Light Induces an Increase in the pH of and a Decrease in the Ammonia Concentration in the Extrapallial Fluid of the Giant Clam *Tridacna squamosa*

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ABSTRACT

The objective of this study was to examine whether 12 h of light exposure would lead to an increase in the pH of and a decrease in the concentration of total ammonia in the extrapallial fluid of the giant clam *Tridacna squamosa*. We also aimed to elucidate indirectly whether movements of ammonia and/or protons (H^+) occurred between the extrapallial fluid and the outer mantle epithelium. The pH of the extrapallial fluid of *T. squamosa* exposed to 12 h of light was significantly higher than that of clams exposed to 12 h of darkness. Conversely, the total ammonia concentration in the extrapallial fluid of the former was significantly lower than that of the latter. In addition, the glutamine content in the mantle adjacent to the extrapallial fluid of clams exposed to 12 h of light was significantly greater than that of clams exposed to 12 h of darkness. These results suggest that in the extrapallial fluid of *T. squamosa* exposed to light, NH_3 combined with H^+ as NH_4^+ and that NH_4^+ was transported into the mantle and used as a substrate for glutamine formation. Injection of NH_4Cl into the extrapallial fluid led to an instantaneous increase in the total ammonia concentration therein, but the total ammonia concentration decreased subsequently and returned to the control value within 1 h. This is in support of the proposition that NH_4^+ could be transported from the extrapallial fluid to the mantle. Injection of HCl into the extrapallial fluid led to an instantaneous decrease in the pH of the extrapallial fluid. However, there was a significant increase in pH within 1 h in light or darkness, achieving a partial recovery toward the control pH value. The increase in pH within this 1-h period in light or darkness was accompanied by a significant decrease in the total ammonia concentration in the extrapallial fluid, which supports the proposition that H^+ could be transported in combination with NH_3 as NH_4^+. Therefore, our results prompt a reexamination of the previous proposition that the removal of H^+ by NH_3 can facilitate calcification in molluscs in general and an investigation of the relationship between H^+ removal through NH_4^+ transport and light-enhanced calcification in *T. squamosa*.

Introduction

The major nitrogenous excretory product in bivalves is ammonia (Bishop et al. 1983; Heavers and Hammen 1985). In aqueous solution, ammonia exists as NH_3 and NH_4^+; the equilibrium reaction can be written as $NH_3 + H_3O^+ \Leftrightarrow NH_4^+ + H_2O$. Because the pK of this reaction is around 9.0–9.5, NH_3 in water acts as a base and binds with H^+ to form NH_4^+ at neutral pH. That means at typical environmental and physiological pHs, approximately 99% of ammonia is present as NH_4^+. Biological membranes are more permeable to NH_3 than to NH_4^+; thus, in most cases, ammonia crosses membranes as NH_3. These two species of ammonia can participate in the acid-base balance of extracellular fluids in bivalves during hypoxia (Bayne et al. 1976; Shick et al. 1988) and restrict the rate of shell decalcification in bivalves exposed to low-oxygen conditions (Shick et al. 1988). In this report, NH_3 represents unionized molecular ammonia, NH_4^+ represents the ammonium ion, and total ammonia refers to the sum of NH_3 and NH_4^+.

Ammonia excretion in giant clams is of particular interest because these clams absorb ammonia from the surrounding

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waters instead of excreting ammonia. Giant clams belong to the family Tridacnidae; they inhabit shallow coral reefs throughout most regions of the tropical Indo-Pacific (Rosewater 1965). Like corals and anemones, these clams live in symbiosis with zooxanthellae (*Symbiodinium* spp). In giant clams, zooxanthellae live extracellularly in a branched tubular system, originating in the stomach, that splits into small and very thin secondary and tertiary tubes dorsally into the root of the siphonal mantle. The tertiary tubes are directly under the surface of the mantle tissues (Norton and Jones 1992; Norton et al. 1992), allowing sufficient light to penetrate for algal photosynthesis. The symbiotic zooxanthellae are capable of transferring parts of their photosynthetic products, such as glucose and glycerol, to the animal tissues (Muscatine 1967; Streamer et al. 1988; Fitt 1993). Thus, the availability of light is a critical factor affecting the growth of giant clams (Lucas et al. 1989).

Zooxanthellae in tridacnids are thought to be nitrogen limited, based on their ability to take up ammonia and nitrate (Wilkerson and Trench 1986), observations of increased photosynthesis with additions of ammonia (Summons et al. 1986), and increased growth rates of clams with the addition of dissolved inorganic nitrogen (Hastie and Heslinga 1988; Onate and Naguit 1989; Hastie et al. 1992). Results obtained by Hawkins and Klumpp (1995) indicate that ammonia-N produced by the host is freely available for assimilation and recycling by the zooxanthellae in *Tridacna gigas*. Rees et al. (1993*a*, 1993*b*) reported that the immediate source of dissolved inorganic nitrogen for zooxanthellae in *T. gigas* was the hemolymph, which always has detectable levels of ammonia (Fitt et al. 1993). As a result, tridacnids excrete little or no ammonia, except when incubated in darkness (Cates and McLaughlin 1976; Muscatine and D'Elia 1978; Wilkerson and Muscatine 1984; Szmant and Gassman 1990).

