Circadian period-heterogeneity is governed by clonal inheritance of variable gene expression

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ABSTRACT

A ubiquitous feature of circadian clock across life forms is its organization as a network of coupled oscillators. Individual oscillators of the network often exhibit a considerable degree of heterogeneity in their intrinsic periods. While the presence of inter-oscillator coupling is hypothesized to counterbalance such heterogeneity in circadian clock networks, our knowledge of mechanisms governing network heterogeneity remains elusive. In this study, we aimed to explore the principles that might underlie inter-oscillator period variation in circadian clock networks (period-heterogeneity). We adopted a laboratory selection approach and derived a panel of 25 clonal populations exhibiting circadian periods ranging from 22 h to 28 h. We report that while a single parent clone can produce progeny clones with a wide distribution of circadian periods, this period-heterogeneity is not stochastically driven but has a strong heritable component involving the inheritance of variation in gene expression patterns. We then measured expression of 20 circadian clock and clock-associated genes across our panel of 25 clones and identified that inheritance of differential expression patterns in at least five clock genes may govern period-heterogeneity in circadian clock networks.

INTRODUCTION

The majority of life forms on earth exhibit ~24 h (circadian) behavioural and physiological rhythms generated by endogenous time-keeping mechanisms termed circadian clocks. In addition to driving such endogenous rhythms, circadian clocks facilitate synchronization of organisms' rhythms to daily and seasonal changes in the environment to enhance their survivability, thereby functioning as an adaptive mechanism (Kumar, 2017). The fundamental

basis of circadian rhythm-generation across all life-forms are cell-autonomous molecular oscillators comprising evolutionarily conserved auto-regulatory transcription-translation feedback loops (TTFL) (Dunlap, 1999). In higher organisms, such cell-autonomous clocks often function as a network of coupled oscillators, which in unison drive circadian rhythms (Bell-Pedersen *et al.*, 2005). Welsh and co-workers first reported that neurons within the suprachiasmatic nucleus (SCN; the master pacemaker in the hypothalamus of mammals) are surprisingly heterogeneous in their intrinsic periods of circadian firing pattern (Welsh *et al.*, 1995). Subsequent studies revealed that such period-heterogeneity is not restricted to the SCN alone, but is also characteristic of mammalian peripheral clock cells (Nagoshi *et al.*, 2004; Leise *et al.*, 2012) as well as of clock cells in Drosophila (Sabado *et al.*, 2017) and plants (Yakir *et al.*, 2011; Muranaka and Oyama, 2016). The ubiquity of this network feature suggests that heterogeneity may be functionally relevant for circadian clocks (Jagota *et al.,* 2000; Schaap *et al.*, 2003; Gonze *et al.*, 2005; Bernar0d *et al.*, 2007; Inagaki *et al.*, 2007; VanderLeest *et al.*, 2007; Gu *et al.*, 2016, 2019), thus likely being a substrate for natural selection. Interestingly, the observed period-heterogeneity among circadian clock cells within an organism cannot be entirely attributed to functional differentiation of cell subtypes as clonal cells of the same subtype also exhibit such variation (Nagoshi *et al.*, 2004; Leise *et al.*, 2016). Clonal-heterogeneity or clonal-phenotypic variability is common in unicellular organisms as well as cancer cells where mechanisms driving such heterogeneity are considered to be adaptively beneficial (Stockholm *et al.*, 2007). Clonal-heterogeneity can stem from various external factors such as stochastic changes in the microenvironment or internal factors like stochastic partitioning of cellular components during cell-division or stochasticity in gene expression (Hilfinger and Paulsson, 2011;

Swain *et al.*, 2002; Stockholm *et al.*, 2007; Raj and van Oudenaarden, 2008). In this study, we intended to explore the possible mechanisms underlying clonal-heterogeneity of circadian period in human circadian oscillator cells.

We hypothesised that clonal period-heterogeneity in mammalian cells is due to a) stochastic variation (Frank and Rosner, 2012) and/or b) heritable variation (Brock *et al.*, 2009). As the term 'stochastic' is used in the context of both non-heritable (external noise and gene expression noise) as well as heritable gene expression variation (epigenetic stochasticity), for the rest of this manuscript we define 'stochasticity' as any non-heritable variation (both internal and external). To test the two possibilities outlined above, we adopted a laboratory selection approach and established a panel of 25 clonal lines (from a common ancestral/founding culture) exhibiting a range of periods between 22 h and 28 h. We observed that while a single parent cell can produce progeny clones with considerable period-heterogeneity; this is not a stochastic process but has a substantial heritable component. In addition, we discovered that heritable variation in gene-expression levels might underlie clonal period-heterogeneity. Finally, we measured expression of 20 clock genes in our panel of 25 clones and observed that variation in gene expression levels of at least five clock genes might underlie clonal period-heterogeneity.

RESULTS

Clonal period-heterogeneity is not stochastically driven but largely inherited

Is the variation in period of individual circadian oscillator cells just due to intrinsic and/or extrinsic stochastic noise? Or is there a heritable component? To test this, we single-cell cloned a

‘founding culture’ of U-2 OS cells (an established model of peripheral circadian clocks) harboring a *Bmal1*-luciferase reporter construct (Maier *et al.*, 2009). Upon reaching confluence, the period of bioluminescence rhythms from these progeny cultures was determined by live-cell bioluminescence recording. As expected, we observed a distribution of circadian periods (from 23.5 h to 27.5 h; Figure 1a top panel). We repeated this protocol for several ‘assay-generations’ by each time selecting short and long period cultures as ‘parents’ for the next assay-generation (Study outline in Supplementary Figure S1).

