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Impacts of Salt Stress on Locomotor and Transcriptomic Responses in the Intertidal Gastropod *Batillaria attramentaria*

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Abstract. Salinity is one of the most crucial environmental factors that structures biogeographic boundaries of aquatic organisms, affecting distribution, abundance, and behavior. However, the association between behavior and gene regulation underlying acclimation to changes in salinity remains poorly understood. In this study, we investigated the effects of salinity stress on behavior (movement distance) and patterns of gene expression (using RNA sequencing) of the intertidal gastropod *Batillaria attramentaria*. We examined responses to short-term (1-hour) and long-term (30-day) acclimation to a range of salinities (43, 33 [control], 23, 13, and 3 psu). We found that the intertidal *B. attramentaria* is able to tolerate a broad range of salinity from 13 to 43 psu but not the acute low salinity of 3 psu. Behavioral experiments showed that salt stress significantly influenced snails' movement, with lower salinity resulting in shorter movement distance. Transcriptomic analyses revealed critical metabolic pathways and genes potentially involved in acclimation to salinity stress, including ionic and osmotic regulation, signal and hormonal transduction pathways, water exchange, cell protection, and gene regulation or epigenetic modification. In general, our study presents a robust, integrative laboratory-based approach to investigate the effects of salt stress on a nonmodel gastropod facing detrimental consequences of environmental change. The current genetic results provide a wealth of reference data for further research on mechanisms of ionic and osmotic regulation and adaptive evolution of this coastal gastropod.

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Abbreviations: AICC, corrected Akaike Information Criterion; ATP, adenosine triphosphate; DEG, differentially expressed gene; FAA, free amino acid; fc, fold change; FDR, false discovery rate; GABA, gamma-aminobutyric acid; Hsp, heat shock protein; LMM, linear mixed-effects model; MDS, multidimensional scaling; RNA-Seq, RNA sequencing.

Online enhancements: supplemental tables.

Correction: This article was reposted on May 14, 2019, to change "Hwanseok Lee" to "Hwanseok Rhee."

Introduction

Salinity is one of the most critical environmental stressors for littoral species, directly affecting metabolism (Spicer and Strömberg, 2003), mortality (Génio *et al.*, 2008; Sameoto and Metaxas, 2008), geographic distribution and abundance (Torres *et al.*, 2006; Arellano *et al.*, 2012), population dynamics (Yen and Bart, 2008; Javanshir, 2013), and behavior (Mann *et al.*, 1991). Littoral organisms are frequently exposed to salt stress associated with cycles of tidal flow, and much research has been devoted to physiological, behavioral, and morphological adaptations that allow marine invertebrates to cope with salinity changes. For example, research on the physiological responses of marine invertebrates to salt stress has yielded insights into oxygen consumption (Flemister and Flemister, 1951; Todd and Dehnel, 1960; Cheung, 1997), uptake of free amino acids (Hammen, 1969), nerve conduction (Tucker, 1970), properties of myosin ATPase (Krishnamoorthy and Venkatramiah, 1971), ionic composition and cell volume (Berger *et al.*, 1978), and ammonia excretion (Cheung, 1997). Documented behavioral responses to salt stress include reduced swimming ability and feeding rate (Mann *et al.*, 1991; Chaparro *et al.*, 2008a), and shelled invertebrates react to changes in salinity by sequestering themselves from the outside environment through valve or operculum closure (Shumway, 1977; Berger and Kharazova, 1997; Sokolova *et al.*, 2000; Kim *et al.*, 2001; Chaparro *et al.*, 2008b, 2009) or by producing corporal mucus (McFarlane, 1980). In

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terms of morphological responses, changes in shell color and volume have been noted (Berger *et al.*, 1978; Sergievsky, 1992; Sokolova and Berger, 2000). However, the relationships between these physiological, behavioral, and morphological responses to salinity are not well understood.

The mud intertidal snail *Batillaria attramentaria* (G. B. Sowerby I, 1855) is the most common *Batillaria* species in the northwest Pacific Ocean. Its range extends from southern Taiwan (Golikov and Scarlato, 1967) and Hong Kong (Golikov and Gulbin, 1978) to southern Primorye, Sakhalin, and northern Kuriles (Golikov and Scarlato, 1967, 1985; Golikov and Gulbin, 1978), including the shorelines of Japan and Korea. Originating from Northeast Asia, the native intertidal *B. attramentaria* was introduced to the bays and estuaries of the Pacific coast of North America, including the United States and Canada, through human-mediated transport coincident with the importation of the Pacific oyster *Crassostrea gigas* from Japan early in the twentieth century (Galtsoff, 1932). *Batillaria attramentaria* has a non-planktonic, direct developmental stage (Yamada, 1982) and low adult mobility (Whitlatch and Obrebski, 1980). Consequently, the species is a poor disperser (Whitlatch and Obrebski, 1980) with a narrow habitable zone about 50 meters in width (Adachi and Wada, 1997); thus, it is unable to avoid environmental stress easily. For these reasons we propose that *B. attramentaria* is an excellent model species for studying the impacts of environmental change on intertidal gastropods.

Several field-based studies have examined locomotor behavior of intertidal molluscs by tracking the distance moved in response to different factors such as microhabitat conditions (Underwood, 1977; Underwood and Chapman, 1989), shore height (Bates *et al.*, 2003), tidal height (Buckman *et al.*, 2005), and parasitism (Curtis, 1990). However, field studies are limited by the inability to control environmental conditions such as shifting sands due to wave exposure or tidal inundation, which may result in uncertainty about movement distance when targeted samples and/or marker rocks are washed away or when organisms' trails are blurred. To overcome these limitations, we developed a laboratory-based culturing system for observing marine snail locomotion—particularly, horizontal crawling—while controlling other environmental conditions such as light and temperature. Using this system, we reared *B. attramentaria* collected from natural habitat conditions (33 psu) and studied behavioral responses to various salinity levels (43, 23, 13, and 3 psu).

In addition to investigating behavioral responses to salt stress, we utilized high-throughput RNA sequencing (RNA-Seq) to discover metabolic pathways and genes that are associated with salinity tolerance or acclimation in *B. attramentaria*. Because there is no reference genome for *B. attramentaria* or its sister species, we used high-throughput sequencing and *de novo* transcriptome assembly, popular methods that have been successfully applied in many marine invertebrates (De Wit and Palumbi, 2012; Du *et al.*, 2012; Lv *et al.*, 2013; Meng *et al.*, 2013; Stefanni *et al.*, 2014; Zhao *et al.*, 2014), including a marine snail (Gleason and Burton, 2015). In the present study, we examined gill tissues because they are a major osmoregulatory organ (Vernberg and Vernberg, 1972; Mangum and Towle, 1977; Willmer, 2006; Rivera-Ingraham *et al.*, 2016) and are commonly used for studying transcriptomic responses to salt stress in intertidal molluscs (Hofmann and Somero, 1995; Gracey *et al.*, 2008; Lockwood and Somero, 2011; Zhao *et al.*, 2012; Meng *et al.*, 2013).

We hypothesized that locomotion would decrease under acute osmotic stress and that a salinity threshold exists for which acclimation is not possible. In addition, we qualitatively assessed six measures of condition over the course of the acclimation experiments. At the end of the acclimation experiments, we examined transcriptional responses to salinity stress to determine which genes were involved in osmotic stress acclimation. We hypothesized that genes associated with ionic and osmotic regulation (Lang *et al.*, 1998; Wehner *et al.*, 2003), genetic modification (Zhao *et al.*, 2012; Lv *et al.*, 2013; Zhang *et al.*, 2016), immune response (Lockwood and Somero, 2011; Zhao *et al.*, 2012; Lv *et al.*, 2013; Zhang *et al.*, 2016), stress response (Werner and Hinton, 2000; Wang *et al.*, 2012), apoptosis (Lockwood and Somero, 2011; Zhao *et al.*, 2013), and cell adhesion and communication (Lockwood and Somero, 2011) would be differentially expressed among treatments.

Materials and Methods

Snail collection and culture

The intertidal gastropod *Batillaria attramentaria* (G. B. Sowerby I, 1855) was collected in June 2016 from Hajeon-ri, Jeollabuk-do (35°32′ N, 126°33′ E), on the southwest coast of the Korean peninsula. The sampling site was a large area of sandy and muddy flats near the low tide line, with a surface salinity of 32–33 psu. We included only adult snails of approximately equivalent length (2.5 cm) to ensure that all individuals would have equal susceptibility to salt stress. We also chose snails with a similar morphological shell pattern (see fig. 1, Ho *et al.*, 2015) because *Batillaria* snails with different shell patterns might be genetically distinct species.

