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Hemolymph osmotic, ionic status, and branchial Na^+/K^+-ATPase activity under varying environmental conditions in the intertidal grapsid crab, *Gaetice depressusd*

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Abstract

Osmo- and ionoregulatory abilities were examined in the intertidal grapsid crab, *Gaetice depressus*, transferred from normal seawater (30 ppt) to low (10 ppt) or high (50 ppt) salinities for 2 and 10 days, in addition to animals kept out of water for 2 days. The results of the hemolymph osmotic and ionic status indicate that *G. depressus* is able to adapt for more than 10 days in these salinities and for 2 days under terrestrial conditions. Especially, the free Ca^{2+} concentration was relatively maintained compared with concentrations of monovalent ions and osmolality values in 10 and 50 ppt, partly using the complexed calcium (total minus free calcium) as an internal reserve in the hemolymph. In 10 ppt, complexed calcium disappeared from the hemolymph after 10 days, indicating that all the hemolymph calcium was ionized. In 50 ppt, free Ca^{2+} was regulated to lower levels than concentrations in the medium, while total calcium increased to higher levels after 2 days. Examination of Na^+/K^+-ATPase activity, which has been implicated in ion transport in many crustaceans, revealed that induction of high Na^+/K^+-ATPase activity varies among the posterior gills in response to salinities. Ten-ppt salinity induces activity in two of the posterior gills (gill numbers 6 and 7, eight in total), albeit with differing degrees of response. In contrast, 50-ppt salinity stimulates the activity primarily in gill number 8, suggesting that this gill may be associated specifically with ion excretion in *G. depressus*. As a euryhaline amphibious crab, this abundant species around Japan will serve as a model to study the osmotic/ionic regulatory mechanisms which operate in crustaceans.

Keywords: Crustacean, Osmoregulation, Salinity

Background

Osmoregulation of decapod crustaceans after hyposaline exposure has been extensively studied. In crabs, the primary site of regulation is the posterior gills which have the highest specific activity of Na^+/K^+-ATPase, the enzyme thought to provide the major driving force for salt uptake, although the underlying cellular components of active ion uptake also include other transport proteins and transport-related enzymes such as a Na^+/H^+ antiporter, a $Na^+/K^+/2Cl^-$ co-transporter, V-ATPases, and carbonic anhydrases

(Bianchini et al. 2008; Lucu and Towle 2003; Pequeux 1995; Henry et al. 2002; Towle et al. 2011, Towle et al. 1997; Ahearn et al. 1999; Serrano and Henry 2008; Towle and Weihrauch 2001). Many terrestrial crabs are capable of directing their urine from their nephropores into their branchial chambers (Morris 2002), and the salt uptake from the urine is also regulated by branchial Na^+/K^+-ATPase (Morris 2001, 2002). Therefore, this branchial Na^+/K^+-ATPase appears to be one of the central players in osmotic and ionic regulation generally in crabs. However, information about the roles of this enzyme at high salinity is limited to some species (the fiddler crab, burrowing crab, and green crab) (Bianchini et al. 2008; Dorazio and Holliday 1985; Lin et al. 2002; Zanders and Rojas 1996; Jillette et al. 2011; Freire et al. 2008). Furthermore, Na^+/Cl^- regulation has been focused on, and little attention has been given to Ca^{2+} handling during the adaptation to different salinities (Freire et al. 2008; Charmantier et al. 2009), while Ca^{2+} homeostasis in the molting cycle of crustaceans has been examined extensively (Wheatly et al. 2002; Ahearn et al. 2004). For example, only the total calcium concentrations in the hemolymph (complexed (protein bound) plus free (unbound) calcium) have been reported as the Ca^{2+} concentration in most of the studies on the responses to different salinities, although it has been known that about 20% of calcium in the hemolymph of intermolt crabs is protein bound unlike other ions (Pequeux 1995; Wheatly 1999; Robertson 1960) and the free moiety which is more relevant can now be measured (Neufeld and Cameron 1992; Wilder et al. 1998).

