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Responses of Two Semiterrestrial Isopods, *Ligia exotica* and *Ligia taiwanensis* (Crustacea) to Osmotic Stress

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ABSTRACT. When immersed in fresh water, *Ligia taiwanensis* is a poorer osmoregulator than *Ligia exotica*, as judged by a lower LD_{50} at 96 hr and by the osmolalities of haemolymph. Animals appear to osmoregulate more efficiently in air. On immersion, both species displayed hyper- and hypo-osmoregulatory ability. Both species subjected less osmotic selection pressure during their inland colonization. The results suggest that a route of terrestrial colonization not involving transitional freshwater stresses had been taken by *L. exotica* and *L. taiwanensis*. COMP BIOCHEM PHYSIOL 118A;1:141–146, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. *Ligia*, osmotic stress, terrestrial colonization

INTRODUCTION

Most extant superfamilies of the order Isopoda are marine, and all terrestrial forms are included in one superfamily, the Oniscidea. Some members of family Ligiidae represent a lesser degree of terrestriality. These species inhabit high-littoral or supralittoral zones where humidity is high, although they require access to water and have been designated "semi-terrestrial" (22); *Ligia exotica* Roux and *Ligia taiwanensis* Lee, for example, spend most of the time on land, but must contact with water frequently. Such contacts allow them to cool down and to rehydrate (3).

Only two *Ligia* species, *L. exotica* and *L. taiwanensis*, have been found in Taiwan (5,11). *L. exotica* is a common species of the supralittoral zone of sheltered and exposed coasts around Taiwan. In contrast, the distribution of *L. taiwanensis* is limited, and they are only found in the strand of hill streams at Heng Chun Peninsula, southern Taiwan. *L. exotica* had long been formerly considered a marine species inhabiting the seashore. However, during a field survey, an isolated population of *L. exotica* was found inhabiting the strand of hill stream about 1 km away from seashore. It offered an opportunity to study the physiological adaptation of these two semiterrestrial isopods to different environmental conditions (the littoral zone and the strand of a hill stream).

One of the most significant changes associated with a transition between these habitats, from marine to terrestrial, is the change in the osmotic pressure (15). The responses of isopods to this change were important to the habitat adaptation. The physiological characteristics of osmoregulation can be used to test some predictions in the physiological evolution (18,24). Water relations, osmoregulation patterns, and environmental tolerances in isopods have been investigated extensively (1,2,4,13,14,16,19,23,26,28,31), but it is rare to compare the responses to various osmotic stresses in different acclimation conditions.

In this study, the responses of *L. taiwanensis* and *L. exotica* to osmolality of media were examined and compared in both aerial exposure and immersion acclimation regimes. The results could provide some clues on the strategies of their terrestrial colonization.

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MATERIALS AND METHODS

Specimens of *L. exotica* and *L. taiwanensis* were collected from the strands of hill streams (about 1 km away from seashore and altitude from 20 to 50 m). *L. exotica* were collected from the littoral rocky shore at Heng-Chen Peninsula. During transportation, animals were maintained in tanks containing the moist substratum from their natural habitat, and covered by a plastic plate to maintain a high humidity. In the laboratory, animals were stored for 2 days in tanks, which were lined with mats of moistened filter paper in the bottom, to allow the osmolality of body fluid to achieve a balance and to clean the guts before the experiments (30). To get sufficiently large haemolymph samples, only the larger animals (>15 mm in body length) were used.

The Responses of the Haemolymph Osmolality to the Osmotic Stress

Two acclimation regimes were employed, animals were 1) immersed in aerated water, and 2) exposed in air and allowed free access to water. In the submersed acclimation regimes, 10 l of medium of each salinity was added to each tank and aerated. Tanks with smooth walls were used to prevent the animals emerging from the aerated water. In the aerial exposure regime, only about 100 ml of medium was provided.

Twenty to 50 individuals from each of the three populations were sampled randomly from stock animals and placed in each tank. Media were prepared by mixing different amounts of sea water and fresh water, and the osmolalities were measured. A wide range of medium osmolality, from the fresh water to concentrated sea water (1645 mOsm/kg), was used in this experiment. All data were ultimately expressed as osmolality (mOsm/kg). Both sea water and fresh water were taken from the sampling sites. The media were aerated throughout the experiment period. Relative humidity >75% in an incubation room was maintained at a temperature 25°C. To achieve steady osmolalities of haemolymph, the animals were acclimated for 1 week. Following acclimation, the haemolymph osmolality of each live individual was measured using a Micro-osmometer 3MO PLUS, ADVANCED. Haemolymph samples (at least 20 μl) were obtained by puncturing the arthrodial membrane between the second and third dorsal plates using a fine capillary, following the method of Morritt (18).

