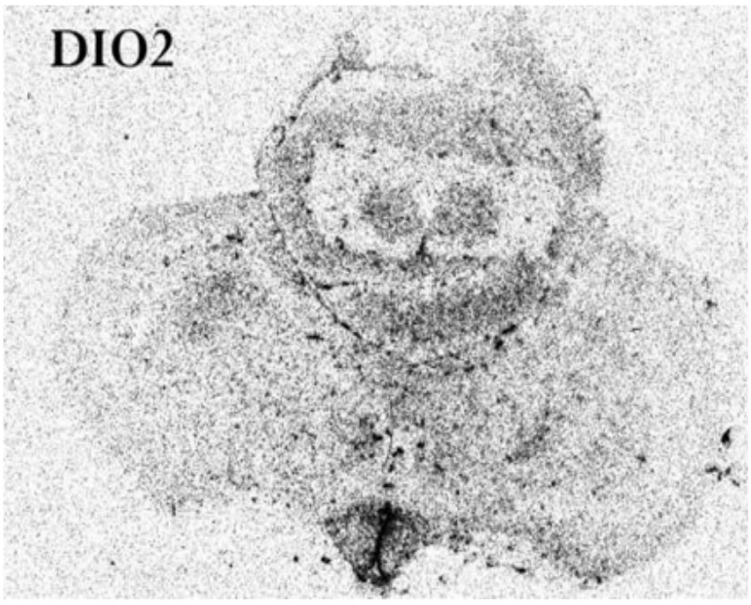
UiT The Arctic University of Norway

Faculty of Biosciences, Fisheries and Economics
Department of Arctic and Marine Biology

Circadian-based processes in the High Arctic: activity, thermoregulation and photoperiodism in the Svalbard ptarmigan (*Lagopus muta hyperborea*)

Daniel Appenroth

A dissertation for the degree of Philosophiae Doctor, January 2021



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Usett og ubetrådt, i dødens mektige ro, slumret de stivnede polaregne under sin uplettede iskåpe fra tidenes morgengry. Hyllet i sitt hvite skrud strakte den veldige jette sine klamme is-lemmer utover og ruget over årtuseners drømmer.

Tidene gikk – dyp var stillheten.

Så, i historiens demring, fjernt i syd, løftet den våknende menneskeånd sitt hode og så over jorden; mot syd møtte den varme, mot nord kulde, og bak det ukjentes grenser la den da de to riker, den altfortærende hetes, den drepende frosts.

Men for menneskeåndens trang, den stetse voksende, mot lys og viden, måtte det ukjentes grenser vike skritt for skritt, inntil de stanset i nord ved dørstokken til naturens store iskirke, polaregnenes endeløse stillhet.

Fridtjof Nansen
Fram over Polhavet

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I. Acknowledgements

This thesis would not have been possible without the help of many people, some of which I would like to thank here.

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Science is by its nature built upon the shoulders of others. In the case of my ptarmigan research the shoulders I built upon belong to Arnoldus Blix, Karl-Arne Stokkan, Alte Mortensen, Erik Reierth and Kjell Lindgård. Over 40 years of their combined efforts have led to a vast knowledge about the bird I wrote my thesis about.

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Last but not least, I like to thank you dear reader for taking the time and showing the interest in reading this thesis.

Daniel Appenroth, Tromsø (January 2021)

II. Thesis abstract

This thesis addresses aspects of the circadian and photoperiodic system in a High Arctic bird: the Svalbard ptarmigan (*Lagopus muta hyperborea*, Sundevall 1845). The most northern resident bird inhabits a unique photic environment; 2/3 of its year it spends either under a night without sunrise (polar night) or under a never setting Sun (polar day). Studies so far suggest a temporal loss of circadian control over behaviour during these constant photic conditions, allowing opportunistic rather than circadian dictated behaviour (Reierth and Stokkan, 1998a; Reierth et al., 1999; Stokkan et al., 1986a). Yet, circadian control extends beyond temporal organisation of behaviour, and other aspects of this Arctic-adapted circadian machinery have received less attention.

