Blood Osmoregulation and Ultrastructure of the Gas Windows ('Tympana') of Intertidal Ocypodid Crabs: *Dotilla* vs. *Scopimera*

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Abstract: Intertidal ocypodid crabs of the two genera *Dotilla* and *Scopimera* have unique membranous windows ('tympana') on their legs for aerial gas exchange, and the former also has them on its thoracic sternum. The osmoregulatory capabilities of *Dotilla myctiroides* and *Scopimera pilula* in dilute external media were compared, and microstructure of their tympana was examined to clarify whether they have an osmoregulatory function as well as a respiratory one, because respiratory surfaces are probable sites for osmoregulation or of water/ion loss. Although both *D. myctiroides* and *S. pilula* were hyper-osmoregulators, the former species was much the weaker in this respect. Histological results showed that the tympana have simple, thin respiratory epithelia in both of these species as well as *S. proxima*, which indicates that the tympana are probably sites of water/ion loss. The thicker cuticle in *Scopimera* species than in *D. myctiroides* may be an adaptive characteristic for maintaining blood osmolality in dilute media, and this was confirmed by silver staining, which indicated that the loss of Cl⁻ through tympana was smaller in *Scopimera* than in *Dotilla*. However, at the same time, the longer blood/gas diffusion distance due to the thickened cuticle must result in lower efficiency of gas exchange. Hence, a trade-off between respiratory efficiency and water/ion retention is involved in the development of tympana. From this viewpoint, the presence/absence of tympana among species of the subfamily Dotillinae, which includes *Dotilla* and *Scopimera*, is reviewed with reference to some taxonomic and phylogenetic considerations.

Key words: blood/gas diffusion distance, cuticle thickness, *Dotilla*, ion/water permeability, osmoregulation, *Scopimera*, terrestrial adaptation, ultrastructure of tympanal epithelia

INTRODUCTION

Semi-terrestrial crabs of the family Ocypodidae provide interesting perspectives for considering the adaptation of aquatic invertebrates to life on land (Powers & Bliss 1983). These crabs mainly inhabit intertidal

areas and exhibit a distinct zonation of genera and species that reflects their differing degrees of terrestriality. Reduced salinity due to freshwater inflows is one of the major problems faced by such semi-terrestrial crabs. Since variation in salinity increases with height on the shore as the moderating influence of the tides diminishes, higher shore species have stronger physiological mechanisms to protect against salinity reduction/fluctuation than lower shore inhabitants (Macintosh 1979).

To address this problem, comparative studies of crustacean species, including semi-terrestrial crabs, have focused largely on the characteristics of respiratory organs (e.g., surface area and morphology) in relation not only to osmoregulatory but also respiratory functions. This is because respiratory surfaces, which require thin membranes for gas exchange, are likely to be more permeable to water and ions than other body surfaces (Greenaway 1988; Lockwood & Bolt 1989). Some previous studies have revealed that the gill, a major respiratory organ of crustaceans, has ion-transporting as well as respiratory functions in a variety of crustacean groups that encounter reduced salinity (Barra et al. 1983; Dickson et al. 1991; Farrelly & Greenaway 1992; Kikuchi & Matsumasa 1993a, b; Takeda et al. 1996a; Matsumasa et al. 1998; Tsubokura et al. 1998). However, little work has been done on other types of respiratory organs, although respiratory surfaces other than gills are seen in a variety of crustaceans (McLusky 1968; Bliss 1979; Maitland 1986; Maitland & Maitland 1992; Kikuchi & Matsumasa 1993a, 1995, 1997).

All members of the subfamily Dotillinae (Crustacea: Brachyura: Ocypodidae) except some species of *Ilyoplax* possess large membranous disks ('tympana') on the meral segment of the legs (Kemp 1919). Species of *Dotilla* and *Scopimera* that inhabit sandy shores have these tympana, and species of the former genus also have tympana on their thoracic sterna. Maitland (1986) carried out an excellent work on the function of tympana and revealed that the surfaces are primarily gas windows for aerial gas exchange. In the present study, we compare *Dotilla myctiroides* as a lower shore inhabitant and *Scopimera* spp. as a higher shore dweller (see Kemp 1919, Hartnoll 1973) in terms of their osmoregulatory capabilities in dilute external media and the microstructures of their tympana, in order to clarify whether the gas window also has an osmoregulatory function. In addition, we propose a functional reason for the restriction of this unique respiratory organ to only some semi-terrestrial crabs, namely, members of the Dotillinae, referring to previous taxonomic studies (Kemp 1919; Serene & Lundoer 1974) and a recent phylogenetic analysis (Kitaura et al. 1998) of this subfamily.

