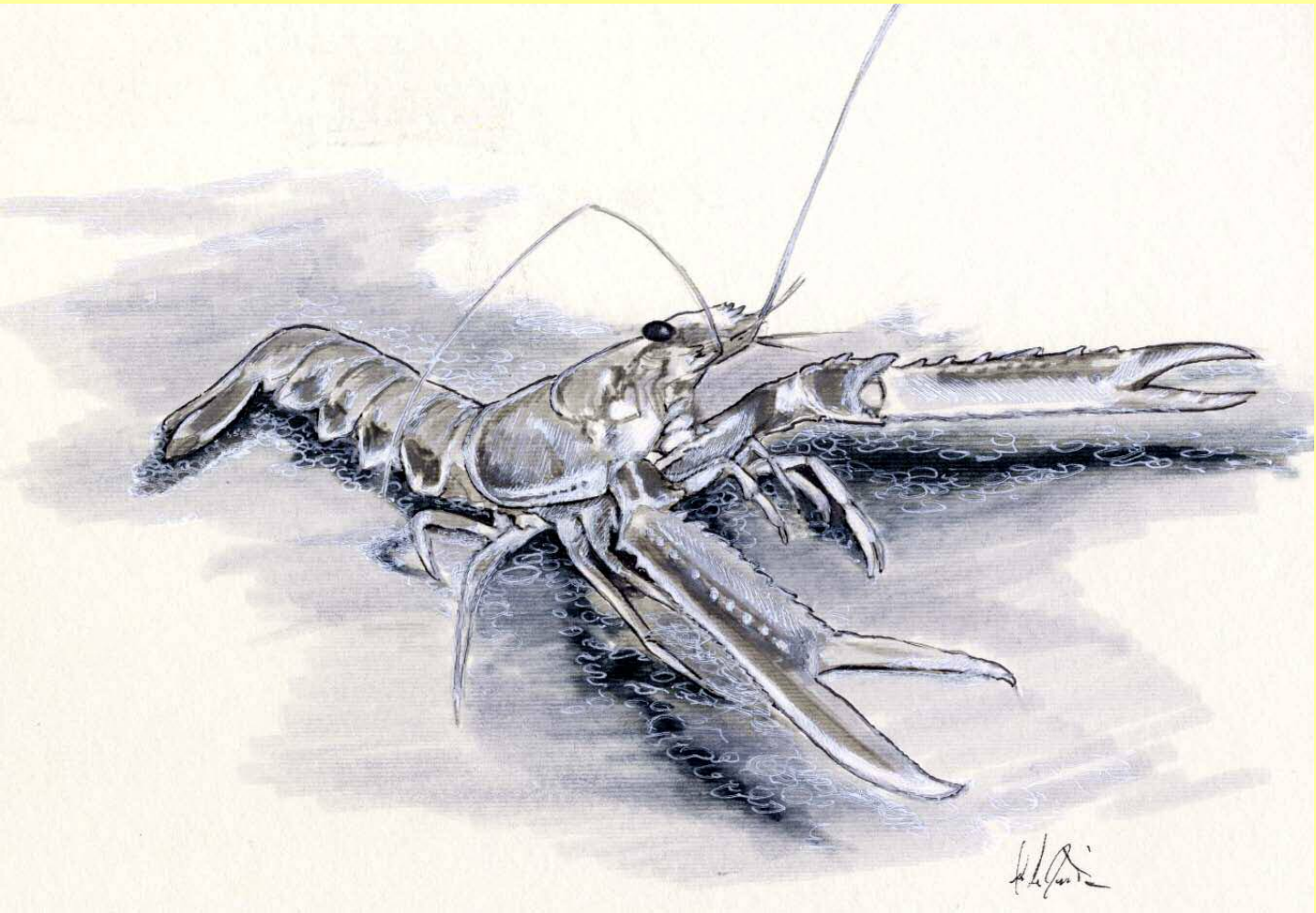
BIOLOGICAL RHYTHMS IN THE NORWAY LOBSTER (*NEPHROPS NORVEGICUS* L.):

Ecological modulation and genetic basis



VALERIO SBRAGAGLIA 2015

BIOLOGICAL RHYTHMS IN THE NORWAY LOBSTER (*NEPHROPS NORVEGICUS* L.): ECOLOGICAL MODULATION AND GENETIC BASIS

Ritmos biológicos en la cigala (*Nephrops norvegicus* L.): modulación ecológica y bases genéticas

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"Biological rhythms in the Norway lobster
(*Nephrops norvegicus*, L.): ecological modulation
and genetic basis"

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The graphic art in the cover is a reproduction of the original work by Roberto la Mantia

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ABSTRACT

Nephrops norvegicus is an important fishery resource for Europe. Its rhythmic burrowing behavior is strictly related to catchability. Here I studied such behavior under laboratory conditions. I investigated the combined effect of light and current cycles demonstrating that tidal current is an important parameter to take in account in fishery management plan not only for *Nephrops.* Then I used a transcriptomics and RT-qPCR approach on cDNA extracted from the eyestalk to elucidate the putative molecular genetics mechanisms underlying circadian gene regulation. My data are in accordance with the current knowledge of the crustacean circadian clock, reinforcing the idea that the molecular clockwork of this group shows some differences with the established model in *Drosophila melanogaster*. Finally, I studied the burrow emergence behavior in group of 4 lobsters organized in dominance hierarchy demonstrating that lower ranks are more vulnerable to trawling. I hypothesized common neural mechanisms for agonistic and non-agonistic behaviors.

RESUMEN

Nephrops norvegicus es un importante recurso pesquero. La emergencia rítmica de la madriguera afecta las capturas. He estudiado dicho comportamiento en laboratorio investigando el efecto de ciclos lumínicos y de corrientes demostrando que las mareas es un factor importante para gestionar el estado del recurso. Mediante técnica de secuenciación masiva y PCR en tiempo real sobre cDNA procedente del pedúnculo ocular he elucidado el presunto mecanismo molecular detrás de la regulación circadiana. Los resultados están de acuerdo con el conocimiento actual de relojes biológicos en crustáceos, reforzando la idea que la maquinaria molecular de este grupo muestra algunas diferencias respecto el modelo consolidado de *Drosophila melanogaster*. Finalmente, he estudiado el comportamiento de emergencia en grupos de 4 cigalas organizadas en una jerarquía de dominancia demostrando que los rangos más bajos son más vulnerables a ser capturados. Además he supuesto la existencia de mecanismos neuronales comunes entre comportamiento agonístico y non agonístico.

KEYWORDS: *Nephrops norvegicus*, biological rhythms, burrow emergence, catchability, tidal currents, clock genes, gene expression, dominance hierarchy, agonistic interactions

PREFACE

"*The truth is like the beauty.....it is a never ending story"*

Tiziano Terzani, One More Ride On The Merry Go Round (2004)

"It may be that while we think we are masters of the situation we are merely pawns being moved about on the board of life by some superior power."

Sir Alexander Fleming's speech at the Nobel Banquet in Stockholm, December 10, 1945

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Introduction

1

BIOLOGICAL RHYTHMS

Oscillatory processes are a common feature of life; they are broadly referred to as biological rhythms. They can span from the neuronal spontaneous firing rate with a periodicity of 100 milliseconds up to the decadal (10 years) rhythm of lynx population (Refinetti 2012). Biological rhythms may be directly triggered by environmental cycles or may be generated by an endogenous timekeeping (Dunlap et al. 2004, Refinetti 2006).

The presence of an endogenous timekeeping (hereafter referred to as biological clock) implies a 3-step mechanism: (i) input pathway, (ii) processing system, and (iii) output pathway. The first is the sum of all the sensory systems that process the environmental information. The second is represented by the peacemaker, a functional entity (based on biochemical feedback loops) that is able to generate a self-sustained oscillation. The third is represented by the rhythm itself (e.g. neural firing rate, hormone secretion, feeding activity, or locomotion). Daily (24 h based) rhythms sustained by a biological clock are referred as circadian (from the Latin: *circa*-around and *dies*-days) rhythms.

The formal properties of a circadian clock are: (i) the persistence of an overt circadian rhythm in constant temperature and constant light or constant darkness conditions with a free-running period of approximately 24 h, (ii), the free running period lengths must be similar when measured at different temperature, (iii) endogenous rhythms of approximately 24 h can be entrained (i.e. synchronized) by certain 24 h environmental cues, such as light-darkness cycles.

The nature and location of the pacemaker is a current theme of investigation in many marine species. That structure is of neural nature and in vertebrates is located in the suprachiasmatic nucleus of the brain (Stephan and Zucker 1972). In invertebrates, where the great part of the knowledge is based on the model species *Drosophila melanogaster*, it is located in the brain hemispheres (Peschel and Helfrich-Forster 2011). However, in crustaceans, no master clock has been yet identified, but a model of distributed clockworks has been proposed as made by different oscillators distributed in the retinular cells, neurosecretory and nervous systems (Aréchiga and Rodríguez-Sosa 2002, Strauss and Dircksen 2010).

The clock is driven by molecular machinery based on transcription and translation feedback loops. The genes involved in such machinery have shown a high level of conservation along evolution (Bell-Pedersen et al. 2005). The most evident advantage to possess an endogenous timekeeper instead of responding directly to environmental cycles is to anticipate environmental periodical changes, both in physiology and behavior, and choose the right time for a given response (Kronfeld-Schor and Dayan 2003).

NEPHROPS NORVEGICUS AND THE BURROWING BEHAVIOR

The Norway lobster, *Nephrops norvegicus* (hereafter referred to as *Nephrops*) is a burrowing decapod inhabiting muddy bottoms of continental shelves and slopes of the Mediterranean and the European Atlantic (Sardà 1995, Bell et al. 2006). *Nephrops* is an important resource for European fishery where landed tons per year are around 70000 (Ungfors et al. 2013).

The burrow emergence behavior of *Nephrops* has a strong influence on the catch patterns. In fact, animals can be captured by trawling nets only when they are out of the burrow (Main and Sangster 1985, Newland and Chapman 1989). Catch patterns are different at different depths, but they conserved a daily (24 h) pattern. On the upper continental shelf (10-50 m), captures are usually high at night, and emergence behavior appears to be influenced by moonlight (Chapman and Rice 1971, Chapman and Howard 1979). This pattern becomes crepuscular on the lower shelf (50-200 m), with bimodal dusk and dawn peaks of catches (Farmer 1975). Finally, the pattern is fully diurnal on the upper slope at 200-430 m (Hillis 1971, Aguzzi et al. 2003).

In order to understand the proximate causation of the depth-dependent temporal switch observed in catch patterns, *Nephrops* behavior was extensively studied in laboratory (Farmer 1975, Sardà 1995, Bell et al. 2006, Aguzzi and Sardà 2008, Katoh et al. 2013). Research questions were addressed to understand the role of light in the exogenous and endogenous modulation of burrow emergence behavior. Laboratory studies investigated the locomotor activity and burrow emergence of individuals in response to 24-h light-darkness cycles of different intensities (e.g. Atkinson and Naylor 1976, Hammond and Naylor 1977, Aguzzi et al. 2004, Sbragaglia et al. 2013b). These studies revealed that *N. norvegicus* activity was controlled not merely by optimum level of light intensity, as formerly hypothesized by Chapman (1972). The presence of diurnal catchability in Mediterranean slope populations suggested that blue light (approx. 470-480 nm) might be the most effective trigger of burrow emergence (Aguzzi et al. 2003, Aguzzi et al. 2009b). In fact, recent laboratory experiments with artificial burrows have demonstrated that the emergence behaviour is controlled by the circadian clock that can be entrained by monochromatic blue light cycles of different intensities (as a proxy of the depth) producing a shift from nocturnal phenotype to a diurnal one as observed in the wild by trawling (Chiesa et al. 2010).

In general all laboratory studies on burrow emergence behavior of *Nephrops* have investigated the response of isolated individuals to light-darkness cycle. However, life in the wild is not the same of life in a tank in the laboratory and the behavior of organisms can be deeply modified by the ecological factors that are not taken into account in the laboratory. On the contrary, laboratory experiments on behavior are fundamental to discern and understand the proximate mechanisms of it. *Nephrops* burrow emergence behavior was recently reviewed (Bell et al. 2006, Aguzzi and Sardà 2008, Sardà and Aguzzi 2012, Katoh et al. 2013) and in all the cases the authors

conveyed that, even if the light seems to be the most important synchronizer for the burrow emergence rhythm, there are other important factors (e.g. water movement at the sea bottom, social interactions) that can deeply affect the behavior of the species in the wild.

One of these factors could be the periodical water movement at the sea bottom. So far, researchers hypothesized that the periodical fluctuations of water movements at the sea bottom (sustained by the tidal movement of water masses) could have affected *Nephrops* behavior, but an experimental study is still missing (Storrow 1912). As depth increases, light fades out, especially in areas where water turbidity is elevated, so other geophysical cycles, such as periodical water currents, could modulate behavioral rhythms of deep water species (Wagner et al. 2007). *Nephrops* possesses mechanoreceptors that are very sensible both to water speed and directions, a sensorial equipment that is probably adapted to its habitat where vision can be often compromised by the reduced light and strong turbidity (Katoh et al. 2013). The sole laboratory evidence that *Nephrops* react to water currents is published by Newland et al. (1988), but whether the current stimulus can modulate the burrow emergence behaviour is still unknown.

Nephrops (as many others animals) display aggressive behavior and fighting occurs over limited resources such as mating and shelter. Chapman and Rice (1971) observed for the first time fighting behavior of *Nephrops* in the wild, during such observation a lobster approached a burrow already occupied from another lobster triggering its aggressive response. The observed ritualized fight was then carefully described in the laboratory by Katoh et al. (2008). Chapman and Rice (1971) also reported that a high proportion of *Nephrops* that were caught by creels in the field have circular indentations or holes in the claws. Due to the nature of these wounds, they were likely inflicted by claws of conspecifics. Those information together suggested that fighting is a common event in natural population of *Nephrops* and could have a paramount role in controlling burrowing behavior, but after more than four decades nobody investigated the effect of agonistic behavior on burrow emergence rhythm.

OVERVIEW AND OBJETIVES

The general aim of this thesis is to investigate the burrow emergence rhythm of *Nephrops* using a laboratory-based approach providing new insights into unknown areas of research. I focused my attention on those factors controlling emergence behavior that have not been previously taken into account by others researchers. So, I simulated light-darkness conditions together with periodical water currents. Then, I provided the first insight into the molecular machinery controlling the rhythmic burrow emergence behavior of *Nephrops*. Finally, I have studied the effect of the formation and maintenance of a dominance hierarchy on burrow emergence behavior. I focused my attention on two different aspects of *Nephrops*' research: I asked specific questions to collect

information that are valuable for the fishery management of the species; and I formulated hypothesis with a wider range of interest for biology and in particularly chronobiology, using *Nephrops* as a model species representing deep-sea (> 200 m) habitats.

A key point in experimental biology is the realization of laboratory devices that allow researchers to test experimental hypotheses simulating the conditions that the model species encounters in the wild. The biggest technical challenge in my thesis has been the realization of a flume tank to simulate periodical water currents and concomitant light cycles in the laboratory. **Chapter 2** is entirely dedicated to methodology; I presented the realization of en experimental flume tank that I used to simulate such habitat fluctuations to study the behavioral response of *Nephrops*.

In **Chapter 3** I investigated what is the effect of periodical water currents and light cycles on the burrow emergence behavior of *Nephrops*. Periodical water currents are simulated in laboratory to reproduce the tidal currents that *Nephrops* experience in its natural habitat. I focused on the effects that the response of lobsters could have on the catchability patters and hence stock assessment. Furthermore, I provided some new information on the interplay of two concomitant environmental cycles (light and water cycles) on the circadian system output.

In **Chapter 4** I provided the first assembled transcriptome of *Nephrops* eyestalk. Then, I identified and characterized four clock genes and studied their daily pattern of expression. This chapter investigated the basis of the proximate causation of *Nephrops* burrowing behavior. It provided important data into the localization and functioning of the circadian molecular mechanisms of crustacean decapods' biological clock. Even if it is not strictly related to the stock management of the species, this information was needed to deeply understand the physiological mechanisms that drive the burrow emergence in this species.

The agonistic behavior of *Nephrops* is described in **Chapter 5** where is provided the first insight into the formation and maintenance of dominance hierarchy and how it modulated the daily burrow emergence behavior. The importance of social interactions in the ecology of species is dramatic, but *Nephrops* studies on burrow emergence have never considered this ecological factor. I showed for the first time here, that all the laboratory information collected during individual experiments could be not representative of the real behavior of lobsters in the wild. In fact, taking into account social behavior, the perception of population dynamic could change dramatically. This key point regarding the ecology of *Nephrops* is very important for the fishery management of the species. Moreover, *Nephrops* could help to answer (as model species) some open question in the field of chronobiology elucidating the mechanism behind the synchronization of biological clocks in social contexts.

Finally, **Chapter 6** provided the global conclusions of my research with emphasis on two aspects: valuable information for the fishery management of the species and importance of *Nephrops* as a model species for chronobiology, socio-chronobiology and deep-sea ecosystems.

Flume tank

2

INTRODUCTION

One of the most studied areas of biological clock regulation is behavior. Temporal patterning has been detected in all animals studied so far (Refinetti 2006). Behavior of marine species can be measured as locomotor/swimming activity and it is an important parameter to be studied in order to increase the knowledge about temporal changes in community composition and thus apparent biodiversity (Aguzzi et al. 2012). In this scenario, laboratory tests on animals' reaction to simulated geophysical cycles are important to understand the temporal regulation of behavior upon day-night (24 h) and tidal cycles (12.4 h) (Palmer 1974, Reebs 2002, Naylor 2010).

Crustaceans play a central role in laboratory research on behavioral rhythms of marine species and a wide numbers of technological approaches have been used for tracking their locomotor activity: stylus recording on rotating drums (Naylor 1958), photo-electric cells (Williams and Naylor 1969), infra-red light (Naylor and Atkinson 1972, Naylor 1985, Aguzzi et al. 2008), radio frequency identification (Aguzzi et al. 2011b), racetracks and running wheels (Jury et al. 2005), rotational displacement transducers (Johnson and Tarling 2008), time-lapse photography (Enright 1965, Klapow 1972) and more lately automated video-imaging (Aguzzi et al. 2009a, Menesatti et al. 2009). The latter is gaining an increasing attention, due to progresses in automation and efficiency of objects recognition (Obdržálek and Matas 2006). Independently of the device used, most of laboratory research with crustaceans has been carried out with intertidal shallow water species that were exposed, light cycles apart, to oscillations in water presence/absence, temperature, hydrostatic pressure, salinity, and turbulence (Williams and Naylor 1969, Jones and Naylor 1970, Taylor and Naylor 1977, Hastings 1981). Conversely, behavioral rhythms in deep water continental margin species have been mostly ignored (Naylor 2005). Locomotor activity of deep water species could also be regulated by the synergic interplay of day-night and hydrodynamic cycles (e.g. internal tides), but data on these aspects are scant (Wagner et al. 2007, Aguzzi et al. 2009c).

In this study, we present and demonstrate the functioning of a multi flume automated actograph that can simulate and reproduce complex scenarios of concomitant day-night and hydrodynamic cycles (e.g. internal tides). We tested the device by tracking, using automated video imaging, the burrow emergence rhythm of a commercially important species, the Norway lobster (Nephrops norvegicus L.). N. norvegicus could be considered a model species to investigate deep-sea behavioral rhythms, since its bathymetric distribution encompasses European Atlantic and Mediterranean shelves and slopes, 20-800 m depth (Sardà 1995, Bell et al. 2006), where different light intensity and hydrodynamic cycles occur. During the testing trial, we simulated the Atlantic continental shelf scenario (~150 m depth), where lobsters' burrow emergence seems to be influenced by monochromatic blue light cycles (Aguzzi et al. 2009b) and internal tides (Bell et al. 2008).

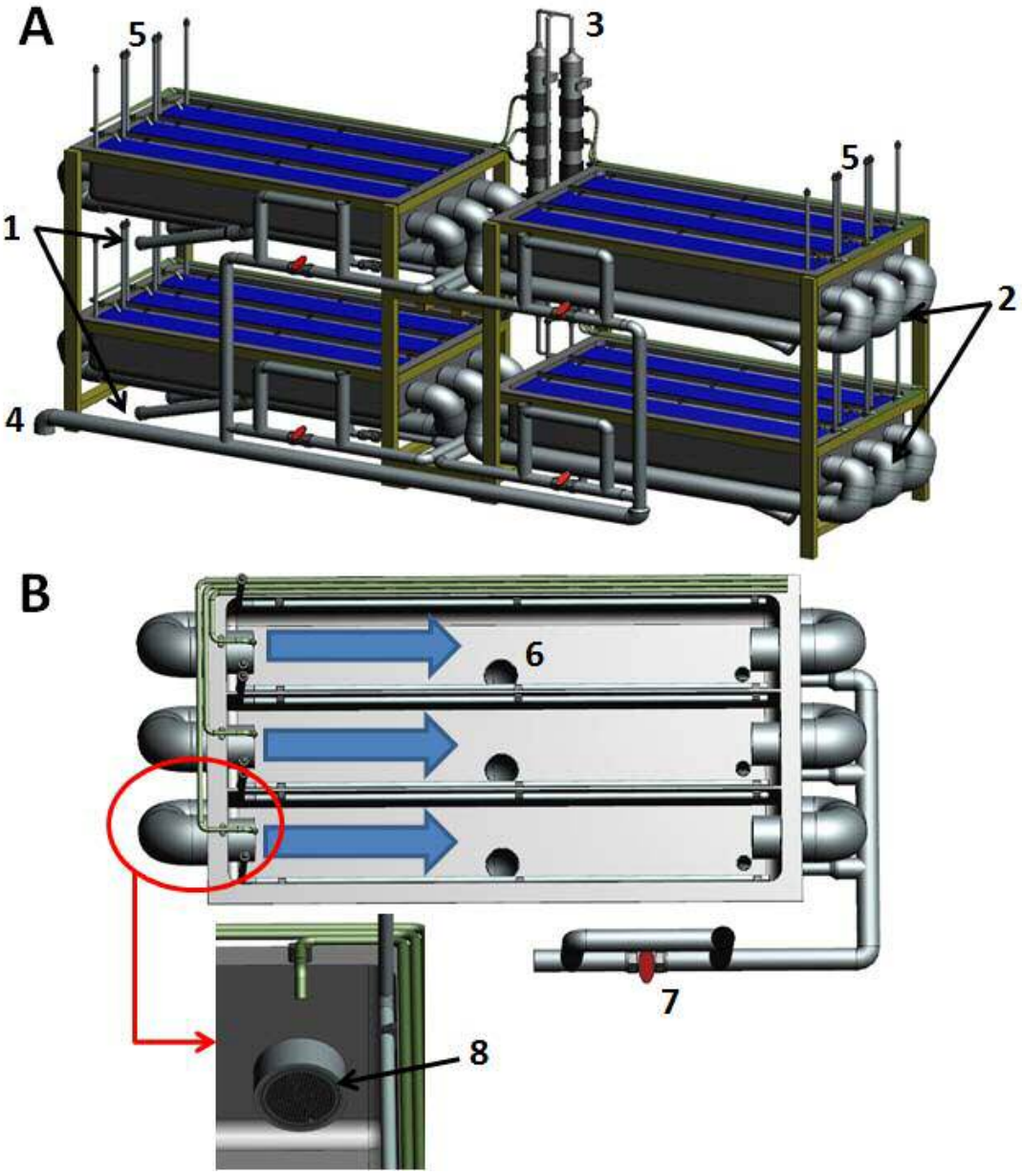


Fig. 2.1 - 3D perspective of the actograph realized without considering the experimental chamber in which is placed. Overall perspective of the actograph (A), top view of a single tank (B) where arrows indicate the current flow direction. **1:** pipes representing the burrow; **2:** pipes forming the loop along the bottom of the tank; **3:** the water lung to obtain a constant flow of incoming water; **4:** the way out of the water; **5:** the L-shaped lighting apparatus for both IR and blue LED's; **6:** overview of the burrow; **7:** the siphon to control the level of water in the tank; **8:** the diffuser.

Fig. 2.2 - Different working phases during the construction of the actograph. **A:** the simulated burrow during the gluing at the bottom of the tank (sand was previously glued inside the burrow); **B:** A particular of the burrow with glued sand; **C:** The view of one painted tank; **D:** Underside the tank with the external down pipe which connects two opposite sides of the tank. **E:** The pump placed inside the pipe. **F:** A close view of the diffuser placed in the opposite side of the pump. **G:** The resulting L shaped submergible lighting system with multiple alimentation apparatus. The dashed line indicates the transparent methyl metacrilate (MM) tube in which was inserted the LEDs strips. The black line represents the smaller PVC tube, containing the cables for LEDs' power supply.

MATERIALS AND METHODS

The actograph

The system consists in four tanks organized over two levels (Fig. 2.1A) with a continuous open flow of filtered and temperature controlled (13 ± 1 °C) sea water. Each tank (150 × 75 × 30 cm) is subdivided in three individual corridors (150 × 25 × 30 cm) (Fig. 2.1B).

Bottom sediment is simulated by sand glued by bicomponent acrylic glue (Fig. 2.2A, B). A burrow was also built in each corridor, assembling PVC pipes (Fig. 2.2A), considering the information we have from field studies (Rice and Chapman 1971). . Since burrow size is correlated to animal's size, we built the artificial burrow according to an average lobster size of 40.00 mm (CL). The internal walls of the burrow were also covered by glued sand (Fig. 2.2A). All the internal sides and elements of the tanks, except the parts with glued sand, were painted in black (Fig. 2.2C) to avoid light reflections and to maximize the efficiency of video-imaging (see below).

In order to create a water flow in each of the 12 corridors, transforming them into flumes, we looped the opposite sides with external bottom pipes, along the entire length of the corridor (Fig. 2.2D). The diameter of the pipes (110 cm) and the pumps utilized for water recirculation were chosen in relation to the average velocity of the current (approximately 10 cm/s) detected in seabed areas where the species is distributed (Puig et al. 2000, Lorance and Trenkel 2006) The pump (HIDOR – Koralia 7) positioned at the entrance of each pipe (Fig. 2.2E) generated the water flow.

Before positioning, pumps were upholstered with a "bushing" system to prevent propagation of noise and vibration to the rest of the tank. At the opposite side, a diffuser was installed inside the pipe to break down the flow turbulences within the corridor. That diffuser was constituted by a tube comprising a mesh (Figs. 2.1B, 2.2F), according to Nowell and Jumars (1987). Furthermore, we placed a plastic net barrier at both sides of the corridor to avoid the direct interaction of animals with the pump and the diffuser. Each corridor was equipped with two different sources of LED's illumination (monochromatic blue: 472 nm, infrared: 850 nm). Monochromatic blue lighting was installed to simulate Light-Darkness (LD) conditions, while infrared (IR) light allowed video-recording during darkness. A strip of LED's photodiodes (Blue LEDs, n = 84; IR LEDs, n = 108) was inserted in a transparent methyl metacrilate (MM) tube of 140 cm long and 16 mm in diameter (Fig. 2.2G). At one of its extremity, we added a smaller PVC tube containing the cables for the LEDs' power supply. L-shaped resulting lighting apparatuses were tested for water resistance and were positioned in the upper part of the corridor, just below the water surface. Once placed in their place, lighting systems were covered in the topside by water resistant black tape, to avoid reflections beneath the water surface that could compromise video imaging analysis.

Four surveillance HD video cameras (Axis P1344-E; Theia SY110A fixed focal megapixel lens), were differently positioned above each tank and their video-imaging efficiency was also compared (see Section 2.3). Two video cameras of the upper tanks were placed in a perpendicular position, while the other two (bottom tanks) were positioned at 45°.

