Circadian cycles are the dominant transcriptional rhythm in the intertidal mussel *Mytilus californianus*

Kwasi M. Connor and Andrew Y. Gracey

Department of Biology, University of Southern California, Los Angeles, CA 90089

Edited by George N. Somero, Stanford University, Pacific Grove, CA, and approved August 18, 2011 (received for review July 8, 2011)

Residents in the marine intertidal, the zone where terrestrial and marine habitats converge, inhabit an environment that is subject to both the 24-h day and night daily rhythm of the terrestrial earth and also the 12.4-h ebb and flow of the tidal cycle. Here, we investigate the relative contribution of the daily and tidal cycle on the physiology of intertidal mussels, *Mytilus californianus*, by monitoring rhythms of gene expression in both simulated and natural tidal environments. We report that >40% of the transcriptome exhibits rhythmic gene expression, and that depending on the specific tidal conditions, between 80% and 90% of the rhythmic transcripts follow a circadian expression pattern with a period of 24 to 26 h. Consistent with the dominant effect of the circadian cycle we show that the expression of clock genes oscillates with a 24-h period. Our data indicate that the circadian 24-h cycle is the dominant driver of rhythmic gene expression in this intertidal inhabitant despite the profound environmental and physiological changes associated with aerial exposure during tidal emergence.

The biology of organisms is closely linked to temporal changes in their environmental surroundings. The earth, revolving every 24 h, exposes animals to highly predictable daily patterns of light and temperature. To match these cycles, most living organisms have evolved biological rhythms that are manifest in daily patterns of behavior and physiology. These biological rhythms are accompanied by large-scale oscillations in gene expression that regulate the timing of many physiological processes (1). For inhabitants of the intertidal, superimposed on this daily cycle are environmental changes linked to the tidal cycle (2). In this paper we explore how environmental changes in this habitat are transduced into coordinated changes in physiological processes by examining gene expression cycles in a sessile intertidal inhabitant, the California ribbed mussel, *Mytilus californianus* Conrad.

**Results and Discussion**

To investigate biological rhythms in intertidal inhabitants, we created a simulated intertidal environment in which mussels were acclimated to alternating high and low tides of 6-h duration, with a 12-h cycle of light and dark. Gill samples from four mussels per time point were collected every 2 h for 96 h, covering eight high tides and eight low tides. The simulation allowed the effects of aerial warming, such as occurs during low tide on a sunny day, to be tested. On day 4 (at the 80-h time point), a warm low tide was simulated in which body temperature was allowed to increase by 7 °C compared with the normal low tide temperature (Fig. 1A).

Relative transcript abundance was measured by hybridization to microarrays, and in the first instance, we identified rhythmically expressed transcripts across the entire 96-h time series using the JTK_CYCLE algorithm (3). At a false discovery rate of <0.05, 2,756 transcripts were found to oscillate with a period of between 10 and 28 h. Two groups of rhythmic transcripts were identified: one group that oscillated with a period of 12 h, and a larger group that oscillated at longer periods with a broad peak centered at 26 h (Fig. 1B). Just 236 transcripts oscillated with what we defined as a tidal rhythm of 10–14 h, whereas 2,365 transcripts followed what we defined as a circadian rhythm of 22–28 h. Thus, there were ~10-fold more transcripts whose rhythm followed a circadian pattern rather than that of the simulated tidal cycle. A similar pattern was detected when the data were analyzed using a second algorithm, COSOPT (4) (Fig. S1). Analysis of the phase of the transcripts that comprised the 12- and 26-h peaks, that is to say the time of day at which expression is highest, reveal that the expression of tidal transcripts tends to peak at the middle of low tide (Fig. 1C), whereas the peak phase of circadian transcripts occurs at dusk and coincides with the first hour of darkness (Fig. 1D). Inspection of a heat map, with the rhythmic expression of each transcript sorted according to phase, shows that circadian transcripts fall into two groups that share similar phases, one expressed around dawn and the other at dusk (Fig. 2A). This pattern is similar to the circadian rhythm observed in plants (5) and contrasts that of mammals in which circadian transcripts show limited bias for phase and peak at all times of the day (6).