Besides the hemolymph, the extrapallial fluid also contributes significantly to the extracellular fluid volume in bivalves, and we suspected that a decrease in the ammonia concentration could also occur in the extrapallial fluid of giant clams exposed to light. Therefore, we hypothesized that the concentration of total ammonia in the extrapallial fluid of giant clams was photodependent. Thus, the first objective of this study was to determine whether the total ammonia concentration in the extrapallial fluid of the local giant clam *Tridacna squamosa* exposed to 12 h of light was significantly lower than that of clams exposed to 12 h of darkness. In addition, because NH_3 and NH_4^+ participate in acid-base balance (see above), the second objective was to elucidate whether changes in the pH of the extrapallial fluid in *T. squamosa* occurred during the dark-light cycle. The hypothesis tested was that exposure to light would lead to a decrease in the concentration of total ammonia in and a simultaneous increase in the pH (due to the removal of NH_3 as NH_4^+) of the extrapallial fluid of *T. squamosa*.

Ammonia assimilation in giant clams is achieved primarily by glutamine synthesis, catalyzed by the enzyme glutamine synthetase (GS; Shepherd et al. 1999). GS has been shown to be present in both the host and the zooxanthellae fractions of several alga-invertebrate associations (Rees 1986; Rees et al. 1989, 1994; Yellowlees et al. 1994). Thus, the assimilation of environmental ammonia by these symbiotic associations, which was once thought to involve primarily the algal symbiont, is apparently facilitated in part by the invertebrate host (Rees 1986; Rees et al. 1994; McAuley 1995). Therefore, the third objective of this study was to determine whether the decrease in total ammonia concentration in the extrapallial fluid was accompanied by an increase in glutamine content in the mantle, specifically in the region adjacent to the extrapallial fluid within the pallial line, of *T. squamosa*.

There is a dearth of knowledge on the movement of ammonia between the extrapallial fluid and the mantle of giant clams. Thus, the fourth objective of this study was to demonstrate indirectly that changes in the total ammonia concentration in the extrapallial fluid could be related to changes in movements of NH_3 and/or NH_4^+ across the outer mantle epithelium and that a relationship exists between the movements of protons (H^+) and NH_4^+ in *T. squamosa*. In this study, a novel approach was adopted that involved the insertion of polyethylene tubing into the extrapallial space between the outer surface of the mantle and the inside surface of the shell of the giant clam. Through this tubing, known quantities of ammonia (as NH_4Cl) or H^+ (as HCl) were delivered directly into the extrapallial fluid to achieve artificially a significant increase in the total ammonia concentration or a significant decrease in pH, respectively. Then, the clearance of the injected ammonia, manifested as a decrease in the total ammonia concentration, and the removal of H^+, manifested as an increase in the pH, were monitored for a period of 6 h in light or darkness. Because the extrapallial fluid was bound on one side by the valve and on the other side by the mantle tissues without any direct link with the external medium, results obtained would indirectly provide information on the direction and magnitude of the NH_4^+ and H^+ movements from the extrapallial fluid to the mantle tissues across the outer mantle epithelium.

Material and Methods

Animals

Specimens of *Tridacna squamosa* (25–40 cm) were collected from Indonesia. Clams were maintained in aerated seawater (31‰–32‰) at 28°C in glass aquaria (80 cm × 40 cm × 40 cm) under a 12L (600 lux) : 12D regime. They were fed live *Artemia* larvae daily, but the food supply was stopped 24 h before experiments. The seawater was changed every 2 d. Clams were acclimated to laboratory conditions for at least 1 wk before experiments. Three series of experiments were performed.

Series 1

Giant clams were exposed to 12 h of light ($n = 4$) or 12 h of darkness ($n = 4$), as stated above. For total darkness, aquaria were covered completely with several layers of black plastic sheeting. At the end of the 12-h period, clams were forced open, and the abductor muscles were cut. The extrapallial fluid was drawn into a syringe, and the pH was recorded immediately using a glass pH electrode (Trizma, Sigma) and an Orion 720A pH meter. The collected extrapallial fluid was centrifuged at 4,000 *g* at 4°C for 10 min and kept at −35°C until analysis (within 1 mo). A portion of supernatant obtained was deproteinized in an equal volume (v/v) of ice-cold 6% trichloroacetic acid and centrifuged at 10,000 *g* at 4°C for 15 min.

