Crosstalk between the circatidal and circadian clocks mediates behavioral adaptation to tidal patterns

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Summary

The ability to anticipate tides is critical for a wide range of marine organisms, but this task is complicated by the diversity of tidal patterns on Earth. Previous findings suggest that organisms whose geographic range spans multiple types of tidal cycles can produce distinct patterns of rhythmic behavior that correspond to the tidal cycles they experience. How this behavioral plasticity is achieved, however, is unclear. Here, we show that *Parhyale hawaiensis* adapts its rhythmic behavior to various naturally occurring tidal regimens through the plastic contribution of its circatidal and circadian clocks. After entrainment to a tidal cycle that deviated only mildly from a regular 12.4h tidal cycle, animals exhibited strong circatidal rhythms. In contrast, following entrainment to more irregularly spaced tides, or to tides that occurred every 24.8h a significant fraction of animals instead synchronized to the light/dark cycle and exhibited circadian behavior, while others showed rhythmic behavior with both circatidal and circadian traits. We also show that the circatidal clock, while able to entrain to various naturally occurring tidal patterns, does not entrain to an unnatural one. We propose that *Parhyale hawaiensis*'s ecological success around the world relies in part on the plastic interactions between the circatidal and circadian clocks, which shape its rhythmic behavior appropriately according to tidal patterns.

Keywords: circatidal rhythms, circadian rhythms, mixed tides, diurnal tides, *Parhyale hawaiensis*, chronobiology, marine biology

Introduction

Biological rhythms allow organisms to follow a schedule optimized for cyclic environmental conditions. Of these rhythms, the best understood are circadian oscillations, which have a 24-hour (h) period and are synchronized by the day/night cycle, primarily through light and temperature perception. Organisms living in the intertidal zone have a rather complex task: in addition to coping with the 24h day/night cycle, they must also anticipate the rising and falling of the tides, usually with a period of ~12.4h. Many marine species exhibit circatidal rhythms, ranging from shallow-water species like the isopod *Eurydice pulchra* to those living in deep-sea vents, such as the mussel *Bathymodius azoricus*[1,2]. In contrast to circadian clocks, circatidal clocks are poorly understood.

A particularly important distinction between circatidal and circadian clocks is the range of entrainment patterns that they must accommodate. A full day/night cycle is always 24h. This is not so for tidal cycles. Depending on the location, and in some cases the time of year or the lunar month, tidal cycles may operate with a period of 12.4h or 24.8h. Variation in tidal cycle by location is due primarily to local differences in the shape of the ocean floor and the way water moves around land masses [3-5]. Variation in the type of tidal cycle over time is more complex. Although the primary force driving tides is the Earth-Moon orbital system, which produces lunar gravitation and centrifugal force on Earth, the magnitude of these forces change over time due to the elliptic and slightly eccentric orbit of the Moon[5]. In addition, Earth also experiences solar gravitational forces, which also change over time due to Earth's elliptical orbit around the sun [5]. There are three general tidal patterns[3,5,6] (Figure 1A,B). Semidiurnal tides are characterized by one high and low tide every 12.4h, with consecutive high or low tides of similar amplitudes. Mixed semidiurnal tides have two high and low tides every 24.8h, however consecutive high or low tides have different heights and/or durations. By contrast, diurnal tides have only one high and one low tide every 24.8h.

A sparse but strong body of evidence shows that wild-caught organisms exposed to all three tidal environments show rhythmic behavior that matches the period and/or shape of the tidal cycle from where they were captured[7-11]. Many species have geographic distributions that cover multiple types of tidal cycles, and as mentioned above, some experience different tidal cycles over the course of a lunar month or a year[5,8,9,12]. Thus, the ability to entrain to tidal cycles should not be genetically hardwired, but also include plasticity of the neural and molecular mechanisms controlling rhythmic behavior. This is supported by studies showing that animals moved between locations with different tidal cycles match their behavior to the current tidal environment, regardless of the cycle at their location-of-origin[13,14]. Klapow evaluated the behavior of populations of *Excirolana chiltoni,* the sand-beach isopod, collected from a California beach where mixed semidiurnal and diurnal tides occurred[15]. He found that freshly caught populations exhibited behavioral rhythms matching the tidal pattern from where they were captured. Furthermore, Klapow was able to induce transient rhythms of activity with a 24.8h period by exposing animals to vibratory stimuli every 24.8h after prolonged isolation in the lab, suggesting that adaptation to different tidal regimens can be studied in a laboratory setting.

In summary, these pioneering studies indicate that tidal species whose native range spans multiple types of tidal cycles can generate different patterns of behavior to match different tidal cycles. However, many mechanistic questions are left unanswered. For example, we do not know how animals adjust the shape and period of their behavioral activity to match the tidal cycle they experience. Is this achieved by a general population-level change in behavior, or is there a spectrum of behavioral phenotypes produced by individual animals in response to different tidal patterns? Many of the studies listed above utilized populations of animals to record behavior and thus do not parse out individual behaviors. Also, it is not clear whether behavioral adaptation to different tidal cycles is achieved by modulating the contributions of the circadian and circatidal clocks. In addition, it is not known whether, for a given species, there is an optimal tidal cycle, one which produces the most precise cycle rhythms and greater amplitudes. Furthermore, does the "optimal" tidal cycle vary between different sub-populations of a given species? Finally, it would also be interesting to determine whether there are limits to the range of tidal entrainment. Answering these questions should help our understanding of how marine organisms shape rhythmic behaviors and their entrainment, and whether it is better for them to maintain flexibility in entrainment to increase the range of conditions to which they can adapt or to optimize entrainment to the conditions they most commonly experience?

Parhyale hawaiensis is an amphipod crustacean that exhibits robust circatidal rhythms of swimming behavior that can be entrained to an artificial semidiurnal tidal regimen in the laboratory[16]. It can also express circadian behavioral rhythms when only entrained to a light/dark (LD) cycle. *P. hawaiensis'* native range is quite broad and includes coastlines that experience all three types of tidal cycle[17,18] (Figure 1A). Therefore, we wondered if we could successfully entrain *P. hawaiensis* to artificial tidal regimens designed to mimic mixed semidiurnal and diurnal tidal cycles as well. Given that *P. hawaiensis'* circatidal behavior does not exhibit circadian modulation after entrainment to semidiurnal tides[16], we were interested in whether circatidal rhythms under non-semidiurnal entrainment might be influenced by the circadian clock.

Here, we report that *P. hawaiensis* exhibits remarkable behavioral plasticity when challenged by different tidal regimens. It can synchronize to all naturally occurring tidal patterns tested, but with waning efficiency as the pattern diverges from being semidiurnal. Interestingly, under diurnal tidal entrainment, or a mixed semidiurnal pattern with severely irregular tides, different behavioral patterns emerge; some individuals rely more heavily on entrainment by the LD cycle suggesting that the circadian clock becomes more influential. This behavioral plasticity is probably crucial to adjust to an ever-changing and remarkably complex environment, and might help explaining the broad distribution of *P. hawaiensis* around the world.

Results

Designing artificial tidal regimens

Parhyale hawaiensis lives in tropical waters around the globe, and can be exposed to any of the three types of tidal patterns: semidiurnal, mixed semidiurnal (referred to as mixed throughout this paper), and diurnal (Figure 1A,B). To determine whether *P. hawaiensis* can entrain to all three types of tidal cycle, we exposed animals to various artificial tidal regimens. We chose to vary the length of the high tide only (Figure 1C), and to keep the short low tides at an equal duration, as used previously in our standard semidiurnal protocol[16]. In addition, we only varied tidal durations and not amplitudes. This allowed us to isolate a single variable associated with tidal cycles, and to prevent animals from experiencing stress due to long dry periods. This approach also reasonably mimicked natural tidal cycles (Figure 1B).

To simulate a mixed tidal pattern that was almost semidiurnal (mild mixed tidal regimen), we exposed the animals to two cycles of high tide/low tide every 24.8 hours (h), with one longer high tide of 12h 15min and one shorter high tide of 8h 15min (Figure 1B,C). To simulate a more irregular mixed pattern, we further lengthened the long high tide to 14h 15min, and decreased the shorter tide to 6h 15min to create a severe mixed tidal regimen. Finally, to simulate a diurnal tidal cycle, we exposed animals to a single high tide every 24.8h, lasting 22h and 35min. Concurrent with the artificial tidal regimen, animals were also exposed to a 12h light/12h dark (LD) cycle, with lights turned on at 08:00, and off at 20:00. Entrainment lasted 10 tidal cycles (5 diurnal tidal cycles), or a little over 5 days. Male animals were then transferred to plastic tubes filled with artificial sea water (ASW), and loaded into

Drosophila population monitors (DPM) to record vertical swimming activity under constant conditions (constant high tide and darkness; vertical swimming was monitored because it is more robustly rhythmic than "roaming" behavior[16]).

To determine whether animals entrain to the LD or the tidal cycle, we varied the phase of the tidal cycle with respect to the LD cycle by 6 hours for each type of tidal regimen (Figure 1D). The last high tide (LHT) on the last day of entrainment occurred either in the early afternoon at 13:20 (this arrangement was termed 13:20LHT) or in the late evening at 19:20 (termed 19:20LHT). If rhythmic behavior following 13:20LHT and 19:20LHT were in phase, then animals would have been entrained by the light-dark cycle. However, if rhythmic behavior following 13:20LHT and 19:20LHT showed a ~6h difference in phase, it would demonstrate tidal entrainment.

Parhyale hawaiensis **entrain efficiently to a mild mixed tidal cycle**

We first entrained *P. hawaiensis* to mild mixed tidal regimens, with last high tides (LHT) at 13:20 and 19:20 (13:20LHT and 19:20LHT, respectively). In both cases, robust behavioral rhythms were observed under constant conditions at the population level, with two peaks every subjective day corresponding to the two expected daily high tides (Figure 2A, B). When the 13:20LHT and 19:20LHT population traces were overlaid, we observed an ~6hr difference in phase, demonstrating tidal entrainment (Figure 2C). This was confirmed when the phase of both daily peaks was quantified in individuals (Figure 2D). We further analyzed the properties of the free-running swimming rhythms of individual animals entrained to both mild mixed tidal regimens. Most animals were rhythmic (Figure 2E) and exhibited both singlet (first harmonic, ca. 12.4h) and doublet (second harmonic, ca. 24.8h) values in their periodogram (Figure 2F), as expected for circatidal rhythms[16,19]. We also measured the power of the individual rhythms (Figure 2G). Overall, rhythms were very similar between the two tidal phase conditions (Figure 2E-G). The only noticeable difference was a slightly longer period for the doublet when the last high tide of the mild mixed tidal regimen occurred at 19:20 (Figure 2F). We conclude that *P. hawaiensis* can efficiently entrain its circatidal behavior to a mild mixed tidal pattern.

P. hawaiensis **shows a spectrum of rhythmic behaviors after entrainment to severe mixed tidal cycles**

Next, we chose to challenge animals with a more severely mixed tidal pattern, in which the two high tides lasted 14h 15min and 6h 15min (Figure 1C). When the last high tide occurred at 13:20, the average activity trace of rhythmic animals appeared to display activity peaks aligned with the expected high tides and troughs aligned with the low tides, but the average trace from animals entrained to the 19:20LHT appeared noisier, with a lower amplitude (Figure 3A, F). Interestingly, there was a notable reduction in individual rhythmicity after entrainment to severe mixed tides – only ~ 50% of animals were rhythmic, significantly less than what we observed after mild mixed tides (Figure 3B). Moreover, a significant fraction of rhythmic animals lacked a singlet period in their periodogram and showed only a doublet period, much more frequently than after mild mixed tides (Figure 3C, S1). We therefore separately plotted the average behavior of animals that had a singlet component detected ("singlet-rhythmic"), and those that only had a doublet component detected ("doublet-only", Figure 3D, E).

When the last high tide occurred at 13:20(13:20LHT), the activity trace of singlet-rhythmic animals appeared quite similar to those observed after mild mixed tidal pattern (Figure 2A, B) or semidiurnal[16] entrainment, with peaks of activity occurring during each expected high tide (Figure 3D). In contrast, the average trace for doublet-only individuals displayed activity rhythms that appeared non-tidal in shape and in phase (Figure 3E. This pattern was similar to the circadian behavior we had previously observed after entrainment to only an LD cycle without tides[16], with a relatively low amplitude average trace and considerable noise. We observed that the timing of the sole activity peak in doublet-only individuals was similar to the timing of activity peaks following a LD cycle[16] (Figure 3E).

