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Temperature regulates the switch between light-synchronized and unsynchronized activity patterns in the subtidal bivalve *Pinna nobilis*

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Abstract

This study provides new information on the biological rhythms of subtidal bivalves, using the fan mussel *Pinna nobilis* as a model. The objective was to determine which factor(s) provoke the change between two different patterns observed in the annual cycle of this species: P1, which is characterized by the individuals opening and closing their valves according to the presence or absence of ambient light and P2, which is characterised by behaviour that is not dependent on the presence of light. Magnetoresistive sensors were installed on 8 fan mussels to record gaping activity in laboratory conditions. Different temperature and light treatments were applied. The results showed that temperature was the factor modulating the change between behavioural patterns. The individuals switched to P1 when temperature reached 24.5ºC. In this pattern, individuals are entrained by light, displaying a circadian rhythm linked to the daily light treatments. During P2, the circadian rhythm was missing or very weak, contrary to in situ observations. The results of this study contribute to the understanding of the biology of the species and planning new conservation strategies. Furthermore, the observed relationship between temperature and P1 is of interest within the framework of fan mussel captive breeding. Altogether, this information is especially relevant in view of the recent mass mortality of *P. nobilis* in the western Mediterranean.

Keywords: Pinnidae; Biomonitoring; Daily rhythm; Marine ecology; Biological rhythm; Fan mussel; Climate change.

Introduction

Gaping behaviour provides a measurable response to stress in bivalves (Andrade *et al*., 2016; Fournier *et al*., 2004). The use of devices to measure valve aperture, is useful to evaluate the effect of environmental factors such as temperature, currents, toxic metals, light and food on bivalves (Garcia-March *et al.*, 2016; Robson *et al.*, 2010a; Rodland *et al*., 2009; Tran *et al*., 2007). Besides the direct response to natural or anthropogenic stressors, bivalve gaping activity usually follows different cycles such as circadian, circatidal or circannual rhythms (Mat *et al*., 2012; Palmer 2000; Rensing *et al*., 2001; Tran *et al*., 2011). These rhythms can be the consequence of an internal clock caused by an environmental factor (zeitgeber) or a physiological response to it (Vaze and Sharma, 2013). If both the rhythms and the relationship between the variation of environmental parameters and gaping activity are accurately understood, the gaping activity of bivalves can be used to develop effective biological early warning systems (Bae and Park, 2014; Borcherding, 2006; Liao *et al*., 2009; Sluyts *et al*., 1996; Sobrino-Figueroa and Caceres-Martinez, 2009; Sow *et al*., 2011). These systems can provide invaluable data and contribute to gaining a better understanding of the biology of the species monitored, but also help to understand the effects of climate change on species autoecology (Andrade *et al*., 2016).

The fan mussel *Pinna nobilis* is a Mediterranean subtidal bivalve that lives in an environment that is unaffected by tidal cycles. In situ, the gaping activity of the fan mussel includes an annual cycle with two patterns (P1 and P2) and a circadian cycle throughout the year (Garcia-March *et al*., 2016; Garcia-March *et al*., 2008); the factors causing the switch between the two annual patterns are yet to be understood. P1 occurs between mid-July/early-August and early-November. During these periods, *P. nobilis* gaping follows the sun and moonlight and has a circadian (c.) 23.7 h rhythm. The valves are closed

during the night on new moon nights, and open if there is ambient light, either produced by the moon during the night or by the sun between sunrise and sunset. P2 occurs from early-November to mid-July/early August. During these periods, gaping is not dependent on the sun and moonlight or any other known environmental factors but still keeps the c. 23.7 h rhythm (Garcia-March *et al*., 2016). In P2, fan mussels keep their valves open most of the time, but produce periodical gap closes of several hours every day. After having studied gaping behaviour in situ, Garcia-March *et al*. (2016) raised a hypothesis regarding the factor(s) potentially inducing such an alternation between P1 and P2. Variations in temperature, oxygen or a combination of these parameters were postulated as the most probable triggers of the switch. Among other benefits, understanding what causes the switch between P1 and P2 could lead to using the gaping activity of the fan mussel to track climate change at local scale. The consistent advance or delay switching time between patterns would indicate the direction of the change if caused by a climatic factor. Furthermore, the fan mussel is an endangered species; it is included in Annex II of the Bern Convention as a strictly protected species and the Barcelona Convention as a threatened or endangered marine species. Its status is being reviewed in Spain to "Critically Endangered" due to recent mass mortality in the western Mediterranean, provoked by a Haplosporidian protozoan parasite (Darriba, 2017; Vázquez-Luis *et al*., 2017). The fan mussel is a typical inhabitant of subtidal *Posidonia oceanica* meadows and the largest bivalve in the Mediterranean Sea (with a maximum shell length of 120 cm, it is one of the largest bivalves in the world) (Basso *et al*., 2015). Understanding its gaping behaviour also provides insight into fan mussel biology, which is useful for planning better conservation strategies for the species. Additionally, the results of this work can help to understand the behaviour and the causes of change in the annual patterns observed in other subtidal bivalves (Mat *et al*., 2012; Schwartzmann *et al*., 2011).

