



# Uncovering deep mysteries: The underwater life of an amphibious louse



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## ARTICLE INFO

### Article history:

Received 1 August 2014

Received in revised form 30 October 2014

Accepted 31 October 2014

Available online 7 November 2014

### Keywords:

Insects

Diving

Ectoparasites

## ABSTRACT

Despite the incredible success of insects in colonizing almost every habitat, they remain virtually absent in one major environment – the open sea. A variety of hypotheses have been raised to explain why just a few insect species are present in the ocean, but none of them appears to be fully explanatory. Lice belonging to the family Echinophthiriidae are ectoparasites on different species of pinnipeds and river otters, i.e. they have amphibious hosts, who regularly perform long excursions into the open sea reaching depths of hundreds of meters (thousands of feet). Consequently, lice must be able to support not only changes in their surrounding media, but also extreme variations in hydrostatic pressure as well as breathing in a low oxygen atmosphere. In order to shed some light on the way lice can survive during the diving excursions of their hosts, we have performed a series of experiments to test the survival capability of different instars of *Antarctophthirus microchir* (Phthiraptera: Anoplura) from South American sea lions *Otaria flavescens*, when submerged into seawater. These experiments were aimed at analyzing: (a) immersion tolerance along the louse life; (b) lice's ability to obtain oxygen from seawater; (c) physiological responses and mechanisms involved in survival underwater. Our experiments showed that the forms present in non-diving pups – i.e. eggs and first-instar nymphs – were unable to tolerate immersion in water, while following instars and adults, all usually found in diving hosts, supported it very well. Furthermore, as long as the level of oxygen dissolved in water was higher, the lice survival capability underwater increased, and the recovery period after returning to air declined. These results are discussed in relation to host ecology, host exploitation and lice functional morphology.

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## 1. Introduction

Insects are the most evolutionarily and ecologically successful group of living animals, being present in virtually all possible habitats (Bradley et al., 2009). Despite this, few species have colonized the ocean and the vast majority of marine insects live closely associated with the sea surface, salt marshes, estuaries, or shallow waters (Cheng, 1976). Much has been argued about the reasons why insects have not successfully colonized the ocean despite their success in land (Ruxton and Humphries, 2008).

Maddrell (1998) postulated that insects failed in colonizing the marine realm because they would be poor competitors. Accordingly, crustaceans have successfully evolved thanks to the development of strategies to avoid predators, such as transparency, and on this point, insects would have failed. Besides, this author suggested

that the insect tracheal system would not be able to support elevated hydrostatic pressure and, in consequence, insects could not survive beyond the first tens of meters of the water column (Maddrell, 1998, and references therein). Yet, some insects manage to survive underwater at great depths and during long immersion periods, e.g. lice.

Sucking lice (Phthiraptera: Anoplura) are obligatory hematophagous insects and permanent ectoparasites of mammals, living into the fur or among the hairs of their hosts. Among sucking lice, those belonging to the family Echinophthiriidae are peculiar in the sense that they infect amphibious hosts, such as pinnipeds (sea lions, walruses, true seals) and river otters (Durden and Musser, 1994). As a consequence, echinophthiriids must be capable of dealing with the challenges of the marine realm. Along the evolutionary time echinophthiriids have developed unique morphological adaptations to cope with the amphibious lifestyle of their hosts. All species possess prehensile tibio-tarsal claws in the second and third pairs of legs adapted to firmly grasping onto hairs and abdominal spiracles with a specialized closing device that preserves atmospheric air and prevents water entering the body during the host's

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immersions. Their membranous abdomen has been proposed as a possible surface for gas exchange underwater (Kim, 1975).

