Chronobiological Studies on the Adaptation to the Tidal Environment in a Freshwater Snail

チリメンカワニナにおける潮汐環境適応の時間生物学的検証

January 2023

Graduate School of Science and Engineering,
Chiba University

Takumi Yokomizo

（千葉大学審査学位論文）

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Abstract

Genetic and non-genetic changes could drive the adaptive modification of traits and contribute to the establishment of populations in novel environments. One of the most fundamental factors that organisms should cope with in the field is the temporal variation of the environment. In a river, the tidal cycle causes complicated environmental variations and creates a steep gradient of salt concentration. Here, I examined the salinity response and endogenous rhythm of the freshwater snail, *Semisulcospira reiniana*, in upstream and downstream populations to reveal the mechanism of tidal adaptation. Behavioral and transcriptome analysis revealed the plastic increase of salinity tolerance and detected biological pathways associated with salinity response. Chronobiological analyses of behavior and gene expression revealed that snails had habitat-specific endogenous rhythms, and even the non-tidal population showed the circatidal rhythm by the entrainment to the simulated tidal cycle. These results suggest that adaptive plasticity plays an important role in coping with tidal environments. I further examined the effect of the simulated tidal cycle on the expression patterns of several genes. My result suggests that Wnt gene expression may be influenced by the water level oscillation. Studies on this thesis would contribute to understanding the mechanism of the adaptation to tidal environments and the genetic basis of circatidal clocks.

General Introduction

Life is ubiquitously established on the earth even in extreme environments. The tremendously wide distribution of organisms is accomplished by the successful range expansion via adaptation and/or accumulation. Adaptation is the evolutionary process whereby an organism becomes better able to live in its habitat or habitats (Dobzhansky 1968), involving genetic changes. Adaptation is powerful but not the only process resulting in the adaptive modification of specific traits. Acclimation through phenotypic plasticity also contributes to coping with environmental factors (Via et al. 1995, Yeh and Price 2004). In addition, adaptive phenotypic plasticity sometimes drives decreased environmental sensitivity, which can result in the constitutive expression of the trait (Waddington 1953, 1961). Phenotypic plasticity could precede and facilitate adaptation, dubbed the plasticity-first hypothesis (West-Eberhard 2003). Altogether, genetic and non-genetic changes play important roles in establishing populations in novel environments.

One of the most fundamental factors which organisms face in the field is the temporal variation of the environment, represented by the day-night cycle. The physical force of the earth, sun, and moon provides a daily cycle, tidal cycle, and seasonal variation. Organisms have endogenous timekeeping system(s) that coordinate their biological processes in rhythmic environments and cope with such environmental cycles. Among the biological timekeeping systems, the circadian clock is the best-characterized one in the history of chronobiology (Sharma 2003). The circadian clock is characterized by three properties: a free-running period of approximately 24 hours, being entrainable to environmental cues, and temperature compensation (Dunlap et al. 2004). Although little is known about the mechanisms of biological clocks other than circadian clocks,

the existence of endogenous rhythms synchronized with tidal, lunar, and annual rhythms has been reported (Gwinner 2003, Tessmar-Raible et al. 2011, Numata and Helm 2014, Kaiser and Neumann 2021). Biological clocks are of great advantage in rhythmic environments for organisms to anticipate environmental changes and prepare accordingly.

In addition to the earth's rotation, tidal forces by the sun and moon have a strong influence on several habitats. Complicated environmental variations caused by the daily and tidal cycle are found in marine environments. The tidal cycle also strongly influences the areas adjacent to the sea. In the estuary, lots of environmental factors such as the water level oscillate with a tidal period of approximately 12.4 hours. Moreover, seawater intrusion due to the tidal cycle provides high salinity. Because the tidal cycle has less influence further away from the sea, there is a steep or stepwise spatial gradient of the strength of the tidal cycle and salt concentration in a river. By focusing on this spatial environment heterogeneity between upstream and downstream areas, I can address the mechanisms of adaptation to the novel, adjacent environment and thus, range expansion. Especially, bridging evolutionary ecology and chronobiology could contribute to uncovering the evolutionary process underlying the adaptation to novel habitats with different environmental cycles. Here, in this thesis, I examined the salinity response and endogenous rhythm in the freshwater/non-tidal and brackish-water/tidal populations of the freshwater snail, *Semisulcospira reiniana*.

In Chapter 1, I investigated behavioral and gene expression responses to salinity in a freshwater and brackish water population of *S. reiniana* using behavioral observations and transcriptome analysis. Salinity is a significant environmental factor affecting the distribution range of aquatic species (Charmantier 1998, Cervetto et al.

1999, Ho et al. 2019). Tolerance to salinity stress is crucial for freshwater snails to successfully inhabit brackish water areas. To answer whether genetic or plastic change contributes to the tolerance to salinity stress and which biological pathways are associated with salinity response, an intraspecific comparison using closely related populations in a freshwater and brackish water area is needed. Investigation into the plasticity of salinity stress and biological pathways related to salinity response could shed light on the mechanisms of coping with brackish water in a freshwater snail. I here revealed the plasticity in salinity tolerance and found biological processes involved in salinity response.

In Chapter 2, I compared endogenous rhythms between the tidal and non-tidal populations of *S. reiniana*. I investigated behavioral and transcriptome rhythmicity using chronobiological analysis. As reported in marine and intertidal species, circatidal rhythm can be advantageous to cope with drastic environmental changes caused by tidal cycles in marine and neighboring habitats (Barnwell 1966, Hastings and Naylor 1980, Satoh et al. 2008). I focused on a tidal area in a river and hypothesized that populations near the estuary showed a circatidal rhythm to synchronize with the tidal cycle. I demonstrated that the tidal population of *S. reiniana* has the circatidal rhythm in activity and gene expression. The present study could reveal the relationship between the modification of endogenous rhythms and range expansion.

Previous researches suggest that some marine species showing the circatidal rhythm plastically switch their endogenous rhythms depending on the dominant environmental cycle (O'Neill et al. 2015, Mat et al. 2016, Tran et al. 2020). Therefore, examining the endogenous rhythm of animals in the field is insufficient to distinguish whether the difference in the endogenous rhythm is caused by plasticity or clock

evolution. In Chapter 3, I investigated whether non-tidal individuals of *S. reiniana* showed the circatidal rhythm after exposure to the simulated tidal environment. My results of this chapter suggest that snails plastically change their endogenous rhythm depending on environmental cycles.

Finally, in Chapter 4, I focus on the molecular mechanisms of the circatidal clock. The discovery of genes governing the circatidal rhythm is still challenging in chronobiology. The circatidal rhythm is suggested to be driven by a different timekeeping system from the circadian rhythm (Zhang et al. 2013). As the first step for identifying candidate genes associated with the circatidal pathway, I examined the gene expression rhythms of tidal individuals after entrainment to the simulated tidal cycle. Detecting the circatidal clock gene would contribute to providing insight into physiological processes and molecular evolution of the circatidal rhythm.

The functional importance of biological clocks in the wild is of interest in evolutionary ecology and chronobiology (Helm et al. 2017, Schwartz et al. 2017). Adaptation to the discontinuous environmental cycles of tidal (12.4 hours), daily (24 hours), semi-lunar (14.8 days), lunar (29.6 days), and annual (1 year) cycles may require drastic and innovative evolution involving a few genes, rather than polygenic and gradual evolution. Evolutionary ecology and chronobiology complement each other and contribute to understanding the mechanism of adaptation to rhythmic environments. Multi-scale analysis of molecular and phenotypic levels focused on natural variations in individuals in different rhythmic environments could be a critical step toward that goal.

In this thesis, I used the freshwater snail, *S. reiniana*, which is an ovoviviparous freshwater snail in a freshwater area in Japan (Fig. 1). This species is found in a wide range of environments from pebble to muddy bottom. Shell height of adult individuals

is described as 26.1 mm on average in previous research (Davis 1969) but is highly variable within species.

All individuals of *S. reiniana* in this thesis were collected in the Kiso River in Japan (Fig. 2). Kiso River is a Class A river flowing through Nagano, Gifu, Aichi, and Mie Prefectures before emptying into Ise Bay. The width of the Kiso River is quite wide and the current is gentle in the lower reaches. Because there is no estuary weir in this river, seawater intrusion reaches 20 km from the estuary. I can study the ongoing process of adaptation by examining populations along an environmental gradient in the Kiso River.

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Figures

Figure 1. An adult individual of *Semisulcospira reiniana* walking in a water tank (a) and an individual in a tidal area at the low tide condition (b).

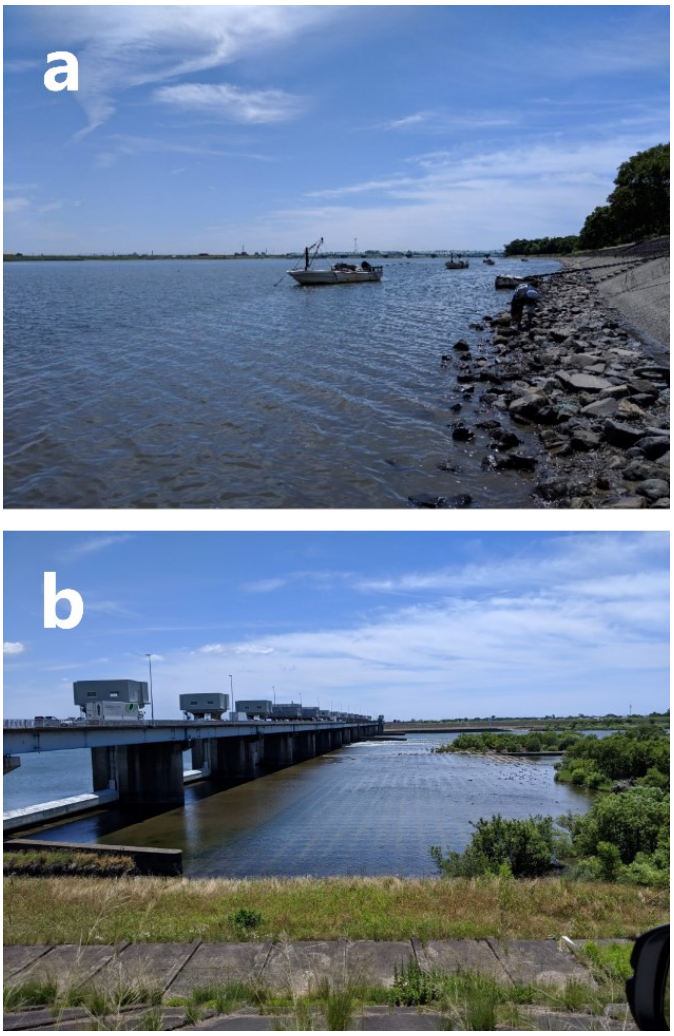

Figure 2. Sampling sites in a tidal area (a) and non-tidal area (b) of the Kiso River.

Chapter 1

Changes in Transcriptomic Response to Salinity Stress Induce the Brackish Water Adaptation in a Freshwater Snail

The content of this chapter has been published in *Scientific Reports*.

(Yokomizo and Takahashi, 2020)

Abstract

Studying the mechanisms of the establishment of a population in a novel environment allows us to examine the process of local adaptations and subsequent range expansion. In a river system, detecting genetic or phenotypic differences between a freshwater and brackish water population could contribute to our understanding of the initial process of brackish water adaptation. Here, I investigated behavioral and gene expression responses to salt water in a freshwater and brackish water population of the freshwater snail, *Semisulcospira reiniana*. Although the individuals in brackish water exhibited significantly higher activity in saltwater than freshwater individuals just after sampling, the activity of freshwater individuals had increased in the second observation after rearing, suggesting that their salinity tolerance was plastic rather than genetic. I found 476 and 1,002 differentially expressed genes across salinity conditions in the freshwater and brackish water populations, respectively. The major biological process involved in the salinity response of the freshwater population was the biosynthesis and metabolic processing of nitrogen-containing compounds, but that of the brackish water population was influenced by the chitin metabolic process. These results suggest that phenotypic plasticity induces adaptation to brackish water in the freshwater snail by modifying its physiological response to salinity.

Introduction

Studies concerning distribution patterns of species or populations along the environmental continuum play a significant role in discovering evidence of environmental adaptations. When a species encounters a novel environment during range expansion, adaptation to the environment could aid in the establishment of a population. Adaptations are achieved via two different processes: adaptive evolution and phenotypic plasticity (Gienapp et al. 2008, Anderson et al. 2012). For instance, invasive species, which are introduced into non-native regions by human activity, have been studied extensively with respect to the ongoing process of their adaptive evolution, as they have acquired various traits that are suitable for a novel environment through rapid evolution (Siemann and Rogers 2001, Bossdorf et al. 2004, Maron et al. 2004). Evolutionary adaptations, of course, contribute to colonization and expansion into a novel environment in native species (Byrne and Nichols 1999, Lee 1999, Linnen et al. 2013). Phenotypic plasticity could enhance adaptation to a novel environment, as well as range expansion, because it should enable a species to cope with unfamiliar conditions (Yeh and Price 2004, Price et al. 2008, Lande 2015). Both theoretical and empirical studies suggest that individuals with high levels of phenotypic plasticity dominate the edges of distribution ranges (Chevin and Lande 2011, Orizaola and Laurila 2016). For example, plasticity in thermal tolerance is an important trait facilitating survival at the range margins for the fruit fly *Ceratitis capitate* (Nyamukondiwa et al. 2010). In general, plasticity makes a significant contribution in adaptation to heterogeneous environments at small spatial scales, or to rapid environmental changes (Richards et al. 2006, Crispo 2008). Adaptive plasticity is hypothesized to provide a rapid response to the environment, and also to precede and facilitate adaptive evolution

(Baldwin 1896, Waddington 1961, Price et al. 2003, Lande 2009, Levis and Pfennig 2016). Therefore, understanding the process of both adaptive evolution and phenotypic plasticity is important for revealing the mechanism of range expansion.

In systems in which different environmental conditions are adjacent to each other at relatively small spatial scales, individuals have opportunities to colonize new habitats outside of their current distribution, and thus range expansion can be achieved if a species or population adapts to the environment. Therefore, I can study the ongoing process of adaptation by examining the evolutionary or plastic change in traits along an environmental gradient. A river is one such system, because a steep gradient of salt concentration, which is a significant environmental factor influencing the distribution range of many aquatic species (Charmantier 1998, Cervetto et al. 1999, Ho et al. 2019), is observed near the estuary due to saltwater intrusion. In freshwater molluscs, tolerance to salinity is critical to populations that reside near estuaries, because salinity affects the osmotic regulation of individuals, and is also involved in their survival, growth, and reproduction (Yang et al. 2018). Therefore, tolerance to salinity can assist freshwater species in successfully inhabiting brackish water areas.

Detecting genetic or phenotypic variation in individuals living in freshwater or brackish water will contribute to our understanding of the mechanisms of adaptation to brackish water. Several studies have evaluated differences in tolerance to salinity between freshwater and brackish water species (Deaton et al. 1989, Jordan and Deaton 1999). However, interspecific comparisons, in general, may not clarify the initial process of adaptation to brackish water. The differences between species include differences that are responsible for adaptation to brackish water, those that accumulated after the adaptation, and those which are totally unrelated to the adaptation to saltwater.

Conversely, differences within species include only the differences responsible for the adaptation to brackish water, because few mutations accumulate in the time following the isolation of populations. Thus, intraspecific comparisons, such as comparisons between a freshwater and a brackish water population within a single species, enable us to clarify the initial process of adaptation to brackish water, and the mechanisms that lead to the expansion of species distributions into brackish water.

The freshwater snail, *Semisulcospira reiniana*, inhabits a broad range of river environments. Although the snail primarily occupies freshwater areas, it also lives in brackish water areas in several rivers of Japan. Here, I investigate the behavioral and gene expression responses to saltwater in adult individuals of the species. First, to examine their activity under salinity stress, I compared activity levels in saltwater using individuals in a freshwater population and its adjacent brackish water population. Second, transcriptome analysis was conducted to find gene(s) differentially responding to saltwater between the populations. Using transcriptome data, I also assessed the genetic distance between the two adjacent populations.

Materials and Methods

Species and Sampling

Semisulcospira reiniana is a common freshwater snail in Japan and mainly inhabits the freshwater areas of rivers. I collected adult individuals of *S. reiniana* from freshwater (35° 15′ 15″ N, 136° 41′ 09″ E) and brackish water (35° 07′ 22″ N, 136° 41′ 27″ E) areas of Kiso River in Japan, in May 2018 and June 2019. While water level fluctuations caused by the tidal cycles occur at both sampling sites, seawater does not reach the freshwater site. The salt concentration of the freshwater site was almost 0%,

while the salt concentration at the brackish water site fluctuates from 0 to 0.5% according to the tidal cycle. Since these two populations are located relatively close to each other (15 km apart), I assume that the two populations have not diverged genetically. The snail specimens were preserved in a container (13 × 13 × 20 cm) maintained at 23°C for one day until the onset of experiments.

Behavioral response to salinity

Individuals collected in freshwater and brackish water populations were examined for activity under concentrations of 0% and 0.4% saltwater. Ten individuals were tested in each salinity condition for each of these two populations (total sample size was 40). Snails were put separately in individual bowls (φ13 × 3.5 cm) filled with 0% or 0.4% saltwater, prepared from decalcified tap water. The bowls were kept under laboratory conditions at a temperature of approximately 23°C. Thirty minutes after putting the individuals into the bowls, they were monitored by a camera (400-CAM061, Sanwa Direct) for two hours to quantify locomotive activity. Images were taken every 30 seconds and used to create time-lapse movies. I tracked the position of each individual using the tracking software UMATracker(Yamanaka and Takeuchi 2018). Using the snail trajectories, the total locomotion distance for two hours was calculated for each individual. After the first behavioral observation, I put individuals back into the containers filled with fresh water (decalcified tap water). I added a crushed oyster shell to the containers to keep the water clear. One week later, I conducted the second behavioral observation following the same procedure and using the same sample set.

Gene expression response to salinity

Three freshwater and three brackish water individuals, reared under the freshwater condition for one week after collection, were exposed to 0%, 0.6%, or 0.8% saltwater for three hours in the laboratory. While 0% saltwater was assumed to be a typical optimum environment for *S. reiniana in the natural population*, 0.6% and 0.8% were expected to be high and extremely high salinity conditions in the tidal cycle, respectively. Soon after exposure, I checked to see whether the snails were active. Individuals were dissected and their epidermis was preserved in 750 μl of RNA*later* Stabilization Solution (Invitrogen). The samples were kept at −80°C until the extraction of total RNA.

Total RNA was extracted using Maxwell 16 LEV Plant RNA Kit with the Maxwell 16 Research Instrument (Promega) according to the manufacturer's instructions. Electrophoresis on 1% agarose gels was performed to check for RNA degradation. RNA concentrations were estimated using a Qubit 2.0 Fluorometer (Invitrogen). RNA purity was estimated using a BioSpec-nano (Shimadzu). The cDNA library was constructed using TruSeq RNA Sample Prep Kits. Paired-end (150 bp) RNA sequencing (RNA-Seq) was performed on the Illumina NovaSeq6000 platform. After the removal of adaptor sequences and low-quality reads using Trimmomatic (version 0.38) (Bolger et al. 2014), I used FastQC ((version 0.11.8; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) for quality control. The remaining high-quality reads were used for *de novo* assembly using Trinity (version 2.6.5) (Grabherr et al. 2011).

To estimate gene expression levels, all reads of each sample were mapped to the reference transcripts using RSEM software (version 1.3.0) (Li and Dewey 2014).

The read count data was used for gene expression analysis. I searched for homologues of every *S. reiniana* gene using BLAST searches for all protein sequences of *Crassostrea gigas*. Genes with the best hit and with an e-value < 0.0001 were used for gene expression analysis. Differentially expressed genes (DEGs) among the three salt concentrations were detected using the TCC package (Sun et al. 2013, Tang et al. 2015). I considered genes with $q < 0.05$ as DEGs. Gene ontology (GO) enrichment analysis of the DEGs was performed using Blast2GO software (Conesa et al. 2005).

F_{ST} calculation

Putative coding regions were extracted from the reference transcripts using TransDecoder (version 5.5.0; https://github.com/TransDecoder/TransDecoder/wiki), providing the reference transcripts only contained coding sequences (CDS). They were clustered based on sequence identities of 90% to remove redundancy, using the CD-HIT program (Li and Godzik 2006). I used STAR for mapping all reads of each sample to the clustered CDS reference. I then used GATK3 (version 3.8.1) (McKenna et al. 2010) to identify SNPs. I used the homologues of *C. gigas,* which were detected in the same manner as described in a previous section. Genes with the best hit and with an e-value < 0.0001 were used to estimate the genetic structure of the two populations. To identify outlier loci which were not selectively neutral between the two populations, I ran the BayeScan program (Foll and Gaggiotti 2008) with default parameters. Putatively neutral loci were used to estimate F_{ST} between the two populations using Arlequin (Excoffier and Lischer 2010).

Statistical analyses

Locomotive distances in 0% and 0.4% saltwater were analyzed by the generalized linear model (GLM) with a Gamma distribution. Population (*i.e.*, freshwater, and brackish water population) and the number of days after collecting (*i.e.*, first and second observation) were included as explanatory variables. Post hoc test for all four pairwise comparisons was conducted using the GLM with a Gamma distribution. Since the analysis was performed four times, I applied the Bonferroni correction to account for multiple comparisons (Bonferroni-adjusted α was 0.0125). All statistical analyses were performed in R 3.4.2.

Results

Genetic differentiation

Information on the number of reads in each sample was summarized in Table 1-S1. I obtained 983,061 transcripts with a mean length of 556 bp via *de novo* assembly. Among the 978 metazoan core gene orthologues, 969 genes (99.1%) were identified completely. I ran the CD-HIT program using 164,656 putative CDS, which provided 94,676 clustered CDS transcripts. Using these CDS transcripts, I identified 5,871 SNPs on the contigs annotated against all protein sequences of *C. gigas* by BLAST search. I used these SNPs for the following population genetic analysis.

The BayeScan program did not identify any outlier loci, indicating that all SNPs were selectively neutral between the two populations. Using all 5,871 SNPs, the F_{ST} value between the freshwater and brackish water populations was estimated at 0.011.

Behavioral response to saltwater

The locomotive activity in 0% saltwater was not significantly different between populations, either in the first observation immediately after collecting, or in the second observation after rearing in experimental freshwater for one week following the first observation (population [P]: $\chi^2 = 0.02$, $P = 0.89$; the number of days after collecting [D]: $\chi^2 = 0.03$, $P = 0.85$; P × D: $\chi^2 = 0.03$, $P = 0.87$, Fig. 1-1a). In contrast, I found interaction effects for the locomotive activity in 0.4% saltwater (P: $\chi^2 = 1.06$, $P = 0.30$; D: $\chi^2 = 0.64$, $P = 0.42$; P × D: $\chi^2 = 11.3$, $P < 0.001$, Fig. 1-1b), indicating a differential response to the experience of being reared in an experimental freshwater setup for one week between populations. Post hoc comparison found significant differences in locomotive activity between populations on the first observation, immediately after sampling. The locomotive activity of the freshwater population in 0.4% saltwater was higher during the second observation, after rearing in freshwater. Although I was not able to identify a statistically significant difference, the locomotive activity of the brackish water population in 0.4% saltwater tended to decrease between the first observation and the second observation. Consequently, no significant difference between populations in locomotive activity in 0.4% saltwater was found at the second behavioral observation.

Gene expression response to salinity

All freshwater and brackish water individuals were active in 0% saltwater. In 0.6% saltwater, all brackish water individuals were active, while three of the four freshwater individuals were not. In 0.8% saltwater, two of the four brackish water individuals were active, but all the freshwater individuals withdrew into their shells to avoid the salinity.

These results supported the hypothesis that brackish water individuals have a higher tolerance to salinity, as shown previously.

Among the164,656 putative CDS, 81,495 (49.5%) of them were annotated against all protein sequences of *C. gigas*, using BLAST search. These annotated sequences comprised 34,816 genes. I used these genes for gene expression analysis.

The number of DEGs among the three salt concentrations was higher in the freshwater population (1,002) than in the brackish population (476), indicating that many more genes were differentially expressed in the freshwater population than in the brackish-water population (Fig. 1-2a). Among these DEGs, 48 genes were shared between the two populations, but the expression patterns of the shared DEGs differed from each other (Fig. 1-2b; Table 1-1). The number of genes highly expressed in 0.8% saltwater was higher than that expressed in other saltwater concentrations, irrespective of the population, probably due to strong salinity stress (Fig. 1-2c).