All individuals were initially maintained for two days in a large aquarium filled with water with a salinity of 33 psu (equivalent to the surface salinity of the seawater at the sampling site) before the different salinity treatments were applied. Saline water was freshly prepared from distilled water that was aerated overnight, and Instant Ocean Sea Salt (United Pet Group, Cincinnati, OH). A total of 120 snails were divided into 6 groups (20 individuals per group) and were cultured in separate plastic aquaria ($40 \times 23 \times 21$ cm^3 in size); this arrangement matched their actual density at the collecting site (52 individuals per area of 50×50 cm^2). All aquaria were set up with a layer of sea sand, which was 20–25° of inclination angle and covered about 2/3 of the bottom, and about 1 L of aerated artificial seawater per tank, as illustrated in Figure A1. The sand was collected from the

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seashore near the sampling site. Each of the 20 individuals in each group was marked using a different color of nail polish (Innisfree Eco Nail Color, Amorepacific, Seoul, Republic of Korea) to keep track of their identity. We maintained all experiments at a constant temperature of 25 °C and on a 12h:12h light:dark photocycle. All animals were fed to satiation every 2 days with excised seaweed (Ottogi, Anyang-si, Gyeonggi-do, Republic of Korea) throughout the 30-day culturing period. Saline water in each tank was changed every two days before adding seaweed for the animals. We are confident that the tank conditions were identical except for salinity, because we cleaned each tank and the sand contained in it with water, removed leftover seaweed and waste from animals, and filled the tank with fresh saline water. This protocol was followed to minimize exposure to biological pathogens and decaying seaweed, which might be potential biological factors resulting in mortality of snails. Additionally, we used the same distilled water and salt to prepare saline water across culturing experiments to control other water quality parameters such as temperature, pH, dissolved oxygen, and so on.

After the behavioral experiments, all snails were genotyped to confirm species identity, using the protocols for genomic DNA extraction and mitochondrial *COI* gene amplification described in our previous study (Ho *et al.*, 2015).

Locomotor behavioral experiments

To evaluate the effects of salt stress on snail locomotion, we consecutively conducted short-term (1-hour observation per individual) and long-term (30-day observation per individual) acclimation experiments under different salinity treatments of 43, 33, 23, 13, and 3 psu (Fig. 1, upper panel). Salinities of 43, 33, and 23 psu may be found in coastal and estuarial areas and are likely encountered by snails in their natural habitats. In addition, to simulate seasonal fluctuations of salinity near estuaries, which commonly occur as a result of heavy rains in the spring and occasional storms in the summer and fall (Nelson *et al.*, 1981; Loder *et al.*, 1983), we also included the lower salinity conditions of 13 and 3 psu.

For the short-term acclimation experiments, we applied two different approaches of salt stress exposure to coastal gastropod *B. attramentaria* exposed to 33 psu saline water for 2 days after collection, as previously described. For short-term acclimation experiment I, we transferred a group of 20 snails to experimental tanks and gradually decreased salinity from 43 to 3 psu in 10-psu increments (Fig. 1A, upper panel). Per each salinity-lowering step, we recorded locomotion of each animal for one hour before transferring it to the next treatment. For short-term acclimation experiment II, we directly transferred 4 groups of snails from the control (33 psu) to abruptly changed salinities (43, 23, 13, and 3 psu) and kept 1 group at the control salinity (33 psu) (Fig. 1B, upper panel). Each group of 20 snails was recorded for 1 hour during the exposure to their respective salinity treatment; this has been reported as sufficient time for intertidal *Littorina* snails to detect changes in their surrounding environment and protect themselves from the source of stress (Sokolova *et al.*, 2000; Sokolova and Boulding, 2004).

The long-term acclimation experiment was conducted subsequent to the above short-term acclimation experiment II. We bath-cultured all the groups of snails in their respective fixed salinities for 30 days to reduce any effects of past environmental conditions and to ensure that the snails were fully acclimated to the new environment. We recorded and tracked snails' movement trails for one hour every two days throughout the long-term acclimation experiment. To monitor the performance and to track the movement of the snails, each snail was placed in the center of a single disposable petri dish filled with saline water from the snail's tank of origin. Snail performance was recorded using a Sony NXCAM camera (AVCHD Progressive MPEG2 SD, Sony Corporation, Tokyo, Japan). The camera was mounted on a tripod above 20 petri dishes, allowing 20 snails to be recorded at the same time. After an hour of monitoring, all snails were transferred back to their culturing tanks with their respective saline water. Additionally, we examined mortality by observing the response of animals to a metal needle. Each individual was touched using a metal needle every day to check whether it was still alive. Snails were considered dead and were removed from the tank if they did not contract their soft body when touched with a metal needle. Only snails that survived the 30-day culturing period were used for RNA extraction and subsequent transcriptomic analyses (described in *RNA isolation and library construction*). In parallel, we also observed other conditions of the snails, including closing operculum or hermitization, secreting corporal mucus, vertical crawling or climbing, feeding, and stooling. For these conditions, we visually observed and qualitatively estimated how active they were according to the proportion of individuals closing their operculum or exhibiting vertical crawling behavior, or by noting the amount of secreted corporal mucus, amount of consumed seaweed, and amount of animals' waste every day throughout the long-term acclimation experiment.

Video analysis

We used a series of computer programs to estimate the snails' movement distance. First, we increased the playback rate of all videos by a factor of 10, using AVS Video Editor v.7.1.2.262 (Online Media Technologies, London, United Kingdom), and saved the files as H.264/AVC, 1200 Kbps, audio: MP3, 192 Kbps. Next, we cropped video files into smaller videos showing only one petri dish at a time, using Avidemux v.2.6.12 (Mean, 2008), which allowed us to track one snail per video. To estimate the length of each snail's movement trail, we used the spectral time-lapse (STL) toolbox (Madan and Spetch, 2014) implemented in Matlab release R2014a (MathWorks, Natick, MA).

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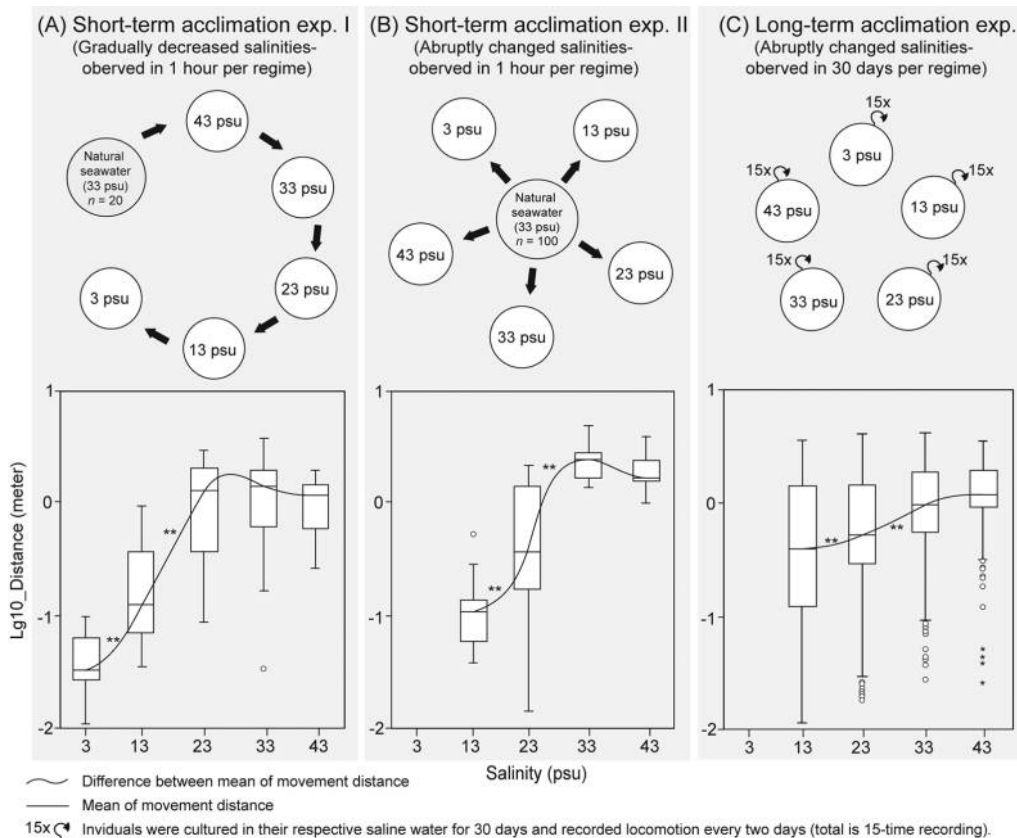

Figure 1. Short- and long-term acclimation experiments of *Batillaria attramentaria* in response to salt stress. The upper panel shows the designs of the short- and long-term acclimation experiments, and the lower panel shows the ANOVA results assessing the effects of salt stress on the movement distance of snails *B. attramentaria* (A) exposed to gradually decreased salinities ($N = 20$, one-way repeated ANOVA), (B) exposed to abruptly changed salinities ($N = 100$, 20 replicates per salinity value, one-way ANOVA), and (C) acclimated to new salinities for 30 days ($N = 100$, 20 replicates per salinity value, linear mixed-effects model for time series data that included movement distance data from 15 recorded times of 20 snails throughout the long-term acclimation experiment [300 data points per each condition]). For the long-term acclimation experiment, the snails were recorded every 2 days throughout 30 days (total is 15 times of recording, labeled as 15×). In the box and whisker plots, the bottoms and tops of the boxes are the 25th and 75th percentiles of snail movement distance, respectively, and the ends of the whiskers represent the minimum and maximum. Curved arrows represent times of recording the locomotion of the snails. Solid black lines represent mean movement. Solid curves represent the difference between the mean of movement distance. Circles are outliers. Asterisks are extreme outliers. Double asterisks are a significant difference between mean movement distance of snails exposed to different salinities.

