Evidence for circadian-based photoperiodic timekeeping in the Svalbard ptarmigan, the northernmost resident bird.

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SUMMARY

The high Arctic archipelago of Svalbard (74 to 81° North) experiences extended periods of uninterrupted daylight in summer and uninterrupted night in winter, apparently relaxing the major driver for the evolution of circadian rhythmicity. Svalbard ptarmigan (*Lagopus muta hyperborea*) are the only year-round resident terrestrial bird species endemic to the high Arctic and are remarkably adapted to the extreme annual variation in environmental conditions. Here we demonstrate that, while circadian control of behaviour disappears rapidly upon transfer to constant light conditions, consistent with the loss of daily activity patterns observed during the polar summer and polar night, Svalbard ptarmigan nonetheless employ a circadian-based mechanism for photoperiodic timekeeping. First, we show the persistence of rhythmic clock gene expression under constant light within the mediobasal hypothalamus and pars tuberalis, the key tissues in the seasonal neuroendocrine cascade. We then employ a “sliding skeleton photoperiod” protocol, revealing that the driving force behind seasonal biology of the Svalbard ptarmigan is rhythmic sensitivity to light, a feature that depends on a functioning circadian rhythm. Hence, the unusual selective pressures of life in the High Arctic have favoured decoupling of the circadian clock from organisation of daily activity patterns, whilst preserving its importance for seasonal synchronisation.

Keywords: Photoperiodism, Circadian, Seasonal reproduction, Pars tuberalis, Svalbard ptarmigan, the Arctic

RESULTS AND DISCUSSION

The rhythmic expression of circadian clock genes in the mediobasal hypothalamus and pars tuberalis of Svalbard ptarmigan persists under constant light

Svalbard ptarmigan (Figure 1A) show diurnal behaviour patterns under daily light-dark cycles, but rapidly become behaviourally arrhythmic in constant light conditions (Figure 1B, Figure S1) [1, 2]. These data, and similar findings in Svalbard reindeer [3, 4], suggest that some circadian phenotypes are weakened in animals isolated in the high Arctic habitat of Svalbard. The Svalbard ptarmigan, however, uses photoperiod to time seasonal changes in its physiology [2, 5-7], and a vast collection of data supports the role of the circadian rhythm in photoperiodic timekeeping [8-18].

We first used radioactive *in situ* hybridization to examine the transcriptional regulation of circadian genes *Cry1* and *Per2* within the mediobasal hypothalamus (MBH) and pars tuberalis (PT) of the pituitary gland, since these sites control the seasonal neuroendocrine response in other gallinaceous species [19-21]. Our results showed that both genes were strongly rhythmic under short photoperiod (SP, L6:D18) and displayed negligible changes in their expression patterns within the first 24 hours after transfer to constant light (LL) (Figure 1C and 1D). Hence, core elements of the avian circadian clock show persistent endogenous rhythmicity in key photoperiodic response tissues.

In temperate and tropical bird species [19-24] the seasonal reproductive response depends on photoperiodic control of thyrotropin beta subunit (*Tshβ*) expression in the PT and consequent thyrotropin receptor-mediated changes in MBH function exemplified by changes in the expression of the thyroid hormone deiodinase genes, *Dio2* and *Dio3*. Similarly, in the Svalbard ptarmigan, *Tshβ* expression in the PT was continuously suppressed under SP, and transfer to LL strongly induced *Tshβ* expression, which peaked 13 h after lights-on, i.e. CT13 (Figure 1E) ($p < 0.0001$ compared to SP control birds by Sidak's multiple comparisons test after 2-way ANOVA, all test details can be found online https://doi.org/10.18710/LUAHFK), before falling back to SP levels 23h after lights on (CT23). Within the MBH, transfer to LL significantly induced the expression of *Dio2* by CT23 ($p = 0.0011$ compared to SP control by Sidak's *post hoc* test), and suppressed the expression of *Dio3* by CT18 (Figure 1E) ($p = 0.0085$ compared to SP control by Sidak's *post hoc* test). These data show that the temporal dynamics of the "first long day" photoperiodic neuroendocrine response is highly conserved between Svalbard ptarmigan and their relatives from temperate latitudes, i.e. Japanese quail (*Coturnix japonica*) [19].

A sliding skeleton photoperiod triggers the long-day seasonal response in Svalbard ptarmigan

In 1936, Erwin Bünning proposed that photoperiodic sensitivity depends on a circadian rhythm in light sensitivity [25]. A wealth of data supports this hypothesis, confirming that short light-pulses given during

a so-called 'photoinducible phase' are sufficient to drive long-day seasonal response [8-14]. In other words, it is not the cumulative duration of light exposure that triggers a long-day response, but the coincidence of light with an endogenously defined circadian phase.

To test the involvement of circadian rhythms in photoperiodic sensitivity of Svalbard ptarmigan, we exposed our birds to either extended SP, an increasing continuous photoperiod (IP) or a sliding skeleton photoperiod (SkP). The SkP-group mimics the extending range of the IP-group, but maintains the same cumulative hours of light in a 24-h period as in the SP-group (Figure 2A and S2). Expression of a long-day phenotype in the SkP would therefore demonstrate a circadian rhythm in photoperiodic sensitivity in Svalbard ptarmigan. To track the development of a long-day phenotype we monitored activity, body mass, food intake and plasma testosterone; variables that are all under photoperiodic control in Svalbard ptarmigan (Figure S3A) [5-7, 26].

We observed a strong diurnal activity preference within all the groups (Figure 2A). The intervening dark periods of the SkP group also negatively masked activity highlighting the repressive effect of darkness, a somewhat surprising response for a species well accustomed to persistent dark winters. The activity profile of the SP-group went unchanged throughout the entire experiment; however, both the IP-group and SkP-group increased their activity between weeks 5 and 7 (Figure 2B) ($p < 0.05$ for all IP vs SP and SkP vs SP between week 5 and 7 by Tukey's multiple comparisons test after 2-way ANOVA). Whereas the activity increase in the IP-group within this period was proportional to the increased hours of light, the activity of the SkP-group showed a marked 3-fold increase in intensity within the restricted light-hours (Figure 2C) ($p < 0.05$ for all SkP vs SP and SkP vs IP in week 7 by Tukey's *post hoc* test), indicating a photoperiodic stimulation of activity.

Associated with these increases in activity, we observed sustained declines in body mass in both the SkP-group and IP-group, continuing until weeks 9 and 11 respectively (Figure 2D, Figure S3B). Food intake was similar between all three groups until week 10 (Figure S3C), suggesting that these responses were either a consequence of increased activity or altered nutrient absorption resulting in a negative energy balance. Longitudinal assessment of plasma testosterone in male birds (Figure S3D) showed a clear stimulation in week 10 in the IP-group ($p < 0.0001$ for IP vs SP and IP vs SkP by Tukey's *post hoc* test), but no statistically significant changes in the other two groups. Hence the intensification of activity in SkP birds and in IP birds prior to week 10 is not a secondary consequence of gonadal changes, but probably reflects photoperiodic induction of pre-breeding territorial behaviour (Figure S3A) [27, 28].

While the activity level of the IP-group continued to rise throughout the experiment, with maximal activity once the birds experienced LL, the activity of the SkP-group reduced after week 7, returning to

SP levels by week 10 (Figure 2B and 2C) ($p > 0.05$ for SkP vs SP at all points from week 10 onwards by Tukey's *post hoc* test). Prior to week 8 we observed a high intensity of activity prior to the longer light pulse in SkP-birds, which we interpret as dawn-anticipatory activity based on corresponding observations in birds on SNP (Figure 2E). From week 8, this anticipatory activity appears to jump to become associated with the short light pulse (Figure 2E). This suggests that the reversal of the photoperiodic response reflects a "phase jump" in the entrainment of the circadian rhythm, so that the extended dark interval following the 4-h light-block re-aligns from subjective day to subjective night [29].

Because Svalbard ptarmigan are unusual in that intense pre-breeding activity precedes gonadal activation by several weeks (Figure S3A), this phase jump effect explains why no significant increase in testosterone was seen in the SkP-birds. Unstable circadian entrainment leads to light exposure failing to coincide with the photoinducible phase when the interval between the two light pulses is extended beyond 16 hours. Overall, these results support the potential involvement of a circadian-based timing mechanism to mediate spring photoperiodic induction of pre-breeding behaviour in Svalbard ptarmigan, with a photoinducible phase some 14 to 16 h after a lights-on signal (ZT 14-16), and dampening characteristics that may lead to unstable entrainment under experimental photoperiods.

A sliding skeleton photoperiod triggers the long-day photoperiodic neuroendocrine cascade in Svalbard ptarmigan

SkP shows a strong photo-stimulatory effect at week 6 where the second (2-h) light-period falls 14 h after the start of the first light-period. We performed a second experiment to determine if these behavioural and physiological responses correspond to classical photoperiodic regulation of the molecular neuroendocrine cascade within the PT/ MBH region. We compared Svalbard ptarmigan under SP to a SkP in which from week 6 onwards the 2-h block of light was held at 14 to 16 h after the start of the 4-h block of light, i.e. to coincide with the photoinducible phase inferred from the previous experiment (Figure 3A). Longitudinal measurements of activity, body mass and food intake, were consistent with our previous experiment, and showed a persistent impact of the ZT 14-16 light-period on the development of a summer phenotype (Figure S4). We sampled brains at ZT0.5-1.5 to consistently collect samples from the light phase between groups. The SkP-group shows increased expression of *Dio2* ($p = 0.0024$ by unpaired t-test) and decreased expression of *Dio3* ($p = 0.0011$ by unpaired t-test) (Figure 3B). This indicates that through light-stimulation of the photoinducible phase we were able to elicit the classically described changes in MBH thyroid hormone metabolism in our experimental birds. This strongly supports the role of the circadian clock in photoperiodic sensitivity in the Svalbard ptarmigan. Radioactive *in situ* hybridization analysis for *Tshβ* showed no change between

treatments (p = 0.2589 by unpaired t-test). The low *Tshβ* expression in the SkP-group is most likely due to the sampling time point when *Tshb* expression is low in both our "first long day" experiment, and comparable experiments in quail (Figure 1E).

