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Discrimination of different rhythmic behavioural patterns in *Nephrops norvegicus* (Linnaeus, 1758), a marine crustacean decapod of commercial interest

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ABSTRACT

Nephrops norvegicus represents one of the most important commercial species for EU trawling fisheries. I investigated the existence of different rhythmic behavioural patterns in 52 *Nephrops* with an innovative actographic method that is able to discriminate 3 geo-referenced components of burrow emergence activity. Recognized patterns were correlated with sex and carapace length and obtained results were extrapolated to the field. The experimental conditions were: 12-12 LD cycle; monochromatic blue (480-nm) light intensity (0.1 lx). The methodological approach produced good time series of locomotor data, since arrhythmic animals are few (8%). Results indicated that *Nephrops* possesses an unexpected plastic exogenous behavioural rhythm, subdivided into 3 categories related to burrow influence (i.e. Burrow-centred, 60%; Burrow-oriented, 17%; Poorly burrow-oriented, 15%). Sex and carapace length did not influence the expression of reported rhythmic behavioural patterns, but we individuated some differences in overall locomotor activity among sexes. *Nephrops* should be considered a good model to investigate rhythmic behavioural patterns of deep-sea crustacean decapods. That investigation revealed its potentiality in aquaculture practice (feeding, reproduction and manipulation) and fishery assessment (extrapolation of laboratory data to the *Nephrops* stock management).

1. INTRODUCTION

The astronomical movement of the Earth in relation to the Sun-Moon axis is responsible for the generation of important temporal fluctuations in selected habitat cues (i.e. light intensity cycle, internal and coastal tides, seasonal variation in temperature, or photophase duration), which in turn drive a consequent patterning in animals' biological functioning through the circadian timing system; namely the biological clock (Aschoff, 1965; DeCoursey, 1989, 2001; Palmer, 2002; Wright, 2002; Dunlap, 2004). The recurrent fluctuation in biological activity in relation to external cyclical habitat conditions is collectively known as biological rhythms. The biological rhythms of animals are expressed at different levels of organization (e.g. from genes and physiology to species and community) (Joshi, 2005). Therefore, these can be studied by considering different kinds of indicators both in the laboratory and in the field (e.g. gene expression, hormones secretion, and behaviour) (reviewed by Aguzzi and Company, 2010).

Biological rhythms are generated by a mechanism that can be described accordingly to a three-step circuit model (reviewed by Tosini and Aguzzi, 2005): (I) input pathway, (II) processing system, and (III) output pathway. The first is the sum of all the sensitive apparatuses processing the environmental information. The second is represented by the circadian peacemaker, a group of cells within the central nervous system that are capable to generate a self-sustained oscillation, the functioning of which can be adjusted (i.e. entrainment) to the periodic environmental fluctuations. The third compartment is represented by the soma, where rhythm can be expressed at a different level of organization (Dunlap, 2004; Refinetti, 2006).

These rhythms can be characterized in terms of period (i.e. the time elapsed for one complete oscillation), and phase (i.e. the displacement between a chosen point and a reference point). Biological rhythms can be operatively classified as endogenous or exogenous,

depending on their degree of persistence under laboratory constant conditions. An endogenous (self-sustained) rhythm is maintained in laboratory under the absence of referencing geophysical cycles (e.g. constant darkness, temperature, or water characteristics). There endogenous fluctuations have a genetic basis, so mutations can affect their expression in terms of periodicity as observed in mutant (*per* and *tim*) *Drosophila* (Konopka and Benzer, 1971). Exogenous rhythms disappear when animals are transferred to constant conditions. These rhythms represent the adaptation of animal physiology to cyclic external fluctuations, whose overwhelming action imposes a masking effect on the true genetic-controlled rhythmcity.

Rhythmic physiology underlying activity patterns is necessary to optimize feeding, growth, and reproductive practice in aquaculture (Boeuf et al., 1999; Felip et al., 2008; Carrillo et al., 2010; Montoya et al., 2010). Also, massive population displacements within sampling windows can consistently alter population/stock and biodiversity assessments. Therefore, there are two fundamental operative scenarios for chronobiological studies: the field and the laboratory (see **Fig. 1**). Within the framework of present challenges to marine exploration, fishery management, aquaculture development, and biodiversity protection studies, species' behavioural rhythms are progressively acquiring importance (Naylor, 2005).

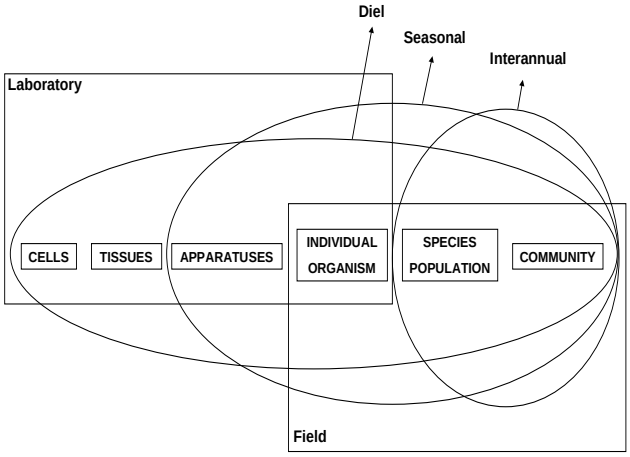


Fig. 1. Schematic representation of the complex relationships that must be considered when researching on biological rhythms at different levels of complexity and at different temporal scales, both in the laboratory and in the field.

Sampling must take into account the cyclically appearing and disappearing of individuals of different species in relation to day-night or seasonal periodicity (Naylor, 2005).

Laboratory tests on individuals for the determination of rhythmic activity patterns could represent the key to extrapolate experimental data to production protocols in aquaculture or at the population level in fishery management. The three-steps model of circadian system organization (i.e. input pathway, processing system, and output pathway) is not of simple elucidation in the majority of species studied up to now (e.g. the presence of multiple input pathways in decapods are represented by eyes, the protocerebrum pigmented area, and the caudal photoreceptor; and, moreover, masking effects must be taken into account). That situation evidences the necessity to implement new technologies for behavioural monitoring and experimental designs more customized according to the different life habits and peculiarity of each targeted species. In this sense, life-habits resembling experimental facilities are important to simulate at best putative geophysical cycles reducing all possible stressors. Furthermore, laboratory tests aiming to extrapolate results in various contexts (i.e. aquaculture and fishery) should employ an elevated number of individuals, in order to obtain representative data.

The Norway lobster, *Nephrops norvegicus* (Linnaeus, 1758), is a burrowing decapod, inhabiting muddy continental margin shelves and slopes of the European Atlantic and Mediterranean coasts (Farmer, 1975). This species represents one of the most important species for EU trawling fishery (Bell et al., 2006). Studies on the possibility to cultivate this species have shown scant results (Figueiredo and Vilela, 1972; Thompson and Ayers, 1989; ICES, 1999, 2000; Dickey-Collas et al., 2000; Morais et al., 2001; Rotlland et al., 2001).

In the field, catches fluctuations (i.e. burrow emergence) at different depths (**Fig. 2**) can be related to the day-night cycle (Oakley, 1979; Chapman and Howard, 1979). *Nephrops* exhibits a diel catchability pattern in relation to its wide depth range of population distribution that is light intensity dependent (Chapman et al., 1975). Whilst in the upper continental shelf of the Atlantic Ocean (10-50 m) the peak capture is nocturnal under the influence of the moon light (e.g. Farmer, 1974; Chapman et al., 1975; Moller and Naylor, 1980), the patterns are crepuscular in the lower shelf (50-200 m) of Atlantic Ocean and Mediterranean Sea (50-200) with peaks at sunset and sunrise (Aguzzi et al., 2003a), whereas, in the upper slope (200-430 m) of Mediterranean Sea peak captures become fully diurnal.

