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1	Moonlight cycles synchronize oyster behavior
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24 Abstract

25 Organisms possess endogenous clock mechanisms that are synchronized to external cues and orchestrate biological rhythms. Internal timing confers the advantage to anticipate 26 27 environmental cycles inherent in life on Earth and to prepare accordingly. Moonlight-28 entrained rhythms are poorly described, being much less investigated than circadian and 29 circannual rhythms synchronized by sunlight. Yet focus on these lunar rhythms is highly 30 relevant to understanding temporal organization of biological processes. Here, we investigate 31 moonlight cycle effects on valve activity behavior of the oyster Crassostrea gigas. Our results 32 show that oysters modulate valve behavior according to both intensity and direction of the 33 lunar illumination cycle. As a consequence, valve opening amplitude is significantly increased 34 at third quarter moons (decreasing lunar illumination) compared to first quarter moons 35 (increasing lunar illumination) despite identical lunar illumination, and this indicates that 36 oyster modulation of valve behavior by moonlight cycles is not a direct response to lunar 37 illumination. We propose that oysters use moonlight cycles to synchronize behavior and also 38 other physiological and ecological aspects of this benthic mollusk bivalve.

39

40 **1. Introduction**

41 Life on earth is influenced by a multitude of cycles resulting from sun, earth and moon 42 trajectories and interactions, including in marine ecosystems. Fitness and survival depend on 43 the capacity to forecast oscillating ecosystem constraints and benefits. By conferring a 44 personal time measurement, internal molecular clock(s) give organisms anticipation [1]. This 45 gives meaning to environmental cues and leads to temporal organization, from cell to 46 ecosystem [1,2]. Lunar rhythms, defined by a 29.5-day period length of moonlight oscillation, 47 has received considerably less attention than circadian rhythms. In marine organisms, 48 moonlight has mostly been observed to act on reproduction, constituting an essential cue for

49 mass synchronous external spawning events [3,4]. For a number of species, this reproductive 50 rhythm is not a direct response to moonlight but requires the action of an endogenous clock 51 [3,4]. Unlike reproductive processes, physiological and behavioral lunar rhythms in marine 52 organisms are largely unknown, despite their potential significance in ecological functioning. 53 The Pacific oyster *Crassostrea gigas* is a benthic marine invertebrate with among the largest 54 of geographical distributions. Diploid and triploid oysters, i.e. organisms with two or three sets of chromosomes, respectively, dominate shellfish production and wild populations are 55 56 increasingly becoming established, with potential impact on ecosystems [5]. Valve activity in 57 bivalve species is closely related to physiological processes and gene expression [6-8]. In C. 58 gigas, valve behavior follows a daily rhythm for which the corresponding circadian clock has 59 been identified [9,10], and an annual rhythm for which endogenous circannual timing has been proposed [9,11]. This behavior also follows a strong tidal rhythm, modulated by neap-60 61 spring tidal (semilunar) and anomalistic moon cycles [12]. A recent study showed that C. gigas behavior might evolve according to a 29.5-day periodicity [11]. However, how oysters 62 63 modulate their valve behavior following lunar cycles has, to our knowledge, never been 64 studied. 65 In this study, we present the first analysis of lunar rhythm-modulated behavior in C. gigas.

Focusing on winter, i.e. minimal reproductive efforts and spawning events, we investigated
effects of both intensity and direction of lunar illumination on this species. We demonstrated
that the lunar rhythm in *C. gigas* is not a direct response to nocturnal light. This finding raises
the question of the underlying mechanism of the lunar-related behavior in oysters.

70

71 **2. Materials and methods**

72 (a) Animals, study area and data collection

73 The present study analyzed valve behavior data of 12 C. gigas oysters (6 diploid and 6 triploid) from 22 December 2014 to 3 April 2015. Two-year old animals (66 ± 1 mm shell 74 length) were positioned in a natural site in the proximity of wild oysters (Arcachon Bay, 75 France, latitude 44.66°, longitude -1.16°) in February 2014, i.e. 9 months before this study 76 77 began. They were in a permanently immersed oyster bag fixed on an oyster table, at a 78 minimum water depth of 1 m. Valve behavior was recorded every 1.6 second using high-79 frequency non-invasive (HFNI) valvometer technology [13]. The astronomical data related to 80 moon cycles was retrieved from the site https://www.timeanddate.com/.