To test the hypothesis raised by Garcia-March *et al*. (2016) about the influence of temperature on the switch between patterns, a manipulative experiment was planned and conducted on 8 fan mussels collected in early spring 2016. Pen shells were kept in experimental tanks for 71 days before relocating them to the sea. During the experimental period, different temperature and light treatments were applied. In order to record the moment of switching between patterns, gaping activity was monitored and the distance of gape aperture was recorded in mm every second.

Material and Methods

Collection and relocation of individuals

Eight fan mussels were collected between the 22nd and the 25th of April 2016 from the marine area adjacent to Peñón de Ifach in Calpe (Western Mediterranean, Alicante, Spain) at a depth of 8-12 meters. Two weeks before collection, sensor brackets were glued to the shells of each individual using epoxy resin (Ivegor®) on each individual (Garcia-March *et al*., 2016; Garcia-March *et al*., 2008). The individuals were extracted gently, avoiding any damage to the byssus threads. This was considered fundamental to improve the relocation of the fan mussels after the experiment. The fan mussels were collected while in P2 behaviour (as confirmed during the first days of the experiment) and, according to the previous experimental data, they were expected to maintain this pattern until mid-July early August (after the experiment was completed). After 71 days, the individuals were returned to their natural habitat. In *P. nobilis*, attachment is made through the byssus (Basso *et al*., 2015) and therefore simple reimplantation would leave them unprotected until they were able to attach themselves again, and exposed to threats such as hydrodynamics and predators. An artificial extension of the byssus was made using plastic net attached to the byssus filaments, firmly fixed to the seabed with plastic camping pegs and covered with sediment. A folded plastic bag (original size 50 x 45 cm and folded size 20 x 15 cm) with 0.5 cm mesh was used for this purpose. This provided sufficient temporal attachment to allow the fan mussels to re-attach themselves naturally, thus increasing survival probability.

Fan mussel maintenance in the laboratory

The fan mussels were divided into two groups of 4 individuals and placed in two 750L closed circuit tanks. Each tank was equipped with an EHEIM professional 3 filter, Bubble Magus Rock SP4000 skimmers and TK 2000 climatizers. Individuals of similar sizes were distributed in both tanks (length range 41.5 to 63.5 cm, Table 1). The biomonitoring system was installed on the 8 fan mussels allowing them 7 days of acclimation before starting the experiment. The tanks were placed in a completely isolated room, where the only source of light available -LED white light- was under experimental control. Water temperature was initially kept at 16.9°C ± 0.6 and saturated of dissolved oxygen. All water parameters were controlled by a ProfiLux 3.1T system, which allowed constant control of light (ON/OFF), temperature (± 0.1 °C), pH (± 0.01), oxygen (± 0.01 mg/l) and salinity (± 0.1 psu). These parameters were recorded at 5 min intervals. The photoperiod was set initially at 12/12h L/D intervals and then modified according to specified experimental

Table 1. Size of each *Pinna nobilis* individual used for the activity experiments.

	1A	2A	3A	4A	1B	2B	3B	4B
Size (cm)	61.3	53.7	55.1	49.3	58.9	63.5	56.8	41.5

treatments (see 2.4. Experimental treatment). The four individuals of each tank were fed automatically using pumps controlled by ProfiLux 3.1T. The daily doses of food for each tank were composed by a mixture of 30 g of mud (10.0% ± 2.4% of organic matter content) and 40 ml of an algae concentrate (Reefphyto®-5 live species phytoplankton) (Trigos, 2016; Trigos *et al*., 2014). The doses were dissolved in 3 l of water and administered in 4 equal doses every 6h.

Biomonitoring of gaping activity

The biomonitoring device consists of magnetoresistive sensors similar to those used by Garcia-March *et al*. (2016) installed on the shells of the fan mussels and connected to a *Pinna-Interface* system, which processes and records the data on a SD card. The *Pinna-Interface* is also connected to a computer equipped with a program —*Pinna*— specially designed not only to record the data, but also to display gaping activities in real time. In case of an electrical blackout, the *Pinna-Interface* continues to register data for 48 hours thanks to its own internal batteries. The *Pinna-Interface* is programmed to record one data/second for every individual. The sensors have a measuring range of 0-30mm and 1mm precision. Each tank had its own temperature sensor synchronized with the activity sensors (i.e. the data is recorded simultaneously in the same file), used to calibrate the gaping positions. This avoids biases due to experimental temperature variation.