After 19:20LHT, the activity of doublet-only animals was similar to that observed after 13:20LHT (Figure 3E, G). However, the average trace of singlet-rhythmic individuals remained noisy and of remarkably low amplitude (Figure 3H). When we looked more closely at the phase of behavior for these singlet-rhythmic individuals, we observed a broad distribution of phases (Figure 3I). This could indicate imprecise entrainment, or the presence of distinct populations with various behavioral phases. To differentiate between these two possibilities, we plotted Peak1 and Peak2 phases for each day (Figure 3J). Interestingly, we observed that phase distribution became progressively more bimodal from day 1 to day 3, with a smaller cohort of animals consistently showing advanced Peak1 and Peak 2 phases (Figure 3J).

Since all individuals that had an average Peak 1 relative phase of 1.33h or earlier also had an average Peak 2 relative phase that was 13.5 or earlier, we used this cutoff to define two subgroups of singlet-rhythmic individuals; the early and late singlets. This grouping resulted in average traces of greater amplitude and coherence, compared to the average trace for all 19:20LHT singlet animals (compare figure 3 L and M with H). Interestingly, animals in the early-phase cohort did not delay the phase of their activity peaks from day to day, while the late-phase cohort animals did (figure 3J). Finally, the interval between activity Peak1 and 2 (Inter-Peak Interval [IPI]) was significantly shorter in the early cohort compared to the late cohort. We therefore conclude that these cohorts represent two distinct behavioral phenotypes (Figure 3K). As a result of their late phase and longer IPI, the more common (~2/3) singlets showed robust activity peaks for each expected high tide, evenly separated by approximately half of the 24.8h cycle (~12.5h). They therefore expressed a similar behavioral pattern as that observed in the singlet-rhythmic cohort seen after severe mixed regimen with 13:20LHT (Figure 3L). In contrast, the early singlets showed activity peaks poorly aligned with the tides, with peaks close to or even within the time at which low tides would be expected (Figure 3M).

In summary, after exposure to severe mixed tidal cycles, three stable behavioral phenotypes emerge: circatidal rhythms aligned with tides, circatidal rhythms misaligned with tides, and a circadian-like behavior with a single activity peak per day.

Behavior after severe mixed tidal cycles reveals distinct entrainment by tidal and LD cycles

We then studied in detail the entrainment of the three different types of behaviors observed after exposure to severe mixed tides. As expected, the animals showing rhythms well-aligned with tides had ~6 hr shifted behavioral phases with the two different tidal schedules, as shown with the overlaid average traces and the quantification of peak phases in individuals (Figure 4A, B). These animals were thus entrained by the tides. In sharp contrast, there was no phase difference between the doublet-only (circadian-like) animals (Figure 4C, D). These animals were thus entrained by the LD cycle, and not the tides. Remarkably, the early singlets animals, misaligned with the tides, had one of their two daily peaks roughly aligned with the doublet-only animals observed after both 13:20 and 19:20LHT (Figure 4D, E). The early singlet cohort thus probably followed the LD cycle as well, supported by the shorter IPI (~11.5h) that might facilitate early singlets' timing of activity peaks around the expected dark phase (Figure 3K). Note that the phase of singlet-rhythmic and doublet-only animals after 13:20LHT is similar, which means that the third behavioral phenotype (singlet-rhythmic that likely follows the LD cycle) would not be distinguishable from properly tidally-entrained singlets-rhythmic animals (Figure S2).

In summary, the severe mixed tidal pattern is a relatively weak Zeitgeber. Almost half of animals are arrhythmic, and a significant fraction of rhythmic animals entrain to the LD cycle. Since the circatidal clock is insensitive to light[16], LD entrainment must occur through the circadian clock, which plays a predominant role in shaping rhythmic behavior under these conditions. This results in most LD-entrained animals showing a single peak of activity per day, and having shorter periodicities (Figure 4G, see also discussion).

Behavioral flexibility also occurs in response to diurnal tidal cycles

We finished our examination of different natural tidal cycles by subjecting animals to a diurnal tidal cycle, comprised of long 22h 35min high tides (Figure 1C). Again, we used both 13:20LHT and 19:20LHT regimes while keeping the LD cycle constant to determine whether animals entrained to tides or to the LD cycle. As with severe mixed tidal cycles, animals struggled to entrain under both diurnal regimens. Only a little over 50% animals were rhythmic (discussed below, Figure 6G). We again observed that a significant proportion of animals showed only doublet periodicities with circadian-like behavior, and accordingly we split behavior into the same cohorts as before: those with a singlet period and those with only a doublet (Figure 5A-F). Remarkably, the behavior of both singlet-rhythmic cohorts exhibited a similar bimodal pattern of activity to that seen previously after mild mixes tides, with one of the two activity troughs occurring just before the expected low tide (Figure 2A, B; 5B, E).

Doublet-only behavior in both cohorts was consistent with LD entrainment, although the pattern of activity was distinct between the two phases (Figure 5C, F). A second peak was detectable in some doublet-only animals after 19:20LHT, and was transiently visible in the doublet-only average trace (red arrow, Figure 5F). This second peak was not, however, detected on period analysis (Figure S3). The overall behavior trace after diurnal 19:20 LHT entrainment seemed more consistent and of higher amplitude than after 13:20 LHT, though this could be due to the low number of doublet-only animals following diurnal 13:20LHT. Notably, following a diurnal tide at 19:20LHT, the IPI between “Peak1” and Peak2” was reduced in these doublet-only individuals compared to singlet-rhythmic animals, again further supporting the existence of distinct behavioral phenotypes with specific properties (Figure 6E, see also discussion). We did not identify the third behavioral phenotype observed with severe mixed tides (tidally misaligned singlet-rhythmic).

We then compared the phase of behavior under the two different diurnal tidal regimes for both the doublet-only and singlet-rhythmic animals. For the latter, phase was clearly different for both peaks, but instead of the expected 6 h phase shift, they were shifted by only 3-4h (Figure 6A, B). In comparison with the phases observed after a mild mixed tide at 13:20LHT and 19:20LHT, the activity peaks following the 19:20LHT diurnal regimen were advanced by about 2-3 hours (Figure 2D, 6B). Intriguingly, this advance brings the timing of the first peak closer to that of the circadian-driven peak (Figure 6D). It therefore appears that singlet-rhythmic animals do not rely solely on tides to entrain their behavior under a diurnal tidal regimen, but can also be influenced by the light-dark cycle, depending on the phase relationship between the tidal and LD cycles.

As expected, the phase of the major peak of activity was very similar for doublet-only animals, although with 19:20LHT there seemed to be a broader phase distribution (Figure 6C, D). Thus, as after severe mixed tides, doublet-only animals synchronized their peak of swimming activity with the LD cycle, rather than to the diurnal tides. However, there was a striking distinction between the two phases of doublet-only behavior: the waveforms were different and thus influenced by the phase of the tides (Figure 5C, F and 6C). After 19:20LHT, activity increased rapidly after its onset , while the increase was much more gradual after 13:20LHT. Indeed, the timing of the onset of activity was close to that of the expected low tide under both tidal regimens (Figure 5 C, F). Additionally, unlike what we had observed after severe mixed tides, we found no indication of period shortening in doublet-only animals (Figure 6F). Finally, the ratio of singlet-rhythmic/circatidally behaving animals to doublet-only/LD-driven animals trended higher with 13:20LHT compared to 19:20LHT, with the difference just below statistical significance (Figure 6G). After diurnal tides with 13:20LHT, only 24% of rhythmic animals followed the LD cycle, much fewer than the 52% observed after 19:20LHT.

In summary, we conclude that a diurnal tidal cycle, like the severe mixed tidal regimen, is not as efficient as regular or mild mixed tides to entrain *P. hawaiensis* swimming rhythms. Animals are less rhythmic overall, and rhythmic animals use both LD and tidal cycles for their entrainment. This results in different patterns of behavior, with different degrees of circadian and circatidal clock contribution.

P. hawaiensis **does not synchronize to an unnatural tidal cycle**

Since we were able to observe entrainment of circatidal rhythms following all types of tidal cycle in at least a fraction of animals, we were curious about the limits of entrainment for the circatidal clock. Could any type of alternating high/low tide regimen entrain 12.4h rhythms? We decided to test this by entraining animals to an entirely artificial 17h tidal cycle comprised of 15h of high tide followed by 2h of low tide. Although animals might experience one or two 17h tidal cycles in the wild as part of the transition between diurnal and mixed semidiurnal tidal cycles, persistent 17h cycles do not occur anywhere on Earth.

We subjected animals to seven 17h cycles, concurrent with a 12:12 LD cycle, first with 13:20LHT and then with 19:20LHT. Under both conditions, free-running rhythmicity was strongly reduced compared to a regular semidiurnal cycle, with only 40.4% and 37.8% of animals exhibiting rhythms (Figure 7A). Surprisingly, the average trace after 13:20LHT seemed to exhibit a ~12.4h rhythmicity, with low amplitude peaks occurring around the time at which the light-on and -off transitions occurred during entrainment (Figure 7B). Activity following 19:20LHT had a similar but noisier pattern, and degraded after the first 3 days of recording (Figure 7C). After either phase of 17h cycles, we observed decreased power of rhythmic animals, as compared to semidiurnal rhythms (Figure 7D), and the variability of singlet and doublet periods was increased significantly (Figure 7E). Unlike after severe mixed or diurnal cycles, however, separating the behavior traces of those individuals lacking a singlet period component and those with one did not produce more coherent traces.

Phase quantification of both daily peaks revealed an additional cause of the noisy appearance as desynchrony between individuals, with phases spread widely across the circatidal period (Figure 7F). Phase averages did not differ significantly between 13:20 and 19:20 LHT. Moreover, when we plotted the two average activity traces over the expected LD cycle, we found that they both showed activity peaks occurring soon after the expected light-to-dark or dark-to-light transitions (Figure 7G). We therefore conclude that a 17h tidal cycle cannot function as a Zeitgeber for the circatidal clock. This is similar to circadian clocks, which also show limits of entrainments to environmental cycles[20-22]. The resultant free-running activity seems to display weak entrainment to the 12:12 LD cycle, but unlike behavior entrained by the LD cycle[16], most animals had a singlet component to their rhythms (81.1%) and the average doublet period was close to 24.8h (Figure 7E). It therefore appears that 17h tidal cycles can activate a circatidal behavioral program weakly synchronized by circadian input (see also discussion).

Discussion

In a recent study, we demonstrated that *Parhyale hawaiensis* harbors both a circadian and a circatidal clock, and that these two clocks are molecularly linked by the transcription factor *Bmal1*[16]. We now show how flexible contributions of both clocks shape durably rhythmic behavior. This remarkable temporal plasticity probably plays a critical role for *P. hawaiensis*' successful colonization of various coastal habitats.

In Kwiatkowski et al. 2023[16], we used the extreme ends of the entrainment spectrum. At one end, we found that a perfectly regular semidiurnal tidal pattern very efficiently entrained circatidal rhythms. Even though a LD cycle was present, these rhythms strictly followed the phase of the tides. On the other extreme, in the complete absence of tides, circadian rhythms were observed, with a peak of activity in the early night[16]. Entrainment to different tidal patterns in this study has now “filled in” the range between the two entrainment extremes. Interestingly, distinct modes of behavioral rhythmicity emerged in response to these challenging, but naturally occurring, tidal regimens, and our results indicate that this plasticity is the result of the adjustable contributions of the circatidal and circadian clocks.

Despite experiencing the same tidal cycle, animals could exhibit either a circadian or a circatidal behavioral phenotype in response to severe mixed and diurnal tidal cycles, and in some cases a behavioral pattern that appeared to be influenced by both clocks. For example, some animals showed two very robust peaks of activity every day, but those were better aligned with the LD cycle than the tidal cycle. Also, circatidal rhythms after diurnal tidal entrainment showed a clear indication of circadian modulation; a 6h shift in tidal phase only resulted in 3-4h behavioral phase shift. This was unexpected, since we had not detected circadian modulation of tidal behavior after a semidiurnal cycle[16]. Combined, these observations suggests that in animals exposed to a semidiurnal or mild mixed tidal regimen, the circatidal clock not only synchronizes behavior at the population level more effectively, but also either controls the pace of the circadian clock, or completely obscures its circadian output. In some animals exposed to diurnal tides, however, we observed the opposite phenomenon: their behavior appeared mostly circadian, but the circatidal clock modulated the onset of activity, and thus the behavioral waveform. Finally, we found that following severe mixed tides, circadian-like animals exhibited shorter periods, but this did not occur following diurnal tides. This suggests again that the circadian and circatidal clock interact to produce rhythmic behavioral outputs, and that their contributions vary with the type of tidal cycle experienced.