Interestingly, by repeating this protocol for several assay-generations we observed a directional divergence of the progeny period-distributions on either side of the ‘founding culture’s’ distribution (Figure 1a). The mean circadian periods of progeny cultures in every assay-generation were always very similar to those of their parental cultures (Figure 1a). Over the course of the selection protocol, the periods of short and long period clonal lines (SCL and LCL) significantly diverged from each other and from the ‘founding culture’. The periods of both SCLs and LCLs differed significantly by assay generation-2 and this difference appeared to reach saturation as the periods did not diverge further (ANOVA followed by Unequal N HSD, $p < 0.001$; Winer, 1991; Figure 1b). At assay generation 4, the circadian periods of LCLs were ~3.4 h longer, and those of SCLs were ~1.7 shorter than the ‘founding culture’ period (Figure 1b; Supplementary Figure S2).

As a measure of period-heritability, we regressed mean periods of the progeny cultures on parental cultures and observed that parental period is a very good predictor of the mean progeny period ($R^2 = 0.96$; Figure 1c) indicating that period-heterogeneity in clonal progeny culture is unlikely to be driven by stochastic variation alone but has a significant heritable component to it.

Inheritance of gene expression levels might underlie period heritability

We further wished to explore the likely basis for heritable variability of the circadian period. During the course of our experiments, we observed that the short and long period clones consistently exhibited low and high bioluminescence intensities/levels respectively (Supplementary Figure S2). This encouraged us to test correlation of the period with other circadian rhythm parameters such as amplitude, damping rate and bioluminescence intensity.

We observed a positive correlation of bioluminescence intensity (Pearson's r = 0.65, p <0.0001) with clone-period; the correlation of relative amplitude with period was negative but not significant (Pearson's r = -0.08, p = 0.19; Figure 2a) and damping rate was not significantly correlated with period either (Spearman's r = 0.26, p =0.06; Figure 2a). We reasoned that mean bioluminescence intensity can, in-principle serve as a proxy for the average expression level of the underlying gene (BMAL1 in this case) and hypothesized that clonal inheritance of average gene expression might underlie the observed period inheritance. This was further supported by the observation that parental bioluminescence intensity was the best predictor of the respective progeny values (R^2= 0.76 Figure 2b), while relative amplitude (R^2= 0.04; Figure 2c) and damping rate (R^2= 0.40; Figure 2d) were only poor predictors.

Clock-gene expression patterns may govern clonal heterogeneity in circadian period

To test, whether differential gene expression correlates with clonal circadian period-heterogeneity, we measured the average expression levels of 20 clock or clock associated genes (Supplementary Table S1) across our panel of 25 single clones, using NanoString multiplex technology. Not surprisingly, we observed a high degree of cross-correlation in expression of the

measured genes (Figure 3a) likely due to the high interconnectivity in the circadian clock molecular loop. We subjected the dataset to Principal Component Analysis (PCA) aiming to extract the major features/genes that might underlie (or is a major contributor to) clonal-period heterogeneity. Based on the Broken-Stick model (Jolliffe , 2011), we retained the first two Principal Components (PCs) which collectively explained 70.2 % of the circadian period variance (Figure 3b). Interestingly, the first two PCs also clustered the panel of clones broadly into 3 groups of short (22.3-23.6 h), intermediate (23.8-26.9 h) and long (27.6-28.2 h) period clones (Figure 3c). PC1 clustered the clones into two groups: i) intermediate periods and ii) the rest including both short and long period clones (non-intermediate). In contrast, PC2 appeared to be important for the formation of the three observed clusters and thus a major determinant of the circadian period (Figure 3c). Based on the cos^2 values (a measure of the quality of representation of the genes on a PC/squared loading values depicted in Figure 3e) and contributions of genes to PC2 (Supplementary Figure S3), we selected the top 25% (ARNTL2, BHLHE40, DBP, NR1D2, PER2) of the genes that we hypothesized might largely account for the period-variation.

We implemented hierarchical clustering of our dataset based on expression levels of the five selected genes and found that the clustering of clones was similar (with one exception) to that by the first two PCs (Figure 3f). The amalgamation schedule suggested a possibility of three clusters (red, blue and green dashed-rectangles, Figure 3f) which was also in agreement with the optimal cluster number reported by five different indices (Supplementary Figure S4).

Clustering-based heat map revealed that the expression of ARNTL2 and BHLHE40 correlated positively with the circadian period, DBP and NR1D2 correlated negatively, while PER2 did not exhibit clear linear trend (Figure 3f, Supplementary Figure S5a). As a control measure, we also

similarly selected the top five genes from PC1 (NR1D1, CLOCK, CSNK1D, CIPC and NFIL3) and, as expected based on PCA (Figure 3c), we observed that these genes were not sufficient to discriminate the short and long periods and resulted in only two clusters – intermediate and non-intermediate (Figure 3g). Interestingly, all five genes from PC1 have higher expression in 'intermediate' period clones and their expression reduced as the period deviates either-way from 'intermediate' (Figure 3g), which also explains their weak linear correlation with period (Supplementary Figure S5b) and will be discussed later.