System architecture and automated routines

To guarantee the maximum safety of operators, all the components potentially in contact with seawater were supplied exclusively with 12 V DC. Uninterruptible Power Supplies (UPSs) guaranteed the constant power of all apparatuses in the case of electricity cut off. IR LEDs were connected to a UPS and a transformer, while fuses guaranteed the protection of each LED's stripe. The hardware of the system was assembled to control all apparatus in use during experiments by a central server (Sarriá et al. 2015) (Fig. 2.3). The central server (UBUNTU O.S.) was the core of the hardware, in which all routines and programs were run, and was placed in the experimental chamber. The central server had the following connections: (i) LAN1, as isolated gigabyte net devoted only to cameras acquisition; (ii) LAN2, as the building net connected to the Network Attached Storage (NAS), placed outside the experimental chamber, also allowing remote access to the system from everywhere; (iii) Bluetooth connection, linking the central server to the control system box. IR LEDs were managed independently from the whole system because they worked continuously to allow acquisition of images during darkness. The control system was designed to integrate and regulate current flow and light (i.e. intensity of light, Light-Darkness cycle, current speed, and current cycle). A Labview interface in the central server was utilized to schedule the

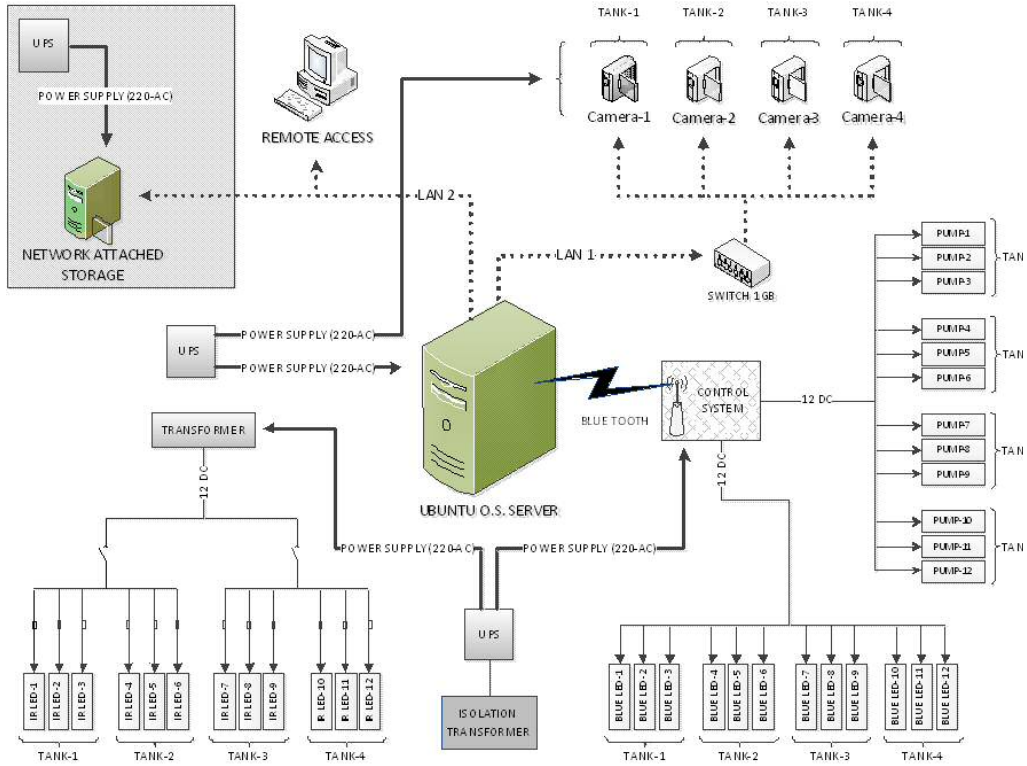


Fig. 2.3 - The architecture of the system. All components are placed in the experimental chamber except for the network attached storage that is placed in an office (grey area) reached by the LAN2. Each tank is equipped with 1 camera, 3 pumps, 3 blue LEDs lighting apparatus, 3 infrared LEDs lighting apparatus. See Sarriá et al. (2015) for further details.

experimental setting in a simple way on a pre-compiled table. LD transitions were gradually achieved within 30 min, in order to avoid Norway lobster's photoreceptors degeneration (i.e. rhabdom deterioration and visual pigments photolysis), as it occurs when animals are subjected to sudden bright light exposure (Gaten et al. 1990).

An automatic routine for data acquisition was created in bash script programming language and a graphical interface allowed us to start and stop experiments. The routine was designed to launch all the processes in a synchronous way: image acquisition for the four video cameras, Matlab functions for automated video-image analysis, monitoring of the system, and data backup. Furthermore, all processes are managed independently in order to prevent system from crashing when one of them failed.

The experimental trial presented in this paper required the running of 13 processes: four to acquire images, four to analyze images, two to control the hardware performance, one as backup and cleaning operations, and two to control the status of whole system. One of the last two processes created an hourly checking report of the status of the system and sent it by a daily email, so the operator had a constant monitoring of the experimental trial.

Automated video-image analysis

Each routine theoretically captured an image every 10 s, which was stored in TIF format with a name code which allowed its identification and retrieval for confrontation with numerical tracking outputs (i.e. year/month/hh/mm/ss.code). That format was a suitable input for the automated video-imaging processing, that was developed within the framework of Matlab 7.1 (The MathWorks, Natick, USA), through the compilation of a script with the Image Processing Toolbox. Images previously saved in a folder were removed and processed in a different folder, which worked at higher speed than the acquisition rate. Inside each image, different Regions Of Interest (ROI), one for each corridor of the experimental tanks, were selected as encompassing each sector of the tank. Space constraints did not permit a complete view of each corridor, so we selected only two ROI per tank reducing the number of specimens from 12 to 8 (Fig. 2.4B).

The video cameras were not perfectly perpendicular to the tank surface hence, before the beginning of the experiment, a metric grid was placed on the bottom of each corridor in order to calculate a map of coefficients (following a polynomial scale), to transform animals' displacements from pixel to metric scale in each ROI. Furthermore, in order to eliminate the darker noise, pixels had 20 subtracted from their grayscale value (this operation transforms all the pixels with values ≤ 20 into zero; i.e. darker noise elimination).

The tracking of animal motion was performed by means of algebraic subtraction of each image (Fig. 2.4C) with a starting image without animals (Fig. 2.4A), as numerical matrixes of pixel values at 236 different levels of grey. A fixed threshold (50) has been applied. A morphologic

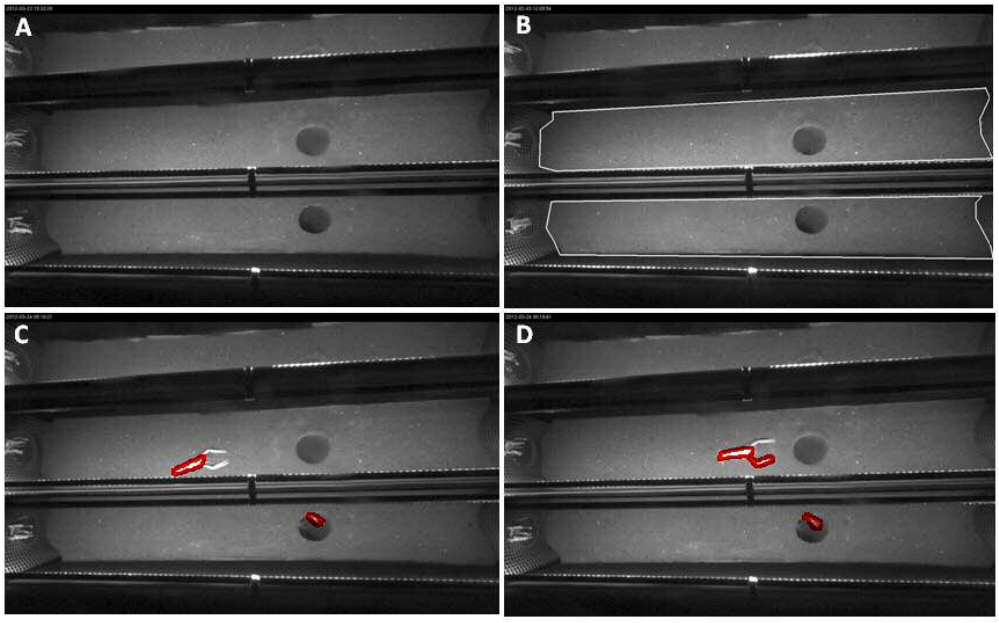


Fig. 2.4 - Four representative images summarizing the automated digital-image analysis routine. **A:** A starting image without animals; **B:** The regions of interest selected for two corridors of one representative tank. The third corridor (upper part of the image) is not used as specified in the text; **C, D:** Two consecutive images showing the detection of the animal evidenced by a red line along the perimeter of the object (in this case the lobster).

threshold of the resulting matrix for the minimum value of body Area (A > 200 pixels) was performed in order to consider only the larger objects as potential animals (Fig. 2.4C). That threshold is a filtering process consisting of the removal of small pixel blocks to determine the potential animal image boundaries. The largest of objects larger than 200 pixel areas were identified as the animal.

The centroid coordinates of the animal were recorded in ASCII file at 1 min frequency. When the animal was not recorded (mainly when residing inside the burrow) the considered position was the one of the previous image. The distance (cm) between two consecutive frames (Fig. 2.4C, D) was then computed. Activity data could be inspected at any time by an interactive script, which directly assessed the ASCII file that extracted a real time graph of the cumulative distance covered by each animal.

Light measurement

The amount of light and its spectral composition are of mportance for entrainment of circadian rhythms (Roenneberg and Foster 1997) and blue light is demonstrated to entrain diel activity cycles in the Norway lobster (Aguzzi et al. 2009b). Also, *Nephrops* show a spectral sensitivity based on different rhodopsins with absorption wavelengths at 425 nm and 515 nm (Johnson et al. 2002). We used a spectroradiometer to determine the underwater spectral power at the bottom of the experimental area for both monochromatic blue and IR LED illumination (Fig. 2.5) Furthermore, we reported the spectral power for lamps utilized for activities during the beginning/end of experiments and for operations on the deck of the trawler during sampling activity in the field. A radiometer (PUV-2500, Biospherical Instruments Inc.) was used to measure the Photosynthetic Active Radiation (PAR; 400–700 nm) at the bottom of the experimental area and,

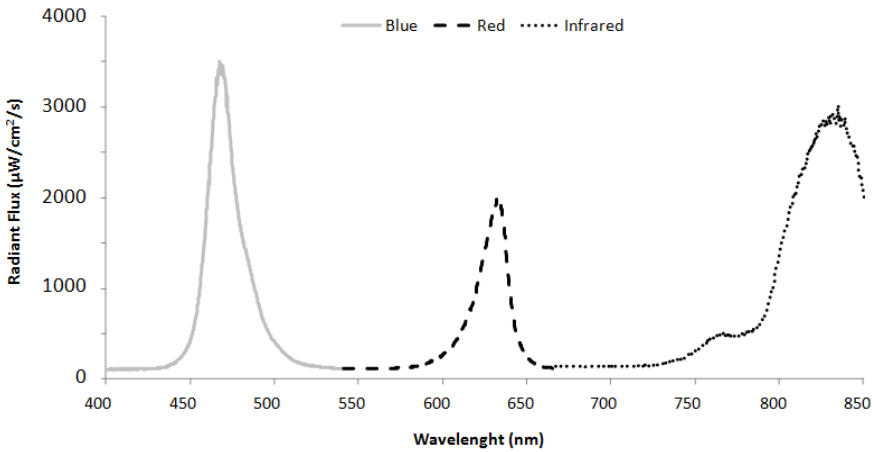


Fig. 2.5 - Spectral power measurements at the bottom of the experimental area of the actograph. Blue LEDs (grey line), red lamps (dashed line), and infrared LEDs (dotted line). Spectral power is expressed in radiant flux (µW/cm^2/s).

since the only radiation present in the PAR range was the blue one, we assumed PAR as a direct measurement of monochromatic blue light. Then, we chose a light intensity equal to 4 10-3 μE/m^2/s considering it as a simulation of lighting regimeat approximately 150 m depth (Jerlov 1968, Morel and Smith 1974).

Animal sampling, acclimation and experimental test

Animals were collected exclusively at night-time by a commercial trawler on the shelf area (100 m) off the Ebro delta (Tarragona, Spain). In order to avoid retinal damage (Gaten 1988) animals were never exposed to sunlight. Once captured (at night) all the operations on the deck of the trawler were performed under dim red light (spectrum is presented in Fig. 2.5). Lobsters were immediately transferred to dark and refrigerated containers and then transported to the laboratory (Aguzzi et al. 2008).

In the laboratory, specimens were transferred to acclimation tanks, hosted within a light-proof isolated chamber under the following conditions: (i) constant temperature of 13 ± 1 °C, as reported for the western Mediterranean continental slope throughout the year (Hopkins 1985); (ii) random feeding time in order to prevent entrainment through food-entraining oscillators, as shown for crustaceans (Fernández De Miguel and Aréchiga 1994); and (iii) LD blue monochromatic regime whose photophase duration matched the natural condition at the latitude of Barcelona (41° 23′ 0 N). Also, light-ON and -OFF, were progressively attained and extinguished within 30 min in order to acclimate animals' eyes to light intensity change. The acclimation facility hosted individual cells (25 × 20 × 30 cm) made with a plastic net of different size in order to allow oxygenation, but not physical contact between animals. Acclimation was carried out at least 1 month prior to behavioral tests.

Table 2.1 - Efficiency values (considering only type 1 errors) obtained in the comparison between the algorithm and the trained operator inspection. Grey and white areas indicate the perpendicular and oblique position of video cameras, respectively, as specified in the second column.

	Position	Number of detections		Efficiency (%)
		Algorithm	Operator	
CAM 1	perpendicular	32278	32618	98.96
CAM 2	oblique	25102	31988	78.47
CAM 3	perpendicular	27797	28278	98.30
CAM 4	oblique	17357	23040	75.33

Eight adult males of *N. norvegicus* with a mean carapace length (CL; average ± standard deviation) of 40.42 ± 4.14 mm were used for a preliminary test over 10 days. The trial was carried out according to the following conditions: current flow of 2 h with a periodicity of 12.4 h mimicking internal tides and 15–9 LD cycle at 4 · 1θ3 μE/m^2/s, simulating a European Atlantic shelf condition. The applied photoperiod matched the natural one at the latitude of Barcelona (onset at 04:21 and offset at 19:24 UTC) during the experimental trial (5–14 of June 2012).

RESULTS

During experiment all the 13 automated routines worked efficiently, including image acquisition and processing. We captured a daily average of 8400 frames. This implies a real frequency acquisition of 10.3 s versus a 10 s theoretical one that would allow us to store 8640 frames. That gap was due to multiple system basic routines into machine that consume CPU time. No type-2 errors (i.e. animal confused with another object) were reported in automated video-imaging efficiency. Differently, type 1 error (no detection of the animal) showed differences among the position of the video cameras. The perpendicular position had an average efficiency of 98.7% while the oblique position 76.9%. A summary of data is presented in the Table 2.1. Failures in the automated detection of the animals occurred only when lobsters stopped close to the walls of the tank. The t-test showed that the different positions of the video cameras (perpendicular and oblique)

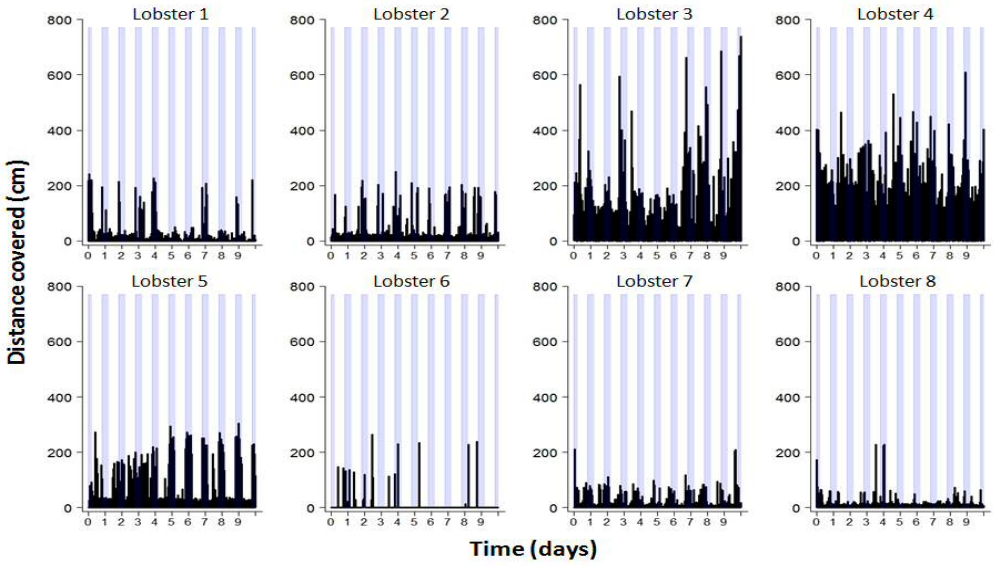


Fig. 2.6 - Time series plots of all the specimens during the 10 days experiment. Each graph is named with the number of the lobster and the activity is expressed as distance (cm) covered during the trial. Grey areas represent dark hours during the experiment.

did not influence the total movement recorded during the experiment (p = 0.329).

The visual inspection of automatically produced time series evidenced robust burrow emergence activity (Fig. 2.6) in all animals. No signs of habituation (i.e. decreasing of movements within days) were detected and there was any linear trend observing the daily distance covered by each lobster (Table 2.2).

DISCUSSION

We describe here the realization and testing of a multi-flume actograph based on automated video-imaging technology. The model species (*N. norvegicus*) we used for the test is a commercially important species (Bell et al. 2006), and a putative model for chronobiological studies in deep waters over the whole continental margin (Aguzzi and Company 2010). We presented the resulting time series of displacements in 8 animals over 10 days, as a valuable output of the performance of the device. The customized automated-video imaging protocol provided a precise tracking of lobsters'movements, also giving the advantages of re-processing acquired images and to visually inspect themwith the possibility to focus on different behavioral aspects. This is an important advantage compared to other actographic technologies used in the past to track behaviour of marine animals (Reid et al. 1989, Last 2003). Visual inspection of acquired images showed slow movements of *Nephrops* in the tank. Therefore, the frequency (10.28 s) of image acquisition was suitable for the tracking of burrow emergence and activity rhythms of the species. The theoretical limit of image frequency acquisition is 25 frames per second (High Definition Movies) that is

Table 2.2 - Daily and total distance (cm) covered during the experimental trial for each lobster. Grey areas indicate the perpendicular position of the video cameras; white areas indicate the oblique position.

	Individual							
Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1	6819	5725	37673	57109	14816	1402	5409	6288
2	5958	10871	25540	40375	20882	4065	6585	3229
3	1829	8771	30468	49670	23579	2396	5269	3633
4	8048	9083	32468	49611	23242	742	5571	4495
5	1817	10373	25386	49178	25064	0	2767	4567
6	794	9990	20814	63051	30927	339	3550	3688
7	2702	9609	37395	61655	23571	0	3611	3538
8	4794	11245	30514	49889	26789	0	4356	4203
9	2622	14980	27348	58486	22961	542	3693	6226
10	2165	7092	33364	48903	22774	0	4176	3986
Sum	**37548**	**97739**	**300971**	**527927**	**234605**	**9487**	**44986**	**43853**

completely dependent from the quality of video cameras. This makes the methodological approach suitable also to track the fast movement of a fish. However the operator should take into account the space (Bytes) to support all the data produced during experiments, and the increasing computational resources required by the machine. The tracking efficiency obtained with the customized script confirms how the automated video-imaging can be used to produce reliable time-series outputs concerning behavior of burrowing species. Although there was discrepancy in detection efficiency between the two different cameras positions (i.e. perpendicular and with 45° angle) this can be ignored when the aim of the study is to investigate the distance covered by lobsters out of the burrow. The t-test showed that the different positions of the video cameras did not influence the total movement recorded during the experiment. In fact, Type-1 errors were reported only when animals stop close to the walls of the tank. Hence, this situation did not influence the computing of distance covered by lobsters because the algorithm used the last position recorded.

Response to water currents

3

INTRODUCTION

Light is the most important zeitgeber (i.e. environmental cue) that synchronizes the biological rhythms of terrestrial organisms (Dunlap et al. 2004). Differently, in the sea, light intensity progressively fades out with depth and other factors can be of importance for the synchronization of rhythmic biological processes (Aguzzi et al. 2011a). In fact, the physical limit for penetration of sunlight (at about 1000 m in the oligotrophic waters) defines a depth range known as the "twilight zone" (Hopkins 1985). Below this depth stratum, tidal currents might represent an environmental cue able of replacing solar light as synchronizer of animal's behaviour and physiology (Wagner et al. 2007, Aguzzi et al. 2010). However, depth limits for light penetration are not sharp (depending on turbidity and other factors), and at the same time tidal regimes has a strong geographical variability depending also from sea bottom orography; the result are numerous possibility of combination between the two cues.

Nephrops has strong burrowing habit, and emergence behaviour can be subdivided in three different phases: door-keeping (lobsters at the burrow entrance with claws protruding out of the burrow, hereafter DK); emergence (totally out the burrow, hereafter OUT); concealment (totally into the burrow, hereafter IN) (Aguzzi and Sardà 2008). Such behaviour is under the control of the circadian system (24 h based). Light-driven burrow emergence behavioural rhythm has been characterized in the wild by temporally scheduled hauling: different diel (24 h) catchability patterns occur at different depths (see chapter 1 for more details).

Rhythmic emergence behaviour in the laboratory has been studied in relation to the day-night modulation without considering the response of lobsters to water currents simulating tidal currents at the sea bottom. The sole laboratory evidence that *Nephrops* react to water currents is published by Newland et al. (1988). This study, conducted with blind lobsters in the absence of an artificial burrow, demonstrated that *Nephrops* assumed a downstream orientation during water currents. On the contrary, some effects of neap and spring tides on catches of *Nephrops* landings were already reported long time ago (Storrow 1912, Thomas 1960). In some areas, the neap/spring state of the tide exert more influence than the time of the day on *Nephrops* overall catches, with spring tides depressing it (Hillis 1971). Bell et al. (2008) also noticed en effect of neap and spring tides on catches of *Nephrops*. In particular, the more is the current speed the less *Nephrops* seems to be caught (Hillis 1996). That observation moved the attention from a cyclic event, neap/spring tides (correlated with the lunar month, 29.5 days) to a direct effect of water currents on the catchability. Thus *Nephrops* represents a good laboratory model for studying the combined effect of light and tidal cycles on the behaviour of deep-water benthos.

Research on how periodic currents and day-night cycles influence behavioural rhythms of species is important to integrate individual behaviour in community and ecosystem dynamic

(Schmitz et al. 2008, Aguzzi et al. 2015). However, laboratory experiments testing putative effects of cyclic environmental variables on the behaviour of deep-water animals are scarce for the intrinsic difficulties in their sampling and laboratory maintenance. In this context, we investigated the effects of periodic water currents (12.4 h) and concomitant blue light-darkness 24 h cycles on *Nephrops* burrowing behaviour.

MATERIALS AND METHODS

The actograph

An actograph was used to track lobsters' behaviour (see chapter 2 for more details; Sbragaglia et al. 2013a). Briefly, the actograph consists in 4 tanks with 2 individual corridors each, in which burrow emergence behaviour of lobster is tracked by automated video image analysis. Each corridor is endowed with glued sand at the bottom, an artificial burrow, a pump, and a monochromatic blue LEDs illumination system (472 nm). The pump, together with a flume system is used to create water currents. The burrow was inclined at about 30º in the opposite direction of the water current. At the same time, we used an open water recirculation system (4 L/min). Blue light was used because this wavelength is used by marine crustacean decapods to synchronize biological clocks (Aguzzi and Company 2010). Infrared illumination was used to allow the recording of lobsters' behaviour during darkness.

Four video cameras were used to track the behaviour of lobsters with a frame acquisition rate of 10 s. Frames were automatically processed by a set of Matlab functions and time series of locomotor activity (cm) were obtained. Moreover, all the frames were assembled into a time-lapse video (hereafter referred to as full length video), for the further characterization of lobsters' behaviour (see below).

Sampling, acclimation and experimental design

Sampling and acclimation protocols are the same of them described in chapter 2. Fifteen adult males with a mean carapace length (CL; average ± standard deviation) of 41.04±4.85 mm were used in this study. Lobsters were never fed during the experiment. Burrow emergence behaviour was studied over 29.5 days (equals to 1 lunar month) under a photoperiod matching the one at the latitude of Barcelona (at about 14-10 hours of Light-Darkness, June-July 2012). Switch-ON/OFF of the blue LEDs was progressive (within 30 min). During light hours the intensity was equals to 4 10^{-3} μE m^{-2} s^{-1}, simulating a lower shelf condition (at about 100-150 m depth; Aguzzi et al. 2003). Water temperature during the experiments was 13±1 °C and dissolved oxygen always above 9 mg/L.

We performed behavioural tests using Mediterranean individuals and exposing them to water currents with a periodicity that is typical of the Atlantic Ocean. My approach is justified by the fact that *Nephrops* has a uniform population without signs of genetic divergence or isolation (Passamonti et al. 1997, Maltagliati et al. 1998, Stamatis et al. 2004). Every 12.4 h, lobsters were exposed to water currents of 2 h duration with a speed of 10 cm s^{-1}. The semi-diurnal periodicity of 12.4 h simulates a periodic intensification of seabed currents' speed that is supposed to entrain physiological rhythms in deep-sea European north Atlantic fishes (Wagner et al. 2007).

Data treatment and behavioural analysis

Time series depicting locomotor activity out of the burrow were binned by 1 min and represented with double plotted actograms (24 h based), in order to discern the effects of both light and current cycles on the behavior of individuals. Chi-square periodogram (Sokolove and Bushell 1978) was used to scan for the presence of significant ($p < 0.05$) periodicity in the range 600–1600 min (equals respectively to 10–27 h) within the total length of the time series (29.5 days) and percentage of variance (%V) was reported as a measure of robustness of rhythmic patterns (Refinetti 2006). Then, periodogram analysis was repeated for the lobsters that have shown a clear effect of currents on locomotor activity in their actograms. Analysis considered a selected number of days during which lobsters maintained synchronization with the water current cycle. At the same time an average waveform (24.8 h based) of the selected days was used to highlight the effect of water currents on locomotor activity.

Average locomotor activity during all the days of experiment was compared between light and darkness and among four different periods of the day: dusk (from 1 h before to 1 h after light OFF); dawn (from 1 h before to 1 h after light ON); day (from 1 h after light ON to 1 h before light OFF); night (from 1 h after light OFF to 1 h before light ON). Then, we compared the sum of locomotor activity 2 h before, during, and after the water current and plot them against the time of the day at which that current started. The overall temporal patterns (before, during, and after) were described using a gamma-family smoothing function.

In a second step, a trained operator visually analyzed the full length videos to characterize *Nephrops*' behaviour during water currents. We quantified the amount of time lobsters spent in each of the three aspects of their diel behavioural rhythm (DK, IN, OUT) during the different periods of time previously identified (dawn, day, dusk, night). Finally, we also characterized *Nephrops*' behaviour out of the burrow in the presence of water currents using three categories: up-stream or down-stream body orientation without or with low displacement (< 1 body length 10 s^{-1}), active displacement (> 1 body length 10 s^{-1}) usually performed with movements from one side to the other of the tank.

We transformed all the data (square root) to satisfy normality test and homoscedasticity of variance. Paired t test and One-way Repeated Measures ANOVA (followed by the Tukey multiple comparison procedure) tests were used to assess significant differences using Sigma Plot (12.5) software. During all analysis we used a confidence interval of 95%.

RESULTS

The inspection of actograms evidenced robust diel burrow emergence activity in all individuals with peaks of locomotor activity at light-OFF (two representative actograms are presented in Figure 3.1). Periodogram analysis on the total length of the time series (29.5 days) detected rhythmic activity in all tested individuals. Twelve lobsters showed diel (24 h) periodicity (mean ± SE = 24.06 ± 0.05 h; 23.37 ± 2.86 %V, *n*=12), while 3 lobsters showed less robust tidal periodicity (mean ± SE; 24.86 ± 0.05 h; 9.50 ± 1.97 %V, *n*=3).