The GO terms that were significantly enriched within the set of DEGs in the freshwater and brackish water populations are listed in Tables 1-2 and 1-3, respectively. I found 62 terms in the freshwater population and 53 terms in the brackish one (Figs. 1-S1, 1-S2) In the freshwater population, 45 terms were unique to the population (Table 1-2), including those involved in the biosynthesis and metabolic processes of nitrogen-containing compounds. In the brackish water population, 36 terms were unique to the population (Table 1-3), including those involved in the chitin metabolic process and urea transport.

Discussion

Either adaptive evolution or phenotypic plasticity, or both, can contribute to the

establishment of a population in a novel environment (Gienapp et al. 2008, Anderson et al. 2012). In a river, salinity gradually increases toward the estuary, creating a steep environmental gradient. Freshwater organisms typically do not possess the physiological mechanisms to cope with high salinity. The distribution of freshwater species is limited to a specific range between brackish and freshwater areas. Therefore, range expansion to brackish areas requires tolerance to salinity, and thus brackish water populations display different salinity responses than freshwater populations. In the present study, I confirmed that individuals from a population living in brackish water displayed different salinity responses from those of a freshwater population, in terms of both activity and physiology. My findings could clarify the understanding of the mechanisms of brackish water adaptations via adaptive evolution and/or phenotypic plasticity.

The F_{ST} value between two populations, which I observed, is very low (0.011) compared with previous studies in snails (Sinclair 2010, Bouétard et al. 2014). This value of F_{ST} is considered to indicate that the two adjacent populations exhibit little genetic differentiation (Hartl and Clark 1997), supporting my assumption. The brackish water population is suggested to have colonized their novel environment recently or regularly receive immigrants from the freshwater population. The brackish water population appears to be in the initial stages of adaptation to brackish water, and thus few mutations have accumulated in the population. Therefore, in my study system, I could observe a phenomenon that occurs in the early stages of brackish water adaptation.

In 0.4% saltwater, I found significantly higher activity levels in individuals in brackish water than in individuals in fresh water immediately after transfer from one environment to the other, indicating that individuals in brackish water can be active

even in conditions of salinity. The lack of significant differences between the activity levels of populations at the second observation is probably due to acclimation to the same rearing environment for one week. The increased activity of freshwater individuals at the second observation may be due to their acclimation to unexpected changes in water conditions, because the 0% saltwater used for keeping individuals may be slightly hypertonic due to the effect of their feces and a crushed oyster shell. The lack of difference between the activity levels of populations in 0.4% saltwater after one week suggests that individuals have the ability to change their activity level according to water conditions. If higher activity in saltwater is derived from high tolerance to salinity, my results suggest that phenotypic plasticity may contribute to tolerance to salinity in individuals in brackish water. The hypothesis that phenotypic plasticity may contribute to salinity tolerance is supported by the absence of outlier loci in the two populations, which showed distinct responses to salinity. However, I cannot rule out adaptive evolution caused by genetic differences, especially in non-coding regions.

The relationship between phenotypic plasticity and adaptation to novel environments has been long studied. Baldwin (1896) suggested that adaptive plasticity plays an important role in the establishment and persistence of populations in new environments (Baldwin 1896). Also, an adaptive phenotype initially accomplished by plasticity is hypothesized to sometimes become genetically-encoded, so that establishment in a new environment is achieved (Waddington 1961, Price et al. 2003). My results indicate that *S. reiniana* can acclimate to salinity even in individuals grown in fresh water. Individuals in brackish water may cope with salinity via phenotypic plasticity, and may be in the process of adaptation to a brackish environment as suggested by Baldwin's hypothesis.

In my transcriptome analysis, I found that the expression level of 48 genes changed with salinity in the two populations, suggesting that these genes could be involved in the basis of saltwater response in *S. reiniana*. I detected four genes encoding proteins with von Willebrand factor domains within the shared DEG set. Von Willebrand factor, which plays a key role in normal hemostasis, is suggested to be expressed when extracellular NaCl levels are elevated (Dmitrieva and Burg 2013). High salinity would have a deleterious effect on freshwater snails, by promoting excess thrombus formation. In the freshwater population I found several genes involved in the biosynthesis and metabolic processing of nitrogen-containing compounds, such as glutamate synthase and glutamine synthetase. Gene ontology analysis also revealed DEGs enriched in nitrogen compound biosynthetic and metabolic processes. Nitrogen containing compounds accumulate in plant species subjected to salinity stress and are involved in osmoregulation (Mansour 2000). Nitrogen metabolism is also involved in the salinity responses of many molluscan species (Somero and Bowlus 1983), indicating that individuals in freshwater have common processes in response to salinity. A different pattern of gene expression was observed in the brackish water population, in which I noted that several genes associated with chitin metabolism were differentially expressed, including chitin synthase C and chitotriosidase-1. While chitin is an essential component of mollusc shells, its function in osmoregulation has not been reported. However, Lv et al. (2013) suggested that chitinase is associated with the response to salinity in crustacea, leading to the hypothesis that a similar process occurs in *S. reiniana* (Lv et al. 2013). DEGs in the brackish water population were enriched not only in chitin metabolism but also in urea transport. Urea functions as an osmolyte, and holds special importance for cell volume preservation in an aquatic snail under hyperosmotic

stress (Wiesenthal et al. 2019). However, it is not clear why genes associated with urea were enriched only in the brackish water population and those associated with nitrogen containing compounds only in the freshwater population; although both of them are osmolytes which act to prevent water loss in hyperosmotic environments. In summary, more than half of the GO terms that were enriched within each DEG set did not overlap, indicating that the biological processes in response to salinity were different in the two populations. These differences in gene expression patterns may be caused by plasticity in brackish water adaptations. Freshwater individuals would acclimate to saltwater in a few days, and individuals in both populations would exhibit similar gene expression patterns.

My results indicate that individuals in fresh water and brackish water show different responses to salinity with respect to activity levels and gene expression patterns. The establishment of a population in a brackish water area requires the activation of biological processes that can cope with high salinity. Given the results of my behavioral and transcriptome analyses, metabolic processing of chitin and urea may lead to higher activity in saltwater in individuals from brackish water. However, my behavioral observations suggest that individuals from fresh water may have enhanced activity under salinity stress. In summary, acclimation to hypertonic conditions and alteration of salinity responses via physiological processes can contribute to the early stage of brackish water adaptation in freshwater snails.

Note that my results do not explain why range expansion to a brackish water environment has not been achieved in all rivers, despite their potential for acclimation to salinity. Evolutionary differences in the strength of plasticity and long-term salinity tolerance may be associated with the success of brackish water adaptations. Further

investigation into the effects of long-term exposure to salinity on the survival, growth, and reproduction of individuals in fresh and brackish water is required.

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Tables and Figures

Table 1-1. Differentially expressed genes in both fresh and brackish water populations. Transcript names are unique names for the *Semisulcospira reiniana* transcriptome dataset. Gene descriptions are referred to UniProtKB/Swiss-Prot and UniProtKB/TrEMBL.

Transcript name	Description
DN104875_c3_g1	Glutamate decarboxylase 2
DN70911_c1_g2	Phospholipase D1
DN96969_c2_g2	Profilin
DN109559_c8_g2	Uncharacterized protein
DN107602_c0_g1	Meprin A subunit beta
DN75608_c1_g2	Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1
DN92446_c4_g1	Complement C1q subcomponent subunit B
DN96940_c6_g1	Dynein heavy chain 5, axonemal
DN81309_c3_g1	Epithelial membrane protein 2
DN106829_c0_g1	Prolow-density lipoprotein receptor-related protein 1
DN73791_c0_g2	VWFA domain-containing protein
DN92722_c0_g1	Beta-1,3-glucan-binding protein
DN72022_c0_g1	Protein SET
DN88881_c1_g1	Uncharacterized protein
DN97201_c3_g2	Sulfotransferase family cytosolic 1B member 1
DN74764_c0_g1	Ras-related protein Rab-8A
DN87228_c4_g1	Myosin regulatory light chain sqh
DN71681_c0_g1	Vitellogenin-6
DN75279_c3_g1	VWFD domain-containing protein
DN85940_c2_g1	Neurogenic locus notch-like protein 2
DN90737_c3_g1	Neurogenic locus notch-like protein 2
DN70840_c2_g1	DnaJ-like protein subfamily A member 1
DN91231_c1_g3	Separin
DN107589_c3_g2	Usherin
DN86757_c0_g1	Elongation factor 1-alpha
DN92429_c0_g1	Elongation factor 1-alpha
DN109604_c1_g1	Apple domain-containing protein
DN96478_c8_g1	Steroid 17-alpha-hydroxylase/17,20 lyase
DN98756_c4_g2	von Willebrand factor D and EGF domain-containing protein
DN93702_c4_g1	Myosin-10
DN100999_c2_g1	Cytochrome b-c1 complex subunit 7
DN89919_c0_g1	Titin
DN107210_c3_g2	Transporter
DN104156_c3_g1	Caprin-2

DN73399_c6_g4	Caprin-2
DN97040_c5_g1	Caprin-2
DN103483_c1_g2	Sodium bicarbonate transporter-like protein 11
DN84475_c5_g2	EB domain-containing protein
DN71942_c0_g1	Collagen alpha-4(VI) chain
DN81464_c3_g4	Heavy metal-binding protein HIP
DN100403_c5_g1	Estrogen sulfotransferase
DN74380_c0_g1	Cathepsin F
DN79716_c2_g1	Tripartite motif-containing protein 2
DN101532_c0_g2	GTPase IMAP family member 7
DN82214_c1_g3	GTPase IMAP family member 7
DN69782_c0_g1	GTPase IMAP family member 7
DN93746_c3_g1	GTPase IMAP family member 7
DN78798_c3_g1	Cytochrome P450 10

Table 1-2. GO terms enriched in the freshwater population. GO terms unique to the freshwater population are shown in bold. BP: biological process, MF: molecular function, CC: cellular component.

Gene ontology ID	Term	Category	FDR
GO:0003735	structural constituent of ribosome	MF	8.06×10^{-13}
GO:0005198	structural molecule activity	MF	1.12×10^{-12}
GO:0043232	intracellular non-membrane-bounded organelle	CC	1.12×10^{-12}
GO:0005840	ribosome	CC	1.21×10^{-12}
GO:0043228	non-membrane-bounded organelle	CC	1.25×10^{-12}
GO:0006518	peptide metabolic process	BP	6.37×10^{-12}
GO:0006412	translation	BP	9.40×10^{-12}
GO:0043043	peptide biosynthetic process	BP	1.88×10^{-11}
GO:1990904	ribonucleoprotein complex	CC	4.46×10^{-11}
GO:0043604	amide biosynthetic process	BP	4.46×10^{-11}
GO:0043603	cellular amide metabolic process	BP	2.79×10^{-10}
GO:1901566	**organonitrogen compound biosynthetic process**	**BP**	$\mathbf{1.49\times 10^{-8}}$
GO:1901564	organonitrogen compound metabolic process	BP	7.24×10^{-8}
GO:0019538	**protein metabolic process**	**BP**	$\mathbf{8.38\times 10^{-7}}$
GO:0032991	**protein-containing complex**	**CC**	$\mathbf{7.07\times 10^{-6}}$
GO:0043229	**intracellular organelle**	**CC**	$\mathbf{1.11\times 10^{-5}}$
GO:0044271	**cellular nitrogen compound biosynthetic process**	**BP**	$\mathbf{1.11\times 10^{-5}}$
GO:0003723	**RNA binding**	**MF**	$\mathbf{1.47\times 10^{-5}}$
GO:0043226	**organelle**	**CC**	$\mathbf{1.93\times 10^{-5}}$
GO:0005622	**intracellular**	**CC**	$\mathbf{2.08\times 10^{-5}}$
GO:0044424	**intracellular part**	**CC**	$\mathbf{2.08\times 10^{-5}}$
GO:0034645	**cellular macromolecule biosynthetic process**	**BP**	$\mathbf{2.39\times 10^{-5}}$
GO:0009059	**macromolecule biosynthetic process**	**BP**	$\mathbf{3.04\times 10^{-5}}$
GO:0004190	**aspartic-type endopeptidase activity**	**MF**	$\mathbf{6.94\times 10^{-5}}$
GO:0070001	**aspartic-type peptidase activity**	**MF**	$\mathbf{6.94\times 10^{-5}}$
GO:0010467	**gene expression**	**BP**	$\mathbf{8.72\times 10^{-5}}$
GO:0051082	**unfolded protein binding**	**MF**	**0.000121**
GO:0044444	**cytoplasmic part**	**CC**	**0.000132**
GO:0034641	**cellular nitrogen compound metabolic process**	**BP**	**0.000181**
GO:0044249	**cellular biosynthetic process**	**BP**	**0.00028**
GO:0005737	**cytoplasm**	**CC**	**0.000363**
GO:0006457	**protein folding**	**BP**	**0.000451**
GO:0004252	serine-type endopeptidase activity	MF	0.000456
GO:0006807	**nitrogen compound metabolic process**	**BP**	**0.000508**
GO:0044238	**primary metabolic process**	**BP**	**0.00059**
GO:0006414	translational elongation	BP	0.00109
GO:1901576	**organic substance biosynthetic process**	**BP**	**0.00109**
GO:0005623	**cell**	**CC**	**0.00111**

GO:0008236	serine-type peptidase activity	MF	0.00111
GO:0017171	serine hydrolase activity	MF	0.00115
GO:0044464	**cell part**	**CC**	**0.00118**
GO:0071704	**organic substance metabolic process**	**BP**	**0.00259**
GO:0009058	**biosynthetic process**	**BP**	**0.00337**
GO:0004175	endopeptidase activity	MF	0.00358
GO:0009987	**cellular process**	**BP**	**0.0038**
GO:0043170	**macromolecule metabolic process**	**BP**	**0.00472**
GO:0044267	**cellular protein metabolic process**	**BP**	**0.00902**
GO:0005856	**cytoskeleton**	**CC**	**0.0148**
GO:0090079	**translation regulator activity, nucleic acid binding**	**MF**	**0.0155**
GO:0008135	**translation factor activity, RNA binding**	**MF**	**0.0155**
GO:0006165	**nucleoside diphosphate phosphorylation**	**BP**	**0.0192**
GO:0046939	**nucleotide phosphorylation**	**BP**	**0.0192**
GO:0072521	**purine-containing compound metabolic process**	**BP**	**0.0204**
GO:0016864	**intramolecular oxidoreductase activity, transposing S-S bonds**	**MF**	**0.0277**
GO:0003756	**protein disulfide isomerase activity**	**MF**	**0.0277**
GO:0045182	**translation regulator activity**	**MF**	**0.0287**
GO:0009132	**nucleoside diphosphate metabolic process**	**BP**	**0.0289**
GO:0045275	**respiratory chain complex III**	**CC**	**0.0289**
GO:0006122	**mitochondrial electron transport, ubiquinol to cytochrome c**	**BP**	**0.0289**
GO:0046034	**ATP metabolic process**	**BP**	**0.0293**
GO:0009116	**nucleoside metabolic process**	**BP**	**0.0416**
GO:1901657	**glycosyl compound metabolic process**	**BP**	**0.0487**

Table 1-3. GO terms enriched in the brackish water population. GO terms unique to the brackish water population are shown in bold. BP: biological process, MF: molecular function, CC: cellular component.

Gene ontology ID	Term	Category	FDR
GO:0008061	**chitin binding**	**MF**	**2.43×10^{-6}**
GO:0006022	**aminoglycan metabolic process**	**BP**	**2.43×10^{-6}**
GO:0006030	**chitin metabolic process**	**BP**	**3.14×10^{-5}**
GO:1901071	**glucosamine-containing compound metabolic process**	**BP**	**4.40×10^{-5}**
GO:0006040	**amino sugar metabolic process**	**BP**	**7.25×10^{-5}**
GO:0017171	serine hydrolase activity	MF	7.47×10^{-5}
GO:0008236	serine-type peptidase activity	MF	7.47×10^{-5}
GO:0004252	serine-type endopeptidase activity	MF	9.06×10^{-5}
GO:0004175	endopeptidase activity	MF	0.000551
GO:0070011	**peptidase activity, acting on L-amino acid peptides**	**MF**	**0.000551**
GO:0005198	structural molecule activity	MF	0.00056
GO:0008233	**peptidase activity**	**MF**	**0.000621**
GO:0003735	structural constituent of ribosome	MF	0.00113
GO:0043603	cellular amide metabolic process	BP	0.00193
GO:0006412	translation	BP	0.00193
GO:0006518	peptide metabolic process	BP	0.00193
GO:0005840	ribosome	CC	0.00193
GO:0043043	peptide biosynthetic process	BP	0.00231
GO:0006508	**proteolysis**	**BP**	**0.00248**
GO:0043604	amide biosynthetic process	BP	0.00294
GO:0004866	**endopeptidase inhibitor activity**	**MF**	**0.003**
GO:0061135	**endopeptidase regulator activity**	**MF**	**0.003**
GO:0006414	translational elongation	BP	0.007
GO:1901564	organonitrogen compound metabolic process	BP	0.00729
GO:0042807	**central vacuole**	**CC**	**0.00733**
GO:0009705	**plant-type vacuole membrane**	**CC**	**0.00733**
GO:0080170	**hydrogen peroxide transmembrane transport**	**BP**	**0.00733**
GO:0030414	**peptidase inhibitor activity**	**MF**	**0.00921**
GO:0061134	**peptidase regulator activity**	**MF**	**0.00952**
GO:0005372	**water transmembrane transporter activity**	**MF**	**0.0109**
GO:0042044	**fluid transport**	**BP**	**0.0109**
GO:0000326	**protein storage vacuole**	**CC**	**0.0109**
GO:0000325	**plant-type vacuole**	**CC**	**0.0109**
GO:0015250	**water channel activity**	**MF**	**0.0109**
GO:0006833	**water transport**	**BP**	**0.0109**
GO:0003746	**translation elongation factor activity**	**MF**	**0.0109**
GO:0009941	**chloroplast envelope**	**CC**	**0.0153**
GO:0019755	**one-carbon compound transport**	**BP**	**0.0162**

GO:0015204	**urea transmembrane transporter activity**	**MF**	**0.0162**
GO:0004857	**enzyme inhibitor activity**	**MF**	**0.0162**
GO:0071918	**urea transmembrane transport**	**BP**	**0.0162**
GO:0015840	**urea transport**	**BP**	**0.0162**
GO:0009526	**plastid envelope**	**CC**	**0.0168**
GO:1990904	ribonucleoprotein complex	CC	0.0168
GO:0043232	intracellular non-membrane-bounded organelle	CC	0.0257
GO:0043228	non-membrane-bounded organelle	CC	0.0305
GO:0005328	**neurotransmitter:sodium symporter activity**	**MF**	**0.0344**
GO:0051181	**cofactor transport**	**BP**	**0.0344**
GO:0005576	**extracellular region**	**CC**	**0.0354**
GO:0004867	**serine-type endopeptidase inhibitor activity**	**MF**	**0.0354**
GO:0015370	**solute:sodium symporter activity**	**MF**	**0.0365**
GO:0052717	**tRNA-specific adenosine-34 deaminase activity**	**MF**	**0.0434**
GO:0005326	**neurotransmitter transporter activity**	**MF**	**0.0441**

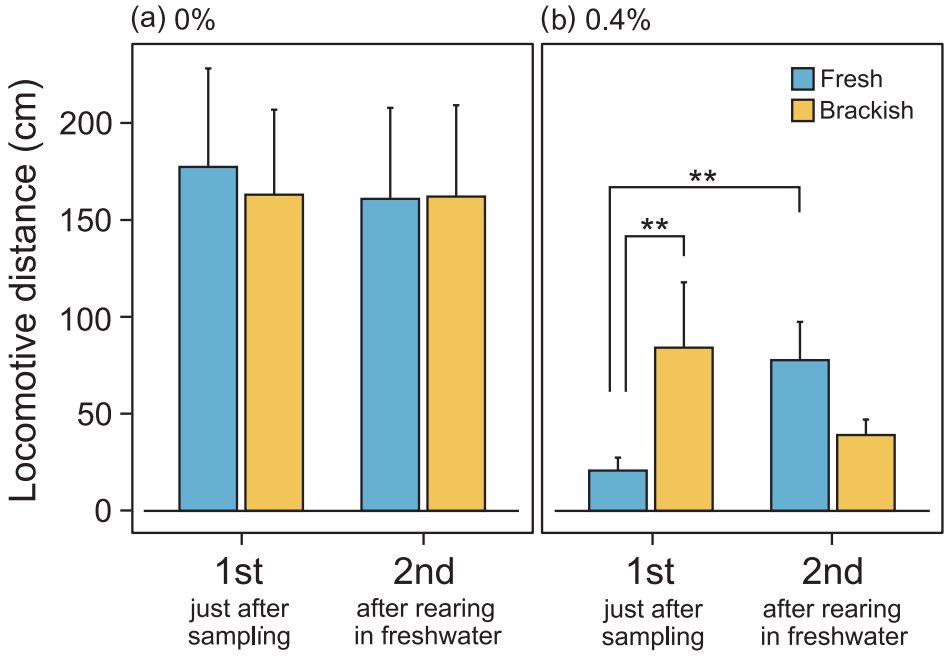

Figure 1-1. Changes in the locomotive distance of individuals collected in freshwater and brackish water area with observation in 0% (a) and 0.4% saltwater (b). The first observation was conducted just after sampling and the second was conducted after rearing in freshwater. Error bars are SEM. **: $P < 0.01$ in post hoc comparison ($\alpha = 0.0125$).

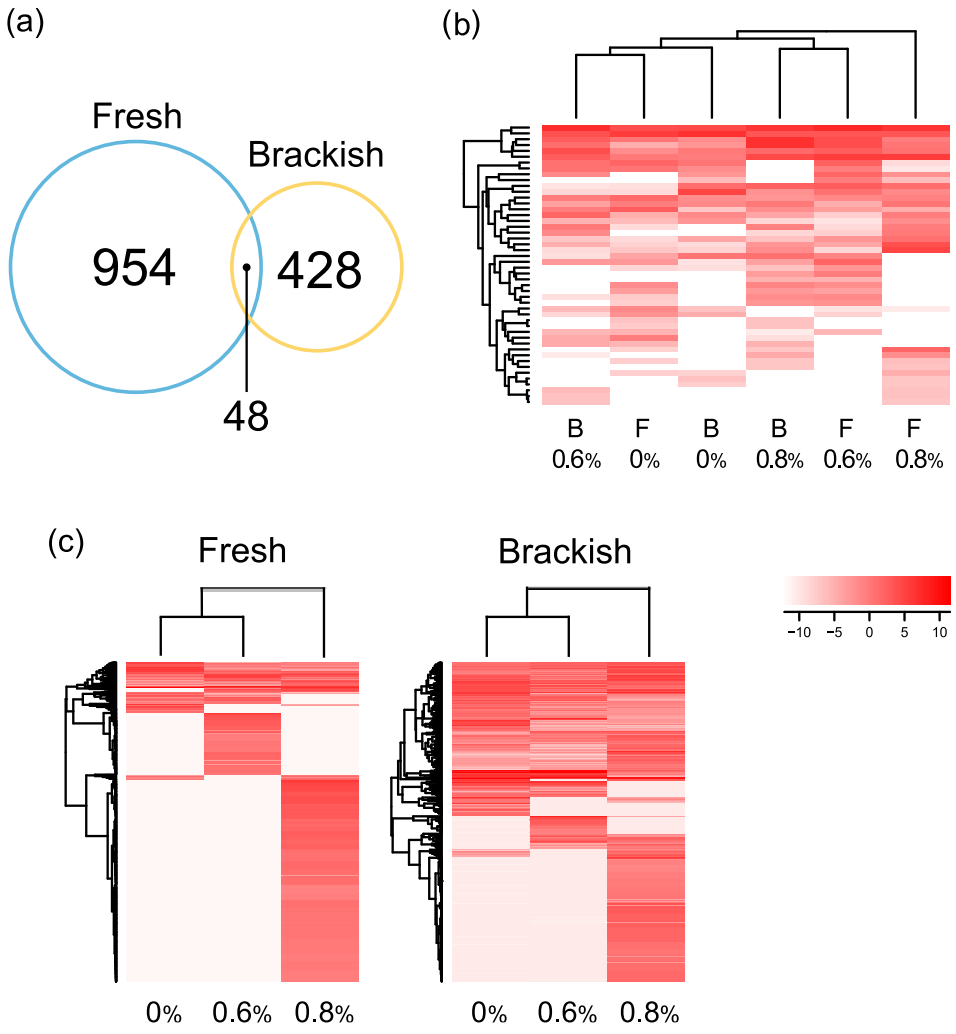

Figure 1-2. The number of differentially expressed genes (DEGs) and their expression patterns. (a) The number of DEGs of the freshwater and brackish water populations. (b, c) The expression patterns of DEGs shared between the two populations (b) and all DEGs in each population (c). Color scale represents the log scaled value of mean FPKM of three individuals in each salt concentration.