Experimental treatment

Initially, the individuals of tank A –individuals 1A, 2A, 3A and 4A– were used as control, whereas the individuals located in tank B –individuals 1B, 2B, 3B, 4B– were used as experimental subjects. To test the hypothesis that variations in water temperature trigger the switch between P1 and P2 and *vice versa*, a treatment (TR) was applied (Table 2) by raising the water temperature of tank B up to a maximum of 25.0 ± 0.1°C at a rate of 0.5°-1°C every two days. All the other parameters were maintained constant. If the hypothesis was correct, it was expected that the fan mussels would start switching between P2 and P1 once the temperature of the water exceeded 23°C.

After applying the conditions of TR for 15 days to tank B (counting from the day maximum treatment conditions were reached), the experimental protocol would be reversed if any response was detected. This meant reducing water temperature to 18°C at a rate of 0.5°-1°C per day. In this scenario, we expected to observe a switch in the gaping activity of the fan mussels, from P1 to P2, below 20°C. To increase the quantity of data, the experiment was replicated applying TR to tank A, using the same settings as those used for tank B.

Table 2. Schedule for the different treatments used (TR, reverse protocol and light treatments).

Date (2016)	Treatment tank A	Treatment tank B
15th April		Apply protocol TR on tank B
10th May		TR conditions reached on tank B
11th May		Individual 1B switch from P2 to P1
15th May		Individual 2B switch from P2 to P1
16th May	i) Reducing light period to 20h/4h L/D	
19th May	Apply protocol TR (up to 23ºC) on tank A	
22-23 May	ii) Full Moon Night	
25th May		Individual 3B switch from P2 to P1
26th May		Apply reverse protocol on tank B
31th May	Continue with protocol TR on tank A	
5th June	TR conditions reached on tank A	
7th June	Individual 3A switch from P2 to P1	
8th June	Individual 2A switch from P2 to P1	
9th June	Individual 1A switch from P2 to P1	
19th June		Reverse protocol conditions reached on tank B
20th June	Apply reverse protocol on tank A	
22th June	iii) Sudden dark	
29th June	iv) Full Moon Night	
5th July	Reverse protocol conditions reached on tank A	

Different light periods were planned to test the relationship between light tracking and the gaping activity of the fan mussels if cycle P1 had occurred after the treatments which are presented in Table 2, and are as follows: i) the daily dark periods were reduced progressively from 12 h (12/12H L/D) to 4 hours (20h/4h L/D). This treatment also prevented exerting excessive stress on the individuals while in cycle P1. It should be noted that when in P1, individuals close the gap in total darkness and, therefore, setting artificially long periods of total darkness for too many days, could lead to excessive fatigue of the fan mussels; ii) the night hours were further decreased to a full moon night (24/0 h) and then the length of the dark period was increased back to 20/4 h; iii) sudden full moon nights were produced (24/0 h) and iv) sudden events of total darkness in the middle of the day were produced.

Reimplantation of the individuals in the field was scheduled to be performed after 10 days in the conditions of the reverse protocol, counting from the first day when the reverse conditions (i.e. back to 18°C) were reached in tank A.

Data visualization and analysis

An updated gapeR program, a data analysis program developed using the open-source R platform (Garcia-March *et al*., 2016), was used for data treatment. This new version of gapeR enables the user to work with temporal data series on an interactive graphic; to visualize all the data of the different individuals and sensors by selecting which ones to show, and for which days; to produce different graphical displays of the data and to perform statistical analysis such as FFT, auto-correlation and cross-correlation. Days are expressed in "experimental days", with the first day of the experiment being experimental day 1, although this parameter can be modified in the program to Julian days.

For data treatment, the time series were fractioned in the two observed patterns (P1 and P2). The statistics were applied both to each individual time series and to the averages of the time series in tank A (avA) and tank B (avB). Averages were calculated only for the days when the behavioural pattern (P1 or P2) of all the individuals of each tank coincided.

A second-order band-pass Butterworth filter (low frequency = 48 h^{-1}, high frequency = 15 h^{-1}) was applied to each fraction of the time series. To overcome the transient effect of the filter, both ends of all-time series were expanded by adding 200 values with their mean. Once the filter was applied, the 200 values in the tails were cut again to keep the original but filtered data. In order to study the main frequencies occurring in the time series, Fast Fourier Transform (FFT) was applied to the raw data for both patterns. An autocorrelation function (acf) was applied in order to view the auto-covariance of each individual both for the whole time series, and for the P1 and P2 fractions. Cross-correlation (cc) between pairs of individuals was also calculated, for lengths of the time series when both individuals displayed the same pattern (P1 or P2). All statistics were applied using the open-source R platform.