Pinnipeds are also diving animals, i.e. most sea lions, fur seals, and phocids usually dive at depths of 150–200 meters (Stewart, 2009). Some species of pinnipeds make deeper and longer dives to reach their prey, e.g. adult southern elephant seal has been recorded to dive up to 1468 meters (Campagna et al., 1999). Moreover, pinnipeds can have their bodies submerged from several weeks to several months (Teilmann et al., 1999; Stewart, 2009). Therefore, during the evolutionary transition of pinnipeds from land to the ocean, echinophthiriids lice have managed to continue exploiting these hosts, some of which may spend more than 80% of the time swimming and performing extreme dives.

The question about how do echinophthiriids survive in deep seawaters remains fully open, albeit the subject has been matter of speculation since more than a century (Enderlein, 1906; Scherf, 1963). Thus, even though there has been collected many data on the biology of pinnipeds and on some aspects of the ecological association with their parasitic partners (see Leonardi and Palma, 2013), the diving physiology of these lice remains unexplained. Considering the above stated, this work is aimed at describing the response of *Antarctophthirus microchir* from South American sea lions, *Otaria flavescens* to immersion, and examining the underwater survival of lice submitted to different conditions of temperature and oxygen in water.

## 2. Materials and methods

### 2.1. Lice

The samples were taken in Punta León rookery (43°04'S, 64°29'W) during the breeding season 2010/11. Nits and lice were collected from *O. flavescens* pups, which were captured with a noose pole and restrained by two people. A third person collected the lice using a fine-tooth comb commonly used for treating human pediculosis (for details see Leonardi, 2014).

Lice were taken from the field to the laboratory immersed in seawater. In order to verify the viability of lice, they were taken out of water and exposed to air at room temperature (ca. 25 °C/77 °F). Those individuals who responded to this change moving their legs and/or antennae were selected for experiments. Eggs were transported separately in plastic tubes. The experiments were carried out no longer than 3 h after the collection.

Different instars and experimental conditions are depicted in Fig. 1. Eggs are usually laid on the back of pups that do not go into the sea. Those of other echinophthiriid louse species do not survive immersion (Murray and Nicholls, 1965). Pups can get wet during high tides, but their back usually dries fast (Leonardi et al., 2012a). First-instar nymphs (N1) usually occupy the back of the host after hatching and slowly migrate to the belly; the number decreases when pups start swimming (Aznar et al., 2009). N1 are the only instar lacking of scales over their body. Second – (N2) and third-instar nymphs (N3) and adults are found together in the ventral side of the host body, even at ages when hosts start

swimming (Leonardi et al., 2012a). So, three categories of experimental subjects were established according to their association with the host: eggs, N1 and N2 + N3 + Adults.

### 2.2. Eggs tolerance

Considering that the exposure of eggs to seawater during their development is reduced because they are laid on young pups, we tested their tolerance to submersion and to different temperatures. To discriminate between these effects, we chose temperatures close to the natural exposure limits (0° and 35 °C/32 °F and 95 °F).

We collected 177 louse eggs from infested sea lion pups. According to their morphological features, eggs were classified as *viable* or *non-viable* following the criteria proposed by Mougabure Cueto et al. (2006), which also apply to *A. microchir*. A total of 84 eggs (47.5%) were considered viable and subjected to the following treatments (Fig. 1): (i) immersion in seawater at 0 °C/32 °F ( $n = 21$ ) and 35 °C/95 °F ( $n = 21$ ) and then exposed to air at 35 °C/95 °F; (ii) exposure to cold air (0 °C/32 °F) during 14 ( $n = 9$ ) and 25 days ( $n = 12$ ) and then transferred to 35 °C/95 °F; (iii) incubated at 35 °C/95 °F in air ( $n = 21$ ). During each treatment, each egg was observed daily and categorized as live or dead.

### 2.3. Submersion tolerance of first-instar nymphs

Three groups of 25 N1 were kept submerged in fresh seawater at 10 °C/50 °F (normal water temperature in the area during the study season) for 24, 48 and 72 h, respectively. After the corresponding immersion period, the insects were individually placed on a filter paper and observed with the aid of a stereomicroscope at different times, i.e. 0, 5, 15, 30, 45, 60, 90, 120, 150 and 180 min. At every stage, lice were categorized as *mobile* (with the ability to walk and/or to move the antennae) or *immobile* (not able to walk and/or to move the antennae). If after 180 min a louse still did not show any sign of recovering, it was considered as dead.