G. depressus, a grapsid crab, is found in subtropical regions and is one of the most abundant northwestern-Pacific species (Kikuchi et al. 1981). This species occurs in intertidal cobble areas and tide pools where the salinities fluctuate considerably due to the effects of rainfall, evaporation, and influx of groundwater (Lohrer et al. 2000; Kawane et al. 2008). Therefore, this amphibious and euryhaline *G. depressus* provides a model for studies on osmotic/ionic responses to various environmental conditions. In this study, we examined changes in the hemolymph osmotic and ionic concentrations and in the activity of branchial Na^+/K^+-ATPase after exposure to high and low salinities as well as after water deprivation in *G. depressus*.

Methods

Collection and maintenance of animals

Adult male individuals of grapsid crab, *G. depressus*, weighing 3 to 6 g were collected from June to September along the shore near the Ushimado Marine Institute in Okayama Prefecture, Honshu, Japan. Crabs were transferred to the Marine Institute and held in undiluted natural seawater at a practical salinity of 30 ppt (448 mM Na^+, 506 mM Cl^-, 9.7 mM Ca^{2+}, 9.7 mM K^+, 994 mOsml kg^{-1}) and a temperature of 24 ± 1°C. A 12:12-h light/dark photoperiod was maintained. Small rocks were placed in each tank to allow animals the opportunity to hide and come out of the water by climbing on them. Animals were acclimated to laboratory conditions for 1 month prior to experimentation and fed a commercial diet ad libitum daily but were not fed for a minimum of 48 h prior to use in the experimentation. Crabs were killed following anesthesia on ice. All procedures were conducted in accordance with the Guidelines for Animal Experimentation established by the Okayama University.

Experimental design

Each crab in 30-ppt seawater was transferred to an individual 2-L aquarium with 10-ppt salinity (seawater diluted with dechlorinated fresh water), 30-ppt salinity (control), or 50-ppt salinity (seawater supplemented with artificial sea salt, GEX, Osaka, Japan) and submerged in these media. The salinity was checked with a refractometer, and its osmolality later confirmed with a vapor pressure osmometer (Wescor Inc. 5500, Logan, UT, USA). The water in the tanks was replaced daily. The eight gills were dissected out from the crabs acclimated for 10 days for the measurement of Na^+/K^+-ATPase activity. The gills were blotted and placed in ice-cold SEI buffer (250 mM sucrose, 10 mM di-sodium EDTA, 50 mM imidazole, pH 7.3) and frozen immediately at −80°C. A separate group of crabs was transferred from 30 ppt to the different salinities or to aquaria without water (terrestrial condition), and subsamples of this group were sampled on day 2 and day 10. Due to a high mortality rate (>50%) 3 days after water deprivation, crabs could only be acclimated to the terrestrial condition for 2 days, and mortality was less than 5% in all groups sampled. Hemolymph (0.2 ml) was withdrawn from the arthrodial membrane of the walking legs, immediately centrifuged (5 min at 6,000×*g*), and the supernatant was analyzed. The posterior gills (G6 to G8) where Na^+/K^+-ATPase activity was high in 10- to 50-ppt salinity (see Results) were used for Na^+/K^+-ATPase analysis. To assess the effects of osmotic conditions on body mass, the crabs were weighed with a Shimadzu AB54 balance (Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan; accuracy ±1 mg) after the visible water had been removed with adsorbent tissue. Only specimens in intermolt stage C were retained for analysis (Drach and Tchernigovtzeff 1967; Fukui 1993; Drach 1939).

Determination of osmolality and ionic concentrations

The osmolality and Cl^- concentration were measured on 5-μl samples (diluted 1:1 with deionized water) using the vapor pressure osmometer and a digital chloride meter (Buchler, Lenexa, KS, USA), respectively. Determinations of Na^+, K^+, and Ca^{2+} were made on 50-μl samples (diluted 1:3 with deionized water) using ion-specific electrodes of an electrolyte analyzer (AVL 984-S, Graz, Austria). In the case of calcium, total (free plus complexed) calcium was also analyzed since about 20% of calcium in the hemolymph of intermolt crabs is a complexed moiety unlike other ions (Wheatly 1999; Robertson 1960). A 5-μl aliquot of hemolymph was diluted 1:400 in deionized H_2O, and total calcium concentrations were determined by an atomic absorption spectrophotometer (Hitachi Z5300, Tokyo, Japan).