Statistical analyses of behavioral experiment

To examine the influence of salt stress on snails' movement distance, we employed (*a*) a one-way repeated analysis of variance (ANOVA) for short-term acclimation experiment I, (*b*) a one-way ANOVA for short-term acclimation experiment II, and (*c*) a linear mixed-effects model (LMM) (Demidenko, 2005) to time series data for the long-term acclimation experiment. For the one-way repeated ANOVA and one-way ANOVA, deviation from normality was tested with the Shapiro-Wilk test. For the LMM analysis, we used a nested block design, where salinity and time were fixed effects and where the individual snail was treated as a random effect. Detection of the most supported covariance structure model for the LMM method was done through model selection procedures, using the corrected Akaike Information Criterion (AICC). The model with the lowest AICC value represents the best compromise between bias and lack of precision; and it is, therefore, used for making inferences. In this study, we chose compound symmetry as the covariance structure model. All statistical tests were performed using IBM SPSS Statistics v.20 (released 2011; IBM, Armonk, NY). The significance level was set at $\alpha = 0.05$ for all statistical tests.

RNA isolation and library construction

After the long-term acclimation experiment, we dissected gills from 12 snails from the 43-, 33-, 23-, and 13-psu salinity regimens (3 replicates per regimen) and used them for transcriptomic analyses. All snails cultured at 3-psu saline water had died as a result of osmotic shock after 16 days, so this group was excluded from the transcriptomic analyses.

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Messenger RNA (mRNA) was extracted from the gills and purified using a RNeasy Mini kit (Qiagen, Venlo, The Netherlands), which was followed by preliminary fluorescence-based quantification and quality check (Nanodrop 1000 Spectrometer, Thermo Fisher Scientific, Wilmington, DE, and Bioanalyzer 2100, Agilent Technologies, Santa Clara, CA). Next, construction of an Illumina-compatible library for each sample, using a Truseq RNA Library Preparation kit (Illumina, San Diego, CA), was conducted. Briefly, the mRNA strands containing poly-A tail were selected, chemically fragmented, and converted into single-strand cDNA strands with random hexamer priming. Subsequently, second cDNA strands were generated to create double-stranded (ds) cDNA, which was then ligated to sequencing adapters. These products were then enriched by quantitative polymerase chain reaction (qPCR) following the Quantification Protocol Guide (Illumina) and qualified using Bioanalyzer 2100 (Agilent Technologies) to build the ultimate library.

Data pre-processing and de novo *assembly*

The cDNA library sequencing was performed using the HiSeq 4000 platform (Illumina) by Macrogen (Seoul, Republic of Korea). Sequence quality was verified with FastQC v.0.10.0 (Andrews, 2010). Before further analysis, all of the adapter fragments from qualified sequencing data were trimmed, and low-quality sequence reads were removed using Trimmomatic v.0.32 (Bolger *et al.*, 2014). Because of the lack of a reference genome for *B. attramentaria*, Trinity v.r20140717 (Haas *et al.*, 2013) was employed to *de novo* assemble short reads into longer fragments or genes without N gaps (Grabherr *et al.*, 2011). These genes were further processed for gene alignment and abundance estimation with Bowtie (Langmead *et al.*, 2009). HTSeq v.0.6.0 (Anders and Huber, 2010) was adopted to generate the count matrix, with default parameters, that would be used for differentially expressed gene identification. A schematic diagram of the Illumina deep sequencing and analysis workflow, with numbers of reads at crucial stages, is shown in Figure 2.

Statistical analyses for differential gene expression

We utilized the two best-performing tools for differentially expressed gene (DEG) identification: DESeq2 (Anders and Huber, 2010; Love *et al.*, 2014) and edgeR (Robinson *et al.*, 2010) from Bioconductor (see Soneson and Delorenzi, 2013 for reviews of DEG tools; Ching *et al.*, 2014; Costa-Silva *et al.*, 2017). All of the DEG analyses were performed using the same count matrix generated by the HTSeq package. For all of the DEG analyses, we estimated the degree of expression of genes from the stressed individuals (43, 23, and 13 psu) compared to the control individuals (33 psu), applying fold change (fc) measurement; and we selected the DEGs with $|fc| \geq 2$ and false discovery rate (FDR) $P < 0.05$. We visualized the similarities among samples through multidimensional scaling (MDS) based on the common DEGs identified by different DEG analyzing tools. Additionally, we also performed a hierarchical clustering analysis using complete linkage and Euclidean distance as a measure of similarity to display the expression patterns of differentially expressed genes ($|fc| \geq 2$, FDR $P < 0.05$). Differentially expressed gene analyses were performed in R software (R Development Core Team, 2011).

Gene annotation

To identify transcripts encoding proteins potentially relevant to salt stress, we compared the six-frame conceptual translation products of nucleotide query sequence (both strands) against five databases including KEGG (Kyoto Encyclopedia of Genes and Genomes) Orthology database (Pacific oyster *Crassostrea gigas* database); DBGET Orthology database (sea hare *Aplysia californica* database); Gene Ontology Consortium v.20150407; National Center for Biotechnology Information non-redundant (nr) protein; and UniProtKB, using BLASTX v.2.4.0 (National Center for Biotechnology Information, Bethesda, MD) with an e-value of $1e^{-5}$.

Results

Behavioral and physiological responses to salt stress

The intertidal snail *Batillaria attramentaria*, from the coastline of the Korean peninsula, was able to acclimate to a range of salinity values (13–43 psu) but unable to tolerate the extremely low salinity of 3 psu. During the long-term acclimation experiment (30 days), no mortality was observed in the groups exposed to salinities of 43, 33, 23, and 13 psu; but in the 3-psu treatment, all snails were dead after the first 16 days. We qualitatively assessed conditions of *B. attramentaria* in response to salt stress. We found that live snails that were exposed to different salinities showed some level of variation in climbing or sticking to the aquarium wall, secreting corporal mucus, closing the operculum, responding to a metal needle, and feeding and stooling responses (Table 1). Individuals of *B. attramentaria* acclimated to 43 psu had activity similar to that at 33 psu (natural condition), while individuals acclimated to the lower salinities of 23 and 13 psu exhibited quick changes and gradually recovered (observed in 23-psu animals) or did not recover (13-psu animals) to the control state (Table 1). Those exposed to 13 psu isolated themselves by tightly closing the operculum over the first 4 days but then returned to an active state. We did not observe any operculum closure due to salt stress in snails exposed to normal (33 psu) or moderately changed salinities of 23 or 43 psu. In contrast, snails that were reared at 3 psu clamped their operculum firmly, stayed stationary, secreted white cloudy corporal mucus, and died after the first 16 days. This suggests that saline water of 3 psu is extremely stressful to *B. attramentaria* and can be considered a lethal level.