Importantly, we found that under any kind of naturally occurring tidal cycle, there was a cohort of animals capable of entraining to the tidal cycle. In these singlet-rhythmic/circatidal-like cohorts, we consistently observed a circatidal period near 12.4h, interpeak interval (IPI) separation close to ~12h, and a behavioral pattern that anticipated expected high and low tides. However, we noticed a shorter IPI in some circadian-like animals under diurnal and severe mixed tidal cycles. The reason for this deviation from the usually regular IPI interval is unclear. Obviously, in the doublet-only animals, the second daily peak of activity is of low amplitude; this could be a tidal peak, dwarfed and perhaps phase-shifted by the circadian clock. In the case of the early singlets arising from severe mixed tides, there is no obvious "weaker" daily peak, suggesting that the contributions of the tidal and circadian clock are more evenly matched in controlling both pattern and phase of behavior, respectively. Thus, there is a level of plasticity that occurs between the circatidal and circadian clock in designing patterns of behavior. This plasticity, however, supports persistent changes in the structuring of activity under constant conditions. Activity patterns appeared quite stable over the course of free-running, suggesting that animals had successfully engaged in a "program" of rhythmic activity and were continuing to follow it.

As mentioned in the introduction, previous studies have characterized rhythmic behavior in the same species under different tidal regimens, relying almost exclusively on wild-caught individuals entrained by environmental cycles in nature[7-11,13,14], with the exception of Klapow in 1972[15]. Within this group, only four studies have compared different tidal regimen-entrained behaviors under free-running conditions, without diurnal temperature or LD cycles[7,11,13,15]. Notably, in these studies, entrained behavior showed no[7,11,15] or very weak[13] evidence of circadian modulation of circatidal rhythms. However, the absence of a circadian component could be due to methodologic constraints: the use of population-level recording could over-represent individuals with the most robust rhythms of activity, which in our study were those with circatidal patterns (Figure 3A,F, Figure 5A,D). Alternately, the intrinsic variability in environmental cues that are experienced by organisms in natural settings could also introduce bias – for instance, crabs captured from deeper waters may experience less robust LD cycles than those living in shallower water[13]. Thus, our results indicate that the relationship between circatidal and circadian rhythms under complex tidal cycles is much more plastic than anticipated, and emphasize the importance of studying behavior (a) at the individual level and (b) under controlled laboratory conditions, in which the contributions of distinct environmental cycles can be better assessed.

A significant question arising from this work is how do individual *Parhyale hawaiensis* “choose” which environmental rhythm to follow? It seems likely that the salience of circadian and circatidal cues is “set” at low and high importance, respectively, since under semi-diurnal or mild-mixed tidal patterns, animals synchronize their behavior to the tidal cues rather than to the LD cycle. However there must be some variability between the relative importance of these specific cues at the individual level. Thus, under challenging tidal regimens, some organisms prioritize cues associated with the light-dark cycle, while others continue to prioritize those associated with the tidal cycle. One possible mechanism might be genetic variation, as is seen in the circadian and circalunar timing of the marine midge *Clunio marinus*. Comparison of different geographic populations of *C. marinus* have shown that certain polymorphisms are associated with distinct behavioral patterns [23,24]. This allows populations to optimally time their adult emergence from the water with the lowest low tides, which occur in a semi-lunar pattern and vary between coastlines. Individual circadian behavior characteristics also appear to have a strong intrinsic component in *P. dumerilii,* which seems at least partially genetically encoded[25]. Regardless of the mechanism, the ability to follow a set pattern of behavior under challenging entrainment conditions, and the inter-individual variation in “choice” of pattern, likely represents an evolutionary adaptation. It should be kept in mind, however, that our artificial cycles did not fully reproduce a natural tidal cycle. Tidal cycles might be entrained by more salient cues in the wild (e.g., vibrations, pressure, temperature etc…), and thus cause more homogenous behavioral patterns. Even if this were the case, the plastic interactions we revealed here likely contribute to tide-dependent behavioral patterns in the wild. The powerful genetic and imaging tools available for *P. hawaiensis* should allow us to understand in detail how two clocks tracking very different environmental cycles can interact to optimize rhythmic behavior. The principles behind this cooperation could also impact circalunar rhythms. Indeed, the combination of a circadian and circatidal clock can be used to generate (semi-) lunar oscillations, and this theory has recently received some experimental support[26]. While we do not know whether *P. hawaiensis* express circa(semi-)lunar rhythms of behavior, such rhythmicity has been observed in closely related amphipod species[27].

Finally, we found that the circatidal clock cannot entrain to an unnatural tidal cycle with a period of 17h. This demonstrates that the circatidal clock is sensitive to the period of the tidal cycles that entrain it, as we had observed circatidal behaviors after every natural tidal cycle we simulated. After exposure to 17h tidal cycles, the animals seemed to phase preferentially their weak activity rhythms with the LD cycle. Surprisingly, most animals exhibited a singlet period value, unlike the circadian-like doublet-only cohorts or animals exposed to LD only[16]. Thus, the presence of an unnatural, non-entraining tide, is not equivalent to the absence of tides. It seems rather that alternating high and low tides promotes tidal behavioral patterns. This again indicates a circatidal system that is capable of obscuring or disrupting the circadian clock's output. This re-structuring of circadian-entrained behavior by the circatidal system is also observed in the early singlet cohort following entrainment to a severe mixed tidal cycle with a last high tide at 19:20. Like that seen after 17h tidal cycles, bimodal behavior occurred while demonstrating entrainment to light-dark cues. Thus, this inter-clock flexibility is not an artifact of entrainment to an unnatural environmental cycle.

Studies in *Nematostella vectensis* and *Drosophila melanogaster* have shown that circadian rhythms can be disrupted to different degrees by "sensory conflict". In *N. vectensis*, exposure to temperature and light-dark cycles that are increasingly out of phase or "misaligned" with each other produces widespread population desynchrony in free-running rhythms, and decreased rhythmicity under entrainment [28]. In *Drosophila*, the phase of circadian behavior and the amplitude of molecular rhythms can also be disrupted by light and temperature cycle misalignments[29-31]. While in nature the phase of photic and thermal cycles have a stable phase relationship, tidal and photic cycles constantly drift in and out of phase. The presence of circadian and circatidal clocks dedicated to track these cycles individually should mitigate sensory conflict, but might not completely eliminate them. Indeed, under challenging tidal cycles, we did not just observe that circadian and circatidal clocks interacted to shape circadian behavior; animals were also more frequently arrhythmic, perhaps because circatidal and circadian outputs were in conflict.

In summary, we propose that circatidal and circadian clocks cooperate to optimize behavior so that *P. hawaiensis* can thrive in complex rhythmic environments. Strikingly, these interactions durably shape rhythmic behavior into behavioral “programs”. It will be critical to determine how this is achieved. It might result from output circuit rearrangements, or direct reciprocal connections between circadian and circatidal clock neurons. Synaptic mechanisms such as long-term potentiation or depression could underlie rhythmic behavior programming. Alternatively, rhythmic structural remodeling could be implicated. Indeed, circadian neurons have been found to rearrange their connectivity as a function of time-of-day[32-34]. It would be quite fascinating if this principle extends to circatidal neurons, as it would provide a combinatorial structural mechanism for shaping rhythmic behavior. Future work, aimed at tracing the circadian and circatidal neural networks, should prove enlightening.

Acknowledgments

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Author Contributions

Conceptualization, E.R.K., P.E. and J.J.C.R; methodology, E.R.K. and P.E.; investigation, E.R.K.; writing – original draft, E.R.K.; writing – review & editing, E.R.K., P.E. and J.J.C.R, funding acquisition, P.E. and J.J.C.R; supervision, P.E.

Declaration of Interests

The authors declare no competing interests.

Figure Titles and Legends

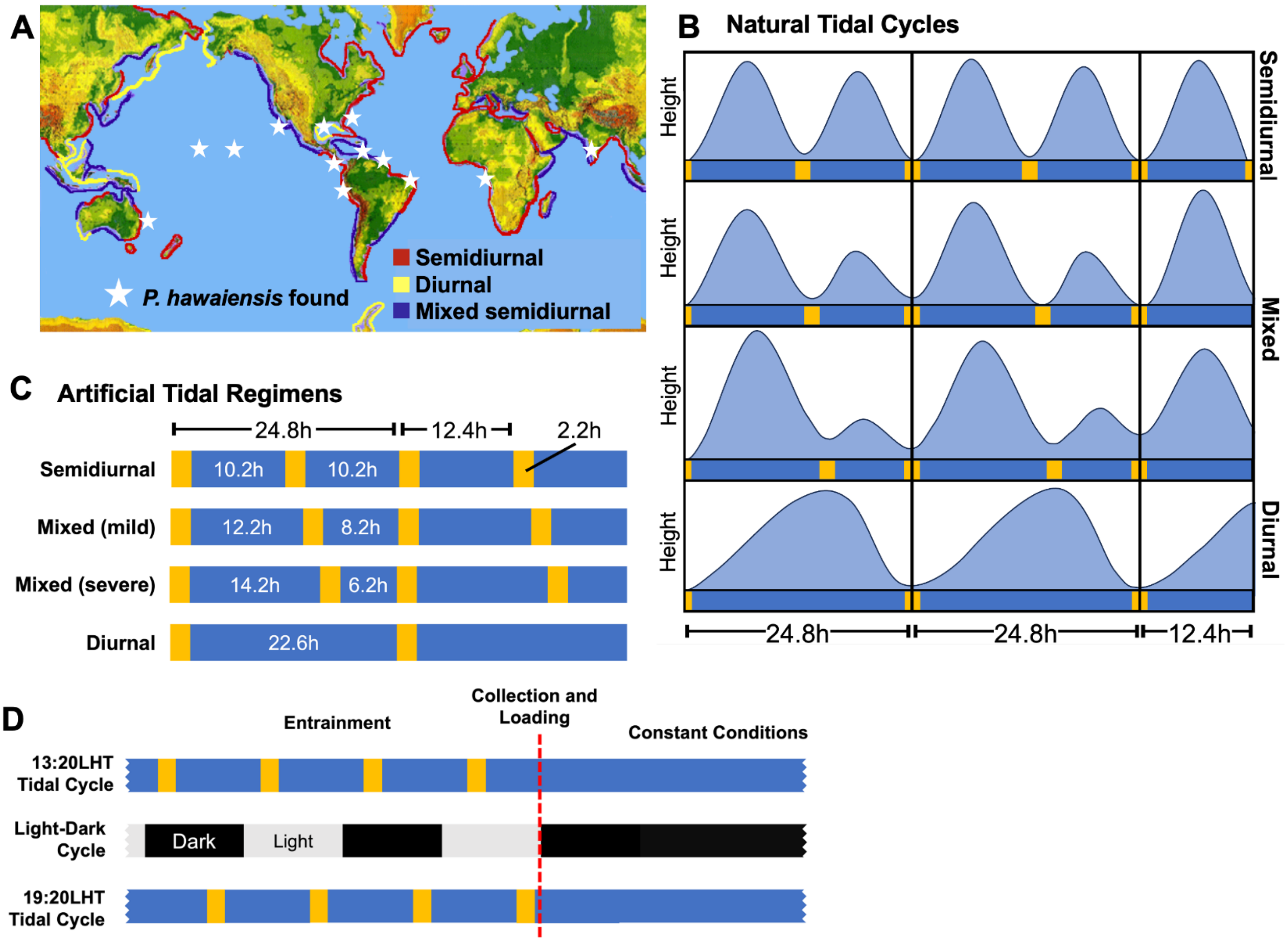

Figure 1. Three types of tidal cycle can be found across different coastlines. (A) Distribution of the three types of tidal cycle around the globe. Coasts outlined in red are those that experience semidiurnal tidal cycles, in yellow are coasts that experience diurnal tides, and in blue those that experience mixed semidiurnal tides. White stars represent where populations of *P. hawaiensis* have been identified[17,18]. Image modified from National Oceanic and Atmospheric Administration (NOAA) (source: oceanservice.noaa.gov). (B) Sample plots demonstrating water height over time for coasts experiencing (from top to bottom): semidiurnal, mild mixed semidiurnal, severe mixed semidiurnal, and diurnal tidal patterns. At the base of each plot is a simplified timeline of when high (blue) and low (gold) tide occurs for each tidal wave plot. Each plot was drawn using NOAA tide predictions from the following sites and times (top to bottom): South Amelia River, FL, USA, 2/9-11/24; Woods Hole, MA, USA, 2/2-4/24; Mahukona, HI, USA, 11/21-23/23; Port Boca Grande, FL, USA, 1/21-23/24. (C) Artificial tidal cycles were composed of one or two high tides (blue) every 24.8h. Low tides (gold) always lasted 2.2h. Depending on the cycle simulated, subsequent high tides were either of the same

duration (for semidiurnal tides, 10.2h; for diurnal, 22.6h) or different durations for mixed-tidal patterns. (D) Alignment of artificial tidal cycles with the light-dark cycle. Lights on occurred in the morning at 08:00, and lights off at 20:00. For animals entrained to regimens with 13:20LHT, the last high tide before the time of loading (indicated with red dotted line) was 13:20, or 1:20PM. For animals entrained to 19:20LHT regimens, the last high tide before loading was 19:20, or 7:20PM. Constant conditions consisted of constant high tide and constant dark.

