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A long photoperiod following a short one, and low temperature, stimulate gametogenesis in the sea cucumber *Holothuria arguinensis*

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ABSTRACT

Temperature and photoperiod are critical regulators of reproduction. However, few studies have evaluated experimentally their specific effects on gametogenesis in sea cucumbers. We tested the effect on gametogenesis of combinations of photoperiod and temperature: “accelerated” (long days following short days; a 12 °C deep in temperature), “constant” (constant short photoperiod; constant Spring/Summer temperature) and “natural” conditions (natural photoperiod and temperature). Experiment 1 started in mid-November (after gametogenesis began) and lasted until March. Experiment 2 started in September, after the spawning season (before gametogenesis started), with gonadal biopsy and spawning trials between March and May. In both experiments, the most advanced gonad development was found in accelerated photoperiod and temperature, with more than 50 % of the sea cucumbers reaching maturity and having the longest and thickest gonadal tubules. However, gametogenesis could not progress in sea cucumbers (i.e., shortest and thinnest gonad tubules) under constant short photoperiod combined with accelerated temperature. This indicates that a constant short photoperiod inhibits gametogenesis and that an accelerated thermal cycle cannot trigger gonad development alone. When accelerated photoperiod was combined with either accelerated or constant temperature, gametogenesis progressed, advancing faster in accelerated than constant temperature, with spawning only seen in these two treatments. This highlights that long days following short days are required for gametogenesis and that temperature has a modulatory role rather than a trigger, with a cool winter period being stimulatory. Altogether, our study provides essential information into environmental factors in sea cucumber reproduction, valuable for broodstock management and sustainable aquaculture.

1. Introduction

Sea cucumbers (Holothuroidea) are ecologically and commercially valuable, with a high demand for consumption and use in traditional medicine in Asian countries (Conand, 2017; Mercier et al., 2024; Purcell, 2014; Purcell et al., 2016; Purcell et al., 2013). As a result, sea cucumber stocks have rapidly decreased and are globally overexploited (Anderson et al., 2011; Purcell et al., 2013). Captive breeding was proposed as an effective solution to sustain the market demand and restock wild populations (Toral-Granda et al., 2008). One of the essential steps in captive breeding is the control of gametogenesis, particularly in species from temperate regions with a limited breeding season. Temperature and photoperiod are proposed proximal factors mediating echinoderm reproduction (Mercier and Hamel, 2009). However, most studies on the role of these environmental factors in gametogenesis have been correlative. The few experimental reports mostly concern higher latitude species of Echinoidea (sea urchins) and Asteroidea (sea stars) and suggested variation in how species respond to these two environmental factors. Some studies found gametogenesis to be triggered by short days, e.g., in *Strongylocentrotus purpuratus*, *Eucidaris tribuloides*, *Pisaster ochraceus*; others by long days, e.g., *Psammechinus miliaris*, *Odondaster validus* (Bay-Schmith and Pearse, 1987; Kelly, 2001; McClintock and Watts, 1990; Pearse and Bosch, 2002; Pearse et al., 1986a). In some species, such as *Pseudocentotrus depressus*, *Hemicentrotus pulcherrimus* and *Heliocidaris crassispina*, temperature, but not photoperiod, has been suggested to stimulate gonadal development (Sakairi et al., 1989; Yamamoto et al., 1988).

Photoperiod and temperature, combined or isolated, have also been implicated in the progression of gametogenesis. For example, increasing temperatures were found to be gametogenic enhancers in some species (*Paracentrotus lividus, Evechinus chloroticus*), while others required cold temperatures (*S. purpuratus*) or long days (*P. miliaris*) to complete their

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gonad development (James and Heath, 2008a; Kelly, 2001; Spirlet et al., 2000; Walker and Lesser, 1998). Many of these experiments also showed that gametogenesis could be advanced or suspended, in comparison with natural conditions, by manipulating photoperiod and temperature (e.g., Bay-Schmith and Pearse, 1987; Bottger et al., 2006; Kirchhoff et al., 2010; McClintock and Watts, 1990; Pearse and Beauchamp, 1986; Pearse and Eernisse, 1982; Pearse et al., 1986b; Pearse and Walker, 1986). To our knowledge, only three studies have experimentally addressed the role of photoperiod and temperature in gametogenesis of sea cucumbers. The first showed that mature *Apostichopus japonicus* could be advanced by two months by increasing seawater temperature by 6 °C above ambient temperature in winter (Wang, 2009). The other two were on *Cucumaria frondosa* where the first showed that gametogenesis did not progress in individuals kept under constant environmental conditions at the time of the summer solstice or 3 weeks before the winter solstice, while if placed under constant conditions 6 weeks after the winter solstice they had normal gonadal development (Hamel and Mercier, 1996). The other showed that placing individuals under constant 24 h light or 24 h dark, or a 4 months advance in photoperiod (when the natural photoperiod was 8 h light) delayed gametogenesis, with a low number of oocytes released at spawning and embryos dying before 10 days post-fertilization (Gianasi et al., 2019).