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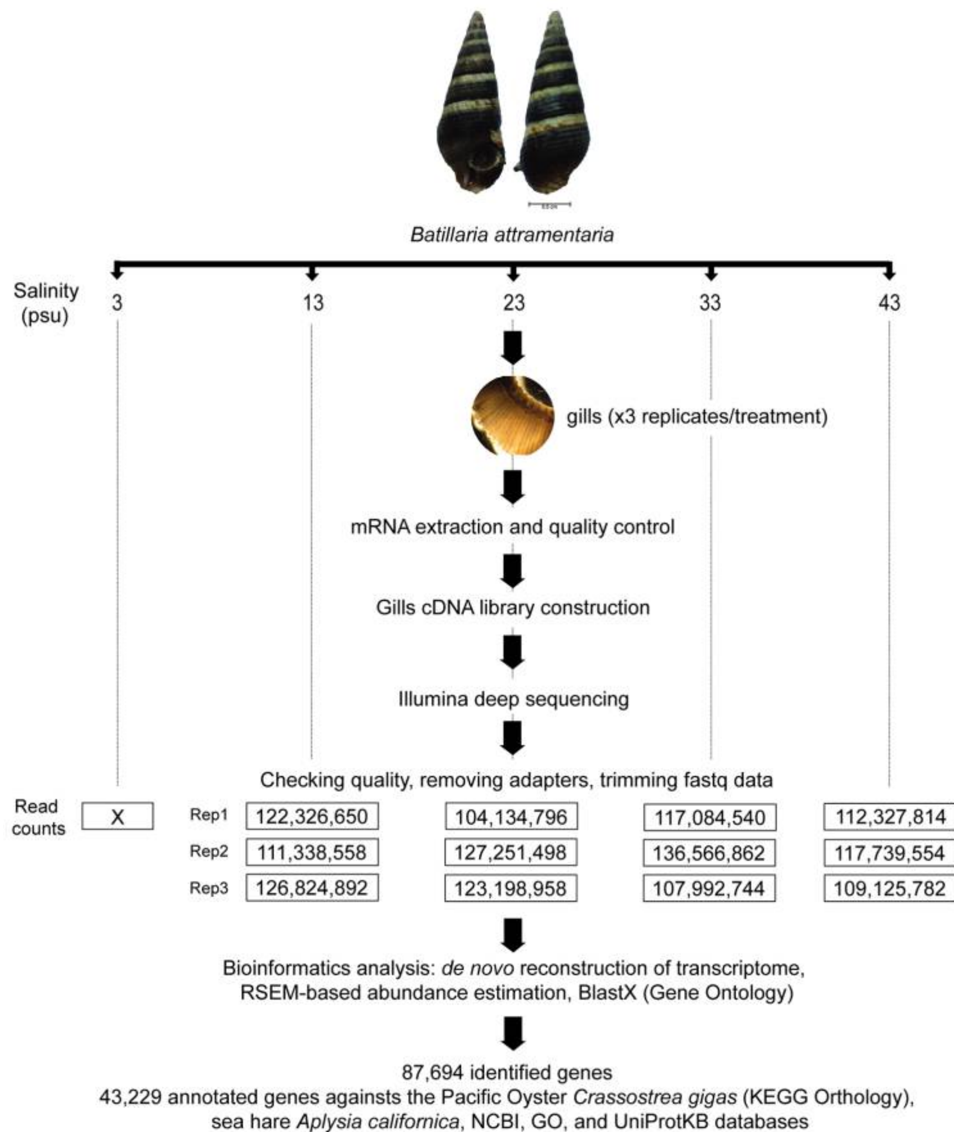

Figure 2. Schematic flow of Illumina deep sequencing and analysis with numbers of reads at critical stages.

Short-term acclimation experiment: locomotor behavior in response to salt stress

To quantitatively assess the effect of salt stress on the snail's movement distance when exposed to gradually decreased salinities (short-term acclimation experiment I), we performed a one-way repeated ANOVA test. Results after Greenhouse-Geisser correction ($\varepsilon = 0.72$) showed that the different salinity levels elicited statistically significant changes in the snails' locomotor performance ($F_{4, 19} = 22.13$, $P < 0.0005$, partial $\eta^2 = 0.75$, Table 2A). The means and standard deviations of the locomotor distance of all snails are listed in order from high to low salinity (Table S1A, available online). These statistics showed that snails were more active at 33 psu, which is similar to their natural salinity. A *post hoc* analysis with a Bonferroni adjustment revealed that movement distance slightly increased and decreased when snails were conveyed from 43 to 33 psu and from 33 to 23 psu, respectively. However, snails exposed to 3 psu moved significantly shorter distances than those exposed to 13 psu (Fig. 1A, lower panel).

We used a one-way ANOVA to assess the effect of salt stress on the snails' movements when exposed abruptly to different salinities (short-term acclimation experiment II). Statistical analysis of movement distance data showed that the salt stress statistically influenced the snails' locomotor performance ($P < 0.0005$, Table 2B). Games-Howell *post hoc* analysis revealed that performance of snails exposed to 33 psu was statistically higher than those exposed to 23 and 13 psu but not to 43 psu (Fig. 1B, lower panel). Snails that were directly ex-

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Table 1

Qualitative observation on conditions of Batillaria attramentaria *in response to salt stress*

Behavior	Salinity (psu) 3	13	23	33	43
Closing operculum	NR	QR	NC	C	NC
Secreting corporal mucus	NR	NC	NC	C	NC
Vertical crawling	NR	NR	QR	C	NC
Responding to a metal needle	NR	GR	QR	C	NC
Feeding	NR	GR	QR	C	NC
Stooling	NR	GR	QR	C	NC

The degree of these conditions of stressed groups was qualitatively estimated and compared to the control group (33 psu), with 20 individuals per treatment. C, control; GR, gradual recovery to the control state; NC, no change in comparison to the control state; NR, no recovery after a quick change from a control state; QR, quick recovery to the control state.

posed to 3 psu did not move throughout short-term acclimation experiment II (1 hour), and all died by the 16th day of the long-term acclimation experiment (30 days). A summary of means and standard deviations of distance moved (see Table S1B, available online) showed that *B. attramentaria* was able to tolerate a salinity range from 43 to 13 psu but tended to decrease its activity when exposed to more stressful conditions.

Long-term acclimation experiment: locomotor behavior in response to salt stress

Snails' movement distance varied substantially across different salinities. Means of the movement distance of different groups were as follows: $d_{43\ \mathrm{psu}} = 1.5$, $d_{33\ \mathrm{psu}} = 1.2$, $d_{23\ \mathrm{psu}} = 1$, and $d_{13\ \mathrm{psu}} = 0.9$ (d = mean of movement distance [in meters], Fig. A2). An LMM test was used to examine the influences of multiple factors of salinity and time on snails' locomotor performance. Results indicated that both salinity ($F_{3,76} = 23.06$, $P < 0.0005$) and time ($F_{14,1064} = 7.55$, $P < 0.005$) were significant predictors of performance throughout the long-term acclimation period of 30 days (Table 3). The parameter estimates of movement distance under both fixed (salinity and time) and random (snail) effects are presented in Table S2, available online. As shown in Figure 1C, snails that acclimated to 43 and 33 psu tended to move significantly farther than those exposed to the lower salinities of 23 and 13 psu. There was no significant difference in movement distance between individuals acclimated to 43 and 33 psu. This indicates that a salinity of 43 psu does not substantially affect snails but that lower salinities of 23 and 13 psu cause slower movement in comparison to the control (33 psu).

De novo *assembly and functional gene annotation*

Twelve cDNA libraries were constructed using mRNA from the gills of 12 acclimated snails used for Illumina sequencing. Reads numbers of 360, 354, 361, and 339 million samples from the 43-, 33-, 23-, and 13-psu treatments were obtained, yielding clean bases of 36, 35, 36, and 34 Gbps, respectively. The total Illumina reads and clean bases for all samples were 1,415,912,648 and 141 Gbps, respectively.

After a *de novo* transcriptome assembly based on all Illumina reads was obtained from all 12 samples, 87,694 genes were identified (Fig. 2). The sequence length distribution of genes and assembly statistics are presented in Figure A3A, B. The average length of all genes was 1009 bp, with the smallest sequence being 201 bp and the largest one 43,901 bp and with N50 and N90 values of 845 and 262 bp. Here, only 43,229 genes (49.3%) were well annotated, with 37,480 genes (86.7%) assigned to KEGG Orthology (Pacific oyster *Crassostrea gigas* database) (Fig. A3C). The taxonomic distributions of top hits from the BLASTX results are summarized in Figure A3C and Table S3, available online.

Gene expression analyses

Differentially expressed gene analyses were performed using two different DEG statistical tools: DESeq2 and edgeR, to identify gene expression changes in the stressed samples (13, 23, and 43 psu) compared to the control (33 psu). Transcriptome-wide gene expression comparisons among individuals obtained by the two different methods were summarized in MDS plots (Fig. 3A). In the MDS graphs, the transcriptome of each individual is presented as a single point, with each mRNA abundance value determining the position of the point in two dimen-

Table 2

(A) Summary results of the one-way repeated ANOVA to assess the effect of salinity stress on snails' movement distance when exposed to gradually decreased salinity from 43 to 3 psu, by steps of 10 psu; (B) summary results of the one-way ANOVA to assess the effect of salinity stress on snails' movement distance when exposed abruptly to new salinities

(A) Variable and source	df	Type III sum of squares	F	P	Partial η^2
Intercept	1	22.13	57.187	<0.0005	0.75
Error	19	7.35			

(B) Variable and source	Sum of squares	df	Mean square	F	P
Between groups	25.05	3	8.349	54.36	<0.0005
Within groups	11.67	76	0.15		
Total	36.72	79			

(A) Results were corrected for sphericity with the Greenhouse-Geisser approach ($\varepsilon = 0.511$). Estimate values were based on log-transformed data. P-values were adjusted by Bonferroni correction. (B) The data were normally distributed for each of five groups of snails transferred to different salinity levels, as assessed by boxplot and Shapiro-Wilk's test ($P > 0.05$). Estimate values were based on log-transformed data. P-values were adjusted by Bonferroni correction.