Mantle tissues were dissected from two regions; one region was adjacent to the extrapallial fluid within the pallial line (mantle 1), and the other region included the extensible middle and inner folds above the pallial line (mantle 2). Samples of adductor muscle, foot muscle, and hepatopancreas were also dissected out. They were blotted dry and immediately freeze-clamped with liquid-nitrogen-precooled aluminum tongs. Frozen samples were kept at −80°C until analysis. The frozen samples were weighed, ground to a powder in liquid nitrogen, and homogenized three times in 5 volumes (w/v) of 6% trichloroacetic acid at 24,000 revolutions min^{-1} for 20 s each, with intervals of 10 s between each homogenization. The homogenate was centrifuged at 10,000 *g* at 4°C for 15 min to obtain the supernatant.

For ammonia analysis, the pH of the deproteinized sample was adjusted to 6.0 with 2 mol L^{-1} $KHCO_3$. The ammonia content was determined by the method of Bergmeyer and Beutler (1985). Freshly prepared NH_4Cl solution was used as the standard for comparison. For free amino acid analysis, the supernatant obtained was adjusted to pH 2.2 with 4 mol L^{-1} lithium hydroxide and diluted appropriately with 0.2 mol L^{-1} lithium citrate buffer (pH 2.2). Free amino acid analysis was performed using a Shimadzu LC-6A amino acid analysis system (Kyoto) with a Shim-pack ISC-07/S1504 Li-type column. Although a complete analysis was performed for each sample, only contents of glutamine and total free amino acid (TFAA) are presented in this report. Results are expressed as micromoles per gram of wet mass tissue or micromoles per milliliter of extrapallial fluid.

In order to estimate the ammonia concentration in mantle 1, the water contents of mantle 1 samples were determined as the difference between the wet mass and dried mass of the sample, obtained before and after being kept in an oven at 95°C for 36 h. To estimate the proportion of NH_3 to NH_4, the intracellular pH of mantle 1 tissue was determined on homogenized frozen tissue using the method described by Pörtner et al. (1990). The frozen sample was ground under liquid nitrogen to a fine powder using a precooled pestle and mortar. A small portion of the sample powder (approximately 150 mg) was rapidly transferred to a preweighed 0.5-mL Eppendorf tube containing 300 μL of inhibitor medium to stop further metabolism. This medium was made up immediately before use and contained 130 mmol L^{-1} potassium fluoride and 6 mmol L^{-1} nitrilotriacetic acid, adjusted to pH 7 with KOH. After reweighing, the Eppendorf tube containing the tissue was filled with a known volume of the inhibitor medium, stirred briefly with a mounted needle to release bubbles, and capped tightly. The contents were mixed mechanically and centrifuged for about 15 s before pH measurements. Intracellular pH was calculated according to the equations of Pörtner et al. (1990).

Series 2

Giant clams ($n = 10$) were anesthetized in 0.05% phenoxyethanol in seawater. After 10–15 min, the clams were removed from seawater. A tiny opening was made along the pallial line between the mantle and the shell with a blunt metal rod. For each giant clam, only one valve was operated on. Polyethylene tubing (PE 190) was inserted through this opening to a depth of approximately 7–8 cm in the extrapallial space. The tubing was secured in position at the pallial line with superglue, which also sealed up the opening. The operation was regarded as successful if extrapallial fluid could be withdrawn into a 10-mL syringe. Then, the free end of the tubing was plugged temporarily, and the clam was returned to seawater. After 2 d of adaptation back to the 12L : 12D regime, extrapallial fluid were sampled through the tubing at 0 h (at the end of 12 h of darkness) and after 1, 6, 9, and 12 h of exposure to light. In order to compensate for the dead volume of the tubing, an aliquot (5 mL) of extrapallial fluid was slowly drawn into the syringe and immediately injected back to the clam before the sampling of 2 mL of fluid for the determination of pH and ammonia content, as described above.

Series 3

This series of experiments was performed with two groups of operated-on clams with inserted tubing. Two days after the operation, one group was injected with NH_4Cl ($n = 6$) and immediately exposed to light ($n = 3$) or darkness ($n = 3$); another group was injected with HCl ($n = 10$) and immediately exposed to light ($n = 5$) or darkness ($n = 5$). Just before the injection of NH_4Cl or HCl, extrapallial fluid (2 mL) was sampled from each individual clam for the determinations of total ammonia or pH, respectively, as described above. These results (as stated in the legends to Figs. 2 and 3) were compared with those obtained immediately after the injection of NH_4Cl (Fig. 2*A*) or HCl (Fig. 3*B*) to verify that a change in ammonia concentration or pH, respectively, had indeed occurred. Efforts were made in the series 1 experiments to determine the volume of extrapallial fluid in a valve of *T. squamosa* (approximately 35 mL for a 30-cm clam) in order to estimate the amount of