MATERIALS AND METHODS

Animals were collected from sand flats at Port Dickson (Pantai), Negeri Sembilan, Malaysia, and in Phuket, Thailand. At Port Dickson, *Dotilla myctiroides* and *Scopimera pilula* were sympatrically distributed. The former species inhabited a bare sand substratum on the

Fig. 1. The Port Dickson site, Malaysia (2° 30'N; 101° 51'E). *Dotilla myctiroides* inhabited the lower part of the shore (A). Many individuals of the species often left their burrows, wandered down to the shoreline, and fed in groups there. This behavior, referred to as 'droving' or 'herding', is characteristic for some ocypodid crabs including this species (B). *Scopimera pilula* was an upper-shore inhabitant, and its habitat was associated with sparse mangroves in this site (C). These photographs were taken in November, 1994.

lower part of the shore, and the latter, a sand area with sparse mangrove vegetation higher up (Fig. 1; see also Takeda et al. 1996b). *Dotilla myctiroides* was also sampled at Ao Nam Bor and Ao Tang Khen, and *S. pilura* and *S. proxima* at Ban Pa Khloh and Sa Pan Hin, respectively, in Phuket. As on the Malaysian beach, *D. myctiroides* was observed in the lower parts of the tidal flats (see Matsumasa et al. 1992, Takeda et al. 1996a). *Scopimera pilura* and *S. proxima* inhabited the higher part of the sandy beaches, although these areas were not associated with mangroves.

Adult males of *D. myctiroides* (mean $\pm$ SD of carapace length: 8.15 ± 0.376 mm; $n = 20$) and *S. pilura* (mean $\pm$ SD of carapace length: 5.83 ± 0.470 mm; $n = 20$) from the Port Dickson site were used in an experiment to determine their blood osmoregulatory characteristics. Four individuals of each species of crab were placed in containers with 20, 30, 60, 95, and 100% seawater from Port Dickson. The depth of water was just 1 cm, so the crabs were able to lift their bodies up into the air. After 18 hrs (room temperature: 22.5 ± 1 °C), blood samples (ca. 100 μ l) were removed with a syringe from the arthrodial membrane at the base of the fourth ambulatory leg of each crab and allowed to clot. Osmolality of the serum and external water samples were determined using a freezing point micro-osmometer (Hermann Roebling, Type 13DR).

Permeable areas on the body surfaces were determined for *D. myctiroides*, *S. pilura*, and *S. proxima* from Phuket by a modified silver staining method (see Kikuchi & Matsumasa 1993b). Living animals of the three species were quickly washed in changes of distilled water to remove any adherent salts and then immersed in 0.2N nitric acid with 1% silver nitrate solution for 1 min. After removal of the excess silver nitrate by rinsing with distilled water, they were soaked in 0.2N nitric acid for 5 min. Then they were washed in distilled water again and put in a solution of photographic developer (Ilford) to blacken the permeable areas of the body surface by reduction of silver ions. The validity of this method for locating the regions of the body surface of crustaceans that are permeable to chloride ions had already been confirmed by element analyses using energy-dispersive X-ray microanalysis (EDX) (Kikuchi & Matsumasa 1993b). In this study, the distribution of silver in the central areas ($80 \mu\text{m} \times 100 \mu\text{m}$) of the tympana was also examined using EDX and compared between *Dotilla* and *Scopimera*. The tympanal areas of the third ambulatory legs of silver-stained *D. myctiroides* and *S. pilura* were cut off, mounted on copper platelets, and then coated with carbon. These were

analyzed by using a Hitachi H-600A scanning-transmission electron microscope equipped with a Kevex 8000 X-ray detector.