Results

Pattern switching

The application of TR (increasing temperature to 25°C) to the experimental tanks sufficed to produce a switch from P2 to P1 in individuals 1B, 2B and 3B (Fig. 1A). These Individuals switched patterns after 1, 5 and 15 days respectively when the temperature reached 24.5°C. Individual 4B, however, maintained P2 during the whole experiment. A week after the first individual reached P1 in tank B, TR was applied to tank A. Because a clear response in three individuals of tank B had been obtained at 24.5°C when temperatures were increased at a rate of 0.5°-1°C every two days, we decided to maintain the water temperature of tank A at 23°C for 5 days, before continuing the experiment (extending the scheduled study by 5 days). By doing this it was possible to check whether the switch of patters was due to the temperature reaching a peak of circa 24.5°C, or to sustained warm temperatures ≥ 23°C despite the maximum temperature reached. No switch was observed after 5 days. Again, individuals 1A, 2A and 3A switched from P2 to P1 2, 3 and 4 days after the temperature reached 24.5°C (Fig. 1C). Like individual 4B, individual 4A maintained P2 during the whole experiment.

Since individuals 4A and 4B did not respond to the treatment, the average for each group was performed only with the individuals who showed both patterns (note that for P2 there are 8 individuals but 6 for P1, so only these 6 where used in both cases to compare patterns). As a consequence, in order to calculate averages, the total of days with all individuals in the same pattern was N = 25 (P1) and N = 41 (P2) for avA and N = 26 (P1) and N = 15 (P2) for avB.

Response to light treatments

The different light treatments produced a positive result, with all the individuals in P1 tracking the presence/absence of light with their gape opening. The progressive reduction of the daily dark period to 20h/4h was mirrored by a progressive change in the moment of gape closing during the hours of darkness (Fig. 1A). Unlike those of tank B, control individuals from tank A showed total lack of response to light, maintaining P2 while not subjected to the temperature treatment (Fig. 1B, Fig. 1D). Once TR was applied to either of the two tanks, individuals 1B, 2B, 3B (Fig. 1D) and 1A, 2A and 3A (Fig. 1C), kept their valves open all night during events of full moon (nights of experimental day 28 and 66 respectively) and closed their valves during events of sudden darkness, even when applied during midday (11:45 to 15:30 of experimental day 59), opening the gape again when light returned (Fig. 1C).