NH_4Cl or HCl to be injected into the extrapallial fluid to achieve a total ammonia concentration of 0.4–0.5 mmol L^{-1} or a pH of 6.2–6.3, respectively. It was important not to lower the pH below 6.2 so that drastic decalcification would not occur. To a 10-mL syringe, approximately 150 μL of 10-mmol-L^{-1} NH_4Cl or 10 μL of 1-mmol-L^{-1} HCl was introduced before the syringe was connected to the free end of the tubing inserted into the clam. Extrapallial fluid (10 mL) was drawn into the syringe to mix with the NH_4Cl solution and then injected back into the clam. This mixing process was repeated twice, which facilitated the complete delivery of the NH_4Cl or HCl solution into the pallial fluid. An aliquot of 2 mL of extrapallial fluid was then collected immediately after the injection for total ammonia and pH determinations (results presented as "injection" in Figs. 2, 3). Samples were also collected at hours 1, 3, and 6 for determination of the total ammonia concentration and pH.

Statistical Analysis

Results are presented as mean ± SEM. Differences between means were evaluated by repeated-measures analysis followed by a Bonferroni test (for figures) or a two-tailed Student's *t*-test (for tables). Any difference with $P < 0.05$ was regarded as statistically significant.

Results

Results obtained from series 1 experiments verified that the pH of the extrapallial fluid of *Tridacna squamosa* ($n = 4$) exposed to 12 h of light was 7.818 ± 0.021, which was significantly higher than that (7.754 ± 0.010) of clams exposed to 12 h of darkness. Conversely, the total ammonia concentration in the extrapallial fluid of the former was significantly lower than that of the latter (Table 1). In contrast, there were no significant differences in total ammonia contents in mantle 1, mantle 2, abductor muscle, foot muscle, and hepatopancreas between these two groups of clams (Table 1). The glutamine and TFAA contents in mantle 1 (the region adjacent to the extrapallial fluid) of clams exposed to 12 h of light were significantly greater than the corresponding values of clams exposed to 12 h of darkness (Table 2). However, no changes in glutamine and TFAA contents were observed in the extrapallial fluid, mantle 2, abductor muscle, foot muscle, and hepatopancreas (Table 2). Mantle 1 tissues had a water content of 79.5% ± 2.6%. The intracellular pH of mantle 1 tissues from clams exposed to 12 h of light (7.478 ± 0.025, $n = 4$) was not significantly different from that of clams exposed to 12 h of darkness (7.421 ± 0.021, $n = 4$).

Results obtained in series 2 experiments with the extrapallial fluid being withdrawn through the tubing reveal that changes in the pH (Fig. 1*A*) of and the total ammonia concentration (Fig. 1*B*) in this fluid occurred within 1 h after *T. squamosa* was exposed to light. Injection of NH_4Cl into the extrapallial fluid led to an instantaneous increase in ammonia concentration therein. However, the total ammonia concentration decreased significantly and returned to the control value within 1 h in light or darkness (Fig. 2*A*). In light or darkness, injection of NH_4Cl had no significant effects on the pH of the extrapallial fluid (Fig. 2*B*). On the other hand, the injection of HCl into the extapallial fluid led to an instantaneous decrease in extrapallial fluid pH (Fig. 3*A*), but there was a significant increase in pH within 1 h in light or darkness, achieving a partial but close to complete recovery of the control pH value (Fig. 3*A*). The increase in pH within this 1-h period in light or darkness was accompanied by a significant decrease in the total ammonia concentration in the extrapallial fluid (Fig. 3*B*).

Table 1: Effects of 12 h of light or 12 h of darkness on the concentrations (μmol mL^{-1} extrapallial fluid or μmol g^{-1} wet mass tissue) of ammonia in *Tridacna squamosa*

	Light	Dark
Extrapallial fluid	.021 ± .001	.041 ± .005*
Mantle 1	.69 ± .14	.44 ± .10
Mantle 2	.42 ± .07	.46 ± .09
Abductor muscle	.73 ± .21	.44 ± .04
Foot muscle	.61 ± .11	.65 ± .09
Hepatopancreas	2.9 ± 1.2	3.7 ± .7

Note. Mantle 1 = mantle within the pallial line; mantle 2 = mantle outside the pallial line. Values represent mean ± SEM ($N = 4$).

* Significantly different from the light condition; $P < 0.05$.