**Effect of Temperature on Circadian and Tidal Rhythms.** As an ectotherm, the body temperature of mussels fluctuates with that of the environment. The ebb and flow of the tide can create thermal cycles, with body temperature at low tide often increasing during the day due to solar heating (7, 8), and declining at night due to colder air temperatures. To investigate the role that a low tide heating episode would have on gene expression rhythms, we imposed a modest heating episode of +7 °C during a low tide on the fourth day of the simulation, and identified a subset of 636 transcripts that lost their periodicity in the time following the heating episode (Fig. 2B and C). In total, we identified 374 and 2,863 transcripts with a tidal or circadian period, respectively, after merging the lists of genes that were periodic over the entire time course, as well as those that lost their periodicity due to heating. Proportionally, tidal transcripts were more affected by heating than circadian transcripts, with 37% of tidal transcripts affected (138/374 transcripts) compared with just 17% of circadian transcripts (498/2,863 transcripts; Fig. 2B). This finding may indicate that the regulatory mechanisms underlying the circadian cycle of mussels is able to compensate better for temperature changes than the pathway that controls tidally regulated gene expression. The data also revealed that moderate heating has a profound effect on gene expression, and 24% of the transcriptome (2,484/10,410 transcripts) exhibited a significant change in expression in the 18 h that followed the onset of the low-tide warming episode (Fig. S2). Elevated environmental temperatures have been shown to have deleterious effects on intertidal mussels, particularly in the context of thermally in-
duced protein denaturation (9), and our data indicate that temperature perturbations have further consequences through their effect on the biological rhythms of intertidal inhabitants.

**Clock Genes Oscillate with a Circadian Rhythm in the Intertidal.**
Circadian oscillations in gene expression have been observed in a wide range of organisms, including mammals (10), plants (11), and flies (12), and are regulated by a set of so-called “clock” genes which comprise a set of core circadian oscillators that activate the transcription of a range of target genes (1), and are themselves often rhythmically expressed (6). To explore the expression patterns of clock genes in mussels, we identified mussel orthologs for the mammalian clock genes CLOCK, BMAL, CRY1, and RORB by sequencing the *M. californianus* transcriptome. We monitored their expression under our simulated tidal conditions using quantitative reverse-transcriptase PCR.

**Fig. 1.** Gene expression profiling reveals tidal and circadian rhythms in a simulated intertidal environment. (A) Representation of the environmental conditions used in the simulated tidal environment. Animals were sampled every 2 h starting at 7:00 AM. Animals were emerged during low tides, which occurred from 12:00 AM to 6:00 AM and from 12:00 PM to 6:00 PM, whereas sunrise and sunset occurred at 6:00 AM and 6:00 PM. At 80 h the mussels were warmed to 24 °C during low tide. (B) Histogram showing the period length of 2,756 statistically significant rhythmic transcripts. (C) Histogram showing the phase of 236 transcripts that had a period of 10–14 h. A phase of zero means that the peak expression of the transcript coincided with the 7:00 AM start of the time course. (D) Histogram showing the phase of 2,365 transcripts that had a period of 22–28 h.
(qRT-PCR) which revealed that the expression of CRY1 and RORB (NR1F2) was rhythmic ($P < 0.05$, JTK_CYCLE; Fig. 2D). Cryptochrome 1 is a key component of the circadian oscillator (13), whereas RAR-related orphan receptor B is a nuclear receptor that coordinates metabolism with the circadian clock (14). The detection of rhythmic clock gene expression is strong evidence that the core circadian oscillator is active in mussels and thus may play a role in orchestrating the circadian transcriptional cycles that are manifest in mussel gill tissue. Further evidence that the circadian rhythm detected in mussels shares characteristics with that of mammals comes from the discovery that histone deacetylase 1 (HDAC1) and nicotinamide phosphoribosyltransferase (NAMPT) display a circadian profile in mussels (Fig. 2D). Circadian control of transcription is believed to be mediated by histone acetylation, regulated in part by HDACs, which by altering the structure of the epigenome can alter the expression of large numbers of genes (15, 16). Circadian regulation of cellular metabolism is mediated through a feedback loop involving NAMPT, which catalyzes the rate-limiting step in NAD$^+$ production (17).