Holothuria arguinensis (Koelher and Vaney, 1906) is a temperate sea cucumber species from the North-East Atlantic Ocean, ranging from Portugal to Mauritania and the Canary Islands, and more recently has been found to colonise the Mediterranean Sea (González-Wangüemert and Borrero-Pérez, 2012; Mezali and Thandar, 2014; Purcell et al., 2023; Rodrigues, 2012; Thandar, 1988). This species has been targeted for fishing and is considered an aquaculture candidate (Domínguez-Godino et al., 2015; González-Wangüemert et al., 2016; Roggatz et al., 2016). In the southern Iberian Peninsula, *H. arguinensis* exhibits an annual reproductive cycle with a spawning period in summer-autumn. Gametogenesis is initiated after the autumnal equinox under short days (<12 h) and decreasing temperatures (<20 °C). In contrast, spawning occurs after the summer solstice when the days are long (>12 h), and the temperatures are increasing (>20 °C) (Marquet et al., 2017). Based on these observations, we hypothesised that *H. arguinensis* requires short days followed by long days and low winter temperatures for gametogenesis to progress. To test this hypothesis, *H. arguinensis* were exposed to a combination of natural, accelerated (long days after short days, a cold temperature period) and constant photoperiod and temperature conditions several weeks before the winter solstice. We found that this species requires a short photoperiod followed by a long photoperiod for gametogenesis to proceed and that a “cold winter” is stimulatory.

2. Material and methods

2.1. Collection and maintenance of sea cucumbers

Sea cucumbers, *H. arguinensis*, were collected, handled, and euthanised under license N°635/2015/CAPT and N°890/2021/CAPT of the ICNF - Instituto da Conservação da Natureza e das Florestas, Portugal.

Reproductively mature *H. arguinensis* (>220 g of total weight) were collected by hand in the intertidal zone of the Ria Formosa (Faro, Portugal; 37°00′35.02″N; 7°59′46.10″O) in November 2015 for Experiment 1 (average total weight ± standard error: 380.04 ± 81.40 g; $n = 48$) and at Farol Island in September 2021 (36°58′28.5”N 7°52′03.6”W) for Experiment 2 (302.14 ± 82.08 g; $n = 72$). At the time of collection, animals were mainly in the recovery (Experiment 1) and partly spawned/spent (Experiment 2) stages, as determined by biopsy of non-experimental animals (Marquet et al., 2017). Animals were kept at the Ramalhete Marine Station (University of Algarve) in outdoor holding tanks (1.2 × 1.0 × 0.6 m) under ambient lighting and temperature and fed with sediment collected from their natural environment for over a couple of weeks before the experiments.

2.2. Experimental setup

The aquaria (70 × 40 × 30 cm, 80 L) were supplied with filtered seawater using a flow-through system, and the bottom was covered with a weekly renewed 3-cm layer of sediment collected from the natural environment. The aquaria were equipped with fluorescent lights (Philips Master TL-D, 36 W/865) with a maximum light intensity of 350 lx recorded using data loggers (Hobo Pendant Temp/Light, 64 K). Each set of replicate aquaria was separated from the others by black plastic sheets to avoid bleeding of light conditions among treatments. The aquaria and storage tank temperature were maintained at the desired level using coolers (Hailea, HC-500 A) and heaters (Aquamedic, 500 W). Both temperature and photoperiod were monitored using controllers (Aquatronica, ACQ115). Animals were fed twice weekly with a mixture of dried *Phaeodactylum* sp. (purchased from Necton, SA, Portugal).

2.3. Experimental design

The experiments, designated 1 and 2, started on 28/11/2015 and 13/09/2021, under shortening days and lasted, respectively, 122 days (experiment 1 ended on 29/03/2016) and 260 days (experiment 2 ended on 31/05/2022).

The two experiments consisted of four treatments (Fig. 1): (1) accelerated photoperiod and accelerated temperature (AP-AT); (2) constant short photoperiod and accelerated temperature (CP-AT); (3) accelerated photoperiod and constant temperature (AP-CT); (4) natural photoperiod and natural temperature (NP-NT).

The accelerated regimes (AT, AP) aimed to expose the same directional changes as natural photoperiod and temperature but earlier and faster than in the annual cycle. The accelerated photoperiod (AP) treatment received a short 8 L:16D (8 h of light and 16 h of darkness) photoperiod for a month in September and November (before the winter solstice), respectively, for Experiment 1 or 2, followed by long (18 L:6D) photoperiod (Figs. 1B, D). The accelerated temperature (AT) treatment was subdivided into four phases and included 1) a gradual decrease of temperature to a minimum of 12 °C (winter temperature), 2) a stabilisation period at this minimum coinciding with the shift from short to long photoperiod, 3) a progressive increase to 22 °C (summer temperature), and 4) maintenance at 22 °C until the end of the experiments. In Experiment 1, 12 °C and 22 °C were reached at the beginning of December and February, respectively, i.e., about two and three months in advance compared to the natural conditions. In Experiment 2, temperatures were shifted one and a half months earlier than in Experiment 1 (Figs. 1A, C).

The constant temperature regime (CT) was 16 and 20 °C in experiments 1 and 2, respectively, corresponding to the ambient temperature when each experiment started (i.e., November in Experiment 1 and September in Experiment 2). A constant short photoperiod (CP) of 8D:16D was used in both experiments. All experimental treatments (AP, AT, CP, CT) were compared to the control, natural photoperiod (NP) and temperature (NT) regimes (Fig. 1).

Individuals were randomly distributed between 8 aquaria (2 replicates per treatment) in Experiment 1 ($n = 48$) and between 12 aquaria (3 replicates per treatment) in Experiment 2 ($n = 72$), in each case with six animals per replicate, and acclimated for seven days before the experiments. *H. arguinensis*, like most sea cucumbers, does not exhibit external sexual dimorphism. Gonadal biopsies were not attempted due to the difficulty of collecting the recovery and spent stages of small-sized gonadal tubules, the dominant stages at the beginning of the experiments, and to avoid additional stress that could affect future gonadal development. The sex ratio in each treatment was determined at the end of the experiments when the animals were dissected.