While the decision of setting a cut-off of 25% was arbitrary, Figures 3a and 3d suggested a reasonably high correlation between BHLHE40 and ARNTL2, and also between NR1D2, DBP and PER2; which might have led to their inclusion in the top 5 contributing genes as an artefact. Therefore, we tested whether the selected five genes is an exhaustive list or could we narrow down to a lower number. To do so, we quantitatively compared clusters generated by top 4 genes with that clustered by top 5 genes (Figure 3f) using the Bk index (Fowlkes and Mallows, 1983) as a measure of similarity between clusters. We observed that choosing four instead of five genes resulted in a reduction in cluster similarity and further collapsing the cluster number to 2 (Supplementary Figure S6a). In addition, there was a large disagreement among the five indices in identifying an optimal cluster number thus suggesting that clustering by four genes might result in unstable clusters (Supplementary Figures S6b-f). On the contrary, increasing the number of selected genes to six resulted in clusters identical to that by the five genes (Supplementary Figures S6b-c).

Together, these results suggest that heritable variation in expression of at least five circadian clock genes might govern period-heterogeneity.

DISCUSSION

We used human U-2 OS cells to investigate whether period-heterogeneity in circadian clock network stems from intrinsic/extrinsic non-heritable stochasticity or whether it has a heritable component. We adopted a laboratory selection protocol (not to be confused with experimental evolution; Garland and Rose, 2009) to select for clonal populations exhibiting short and long circadian periods from which we derived a panel of 25 clonal populations exhibiting circadian periods between 22 h to 28 h.

We observed a directional response to our selection protocol and that parental clones always produced progeny clones with mean periods closely resembling the former (Figure 1a). Consistently, the period of parental cultures was a very good predictor (R^2 = 0.96) of the progeny's mean periods (Figure 1c). Taken together, these results suggest that period-heterogeneity in circadian clock networks is unlikely to be stochastically driven and has a strong heritable component. This raises an interesting question: why would natural selection favour the evolution of heritable mechanisms to drive period-heterogeneity over stochastically driven heterogeneity? While this question falls within the realms of 'ultimate' questions (Laland *et al.*, 2011), we present a possible hypothesis. Although period heterogeneity may be beneficial for circadian clock functions (Jagota *et al.*, 2000; Schaap *et al.*, 2003; Gonze *et al.*, 2005; Bernard *et al.*, 2007; Inagaki *et al.*, 2007; VanderLeest *et al.*, 2007; Gu *et al.*, 2016, 2019), very large heterogeneity can also lead to reduction in amplitude, synchronization and entrainability of the clock (Gonze *et al.*, 2005; Bernard *et al.*, 2007; Gu *et al.*, 2016). Stochastic mechanisms may lead to very large variation in inter-cellular/oscillator period which would be detrimental to the network dynamics, whereas heritable mechanisms may impose phenotypic constraints (Wagner,

2011) within which period-heterogeneity can be maintained thereby being favoured by natural selection.

Over the course of our experiments, we observed that long-period clones often exhibited higher bioluminescence intensity compared to the short-period clones (Figure 2a; Supplementary Figure S2a). Further, the parent-progeny regression revealed that the parent bioluminescence intensity was a good predictor of progeny bioluminescence intensity but this was not the case for either relative amplitude or the damping rate (Figure 2). We reasoned that bioluminescence intensity could serve as a proxy measure for *bmal1* expression; therefore, we hypothesised that the period heritability that we observe is likely to be due to the inheritance of average gene expression from parental clones. To further explore this, we measured average expression of 20 circadian clock and clock-associated genes (Supplementary Table S1) across all 25 clones of our panel. By employing principal component analysis, we identified five genes (ARNTL2, BHLHE40, DBP, NR1D2 and PER2) that grouped the clones into three distinct clusters – short, intermediate and long periods (Figures 3b-f, Supplementary Figures S3a-e). The expression of these genes exhibited a significant linear but inverse correlation with each other and with the period (Figure 3f, Supplementary Figure S5a). While ARNTL2 (BMAL2) and NR1D2 (REVERB-β) strongly correlated with the circadian period, the correlation of BHLHE40 (DEC1), DBP and PER2 expression with period was relatively weak (Figure 3f, Supplementary Figure S5a). Acknowledging that the results of our analysis are mostly suggestive and not confirmatory, we refrain from discussing the possible molecular mechanisms underlying period-heterogeneity; instead, in the following sections we highlight some intriguing observations of our study.

Expression patterns of the five selected genes could individually account for 20-50% of period-variation (Supplementary Figure S5a). In addition, expression patterns of all five genes appeared to be crucial and sufficient for effective clustering of periods while the expression patterns of other genes failed to differentiate between long and short period clones (Figure 3f-g, Supplementary Figure S6). Taken together, these results suggest that that circadian period variation is a multi-gene trait involving a consortium of multiple medium-effect genes. Notably, four of the five above-mentioned genes, with the exception of PER2 (Albrecht *et al.*, 1997) are transcription factors that are either regulated by and/or act on E-boxes in both the core and auxiliary molecular clock loops (Lopez-Molina *et al.*, 1997; Ikeda *et al.*, 2000; Okamura *et al.*, 2002; Kawamoto *et al.*, 2004; Guillaumond *et al.*, 2005; Nakashima *et al.*, 2008; Sasaki *et al.*, 2009; Shi *et al.*, 2010; Takahashi, 2017). This reinforces the idea that while persistence of circadian oscillation requires a functional core clock loop involving negative feedback by the PER-CRY family, modulation of clock period might be governed by communication between multiple loops coupled by E-box associated transcription factors (Zhang and Kay, 2010; Relógio *et al.*, 2011). Another notable gene that our analysis revealed happens to be one of the relatively less studied circadian clock genes ARNTL2 (BMAL2). While ARNTL2 is a functional paralog of the core clock gene ARNTL1 (BMAL1), its precise role in the clock loop remains largely elusive (Ikeda *et al.*, 2000; Sasaki *et al.*, 2009; Shi *et al.*, 2010). Interestingly, we find that ARNTL2 is the highest contributor to principal component-2 that segregates the clones based on period (Figure 3c-e, Supplementary Figure S3) and is also highly correlated with circadian period, independently explaining 49 percent of variance in period (Supplementary Figure 4a) thus emphasizing a potential role of ARNTL2 in circadian period-modulation which awaits further exploration.