Supplementary Information

Table 1-S1. The number of raw reads in each sample.

Sample	Population	Salinity	Reads
mak_18	Freshwater	0%	11808065
mak_19	Freshwater	0%	12605106
mak_20	Freshwater	0%	12253500
mak_21	Freshwater	0.6%	14083809
mak_22	Freshwater	0.6%	10594428
mak_23	Freshwater	0.6%	10421054
mak_24	Freshwater	0.8%	11769869
mak_25	Freshwater	0.8%	12915424
mak_26	Freshwater	0.8%	12033359
nis_18	Brackish water	0%	11113904
nis_19	Brackish water	0%	10222034
nis_20	Brackish water	0%	11269184
nis_21	Brackish water	0.6%	9818309
nis_22	Brackish water	0.6%	11867340
nis_23	Brackish water	0.6%	12041622
nis_24	Brackish water	0.8%	12102968
nis_25	Brackish water	0.8%	10714084
nis_26	Brackish water	0.8%	11751277

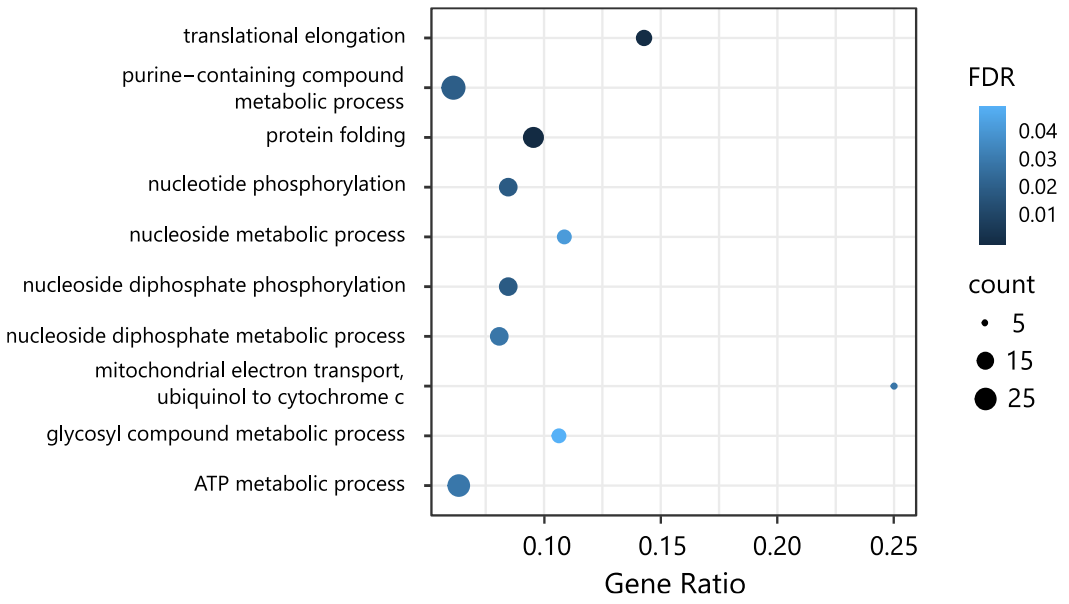

Figure 1-S1. GO enrichment analysis of the freshwater population. Gene ratio is referred to the ratio of the number of DEGs to the total number of genes with a specific GO term. The size of the circles represents the count of DEGs. Circles are colored based on the adjusted *P*-value. Enriched GO terms of Biological Process tagged to more than 10 and less than 1,000 genes are shown.

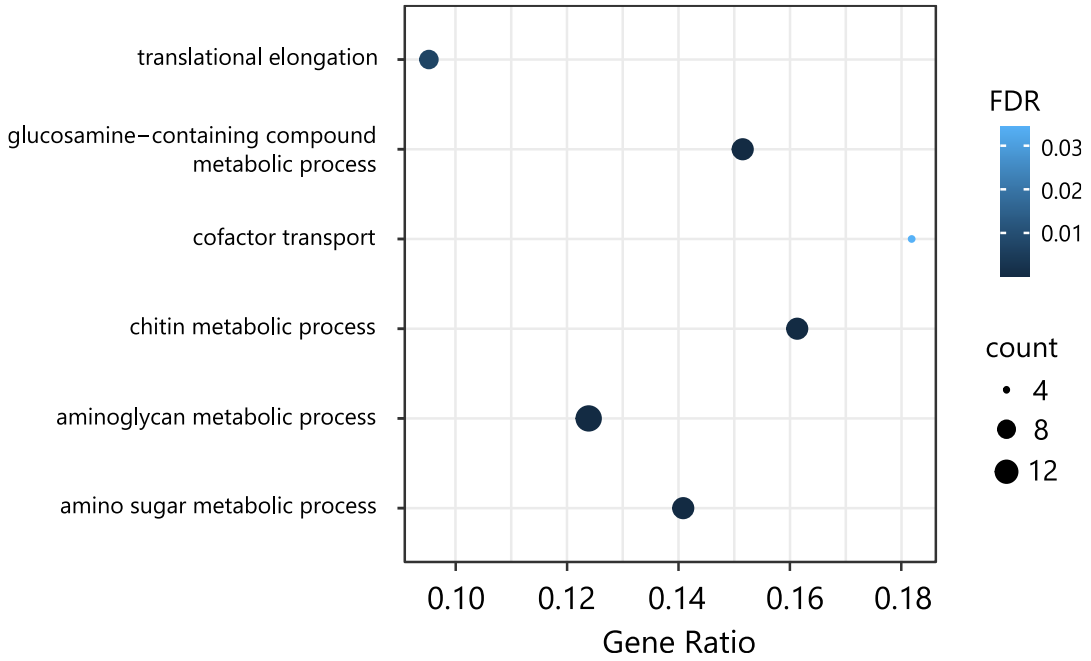

Figure 1-S2. GO enrichment analysis of the brackish water population. Gene ratio is referred to the ratio of the number of DEGs to the total number of genes with a specific GO term. The size of the circles represents the count of DEGs. Circles are colored based on the adjusted *P*-value. Enriched GO terms of Biological Process tagged to more than 10 and less than 1,000 genes are shown.

Chapter 2

Endogenous Rhythm Variation and Adaptation to the Tidal Environment in the Freshwater Snail, *Semisulcospira reiniana*

The content of this chapter has been published in *Frontiers in Ecology and Evolution*. (Yokomizo and Takahashi, 2022)

Abstract

Organisms have endogenous timekeeping system(s) to coordinate their biological processes with environmental cycles, allowing adaptation to external rhythmic changes in their environment. The change in endogenous rhythms could contribute to range expansion in a novel rhythmic environment. I hypothesized that populations of the freshwater snail near estuaries show a circatidal rhythm to synchronize with the tidal cycle. I compared the behavioral and gene expression rhythms between non-tidal and tidal populations of the freshwater snail, *Semisulcospira reiniana*. Individuals inhabiting tidal areas exhibited a rhythmic activity pattern coordinated with the tidal cycle under both field and laboratory conditions, but individuals inhabiting upstream non-tidal areas showed a circadian activity pattern. The proportion of circadian oscillating genes was greater in non-tidal than in tidal individuals, while that of circatidal oscillating genes was greater in tidal than in non-tidal individuals. Additionally, transcriptome-wide population genetic analyses revealed that these two adjacent populations can be clearly distinguished genetically, though the genetic distance was very small. My results provide evidence of the shift in an endogenous rhythm via range expansion to a novel rhythmic environment. The changes in a small number of genes and/or phenotypic plasticity may contribute to the difference in the endogenous rhythms between non-tidal and tidal populations.

Introduction

Life on Earth is exposed to a variety of periodic environmental changes. The Earth's rotation provides a daily cycle and seasonal variation, resulting in periodic changes in environmental factors, such as light and temperature. In marine environments, the rise and fall of sea levels every 12.4 h and the neap-spring tidal cycle are caused by the combination of the inertial force of the Earth and the gravitational forces of the sun and moon. Organisms have endogenous timekeeping system(s) that coordinates their biological processes in rhythmic environments. Circadian clocks are the best characterized among biological clocks and are ubiquitous in organisms (Pittendrigh 1960, Sharma 2003). Even in the absence of environmental time cues, circadian clocks sustain a period of approximately 24 h. Circadian clocks can be entrained to the environmental cues and are temperature-compensated (Dunlap 1999, Bell-Pedersen et al. 2005). To adapt to complicated environmental cycles, some species have endogenous rhythms other than circadian rhythms. Some marine species show rhythmic behaviors with a period of approximately 12.4 h synchronized to the tidal cycle (*i.e.,* circatidal rhythm) (Barnwell, 1966; Chabot et al., 2004; Satoh et al., 2008; Zhang et al., 2013). Several species exhibit semi-lunar or lunar rhythmicity (Takemura et al. 2010, Zantke et al. 2014) and annual rhythmicity (Heideman and Bronson 1994, Guyomarc'h and Guyomarc'h 1995).

Biological clocks could contribute to adaptation to rhythmic environments by allowing organisms to anticipate environmental changes and prepare accordingly (Yerushalmi and Green 2009). For instance, *Apteronemobius asahinai*, which inhabits mangrove forest floors, can anticipate high tides by the endogenous clock and escape from submergence (Satoh et al. 2008). Biological clocks also play important roles in the

regulation of genes known as clock-controlled genes (CCGs). They are associated with the temporal regulation of many physiological processes synchronized with periodic environmental variation (Miller et al. 2007, Bozek et al. 2009, Gamble et al. 2014). Given that phase and period of biological clocks are suggested to affect fitness in the natural condition (Yerushalmi et al. 2011, Rubin et al. 2017, 2018), modification of these parameters could coincide with the range expansion to a novel rhythmic environment. The mutation of genes governing biological clocks causes the adaptive evolution of phenological traits and could lead to range expansion to novel habitats with different rhythmic environments (Urbanski et al. 2012, Zakhrabekova et al. 2012). In addition, the genetic and/or non-genetic modification of endogenous rhythm should contribute to local adaptation and subsequent range expansion as well (Kaiser 2014, Kaiser et al. 2021).

Individuals may have endogenous rhythms specific to dominant environmental cycles in their habitats. Because a river is adjacent to the sea, there is a steep gradient of the strength of periodic variation caused by tidal cycles. In addition to the light-dark (LD) cycle of 24 h period and daily changes in water temperature, animals near the estuary are exposed to an environmental cycle of a 12.4 h period in salinity and water level. Because the tidal cycle causes drastic environmental changes in both biotic and abiotic factors, it is advantageous for a given species to anticipate tidal changes by the circatidal clock (Tessmar-Raible et al. 2011). Thus, I hypothesized that populations near the estuary showed a circatidal rhythm to synchronize with the tidal cycle. The relationship between the modification of endogenous rhythms and range expansion can be revealed by focusing on the adaptation of freshwater species to tidal areas in a river.

To investigate the accurate endogenous rhythm, the analysis of gene expression rhythms should be informative for us because the activity rhythm is sometimes irregular owing to the effect of environmental variables other than zeitgeber, a process termed masking (Mrosovsky 2009, Helm et al. 2017, Kronfeld-Schor et al. 2017). Unfortunately, little is known about the molecular basis of circatidal rhythm despite recent advances in molecular techniques (Zhang et al., 2013). However, examining transcriptome dynamics helps in understanding the rhythmicity of gene expression and detecting candidate circatidal clock genes or associated genes. The identification of oscillators that generate circatidal rhythmicity can be a key step in understanding the molecular mechanism by which the circatidal clock regulates the rhythmic process of physiology and activity.

The freshwater snail *Semisulcospira reiniana* mainly inhabits freshwater areas in Japan, but it can also inhabit tidal areas in several rivers. To evaluate whether populations established near the estuary have a circatidal rhythm, I compared the endogenous rhythms of snails between non-tidal and tidal populations. First, I investigated the activity rhythms of each population in the field and the laboratory. I then performed a transcriptome analysis to reveal the gene expression rhythm of each population under constant conditions. Finally, I investigated the genetic differentiation between the two populations using single nucleotide polymorphism (SNP) data obtained from the transcriptome analysis.

Materials and Methods

Species and sampling sites

Semisulcospira reiniana is a common freshwater snail that inhabits freshwater areas in Japan. Sampling sites were set in non-tidal (3–5 m above sea level) and tidal areas (< 1 m above sea level) of the Kiso River in Japan. In the present study, I refer to river areas where the water level fluctuates due to the tidal cycle as tidal areas and individuals inhabiting there as tidal individuals. These populations were approximately 20 km apart along the river. Fluctuations in the water level and salt concentration caused by the tidal cycle occur only in tidal areas. While snails are submerged at high tide, they go under the mud at low tide because the riverbed is exposed to air. The salt concentration is nearly 0% but slightly increases at high tide. Therefore, snails in tidal areas are exposed to both daily and tidal environmental cycles. In May 2018, June 2019, and June 2021, I collected snails from two areas of the Kiso River for behavioral assays and transcriptome analysis in the laboratory. In August 2020, I collected snails and conducted behavioral observations in the field. The animal study was reviewed and approved by the ethics committee of Chiba university.

Activity rhythm in the field

To investigate the activity patterns of adult *S. reiniana* exposed to field conditions, the activity of individuals collected from non-tidal and tidal areas was examined in August 2020. To collect non-tidal and tidal individuals whose habitats were located 20 km apart from each other just before the onset of the series of observations, the non-tidal individuals were kept in tanks in the tidal area near my research base. The tanks were filled with river water collected from a non-tidal area and kept in the shade on the

riverbank. The tidal individuals collected were enclosed in mesh bags, which were placed in the river to expose the stimulus of the tidal cycle. Ten individuals of non-tidal and tidal populations respectively were collected simultaneously for behavioral observations at 13:30 (low tide), 19:30 (high tide), 1:30 (low tide), and 7:30 (high tide). Snails between 23 and 33 mm in shell height were used for subsequent analysis. All observations were conducted at room temperature (approximately 25°C) without tidal stimulation. The first and fourth observations were conducted under light conditions, and the second and third observations were conducted under dark conditions corresponding to the natural photoperiod. Individuals were separately placed into individual bowls (φ13 × 3.5 cm) filled with freshwater from the river and monitored using a model 400-CAM061 camera (Sanwa Direct) for 60 min. Images were taken every 30 s and were used to create time-lapse movies. I tracked the position of each individual using UMATracker tracking software (Yamanaka and Takeuchi 2018). Using the snail trajectories, the total locomotion distance traveled in the hour was calculated for each individual.

Activity rhythm under constant conditions

To investigate the endogenous activity rhythm of *S. reiniana*, individuals collected from non-tidal and tidal populations were examined under constant laboratory conditions. Eight snails per population were individually placed in containers (13 × 11.7 cm, 4.7 cm in height) filled with freshwater. Snails were observed under the DD condition at a temperature of 23°C in the laboratory. No food was supplied during the observation. Water was circulated using a pump to keep the water clean. Individuals were monitored for 120 h (10 tidal cycles) and images were taken every 30 s. After averaging the

coordinates of each individual every three frames, the locomotion distance of each individual for every hour was calculated using the same procedure as the activity rhythm in the field.

RNA sampling

Adult individuals were collected from non-tidal and tidal areas around the time of low tide. They were preserved in containers without water under the DD condition at a constant temperature (23°C) for tissue collection. Starting at subjective high tide in the evening on the same day, three individuals per population were dissected every 3.1 h for 49.6 h (i.e., 17 sampling time points). The epidermis of each individual was preserved in 750 μL of RNA*later* Stabilization Solution (Invitrogen). The samples were stored at 4°C for approximately 3 h and then stored at −80°C until total RNA was extracted.

RNA extraction and sequencing

Total RNA was extracted using the Maxwell 16 LEV Plant RNA Kit with the Maxwell 16 Research Instrument (Promega), according to the manufacturer's instructions. Electrophoresis was performed on 1% agarose gel to evaluate RNA degradation. RNA concentrations were estimated using a Qubit 2.0 fluorometer (Invitrogen). RNA purity was estimated using BioSpec-nano (Shimadzu). Since my experiment was not designed to compare the expression level of genes between groups, biological replicates for each time point were not sampled. However, to reduce variations between individuals, three individuals were pooled per sample. Total RNA from three individuals in the same population at one sampling time point was pooled in equal amounts prior to RNA sequencing (pooled RNA-seq). RNA integrity was checked by Bioanalyser. A cDNA

library was constructed using the TruSeq RNA Sample Prep Kit. Paired-end (150 bp) pooled RNA-seq was performed using the Illumina NovaSeq6000 platform. After the removal of adaptor sequences and low-quality reads using Trimmomatic (version 0.38) (Bolger et al. 2014), quality control was performed using FastQC (version 0.11.8; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The remaining high-quality reads of all samples were used for *de novo* assembly using Trinity software (version 2.6.5) (Grabherr et al. 2011). To estimate gene expression levels, all reads from each sample were mapped to the reference transcripts using RSEM software (version 1.3.0) (Li and Dewey 2014), providing the FPKM of each gene. Putative coding regions (CDS) were extracted from the reference transcripts using TransDecoder (version 5.5.0; https://github.com/TransDecoder/TransDecoder/), providing reference transcripts containing only CDS. Using all CDS transcripts, I searched for homologs of every *S. reiniana* gene using BLAST for all protein sequences of *Crassostrea gigas* (oyster_v9, Wang et al., 2012). Genes with the best hit and an e-value < 0.0001 were used to analyze gene expression rhythmicity.

Population genetics

Reference transcripts containing only CDS were clustered based on sequence identities of 90% to remove redundancy using the CD-HIT program (Li and Godzik 2006). All reads from each sample were mapped to the clustered CDS reference using the STAR program. I then used the GATK4 (version 4.2.0) (McKenna et al. 2010) for SNPs calling. Because three individuals were pooled per sample, the sample ploidy option of GATK4 was set to 6. Homologs of *C. gigas* detected in the same manner as described in the previous section were used to estimate the genetic structure of the two populations. I

performed DAPC and PCA using adegenet (Jombart 2008), and calculated F_{ST} between the two populations using PopGenome (Pfeifer et al. 2014).

Statistical analyses

I evaluated whether the locomotor distances of the snails in the field differed between the times corresponding to high and low tides using the general linear model. Because the activity level tended to increase with time after the onset of the experiment (i.e., the time after the enclosure in a tank or mesh bags), irrespective of the population, probably because of acclimation, the residuals of the regression of the activity level at the time after the onset of the experiment were used to detect the daily rhythm of activity. The LD condition and tidal levels were included as explanatory variables. The activity rhythm of each population in the laboratory was determined via Lomb-Scargle periodogram using ActogramJ, a software package based on ImageJ for the analysis and visualization of chronobiological data (Schmid et al. 2011), using the mean locomotor distance of all individuals for each population. Gene expression rhythmicity was analyzed using two programs, JTK_CYCLE (Hughes et al. 2010) and RAIN (Thaben and Westermark 2014) to identify rhythmic components and estimate their phase. FPKM data of genes with average value > 0 and oscillation amplitude (peak/trough) ≥ 1.3 were used. Genes with significant rhythmic expression in the 12.4 h and 24.8 h period ($P < 0.01$) detected by at least one of two programs were identified as circatidal oscillating genes and circadian oscillating genes, respectively. To investigate whether pathways in salinity response are regulated by circatidal rhythm, I listed circatidal oscillating genes involved in salinity response using transcriptome data from my previous study (Yokomizo and Takahashi 2020), referring to data in the Dryad Data

Archive at https://doi.org/10.5061/dryad.jdfn2z37w. All statistical analyses were performed using R 4.0.4.

Results

Activity rhythm in the field

The activity of individuals from the tidal area was relatively higher at 19:30 and 7:30, and lower at 13:30 and 1:30 (Fig. 2-S1). The level of activity corresponded to the tidal cycle rather than the LD cycle, and it was higher during high tide (tide: $F = 4.27$, $P = 0.05$; LD: $F = 4.81$, $P = 0.91$; tide × LD: $F = 0.01$, $P = 0.93$; Fig. 2-1a). In contrast, non-tidal individuals were more active at 19:30, 1:30, and 7:30 than at 13:30 (Fig. 2-S1). The level of activity changed with the LD cycle rather than the tidal cycle and was higher under dark conditions (tide: $F = 2.56$, $P = 0.12$; LD: $F = 0.01$, $P = 0.04$; tide × LD: $F = 0.14$, $P = 0.71$; Fig. 2-1a), indicating nocturnal activity patterns.

Activity rhythm in the laboratory

Snails exhibited rhythmic activity patterns in the laboratory under constant dark (DD) conditions. Non-tidal individuals exhibited circadian activity rhythm with a period of 23.6 h (Fig. 2-1b, c), indicating that their activity patterns were synchronized with the daily cycle, but not with the tidal cycle. In contrast, the activity of individuals from the tidal population increased with the time of high tide in the laboratory under DD conditions, despite no tidal stimulation (Fig. 2-1b). The significant periodicities of 12.1 h and 23.6 h were detected in the mean locomotor distance, indicating that endogenous activity rhythms were synchronized with both the daily and tidal cycles (Fig. 2-1c).

Gene expression rhythm under constant conditions

Information on the number of reads in each sample was summarized in Table 2-S1. I obtained 1,686,925 contigs, with a mean length of 556 bp. Among the 978 metazoan core gene orthologs, 975 (99.1%) were identified. Using all the contigs, 225,139 putative coding DNA sequences (CDS) were estimated using TransDecoder. The CDS contained 99,784 contigs annotated against all protein sequences of *C. gigas*. These annotated sequences comprised 40,506 genes, of which 11,967 and 13,044 in non-tidal and tidal populations, respectively, met the requirements mentioned in the Method section.

I detected 546 (4.0%) and 333 (2.3%) circadian oscillating genes in non-tidal and tidal samples, respectively (Fig. 2-2a, b). The proportion of genes oscillating in the circadian period (i.e., 24.8 h) was greater in non-tidal individuals than in tidal individuals (Fisher's exact test, $P < 0.001$). Eight circadian oscillating genes kept the same period in the two populations (Fig. 2-S2). The distribution of peak phase estimated by the JTK_CYCLE and RAIN programs revealed that genes whose expression peaked in the evening were most frequent among the circadian oscillating genes (Fig. 2-2c). In the non-tidal population, peak expression was coordinated with their activity patterns. I detected 303 (2.2%) and 364 (2.6%) circatidal oscillating genes in non-tidal and tidal samples, respectively (Fig. 2-2a, b). The proportion of genes oscillating in a circatidal period (i.e., 12.4 h) was greater in tidal individuals than in non-tidal individuals (Fisher's exact test, $P = 0.03$). Eight circatidal oscillating genes kept the same period in the two populations (Fig. 2-S2). The expression peaks of most circatidal oscillating genes in the tidal population coincided with subjective high tide (Fig. 2-2c), which was synchronized with the activity pattern shown in Fig. 2-1. In non-tidal individuals, the

majority of circatidal oscillating genes showed the expression peak of time of low or rising tide. These timings correspond to midnight and early afternoon.

I identified some core clock genes. None showed circadian rhythmicity in either population (Fig. 2-3 and 2-S3). Among significantly oscillating transcripts, sixteen genes exhibited circadian rhythmicity in their expression in the non-tidal population, but circatidal rhythmicity in the tidal population (Fig. 2-3 and Fig. 2-S4). Three genes with clearly different rhythmicity between populations are shown in Fig. 2-3 (Wnt glycoprotein, chromatin remodeling ATPase INO80, and VPRBP protein). The Wnt glycoprotein functions as a ligand for the Wnt pathway. This glycoprotein is involved in a variety of cellular responses (Arend et al. 2013). Chromatin remodeling ATPase INO80 is associated with chromatin remodeling by either sliding nucleosomes along with DNA or exchanging histones within nucleosomes (Bao and Shen 2011). VPRBP is a component of the DDB1-CUL4A^{VPRBP} E3 ubiquitin ligase complex and is involved in the induction of G2 arrest (Belzile et al. 2007). As these genes do not function as clock genes, they are likely to be CCGs. The presence of these genes indicates the existence of different biological clocks in non-tidal and tidal populations or indicates that they reflect the dominant cycle that snails usually face in their environment.