Assay of Na^+/K^+-ATPase enzyme activity

The Na^+/K^+-ATPase activity was determined with a linked pyruvate kinase/lactate dehydrogenase-NADH assay (McCormick 1993). Gill tissue was homogenized in ice-cold 0.1%-deoxycholate SEI buffer (1:9 *w*/*v*) and centrifuged at 5,000×*g*. The resulting supernatant was diluted and assayed for Na^+/K^+-ATPase activity. Each sample of gill homogenate was plated in quadruplicates of 10 μl, two contained 2.8 mM ouabain and two did not. Fifty microliters of salt solution (50 mM imidazole, 189 mM NaCl, 10.5 mM $MgCl_2$, and 42 mM KCl) and 150 μl of assay mixture (50 mM imidazole, 2 mM phosphoenolpyruvate, 0.16 mM nicotinamide adenine dinucleotide, 0.5 mM adenosine

triphosphate, 3.3 U/ml lactic dehydrogenase, and 3.6 U/ml pyruvate kinase) were added to each well. The kinetic assay was read at a wavelength of 340 nm at 24°C with a run time of 10 min and intervals of 10 s. The difference between the kinetic reading with and without ouabain is the Na^+/K^+-ATPase activity and is expressed as micromoles ADP per milligram protein per hour. Total protein in homogenates was measured using a BCA Protein Assay kit (Pierce Chemical Co., Rockford, IL, USA). Assays were run on a microplate reader (Multiskan Ascent, Thermo Electron Corporation, Vaanta, Finland).

For validation of this system, standard conditions described above were employed, varying one factor while keeping all the other parameters constant. Inhibition by ouabain corresponding to the actual measurement of Na^+/K^+-ATPase activity, as a function of ouabain concentration in the reaction mixture, was first examined. Under the standard conditions, final concentrations of ouabain in the reaction mixture varied from 0, 0.5, 1.4, 2.8, and 5.0 mM in wells, and maximal inhibition was observed at 2.8 mM ouabain, and thus this concentration was fixed in the examination of other parameters in the remainder of the validation. Optimal conditions for actual analyses of response to changing environmental condition were set according to such results. In examinations of the effects of gill protein concentration on the enzymatic activity, it was seen that in a sample consisting of 0.1 mg protein/10 μl of 0.1%-deoxycholate SEI buffer diluted from 2- to 16-fold, activity decreased linearly in proportion to the protein quantity. Therefore, measurements were valid for samples diluted at least twofold, but samples were usually diluted tenfold in this investigation.

Statistical analyses

Statistics were performed using Statview 4.11 (Abacus Concept). Since there was a significant interaction between the treatment (environmental condition) and time by two-way ANOVA, data for day 2 and day 10 were analyzed separately by the appropriate *post hoc* test to determine the differences between the control (30 ppt) and treatments (different environmental conditions). All data were checked for normality and equal variances. Where assumptions of normality or equal variances were not satisfied, equivalent nonparametric tests were used.

Results

Body mass

Exposure of *G. depressus* to salinities of 10, 30, or 50 ppt did not result in any significant change in body mass ($P > 0.05$), while the loss of 10% in the crabs after exposure to terrestrial conditions was significant ($P < 0.001$, Figure 1a).