For morphological study, meral segments of the second and third ambulatory legs of the three species and sterna (for *Dotilla* only) were fixed in a mixed solution of 2.5% glutaraldehyde and 2% formaldehyde buffered with 0.2M phosphate at pH 7.4. They were rinsed several times in 0.2M phosphate buffer and then post-fixed for 1 hr at room temperature in 2% osmium tetroxide in the same buffer solution. After dehydration in an ascending ethanol series, the specimens were embedded in an epoxy resin (Spurr). Ultrathin sections double-stained with uranylacetate and lead citrate were examined for five individuals of each of the three species with a JEOL JEM-100B transmission electron microscope. Epithelial and cuticle thicknesses were measured using a ruler at ten haphazardly selected places on photographs of the cross sections (magnification: 1×10^4) for each individual; their sum was referred to as the 'total blood/gas diffusion distances' across the tympana. Because a Bartlett test indicated heterogeneities in variances for the cuticle thickness and the blood/gas diffusion distance, $\log(x + 1)$ -transformed data were examined by ANOVA using SYSTAT (Wilkinson 1992). Bonferroni tests were done for multiple comparisons among means.

Energy-dispersive X-ray analysis was also used to compare the relative abundance of Ca as an index of cuticle calcification between tympanal areas of *Dotilla* and *Scopimera*. The tympanal areas and surrounding cuticles were cut from the third ambulatory legs of individuals of *D. myctiroides* and *S. pilura* that had been fixed in 70% ethanol. These were dried in air, mounted on copper platelets, and coated with carbon. After detecting the elements recognized as peaks, the elemental proportions in terms of atoms were calculated for Mg, Si, P, S, Cl, Ca, and Fe under the following conditions: acceleration voltage, 25 kV; beam current, 100 nA; and beam diameter, 1 μm . The differences in elemental proportion of Ca between analyzed areas (i.e. the tympanal and the surrounding areas of the two species) were examined by a *G*-test.

RESULTS

Eighteen hours after exposure to different salinities, none of the *Dotilla* survived at the lowest salinity (20% SW), whereas three of four did at 30% SW (Fig. 2). On the other hand, all the *Scopimera* survived in the

Fig. 2. The blood osmolality controls of *Dotilla myctiroides* (solid line) and *Scopimera pilura* (dotted line). Vertical bars show $\pm$ SE ($n = 4$). The value for *Dotilla* is missing at 20% SW (176 mOsm/kg) and is based on three samples at 30% SW (316 mOsm/kg), since none survived at the lowest salinity and only three of four individuals survived at 30% SW.

various test salinities. *Scopimera* kept its blood osmolality much higher than the dilute external media of 176–622 mOsm/kg (20–60% SW) and became isotonic to the external media above 900 mOsm/kg. *Dotilla* also maintained its hyperosmotic state in the dilute media, but its capability to do so was much lower than that of *Scopimera*. The blood osmolality of *Dotilla* was 531 ± 7.0 mOsm/kg (mean $\pm$ SE) in an external medium of 316 mOsm/kg, whereas that of *Scopimera* was 780 ± 27.3 mOsm/kg.

Tympana on both the meral segments of the legs and the thoracic sterna of *Dotilla myctiroides* were silver-stained more strongly than the surrounding areas (Fig. 3). In contrast, the tympana of the two species of *Scopimera* were scarcely stained at all. Energy-dispersive X-ray analysis for silver in the central areas ($80 \mu\text{m} \times 100 \mu\text{m}$) of the leg tympana clearly showed a difference in staining between *D. myctiroides* and *S. pilura* (Fig. 4). This confirmed that the permeability of the leg and sternal tympana of *D. myctiroides* to Cl^- is higher than that of the leg tympana of *S. pilura*.