Rhythms in core body temperature (T_b) have not been thoroughly studied in Svalbard ptarmigan and in Arctic birds in general and the extent of circadian control over this physiological parameter is unknown. In **paper I**, we have investigated the T_b rhythm alongside activity in captive Svalbard ptarmigan under short photoperiod (SP), long photoperiod (LP) as well as under constant light (LL) and constant darkness (DD). While birds under SP and LP showed clear diurnal activity and T_b patterns, these rhythms seemed to be lost under LL and DD. However, under SP we noticed nocturnal rise in T_b in anticipation to the light-on signal, a rise which also preceded rise in activity. Anticipation is a hallmark of circadian rhythmicity and indicates circadian control of thermoregulation in Svalbard ptarmigan.

In an additional experiment (unpublished), we transferred Svalbard ptarmigan entrained to L:D 12:12 into either LL or DD and measured dampening in T_b cycles by sine wave and periodogram analysis. The results show that T_b cycles dampened under DD and LL before becoming arrhythmic. It is further shown that the T_b rhythm dampened faster under LL (rhythm dampened by half after 1d 23h in LL) than under DD (rhythm dampened by half after 5d 12h).

While T_b and activity might be useful parameters to characterise circadian organisation, the primary importance of circadian rhythms in the Arctic might not be found in behavioural and physiological synchronisation over the 24-h timescale. Photoperiodism describes the mechanism by which organisms receive and respond to changes in day length (photoperiod) in order to achieve synchrony with a seasonal environment. This process is theorised to be based on circadian rhythmicity at least in mammals and birds (Bünning, 1936; Follett, 1973; Wood et al., 2020). In the weak daily rhythmicity but strong seasonal rhythmicity of the High Arctic, the true importance of circadian rhythms might, therefore, lie in its participation in photoperiodism rather than in daily organisation of behaviour and physiology.

In order to explore whether photoperiodism is circadian-based within Arctic animals, we have studied the neuroendocrine centre for photoperiodic responses: the mediobasal hypothalamus (MBH) and the adjacent pars tuberalis (PT) in captive Svalbard ptarmigan under various photoperiodic treatments. In **paper II**, we established that processes within the MBH and PT of an Arctic animal are identical to temperate species. We also showed that a

photoperiodic response in the MBH, PT and in gonadal maturation can take place when birds are transferred directly from DD to LL, i.e. without light-mediated entrainment and despite behavioural arrhythmicity in both conditions. This suggests that the rhythm necessary for photoperiodic induction was either sustained or rapidly initiated under these conditions.

In **paper III**, we measured expression of clock genes and key genes of the photoperiodic response pathway within the PT and MBH for 24 h after a direct transfer from SP into LL. Svalbard ptarmigan retained pronounced clock gene expression in the first day of LL and showed appropriate expression of photoperiodic key genes. In the second part of the experiment, we showed that Svalbard ptarmigan can photoperiodically respond to skeleton photoperiods in terms of activity, body mass and photoperiodic key genes. Both parts suggest circadian-based photoperiodism in our High Arctic model organism.

The sum of these studies show that Svalbard ptarmigan are able to escape circadian hegemony in behaviour and thermoregulation under arrhythmic conditions but are able to produce rhythm-based photoperiodic responses under various experimental light schedules. This establishes the importance of circadian rhythms in the Arctic as basis for seasonal responses.