In Experiment 2, gonadal development was monitored through biopsies of three random individuals in each treatment after the first sampling (see below) to decide when to perform spawning trials on the 36 remaining sea cucumbers. This coincided with the temperature

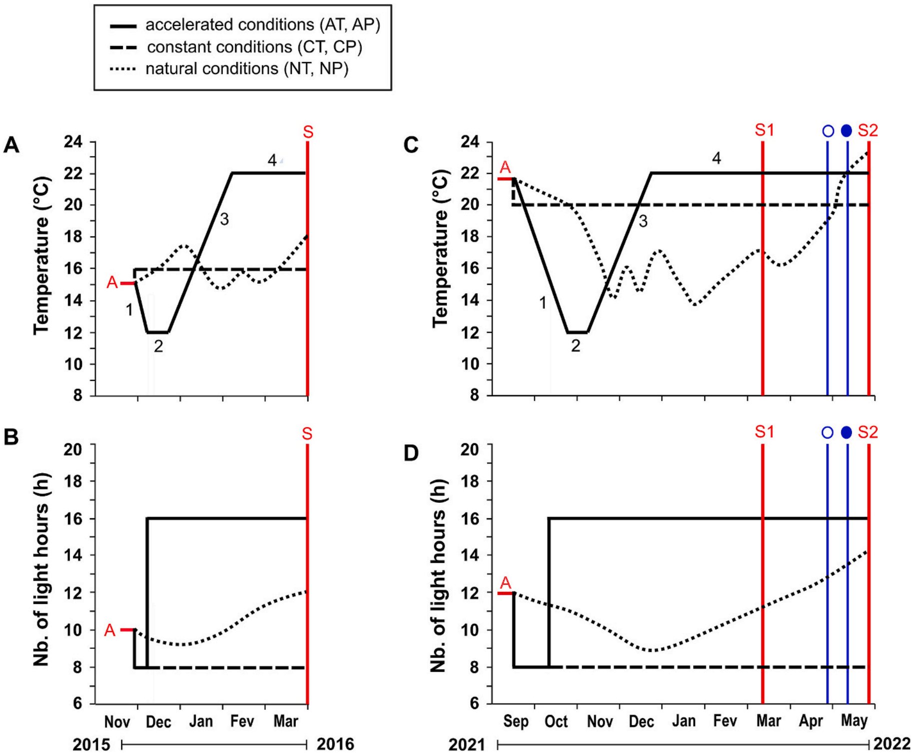

Fig. 1. Temperature and photoperiod (i.e., nb: number of light hours) used in each regime (accelerated, constant, natural) and experiment (1,2). Temperature regimes were used in Experiment 1 (A) and Experiment 2 (C), and photoperiod regimes were used in Experiment 1 (B) and Experiment 2 (D). Filled circles represent the full moon and open circles the new moon.

reaching 22 °C in the AT regime (hypothesised as being the most advanced). These trials aimed to verify if the animals that had reached maturity could spawn. Spawning was attempted in all treatments (on the 9 remaining individuals per treatment after the first sampling) on days close to the new (28–30 April 2022) and full moon (14–16 May 2022), when natural spawning is expected to be more frequent (Figs. 1C, D). For spawning induction, batches of three individuals from each replicate were transferred for 10 min into a 15 L bucket containing water 5 to 6 °C cooler before being placed in an aquarium (40 × 40 × 40 cm) at the same temperature as their origin. Signs of spawning behaviours were monitored for a maximum of two hours, as previously described (Marquet et al., 2018).

2.4. *Morphometry and gonadal maturity staging*

Individuals were euthanised at the end of Experiment 1 ($n = 48$, S in Figs. 1 A, B) and at the pre-spawning ($n = 36$; 15–18 March 2022, S1 in Figs. 1C, D) and post-spawning trials (n = 36; 26–31 May 2022, S2 in Fig. 1C, D) in Experiment 2. The gonadosomatic index (GSI) was calculated as GW/GBW * 100, in which GW is the gonad weight (g), and GBW is the gutted body weight (g), i.e., body weight without internal organs and coelomic fluid.

The diameter and length of 5 to 10 complete gonadal tubules stored directly in 70 % ethanol were measured under a stereomicroscope (Leica MZ6) using a ruler and an ocular micrometre. A small piece of tubule (around 2 cm) was fixed in 4 % paraformaldehyde for 24 h, followed by 70 % ethanol, dehydration and embedding, and haematoxylin and eosin (H&E) staining on paraffin sections. The maturity stage of each gonad was assigned based on the histology and dimensions of gonadal tubules according to the following classification: 1. Spent, 2. Recovery, 3. Growing, 4. Mature and 5. Partly-spawned (Marquet et al., 2017).

The maturity index (MI), representing the mean gonadal stage, was calculated for each treatment as $\Sigma\ [C_i\ n_i\ /\ n]$, where C is the maturity stage (from 1 to 5), n_i is the number of individuals from one treatment at that maturity stage, and n is the total number of individuals from that treatment. MI was converted into circular data and represented graphically using polar coordinates (Zar, 1996), in which vector direction represents the mean value and vector length is proportional to the homogeneity of the sample.