The histological examination revealed that the epithelia in the areas of the leg tympana of *D. myctiroides* and *S. pilura* are quite thin, with few mitochondria and neither apical nor basolateral infoldings of cell membranes. Under the thin epithelia, large hemocoels were observed (Fig. 5). A similar structure was observed for epithelia of the sternal tympana of *D. myctiroides* and of the leg tympana of *S. proxima*. These structural characteristics, being common to the tympana of the three species, suggest that these organs have a respiratory function both in *Dotilla* and *Scopimera*, but osmoregulatory one. Although the epithelial thickness of *S. proxima* is greater than those of the other species (Table 1; Bonferroni test, $P < 0.001$), and that for *S. pilura* was greater than that of *D. myctiroides* ($P < 0.05$), even the epithelia of *Scopimera* species were too thin to develop intracellular structures for ion-transport, such as frequent membrane foldings and an abundance of mitochondria. However, the cuticle was much thicker in the two species of *Scopimera* than in *D. myctiroides* (Table 1; Bonferroni test, $P < 0.001$), so the blood/gas diffusion distance across the tympana is greater for *Scopimera* than *Dotilla* ($P < 0.001$). Significant differences were also detected in cuticle thickness and blood/gas diffusion distance between the leg and sternal tympana of *D. myctiroides* ($P < 0.001$).

Table 1. Epithelial thickness, cuticle thickness, and total blood/gas diffusion distance (μm) across the tympana of *Dotilla myctiroides* and two species of *Scopimera* (mean $\pm$ SE, $n = 5$). Values for leg tympana of the three species and for sternal ones of *Dotilla* were obtained from the 3rd ambulatory legs and from the 5th thoracic sternites, respectively. Carapace widths (mean $\pm$ SE) of crabs examined were 6.9 ± 0.21 cm (*Dotilla myctiroides*), 6.6 ± 0.04 cm (*S. pilura*), and 5.1 ± 0.04 cm (*S. proxima*). Underlines indicate no significant differences between means (Bonferroni test; $P > 0.05$).

Region	<i>D. myctiroides</i>		<i>S. pilura</i>	<i>S. proxima</i>
	leg	sternum	leg	leg
Epithelium	<u>0.10 ± 0.007</u>	<u>0.09 ± 0.003</u>	0.12 ± 0.004	0.20 ± 0.005
Cuticle	0.80 ± 0.038	1.63 ± 0.040	<u>3.53 ± 0.175</u>	<u>3.85 ± 0.087</u>
Total (blood/gas diffusion distance)	0.90 ± 0.037	1.72 ± 0.038	<u>3.65 ± 0.179</u>	<u>4.05 ± 0.091</u>

Fig. 3. Silver-stained tympana. The leg and sternal tympana of *Dotilla myctiroides* were darkly stained (A, B), but the leg tympana of *Scopimera pilura* (C) and *S. proxima* (D) were scarcely stained at all. (A) Posterior face of the meral segment of the 4th right ambulatory leg of *D. myctiroides*; the oval part (arrow) near the distal end of the meral segment is also darkly stained. Such oval parts are observed on the meral segments of all of the ambulatory legs. (B) Thoracic sternites of *D. myctiroides*. (C) Posterior face of the meral segment of the 4th right ambulatory leg of *S. pilura*. (D) Anterior face of the meral segment of the 4th right ambulatory leg of *S. proxima*; all of the tympana on the meral segments of the walking legs, with the exception of those on the posterior surfaces of the last pair of legs, are longitudinally divided by a narrow ridge. Scale bar = 1 mm.

The patterns of elemental proportions in terms of atoms were quite similar for the leg tympana of *Dotilla myctiroides* and *Scopimera pilura* (Fig. 6), but they differed between the tympanal area and its surrounding cuticle in both species, the relative percentage of Ca in the tympana being lower (*G*-test, $P < 0.001$). There was no difference in this respect between *Dotilla* and *Scopimera* ($P = 0.887$).

DISCUSSION

Both *Dotilla myctiroides* and *Scopimera pilura* are hyper-osmoregulators, and the osmoregulatory ability of the latter species is stronger than that of the former

Fig. 4. Silver distributions on the silver-stained tympana of the 3rd ambulatory legs of *Scopimera pilura* (A) and *Dotilla myctiroides* (B). The white dots show silver detected by energy-dispersive X-ray analysis, and the detected silver indicates leaks of chloride ions from the tissue. Scale bar = 20 μm .