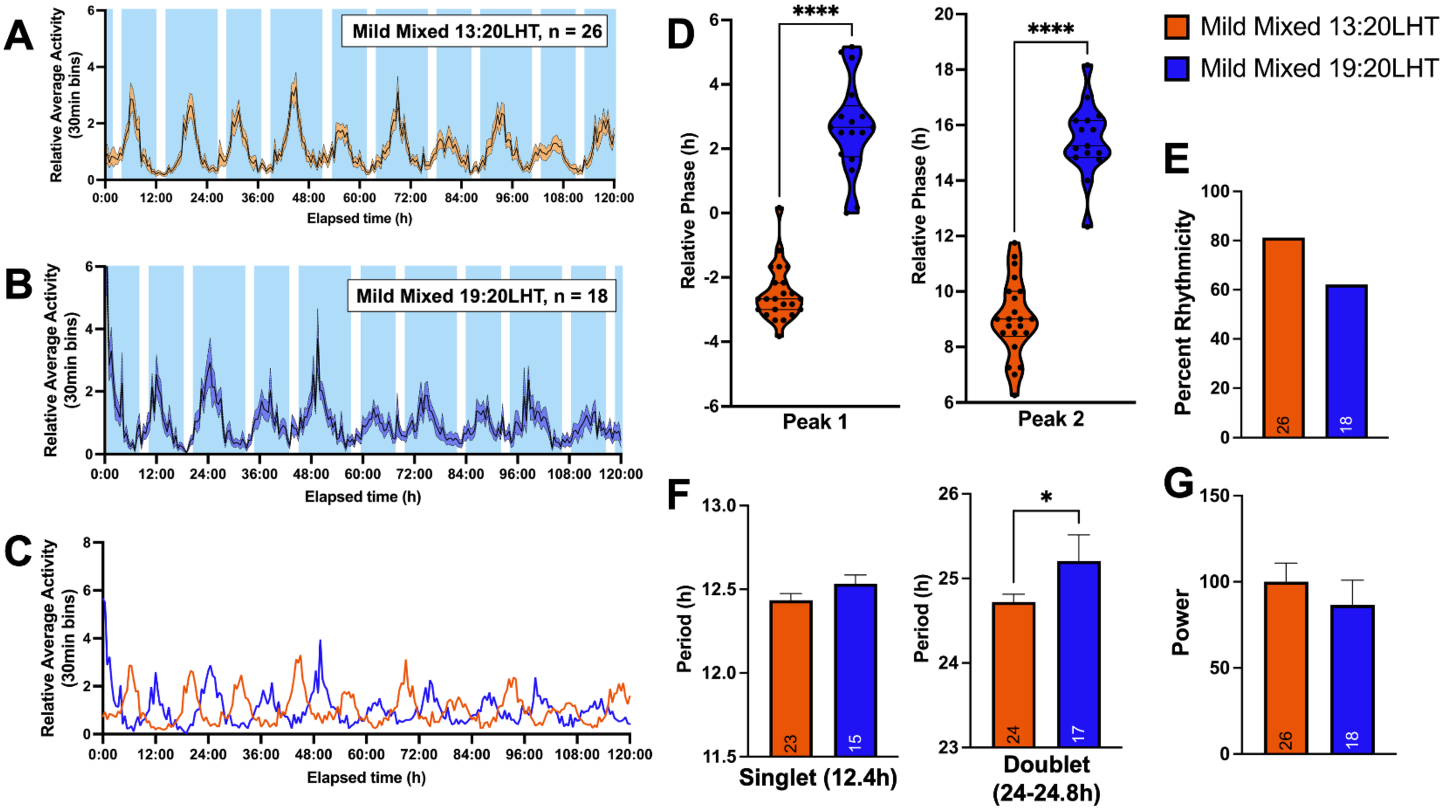

Figure 2. ***Parhyale hawaiensis*** **exhibits robust circatidal rhythms of activity after entrainment to a mild mixed tidal cycle.** (A, B) Relative average activity in constant conditions of rhythmic animals after entrainment to a mild mixed tidal cycle with a last high tide (LHT) of 13:20 (A) and 19:20 (B). Activity is plotted beginning at midnight (4 hours after lights off), standard error is indicated with orange or dark blue shading, and light blue and white shading represents subjective high and low tides, respectively (as they will throughout this paper). (C) Overlay of average relative activity traces of rhythmic animals after entrainment to a mild mixed tidal regimen with LHT 13:20 (in dark orange)

and LHT 19:20 (in blue). (D) Phase quantification for the two daily peaks of activity. The Y-axis represent relative phase values, quantified from individual rhythmic animals. The phase of both peaks significantly differs between phases of entrainment (Peak 1: Mann-Whitney test, $p < 0.0001$ (****); Peak 2: unpaired t-test, $p < 0.0001$ (****)). Ns for each (written as Peak 1/Peak 2): 13:20LHT – 23 / 22; 19:20LHT – 17 / 15. (E) The prevalence of rhythmicity does not significantly differ between animals entrained to either phase of mild mixed tidal regimen (Chi-Square: $p = 0.0952$). (F) Average periods for singlets and doublets. The only significant difference is between the doublet values of the two mild mixed tidal patterns (singlets: unpaired t-test, $p = 0.1361$; doublets: Mann Whitney test, $p = 0.0172$ (*)). (G) Power did not significantly differ between animals entrained to mixed mild or semidiurnal regimens (Mann Whitney test, $p = 0.3619$. Error bars represent SEM and values at bottom of bars represent Ns, and will throughout this paper.

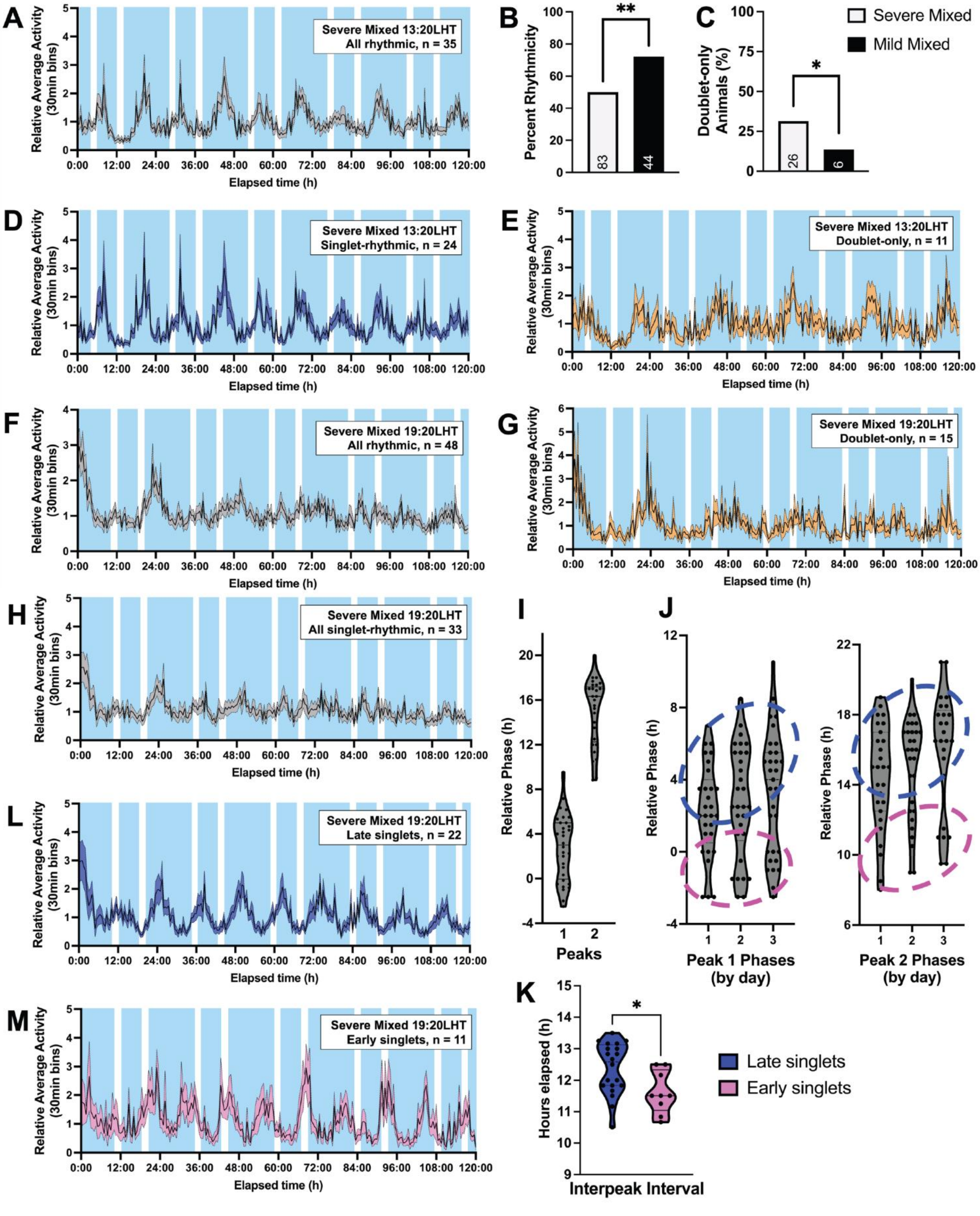
A
Severe Mixed 13:20LHT
All rhythmic, n = 35
Relative Average Activity (30min bins)
Elapsed time (h)
B
Percent Rhythmicity
**
83
44
C
Doublet-only Animals (%)
*
26
6
Severe Mixed
Mild Mixed
D
Severe Mixed 13:20LHT
Singlet-rhythmic, n = 24
E
Severe Mixed 13:20LHT
Doublet-only, n = 11
F
Severe Mixed 19:20LHT
All rhythmic, n = 48
G
Severe Mixed 19:20LHT
Doublet-only, n = 15
H
Severe Mixed 19:20LHT
All singlet-rhythmic, n = 33
I
Relative Phase (h)
Peaks
J
Peak 1 Phases (by day)
Peak 2 Phases (by day)
L
Severe Mixed 19:20LHT
Late singlets, n = 22
M
Severe Mixed 19:20LHT
Early singlets, n = 11
K
Hours elapsed (h)
*
Interpeak Interval
Late singlets
Early singlets

Figure 3. Rhythmic behavioral plasticity is observed after entrainment to severe mixed tidal regimens. (A) Relative average free-running activity of all rhythmic animals after entrainment to a severe mixed tidal cycle with 13:20LHT. SEM and expected tidal cycle as represented in Figure 2. (B) Rhythmicity after severe mixed entrainment was significantly reduced compared to that after mild mixed entrainment (Chi-Square: severe mixed vs. mild mixed, p = 0.0029(**)). (C) Frequency of animals with no singlet component in their periodogram was increased after a severe mixed tide compared to after mild mixed tides (Chi-Square: severe mixed vs. mild mixed, p = 0.0289 (*)). (D, E) Relative average activity of rhythmic animals with a singlet component (D) (singlet-rhythmic), or only a doublet component (E) (doublet-only) after entrainment to a severe mixed tidal cycle with 13:20LHT. (F) Relative average free-running activity of all rhythmic animals after entrainment to a severe mixed tidal cycle with 19:20LHT. (G, H) Relative average free-running activity of animals exhibiting only a doublet component (G, doublet-only) or a singlet component (H, singlet-rhythmic) after entrainment to a severe mixed tidal cycle with LHT 19:20. (I) Plotting activity phase of singlet-rhythmic animals after 19:20LHT reveals a large distribution for both peaks. Ns: peak 1, 32; peak 2, 29. (J) Plotting individual phases of activity peaks day by day reveals two “cohorts” of singlet-rhythmic animals: one in which the timing of activity has an earlier phase and does not delay relative to the LD cycle (i.e individuals have a period close to 24h, circled in pink) and one in which activity has a slightly later phase and delays (consistent with a >24h period) from day 1 to 3 (circled in blue). Peak 1 n’s: 29, 30, 31. Peak 2 n’s: 27, 29, 25. (K) The average interpeak interval significantly differs between these two cohorts (unpaired t-test, p = 0.0197 (*)). Interpeak interval n’s: early singlets: 9; late singlets: 20. (L, M) Relative average free-running activity of singlet-rhythmic animals exhibiting the later phase of activity (L, late singlets) or the earlier phase (M, early singlets).