Fig. 2. Gene expression patterns correspond to tidal height and dusk and dawn. (A) Heat map showing the rhythmic expression of tidal and circadian transcripts that were identified as rhythmic across the entire 96-h time-course or (B) across the first 78 h only. Yellow or blue color indicates that the expression of a transcript was greater or less than the median expression of the transcript, respectively. Transcripts were grouped according to period and then ordered according to their phase. (C) Venn diagram shows the overlap between the lists of transcripts that were rhythmic across the 96-h time course vs. transcripts, were rhythmic only before the heating episode at 80 h. (D) Gene expression profiles of RORB and CRY1 determined by qRT-PCR, and NAMPT vs. HDAC1 quantified by microarray.
During low tide, *M. californianus*, like most bivalves, close their shells, enter hypoxia, and switch to anaerobic metabolism (22), and our data revealed a number of transcripts whose expression could be linked to hypoxia. For example, we observed that transcript levels of two isoforms of carbonic anhydrase (CA2, CA13) were elevated during low tide (Fig. 3E). Members of this gene family are hypoxia inducible in mammals (23), and their induction at low tide suggests that they may play a functional role in counteracting tissue acidosis that has been reported during emergence (24) by catalyzing the conversion of CO2 and protons to bicarbonate. Transcripts for cAMP-responsive element binding protein-like 2 (CRBL2) exhibited particularly robust tidal oscillations (Fig. 3F), suggesting that the cAMP-dependent pathway is activated during aerial emergence, consistent with the role of cAMP as a signal of redox state (25) and as a key signaling molecule in bivalves (26).

**Concordance Between Simulated and Field Intertidal Environments.** Next, we sought to verify that the rhythms of gene expression observed under simulated tidal conditions are evident under natural tidal regimes in the marine environment. To accomplish this, mussels were acclimatized in the field to midintertidal conditions, which ensured that mussels spent approximately half the duration of each tidal cycle submerged and half emerged (Fig. 4A and Fig. S3). Gill samples from four mussels per time point were collected every 2 h for 50 h, and a search for periodic genes identified 697 rhythmic transcripts, of which 72 exhibited a tidal periodicity of 10–14 h, and 501 exhibited a circadian rhythm of 22–28 h. A priori, we predicted that transcripts in the field would not exhibit a constant tidal period because the time and height of each high and low tide shifts with each tidal cycle (Fig. 4A). Despite this, 28 of the 72 transcripts that exhibited a statistically significant 10- to 14-h period in the field also exhibited
Cycles of gene expression show a strong circadian rhythm in Fig. 4. By the red line, and the predicted tidal cycle by the blue line. Dashed lines which the animals were sampled. Estimated body temperature is illustrated to the sampling period. (Fig. 4B and Table S3). Closer inspection of the field data revealed that a number of the transcripts that were identified as tidal in the simulated experiment displayed rhythmic transcriptional oscillation in the field, and that their profile matched the tidal cycle observed in the field. Using the profile of these transcripts as a guide, we identified an additional 37 transcripts whose expression follows a tidal rhythm in the field, yielding a total of 109 tidal genes. Thus, there are >4× as many genes that follow a circadian vs. a tidal rhythm at this particular intertidal location. Overall, we identified fewer rhythmic transcripts in the field time-course compared with the laboratory simulation; we speculate that this is attributable in part to the higher temperature variation, which can affect the periodicity of some transcripts, as evident in our simulated dataset.

The alternating bouts of submergence and aerial emergence represent candidate cues responsible for the tidal pattern of rhythmic gene expression. To investigate this we collected a corresponding set of gene expression data from field animals that were held under subtidal conditions of almost constant temperature (16–17 °C) and that never experienced aerial emergence (Fig. 4C). These data revealed that just 41 transcripts exhibited an expression cycle with a tidal period, of which just one transcript overlapped with those identified as tidal in both the simulated or field intertidal datasets (Fig. 4D and Table S4). These data support the hypothesis that the dominant driver of tidal gene expression rhythms is the episodes that intertidal inhabitants spend in and out of the water. In contrast, the molecular signature of subtidal animals was dominated by a pattern of circadian gene expression with over twice as many genes exhibiting a circadian rhythm in subtidal vs. intertidal field animals (1,224 vs. 501 transcripts respectively). Interestingly, the heat maps reveal that the phase of circadian gene expression was similar in both subtidal and intertidal field mussels, suggesting that the same cues may be driving circadian expression cycles in both habitats.