Hemolymph osmotic and ionic status

In crabs exposed to 10-ppt salinity (331 mOsm), hemolymph osmolality decreased after 2 days to its new acclimation level at 580 mOsm ($P < 0.001$, Figure 1b) and then did not change significantly ($P > 0.05$) thereafter. After exposure to 50 ppt (1,660 mOsm) and terrestrial conditions, hemolymph osmolality increased ($P < 0.001$), although the

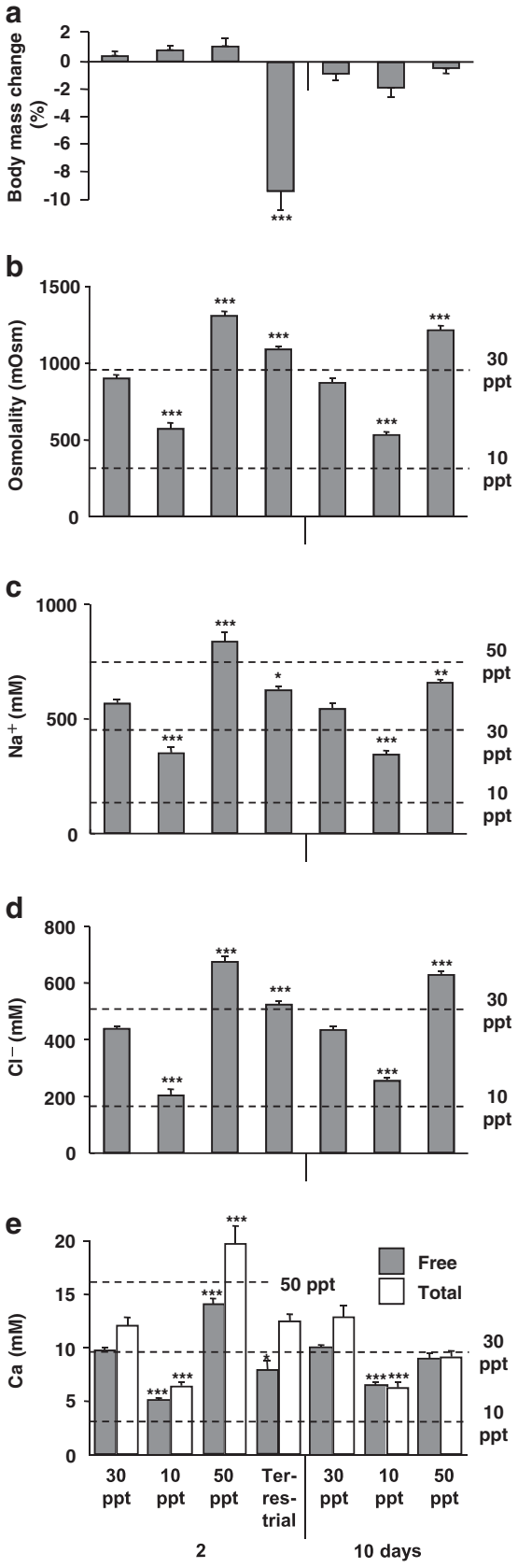

Figure 1 (See legend on next page.)

(See figure on previous page.)
Figure 1 The wet body weight (a) as well as hemolymph osmotic and ionic concentrations (b to f) of *G. depressus*. Change in the wet body weight (%) as well as osmotic and ionic concentrations in the hemolymph of *G. depressus* under various environmental conditions for 2 and 10 days after being transferred from 30-ppt seawater. Dotted line indicates osmotic and ionic concentrations of the media. Mean ± SE (N = 4 to 10) is indicated. Means with asterisks are significantly different from the means of the control (30 ppt) on the same day (asterisk indicates $P < 0.05$; double asterisk, $P < 0.01$; triple asterisk, $P < 0.001$).

levels in 50-ppt seawater also stabilized by day 10 to new acclimated values which were about 350 mOsm hypoosmotic to the medium. The osmoregulatory performance of crabs acclimated for 10 days in various salinity media is shown in Figure 2a.

At 30 ppt, hemolymph was isoionic to the medium in the case of the three ions, Na^+, Cl^-, and Ca^{2+} (Figure 1c,d,e). A virtually identical pattern was seen for the major hemolymph ions, Na^+ and Cl^-. The concentrations of these ions decreased and increased 2 days after being transferred to 10 and 50 ppt, respectively ($P < 0.001$). Thereafter, they appeared to stabilize at new acclimated values, although the Na^+ levels after transfer to 50 ppt decreased on day 10 compared to those on day 2 (Figure 1c,d). Changes in hemolymph K^+ concentrations also paralleled to those of osmolality, Na^+ and Cl^- (data not shown).