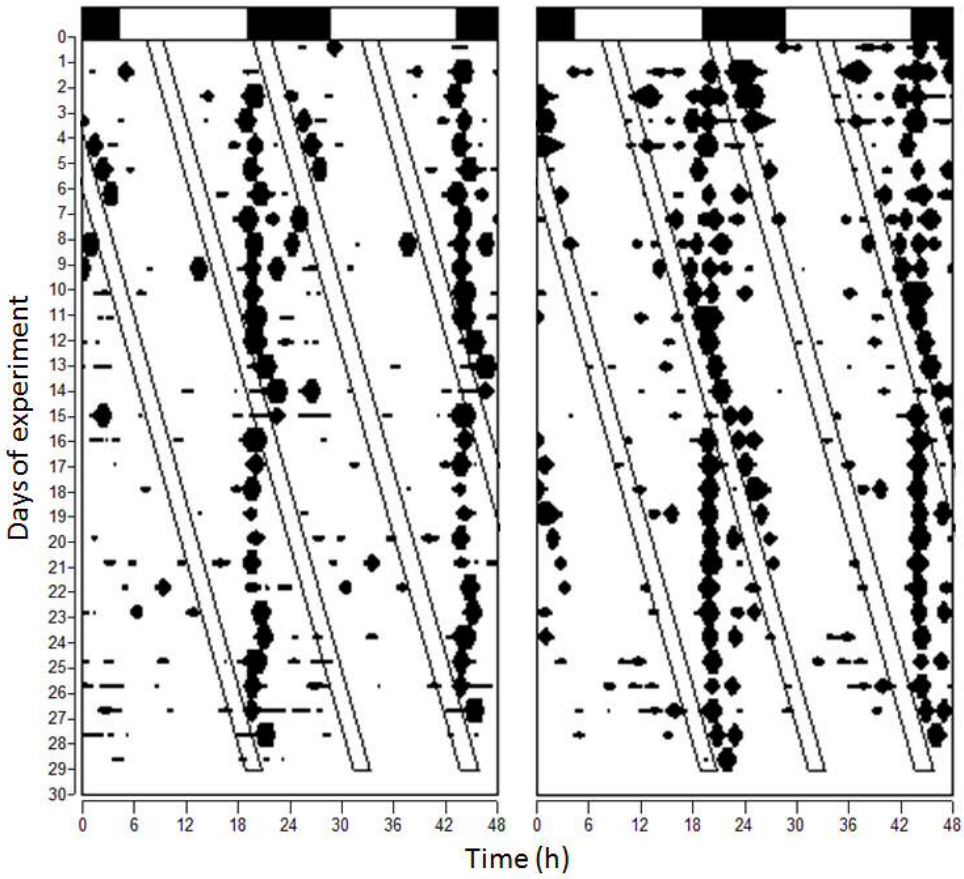


Fig. 3.1 - Double plotted (two days per line) actograms of two representative *N. norvegicus*. Locomotor activity is represented by cm covered out of the burrow for 29.5 days. White-dark bars at the top represent Light-Darkness (LD: 14–10 h) cycle. Current cycle (12.4 h) is identified by white oblique rectangles in the plot.

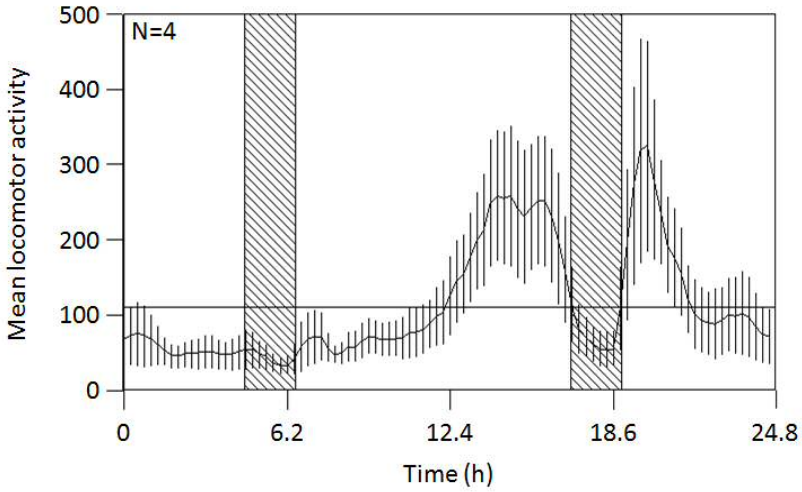


Fig. 3.2 – Mean waveform (24.8 h based) for the 4 lobsters that have shown a clear synchronization with the water current cycle in their actograms. Data represent the average activity of the lobsters during the selected days indicated in Figure S1. Mean locomotor activity is identified by the curve while vertical lines represent the standard error. The horizontal line represents the Midline Estimating Statistic Of Rhythm (MESOR). Periodic water currents are identified by the vertical shadowed areas. The inhibition effect of water currents is identified with a clear drop of locomotor activity below the MESOR.

Moreover, actograms output indicated the presence of 4 lobsters with an evident synchronization to the water current cycle. We selected the days during which the 4 lobsters maintained a clear synchronization with the water current cycle (see Figure A3.1). Periodogram analysis indicated a more robust tidal periodicity (mean ± SE = 24.85 ± 0.05 h; 55.92 ± 4.90 %V, n=4) than the values previously observed. The average (n=4) waveform (24.8 h based) of the selected days was used to highlight the effect of water currents on locomotor activity (Fig. 3.2).

Locomotor activity of lobsters was significantly (Paired t test, $t14 = 5.432$, $P < 0.001$; Table 1) higher during darkness (mean ± SE = 10.30 ± 2.35 cm, n=15) than light (mean ± SE = 5.96 ± 1.47 cm, n=15). When we look in more details, lobsters were more active at dusk (mean ± SE = 13.04 ± 2.95 cm, n=15) and night (mean ± SE = 9.78 ± 2.37 cm, n=15) than at dawn (mean ± SE = 6.18 ± 1.56 cm, n=15) and day (mean ± SE = 5.78 ± 1.41 cm, n=15) with significant differences among periods (ANOVA, $F(3,14) = 22.61$, $P < 0.001$) (Table 1).

The comparison of the sum of locomotor activity 2 hours before, during, and after the onset of water currents highlighted a behavioural locomotor response modulated by the time at which the currents stimuli were applied (Fig. 3.3). When the onset of currents occurred during the first hours of light (when lobsters were not active), there were not great differences in the resulting smoothing curves. When the current onset was close to light-OFF (and lobsters began to be more active out of the burrow), the level of activity before and during the currents was the same, but the activity after the currents reached its maximum. The level of activity after the water currents started to decrease

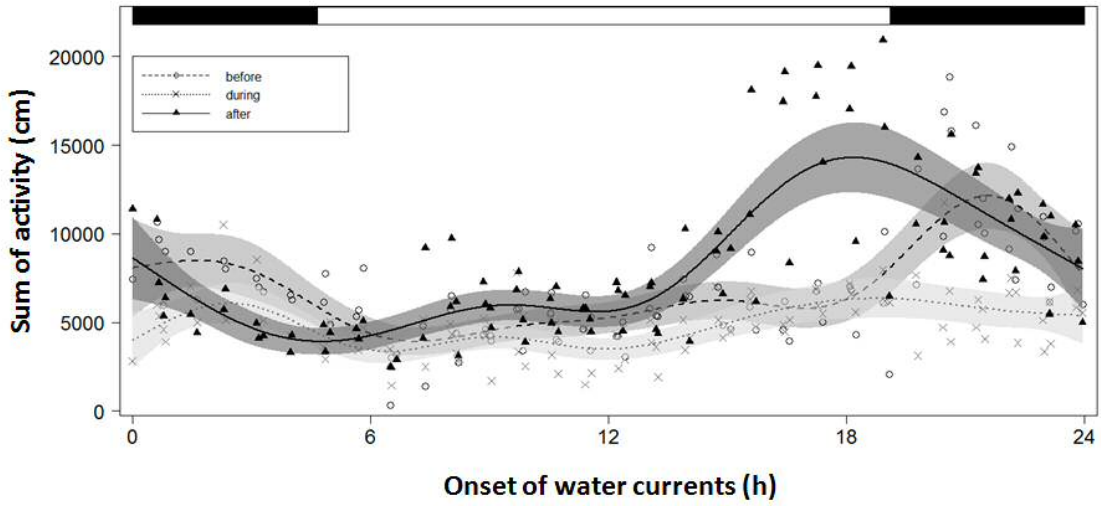


Fig. 3.3 – Plot of the locomotor activity 2 h before (empty circles), during (crosses) and after (solid triangles) water currents plotted against the time of water currents onset. Data are presented along with a Gamma-family smoothing function (indicating mean as: dashed line for empty circles, dotted line for crosses, solid line for solid triangles) and 95% confidence interval as shaded grey-scale contours.

when water currents onset occurred at the first hours of darkness, while the activity before the currents started to increase up to reaching its peak. Notice that during the hours of darkness there were two distinct peaks of activity, before and after the water current stimulus (Figs. 3.1-3.3). The activity after water currents reached its minimum when the onset of currents was close to light-ON, while the activity before the water currents was still greater than the activity during currents (indicating inhibition of activity by water current).

Table 3.1 - Out of burrow locomotor activity expressed in cm (Mean ± SEM) covered by lobsters in relation to the LD cycle at dawn, day, dusk, and night periods. **t/F** represents the value of the statistics used to assess significant differences together with the probability (***p***) and the sample size (**N**). Letters indicate the output of the multiple comparison post-hoc test (a>b>c).

Locomotor activity	cm covered (mean±SEM)	t / F	*P*	N
darkness (a)	10.30±2.35	5.432	< 0.001	15
light (b)	5.96 ±1.47			
dawn (c)	6.18±1.56	22.608	< 0.001	15
day (c)	5.78±1.41			
dusk (a)	13.04±2.95			
night (b)	9.78±2.37			

Table 3.2 – Mean ± SEM of the percentage of time spent by lobsters in different phases of burrow emergence during currents (**behavior during current**), and in different orientation in the presence of the currents (**body orientation during currents**). **t/F** represents the value of the statistics used to assess significant differences together with the ***P*** value and the **N**. Letters indicate Letters indicate the output of the multiple comparison post hoc test (a>b). ***** indicate that the power of the test is below the desired value.

Behaviour during current	Percentage of Time (mean±SEM)	t / F	*P*	N
dawn-DK (b)	11.00±3.68			
dawn-IN (a)	83.59±5.62	63.208	< 0.001	15
dawn-OUT (b)	5.41±2.67			
day-DK (b)	14.23±7.17			
day-IN (a)	76.91±9.26	17.505	< 0.001	15
day-OUT (b)	8.86±5.63			
dusk-DK (b)	13.25±3.53			
dusk-IN (a)	68.50±8.45	9.628	< 0.001	15
dusk-OUT (b)	18.24±8.17			
night-DK (b)	14.67±4.48			
night-IN (a)	69.88±8.96	11.400	< 0.001	15
night-OUT (b)	15.45±7.51			
dawn-OUT (b)	5.41±2.67			
day-OUT (b)	8.86±5.63	5,893	0.002	15
dusk-OUT (a)	18.24±8.17			
night-OUT (a)	15.45±7.51			
Body orientation during current	**Percentage of Time (mean±SEM)**	**t / F**	***P***	**N**
dawn-upstream	27.62±18.90			
dawn-downstream	58.30±20.09	1.331	0.332*	4
dawn-moving	14.07±3.31			
day-upstream	35.10±12.48			
day-downstream	52.69±11.43	2.805	0.119*	5
day-moving	12.22±2.67			
dusk-upstream	20.39±8.78			
dusk-downstream	62.91±11.08	1.828	0.203*	7
dusk-moving	28.60±12.23			
night-upstream (b)	12.16±2.58			
night-downstream (a)	70.32±3.59	66.332	< 0.001	9
night-moving (b)	17.52±6.17			

The full length videos indicated that lobsters exposed to water currents always spent a significantly higher amount of time into the burrow than at door-keeping or out of the burrow (ANOVA, dawn: $F(2,14) = 63.21$, $P < 0.001$; day: $F(2,14) = 17.51$, $P < 0.001$; dusk: $F(2,14) = 9.63$, $P < 0.001$; night: $F(2,14) = 11.40$, $P < 0.001$; Table 2, Fig. 4). Activity out of the burrow during water currents was higher at dusk and night than dawn and day (ANOVA, $F(3,14) = 5.90$, $P = 0.002$; see Table 2 and Figure 3.4).

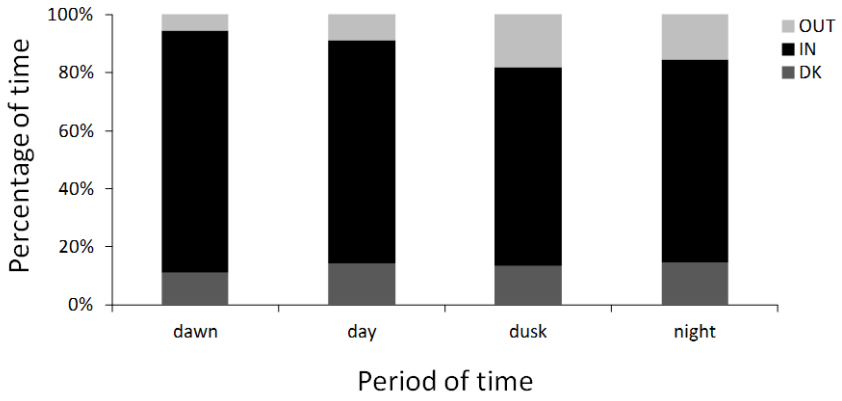


Fig. 3.4 – Bars of the percentage of average time spent by lobsters out of the burrow, into the burrow or at the burrow mouth in the presence of water currents at different periods of time. Light-grey represent the percentage of time spent out of the burrow (OUT), dark bars represent the percentage of time spent into the burrow (IN), dark-grey bars represent the percentage of time spent at the burrow mouth (DK).

We also characterized the body orientation of the lobsters during water currents watching the full length videos. There were no significant differences in the percentage of time they spent moving or orientated up- and down-stream at dawn, day, and dusk (even if the power of the statistic test suggested caution interpreting such results, see Table 2). However, when lobsters were out of the burrow at night they spent a significant greater amount of time orientated downstream than upstream or moving (mean ± SE = upstream: 12±2.58%, moving: 17.52±6.17%, downstream: 70.32±3.59%, n=9; ANOVA, $F(2,8) = 66.33$, $P < 0.001$; see Table 2 and Fig. 3.5).

Finally, 4 out of 15 lobsters showed signs of entrainment to the periodic water currents (Figure A3.1). In the actogram on the left (see Figures 3.1 and 3.6), an individual showed two components of activity (i.e. peaks) during days 2-5, one in correspondence of the light-OFF and the other just after the current offset. When the current stimulus was too far from the light-OFF (more than 6:23 h, see Figure 3.6), the lobster showed only the component of activity at light-OFF (day 6). Interestingly, during days 7-9 the component of activity previously synchronized to the current offset, showed transients (i.e. it drifted to the left) that allowed it to resynchronize the phase with the major peak of activity at light-OFF. In fact during days 10-11 the lobsters showed only one peak of activity. In the actogram of the right during days 11-15 there was only one component of activity after the current offset (the diel peak of activity at light-OFF was inhibited). During days 16-21, two components of activity were visible: at light-OFF and after the current offset. During days 22-24, when the current stimulus was too far from the light-OFF (more than 7:24 h, see Figure 3.6), lobster's activity showed only one major peak of activity at light-OFF, while the component of activity previously synchronized to the current offset showed transients (as observed for the other individual).

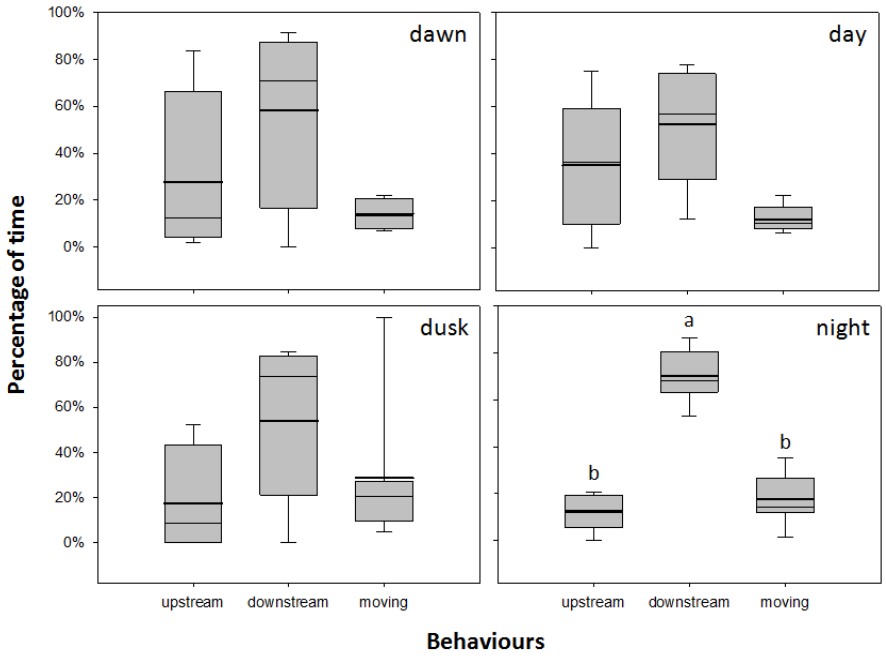


Figure 3.5 – Box plot of the percentage of the average time spent by lobsters orientated up-stream, down-stream, or moving during the 4 period of time. Bold line represents the mean. Normal line represents the median. The grey box represents the first quartile. Lines extending vertically from the boxes (whiskers) indicating variability outside the upper and lower quartiles. Letters indicate the output of the multiple comparison post hoc test (a>b).

DISCUSSION

We demonstrated that periodic water stimuli (as proxy of seabed tidal currents) influenced *Nephrops* burrow emergence behaviour with a strength that is dependent on the phase relationship with the light-darkness cycle. Also, water currents could act as putative synchronizer for one of the component of the circadian oscillator. My results introduced new information regarding the response of the Norway lobster to periodic hydrodynamic stimuli. Firstly, *Nephrops* preferred to remain into the burrow in the presence of water currents. Secondly, during water currents some lobsters spent a reduced amount of time out of the burrow; this is higher at dusk and night, when lobsters are more active out of the burrow. Finally, lobsters spent more time orientated downstream during darkness hours.

The response of lobsters to water currents was strictly dependent on the time at which the hydrodynamic stimulus was applied (see Figure 3.3). The highest rate of locomotor activity after the water currents was observed when currents started within the two hours before the light-OFF. Then, the activity progressively decreased reaching its minimum around light-ON. On the other hand, the locomotor activity before currents reached its maximum when currents onset was at about 3 hours

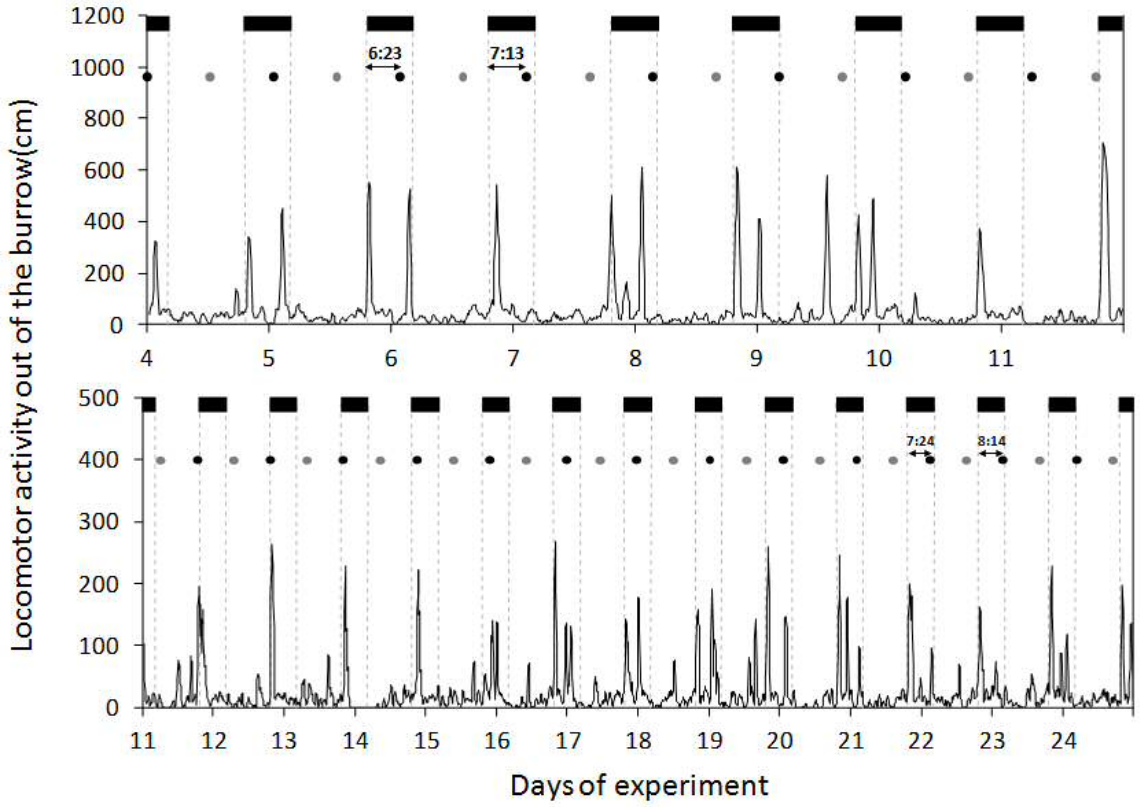


Fig. 3.6 – Plot of an extract of the data presented in the double plotted actograms of Figure 1. The plot above represents days 4-11 from the actogram on the left, while the plot below represents days 11-25 of the actogram on the right. Locomotor activity is binned at 10 min and a 3 steps moving average is applied. Grey dashed line represents the light-darkness cycle (dark bars stays for darkness). Points represent the offset of water currents; these are black to indicate the water currents to which the lobsters are synchronized. Black arrows indicate the distance (h:mm) between the light-OFF and the offset of water currents.

after light-OFF. Lobsters showed the highest peak of diel activity around light-OFF (see Table 3.1 and Figure 3.1), as already observed in previous studies (Atkinson and Naylor 1976, Hammond and Naylor 1977, Sbragaglia et al. 2013b). Furthermore, lobsters activity out of the burrow in the presence of water currents was higher at dusk and night (see Table 3.2). Taken together, these data indicated that light-OFF is a crucial cue for the synchronization of burrowing behaviour of *Nephrops*. However, the light cycle is a more powerful synchronizer than the water current cycle.

It is important to notice that when the water currents coincided with the light-OFF, we observed a negative masking on locomotion (i.e. suppression; *sensus* Mrosovsky (1999)). In other words, the locomotor activity is inhibited and lobsters shifted their activity out of the burrow just after the offset of currents (see Figures 3.1-3.3). Complex patterns of behaviour were already observed when light-darkness and tidal cycles were studied simultaneously in marine organisms, and behavioural output usually depended from the relative phase between the two cycles (Gibson 1992, Krumme 2009, Last et al. 2009, Naylor 2010, Watson and Chabot 2010, Refinetti 2012).

Laroche et al. (1997) and Krumme et al. (2004) observed that recurring fish assemblages followed the combinations between tidal and light cycles in mangrove habitats. *Nephrops* is a generalist predator and scavenger and stable isotope studies indicated its role as secondary consumer (Loc'h and Hily 2005, Johnson et al. 2013). Its behavioural patter could affect the structure of the benthic community and consequently the coupling with the benthopelagic compartment, thus periodically modify biodiversity and trophic flow (Aguzzi et al. 2015). Krumme (2009) suggested considering short-term variation caused by the interplay of tidal and light cycles during long term monitoring programs in intertidal zones. My data indicated that the relative combination of tidal (12.4 and 24.8 h) and light cycles (24 h) could be an important parameter also for deep-water benthic community, suggesting the same attention at the moment to design monitoring program.

Results indicated that water currents can have some effects on the output of the circadian system. We identified the presence of two distinct peaks during darkness hours, suggesting that the water currents could affect the phase of the circadian clock, rather than simply masking its output. In fact, my data showed how water currents can be considered as a putative zeitgeber for one of the components of the circadian oscillator. This result is of relevance for benthic species that are distributed on shelf and upper slope where light is supposed to be the predominant zeitgeber for the synchronization of biological activity (Aguzzi et al. 2011a). In a previous study in the eastern North Atlantic, Wagner et al. (2007) demonstrated at depths at about 2700 m that 12.4 h periodic peaks of currents speed (similar to those we simulated here) may act as zeitgeber for demersal fish. *Nephrops* posses mechanoreceptors distributed throughout the body (cuticular setae, first second antennae, and statocysts) that are used for tactile exploration, perception of water movement, and detection of acoustic stimuli (Katoh et al. 2013). In decapod crustaceans hydrodynamic stimuli and flow information are integrated by very sensitive mechanoreceptive neurons and interneurons connected to statocysts (Wiese 1976, Breithaupt and Tautz 1990, Katoh et al. 2013). Mechanoreceptors may also represent one of the input pathways to convey hydrodynamic information to the circadian system.

To my best knowledge, this is the first evidence that periodic water currents showed an effect on the circadian system output in a deep water crustacean. The presence of an oscillator synchronized to the light-OFF, splitting into 2 components in presence of periodic current stimuli, provides an insight into the mechanism behind the spectral coordination (i.e. integration of various rhythms within an organism, sensus Refinetti 2012) of diel and tidal rhythms in this species. Aguzzi et al. (2011a) presented a model of *Nephrops* circadian peacemaker assuming the presence of a population of oscillators that possess two basic properties: 6 h phase locked-coupling and dumping. The data presented in Figure 3.1 and 3.6 partially fell into this model with the presence of a circadian oscillator which is able to split into 2 components, one that preserves its synchronization

to the light-OFF and the other one that synchronizes with the periodic current stimulus. My data suggested that the phase coordination between the two components may be higher than 6 h reaching values of 7:24 h (before the lost of synchronization; see Figure 3.6). Such phenomenon is clearly observed only in few individuals (N=4). Interestingly, also splitting (the presence of two separate phase components 180° apart) in mammalian model organisms is usually observed in a minority of tested individuals (Refinetti 2006). The optimization of physiological processes through the spectral coordination of diel and tidal rhythms has not received enough attention (Refinetti 2012), but it could be determinant at the moment to assess the ecological significance of biological rhythms.

Water currents induced *Nephrops*' concealment (see Figure 3.4 and Table 3.2). This behaviour may be of adaptive significance in order to minimize the risk of predation. Predation risk experienced by *Nephrops* during peaks of current speed could be higher because predators swimming activity may also be affected by water currents (Arnold 1981, Gibson 1992). For example, some deep-water continental margin fishes adjust their swimming behaviour in relation to current's speed (Lorance and Trenkel 2006). The most common predator of *Nephrops* in the Atlantic is the cod (*Gadus morhua*), the haddock (*Melanogrammus aeglefinus*), the dogfish (*Scyliorhinus canicula*), the thornback ray (*Raja clavata*), as well as cephalopods (Farmer 1975, Chapman 1980, Bell et al. 2006, Johnson et al. 2013). Among *Nephrops'* predators, the cod seems to be the most efficient and it is demonstrated that its horizontal and vertical displacements can be affected by tidal currents (Arnold et al. 1994, Michalsen et al. 1996, Pinnegar and Platts 2011). However, predation success by fish on *Nephrops* is usually low (Serrano et al. 2003), suggesting that a sudden retreat into the burrow can be a successful anti-predator strategy.