A seasonal imperative for Arctic circadian rhythmicity

Surveys of diel organisation across diverse Arctic species have revealed a diversity of behavioural patterns during the solsticial phases, with rhythmicity largely maintained in some species (e.g. arctic ground squirrel, polar bear, copepod and several migrating birds) [30-35], but largely lost in others (e.g. Svalbard reindeer, Svalbard ptarmigan) [1, 3, 4, 36-38]. This diversity indicates that the moulding effect of Arctic life on the circadian network in relation to diel patterning is highly life-history and ecotype dependent, and therefore likely to resist a unifying rationale for involvement or exclusion. Contrastingly, all species resident in the Arctic must synchronise annual changes in physiology and behaviour to survive throughout the year. Our examination of the Svalbard ptarmigan clearly implicates the circadian system in this seasonal synchronisation: It maintains a rhythmic molecular clock in tissues of the seasonal neuroendocrine axis and functionally employs a circadian rhythm to set a photoinducible phase (Figure 4). We suggest that seasonal synchrony will emerge as a conserved imperative for Arctic circadian organisation.

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AUTHOR CONTRIBUTIONS

Conceptualization, all; Methodology, all; Validation, DA & GCW; Formal analysis, DA & ACW; Investigation, all; Resources, GCW & DGH; Data curation, DA; Writing – Original draft, DA & ACW; Writing Review & Editing, all; Visualization, DA, GCW & ACW; Supervision, DGH, GCW & ACW; Project Administration, all; Funding Acquisition, DGH.

INCLUSION AND DIVERSITY

We worked to ensure sex balance in the selection of non-human subjects.

DECLARATION OF INTERESTS

The authors declare no competing interests.

FIGURE LEGENDS

Figure 1. Persistence of circadian rhythmicity in the pars tuberalis and mediobasal hypothalamus of Svalbard ptarmigan

(A) Svalbard ptarmigan in different plumages. On the left a male in white winter plumage and on the right a female in brown summer plumage (© Ida-Helene Sivertsen).

(B) Representative double plotted actogram of captive Svalbard ptarmigan. SP (5L:19D) entrained birds were directly transferred into LL. The actograms shows 10 days in 5L:19D and 10 days in constant light. Activity was monitored by passive infrared sensors and normalized against its 99th percentile. Grey shading indicates periods of darkness. Additional actograms (n = 25) can be found in Figure S1. Activity was measured in birds unrelated to the gene expression experiment.

(C) Experimental design. Birds entrained to SP (6L:18D) either remained in SP or were transferred directly into LL. Samplings are indicated by arrows and are given in Zeitgeber time (ZT) or circadian time for the LL-group (CT). Both groups were sampled at ZT/ CT 8, 13, 18 and 23. The SP-group was additionally sampled at ZT 3 (ZT 3 was used as initial point for plotting both group, but was omitted from statistical analysis). n = 4 for each sampling point in each group (except CT 13 in LL for which n = 3).

(D) Gene expression for *Per2* and *Cry1* in the MBH and PT between the SP-group (dashed line) and LL-group (solid line). Data is displayed as mean optical densities (OD) ± SEM. Asterisks indicate significant differences between the groups at a given ZT/ CT ($p<0.05$ by Sidak's multiple comparison after 2-way ANOVA). A representative radiograph of a coronal PT/ MBH section for *Cry1* is shown underneath.

(E) Gene expression for *Tshβ*, *Dio2* and *Dio3* in the PT and MBH between the SP- (dashed line) and LL-group (solid line). Gene expression was measured by radioactive *in situ* hybridization and is displayed as mean OD ± SEM. Asterisks indicate significant differences between the groups at a given ZT/ CT ($p < 0.05$ by Sidak's multiple comparison after 2-way ANOVA).

All test details are accessible online (https://doi.org/10.18710/LUAHFK).

Figure 2. Physiological and endocrine responses to increasing photoperiod and a sliding skeleton photoperiod

(A) Experimental design. All bird were initially transferred from DD to SP (6L:18D), which marked the start of the experiment. Birds of the SP-group (n = 10) remained under SP for 12 weeks. Birds of the IP-group (n = 12) were subjected to a stepwise increase in photoperiod by extending the lights-off signal by two hours every week. The light-period of the SkP-group (n = 12) was split into two blocks of light at week 2. The long 4-h light-period remained static while the 2-h light-period shifted backwards weekly by two hours. By week 10, the light-period merged again at which point the birds were back to SP but shifted forward by two hours. Representative single-plotted actograms are displayed next to photoperiodic treatments. Grey shading in the actograms indicate periods of darkness. See also Figure S2.

(B) Activity profiles for each group measured as count/day and displayed as means ± SEM.

(C) Activity profiles presented as counts/ day divided by the hours of light. Data is displayed as means ± SEM.

(D) Changes in body mass measured as grams gained or lost from one week to another. Data is presented as means ± SEM. See also Figure S3B.

(E) Activity 1 h before the light-on signal of both light phases in the SkP group. At week 8, activity before the first light phase decreased while it increased before the second light phase, suggesting that the light-on signal of the second light phase was received as dawn signal from week 8 onwards. Data displayed as mean ± SEM (n = 12).

Statistics: Figure 2B-D were analysed by 2-way ANOVA with *post hoc* Tukey's multiple comparisons test. All test details are accessible online (https://doi.org/10.18710/LUAHFK).

Figure 3. Response of photo-induced genes in the PT and MBH to a skeleton photoperiod

(A) Experimental design. Birds entrained to SP (6L:18D) either remained in SP for 8 weeks (n = 5) or experienced a shifting skeleton photoperiod (n = 5). The light-period of the SkP-group was split into a 4-h and a 2-h light-period in week 2. The 2-h light-period was weekly shifted backward by two hours until week 6 at which point the light-period remained at ZT 14-16 for three weeks. All birds were sampled in week 8 at ZT 0.5-1.5. Representative single-plotted actograms are displayed next to

photoperiodic treatment. Grey shading in the actograms indicate periods of darkness. See also Figure S2.

(B) Gene expression of *Tshβ*, *Dio2* and *Dio3* in the PT and MBH, measured by *in situ* hybridization. Data is presented as mean optical densities (OD) ± SEM and asterisks indicate significant differences between the groups. Gene expression between the groups was compared by t-test (*Tshβ* p = 0.259, t= 1.215, df = 8; *Dio2* p = 0.002, t = 4.349, df = 8; *Dio3* p = 0.001, t = 4.966, df = 8). Representative radiographs are displayed under the respective gene and group.

Figure 4. Adaptation of the circadian system to the Arctic

The Japanese Quail (left panel) uses its circadian system to control activity, as it retains a free running rhythm in prolonged constant darkness (DD) [39, 40]. The circadian system is also employed for photoperiodic time measurement. This is supported by studies using skeleton photoperiods that trigger long day responses, e.g. developing gonads, when the second light-period coincides with the photoinducible phase [10, 20, 41, 42]. We show here that its Arctic relative, the Svalbard ptarmigan (right panel), retains its circadian system, sustains a rhythm of photosensitivity and responds to a correctly timed skeleton photoperiod in the same manner as the quail does. However, due to its high latitudinal environment and the special photic conditions there within we propose that the functional circadian system exhibits weak control over behavioural output.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
DEPC	Sigma	D5758
TriReagent	Sigma	93289
Omniscript RT kit	Qiagen	205111
Taq DNA polymerase	Qiagen	201203
pGEM®-T Easy Vector Systems with JM109 Competent Cells	Promega	A1380
UTP-S35 radio-isotope	PerkinElmer	NEG739H001MC
Riboprobe combination system (SP6/T7)	Promega	P1460
G-50 micro spin Columns	GE Healthcare	GE28-9034-08
AquaLight Beta scintillation cocktail	Gammadata	461-035
OCT embedding matrix	CellPath	KMA-0100-00Y
PFA	Sigma-Aldrich	P6148
NaH2PO4 (for PB buffer)	Sigma-Aldrich	04276
NaH2PO4 1H2O (for PB buffer)	Sigma-Aldrich	S9638
Acetic Anhydride	Sigma-Aldrich	A604
Triethanolamine	Sigma-Aldrich	T1502
NaOH	Sigma-Aldrich	71690
Ethanol 97%	VWR	20823.362
Ethanol 99%	VWR	20821.310
Formamide deionized	Sigma-Aldrich	F9037
Dextran sulphate	Sigma-Aldrich	D8906
50X Denhardts	Sigma-Aldrich	30915
Tris	Sigma-Aldrich	T2694
DTT (10g)	Sigma-Aldrich	D9779
0.5M EDTA	Sigma-Aldrich	E7789
tRNA	Roche	10109525001
Tri Sodium citrate	Sigma-Aldrich	C7254
NaCl	VWR	27808.297
Rnase A	Sigma-Aldrich	R5125
Autoradiography GBX developer	Carestream	P7042
Autoradiography GBX fixer	Carestream	P7167
Na-Heparin 5000IE/ ml	LEO Pharma	Lot: 16071809
Critical Commercial Assays		
Testosterone ELISA kit	MyBioSource	MBS9711529
Deposited Data		
Raw data, figure data, statistical tests, overview over experimental birds and riboprobe sequences	DataverseNO	https://doi.org/10.18710/LUAHFK
Experimental Models: Organisms/Strains		
Svalbard rock ptarmigan (*Lagopus muta hyperborea*)	Own breeding/ Svalbard	N/A
Oligonucleotides		
Primer for *in situ* hybridization synthesis	Sigma-Aldrich	https://www.sigmaaldrich.com/norway.html
Riboprobes for *in situ* hybridization (Ptarmigan specific)	Own design	https://doi.org/10.18710/LUAHFK
Software and Algorithms		

GraphPad Prism 8	GraphPad Software	https://www.graphpad.com/
ImageJ 1.51k	Wayne Rasband	https://imagej.nih.gov/ij/
ActogramJ (plugin for ImageJ)	Schmid et al., 2011	https://bene51.github.io/
ClockLab data acquisition software	Actimetrics	https://www.actimetrics.com/products/clocklab/
Other		
Cryostat CM3050 S	Leica Biosystems	14047033534
SupeFrost® Plus microscopic slides	VWR	631-0108
Triathler liquid scintillation counter	Hidex	425-034
V800 transmission scanner	Epson	EPSONV800
BioMax® MR Film	Carestream	Z350370-50EA
Passive infrared activity recorders	Home-built	N/A
Actimetrics CL200 USB interface	Actimetrics	06115
Ptarmigan food	Fiskå Mølle AS	4120 TAU
Fluorescent strip lights	Osram	L 58W 830 Lumilux
Northlight red light bulb, 15 lm	Clas Ohlson	36-6557
PL3000 analytical balance	Mettler	612421
Himac Centrifuge	Hitachi Koki	CT15RE
GloMax Explorer microplate reader	Promega	GM3500

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Alexander West (alexander.west@uit.no).