The changes in K^+, Na^+, and Cl^- showed a relationship with changes in hemolymph osmolality, but free Ca^{2+} showed different patterns after exposure to 50 ppt and terrestrial conditions (Figure 1e). After being transferred to 50 ppt, both the free and total calcium increased significantly ($P < 0.001$) on day 2, but returned to the levels similar to those at 30 ppt on day 10. In crabs kept out of the water, free Ca^{2+} decreased slightly but significantly ($P < 0.05$). Furthermore, the complexed calcium (total minus free calcium) virtually disappeared from the hemolymph on day 10 in 10 and 50 ppt, indicating that all the hemolymph calcium was ionized, and the concentrations appeared to be maintained at acclimated values. The calcium regulatory performance of crabs acclimated for 10 days in various salinity media is shown in Figure 2b.

Branchial Na^+/K^+-ATPase activity

Branchial Na^+/K^+-ATPase activity was heterogenously distributed among the eight gill pairs in crabs acclimated to 30, 10, or 50 ppt for 10 days (Figure 3a). Activity was high in the posterior three gills, G6 to G8. This distribution and the absolute values of the activity reported here were similar to those reported in the literature (see the 'Discussion' section).

The responses of Na^+/K^+-ATPase activity after exposure to various environmental conditions were examined in the posterior gills (G6 to G8) since the above results (Figure 3a) demonstrated the high levels of Na^+/K^+-ATPase activity in these gills after acclimation to 30, 10, or 50 ppt. The Na^+/K^+-ATPase activity in G6 and G7 increased significantly during exposure to 10 ppt by day 10 ($P < 0.05$). The Na^+/K^+-ATPase activity in G8 did not change in 10 ppt ($P > 0.05$) but increased significantly 10 days after transfer to 50 ppt ($P < 0.05$). In G6 to G8 of the crabs kept out of water, there was no significant difference in the Na^+/K^+-ATPase activity ($P > 0.05$), although a threefold increase in the mean activity was observed in G6 (Figure 3b).

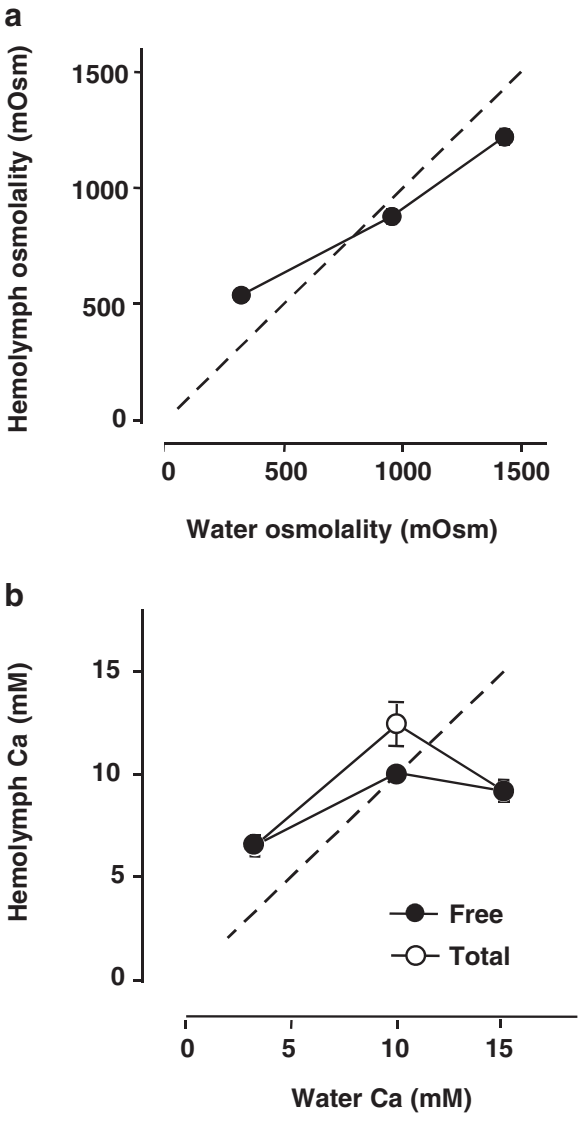

Figure 2 Osmolality (a) and calcium (b) concentration in the hemolymph of *G. depressus*. The crabs were acclimated for 10 days in 10-, 30-, or 50-ppt salinity (mean ± SE, $N = 6$ to 10), in relation to the measured values of the medium. Error bars are shown only when larger than the symbols. Dotted line indicates isosmotic and isoionic relationships, drawn from the data in Figure 1.