Combining the lists of rhythmic transcripts identified under both simulated and natural field conditions revealed that >40% of gill transcripts exhibited rhythmic gene expression in at least one of the environmental conditions studied (3,934 and 476 transcripts with circadian or tidal rhythms, respectively, of 10,410 total transcripts; Fig. 4D). These data indicate that though some transcripts show rhythmic expression in all of the datasets, others are only rhythmic under specific conditions. The source of this variability is unclear, but results obtained in other organisms have reported similar findings of interlaboratory and interexperimental variation in circadian gene expression (27). For example, studies on Arabidopsis have revealed that 89% of the transcriptome cycles under at least one set of environmental conditions, but that <51% of the transcriptome cycles in any single experiment, and that <1% of transcripts cycle in every experiment (5). Together with similar findings in mammals (6, 20), it has become clear that circadian cycles of gene expression are driven by endogenous internal clocks, local or systemic cues, and external environmental factors (1). In the case of the current study, differences in the lists of cycling transcripts are most likely indicate the tidal height inhabited by each set of mussels, and circles the sample time points. (B) Heat map showing the rhythmic expression of tidal and circadian transcripts that were identified as rhythmic in the field intertidal location and (C) subtidal location. Bars above heat map indicate the predicted periods of emergence based on the tidal cycle and the times of dusk and dawn. (D) Venn diagram showing the relative number of tidal and circadian transcripts identified in the simulated intertidal and field intertidal and subtidal datasets.

**Fig. 4.** Cycles of gene expression show a strong circadian rhythm in field intertidal and subtidal environments. (A) Environmental conditions experienced by mussels located in intertidal and subtidal locations in the field. (Upper) Conditions experienced by the mussels during the 3 wk leading up to the sampling period. (Lower) Detailed conditions during the 50 h over which the animals were sampled. Estimated body temperature is illustrated by the red line, and the predicted tidal cycle by the blue line. Dashed lines
Conclusions

Previously we have reported that high-intertidal mussels that experience a single brief episode of submergence per day exhibit temporal compartmentalization of their physiological processes, alternating between states of metabolism, cell division, or thermal stress (19). In the current study we examined mussels living under relatively benign tidal conditions to reveal that most rhythmic gene expression adheres to a 24-h cycle. This finding is surprising given that each episode of aerial emergence is associated with profound physiological changes, including physiological hypoxia and a cessation of cardiac activity (22).

We do not know the extent to which either the circadian or tidal transcriptional rhythms observed in our data are entrained and will persist in the absence of external cues. A number of intertidal organisms, particularly crustaceans, display entrained behavioral rhythms that follow persistent circadian (28) and tidal patterns (29, 30) even when held under constant conditions. Similarly, mussels are reported to show entrained tidal patterns of cell division (31). These findings have led to questions regarding the existence of a tidal clock and the nature of its regulation, with some data suggesting the presence of a circatidal clock with a period of 12.4 h (28), and other data supporting an alternative hypothesis that postulates that two circadian clocks with 24.8-h periods running in antiphase to one another could generate peaks in activity every 12.4 h (32). Though the molecular basis of the tidal clock remains to be elucidated (22), our observation that two clock genes, CRY1 and RORB, exhibit a circadian cycle suggests that the circadian clock apparatus is not co-opted to oscillate with a tidal rhythm in mussels.