2.5. *Statistical analyses*

Unless otherwise stated, data are presented as mean ± standard error of the mean (SEM). Data were tested for the prerequisites for parametric analysis using Shapiro's and Brown-Forsythe's tests. A significance level of 0.05 was used. GSI data with arcsine transformed were analysed using one-way ANOVA with treatment as a fixed factor in Experiment 1 and two-way ANOVA in Experiment 2 using sampling time (i.e., before or after spawning trials) and treatment as fixed factors. One-way ANCOVA and a ranked ANCOVA were used, respectively, in pre- and post-spawning trial data to evaluate the effect of treatment (fixed factor) on gonad weight with gutted body weight (both log-transformed) as a covariate. The Kruskal-Wallis H test followed by pairwise post hoc Tukey's or Dunn's tests were used to compare the length and diameter of gonad tubules among treatments. To test differences in MI (polar transformed values) among treatments, a two-sample non-parametric test, the Wheeler-Watson test based on spacing frequencies and adapted for circular data was used with 5000 bootstrap replicates (Jammalamadaka et al., 2021). The difference in sample homogeneity (corresponding to the vector length) among treatments was tested using a Kruskal-Wallis H test. No statistical analyses were performed on the results obtained during spawning trials due to the low number of individuals available per treatment (n = 9). Data were analysed using SigmaPlot v14.0 (Systat Software, Inc) except for the ranked ANCOVA and circular data (TwoCircles packages) done in R.

3. Results

3.1. Experiment 1

In Experiment 1, there was no statistical difference in GSI among groups (one-way ANOVA, $F(3,44) = 1.42$, $n = 12$, $p = 0.250$; Fig. 2A). However, AP-AT gonad weight was borderline of statistical significance using eviscerated weight as covariate (ANCOVA, $F(3,43) = 2.76$, $p = 0.053$). Gonadal tubules were also longer and thicker in AP-AT compared to all other treatments (Kruskal-Wallis H test, $H = 59.35$ for length, $H = 62.35$ for diameter, $p < 0.001$ in both cases; pairwise Tukey tests, $p < 0.05$ in all comparisons) (Figs. 2 B, C). They were shorter and thinner in CP-AT when compared to the other treatments ($p < 0.01$ in all cases) with the tubule diameters similar to those in NP-NT ($p = 0.087$). There were no differences in tubule length ($p = 0.925$) and diameter ($p = 0.534$) between AP-CT and NP-NT.

The gonads of AP-AT were the most advanced, with more than 50 % of the individuals classified as mature and had the highest MI (MI = 3.42; between 3-growing and 4-mature; Wheeler-Watson test, $p < 0.05$ in all cases) (Figs. 3 A, B). The least advanced treatment was CP-AT, with 68 % of the individuals in the recovery stage, i.e., similar to the beginning of the experiment (MI = 2.33; closer to 2-recovery; $p < 0.05$ in all cases). AP-CT and NP-NT had most individuals in growing stages (around 50 %), indicating slight gametogenesis progression in some individuals and similar MI (AP-CT MI = 2.83; NP-NT MI = 2.75; $p = 0.28$). There were no differences in sample homogeneity, shown by the length of the vectors in Fig. 3B, among all treatments (AP-AT:0.62; CP-AT: 0.63; AP-CT:0.67 and NP-NT: 0.75) (Kruskal-Wallis H test, $H = 3$, $p = 0.392$).

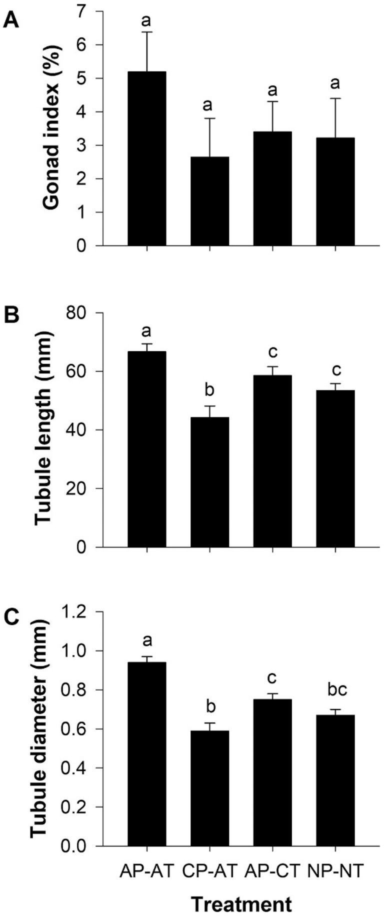

Fig. 2. Gonad index (A), gonadal tubule length (B) and diameter (C) in each treatment in Experiment 1. Data are represented as mean ± SEM.

3.2. Experiment 2

The GSI differed between pre- and post-spawning samples (two-way ANOVA, $F(1,64) = 11.36$, $p < 0.01$) but not among treatments at any of the two sampling points (interaction sampling time*treatment, $F(3,64) = 1.17$, $p = 0.328$) (Figs. 4A, B). Gonad weight was also not significantly different among treatments in the pre-spawned individuals (ANCOVA, $F(3,31) = 0.35$, $p = 0.790$; no effect of the covariate, $p = 0.085$) and in the post-spawned individuals (Ranked ANCOVA, $F(3,31) = 1.03$, $p = 0.393$; effect of the covariate, $p < 0.05$). Spawning trials with the 36 remaining individuals ($n = 9$ per treatment) after the first sampling, end of April/early May, were successful in a few individuals in two treatments: AP-AT (1 individual out of 9) and AP-CT (3 individuals out of 9). Pre-spawning behaviours, such as swaying the anterior body region from one side to the other, were observed in four individuals in AP-AT and three individuals in AP-CT without triggering the release of gametes during the observation period. However, no spawning or pre-spawning behaviours were seen in CP-AT and NP-NT (i.e., water was clear – without sperm - the following day).