Materials Availability

Ptarmigan specific *in situ* hybridization riboprobes generated for this study are available upon request.

Data and Code Availability

All material and data generated during this study are available at DataverseNO (https://doi.org/10.18710/LUAHFK).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

This study was conducted on captive Svalbard ptarmigan (*Lagopus muta hyperborea*). The Svalbard ptarmigan is a subspecies of the Rock ptarmigan (*Lagopus muta*) and is a non-migratory and therefore permanent inhabitant of the high Arctic archipelago of Svalbard (74 to 81 °N). Even though these birds are capable flyers there are isolated from other rock ptarmigan population, which is expressed in low genetic diversity [43] and a different phenotype compared to other ptarmigan populations, e.g. strong body mass cycles [44].

Our facility located at the University of Tromsø operates a breeding program for Svalbard ptarmigan, which is regularly supplemented by birds caught in Svalbard. Experimental birds were hatched from eggs laid by captive Svalbard ptarmigan held in outside-aviaries. Hatching takes place between June and August in each breeding season and chicks are either raised outdoors on the ground or indoors at a photoperiod corresponding to the on- and offset of natural civil twilight in Tromsø (69° 39´N, 18° 57´E). Birds used for our study were transferred into individual cages in light and temperature controlled rooms (ambient temperature kept at 7±3 °C) in September 2017 for the circadian experiment (Figure 1) and in September 2018 for the skeleton photoperiod experiments (Figure 2 and 3). Birds of different sexes were housed together and each room held a maximum of twelve birds for the circadian experiment and a maximum of six bird for the skeleton photoperiod experiments. In both years, the initial photoperiod at transfer was L12:D12 which was thereafter gradually decreased to the respective photoperiodic treatments, which is L6:D18 for the circadian experiment and constant darkness (DD) for the skeleton photoperiod experiments. All birds were fed standardised protein food *ad libitum* (Fiskå Mølle) and provided with fresh water. Light was provided by fluorescent strip lights (Osram) delivering approximately 1000 lux at floor level. Permanent red light illumination (Clas Ohlson) was provided in order to allow husbandry in DD and outside the light hours. Svalbard ptarmigan can handle long-term housing on dim red light or constant dark as they experience many months of constant darkness in their natural environment.

Both sexes were used for the experiments as we have not seen any sex differences in hypothalamic gene expression in our previous study [2]. Similarly, the seasonal rhythm in body mass, activity and food intake is similar between the sexes [5, 26]. A full table with all birds with their respective experimental group and their respective data is available online at DataverseNO (https://doi.org/10.18710/LUAHFK).

All animals were kept in accordance of the EU directive 201/63/EU under licences provided by the Norwegian Food Safety authority (Mattilsynet, FOTS 7971 for the circadian experiment, FOTS 14209 for the skeleton photoperiod experiments).

METHOD DETAILS

Circadian experiment (Figure 1)

Photoperiod was gradually decreased from September 2017 until reaching L6:D18 in mid-November 2017. The circadian experiment took place on the 21st and 22nd December 2017. The experimental birds were divided into two groups. The short photoperiod group (SP-group, n = 20) was kept under L6:D18 while the constant light group (LL-group, n = 16) was directly transferred from L6:D18 into LL on the 21st December. Both groups were then sampled five times with an interval of five hours (the LL group was sampled on the day of transfer). The sampling timed are given in Zeitgeber time (ZT) for the SP-group and CT for the LL-group and are as followed: ZT/ CT 3, 8, 13, 18 and 23 (ZT 0 corresponds to light-on switch for the SP-group). Birds were euthanized and brains were removed within five minutes after. Removed brains were rapidly transferred onto a cooled metal block and ultimately stored at -80 °C until further processing. Brains from four birds were taken per sampling point. However, only three brains could be used for the CT 13 sampling point in the LL-group because one brain was damaged during the sampling procedure. ZT 3 was only sampled ones and was used for plotting of both groups as there is effectively no experimental difference between the groups at this point. ZT 3 was, however, excluded from the statistical analysis. All bird IDs and their corresponding sampling time points is available online at DataverseNO (https://doi.org/10.18710/LUAHFK).

The gene expression data is supplemented by an experiment demonstrating the typical behavioural responses of Svalbard ptarmigan transferred from SP to LL (Figure 1B). In this experiment, we have entrained birds to SP (5L:19D) for three months and transferred them directly into LL. Locomotor activity of all experimental birds was continuously recorded as movement per minute by passive infrared sensors (home-built), mounted on the cage doors. Data were collected by an Actimetrics CL200 USB interface coupled to a PC with the ClockLab data acquisition software version 2.61 (Actimetrics). Activity data was normalized against its 99^{th} percentile for each individual bird (n = 26).

First skeleton-photoperiod experiment (Figure 2)

Photoperiod was gradually decreased from September 2018 until reaching DD (dim red light excepted) on the 13th December 2018 in which they remained until the start of the experiment in the middle of January 2019. On the 19th January 2019 all birds were transferred into L6:D18. This marked the start of this experiment, which lasted 12 weeks. The birds were divided into three groups. The SP control group (n = 10) remained under SP throughout the whole experiment (SP-group). The increasing photoperiod group (n = 12) was subjected to a stepwise increase in photoperiod (IP-group). The light-period was extended by shifting the light-off switch by two hours each week until reaching LL in week

10. Thereafter birds of this group remained in LL for two more weeks until the end of the experiment in week 12.

The skeleton photoperiod group (n = 12) was subjected to a night break protocol in which the initial photoperiodic treatment of L6:D18 was split into two blocks of light from week 2 onwards (SkP-group). The first light-period of four hours remained fixed whereas the second light-period of two hours was shifted weekly backward by two hours. In week 10, the moving block of light joined with the start of the fixed light-period. At this point the light-period was not shifted further and the photoperiod was effectively L6:D18 again, yet shifted forward by two hours compared to the SP-group. Thereafter SkP birds of this group remained in L6:D18 for two more weeks until the end of the experiment in week 12.

We chose skeleton photoperiods over a T cycle design in an attempt to maintain the birds at the same circadian phase. Previous studies on quail demonstrated that T cycle experiments often fail to trigger photoperiodic responses [45, 46] because birds with weak circadian organization re-entrain rather than to track light pulses interrupted by extended dark periods. This confounds the ability of T cycles to show the involvement of a circadian rhythm in some species. We expected our ptarmigan, as a fellow galliformes, to react similarly. The 24h regularity of our skeleton photoperiod design mitigates these factors.

Body mass and voluntary food intake of all birds was measured weekly with an analytical scale (Mettler). VFI was measured once a week from all birds by measuring food eaten within a 24 hours period. In addition blood was taken weekly from four males per group for plasma testosterone measurements at ZT0-4 (blood was not taken in week 11 and 12). Locomotor activity of all experimental birds was continuously recorded as described above.

All bird IDs and their respective photoperiodic treatment is available online at DataverseNO (https://doi.org/10.18710/LUAHFK).

Second skeleton photoperiod experiment (Figure 3)

The second skeleton photoperiod experiment was conducted with birds from the SP-group (n = 10) from the first skeleton photoperiod experiment. This experiment started on 4th April 2019. The birds were separated into two groups. The SP-group (n = 5) further remained under L6:D18 for eight weeks and where sampled at the end of the experiment. The SkP-group (n = 5) went through a similar shifting skeleton photoperiod as described in the previous experiment. However, the two hour light-period was only shifted until reaching ZT 14-16 in week 6 upon which point birds remained on L4:D10:L2:D8 for additional two weeks before they were sampled (ZT 0 corresponds to the lights-on switch from the

fixed four hour light-period). All birds of this experiment were euthanised on week 8 between ZT 0.5 and ZT 1.5. After the euthanasia brains were removed and rapidly transferred onto a cooled metal block until ultimately stored at -80 °C.

Measurements of BM, VFI and activity and blood sampling was conducted in the same manner as in the first skeleton photoperiod experiment and all bird IDs with their respective photoperiodic treatment is available online at DataverseNO (https://doi.org/10.18710/LUAHFK).

Hormone measurement

Blood was taken weekly from four male birds of every group. In the first skeleton experiment, four birds were sampled in each group and each week, except in DD and week 1, in which only a total of four birds were sampled. The data from DD and week 1 was used to plot all groups but was excluded from statistical analysis. In the second skeleton photoperiod experiment, two male birds were sampled in each group and each week. Up to 1 ml of blood was taken with heparinized (LEO Pharma) syringes and transferred into 1.5 ml Eppendorf tubes stored on ice. Within 30 minutes, the blood was centrifuged at 3.000 rpm at 4 °C for 15 minutes (Hitachi Koki). After centrifugation, the plasma was pipetted from the sample and transferred into 60 µl aliquots. The aliquots were frozen at -80 °C until further processing.

Plasma Testosterone concentration was measured with a competitive inhibition ELISA kit (MyBioSource) following the manufacture's manual. Optical density was measured by a microplate reader (Promega) at 450 nm.

cDNA cloning and *in situ* hybridization

Gene expression of seasonal and clock genes in the PT and MBH for the circadian and second skeleton photoperiod experiment was measured by radioactive *in situ* hybridization. All *in situ* hybridization probes (*Tshβ*, *Dio2*, *Dio3*, *Per2* and *Cry1*) are based on RNA extracted from Svalbard ptarmigan brain tissue and were designed using a Icelandic ptarmigan genome [47]. Brain cryo-sectioning, probe synthesis and *in situ* hybridization were performed as reported previously [2, 48] and are described in short as follows.