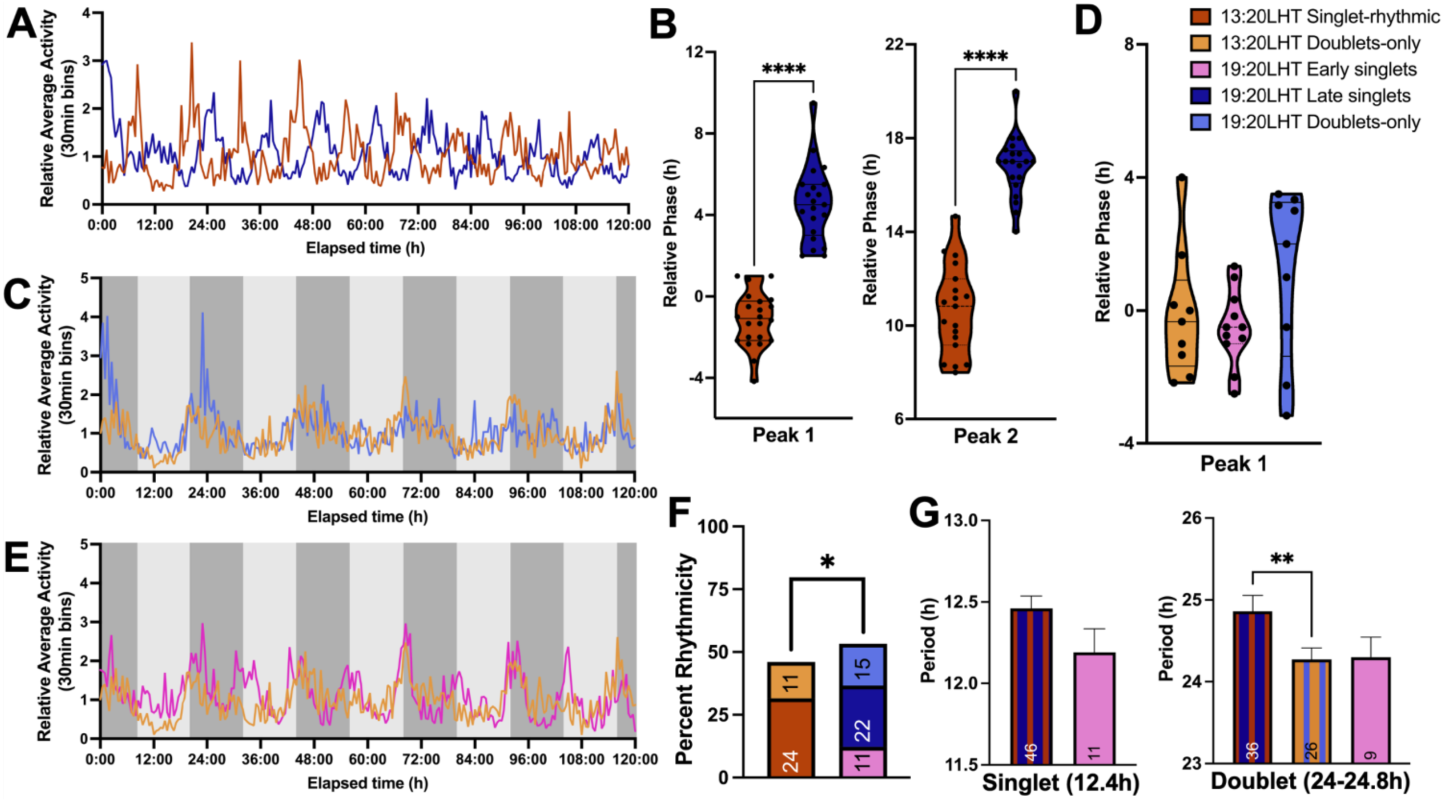

Figure 4. Specific patterns of activity synchronize to distinct environmental cycles. (A) Overlay of average relative activity traces of rhythmic animals with singlet components after entrainment to a severe mixed tidal regimen with 13:20LHT (in dark orange) and 19:20LHT (in blue). (B) Tidal phase dictates the timing of both activity peaks for singlet-rhythmic animals (Peak 1: unpaired t-test, $p < 0.0001$ (****); Peak 2: unpaired t-test, $p < 0.0001$ (****)). N's (Peak 1/2): 13:20LHT, 22/19; 19:20LHT, 21/20. (C) Overlay of average relative activity traces of rhythmic doublet-only animals after entrainment to a severe mixed tidal regimen with 13:20LHT (in gold) and 19:20LHT (in light blue). Alternating dark and light gray stripes indicate expected dark and light periods, respectively (and will throughout this paper). (D) Timing of activity peak does not significantly differ between tidal phase for doublet-only animals or late-singlet animals (one-wayANOVA, $p = 0.1705$). Ns: 13:20LHT doublets-only, 9; 19:20LHT early singlets, 11; 19:20LHT doublets-only, 9. (E) Overlay of average activity traces of doublet-only animals after entrainment to a severe mixed tidal cycle with 13:20LHT (gold) and late-singlet animals after severe mixed cycle with 19:20LHT (pink). (F) The distribution of animals as

arrhythmic, late-singlet or early singlet, or doublet-only after severe mixed entrainment significantly varied by phase of the tidal cycle (Chi-Square test, p = 0.0140 (*)). (G) Singlet periods tended to be shorter in early-singlet than in singlet-rhythmic animals, but were not significantly different (Mann-Whitney test, p = 0.0933). Average doublet period observed after severe mixed entrainment was significantly longer in singlet-rhythmic and late-singlet individuals compared to that of the doublet-only animals (Kruskal-Wallis ANOVA, p = 0.0036 (not shown); subsequent Dunn's multiple comparisons test: singlet-rhythmic vs. doublet-only, p = 0.0035 (**); other comparisons, p > 0.05).

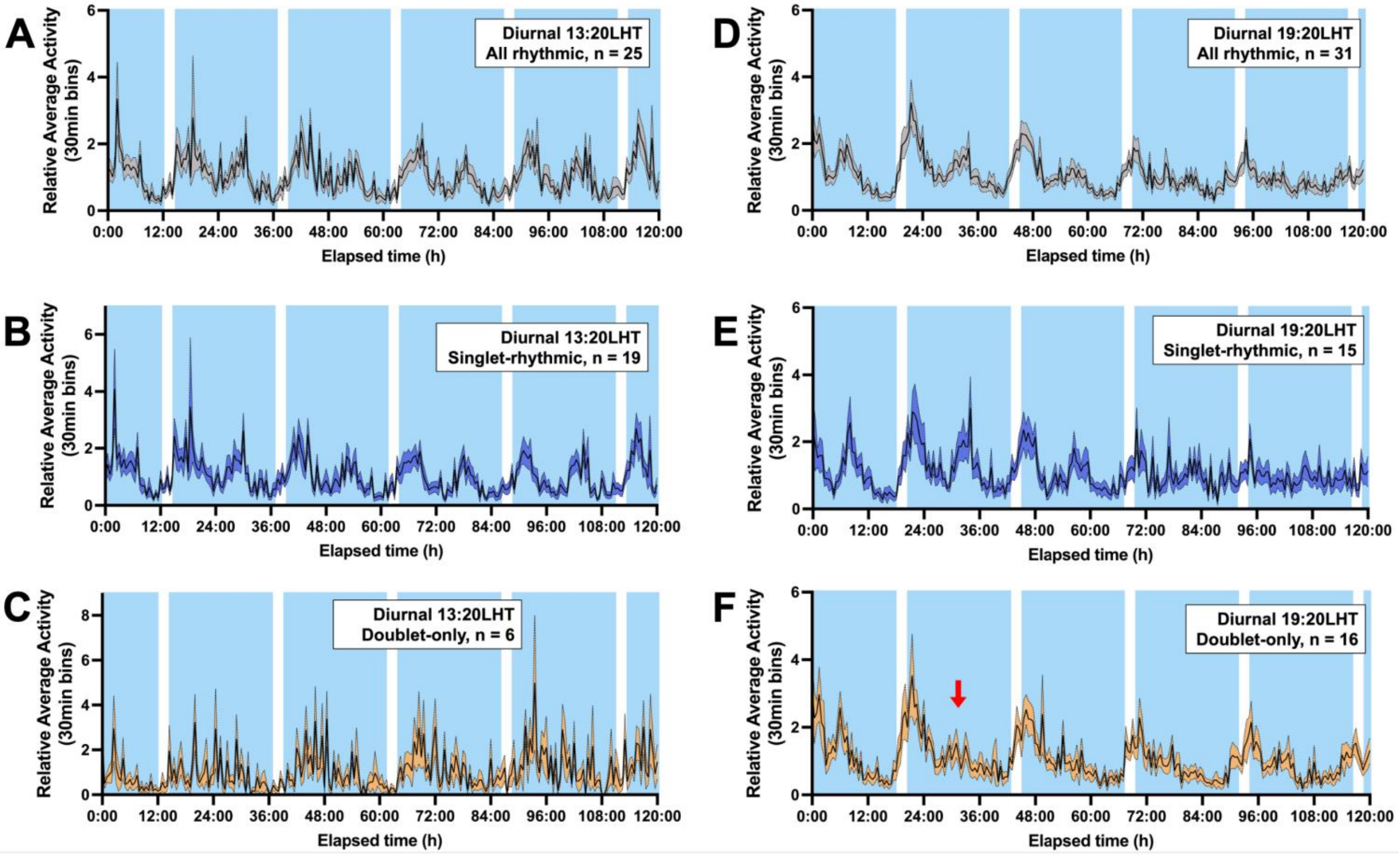

Figure 5. Bimodal behavioral plasticity is observed after entrainment to a diurnal tidal regimen. (A, B, C) Relative average free-running activity of all rhythmic animals (A), and animals exhibiting activity rhythms with a singlet component (B) (singlet-rhythmic) or only a doublet component (C) (doublet-only) after entrainment to a diurnal tidal cycle with 13:20LHT. SEM and expected tidal cycle as represented in Figure 2. (D, E, F) Relative average free-running activity of all rhythmic animals (D), and animals exhibiting activity rhythms with a singlet component (E) (singlet-

rhythmic), or only a doublet component (F) (doublet-only) after entrainment to a diurnal tidal cycle with 19:20LHT. (F) Red arrow indicates transient “second” peak described in Figure 6 and text.