Here, we investigated 3 different behavioural responses to water currents when lobsters were out of the burrow: up-stream or down-stream body orientation, or active displacement. Significant behavioural differences were found only during hours of darkness, when lobsters spent more time orientated down-stream (see Figure 3.5). There were no significant differences in these behaviours during dawn and day but that result should be interpreted carefully because of the reduced number of individuals observed moving out of the burrow in those periods (see Table 3.2). Significant differences should also have been expected at dusk, when lobsters were significantly more active out of the burrow (see Table 3.1), in fact the variability outside the upper quartile (see top whisker in Figure 3.5) is higher compared to the other periods of time. However, dusk (light-OFF) was also the time in which water currents exerted negative masking on locomotion (the highest level of inhibition, see above). Such behavioural response of *Nephrops* can be also of ecological relevance in relation to its predators. Blind *Nephrops* in laboratory orientated down-stream in the presence of water currents of speed within 7-20 cm s^{-1} (Newland et al. 1988). In the field, underwater television surveys documented a down-stream orientation of *Nephrops* when current velocity showed a tidal periodicity with peaks at 10 cm s^{-1} (Newland and Chapman 1989).

Newland et al. (1988) demonstrated that a down-stream orientation in *Nephrops* reduced drag forces on its body and may increase the probability to detect fish predators that preferentially move upstream in water flow (Arnold 1981). Differences in body orientation could be also related to the efficiency of lobsters in detecting odours plumes. *Nephrops* probably relies on chemoreception for food search and assess predation risk (Katoh et al. 2013). Chemoreception is strictly correlated to the way in which antennules are deployed in relation to the water flow because it modifies the efficiency of aesthetascs (i.e. chemosensory hairs) to detect odours through the water (Koehl 2011). However, the way in which the orientation of the body influences chemical sensing in *Nephrops* is not known and could be an interesting question to address in future investigations.

ANNEX 3A

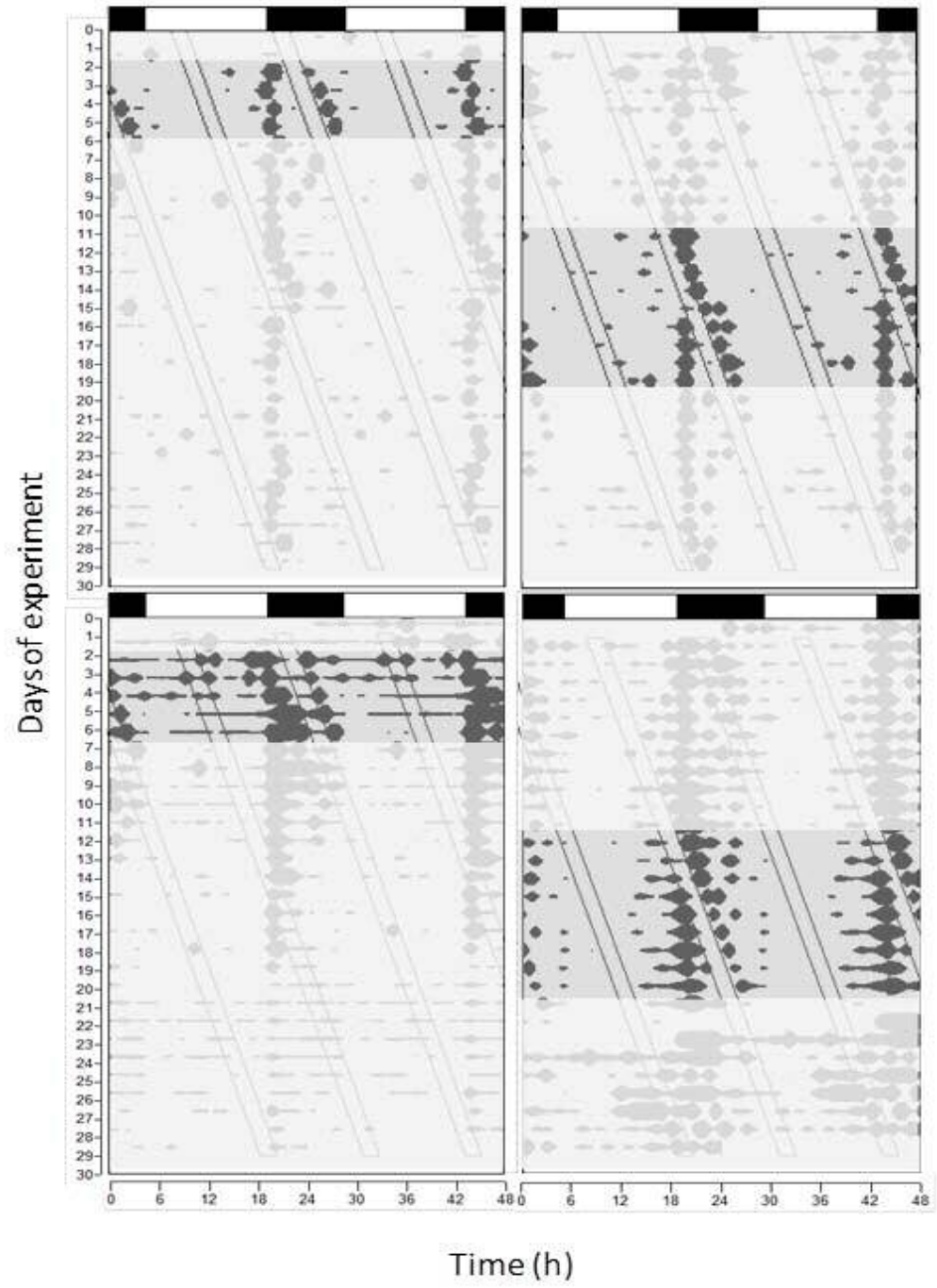


Fig. A3.1 – Double plotted (two days per line) actograms of the 4 lobsters with evident synchronization to currents. Locomotor activity is represented by cm covered out of the burrow for 29.5 days. White-dark bars at the top represent Light-Darkness (LD: 14–10 h) cycle. Current cycle (12.4 h) is identified by white oblique rectangles in the plot. Grey transparent areas represent the selected used to plot the average waveform of Figure 3.2.

Clock genes daily pattern

4

INTRODUCTION

The current knowledge of *Nephrops* circadian biology (and of crustaceans in general) is merely phenomenological, with very few insights on the molecular mechanisms regulating this behavior (Strauss and Dircksen 2010, De Pitta et al. 2013, Zhang et al. 2013). The molecular architecture of the circadian system in decapod crustaceans is indeed poorly known (Aréchiga and Rodríguez-Sosa 2002, Escamilla-Chimal et al. 2010) when compared to what has been achieved so far in other arthropods such as the fruitfly *Drosophila melanogaster* (Peschel and Helfrich-Forster 2011). In crustacean decapods, the eyestalks and their optic ganglia play a crucial role in the modulation of neuroendocrine and behavioral rhythms. They are an important source of neuropeptides including red pigment concentrating hormone, crustacean hyperglycemic hormone, pigment dispersing hormone, typically released by X-organ sinus gland complex, as well as of small molecules, such as serotonin and melatonin, both involved in circadian regulation (Aréchiga et al. 1985, Garfias et al. 1995, Escamilla-Chimal et al. 2001, Rao 2001, Böcking et al. 2002, Hardeland and Poeggeler 2003). Hence, the eyestalks are a good candidate for the search of genes involved in circadian regulation (clock genes) and their temporal pattern of expression.

The striking level of conservation of the molecular architecture of the circadian system among eukaryotes implies that putative clock genes of *Nephrops* could show homology with those of the phylogenetically closest arthropod model organism such as the fruitfly *Drosophila melanogaster* (Bell-Pedersen et al. 2005). On the other hand, the advent of next-generation sequencing (NGS) technologies allows delivering new, fast, and accurate information of wide portions of organisms' genomes, providing large number of reads in non-model species in which previous genomic information is unavailable (Metzker 2009, Morozova et al. 2009). Recent advances in assembly algorithms allow using NGS technologies that produce short reads; computational time in reads assembly is reduced by using a paired-end protocol without decreasing accuracy (Rodrigue et al. 2010, Martin and Wang 2011). RNA-sequencing (RNA-seq) is a method that uses NGS to gain information on transcriptomes (the transcribed portion of the genome). The fact that RNA-seq is not based on a hybridization-based approach using preexisting sequences (e.g. microarrays) makes this technique less biased and very attractive for non-model species such as the Norway lobster, where reference genomic data are not available.

In the present chapter, I sequenced and de novo assembled the *Nephrops norvegicus* eyestalk transcriptome. Because of the paramount role that clock genes likely have in regulating the rhythmicity of burrow emergence behavior, we annotated canonical clock gene homologs (e.g. *period*, *timeless*, *clock*, *bmal1*) and assessed their daily pattern of expression (RT-qPCR) in relation to the burrow emergence rhythms.

MATERIALS AND METHODS

Sampling and housing

Animals were collected following protocols reported in chapter 2 (See also Aguzzi et al. 2008). In the laboratory, animals were acclimated within a light-proof isolated chamber (See chapter 2 and Sbragaglia et al. 2013b). Burrow emergence rhythm was tracked in the laboratory using an actograph under monochromatic blue light (472 nm) and equipped with an artificial burrow. Automated video image analysis quantified animal displacements out of their burrows, for further details see (Chapter 2 and Sbragaglia et al. 2013a).

The 14 individuals used in this study were adult intermoult *Nephrops* males. Animals were acclimated for at least 40 days. Behavioral tests were carried out as follow: 12h-12h LD cycle (lights-ON at 08:00 h and lights-OFF at 20:00 h), with an intensity during light hours of $4 \cdot 10^{-3}$ $\mu E/m^2/s$, simulating light intensity at depth of about 150 m. During darkness hours video recording was accomplished using infrared (850 nm) light. Blue lights-ON and -OFF were progressively attained and extinguished within 30 min. All trials were conducted under constant temperature (13±1°C) for 10 days. Eyestalks were dissected during the last day of the experiment. Sampling and laboratory experiments followed the local legislation regarding animal's welfare.

Behavioral data analysis

Behavioral analyses were performed using the software Eltemps (www.el-temps.com). Chi-square periodogram (Sokolove and Bushell 1978) was used to scan for the presence of significant ($p < 0.05$) periodicity in the range 10-28 h and percentage of variance (%V) explained by each period is reported as a measure of rhythms' robustness (Refinetti 2006). Waveform analysis (24-h based) was carried out in order to identify the behavioral phenotype (nocturnal or diurnal) and the "midline estimating statistic of rhythm" (MESOR) was also computed. The percentage of the activity (area under the waveform curve) during darkness was calculated to determine the nocturnal or diurnal phenotype of the lobsters. Lobsters were considered nocturnal when more than 60% of locomotor activity was concentrated during darkness.

Transcriptome: eyestalk dissection and RNA extraction

Lobsters were anesthetized on ice for 15 minutes and their eyestalks dissected at the middle of the photophase (n=4) and at the middle of the scotophase (n=4). The cuticle and the retina were rapidly eliminated using a stereoscope under dim red light and the remaining tissue immediately transferred to RNA-later tissue collection (Invitrogen Inc.) and stored at -80°. Total

RNA isolation was performed using the column-based RNeasy® Mini Kit (Qiagen Inc.) following manufacturer instructions.

Transcriptome: sequencing and quality check

Before sequencing, the quality of the RNA integrity was checked using Agilent Technologies 2100 Bioanalyzer. Two samples (one for the photophase: NEP-L; and the other for the scotophase: NEP-D) were chosen for the construction of non-normalized cDNA libraries. The mRNA fraction of the total RNA was converted into a library of template molecules suitable for subsequent cluster generation using the reagents provided in the Illumina TruSeq RNA Sample Preparation Kit. Sequencing was performed using one channel of a HiSeq 2000 Illumina Sequencing System (paired-end, 100bp). FastQC (v0.10.0) was used to provide the quality control checks on raw sequence data coming from high throughput sequencing pipelines (http://www.bioinformatics.babraham.ac.uk).

Transcriptome: data analysis

Trinity r2011-11-26 (http://trinityrnaseq.sourceforge.net) was used for the de novo reconstruction of transcriptomes from the read data (Grabherr et al. 2011, Haas et al. 2013). Transcriptomes were first assembled separately (NEP-L and NEP-D) and then together (NEP-comb). In order to get the species distribution of the annotated hits of transcripts the combined transcriptome (NEP-Comb) was blasted against the Uniprot database (http://www.uniprot.org) using a stand-alone version of the blastX tool (v20120420) and setting the E-value cutoff to 10-6. BlastX translates the query sequence in all six possible reading frames and provides combined significance statistics for hits to different frames (http://blast.ncbi.nlm.nih.gov). Then, to assign putative gene functions, contigs from NEP-L and NEP-D were blasted separtely. Estimates of the numbers of annotated contigs that matched to known genes from the NCBI non-redundant protein sequence database were made and functional categories of the predicted genes were obtained by extracting the relative Gene Ontology (GO) terms from the blastX output (http://www.geneontology.org) (Ashburner et al. 2000). The grouped sets of GO terms was then subjected to a Fisher's exact test, using False Discovery Rate (FDR) p-value correction for multiple comparisons ($p < 0.05$), in order to find under- or over-represented terms between the two transcriptomes. In order to find transcripts in my dataset that could be considered as putative clock genes or genes related to the circadian system, we screened the description of the annotated sequences looking for the following key terms: "*circadian*", "*rhythmic*", "*entrainment*".

RT-qPCR: RNA extraction and cDNA synthesis

The eyestalks were dissected in 12 lobsters at four different time points (three individuals at each time point): 07:30 (just before lights-ON), 13:30 (at the middle of photophase), 19:30 (just before lights-OFF), and 01:30 (at the middle of scotophase). The cuticle and the retina were rapidly eliminated using a stereoscope under dim red light and the remaining tissue immediately frozen in liquid nitrogen and then stored at -80°. Eyestalk tissue was homogenized with 0.5 mL of Trizol and total RNA was extracted with chloroform, precipitated with isopropanol and washed with 75% ethanol. Pellets were suspended in 25 µL DEPC-water and stored at -80˚C. The quality of RNA was checked on gel electrophoresis and by absorbance ratio (A260/A280 nm) > 1.8. No signs of DNA contamination were found so we decided to not apply a DNase treatment. Concentration was assessed by absorbance at 260 nm, using a ND-1000 spectrophotometer (NanoDrop Technologies). One µg of total RNA was reverse transcribed into cDNA using Superscript III (Invitrogen) and random hexamers following manufacturer's instructions.

RT-qPCR: cloning and sequencing

We used PCR amplification to clone the putative *Nephrops* clock genes previously identified by blasting the de novo assembled transcriptome in Uniprot. This step also allowed assessing the fidelity of the assembling provided by trinity. We selected the contigs that matched with canonical clock genes *timeless*, *period*, *clock* and *bmal1* in order to study their expression using quantitative RT-qPCR. We retrieved a cDNA sequence of *Homarus americanus* (accession AF399872) encoding *α-actin* as housekeeping gene. Primers used for cloning were designed using MacVector 11.1.2 (for details see Table A4.1). cDNAs for cloning and sequencing were obtained from a pool of eyestalks dissected during light and darkness conditions following the protocol described above.

The PCR protocol consisted of one denaturating step at 94°C for 2 min followed by 30 to 35 cycles each consisting of 94°C for 30 s, annealing temperature (see Table A4.1) for 30 s and 72°C for 40 to 100 s (60 s per kb). The PCR product was then cloned using the StrataClone PCR cloning kit (Agilent Technologies, USA) following manufacturer's instructions. Plasmids were then purified using the PureLink Quick Plasmid Miniprep Kits (Invitrogen, USA) and sequenced for both strands using the T3 and T7 universal primers (Genewiz, Inc., USA).

Alignments between assembled and cloned sequences (as well as *α-actin* from *H. americanus* and *N. norvegicus*) were performed using EMBOSS Water open software suit (CluscalW2, http://www.ebi.ac.uk). BlastX was used to compare the translated protein products of the contigs against NCBI database. SMART (http://smart.embl-heidelberg.de) was used to identify conserved domains and structural motifs of protein (Schultz et al. 1998, Letunic et al.

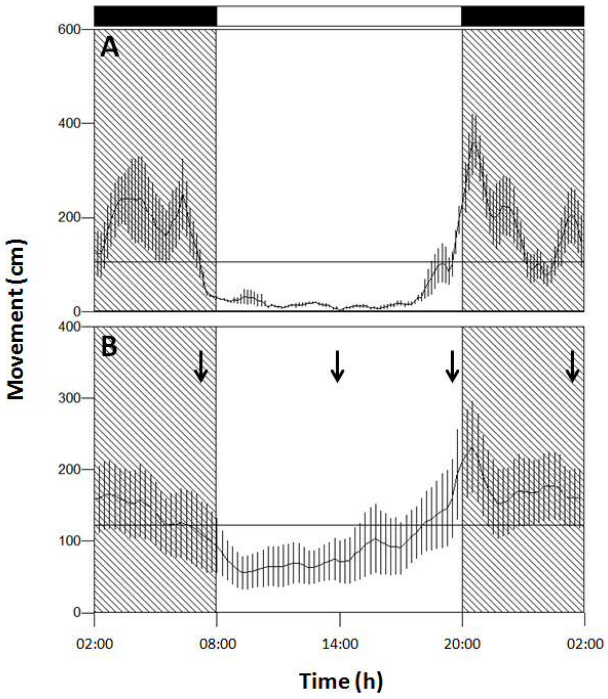


Fig. 4.1 – Waveform (24 h) analysis for a representative lobster (**A**) and averaged for the 14 lobsters used during the study (**B**). Activity is reported as displacement (cm) out of the burrow. Black and white bars represent darkness and light hours, respectively. Shadowed areas represent scotophase. Vertical lines represent the standard error of the mean and the horizontal line represents the MESOR. Arrows in **B** stay for the sampling points at which eyestalk were dissected for the RT-qPCR experiment.

2009), then amino acids sequences were blasted against NCBI data base (http://blast.ncbi.nlm.nih.gov/Blast.cgi) directly from SMART (Altschul et al. 1997).

RT-qPCR: daily expression of clock genes

We have tried to report all the fundamental information regarding the RT-qPCR experiment as suggested by Bustin et al. (2009). RT-qPCR reactions were carried out using an ABI 7900 HT (Applied Biosystems). Primers for *timeless*, *period*, *bmal1*, *clock*, *α-actin* (this latter as endogenous reference gene to standardize the expression levels) were designed using MacVector on the cloned sequences (Table, A4.2). Primers were tested before using a RT-PCR touch-down protocol with the following settings: 94˚C for 5 min, 10 cycles (94˚C for 30 s, 55˚C + 0.5˚C each cycle for 30 s, 72˚C for 30 s), 30 cycles (94˚C for 30 s, 55˚C for 30 s, 72˚C for 30 s), 72˚C for 7 min. Primers amplification efficiencies were tested by linear regression analysis from a cDNA dilution series and by running a melting curve (95˚C for 15 s, 60˚C for 15 s and 95˚C for 15 s). Efficiency (E=10(-1/slope)), showed values between 1.9 and 2.3, standard curves ranging from –2.5 to –3.6 and linear correlations (R^2) higher than 0.97 were recorded. cDNA was diluted 1:10 for all genes.

Table 4.1 - Descriptive statistics for the Illumina sequencing run and the assembly of the two *de novo* transcriptomes.

SAMPLES		NEP-L	NEP-D	NEP-comb
RAW SEQUENCES	Read type	Paired-end		-
	Read length (bp)	101		-
	Number of total reads	87'830'082	91'938'198	-
	Total (bp)	8'870'838'282	9'285'757'998	-
TRANSCRIPTOME ASSEMBLY	Total length of contigs	94'950'636	109'100'701	97'192'541
	Total number of contigs	106'256	114'235	108'599
	Max length	13'517	26'988	13'280
	Min length	201	201	201
	N90	311	322	310
	N80	532	571	530
	N70	864	956	875
	N60	1'300	1'468	1'305
	N50	1'796	2'055	1'810

Cycling conditions of the RT-qPCR were: decontamination step (50°C for 2 min), activation step (95°C for 10 min), 40 cycles of denaturation (95˚C for 15 s) and one annealing/extension step (60˚C for 1 min). A final dissociation step was also added (95˚C for 15 s and 60˚C for 15 s). Each sample was run in triplicate in 384-well plates. The reaction volume (10 µL) was composed by 2 µL of 5x PyroTaq EvaGreen qPCR Mix Plus, ROX (Cultek Molecular Bioline), 6 µL distilled water, 1 µL primer mix at a 10 mM concentration and 1 µL of cDNA. Duplicate negative controls were also run. SDS 2.3 software (Applied Biosystems) was used to collect raw data. . The transcript levels of the target genes were normalized to the reference gene *α-actin* and fold change was calculated following the $2^{\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The fold change was calculated using one of the sampling point (07:30) as a control or calibrator. Statistical analysis was performed using the 2ΔCT values. Results from 2ΔCT calculation were then checked for normality (Shapiro-Wilkoxon test), homoscedasticity of variance (Levene's mean test) and a one-way ANOVA test was used to assess differences among sampling times using the Sigma Plot (12.5) software.

RESULTS

Behavioral analysis

Fig. 4.1A shows the analysis of behavioral activity rhythms for one representative lobster used in the study. The Chi-square periodogram analysis identified a significant periodicity in burrow emergence rhythms of all animals, mean±SEM: 24.09±0.12h (37.37±4.68 %V). Waveform analysis showed a nocturnal burrow emergence activity for all lobsters (mean±SEM: 71.27±3.35 % of locomotor activity during darkness). The average waveform for

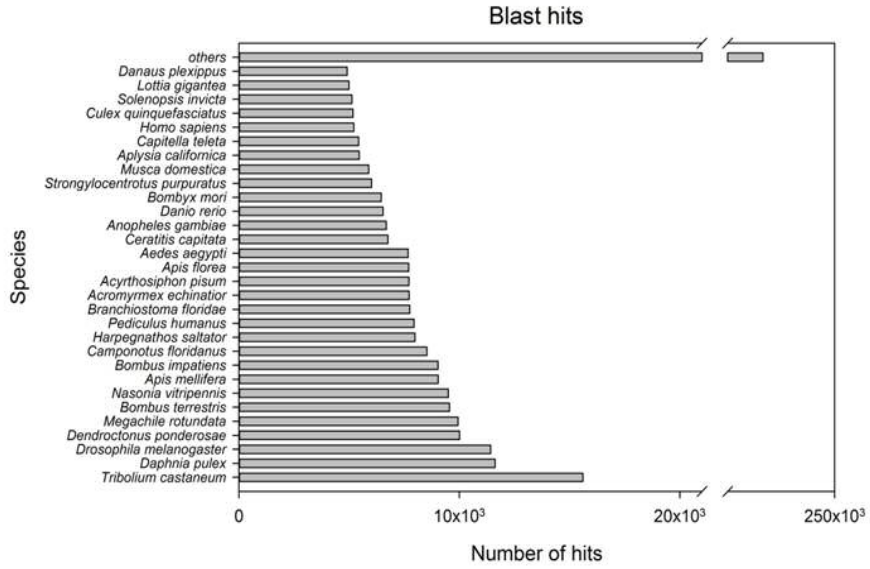


Fig. 4.2 – The species distribution of the annotated hits of transcripts of NEP-Comb against the NCBI non-redundant protein sequence database (E-value cutoff to 10^{-6}). Horizontal bars depict the number of hits for each one of the species. Only 30 species in order of number of annotated hits were presented, while the hits of all the other species are summed into the bar "*others*".

all 14 individuals (Fig. 4.1B) revealed an anticipatory peak of activity just before lights-OFF, which was also evident in the waveform for each individual (Fig. 4.1A).

Transcriptome analysis

The sequencing of libraries produced a total of approximately 88 and 92 millions of paired-end reads for NEP-L and NEP-D respectively (Table 1). The de novo assembly of NEP-Comb produced 108,599 contigs with a N50 (i.e. the size at which half of all assembled bases reside in contigs of this size or longer) of 1,810. The de novo assembly of NEP-L produced 106,256 contigs with a N50 of 1,796, while for NEP-D the number of contigs was 114,235 with an N50 of 2,055. The species distribution of the annotated hits of transcripts of NEP-Comb against the NCBI non-redundant protein sequence database is presented in Fig. 4.2. At about 73% of the first 30 species in order of number of annotated hits were insects, while the second species was the crustacean *Daphnia pulex*. The annotation of transcript sequences of NEP-L and NEP-D against the GO vocabulary produced 81,711 (77%) of no hits in NEP-L and 87,312 (76%) in NEP-D.. The positive hits and following assignment of functional categories were distributed as follows: biological processes 8,522 (8%), cellular component 6,944 (6%), molecular function 9,079 (9%) for NEP-L; biological process 9,586 (8%), cellular component 7,583 (7%), molecular function 9,754 (9%) for NEP-D (Fig. 4.3). The Fisher's exact test indicated that the 62 functional groups are equally represented among the two transcriptomes,

so we reported the detailed percentage of GO annotation for both samples (Fig. 4.3). The screening for putative clock genes produced 140 positive matches. Different sequences showed positive hits with genes related to the circadian system: *timeless, period, clock, cycle, bmal1, cryptochrome, double time, vrille, clockwork orange* and *jetlag.* Further details concerning the annotation are reported in Table A4.3.

Cloning and RT-qPCR

The partial sequences of putative *Nephrops* clock genes obtained by cloning exhibited high levels of identity and similarity with the contigs obtained by the *de novo* assembly of the transcriptome (Table 4.2). We cloned a fragment of 4,754-bp for the putative *Nephrops period* gene (accession KP943777) that showed a 98.3% of similarity and 1.3% of gaps with the assembled contig on which we designed the primers. The cloned fragment for the putative *Nephrops timeless* gene (accession KP943778) had 2,137-bp and showed a 100% similarity. The fragments cloned for the *clock* (accession KP943779) and *bmal1* (accession KP943781) genes were shorter, 272-bp and 222-bp respectively, and had 100% similarity in both cases. We

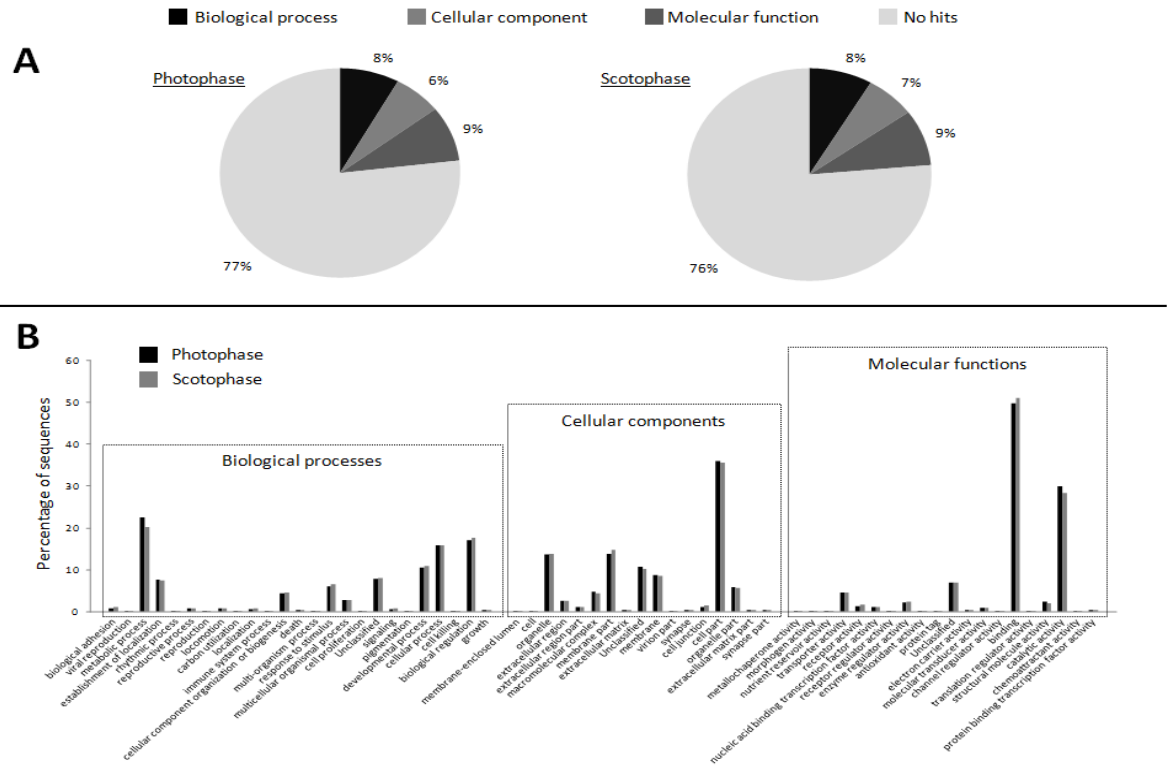


Fig. 4.3 - Gene ontology (GO) annotation of the assembled transcriptomes. **A:** The percentage distribution of functional categories between the two transcriptomes together with the proportion on no hits. **B:** The percentage of sequences distributed among 62 different functional groups of both samples (black columns: photophase; grey columns: scotophase).