Pre-spawning individuals had longer and larger gonad tubules in AP-AT compared to those in all other treatments (Kruskal-Wallis H test, $H = 30.60$ for length, $H = 60.54$ for diameter, $p < 0.001$ in both cases; pairwise Tukey test, $p < 0.01$ in all cases). However, there were no differences in length and diameter between those sampled in AP-CT and NP-NT ($p = 0.929$ for diameter, $p = 0.180$ for length), AP-CT and CP-AT ($p = 0.973$ for diameter, $p = 0.978$ for length) and CP-AT and NP-NT ($p = 0.726$ for diameter, $p = 0.369$ for length) (Fig. 4C, E). After the spawning trials, the gonad tubules were longer and thicker in NP-NT than in AP-CT and CP-AT (Kruskal-Wallis H test, $H = 42.57$ for length, $H = 59.86$ for diameter, $p < 0.001$ in both cases; pairwise Dunn's test, $p < 0.001$ in all cases) (Figs. 4D, F). However, they were similar in length ($p = 0.854$) but not in diameter ($p < 0.05$) to those in AP-AT. Tubules were longer in AP-AT than in CP-AT ($p < 0.001$) and in AP-CT ($p < 0.01$ in both cases). However, they had similar diameters to those in CP-AT ($p = 0.131$) but not those in AP-CT ($p < 0.05$). There were no differences in length and diameter between CP-AT and AP-CT ($p = 0.123$ for diameter, $p = 0.808$ for length).

Before the spawning trials, more than 50 % of AP-AT individuals were identified as mature, indicating clear signs of gametogenesis progression (Fig. 5A). This treatment had the highest MI (3.21; between 3-growing and 4-mature) compared to all other treatments (Wheeler-Watson test, $p < 0.01$ in each case) (Fig. 5B). Most CP-AT individuals (>75 %) were in partly spawned and spent stages and the MI (between 5- partly spawned and 1- spent) was significantly different from all other treatments ($p < 0.01$ in all comparisons). As partly spawned/spent stages were the main stages found at the beginning of the experiment, this indicates that gametogenesis did not proceed significantly in this treatment. AP-CT and NP-NT had similar MI (between 2- recovery and 3-growing, $p = 0.35$) and were dominated by individuals in the recovery stage (67 % in AP-CT and 56 % in NP-NT), showing only a slight progression in gametogenesis. As indicated by shorter vector lengths, individuals in AP-AT (0.44) and CP-AT (0.56) were more heterogeneous (with at least three maturity stages) than in AP-CT (0.83) and NP-NT (0.81) (two maturity stages). However, these differences were not significant (Kruskal-Wallis H test, $H = 3$, $p = 0.391$).

After the spawning trials, gametogenesis continued progressing in NP-NT, with 33 % classified as mature. This treatment had an MI close to 3-growing while all the other treatments were between 1-spent and 2-recovery; however, the MI in NP-NT was only significantly higher than that in AP-CT (Wheeler-Watson test, $p < 0.01$; Figs. 5C, D). In AP-CT, most individuals were in partly spawned/spent stages (78 %), with an MI of about 1-spent, similar to CP-AT ($p = 0.07$) and AP-AT ($p = 0.32$). As with AP-CT, 55 % of CP-AT individuals were in spent stages, and the MI was similar to the other groups (CP-AT vs. NP-NT: $p = 0.63$; vs. AP-CT: $p = 0.32$; vs. AP-AT: $p = 0.83$). Unlike AP-CT, most CP-AT individuals sampled before spawning trials were in partly spawned/spent

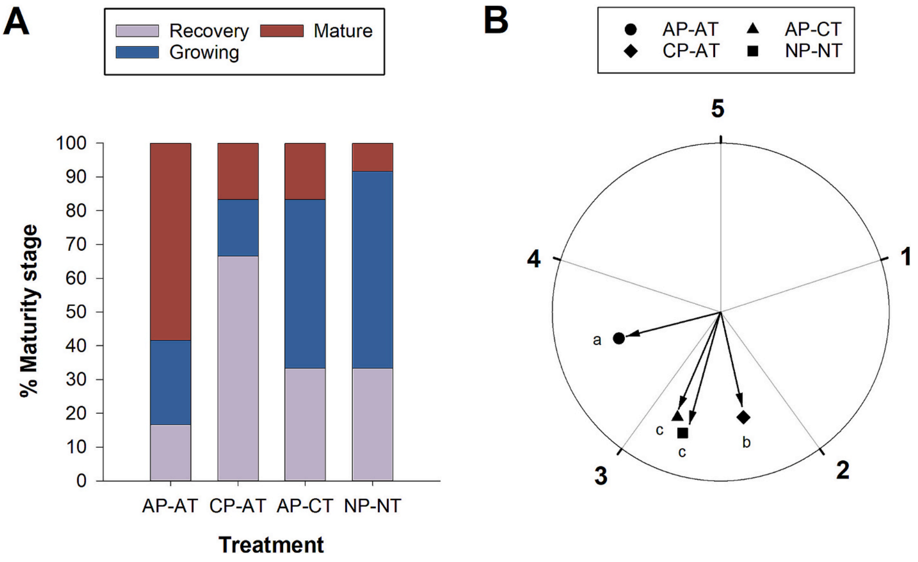

Fig. 3. Percentage of each maturity stage (recovery, growing, mature, partly-spawned, spent) in each treatment (A) of the Experiment 1, and circular representation of the MI after polar transformation of the data (B). The direction of the vector represents the mean value of the MI, and the length characterised the homogeneity of the samples.