Fig. 5. TEM of the leg tympana of *Scopimera pilura* (3rd ambulatory leg; A) and *Dotilla myctiroides* (2nd ambulatory leg; B). The epithelia (arrows) are quite thin and have few intracellular organelles in both species. C, cuticle; H, hemocoel; He, hemocyte. Scale bar = 1 μ m.

Fig. 6. Relative abundances of elements in tympana (TYM) and surrounding cuticles (SUR) of *Dotilla myctiroides* and *Scopimera pilura*. Percentages of atoms were calculated for the seven elements that were detected by energy dispersive X-ray analyses.

in dilute media (Fig. 2). This coincides with the fact that, in the tropical Indo-West Pacific, species of *Scopimera* generally inhabit higher levels of sandy shores than do species of *Dotilla* (MacNae & Kalk 1962; Silas & Sankarankutty 1967; Hartnoll 1973; Matsumasa et al. 1992; Takeda et al. 1996b). Higher up, rainfall and other freshwater inflow often reduce the environmental salinity.

Our histological results showed that the epithelia of the tympana of *Dotilla* and *Scopimera* are thin, simple, respiratory surfaces with few mitochondria and covered with relatively thin and weakly calcified cuticle layers (Fig. 5). Although some other types of respiratory organs including gills often function as osmoregulatory sites in intertidal, brackish, and freshwater crustaceans (Mantel & Farmer 1983; Gilles & Péqueux 1985; Péqueux & Gilles 1988; Kikuchi & Matsumasa 1997), the tympana do not. The tympana are exposed directly to air during surface activities, while the gills of semi-terrestrial crabs are in branchial chambers,

into which urine is excreted from antennal glands and external water is taken up from the substrate through specialized setae at the bases of the walking legs (see Greenaway 1988; Farrelly & Greenaway 1992). These differences in localization of the respiratory organs suggest that the tympana would be less suitable sites for ion transport than the gills. As a result, without ion-transporting epithelia, the tympanal areas would be one of the most probable sites for ion-loss from the body in dilute external media. In fact, the results of silver staining revealed that in *Dotilla*, the tympanal area is more permeable to Cl^- than the surrounding body surface (Fig. 3). However, the tympana of the two examined species of *Scopimera* were scarcely stained at all, indicating that they were far less permeable than the leg and thoracic tympana of *Dotilla* (Figs. 3, 4). Although the silver-staining method has usually been used to identify ion-transporting surfaces by staining these more darkly than ordinary respiratory surfaces, the absence of any ion-transporting epithelia in the tympana of *Dotilla* shows that the stained area does not always function as an osmoregulatory site. As mentioned in some previous studies (Farrelly & Greenaway 1992; Kikuchi 1992; Kikuchi & Matsumasa 1993b), the cell types underlying silver-stained areas should always be identified ultrastructurally. As physiological adaptations in brackish and freshwater crustaceans, Lockwood & Bolt (1989) pointed out (1) the reduction in the permeability of the body surface to water/ions and (2) the improvement of effectiveness in ion uptake from low external salinities. The less-stained tympana of *S. pilura* and *S. proxima* with simple respiratory epithelia are involved with consideration (1) above, whereas gills with ion-transporting epithelia in some semi-terrestrial crabs (e.g. Takeda et al. 1996a) are concerned with consideration (2).

The thickness and density of the cuticle (e.g. the degree of calcification) are considered to be particularly important variables with respect to water/ion permeability, because the well-developed surface layer of lipids typical of insects has not been observed in crustaceans (Greenaway 1988). The tympanal cuticles of the two species of *Scopimera* were significantly thicker than that of *Dotilla myctiroides* (Bonferroni test: $P < 0.001$) (Table 1), in accordance with the differences in silver staining between the species. On the other hand, the relative percentage of Ca was not significantly different between the tympanal areas of *Dotilla* and *Scopimera* (G -test: $P = 0.887$), although the values were much lower than those for the normal exoskeleton of the legs (Fig. 6). These results indicate that cuticle