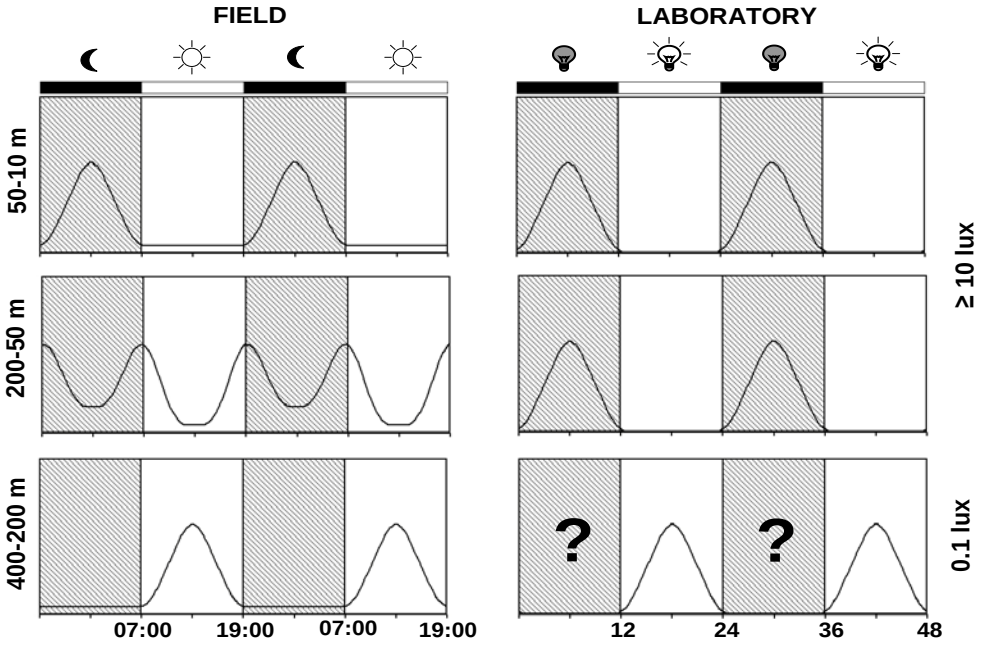


Fig. 2. The comparison of *Nephrops* locomotor activity rhythms in the field and in the laboratory. Data modelling is presented over two consecutive day-night cycles as a summary of obtained data. In the field (FIELD) the locomotor activity rhythm is inferred from catches fluctuations obtained during hourly-scheduled trawling at three different depth ranges of shelves (10-200 m) and slopes (200-400 m). In the laboratory, (LABORATORY) the locomotor activity of animals processing from shelf and slope areas is recorded with actographic methods in individual aquaria, during experimental tests with different light intensity conditions (lx). Photoperiod utilized has been in most of cases of standard (i.e. 12:12 LD) type. Dark/white areas indicate night and day phases, respectively.

Laboratory tests in constant darkness indicate that *Nephrops* displays an endogenous regulation of the locomotor activity with peaks always at subjective night, independent from the shelf or slope depths of animals sampling (reviewed by Aguzzi and Sardà, 2008). The same pattern occurs in other physiological variables as cardiac rate and oxygen consumption (Aguzzi et al., 2003b, 2004).

In order to clarify disagreement between laboratory and field data, several investigation on photoperiod cycles and different light intensities (i.e. simulating field scenario of depth increase from the shelf to the slope) were carried out recently (Aguzzi et al., 2009; Chiesa et al., 2010). Results showed that *Nephrops* preserve nocturnal peaks of locomotor activity (**Fig. 2**) if exposed to laboratory light cycle of intensity higher or equals to 10 lx (i.e. simulating shelf). If light intensity decreased to 0.1 lx (i.e. simulating slope) the out of burrow activity timing shifts to day-hours. However, these results should be considered as preliminary, since the reduced number of individuals tested creates difficulties for the comparison to the field data.

The cross-comparison of field and laboratory results indicates that *Nephrops* locomotor activity patterns at the base of burrow emergence are not merely the result of light entrainment. Apparently, a complicated and still largely uncharacterized ecological modulation of burrowing activity can stimulate or inhibit particular traits of that behaviour as reported in other species tested with similar experimental protocols (Mrosovsky and Hattar, 2005).

Data on mechanisms governing rhythmic phenomena in aquatic species, both in the field and in the laboratory, are scarce (Naylor, 2005). In this context, *Nephrops* behavioural rhythms have been investigated in the field (catchability by trawling nets) and in the laboratory in the last 40 years, but the data obtained has still not provided sufficient information on the environmental or demographic factors modulating the burrow emergence rhythms and hence obtainable demographic estimates by trawling (reviewed by Aguzzi and Sardá, 2008). It is yet unclarified whether emergence behaviour (and therefore catchability) is controlled by complex endogenous and exogenous mechanisms. As a result, temporally scheduled trawling may underestimate the population size due to the suppression of emergence in an unknown proportion of individuals. Accordingly, the objective of my study is to verify the presence of different exogenous rhythmic behavioural patterns in locomotor activity of *Nephrops norvegicus*. Using IR geo-referenced actographic technology I investigated 3 different components of burrowing behaviour in relation to sex and carapace length. Further, I tried to extrapolate results to population dynamics in the field.

2. MATERIAL AND METHODS

Data shown in this thesis were obtained during the NORIT project (CTM20055-02034/MAR) founded by the Ministry of Science and Innovation, 2007-2010 (MICINN). I present results from five experiments in which only a small proportion of behavioural data was analyzed. Experiments were run in the facilities of the Zone of Experimental Aquaria (ZAE) of the Marine Science Institute (ICM-CSIC) in Barcelona.

Abbreviation list:
DK: Door-Keeping; PE: Proximal-Emergence; DE: Distal-Emergence; TLB: Total Locomotor Bouts; LD: Light-Darkness

2.1 Animals sampling and transport

Sampling was carried out by a commercial trawler on the shelf area off the Ebro delta (80 m depth). Animals were collected at night-time. During the operations on the deck a dim red light was applied to not damage either animals' eyes or circadian photoreceptors (Gaten, 1988;

Gaten et al., 1990). Chapman et al. (2000) demonstrated that light induced eye damage in *Nephrops* is irreversible, even if such damage does not seem to influence long-term survival, growth and reproduction. Therefore, we avoided exposure of the animals to sunlight in order to discharge any potential effect upon the functioning of photoreceptors.

Soon after the hauling, specimens were immediately transferred to dark and refrigerated containers (i.e. water temperature of 5±1 °C), provided with a constant aeration of the water. During the transport specimens were maintained separately in order to avoid aggressive social interactions potentially leading to severe body damage for fighting.