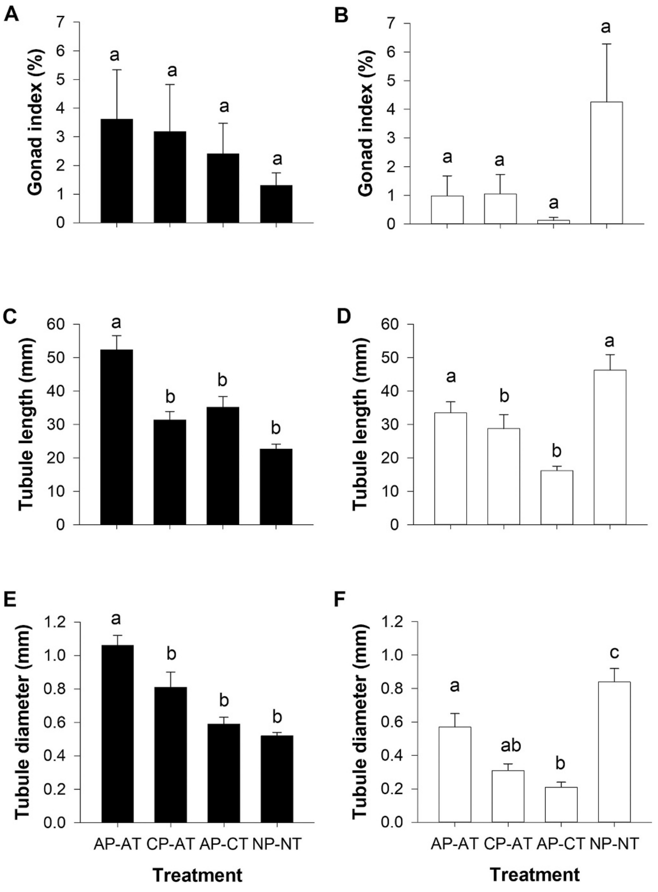

Fig. 4. Gonad index (A, B), gonadal tubule length (C,D) and diameter (E,F) in each treatment at the two collection times: before (A,C,E) and after (B, D, F) spawning trials in Experiment 2. Data are represented as mean ± SEM.

stages (Fig. 5A). As no spawning was seen in CP-AT, it suggests that the gametogenesis in this treatment did not progress from the beginning of the experiment. In AP-AT, around 60 % were in recovery, 25 % were mature, and 12 % were spent. There were no differences in MI post-spawning between AP-AT and the other treatments (AP-AT vs. NP-NT: $p = 0.56$; vs. AP-CT:0.32; vs. CP-AT: 0.14). No differences were found in sample homogeneity between treatments (AP-AT:0.45; CP-AT:0.56; AP-CT:0.69; NP-NT:0.34) (Kruskal-Wallis H test, $H = 3$, $p = 0.392$).

4. Discussion

Photoperiod is the fundamental zeitgeber for gametogenesis in *H. arguinensis,* with temperature having a modulatory role. Long days following short days were stimulatory for *H. arguinensis* gametogenesis, and a low winter temperature of around 12 °C, coinciding with the transition from short to long photoperiod as seen in the wild, appeared to have a synergistic effect. In the two experiments, the hypothesised accelerated regimes of photoperiod and temperature (AP-AT) initiated and accelerated gametogenesis, with more than 50 % of individuals reaching gonadal maturity in March in each case, compared to few (<10 %) to none in control conditions (NP-NT). This represents an advance of about 2–2.5 months compared to that seen in the wild in the same region (Ria Formosa) (Marquet et al., 2017). Previous studies testing advanced environmental conditions in echinoderms generally consisted of simulations of seasonally changing conditions 4 to 6 months out-of-phase with the environment (e.g., Kirchhoff et al., 2010; McClintock and Watts, 1990; Pearse and Bosch, 2002; Pearse and Eernisse, 1982; Pearse and Walker, 1986; Walker and Lesser, 1998; Yamamoto et al., 1988). Recently, other methods, such as compressing the seasonal variations in day length between two winter solstices, already used in shellfish and fish, have been tested in sea urchin (Ishii et al., 2022; Joyce et al., 2013; Watanabe et al., 2006). However, in the sea cucumber *C. frondosa,* no differences in gametogenesis rates were seen between animals exposed to a 4-month advanced photoperiod and those in the ambient group (Gianasi et al., 2019). Nevertheless, this must be interpreted cautiously due to the lack of detailed information about the experimental starting date.

Our approach, using abrupt change in photoperiods, suggests that *H. arguinensis* (and probably other echinoderms) respond to the direction of photoperiod change in a similar way to teleost fishes (Randall and Bromage, 1998). This is supported by experimental studies conducted in other echinoderms. For example, the sea urchin, *S. purpuratus* from the coast of California (37° 10′ 54" N), starts gametogenesis in summer when days are decreasing and spawning starts in January (Pearse et al., 1986a). When subjected in June (14 h:20 min L:9 h 40 min D natural