Discussion

The Concentration of Ammonia in the Extrapallial Fluid Was Low

The concentration of total ammonia in the extrapallial fluid of *Tridacna squamosa* ranged between 0.02 and 0.04 μmol mL^{-1} (Table 1). Since the extrapallial fluid had a pH of approximately 7.66 (for clams kept in darkness; Fig. 1), the P_{NH_3} and NH_4^+ concentration were estimated to be 5–10 mmHg and 0.018–0.037 mmol L^{-1}, respectively. In comparison, the mantle of *T. squamosa* had a total ammonia content of 0.44–0.69 μmol g^{-1} (Table 1). Based on a water content of 80%, the estimated total ammonia concentration was 0.55–0.86 μmol mL^{-1}. Because the tissue of mantle 1 had an intracellular pH of 7.4, the estimated P_{NH_3} and NH_4^+ therein were 21–32 mmHg and 0.52–0.82 mmol L^{-1}, respectively. Thus, ΔP_{NH_3} was driving NH_3 from the outer mantle epithelium to the extrapallial fluid. Simultaneously, a positive gradient of NH_4^+ existed between the outer mantle epithelium and the extrapallial fluid. These results indicate that ammonia concentrations in the extrapallial fluid and mantle tissues were not in equilibrium, and therefore it became essential to understand how and why the steady state concentration of ammonia in the extrapallial fluid of *T. squamosa* was maintained at a low level.

It is important to distinguish the steady state concentration

Table 2: Effects of 12 h of light or 12 h of darkness on the concentrations (μmol mL^{-1} extrapallial fluid or μmol g^{-1} wet mass tissue) of glutamine and total free amino acid (TFAA) in *Tridacna squamosa*

	Glutamine		TFAA	
	Light	Dark	Light	Dark
Extrapallial fluid	.15 ± .08	.095 ± .018	.31 ± .11	.44 ± .04
Mantle 1	2.0 ± .5	.63 ± .20*	9.0 ± 1.5	3.8 ± .9*
Mantle 2	2.09 ± .43	2.11 ± .41	13.4 ± .54	13.1 ± 3.9
Abductor muscle	9.0 ± 2.2	12 ± 3	95 ± 17	113 ± 8
Foot muscle	1.4 ± .4	1.2 ± .2	19 ± 4	24 ± 10
Hepatopancreas	.64 ± .26	.65 ± .18	15 ± 3	15 ± 3

Note. Mantle 1 = mantle within the pallial line; mantle 2 = mantle outside the pallial line. Values represent mean ± SEM ($N = 4$).

* Significantly different from the light condition; $P < 0.05$.

of total ammonia in the extrapallial fluid from movements of ammonia (NH_3 and/or NH_4^+) into (influx) and out of (efflux) this compartment. Low concentrations of ammonia do not necessarily equate with low ammonia fluxes, although fluxes are involved in maintaining concentrations. Because a positive ΔP_{NH_3} existed between the mantle tissues and the extrapallial fluid of *T. squamosa* and because gaseous NH_3 can permeate biomembranes freely, it can be deduced that there was a great influx of NH_3 from the mantle tissues to the extrapallial fluid. Therefore, from the low steady state concentration of total ammonia ($NH_3 + NH_4^+$) in the extrapallial fluid, we can further deduce that there was an efflux of ammonia (NH_3 and/or NH_4^+) of similar magnitude from the extrapallial fluid back to the outer mantle epithelium. While NH_3 could diffuse passively from the outer mantle to the extrapallial fluid down a positive ΔP_{NH_3}, it could not move from the latter to the former spontaneously because of the negative ΔP_{NH_3}, not even when the pH of the extrapallial fluid increased by 0.08 (Fig. 1) in clams exposed to light. Thus, the only way to maintain the low level of total ammonia in the extrapallial fluid was through the active transport of NH_4^+ from the extrapallial fluid into the adjacent mantle tissue. In fact, a net uptake of ammonia from the medium is known to occur in giant clams exposed to environmental ammonia concentrations as low as 10 μmol L^{-1} or less (Fitt et al. 1993), which supports the proposition that giant clams have the ability to actively transport NH_4^+ despite the lack of information on the nature and location of the mechanism(s) involved. To date, active NH_4^+ transport is known to occur in the inner mitochondrial membrane (Campbell 1991), the gills of crabs (Weihrauch et al. 1999, 2002), the gills of the giant mudskipper *Periophthalmodon schlosseri* (Randall et al. 1999; Chew et al. 2003; Ip et al. 2004*a*), epithelial tissues of the African catfish *Clarias gariepinus* (Ip et al. 2004*b*), and certain regions of mammalian kidney tubules (Good and Knepper 1985; Knepper et al. 1989). If active NH_4^+ transport indeed occurred across the outer mantle epithelium of *T. squamosa*, it would imply that NH_3 has to combine with H^+ in the extrapallial fluid before being transported into the mantle.