RNA from Svalbard ptarmigan brain samples was extracted using TriReagent (Sigma-Aldrich) and the extracted RNA was converted into cDNA using the Omniscript RT kit from Qiagen. Subsequent PCR was performed with primers (Sigma-Aldrich) based on the Icelandic rock ptarmigan genome and Taq DNA polymerase (Qiagen). Correct sized PCR products were extracted, cloned into pGEMT easy vectors (Promega), sequenced and verified against the reference genome. Riboprobe sequences are available online at DataverseNO (https://doi.org/10.18710/LUAHFK).

Vectors were linearized and transcribed with a Promega T7/ SP6 Riboprobe combination system in combination with a 35S-UTP isotope (PerkinElmer). Radioactively labelled riboprobes were subsequently purified with G-50 micro spin columns (GE healthcare) and incorporation of the radionucleotide into the riboprobe was measured as counts per minute by a liquid scintillation counter (Hidex, scintialtion cocktail form Gammadata).

Frozen brains were cryo-sectioned coronally (Leica Biosystems) on the level of the pars tuberalis and the mediobasal hypothalamus and were mounted onto pre-coated adhesion microscopic slides (VWR). Sections were fixed in 4 % PFA (in 0.1 M PB) for 20 minutes on ice. Sections were rinsed twice with 0.1 M PB for 5 minutes after fixation. Next sections were acetylated with 3.75 % v/v of acetic anhydride in 0.1 M triethanolamine buffer (0.05 N NaOH). Slides were rinsed twice with 0.1 M PB for 5 minutes after acetylation, dehydrated with stepwise increasing ethanol solutions (50 %, 70 %, 96 %, 100 % for 3 minutes each) and dried under vacuum for at least 1 hour. Dried sections were hybridized overnight at 56°C with radioactively labelled riboprobe in hybridization buffer (50 % deionised formamide, 10 % dextran sulfate, 1 x Denhardt's solution, 300 mM NaCl, 10 mM Tris, 10 mM DTT, 1 mM EDTA, 500 µg/ml tRNA). The amount of added riborpobe equals 10^6 counts per minute for each microscopic slide. Hybridized sections were washed with 4 x saline sodium citrate (SSC) solutions (3 x 5 minutes) and treated with RNase-A solution (20 µg/ml RNase A, 500 mM NaCl, 1 mM Tris, 1 mM EDTA) for 30 minutes at 37 °C. After RNase-A treatment stringency washes were performed with SSC of decreasing concentration: 2 x SSC (2 x 5 minutes), 1 x SSC (1 x 10 minutes), 0.5 x SSC (1 x 10 minutes), 0.1 x SSC (30 minutes at 60°C), 0.1 x SSC (rinse). SSC solutions were each supplemented with 1 mM DTT.

After stringency washing slides were dehydrated in stepwise increasing ethanol solutions (50 %, 70 %, 96 %, 100 % for 3 minutes each) and dried under vacuum. Once sections were dry, they were exposed to autoradiographic films (Carestream) for 10 to 25 days. Exposed films were developed (Carestream), fixed (Carestream) and digitalised with a transmission scanner (Epson). Optical density (OD) was measured with ImageJ (Wayne Rasband).

Quantification and Statistical Analysis

All graphs and statistical test were prepared in GraphPad Prism (Version 8.3.0, San Diego, CA, USA). Seasonal and clock gene expression of the circadian experiment was analysed with 2-way ANOVA with *post hoc* Sidak's multiple comparisons test (Figure 1D-E). 2-way ANOVA with *post hoc* Tukey's multiple comparisons test was used to examine changes in body mass, activity (in activity/ day and in activity/ day divided by the photoperiod in h), plasma testosterone and food intake in the first skeleton-photoperiod experiment (Figure 2B-E and S3). Activity, body mass and food intake of the second skeleton photoperiod experiment was analysed by 2-way ANOVA with *post hoc* Sidak's multiple

418 comparison test (Figure S4). Relative gene expression between the SP-group and SkP-group of the
419 second skeleton photoperiod experiment was tested with unpaired t-tests.

420 Activity was normalized by dividing counts per minute of each bird by its 99th percentile and actograms
421 (Figure 2A, 3A, S1 and S2) were plotted with ActogramJ [49], a plugin for ImageJ (Wayne Rasband).

422 Results of statistical tests are available online at DataverseNO (https://doi.org/10.18710/LUAHFK).

SUPPLEMENTAL INFORMATION

Figure S1. Double plotted actograms of captive Svalbard ptarmigan transferred from 5L:19D into LL.

SP entrained birds (n = 26) were directly transferred from 5L:19D into LL. The actograms shows 10 days in 5L:19D and 10 days in constant light. Activity was monitored by passive infrared sensors and normalized against its 99th percentile. Grey shading indicates periods of darkness. Activity was measured in birds unrelated to the gene expression experiment.

Figure S2. Response in body mass and food intake to increasing and skeleton photoperiod.

(A) Simplified response of Svalbard ptarmigan to increasing vernal photoperiod. Svalbard loose body mass while increasing in activity due to pre-breeding behaviour early in the spring. The response in reproduction is delayed and egg laying takes place mid-June in their natural habitat.

(B) Weekly body mass and is displayed as mean ± SEM

(C) Weekly voluntary food intake measured as grams of food eaten in a 24-h period. Data is presented as mean ± SEM.

(D) Plasma testosterone of male birds measured as ng/ml and displayed as means ± SEM.

Figure S3. Physiological and endocrine responses in the second skeleton photoperiod experiment.

(A) Activity measured as counts/ day and displayed as mean ± SEM

(B) Weekly body mass is displayed as mean ± SEM.

(C) Weekly body mass changes displayed as mean ± SEM.

(D) Weekly voluntary food intake measured as grams eaten in a 24-h period and displayed as mean ± SEM.

(E) Weekly plasma testosterone in male birds measured in ng/ml and displayed as mean ± SEM.

Figure S4. Double plotted actograms of all experimental birds of the skeleton photoperiod experiments.