In contrast to the above-discussed genes, we find another category among the assayed genes that intriguingly exhibit an inverted-U shaped relationship with period. The expression of these genes (NR1D1, CSNK1D, NFIL3, CLOCK, CIPC) is high in all clones with intermediate periods (23.8-26.9 h) and is drastically reduced in clones with periods deviating from the intermediate range (Figure 3g). Such inverted-U shaped responses (Hormesis) is observed in various biological systems and is regarded as a control system/homeostatic mechanism to prevent cellular/organismal phenotypes from largely deviating from their optimal range (Calabrese and Baldwin, 2001; Baldi and Bucherelli, 2005; Zhang *et al.*, 2008). As discussed earlier, since a higher degree of period-heterogeneity can be detrimental to the circadian clock network, we hypothesize that while there are mechanisms within the clock circuitry that promote period-heterogeneity, the circuit might also harbour hormesis-based mechanisms which impose constraints on the range of period that the circadian clock can exhibit (Baldi and Bucherelli, 2005; Zhang *et al.*, 2008). Such mechanisms may also explain why we observe a saturation of period divergence after assay generation 2 (Figure 1b).

While evidence thus far strongly suggests that clonal period-heterogeneity is driven by differences in clock gene expressions, this raises another interesting question: what is the source of these expression differences? One possible explanation for this is the occurrence of random mutations in our short and long period clones during the course of our selection protocol. There is a possibility that the short and long period clones might have accumulated random mutations resulting in period change and subsequently selected by us. However, we reason that this is highly unlikely because – a) With a mutation rate of ~2.5×10^{-8}/nucleotide in human cells (Nachman and Crowell, 2000), the probability of occurrence of at least two kinds of mutations

within a small fraction of the genome (comprising clock genes) driving short and long periods is extremely low b) We see significant trends in expression of the same subset of genes across all short and long period clones (Supplementary Figure S4). This presupposes that mutations driving short and long periods have occurred within the same genes, which further drastically reduces the probability that the observed period differences stem from random mutations, and c) Even if the mutation rate is higher than we estimate, the saturation of divergence in period over the last 3 assay generations (Figure 1b) cannot be entirely accounted for by mutations since the periods would continue to diverge due to further accumulation of mutations. Therefore, we argue that the observed period-heterogeneity is unlikely to be due to random mutations, which leaves us with another alternative – epimutations. Epimutations are heritable changes in expression of genes and are not associated with DNA mutations. Epimutations are often associated with changes in methylation states of genes or other heritable chromatin modifications (Holliday, 2006). The rates of epimutations are observed to be order of magnitude higher than DNA mutation rates (van der Graaf *et al.*, 2015) and successfully explains phenotypic heterogeneity in many life forms including clonal populations (Kaufmann *et al.*, 2007; Stockholm *et al.*, 2007; Neildez-Nguyen *et al.*, 2008; Taudt *et al.*, 2016; Springer and Schmitz, 2017). Thus, in light of the differences in gene expression patterns in our panel of clones, we hypothesize that epimutations-driven gene expression variation may underlie clonal heterogeneity in circadian period. Further studies exploring the effect of treatment with epigenetic modifiers on the period heterogeneity in our clones can shed more light on this aspect.

In conclusion, our study reports that the heterogeneity in periods observed within circadian clock networks in mammals is not stochastically driven but has a heritable basis and that, this is likely

to be a multi-gene trait. We identified that differential modulation of at least five circadian clock and clock associated genes (including the relatively less studied ARNT2), majority of which are E-box associated transcription factors might govern period-heterogeneity in circadian clock networks. In addition, we also observed a subset of genes that exhibit hormetic gene-expression patterns, which we hypothesize is part of homeostatic mechanisms that may constrain circadian clocks from deviating largely from their optimal period range. Further studies on other models will help address whether this is phenomenon is a ubiquitous feature of mammalian peripheral clocks or circadian clock networks in general across all organisms.

MATERIALS AND METHODS

Clone selection protocol

All clones used in this study were U-2 OS cells (human, ATCC # HTB-96) stably expressing firefly luciferase from a 0.9-kb *Bmal1* promoter (Maier *et al.*, 2009), cultured and maintained in DMEM containing 10% fetal bovine serum, antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). See Supplementary Figure S1 for a pictorial description of the selection protocol. Briefly, cells from 'founding culture' expressing a circadian period of 24.6 ± 0.16 h (mean ± SD) were plated as single-cell clones in 96-well 'parent plates' and grown to confluency. Upon reaching confluency, an 'assay plate' was established for every 'parent plate' by splitting cells from the latter. The period of bioluminescence rhythms from cells in 'assay plates' were recorded (see below for recording protocol) and clones exhibiting short or long periods (tails of the period-distribution) were selected. This protocol was repeated 2-3 times and only clones that consistently exhibited

shot/long periods were selected. Following the selection of clones, corresponding clones from the 'parent plate' were single-cell cloned in 96-well plates, and the procedure was repeated for four assay generations by selecting short and long period clones every generation.