2.2 Animals acclimation

Once at the laboratory, animals were transferred to the acclimation tanks. These tanks were hosted within the acclimation facilities of the ZAE, inside a light-proof isolated chambers in which specimens were maintained for 1 month under the following conditions: (I) constant temperature of 13±0.5 °C, as reported for the Western Mediterranean continental slope throughout the year (Hopkins, 1985; Salat, 1995), (II) random feeding time (i.e. once per week at different hours) in order to prevent entrainment through peripheral food-entraining oscillators, as shown for both crustaceans (Fernandéz de Miguel and Aréchiga, 1994; Clemens et al., 1998) and mammals (Stephan, 2001), and (III) LD blue monochromatic photoperiod regime (photophase duration matched the natural condition at the latitude of Barcelona, 41º 23' 0'' N; 2º 11' 0'' E). In relation to that latter condition, monochromatic blue light (i.e. 480-nm) LD regime matches the life habitat conditioning of the species. That radiation fits the maximum absorbance of *Nephrops* visual pigments (Loew, 1974) and its presence with different intensities can recreate photic conditionings experienced by populations of shelves and slopes. In fact, this radiation is the sole invariantly present within the whole twilight zone range (Jerlov, 1968; Herring, 2002). Given to its presence over the entire light range of oceans, this blue light cycle likely entrains crustaceans biological clocks of the majority of marine organisms (Aguzzi and Company, 2010), being blue light photoreceptors ubiquitous in the animal kingdom (Hankins et al., 2008). Also, light-ON and -OFF, were progressively attained and extinguished within 30 min in order to acclimate animals' eyes to light intensity change. That was required to avoid photoreceptors degeneration (i.e. rhabdom deterioration and visual pigments photolysis) as it occurs at sudden eyes light exposure (see also Section 2.1) (Gaten et al., 1990).

The acclimation facility hosted individual cells (25x20x30 cm) made with a plastic net of different sizes in order to allow oxygenation, but not the contact between animals (**Fig. 3**). Soon before each experiment the animals were transferred from the acclimation facility to the experimental room by dark containers.

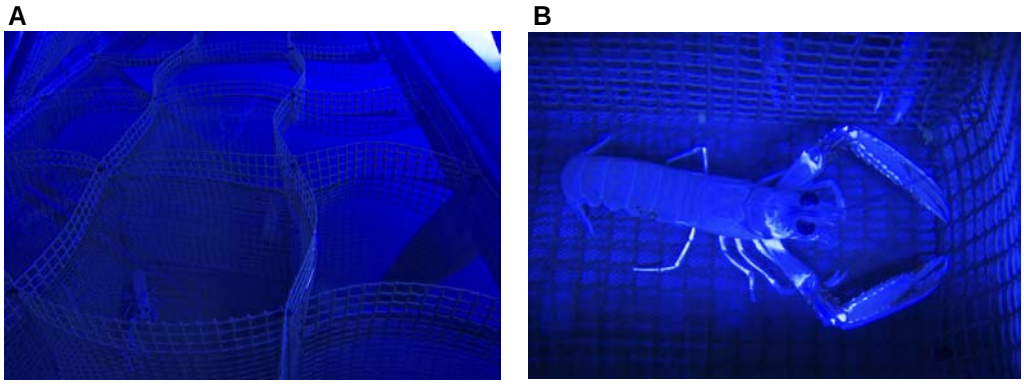


Fig. 3. Animals within the acclimation facility of ZAE (ICM). **A:** A detail of separation cells hosting individual in an isolated fashion, under the blue photophase. **B:** A zoom of an individual cell containing an animal. Another individual from the near cell is trying to enter its claws. That, testify the aggressive territorial behaviour.

2.3 The actographic aquarium and the management system for data recording and geophysical cycles simulation

In this study we used locomotor data obtained with a new technological approach for the study of the circadian locomotor activity of burrowing decapod crustaceans of commercial and aquaculture relevance. A new individual actographic aquaria (60×25×20 cm) made of transparent polycarbonate was recently designed (**Fig. 4**) (Aguzzi et al., 2008). Each experimental aquarium was constructed in order to recreate environmental features of *Nephrops* typical habitat, such as the burrow (entrance and tunnel diameters of 10 cm and 7 cm, respectively; tunnel length of 25 cm; angular inclination of burrow entrance of 20º) with a terminal ventilator shaft (0.4 cm of diameter) and substratum simulating the presence of the sediment, obtained by pasting sediment at the aquarium base floor (Aguzzi et al*.,* 2008). The aquarium automated functioning in terms of data collection and light intensity cycles' generation was derived from actographers used in mammalian chronobiology (**Fig. 4**). *Nephrops* locomotor bouts were tracked when specimens crossed infrared (IR) vertical detection barriers, creating a drop in intensity at IR receiving receptors. This actographer was endowed with three progressively distantly located detection IR barriers. That methodological approach permits to discriminate three different components of rhythmic behaviour, which could be endogenously (i.e. light controlled) of exogenously (i.e. demographically controlled; e.g. sex and size) modulated. The barrier at the burrow entrances measured the DK behavior as the activity of the animal at the burrow entrance. The barrier located at the intermediate zone of the aquarium measured the PE as the activity of excursion of the animal; and finally, at the opposite and more distant side, the DE measured the full excursion of the animal. Each aquarium barrier was equipped with the following LEDs asset (see **Fig. 4**). IR receiving LEDs were: n=5 at the DK barrier, n=16 at PE barrier, and n=16 at the DE barrier. Emitting IR photodiodes were positioned on the top of each barrier as follows: n=2 at the DK barrier, n=8 at both the PE and DE barrier. The use of infrared detection wavelengths does not constitute a photic interference for the circadian photoreceptors of *Nephrops*, which possess an eye adapted for vision in dim-light conditions, with a peak of absorbance at 498 nm (Loew, 1974). Blue monochromatic (480-nm) LEDs were integrated with IR ones in order to provide the light cycle utilized for experimental

tests. 4 blue LEDs were integrated within PE and DE barriers, illuminating each aquarium from above (see **Fig. 4**).

The complete data series was calculated for each aquarium and barriers by a central computer unit that at the same time managed the control of intensity and duration of the blue light photoperiod regime (see **Fig. 4**). I applied a LabView application that could control up to a 12 different aquaria at once (Aguzzi et al., 2008; Mànuel and del Río, 2005). The light cycle was also generated by a microcontroller circuit managed by the central computer.