LD_{50}

Based on a pre-test, seven medium osmolalities (104,124, 143,155,167,196, and 221 mOsm/kg) were used to examine LD_{50} (the lower medium osmolality which was lethal to 50% of animals) at 96 hr. Among 30 to 50 individuals from three sources were placed in each tank, and immersed in 101 medium. The experiment of each osmolality was executed in triplicate. Mortality rates of isopods were examined at 2-hr intervals on the first day, at 6-hr intervals in the following 3 days, and dead animals were removed immediately. LD_{50} was calculated using the program of Trevors and Lusty (27). The same procedure was replicated six times.

The Haemolymph Osmolalities of Dying Animals

The haemolymph osmolalities of dying animals (in which the beating of pleopods slowed down significantly, and became insensitive to the touch by a probe) immersed in fresh water were also examined as a reference of the tolerance to osmotic stress. Animals (35 individuals) from each population were immersed in fresh water. The individuals were removed and the haemolymph osmolalities of these individuals were measured, as soon as they were dying.

Data Analysis

The relationships between haemolymph and ambient medium osmolality were described by regression, and a parallelism test between the regression equation slopes was carried out by analysis of covariance (ANCOVA). ANOVA and Tukey test (32) were used to test the difference of the LD_{50} and the haemolymph concentration among the three *Ligia* populations.

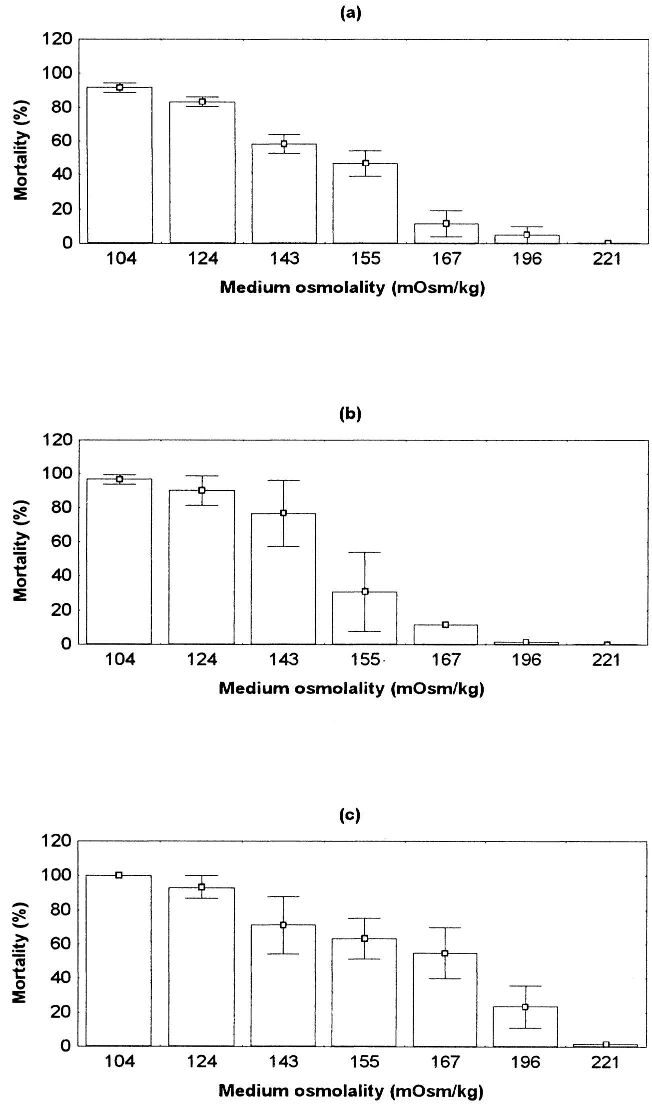

FIG. 1. Mean and standard deviation (mean ± SD) of percent mortality of isopods immersed for 96 hr in media with different osmolalities (n = 6): (a) littoral *Ligia exotica*, (b) hill *Ligia exotica* and (c) hill *Ligia taiwanensis*.