Bioluminescence recording

Cells were plated in white 96-well plate (20×10^3 cells/well), and after 72 hours, cells were synchronized with dexamethasone (1 µM) for 30 minutes, washed with PBS and cultured in Phenol-Red-free DMEM containing 10% fetal bovine serum, antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) and 250 µM D-luciferin (Biothema, Darmstadt, Germany). Bioluminescence was recorded at 37°C in a 96-well plate luminescence counter (TopCount, PerkinElmer, Rodgau, Germany) for up to 7-days. ChronoStar software (Maier *et al.*, *in press*) was used for data analysis and estimation of rhythms parameters including period, decay constant (damping), relative amplitude and average bioluminescence (MESOR) of the oscillation as described previously (Abraham *et al.*, 2010).

RNA preparation and NanoString based gene expression analysis

Five days before the RNA extraction, cells were plated at a density of ~20×10^3 cells in 24-well plate with DMEM containing 10% fetal bovine serum, antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Since we intended to measure average gene expression levels, the culture medium was not replaced for five days to prevent accidental synchronization of cells. On day-5 the medium was removed, 100 µl/well iScript™ RT-qPCR Sample Preparation Reagent (Biorad) was added on top of the cell-layer and incubated at 37 ^{0}C for 5 min. 3µl of the sample

was withdrawn without disturbing the cell-layer and used for further downstream analysis as per manufacturer's instructions.

A previous study of ours combined whole-genome transcriptomics with machine learning and identified genes that could serve as reliable circadian time-telling markers (Wittenbrink *et al.*, 2018). Based on this, we designed a 24-plex NanoString probe panel comprising 20 circadian clock and clock associated genes and 4 housekeeping genes (Supplementary Table S1). The custom-designed probes included a 3'-end biotinylated capture probe and a 5'-fluorescence-barcoded reporter probe for each gene target. Hybridization of probes and gene expression-count reading was according to the manufacturer's instructions. Raw expression data was acquired by a NanoString nCounter Digital Analyzer (NanoString Technologies), QC processed and analysed by nSolver™. QC analysis flagged reads from one (CIART) of the 24 genes in the panel as unsuitable for analysis and was not considered. Data normalization involved three steps: (a) normalization by the arithmetic mean of the positive spike-in controls, (b) subtraction of the mean of the negative controls, and (c) normalization by the geometric mean of the four housekeeping genes.

Principal Component Analysis and Clustering

Log_2-transformed gene expression data were first subjected to Bartlett's Test of Sphericity (Snedecor and Cochran, 1989) to validate its adequacy for Principal Component Analysis (PCA) following which correlation-based PCA was implemented in R (R Core Team, 2018) using factoextra and FactoMineR packages (Kassambara, 2017). Broken-Stick model (Jolliffe, 2011) was used to determine the number of retainable Principal Components (PCs). Determining the optimal cluster-number is often a complication in unsupervised exploratory data analysis. Unlike

many studies in biology that employ PCA to identify genes based on expression differences between known cell-types (which can be used to estimate the optimal number of clusters), our study employs a panel of clones with a continuous distribution of phenotypes (period) and thus cannot be categorized trivially. Hence, we adopted two schemes for optimal cluster-number determination. (a) For agglomerative hierarchical clustering, we assessed the agglomeration schedule of the dendrogram to identify the possible number of clusters (Yim and Ramdeen, 2015). (b) In addition, we also performed k-means clustering for different values of k (1-10) and used 5 different methods (indices) – 'silhouette method' (Rousseeuw, 1987), 'elbow method' (Thorndike, 1953), 'gap-statistic' (Tibshirani *et al.*, 2001), 'Calinski-Harabasz criterion value (variance-ratio method)' (Caliñski and Harabasz, 1974) and Bayesian Information Criterion (BIC; Fraley and Raftery, 2002) to assess the optimal cluster-number. We selected the optimal cluster number based on agreement between (a) and (b). Heatmapper (Babicki *et al.*, 2016) and 'dendextend' (Galili, 2015) were used for hierarchical clustering analysis based on 'euclidean-distance' and 'complete-linkage' measures (Patrik, 2005). 'Nbclust' (Charrad *et al.*, 2015) and 'mclust' (Scrucca *et al.*, 2016) were used for k-means based clustering analysis while for all other statistical analysis and graphing was performed using Prism version 8.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

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AUTHOR CONTRIBUTIONS

Conceptualization: AK and SK; Experiments and data acquisition: NKL and SK. Data analysis: NKL; Manuscript preparation: NKL and AK.

COMPETING INTERESTS

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1: Clonal period-heterogeneity is not stochastically driven but largely inherited

(a) Divergence of the period-distributions of short (red) and long (blue) period clones from a common founding culture (green) across multiple assay generations. Dashed black lines depict the mean of respective period distributions. The grey dashed line extended from assay generation-1 depicts mean period of the founding culture (assay generation 0) for visual assessment of the period divergence. Red (short period clone) and blue (long period clone) arrows indicate the means periods of representative clones selected for the successive generation **(b)** Divergence of the mean period among three representative clonal lines each for long-period clonal line (LCL) 1-3 and short-period clonal line (SCL) 1-3. Error bars are SEMs (n = 3-5 experiments). ¤ indicates that the period of all three SCLs differs significantly from all three LCLs for the given assay generation. Asterisks (*) on top represent LCLs and those at the bottom represent SCLs. Asterisks of different colours indicate that the period of the three clones in that generation is significantly different ($p < 0.001$; n = 3-5) from the periods in other generations, while those with the same colour do not differ significantly. For example, the periods of SCLs 1-3 in generation 2 differ significantly from their periods in assay generation 1 and from the founding culture in assay generation 0, but not from assay generations 3 and 4. **(c)** Regression of parental culture periods across the mean periods of their respective progeny cultures as a proxy-estimate of heritability. Each data point is an average of periods measured across 3-5 experiments. Blue solid line is the linear regression fit with its 95 % CI (blue dots).