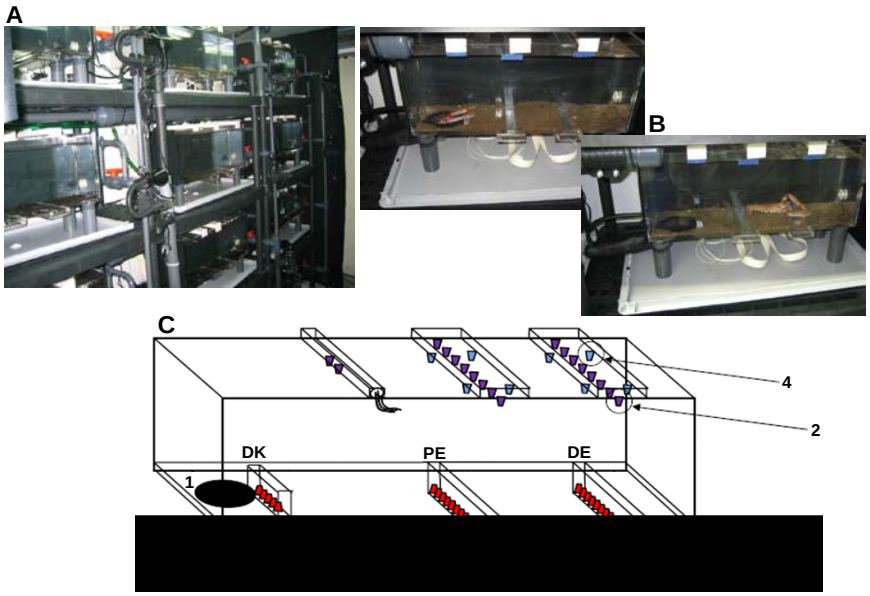


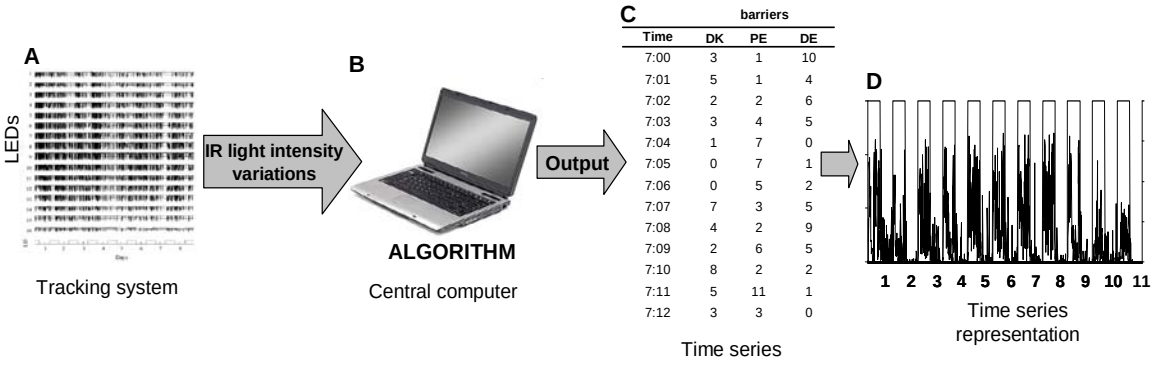
Fig. 4. The experimental IR actographic aquarium utilized for burrow emergence tests in *Nephrops*, as carried out during the previous Spanish National research project, the NORIT (adapted from Aguzzi et al., 2008). **A**, aquaria in series within the ZAE (ICM-CSIC) refrigerated chamber; **B**, pictures depicting two consecutive moments of burrow emergence behaviour in a tested specimen; and finally, **C**, the scheme depicting the different parts of the aquaria with the three detection IR barriers (DK, PE, and DE): (1) burrow mouth location at one extremity and details of the barriers assemblage (2 and 3, IR emitting and receiving LEDs, as well as 4, monochromatic blue light LEDs for the LD cycle generation).

The intensity of light gradually increased and decreased during the cyclically transition between scotophase and photophase in order to simulate sunrise and sunset illumination light conditions.

The central computer send the command to check the status of all LEDs within all barriers by 5-s. The tracking system transmitted data about IR light intensity variations (i.e. at animal presence) (**Fig. 5A**) from the receiving photodiodes to the central computer, but an algorithm was required (**Fig. 5B**) in order to: I. discard incorrect detections (e.g. water turbidity or impurities near the sensors); II. eliminate signals of photodiodes that did not properly work; and, III. transform the signal into a movement record. Additionally, the algorithm permitted: I. the filtration of data in order to exclude movement detection caused by claws and antennae; and II. the binning of IR 5-s time series of data from all LEDs of each barrier into numbers of locomotor bouts by minute. The result of the tracking system was a time series in which was reported the number of specimens' locomotor bouts per minute for each detection barrier (**Figs. 5C, D**).

2.4 Experimental settings and data acquisition

52 adult animals (37 males and 15 females) with mean carapace lengths (mm±sd) of 37.87±4.97 were used in our experimental tests. Sex was determined before the experiments by visual analysis. In order to consider a homogeneous data sample, we considered the first 11 days of locomotor time series. In fact, the treatments applied during the different experiments of NORIT project varied after that temporal window. Homogeneous experimental conditions were therefore similar to those used for acclimation (see Section 2.2): *i.* Blue monochromatic light (480-nm) intensity cycle; *ii.* standard photoperiod duration of 12-12 LD with light-ON at 07:00 and light-OFF at 19:00 and progressive intensity maximum attainment and extinguishment within 30 min; *iiii.* light intensity of 0.1 lx simulating depth greater than 200 m (see **Fig. 2**); and finally, *iiiii.* no feeding to prevent other sources of synchronization other than light.



Time	DK	PE	DE
7:00	3	1	10
7:01	5	1	4
7:02	2	2	6
7:03	3	4	5
7:04	1	7	0
7:05	0	7	1
7:06	0	5	2
7:07	7	3	5
7:08	4	2	9
7:09	2	6	5
7:10	8	2	2
7:11	5	11	1
7:12	3	3	0

Fig. 5. Schematic representation of the process of locomotor data acquisition and treatment at the three detection barriers of each actographic aquaria (DK, PE, and DE). **A:** Light intensity variation recorded by 16 receiving LEDs at the Proximal-Emergence detection barrier. **B:** Central computer in which data were pre-processed by an algorithm for filtering. **C:** Time series resulting from data pre-processing. Locomotor bouts were reported per min for each of the three barriers. **D:** Time series representation shows the rhythmic behaviour of an animal over consecutive days at the PE barrier. That behavioural pattern is diurnal at 0.1 lx. The quadratic line represents the blue monochromatic light intensity fluctuation.

2.5 Data analysis

Time series were transformed from number of locomotor bouts per min to number of locomotor bouts per 10 min. Time series of locomotor data were represented over the time and resulting patterns were utilized for a primarily inspection.

In order to ascertain the occurrence of global differences in locomotor activity at the three barriers depending form their distance from the burrow entrance, we calculated the total activity for each animal at each barrier as the sum of all recorded values during 11 days. TLB were ranked at discontinuous intervals of 2000. Then we counted the number of individuals per bouts rank and produced frequency distribution histograms for each barrier. Furthermore, we computed the total diurnal and nocturnal activity as the TLB during photophase and scotophase, respectively.

In order to assess the number of rhythmic animals and their periodicity in locomotor activity at each barrier, I firstly screened all time series by Sokolove-Bushell periodogram analysis by the software El Temps (Prof. Díez-Noguera, Univ. Barcelona-UB). In output plots periodicity is indicated by the most significant ($p<0.05$) peak value expressed in min (Sokolove and Bushell, 1978). Time series were screened within the range 600 min (i.e. equals to 10-h) and 1600 min (i.e. equals to 27-h), in order to detect both inter-diel and diel oscillations

(Refinetti, 2006). We utilized the percentage of variance (%V) of each significant periodogram peak as measure of rhythm fluctuation stability and strength (Carpentieri et al., 2006; Chiesa et al., 2006).

The diurnal or nocturnal phase of reported locomotor patterns of rhythms was assessed by waveform analysis in order to support the translation of results in the field. That analysis consisted in producing averaged activity profiles that are calculated on a 24-h basis as follows: we subdivided time series in columns of 144 values (i.e. corresponding to 24-h); all temporally coinciding values were averaged throughout all subsets (i.e. days). The resulting curve was represented over 48-h (i.e. double plotted), along with standard error of the mean (SEM). Significant activity (i.e. the waveform peak) was estimated by computing the Midline Estimating Statistic Of Rhythm (MESOR). MESOR was computed by re-averaging all waveform values and represented by a horizontal line in the waveform plot. All values above the MESOR depicted the significant increase in averaged locomotor pattern (Hammond and Naylor, 1977; Aguzzi et al., 2005). The temporal positioning of the mayor peak is chose to characterize the phase of the rhythm (i.e. diurnal or nocturnal), whose amplitude can be assumed as the distance between the MESOR and the peak. Consequently, considering the potentially complex modulation in burrow emergence rhythms, I grouped animals according to similitude in their behavioural dynamic at the three barriers. The division was carried out by considering for each animal the waveforms profiles for DK, PE, DE barriers at the same time and trying to establish a trajectory description of the emergence behaviour during the 24-h cycle. Finally, a general mean waveform was computed for each recognized group of animals in order to resume activity into different typologies that can be used for comparison with field data. General mean waveforms were also double plotted to better visualize the diel fluctuation.