Table 4.2 – Similarities observed during the alignment between the cloned sequences and the corresponding assembled contigs (*period*, *timeless*, *clock* and *bmal1*). For *α-act* the alignment was between the cloned sequence and the sequence retrieved from *H. americanus*.

Genes	alignment	length (bp)	similarity (%)	gaps (%)
NnPeriod	assembling vs cloning	4754	98.3	1.3
NnTimeless	assembling vs cloning	2137	100	0
Nnclock	assembling vs cloning	272	100	0
Nnbmal1	assembling vs cloning	222	100	0
a-act	*H.americanus* vs *N.norvegicus*	1036	96.9	0.4

also cloned a 1036-bp fragment of the *Nephrops α-actin* gene (accession KP943780) that showed a 96.9% of similarity with the homologous gene of *H. americanus* with a 0.4% of gaps.

The whole open reading frame of the contigs of the four putative clock genes were blasted on NCBI using the tool blastx to compare the translated protein products and results of representative best matches are reported in Table 4.3. The cDNA fragments were conceptually translated in peptides with the following amino acid (aa) lengths: PERIOD, 1,654aa; TIMELESS, 842aa; CLOCK, 148aa; BMAL1, 74aa. *Nephrops* putative clock proteins showed higher levels of similarity with other Crustaceans of the order Decapoda (e.g. the Signal crayfish, *Pacifastacus leniusculus* and the giant river prawn, *Macrobrachium rosenbergii*), Isopoda (e.g. the speckled sea louse, *Eurydice pulchra*) and with insects of different orders such as Diptera (e.g. the fruit fly, *Drosophila melanogaster*) and Orthoptera (e.g. the mangrove cricket, *Apteronemobius asahinai*).

We also blasted a contig (comp1618_c0_seq1) that was annotated against the GO database to *cryptochrome 1* (from *Homo sapiens*). The blastx of the contig against the NCBI database produced high level of identities with crustaceans of the Class Malacostraca, in particular with *cryptochrome* of *E. superba* (82%), and with *cryptochrome 2* of *E. pulchra* (79%) and *T. saltator* (79%) (see Table A4.4).

The conceptual translation of canonical clock genes cDNAs indicated the presence of conserved domains (Table A4.5). These were identified by SMART and blasted against NCBI database. *Nephrops* putative PERIOD has two PAS domains (from 229-296aa and from 373-442aa) and a PAC motif (from 450-493aa) that showed a high level of homology (expressed as identity) with conserved domains on the PERIOD protein of the isopod of *E. Pulchra* (PAS: 229-296aa, 85%; PAS: 373-442aa, 74%; PAC, 84%). *Nephrops* BMAL1 has the basic helix-loop-helix (bHLH, from 41-74aa) conserved domain that showed high level of homology with

the bHLH domain on the protein BMAL1 of the decapod *P. leniusculus* (100%) and the isopod *E. Pulchra* (97%) (Table A4.5). No conserved domains were identified in *Nephrops* TIMELESS and CLOCK.

The melting curves of the RT-qPCR indicated the presence of a single peak, suggesting no signs of contamination by DNA (as already supported by gel electrophoresis and absorbance ratio, see above). Among the canonical *Nephrops* clock genes, only *timeless* has a significant ($F = 10.470$; $P < 0.01$) pattern of expression with a peak just before the light-OFF (late day, Fig. 4.4). The other transcripts did not show significant differences of expression among the different sampling time points: *period*, ($F = 2.020$; $P = 0.19$); *clock*, ($F = 1.354$; $P = 0.32$); *bmal1*, ($F = 1.342$; $P = 0.33$. Despite its lack of significant rhythmicity, *period* expression pattern appeared to be similar to *timeless*.

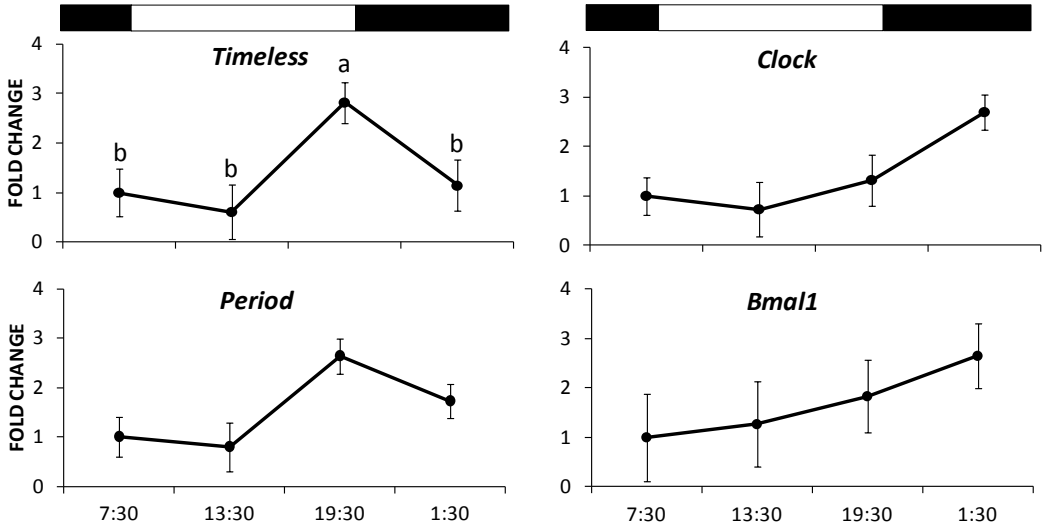


Fig. 4.4 – Canonical clock genes expression in *Nephrops* eyestalk. Measurements (n=3 each time point) were normalized to *a-act* and expressed as fold change respect to a control time point (7:30). Vertical bars represent the confidence limits. Black and white bars represent darkness and light hours, respectively. *Timeless* shows a significant pattern of expression (ANOVA, $P < 0.05$). Letters indicate the output of the Tukey's post hoc test (a>b).

Table 4.3 – The most representative match of the blastx against NCBI database of the putative canonical clock genes of *Nephrops*.

Genes	Species	Phylum - Class -Order	Protein product	Identity
NnPeriod	*Eurydice pulchra*	Arthropoda - Malacostraca - Isopoda	period	520/1101
	Blattella germanica	Arthropoda - Insecta - Blattodea	circadian clock protein period	360/1099
	Apteronemobius asahinai	Arthropoda - Insecta - Orthoptera	period isoform1	356/1100
	Laupala cerasina	Arthropoda - Insecta - Orthoptera	period	317/1031
	Rhyparobia maderae	Arthropoda - Insecta - Blattodea	period	201/538
NnTimeless	*Eurydice pulchra*	Arthropoda - Malacostraca - Isopoda	timeless	471/799
	Thermobia domestica	Arthropoda - Insecta - Thysanura	timeless	203/456
	Drosophila melanogaster	Arthropoda - Insecta - Diptera	timeless	196/465
	Clunio marinus	Arthropoda - Insecta - Diptera	timeless	192/456
	Belgica antarctica	Arthropoda - Insecta - Diptera	timeless	188/459
Nnclock	*Pacifastacus leniusculus*	Arthropoda - Malacostraca - Decapoda	clock-like protein	34/55
	Anopheles darlingi	Arthropoda - Insecta - Diptera	clock-like protein	34/41
	Macrobrachium rosenbergii	Arthropoda - Malacostraca - Decapoda	clock	28/29
	Thermobia domestica	Arthropoda - Insecta - Thysanura	clock	25/29
	Eurydice pulchra	Arthropoda - Malacostraca - Isopoda	clock 1-7	25/29
Nnbmal1	*Pacifastacus leniusculus*	Arthropoda - Malacostraca - Decapoda	bmal1a	72/75
	Eurydice pulchra	Arthropoda - Malacostraca - Isopoda	brain and muscle arnt-like protein-1	59/75
	Tribolium castaneum	Arthropoda - Insecta - Coleoptera	cycle protein	46/73
	Phyllotreta striolata	Arthropoda - Insecta - Coleoptera	cycle protein, partial	46/61
	Culex quinquefasciatus	Arthropoda - Insecta - Diptera	circadian protein clock/arnt/bmal/pas	45/72

DISCUSSION

Here we assembled for the first time the eyestalk transcriptome of *Nephrops norvegicus* and annotated putative clock genes. We confirmed the fidelity of *de novo* assembly of canonical clock genes by cloning them. Conceptually translated protein products of partial fragments of *N. norvegicus period, timeless, clock and bmal1* showed high similarities with these genes in crustaceans and insects, with the presence of characteristics conserved domains (PAS and bHLH). Other putative homologous of clock genes include *cryptochrome 2*. Interestingly, results of the RT-qPCR experiment indicated that *timeless* oscillates with a diel rhythm and could be considered a suitable genetic marker of the molecular circadian clockwork controlling *Nephrops* locomotor activity rhythm. Together, my results are consistent with the notion that the eyestalk in decapod crustaceans houses a circadian oscillator involved in the regulation of behavioral and physiological rhythms.

The amount and quality of the reads produced for the present study are consistent with the average throughput produced by Illumina HiSeq 2000 platform. The assembly statistics are in line with those produced by other studies using a similar approach (Crawford et al. 2010). The similar distribution of the 62 functional groups during GO analysis between the light and dark phase of the LD cycle suggests that there is no difference in gene expression in terms of broad functional categories. The screening of the *de novo* assembled transcriptome identified 140 transcripts encoding for putative circadian proteins, demonstrating its power when applied to non-model species where scarce or absent previous genomic knowledge is available.

The similarities observed comparing the cloned and assembled cDNAs fragments of putative canonical clock genes of *Nephrops* validates a high fidelity of the assembly performed with trinity, as previously demonstrated in yeast, mouse and non-model organisms such as the whiteflies (Grabherr et al. 2011). The blastx of the contigs of the putative clock genes of *Nephrops* against NCBI database revealed highest identities with the translated proteins for the full length cDNAs sequences from *E. pulchra* (*period*: 47%, *timeless*: 59%, *bmal1*: 79%) and *M. rosenbergii (clock*: 100%*)*. The homology with other crustacean clock proteins strongly suggest that the partial fragments cloned in this study could be considered homologous of canonical clock genes and hence part of the transcriptional-translational feedback loop that constitutes the molecular circadian machinery in all metazoans studied so far (Peschel and Helfrich-Forster 2011). This notion is reinforced by the presence of some characteristic conserved domains of clock proteins; *Nephrops* PERIOD has the PER-ARNT-SIM (PAS) domains, while *Nephrops* BMAL1 showed the basic helix-loop-helix (bHLH) domain. These domains are fundamental for the expression of protein PERIOD and TIMELESS and their activation by the heterodimeric bHLH-PAS transcription factors CLOCK and BMAL/CYCLE (Taylor and Zhulin 1999, Scheuermann et al. 2007, Hennig et al. 2009).

Among the four genes studied with RT-qPCR only *timeless* had an oscillating pattern of expression. My results are consistent with a recent study on *E. pulchra* where *period*, *timeless*, *bmal1*, *clock* and *cryptochrome 2* were studied using RT-qPCR (Zhang et al. 2013). Those authors reported that only *timeless* gave a robust and reliable circadian expression in whole head tissue, with a peak late in the subjective day. We didn't expose lobsters to constant conditions, but previous studies have demonstrated that *Nephrops* burrow emergence is under the control of circadian system and can be entrained by blue light (Chiesa et al. 2010). The peak of *timeless* transcripts is observed in lobsters sampled 30 min before light-OFF when animals also showed anticipation (increase of activity before any change in light intensity, see Fig. 4.1), suggesting that the observed oscillation of *timeless* transcripts is endogenous. We also did not observe a significant daily pattern of expression for *clock*. Yang et al. (2006) showed similar results for *M. rosembergii clock (Mar-clock)* under LD conditions; using semi-quantitative RT-PCR and *β-actin* as internal control, the authors did not observed diel patterns of expression either in the central nervous system or peripheral tissues. However the expression tended to increase at night, as observed in this study.

Finally, a contig of 3,239 bp matched with the vertebrate-like *cryptochrome 2*. In this study we did not focus on its cloning and expression, but the blastx of the contig against the NCBI database produced high level of identities with the crustaceans *E. superba* (82%), *E. pulchra* (79%), *T. saltator* (79%) (see Table S6). *Cryptochrome 2* was initially described for non-drosophilid insect species and proposed as a transcriptional repressor for the clock molecular machinery (Zhu et al., 2005). Its expression has daily oscillations in *E. superba* both in laboratory (Teschke et al. 2011) and in natural conditions (Mazzotta et al. 2010); recently it has been suggested as a major negative regulator also for the circadian clock of the crustacean *E. pulchra* (Zhang et al. 2013). Future studies could determine whether the same is true in *Nephrops*.

ANNEX 4A

Table A4.1 – Primers used for cloning.

Genes	Primer	Position (bp)	Primer sequence (5' -> 3')	Ann. Temp. (°C)
Period	C-per-F1	12-37	CCCAGAGTTAGTGGAGTAAAGGTGTG	56.7
	C-per-R1	1262-1241	TGTGGCAGATGACCCAGGTAGG	
	C-per-F2	315-337	TGGCGACAATGCTGATTTTAGTG	57.2
	C-per-R2	2103-2078	TGAGAGAGTCTGTGAGTGTGATAGCG	
	C-per-F3	2020-2039	AGCACCCATCCAGCCTTTTC	57.8
	C-per-R3	3260-3238	CGGTTCATCTCAGAATCCTTTGG	
	C-per-F4	3058-3077	CGTTCCACCACTAACCTGCG	56.1
	C-per-R4	4095-4073	CCTTGAGCCACCTATTGCCATAC	
	C-per-F5	3658-3678	AGATTACGACAGCCTGCCTGG	54.1
	C-per-R5	4850-4831	TCCGTTCTTTTTTTTCGCCC	
Timeless	C-tim-F2	752-773	TCGGACAGTTGGTAGAGGTGCG	56.5
	C-tim-R2	2304-2283	TGTTTGAGGATTCGTCGTCGTG	
	C-tim-F3	168-192	GCACCATCAGAAGCCTCATAAAATG	56.4
	C-tim-R3	801-778	CAAGCGAATCAGCAACACAAATAG	
clock	C-clk-F3	122-149	AGTTTAGTGATAACCAGGGAGTAAGAGC	54.5
	C-clk-R3	393-372	CGGACAGTTCGTTGATGAGGAG	
bmal1	C-bmal1-F1	1-24	TCCTTCTCCTCTGATGGCTCTAAG	52.1
	C-bmal1-R1	222-200	TGTCAGTTTGTCAAGCTTCCGAG	
a-act	C-aact-F1	300-322	CACTCCTTCTACAACGAACTGCG	58.1
	C-aact-R1	1160-1141	GCACTTGCGGTGGACAATGC	
	C-aact-F3	127-146	TATTCCCCTCCATCGTCGGC	57.7
	C-aact-R3	398-378	GGTCATCTTCTCACGGTTGGC	

Table A4.2 – Primers used for RT-qPCR.

Genes	Primer name	Position on cloned fragment	Primer sequence (5' -> 3')	Amplicon size (bp)	Efficiency (E)	R^2
NnPeriod	per-F1	364-388	TGGAAGAAGTTGAAGGAGAAGACCG	151	2.2	0.969
	per-R1	515-493	CAATACTGCTGGCTGTTTCGCTG			
NnTimeless	tim-F1	256-280	GCCCTATCAGATTGACCTGGACAAG	154	1.9	0.969
	tim-R1	409-385	CATCACCACTCCCTCATACACCAAG			
Nnclock	clk-F1	33-57	TGGTGGTGGTGTGAAGTGGATTTAC	132	2.5	0.970
	clk-R1	164-142	CAGATTTGCCAGGTGATGTTTCG			
Nnbmal1	bmal1-F1	1-21	TCCTTCTCCTCTGATGGCTCT	108	2.2	0.968
	bmal1-R1	108-89	TTTATTCCAATCCCCAGCAG			
Nnaact	aAct-F2	414-438	GGTTATTGTCTCCCACACGCTATCC	136	2.0	0.979
	aAct-R2	549-527	TGATGTCACGAACGATTTCTCGC			

Table A4.3 – List of candidate genes in *Nephrops norvegicus* that can be considered as putative clock genes or genes related to the circadian system.

Transcript	Length (bp)	Match	Start	End	Species	Access number
comp65743_c0_seq1	304	48 related 2	3	299	*Drosophila melanogaster*	FB|FBgn0038402
comp8903_c0_seq1	165	5'-AMP-activated protein kinase catalytic subunit alpha-2	135	1646	*Pongo abelii*	UNIPROTKB|Q5RD00
comp6931_c0_seq1	3892	Alpha-N-acetylglucosaminidase	3549	1936	*Homo sapiens*	UNIPROTKB|P54802
comp6931_c0_seq2	3710	Alpha-N-acetylglucosaminidase	3255	1936	*Homo sapiens*	UNIPROTKB|P54802
comp48442_c0_seq1	219	AT5G63860	2	193	*rabidopsis thaliana*	TAIR|locus:2163986
comp1846_c0_seq1	6297	ATP-binding cassette, sub-family B (MDR/TAP), member 1A	352	3969	*Rattus norvegicus*	RGD|619951
comp1846_c0_seq10	2755	ATP-binding cassette, sub-family B (MDR/TAP), member 1A	54	1505	*Rattus norvegicus*	RGD|619951
comp1846_c0_seq11	2300	ATP-binding cassette, sub-family B (MDR/TAP), member 1A	733	1050	*Rattus norvegicus*	RGD|619951
comp1846_c0_seq12	1899	ATP-binding cassette, sub-family B (MDR/TAP), member 1A	137	649	*Rattus norvegicus*	RGD|619951
comp1846_c0_seq2	5387	ATP-binding cassette, sub-family B (MDR/TAP), member 1A	352	4137	*Rattus norvegicus*	RGD|619951
comp1846_c0_seq5	3804	ATP-binding cassette, sub-family B (MDR/TAP), member 1A	517	1476	*Rattus norvegicus*	RGD|619951
comp1846_c0_seq6	3665	ATP-binding cassette, sub-family B (MDR/TAP), member 1A	54	1337	*Rattus norvegicus*	RGD|619951
comp1846_c0_seq8	2894	ATP-binding cassette, sub-family B (MDR/TAP), member 1A	517	1644	*Rattus norvegicus*	RGD|619951
comp65830_c0_seq1	855	beta-transducin repeat containing	2	853	*Rattus norvegicus*	RGD|1359721
comp1689_c0_seq1	5472	cAMP-dependent protein kinase 1	670	1614	*Drosophila melanogaster*	FB|FBgn0000273
comp1689_c0_seq2	5177	cAMP-dependent protein kinase 1	267	1319	*Drosophila melanogaster*	FB|FBgn0000273
comp33327_c0_seq1	1296	cAMP-dependent protein kinase R2	1156	44	*Drosophila melanogaster*	FB|FBgn0022382
comp78077_c0_seq1	983	Caveolin-1	947	459	*Ornithorhynchus anatinus*	UNIPROTKB|Q07E02
comp30303_c0_seq1	535	CG2650	510	91	*Drosophila melanogaster*	FB|FBgn0000092
comp1595_c0_seq13	3033	circadian trip	1616	426	*Drosophila melanogaster*	FB|FBgn0260794
comp1595_c0_seq14	3021	circadian trip	1616	426	*Drosophila melanogaster*	FB|FBgn0260794
comp23855_c0_seq2	445	Clock	122	33	*Drosophila melanogaster*	FB|FBgn0023076
comp10496_c0_seq1	3957	clockwork orange	2893	2486	*Drosophila melanogaster*	FB|FBgn0259938
comp10496_c0_seq2	3936	clockwork orange	2872	2465	*Drosophila melanogaster*	FB|FBgn0259938
comp10496_c0_seq3	3242	clockwork orange	3049	2486	*Drosophila melanogaster*	FB|FBgn0259938
comp10496_c0_seq4	3221	clockwork orange	3028	2465	*Drosophila melanogaster*	FB|FBgn0259938
comp1618_c0_seq1	3239	Cryptochrome-1	197	1666	*Homo sapiens*	UNIPROTKB|Q16526
comp25352_c0_seq1	1976	curled	231	1316	*Drosophila melanogaster*	FB|FBgn0261808
comp25352_c0_seq2	1786	curled	83	1126	*Drosophila melanogaster*	FB|FBgn0261808
comp25352_c0_seq3	1429	curled	302	769	*Drosophila melanogaster*	FB|FBgn0261808
comp25352_c0_seq4	980	curled	231	854	*Drosophila melanogaster*	FB|FBgn0261808

comp54873_c0_seq1	222	cycle	55	222	*Drosophila melanogaster*	FB|FBgn0023094
comp1099_c0_seq1	7845	discs large 1	1033	3504	*Drosophila melanogaster*	FB|FBgn0001624
comp1099_c0_seq2	7827	discs large 1	1033	3486	*Drosophila melanogaster*	FB|FBgn0001624
comp21486_c0_seq1	706	discs large 1	379	134	*Drosophila melanogaster*	FB|FBgn0001624
comp16468_c0_seq1	6864	Dopa decarboxylase	6738	5344	*Drosophila melanogaster*	FB|FBgn0000422
comp16468_c0_seq3	3218	Dopa decarboxylase	3092	1698	*Drosophila melanogaster*	FB|FBgn0000422
comp49424_c0_seq1	527	Dopamine transporter	526	14	*Drosophila melanogaster*	FB|FBgn0034136
comp69404_c0_seq1	229	Dopamine transporter	21	155	*Drosophila melanogaster*	FB|FBgn0034136
comp73842_c0_seq1	482	Dopamine transporter	480	4	*Drosophila melanogaster*	FB|FBgn0034136
comp27457_c0_seq1	3891	Dttg protein	439	837	*Drosophila* sp.	UNIPROTKB|P91608
comp27457_c0_seq2	3828	Dttg protein	439	915	*Drosophila* sp.	UNIPROTKB|P91608
comp11431_c0_seq1	4284	dunce	267	2375	*Drosophila melanogaster*	FB|FBgn0000479
comp11431_c0_seq2	4263	dunce	267	2354	*Drosophila melanogaster*	FB|FBgn0000479
comp11431_c0_seq3	4260	dunce	267	2351	*Drosophila melanogaster*	FB|FBgn0000479
comp11431_c0_seq4	4239	dunce	267	2330	*Drosophila melanogaster*	FB|FBgn0000479
comp11431_c0_seq5	3575	dunce	116	1666	*Drosophila melanogaster*	FB|FBgn0000479
comp11431_c0_seq6	3554	dunce	116	1645	*Drosophila melanogaster*	FB|FBgn0000479
comp12887_c0_seq1	1705	dusky	1464	676	*Drosophila melanogaster*	FB|FBgn0004511
comp89314_c0_seq1	408	dusky	102	365	*Drosophila melanogaster*	FB|FBgn0004511
comp9690_c0_seq16	2331	ebony	596	2200	*Drosophila melanogaster*	FB|FBgn0000527
comp9690_c0_seq22	1692	ebony	432	1061	*Drosophila melanogaster*	FB|FBgn0000527
comp9690_c0_seq27	802	ebony	76	171	*Drosophila melanogaster*	FB|FBgn0000527
comp9690_c0_seq6	3245	ebony	1738	3114	*Drosophila melanogaster*	FB|FBgn0000527
comp9690_c0_seq8	3022	ebony	432	2891	*Drosophila melanogaster*	FB|FBgn0000527
comp9690_c0_seq9	2739	ebony	1277	2608	*Drosophila melanogaster*	FB|FBgn0000527
comp43073_c0_seq1	731	Ecdysone receptor	452	75	*Drosophila melanogaster*	FB|FBgn0000546
comp45891_c0_seq1	264	Ecdysone receptor	263	159	*Drosophila melanogaster*	FB|FBgn0000546
comp50197_c0_seq1	448	Ecdysone receptor	1	420	*Drosophila melanogaster*	FB|FBgn0000546
comp59204_c0_seq1	513	F-box and WD repeat domain containing 11	7	513	*Rattus norvegicus*	RGD|1309121
comp8570_c0_seq1	3139	glass	1887	925	*Drosophila melanogaster*	FB|FBgn0004618
comp11966_c0_seq1	820	Heat shock protein 83	2	820	*Drosophila melanogaster*	FB|FBgn0001233
comp19340_c0_seq1	2213	jetlag	2174	1398	*Drosophila melanogaster*	FB|FBgn0031652
comp83131_c0_seq1	291	KaiRIA	1	291	*Drosophila melanogaster*	FB|FBgn0028422
comp4180_c0_seq3	4236	Large proline-rich protein BAG6	1313	87	*Ornithorhynchus anatinus*	UNIPROTKB|A7X5R6

comp10165_c0_seq1	1259	lark	1258	575	*Drosophila melanogaster*	FB|FBgn0011640
comp10165_c0_seq2	1227	lark	1226	543	*Drosophila melanogaster*	FB|FBgn0011640
comp10165_c0_seq3	1185	lark	1123	575	*Drosophila melanogaster*	FB|FBgn0011640
comp10165_c0_seq4	1153	lark	1091	543	*Drosophila melanogaster*	FB|FBgn0011640
comp10165_c0_seq5	1095	lark	1094	498	*Drosophila melanogaster*	FB|FBgn0011640
comp10165_c0_seq6	1063	lark	1062	466	*Drosophila melanogaster*	FB|FBgn0011640
comp10165_c0_seq7	1021	lark	959	498	*Drosophila melanogaster*	FB|FBgn0011640
comp10165_c0_seq8	989	lark	927	466	*Drosophila melanogaster*	FB|FBgn0011640
comp10165_c0_seq9	335	lark	334	212	*Drosophila melanogaster*	FB|FBgn0011640
comp1808_c0_seq1	1440	lark	101	688	*Drosophila melanogaster*	FB|FBgn0011640
comp1808_c0_seq2	902	lark	101	301	*Drosophila melanogaster*	FB|FBgn0011640
comp29701_c0_seq1	1374	lark	1112	639	*Drosophila melanogaster*	FB|FBgn0011640
comp6509_c0_seq1	1127	lark	471	115	*Drosophila melanogaster*	FB|FBgn0011640
comp6509_c0_seq2	959	lark	303	1	*Drosophila melanogaster*	FB|FBgn0011640
comp6509_c0_seq3	747	lark	471	115	*Drosophila melanogaster*	FB|FBgn0011640
comp6509_c0_seq4	579	lark	303	1	*Drosophila melanogaster*	FB|FBgn0011640
comp3068_c0_seq1	3907	minibrain	1730	2935	*Drosophila melanogaster*	FB|FBgn0259168
comp3068_c0_seq2	3864	minibrain	1730	2935	*Drosophila melanogaster*	FB|FBgn0259168
comp5354_c0_seq1	3603	Myocyte enhancer factor 2	71	1216	*Drosophila melanogaster*	FB|FBgn0011656
comp5354_c0_seq2	3591	Myocyte enhancer factor 2	71	1237	*Drosophila melanogaster*	FB|FBgn0011656
comp34215_c0_seq1	967	narrow abdomen	2	484	*Drosophila melanogaster*	FB|FBgn0002917
comp66820_c0_seq1	451	narrow abdomen	451	2	*Drosophila melanogaster*	FB|FBgn0002917
comp85336_c0_seq1	285	narrow abdomen	37	285	*Drosophila melanogaster*	FB|FBgn0002917
comp96661_c0_seq1	223	narrow abdomen	221	3	*Drosophila melanogaster*	FB|FBgn0002917
comp97497_c0_seq1	329	narrow abdomen	2	328	*Drosophila melanogaster*	FB|FBgn0002917
comp8495_c0_seq1	8857	Neurofibromin 1	65	7399	*Drosophila melanogaster*	FB|FBgn0015269
comp8495_c0_seq2	8845	Neurofibromin 1	65	7387	*Drosophila melanogaster*	FB|FBgn0015269
comp8495_c0_seq3	8686	Neurofibromin 1	65	7399	*Drosophila melanogaster*	FB|FBgn0015269
comp8495_c0_seq4	8674	Neurofibromin 1	65	7387	*Drosophila melanogaster*	FB|FBgn0015269
Comp841_c0_seq1	4029	no receptor potential A	474	3737	*Drosophila melanogaster*	FB|FBgn0262738
comp8804_c0_seq1	2651	numb	281	1441	*Drosophila melanogaster*	FB|FBgn0002973
comp8804_c0_seq2	2614	numb	244	1404	*Drosophila melanogaster*	FB|FBgn0002973
comp8804_c0_seq3	2585	numb	281	1375	*Drosophila melanogaster*	FB|FBgn0002973
comp8804_c0_seq4	2573	numb	203	1363	*Drosophila melanogaster*	FB|FBgn0002973