(A) Actograms correspond to experimental design of Figure 2A

(B) Actograms correspond to experimental design of Figure 3A

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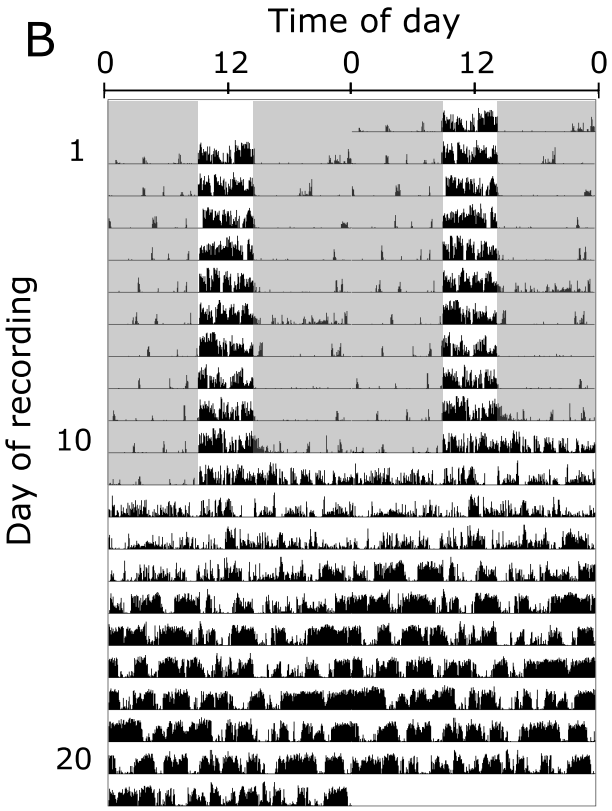
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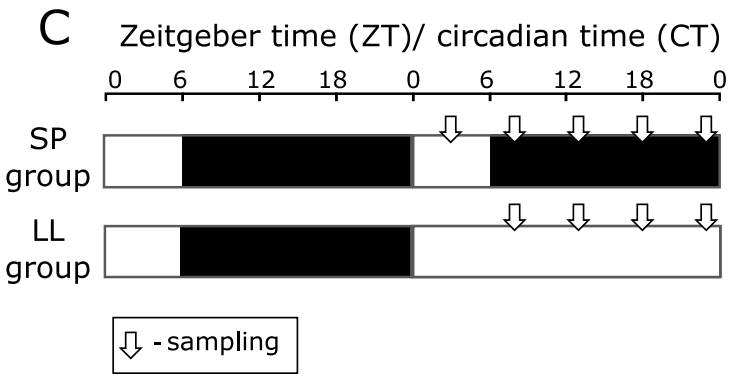
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SP group
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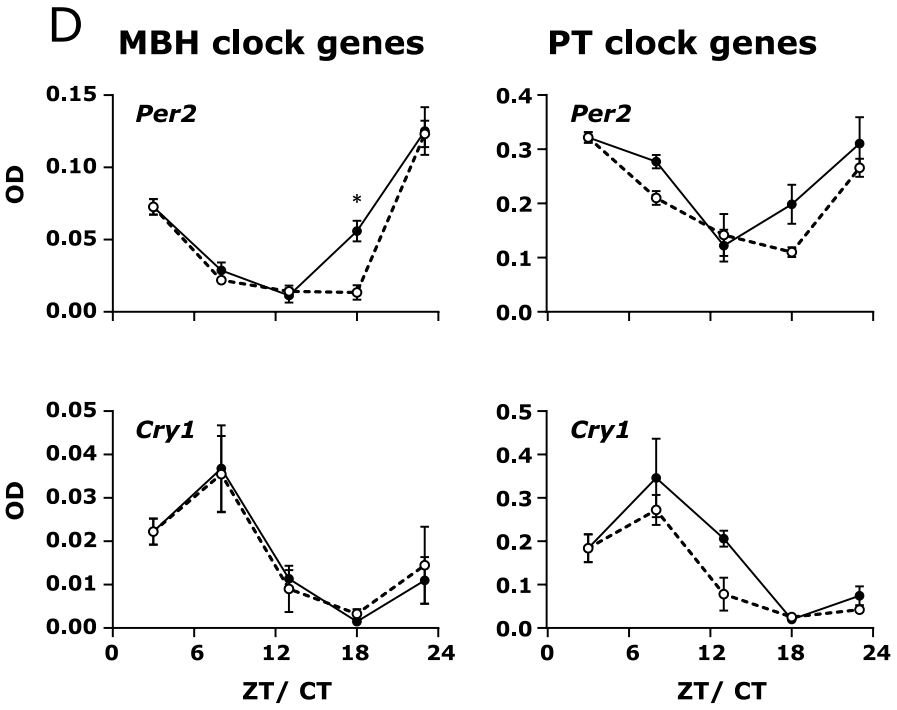
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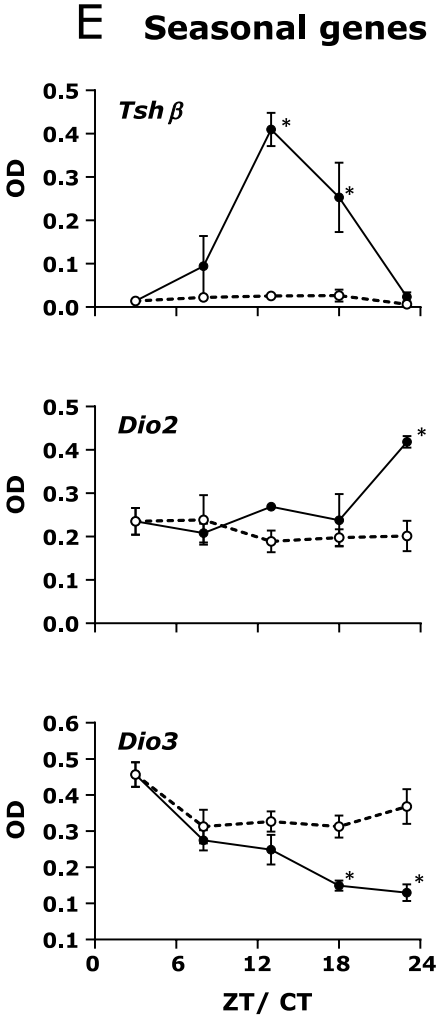
PT clock genes
Per2
Cry1
OD
ZT/ CT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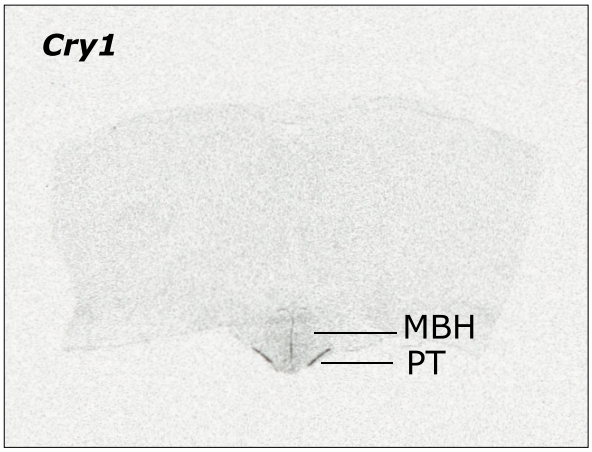
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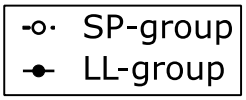
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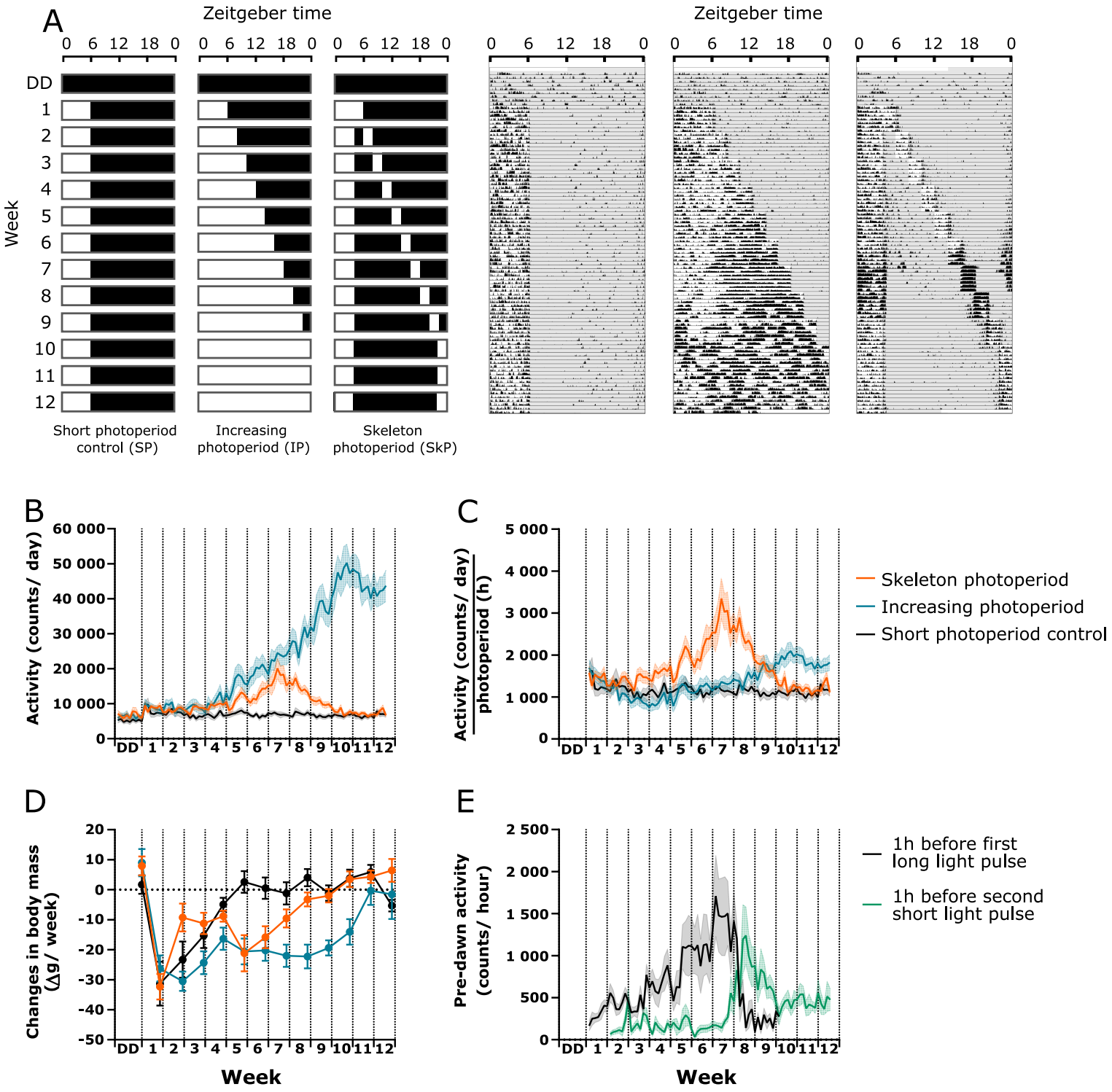
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Increasing photoperiod (IP)
Skeleton photoperiod (SkP)
B
Activity (counts/ day)
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50 000
40 000
30 000
20 000
10 000
0
C
Activity (counts/ day) / photoperiod (h)
5 000
4 000
3 000
2 000
1 000
Skeleton photoperiod
Increasing photoperiod
Short photoperiod control
D
Changes in body mass (Δg/ week)
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10
0
-10
-20
-30
-40
-50
Week
E
Pre-dawn activity (counts/ hour)
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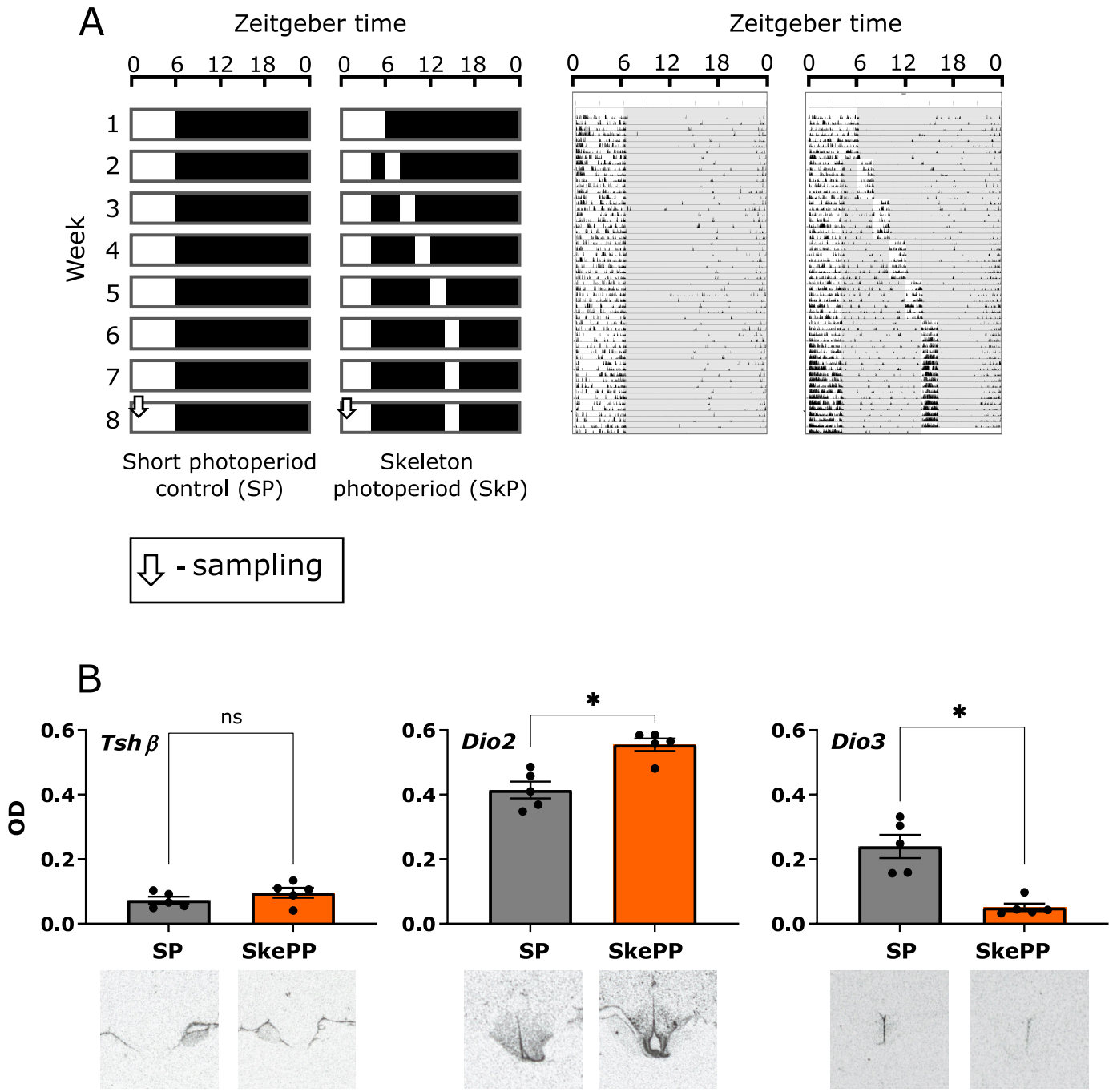
1h before second short light pulse
Week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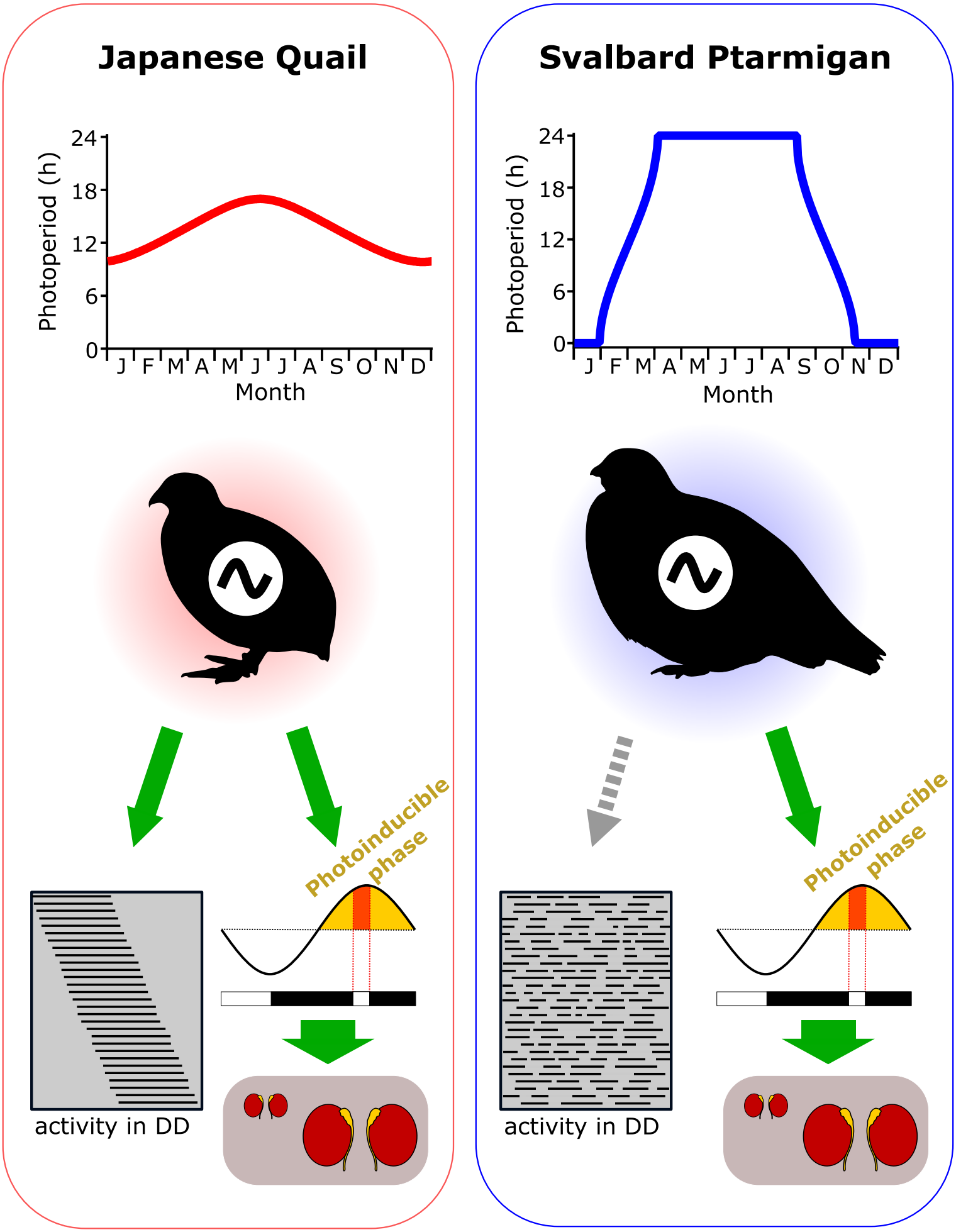
Japanese Quail
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activity in DD
Svalbard Ptarmigan
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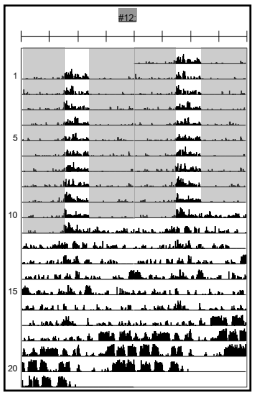
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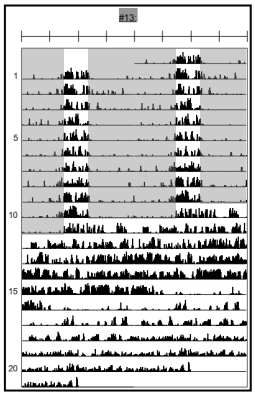
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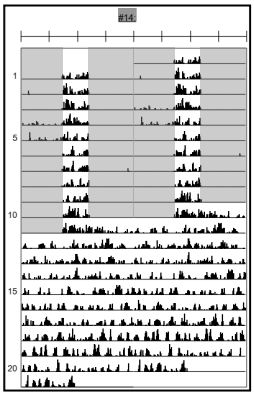
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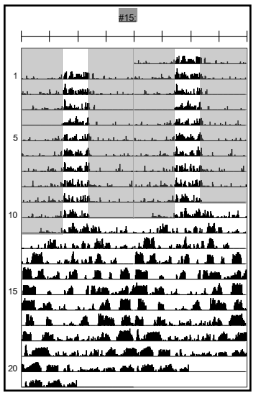
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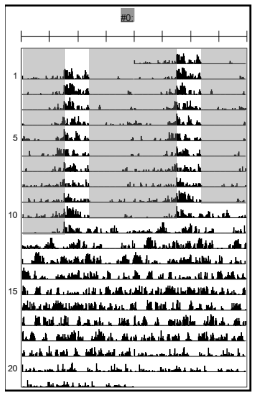