The abundance of circadian transcripts raises questions regarding the nature of the zeitgeber or exogenous cue that is responsible for synchronizing the mussel’s transcriptional rhythm to the 24-h rotation of the earth. The general consensus is that bi-valves possess photoreceptor cells (33) and will respond to light (34), and so we speculate that light is the most plausible exogenous cue for synchronizing the mussel’s transcriptional rhythm to the 24-h rotation of the earth. Further experiments that include light/dark and tidal manipulations will be required to elucidate further the nature of endogenous rhythms in intertidal organisms and their relationship to environmental cues. Overall, our results emphasize the central role that the circadian rhythm plays in physiology, even in organisms that experience strong subdaily external cues arising from the tidal cycle.

Materials and Methods

*M. californianus* were acclimated to laboratory-simulated or natural tidal cycles for >4 wk before sampling. Pooled total RNA was prepared from four mussels sampled at 2-h intervals, amplified, and hybridized to cDNA microarrays. Periodic transcripts were detected using JTK_CYCLE (3). Over-represented classes of genes were identified using DAVID (35) by mapping the mussel sequences to their putative human orthologs. Full details of the methods used can be found in SI Materials and Methods.

ACKNOWLEDGMENTS. This research was supported by National Science Foundation Award IOS 0745451 (to A.Y.G.).
Supporting Information

Connor and Gracey 10.1073/pnas.1111076108

SI Materials and Methods

Tidal Simulation and Sampling. Mytilus californianus of 4–5 cm length were collected at Zuma Beach, north of Los Angeles, CA. To simulate intertidal conditions in the laboratory, the mussels were maintained in aquaria and grown as a single layer of animals on shelves at the midlevel height of the aquarium. The ebb and flow of the tide was simulated using computer-controlled water pumps that regulated the depth of water in the aquaria by pumping seawater in and out of the aquarium. The room was maintained at a constant temperature of 17 °C to ensure that the body temperature of the mussels remained constant whether submerged or emerged in air. Ceramic heat lamps suspended above the shelves were used to simulate the solar radiation and warming that the mussels would experience during a midday summer low tide. The output from the lamps was controlled by a computer program that defined the rate of warming and upper limit and duration of heating episodes, and this was fine-controlled using a feedback sensor comprised of a thermocouple surgically inserted into the body cavity of one of the mussels. Food as liquid algal cultures was continuously added to the water during each episode of high-tide submergence. The seawater in the aquaria was constantly replaced such that 10% of the volume of the system was changed every day.

A tidal regime of alternating periods of 6 h in and 6 h out of the water was established, and mussels were acclimated to this regime for 4 wk before the commencement of sampling. Low tides occurred from 12:00 AM to 6:00 AM and 12:00 PM to 6:00 PM. A light/dark cycle was imposed with period of darkness occurring from 6:00 PM to 6:00 AM. Animals were sampled every 2 h over a period of 96 h with the initial sample collected at 7:00 AM on day 1 of the experiment. This sampling regime ensured that three samples were collected per episode of low or high tide, with the first sample collected 1 h into the episode, the second sample taken at the middle 3-h time point, and the third sample taken 1 h before the change in tidal episode. Four individual mussels were collected at each time point. A warming event was simulated on day 4 of the experiment by warming the mussels with the ceramic lamps during a low-tide episode that started at 12:00 PM. The body temperature of the animals increased from 17 °C to 24 °C during this low-tide episode.

Field Sampling. Mussels collected from Zuma Beach were acclimatized to intertidal and subtidal conditions in cages suspended from the dock at Wrigley Marine Science Center, Catalina Island, CA (Fig. S3). The tidal height of the intertidal cage was 0.92 m and the subtidal cage was ~0.85 m. Mussel body temperatures at each site were approximated by the deployment of “robo-mussels” that comprised a temperature data logger embedded in silicon sealant inside a mussel shell (1). Mussels were simultaneously sampled from both cages every 2 h over a period of 50 h. Four individual mussels were collected at each time point.