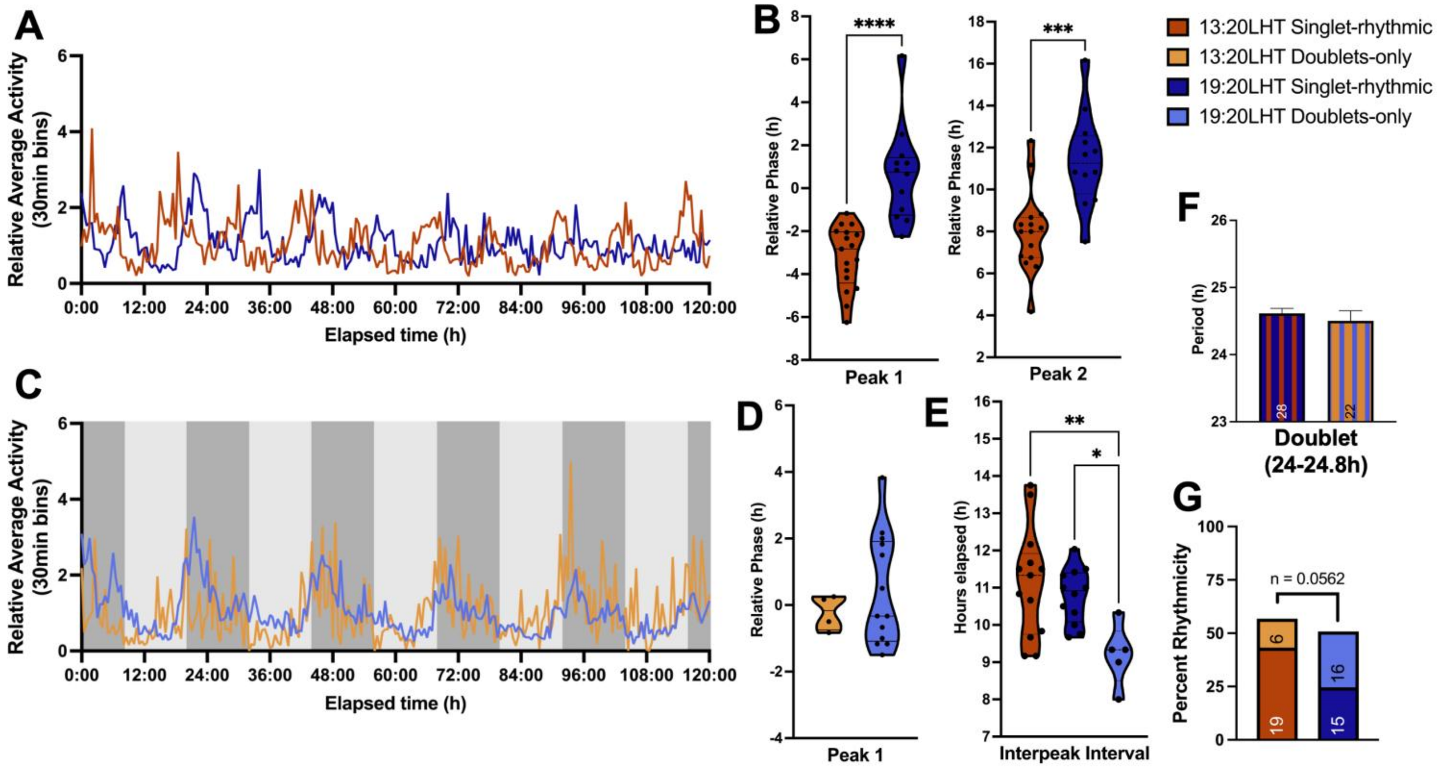

Figure 6. Behavioral plasticity under diurnal tidal cycles shows cooperation between clocks. (A) Overlay of average relative activity traces of rhythmic animals with singlet components after entrainment to a diurnal tidal regimen with 13:20LHT (in dark orange) and 19:20LHT (in blue). (B) Timing of both activity peaks significantly differs by the phase of the tidal cycle for singlet-rhythmic animals (Mann-Whitney test, Peak 1, $p < 0.0001$ (****); unpaired t-test, Peak 2, $p = 0.0003$ (***)). N’s for Peak 1/2: LHT 13:20, 17/15; LHT 19:20, 12/12. (C) Overlay of average relative activity traces of rhythmic animals without singlet components (doublet-only) after entrainment to a diurnal tidal regimen with LHT 13:20 (in gold) and LHT 19:20 (in light blue). Shading as described in Figure 4. (D) Timing of activity peak does not significant differ based on tidal phase for doublet-only animals (unpaired t-test, $p = 0.4534$). N’s: LHT 13:20, 4; LHT 19:20, 13. (E) Interpeak interval differs between the singlet-rhythmic groups from either phase and the doublet-only cohort with 19:20LHT (one-way

ANOVA, p = 0.0112 (not shown); subsequent Tukey's multiple comparisons: 13:20LHT singlet-rhythmic vs. 19:20LHT doublet-only, p = 0.0087 (**), 19:20LHT singlet-rhythmic vs. 19:20LHT doublet-only, p = 0.0374 (*), 13:20LHT singlet-rhythmic vs. 19:20LHT singlet-rhythmic, p = 0.7194). N's: LHT 13:20, 13; LHT 19:20 singlet-rhythmic, 12;, LHT 19:20 doublets-only, 5. (F) Average doublet period observed after diurnal entrainment does not differ between singlet-rhythmic and doublet-only cohorts (Welch's t test, p = 0.5128). (G) Distribution of animals as arrhythmic, singlet-rhythmic, or doublet-only after diurnal entrainment. The variation in distribution between the two phases of entrainment was just above the significance threshold (Chi-Square test, p = 0.0562).

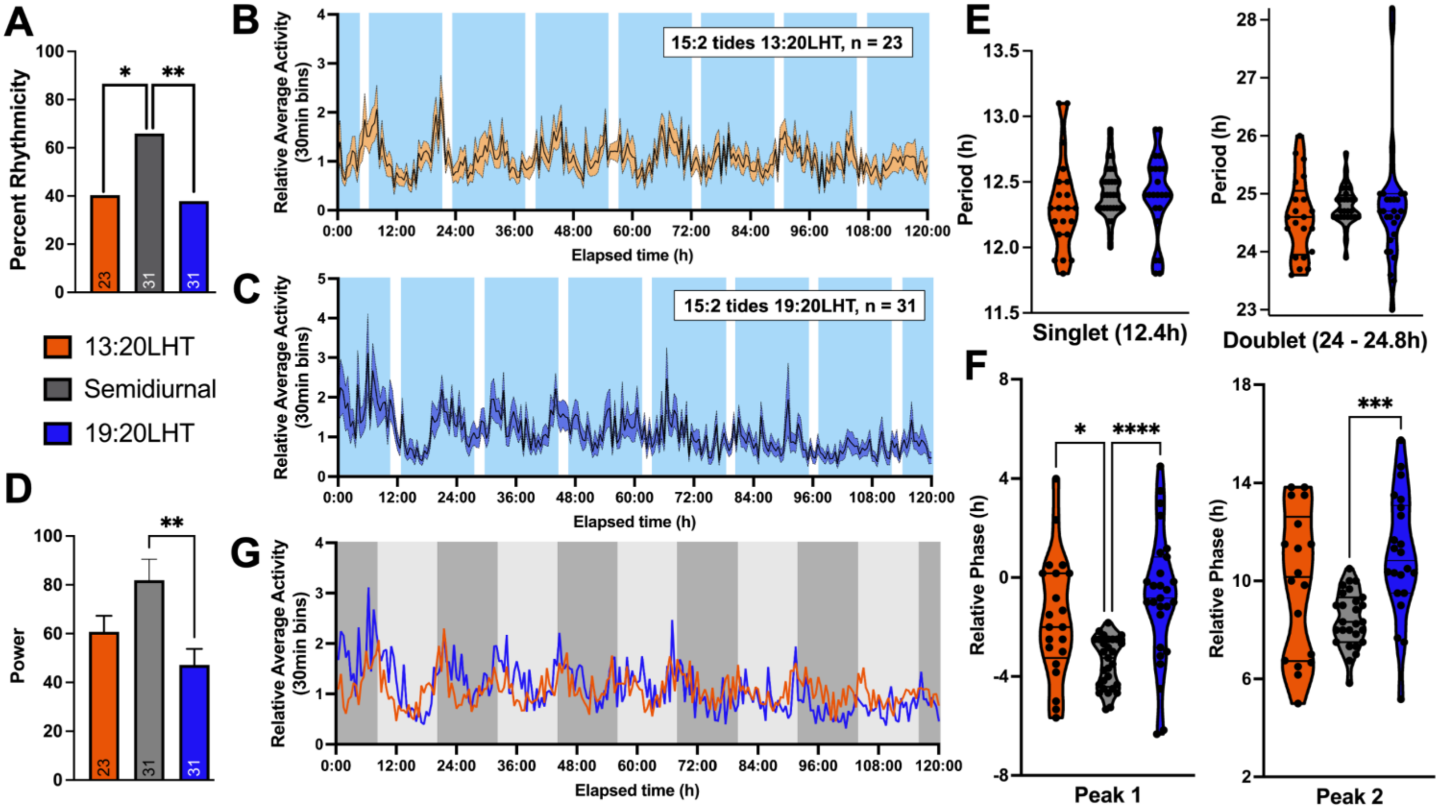

Figure 7. *Parhyale hawaiensis* do not entrain to an unnatural tidal cycle. (A) Rhythmicity is significantly decreased after entrainment to 15h high tide:2h low tide "tidal cycles" compared to semidiurnal entrainment (Chi-Square, p = 0.0046 (not shown), subsequent Fisher's exact tests: 15:2 LHT 13:20 vs. semidiurnal, p = 0.0110 (*); 15:2 LHT 19:20 vs. semidiurnal, p = 0.0020 (**)). (B, C) Relative average activity in constant conditions of animals exhibiting rhythmic activity after

entrainment to a 15h:2h "tidal cycle" with 13:20LHT (B) and 19:20LHT (C). SEM and expected tidal cycle as represented in Figure 2. (D) After entrainment to a 15:2 tidal cycle with 19:20LHT, but not 13:20LHT, average power was significantly reduced compared to semidiurnal entrained animals (Kruskal-Wallis ANOVA, $p = 0.0013$ (not shown); subsequent Dunn's multiple comparisons: 15:2 LHT 19:20 vs. semidiurnal, $p = 0.0032$ (**); other comparisons, $p > 0.05$). (E) Both singlet and doublet periods observed after entrainment to either phase of 15:2 tides have a significantly broader distribution than those observed after entrainment to a semidiurnal tidal cycle, but the average period does not differ (Singlet: Brown-Forsythe ANOVA, $p = 0.5420$; F test to compare variances: 15:2 13:20LHT vs. semidiurnal, $p = 0.0035$; 15:2 19:20LHT vs. semidiurnal, $p = 0.0218$) (Doublet: Kruskal-Wallis ANOVA, $p = 0.3755$; F test to compare variances: 15:2 13:20LHT vs. semidiurnal, $p = 0.0018$, 15-2 19:20LHT vs. semidiurnal, $p < 0.0001$). Ns for periods (written as singlet n / doublet n): 15:2 13:20LHT, 21/21; 15:2 19:20LHT, 23/28; semidiurnal, 31/28. (F) The timing of peak one does not differ between different phases of 15:2 tidal cycle, but do significantly differ from that produced by a semidiurnal entrainment with 13:20LHT (Peak 1: Kruskal-Wallis ANOVA, $p < 0.0001$ (not shown); subsequent Dunn's multiple comparisons: 15:2 13:20LHT vs. semidiurnal, $p = 0.0127$ (*); 15:2 19:20LHT vs. semidiurnal, $p < 0.0001$ (****); 15:2 13:20LHT vs. 15:2 19:20LHT, $p = 0.8223$). The timing of peak two only differs between 19:20LHT 15-2 and that generated by a semi-diurnal regimen with 13:20LHT (Peak 2: Welch's ANOVA, $p = 0.0003$ (not shown); Brown-Forsythe ANOVA, $p = 0.0021$ (not shown); subsequent Dunnett's T3 multiple comparisons test: 15:2 19:20LHT vs. semidiurnal, $p = 0.0003$ (***); 15:2 LHT 13:20 vs. semidiurnal, $p = 0.1634$; 15:2 13:20LHT vs. 15:2 19:20LHT, $p = 0.4888$). Additionally, the observed variance in peak timing was significantly different between animals entrained to 15:2 and semidiurnal regimens (Peak 1: F test for variance: 15:2 13:20LHT vs. semidiurnal, $p < 0.0001$; 15:2 19:20LHT vs. semidiurnal, $p < 0.0001$) (Peak 2: F test for variance: 15:2 13:20LHT vs. semidiurnal, $p < 0.0001$; 15:2 19:20LHT vs. semidiurnal, $p = 0.0002$). N's for each (written as Peak 1/Peak 2): 15:2 LHT 13:20 – 21 / 18; 19:20 – 27 / 22; semidiurnal – 29 / 27. (G) Overlay of average relative activity traces of rhythmic animals after entrainment to a 15:2 tidal regimen with 13:20LHT (in dark orange) and 19:20LHT (in blue). Shading as described in Figure 4.