thickness is a more important variable with respect to the tympanal permeability than the degree of cuticle calcification is. The extremely thin ($0.8 \mu\text{m}$) cuticle of the leg tympana of *D. myctiroides* is comparable with those of gills and lungs in the branchial chambers of semi-terrestrial crabs (Maitland 1986; McMahon & Burggren 1988; Kikuchi 1992; Takeda et al. 1996a). An Australian sand bubbler, *Scopimera inflata*, also has extremely thin ($0.6 \mu\text{m}$) cuticle in its leg tympana (Maitland 1986). But *S. pilura* and *S. proxima* in this study have much thicker cuticles ($> 3.5 \mu\text{m}$). Hence, there appears to be some degree of variation in thickness of tympanal cuticles among species of the same genus. This should be examined more closely, together with an examination of the relationships between osmoregulatory ability and habitat characteristics (e.g. elevation, salinity, and sediment conditions).

The thicker, relatively less permeable tympana of *Scopimera pilura* and *S. proxima* have an advantage over those of *Dotilla myctiroides* in maintaining its blood osmolality. A thick tympanum is probably important for reducing not only the loss of ions from crab bodies in dilute media, but also the loss of water during aerial activities in arid environments (Greenaway 1988). However, the resultant longer blood/gas diffusion distances for the leg tympana of *S. pilura* and *S. proxima* (Table 1) no doubt also result in lower air-breathing efficiency and a reduced absolute contribution of the tympana to aerial gas exchange (see Farrelly & Greenaway 1992 for gills). The blood/gas diffusion distances for these species of *Scopimera* are more than four times longer than that ($0.9 \mu\text{m}$) for leg tympana of *D. myctiroides*, which is comparable to those ($< 1 \mu\text{m}$) for respiratory gills (not osmoregulatory ones) and for lungs of similar-sized crabs (Maitland 1986; McMahon & Burggren 1988). This possible trade-off between efficiency in air breathing and in water/ion retention, which is related mainly to the cuticle thickness of the tympana, indicates a physiological limitation for the tympana as air-breathing organs in semi-terrestrial environments where the influence of reduced salinity is potent. That is, the gas window that had evolved and developed during the invasion from marine to land habitats may be degenerative in some euryhaline, higher-shore species of the subfamily Dotillinae. From this viewpoint, another genus of Dotillinae, *Ilyoplax*, is interesting, because this genus includes both species with and without tympana on their legs (Kemp 1919). Recently, based on analyses of 1,416-base-pair nucleotide sequences from the mitochondrial 12S to 16S rRNA genes, Kitaura et al.

(1998) figured the relationships between 21 species of the family Ocypodidae, including 15 species of the subfamily Dotillinae, in a phylogenetic tree. Although they did not mention the distribution of tympana among the species of Dotillinae, their excellent study revealed some important phylogenetic relationships. For example, *Dotilla wichmanni*, a species close to *D. myctiroides* in this study, formed a clade with an *Ilyoplax* lineage in which the species do not possess any gas windows (see Serene & Lundoer 1974). Since *D. wichmanni* is the most basal among the species in the clade, it is supposed that the *Ilyoplax* species without tympana might be derived from an ancestral species with tympana. Without the knowledge of the tympanal function, Kemp (1919) had already stated, "Most of the species of *Dotilla* and *Scopimera* live on the sea-shore. ... Most species of *Tympanomerus* [= *Ilyoplax*] are found in estuaries, often near or even beyond the extreme limit of tide influences. Both species of *Dotillopsis* are essentially estuarine, ..." and, "The tympana are well-defined and usually conspicuous in *Scopimera* and *Dotilla*, ill-defined in *Dotillopsis* and ill-defined or absent in *Tympanomerus*." The ill-defined tympana of *Dotillopsis brevitarsis* has a thicker (ca. 6–7 μm) cuticle than the tympana of *Dotilla* and *Scopimera*, which are associated with similar simple respiratory epithelia (Matsumasa et al., unpublished data). Therefore, the increase in cuticle thickness would make tympana appear ill-defined. If a fully resolved phylogenetic tree were available for this subfamily, we believe that more detailed investigations of relationships between degrees of tympanal development/degeneration and the functional significance of tympana (e.g. contributions of the gas windows vs. gills/lungs to total oxygen consumptions) in various habitats will provide us with further insights.

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