81

82 (b) Data treatment and statistical analyses

83 Daily valve opening amplitude (daily VOA, 54000 averaged data, %, more details in [11]) 84 was analyzed individually and averaged based on ploidy status. Mean VOA values for the 5 days around each new moon (NM), full moon (FM), first and third quarter moon (first and 85 86 third QM) phases were calculated. Because of the non-normality of the data, ANOVA on 87 ranks of the data followed by Dunn's method were applied to test moon phase effects. An 88 illuminated moon surface value (%) was associated with each daily VOA, as a proxy of lunar 89 illumination. A random components mixed model for repeated measures was applied to test 90 lunar illumination, direction of lunar illumination and ploidy effects on individual daily VOA 91 values, using XLSTAT 2018 software (Addinsoft. New York, USA). All statistical analyses 92 were performed after checking assumptions (normality and equal variance; p-value < 0.05 93 considered significant) and using Sigma Plot software (Version 13.0; Systat Software, USA).

94

95 **3. Results**

Figure 1 presents daily valve opening amplitude (VOA, %) of diploid and triploid oysters
during the entire winter season, i.e. three and a half moon cycles. In figure 2, ANOVA on

ranks showed significant daily VOA differences according to moon phase in both diploid and
triploid oysters (*p* < 0.001 for both). Daily VOA was maximal during NM and minimal during
first QM, and increased at FM and third QM (figure 2). Finally, daily VOA was increased by
16 % and 19 % at third QM compared to first QM, in diploid and triploid oysters, respectively
(figure 2).

- 103 In table 1, the entire daily VOA dataset was used. The mixed model showed a significant
- 104 random effect for oyster individuals (p = 0.007). Moreover, this model showed significant
- 105 effect of the fixed parameters: lunar illumination (p < 0.0001) and direction of lunar
- 106 illumination (p < 0.0001) on daily VOA. No significant effect was shown for the ploidy (p =
- 107 0.2916). There was a significant interaction between lunar illumination and direction of lunar
- 108 illumination (p < 0.0001) independent of ploidy status.

109

110 **4. Discussion**

111 Our findings clearly show behavioral modulations in C. gigas which follow lunar cycles, and 112 allow innovative hypotheses. First, we indicate that oysters, independent of ploidy, are able to 113 sense moonlight despite its extremely low intensity compared to sunlight. Secondly, we 114 demonstrate that ovsters can detect if the moonlight is increasing or decreasing. Finally, we 115 show that ovsters combine these two pieces of information to adjust their behavior. 116 The moonlight sensitivity observed in C. gigas is consistent with a moonlight response in 117 another bivalve, the mussel Pinna nobilis [8,14]. In contrast to our results, P. nobilis valves 118 were more open when the moon was more than 50 % illuminated [8]. Yet both studies imply 119 that these mollusks possess light receptor(s) able to perceive dim nocturnal light. In other 120 phyla, photoreceptor families such as cryptochromes or opsins, also present in oysters [10], 121 have received significant attention, but their functional involvement in moonlight perception 122 remains unclear [3,4].