RESULTS

Mortality and Tolerance

All individuals exposed in air or immersed in aerated full strength sea water had 100% survival rates. In contrast, all animals immersed in aerated fresh water died within 4 days. Figure 1 shows the mortality variations of three sources of *Ligia* when they were immersed in media with different os-

TABLE 1. Analysis of the tolerance to osmolalities (LD_{50} for 96 hr, immersed) and the haemolymph osmolarity of dying animals (immersed in fresh water) among three sources of animals: littoral *Ligia exotica,* hill *Ligia exotica,* and *Ligia taiwanesis*

Species and habitats	LD_{50} (mOsm/kg)	*F*-test	Tukey test	Haemolymph osmolality of dying animal (mOsm/kg)	*F*-test	Tukey test
Ligia exotica	149 ± 9		a	559 ± 35		a
Marine (littoral)	(n = 6)			(n = 35)		
Ligia exotica	146 ± 26	$p < .01$	a	560 ± 27	$p < .001$	a
Stream (strand)	(n = 6)			(n = 35)		
Ligia taiwanensis	170 ± 16		b	614 ± 23		b
Stream (strand)	(n = 6)		$p < .001$	(n = 35)		$p < .001$

a, b are significantly by Tukey test, $p < 0.001$.

molalities. In the immersion experiments, the lower lethal osmolalities for 50% of the animals were 140 ± 9 mOsm/kg for littoral *L. exotica*, 146 ± 26 mOsm/kg for hill *L. exotica*, and 170 ± 16 mOsm/kg for *L. taiwanensis*. The LD_{50} for *L. taiwanensis* was significantly higher than that of *L. exotica*; no significant difference was found between two populations of *L. exotica* (Table 1).

Although *L. taiwanensis* was only found at hill stream far from the seashore, the ability to tolerate low external osmotic pressure was slightly reduced, compared with that of littoral or of strand *L. exotica.* To confirm this, the characterization of capacity to tolerate the lower haemolymph osmolality was estimated by examining the haemolymph osmolality of dying individuals, which were immersed in fresh water. The average haemolymph osmolalities of dying animals were 559 ± 35, 560 ± 27, and 614 ± 23 mOsm/kg for littoral *L. exotica*, hill stream *L. exotica*, and *L. taiwanensis*, respectively. The result corresponded to the LD_{50} (Table 1).

Relationship Between Haemolymph and Medium Osmolality

A simple linear regression model was used to show the relationships between internal and external osmolalities. The equations are given in Figs 2 and 3. All three populations showed significant differences in slopes between two acclimation regimes ($p < 0.001$ for littoral *L. exotica*; $p < 0.001$ for hill *L. exotica*, $p < 0.001$ for hill *L. taiwanensis*, ANCOVA). In the aerial exposure regime, the three populations showed a common slope ($p > 0.5$, NS). In immersed regimes, the slopes were different ($p < 0.01$) between the species; there was no difference between the two populations of *L. exotica* ($p > 0.5$, NS).

Upon immersion, all three populations displayed both hyper- and hypo-osmoregulatory abilities. The ability of osmoregulation seemed to be better in lower salinity media than that of in higher salinity media (osmolality levels above 1300 mOms/kg) (Fig. 2). In the exposed regimes, the haemolymph concentrations were less affected by media, compared to those immersed (Figs 2 and 3). The osmoregulatory patterns of both species were similar, yet the ranges tolerated were different.

DISCUSSION

Lethal Effect of Immersion

The retention of exosomatic water (such as the water existing in the body grooves, between the pleopods, and in the capillary uropods) was a key to success of many semiterrestrial amphipods and isopods (including the supralittoral *Ligia*) invading land, and this exosomatic water allows "aquatic" methods of gas, water, and ion exchange (8,10,24,25). As such, it is not surprising that *L. exotica* and *L. taiwanensis* can survive when immersed in aerated sea water. A terrestrial isopod, *Armadillidium album* Dollfuss, also showed to be very tolerant toward immersion in sea water (28). Similar results were also found in some littoral