I tested the grouping of the different rhythmic behavioural patterns comparing them with a multidimensional model described by a Principal Component Analysis (PCA) in which were introduced two representative variables (TLB and periodicity). Furthermore the PCA was utilized to determine whether the three recorded components of the burrow emergence rhythm (DK, PE, and DE) are different modulated sex. Additive ANOVA was utilized to individuate differences in carapace length among sexes and among the different rhythmic behavioural patterns. Two way ANOVA (Repeated measures analysis of variance) is utilized to assess differences (in terms of TLB and sex) among: (I) TLB at different barriers; (II) TLB during day and night. Finally, X^2 test was performed in order to establish if the expression of the different rhythmic behavioural patterns varies regarding to sex.

3. RESULTS

With the new actographic system, clear rhythmic activity patterns were reported in the majority of tested animals during 11 days under monochromatic blue 12-12 LD photoperiod conditions of intensity equals to 0.1 lx. Locomotor patterns were efficiently discerned at the three barriers allowing further time series analysis with methods of chronobiology. A visual inspection of recorded data patterns often allowed the individuation of diel oscillations in the time series in relation to the LD cycle.

Time series globally showed inter-day variations within the same individuals. Also, activity levels varied among individuals. As an example we introduced the time series plots for 3 individuals **(Fig. 6**) that exhibited different rhythmic behavioral patterns at the three barriers.

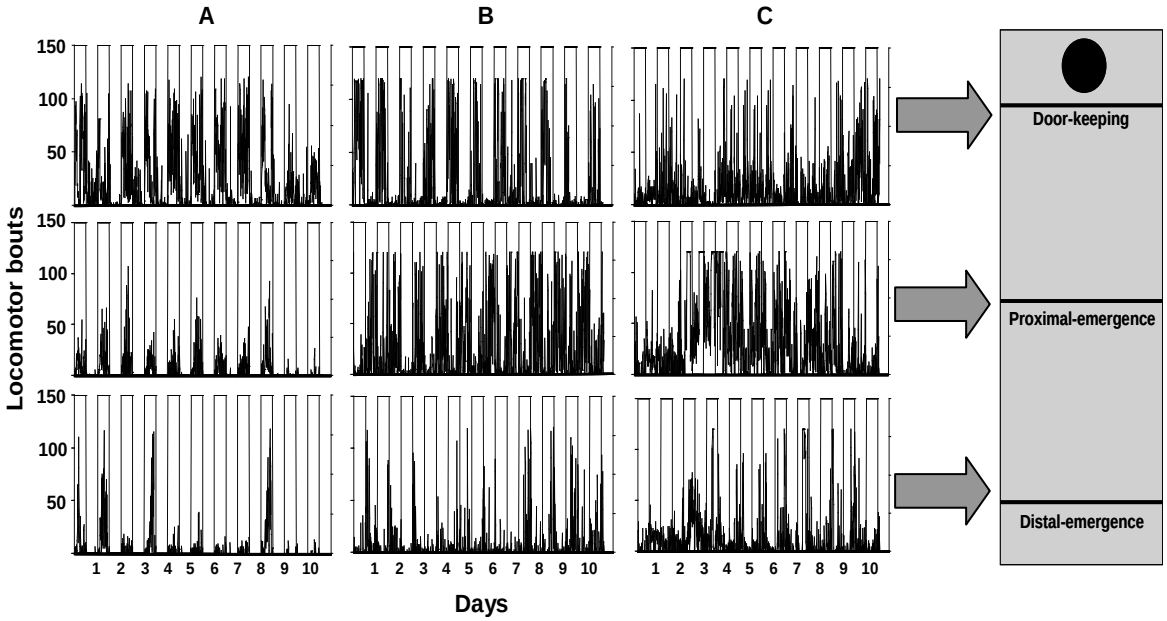


Fig. 6. Time series for three different individuals (A, B, and C) are reported at the three barriers (DK, PE, and DE) as an example of recorded locomotor patterns in the whole set of animals. Time series were linked to the barrier positioning by presenting on the right part of the schematic representation of the experimental aquarium these barriers are indicated. The quadratic line represents the light intensity cycle.

3.1 Total activity analysis

Total locomotor activity of *Nephrops* at the three different barriers (i.e. DK, PE, and DE) showed a wide range of variation in its magnitude. Activity decreased with distance of the barrier from the burrow entrance. The distribution of animals in relation to ranked values of TLB as computed during 11 days is presented in **Fig. 7**. From the comparison of the three histograms we can observe a trend in locomotor bouts reduction from DK to DE. The number of *Nephrops* that presented values of TLB smaller than $30x10^3$ was 29 at DK, 40 at PE, and 46 at DE. Animals showing high activity levels (TLB>$85x10^3$) were present only at DK. The global sum of the activity of all animals at each barrier also showed applying two way ANOVA a significant decrement ($F_{2.92}$=6.19; p<0.01) of TLB according to distance, but no differences among sexes were detected.

Calculating the TLB separately for photophase and scotophase at each barrier. Animals showed the same pattern observed for TLB, since during photophase the values of locomotor bouts significantly decrease ($F_{2.92}$=10.67; p<0.01) with the distance from the burrow. The same decrement is observed during scotophase, but without significant differences. No differences among sexes were detected in either case.

Furthermore, TLB at DK barrier during photophase ($922x10^3$) is significantly larger ($F_{1.46}$=17.93; p<0.01) than that reported during scotophase ($540x10^3$). At PE and DE barriers TLB did not show significant differences with a trend to decrease during scotophase. At the PE barrier I observed an opposite trend in females which are significantly ($F_{1.46}$=4.53; p<0.05) more

active than males during night. In none of the barriers a recognizable variation in TLB could be observed through 11 consecutive days.

3.2 Periodogram analysis

Periodogram analysis detected significant locomotor rhythmic patterns at each barrier in the majority of tested animals. Significant periods were found in: 38 animals at DK with an average period of 23:59±00:43; 37 animals at PE barrier with an average period of 23:57±00:46; 37 animals at DE barrier with an average period of 23:48±01:10. We also observed at the three barriers few individuals that exhibited periods close to 20-h periodicity (i.e. an individual for PE at 20:00 h and two ones for DE at 18:50 and 21:35 h). Four animals did not show periodicity at any barriers, thus had to be eliminated in the following analysis.

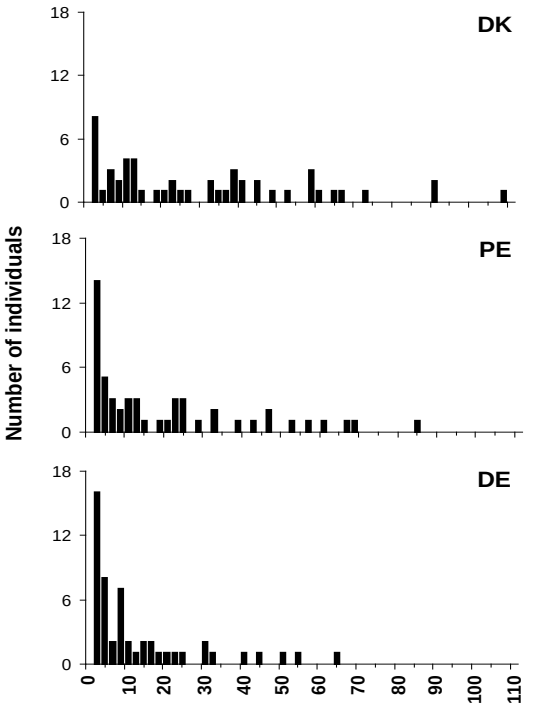


Fig. 7. Frequency distribution of *Nephrops* (n=52) clustered by the sum of the TLB at the three barrier (DK, PE, and DE). In each graphic is also indicated the sum of TLB of *Nephrops* all together (n=52) at each barrier.