123 Moonlight sensitivity in C. gigas could lead to a direct behavioral response to lunar 124 illumination cycles. However, our results indicated significant VOA differences between first 125 and third QM despite similar moonlight intensity. Alternatively, moonlight could influence 126 the circadian clock mechanism, usually synchronized by sunlight, and known to regulate 127 valve behavior in C. gigas [6,7,10]. Another hypothesis could be that a specific dedicated 128 circalunar clock is behind the moonlight rhythm [3,4]. To answer to the question if the lunar-129 related behavior is internally or externally regulated, further laboratory experiments should be 130 carried out in constant light and food supply, and in simulated moonlight entrainments. 131 Internal timing offers major advantages compared to solely responding to the immediate 132 external environment [1]. A recent study indicated that moonlight cycles drive vertical 133 migration of pelagic zooplankton species, influencing predation success and risk [15]. Our 134 analyses show that oyster VOA is globally higher when lunar illumination is low. This 135 corresponds well with the nocturnal pattern of oyster circadian rhythms during winter [9]. Previous experiments attest that circadian behavior in C. gigas is in accordance with digestive 136 137 rhythms [6], and we also hypothesize that the global increase in oyster VOA in the absence of 138 light (night and NM) could optimize their access to food, which is of low abundance in 139 winter. Indeed, as a sessile and benthic species, oyster rhythms could be an adaptive 140 mechanism allowing e.g., synchronization with light-dependent vertical migration of 141 phytoplankton in the water column [16]. Previous experiments also show that C. gigas daily 142 behavior is associated with circadian expression of at least 6.2 % of the genes in gills [7]. By 143 analogy, behavioral lunar rhythms observed in this study could reflect a lunar rhythmicity of 144 some other physiological functions, as observed in corals where gene expression was 145 modulated over a lunar month [17]. Finally, a seasonal modulation of phase and intensity of 146 oysters' lunar rhythm could be hypothesized, as observed concerning daily and tidal rhythms

in this species [9,11]. In this sense, it has been observed that moonlight response in the mussel *Pinna nobilis* depend on periods of the year [14].

149 Temporal organization of physiological processes at a species level may have significant 150 effects on ecosystems. It has been argued that lunar rhythms of zooplankton vertical migration 151 could have a large influence on biogeochemical cycles by facilitating monthly pulses of 152 carbon remineralization [15]. Moreover, it has been proposed that vertical migrations at one 153 trophic level can affect vertical migrations at the next one, leading to "cascading migrations", 154 from phytoplankton to fish [18]. By the oysters capacity to modulate several biogeochemical 155 properties and processes, including phytoplankton assemblages [5], oyster lunar rhythms 156 could have a non-negligible ecological impact both on benthic and pelagic areas. 157 158 **Ethics:** All experiments complied with the laws in effect in France and they conformed to 159 international ethical standards. 160 Data accessibility: The data underlying this study are available in supplementary material 161 (SM_Payton&Tran_2018). 162 Author contributions: L.P. and D.T. conceived the study, analyzed the data, drafted and 163 revised the manuscript. Both authors agree to be held accountable for the content therein and 164 approve the final version of the manuscript. 165 **Competing interests:** We have no competing interests. 166 Funding: This work was supported by the financial support of the French National Research 167 Agency (ANR), ACCUTOX project 13-CESA-0019 (2013-2017) and WAQMOS project 15-168 CE04-0002 (2015-2020).

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231	Legends

Figure 1. In situ profiles of individual (thin lines) and mean (bold line) daily valve opening amplitude (VOA, %) of diploid (blue, n = 6) and triploid (green, n = 6) oysters during the winter season in Arcachon Bay (France). Moon phases are reported at the top (new moon: black circle; full moon: white circle; first quarter moon: half black and half white circle; third quarter moon: half white and half black circle). **Figure 2.** Mean daily VOA and standard error of diploid (blue, n = 6) and triploid (green, n =6) oysters according to the moon phases. NM: new moon; First and third QM: first and third quarter of the moon; FM: full moon. Identical letters indicate no significant differences of mean daily VOA (p-value = 0.05). Table 1. Random components mixed model for repeated measures applied to test the effects of lunar illumination (i.e. over or under 50 % of illuminated moon surface), direction of lunar illumination (i.e. increasing or decreasing illumination) and ploidy of oysters (diploid or triploid) on individual daily VOA. (** and ***) indicate *p*-value < 0.01 and < 0.001, respectively.

257 Table 1: Payton & Tran

Random components mixed model for repeated measures

Covariance parameters	Z		<i>p</i> -value
Random effects	2.452		0.007**
Repeated factors	16.831		< 0.0001***
Source of variation (fixed parameters)	NumDF	F	<i>p</i> -value
Lunar illumination	1	63.446	< 0.0001***
Direction of lunar illumination	1	65.204	< 0.0001***
Ploidy	1	1.240	0.2916
Interactions	_		
Illumination x Ploidy	1	3.332	0.0682
Direction x Ploidy	1	0.005	0.9428
Illumination x Direction	1	20.501	< 0.0001***



Figure 1 : Payton & Tran



Figure 2 : Payton & Tran