comp8804_c0_seq5	2548	numb	244	1338	*Drosophila melanogaster*	FB|FBgn0002973
comp8804_c0_seq6	2507	numb	203	1297	*Drosophila melanogaster*	FB|FBgn0002973
comp2789_c0_seq1	4961	period	4220	2901	*Drosophila melanogaster*	FB|FBgn0003068
comp24010_c0_seq1	1436	Pigment-dispersing factor receptor	81	1259	*Drosophila melanogaster*	FB|FBgn0260753
comp33631_c0_seq1	4066	Pigment-dispersing factor receptor	722	2005	*Drosophila melanogaster*	FB|FBgn0260753
comp2319_c0_seq1	621	Prokineticin-2	425	177	*Bos taurus*	UNIPROTKB|Q863H5
comp2319_c0_seq2	578	Prokineticin-2	382	134	*Bos taurus*	UNIPROTKB|Q863H5
comp17940_c0_seq1	1678	Protein lin-52 homolog	315	551	*Oncorhynchus mykiss*	UNIPROTKB|Q6X4M3
comp17940_c0_seq2	1573	Protein lin-52 homolog	105	446	*Oncorhynchus mykiss*	UNIPROTKB|Q6X4M3
comp17014_c0_seq1	700	Protein quiver	274	576	*Drosophila mojavensis*	UNIPROTKB|B4KR21
comp17014_c0_seq2	492	Protein quiver	277	492	*Drosophila mojavensis*	UNIPROTKB|B4KR21
comp43724_c0_seq1	2037	Protein timeless homolog	1732	161	*Homo sapiens*	UNIPROTKB|Q9UNS1
comp10628_c0_seq1	2883	quasimodo	459	1292	*Drosophila melanogaster*	FB|FBgn0028622
comp62503_c0_seq1	366	Rhythmically expressed gene 5	226	336	*Drosophila melanogaster*	FB|FBgn0015801
comp54599_c0_seq1	573	RNA-binding protein 4B	572	279	*Sus scrofa*	UNIPROTKB|F1RUT7
comp573_c0_seq1	3368	RNA-binding protein 4B	129	371	*Sus scrofa*	UNIPROTKB|F1RUT7
comp573_c0_seq2	3044	RNA-binding protein 4B	129	371	*Sus scrofa*	UNIPROTKB|F1RUT7
comp573_c0_seq3	2925	RNA-binding protein 4B	129	371	*Sus scrofa*	UNIPROTKB|F1RUT7
comp573_c0_seq4	2601	RNA-binding protein 4B	129	371	*Sus scrofa*	UNIPROTKB|F1RUT7
comp24954_c0_seq1	3152	roundabout	362	2737	*Drosophila melanogaster*	FB|FBgn0005631
comp24954_c0_seq2	3134	roundabout	362	2764	*Drosophila melanogaster*	FB|FBgn0005631
comp24954_c0_seq3	2854	roundabout	46	2439	*Drosophila melanogaster*	FB|FBgn0005631
comp24954_c0_seq4	2836	roundabout	46	2466	*Drosophila melanogaster*	FB|FBgn0005631
comp26721_c0_seq1	7012	roundabout	442	2991	*Drosophila melanogaster*	FB|FBgn0005631
comp26721_c0_seq2	5448	roundabout	3	1445	*Drosophila melanogaster*	FB|FBgn0005631
comp45935_c0_seq1	1373	roundabout	1362	130	*Drosophila melanogaster*	FB|FBgn0005631
comp60778_c0_seq1	488	roundabout	2	394	*Drosophila melanogaster*	FB|FBgn0005631
comp67560_c0_seq1	506	roundabout	340	486	*Drosophila melanogaster*	FB|FBgn0005631
comp45706_c0_seq1	482	Serotonin receptor 1A	3	386	*Drosophila melanogaster*	FB|FBgn0004168
comp90243_c0_seq1	327	Serotonin receptor 1A	325	86	*Drosophila melanogaster*	FB|FBgn0004168
comp96775_c0_seq1	235	Shaker	234	1	*Drosophila melanogaster*	FB|FBgn0003380
comp19976_c0_seq1	1351	slowpoke	1053	910	*Drosophila melanogaster*	FB|FBgn0003429
comp29144_c0_seq1	219	slowpoke	218	87	*Drosophila melanogaster*	FB|FBgn0003429
comp62466_c0_seq1	411	slowpoke	275	409	*Drosophila melanogaster*	FB|FBgn0003429

comp4789_c0_seq1	2620	timeless	2545	1181	*Drosophila melanogaster*	FB|FBgn0014396
comp4789_c0_seq2	2526	timeless	2451	1087	*Drosophila melanogaster*	FB|FBgn0014396
comp6413_c0_seq1	2451	timeless	1551	196	*Drosophila melanogaster*	FB|FBgn0014396
comp6413_c0_seq2	1966	timeless	1551	196	*Drosophila melanogaster*	FB|FBgn0014396
comp39652_c0_seq1	1137	TIMELESS (Uncharacterized protein)	1135	458	*Sus scrofa*	UNIPROTKB|F1SLB6
comp67299_c0_seq1	309	timeout	42	260	*Drosophila melanogaster*	FB|FBgn0038118
comp7837_c0_seq1	1531	vrille	470	832	*Drosophila melanogaster*	FB|FBgn0016076

Table A4.4 – Blastx of the contig annotated to *cryptochrome*.

Genes	Species	Phylum - Class -Order	Definition	Identity	Gaps	Accession
Cry2	*Euphausia superba*	Arthropoda - Malacostraca - Euphasiacea	cryptochrome	441/539	3/539	CAQ86665
	Eurydice pulchra	Arthropoda - Malacostraca - Isopoda	cryptochrome 2	421/541	4/541	AGV28717
	Talitrus saltator	Arthropoda - Malacostraca - Anphipoda	cryptochrome 2	396/499	0/499	AFV96168

Table A4.5 – Conserved domains of canonical clock genes in *Nephrops norvegicus*.

Protein	Conserved domain	Position (aa)	Aminoacid sequence
PERIOD	PAS domain	229-296	AAFLKSFKSTRGFTVAISVQDGTVLQVSPAITDVLGFPKDMLIGQSFIDFVYPKDSINLSSKIIHGLN
	PAS domain	373-442	ESIYTVPEETPAMGSFSIRHSASCNFSEYDPEAIPYLGHLPQDLTGNSVFDCYHXEDLPLLKAVYEGMVR
	PAC motif	450-493	SKPYRFRTFNGSYVTLQTEWLCFVNPWTKRIDSIIGQHRVLKGP
BMAL1	bHLH	41-74	SEIEKRRRDKMNTYIMELSSIIPVCTSRKLDKLT

Dominance hierarchy

5

INTRODUCTION

The fighting behaviour and the consequent formation of dominance hierarchies in groups of clawed decapod crustaceans have always attracted the attention of researchers (Edwards and Herberholz 2005). Dominance was defined by Drews (1993) as an attribute of the pattern of repeated agonistic interactions between two individuals, characterized by a consistent outcome in favour of the same dyad member, while dominance ranks refers to the position of one individual in a dominance hierarchy. Dominance hierarchies can be characterized by two properties: linearity and steepness (De Vries 1995, De Vries et al. 2006). The formation and maintenance of dominance hierarchies is accompanied with changes in behaviour, polarization of dominance ranks and consequent reduction of frequency and duration of agonistic interactions (Chase 1982; Goessmann et al. 2000; Herberholz et al. 2007), such characteristics, as demonstrated by modelling, imply a stabilization of the hierarchy (Hemelrijk 1999). Pheromones released with urine are considered one of the most important chemical signals for the maintenance of dominance hierarchies in lobsters and crayfish (Karavanich and Atema 1998, Breithaupt and Atema 2000, Aggio and Derby 2011, Breithaupt 2011). In the case in which the members of the hierarchy have no previous experience (prior residence in other contexts, prior agonistic encounters, different diets, knowledge of resource value), fighting success is usually associated with physical superiority (Ranta and Lindström 1992, 1993, Rutherford et al. 1995, Barki et al. 1997, Goessmann et al. 2000).

Almost all living animals organize their behavioral activities and physiology on a 24 h basis, by means of endogenous timekeeping or biological clock (Dunlap et al. 2004). On the base of daily activity patterns we can recognize nocturnal, diurnal of crepuscular (active at sunset and sunrise) animals. The selective forces and constraints affecting evolution of activity patterns underlie the partitioning of time as a resource (Kronfeld-Schor and Dayan 2003). However, activity patterns are not rigid and respond to changes in photoperiod, food resources, temperature, as well as to other organisms. The effect of social interactions on circadian clocks has been reviewed several times (Regal and Connolly 1980, Davidson and Menaker 2003, Mistlberger and Skene 2004, Favreau et al. 2009, Castillo-Ruiz et al. 2012, Bloch et al. 2013), but the mechanisms controlling the synchronized clocks network are still not totally understood.

Almost no attention has been devoted to the fact that many animals society are organized in a hierarchy. What could happen to the biological rhythms of individuals of the same species that have a clear dominant/subordinate relationship? Bovet (1972) demonstrated that when a group of 4 long-tailed field mice (*Apodemus sylvaticus*) were co-housed for a long period of time, the dominant individual has a different daily activity pattern compared to the other 3 subordinates. Long term changes in daily rhythms were also observed when rats

experienced subordination for one hour (Meerlo et al. 1999). Another example is provided by Hansen and Closs (2005) that studied the daily activity the giant kokopu (*Galaxias argenteus*).

The ecological significance to form dominance hierarchies is related to the partitioning of limited resources such as food, shelter, and mating opportunities (Wilson 1975). For example, in groups of three crayfish (*Procambarus clarkii*) higher dominance indices were significantly correlated with increased access to food (Herberholz et al. 2007). So, if time is considered a limited resource (Kronfeld-Schor and Dayan 2003), a group of social animals organized in a stable dominance hierarchy should distribute the access to the resource "time" according to their rank into the hierarchy.

Like others clawed decapod crustaceans, the Norway lobster, *Nephrops norvegicus*, forms lasting dyadic dominance relationships based on the assessment of chemical signals release with urine (Katoh et al. 2008), but nothing is know on the formation and maintenance of dominance hierarchies. Here, I studied the formation and maintenance of a dominance hierarchy into a group of 4 *Nephrops;* I have also recorded the time that lobsters spent into the burrow and their individual locomotor activity out of the burrow. My hypothesis was that in a stable dominance hierarchy the burrow and the time of emergence (i.e. locomotor activity) are important resources and their access is distributed according to the rank of lobsters.

MATERIALS AND METHODS

Animals' sampling and acclimation

Animals were collected at night-time by a commercial trawler on the shelf area (100 m depth) off the Ebro delta (Tarragona, Spain). All sorting operations on the deck and the transportation of individuals to the laboratory, followed the methodology described in Aguzzi et al. (2008). In the laboratory, specimens were transferred to acclimation tanks, hosted within a light-proof isolated chamber under the following conditions: *i*. constant temperature of 13±1 °C, as reported for the western Mediterranean continental slope throughout the year (Hopkins 1985); *ii*. random feeding time, in order to prevent entrainment through food-entraining oscillators, as shown for crustaceans (Fernández De Miguel and Aréchiga 1994); and *iii*. Light (L)-Darkness (D) cycle, matching the duration of photoperiod at the latitude of Barcelona (41º 23' 0" N). Also, light-ON and -OFF, were progressively attained and extinguished within 30 min, in order to acclimate animals' eyes to light intensity change. The acclimation facility hosted individual cells (25x20x30 cm) made with a plastic net of different sizes, allowing full oxygenation and recirculation of the water, but not the contact between animals.

Experimental tank

The experimental tank used in this study was previously developed by (Aguzzi et al. 2011b). Briefly, the tank (150×70×30 cm) was endowed with 4 burrows of 25 cm length and an approximately inclination of 20° of the entrance (Fig. 5.1). Sand was glued at the bottom of the tank and on the internal walls of the burrows. The rest of the tank was painted in black to facilitate video image analysis. The tank was equipped with two different sources of LED's illumination (monochromatic blue: 472 nm, infrared: 850 nm). Monochromatic blue lighting was installed to simulate Light-Darkness (LD) conditions, while infrared (IR) light allowed video-recording during darkness. A strip of LED's photodiodes (Blue LEDs, n = 84; IR LEDs, n = 108) was inserted in a transparent methyl metacrilate (MM) tube of 140 cm long and 16 mm in diameter. At one of its extremity, I added a smaller PVC tube containing the cables for the LEDs' power supply. L-shaped resulting lighting apparatuses were placed in the longer sides of the tank (1 blue and 1 IR each side) to get a uniform illumination (See Chapter 2 and also Sbragaglia et al. (2013a)).

Light cycle was controlled using the Arduino Board *Arduino uno*. Arduino is an open-source electronics platform based on easy-to-use hardware and software (http://www.arduino.cc/). The behavior of lobster was recorded using a digital camera (UI-1545LE-M, IDS), with a 1280×1024 pixels resolution and a wide-angular objective of 6.0 mm, and F1.4 screw C 1/2 (IDS) lens, and a polarized filter. The camera was placed on a tripod at 1.5 m directly above the tank. The camera acquisition was controlled by ISPY that is an open source camera security software (http://www.ispyconnect.com/). All experimental trials were run (June 2013-July 2014) at the aquaria facility of the Marine Science Institute in Barcelona (Spain) at an environmental temperature of 13 ± 0.5 ºC. The tank was provided with a continuous open flow (4L/min) of filtered sea water at 13 ± 1 ºC.

Animals and experimental design

The intermoult males lobsters used during the study had a cephalothorax length (mean±SD) of 45.35±2.92 mm (n=32). The right claw was 67.05±8.90 mm and the left claw was 67.12±9.58 mm. When possible, individuals were distributed among the different groups taking into account their CL and trying to maintain the differences in size to the minimal levels (See Table 1). The day before each experimental trial a group of 4 intermoult male lobsters were selected from the acclimation tanks and different tags (Fig. 4.1) were fixed on the superior part of the animals' cephalothorax with fast-acting glue, which was removable at the end of the experiment without damage. Then animals were left in isolation for 24 hours

Table 5.1 – The distribution of individuals among the different groups taking into account their cephalothorax length (**CL**), the size of the right (**RC**) and left (**LC**) claws. The difference in CL (expressed as percentage) between the larger and the smaller lobster is reported for each group. All the values are expressed in mm.

Group	Tag	CL (mm)	RC (mm)	LC (mm)	Difference CL (%)
1	C	39.82	53.11	54.93	10.42
	Cp	42.36	63.28	60.47	
	T	43.97	69.02	69.00	
	Tp	43.90	68.35	64.56	
2	C	40.84	60.41	62.03	7.35
	Cp	41.63	63.04	67.09	
	T	40.80	58.38	59.19	
	Tp	43.80	68.31	70.34	
3	C	47.00	88.78	92.57	12.64
	Cp	45.50	75.61	76.82	
	T	43.50	66.89	67.30	
	Tp	49.00	85.50	85.97	
4	C	43.20	71.65	71.82	3.80
	Cp	42.70	62.60	58.70	
	T	43.30	71.90	68.32	
	Tp	42.10	68.70	61.10	
5	C	43.50	67.90	69.20	5.67
	Cp	44.70	67.50	68.20	
	T	43.80	64.20	68.70	
	Tp	42.30	69.10	66.40	
6	C	44.79	63.00	61.40	5.48
	Cp	45.82	68.20	70.20	
	T	43.44	69.90	64.00	
	Tp	44.77	59.00	62.00	
7	C	45.56	72.10	68.80	35.23
	Cp	38.20	58.70	58.30	
	T	33.69	47.60	43.70	
	Tp	39.84	51.70	54.50	
8	C	44.79	76.50	79.50	6.87
	Cp	45.92	63.60	69.40	
	T	47.86	82.30	83.70	
	Tp	44.78	68.80	69.70	

The experimental trials started between 11:00 and 14:00 when lobsters were transferred from isolation to the experimental tank into individual plastic container and gradually (15 min) acclimated to the new environment before leave them free to interact. Each trial lasted 5 days during which lobsters were exposed to a 12-12 Light-Darkness blue light cycle. LD transitions were gradually achieved within 30 min, in order to avoid lobster's photoreceptors degeneration (i.e. rhabdom deterioration and visual pigments photolysis), as it occurs when animals are

subjected to sudden bright light exposure (Gaten et al. 1990). For each experimental trial a time lapse video was created at a frequency of acquisition of 1 frame /sec.

Behavioural analysis

The time lapse video were analysed manually by a trained operator, determining (i) the winner and loser of each agonistic interaction (Atema and Voigt 1995), (ii) the number of evictions (fighting to get possession of the burrow of others lobsters), and (iii) the burrow occupied by each lobster. In particular, an interaction was considered when two lobsters approached frontally within a distance of less of one body length and one of them react (e.g. change in moving trajectory, moving backward or accelerating the locomotion) to the other's presence. I considered two consecutive approaches as two distinct interactions when the time gap between them was at least 15 s. As regarding eviction events, I considered a success when a lobster (intruder) approached a burrow in which there was another lobster (resident) and the resident left the burrow suddenly or after has fought for it. On the contrary, I considered a failed eviction when an intruder fought with a resident for at least 2 min and then left the fight without succeed to evict the resident. The core output of this behavioural analysis was a 4x4 socio matrix for each representative day (1, 3, 5) reporting the number of wins for all possible dyads.

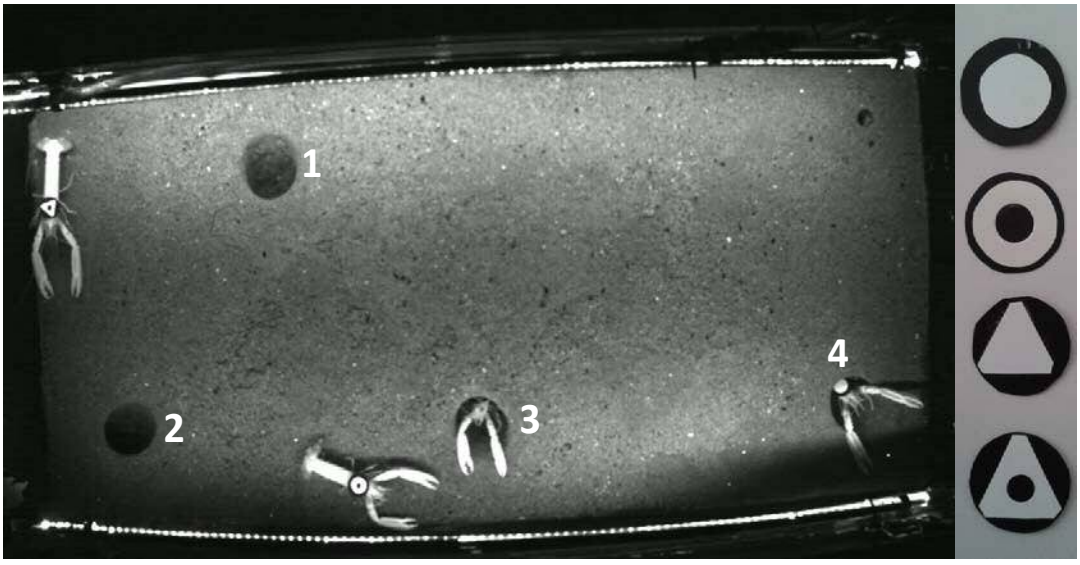


Fig. 5.1 – The field of view of the video camera used to film the behavioural activity of lobsters in the experimental tank. The white numbers indicated the four artificial burrows. The different tags used to identify the lobster with the video imaging analysis are presented on the right.

Automated Behavioural tracking

The time lapse videos were also analyzed by using automated behavioural tracking routine developed in *Phyton* using *OpenCV* libraries (Python Language Reference, version 2.7; Available at http://www.python.org). The algorithm works using different logic steps in order to automatically track the locomotor activity and at the same time identify the different tags of lobsters (Fig. S1). Key steps of the process are described as follow. First of all, the differences among two consecutive frames are evaluated using a background subtraction (OpenCV). The objects into one frame are identified focusing into a previously selected main Region Of Interest (ROI); the area of each object is analyzed dividing the main ROI into smaller areas (sROIs) adapted to the size of the object. Then, the image is binarized (two possible values each pixel) using the algorithm *Otsu* to extract the profiles of all the forms (in this case the different tags of the lobsters) that are recognized in each sROIs. The recognized forms are compared by the moments extraction procedure (the geometric properties of each form) with a training library of images previously created using the tags presented in Figure 1; a form (tag) is associated to an object (lobster) when there is a positive match with one of the form of the training library otherwise the object is discarded. Finally, the centroid (position in a *xy* plan) of the object with the associated tag is recorded. The positions of the centroid is used to calculate the distance covered by the object between consecutive frames; in the case that there are no consecutive detections ofthe same object, the last detection is always considered as the last position of the object. The data are stored in a MySQL database and time series are created using a SQL script.

The final output of the behavioural tracking is a time series of movement (cm) for each lobster binned at 10 minutes intervals. The average movements were also calculated according to 4 different periods of the experiment: day (from 1 hour after light-ON to 1 hour before light-OFF), dusk (from 1 hour before to 1 hour after light-OFF), night (from 1 hour after light-OFF to 1 hour before light-ON), dawn (from 1 hour before to 1 hour after light-ON).

Sociomatrix treatment and statistics

Each socio-matrix was analysed to assess the formation and maintenance (during day 1, 3, 5) of a dominance hierarchy among the four lobsters using the normalized David's Score (nDS) (De Vries et al. 2006). Then, I evaluated the stability of the hierarchy calculating the value of its steepness. In a dominance hierarchy steepness is defined as the size of the absolute differences between adjacently ranked individuals in the overall success in winning dominance encounters (i.e. dominance success; De Vries et al. 2006). The dominance hierarchy was considered stabile whether after a randomization test with 10000 runs the steepness had a p value < 0.05. All matrix analysis was performed using the R Package "DyaDA" (Leiva et al. 2010).

Data treatment

Data were structured according to a *person-period* format to estimate the longitudinal models (see below) focusing on the following variables: (i) the steepness value during day 1, 3, and 5 (the value of steepness was eliminated from 2 groups at day 5 because of the presence of observational zeros into the matrix, in both cases one lobsters remain for the whole 24 hours of observation into the burrow); (ii) the average number of agonistic interactions was calculated during the different periods of the experiment (day, dawn, dusk, night) for day 1, 3 and 5; (iii) the average movement during the different periods of the experiment (day, dawn, dusk, night) for day 1, 3 and 5 (these data were Log transformed to smooth the effect of some outliers); (iv) The amount of time each lobster, identified by its rank, spent into the burrow during day 1, 3 and 5 (analogously to average movement, Log transformation was required when modelling this variable); (v) the numbers of times each lobster, identified by rank, succeeded in evict other lobsters; (vi) the numbers of time each lobster, identified by rank, has been evicted from the burrow.

Multilevel longitudinal models

I was interested in detecting developments or systematic changes in the behavior of lobsters during the formation and stabilization of the dominance hierarchy. For this reason I decided to use multilevel longitudinal models instead of a classical cross-sectional approach. I modeled average movements, burrow occupancy and evictions over time using three-level models: level 1 (the different days of observation: 1, 3 and 5); level 2 (the individuals: 1-4); level 3 (the groups: 1-8). Then, I modeled steepness and agonistic interactions using two-level models: level 1 (the different days of observation: 1, 3 and 5); level 2 (the groups: 1-8). In all cases the starting point was an unconditional means model from which subsequent models included growth terms (linear and quadratic) as well as time-varying predictors (normalized David's Scores, light period or ordinal rank). Table A5.1 describes the structure of all the models elaborated in the current study using the last version of the R packages "nlme" (Pinheiro et al. 2007) and "lmer" (Bates et al. 2013).

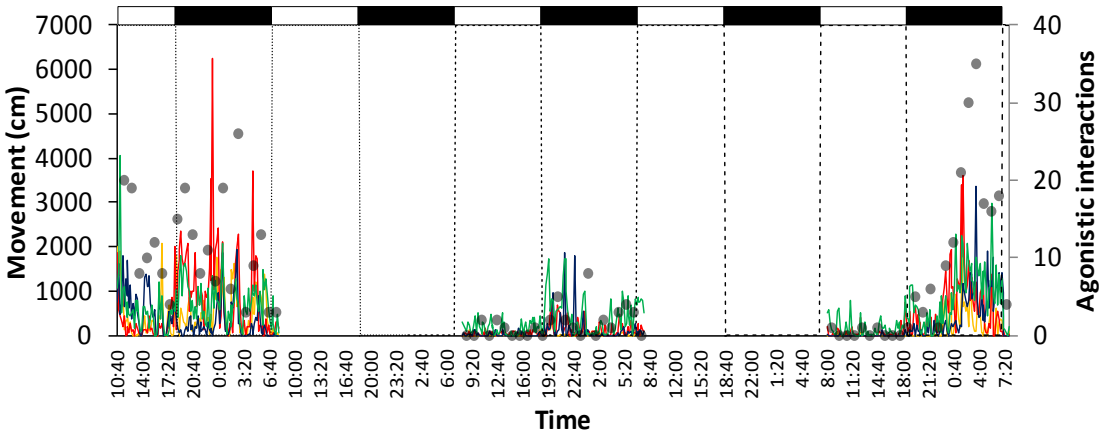


Fig. 5.2 – The locomotor activity and agonistic interactions of a representative group of 4 lobsters during day 1, 3 and 5. The locomotor activity out of the burrow is represented by the coloured lines and expressed as cm covered (binned at 10 m). The points represent the number of agonistic interactions and are binned at 1 h. White and black bars at the top represent Light and Darkness hours.

RESULTS

During this study I scored a total of 5013 dyadic agonistic interactions during which both members were out of the burrow. As an example I reported the data from one group where the locomotor activity at day 1, 3 and 5 is shown together with the number of agonistic interactions out of the burrow (Fig. 5.2). I also scored 385 agonistic interactions to get possession of burrows already occupied from other lobsters: in 198 cases the intruder failed to get possession of the burrow, while in 187 it succeeded.

The evolution of the average number of agonistic interactions for each of the 8 groups is shown in Figure 5.3 together with the values of steepness. A visual inspection of the graphs indicated a decreasing trend for the average agonistic interactions and an increasing trend for the steepness values. The dominance index of each lobster, expressed with the normalized David's Score, is reported in Figure 5.4 for each of the 8 groups (see also Table A5.2). The ranks of lobsters changed during the experiment (i.e. rank inversions) but looking into more details at Figure 5.3 the α lobster (the dominant) did not change its rank from day 3 to 5 (there is only one exception in group 3). The same is also true for the σ lobster (the lower into the dominance rank position).