Since salt concentration in the sampling site of the tidal population slightly fluctuates by the tidal cycle, salinity response pathways may be regulated by the circatidal rhythm. Among the circatidal oscillating genes, 41 were found in the list of genes involved in the salinity response (Fig. 2-S5 Table 2-S2). Most of these genes were highly expressed under high salinity conditions. Although I found rhythmic genes with

various expression phases, there were few genes with expression peaks during rising tide.

Population differentiation

I identified 35,864 SNPs on the contigs annotated against all protein sequences of *C. gigas* using a BLAST search. Composition plot by DAPC using these SNPs distinguished non-tidal and tidal populations (Fig. 2-S6). The two populations separated along PC1 and overlapped along PC2 (Fig. 2-4b). Although PCA indicated that non-tidal and tidal populations could be distinguished, the proportion of variance of PC1 was small (4.8%). The BIC value was lowest when one cluster was estimated, indicating that the most likely number of groups in the sample was one (Fig. 2-S7). F_{ST} value between the non-tidal and tidal populations was estimated at 0.018, indicating quite low genetic differentiation.

Discussion

Biological clocks could contribute to the improvement of fitness via the entrainment of biological processes and the environmental cycle (Yerushalmi and Green 2009). Circadian clocks are considered adaptive in an environment with a diurnal cycle. An additional or modified timekeeping system synchronized with the tidal cycle can be helpful in the tidal environment. Although much evidence suggests that the circatidal rhythm is adaptive in intertidal organisms (Tessmar-Raible et al. 2011, Goto and Takekata 2015, Häfker and Tessmar-Raible 2020), the mechanism of the evolution of the circatidal clock during tidal adaptation remains unclear. In the present study, I focused on freshwater organisms, which have expanded their distribution to tidal

reaches, and investigated their endogenous rhythms. My results concerning the difference in activity and gene expression rhythm between populations could be evidence of the expression of habitat-specific endogenous rhythms in a single species. The small genetic distance between the two populations suggests that they have a similar genetic basis of clocks and plastically show different endogenous rhythms depending on the environmental cycle. Although I cannot deny that the evolution of a small number of genes contributing to distinguishing two populations results in differentiated clocks, the further experiment would be needed to prove the hypothesis.

First, I clarified the activity patterns of *S. reiniana* under natural and DD conditions. Snails in a non-tidal population exhibited nocturnal activity patterns in the field, consistent with previous research (Urabe 1998). Given that this observation was conducted in summer, the low daytime activity could be attributed to high temperatures. To assess the endogenous activity rhythms of non-tidal individuals, I conducted behavioral observations under DD conditions. Periodogram analysis detected unimodal rhythmicity of the circadian period, suggesting that non-tidal individuals have an endogenous rhythm corresponding to the natural LD cycle. Snails in tidal populations tended to be active at high tide, suggesting that they responded to the rising water level caused by the tidal cycle. This finding also suggests that a slight increase in salt concentration at high tide does not reduce snails' activity. This activity pattern was also observed under DD conditions in the laboratory, with no tidal stimulation. I detected a free-running period close to the natural tidal cycle in addition to the circadian period, supporting my hypothesis that snails in tidal populations have a circatidal rhythm. Additionally, tidal individuals exhibited an activity rhythm with a period of 24 h. Although I cannot rule out the possibility that this periodicity appears computationally

because 24 is twice as large as 12, this is more likely to be a circadian rhythm since activity at night was higher than during the day. Taken together, the data indicate that *S. reiniana* expresses habitat-specific endogenous rhythm.

In addition to the activity rhythm, the present study focused on gene expression rhythms under DD conditions with no tidal stimulation. The proportion of circadian oscillating genes was greater in non-tidal individuals than in tidal individuals, whereas the opposite was true for circatidal oscillating genes. I identified some genes whose expression showed circadian rhythmicity in the non-tidal population but showed circatidal rhythmicity in the tidal population. These findings suggest that endogenous rhythms could differ in their periods between non-tidal and tidal populations, and some of the CCGs are regulated by putative different clocks. In the non-tidal population, however, I also found hundreds of rhythmic transcripts with a 12.4-h period. This might be a conservative rhythm among species derived from the circadian clock, as reported in mammals (Hughes et al. 2009, Zhu et al. 2017, Zhu 2020). Rhythmicity with a period of 12 h in mammalian clocks is suggested to be harmonics of the circadian clock (Hughes et al. 2009). In my experiments, the beginning of the dark phase and the main high tide coincide, and thus, I could not distinguish between genes with 12 h periodicity driven by harmonics and those synchronized with tides.

I did not find any significant rhythmicity in the identified core clock genes in either population. This finding is not surprising in tidal populations, as the lack of circadian rhythmicity in core clock genes has also been reported in other intertidal animals (Zhang et al., 2013; Schnytzer et al., 2018; Satoh and Terai, 2019). Alternatively, the absence of circadian rhythmicity in core clock genes was due to the weak peripheral clocks in the epidermis or samples including more than one tissue with not

synchronized peripheral clocks leading to a masking effect. Few common circadian or circatidal oscillating genes in the two populations may also result from this limitation. Although homologs of the core clock genes identified here might lack the function associated with the circadian clock, the circadian activity rhythm under constant conditions suggests the presence of a circadian timekeeping system in *S. reiniana*. It is unlikely that they have a novel timekeeping system without circadian clock genes. I cannot deny that the arrhythmic expression pattern in core circadian clock genes is caused by the lack of information on variability between biological replicates due to the pooled RNA-seq. the mechanism that governs the activity rhythm with a period of 23.6 h in the non-tidal population is unclear. Transcriptome analysis revealed that some genes exhibited different expression rhythms between populations. I found 16 genes, including those encoding Wnt, which showed circadian and circatidal oscillations in non-tidal and tidal populations, respectively. Previous studies have reported that the Wnt signaling pathway is regulated by circadian clock genes (Guo et al. 2012, Fuwei et al. 2013). The fact that genes directly regulated by the circadian clock exhibit circatidal rhythmicity in their expression supports the hypothesis of a shift in the endogenous rhythm between populations. Considering the results of the behavioral assay, *S. reiniana* in the tidal area likely has a circatidal rhythm.

In addition to the water level, the salt concentration at the tidal site fluctuated from 0 to 0.5%, according to the tidal cycle. I detected 41 genes that were related to the salinity response and oscillated in circatidal rhythmicity. Given that *S. reiniana* is active at high tide and low salinity conditions (Yokomizo and Takahashi 2020), the one gene that was highly expressed under such conditions should be associated with locomotor function. In contrast, the 10 genes highly expressed under low tide and high salinity

conditions should be expressed when snails are inactive. I found eight genes that were highly expressed under high tide and high salinity conditions, indicating that they are related to a direct response to salinity. These findings suggest that some of the pathways involved in the salinity response are controlled by the circatidal rhythm. Therefore, I hypothesized that *S. reiniana* in a tidal area could anticipate the variation in salt concentration due to the tidal cycle and regulate gene expression via the circatidal rhythm. Further investigation is needed on the relationship between circatidal rhythm and salinity response in freshwater snails.

Although my study indicates that *S. reiniana* in a tidal area has a circatidal rhythm, the nature of the circatidal clock remains controversial in any organism. Based on previous studies of tidal animals, three major hypotheses have been proposed to explain tidally synchronized biological rhythms. The first hypothesis is that there is a biological clock with a 12.4-h period other than the circadian clock (Naylor 2010). In the second hypothesis, Palmer (1995) argued for the existence of two unimodal clocks with 24.8-h coupled in antiphase, termed circalunidian clocks. Finally, the third hypothesis suggests that a single oscillator governs both circadian and circatidal patterns (Enright 1976, Mat et al. 2016). I could not evaluate whether the circadian and circatidal rhythms of snails are regulated by a single or multiple biological clock(s). Previous research in mangrove crickets showed that silencing the circadian core clock genes does not disrupt the circatidal rhythm (Takekata et al. 2012, 2014), and molecular mechanisms different from the circadian clock might exist in intertidal animals. In contrast, *C. gigas* under subtidal field conditions exhibits tidal rhythmicity of circadian clock genes, suggesting that a single clock could entrain behavioral patterns at tidal and daily rhythms (Tran et al. 2020). Revealing the molecular mechanisms of the circatidal

clock is an ongoing challenge in chronobiology. Given that the first proto-clocks evolved in an aquatic environment, developing a comprehensive understanding of the circatidal clocks should contribute to a more holistic understanding of the evolution of the biological clock (Rock et al. 2022).

My results revealed a circatidal rhythm only in snails in tidal areas. Although genetic differentiation estimated by F_{ST} was quite small, DAPC and PCA indicated that non-tidal and tidal populations were apparently isolated, suggesting differentiation of a small number of genes. Therefore, my results from the population genetic analyses suggest recent isolation and subsequent slight divergence between the two populations. I provide two hypotheses for the fact that different endogenous rhythms were observed between tidal and non-tidal populations. The first hypothesis is that only the tidal population has the circatidal clock resulted from the adaptive evolution of a small number of genes. Such genetic adaptation may lead to the clear but slight genetic differentiation between tidal and non-tidal populations. The second hypothesis is that differences in environmental cycles in habitats plastically determine the endogenous rhythm of *S. reiniana*. Even non-tidal individuals have the potential to show the circatidal rhythm when exposed to a tidal environment. If it is true, however, I cannot deny that the ability of plasticity of biological clocks evolved only in the river studied or only in the lower reaches of the river. Further investigations on the ability of plasticity on endogenous rhythm in the individuals derived from other populations of Kiso River and populations in the independent rivers can provide a deeper understanding of the evolution of biological clocks.

References

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Figures

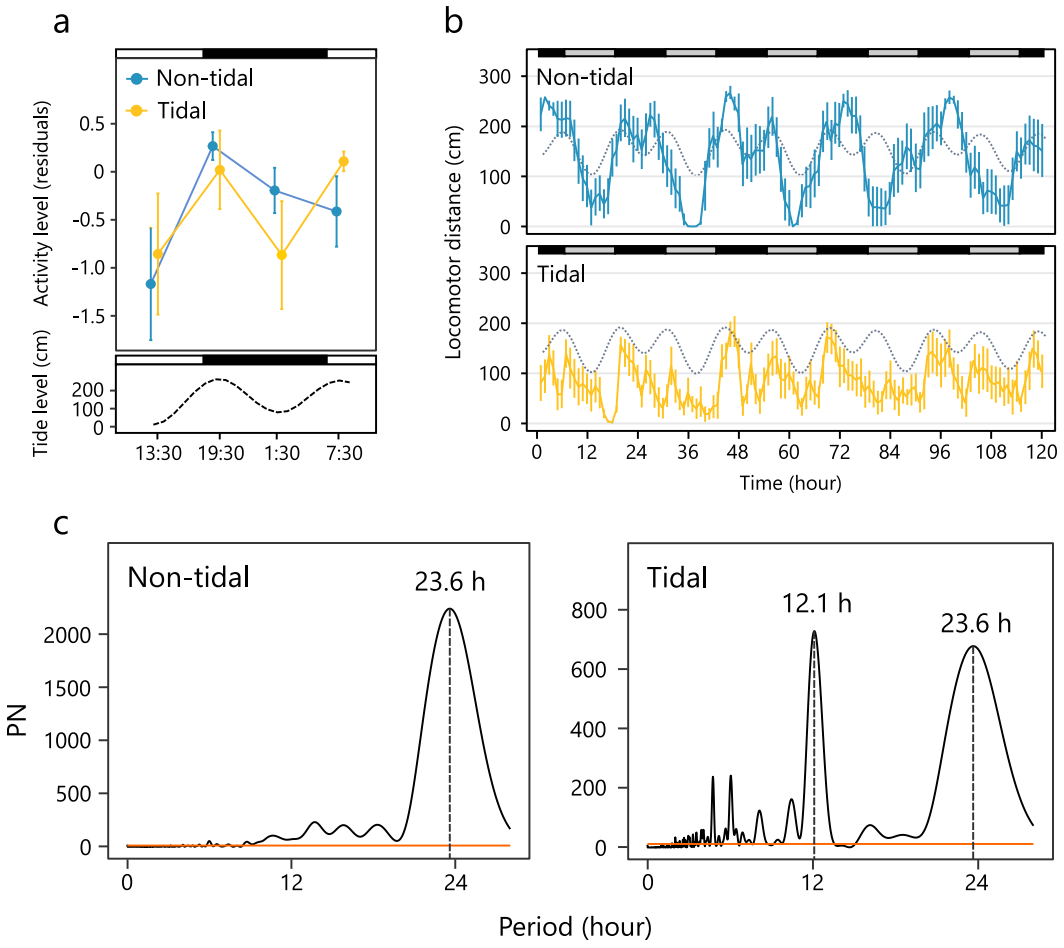

Figure 2-1. Activity patterns of non-tidal and tidal individuals. (a) Daily changes in the activity level (residuals of the regression of locomotion distance on the time after the onset of the experiment) in the field. Error bars depict the standard error of the mean (SEM). The non-tidal and tidal populations in each observation time are shown in blue and yellow, respectively. The white and black bars represent light and dark conditions, respectively. The lower panel represents a tidal level. (b) Locomotor distance of the non-tidal and tidal population for 5 days under the DD condition in the laboratory. Error bars are SEM. Subjective day and night are shown as grey and black bars above. The tide level of the natural environment is shown as a gray dotted line. (c) Lomb-Scargle periodogram analysis. Peaks in the circatidal and circadian range are noted.

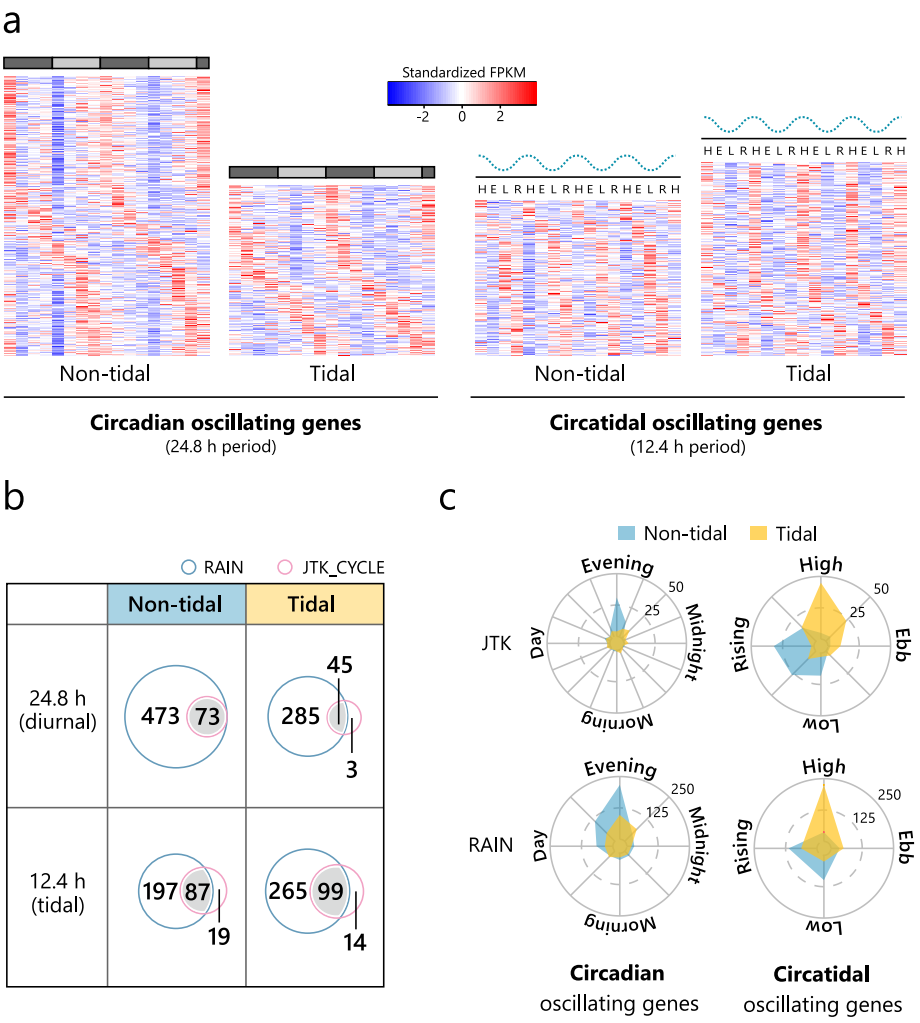

Figure 2-2. Rhythmic transcripts with circadian and circatidal period in the non-tidal and tidal individuals. (a) Heatmaps of standardized expression patterns of rhythmic transcripts with 24.8 h (diurnal) or 12.4 h (tidal) period in the non-tidal and tidal individuals under DD conditions detected by JTK_CYCLE or RAIN. The dotted wavy lines above the heatmaps of tidal transcripts represent the tide level of the natural environment. H: subjective high tide, E: subjective ebb tide, L: subjective low tide, R: subjective rising tide. The grey and black bars above the heatmaps of diurnal transcripts represent the subjective day and night, respectively. (b) Venn diagrams detailing the number of rhythmic transcripts with 24.8 h (diurnal), or 12.4 h (tidal) period detected by JTK_CYCLE (pink circle) and/or RAIN (light blue circle). (c) The distribution of the phase of circadian and circatidal oscillating genes in the non-tidal and tidal individuals. Oscillating genes detected by both JTK_CYCLE and RAIN were included in both upper and bottom radar charts.

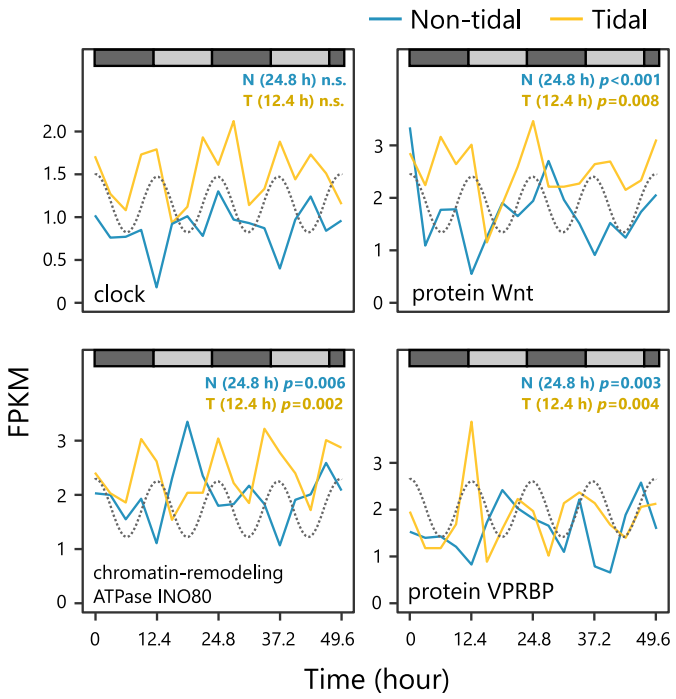

Figure 2-3. Examples of the expression patterns of the circadian clock gene (upper left) and oscillating genes with a different period between non-tidal and tidal populations (upper right, bottom right, and bottom left). The tide level of the natural environment is shown as a gray dotted line. The grey and black bars represent the subjective day and night, respectively. Rhythmicity of a period of 12.4 h and 24.8 h were tested using RAIN for tidal (T) and non-tidal (N) populations, respectively.

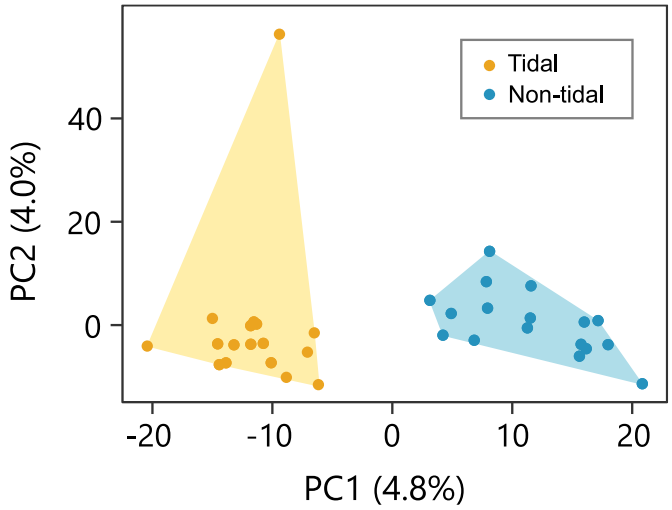

Figure 2-4. Genetic structure of the non-tidal and tidal population. Samples collected in the tidal area and non-tidal area are shown in yellow and blue, respectively. PCA on the non-tidal and tidal individuals. The two populations can be distinguished along the PC1.

Supplementary Information

Table 2-S1. The number of raw reads in each sample.

Sample	Population	Time	Reads
nkj_1	Non-tidal	0	12692422
nkj_2	Non-tidal	3.1	13717368
nkj_3	Non-tidal	6.2	15453765
nkj_4	Non-tidal	9.3	17206495
nkj_5	Non-tidal	12.4	14211764
nkj_6	Non-tidal	15.5	14785763
nkj_7	Non-tidal	18.6	12620021
nkj_8	Non-tidal	21.7	14586008
nkj_9	Non-tidal	24.8	15950966
nkj_10	Non-tidal	27.9	15085466
nkj_11	Non-tidal	31.0	12663669
nkj_12	Non-tidal	33.1	19539652
nkj_13	Non-tidal	37.2	15814246
nkj_14	Non-tidal	40.3	12116846
nkj_15	Non-tidal	43.4	16057902
nkj_16	Non-tidal	46.5	15215401
nkj_17	Non-tidal	49.6	12112989
nis_1	Tidal	0	12152653
nis_2	Tidal	3.1	13405344
nis_3	Tidal	6.2	12838312
nis_4	Tidal	9.3	12526128
nis_5	Tidal	12.4	12502257
nis_6	Tidal	15.5	11647309
nis_7	Tidal	18.6	11008156
nis_8	Tidal	21.7	12436216
nis_9	Tidal	24.8	12009415
nis_10	Tidal	27.9	11720062
nis_11	Tidal	31.0	10191342
nis_12	Tidal	33.1	11955890
nis_13	Tidal	37.2	10138871
nis_14	Tidal	40.3	12446976
nis_15	Tidal	43.4	13015139
nis_16	Tidal	46.5	13108881
nis_17	Tidal	49.6	13120598

Table 2-S2. Circatidal oscillating genes related to salinity response. Salinity at high expression is the relative salt concentration at which each gene is highly expressed. "Low" is 0%, and "High" is 0.6% and/or 0.8%. The tidal phase is the peak phase of expression in circatidal rhythmicity.