### 2.4. Immersion and recovery of diving instars

Groups of 18 lice including second- and third-instar nymphs and adults were placed into glass vials (5 ml) and submerged during 1–15 days in seawater with different oxygen saturations (i.e.  $n = 18 \times 2$  conditions  $\times$  15 time periods,  $N = 540$ ). To test oxygen dependence, trials were conducted into 1-liter identical aquaria on different conditions each. Half of the lice were placed in seawater constantly bubbled with ambient air by a pump, here indicated as “normoxic”. The other half was kept into seawater previously boiled for 15 min and without aeration, here called “hypoxic”. Both aquaria were closed so that equilibrium with atmospheric air would not be reached. Experiments were conducted at 10 °C/50 °F, approximately the mean temperature of seawater at Punta León latitude during the reproductive season of sea lions. The survival was determined following the same criterion that was used with the first-instar nymphs. In addition, the recovery time, i.e.

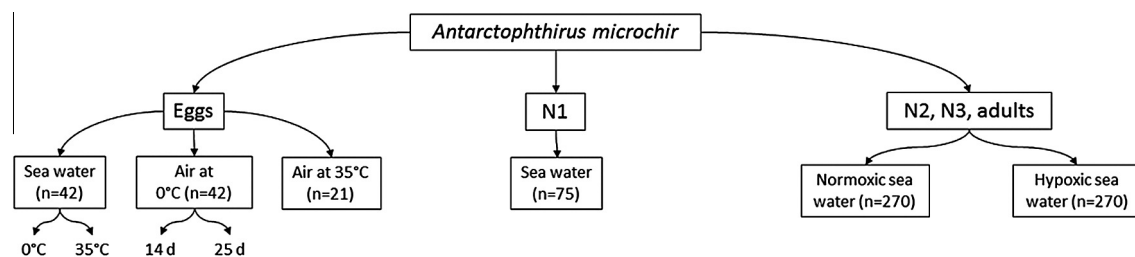


Fig. 1. Summary of the experiments conducted with *Antarctophthirus microchir*.

the time elapsed up to the appearance of the first movements, was recorded individually using the same criteria as for nymphs 1.

### 2.5. Underwater behavior

To determine the activity of submerged *A. microchir*, replicates of 61 lice (5 males, 5 females, 25 N1, 13 N2 and 13 N3) were placed in vial glasses (5 ml) and submerged into fresh seawater at room temperature (around 25 °C/77 °F). During the immersion, each louse was observed at short (1, 5, and 15 min), intermediate (60 and 120 min) and long intervals (24, 48, 72 h). At each observation stage, individuals were categorized as mobile or immobile.

### 2.6. Statistical analysis

Survival data were analyzed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA 92121) to produce survival curves depicting survival time rates using the Kaplan–Meier method and the Greenwood method for computing standard errors (Daniel, 2005). The comparison of survival curves was made with a variation of the Mantel–Haenszel method, i.e. the log-rank test. Chi square goodness of fit (Zar, 1994) was used to assess statistical significance of recovery periods in normoxic and hypoxic seawater.