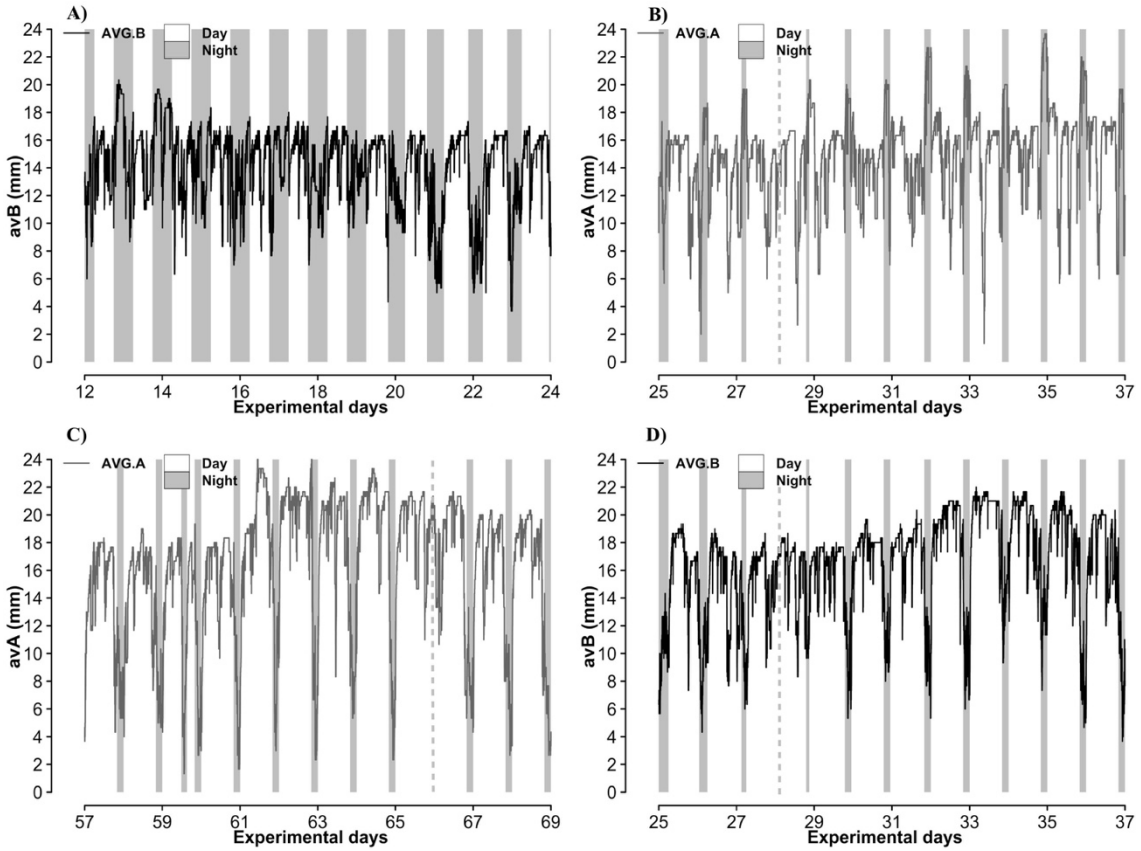

Fig. 1: Examples of gaping activity-averaged raw data. A) Average of tank B (avB) (n = 3) during the change from P2 to P1. B) Average of tank A (avA) (n=3), control in P2 during the same period of Figure 1D. C) avA (n = 3) during P1 showing a period of sudden darkness in experimental day 59. D) avB (n = 3) during P1, showing a period of full moon in experimental day 28. Dashed lines point the time were a darkness period should have occurred according to previous light cycles, but lights were kept on. Note that individual 4A and 4B were not selected to calculate averages because they did not change from P2 to P1 during the whole experiment.

Excluding individuals 4A and 4B, which always maintained P2, the rest of the fan mussels maintained P1 despite the application of the reverted protocol (i.e. reducing water temperature by 0.5º-1ºC per day up to 18°C) even when maintaining this setting for 10 days.

Individuals of tank A and B were reimplanted in the field after 10 and 15 days respectively in the reverse protocol. It should be noted that 5 days were needed to reimplant the individuals of tank A, before proceeding with those of tank B. Even though tank B specimens passed 5 more days than individuals of tank A at 18°C in the reverse protocol, they maintained P1 following the light cycles.

Statistical analysis

The Fast Fourier Transform (FFT) analysis revealed a clear peak of 23.11 h and 23.15 h, respectively, for avA and avB during P1. Only a peak of 5.96 h signal was detected during P2 for avA (Table 3 and Fig. 2B).

The autocorrelation coefficient for avA and avB was 0.69 and 0.66, respectively, during P1, displaying a clear rhythm (Fig. 3C and 3D). Lower values were obtained for P2, 0.28 and 0.48 respectively (Fig. 3A and Fig. 3B). A small peak corresponding to the peak detected by the FFT every 5.96 h during P2 for avA (Fig. 3B) was also observed. The autocorrelation coefficients of each individual during each pattern was always higher for P1, with the exception of individual 1A, which showed a weak coefficient –for both P1 and P2 (Table 3)–.

The cross-correlation coefficients showed a strong correlation between the individuals of each group during P1, both in raw and filtered data. Only the correlation between individuals 1B and 3B was higher for P2 than for P1 (Table 4).

Discussion

The observed results show that water temperature triggers the change from P2 to P1 in *P. nobilis*. Despite the number of individuals (8) being limited due to the protected status of the species, the responses of 6 out of 8 individuals match predictions, based on *in situ* observations, of Garcia-March *et al*. (2008) and Garcia-March *et al*. (2016). The probability of this behaviour match being a result of chance or a random activity of the fan mussel

Table 3. Dominant period in hours (h) obtained from Fast Fourier Transform (FFT), period of maximum autocorrelation (acf lag) and auto correlation coefficient (acf) for each individual, average of all individuals in tank A (avA) and average of all individuals in tank B (avB).

Pattern	1A	2A	3A	4A	1B	2B	3B	4B	avA	avB
					FFT (h)					
P2	-	-	5.96*	-	-	-	-	-	5.96*	-
P1	23.11	23.11	23.11	-	23.56	23.53	23.15	-	23.11	23.15
					acf lag (h)					
P2	23,89	23,35	18,02	23,97	13,96	24,02	19,52	24	23,98	23,99
P1	23,28	23,96	24,01	-	24,00	23,99	23,86	-	23,97	24,00
					acf					
P2	0.38	0.31	0.29	0.29	0.16	0.27	0.18	0.38	0.48	0.28
P1	0.34	0.67	0.66	-	0.54	0.59	0.49	-	0.69	0.66

*Peak related to food pulses.