ID	Description	Circatidal phase	Salinity at high expression
DN89491_c1_g1	Matrix metalloproteinase-19	High	High
DN90370_c3_g1	Cytoplasmic FMR1-interacting protein	High	High
DN52795_c0_g2	Transport protein Sec61 subunit alpha isoform 2	High	High
DN72335_c0_g2	Far upstream element-binding protein 3	High	High
DN73791_c0_g2	VWFA domain-containing protein	High	High
DN108857_c1_g1	Transposon Ty3-I Gag-Pol polyprotein	High	High
DN100480_c0_g1	G2/M phase-specific E3 ubiquitin-protein ligase	High	Low
DN71854_c0_g1	Serine hydroxymethyltransferase, EC 2.1.2.1	High	High
DN46741_c0_g1	Nucleosome assembly protein 1-like 4	High	High
DN100547_c4_g2	Caprin-2	Ebb	High
DN73399_c6_g4	Caprin-2	Ebb	High
DN97040_c5_g1	Caprin-2	Ebb	Low
DN73497_c7_g1	Tripartite motif-containing protein 45	Ebb	High
DN107589_c3_g2	Usherin	Ebb	Low
DN101763_c1_g1	Cubilin	Ebb	High
DN97143_c3_g1	Tubulin beta chain	Ebb	High
DN82214_c1_g4	GTPase IMAP family member 4	Ebb	High
DN101532_c0_g2	GTPase IMAP family member 7	Ebb	High
DN82214_c1_g3	GTPase IMAP family member 7	Ebb	High
DN69782_c0_g1	GTPase IMAP family member 7	Ebb	Low
DN93746_c3_g1	GTPase IMAP family member 7	Ebb	High
DN82125_c1_g2	TIR domain-containing protein	Ebb	Low
DN85429_c3_g2	TIR domain-containing protein	Ebb	Low
DN95082_c8_g4	Ankyrin-2	Ebb	High
DN70911_c1_g2	Phospholipase, EC 3.1.4.4	Ebb	High
DN71112_c0_g1	Phospholipase, EC 3.1.4.4	Ebb	High
DN74391_c1_g1	Phospholipase, EC 3.1.4.4	Ebb	High
DN70911_c1_g1	Phospholipase, EC 3.1.4.4	Ebb	High
DN85143_c3_g1	Carbonic anhydrase, EC 4.2.1.1	Low	High
DN106112_c0_g4	Putative tyrosinase-like protein tyr-1	Low	High
DN96678_c0_g1	Putative tyrosinase-like protein tyr-1	Low	High
DN104929_c1_g1	Macrophage mannose receptor 1	Low	High
DN71366_c0_g1	Neurotrypsin	Low	Low
DN100647_c0_g1	Putative tyrosinase-like protein tyr-3	Low	High
DN96678_c0_g2	Kielin/chordin-like protein	Low	High
DN71937_c0_g1	Transgelin	Low	High

DN77451_c1_g1	Inter-alpha-trypsin inhibitor heavy chain H3	Low	High
DN74689_c0_g3	Exoglucanase xynX	Low	High
DN92213_c2_g4	Exoglucanase xynX	Low	High
DN109728_c9_g1	Thrombomodulin	Rising	High
DN71166_c1_g1	60S ribosomal protein L3	Rising	High

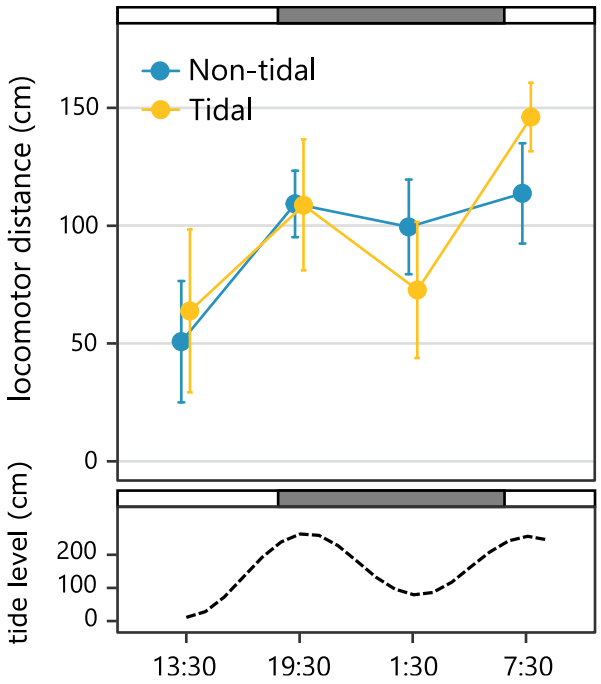

Figure 2-S1. Locomotive distance and tide level at each observation time. Error bars are SEM. The freshwater and brackish water population are shown in blue and yellow, respectively. The white and black bars represent light and dark conditions, respectively.

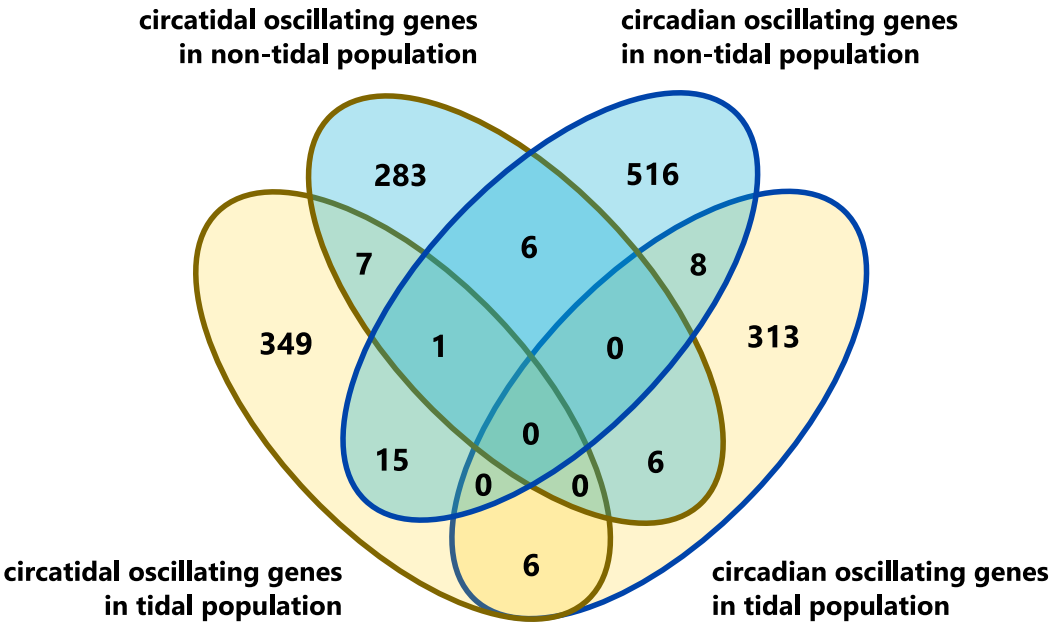

Figure 2-S2. Venn diagrams of oscillating genes in the tidal and non-tidal populations. The ovals bordered in blue and yellow represent the circadian and circatidal oscillating genes, respectively. The ovals filled in blue and yellow represent the non-tidal and tidal populations, respectively.

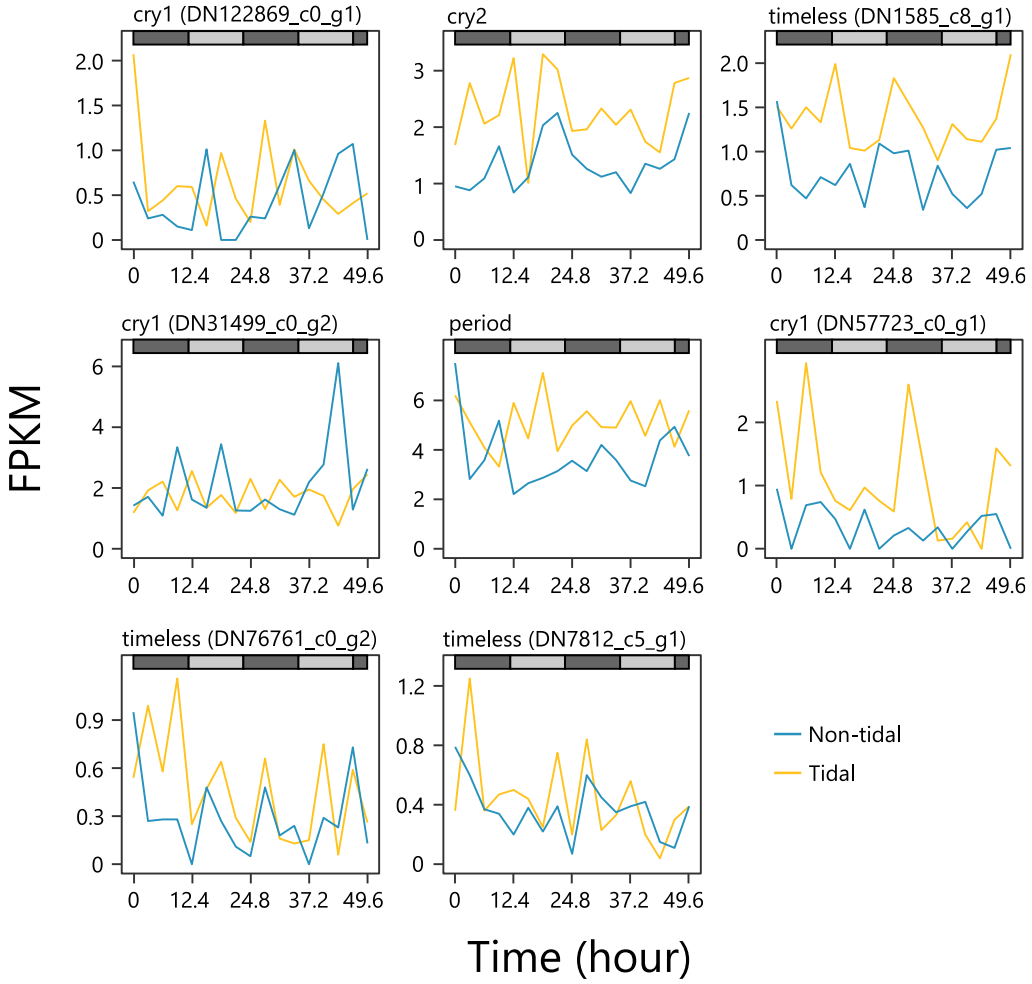

Figure 2-S3. The expression patterns of circadian clock genes of the freshwater (blue) and brackish water (yellow) individuals under the DD condition.

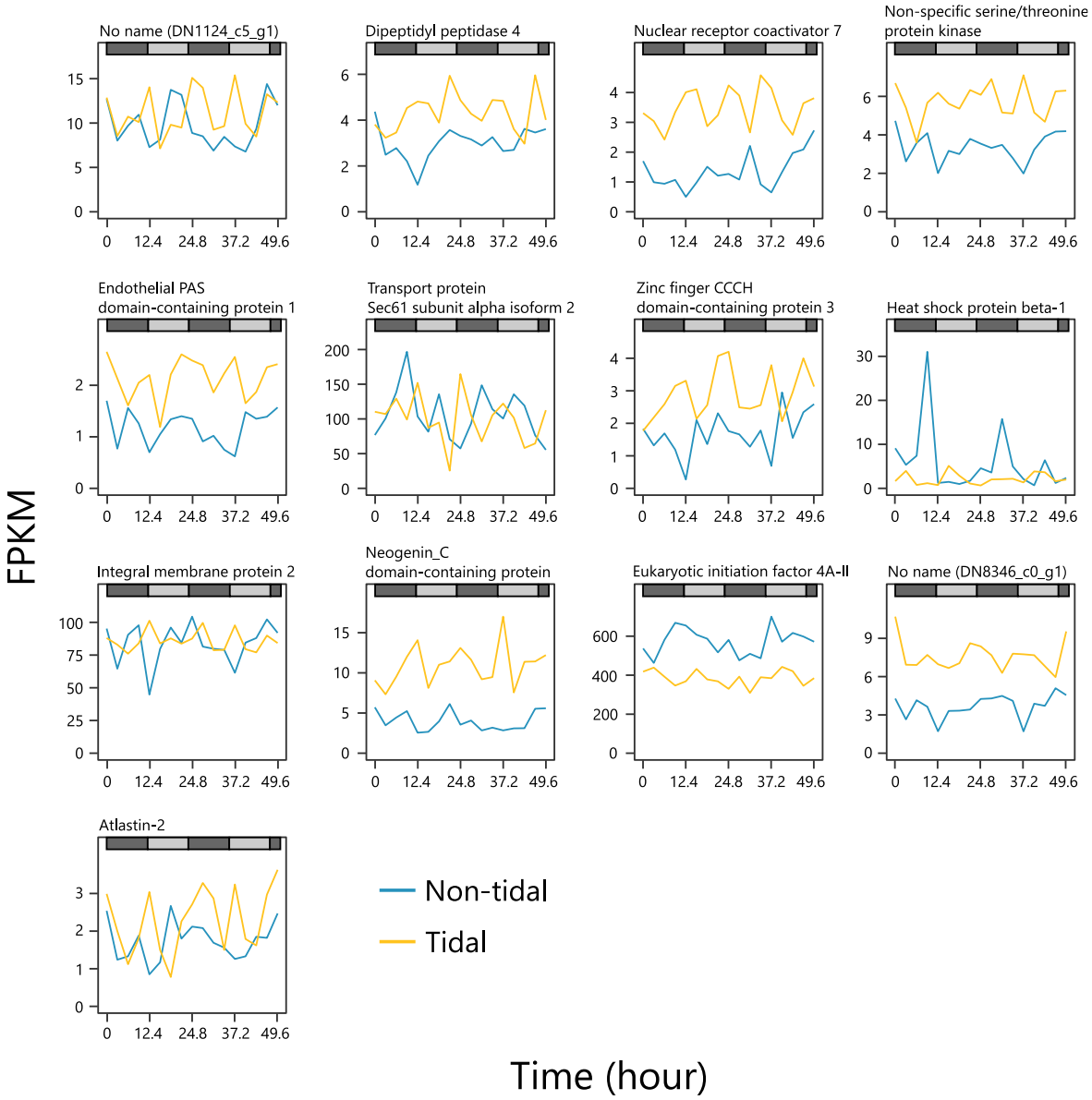

Figure 2-S4. The expression patterns of genes with circadian and circatidal period in the freshwater and brackish water population, respectively.

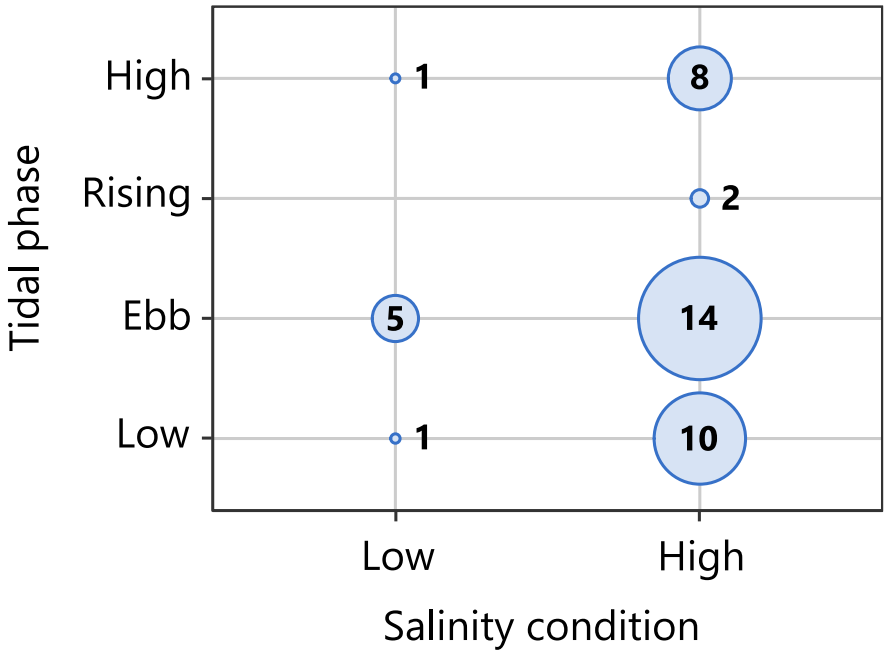

Figure 2-S5. Circatidal oscillating genes related to salinity response. The circles and numbers represent circatidal oscillating genes highly expressed in each tidal phase and salinity conditions. In salinity condition, “Low” is 0%, and “High” is 0.6% and/or 0.8%.

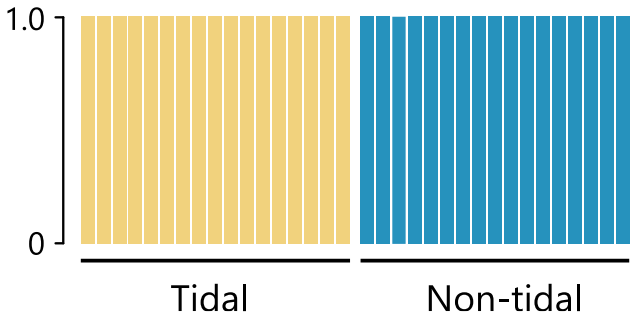

Figure 2-S6. Composition plot by DAPC on the non-tidal and tidal individuals. Samples were separated depending on sampling site. Samples collected in the tidal area and non-tidal area are shown in yellow and blue, respectively.

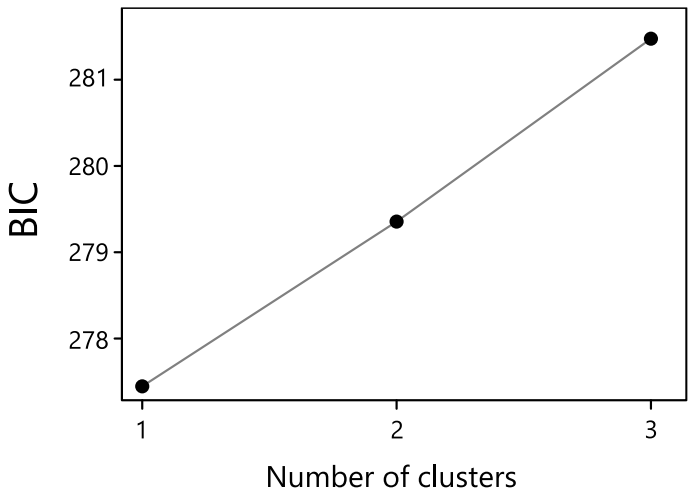

Figure 2-S7. The BIC value of each number of clusters.

Chapter 3

Plasticity of the Endogenous Rhythm and Transcriptomic Dynamics Depending on Environmental Cycles in a Freshwater Snail

Abstract

Biological clocks contribute to the adaptation to the periodic variation of environments. The adaptive significance of the synchronization of biological rhythms with environmental cycles would result in the diversity of the endogenous rhythm depending on the environment. In some marine species, the advantages of anticipation of tidal changes have favored the evolution of circatidal rhythm. I have demonstrated that in the freshwater snail, *Semisulcospira reiniana*, individuals in a non-tidal area exhibited the circadian rhythm but those in a tidal area exhibited both circadian and circatidal rhythms. Here, I investigated whether the circatidal rhythm of individuals in a tidal area is determined genetically or induced by environmental cycles. After exposure to a simulated tidal environment, individuals in both tidal and non-tidal populations exhibited the circatidal rhythm in the activity, suggesting that the endogenous rhythm plastically changed depending on the environmental cycle. On the other hand, analyses of gene expression rhythm suggest that genes with circatidal oscillation increased only in the tidal population. In addition, GO analysis detected biological pathways enriched in circatidal oscillating genes only in the tidal population. My findings suggest that there is the plasticity of the endogenous rhythm but genes or biological pathways regulated by circatidal clocks have increased in the tidal population. The plasticity of the endogenous rhythm and subsequent genetic changes in a molecular network between clocks and several genes may have contributed to the adaptation to the tidal environment.

Introduction

Adaptation to the periodic variation of environments has been one of the challenges for organisms since ancient times. The rotation and inertial force of Earth and the gravitational forces of the sun and moon provide environmental variation in multiple timescales such as day-night, tidal, lunar, and seasonal cycles. To cope with those periodic variations, organisms have timekeeping system(s) that is self-sustained, temperature compensated, and capable of entrainment to environmental cues (Dunlap 1999, Bell-Pedersen et al. 2005, Yerushalmi and Green 2009). The circadian rhythm is conserved in various organisms ranging from bacteria (Kondo et al. 1993) to mammals (Takahashi 1995) for the adaptation to day-night cycles. Biological clocks allow organisms to anticipate environmental changes and regulate physiological processes at appropriate timing. Parameters of the clock, such as phase and period of biological clocks coordinated to environmental cycles, could enhance fitness, playing an important role in adaptation to rhythmic environments (Yerushalmi et al. 2011, Rubin et al. 2017, 2018).

The adaptive significance of the synchronization of biological rhythms with environmental cycles would result in variations in the endogenous rhythm depending on the circumstance. As reported in *Neurospora discreta*, local adaptation to different rhythmic environments involving circadian clock regulation causes the habitat-specific endogenous rhythm and influences reproductive success (Koritala et al. 2020). Variations in the endogenous rhythm are also induced by plasticity depending on social roles. Honeybee shows the social environment-dependent plasticity in the circadian rhythm within a single population (Bloch and Robinson 2001, Shemesh et al. 2007).

These studies suggest that genetic and/or non-genetic changes in the endogenous rhythm could contribute to enhancing the performance of individuals.

Organisms in marine or intertidal habitats face drastic and complicated environmental changes, such as water level, salinity, temperature, light, and food availability, caused by the daily and tidal cycles. The circatidal rhythm is a tidally synchronized biological rhythm with an approximately 12-hour period (Barnwell 1966, Satoh et al. 2008, Tessmar-Raible et al. 2011, Chabot et al. 2016). Because anticipation of tidal changes is of critical importance for marine species, selective pressure by the tidal cycle could drive the adaptive evolution of circatidal clocks. Although the circadian and circatidal rhythms have different periodicity and zeitgebers, whether these two rhythms are generated by genetically different clocks is controversial. Several studies on marine species have shown plasticity in rhythm expression within the same individuals or populations depending on environmental conditions (Mat et al. 2014, O'Neill et al. 2015, Schnytzer et al. 2018). Interestingly, some circadian clock genes in the pacific oyster could oscillate at a tidal frequency under tidal conditions (Mat et al. 2016, Tran et al. 2020). These studies provide the hypothesis that the circadian and circatidal rhythms could be generated by a single biological timekeeping system. Alternatively, previous researches on the mangrove cricket suggest that the circatidal timekeeping system is independent of circadian clocks (Takekata et al. 2012, 2014). The dissociation of the circadian and circatidal clock in the cricket may be caused by the adaptative evolution of a novel timekeeping system via establishment in a mangrove habitat. Together, both plasticity and genetic change could play important roles in the oscillation of circatidal rhythms in tidal environments.

A river is a complicated rhythmic system that is partially influenced by the tidal cycle. While there is a light-dark (LD) cycle of 24 h period throughout a river, the tidal cycle results in an environmental cycle of a 12.4 h period in the downstream area. To investigate variation in the endogenous rhythm by habitats with different environmental cycles, I focused on the freshwater snail *Semisulcospira reiniana*, which inhabits both non-tidal and tidal areas of a river. I have demonstrated that individuals in a non-tidal area exhibited the circadian rhythm but those in a tidal area exhibited both circadian and circatidal rhythms (Yokomizo and Takahashi 2022). However, whether the differential rhythmicity is caused by clock evolution or plasticity depending on environmental cycles remains unrevealed. I here evaluated whether individuals in a non-tidal area plastically express the circatidal rhythm by entraining to the tidal cycle. I examined the changes in the endogenous rhythm of *S. reiniana* caused by exposure to the simulated tidal environment using behavioral observation and transcriptome analysis.

Materials and Methods

Species and sampling sites

Semisulcospira reiniana is a common freshwater snail that mainly inhabits freshwater areas in Japan. I collected adult snails in non-tidal (3–5 m above sea level, 35° 15′ 15″ N, 136° 41′ 09″ E) and tidal areas (< 1 m above sea level, 35° 08′ 49″ N, 136° 40′ 37″ E) of the Kiso River in Japan in June and November 2021. These two populations were approximately 20 km apart along the river. Collected snails were kept in the freshwater and acclimated in the laboratory under 23°C and 12L12D conditions for at least one month.

Entrainment to the simulated tidal cycle

After acclimation, individuals in tidal and non-tidal populations were respectively divided into control and treatment groups. Individuals in the treatment group were exposed to the simulated tidal cycle simulated by raising and lowering the water level in the water tank (60 × 30 × 45 cm) (Fig. 3-1). This tidal simulation was conducted twice in the laboratory under 23°C and 12L12D conditions. Containers (φ7 × 9 cm) with water vents were set at 32 cm from the bottom of the water tank. Snails were put into the containers (four individuals per container). To generate the high tide condition, water was filled to a depth of 39 cm, in which snails were completely submerged. To simulate the low tide condition, the water level dropped to a depth of 24 cm, in which snails were completely exposed to air. Water supply to the tank and draining from it were programed and controlled by timers. In the first simulation, the switches from water supply to drainage were 3:00 and 15:00, and those from drainage to supply were 9:00 and 21:00. In the second simulation, the switches from water supply to drainage were 5:00 and 17:00, and those from drainage to supply were 11:00 and 23:00. The treatment of entrainment was conducted for four weeks. Individuals in the control group were kept in the freshwater without water level oscillation under 12L12D conditions. Snails in the first tidal simulation were used for behavioral observations and those in the second simulation were used for gene expression analysis.

Behavioral observation

To investigate the effect of the water level oscillation of a 12 h period on the endogenous activity rhythm of *S. reiniana*, individuals in non-tidal and tidal populations of the control and treatment groups were examined under constant laboratory conditions.