Figure 2: Inheritance of gene expression levels might underlie period heritability

(a) Correlations between clone-period and rhythms parameters – relative amplitude (Pearson's r = -0.08, p = 0.19), bioluminescence intensity (Pearson's r = 0.65) and damping rate (Spearman's r = 0.26, p = 0.06). While mean bioluminescence and relative amplitude values were normally distributed (KS distance = 0.08, $p > 0.10$ and KS distance= 0.07, $p > 0.10$ respectively), damping rate was not (KS distance = 0.21, $p < 0.0001$), hence non-parametric Spearman correlation was used for damping rate. Linear regression analysis of mean progeny values across parent values for **(b)** bioluminescence intensity (R^2 = 0.76), **(c)** relative amplitude (R^2 = 0.04) and **(d)** damping rate (R^2 = 0.40). Each data point is an average of periods measured across 3-5 experiments. Blue solid line is the linear regression fit with its 95 % CI (blue dots). (****: $p < 0.0001$).

Figure 3: Clock-gene expression patterns may govern clonal heterogeneity in circadian period

(a) Cross-correlation of average expression values between the 19 analysed genes across all 25 clones indicates a high degree of inter-gene correlation. The colour and size of the circles represent the strength of correlation (Pearson's r). **(b)** Scree plot depicting the percentage of variance explained by the 19 principal components (black bars) and the expected values based on the Broken-Stick model (red line). **(c)** Factor map of individual clones plotted across the principal components (PCs) 1 and 2. PCs 1 and 2 cluster the clones in three clusters of short (red), intermediate (green) and long (blue) period clones. **(d)** Correlation circle depicting the loading of 19 genes across PCs 1 and 2. **(e)** Cos^2 values (squared loadings as a measure of the quality of representation of a gene on a PC) of the 19 genes for PCs 1-5. The colour and size of circles represent the magnitude of Cos^2 value. **(f)** Hierarchical clustering based on the expression of top 5 genes selected from PC2. With the exception of one clone, all others clustered into 3 groups of

short, intermediate and long clones (red, green and blue dashed rectangles respectively). **(g)** Hierarchical clustering based on the expression of top 5 genes from PC1 resulted in 2 clusters – i) intermediate period (green dashed rectangle) and ii) short and long period (blue dashed rectangle). The colour coding of clones in (f) and (g) is the same as in (c).