### 2.7. Ethics statement

All necessary permits for the described field studies were obtained from *Subsecretaria de Turismo y Áreas Protegidas* and *Dirección de Fauna y Flora Silvestre* (Chubut Province, Argentina). Animals were manually restrained to avoid the use of anesthesia. To collect lice, each pup was restrained by two people while a third person (the same one in all collections to minimise biases) combed the pelage with fine-tooth combs of the type used for treating human pediculosis (see details in Leonardi, 2014). The mean manipulation time was  $3'25'' \pm 30''$  ( $n = 25$ ). Manipulation included measuring, weighing, tagging and combing the pup. Combing took about half of the entire manipulation time and ended when no more lice were found. Once pups were released, their mothers readily accepted and nursed them, and all manipulated pups survived the study. The samples were taken in January, 2011. Fieldwork started at 10 a.m. and finished at 6 p.m. This study was conducted in the sea lion rookery of Punta León, Chubut Province, Argentina. Punta León is a Provincial Scientific Reserve located 10 km south of Punta Ninfas, on the southern side of the mouth of Golfo Nuevo gulf.

Lice were collected from 18 South American sea lions, *O. flavescens*, pups (7 females, 11 males, average weight = 15.125 kg, average length = 82 cm, average age = 14 days).

## 3. Results

### 3.1. Eggs tolerance

Regardless of the temperature of the water, all the submerged eggs died during the first 24 h of treatment. However, after 14 days exposed to cold air, all eggs were viable and 77.8% of them hatched in about nine days after being transferred to 35 °C. Conversely, all eggs that remained under cold conditions for 25 days died during the treatment. When kept at 35 °C during their whole development, eggs hatched after a mean time of 9.7 days with 76.2% of survival.

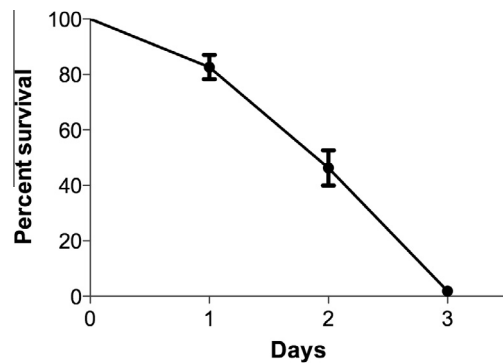


Fig. 2. Survival of first nymphal stage (N1) of *Antarctophthirus microchir* submerged in normoxic seawater. Results are presented as the mean survival per day of treatment and standard errors computed using Kaplan–Meier and Greenwood methods ( $n = 25$  N1 per treatment, 75 N1 1 total).

### 3.2. Tolerance to submersion of first-instar nymphs

The median survival time (i.e. the time at which 50% of the lice died) of N1 submerged in seawater was two days, and at the third day all the specimens were already dead (Fig. 2). The recovery time of surviving N1 ranged from 15 to 90 min after 24 and 48 h submerged and from 45 to 90 min in lice submerged for 72 h.

### 3.3. Immersion and recovery of diving instars

The Fig. 3 depicts the survival of lice under different treatment conditions, i.e. different immersion times in normoxic or hypoxic water. For both treatments, increasing immersion time decreased survival rate. Furthermore, survival curves were significantly different for lice kept in normoxic or hypoxic water ( $X^2 = 11.13$ , d.f. = 1,  $p < 0.001$ ). The median survival time was 7 days for lice in normoxic water and 6 days for those in hypoxic water. In both cases, no louse survived beyond the tenth day.

Recovery times of lice ranged from instantly to 90 min (Fig. 4) depending on both, the type of water (normoxic or hypoxic) and the time at which they were submerged. In both types of water after 24 h of treatment, all the surviving lice reactivated in five minutes at ambient temperature. However, the longer the time submerged, the longer the time they needed for recovery, particularly in hypoxic water (Fig. 4). Beyond 90 min, no louse showed signs of recovering.