III. List of papers

Paper I

Body temperature and activity rhythms under different photoperiods in High Arctic Svalbard ptarmigan (*Lagopus muta hyperborea*)

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Manuscript

Paper II

Photoperiodic induction without light-mediated circadian entrainment in a High Arctic resident bird

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Paper III

Adaptive value of circadian rhythms in High Arctic Svalbard ptarmigan

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IV. Glossary of abbreviations

Arntl	Aryl hydrocarbon receptor nuclear translocator-like protein 1
Bmal1	Brain and Muscle ARNT-Like 1
Bmal2	Brain and Muscle ARNT-Like 2
cAMP	cyclic adenosine monophosphate, second messenger molecule
Clock	Circadian Locomotor Output Cycles Kaput
Cry1	Cryptochrome Circadian Regulator 1
Cry2	Cryptochrome Circadian Regulator 1
d	day(s)
DD	constant darkness
DIO2	type II iodothyronine deiodinase (enzyme)
Dio2	type II iodothyronine deiodinase (gene)
DIO3	type III iodothyronine deiodinase (enzyme)
Dio3	type III iodothyronine deiodinase (gene)
e.g.	*exempli gratia*, "for example"
E-box	enhancer box, DNA response element bound by transcription elements
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
h	hour(s)
i.e.	*id est*, "that is"
IP	increasing photoperiod, referred to as experimental group in paper III
L:D	light:dark, often referred to as hours of light and dark in a period
LH	luteinizing hormone
LL	constant light
LP	long photoperiod
m	minutes
MBH	mediobasal hypothalamus
ME	median eminence
mRNA	messenger ribonucleic acid
Per2	Period Circadian Regulator 2
Per3	Period Circadian Regulator 2
POMC	pro-opiomelanocortin
PT	pars tuberalis
qPCR	quantitative polymerase chain reaction
Raldh1	retinaldehyde dehydrogenase 1
SCN	supra-chiasmatic nucleus
SD	standard deviation
SEM	standard error of the mean
SkP	skeleton photoperiod, referred to as experimental group in paper III
SNP	simulated natural photoperiod, referred to as experimental group in paper II
SP	short photoperiod
$t_{1/2}$	half-life time, here referred to as time in which a rhythm dampens by half
T_3	triiodothyronine

T_4	thyroxine
T_b	core body temperature
TTFL	transcriptional-translational feedback loop
TSH	thyroid-stimulating hormone
Tshβ	β subunit of thyroid-stimulating hormone (gene)
VA opsin	vertebrate ancient opsin
ZT	zeitgeber time, in which ZT 0 refers to light-onset

1. Introduction

This thesis addresses aspects of the circadian and photoperiodic system in a High Arctic bird, the Svalbard ptarmigan. Here, I give a short overview about circadian rhythms followed by a description of photoperiodism with a highlight on its circadian basis in mammals and birds. The last part of this introduction discusses the role of circadian and photoperiodic processes in Arctic environments.

1.1 Circadian rhythms and circadian clocks

The Earth rotates around its own axis driving cyclic changes in local conditions with a period of 24 h (a day). These changes include abiotic factors such as light exposure, temperature, humidity and ultimately also biotic factors including food availability and predation risk. Most organisms have adapted to exploit certain periods of the day while avoiding others. For example, many plants swell leaves to maximise light exposure and to increase photosynthetic capacities during the day. Also many animals are active during portion of the day when conditions are favourable and rest during unfavourable conditions (activity cycle). These biological cycles persist in many organisms even under constant environmental conditions (Beling, 1929; de Mairan, 1729). This indicates that they are not merely driven by the environment but are generated by internal self-sustained processes. These self-sustained biological cycles have been coined circadian rhythms (from the Latin words circa “around” and dies “day”) (Halberg, 1959) and are defined by three main criteria. First, circadian rhythms must be endogenous and must be free-running (run in constant conditions) with a period approximating 24 h. Second, they must be entrainable or be able to synchronise to the external environment, and third, their period length must be independent of temperature.