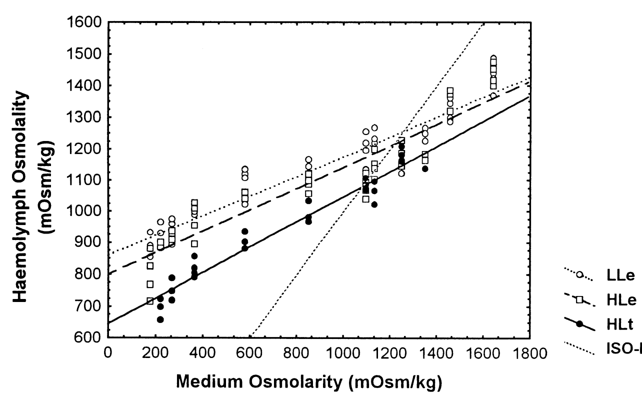

FIG. 2. Graphs plotting haemolymph osmolality against external osmolality for the littoral *Ligia exotica* (LLe); hill *L. exotica* (HLe); hill *L. taiwanensis* (HLt) in the submersion acclimation regimes, and iso-osmotic line (ISO-L). The equations of linear regression are: LLe $Y = 859.8 + 0.3X$ ($n = 44$; $F = 374.5$; $R = .948$; $P < .001$; $t_{intercept} = 54.7$; $t_{slope} = 19.3$), SLe $Y = 803.8 + 0.3X$ ($n = 44$; $F = 303.1$; $R = .937$; $P < .001$; $t_{intercept} = 43.4$; $t_{slope} = 17.4$); Lt $Y = 671.0 + 0.4X$ ($n = 36$; $F = 272.7$; $R = .949$; $P < .001$; $t_{intercept} = 32.1$; $t_{slope} = 16.5$).

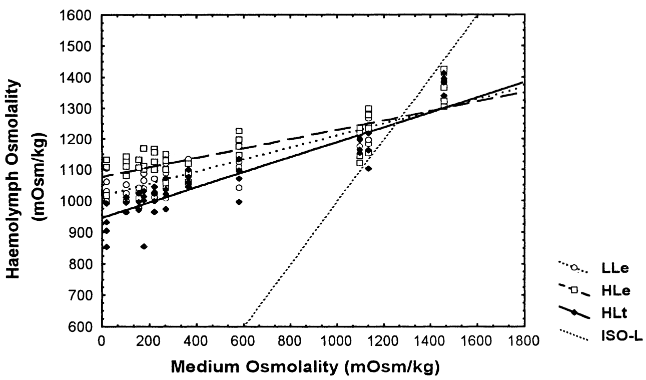

FIG. 3. Graphs plotting haemolymph osmolality against external osmolality for the littoral *Ligia exotica* (LLe); hill *L. exotica* (HLe); hill *L. taiwanensis* (HLt) in the aerial exposure acclimation regimes, and iso-osmotic line (ISO-L). The equations of linear regression are: LLe $Y = 1015.6 + 0.2X$ ($n = 44$; $F = 183.0$; $R = .902$; $P < .001$; $t_{intercept} = 101.1$; $t_{slope} = 13.5$); SLe $Y = 1077.6 + 0.2X$ ($n = 44$; $F = 82.154$; $R = .813$; $P < .001$; $t_{intercept} = 91.8$; $t_{slope} = 9.1$); Lt $Y = 946.8 + 0.2X$ ($n = 44$; $F = 176.6$; $R = .899$; $P < .001$; $t_{intercept} = 74.7$; $t_{slope} = 13.1$).

and terrestrial amphipods (18). Another field observation found that *Ligia italica* had to emerge from an intertidal pool at intervals to breathe in air (7). This emergence of *L. italica* may be related to the insufficient dissolved oxygen in an intertidal pool. In our experiments, aeration was very important for the survival of animals immersed in water. Morritt (18) found that the mortalities of some littoral and terrestrial talitroidean amphipods were increased in non-aerated water (18). In this study, none of the isopods could survive when they were immersed in water without aeration.

Animals immersed in freshwater were all dead within 96 hr, yet those immersed in sea water all survived under the same aerated condition. It seems likely that this immersion in aerated water itself did not have any lethal effect. Therefore, the reduction of survival rate is not likely to be related to the inability to maintain a sufficiently high rate of oxygen consumption under conditions of immersion. Accordingly, the change of osmotic pressure in the medium may be the main factor that affected the survival of the animals.

Tolerance to Osmotic Stresses

None of the animals could withstand the osmotic stress in fresh water, although they showed a wide range in salinity tolerance. The similar range of salinity tolerance was also observed in the euryhaline isopod *Sphaeroma serratum* (4). Osmoregulatory ability of *L. taiwanensis* immersed in fresh water was lower than that of *L. exotica*, as demonstrated by the LD_{50} at 96 hr and based on the haemolymph osmolalities (Table 1).

It is interesting that the individuals of *L. taiwanensis*, only inhabiting hill streams, were less tolerant to osmotic stress of fresh water compared to littoral *L. exotica* when immersed (Table 1). In this case, this lesser tolerance to fresh water of *L. taiwanensis*, seems unlikely to have evolved secondarily. It is unfavorable for *L. taiwanensis* inhabiting hill streams to maintain haemolymph concentration at such a high level. However, metabolic cost of osmoregulation is substantial (20). The most probable explanation is that this inability to tolerate low osmolality of fresh water and the high level of haemolymph osmolality, are traits of their marine ancestors.