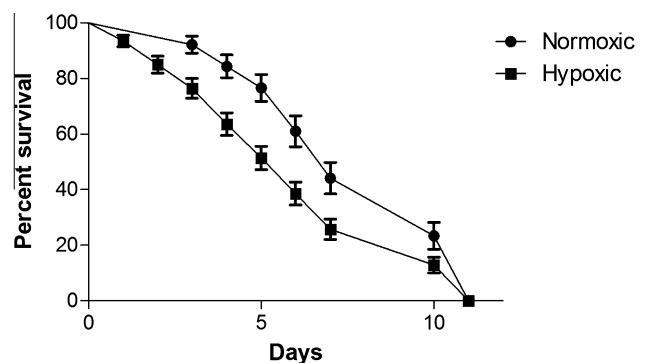
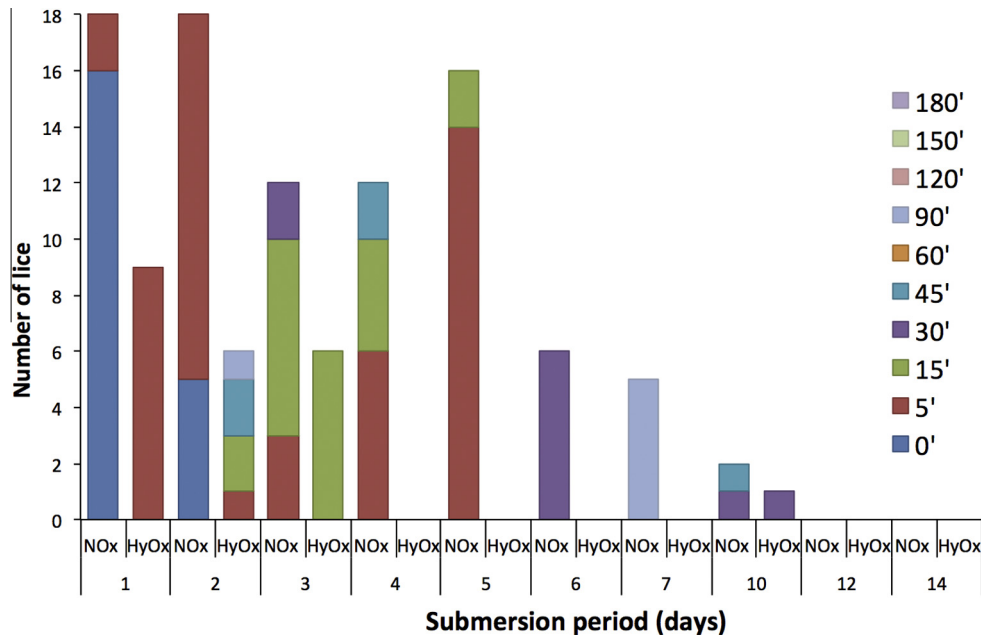


Fig. 3. Survival of *Antarctophthirus microchir* (N2, N3, and adults) submerged in normoxic (●) and hypoxic (■) seawater. Results are presented as the mean survival per day of treatment and standard errors computed using Kaplan–Meier and Greenwood methods ( $n = 18$  lice per treatment, 540 lice total).



**Fig. 4.** Recovery times for *Antarctophthirus microchir* after submersion in normoxic and hypoxic seawater. Results presented as number of recovered lice for each day of treatment.

### 3.4. Underwater behavior

All the lice observed remained completely motionless as soon as they came in contact with seawater. No movement of legs and/or antennae was verified at any of the observation times, notwithstanding whether the lice eventually died or not.

## 4. Discussion

The results presented here unravel several interesting aspects of the underwater life of lice. Our experiments showed that whereas eggs and first-instar nymphs (N1) are unable to tolerate immersion in water, further instars and adults tolerate it very well. In addition, as the level of oxygen saturation increments, the lice survival capability underwater increases, and the recovery period after returning to air shortens.