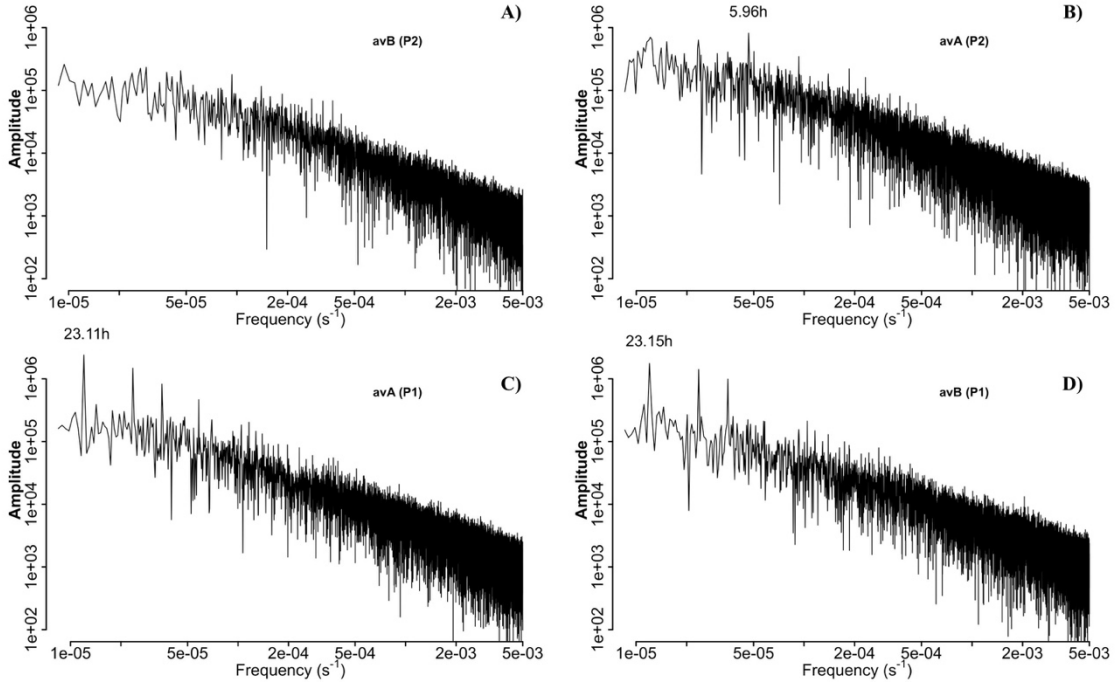

Fig. 2: Fast Fourier Transform of averaged raw data. A) Average of tank B (avB) (n = 3) during P2. B) Average of tank A (avA) (n = 3) during P2. C) Average of tank A (avA) (n = 3) during P1. D) Average of tank B (avB) (n = 3) during P1. Note that during P2 (boxes A and B), the c. 23 h frequency spike is missing. In B, a spike is observed at 5.96 h, the timing of food pulses. Individual 4A and 4B were not selected to calculate averages because they did not change from P2 to P1 during the whole experiment.

is unlikely and dismissible. Furthermore, although it is known that laboratory conditions can alter bivalve activity (Robson *et al*., 2010b; Williams and Pilditch, 1997), the knowledge acquired from previous *in vivo* studies on the natural behaviour of this species (Garcia-March *et al*., 2016; Garcia-March *et al*., 2008) was used to establish whether the behaviour observed in the tanks was a reaction to the treatments and thus a reliable result. The limited sample size, however, imposes replication of the experiment in another population in order to confirm that behaviour is also regulated by water temperature as observed in this study. Reducing oxygen concentration to check the response of the individuals was discarded due to the protection status of the species. Future experiments