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Table 3

Summary results of the linear mixed-effects model test of the effects of salinity and time on snails' movement distance throughout the long-term acclimation experiment

Fixed effects	Numerator df	Denominator df	*F*	*P*
Intercept	1	76	42.14	<0.005
Salinity	3	76	23.06	<0.005
Time	14	1064	7.55	<0.005
Salinity × time	42	1064	8.05	<0.005

Estimated values were based on log-transformed data. *P*-values were adjusted by Bonferroni correction.

sions. Both DESeq2 and edgeR MDS graphs reveal clustering of transcriptional profiles among groups treated with different salinities (Fig. 3A). Figure 3A shows overall average expression level between $\log_2 |fc|$ of all the genes found by the two DEG tools. The genes were considered statistically significant DEGs if the $|fc|$ value was greater than 2 with an FDR *P*-value less than 0.05, as estimated by both DEG tools. Based on these criteria, the DESeq2 method was able to identify more DEGs, with 4863 genes, while edgeR was able to find only 2899 genes. Expression of all of the DEGs obtained from the two tools is illustrated using heat maps (Fig. 3B), and DEGs obtained from the comparisons between the control (33 psu) and stressed (13, 23, and 43 psu) individuals are presented in smear plots (Fig. 3B).

To examine the extent of salinity tolerance of *B. attramentaria*, we analyzed the clear majority of common DEGs (Table S4A, available online) identified by both methods, although some of them did not show agreement in the direction and magnitude of the fold change. Combined DEG analyses revealed a total of 2683 significant DEGs of snails exposed to salt stress (43, 23, and 13 psu) in comparison to the control (33 psu), but only 1173 genes among them were annotated (Fig. 4A; Table S4A, available online). At $|fc| \geq 2$ and FDR $P < 0.05$, 2592 genes displayed significant differential expression levels in 33 psu compared with 13 psu, but only 85 in 33 psu compared with 23 psu and 94 in 33 psu compared with 43 psu (Fig. 4A). The number of up- and downregulated genes obtained from the comparisons can be seen in Figure 4A and Table S4, available online.

Biological functions related to snail response to salt stress

By comparing the occurrence and expression of transcripts obtained from the stressed snails and the control group, a list of transcripts responding to salt stress was created (Table S4, available online). We particularly focused on biological functions of *B. attramentaria* that may characterize its responses to strong hypo-osmotic stress (13 psu) (Table S4B, available online; Fig. 4B). Ionic regulation is implicated by the upregulation of several genes encoding for ion channels such as Na^+/K^+ ATPase, K^+ channel, Ca^{2+} channel, and H^+ channel; ion transporters of Na^+-coupled monocarboxylate, organic cations, Na^+/Cl^- dependent gamma-aminobutyric acid (GABA) and glycine; and cotransporters of Na^+/glucose and Na^+/myo-inositol. Simultaneously, osmotic regulation is also implied by the upregulation of genes encoding for the enzymes glycine N-acyltransferase, glutamate dehydrogenase, and taurocyamine kinase, which degrade intracellular free amino acids; transporters of H^+-coupled amino acids and vesical amino acids; and solute carriers. Genetic modification is also noted in the regulation of several methyltransferase genes encoding betaine-homocysteine S-methyltransferase, histone-lysine N-methyltransferase, and 28S rRNA (cytosine-C(5))-methyltransferase. In addition, immune response is characterized by genes relating to calcium-dependent signal transduction and hormonal signaling pathways, including upregulation in the genes coding cadherin G-type receptor, dopamine β-hydroxylase, and thyrotropin-releasing hormone receptor. Stress proteins, such as heat shock proteins Hsp 68 and Hsp 70, were also found to be upregulated. Cell adhesion and communication are mainly activated through upregulation of the genes relating to protocadherin and cadherin. Apoptosis was also notable not only by upregulation of genes relating to apoptosis inhibition but also by downregulation in genes relating to apoptosis induction.

Discussion

Our study reveals impacts of salt stress on the intertidal snail *Batillaria attramentaria* on aspects of both locomotion and gene expression. The typical behavioral response to acute reductions in salinity was a decrease in locomotion. Increases in salinity (43 psu) did not produce a significant change in locomotion. The lowest salinity treatment (3 psu) resulted in the death of all individuals in the long-term acclimation experiment, suggesting that the lethal salinity threshold for these snails is between 3 and 13 psu. Qualitative observations of snail conditions (responding to physical impact, vertical crawling, feeding, and stooling) also suggested that individuals fully acclimated to osmotic stress for some treatments (23 and 43 psu). Indeed, numerous osmotic stress genes hypothesized to contribute to physiological acclimation were found to be differentially expressed in individuals acclimated to osmotic stress for 30 days.

Behavioral responses to salt stress

Despite the well-known broad tolerance of intertidal snails to a wide range of salinity (Fretter and Graham, 1962; Hedgpeth, 1967), little is known about their behavioral and transcriptional responses to salt stress. In the present study, we qualitatively observed various behaviors indicative of condition such as closing the operculum, secreting corporal mucus, crawling, responding to a metal needle, feeding, and stooling; and we quantitatively examined the locomotion behavior of *B. attramentaria* in response to salt stress. In general, the results from these observations indicated a high tolerance to a range

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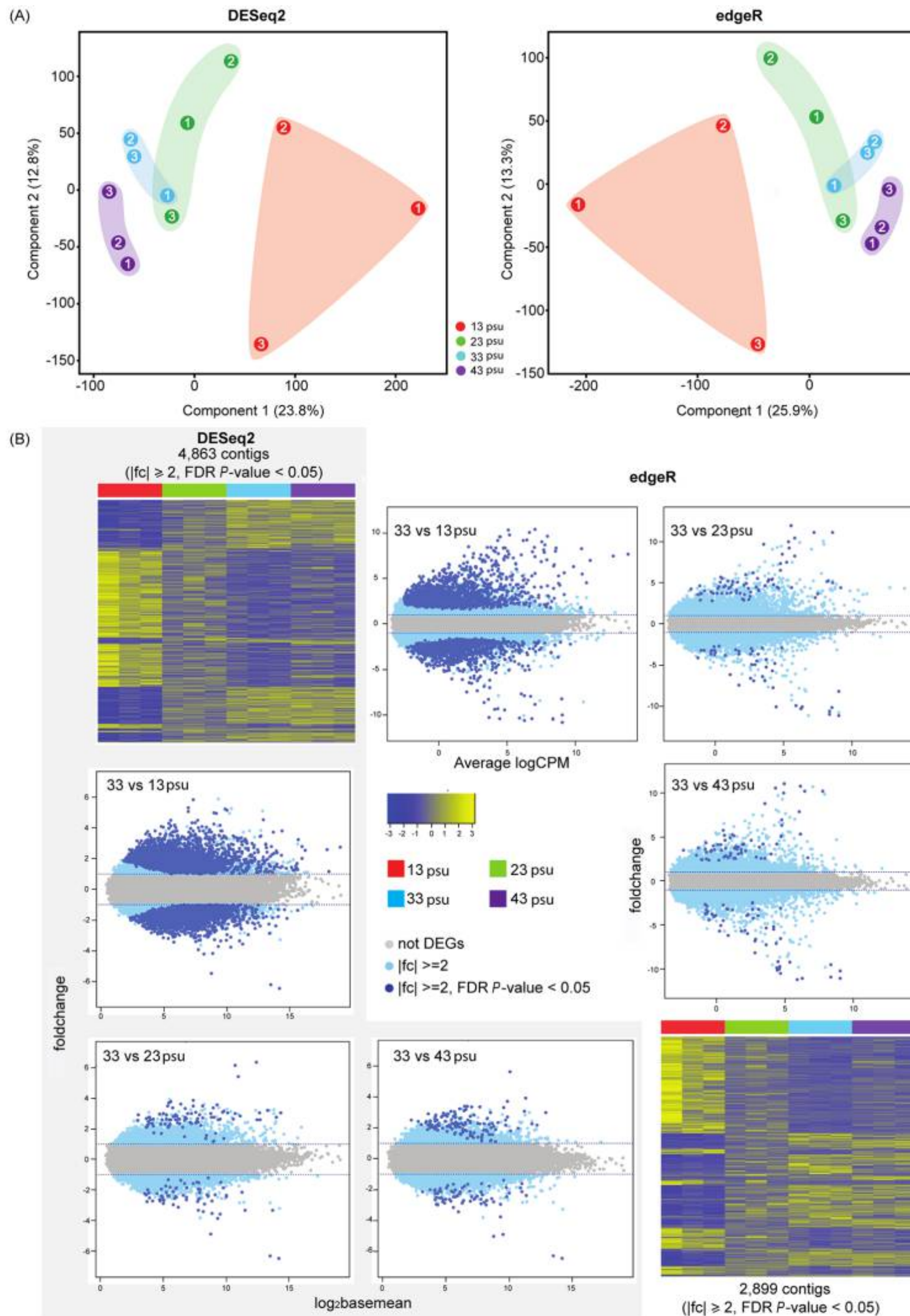

Figure 3. (A) Multidimensional scaling (MDS) plot visualization of distances between the wide transcriptomes of *Batillaria attramentaria* individuals acclimated to different salinity treatments generated by the tools DESeq2 and edgeR. Each circle is an examined sample. Different colors represent different salinity treatments. Numbers inside circles indicate the biological replicates (number of samples) from each treatment. (B) Differentially expressed genes (DEGs) found by DESeq2 and edgeR from *B. attramentaria* exposed to low and high salinities. The heat map displays $\log^2$ fold change (fc) in average expression of gene models (horizontal axis, $|fc| \geq 2$, false discovery rate [FDR] $P < 0.05$) showing significant differential expression across treatments. Genes upregulated from the average are shown in yellow, and genes downregulated are shown in purple. The smear plots show overall expression level of genes expressed in stressed individuals (13, 23, and 43 psu) in comparison to the control (33 psu). Gray dots, or "Not DEGs," indicate not differentially expressed genes, while light and dark blue dots represent insignificant and significant DEGs with $|fc| \geq 2$ and $|fc| \geq 2$ + FDR $P < 0.05$, respectively. Smear plots in the white frame were generated by edgeR, and those in the gray frame were generated by DESeq2.