M. californianus Microarray Construction. The M. californianus cDNA microarray was constructed from PCR amplicons derived from cDNAs picked from seven high-quality cDNA libraries prepared from adult gill, adductor muscle, mantle tissues, and early larval RNA samples. To ensure a good representation of environmentally regulated genes, the adult tissue mRNA was isolated at two time points following exposures on the animals to either heat, cold, hypoxia, hypo-osmotic stress, aerial emergence, or oxidative stress. The libraries were normalized and serially subtracted so that cDNAs that had been isolated from previous libraries were physically subtracted during the construction of each new library (2). The 5’ and 3’ EST sequences were generated from the clones and the sequences annotated using EST-Ferret, a custom annotation pipeline (3). The EST sequences were clustered with CAP3, and the resulting nonredundant contigs were queried against the public protein databases using BLASTX. Each cDNA was assigned a putative identity based on search results against the SwissProt and RefSeq protein databases, and sequences that yielded a hit with an E-value ≤1e−5 were annotated using the name of the gene for which it had the greatest identity. Gene ontology terms were assigned to cDNAs with hits in SwissProt by parsing the SwissProt GOA association file with the SwissProt IDs obtained by the BLASTX search. A putative nonredundant set of 10,410 cDNAs was identified and printed on in-house prepared poly-l-lysine-coated microarray slides.

RNA Isolation and Microarray Hybridization. Total RNA was isolated from gill tissue using TRIZol (Invitrogen) according to the manufacturer’s instructions. The total RNA was purified further across glass-fiber filter columns (Qiagen) according to the manufacturer’s instructions. An equal amount of total RNA from four individual animals sampled at each time point was pooled, and amplified RNA was prepared as previously described (4). Briefly, double-stranded cDNA was prepared by reverse transcribing 2 μg pooled total RNA in a 20-μL reaction containing 20 pmols of T7-dT15 VN primer, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 50 μMs DNTP, 20 units of RNaseOut (Invitrogen) and 100 units of M-MLV reverse transcriptase (Epigenzyme). The RNA and primer mixture was heated denatured at 65 °C for 10 min, the remaining components added and the reaction incubated at 40 °C for 2 h, and then stopped by heating to 65 °C for 15 min. Next, 60 μL of in vitro transcription reaction mixture, containing 53 mM Tris-HCl (pH 7.5), 13 mM NaCl, 8 mM MgCl₂, 5.5% PEG 8000, 2.6 mM spermidine, 3.33 mM each (ATP, GTP, CTP), 2.5 mM UTP, 0.83 mM amino-allyl UTP (Epigenzyme), 0.12 units organic pyrophosphatase, 20 units RNaseOut, 500 units T7 RNA polymerase (Epigenzyme), was added and the reaction incubated at 40 °C for 18 h. The resulting aRNA was purified using a Qiagen RNeasy kit, and half the aRNA was labeled with Cy5 and the other half with Cy3. Fluorescently labeled antisense RNA samples were hybridized to the M. californianus array using an interwoven loop design that yielded a balanced design in which each RNA sample was hybridized to either two or four arrays with fluor reversal. This loop hybridization design yields improved statistical inference of microarray data (5).

Gene Expression Analysis. Tagged image file format images of hybridized arrays were captured with an Agilent scanner (Agilent Technologies) and spot intensities quantified with Agilent Feature Extraction software (version 9.5.1). Spot median pixel intensities without background correction were collected, spatial intensity trends removed, and individual channels normalized using joint lowess transformation. Relative expression of each gene in each hybridization loop was estimated using MAANOVA version 0.98-7 for R using an ANOVA model in which dye and sample were treated as fixed effects, and array was treated as random effects (6). Gene expression data were centered by dividing the relative expression of each gene by the median expression of that gene across the samples in the dataset.

Connor and Gracey www.pnas.org/cgi/content/short/1111076108
Genes that exhibited rhythmic expression were identified using JTK_CYCLE (7) or COSOPT (8), and P values were corrected for a false discovery rate of <0.05 using a standard method (9). Genes whose expression was altered as a result of the heating event were identified using MAANOVA (version 0.98-7) in a model in which dye was treated as a fixed effect, array was treated as a random effect, and testing for differences in expression between samples collected during the first 78 h vs. samples collected postheating at time points 80–96 h. The P values were adjusted for false discovery rate (10) and a statistical threshold of P < 0.01 defined a list of genes that were statistically significantly differentially expressed between the samples collected before and after the heating event. To identify additional rhythmic transcripts, we selected five genes that exhibited highly significant periodic expression in the simulated tidal dataset and used these as guide genes to identify a set of transcripts that shared a Pearson correlation coefficient >0.7 in the field intertidal dataset.