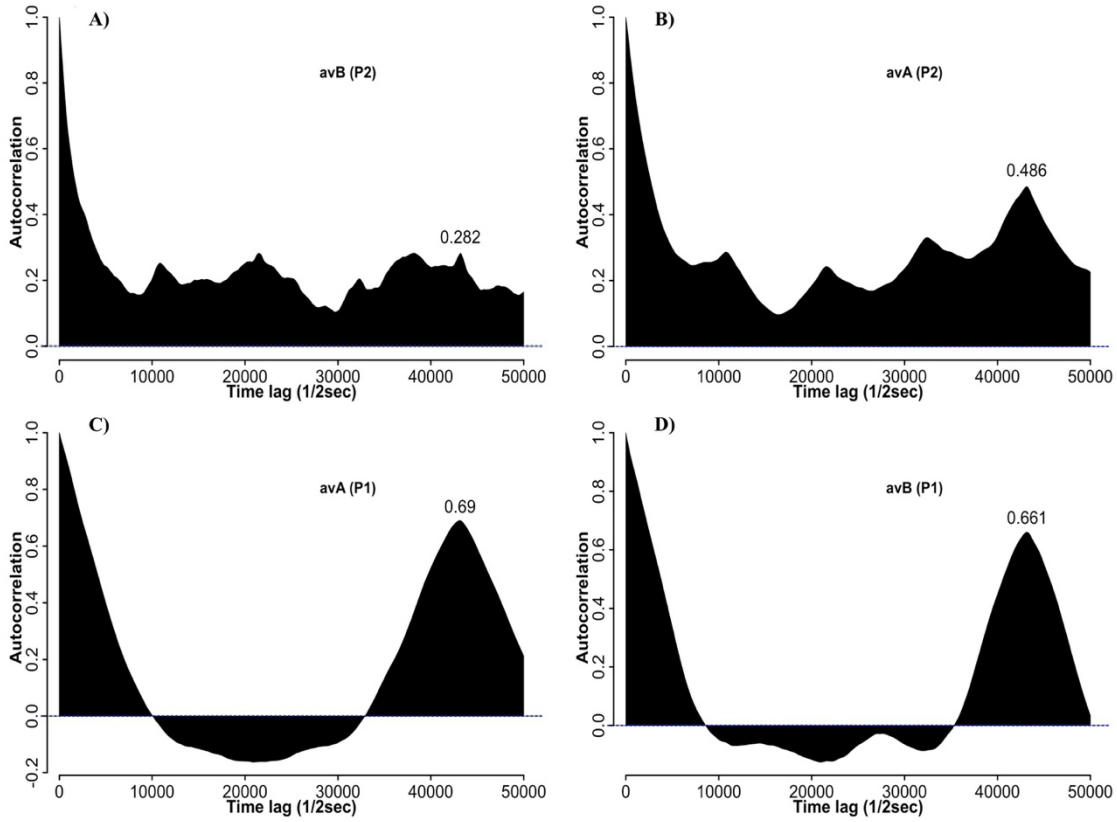

Fig. 3: Autocorrelation function of averaged raw data. A) Average of tank B (avB) (n = 3) during P2. B) Average of tank A (avA) (n = 3) during P2. C) Average of tank A (avA) (n = 3) during P1. D) Average of tank B (avB) (n = 3) during P1. Note that iIndividual 4A and 4B were not selected to calculate averages because they did not change from P2 to P1 during the hole experiment.

Table 4. Coefficient obtained from cross-correlation function between pairs of *Pinna nobilis* individuals.

	RAW	Smoothed	RAW	Smoothed
	P1 / P2	P1 / P2	P1 / P2	P1 / P2
	2A		3A	
1A	0.55 / 0.36	0.68 / 0.39	0.45 / 0.26	0.57 / 0.21
2A	-	-	0.70 / 0.24	0.88 / 0.35
	2B		3B	
1B	0.59 / 0.23	0.73 / 0.37	0.42 / 0.12	0.44 / 0.64
2B	-	-	0.30 / 0.10	0.42 / 0.13

with oxygen as a variable would help to understand the role, if any, played by oxygen in fan mussel behaviour.

The switch between patterns occurred when the temperature reached 24.5ºC. All the individuals of tank A maintained P2 despite water temperature being kept at 23.0ºC for 5 days. The individuals of both tanks only started switching patterns one or two days after reaching 24.5ºC. This fact supports the idea that in the field it is the peak summer water temperatures that provoke this pattern switch, instead of sustained warm temperatures. Six out of eight individuals followed the light and dark cycles as expected in the hypothesis, effectively switching from P2 to P1. They opened their valves with light and closed them during dark periods, even during events of sudden, and therefore unexpected, simulated full moon days or total darkness. This confirms the importance of light when the individuals are in P1, and that fan mussels do not predict light cycles, but respond directly to the presence/absence of light. The benefits of tracking light with gaping activity patterns during the warm months for marine bivalves are not clear. According to Ortmann and Grieshaber (2003), shell closure at night could be a way of saving energy when food is scarce. However, in our experiment food was provided on a regular basis, and the fan mussels maintained their gap closed when in total darkness while in P1 despite the presence of food. Furthermore, during P1 in natural conditions, *P. nobilis* gaping activity is also independent of food (phytoplankton spikes) (Garcia-March *et al*., 2016). Alternatively, light tracking with gaping activity during P1 effectively helps synchronize the behaviour of individuals within a population. This synchronization could have reproduction benefits during summer months. Indirect observations by Cabanellas-Reboredo *et al*. (2009), Kersting and Gar-

cia-March (2017) suggested that *P. nobilis* reproduction mostly occurred between June and August. A histological analysis performed by Deudero *et al*. (2017) showed that the beginning of spawning occurs in May with a higher peak in June-July. The differences observed could be due to the depth where the populations live, the position of the thermocline, latitudinal gradients or other local factors. Synchronization with light could help to signal when spawning would be more beneficial for successful fecundation. However, this possibility will have to be demonstrated in future studies.

Individuals 4A and 4B followed pattern P2 throughout the experiment. If tracking light with gaping activity during P1 were related to reproduction, it is possible that individuals 4A and 4B, the smallest of all (Table 1), were immature. This status would make synchronization with the rest of the individuals unnecessary and hence, light tracking a burden more than a benefit. However, according to Trigos (2016), these individuals were large enough to produce viable gametes. Due to the fact that *P. nobilis* is an endangered species the individuals were not sacrificed to test their maturity status. Moreover, Garcia-March *et al*. (2016) also postulated oxygen as a possible trigger for the switch between patterns. For this experiment, only temperature variations were applied. Temperature variations, however, induce direct variations in dissolved oxygen. This could explain why Garcia-March *et al*. (2016) found a potential correlation with temperature and also with oxygen at the time of the switch between the behavioural patterns of fan mussels. Further studies on gaping activity are necessary to understand what prevents some individuals from changing behavioural patterns with high temperatures. The relationship between the maturity status of fan mussels and seasonal activity patterns should be evaluated in this respect.