In both groups, eight snails per population were individually placed in containers (13 × 11.7 cm, 4.7 cm in height) filled with freshwater. Snails were observed under the constant dark (DD) condition at a temperature of 23°C in the laboratory for 96 and 84 hours in the control and treatment groups, respectively. The short observation period in the treatment group was due to a camera malfunction. Water was circulated using a pump to keep the water clean. Images were taken every 30 seconds using a model 400-CAM061 camera (Sanwa Direct, Japan) and were used to create time-lapse movies. I tracked the position of individuals using UMATracker tracking software (Yamanaka and Takeuchi 2018). After averaging the coordinates of individuals every three frames, the total locomotion distance traveled in the hour was calculated using the trajectories.

RNA sampling

To investigate the effect of the water level oscillation of a 12 h period on the endogenous gene expression rhythm of *S. reiniana*, I performed time course RNA-seq using individuals in non-tidal and tidal populations of the control and treatment groups. Snails were preserved in containers without water under the DD condition at a constant temperature (23°C) for tissue collection. Starting at subjective high tide, three individuals per population were dissected every 3.1 hours for 49.6 hours (i.e., 17 sampling time points) in both control and treatment groups. The epidermis of each individual was preserved in 750 μL of RNA*later* Stabilization Solution (Invitrogen). The samples were stored at 4°C for approximately 3 hours and then stored at −80°C till the extraction of total RNA.

RNA extraction and sequencing

Total RNA was extracted using the Maxwell 16 LEV Plant RNA Kit with the Maxwell 16 Research Instrument (Promega, USA), according to the manufacturer's instructions. Electrophoresis was performed on 1% agarose gel to evaluate RNA degradation. RNA concentrations were estimated using a Qubit 2.0 fluorometer (Invitrogen). RNA purity was estimated using NanoDrop Lite (Thermo Scientific). Total RNA from three individuals in the same population at one sampling time point was pooled in equal amounts prior to RNA sequencing (pooled RNA-seq). A cDNA library was constructed using the TruSeq RNA Sample Prep Kit. Paired-end (150 bp) pooled RNA-seq was performed using the Illumina NovaSeq6000 platform. After the removal of adaptor sequences and low-quality reads using Trimmomatic (version 0.38) (Bolger et al. 2014), I performed quality control using FastQC (version 0.11.8; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The remaining high-quality reads of all samples were used for *de novo* assembly using Trinity software (version 2.9.1) (Grabherr et al. 2011). To estimate gene expression levels, all reads from each sample were mapped to the reference transcripts using RSEM software (version 1.3.0) (Li and Dewey 2014), providing the TPM of each gene. The reference transcripts were used to create supertranscripts. Using the supertranscripts, I searched for homologs of every gene by BLAST for all protein sequences of *Crassostrea gigas* (cgigas_uk_roslin_v1; Peñaloza et al., 2021). Genes with the best hit and an e-value < 0.0001 were used to analyze gene expression rhythmicity.

Statistical analyses

I examined the activity rhythm of individuals of the control and treatment groups by Lomb-Scargle periodogram using ActogramJ, a software package based on ImageJ for the analysis and visualization of chronobiological data (Schmid et al. 2011). The mean locomotor distance of survived individuals was used for rhythm detection. Because most individuals in the treatment group hardly moved at the end of the observation, activity data for 72 hours from the onset was used for periodogram analysis. Gene expression rhythmicity was analyzed using two programs, JTK_CYCLE (Hughes et al. 2010) and RAIN (Thaben and Westermark 2014), non-parametric algorithms to identify rhythmic components in large, genome-scale biological datasets. To remove genes with low expression, genes with average TPM value > 0 and oscillation amplitude (peak/trough) ≥ 1.3 were used. I identified circatidal (12.4 ± 3.1 h) and circadian (24.8 ± 3.1 h) oscillating genes detected by at least one of two programs ($P < 0.01$). To estimate the differential rhythmicity of gene expression between the control and treatment groups, I calculated a differential rhythmicity score (S_{DR}) for each gene. I tested a periodicity of 12 h for each gene using JTK_CYCLE, and then genes with P-value < 1 and estimated amplitude > 0 were used for the calculation of S_{DR}. S_{DR} was defined as

$$S_{DR} = \frac{Z_P + Z_R}{\sqrt{2}},$$

where Z_P and Z_R are the Z-score for changes in periodicity and amplitude between groups, respectively (Kuintzle et al. 2017). Changes in periodicity are defined as log (P-value of the control group) –log (P-value of the treatment group). Changes in amplitude are defined as $\log_2$ (amplitude of treatment group / amplitude of control group). I computed the P-value for S_{DR} using a Gaussian distribution based on the fit to the empirical distribution. The P-value was adjusted by Benjamini & Hochberg procedure

for multiple testing. To identify functional enrichment of biological processes in circatidal oscillating genes or differential rhythmic genes based on S_{DR}, I performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway over-representation analysis and KEGG gene set enrichment analysis (KEGG-GSEA) by clusterProfiler (Yu et al. 2012). Circatidal oscillating genes detected by JTK_CYCLE and RAIN in the treatment group were used for a test set of KEGG pathway over-representation analysis. The gene list for GSEA was generated according to S_{DR}.

Results

Activity rhythm

Snails exhibited rhythmic activity patterns in the laboratory under DD conditions while the activity of individuals in the treatment group gradually decreased. Individuals in the control group exhibited the circadian activity rhythm of a period of 21.6 h in the non-tidal population and 22.5 h in the tidal population under laboratory DD conditions (Fig. 3-2a, b). Individuals in the tidal population did not exhibit the circatidal rhythm, indicating that their activity rhythm was synchronized with the laboratory 12L12D condition. In contrast, individuals in the treatment group exhibited the circatidal activity rhythm of a period of 12.4 h in the non-tidal population and 11.9 h in the tidal population under laboratory DD conditions (Fig. 3-3a, b), indicating that they were entrained to the simulated tidal cycle. The peak timing of activity preceded the expected peak of high tide.

Transcriptome rhythm

Information on the number of reads in each sample was summarized in Table 3-S1. I obtained 1,578,392 contigs, with a mean length of 513 bp. Among the 954 metazoan core gene orthologs, 948 (99.3%) were identified. I obtained 908,817 supertranscripts containing 58,952 contigs annotated against all protein sequences of *C. gigas*. The number of genes with average TPM value > 0 and oscillation amplitude (peak/trough) ≥ 1.3 in the tidal population was 13,929 and 14,508 in the control and treatment groups, respectively. I also obtained 13,448 and 13,997 genes meeting the same criteria in the non-tidal population.

In the non-tidal population, I detected 344 (2.6%) and 314 (2.2%) circatidal (12.4 ± 3.1 h) oscillating genes in the control and treatment groups, respectively (Fig. 3-4a, S1). The proportion of genes oscillating in the circatidal period was not significantly changed between the control and treatment groups (Fisher's exact test, $P = 0.10$). In the tidal population, I detected 283 (2.0%) and 354 (2.4%) circatidal (12.4 ± 3.1 h) oscillating genes in the control and treatment groups, respectively (Fig. 3-4a, S1). Unlike the non-tidal population, the proportion of genes oscillating in the circatidal period was greater in the treatment than in the control group (Fisher's exact test, $P = 0.03$), suggesting that the circatidal gene expression was induced by the entrainment. No identified circadian clock genes showed circadian or circatidal rhythmicity in the control and treatment groups (Figs. 3-S2, 3-S3).

Differential rhythmicity analysis revealed the changes in rhythmicity in transcriptome between the control and treatment groups (Fig. 3-4b). Genes in the first quadrant showed clearer periodicity and larger amplitude in the treatment group than in the control group, indicating increased circatidal rhythmicity. In the non-tidal population,

four genes showed significantly (FDR < 0.1) increased rhythmicity of 12.4 h period in the treatment group, only one of which was included in the circatidal oscillating genes (Table 3-S2). In the tidal population, seven genes showed significantly (FDR < 0.1) increased rhythmicity of 12.4 h period in the treatment group, five of which were included in the circatidal oscillating genes (Table 3-S3). Almost all of the transcripts did not show significant differential rhythmicity in expression pattern in both tidal and non-tidal populations, indicating that few genes increased the rhythmicity of the 12.4 h period via the tidal cycle and the pattern of differential rhythmicity in transcriptome was similar between populations.

KEGG pathway over-representation analysis revealed that circatidal oscillating genes in the treatment group of the tidal population were enriched for pathways of "Aminoacyl-tRNA biosynthesis" (KO: crg00970) and "Histidine metabolism" (KO: crg00340) (Fig. 3-5a). KEGG-GSEA based on S_{DR} showed that biological processes including "Lysosome" (KO: crg04142) and "Glycosaminoglycan degradation" (KO: crg00531) were enriched for genes with increased rhythmicity of 12.4 h period in the tidal population (Fig. 3-5b). No enriched pathways were detected in the non-tidal population by either KEGG pathway over-representation analysis or KEGG-GSEA.

Discussion

The coordination of biological processes with environmental cycles is regulated by biological clocks represented by the circadian clock, enhancing fitness in organisms in rhythmic environments (Sharma 2003). In marine or intertidal habitats, the tidal cycle provides complicated and drastic environmental variations, which is different from terrestrial and inland water habitats. The freshwater snail, *S. reiniana,* in a tidal area

exhibits the circatidal rhythm, which is thought to be adaptive in the tidal environment (Yokomizo and Takahashi 2022). Here, I investigated whether differential rhythmicity in *S. reiniana* between the tidal and non-tidal populations is caused by clock evolution or plastic rhythm expression depending on the dominant environmental cycles. I demonstrated that even individuals in the non-tidal population could show the circatidal rhythm by the entrainment to the tidal cycle. On the other hand, my results concerning the transcriptome rhythm suggest that more genes or pathways are influenced by the tidal cycle in the tidal population compared to the non-tidal population.

First, I examined the influence of the simulated tidal cycle on the activity rhythm under constant conditions in each population. Free-running period of individuals of the control group in DD condition was shorter than 24 hours, irrespective of the population. Because *S. reiniana* is a nocturnal animal (Urabe 1998), this short circadian period could be predicted by Aschoff's rule, in which the free-running period in DD is longer than 24 h for diurnal animals and shorter than 24 h for nocturnal animals (Beaulé 2008). Although individuals in the control group exhibited the circadian activity rhythm, the periodicity was not clear (Fig. 3-2a). Because only the light condition was regulated in the 24 h period in the laboratory, the lack of other circadian zeitgebers, such as the daily cycle of temperature, may have emphasized a noise or ultradian rhythms. The activity rhythms of individuals drastically changed by exposure to the simulated tidal cycle. Individuals in the treatment group exhibited the circatidal activity rhythm irrespective of the population (Fig. 3-3), suggesting that the circatidal rhythm was plastically expressed by the tidal cycle even in the individuals in the non-tidal population. Because I manipulated only water level in the simulation of the tidal cycle, cyclic change in salt concentration could not be a zeitgeber of the circatidal rhythm. The

peak of the activity rhythm was a bit different from individuals in the natural tidal condition. While snails immediately after capturing in a tidal area show the rhythmic activity pattern with a peak of subjective high tide (Yokomizo and Takahashi 2022), individuals in the treatment group in the present study had peaks preceding the expected high tide. The advance in the peak timing of activity probably resulted from the earlier arrival of high tide than the programmed switching time because the rate of water supply was set to reach the high tide in a little less than 6 hours. Periodogram analysis also detected peaks around 19 h in the activity rhythms of individuals in the treatment group. Although this periodicity may be derived from the shortened circadian rhythm or the combination of circadian and circatidal rhythm, the biological process generating periodicity of 19 h cannot be identified.

I then examined the effect of the simulated tidal cycle on the gene expression rhythm. In the non-tidal population, I found no significant difference in the proportion of circatidal oscillating genes between the control and treatment groups. In the tidal population, however, circatidal oscillating genes significantly increased in the treatment group. These results suggest that individuals in the tidal population were entrained to the simulated tidal cycle and circatidal gene expression was activated. The population-specific increase in the circatidal oscillating genes suggests that the number of circatidal clock-controlled genes (CCGs) is greater in the tidal population. However, I detected hundreds of circatidal oscillating genes in the control group of both tidal and non-tidal populations. The expression rhythm of a 12.4 h period in these genes might be a conservative rhythm derived from the harmonics of circadian clocks, as reported in mammals (Hughes et al. 2009, Zhu et al. 2017, Zhu 2020). Although I detected hundreds of genes with circatidal oscillation, circadian clock genes were not included in

the circatidal oscillating genes. Unlike the pacific oyster, the tidal cycle did not result in circatidal rhythmic expression in circadian clock genes. In addition, none of the circadian clock genes identified here exhibited the circadian rhythm in their expression, probably because of the weak peripheral clocks in the epidermis or asynchronization between tissues in samples. On the other hand, my results are consistent with the findings that several intertidal organisms do not show the circadian rhythm in the clock genes (Zhang et al. 2013, Schnytzer et al. 2018, Satoh and Terai 2019). The oscillations of the circatidal clock might underlie the absence of circadian rhythmicity in circadian clock genes.

To evaluate to what extent the tidal cycle increased the circatidal rhythmicity in the transcriptome, S_{DR} was calculated for each gene in the two populations. The distribution of S_{DR} of the tidal population was similar to that of the non-tidal populations. The number of genes with significantly increased circatidal rhythmicity did not greatly differ between populations. These results suggest that the degree of changes in the circatidal rhythmicity in the transcriptome in individuals exposed to the tidal cycle is not different between the tidal and non-tidal populations. Although almost all genes did not significantly change the strength of rhythmicity of a 12.4 h period between the control and treatment groups, four and seven genes significantly increased rhythmicity in the treatment group of the non-tidal and tidal populations, respectively. These genes are suggested to be entrained to the simulated tidal cycle and enhanced circatidal rhythmicity.

To detect biological processes enriched in the circatidal oscillating genes or differential rhythmic genes, I performed KEGG over-representation analysis and KEGG-GSEA. Totally four pathways were detected in the tidal population, while no

pathways were detected in the non-tidal population. Taken together with the fact that the increase in the circatidal oscillating genes in the treatment group was found only in the tidal population, individuals in the tidal population should have circatidal CCGs and biological processes regulated by the circatidal clocks more abundantly than individuals in the non-tidal population. I found that "Aminoacyl-tRNA biosynthesis" (KO: crg00970) pathway was enriched in the circatidal oscillating genes in the tidal population. Aminoacyl-tRNA is responsible for delivering amino acids for the mRNA-guided synthesis of proteins at the ribosome (Moutiez et al. 2017). "Histidine metabolism" (KO: crg00340) pathway was also detected, suggesting that proteins containing histidine were associated with response to the tidal cycle. It also should be noted that the "Lysosome" (KO: crg04142) pathway was enriched in the differential rhythmic genes. Lysosomes respond to a broad spectrum of physical and chemical stressors. Lysosomal membrane stability is known as a biomarker of natural stress factors, including the tidal cycle and air exposure (Tremblay and Pellerin-Massicotte 1997, Brenner et al. 2012). Increased circatidal rhythmicity of genes associated with the lysosome pathway in individuals in the tidal population suggests that stress responses to the cyclic exposure to air due to the simulated tidal cycle would periodically be regulated by clocks. In contrast, individuals in the non-tidal population may have directly responded to the stress factors during the tidal simulation.

My results concerning the activity rhythm revealed the plasticity of the endogenous rhythm in individuals in both tidal and non-tidal populations. The genetic basis of circatidal rhythm may be conserved in the genome and tidal zeitgeber resulted in the expression of circatidal rhythm in individuals in the non-tidal population. The plasticity of the endogenous rhythm may have contributed to inhabiting a tidal area of a

river. I have revealed that genetic differentiation between the two populations in the present study was small (Yokomizo and Takahashi 2022), supporting the contribution of plasticity to the range expansion. By focusing on other rivers where the distribution of *S. reiniana* is limited in a non-tidal area, I will further examine a relationship between the plasticity of the endogenous rhythm and establishment in a tidal area. Although my findings suggest that the genetic basis of circatidal rhythm exists in individuals in the non-tidal population, it remains unclear whether they have independent circatidal clocks from circadian clocks or a single timekeeping system governing both circadian and circatidal rhythms. The existence of the later system is a growing idea, especially in studies on biological clocks in marine molluscs (Schnytzer et al. 2018, Tran et al. 2020, Mat et al. 2020). Further studies are needed to determine whether the lack of circadian activity rhythm in the individuals in the treatment group resulted from changes in the periodicity of one biological clock or masking of the circadian rhythm. Revealing the molecular relationship between circadian and circatidal clocks would help us to test the controversial hypotheses on the mechanism of generation of circatidal oscillation (Enright 1976, Hastings and Naylor 1980, Palmer 1995).

My study suggests that even individuals in the non-tidal population have the circatidal clock and express the circatidal rhythm when exposed to the tidal cycle. Nevertheless, the results of transcriptome analysis suggest that individuals in the tidal population increased regulation by the circatidal clock in biological processes. Taken together, the plasticity of the endogenous rhythm and subsequent genetic changes in a molecular network between clocks and several genes may contribute to the adaptation to the tidal environment. Examining the parallel evolution of biological clocks using individuals in different rivers would provide a comprehensive understanding of the

mechanisms of adaptation to tidal environments via genetic and/or non-genetic changes in the biological clocks.

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Figures

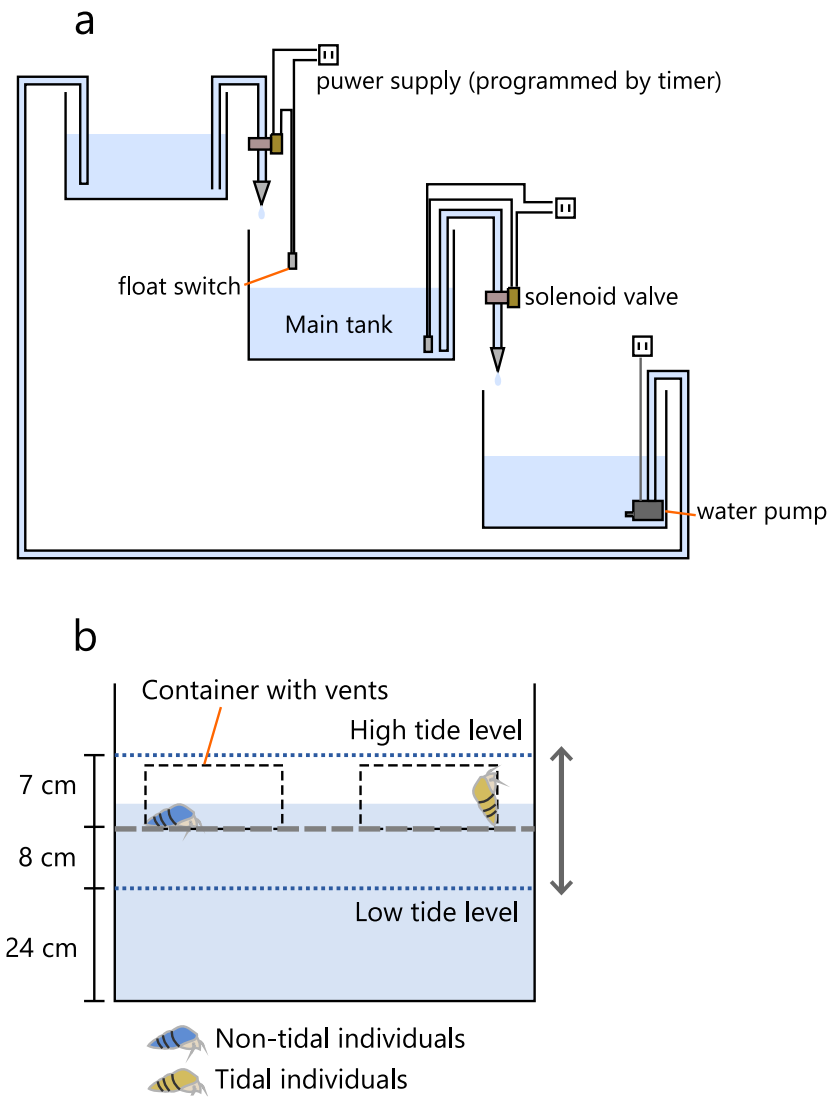

Figure 3-1. The simulation of the tidal cycle and the structure of the water tank. (a) Outline of the tidal simulation system. Snails were placed in the central tank (Main tank) and entrained to the tidal cycle. The water levels of the high and low tides were determined by the float switches. Water was siphoned from the upper tank to the central tank and from the central tank to the lower tank. Water was pumped from the lower tank to the upper tank. The opening and closing of the solenoid valves were programmed by timers. (b) The structure of the central tank (Main tank). Snails were placed in the containers at the height of the dashed line. The dotted lines represent water levels of the high and low tides.

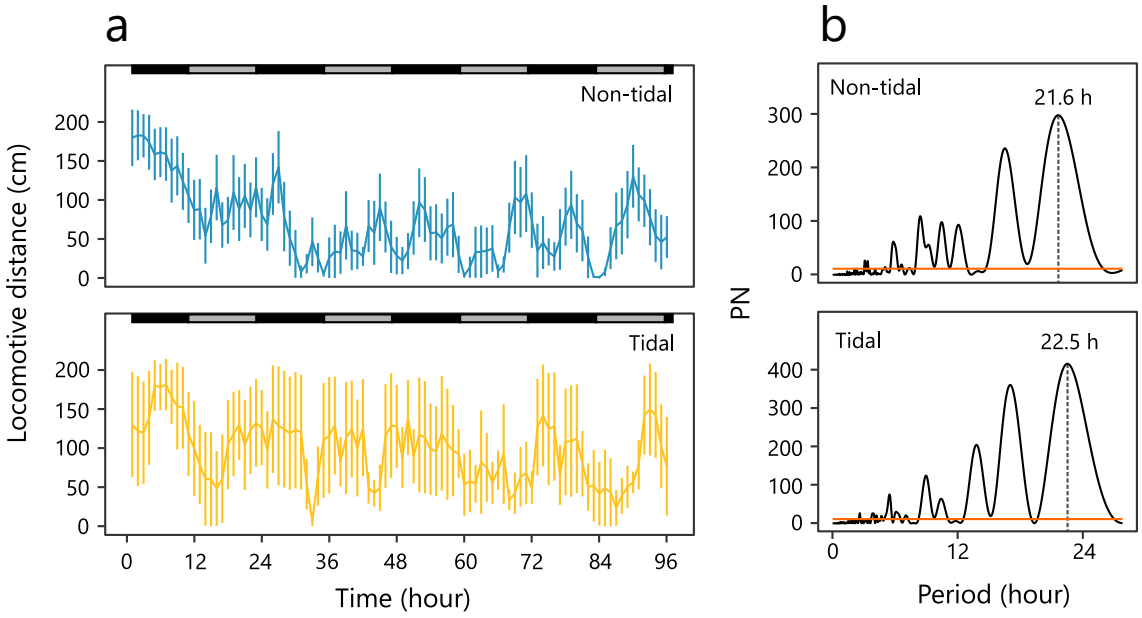

Figure 3-2. Activity patterns of individuals in non-tidal and tidal populations of the control group under laboratory DD conditions. (a) Locomotor distance of the non-tidal and tidal population for 96 hours. Error bars are SEM. Subjective day and night are shown as grey and black bars above. (b) Lomb-Scargle periodogram analysis. Peaks in the circadian range are noted.

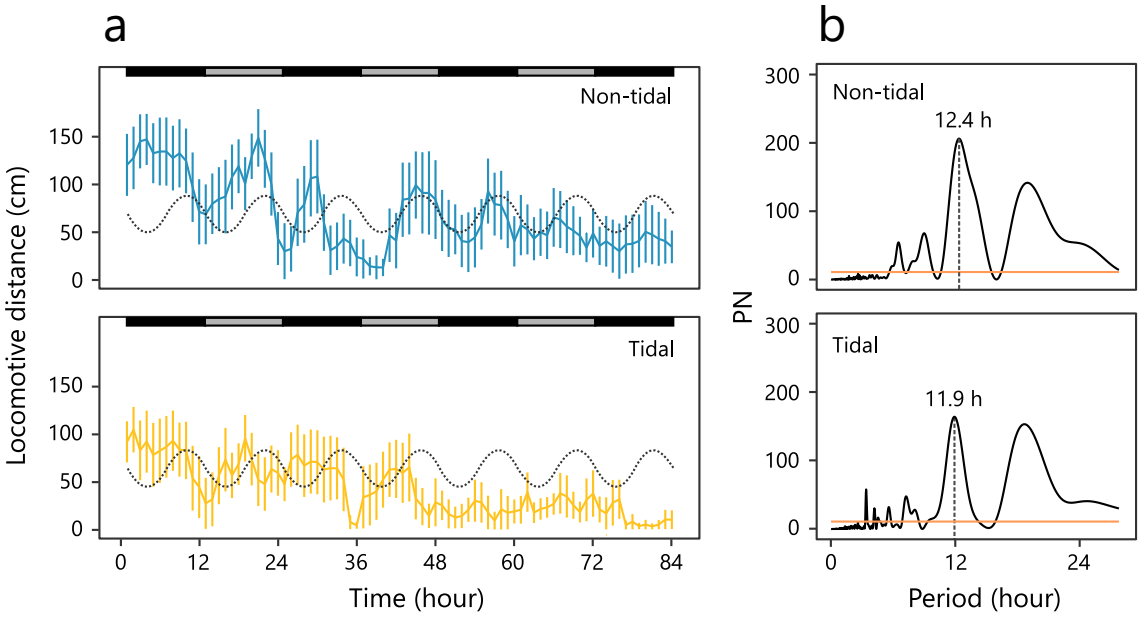

Figure 3-3. Activity patterns of individuals in non-tidal and tidal populations of the treatment group under laboratory DD conditions. (a) Locomotor distance of the non-tidal and tidal population for 84 hours. Error bars are SEM. Subjective day and night are shown as grey and black bars above. The expected tide level in the laboratory is shown as a gray dotted line. (b) Lomb-Scargle periodogram analysis. Peaks in the circatidal range are noted.