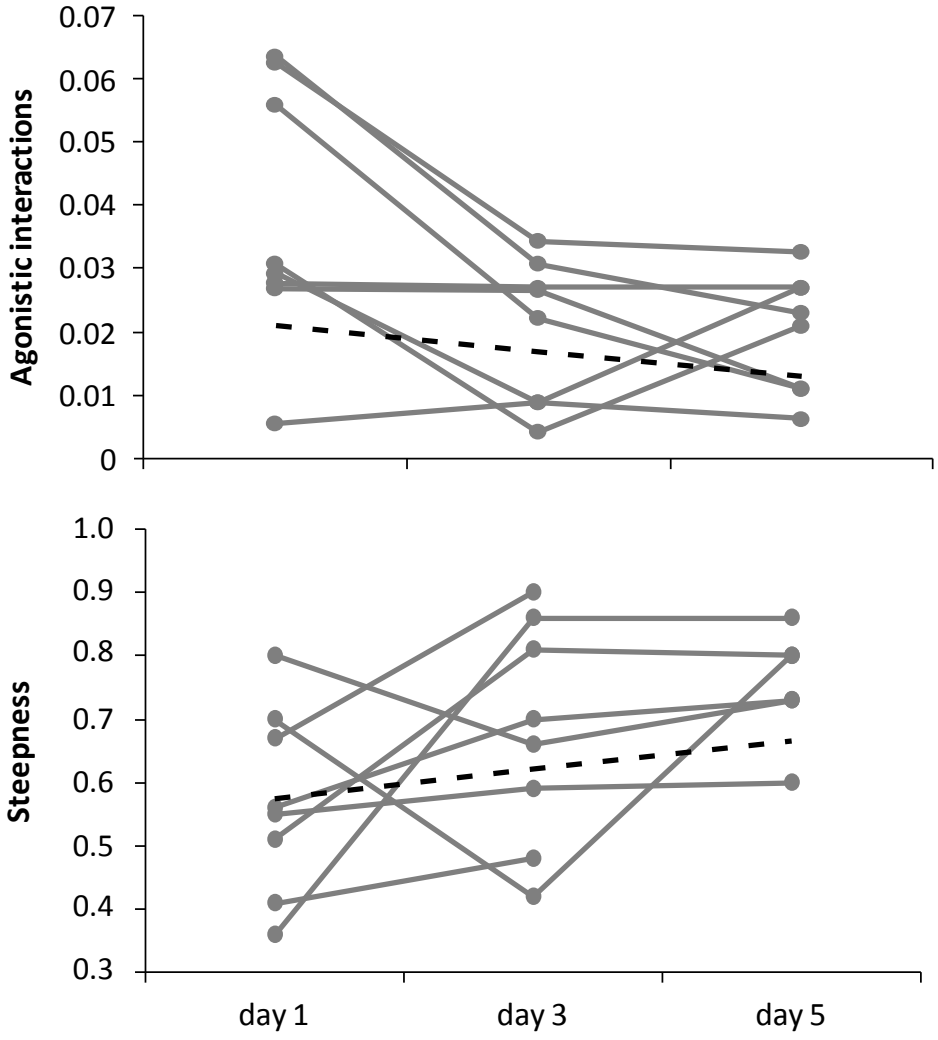


Fig. 5.3 – The evolution of the average number of agonistic interactions (up) and of the steepness values (down) throughout day 1, 3 and 5. Each grey line represents one of the eight groups analyzed in this study. The dashed black line represents the output of the model.

I observed a general nocturnal behaviour of the lobsters by visual inspections of time lapse videos (this is also supported by visual analysis of locomotor activity tracking outputs); they spent the great part of the day into their burrow, but with some exceptions (See Table A5.3 for more details). Some lobsters showed no interest in concealment into the burrow. Such observations gave us some cues on the possible effects of the dominance hierarchy on lobster's behaviour.

The evolution of the hierarchy in terms of steepness is explained by the model B (Table A5.4); the estimated coefficients for intercept (γ_{00}) was 0.575 ($p < 0.001$). The rate of change followed a positive linear trajectory (γ_{10} = 0.046; $p < 0.05$). It means that there is a positive

increment of 0.046 during the observations at level 1 (days). A graphical representation of the output of the variable is presented in Figure 5.3.

The evolution of the average number of agonistic interactions is described in relation to the results of the model D (Table A5.5 and Fig. 5.3); it is the more complex model that takes into account the time of the day (dusk, night, day, dawn) with a reasonable goodness of fit. The coefficients of intercept are significantly different (γ_{00} = 0.021; $p < 0.01$) and the rate of change followed a negative linear pattern (γ_{10} = 0.004; $p < 0.05$), but not a quadratic trajectory. There was a significant interaction with dusk (γ_{30} = 0.022; $p < 0.001$) and night (γ_{30} = 0.025; $p < 0.001$); differences among period of the experiment are calculated using the period day as a control. Take into account that the values reported are expressed as average number of interactions during the observational period (it justified the small values).

The results of the different models applied to the average movement of lobsters out of the burrow are presented in table A5.6. I have used the model E to explain the data, considering both the significance threshold of the predictors and the goodness of fit. Locomotor activity rates follow a significant quadratic trajectory. Estimated coefficients for intercept (γ_{000}), instantaneous rate of change (γ_{100}) and curvature (γ_{200}) were 5.662 ($p < 0.001$), -0.685 ($p < 0.001$) and 0.137 ($p < 0.001$), respectively. Thus, logarithm of the movement decreased from the initial value through the observations, then the decrement smoothed and then rose again (a simplified representation of the data is presented in Fig. 5.5). The normalized David's scores predicted the values of locomotor activity (γ_{300} = -0.186; $p < 0.10$); the higher is the rank the lower is the locomotor activity. As expected the locomotor activity is predicted by the period of the experiment, with significant values during night (γ_{400} = 1.121; $p < 0.001$), dusk (γ_{400} = 1.222; $p < 0.001$) and dawn (γ_{400} = 0.291; $p < 0.05$); remember that the period day is used as the reference category for the different comparisons. Model F indicated that there were no significant interactions between period of the experiment and David's score (i.e. rank), it means that there are not abrupt switches in the daily locomotor activity that can be explained by the rank of lobsters.

The burrow occupancy data indicated that the model D can be used to describe the evolution of the variable (Table A5.7). The Log of the rate of burrow occupancy followed a significant quadratic trajectory. Estimated coefficients for intercept (γ_{000}), instantaneous rate of change (γ_{100}) and curvature (γ_{200}) were 8.007 ($p > 0.10$), 0.383 ($p < 0.05$) and -0.056 ($p < 0.05$), respectively. There were no significant differences in the initial intercepts and the negative coefficient of the curvature indicated that occupancy increased from the initial value through the observations, the increment smoothed and then decreased again (the opposite of what observed for Log of movement; see Fig. 5.5). The rank of lobsters predicted the trajectory

of the variable (γ_{300} = -0.144; p < 0.10), it means that a decrease of a rank position corresponded to a small amount of time (-0.144) spent into the burrow.

The following two variables (tables A5.8 and A5.9) are count variables and followed a Poisson distribution; it means that the estimated coefficient reported in the tables represent the exponent of the mathematical constant e = 2.718. The relation between rank of the lobsters and success evicting other lobsters from the burrow is presented in Table A5.8. Model D is the one used to explain the data that did not follow either a linear or a quadratic trajectory. The estimated coefficient for intercept (γ_{000}) was 0.828 (p < 0.10). However, the rank strongly predicted the evolution of the variable (γ_{300} = -0.471; p < 0.001), it means that for a decrease of

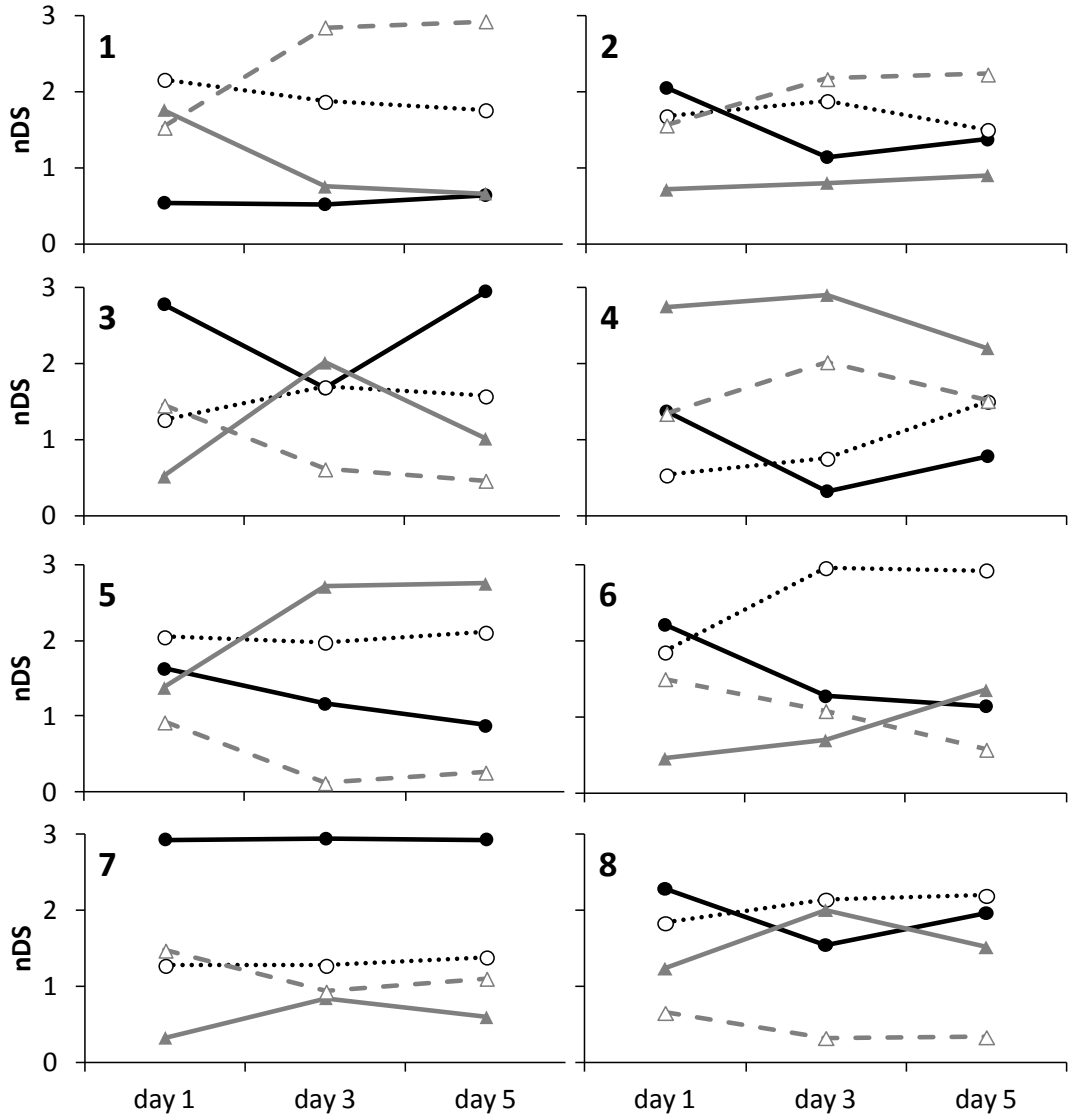


Fig. 5.4 – The evolution of the dominance index expressed with the normalized David's scores throughout day 1, 3 and 5 for each of the 8 groups analyzed in this study.

the rank position the number of evictions with a success abruptly decrease. Interestingly, the opposite was true observing the evolution of the evictions suffered by lobsters in relation to the rank (Table A5.9). In this case the data again did not follow either a linear or a quadratic trajectory. The estimated coefficient for intercept (γ_{000}) was -1.248 ($p < 0.05$) and the rank strongly predicted the evolution of the variable ($\gamma_{300} = 0.519$; $p < 0.001$), it means that for a decrease of the rank position the number of suffered evictions abruptly increase.

DISCUSSION

Here I described for the first time the formation and maintenance of a dominance hierarchy in *Nephrops norvegicus*. The stabilization of the hierarchy was described trough the evolution of its steepness. I have correlated the developments and rate of change of non-social (locomotor activity and burrow occupancy) and social (agonistic interactions and evictions) behaviours to the process of stabilization of the hierarchy. Burrowing behaviour and locomotor activity showed to be affected by the positions of lobsters into the dominance hierarchy, but without abrupt partitioning of daily locomotor activity out of the burrow among different ranks. The rank of lobsters strongly predicted the success of lobsters to evict conspecifics and the probability to be evicted.

The steepness increased following linear trajectory through observations, indicating the tendency of the system to reach stability that in this study is evaluated through the polarization of dominance ranks. This is also supported by the linear decrease of agonistic interactions throughout the experiment, indicating that the more the system is stable the less is the frequency of fights. Interestingly these two variables both followed a lineal trajectory but with opposite slopes. Previous studies have already demonstrated that when the dominance relationships in a group evolved towards stability they were parallel changes in others behaviours (e.g. Goessmann et al. 2000). However, in the great part of literature on decapod crustaceans the dominance hierarchy is characterized by its degree of linearity (e.g. Allee and Douglas 1945, Hazlett 1968, Cobb and Tamm 1975, Sastry and Ehinger 1980, Vannini and Gherardi 1981), although in small groups (less than 6) the probability to get significant results due to chances is very high and there are no available statistical test to check for the *p* values. Steepness is definitively the best option to characterize the hierarchy formation in small groups of animals (De Vries et al. 2006) and the available R package DyaDA make it easy to calculate (Leiva et al. 2010). In this study the steepness followed a linear trajectory, but an extension of the number of observations could lead to a more stable point of the hierarchy (higher values of steepness) and also allowed to model the dynamic of the systems with more suitable trajectories; It could be interesting to observe the moment in which steepness values reach a plateau. Finally, I observed

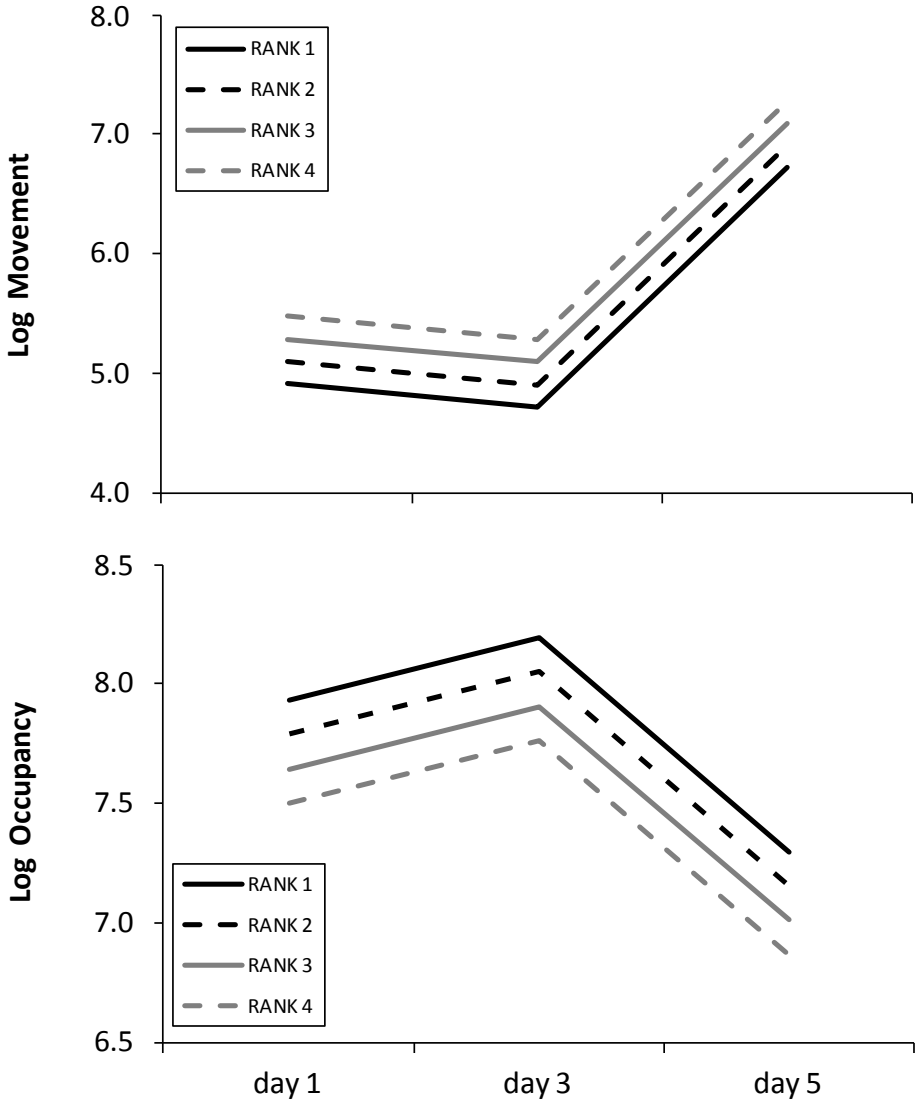


Fig. 5.5 – The evolution of the Log of the average movement (up) and occupancy of burrow (down) throughout day 1, 3 and 5. Each line represents the output of the model for the lobsters organized according to their rank as specified in the legend.

frequent rank reversals that seemed to not prevent the hierarchy to reach stability as already observed by (Oliveira and Almada 1996, Goessmann et al. 2000).

The model applied to describe the changes in locomotor activity of the lobster during the formation of the hierarchy indicated that there were different starting points (i.e. intercepts) and then the variable evolved in a quadratic way. The most interesting result is that the rank of the lobsters predicted their level of activity although the effect was not so strong ($p < 0.10$). As expected the activity during night and dusk was higher than day. Unfortunately the first part of my hypothesis must be rejected because the rank of lobsters did not predict switches in daily locomotor activity during the stabilization of the hierarchy (model F in Table A5.7). There are

no many studies that have studied biological rhythms of animals when they are organized in a dominance hierarchy. Bovet (1972) demonstrated that when a group of 4 long-tailed field mice (*Apodemus sylvaticus*) were co-housed for a long period of time, the dominant individual has a different daily activity pattern compared to the other 3 subordinates. Long term changes in daily rhythms were also observed when rats experienced subordination for one hour (Meerlo et al. 1999). Another example is provided by Hansen and Closs (2005) that studied the daily activity the giant kokopu (*Galaxias argenteus*) in group of 4 fish with a dominance relationships. Authors observed that the dominant fish (the biggest) shifted its daily activity from night to day in the presence of a food limited environment. Interestingly the closest subordinate (in terms of rank) was the more impacted individual into the hierarchy, indicating that directionality of aggression could have a great influence in the partitioning of daily activity. Unfortunately, the above mentioned studies did not properly characterize the properties of the hierarchy (e.g. steepness or linearity) giving just descriptive information on the relationships among individuals. There could be different reasons why I failed to observe a daily activity switch in locomotor activity. First of all the period of observation could have been too short and extending it could led to a higher stability of the system and maybe to a clear switch of daily activity. Secondly, it could be that *Nephrops,* despite showing a niche switching driven by light intensity (Chiesa et al. 2010), did not have the same circadian plasticity related to social interactions. Finally, the introduction of food as a limited resource could be more effective in triggering competition among lobsters and maybe a more evident switch in the daily activity related to rank position.

The model applied to describe the rate of burrow occupancy showed that the starting point is the same but then the variable followed a quadratic negative trajectory. As demonstrated for locomotor activity (see above) the rank of the lobsters predicted their level of burrow occupancy ($p < 0.10$). In this case the second part of my hypothesis is accepted because the burrow occupancy (imagined as a resource) is distributed according to the rank of the lobster. *Nephrops* has a strong burrowing habit and as suggested in chapter 3 it could make the difference at the moment to escape from a predator (or from a trawling net). So, it makes sense that the more the lobster is dominant the more it has access to burrows. Previous studies in the crayfish *Procambarus clarkii*, have already demonstrated that an increase in burrowing behavior marked the ascendancy of the dominant while an immediate suppression of burrowing paralleled the inhibition of aggressive behavior in the new subordinate (Herberholz et al. 2003). These results are important for the fishery management of *Nephrops*, because if the rank affect the time spent into the burrow, it could also have an impact on the vulnerability of lobsters to trawling.

In this study the lobsters were provided with one burrow each. So, in principle it was not a limited resource. However, as demonstrated by the results of the modeling in Tables A5.8

and A5.9 the rank strongly predicted the probability to evict of get evicted. So, the decrease of burrow occupancy is also explained by the parallel dynamic of evictions events. In summary: The α lobster (more dominant) spent more time into the burrow, evicted more and is less evicted. Then, these variables changed through observations with a rate described by their respective models. The lobster σ (the more subordinate) is the lobster that spent less time into the burrow, evicted less and is more evicted. Fero and Moore (2008) have already observed that dominant crayfish also performed significantly more shelter evictions during hierarchy formation, a behavior that seemed to be related to territorial behavior or competition for space which neighbors. In fact, burrow ownership is suggested to contribute to social status in crayfish (Gherardi and Daniels 2004). During time lapse observation I have also recorded the movements of lobsters among different burrows, but data are not presented here. Future studies will focus on the ownership of burrows with a focus on the mechanisms of space distribution dynamics in relation to rank.

After more than 40 years that Chapman and Rice (1971) observed for the first time fighting behaviour of *Nephrops* in the wild, I described the formation and maintenance of dominance hierarchies in this species. I observed ritualized fights as described in the laboratory by (Katoh et al. 2008). Although the observation in laboratory are the output of forced encounters in a delimited perimeter (the tank), I can hypothesized that the mechanisms described are fundamental properties of the organization of *Nephrops* society. As demonstrated for the American lobster (*Homarus americanus*), the frequency and level of agonistic interactions in wild or under more natural conditions are lower than in laboratory conditions, but they are fundamental for ecological purposes such as mating and space distribution (Stein et al. 1975, Atema et al. 1979, Karnofsky et al. 1989, Karnofsky and Price 1989, Atema and Voigt 1995, Atema and Steinbach 2007).

The data set produced during this chapter was rich of information and I struggled to filter the most valuable information to present them here. I gave attention to the results that were more reliable to connect my experiments to catchability patterns in the wild and then I focused on the study of parallel changes of social and non-social behavior to provide insights into the putative relationships of related neural mechanisms. Both these aspects are remarked in the following chapter where I draw the conclusions of my thesis.

ANNEX 5A

Table A5.1 – The summary of the different multi-level models estimated.

Variable	Model	Composite model	Description
Average movement (Mov)	Model A	$\log(Mov_{ijk}) = [\gamma_{000}] + [\zeta_{0jk} + \xi_{00k} + \epsilon_{ijk}]$	Unconditional means model
	Model B	$\log(Mov_{ijk}) = [\gamma_{000} + \gamma_{100}Day_{ijk}] + [\zeta_{0jk} + \xi_{00k} + (\zeta_{1jk} + \xi_{10k})Day_{ijk} + \epsilon_{ijk}]$	Unconditional growth model (linear)
	Model C	$\log(Mov_{ijk}) = [\gamma_{000} + \gamma_{100}Day_{ijk} + \gamma_{200}Day_{ijk}^2] + [\zeta_{0jk} + \xi_{00k} + (\zeta_{1jk} + \xi_{10k})Day_{ijk} + \epsilon_{ijk}]$	Unconditional growth model (quadratic)
	Model D	$\log(Mov_{ijk}) = [\gamma_{000} + \gamma_{100}Day_{ijk} + \gamma_{200}Day_{ijk}^2 + \gamma_{300}DS_{ijk}] + [\zeta_{0jk} + \xi_{00k} + (\zeta_{1jk} + \xi_{10k})Day_{ijk} + \epsilon_{ijk}]$	Time-varying predictor DS included
	Model E	$\log(Mov_{ijk}) = [\gamma_{000} + \gamma_{100}Day_{ijk} + \gamma_{200}Day_{ijk}^2 + \gamma_{300}DS_{ijk} + \gamma_{400}Light_{ijk}] + [\zeta_{0jk} + \xi_{00k} + (\zeta_{1jk} + \xi_{10k})Day_{ijk} + \epsilon_{ijk}]$	Time-varying Light included
	Model F	$\log(Mov_{ijk}) = [\gamma_{000} + \gamma_{100}Day_{ijk} + \gamma_{200}Day_{ijk}^2 + \gamma_{300}DS_{ijk} + \gamma_{400}Light_{ijk} + \gamma_{500}DS_{ijk} \times Light_{ijk}] + [\zeta_{0jk} + \xi_{00k} + (\zeta_{1jk} + \xi_{10k})Day_{ijk} + \epsilon_{ijk}]$	Interaction DS-Light included
Time in Burrows (TB)	Model A	$\log(TB_{ijk}) = [\gamma_{000}] + [\zeta_{0jk} + \xi_{00k} + \epsilon_{ijk}]$	Unconditional means model
	Model B	$\log(TB_{ijk}) = [\gamma_{000} + \gamma_{100}Day_{ijk}] + [\zeta_{0jk} + \xi_{00k} + (\zeta_{1jk} + \xi_{10k})Day_{ijk} + \epsilon_{ijk}]$	Unconditional growth model (linear)
	Model C	$\log(TB_{ijk}) = [\gamma_{000} + \gamma_{100}Day_{ijk} + \gamma_{200}Day_{ijk}^2] + [\zeta_{0jk} + \xi_{00k} + (\zeta_{1jk} + \xi_{10k})Day_{ijk} + \epsilon_{ijk}]$	Unconditional growth model (quadratic)
	Model D	$\log(TB_{ijk}) = [\gamma_{000} + \gamma_{100}Day_{ijk} + \gamma_{200}Day_{ijk}^2 + \gamma_{300}Rank_{ijk}] + [\zeta_{0jk} + \xi_{00k} + (\zeta_{1jk} + \xi_{10k})Day_{ijk} + \epsilon_{ijk}]$	Time-varying predictor Rank included
Steepness	Model A	$Steepness_{ij} = [\gamma_{00}] + [\zeta_{0j} + \epsilon_{ij}]$	Unconditional means model
	Model B	$Steepness_{ij} = [\gamma_{00} + \gamma_{10}Day_{ij}] + [\zeta_{0j} + \zeta_{1j}Day_{ij} + \epsilon_{ij}]$	Unconditional growth model (linear)
	Model C	$Steepness_{ij} = [\gamma_{00} + \gamma_{10}Day_{ij} + \gamma_{20}Day_{ij}^2] + [\zeta_{0j} + \zeta_{1j}Day_{ij} + \zeta_{2j}Day_{ij}^2 + \epsilon_{ij}]$	Unconditional growth model (quadratic)
Agonistic Interactions (AI)	Model A	$AI_{ij} = [\gamma_{00}] + [\zeta_{0j} + \epsilon_{ij}]$	Unconditional means model
	Model B	$AI_{ij} = [\gamma_{00} + \gamma_{10}Day_{ij}] + [\zeta_{0j} + \zeta_{1j}Day_{ij} + \epsilon_{ij}]$	Unconditional growth model (linear)
	Model C	$AI_{ij} = [\gamma_{00} + \gamma_{10}Day_{ij} + \gamma_{20}Day_{ij}^2] + [\zeta_{0j} + \zeta_{1j}Day_{ij} + \zeta_{2j}Day_{ij}^2 + \epsilon_{ij}]$	Unconditional growth model (quadratic)
	Model D	$AI_{ij} = [\gamma_{00} + \gamma_{10}Day_{ij} + \gamma_{30}Light_{ij}] + [\zeta_{0j} + \zeta_{1j}Day_{ij} + \epsilon_{ij}]$	Time-varying predictor Light included

Success in Evictions (SE)	Model A	$SE_{ijk} = [\gamma_{000}] + [\zeta_{0jk} + \xi_{00k} + \epsilon_{ijk}]$	Unconditional means model
	Model B	$SE_{ijk} = [\gamma_{000} + \gamma_{100}Day_{ijk}] + [\zeta_{0jk} + \xi_{00k} + \zeta 1jk + \xi 10kDayijk + \epsilon ijk$	Unconditional growth model (linear)
	Model C	$SE_{ijk} = [\gamma_{000} + \gamma_{100}Day_{ijk} + \gamma_{200}Day_{ijk}^2 + \gamma_{300}DS_{ijk}] + [\zeta_{0jk} + \xi_{00k} + (\zeta_{1jk} + \xi_{10k})Day_{ijk} + (\zeta_{2jk} + \xi_{20k})Day_{ijk}^2 + \epsilon_{ijk}]$	Unconditional growth model (quadratic)
	Model D	$SE_{ijk} = [\gamma_{000} + \gamma_{100}Day_{ijk} + \gamma_{300}Rank_{ijk}] + [\zeta_{0jk} + \xi_{00k} + (\zeta_{1jk} + \xi_{10k})Day_{ijk} + \epsilon_{ijk}]$	Time-varying predictor Rank included
Get Evicted (GE)	Model A	$GE_{ijk} = [\gamma_{000}] + [\zeta_{0jk} + \xi_{00k} + \epsilon_{ijk}]$	Unconditional means model
	Model B	$GE_{ijk} = [\gamma_{000} + \gamma_{100}Day_{ijk}] + [\zeta_{0jk} + \xi_{00k} + \zeta 1jk + \xi 10kDayijk + \epsilon ijk$	Unconditional growth model (linear)
	Model C	$GE_{ijk} = [\gamma_{000} + \gamma_{100}Day_{ijk} + \gamma_{200}Day_{ijk}^2 + \gamma_{300}DS_{ijk}] + [\zeta_{0jk} + \xi_{00k} + (\zeta_{1jk} + \xi_{10k})Day_{ijk} + (\zeta_{2jk} + \xi_{20k})Day_{ijk}^2 + \epsilon_{ijk}]$	Unconditional growth model (quadratic)
	Model D	$GE_{ijk} = [\gamma_{000} + \gamma_{100}Day_{ijk} + \gamma_{300}Rank_{ijk}] + [\zeta_{0jk} + \xi_{00k} + (\zeta_{1jk} + \xi_{10k})Day_{ijk} + \epsilon_{ijk}]$	Time-varying predictor Rank included

Table A5.2 – The evolution of the dominance hierarchy in each of the eight groups throughout day 1, 3 and 5. The position of the lobsters i ranks (**Rank**) and also with cardinal rank (**Normalized David's score**). Then, the steepness of the hierarchy is reported for each day toget used to evidenced when the p value is < 0.05: ** p value < 0.01; *** p value < 0.001.