STAR Methods

Resource Availability

Lead contact: Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Patrick Emery [patrick.emery@umassmed.edu].

Materials availability: This study did not generate new unique reagents.

Data and code availability: original data reported in this paper will be shared by the Lead Contact upon request. This paper does not report original codes. Any additional information required to reanalyze the data reported in this paper will be made available by the lead contact upon request.

Experimental Model and Study Participant Details

Animal Husbandry

Wild-type *P. hawaiensis*, of the Chicago F strain, were obtained from N. Patel's lab at the Marine Biological Laboratory and maintained as detailed previously in Kwiatkowski et al 2023. Briefly, animals were kept in polycarbonate tanks containing ~1.5cm of crushed coral substrate and filled with 5 cm of artificial seawater (Instant Ocean Sea Salt, Instant Ocean, China). Tanks were kept in a temperature-controlled room at 25^o C and constantly exposed to a 12h:12h light:dark regimen with ZT0 at 8AM. Water salinity was maintained at a specific gravity of 1.025, and an air pump (TOM Aquarium Stellar Air Pump W-40, Koller Products, Shawnee, KS) and a submerged airstone provided constant oxygenation.

Method Details

Tidal entrainment and behavior recording

Tidal entrainment consisted of either ten artificial tidal cycles (for semidiurnal, mild, and severe mixed regimens) or five artificial tidal cycles (for diurnal regimens) (Figure 1C). Semidiurnal cycles consisted of 10.2:2.2 h high tide: low tide, mild mixed consisted of alternating 8.2h:2.2h and 12.2h:2.2h high:low tides, and severe mixed consisted of alternating 6.2h:2.2h and 14.2h:2.2h high:low tides. Diurnal regimens consisted of 22.6h:2.2h high:low tides. Generation of low and high tides and recording of activity in constant conditions was performed as previously described[16].

Tubes with male animals were then loaded into vertically oriented *Drosophila* Population Monitors (DPMs) or Locomotor Activity Monitors (LAMs, TriKinetics, Waltham MA) located in a temperature and light-controlled incubator (I36LL, Percival Scientific, Iowa). Only males were used as we have previously shown that females are significantly less likely than males to exhibit free-running rhythms of activity following exposure to a tidal regimen[15]. Swimming behavior was monitored under constant conditions (constant high tide and darkness) using the TriKinetics Drosophila Activity Monitoring (DAM) system, with activity counts sampled every minute. Animals that died during the experiments were excluded from analysis.

Behavior analysis

Breaks in the infrared beams of the DPM or LAM was used as a surrogate to measure swimming activity. Beam break counts were collected in 30-minute bins and plotted over time. Analysis of rhythmicity and period length were made using the FaasX software (https:// neuropsi.cnrs.fr/en/cnn-home/francois-rouyer/faas-software/, courtesy of F. Rouyer, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France). Animals were considered rhythmic if they exhibited a period shorter than 30 h, the ‘‘power’’ reached at least 10, and ‘‘width’’ at least 2 (= 1 h), as determined from chi-square periodogram analysis. Power and width are defined as the height and width (in number of 30 min bins or hours) of a periodogram peak above the statistical significance line. The filter for low frequencies was on.

Average activity plots were generated with Graphpad/PRISM. For average plots, behavioral activity was normalized within each animal by dividing each 30-min bin value by the bin average activity. These relative average values were then averaged to generate average activity plots for a group of animals. Phase quantifications were performed manually by an observer blind to entrainment conditions, using individual traces smoothened with a 4h moving average of the raw activity counts. Identifiable activity peaks occurring between hours 18:00 and 96:00 of the free running period were quantified, and the timing of the peak on the first day was used to determine which grouping they were part of (i.e., peak 1 or peak 2). At least two peaks had to be quantified to generate a data point. Thus, a small fraction of rhythmic flies was not included in the quantification.

For each condition (ex: mild mixed tides at 13:20LHT), at least two independent experiments were performed with a minimum of 18 individual rhythmic animals recorded.

Quantification and Statistical Analysis

GraphPad Prism 10.2 was used to plot graphs and compare independent groups of data.

Bar graphs represent the means ± S.E.M. (unless otherwise mentioned in figure legends). Violin plots are labeled with median value (solid line) and quartiles (dotted lines). Data were pooled from at least two independent sets of experiments, and 'n' rep- resents the biological replicates or total numbers of animals. All the data were tested for normality using the D'Agostino and Pearson omnibus test if the sample size was greater than 20, otherwise, the Shapiro-Wilk test was used. In the case of normally (Gaussian) distributed data, a two-tailed unpaired t-test with or without Welch's correction was used for two groups; for multiple groups, either a one-way ANOVA or Welch's ANOVA (for groups with unequal variances) was used, followed by either a Dunnett's multiple comparisons or Sidak's Multiple Comparison's test for multiple groups. For non-Gaussian distribution, a Mann–Whitney U-test was performed for two groups, while for multiple groups, a Kruskal-Wallis test with Dunn's post-hoc test was performed. For contingency table analyses, for two groups, Chi-square was used if all $n > 5$, otherwise Fisher's exact test was used, and for multiple groups, Chi-square was used for multiple groups, with subsequent Fisher's exact test. Statistical significance is demonstrated as ****$P < 0.0001$; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

References

1. Zhang, L., Hastings, M.H., Green, E.W., Tauber, E., Sladek, M., Webster, S.G., Kyriacou, C.P., and Wilcockson, D.C. (2013). Dissociation of circadian and circatidal timekeeping in the marine crustacean Eurydice pulchra. Curr Biol *23*, 1863-1873. 10.1016/j.cub.2013.08.038.
2. Mat, A.M., Sarrazin, J., Markov, G.V., Apremont, V., Dubreuil, C., Eche, C., Fabioux, C., Klopp, C., Sarradin, P.M., Tanguy, A., et al. (2020). Biological rhythms in the deep-sea hydrothermal mussel Bathymodiolus azoricus. Nat Commun *11*, 3454. 10.1038/s41467-020-17284-4.
3. Nybakken, J.W. (2001). Marine Biology: an Ecological Approach., 5 Edition (Benjamin Cummings).
4. Naylor, E. (2010). Chronobiology of Marine Organisms. (Cambridge University Press).
5. McCully, J.G. (2006). Beyond the Moon: A Conversational, Common Sense Guide to Understanding the Tides (World Scientific Publishing Co. Pte. Ltd.).
6. Castro, P., and Huber, M.E. (2003). Marine Biology, 4th Edition (McGraw Hill).
7. Enright, J.T. (1963). The tidal rhythms of activity of a sand-beach amphipod. Zeitschrift fur vergleichende Physiologie *46*, 276-313.
8. Morgan, S.G., Christy, J.H. (1994). Plasticity, constraint, and optimality in reproductive timing. Ecology *75*, 2185-2203.
9. Kellmeyer, K., and Salmon, M. (2001). Hatching rhythms of Uca thayeri Rathbun: timing in semidiurnal and mixed tidal regimes. J Exp Mar Biol Ecol *260*, 169-183. 10.1016/s0022-0981(01)00259-3.
10. Stillman, J.H., and Barnwell, F.H. (2003). Relationship of daily and circatidal activity rhythms of the fiddler crab, Uca princeps, to the harmonic structure of semidiurnal and mixed tides. Marine Biology *144*, 473-482.
11. Lopez-Duarte, P.C., and Tankersley, R.A. (2007). Circatidal swimming behaviors of fiddler crab Uca pugilator larvae from different tidal regimes. Marine Ecology Progress Series *343*, 207-220.
12. Krumme, U. (2014). Diel and tidal movements by fish and decapods linking tropical coastal ecosystems. In Ecologiccal Connectivity among Tropical Coastal Ecosystems, I. Nagelkerken, ed. (Springer Science), pp. 271-324.
13. Forward Jr., R.B., Lohmann, K., and Cronin, T.W. (1982). Rhythms in larval release by an estuarine crab (Rhithropanopeus harrisii). Biological Bulletin *163*, 287-300.
14. Morgan, S.G. (1996). Plasticity in reproductive timing by crabs in adjacent tidal regimes. Marine Ecology Progress Series *139*, 105-118.
15. Klapow, L.A. (1972). Natural and artificial rephasing of a tidal rhythm. Journal of Comparative Physiology *79*, 233-258.
16. Kwiatkowski, E.R., Schnytzer, Y., Rosenthal, J.J.C., and Emery, P. (2023). Behavioral circatidal rhythms require Bmal1 in Parhyale hawaiensis. Curr Biol *33*, 1867-1882.e1865. 10.1016/j.cub.2023.03.015.
17. Shoemaker, C.R. (1956). Observations on the amphipod genus Parhyale. Proceedings of the United States National Museum *117*, 459-552.
18. Barnard, J.L. (1965). Marine amphipods of atolls in Micronesia. Proceedings of the United States National Museum *106*, 345-358.
19. Neumann, D. (2014). Timing in Tidal, Semilunar, and Lunar Rhythms. In Annual, Lunar, and Tidal Clocks, H.N.a.B. Helm, ed. (Springer), pp. 3-24.
20. Pittendrigh, C.S., and Daan, S. (1976). A Functional Analysis of Circadian Pacemakers in Nocturnal Rodents. IV. Entrainment: Pacemaker as Clock. . Journal of Comparative Physiology *106*, 291-331.
21. Yoshii, T., Sakamoto, M., and Tomioka, K. (2002). A temperature-dependent timing mechanism is involved in the circadian system that drives locomotor rhythms in the fruit fly Drosophila melanogaster. Zoolog Sci *19*, 841-850. 10.2108/zsj.19.841.

22. Udo, R., Hamada, T., Horikawa, K., Iwahana, E., Miyakawa, K., Otsuka, K., and Shibata, S. (2004). The role of Clock in the plasticity of circadian entrainment. Biochem Biophys Res Commun *318*, 893-898. 10.1016/j.bbrc.2004.04.113.
23. Fuhrmann, N., Prakash, C., and Kaiser, T.S. (2023). Polygenic adaptation from standing genetic variation allows rapid ecotype formation. Elife *12*. 10.7554/eLife.82824.
24. Kaiser, T.S., Poehn, B., Szkiba, D., Preussner, M., Sedlazeck, F.J., Zrim, A., Neumann, T., Nguyen, L.T., Betancourt, A.J., Hummel, T., et al. (2016). The genomic basis of circadian and circalunar timing adaptations in a midge. Nature *540*, 69-73. 10.1038/nature20151.
25. Hafker, N.S., Holcik, L., Mat, A.M., Coric, A., Vadiwala, K., Beets, I., Stockinger, A.W., Atria, C.E., Hammer, S., Revilla, I.D.R., et al. (2024). Molecular circadian rhythms are robust in marine annelids lacking rhythmic behavior. PLoS Biol *22*, e3002572. 10.1371/journal.pbio.3002572.
26. Cheeseman, J.F., Fewster, R.M., and Walker, M.M. (2017). Circadian and circatidal clocks control the mechanism of semilunar foraging behaviour. Sci Rep *7*, 3780. 10.1038/s41598-017-03245-3.
27. Kaiser, T.S., and Neumann, J. (2021). Circalunar clocks-Old experiments for a new era. Bioessays *43*, e2100074. 10.1002/bies.202100074.
28. Berger, C.A., and Tarrant, A.M. (2023). Sensory conflict disrupts circadian rhythms in the sea anemone Nematostella vectensis. Elife *12*. 10.7554/eLife.81084.
29. Currie, J., Goda, T., and Wijnen, H. (2009). Selective entrainment of the Drosophila circadian clock to daily gradients in environmental temperature. BMC Biol *7*, 49. 10.1186/1741-7007-7-49.
30. Yoshii, T., Hermann, C., and Helfrich-Forster, C. (2010). Cryptochrome-positive and -negative clock neurons in Drosophila entrain differentially to light and temperature. J Biol Rhythms *25*, 387-398. 10.1177/0748730410381962.
31. Harper, R.E.F., Dayan, P., Albert, J.T., and Stanewsky, R. (2016). Sensory Conflict Disrupts Activity of the Drosophila Circadian Network. Cell Rep *17*, 1711-1718. 10.1016/j.celrep.2016.10.029.
32. Fernandez, M.P., Berni, J., and Ceriani, M.F. (2008). Circadian remodeling of neuronal circuits involved in rhythmic behavior. PLoS Biol *6*, e69. 10.1371/journal.pbio.0060069.
33. Fernandez, M.P., Pettibone, H.L., Bogart, J.T., Roell, C.J., Davey, C.E., Pranevicius, A., Huynh, K.V., Lennox, S.M., Kostadinov, B.S., and Shafer, O.T. (2020). Sites of Circadian Clock Neuron Plasticity Mediate Sensory Integration and Entrainment. Curr Biol *30*, 2225-2237.e2225. 10.1016/j.cub.2020.04.025.
34. Gunawardhana, K.L., and Hardin, P.E. (2017). VRILLE Controls PDF Neuropeptide Accumulation and Arborization Rhythms in Small Ventrolateral Neurons to Drive Rhythmic Behavior in Drosophila. Curr Biol *27*, 3442-3453 e3444. 10.1016/j.cub.2017.10.010.