The pH of and the Concentration of Total Ammonia in the Extrapallial Fluid Were Photodependent

The extrapallial fluid of *T. squamosa* exposed to 12 h of light was significantly more alkaline than that of clams exposed to

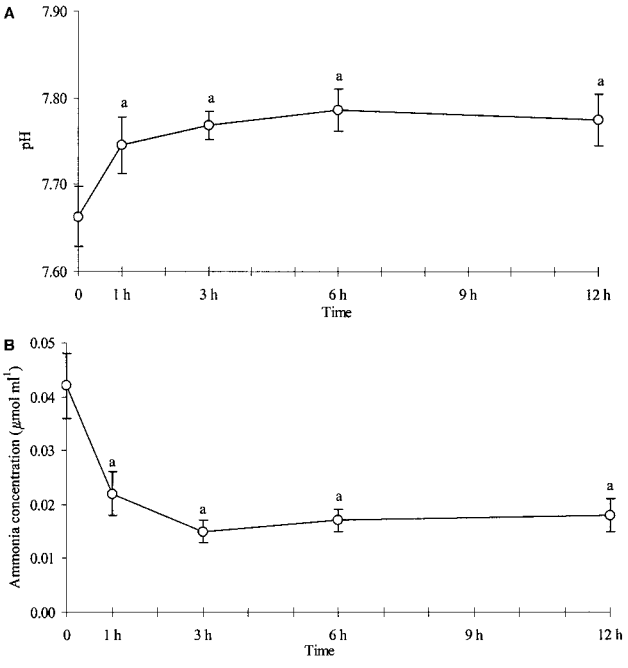

Figure 1. Effects of exposure to 12 h of light on the pH of (*A*) and the ammonia concentration (μmol mL^{-1}) in (*B*) the extrapallial fluid of *Tridacna squamosa*. Error bars represent SEM ($N = 10$). *a* = significantly different from the value for the 0-h-dark condition; $P < 0.05$.

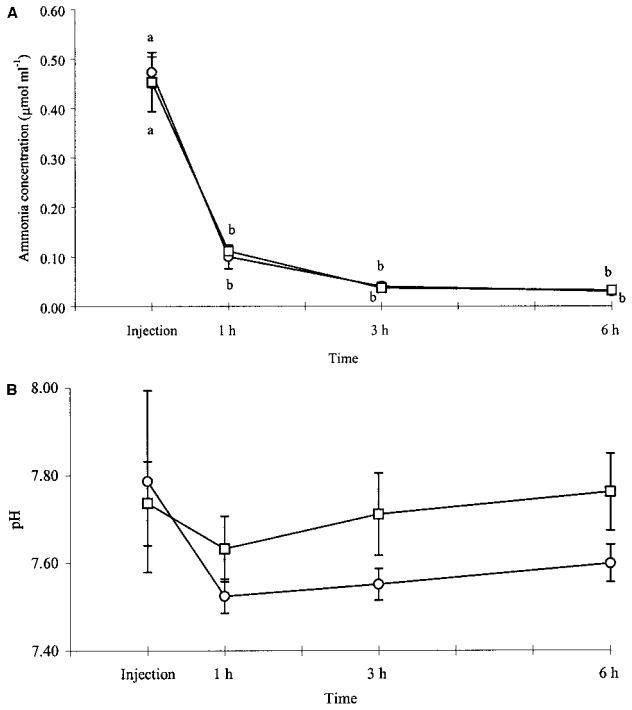

Figure 2. Effects of injection of NH_4Cl solution (pH 7.0) into the extrapallial fluid of *Tridacna squamosa* followed by 6 h of exposure to light (*squares*) or darkness (*circles*) on the ammonia concentration (μmol mL^{-1}) in (*A*) and the pH of (*B*) the extrapallial fluid. Error bars represent SEM ($N = 3$). The ammonia concentration in the extrapallial fluid sampled in the dark just before the injection with NH_4Cl was 0.049 ± 0.01 μmol mL^{-1}. *a* = significantly different from the value of 0.049 ± 0.01 μmol mL^{-1} just before the injection with NH_4Cl; $P < 0.05$. *b* = significantly different from the value immediately after the injection of NH_4Cl; $P < 0.05$.

12 h of darkness. Because the increase in the pH of the extrapallial fluid was associated with a significant decrease in the total ammonia concentration therein, these results support the proposition that NH_3 could combine with H^+ in the extrapallial fluid to form NH_4^+, which was subsequently actively transported back to the outer mantle. In essence, the removal of NH_4^+ led to the removal of H^+. Thus, the significantly lower steady state concentration of ammonia in the extrapallial fluid of clams exposed to 12 h of light, as compared with those exposed to 12 h of darkness, indicates that there was an increase in the removal of ammonia from this compartment to the mantle tissues. Because the mantle tissues had higher concentrations of NH_3 and NH_4^+, it can be concluded indirectly that the increased removal of ammonia from the extrapallial fluid involved an increase in active NH_4^+ transport through the outer mantle epithelium into the mantle tissues of *T. squamosa* in the presence of light.