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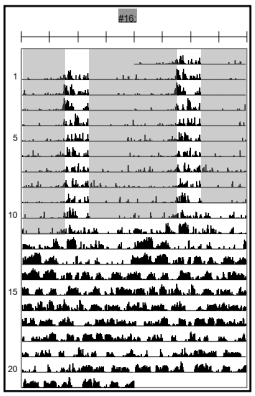
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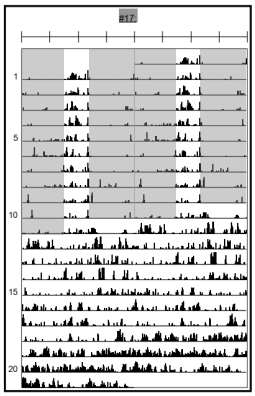
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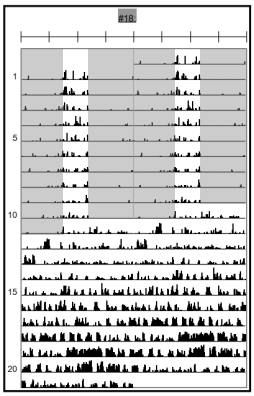
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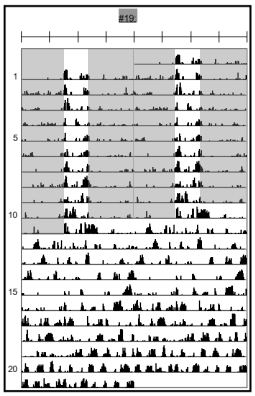
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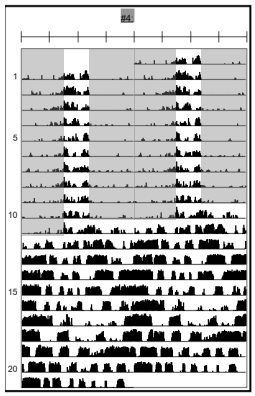
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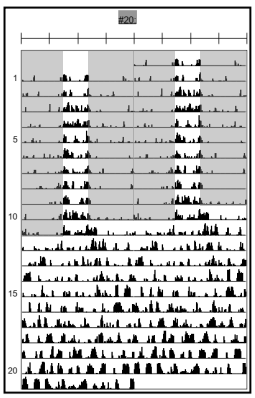
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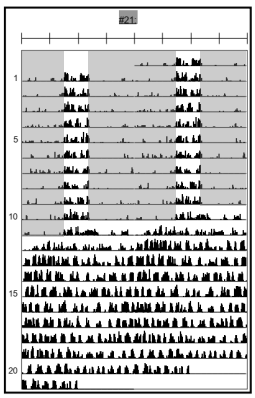
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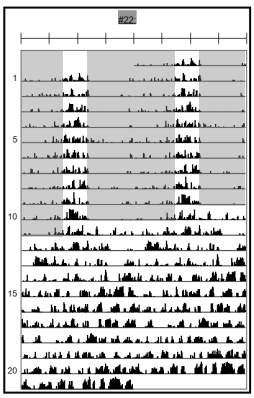
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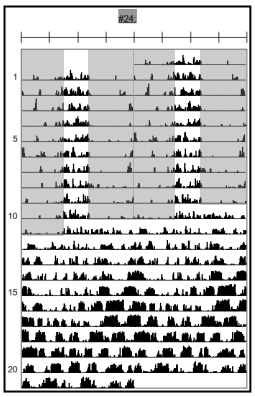
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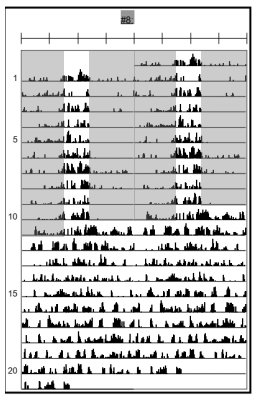
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15
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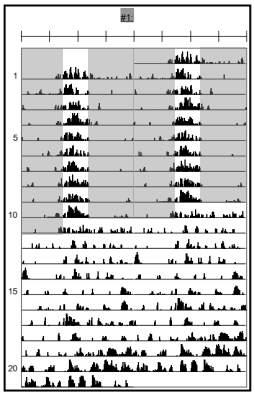
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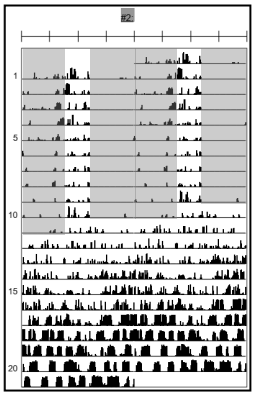
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1
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15
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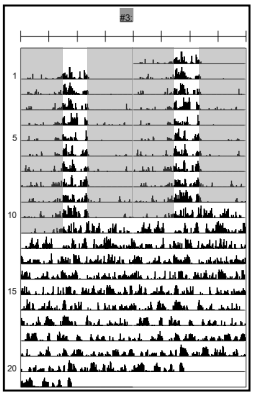
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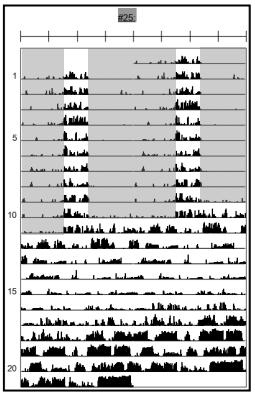
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1
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15
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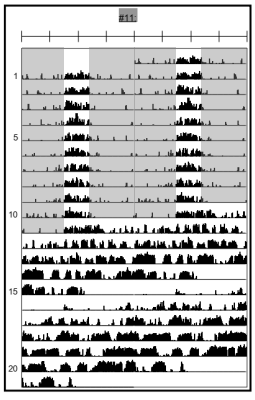
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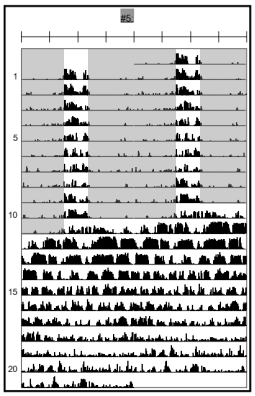
#5
1
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10
15
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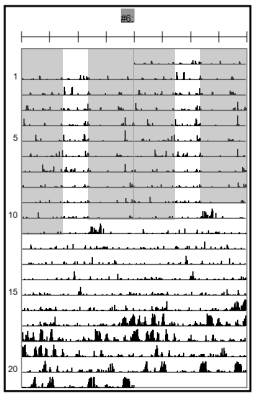
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10
15
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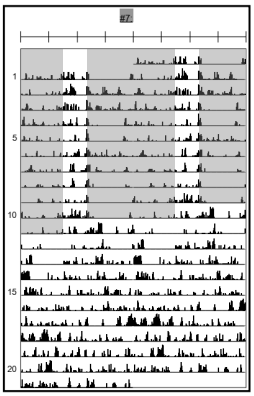
#7
1
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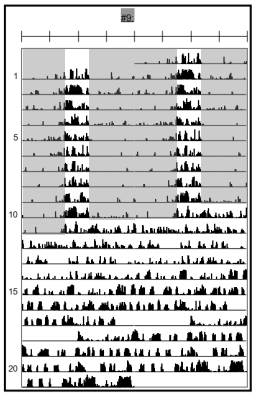
10
15
20