We used DAVID (11) to investigate whether particular GO biological processes categories were enriched in the lists of transcripts with a tidal rhythm. Putative human orthologs of the *Mytilus* genes were identified using BLASTX homology searches between the arrayed mussel 5′ EST sequences and the human protein collection in the SwissProt database. We assigned the mussel genes with a putative human gene identifier and used the lists of arrayed genes and mortality signature genes as the reference and query gene lists in the analysis. The lists were first dereplicated so that only one instance of a gene identifier was input into the analysis. A right-tailed Fisher’s exact test was used to calculate a P value that was adjusted using the Benjamini–Hochberg false discovery rate procedure to yield a corrected P value that determined the probability that an enriched GO was due to chance alone.

**qRT-PCR Analysis.** For qRT-PCR, 2 μg of each experimental RNA sample was reverse transcribed with T<sub>P</sub>VN (1 μg each per reaction) in a 20-μL reaction. The product was diluted to 100 μL with water, and aliquots of 2 μL of the resulting diluted cDNA were amplified in the presence of SYBR Green (Takara PreMix ExTaq II); intercalation of the dye was monitored on a Stratagene MX4000 thermal cycler. Sequences of the mussel CLOCK genes were identified from 60 million single-ended 100-bp RNA-seq reads generated from *M. californianus* gill tissue on the Illumina platform. Reads were assembled with Velvet using multiple k-mer lengths of 31–81. Clock orthologs were identified by BLASTX analysis between the resulting contigs and the human clock gene sequences in the SwissProt database. Forward and reverse primers for *RORB* were 5′-3′ AAACCGTGCACCCGTTCACA and CCCACGTTAACCCCATGAAA; for CRY1 were 5′-3′ GTGTTCGTGCCAGTTGGAT and CCTTCAACACGGGCAAGTAT; and for α-tubulin were 5′-3′ TCCAAGATCCGGGCAATACCA and TGGAAACCAGTTGGACACCA. All primers pairs exhibited an amplification efficiency between 95% and 105%, and relative expression was measured using the ΔΔC<sub>T</sub> method, using α-tubulin as the reference transcript.

**Accession Numbers.** Accession numbers associated with the tidally regulated genes are provided in Tables S1–S4. The ArrayExpress accession number for the microarray data is E-MTAB-777.

Fig. S1. Periodicity analysis using the COSOPT algorithm. (A) Heat map showing the rhythmic expression of tidal and daily transcripts that were identified as rhythmic across the entire 96-h time course using COSOPT. Yellow or blue indicates that the expression of a transcript was greater or less than the median expression of the transcript, respectively. Transcripts were grouped according to period and then ordered according to their phase. (B) Heat map showing the rhythmic expression of tidal and daily transcripts that were only identified as rhythmic across the first 78 h of the time course. (C) Venn diagram shows the overlap between the lists of transcripts that were identified as having a tidal rhythm by JTK_CYCLE vs. COSOPT. (D) Venn diagram shows the overlap between the lists of transcripts that were identified as having a daily rhythm by JTK_CYCLE vs. COSOPT.
Fig. S2. Modest heating results in the differential expression of 24% of the transcriptome. Heat map showing the 2,484 transcripts whose expression is significantly elevated following a 7 °C warming event at the 80-h time point (FDR corrected, P < 0.01).

Field Location:
Wrigley Marine Science Center,
Catalina Island, CA

Cages used as surrogate tidal habitats. Cage in upper part of picture served as intertidal habitat and lower cage served as subtidal habitat

Cages were suspended by rope at appropriate height from dock. Rope allowed cages to be quickly hauled up allowing easy fast access to samples.

Fig. S3. Implementation of high-resolution sampling in the field. Photographs show the location and design of the field-sampling experiment.
Other Supporting Information Files

Table S1 (DOCX)
Table S2 (DOCX)
Table S3 (DOCX)
Table S4 (DOCX)