Discussion

The results of the transfer to 10- and 50-ppt salinities indicate that *G. depressus* is able to osmoregulate and survive at least for 10 days in these salinities as a hyper-hypoosmoregulating marine crab, and are consistent with the previous reports (Charmantier et al. 1998, 2002) on hemolymph osmolality in the other grapsid crabs (*Armases miersii* and *Chasmagnathus granulata*) as a function of the ambient salinity. Together with the results of the terrestrial exposure showing that hemolymph osmolality was in the same physiological range after 2 days despite dehydration, *G. depressus* can be considered as an appropriate model to study the osmotic/ionic regulatory mechanisms supporting salinity/terrestrial acclimation. In this investigation, it was considered necessary to obtain more basic information concerning the ionic status in

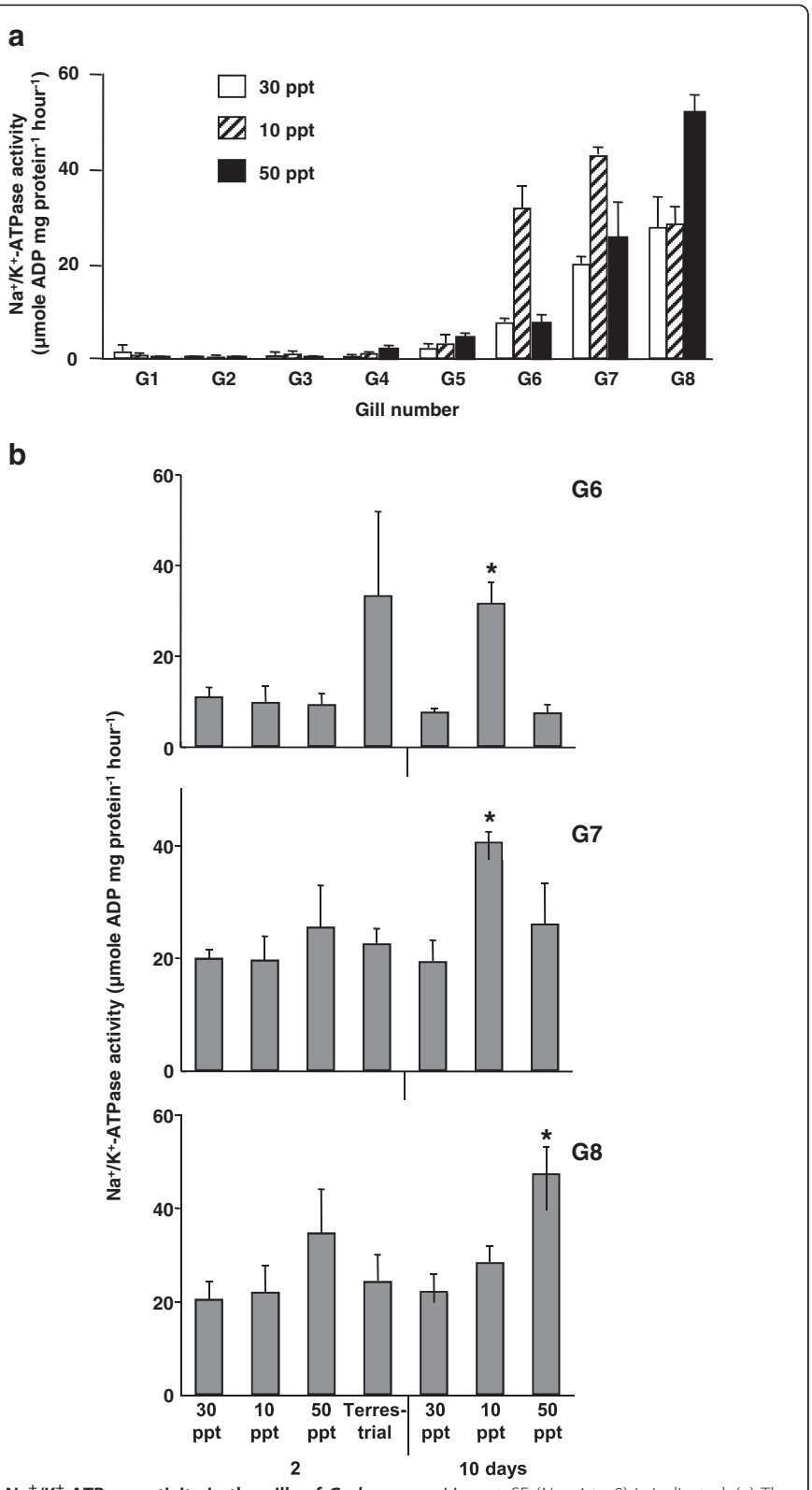

Figure 3 Na^+/K^+-ATPase activity in the gills of *G. depressus*. Mean ± SE (N = 4 to 9) is indicated. (**a**) The activity in the gills of crabs acclimated for 10 days in 10-, 30-, or 50-ppt salinity after being transferred from the 30-ppt seawater. (**b**) The activity in the posterior gills (G6 to G8) of crabs under various environmental conditions on day 2 and day 10 after being transferred from 30 ppt; means with an asterisk are significantly different from the mean of the control (30 ppt) on the same day ($P < 0.05$).

the hemolymph as well as the branchial Na^+/K^+-ATPase activity as a prerequisite for further studies.

Regarding ionic changes in the hemolymph, changes in Na^+ and Cl^- generally paralleled those in osmotic concentrations in response to high and low salinity exposure as well as to water deprivation, and it is likely that altered osmolality of the hemolymph under varying environmental conditions was based for the most part on altered levels of Na^+ and