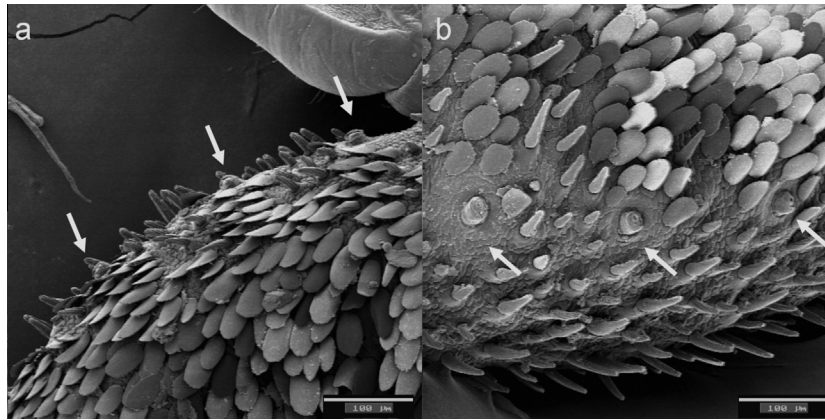
The fact that eggs do not survive submerged could constitute a main restriction to the life history of echinophthiriids as parasites of amphibious hosts. Consequently, lice reproduction could only occur when their hosts remain on land enough time. Therefore, the number of lice generations per year should be constrained by the duration of haul out periods of their hosts. In the case of *A. microchir*, the only moment when the host spend enough time ashore is during its reproductive season and only if the pups remain outside the water. Therefore, *A. microchir* would have approximately one month to reproduce in newborn pups. Afterwards, pups start swimming and molting, and both events may kill lice eggs (Murray, 1976, 1987). On the other hand, the reproductive season only occurs from December to early February, i.e. during the Austral summer, and temperatures do not decrease as much as in our experiment – 0 °C/32 °F – nor for enough time – 25 days in our study – to kill the eggs. The lifetime of adult lice is not known, but provided that females transfer lice from the previous season to pups, we can conclude that they survive for at least one year in the water (Leonardi et al., 2013).

Murray and Nicholls (1965) provided the first evidence on underwater lice survival by means of simple experiments con-

ducted in Antarctica, consisting in submerging one infected hind flipper of a Weddell seal into seawater, while the other one remained dry. The authors reported the death of eggs, but the survival of advanced nymphs and adults; N1 were not included in the experiment. Our results demonstrated that N1 survived submersion only for a couple of days. The reduced tolerance to immersions of N1 as compared to more advanced instars has been previously supposed by Aznar et al. (2009), based on the reduction of N1 in South American sea lion pups population when they started to swim. Murray and co-workers arrived at a similar conclusion (Murray and Nicholls, 1965; Murray et al., 1965) and by Kim (1975) regarding lice on northern fur seals from the absence of N1 in old pups and adult pinnipeds. The incapacity of N1 to survive underwater was suggested to be associated with the absence of abdominal scales (Kim, 1975; Aznar et al., 2009; Leonardi et al., 2012b), which are present over the body of the other instars.

The presence of scales covering the thorax and the abdomen is characteristic of species of echinophthiriids infecting sea lions, seals and walruses, but they are absent in lice associated with fur seals, i.e. host having a waterproof pelage. Accordingly, the insulation strategy adopted by the pinniped host would represent an important factor determining adaptation. Fur seals have developed a dense long-haired pelage that creates a layer of trapped air when the animal is submerged. This insulation mechanism would generate a virtual aerial environment for lice (Kim, 1975). Conversely, sea lions, phocids, and walruses rely on a blubber layer for insulation and present a wettable pelage (Mostman Liwanag, 2008). As a consequence, echinophthiriids infesting sea lions, phocids and walruses are exposed to real aquatic conditions, and this would result in further morphological, and presumably physiological, adaptations for surviving when the host submerges (Hinton, 1976a; Murray, 1976; Mehlhorn et al., 2002; Leonardi et al., 2012b).