Comparison of Haemolymph Osmolality

Barnes (1) had emphasized the wide variation in osmotic pressure of blood found in crustaceans. Little (15) suggested that many terrestrial invertebrates with a relatively high osmotic pressure probably have a direct marine origin, while those with a low osmotic pressure and little tolerance of internal change were probably descended from freshwater ancestors. Comparing the blood osmolality with the other isopods (16,19), the haemolymph osmolality of *L. taiwanensis* is much higher than that of true freshwater species, *Ligia aquaticus* (L), and closer to those of littoral species, *Ligia oceanica* (L.) and *L. exotica*, and a terrestrial species with a presumed marine origin, *Porcellio scaber* (13) (Table 2). Similar phenomena have been reported for freshwater crabs (29). These authors noted that "Although the blood osmoconcentration in freshwater crabs is significantly above that of the ambient fluid, it is less than that of marine crabs."

The haemolymph osmolality of hill *L. exotica* is only slightly lower than that of seashore *L. exotica* (Table 2). It seems likely that *L. exotica* subjected less osmotic selection pressure during their inland colonization. This result supports the remarks by Potts (21). "Once on land, the blood concentration is no longer subject to strong selection". This presumption of the marine origin, might also provide a good

TABLE 2. Comparison of the haemolymph osmotic pressure in some isopods residing in different habitats

Species	Natural habitat	Blood osmolality (mOsm/kg)
Asellus acquaticus	Freshwater (aquatic)	120[a]
Porcellio scaber	Moist substrate (terrestrial)	700[b]
Ligia oceanica	Sea water (littoral)	1160[c]
Ligia exotica	Sea water (littoral)	1185 ± 32[d]
Ligia exotica	Freshwater (hill)	1095 ± 53[d]
Ligia taiwanensis	Freshwater (hill)	921 ± 57[d]

[a](16).
[b](13).
[c](19).
[d]This study.

explanation for the high haemolymph osmolality of *L. taiwanensis*.

Osmoregulation

In order to compare the relationships between medium and haemolymph osmolalities of three populations easily, a regression model was used. A second-order polynomial regression model had been used previously to describe the relationships between haemolymph and medium osmolality of amphipods (18). In this study, the estimates of regression coefficient of second-order term were not significant. Therefore, a simple linear regression model was used.

All three populations show different slopes between different acclimation regimes. Price and Holdich also found differences in osmoregulatory ability of *L. oceanica* during aerial desiccation and immersion (23). In aerial exposure regimes, the haemolymph osmolality of animals (both species) was less affected by the ambient medium compared to that of immersion. It seems that the animals osmoregulated more efficiently in the air.

Although the ability to regulate hypoosmotically was weaker compared to that of hyper-osmoregulation, when immersed both species demonstrated both hyper- and hypo-osmoregulatory abilities (Fig. 2). The ability for hypo-osmoregulation has been regarded as a possible pre-adaptation to terrestrial life (6,17), and has been demonstrated in *Ligia occidentalis* and *L. pallasii* (31). Some littoral and supralittoral *Ligia* species maintain relatively high internal concentrations, even in low salinity media (19,26,31), and can also osmoregulate at high salinity (2,12). Although the ability of osmoregulation might be an advantage for *L. exotica* and *L. taiwanensis* to resist the extreme osmotic stresses, it might be too expensive energetically for long-term survival in such an extreme environment (20).

Land Colonization

Bliss and Mantel (3) suggested that certain species, though closely related, may have approached land *via* different routes: across sandy beaches or the rocky intertidal zone, through mangrove swamps, or by way of freshwater lakes and streams. Several possible scenarios might be suggested for *L. taiwanensis* and *L. exotica* to colonize in the strand of hill brooks.

The osmotic pressure of haemolymph may reflect the ancestral type of terrestrial isopods because the blood concentration might subject less selected pressure and maintained at the previous levels once on land (21). Little (15) suggested that blood osmolality is probably the most widely available physiological character reflecting the ancestry of animals. The results of our physiological study on osmoregulation presume that *L. taiwanensis* and *L. exotica* took route of terrestrial invasion not involving great transitional freshwater stresses. The similar result that Ligiidae were derived from marine ancestors, was also suggested by Edney (9) based on morphological characteristics.

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