Cl^- (Wilder et al. 1998). An interesting finding of this study, however, is the pattern of hemolymph calcium levels, particularly for those of complexed calcium. Slightly lower levels of hemolymph free Ca^{2+} in crabs kept out of water seems to be related to the fasted condition since intermolt terrestrial crabs regulate hemolymph calcium by controlling intake of dietary calcium (Wheatly 1999; Zanotto and Wheatly 2002). In crabs acclimated to 10-ppt salinity, free Ca^{2+} was maintained at twofold higher values than concentrations of the medium, and complexed calcium disappeared from the hemolymph after 10 days. In crabs exposed to 50-ppt salinity, free Ca^{2+} was regulated to the lower levels than those in the medium through the experiment while total calcium increased to higher levels after 2 days. These responses may indicate that hemolymph complexed calcium could serve as an internal reserve for maintaining free Ca^{2+} levels in the hemolymph. On the other hand, the complexed calcium decreased dramatically and disappeared from the hemolymph after 10 days in 50 ppt, and we speculate that this represents a surplus (unnecessary) calcium reservoir in the hemolymph for prolonged period in higher Ca^{2+} environment. Calcium regulation in various environments has been studied in crustaceans, mostly with respect to the control of epithelial calcium transport (Freire et al. 2008; Ahearn et al. 2004; Wheatly 1999; Zanotto and Wheatly 2002), and the role of complexed calcium in the hemolymph as a reserve for free Ca^{2+} is unknown. Our findings, therefore, suggest a new control mechanism of hemolymph free Ca^{2+} and imply that hemolymph concentrations of both total and free calcium need to be analyzed. At any rate, it appears that it is necessary to regulate free Ca^{2+} to a specific range and that this control is separate from the osmoregulatory mechanisms.