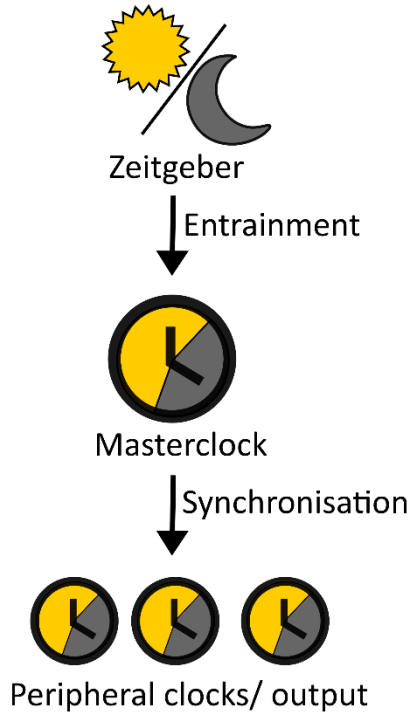


Figure 1. The organisation of the circadian system. One or multiple master clocks entrain to rhythmic zeitgebers in the environment (e.g. the light-dark cycle) and generate an endogenous and self-sustained circadian rhythm. The master clock(s) imposes its rhythm on peripheral clocks and tissues, ultimately leading to rhythmic output, e.g. activity cycles.

Observable circadian rhythms in, for example, locomotor activity or leaf movement are merely the output of a whole circadian system (**Figure 1**) at the base of which biochemical oscillators produce rhythmicity within cells by transcriptional-translational feedback loops (TTFL). In

TTFLs, so-called clock genes translate into proteins that drive complex negative feedback loops, producing a self-sustained rhythm of gene expression and inhibition with a period of approximately 24 h (**Box 1**) (Dunlap, 1999). Most cells of an organism contain clock genes and express an approximately 24-h rhythm based on TTFLs (Whitmore et al., 1998; Yamazaki et al., 2000), but in order to coordinate all clocks and to entrain them to the environment the circadian system must also contain one or multiple master clocks. A master clock is often defined as a specific tissue in which cells and their circadian rhythms are tightly coupled. Master clocks, in contrast to peripheral clocks, can entrain or synchronise to the external environment by cyclic cues, called zeitgebers (from German "timer-giver"). Zeitgebers must themselves be rhythmic and must be a reliable representation of the passing of a day. Cues like ambient temperature can act as zeitgebers (Aschoff and Tokura, 1986) but the most potent, because most reliable, cue is the light-dark cycle (Aschoff, 1954). The self-sustained and entrained master clocks are able to synchronise peripheral clocks through neurological and hormonal pathways, e.g. through melatonin or the autonomic nervous system (Buijs et al., 2016). The rhythm in body temperature, a process under circadian control, was also suggested to be able to entrain peripheral clocks and tissues (Brown et al., 2002; Buhr et al., 2010). Ultimately, the sum of synchronised clocks produce rhythmic behaviour, physiology and metabolism.

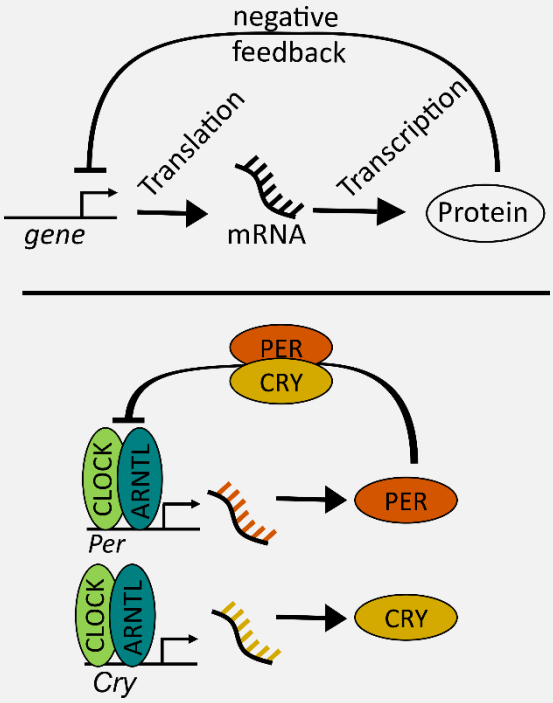
The organisation of the circadian system differs between animal classes. In mammals, a single master clock within the suprachiasmatic nucleus (SCN) of the hypothalamus receives light input through the eyes via the retinohypothalamic tract and entrains all peripheral clocks. In contrast, the avian circadian system comprises at least three master clocks within the pineal gland, the retina and the avian SCN, each of which is able to receive light information and produce a self-sustained rhythm (Brandstätter, 2003; Cassone, 2014).