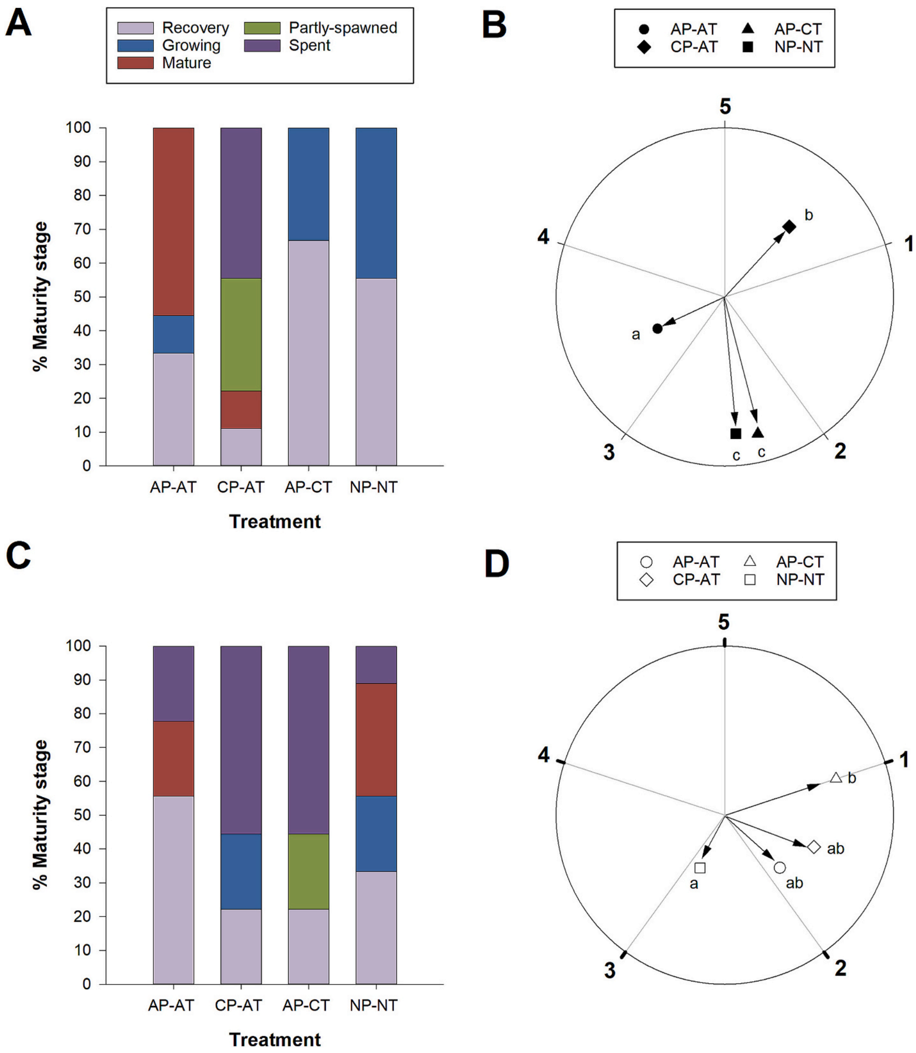

Fig. 5. Percentage of each maturity stage (recovery, growing, mature, partly-spawned, spent) in each treatment (A, C) in Experiment 2, and circular representation of the MI after polar transformation of the data (B, D) before (A, B) and after (B, D) spawning trials. The direction of the vector represents the mean value of the MI, and the length characterised the homogeneity of the samples.

photoperiod) to a constant long photoperiod (16 L:8D), a "neutral" photoperiod (12 L:12D) or a short photoperiod (8 L:16D), gametogenesis was inhibited in the long photoperiod regime and stimulated (similar to natural photoperiod control) in the other two, in which there was a decrease in photoperiod compared to the start (Bay-Schmith and Pearse, 1987). This means that *S. pupuratus* is sensitive to shortening days and can distinguish changes of at least 2 h of light (i.e., the difference in natural hours at the start of the experiment in June and the neutral treatment that was stimulatory, recognized as decreasing day length). Also, constant short days (9 L:15D) were stimulatory and continuous long days (15 L:9D) were inhibitory in the tropical sea urchin *Eucidaris tribuloides,* collected in June (13:30 h light) in Florida (24°40′01" N). The process was also postponed in a 6 months out-of-phase treatment until winter started, suggesting that short days or long nights trigger gametogenesis (McClintock and Watts, 1990). Unfortunately, published studies on photoperiod are often correlational and generally lack sufficient detail about the progression of gametogenesis to infer mechanisms. Since other factors may also influence this process (Mercier and Hamel, 2009), it is difficult to determine the specific role of photoperiod.

Our results clearly show that histological and morphological parameters are more sensitive than the GSI in determining the progression of gametogenesis. This index assumes an isometric relationship to body size; however, this may not always be the case (Gonor, 1972; Ebert et al., 2010), and variation in gonad development among individuals may accentuate the bias. This is noticeable in our results where heterogeneity was sometimes significant (i.e., a mature individual with large gonads could be found in the same treatment as an individual with small gonads in the recovery stage) and was partly overcome by using gonad weight with eviscerated body weight as a covariate. However, direct analysis of gonadal stages and gonadal tubule length and diameter proved to be the most sensitive parameters to determine gametogenesis progression. This agrees with other studies that showed a strong correspondence between tubule morphometry and gonadal stages, while GSI gives limited information about the processes occurring within the gonads (e.g., Mercier and Hamel, 2009; Pasquini et al., 2022; Ramofafia et al., 2003; Shiell and Uthicke, 2006). Accordingly, these data will be mainly discussed below.