Figure 1

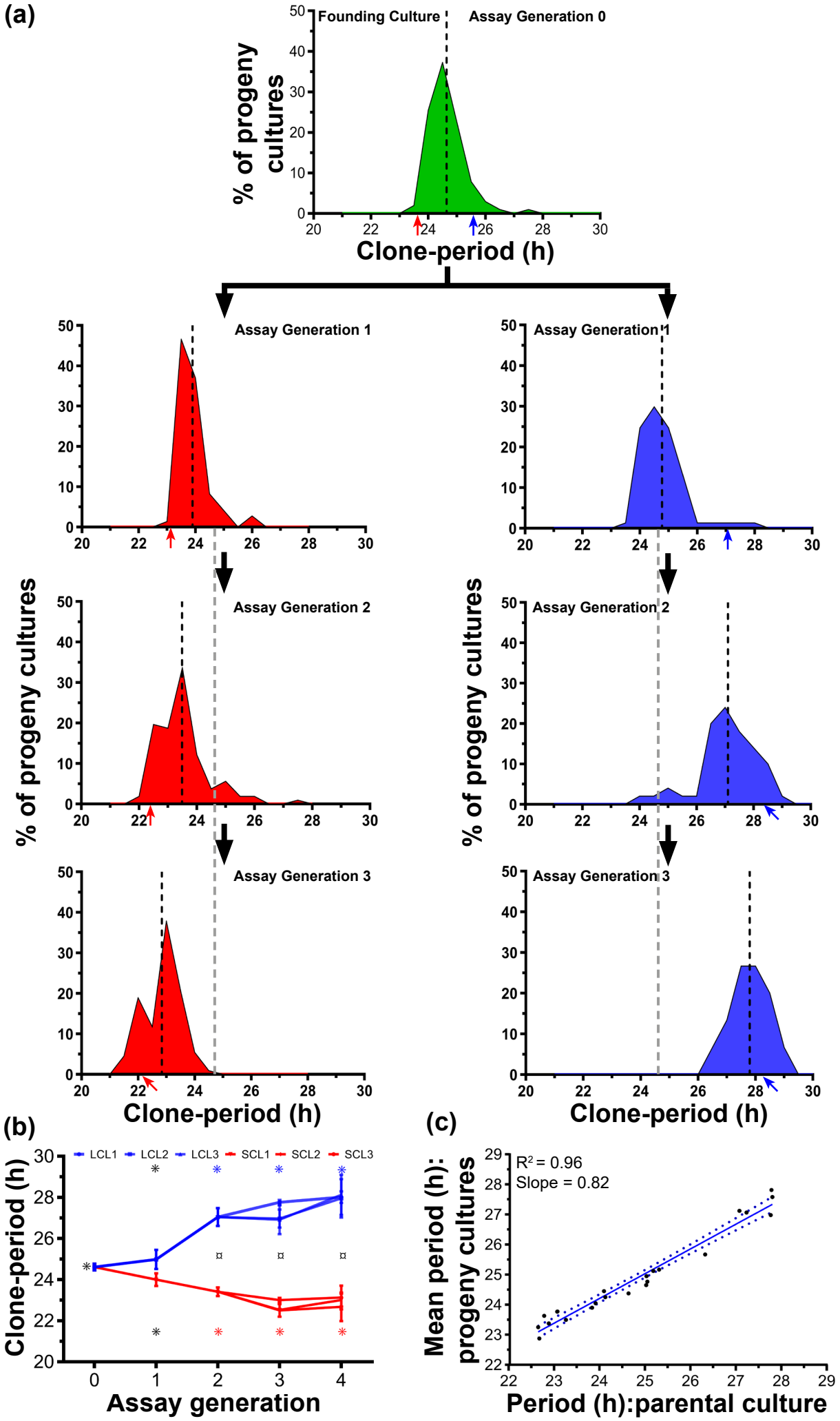

(a)
Founding Culture
Assay Generation 0
% of progeny cultures
Clone-period (h)
Assay Generation 1
Assay Generation 2
Assay Generation 3
(b)
LCL1
LCL2
LCL3
SCL1
SCL2
SCL3
Clone-period (h)
Assay generation
(c)
R² = 0.96
Slope = 0.82
Mean period (h): progeny cultures
Period (h):parental culture

Figure 2

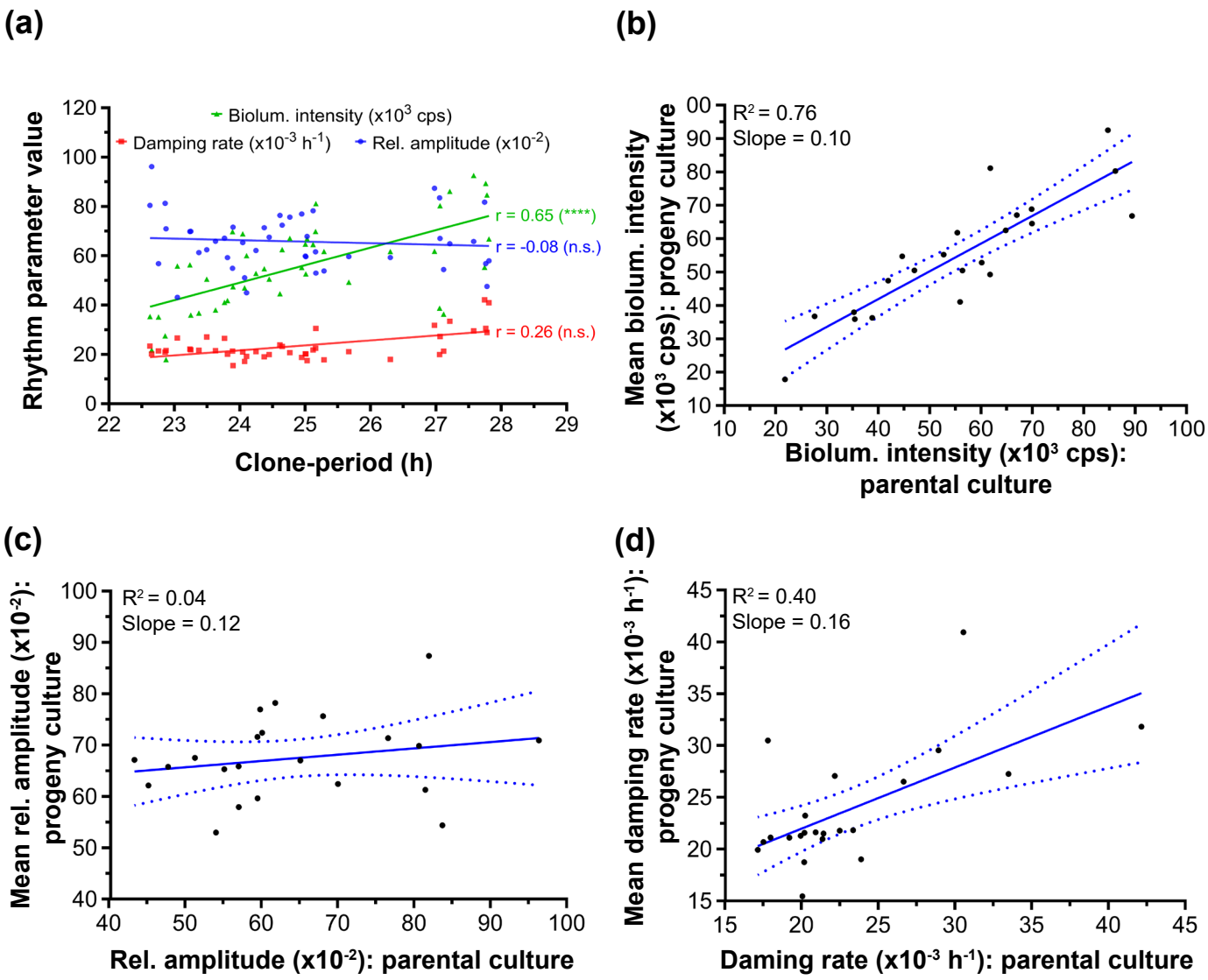
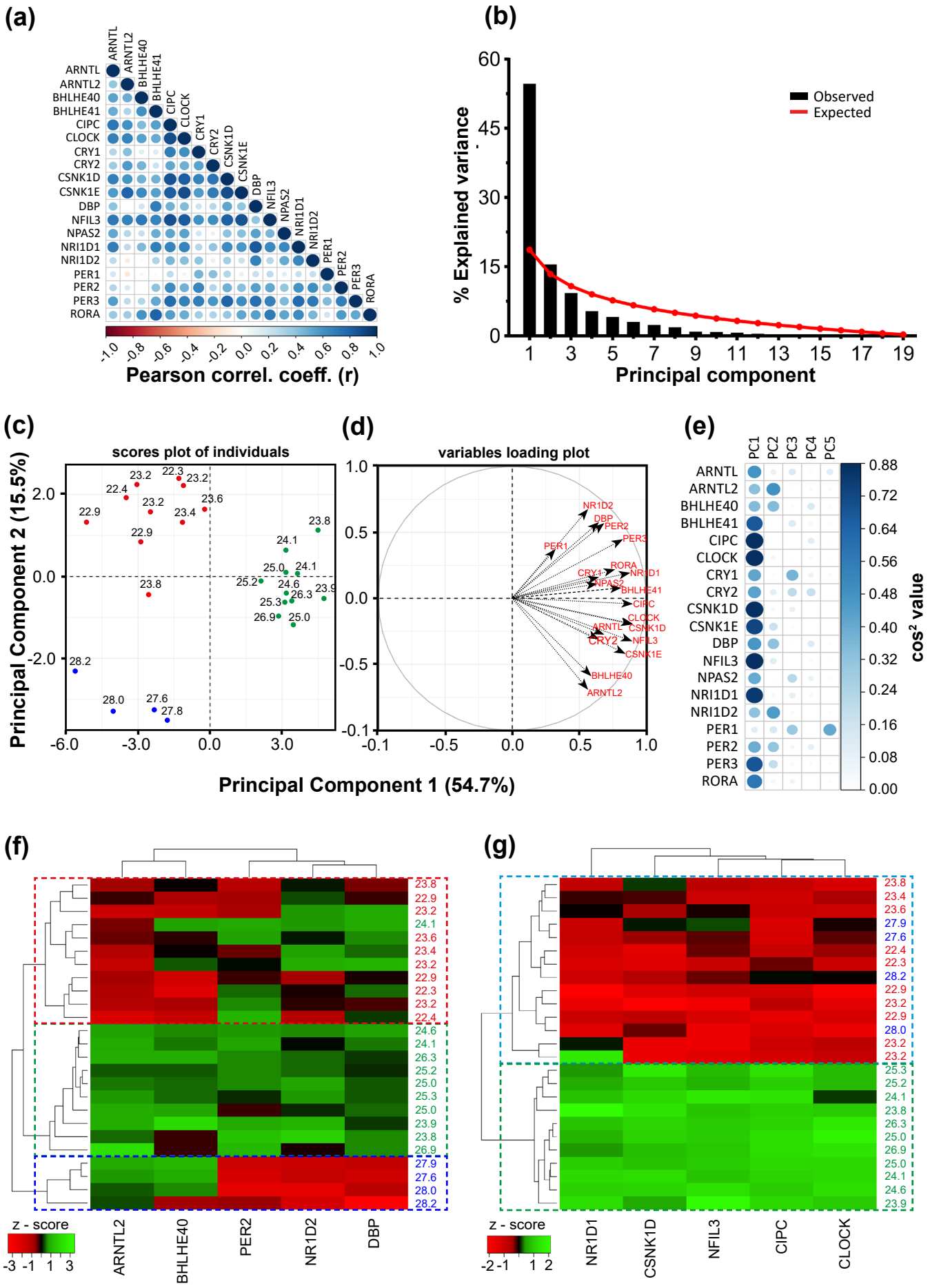