3.3 Waveform analysis

Waveform analysis produced consensus 24-h averaged activity patterns of strength that differed among animals and within each animal at the three barriers. That data is in agreement with total activity analysis and periodogram outputs. Waveform plots of animals of **Fig. 6** are presented in **Fig. 8** as an example of analysis outputs. The three individuals showed very different behavioural patterns. The first animal (**Fig 8A**) showed a diurnal behavioural pattern at the three barriers. The second animal (**Fig. 8B**) exhibited a diurnal behavioural pattern at DK barrier, a nocturnal one at PE barrier as well as a weak crepuscular pattern at the DE barrier. The third individual (**Fig. 8C**) showed a nocturnal activity at DK barrier, a diffuse nocturnal activity at PE barrier, and a diurnal activity at DE barrier. We visually recognized three groups of animals according to their common pattern of rhythmic behaviour, comparing the movement at the three barriers.

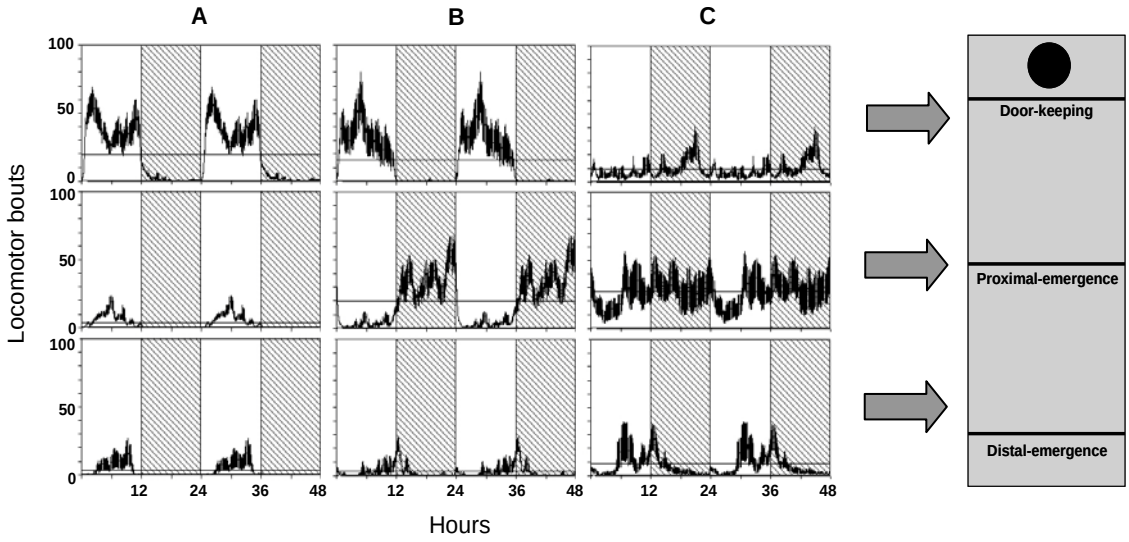


Fig. 8. Double-plotted waveforms as an example of three different behavioural patterns (A, B, and C of **Fig. 6**) at the three barriers (DK, PE, and DE). White areas represent photophase, while shadowed ones represent scotophase. The horizontal line represents the MESOR. In the right part of the graphic a schematic representation of the experimental aquarium indicate the spatial distribution of the barriers.

We defined these patterns in relation to the strength of relationship with the burrow: (I) the specimen presented a locomotor activity always influenced by the burrow (Burrow-centred; n=31); (II) the specimen locomotor activity is partly influenced by the burrow (Burrow-oriented; n=8); and (III) the specimen locomotor activity is poorly influenced by the burrow (Poorly burrow-oriented; n=9). Mean waveforms at each barrier were computed for each of the three patterns by averaging individual curves in order to enhance the occurrence of global differences among the three rhythm typologies observed (**Fig. 9**).

Individual waveform analysis showed the occurrence of variable nocturnal or diurnal phase for each animal at each barrier. Individual waveform output plots were analyzed to determine diurnal or nocturnal behavioural patterns for all individuals at the three barriers. Individuals were also subdivided in relation to the rhythmic behavioural patterns previously recognized (**Table 1**). Some animals showed unclassifiable behavioural patterns as follow (3, 2 and 3 individuals at DK, PE, and DE barriers, respectively for Burrow-centred).

The observed plasticity of rhythmic behavioural patterns can be simplified in order to translate that information to the field population dynamics. Animals can be divided in: fishable that identifying animals out of the burrow (i.e. DE barriers) and thus vulnerable to trawling; not fishable that are animals near the burrow (i.e. at DK barrier) or at an intermediate position (i.e. at PE barrier) that are probably not vulnerable to the trawling (**Table 2**).