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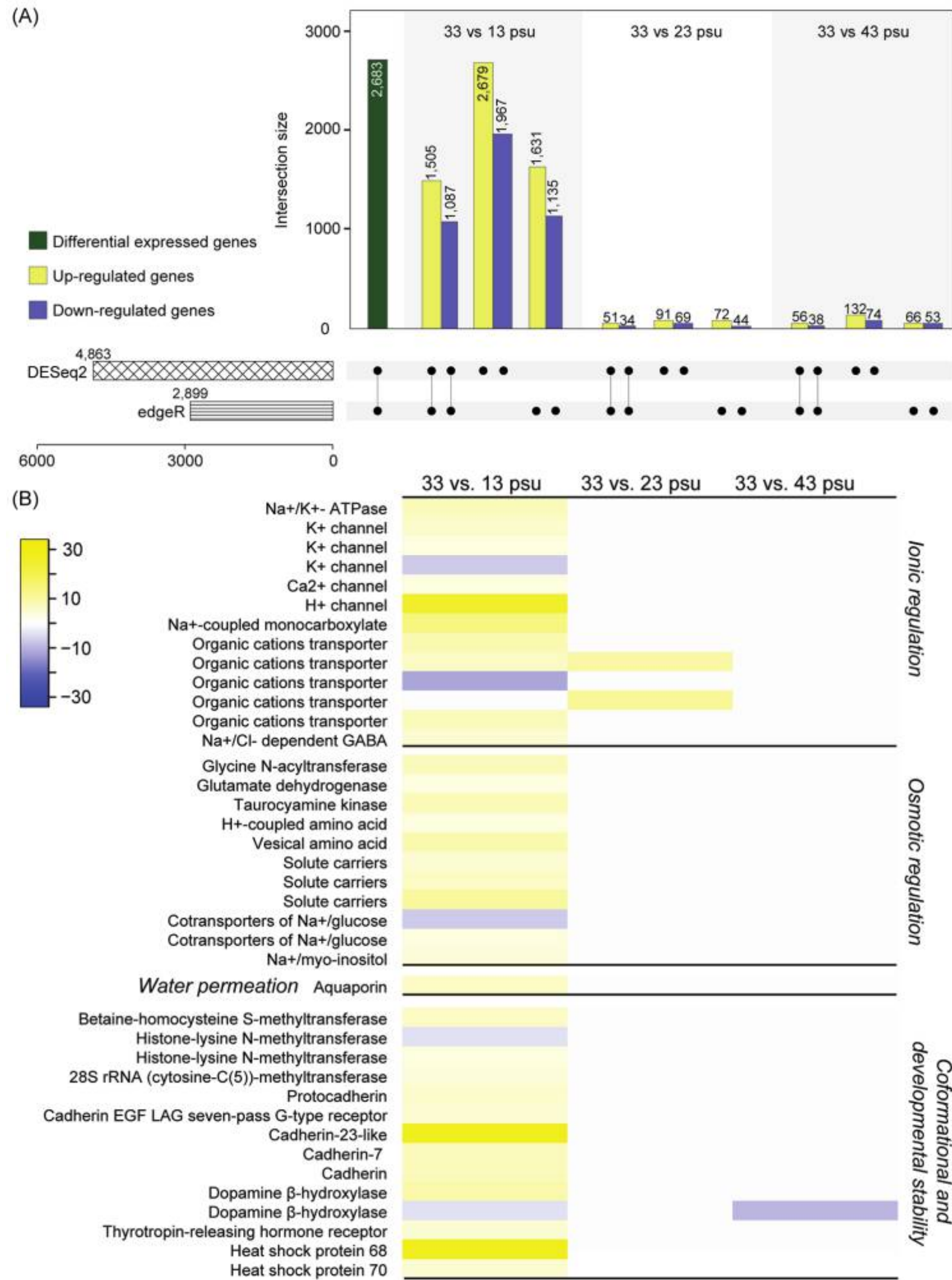

Figure 4. Matrix for intersections of pairwise comparisons between differentially expressed genes (DEGs) of *Batillaria attramentaria* found by the DESeq2 and edgeR tools. (A) The horizontal bar graph in the bottom left corner presents the amount of DEGs found by DESeq2 (4863 genes) and edgeR (2899 genes). Black dots within the bottom matrix indicate sets of DEGs that are found by DESeq2 and/or edgeR. The black dots that indicate DEG sets commonly found by the two methods are connected by a straight line. The dark green column represents the number of common DEGs found by the two methods, counting all DEGs found from the three different comparisons (33 *vs.* 13 psu, 33 *vs.* 23 psu, and 33 *vs.* 43 psu). The yellow and purple columns represent the up- and downregulated genes found by DESeq2 and edgeR, respectively. (B) Heat map of annotated DEGs with $|fc| \geq 2$ + false discovery rate (FDR) $P < 0.05$. The dendogram on the right side shows the gene expression pattern. The color scale bar indicates fold change, with purple indicating downregulation, yellow indicating upregulation, and white indicating no change in expression compared to the control (33 psu) and salt stress samples (13, 23, and 43 psu). Fold changes were calculated by averaging the values estimated by DESeq2 and edgeR. Sample size $N = 12$ for both control and treatment groups.

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of salt stress from 13 to 43 psu in laboratory conditions. Nevertheless, the degree of tolerance in this species is narrower than other intertidal molluscs such as the snail *Hydrobia ulvae* (5–45 psu, Khlebovich and Kondratenkov, 1973), oyster *Crassostrea gigas* (2–50 psu, Goulletquer, 1997), and mussel *Mytella charruana* (2–40 psu, Walters *et al.*, 2010). Individuals of *B. attramentaria* quickly responded to suddenly altered salinity conditions, as shown in the short-term acclimation experiments, and appeared to tolerate stressful conditions through the 30th day of salinity treatment in the long-term experiment. Notably, snails immediately responded to the harsh change of salinity (3 and 13 psu) through hermitization (closing of the mantle cavity) in order to impede water-salt exchange with the external saline water. Several previous studies indicated that hermitization is controlled by the activity of peripheral detectors located on head tentacles, mantle ridges, and gill surfaces, including osmoreceptors and other receptors (Freeman and Rigler, 1957; Davenport, 1979, 1981). Hermitization allows molluscs to withstand hours of salinity changes without spending metabolic energy on maintaining internal homeostasis (Hoyaux *et al.*, 1976; Shumway, 1977; Drouin *et al.*, 1985; Berger and Kharazova, 1997). The 3-psu snails closed the operculum and secreted corporal mucus as observed in the marine gastropod *Onchidium verruculatum* (McFarlane, 1980); they exhibited no climbing, no responding to a metal needle, no feeding, and no stooling at all. However, the 13-psu animals started to open the operculum and to become more active in a few days; they started responding to a metal needle, feeding, and stooling, but still exhibited no climbing (Table 3). In contrast, *B. attramentaria* was not strongly affected by moderate changes in salinity (23 and 43 psu) and stayed as opened and active as it did in the control (33 psu); it was similarly active in climbing, responding to a metal needle, feeding, and stooling (Table 3).

Climbing activity has been recorded in intertidal gastropods as an avoidance behavior from subtidal predation (Warren, 1985; Main, 1987; Vaughn and Fisher, 1988; McKillup and McKillup, 1993; Duncan and Szelistowski, 1998) and harsh physical conditions such as ambient temperature (McBride *et al.*, 1989; Williams and Appel, 1989; McGuinness, 1994). Previous studies also indicated that climbing activity is dependent on food resources (Little and Nix, 1976; Byers, 2000). Various studies have described a complex relationship between a snail's feeding activity and environmental or biological factors such as quality and size of food (Barnes and Greenwood, 1978; Forbes and Lopez, 1989), concentration of chlorophyll *a* (Levinton and Lopez, 1977; Bianchi and Levinton, 1984; Morrisey, 1988), and snail density (Levinton and Lopez, 1977; Blanchard *et al.*, 2000; Byers, 2000). However, in the present study, we only roughly estimated, but did not carefully quantify, the consumed seaweed and stooling activity by snails in different tanks. Therefore, we suggest that further studies on feeding and stooling activity in response to salt stress should be conducted to gain more understanding about the reaction of animals to salt stress through these behaviors.