#9
1
5
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15
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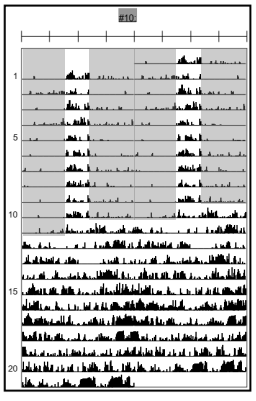

#10
1
5
10
15
20

Figure S1. Double plotted actograms of captive Svalbard ptarmigan transferred from 5L:19D into LL, Related to Figure 1.

SP entrained birds (n = 26) were directly transferred from 5L:19D into LL. The actograms show 10 days in 5L:19D and 10 days in constant light. Activity was monitored by passive infrared sensors and normalized against its 99th percentile. Grey shading indicates periods of darkness. Activity was measured in birds unrelated to the gene expression experiment.

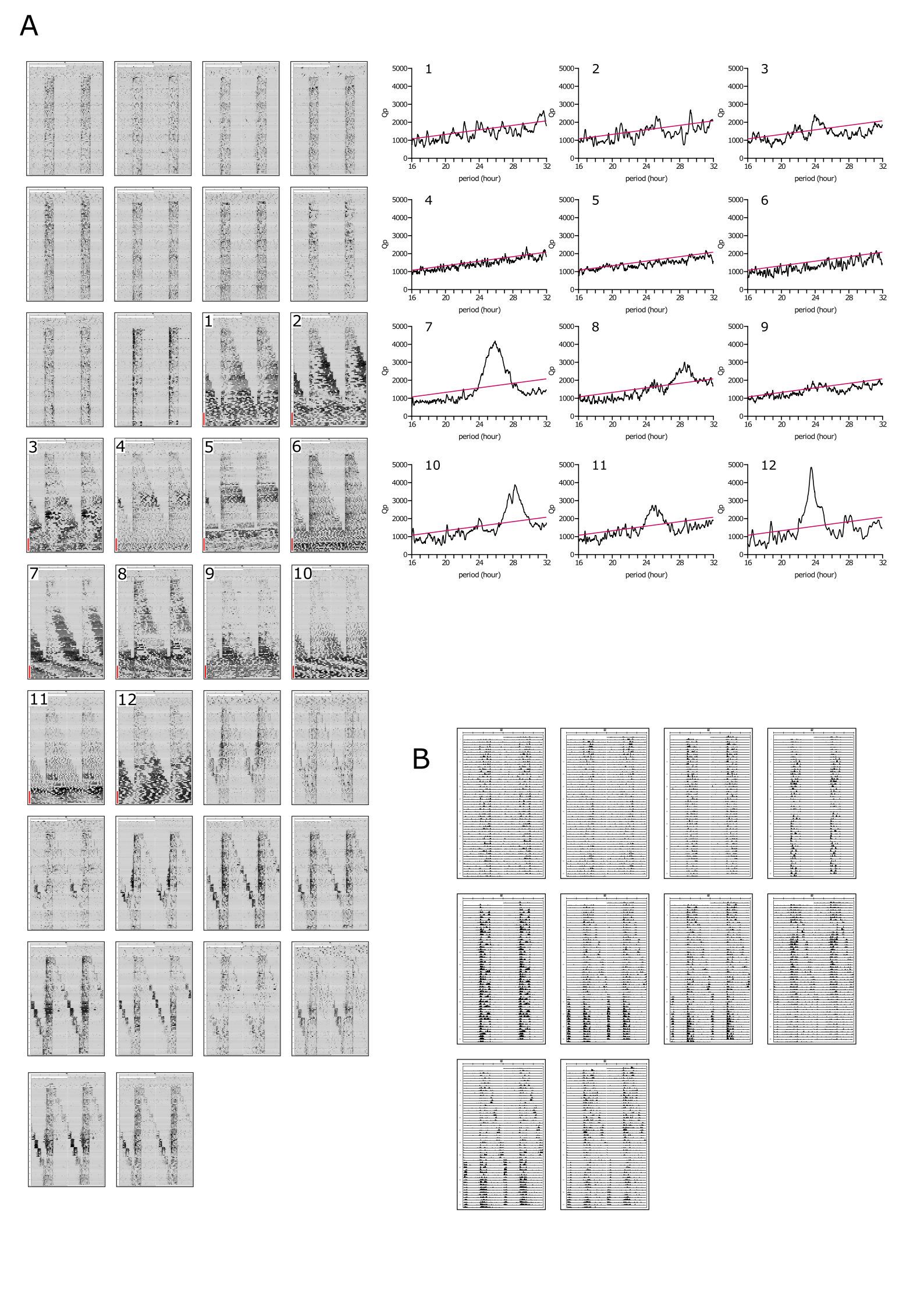
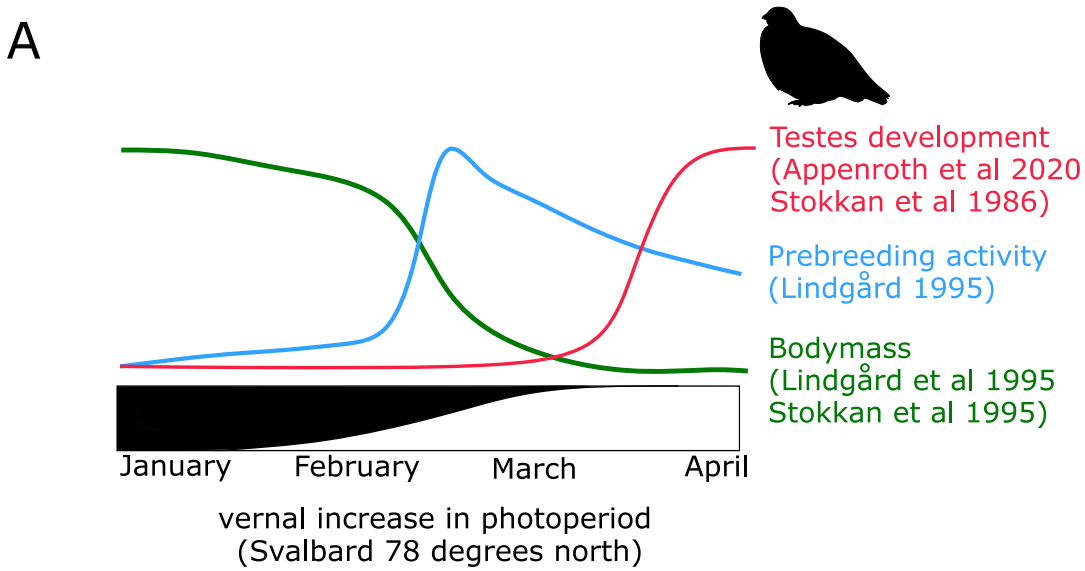

A
1
2
3
4
5
6
7
8
9
10
11
12
Qp
period (hour)
B

Figure S2. Double plotted actograms of all experimental birds of the skeleton photoperiod experiments, Related to Figures 2 and 3.