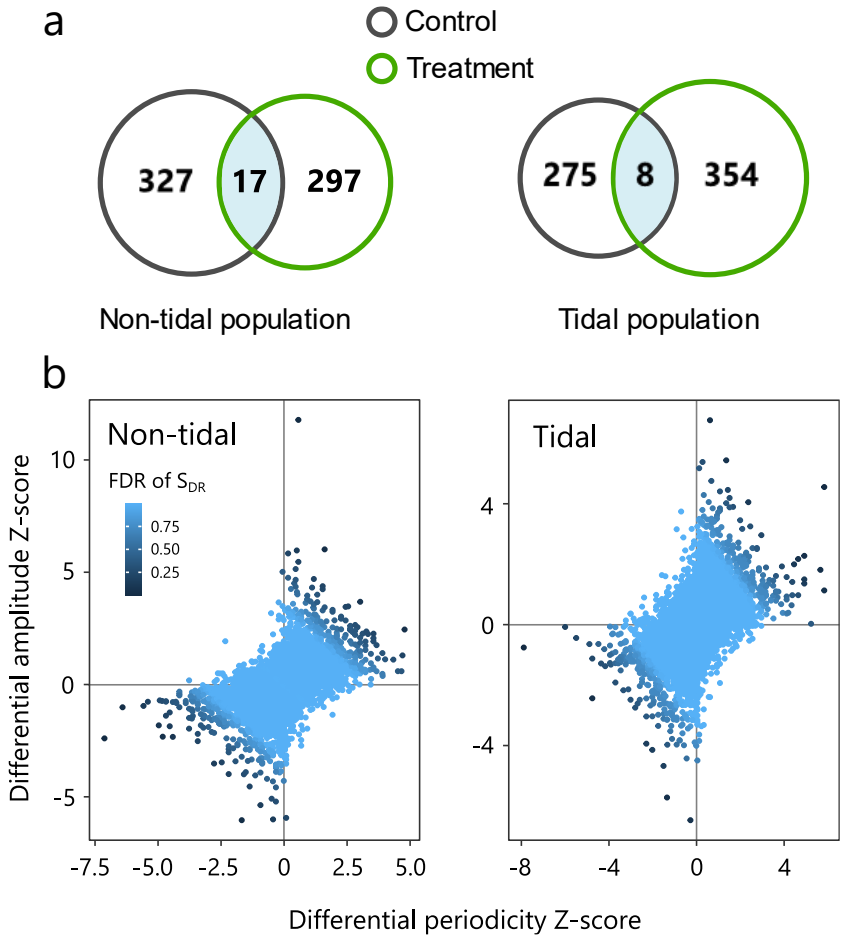

Figure 3-4. Transcriptome rhythms of individuals in non-tidal and tidal populations under laboratory DD conditions. (a) Venn diagrams detailing the number of circatidal oscillating genes detected in the control and treatment groups. (b) Differential rhythmicity of 12.4 h period in the transcriptome between the control and treatment groups. Genes with greater differential periodicity Z-score (horizontal axis) show stronger cyclical oscillation patterns of a 12.4 h period in the treatment group. Genes with greater differential amplitude Z-score (vertical axis) show larger differences between their expression peak and trough in the treatment group. Genes are colored based on their FDR of S_{DR}. Genes with positive S_{DR} show increased rhythmicity in the treatment group, while genes with negative S_{DR} show increased rhythmicity in the control group.

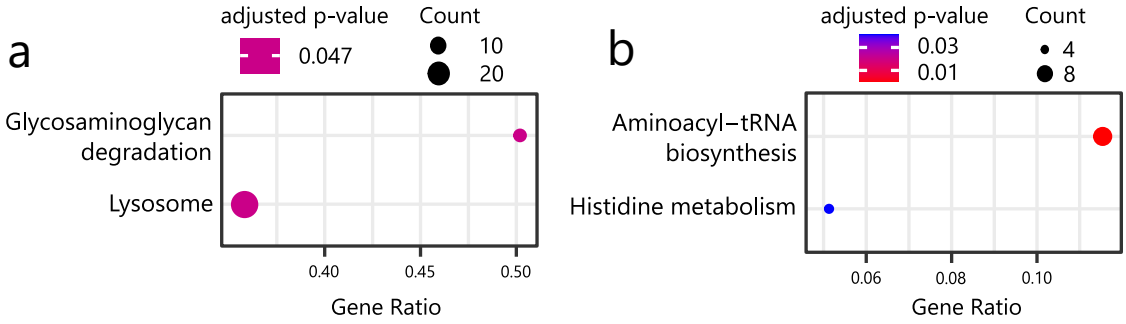

Figure 3-5. KEGG pathway analysis of the tidal population. (a) KEGG pathway over-representation analysis of the tidal population. Gene ratio is referred to the ratio of the number of DEGs to the total number of genes in a specific pathway. The size of the circles represents the count of DEGs. Circles are colored based on the adjusted *p*-value. No pathways were enriched in the non-tidal population. (b) KEGG-GSEA of the tidal population. Gene ratio is referred to the ratio of the number of the core enriched genes to the total number of genes in a specific pathway. The size of the circles represents the count of the core enriched genes. No pathways were enriched in the non-tidal population.

Supplementary Information

Table 3-S1. The number of raw reads in each sample.

Sample	Population	Experiment	Time	Reads
mak_1c	Non-tidal	control	0	11637217
mak_2c	Non-tidal	control	3.1	14517088
mak_3c	Non-tidal	control	6.2	12876528
mak_4c	Non-tidal	control	9.3	13158385
mak_5c	Non-tidal	control	12.4	12338172
mak_6c	Non-tidal	control	15.5	11384899
mak_7c	Non-tidal	control	18.6	13883070
mak_8c	Non-tidal	control	21.7	13259870
mak_9c	Non-tidal	control	24.8	13053790
mak_10c	Non-tidal	control	27.9	12874985
mak_11c	Non-tidal	control	31.0	13662857
mak_12c	Non-tidal	control	33.1	12385653
mak_13c	Non-tidal	control	37.2	11873204
mak_14c	Non-tidal	control	40.3	11778180
mak_15c	Non-tidal	control	43.4	11515721
mak_16c	Non-tidal	control	46.5	11516692
mak_17c	Non-tidal	control	49.6	12851196
mak_18t	Non-tidal	treatment	0	10738672
mak_19t	Non-tidal	treatment	3.1	10473783
mak_20t	Non-tidal	treatment	6.2	12469639
mak_21t	Non-tidal	treatment	9.3	11851404
mak_22t	Non-tidal	treatment	12.4	12567758
mak_23t	Non-tidal	treatment	15.5	12238422
mak_24t	Non-tidal	treatment	18.6	10183881
mak_25t	Non-tidal	treatment	21.7	13026176
mak_26t	Non-tidal	treatment	24.8	10178131
mak_27t	Non-tidal	treatment	27.9	14377518
mak_28t	Non-tidal	treatment	31.0	11302195
mak_29t	Non-tidal	treatment	33.1	10926096
mak_30t	Non-tidal	treatment	37.2	10994254
mak_31t	Non-tidal	treatment	40.3	10330389
mak_32t	Non-tidal	treatment	43.4	12231522
mak_33t	Non-tidal	treatment	46.5	13839002
mak_34t	Non-tidal	treatment	49.6	10985898
tat_1c	Tidal	control	0	14193317
tat_2c	Tidal	control	3.1	14707953
tat_3c	Tidal	control	6.2	13724581
tat_4c	Tidal	control	9.3	12917632
tat_5c	Tidal	control	12.4	14769246

tat_6c	Tidal	control	15.5	11551791
tat_7c	Tidal	control	18.6	12673541
tat_8c	Tidal	control	21.7	12343878
tat_9c	Tidal	control	24.8	13471394
tat_10c	Tidal	control	27.9	12894287
tat_11c	Tidal	control	31.0	13922733
tat_12c	Tidal	control	33.1	16295868
tat_13c	Tidal	control	37.2	11858458
tat_14c	Tidal	control	40.3	11634813
tat_15c	Tidal	control	43.4	13437996
tat_16c	Tidal	control	46.5	13235442
tat_17c	Tidal	control	49.6	13847547
tat_18t	Tidal	treatment	0	13584585
tat_19t	Tidal	treatment	3.1	15781059
tat_20t	Tidal	treatment	6.2	14143894
tat_21t	Tidal	treatment	9.3	13128863
tat_22t	Tidal	treatment	12.4	11621822
tat_23t	Tidal	treatment	15.5	11531164
tat_24t	Tidal	treatment	18.6	12843546
tat_25t	Tidal	treatment	21.7	16007631
tat_26t	Tidal	treatment	24.8	15515239
tat_27t	Tidal	treatment	27.9	12808593
tat_28t	Tidal	treatment	31.0	12518777
tat_29t	Tidal	treatment	33.1	11375355
tat_30t	Tidal	treatment	37.2	10926448
tat_31t	Tidal	treatment	40.3	10411385
tat_32t	Tidal	treatment	43.4	13037979
tat_33t	Tidal	treatment	46.5	11425600
tat_34t	Tidal	treatment	49.6	11877840

Table 3-S2. Genes that significantly increased rhythmicity of 12 h period in the treatment group of the non-tidal population. FDR is computed for S_{DR} using a Gaussian distribution based on the fit to the empirical distribution. Circatidal oscillating genes in the treatment group are shown as Yes.

ID	S_{DR}	FDR	Circatidal oscillation	Description
DN12910_c0_g1	5.39	0.024	No	zonadhesin-like isoform X1
DN3625_c0_g1	8.72	9.63×10^{-9}	No	alpha-amylase
DN3735_c6_g1	5.11	0.044	Yes	histone acetyltransferase type B catalytic subunit
DN51920_c1_g1	4.74	0.097	No	uncharacterized protein LOC105317097 isoform X1

Table 3-S3. Genes that significantly increased rhythmicity of 12 h period in the treatment group of the tidal population. FDR is computed for S_{DR} using a Gaussian distribution based on the fit to the empirical distribution. Circatidal oscillating genes in the treatment group are shown as Yes.

ID	S_{DR}	FDR	Circatidal oscillation	Description
DN100171_c0_g1	7.33	1.58×10^{-5}	Yes	craniofacial development protein 2-like
DN10917_c2_g2	4.81	0.071	Yes	uncharacterized protein LOC117681628
DN12119_c0_g1	4.92	0.058	Yes	Golgi pH regulator isoform X4
DN3825_c0_g1	5.20	0.042	No	arylsulfatase B
DN4823_c1_g1	4.79	0.071	No	uncharacterized protein LOC109618738
DN64_c11_g1	5.27	0.042	Yes	gamma-tubulin complex component 5 isoform X2
DN67461_c2_g1	5.08	0.043	Yes	zinc finger protein 665

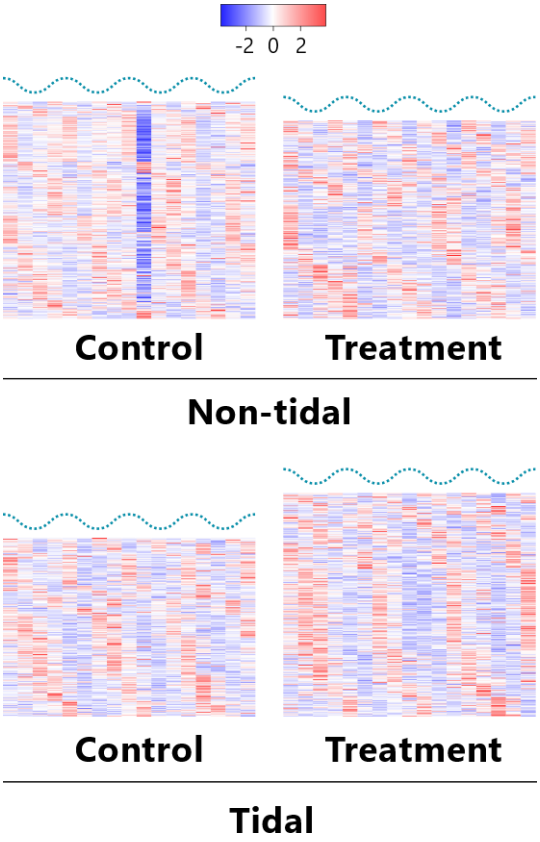

Figure 3-S1. The heatmaps of standardized expression patterns of circatidal rhythmic transcripts in individuals of the control and treatment groups of the non-tidal and tidal populations. The dotted wavy lines above the heatmaps represent the water level of the simulated tidal cycle.

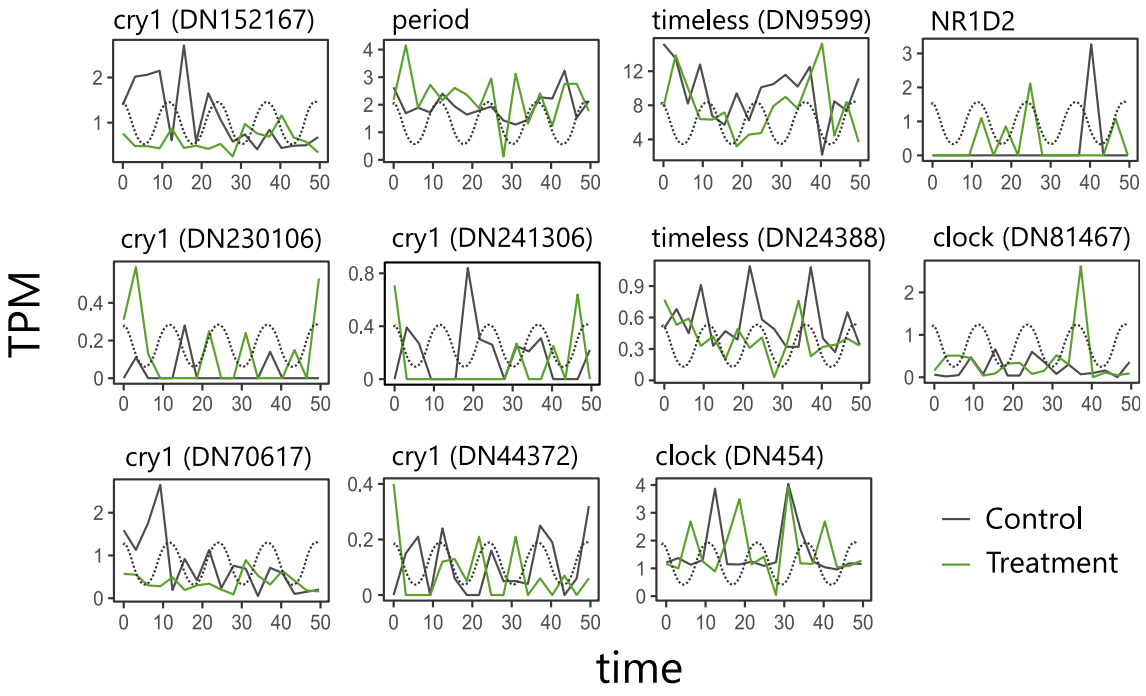

Figure 3-S2. The expression patterns of circadian clock genes of the control (gray) and treatment (green) groups of the tidal population under the DD condition. The simulated tidal cycle is shown as a gray dotted line.

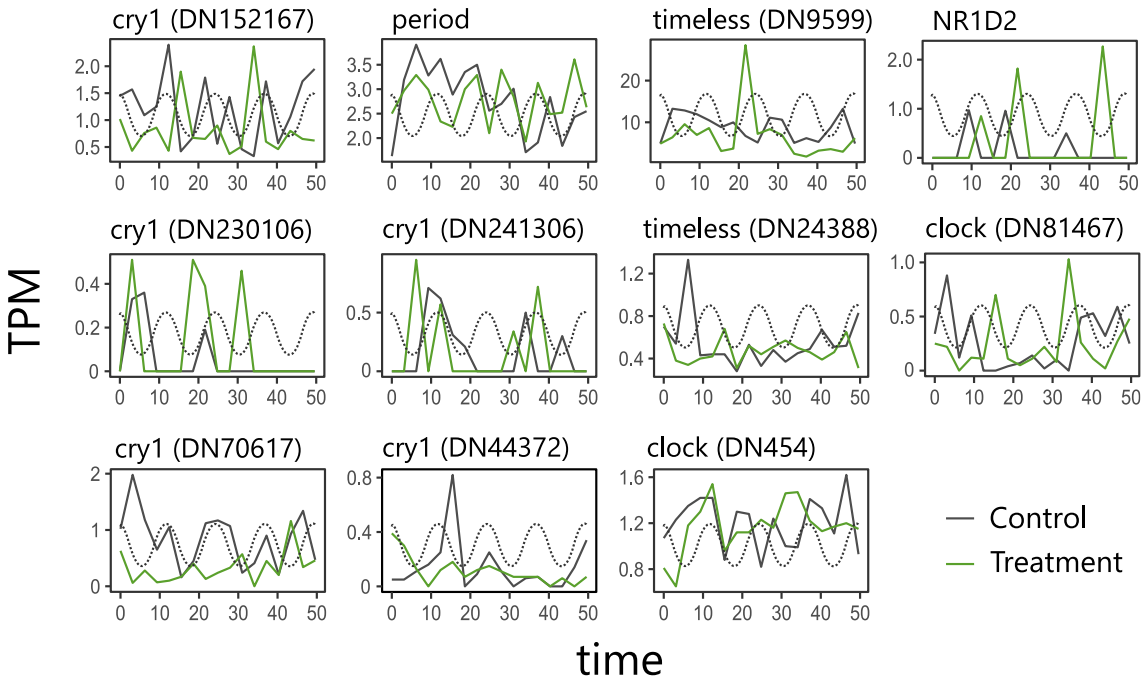

Figure 3-S3. The expression patterns of circadian clock genes of the control (gray) and treatment (green) groups of the non-tidal population under the DD condition. The simulated tidal cycle is shown as a gray dotted line.

Chapter 4

Effect of Water Level Oscillation on Gene Expression Patterns in a Freshwater Snail

Abstract

Periodic environmental changes favored the evolution of biological clocks. Although there are a lot of evidence of the existence of circatidal rhythm in many marine species, little is known about its molecular mechanism. My previous study revealed the circatidal gene expression in individuals of the tidal population of the freshwater snail, *Semisulcospira reiniana*. To identify candidate circatidal-related genes, I examined whether some of circatidal oscillating genes synchronized with the simulated tidal cycle. After individuals of the tidal population were exposed to the simulated tidal cycle, the expression patterns of *dual oxidase 2*, *mitogen-activated protein kinase 1* (*MAPK1*), and *protein Wnt-5b* were estimated using RT-qPCR. Although none of the four genes exhibited synchronized expression with the tidal cycle, the circatidal rhythmicity of the expression of Wnt-5b was marginally significant. Genes tested in this study may have no interaction with circatidal clocks or respond to other tidal zeitgebers, such as cyclic variations in the salt concentration. On the other hand, weak rhythmicity in the expression of protein Wnt-5b emphasizes the necessity of the functional analysis of this gene to reveal the roles in the response to the tidal cycle. Focusing on the synchronization between gene expression and tidal cycles would shed light on the molecular basis of the circatidal clocks.

Introduction

Biological clocks serve as an adaptation for a rotating world. Because the diurnal cycle influences organisms living in various habitats, including aquatic environments, circadian clocks are conserved in a wide range of a taxon. The molecular mechanism of the circadian clock has been explored in recent decades. Since the first report of clock gene mutant of *Drosophila melanogaster* (Konopka and Benzer 1971), many circadian clock genes have been identified (Bargiello et al. 1984, Zehring et al. 1984, Vitaterna et al. 1994, Emery et al. 1998, Bunger et al. 2000). In 2014, CHRONO, believed to be the last remaining component of the mammalian circadian clock, was discovered (Goriki et al. 2014). Circadian clock genes compose transcriptional-translational feedback loops and generate the oscillation of approximately 24 h period (Hardin et al. 1990, Duong et al. 2011). The oscillation of clock genes regulates the rhythmic expression of downstream genes, known as clock-controlled genes, and underlies the rhythmic phenotype, such as activity, metabolism, and immunity (Rutter et al. 2003, Scheiermann et al. 2013, Panda 2016).

Complex environmental cycles on the earth favored the evolution of diverse biological rhythms. The circatidal rhythm, synchronized with the tidal cycle of a 12.4 h period, is one of the biological clocks other than the circadian clock. The existence of circatidal clocks is evident in many species, including crustaceans, molluscs, insects, and so on (Hastings and Naylor 1980, Kim et al. 1999, Satoh et al. 2008). However, little is known about the molecular basis of circatidal rhythm (Zhang et al. 2013, Rock et al. 2022). A classic hypothesis for the circatidal oscillator was proposed by Enright (Enright 1976). This hypothesis states that a single oscillator governs both circatidal and circadian rhythms depending on the dominant environmental cycles. Other hypotheses

expect that the circatidal rhythm is generated by an independent oscillator(s) from circadian clocks (Palmer 1995, Naylor 2010). Palmer (1995) argued that the circatidal behavior results from dual circalunidian clocks with 24.8 h coupled in antiphase, while Naylor (2010) explained the circatidal rhythm is produced by a distinct circatidal clock of 12.4 h period. While functional analyses to test those hypotheses are still lacking, several studies to investigate the molecular or physiological mechanism of the circatidal rhythm have been reported. For instance, silencing some of the circadian clock genes in the mangrove cricket revealed the dissociation of circatidal clocks from circadian clocks (Takekata et al. 2012, 2014). Clock-related genes in an intertidal crab were found by rhythmic phenotype rescue in transgenic flies (Beckwith et al. 2011).

My previous studies revealed the circatidal rhythm in a freshwater snail, *Semisulcospira reiniana* (Yokomizo and Takahashi 2022). In this study, many oscillating genes with a period of 12.4 hours in a tidal population were detected by transcriptome analysis, supporting Naylor's hypothesis. However, not all circatidal oscillating genes should be regulated by the circatidal clock. The oscillation of a 12 h period is suggested to be generated by circadian clocks and found in terrestrial mammals (Hughes et al. 2009). These 12 h oscillations are considered the second harmonic of the circadian rhythm because they are apparently synchronized with/by circadian clocks. Therefore, circatidal oscillating genes include genes resulting from the harmonics of circadian clocks as well as those synchronized with a tidal cycle. In the present study, I aimed to detect genes synchronized with a tidal cycle to identify candidate circatidal-related genes. To eliminate non-tidal environmental factors such as precipitation and temperature cycle, individuals collected from a tidal area were exposed to the simulated tidal cycle in the laboratory. I focused on several circatidal

oscillating genes detected in the previous study and examined their expression under constant conditions using RT-qPCR.

Materials and Methods

Species and Sampling

I collected adult individuals of *S. reiniana* in a tidal area (< 1 m above sea level, 35° 08′ 49″ N, 136° 40′ 37″ E) of the Kiso River in Japan in May 2019. Collected snails were kept in the freshwater and acclimated in the laboratory under 23°C and 12L12D conditions for five months.