The possible implication of scales in echinophthiriids breathing has been matter of speculation. Hinton (1976a) proposed that scales could form a plastron, i.e. a surface to retain an air layer, creating a water-air interface that acts as a physical gill. However, Murray (1976) argued that the scales could not be related to aqua-



**Fig. 5.** Scanning electron microscope images of the abdomen of an adult female of *Antarctophthirus microchir*: (a) lateral view of abdominal spiracles (Bar = 100 µm); (b) abdominal scales are not disposed around spiracles (Bar = 100 µm). White arrows indicate the spiracles.

tic respiration because they have no connection with the tracheal system, i.e. spiracles are free from scales (see Leonardi et al., 2012b). This author suggests then that lice take oxygen directly from water by cutaneous exchange, proposing that scales would provide mechanical protection (Murray, 1976). In *A. microchir*, scales cover the whole abdomen and are disposed in an imbricated pattern (Leonardi et al., 2012b), an arrangement that could allow the retention of an air film. However, in this species, scales are not particularly disposed around the spiracles (Fig. 5). The theoretical analysis of the insect plastron has unravelled relevant aspects of their performance in terms of resistance to hydrostatic pressure and of gas exchange capability (Hinton, 1976b; Flynn and Bush, 2008). These models are based, however, on plastrons formed by cylindrical hairs and not by scales, making it difficult to speculate about the properties and performance of a scale plastron. Our results support the idea that echinophthiriids are able to survive submerged during several days because they would be able to use the oxygen dissolved in seawater (Fig. 3).

The prolonged tolerance to immersion seems also to depend on a reflex activity reduction, i.e. quiescence, triggered by the contact with water. Kim (1975) was the first to suggest that the tolerance of lice to diving could be associated with a reduction of their metabolic activity, but this author did not analyze the behavior of echinophthiriids while submerged. We observed that as soon as *A. microchir* came into contact with the seawater, they instantly adopted a state of quiescence, and no movement of the legs or antennae could be detected. Quiescence is defined as an immediate response to a decline in any limiting environmental factor (e.g. temperature, oxygen concentration, humidity) below a physiological threshold (Košťál, 2006) and it is usually associated with a decrease in the animal metabolic rate. When echinophthiriids are submerged they confront a decrease in oxygen concentration, as well as in temperature and an increase in the hydrostatic pressure. Because the experiments were realized at the sea level, we can exclude hydrostatic pressure from being responsible of the induced quiescence. Temperature, wetting and low oxygen availability could then trigger akinesis, and probably low metabolic activity, decreasing the needs of lice for nutrients and oxygen.

It is worth remembering, however, that pressure may constitute a major selective pressure for morphological and physiological adaptation in echinophthiriids, because it can be extremely high during diving, as in the case of *Lepidophthirus macrorhini*, a louse infecting southern elephant seals. These seals are the most extraordinary divers among marine mammals (Campagna et al., 2007). They are used to diving at an average depth of 500 meters/1600 feet for 20 min. Some animals have been recorded to remain

at 1500 meters/4900 feet depth for two hours, which means that hosts and lice are submitted to a hydrostatic pressure of about 150 atm, i.e. more than 150 kg per square centimeter! Therefore, it seems clear that lice from elephant seals must have developed extraordinary physiological and anatomical adaptations to survive such prolonged dives at great depths. These adaptations constitute one of the most interesting aspects of the biology of this particular group of insects that still remains to be studied.

#### Acknowledgments

The authors thank D. Vales, M.F. Grandi, L. Silva, M. Feijoo, M. Degrati, N.A. García, G. Giardino, M.J. Klaich and A. Mandiola for their invaluable assistance in the fieldwork. Institutional and logistic support was provided by the *Centro Nacional Patagónico* (CONICET, Argentina) under permits of the *Secretaría de Áreas Protegidas y Turismo* and the *Dirección de Fauna y Flora Silvestre*, Chubut Province (Argentina). Thanks are also given to the *Ministerio de Ciencia, Tecnología e Innovación Productiva de la Nación* for the promotion of the scientific Argentinean program and the support to public education. The collaboration between the authors was possible thanks to a Travel Fellowship of *Journal of Experimental Biology* to M.S. Leonardi.

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