In our experiments, we chose to start the experiments when the photoperiod was decreasing and at two periods: during the spawning season (September) and around the time gametogenesis starts in nature (November) (Marquet et al., 2017). Only the AP-AT treatment caused a clear advance in gametogenesis (>50 % of mature individuals), with the highest MI and tubule length and diameter in both experiments. In contrast, maintaining a constant short photoperiod (8 L:16D) in CP-AT before the winter solstice was inhibitory (lowest MI with mostly no gametogenesis progression) while maintaining the temperature above

14 °C in AP-CT slowed down the progression compared to AP-AT but was similar to NP-NT (similar MI, tubules' length and diameter). This demonstrates that *H. arguinensis* can detect the direction of photoperiod change, and a long photoperiod stimulates gametogenesis after a short photoperiod. It also explains why compressed photoperiods tend to accelerate gametogenesis and how out-of-phase photoperiods, commonly used in echinoderm research, can initially have either stimulatory or inhibitory effects (e.g, Pearse and Bosch, 2002; Pearse et al., 1986a; Xu and Barker, 1990). Our results follow similar observations in the *C. frondosa* sea cucumbers, where gametogenesis was inhibited under constant conditions when collected before the winter solstice. However, *C. frondosa* could progress normally when captured afterwards, indicating that increasing day length is essential to initiate their gametogenesis (Hamel and Mercier, 1996). Sensitivity to environmental modifications was also suggested to depend on the developmental stage of the gonad at the start of the experiments (Bouland and Jangoux, 1988). Some reports show that gametogenesis cannot be halted by constant photoperiod, irrespective of length, once it has already begun (Dumont et al., 2006; Pearse and Bosch, 2002). However, in our study, gametogenesis inhibition with a constant short photoperiod occurred with individuals who were both in recovery/growing (Experiment 1; when gametogenesis already started) and partly-spawned/spent stages (Experiment 2; before gametogenesis) at the beginning of the experiment.

Our experiments show that temperature plays a modulatory role in gametogenesis rather than a trigger. In CP-AT, most individuals stayed in the same gametogenic stage throughout the experiment (i.e., recovery in Experiment 1; partly spawned/spent in Experiment 2). After the spawning trials, individuals in Experiment n°2 were mainly in spent stages, the same as at the beginning of the experiment, while no spawning was seen. This indicates that an accelerated thermal cycle is insufficient to trigger gametogenesis. However, gonad development progressed under a stimulatory photoperiod even if the temperature was held constant (AP-CT or AP-AT). This indicates that photoperiodic stimulation is required to initiate gametogenesis and that an appropriate temperature may be required for normal development. In Experiment n°2, at least three individuals in AP-CT were seen to spawn in April/May, while no mature individuals were found in March (first sampling). This suggests that maturation was accelerated in this group compared to the control, where no sea cucumbers spawned. The absence of spawning in the control, compared to AP-CT, might be due to the lower average temperature observed until the first sampling, suggesting that maturity might have been reached later (i.e., between the spawning trials and the second sampling). Further studies of the effects of temperature and different stages are needed.

The following steps in this research should be dedicated to understanding how sea cucumbers transduce photoperiodic information into seasonal reproductive cycles. Melatonin, whose production is influenced by photoperiod, is known to play a fundamental role in the seasonal timing of reproduction in vertebrates (e.g. Szpręgiel and Wronska, 2020). As echinoderms and chordates are phylogenetically related to vertebrates, and melatonin is present in both, McClintock and Watts (1990) hypothesised that melatonin could also regulate gametogenesis in echinoderms. However, this hypothesis has not been thoroughly investigated. Additionally, understanding the mechanisms for obtaining early maturation needs to be addressed. Walker and Lesser (1998) suggested that advancing photoperiod might lead to the activation of gonial cell mitosis directly through a mitogen-induced mechanism or might result in the mobilisation of nutrients from nutritive phagocytes, stimulating oogonial or spermatogonial mitosis. To our knowledge, these effects remain, nevertheless, unclear and do not consider the role of endocrine factors. Another explanation could come from the feeding activity that, in some echinoderms, is influenced by photoperiod (e.g., Grosjean and Jangoux, 1994; Sun et al., 2020; Walker and Lesser, 1998). As food quality and quantity are important factors for successful gametogenesis (e.g., James and Heath, 2008b; Mercier and Hamel, 2009; Pearse et al., 2002), determining if these photoperiodic fluctuations affect the feeding behaviour in *H. arguinensis* might indirectly help in understanding how they influence gametogenesis progression.

5. Conclusion

The reproductive cycle of *H. arguinensis* has been successfully altered by the experimental treatments, with the highest percentage of mature individuals found in accelerated conditions (AP-AT). Effective spawning was seen in both treatments wherein photoperiod was accelerated (AP-AT and AP-CT). We demonstrated that gametogenesis in this species is stimulated by long days following short days and inhibited by constant short days. The inhibition occurred directly after spawning or when gametogenesis had already started. It still needs to be determined whether this process can be halted at any moment of the cycle using constant short photoperiod or temperature manipulation to maintain maturity over a more extended period, similar to what has been done in *S. droebachiensis* using constant spring conditions (Kirchhoff et al., 2010). Temperature change alone was insufficient to trigger gametogenesis but had either a neutral or accelerated effect on gametogenesis and, therefore, had a modulatory role. Overall, our results provide valuable insights into the environmental control mechanisms controlling the reproductive cycle, which are essential for obtaining out-of-phase mature sea cucumbers.

CRediT authorship contribution statement

Nathalie Marquet: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Adelino V.M. Canário:** Writing – review & editing, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Raw data have been deposited on Zenodo (https:// doi.org/10.5281/zenodo.13907987).

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