spawning, that phytoplankton might be such a cue (Himmelman 1975, Starr et al. 1993, Gaudette et al. 2006, Reuter & Levitan 2010, Zhadan et al. 2016). The present study, based on high temporal resolution recording of spawning events and environmental conditions, directly answers the question of whether an increase in phytoplankton concentration is the main environmental factor triggering spawning in *S. intermedius* populations. In addition, we included a new driver, circadian rhythm, in the hierarchy of the drivers of sea urchin spawning activity.

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