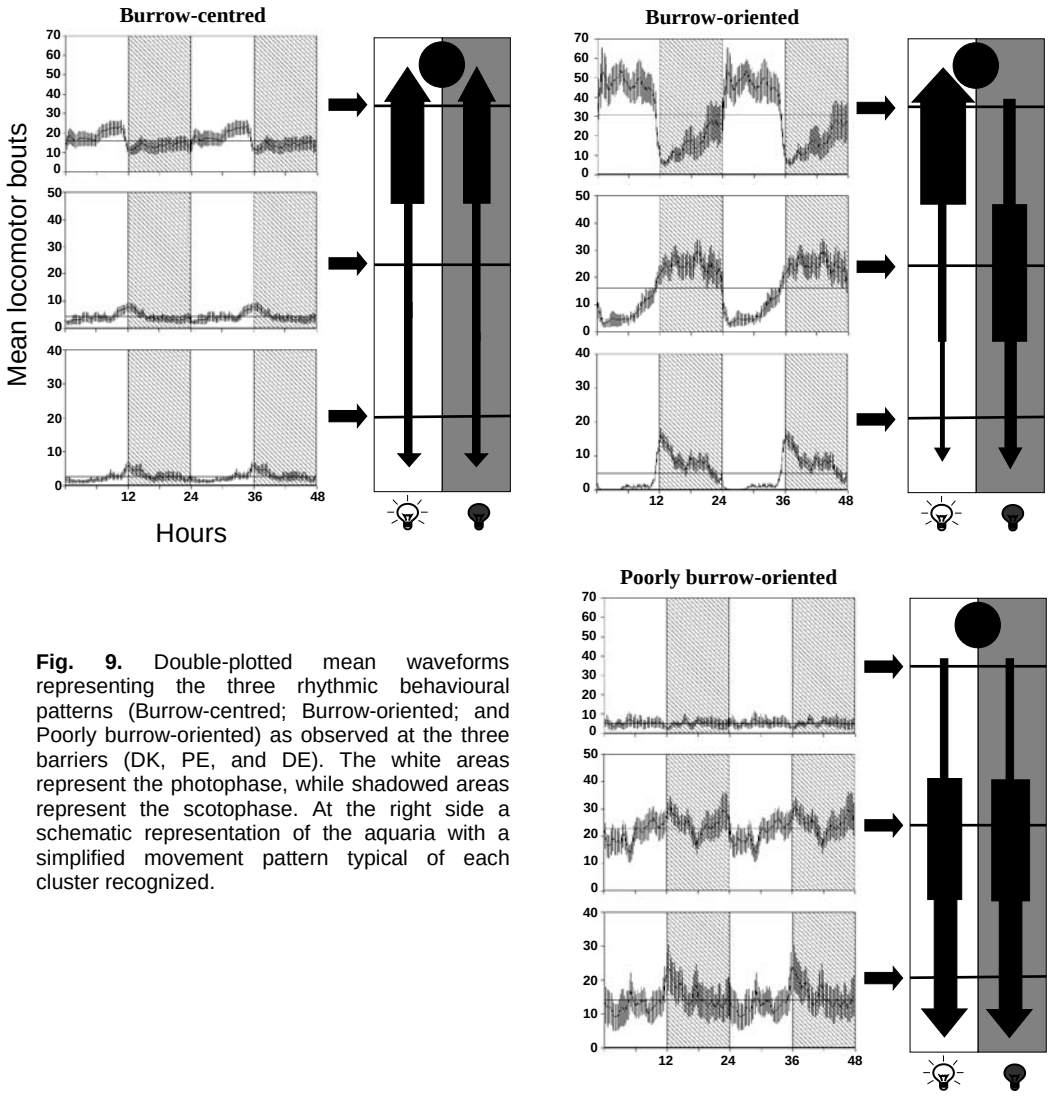


Fig. 9. Double-plotted mean waveforms representing the three rhythmic behavioural patterns (Burrow-centred; Burrow-oriented; and Poorly burrow-oriented) as observed at the three barriers (DK, PE, and DE). The white areas represent the photophase, while shadowed areas represent the scotophase. At the right side a schematic representation of the aquaria with a simplified movement pattern typical of each cluster recognized.

Table 1. Diurnal and nocturnal patterns of all animals tested in waveform analysis. The different pattern is expressed for each barrier (DK, PE, and DE).

Rhythmic behavioural patterns		DK		PE		DE	
	n	*Diurnal*	*Nocturnal*	*Diurnal*	*Nocturnal*	*Diurnal*	*Nocturnal*
Burrow-centred	***31***	21	7	13	16	10	18
Burrow-oriented	***8***	7	1	0	8	1	7
Poorly burrow-oriented	***9***	5	4	4	5	4	5

Table 2. Distribution of the animals in relation to their hypothesized vulnerability to trawling within each rhythmic behavioural pattern.

LABORATORY		FIELD			
Rhythmic behavioural patterns		**Fishable**		**Not fishable**	
	n	Day	Night	Day	Night
Burrow-centred	***31***	10			18
Burrow-oriented	***8***	1	7		
Poorly burrow-oriented	***9***	4	5		

3.4 Principal Component Analysis

The variables introduced in the PCA are considered the most representative for discrimination of different rhythmic behavioral patterns. The starting screen plot obtained let us considered only the first 2 factorial axis, with the first two (PC1 and PC2) that represented the 59.16% of total variance. The first factorial axis (PC1) is most characterized by TLB at PE barrier during photophase (-0.91) and at DE barrier during scotophase (-0.93). The second factorial axis (PC2) is most characterized by TLB at DK barrier during photophase (-0.87) and during scotophase (-0.74).

The projection of the individuals in the factor plane (PC1xPC2) permitted to evaluate how their rhythmic behavior is characterized by the two factorial axes (**Fig. 10**).

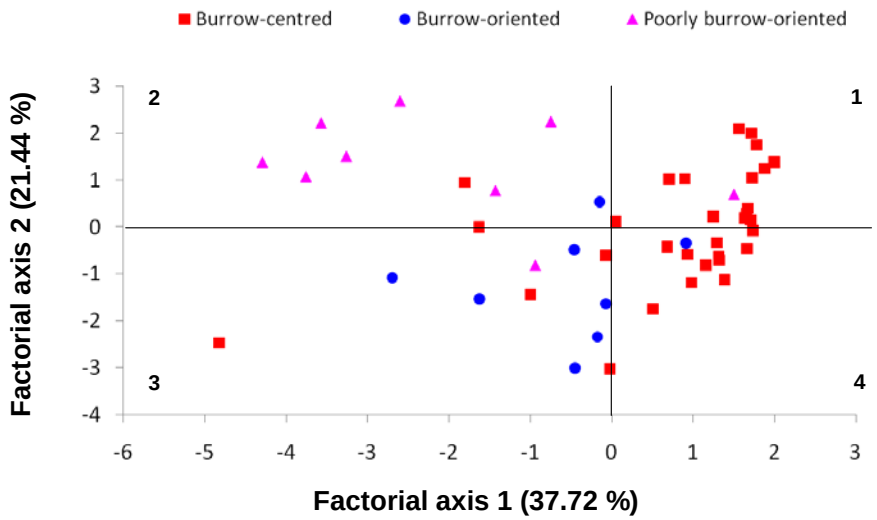


Fig. 10. The representation of the factorial plane depicted by the factorial axis 1 and 2. Individuals are distributed in the plane and symbols of different colors indicate the rhythmic behavioural pattern recognized in the previous section (Burrow-centred, Burrow-oriented, and Poorly burrow-oriented).

Animals previously grouped as Burrow-centred pattern are grouped in the first and fourth quadrant that are characterized by few TLB at PE and DE barriers. Animals with Burrow-oriented pattern are distributed in the third quadrant that is characterized by great values of TLB at DK barrier during photophase and scotophase. Animals previously grouped as Poorly burrow-oriented pattern are distributed in the second quadrant that is characterized by few movements at DK barrier during photophase and scotophase.

Female individuals are distributed in the first and fourth quadrants (except for one individual, see **Fig. 11**) that are characterized by low values of TLB at PE and DE barriers. Males seem to be equally distributed in the plane depicted by PC1 and PC2. Males and females do not express different rhythmic behavioural patterns (X^2_5=3.57; p>0.61). Moreover, the additive ANOVA demonstrated that carapace length does not differ among the rhythmic behavioural patterns recognized (F_2=0.89 with p>0.42), but females results smaller than males (F_1=11.49 with p<0.01).

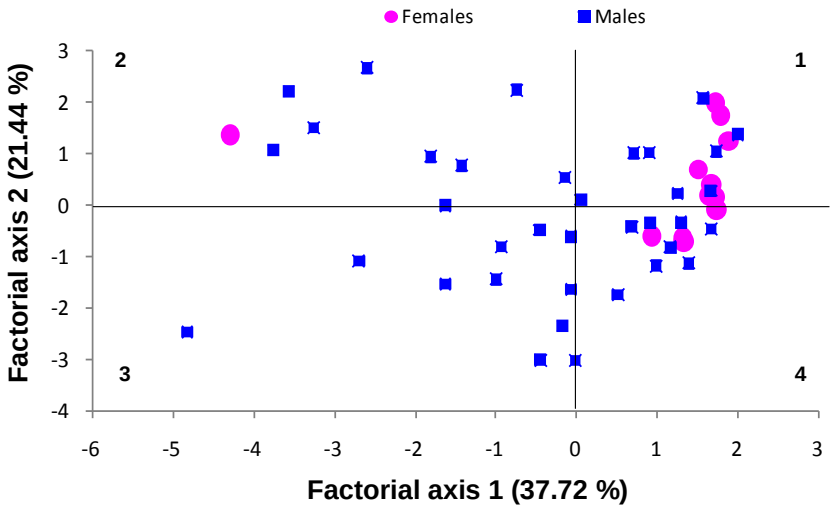


Fig. 11. The representation of the factorial plane depicted by the factorial axis 1 and 2. Individuals are distributed in the plane and letters indicate the different sexes.