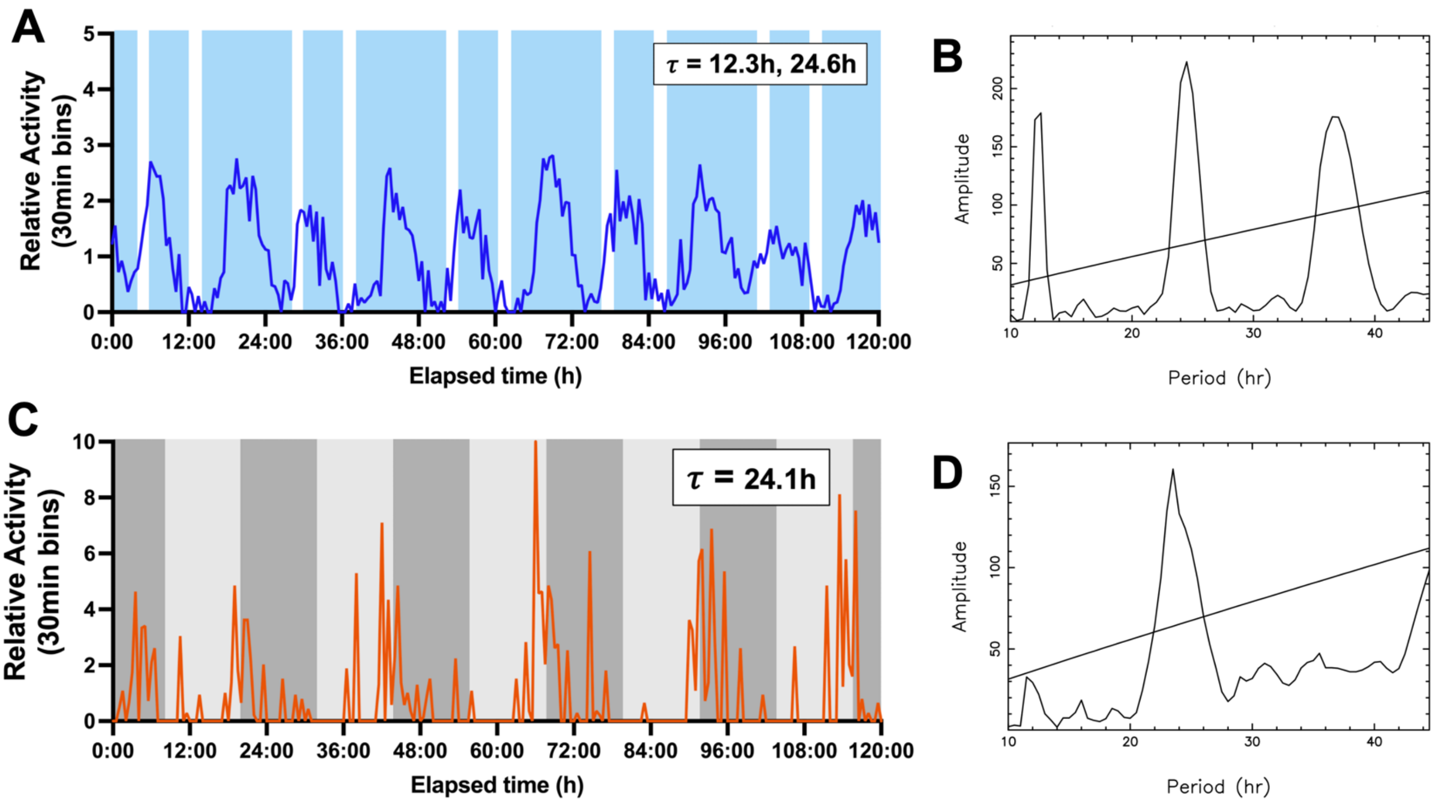

Figure S1. Examples of singlet-rhythmic and doublet-only behavior traces and periodograms, related to Figure 3. (A,C) Relative activity in constant conditions of rhythmic animals after entrainment to a severe mixed tidal cycle. (B.D) Corresponding periodograms. (A,B) Rhythmic animal with a significant singlet and doublet period value or singlet-rhythmic. (C,D) Rhythmic animal with only a significant doublet period value, or doublet-only. Activity is plotted beginning at midnight (4 hours after lights off). Light blue and white shading represent subjective high and low tides, respectively, while gray and light gray shading represents subjective dark and light periods, respectively.

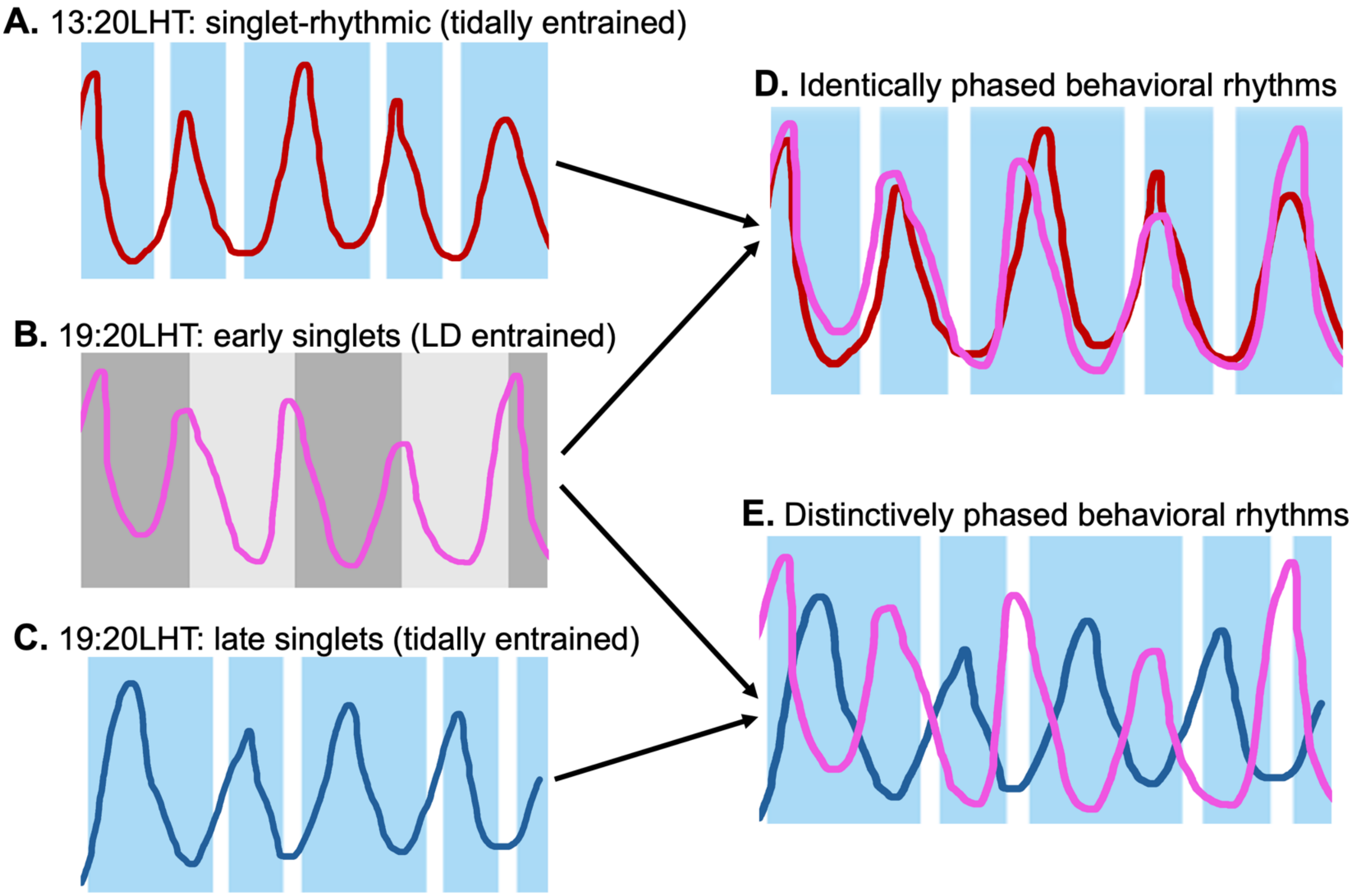

Figure S2. Comparing the phase of "early singlet" behavior trace with the "late singlet" from 19:20LHT and the "singlet-rhythmic" from the 13:20LHT cohort, related to Figure 3. (A) Recreation of the relative average activity of singlet-rhythmic animals after entrainment to a severe mixed tide with 13:20LHT. (B,C) Recreation of the relative average activity of early-singlet (B) and late-singlet (C) animals after entrainment to a severe mixed high tide with 19:20LHT. (D) Overlay of the 13:20LHT singlet-rhythmic and 19:20LHT early-singlet traces reveals similar phasing. Since a LD-entrained singlet-rhythmic cohort should show the same phase as that observed after 19:20 LHT, it would not be distinguishable from tidally-entrained singlet-rhythmic after 13:20LHT. (E) Overlay of the 19:20LHT early and late singlets reveals a distinct relationship, as noted during phase quantification of the 19:20LHT severe mixed singlet-rhythmic individuals.

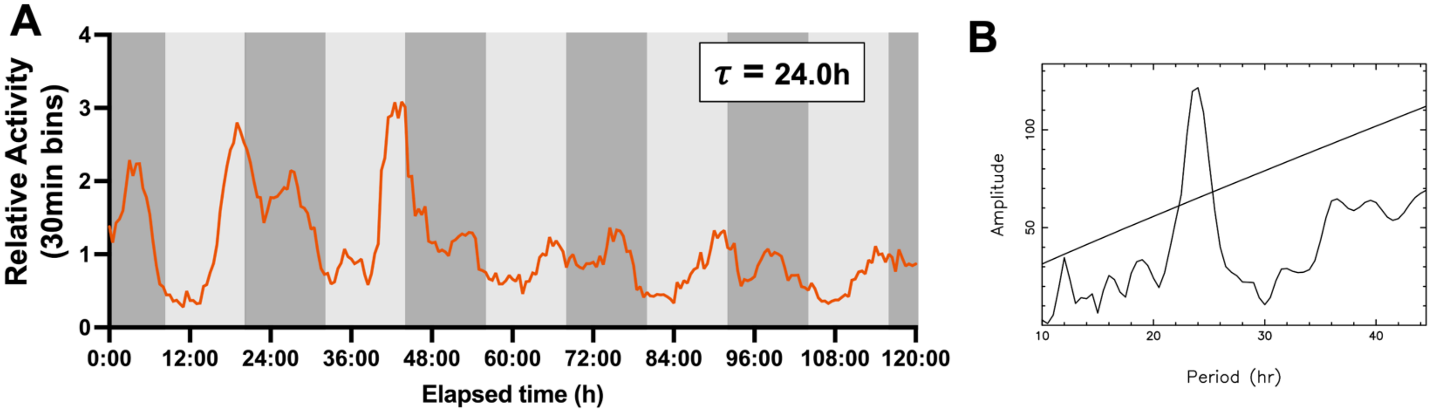

Figure S3. Representative activity trace of a doublet-only individual with two detectable activity peaks, related to Figures 5 and 6. (A) Relative activity trace of a single animal with only a doublet period after entrainment to a diurnal regimen with 19:20LHT. Activity is plotted as a 4h moving average, as was used to define timing of peaks for phase analysis. (B) Periodogram of the activity in (A) demonstrates that despite the appearance of a second activity peak, there was no statistically significant singlet period near 12.4h.