A similar P1 behaviour in gaping activity has been also observed in the subtidal giant clam *Hippopus hippopus* (Schwartzmann *et al*., 2011). This tropical species always lives in high temperatures with little seasonal variation (21-28 ºC). The lack of cold water temperatures could perhaps keep the giant clam in P1. This behaviour is disrupted when water temperatures are maintained at 27-28ºC for several weeks, provoking abundant gape closures and valve movements during the day, but still maintaining night closures (Schwartzmann *et al*., 2011). If the giant clam is always in P1 due to water temperature, it should change to P2 behaviour if maintained artificially at low water temperatures (18-20ºC) for several weeks. Despite the fact that the gaping behaviour of *Crassostrea gigas* is mainly circatidal, Mat *et al*. (2012) also observed a dual rhythm in relation to the photoperiod in their natural environment. The species is diurnal in spring and summer and nocturnal during autumn and winter. However, this species switched patterns gradually and at constant temperatures in the laboratory, thus suggesting that the switch is provoked by endogenous causes and that temperature does not significantly affect seasonal changes in the gaping activity of *C. gigas*. The similarities between *P. nobilis* and *H. hippopus*, but not *C. gigas*, suggest that the influence of water temperature in the response of bivalves to light is probably a common feature, but not universal in bivalves. More research on other bivalves could help understand the evolutionary benefits of this influence.

During the application of the reverse protocol, the individuals maintained P1 despite temperature being kept at 18ºC for 10 and 15 days in tank A and B, respectively. According to field data, the switch between P1 and P2 was expected to occur below 20 ºC (Garcia-March *et al*., 2016). If synchronization for reproduction purposes regulates the switch between P2 and P1, as suggested above, the fan mussels would need a minimum time in P1 before returning to P2, to complete gonad maturation and spawning. In this scenario, temperature would be overshadowed by the necessity of completing the reproduction cycle. Alternatively, the fan mussels could need more time to switch between patterns due to the intrinsic physiological requirements for the switch. In this case, the experimental settings would prevent the individuals to prepare for the planned short time switches between patterns in the current study. To identify the factors influencing the switch from P1 to P2, the time that fan mussels are kept in each pattern should be increased in future experiments.

The spectral analysis (FFT) shows a periodicity of 23.11 h and 23.15 h for avA and AvB, respectively, during P1. The observations are similar to those of Garcia-March *et al*. (2016) when the individuals were in P1 in the field studies. However, when the individuals were in P2 in the laboratory, the circadian cycle observed *in situ* was missing and only a 5.96h cycle was detected for avA.

Autocorrelation confirms a strong constant periodicity of 23.11 h and 23.15 h linked to the light cycles during P1 (0.69 and 0.66 for avA and avB respectively, Fig. 3C and 3D). Nevertheless, the autocorrelation of the 5.96h peak detected by FFT (10728 seconds in the time lag scale 1/2sec) during P2 for avA, was lower than 0.28 (Fig. 3B). The food cycles (6h) produced an inconstant response in the fan mussels. Individuals sporadically noticed the food pulses and varied their gape opening inconsistently when food was provided. Autocorrelation during circadian cycles was lower than observed *in situ* by Garcia-March *et al*. (2016), as suggested by the lack of spikes for this frequency in the FFT. It is possible that laboratory conditions altered the weaker rhythm of P2 (Garcia-March *et al*., 2016). If the rhythm of P2 is not caused by zeitgebers, as suggested by the field studies, it would be more sensitive to disruptive external factors. These could be the artificial feeding cycles, which could explain the 5.96 h peak detected for avA, or small nuisances such as random disturbances easily occurring in laboratory conditions. Altogether, they could shadow the internal rhythms during P2. Performing further studies with free running conditions in both patterns, i.e. keeping constant light or darkness and a constant supply of food to avoid entrainment, would help to gain a better understanding of these inner cycles.

The high cross-correlation between pairs of individuals during P1 shows synchronization among fan mussels that track the light cycles with their gape opening. For P2, the values were again lower than expected (Garcia-March *et al*., 2016), which reinforces the idea of an internal clock governing both patterns; however, while light entrains and strengthens P1 rhythmicity, P2 would be more easily disturbed, due to the fact that it is self-sustained.

The close relationship between gaping behaviour and temperature in *P. nobilis* makes the species a potential biomonitor in the context of climate change. Monitoring individuals to check the variation in the timing of the switch could help to understand the direction of the change and its potential effects at community level. If *P. nobilis* are affected by this change, other organisms most probably are feeling the variation as well. The combination of real-time field gaping activity data with schlerochronological analysis of shells could help in deciphering the accumulated information in the shells, providing longer time series of local climatological data in areas where these are missing. Further studies combining these two approaches will help to calibrate the schlerochronological data in order to obtain longer local temperature time series (Garcia-March *et al*., 2011).

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