Group	day	Rank				Normalized David score				St
1	1	α	β	γ	δ	2.16	1.76	1.53	0.54	
	3	β	γ	α	δ	1.87	0.75	2.85	0.52	0
	5	β	γ	α	δ	1.76	0.66	2.93	0.64	0
2	1	α	β	γ	δ	2.05	1.68	1.56	0.71	
	3	γ	β	α	δ	1.14	1.88	2.17	0.80	
	5	γ	β	α	δ	1.37	1.50	2.23	0.90	
3	1	α	β	γ	δ	2.78	1.45	1.26	0.51	
	3	γ	δ	β	α	1.67	0.61	1.69	2.01	
	5	α	δ	β	γ	2.95	0.46	1.57	1.01	0
4	1	α	β	γ	δ	2.75	1.37	1.34	0.53	
	3	α	δ	β	γ	2.90	0.32	2.02	0.75	0
	5	α	δ	β	γ	2.20	0.78	1.51	1.50	
5	1	α	β	γ	δ	2.05	1.63	1.38	0.93	
	3	β	γ	α	δ	1.98	1.17	2.71	0.13	0
	5	β	γ	α	δ	2.11	0.88	2.75	0.27	0
6	1	α	β	γ	δ	2.21	1.85	1.50	0.45	
	3	β	α	γ	δ	1.27	2.96	1.08	0.69	
	5	γ	α	δ	β	1.14	2.93	0.57	1.35	0
7	1	α	β	γ	δ	2.93	1.47	1.27	0.32	0
	3	α	γ	β	δ	2.94	0.93	1.27	0.84	
	5	α	γ	β	δ	2.93	1.10	1.38	0.59	
8	1	α	β	γ	δ	2.28	1.83	1.23	0.65	
	3	γ	α	β	δ	1.54	2.14	2.00	0.32	
	5	β	α	γ	δ	1.96	2.19	1.51	0.33	0

	δ	17.23	35.43	34.51
3	α	71.53	95.71	86.16
	β	0.00	94.18	80.49
	γ	62.67	95.82	75.28
	δ	49.66	2.81	0.20
4	α	75.64	99.42	97.79
	β	0.00	79.35	66.13
	γ	20.98	19.41	99.99
	δ	22.57	18.80	38.80
5	α	68.56	99.76	99.97
	β	32.59	90.50	66.37
	γ	59.35	38.46	99.46
	δ	42.07	3.92	7.06
6	α	19.83	71.55	77.59
	β	64.50	44.33	92.12
	γ	26.91	71.94	49.88
	δ	51.17	80.20	52.07
7	α	69.29	89.18	93.61
	β	2.75	85.07	90.61
	γ	17.96	19.17	74.05
	δ	9.83	2.59	0.47
8	α	83.98	98.51	99.41
	β	80.84	99.12	93.59
	γ	83.14	92.64	98.90
	δ	40.08	66.25	11.84

Table A5.4 - The results of the different models for the steepness values. Model A: unconditional means model; Model B: unconditional growth model; Model C: non-linear unconditional growth model. † $p < 0.10$; * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$. The values between parentheses represent the error. AIC represents the Akaike information criterion; BIC represents the Bayesian information criterion.

		Parameter	Model A	Model B	Model C
Fixed effects					
Initial status	Intercept	γ_{00}	0,659*** (0,034)	0,575*** (0,046)	0,570** (0,052)
Rate of change					
	Experiment (linear)	γ_{10}		0,046* (0,019)	0,061 (0,076)
	Experiment (quadratic)	γ_{20}			-0,004 (0,017)
Variance component					
Level 1	Within-subject	σ_ϵ^2	0.025	0	
Level 2	Initial status	σ_0^2	0	0	
	Experiment (linear)	σ_1^2		0	
	Experiment (quadratic)	σ_0^2			
	Covariance Initial-Experiment (linear)	σ_{01}		0	
	Covariance Initial-Experiment (quadratic)	σ_{01}			
	Covariance linear-quadratic terms	σ_{12}			
Goodness-of-fit					
	Deviance		-15	-14	-11
	AIC		-9	-2	9
	BIC		-6	4	19

Table A5.5 - The results of the different models for number of agonistic interactions. Model A: unconditional means model; Model B: unconditional growth model; Model C: non-linear unconditional growth model; Model D: Period as a time-varying predictor included. † $p < 0.10$; * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$. The values between parentheses represent the error. AIC represents the Akaike information criterion; BIC represents the Bayesian information criterion.

		Parameter	Model A	Model B	Model C	Model D
Fixed effects						
Initial status	Intercept	γ_{00}	0,025*** (0,003)	0,033*** (0,006)	0,034*** (0,006)	0,021** (0,006)
Rate of change						
	Experiment (linear)	γ_{10}		-0,004* (0,002)	-0,008† (0,005)	-0,004* (0,002)
	Experiment (quadratic)	γ_{20}			0,001 (0,001)	
	Dusk	γ_{30}				0,022*** (0,005)
	Night	γ_{30}				0,025*** (0,005)
	Dawn	γ_{30}				0,001 (0,005)
Variance component						
Level 1	Within-subject	σ_{ϵ}^2	0	0	0	0
Level 2	Initial status	σ_0^2	0	0	0	0
	Experiment (linear)	σ_1^2		0	0	0
	Experiment (quadratic)	σ_2^2		0	0	0
	Covariance Initial-Experiment (linear)	σ_{01}			0	0
	Covariance Initial-Experiment (quadratic)	σ_{02}			0	
	Covariance linear-quadratic terms	σ_{12}			0	
Goodness-of-fit						
	Deviance		-449	-448	-437	-458
	AIC		-443	-436	-417	-440
	BIC		-435	-421	-392	-418

Table A5.6 – The results of the different models for the Log of the average movement of lobsters out of the burrow. Model A: unconditional means model; Model B: unconditional growth model; Model C: non-linear unconditional growth model; Model D: David's scores as time-varying predictor included; Model E: light condition as time-varying predictor included; Model F: interaction between time-varying predictors included. † $p < 0.10$; * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$. The values between parentheses represent the error. AIC represents the Akaike information criterion; BIC represents the Bayesian information criterion.

		Parameter	Model A	Model B	Model C	Model D	Model E	Model F
Fixed effects								
Initial status	Intercept	γ_{000}	5.589*** (0.266)	5.862** (0.299)	6.042*** (0.304)	6.330*** (0.357)	5.662*** (0.356)	5.801*** (0.390)
Rate of change								
	Experiment (linear)	γ_{100}		-0.137** (0.043)	-0.681*** (0.125)	-0.682** (0.125)	-0.685*** (0.109)	-0.685*** (0.110)
	Experiment (quadratic)	γ_{200}			0.136*** (0.029)	0.136* (0.029)	0.137* (0.025)	0.137* (0.025)
	David's scores	γ_{300}				-0.193 (0.124)	-0.186† (0.112)	-0.279† (0.155)
	Dusk	γ_{400}					1.121*** (1.121)	0.959** (0.293)
	Night	γ_{400}					1.222*** (0.134)	0.988*** (0.293)
	Dawn	γ_{400}					0.291* (0.136)	0.132 (0.296)
	David's scores x Dusk	γ_{500}						0.108 (0.174)
	David's scores x Night	γ_{500}						0.157 (0.174)
	David's scores x Dawn	γ_{500}						0.106 (0.175)
Variance component								
Level 1	Within-subject	σ_ϵ^2	1.298	1.23	1.159	1.164	0.861	0.867
Level 2	Initial status	σ_0^2	0.61	0.51	0.514	0.581	0.605	0.604
	Experiment (linear)	σ_1^2		0.002	0.002	0	0	0
	Covariance Initial-Experiment (linear)	σ_{01}		0.001	0.001	0	0	0
Level 3	Initial status	σ_{k0}^2	0.388	0.525	0.537	0.526	0.526	0.531
	Experiment (linear)	σ_{k1}^2		0.005	0.005	0.006	0.006	0.008
	Covariance Initial-Experiment (linear)	σ_{k01}		-0.002	-0.002	-0.002	-0.002	-0.002
Goodness-of-fit								
	Deviance		1247	1235	1219	1220	1122	1127
	AIC		1255	1253	1239	1242	1150	1161
	BIC		1271	1288	1278	1285	1205	1228

Table A5.7 – The results of the different models for the amount of time each lobster spent into the burrow. Model A: unconditional means model; Model B: unconditional growth model; Model C: non-linear unconditional growth model; Model D: Rank as time-varying predictor included. † $p < 0.10$; * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$. The values between parentheses represent the error. AIC represents the Akaike information criterion; BIC represents the Bayesian information criterion.

		Parameter	Model A	Model B	Model C	Model D
Fixed effects						
Initial status	Intercept	γ_{000}	8.187*** (0.183)	8.069*** (0.224)	7.682*** (0.271)	8.077 (0.345)
Rate of change						
	Experiment (linear)	γ_{100}		0.046† (0.068)	0.402* (0.156)	0.383* (0.159)
	Experiment (quadratic)	γ_{200}			-0.059* (0.023)	-0.056* (0.024)
	Rank	γ_{300}				-0.144† (0.078)
Variance component						
Level 1	Within-subject	σ_ϵ^2	0.502	0.211	0.179	0.188
Level 2	Initial status	σ_0^2	0.904	0.396	0.452	0.444
	Experiment (linear)	σ_1^2		0.091	0.091	0.093
	Covariance Initial-Experiment (linear)	σ_{01}		-0.069	-0.075	-0.101
Level 3	Initial status	σ_{k0}^2	0	0.218	0.216	0.226
	Experiment (linear)	σ_{k1}^2		0.008	0.008	0.008
	Covariance Initial-Experiment (linear)	σ_{k01}		-0.04	-0.04	-0.041
Goodness-of-fit						
	Deviance		261	240	240	240
	AIC		269	258	260	262
	BIC		279	281	285	290

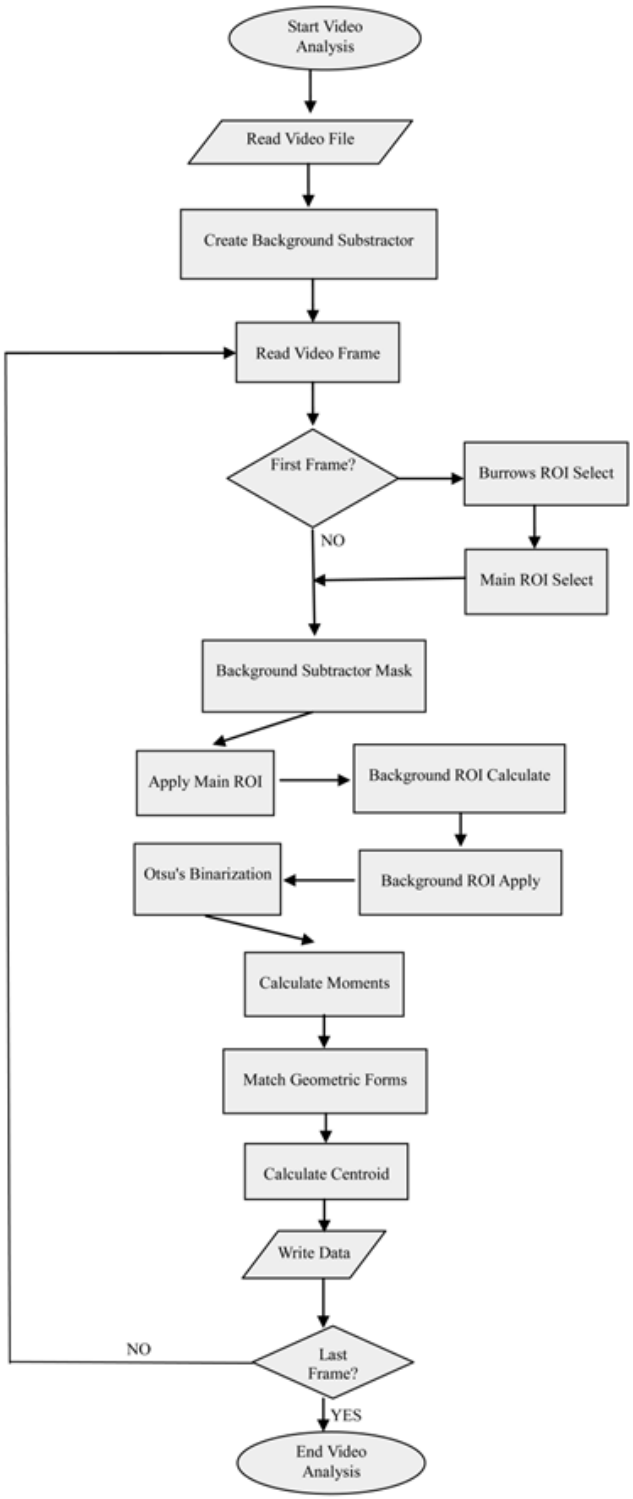
Table A5.8 - The results of the different models for the numbers of times each lobster, identified by rank, succeeded in evict other lobster from the burrow. Model A: unconditional means model; Model B: unconditional growth model; Model C: non-linear unconditional growth model; Model D: Rank as time-varying predictor included. σ_ϵ^2 is not reported by glmer. † $p < 0.10$; * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$. The values between parentheses represent the error. AIC represents the Akaike information criterion; BIC represents the Bayesian information criterion.

		Parameter	Model A	Model B	Model C	Model D
Fixed effects Initial status Rate of change	Intercept	γ_{000}	-0,358 (0,322)	-0,216 (0,362)	-0,263 (0,390)	0,828† (0,434)
	Experiment (linear)	γ_{100}		-0,184 (0,148)	-0,694 (0,648)	-0,064 (0,134)
	Experiment (quadratic)	γ_{200}			0,143 (0,161)	
	Rank	γ_{300}				-0,471*** (0,118)
Variance component						
Level 1	Within-subject	σ_ϵ^2				
Level 2	Initial status	σ_0^2	2.262	1.765	1.739	1.543
	Experiment (linear)	σ_1^2		0.154	1.47	0.039
	Covariance Initial-Experiment (linear)	σ_{01}^2		-0.002	-0.07	0.003
	Experiment (quadratic)	σ_2^2			0.052	
	Covariance Initial-Experiment (quadratic)	σ_{02}^2			0.016	
	Covariance linear-quadratic terms	σ_{12}^2			-0.277	
Level 3	Initial status	σ_{k0}^2	0	0.103	0.147	0.184
	Experiment (linear)	σ_{k1}^2		0.016	0.731	0.029
	Covariance Initial-Experiment (linear)	σ_{k01}^2		-0.04	-0.162	-0.074
	Experiment (quadratic)	σ_{k2}^2			0.055	
	Covariance Initial-Experiment (quadratic)	σ_{k02}^2			0.034	
	Covariance linear-quadratic terms	σ_{k12}^2			-0.199	
Goodness-of-fit						
	Deviance		351	323	306	307
	AIC		357	339	336	325
	BIC		365	359	374	348

Table A5.9 - The results of the different models for the numbers of times each lobster, identified by rank, has been evicted from the burrow. Model A: unconditional means model; Model B: unconditional growth model; Model C: non-linear unconditional growth model; Model D: Rank as time-varying predictor included. σ_ϵ^2 is not reported by glmer. † $p < 0.10$; * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$. The values between parentheses represent the error. AIC represents the Akaike information criterion; BIC represents the Bayesian information criterion.

		Parameter	Model A	Model B	Model C	Model D
Fixed effects Initial status Rate of change	intercept	γ000	0,188*** (0,002)	0,149 (0,321)	-0,126 (0,447)	-1,248* (0,508)
	Experiment (linear)	γ100		-0,076 (0,136)	-0,014 (0,528)	-0,044 (0,124)
	Experiment (quadratic)	γ200			0 (0,124)	
	Rank	γ300				0,519*** (0,134)
Variance component						
Level 1	Within-subject	σ_ϵ^2				
Level 2	Initial status	σ_0^2	1.969	1.435	1.75	1.281
	Experiment (linear)	σ_1^2		0.136	0.836	0.095
	Covariance Initial-Experiment (linear)	σ_{01}^2		-0.001	-0.117	0.035
	Experiment (quadratic)	σ_2^2			0.017	
	Covariance Initial-Experiment (quadratic)	σ_{02}^2			-0.017	
	Covariance linear-quadratic terms	σ_{12}^2			-0.119	
Level 3	Initial status	σ_{k0}^2	0	0.087	0.372	0.083
	Experiment (linear)	σ_{k1}^2		0.036	0.871	0.028
	Covariance Initial-Experiment (linear)	σ_{k01}^2		-0.056	-0.557	-0.048
	Experiment (quadratic)	σ_{k2}^2			0.056	
	Covariance Initial-Experiment (quadratic)	σ_{k02}^2			0.133	
	Covariance linear-quadratic terms	σ_{k12}^2			-0.217	
Goodness-of-fit						
	Deviance		428	386	367	369
	AIC		434	402	397	387
	BIC		442	422	435	410

Fig. A1 – The different logic steps of the algorithm



Conclusions

6

METHODOLOGIAL APPROACH

The methodological approach presented in chapter 2 has shown to be suitable for experimental biologists. The video imaging analysis has the advantage that once the frames are acquired they can be further re-processed changing parameters such as algorithm sensibility and different location of the region of interest. This allows studying different aspects of *Nephrops*' behavior such as activity at the burrow entrance and exploratory activity out of the burrow.

In addition, flume tanks have been already used to investigate different ecological aspects of decapods crustaceans: larval settlement, swimming behavior, habitat selection, and response to odor plumes. These aspects are not fully understood in the Norway lobster but they could be in the next future, and the system presented here might be pivotal for these discoveries.

In chapter 5 I have used a different methodological approach for video imaging analysis and for controlling experimental variables. Open source easy-to-use hardware and software are developed for anyone making interactive projects. I strongly believed that this kind of approach will have dramatic potentialities in the experimental biology labs in the next future, for two basic reasons: It is usually a very cheap technology or even free; the open source philosophy is that the software can be modified changing the code itself, and finally, the most important feature, it can be shared among people and researchers.

CHAPTER 3

Fishery implications

The results presented in this chapter suggested that the phase of the diurnal or semi-diurnal tides is important parameters to take in account at the moment to interpret *Nephrops* fishery-dependent and underwater television surveys data. The presence of currents can strongly inhibit the burrow emergence behaviour with the possibility to create important errors at the moment to evaluate the state of the resource. These findings are not only important for *Nephrops*, but in general give an interesting example for deep-water benthic community.

General Biology

I have shown that periodic water currents simulated in the laboratory can modulate the behavioural rhythm of a deep water species. Currents were able not only to mask but also to modify the phase of the circadian system output, suggesting their partial role as zeitgeber at depths where the sunlight is still present. Such results are promising for future research to understand how the organization of circadian biology changes from shallow to deep water.

CHAPTER 4

Fishery implications

This chapter did not provide information with a direct interest for fishery management, but will allow researchers to exploit the assembled transcriptome for different purposes concerning *Nephrops* biology and ecology. However, future investigations on the molecular mechanisms of circadian clock of *Nephrops* could lead to a more precise understanding of population dynamics and niche switching by light intensity with more direct application for stock assessment plans.

General Biology

I identified several putative clock genes in *Nephrops*. The finding that *timeless* is the only oscillating transcript for *Nephrops norvegicus* (at least in eyestalk) is consistent with the current knowledge on crustaceans' circadian clocks, suggesting that the molecular clockwork of this group of arthropods may differ from that in *Drosophila*. This is also reinforced by the identification of a *Nephrops* homolog of the vertebrate-like *cryptochrome*. The results presented here, although preliminary, could become the basis for future research aimed at elucidating the crustacean molecular clockwork, with a particular emphasis on decapod crustaceans in the deep-water marine environment (Aguzzi and Company 2010).

CHAPTER 5

Fishery implications

I demonstrated that *Nephrops* can form steep and stable dominance hierarchies. This aspect could open a new scenario into the aspects controlling its burrowing behavior and hence catchability. From the data presented here, the probability to be caught by trawling increased with a decrease in rank position (lower rank spent more time out of the burrow). However these results must be interpreted with caution because the dynamic of burrow occupancy in a close tank could be different respect to the wild where lobsters are free to change burrows, areas or even build a new burrow.

General Biology

I observed a parallel development of both agonistic and non-agonistic behaviors with a clear predictive value of the dominance rank. Herberholz et al. (2003) has previously documented the: "*the strikingly similar changes in both agonistic and non-agonistic behaviors following the decision on rank status...*". Here, I went deeper, in fact I characterized and quantified the rate of change throughout the development of the two categories of behaviors and I also determined the rate of

change associated to the rank. My data reinforced the hypothesis by Herberholz et al. (2003) supporting the idea of common neural mechanisms for the categories of behaviors observed in this study.

GENERAL OVERVIEW AND FUTURE DIRECTIONS

Results presented in this thesis added important information to the general knowledge of *Nephrops* rhythmic emergence behavior. I have demonstrated in laboratory that *Nephrops* burrowing behavior is affected by periodical water currents. Moreover, I provided the first transcriptomics database and preliminary insight on the genetic mechanism governing circadian locomotor activity rhythms. Finally, I showed how the organization of N*ephrops* society modulates the burrowing behavior. However, *Nephrops* is just one of the species belonging to the benthic community that as showed by (Aguzzi et al. 2015) has a clear coupling with the benthopelagic compartment. The result in physical terms is a complex network system of numerous circadian oscillators (individuals) organized in clusters (species) that interact in a rhythmic, complex and hierarchical way to finally produce a harmonious synchronized dynamic whose functioning mechanisms are unknown. The discovery of the underpinning mechanisms of such complex systems will increase the understanding of the deep water ecosystems and increase the efficiency of the renewable resources management plans. Finally, as stated by Bloch et al. (2013): *"if cells in circuits and animals in communities share some network design principles for synchronizing their circadian oscillators, then insights made at one level may inform understanding at the other"*.

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UNKNOWLEDGMENTS & SCIENTIFIC PRODUCTION

In this last section I would like to report the list of all the collaborators that helped me during the work on each chapter. Furthermore, I would like to provide a brief list of the scientific production published in the context of each chapter.

Chapter 1 – INTRODUCTION

Collaborators

Thomas Breithaupt (University of Hull, UK)

Scientific production

E. Katoh, **V. Sbragaglia**, J. Aguzzi, T. Breithaupt (2013). Sensory biology and behaviour of *Nephrops norvegicus*. In: Johnson M., Johnson M. (eds), The ecology and biology of *Nephrops norvegicus*. *Advances in Marine Biology* 64: 65-106.

Chapter 2 – FLUME TANK

Collaborators

Francesc Sardà, José Antonio García, Marta Vilaró (ICM-CSIC, Spain)

David Sarriá, Spartacus Gomariz, Antoni Manuel (UPC, Spain)

Corrado Costa and Paolo Menesatti (CRA-ING, Italy)

Scientific production

V. Sbragaglia, J. Aguzzi, J.A. García, D. Sarriá, S. Gomariz, C. Costa, P. Menesatti, M. Vilaró, A. Manuel, F. Sardà (2013). An automated multi-flume actograph for the study of behavioral rhythms of burrowing organisms. *Journal of Experimental Marine Biology and Ecology* 446: 177-185.

D. Sarriá, **V. Sbragaglia**, S. Gomáriz, J.A. García, C. Artero, J. Aguzzi, F. Sardà, A. Mànuel. Light and current generation system for measuring the behaviour of the Norway lobster. *Measurement* 69:180-188

- 2 posters and 1 oral communication

Chapter 3 – RESPONSE TO WATER CURRENTS

Collaborators

Francesc Sardà, José Antonio García, Marta Vilaró (ICM-CSIC)

David Sarriá, Spartacus Gomariz, Antoni Manuel (UPC)

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V. Sbragaglia, J.A. García, J. Aguzzi. The effect of simulated tidal currents on burrow emergence rhythms of the Norway lobster (*Nephrops norvegicus*, L.). *Marine Biology (addressing reviewers comments)*

- 1 poster and 2 oral communications

Chapter 4 – CLOCK GENES DAILY PATTERN

Collaborators

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V. Sbragaglia, F. Lamanna, A.M. Mat, G. Rotllant, S. Joly, V. Ketmaier, H.O. de la Iglesia, J. Aguzzi. Identification, characterization, and diel pattern of expression of canonical clock genes in *Nephrops norvegicus* (Crustacea: Decapoda) eyestalk. *Plos One (addressing reviewers comments)*

- 1 poster

Chapter 5 – DOMINANCE HIERARCHIES

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Thomas Breithaupt (University of Hull, UK)

José Antonio García, Anna Arias (ICM-CSIC, Spain)

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J. Aguzzi, **V. Sbragaglia**, D. Sarriá, J.A. García, C. Costa, J. Río, A. Mànuel, P. Menesatti, F. Sardà (2011). A new laboratory radio frequency identification (RFID) system for behavioural tracking of marine organisms. *Sensors* 9532-9548.

V. Sbragaglia, D. Leiva, J.A. García, A. Arias, J. Aguzzi, T. Breithaupt. The formation and maintenance of dominance hierarchies in *Nephrops norvegicus* (Crustacea: Decapoda): does it influence the daily burrow emergence rhythm? *(in preparation)*

- 1 poster 1 oral communication

Chapter 6 – CONCLUSIONS

J. Aguzzi, **V. Sbragaglia**, S. Tecchio, J. Navarro, J.B. Company. Rhythmic behaviour of marine benthopelagic species and the synchronous dynamics of benthic communities. *Deep-Sea Research I* 95:1-11.