(a)
Biolum. intensity (x10³ cps)
Damping rate (x10⁻³ h⁻¹)
Rel. amplitude (x10⁻²)
r = 0.65 (****)
r = -0.08 (n.s.)
r = 0.26 (n.s.)
Rhythm parameter value
Clone-period (h)
(b)
R² = 0.76
Slope = 0.10
Mean biolum. intensity (x10³ cps): progeny culture
Biolum. intensity (x10³ cps): parental culture
(c)
R² = 0.04
Slope = 0.12
Mean rel. amplitude (x10⁻²): progeny culture
Rel. amplitude (x10⁻²): parental culture
(d)
R² = 0.40
Slope = 0.16
Mean damping rate (x10⁻³ h⁻¹): progeny culture
Daming rate (x10⁻³ h⁻¹): parental culture

Figure 3


(a)
ARNTL
ARNTL2
BHLHE40
BHLHE41
CIPC
CLOCK
CRY1
CRY2
CSNK1D
CSNK1E
DBP
NFIL3
NPAS2
NRI1D1
NRI1D2
PER1
PER2
PER3
RORA
-1.0 -0.8 -0.6 -0.4 -0.2 0.0 0.2 0.4 0.6 0.8 1.0
Pearson correl. coeff. (r)
(b)
% Explained variance
Observed
Expected
Principal component
(c)
scores plot of individuals
Principal Component 2 (15.5%)
(d)
variables loading plot
Principal Component 1 (54.7%)
(e)
PC1 PC2 PC3 PC4 PC5
cos² value
(f)
z - score
ARNTL2 BHLHE40 PER2 NR1D2 DBP
(g)
z - score
NR1D1 CSNK1D NFIL3 CIPC CLOCK