Entrainment to the simulated tidal cycle

After acclimation, snails were divided into control and treatment groups. Individuals in the treatment group were exposed to the tidal cycle, simulated by raising and lowering the water level in the water tank (60 × 30 × 45 cm). The tidal simulation was conducted in the laboratory under 23°C and 12L12D conditions. Containers with water vents (φ7 × 9 cm) were set at 10 cm from the bottom of the water tank. Snails were put into the containers (three individuals per container). To generate the high tide condition, water was filled to a depth of 17 cm, in which snails were completely submerged. To simulate the low tide condition, the water level dropped to a depth of 5 cm, in which snails were completely exposed to air. Water supply to the tank and draining from it were programmed and controlled by timers. The switches from water supply to drainage were 7:00 and 19:00, and those from drainage to supply were 1:00 and 13:00. The treatment of entrainment was conducted for ten days. Individuals in the control group were kept in the freshwater without water level oscillation under 12L12D conditions.

RNA sampling

To investigate the endogenous gene expression rhythm, I obtained time series RNA samples of individuals of control and treatment groups. Individuals in the control group were preserved in containers without water under the DD condition at a constant temperature (23°C) for tissue collection. Starting at the time of the rising tide in the sampling site, three individuals were dissected every 3.1 hours for 49.6 hours (i.e., 17 sampling time points). The epidermis of each individual was preserved in 750 μL of RNAlater Stabilization Solution (Invitrogen). The samples were stored at 4°C for approximately 3 hours and then stored at −80°C till the extraction of total RNA. RNA sampling of individuals in the treatment group was conducted immediately after the tidal simulation in the same procedure as the control group. The onset of RNA sampling corresponded to the subjective rising tide of tidal simulation.

RNA extraction and reverse transcription

Total RNA was extracted using the Maxwell 16 LEV Plant RNA Kit with the Maxwell 16 Research Instrument (Promega), according to the manufacturer's instructions. Electrophoresis was performed on 1% agarose gel to evaluate RNA degradation. RNA concentrations were estimated using a Qubit 2.0 fluorometer (Invitrogen). RNA purity was estimated using NanoDrop Lite (Thermo Scientific). Total RNA was converted to cDNA using High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems).

qPCR analysis

Candidate circatidal-related genes were selected from circatidal oscillating genes in the tidal population detected in my previous research (Yokomizo and Takahashi 2022). I focused on cyclic genes with high expression levels throughout sampling time and low p-value of periodicity. In the present study, I examined *dual oxidase 2*, *mitogen-activated protein kinase 1* (*MAPK1*), and *protein Wnt-5b*. To investigate the expression rhythm of the circadian clock gene, I also examined the expression pattern of *clock*. *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was used as a reference gene for the standard curve method of dilutions 1∶30, 1∶120, 1∶480, 1∶1920, 1∶7680, and 1:30720. Referring to the *de novo* assembled sequences in my previous research (Yokomizo and Takahashi 2022), the primers of each gene for qPCR analysis were designed (Table 4-S1). qPCR analysis was performed using 7300 Real Time PCR System (Applied Biosystems) with *Power* SYBR™ Green PCR Master Mix (Applied Biosystems), according to the manufacturer's instructions.

Statistical analyses

Time course relative expression data of candidate genes with three biological replicates at each time point was obtained by qPCR analysis. Gene expression rhythmicity of 12.4 or 24.8 h period was analyzed using RAIN, a robust nonparametric method for detecting rhythms of prespecified periods in biological data (Thaben and Westermark 2014). The peak phase of expression was also determined by RAIN.

Results

There was temporal variation in the expression levels of the genes that I examined. I found correlations between the expression patterns of several genes (Figs. 4-1, 4-2). Notably, the expression of *clock* and *MAPK1* strongly correlated in both the control and treatment groups.

Circadian or circatidal rhythmic expression of the genes was not significant in both the control and treatment groups (Fig. 4-3; Table 4-1). However, *P*-values of circatidal periodicity tended to be small in all the studied genes. Circatidal rhythmicity of the expression of Wnt-5b was marginally significant ($P = 0.10$). The peak phases were relatively synchronized in the treatment group. All genes peaked at the time of the rising or high tide. For *clock*, the expression pattern did not show circadian rhythmicity in both control ($P = 0.74$) and treatment ($P = 0.27$) groups.

Discussion

Numerous studies on the endogenous rhythm of marine species have left no doubt about the existence of the circatidal rhythm (Barnwell 1966, Hastings and Naylor 1980, Satoh et al. 2008, Chabot et al. 2016). While the molecular mechanism of the circadian clock is progressively being understood, the molecular or physiological mechanisms of the circatidal clock remain unclear. In my previous research, I detected hundreds of genes with circatidal rhythmic expression in the freshwater snail, *S. reiniana* (Yokomizo and Takahashi 2022). Here, I examined the effect of the tidal cycle on the expression rhythm of some circatidal oscillating genes to evaluate response to the tidal cycle. Although no genes tested here exhibited circatidal rhythmicity in their expression even after exposure

to the simulated tidal cycle, *protein Wnt-5b* showed a marginally significant periodicity of 12.4 h. *Wnt-5b* might be weakly regulated by the circatidal clock.

Among the circatidal oscillating genes, I focused on *dual oxidase 2*, *mitogen-activated protein kinase 1* (*MAPK1*), and *protein Wnt-5b*. Dual oxidase 2 is a glycoflavoprotein that regulates the production of reactive oxygen species involved in thyroid hormone biosynthesis (Ameziane-El-Hassani et al. 2005). MAPK1 is a member of the MAP kinase family, which functions as integration points for multiple biochemical signals and regulates many fundamental cellular processes, including proliferation, differentiation, transcriptional regulation, and development (Kolch 2005). MAPK signaling also functions as input of circadian clocks, producing changes in the phasing of clock gene expression (Coogan and Piggins 2004, Goldsmith and Bell-Pedersen 2013). Wnt-5b is a ligand for the Wnt signaling pathway implicated in various developmental processes and is regulated by circadian clocks (Soták et al. 2014).

I found positive correlations between the expression patterns of several genes. The correlations between *clock* and other genes indicate the regulation by circadian clocks. Notably, the expression of *clock* and *MAPK1* significantly correlated in both control and treatment groups (Figs. 4-1, 4-2), suggesting positive interaction between circadian clocks and MAPK pathways. Although the expression of *Wnt-5b* did not correlate with that of *clock*, the expression of *Wnt-5b* and *MAPK1* correlated as a result of the tidal simulation (Fig. 4-2). The expression patterns of all examined genes were arrhythmic both in the control and treatment groups (Fig. 4-3). However, I note that the expression pattern of *protein Wnt-5b* was close to a 12.4 h period ($P = 0.10$) in the treatment group. If a weak peripheral clock in the samples obscured the circatidal rhythm in the *protein Wnt-5b*, examining tissues with the central clock could discover

cyclic expression. Further molecular biological analyses are needed to evaluate the roles of Wnt proteins in the response to tidal cycles. I also found the lack of circadian expression in *clock*, which is consistent with my result in Chapter 3 of this thesis (Fig. 3-S2). The arrhythmic expression of a circadian clock gene may reflect technical limitations, such as the paucity of biological replicates in the experiment.

My results showed no rhythmic expression in the examined genes. The genes tested in this study are suggested not to synchronize with water level oscillation. However, I cannot deny that those genes respond to other tidal zeitgebers, such as cyclic variations in the salt concentration, and show circatidal rhythmic expression. Alternatively, the tidal simulation of 10 days might be insufficient to express the circatidal rhythm in gene expression. Therefore, long-term entrainment to the natural tidal cycle would be needed to conclude the association of the studied genes with the circatidal rhythm. Investigating gene expression response to tidal stimuli should help identify circatidal clock genes from transcriptome data. A combination of comprehensive analysis and functional analysis of the specific gene would provide insight into the molecular mechanisms of the circatidal clock.

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Tables and Figures

Table 4-1. Results of periodic analysis by RAIN. The phase represents the peak timing of expression.

	Control		**Treatment**	
	p-value	phase	p-value	phase
clock	0.82	12.4	0.41	12.4
dual oxidase 2	0.85	9.3	0.53	12.4
MAPK1	0.76	3.1	0.66	9.3
Wnt-5b	0.66	6.2	0.10	9.3

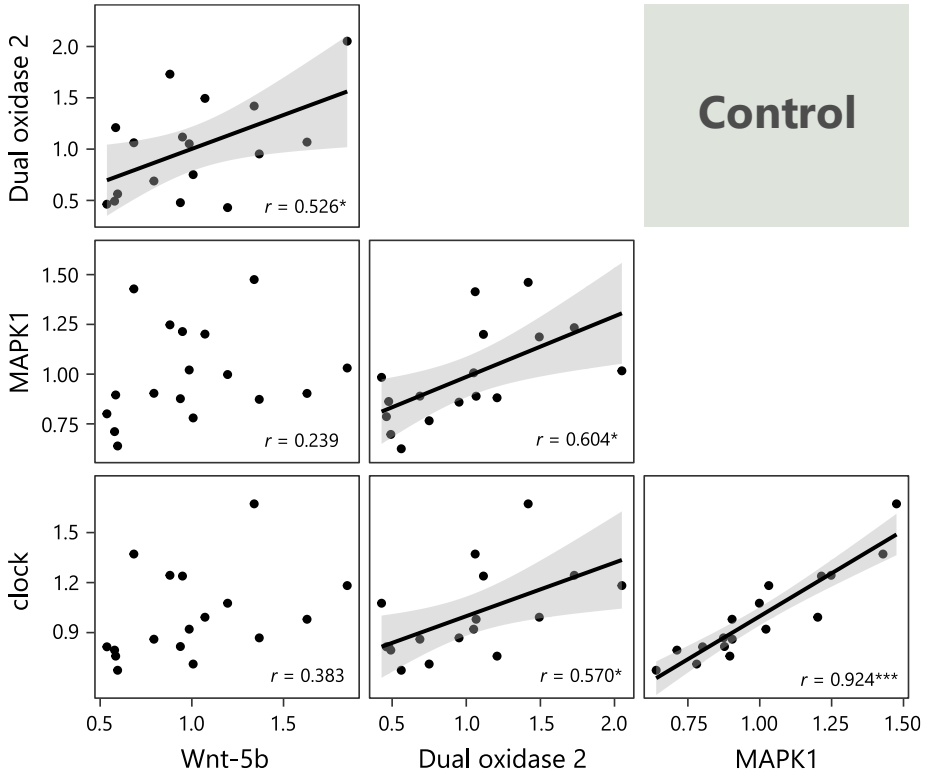

Figure 4-1. The correlations between relative expressions of each gene in the control group. The gray shaded areas represent 95% confidence intervals.

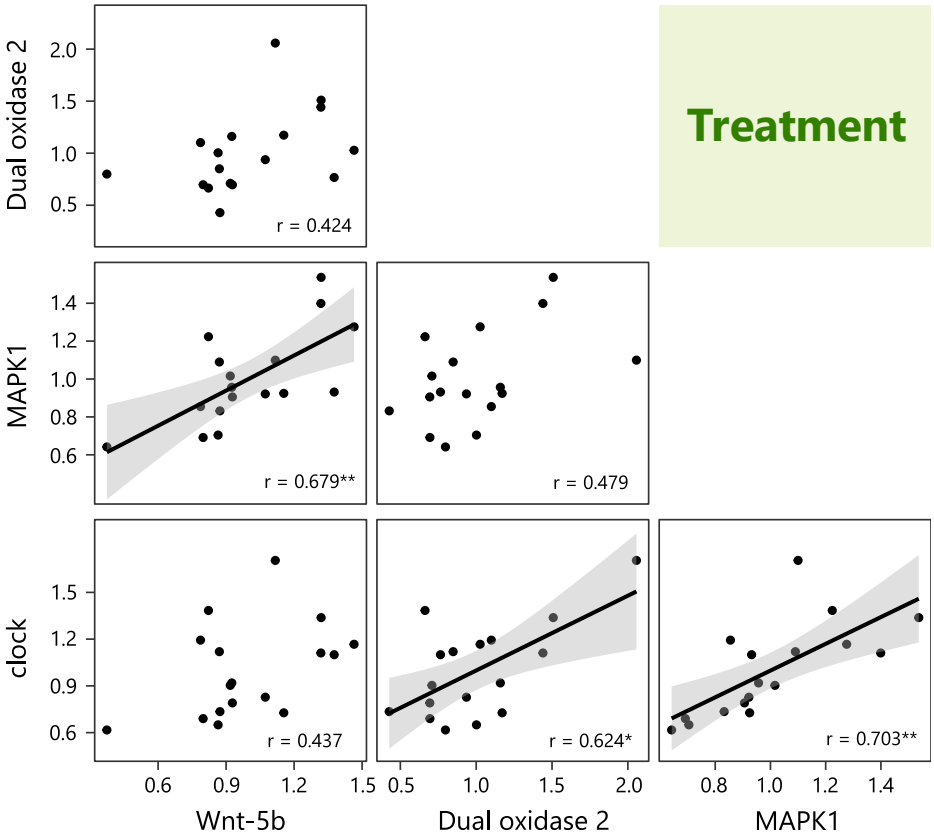

Figure 4-2. The correlations between relative expressions of each gene in the treatment group. The gray shaded areas represent 95% confidence intervals.

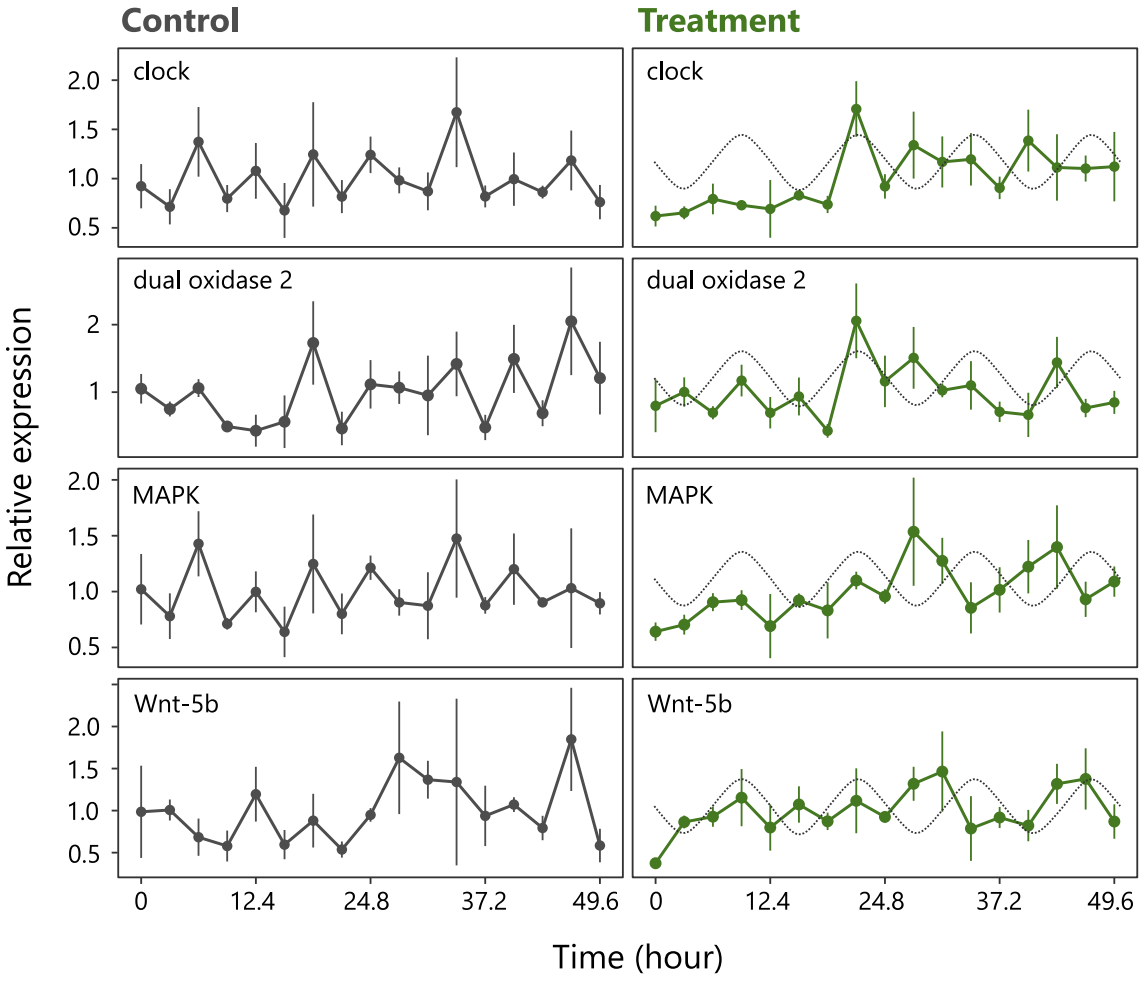

Figure 4-3. The expression patterns of *clock*, *dual oxidase 2*, *MAPK*, and *Wnt-5b* in individuals of the control and treatment groups. The simulated tidal cycle in the treatment group is shown as a gray dotted line.

Supplementary information

Table 4-S1. Primer information used in the qPCR analysis.

Gene name	Left primer	Right primer
GAPDH	GCCATTGACGACCAGTTTGT	CCGGCTCCTTCACATATAACAC
clock	ATCCACTGCCTCCAACATCA	TCAATGGTGGCTGCAAACAA
dual oxidase 2	GTCACCGAGAGATCACGTGA	ACGATTTTCTGATACTGCGCA
MAPK1	ATTATCAACGACAAGGCCCG	TCCAGCTCCATCTCAAACGT
Wnt-5b	GTGTCGGGAGGGAGAGTTAG	CGTAGTTGTACACCGCCTTG

General Discussion

Adaptive evolution and phenotypic plasticity could allow organisms to establish populations in tremendously diverse environments on Earth. Organisms should cope with both spatial and temporal variations of environmental conditions in their habitats. Previous studies have discussed the adaptive significance of biological clocks in periodic environments (Sharma 2003, Yerushalmi and Green 2009, Bulla et al. 2017, Kronfeld-Schor et al. 2017). The progress of our understanding of circadian clocks helps to discuss the adaptation to daily temporal variation. However, to understand biological rhythms in the real world, focusing on non-daily cycles, including tidal, lunar, and seasonal cycles, should also be emphasized (Numata and Helm 2014). In this thesis, I investigated adaptation to salinity and environmental variations caused by the tidal cycle using the freshwater snail *S. reiniana*.

Salinity is a significant environmental factor influencing the distribution range of many aquatic species (Charmantier 1998, Cervetto et al. 1999, Ho et al. 2019). The tidal cycle creates a steep gradient of salt concentration in a river. In Chapter 1, I demonstrated that individuals in a brackish area had high salinity tolerance and those in a freshwater area plastically enhanced salinity tolerance. This finding indicates that adaptive phenotypic plasticity in salinity response could contribute to coping with a brackish environment. Transcriptome analysis also revealed a difference in biological processes responding to salinity between individuals in a freshwater and brackish water area. The activation of the chitin metabolic process, specifically found in a brackish water population, suggests that chitin plays some roles in salinity response, as reported in crustacea (Lv et al. 2013). Examining gene expression patterns in snails acclimated to

brackish water would reveal whether similar biological processes between the two populations would be activated in response to salinity.

Because the tidal cycle causes periodic environmental changes in both biotic and abiotic factors, it is advantageous for a given species to anticipate tidal changes (Tessmar-Raible et al. 2011). Circatidal clocks produce a tidally synchronized endogenous rhythm with a 12 h period. In Chapter 2, I revealed that individuals in a tidal area exhibited the circatidal rhythm in the activity and gene expression peaking at subjective high tide, suggesting that snails could anticipate tidal changes by circatidal clocks and be active at high water levels. Interestingly, several genes involved in salinity response showed circatidal rhythmicity in their expressions. Snails in a tidal area may anticipate periodic variations in salt concentration and regulate gene expression via circatidal clocks. My results could be evidence of the expression of circatidal rhythms in a population established in a tidal environment.

Several studies on marine species have revealed plasticity in rhythm expression within a single individual or population depending on environmental conditions (O'Neill et al. 2015, Mat et al. 2016, Schnytzer et al. 2018). In Chapter 3, I investigated the plasticity of the endogenous rhythm in *S. reiniana* by entraining to the simulated tidal cycle. My results suggest that even individuals in a non-tidal area plastically show the circatidal rhythm in the activity. Population genetic analyses in Chapters 1 and 2 revealed slight genetic differentiation between the tidal and non-tidal populations, supporting that the timekeeping systems of individuals in the two populations are not genetically differentiated. Taken together, at least in the studied populations of *S. reiniana*, the endogenous rhythm would be determined by the dominant environmental cycle in their habitat. However, analyses of gene expression rhythm suggest that genes

or biological pathways regulated by circatidal clocks increased in the tidal population. A few genes may be differentiated between populations and have different regulations by biological clocks.

Despite the ecological and evolutionary importance of circatidal clocks, little is known about their molecular basis (Zhang et al. 2013, Rock et al. 2022). In Chapter 4, I examined some circatidal oscillating genes detected in Chapter 2 to identify circatidal-related genes. Unfortunately, none of the genes studied in Chapter 4 showed circatidal rhythmic expression after the tidal simulation. On the other hand, marginal rhythmicity found in *wnt-5b* expression could leave a possibility of circatidal regulation. Comprehensive and functional analyses would be needed to detect oscillating genes that respond to the tidal cycle, leading to the identification of circatidal clock genes.

Although my results demonstrated that the circatidal rhythm is plastically expressed in response to the tidal cycle, whether the single biological clock switched circadian and circatidal rhythm remains unclear. The independence of circadian clocks and circatidal clocks is currently controversial (Takekata et al. 2012, 2014, Zhang et al. 2013, Tran et al. 2020). Moreover, several hypotheses have been proposed for the mechanism of oscillation of the circatidal rhythm (Enright 1976, Palmer 1995, 1997, Naylor 2010). Integrated studies of evolutionary ecology and chronobiology could reveal the genetic basis for the circatidal rhythm and shed light on the evolutionary mechanism of adaptation to tidal environments.

I here demonstrated that plasticity of the salinity response and endogenous rhythm play important roles in range expansion to tidal environments in a freshwater snail (Figure 3). Notably, my findings of changes in the endogenous rhythm depending on the environmental cycles suggest the importance of plasticity in heterogeneous

rhythmic environments. On the other hand, differences in circatidal regulation in transcriptome between the populations may be caused by genetic differentiation. If it is true, the present study shows establishment in tidal habitats driven by adaptive evolution and phenotypic plasticity. In the future, adaptive phenotypic plasticity could drive decreased environmental sensitivity and result in the constitutive expression of adaptive traits, facilitating adaptive evolution (Waddington 1953, 1961).

Evolutionary changes have been recognized as a significant process in range expansion to novel environments, and in addition, adaptive plasticity also plays important roles at range limits (Sexton et al. 2009). The results in this thesis revealed the expression of the adaptive phenotype and the phenotypic plasticity in traits associated with the tidal cycle in an edge population of *S. reiniana*. Assessing the role of adaptive evolution and plasticity in fostering the establishment of populations would provide insight into the evolutionary mechanisms of adaptation to spatially and temporally novel environments.

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Figure

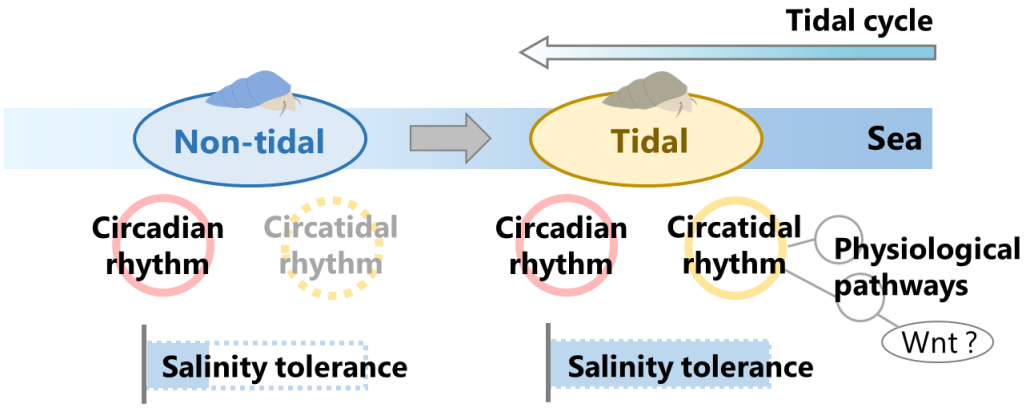

Figure 3. Graphical summary of the present study. Individuals in the non-tidal population have plasticity in the circatidal rhythm and salinity tolerance. Physiological pathways regulated by the circatidal rhythm were detected only in individuals in the tidal population. The pathways regulated by the circadian rhythm were not shown in both populations.

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