(A) Actograms correspond to experimental design of Figure 2A

(B) Actograms correspond to experimental design of Figure 3A


A
Testes development
(Appenroth et al 2020
Stokkan et al 1986)
Prebreeding activity
(Lindgård 1995)
Bodymass
(Lindgård et al 1995
Stokkan et al 1995)
January
February
March
April
vernal increase in photoperiod
(Svalbard 78 degrees north)

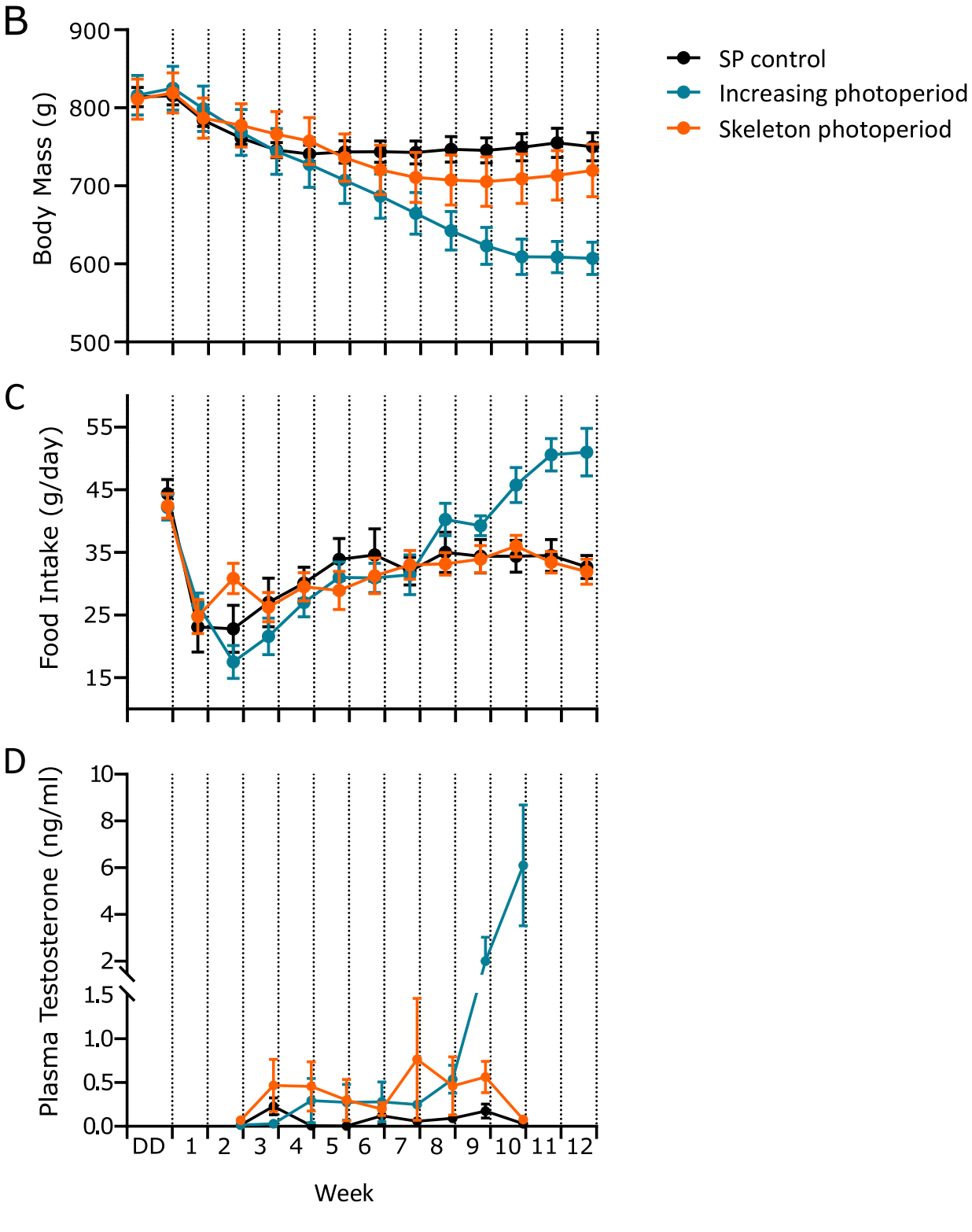

B
Body Mass (g)
900
800
700
600
500
SP control
Increasing photoperiod
Skeleton photoperiod
C
Food Intake (g/day)
55
45
35
25
15
D
Plasma Testosterone (ng/ml)
10
8
6
4
2
1.5
1.0
0.5
0.0
DD
1
2
3
4
5
6
7
8
9
10
11
12
Week

Figure S3. Response in body mass and food intake to increasing and skeleton photoperiod, Related to Figure 2.

(A) Simplified response of Svalbard ptarmigan to increasing vernal photoperiod. Svalbard loose body mass while increasing in activity due to pre-breeding behaviour early in the spring. The response in reproduction is delayed and egg laying takes place mid-June in their natural habitat.

(B) Weekly body mass and is displayed as mean ± SEM

(C) Weekly voluntary food intake measured as grams of food eaten in a 24-h period. Data is presented as mean ± SEM.

(D) Plasma testosterone of male birds measured as ng/ml and displayed as means ± SEM.

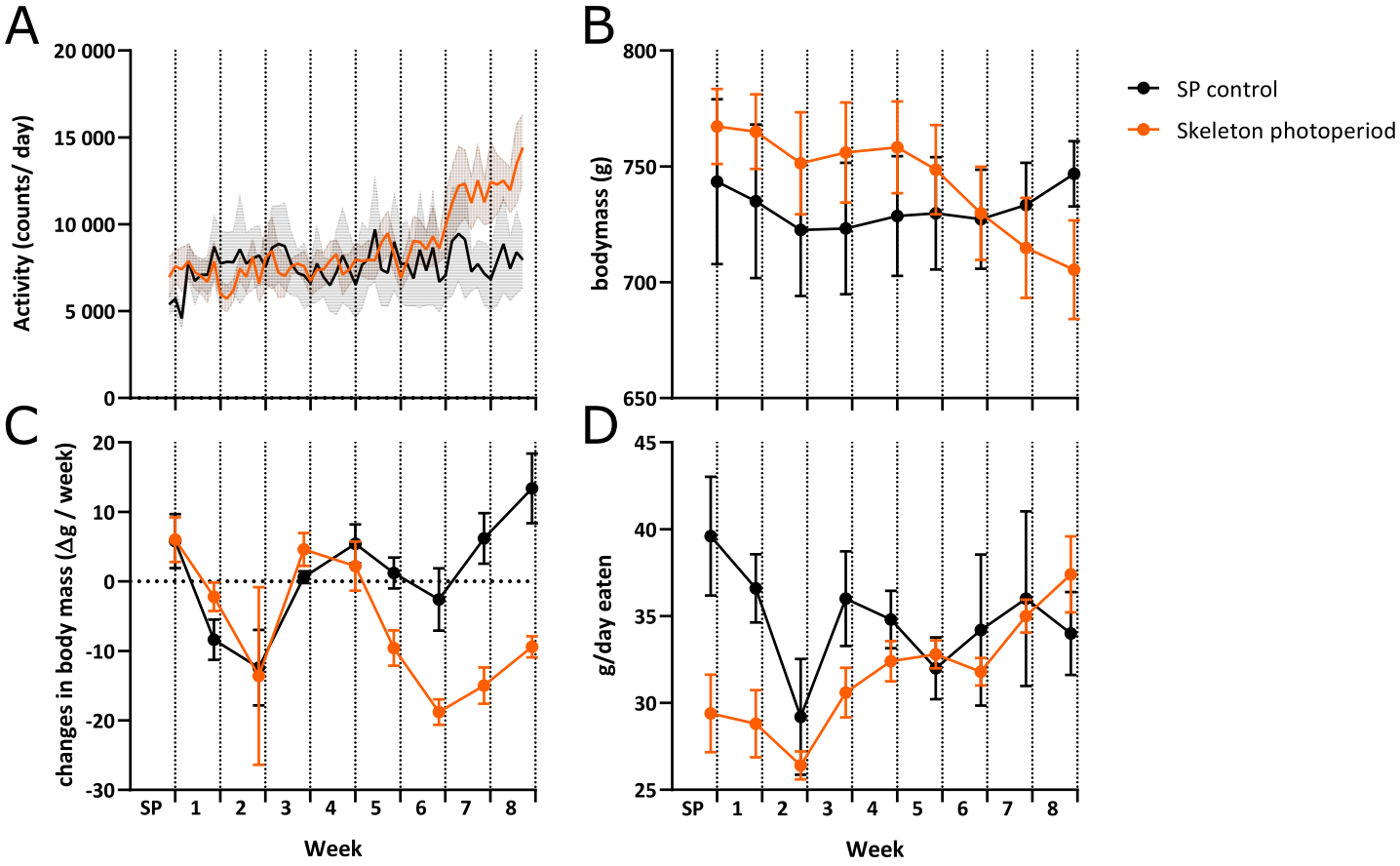

A
Activity (counts/ day)
20 000
15 000
10 000
5 000
0
B
bodymass (g)
800
750
700
650
C
changes in body mass (Δg / week)
20
10
0
-10
-20
-30
SP
1
2
3
4
5
6
7
8
Week
D
g/day eaten
45
40
35
30
25
SP
1
2
3
4
5
6
7
8
Week
SP control
Skeleton photoperiod

Figure S4. Physiological and endocrine responses in the second skeleton photoperiod experiment, Related to Figure 3.

(A) Activity measured as counts/ day and displayed as mean ± SEM

(B) Weekly body mass is displayed as mean ± SEM.

(C) Weekly body mass changes displayed as mean ± SEM.

(D) Weekly voluntary food intake measured as grams eaten in a 24-h period and displayed as mean ± SEM.

(E) Weekly plasma testosterone in male birds measured in ng/ml and displayed as mean ± SEM.