Clearance of NH_4^+ Injected into the Extrapallial Fluid

When NH_4Cl was injected into the extrapallial fluid of *T. squamosa* in light or darkness, the ammonia concentration increased instantaneously and then decreased drastically back to the control level within 1 h. These results confirm that NH_4^+ could indeed be removed from the extrapallial fluid by the clam tissues. Therefore, attempts should be made in the future to elucidate the mechanisms involved in active NH_4^+ transport in the outer mantle epithelium of *T. squamosa* and other bivalves.

Because ammonia was injected into the extrapallial fluid as NH_4Cl in water at pH 7.0, in which 99% of the ammonia was represented by NH_4^+, the situation was different from that created by the movement of NH_3 into this compartment from the mantle tissue. NH_4^+ behaves as a weak acid and would dissociate to produce a proton instead of combining with a proton to remove it. Therefore, results obtained in this experiment did not give indications as to whether the removal of NH_4^+ from the extrapallial fluid would lead to a removal of H^+.

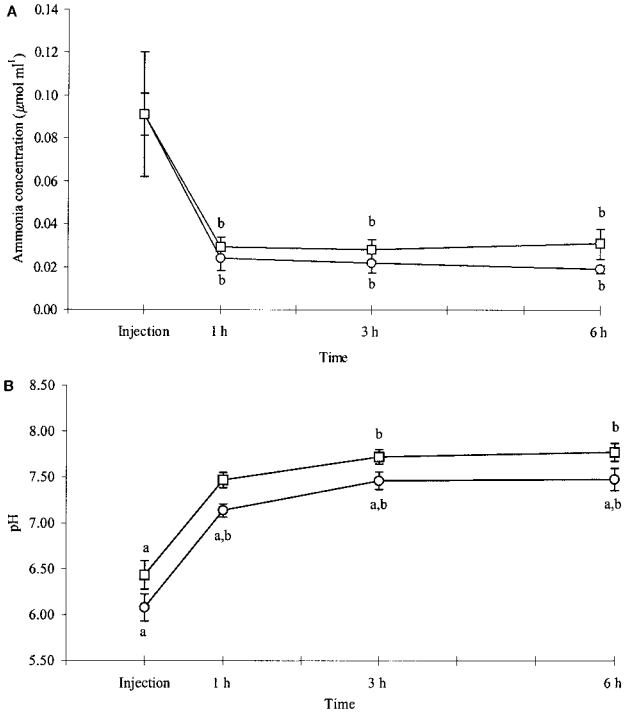

Figure 3. Effects of injection of HCl into the extrapallial fluid of *Tridacna squamosa* followed by 6 h of exposure to light (*squares*) or darkness (*circles*) on the ammonia concentration (μmol mL^{-1}) in (*A*) and the pH of (*B*) the extrapallial fluid. Error bars represent SEM ($N = 5$). The pH of the extrapallial fluid sampled in the dark just before the injection with HCl was 7.900 ± 0.115. *a* = significantly different from the value of 7.900 ± 0.115 just before the injection with HCl; $P < 0.05$. *b* = significantly different from the value immediately after the injection of HCl; $P < 0.05$.

Clearance of H^+ Injected into the Extrapallial Fluid and Its Relationship with NH_4^+ Movement

The definitive evidence supporting the proposition that changes in pH in the extrapallial fluid are related to the movement of NH_4^+ was derived from the injection of H^+ as HCl into the extrapallial fluid of *T. squamosa* in light or darkness. After the injection of HCl, the pH of the extrapallial fluid decreased instantaneously to 6.20–6.40 but returned to >7.50 within 3 h, although it did not fully recover to the control level. The partial recovery of the extrapallial fluid pH to >7.50 could not be a result of the buffering capacity of $CaCO_3$ because the pK of dissolution of $CaCO_3$ was approximately 6.7. Hence, H^+ must be removed as H^+ per se (possibly through H^+-ATPase) or as NH_4^+ from the extrapallial fluid. It is well established that epithelial H^+ transport is an important factor in the mineralization and demineralization of calcified tissues that occur during vertebrate bone mineral turnover (Blair et al. 1989) and the formation of shell structures in crustaceans (Cameron 1989; Ziegler et al. 2004). While our results do not rule out the involvement of H^+-ATPase in H^+ removal in *T. squamosa*, they support the proposition that NH_4^+ was involved in this process, because the increase in pH of the extrapallial fluid was accompanied by a decrease in the total ammonia concentration during the 1-h period after the injection with HCl. However, in this set of experiments, no difference in the rates of removal of H^+ and NH_4^+ was observed between clams exposed to light and clams exposed to darkness. This could be due to the unnatural amount of H^+ introduced into the extrapallial fluid, which initiated a high rate of H^+ and NH_4^+ removal, masking the small differences observed under normal light : dark conditions.