4. DISCUSSION

In this study, I reported the presence of different exogenous rhythmic locomotor patterns in the burrow emergence activity of *Nephrops norvegicus* with a new actographic IR technology and I clarified the role of two important demographic traits (carapace length and sex) in influencing the proportions of these patterns in a relative large number of animals (n=52). The majority of animals exhibited clear 24-h locomotor rhythms with low total arrhythmia (i.e. only in four animals with no periodicity at any barrier), under the imposed photoperiod regime (i.e., 12:12 LD of monochromatic blue light at 0.1 lx). The possibility to study emergence rhythms with 3 different geo-referenced barriers (DK, PE, and DE) permitted to: (I) observe the occurrence of never reported before rhythmic behavioral patterns in relation to burrow distance;

(II) propose a novel functional ecological justification to the burrowing habit; (III) hypothesized some potential driver for future improvements in aquaculture research and fishery management.

A significant decrease in TLB was reported in relation to the increase of the barriers' distance from the burrow. That observation is in accordance to what reported by Aguzzi et al. (2008) in relation to the strong burrow related behavior of *Nephrops*. Moreover, TLB time series did not decrease over consecutive days (i.e. habituation), although this phenomenon has been already observed with other decapods in similar experiments (e.g. Basil and Samdeman, 2000; Palomar et al., 2005; Aguzzi et al., 2008). Some demographic traits (i.e. sex and carapace length), seems to play an important role in TLB levels reported at each barrier. Female individuals expressed a lower overall activity respect to males (even if that difference is not significant). This could be due to the lower size of animals within that group (additive ANOVA). Aguzzi et al. (2008) in experiments with the same photoperiod but light intensity of 5 lx observed the same situation for wandering excursion (i.e. PE and DE barriers) where females exhibited a reduction of overall activity.

The analysis of TLB during photophase and scotophase, respectively, highlights a different modulation of behavior at the 3 barriers. The diurnal increase of TLB at DK barrier and its nocturnal decrease at PE and DE barriers (even if not significantly) could be explicated by an ecological point of view. While a suppression of the locomotor activity at more distal barriers (i.e. PE and DE) is in accordance to visual predators avoidance, the activity at the burrow mouth can be related to territorial control (Aguzzi and Sardà, 2008; Menesatti et al., 2009; Chiesa et al., 2010) and to burrow maintenance operations (Atkinson and Naylor 1976; Palomar et al, 2005). I reported a diurnal increment in TLB at burrow mouth and a nocturnal increment in this variable at the two other distant barriers. As stated before, activity out from the burrow may be correlated to vulnerability to trawling. That occurred under a photoperiod regime that simulate the slope depths (i.e. light intensity cycles of 0.1 lx) where peaks in catches are predominantly diurnal (Aguzzi et al., 2003). That disagreement seems to be the product of uncontrolled masking factors in the laboratory that shift emergence at times not corresponding to those of peak in catches in the field. The reasons for that are presently still under research. Anyway, this phenomenon has already been observed in *Homarus americanus* (Jury et al., 2005; Golet et al., 2006) whose diel behavioural rhythms are of variable phase for a combination of endogenous and exogenous influences.

Many authors investigated the nocturnal or diurnal behavior of *Nephrops* under different laboratory conditions (Aréchiga and Atkinson 1975; Atkinson and Naylor, 1976; Hammond and Naylor, 1977; Aguzzi and Sardà, 2008; Chiesa et al., 2010). In this study, I investigated on the resolution of *Nephrops* behaviour in 3 geo-referenced components and the elevated number of tested individuals revealed a more complex scenario with both nocturnal and diurnal animals. By linking together the waveforms of each animal at the 3 barriers, I could recognize different rhythmic patterns: Burrow-centred, Burrow-oriented, and Poorly burrow-oriented (see **Fig. 9**). That grouping matched *a posteriori* with the outputs of the PCA modeling (see **Fig. 10**) confirming hence the goodness of the initial grouping. There are animals that emerge only once per day with a great activity at DK barrier and go to more distal barriers at the shift between

photophase and scotophase (i.e. Burrow-centred). Differently, other animals perform a great activity during photophase at DK barrier and go at distal barriers during scotophase (i.e. Burrow-oriented). Finally, a group of animals, showed great values of locomotor activity at more distal barriers and scarce occupancy of the burrow position (i.e. Poorly burrow-oriented).

There are no directly studies relating diel catchability variations in the field to individual rhythmic behavior in the laboratory. The extrapolation of present results to population catchability dynamics permit to improve the knowledge on demographic assessment biases by trawling performed at different times of the day (Aguzzi and Sardà, 2008). The difficulties to estimate the stock size (i.e. number of individuals and biomass) of this fishery resource relies in the uncertainty on the true number of emerging animals at a diel base. In order to evaluate the proportion of non fishable individuals, extrapolating laboratory results to field context, I considered: (I) the "Serola" bank as a fishery ground model, since the light intensity and photoperiod regime I used, simulated a typical equinox day (e.g. 12-12 LD) on the slope (see **Fig. 1**; Aguzzi et al 2003a); (II) "Serola" demographic sizes (i.e. a total of 485240 individuals for 9884 Kg in biomass; Sardà and Lleonart, 1993); and finally, (III) I counted animals with diurnal or nocturnal waveform at the DE barrier which should approximate the maximum vulnerability of specimens to trawling during burrow emergence phase. From the results of these assumptions emerged that the daytime trawling (i.e. fishery is diurnal in Cataluña) may capture only 31.3% of individuals in the population (i.e. 15 out of 48 individuals from our experiments should be vulnerable during day). Translated to the population level, 151637 out of 485240 can be captured with a biomass ratio of 3089/9884 Kg. This methodological approach is only a merely approximation of the real situation, but it should be seen as the first step for future investigations.

Chronobiological research on marine crustacean decapods such as *Nephrops* can be of great interest in marine research in relation to fishery management and conservation. Anyway, it should be taken into account that the Norway lobster may be used to improve other research aspects more related to aquaculture productivity, which are presently not fully investigated. For example individual daily scheduling of reproductive activity may affect the potential number of mates, and it's not clear whether mating has a high probability at particular times of the day. In this context is of fundamental importance known the rhythmic activity pattern of the reproductors (Waddy et al., 1995). The results obtained in this study could be used in the future to improve production efficiency of other crustacean decapods of similar burrowing behaviour (e.g. Penaeids; Rothlisberg, 1998) optimizing species-specific scheduled treatments in terms of feeding, manipulation, and reproduction.

5. CONCLUSIONS

Nephrops is a good model to investigate rhythmic behavioural patterns of crustacean decapods for the following reasons: (I) its high survival rate during experiments; (II) its good locomotor patterns; and finally, (III) its well known ecology. Moreover the possibility to interpret fishing data permit to compare and best understand the relationship between endogenous and exogenous influence. This kind of investigation revealed its potentiality in aquaculture practice

and fishery management revealing that scheduled treatment must considered behavioural plasticity to obtain good results in rearing practice. More investigation is required to understand the masking effects related to light and interspecific interactions, and to comprehend eventually size related effects in the plasticity of rhythms. Finally, the extrapolation presented in the final part of this study should be a starting point of discussion for further debates upon the complicated theme of translation of laboratory tests in elation to fishery management.

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