One of the ion transporters that has received the most intensive studies in osmoregulating crustaceans is Na^+/K^+-ATPase (Bianchini et al. 2008; Lucu and Towle 2003; Pequeux 1995; Henry et al. 2002; Towle et al. 1997, 2011; Ahearn et al. 1999; Serrano and Henry 2008; Towle and Weihrauch 2001). In addition to the Ca^{2+} channel, Ca^{2+}-ATPase, and Na^+/Ca^{2+} exchanger, the transportation of Ca^{2+} in the hemolymph of crustaceans is also affected by the potential energy of the Na^+ gradient, established by Na^+/K^+ ATPase activity (Roer and Dillaman 1993). In this study, we observed higher Na^+/K^+-ATPase activity in the posterior gills when compared with the anterior gills (see Figure 3a), consistent with the molecular biological and physiological studies on many crab species (Bianchini et al. 2008; Lucu and Towle 2003; Pequeux 1995; Towle and Weihrauch 2001; Freire et al. 2008; Charmantier et al. 2009; Siebers et al. 1982; Onken and Putzenlechner 1995), which have designated the posterior gill epithelium, with its high abundance of Na^+/K^+-ATPase activity, as the principal site of osmoregulatory ion transport. These differences constitute the basis of the paradigm that anterior gills are structurally and functionally specialized for respiratory gas exchange, while the posterior gills have become specialized for active ion absorption counterbalancing passive losses in dilute media (Freire et al. 2008; Charmantier et al.

2009) as reflected in the significant increases in the Na^+/K^+-ATPase activity of G6 and G7 after acclimation to 10-ppt salinity (Figure 3b).

Following these observations, the present investigation provides an interesting finding that acclimation to 50-ppt salinity for 10 days, in which hypo-ionoregulation occurred, was accompanied by increased Na^+/K^+-ATPase activity exclusively in G8 (Figure 3b), suggesting that G8 may participate in ion excretion into the concentrated media. These different responses of the enzyme activity among the individual gills indicate a gill-specific pattern of the regulation and a higher degree of specialization in gill function in *G. depressus*. Together with the differences between the terrestrial condition and 50-ppt salinity, the increased Na^+/K^+-ATPase activity is not simply part of a cellular regulation since the cells were exposed to the similar osmo/ionic stresses. A study with the marble shore crab (*Pachygrapsus marmoratus*) also showed that the abundance of Na^+/K^+-ATPase mRNA induced in all nine gills following the transfer of crabs to low salinity but increased only in G7 after being transferred to high salinity (Jayasundara et al. 2007). Our enzyme activity results for *G. depressus* support the notion that individual gills do indeed play distinct osmoregulatory roles in euryhaline crustaceans. Other transport proteins and transport-related enzymes in gills, including a Na^+/H^+ antiporter, carbonic anhydrase, and $Na^+/K^+/2Cl^-$ cotransporters (Bianchini et al. 2008; Pequeux 1995; Henry et al. 2002; Towle et al. 1997, 2011; Serrano and Henry 2008; Jillette et al. 2011; Ahearn et al. 2004; Wheatly 1999), might be involved in the specificity of function. For example, a basolateral $Na^+/K^+/2Cl^-$ cotransporter involved in NaCl excretion appears to be induced during acclimation to concentrated seawater (Freire et al. 2008; Luquet et al. 2005). To further develop *G. depressus* as a new model for the study of salinity acclimation in crabs, future investigations will examine the role of these transporters and possible ionocytes in the acclimation responses of this euryhaline species.

Conclusions

The hemolymph osmotic and ionic status of *G. depressus* indicates that this intertidal grapsid crab is a hyper/hypo-ionoregulating amphibious species. Especially, the free Ca^{2+} concentration was well-maintained partly by the hemolymph complexed calcium as an internal reserve. Induction of Na^+/K^+-ATPase activity in response to salinities varies between the gills. This abundant species around Japan will serve as a model to study the crustacean osmotic/ionic regulation.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TS conceived of the study, participated in its design and coordination, and drafted the manuscript. TN participated in the design of the study and performed the experiments and analyses. HT and TA participated in the design of the study, helped perform the experiments, and drafted the manuscript. WG helped perform the experiments. MO, HS, and NT helped draft the manuscript. All authors read and approved the final manuscript.

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