Within the scope of this study, 13 psu can be considered a lower salinity threshold because of two reasons. The first is that snails reared in the extremely low salinity condition (3 psu) died after the first 16 days of culture, and the second is that snails reared in 13 psu were still active but less so than those reared in higher salinity conditions. In addition, we noticed that the lower salinity (13 psu) had a more substantial effect on snails' activity than the higher salinity (Fig. 1; Table 3), which likely indicates that the low-psu saline water is more harmful to the coastal snails than the high-psu saline water.

On the other hand, the results of short-term acclimation experiments I and II show that snails exposed to the salinity level of their natural origin (33 psu) traveled farther than those exposed to hypo-osmotic conditions (13 and 23 psu, Fig. 1, lower panel). However, variation in the means of movement distance among groups exposed to gradually decreased salinities (short-term acclimation experiment I) was smaller than those exposed to abruptly changed salinities (short-term acclimation experiment II) (Fig. 1A, B). In addition, we observed that all snails ($N = 20$) abruptly exposed to 3 psu clamped tightly shut and stayed stationary until death, while several individuals (7 of 20 snails) moved slightly when conveyed step by step from 43 to 3 psu. We interpret this to mean that gradually decreasing salinity allows snails to adjust appropriately and triggers less osmotic shock than an abrupt change in salinity. In summary, methods of salt stress application (gradual *vs.* abrupt salinity change) influenced the snail's locomotor performance differently.

Our study of movement distance and gene expression revealed that snails respond to salt stress through changes in locomotion and transcription (Figs. 1, 4; Table 3). The significant reduction in snails' movement when exposed to low salinities likely preserves energy for additional metabolic and ionic osmotic regulatory mechanisms, as it does in sea urchin larvae (Stumpp *et al.*, 2012) and marine worms (Pörtner *et al.*, 2004) when challenged with acidified seawater. On the other hand, the decrease in locomotion also could be explained by a decrease in intracellular adenosine triphosphate (ATP) concentration through ATP depletion or ATP extrusion from the cells; however, this has not been well studied yet.

Transcriptomic responses to salt stress

In this study, we utilized *de novo* transcriptome sequencing (RNA-Seq) to investigate the potential contribution of differential genes that may play significant roles in physiological tolerance of the intertidal snail *B. attramentaria* to salt stress. We compared gene expression between the control group (33 psu) and the stressed samples (43, 23, and 13 psu) and found that the number of DEGs from the 13-psu samples in comparison to the control (33 *cf.* 13 psu) was largest, with 2592 genes. Nevertheless, the number of DEGs from the comparisons of 33 and

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23 psu and of 33 and 43 psu were similar to each other (Fig. 4A; Table S4, available online). This result suggests that snails tend to express more genes at relatively low salinity (13 psu) in their osmoregulatory organs, such as gills, in order to acclimate to stress conditions. Functional gene annotation revealed genes associated with ionic and osmotic regulation, water permeation, and conformational developmental stability (Fig. 4B; Table S4). These genes and their functional pathways likely assist the snails to tolerate and acclimate to salt stress.

Ionic regulation

In general, when challenged by salt stress, living organisms must regulate their cell volume through activation of volume-regulatory mechanisms (Florkin, 1962; Wehner *et al.*, 2003; Freire *et al.*, 2008; Foster *et al.*, 2010; Florkin and Schoffeniels, 2013). Cell volume can be regulated through gaining osmotically active solutes (primarily, inorganic ions such as Na^+, K^+, and Cl^-) or by losing active solutes (Kirschner, 1991, 1997, 2004; Willmer, 2006). These mechanisms may be achieved by activating Na^+/H^+ and Cl^-/HCO^{3-} exchangers or Na^- $K^{-2}Cl$ cotransporters, in cooperation with Na^+, K^+ channels, energized by Na^+/K^+-ATPase, in order to take up the extra salt needed to counter the inevitable passive outflow (Riestenpatt *et al.*, 1996; Kirschner, 2004). These ion-transporting mechanisms are localized to the osmoregulatory or even excretory organs at the cell and blood interface. In the present study, genes encoding the ion transporters and channels Na^+/K^+-ATPase (crg [*Crassostrea gigas*]:105333474 [KEGG ID]), K^+ channel (crg:105318217), K^+ channel (crg:105334111), Ca^{2+} channel (crg:105338822), and H^+ channel ([*Gallus gallus*]: HVCN1_CHICK [UniProtKB ID]) were found to be upregulated only in the snails acclimated to low salinity (13 psu) (Fig. 4B; Table S4, available online). Apart from genes relating to ion channels, several genes relating to transporters of Na^+-coupled monocarboxylate (crg:105322721), organic cations (crg:105324636, crg:105332925), and Na^+/Cl^--dependent GABA (crg:105348180) were also found regulated in snails acclimated to 13 psu; but only some of them were found upregulated in those acclimated to 23 psu (Fig. 4B; Table S4). These genes also have been reported in other estuarine and brackish-water invertebrates such as shrimp *Macrobrachium amazonicum* (Boudour-Boucheker *et al.*, 2016), European green crab *Carcinus maenas* (Shetlar and Towle, 1989; Towle and Weihrauch, 2001), and blue crab *Callinectes sapidus* (Towle, 1997; Towle and Weihrauch, 2001).

Osmotic regulation

When exposed to osmotic shock, aquatic organisms tend to maintain constant cell volume through the degradation or production of osmolytes, such as intracellular free amino acids (FAAs), sugars (trehalose), and other small organic molecules of polyols or methylamines (Lang *et al.*, 1998; Wehner *et al.*, 2003; Friedrich *et al.*, 2006; Pasantes-Morales *et al.*, 2006; Willmer, 2006). With respect to regulation of cell volume by lowering intracellular FAAs, we found several upregulated genes exclusively in the 13-psu group that encode for glycine N-acyltransferase (crg:105329920), glutamate dehydrogenase (crg:105348957), and taurocyamine kinase (crg:105320161) (Fig. 4B; Table S4, available online), which act to decrease the concentration of FAAs such as glycine, glutamate, taurine, and hypotaurine. In addition, we also found various upregulated genes relating to transporters of H^+-coupled amino acid (crg:105338428), vesical amino acid (crg:105330890), B(0,+)-type amino acid (crg:105321809), Y+L amino acid (crg:105319319), vesicular glutamate (crg:105342017), and solute carriers (crg:105327127, crg:105335737, crg:105317954, crg:105332163), as well as cotransporters of Na^+/glucose (crg:105328887) and Na^+/myo-inositol (crg:105333119) (Fig. 4B; Table S4A), which help the cell retain ions and eliminate FAAs. No gene expression changes were found relating to synthesis and/or acquisition of FAAs, essential ions, sugars, and organic molecules when the snails were acclimated to moderately changed salinities (23 and 43 psu). A possible explanation for this observation might be that 43 psu did not create a strong enough hyperosmotic shock to alter cell volume, so snails did not initiate osmolyte accumulation to protect cell volume. This interpretation is indirectly supported by the insignificant difference in movement distance among snails acclimated to 43 and 33 psu (Fig. 1B). Altogether, these results show that *B. attramentaria* utilizes FAA degradation and extrusion to deal with low salinity stress. This is consistent with the crab *Portunus tritubucuberlatus* (Lv *et al.*, 2013) and the White Sea periwinkle *Littorina* (Kuznetsov, 1960), which also reduce intracellular FAAs when exposed to low salinity conditions.

Water permeation

While the transportation mechanisms for ions and osmolytes have been discussed for decades, the molecular pathway of compensatory water fluxes *via* regulating aquaporins to deal with osmotic stress remains poorly understood. Aquaporins are integral membrane proteins that serve as channels for the transference of water and small solutes across the membrane (Agre *et al.*, 1993; Takata *et al.*, 2004). Interestingly, an earlier study reported that aquaporins might play important roles in many water transport processes because the aquaporin transcript appears to change widely in various organs (such as digestive tract, cerebral ganglia, kidney, reproductive system, and foot) of aquatic snails exposed to salt stress (Pieńkowska *et al.*, 2014). In the current transcriptional data, we found an upregulated gene relating to aquaporin (crg:105341812) in individuals reared at 13 psu (Fig. 4B; Table S4, available online). This finding is consistent with previous transcriptomic studies of the oyster *Crassostrea gigas* and crab *P. tritubucuberlatus*, which showed upregulation in genes relating to aquaporins under both hyper- and hypo-osmotic shock (Zhao *et al.*, 2012; Lv *et al.*, 2013; Meng *et al.*, 2013). Because the pathway of

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compensatory water fluxes through aquaporins during osmotic shock is still poorly understood for marine snails, further studies are warranted to investigate these mechanisms.