Removal of NH_4^+ from the Extrapallial Fluid Might Facilitate Calcification

The rate of calcification can be enhanced by the removal of H^+, because H^+ is released during the calcification process: $Ca^{++} + HCO_3^- \rightarrow CaCO_3 + H^+$. Two methods have been proposed for accomplishing H^+ removal to facilitate calcification in molluscs in general. Wheeler (1975) has proposed that H^+ reacts with HCO_3^-, forming CO_2 within the extrapallial fluid. CO_2 then diffuses down the gradient from the extrapallial fluid into the mantle tissues and hemolymph. Carbonic anhydrase present in the mantle can function to increase the rate of proton and CO_2 removal (Wheeler 1975; Sikes and Wheeler 1983). Traditionally, it has been proposed that the function of zooxanthellae in enhancing calcification rate in alga-invertebrate associations is related to the removal of CO_2 through photosynthesis (Goreau 1959; Simkiss 1976).

On the other hand, Campbell and Speeg (1969) proposed that calcification could be enhanced by the removal of H^+ by NH_3 produced through the action of urease on urea. NH_3 released through deamination of purine, purine nucleotides, and purine nucleosides could also be involved (Campbell and Boyan 1976; Loest 1979). From the activities of adenosine deaminase and urease in 14 mollusc species examined, Loest (1979) calculated that the rate of ammonia formation was more than adequate to react with H^+ released with shell growth. However, not realizing the existence of active NH_4^+ transport phenomena (Good and Knepper 1985; Knepper et al. 1989; Randall et al. 1999; Weihrauch et al. 1999, 2002; Chew et al. 2003; Ip et al. 2004*a*, 2004*b*), Simkiss (1976) suggested that ammonia production was a poor mechanism for removing H^+ because the NH_4^+ formed would penetrate plasma membranes with difficulty and so would accumulate in the extrapallial fluid. Contrary to Simkiss's (1976) suggestion, we demonstrated in this study that NH_4^+ injected into the extrapallial fluid of *T. squamosa* did not accumulate therein but was removed quickly.

Thus, the possible role of NH_3 in enhancing calcification in molluscs (Campbell and Speeg 1969) should be reexamined. At least in the case of *T. squamosa*, we demonstrated that H^+ could be removed from the extrapallial fluid after combining with NH_3 to form NH_4^+. The enhanced removal of H^+ as NH_4^+ from the extrapallial fluid in the presence of light correlates well with the fact that the rate of calcification in *T. squamosa* is light dependent. Therefore, our results in effect augment existing theories on CO_2 removal (Goreau 1959; Simkiss 1976) to explain how light-enhanced calcification occurs, and it is highly probable that multiple mechanisms are involved in light-enhanced calcification in these associations.

Possible Relationship between Glutamine Synthesis and Light-Enhanced Calcification

The ammonia-assimilating enzyme GS, which catalyzes the formation of glutamine from NH_4^+ and glutamate in the presence of ATP, has been detected in host tissues from a number of alga-invertebrate associations (Rees 1986; Rees et al. 1989; Yellowlees et al. 1994). Rees et al. (1994) reported that host GS activity decreased by 80% in gill tissues and by 45% in mantle tissue of *Tridacna gigas* maintained in continuous darkness for 8 d. Similar effects were found when clams were kept in light in the presence of elevated ammonia concentrations. Rees et al. (1994) suggested that both host and symbionts were nitrogen deficient and that host GS played a role in ammonia assimilation by the intact association. Our results support this contention, because the mantle adjacent to the extrapallial fluid (mantle 1) of *T. squamosa*, in spite of the apparent lack of photosynthetic pigments, is capable of converting NH_4^+ to glutamine. The glutamine content (Table 2) in mantle 1 of clams exposed to 12 h of light (2.00 μmol g^{-1}) was significantly greater than that of clams exposed to 12 h of darkness (0.63 μmol g^{-1}). A similar phenomenon was observed for TFAA. It is important to note that glutamine accumulation occurred only in the region of the mantle adjacent to the extrapallial fluid (man-

tle 1) and not in the extensible region of the mantle outside the pallial line (mantle 2). In addition, there were no glutamine accumulations in the foot and adductor muscles and the hepatopancreas of these clams. Because the region of the mantle adjacent to the extrapallial fluid is involved directly in the calcification process, our results suggest that light-enhanced calcification in *T. squamosa* could involve the removal of H^+ as NH_4^+ from the extrapallial fluid and the subsequent formation of glutamine in the mantle tissues. How these processes, which occur in the animal tissues, are related to photosynthesis in symbiotic zooxanthellae is uncertain, and the elucidation of these relationships awaits future study.

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