Conformational or developmental stability

The mechanisms described thus far help the cell to maintain relatively constant and less dilute conditions than its surroundings. However, to acclimate to changes in salinity, intertidal organisms, including *B. attramentaria*, must also perform additional tasks involved in protein conformational or developmental stability through genetic modification, signaling transduction, and/or stress detection.

From the current genetic profiles, we found several regulated genes belonging to the methyltransferase family, including betaine-homocysteine S-methyltransferase (crg:105339575), histone-lysine N-methyltransferase (cfo:105258654), and 28S rRNA (cytosine-C(5))-methyltransferase (crg:105327705), in the individuals exposed to 13 psu but not those exposed to other salinities (Fig. 4B; Table S4, available online). These genes are believed to have pivotal roles in responding to environmental stress and regulating expression of specific genes (Elango *et al.*, 2009; Bonasio *et al.*, 2012; Wang *et al.*, 2013). Similar findings have been reported in the Japanese blue crab *P. trituberculatus* exposed to salt stress (Lv *et al.*, 2013).

We also found at least one gene relating to protocadherin (crg:105331301), cadherin EGF LAG seven-pass G-type receptor ([*Aplysia californica*]: XP_012939635.1 [UniProtKB ID]), and cadherin ([*Rhinolophus ferrumequinum*]: CADH2_RHIFE [UniProtKB ID]) to be upregulated in individuals exposed to 13 psu (Fig. 4B; Table S4, available online). Additionally, several genes encoding for dopamine β-hydroxylase (crg:105343978) and relating to hormone activity (thyrotropin-releasing hormone receptor, crg:105332602) were found to change in expression level in individuals exposed to both 13 and 43 psu. These signal transduction pathways, including calcium-dependent pathways and hormonal signaling pathways, were recently reported as osmotic-responsive pathways in the Pacific oyster *C. gigas* (Zhao *et al.*, 2012; Zhang *et al.*, 2016) and crab *P. trituberculatus* (Lv *et al.*, 2013).

In the present study, we found several Hsp genes, including Hsp 68 (crg:105334510) and Hsp 70 (crg:105348304), that were upregulated exclusively in the individuals exposed to the acute low salinity of 13 psu (Fig. 4B; Table S4, available online). Since we controlled the temperature and artificial seawater conditions during the whole acclimation period, we suspect that the changes in Hsp expression were due to changes in concentrations of ions and osmolytes, and possibly cellular energy depletion, directly caused by exposure to low salinity (13 psu). Among various genes encoding for chaperone proteins, we particularly focus on Hsp genes because they are well known as salt stress inducible and function to protect cells from the damaging effects of heat, cellular energy depletion, and other stressors (Feder and Hofmann, 1999; Deane *et al.*, 2002; Deane and Woo, 2004). This finding is also supported by previous studies that show a correlation between Hsp expression and salt stress in intertidal molluscs such as the bivalve *Potamocorbula amurensis* (Werner and Hinton, 2000; Werner, 2004) and Pacific oyster *C. gigas* (Zhang *et al.*, 2012).

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Data Accessibility

We deposited the short-read (Illumina HiSeq4000) data in the Short Read Archive (SRA) of the National Center for Biotechnology Information with the study accession number SUB2798442. We also archived all footage videos achieved from behavioral experiments and transcriptomic assembly files in Dryad Digital Repository, https://doi.org/10.5061/dryad.455mv2m.

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Appendix

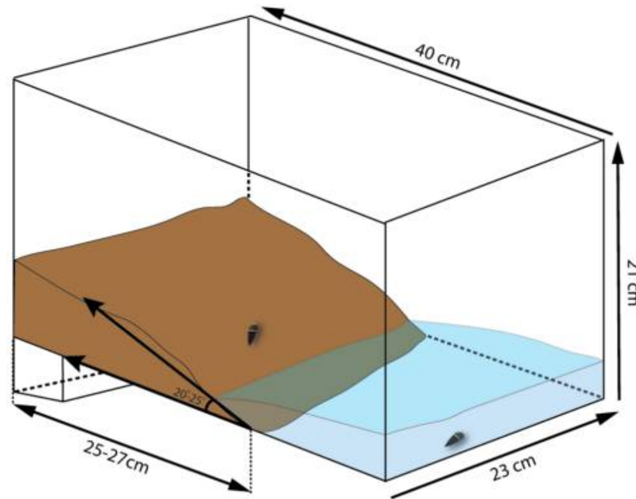
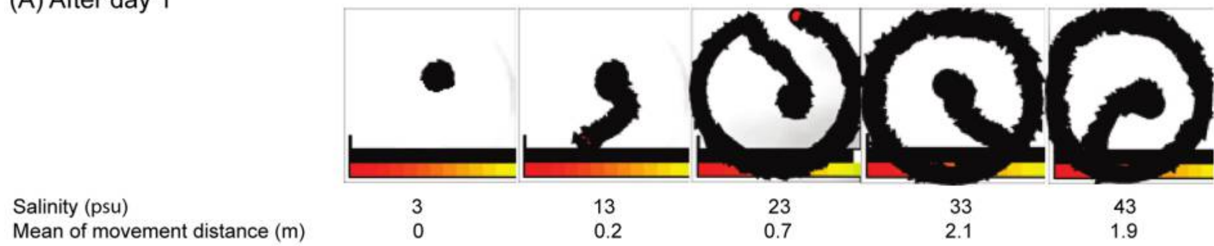


Figure A1. Laboratory-based culturing system designed for the intertidal gastropod *Batillaria attramentaria*.



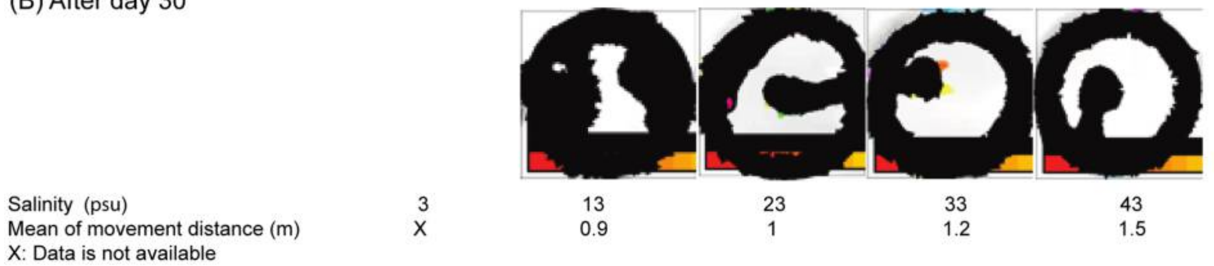


Figure A2. Mean of movement distance and representative images of cumulative *Batillaria attramentaria* trails over one hour at different salinities after (A) day 1 (short-term acclimation experiment II) and (B) day 30 (long-term acclimation experiment).

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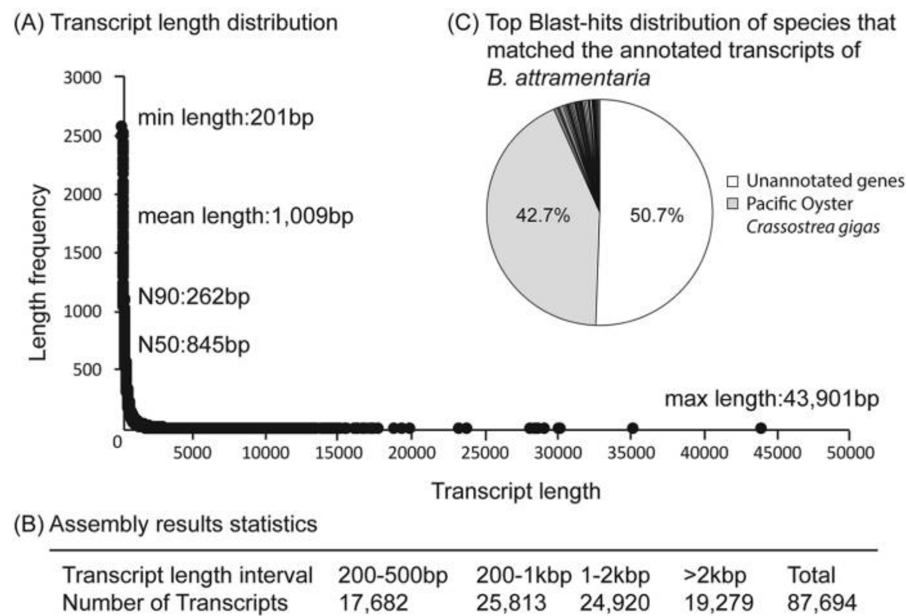

Transcript length interval	200-500bp	200-1kbp	1-2kbp	>2kbp	Total
Number of Transcripts	17,682	25,813	24,920	19,279	87,694

Figure A3. (A) Length distribution of genes assembled from Illumina reads, (B) assembly result statistics, and (C) top BLAST hit distribution of species that matched the annotated transcripts of *Batillaria attramentaria*.

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