The evolutionary drivers of circadian rhythms are thought to derive from three adaptive values. External synchronisation, internal synchronisation and forming the basis for photoperiodism (Hut and Beersma, 2011; Vaze and Sharma, 2013). External synchronisation describes the function of producing rhythmic behaviour and physiology in order to be in synchrony with environmental cycles and to anticipate and to exploit upcoming favourable conditions. Internal synchronisation describes the harmonisation of internal processes to allow efficient energy metabolism and to avoid opposing processes to coincide. The third function, being a major topic of this thesis, will be addressed in further detail in the following chapters.

Box 1. Transcriptional-translational feedback loop (TTFL) and clock genes

TTFLs are at the core of circadian clocks. In a TTFL, genes are first transcribed into their mRNA and subsequently translated into their respective amino acid sequence. The produced protein then inhabits its own transcription. In absence of freshly produced mRNA, the concentration of the protein decreases, eventually lifting inhibition and allowing the cycle to start anew.

Circadian clocks contain numerous clock genes that are responsible of generating these TTFLs in an approximately 24-h cycle and to connect the produced rhythm to various outputs. The core cycle contains the clock genes: *Per* and *Cry* and their respective homologs as well as *Clock* and *Arntl* (also known as *Bmal1*).



In the simplified form, a complex of the proteins CLOCK and ARNTL is binding the E-boxes (transcriptional response elements) of the genes *Per* and *Cry*, which promotes their expression (Kume et al., 1999; Shearman et al., 1997). Consequently, *Per* and *Cry* are transcribed and translated into their respective proteins. The PER and CRY proteins heterodimerise and inhibit the action of the CLOCK-ARNTL complex, thereby indirectly inhibiting their own transcriptions (Lee et al., 2001). The inhibited gene expression leads to a gradual depletion of CRY and PER until inhibition on the CLOCK-ARNTL complex is lifted and can activate transcription of *Per* and *Cry* anew. This core cycle is supplemented by additional regulators and elements, such as an TTFL controlling expression of *Arntl* (Preitner et al., 2002). The rhythmic expression of clock genes drives rhythmic expression of numerous other genes, e.g. through the action of CLOCK-ARNTL (Cox and Takahashi, 2019; Menet et al., 2014), leading ultimately to rhythmic processes across all tissues.

1.2 Circadian basis of seasonal timing

The Earth revolves around the Sun (Copernicus, 1543) with an axial tilt of 23°26' (Sédillot, 1853). This astronomical combination causes a latitude-dependent annual variation of light exposure ultimately causing the seasons. In temperate and high latitudes, the seasons shape the environment and cause pronounced annual cycles in temperature, precipitation and day length. Inhabitants of seasonal habitats have evolved to be in synchrony with these annual cycles and express various seasonal traits to cope with the interplay between favourable and unfavourable conditions. Examples of the seasonal life traits are seasonal reproduction, migration, moulting, flowering and hibernation. One primary process by which organisms anticipate upcoming seasonal conditions and time these metabolically costly traits is called photoperiodic time measurement. Photoperiodic time measurement, here after referred to as photoperiodism, can be defined as the ability of an organism to perceive and to measure the annual change in photoperiod (day length) and to use this to synchronise cycles of physiology and behaviour to the solar year. Past and current research focuses to uncover the fundamental mechanisms of photoperiodism, i.e. how the organism translates the changing photoperiod into a signal that ultimately leads to the expression of the appropriate seasonal phenotype.

Erwin Bünning (**Figure 2A**) was the first to propose that photoperiodism is based on a circadian rhythm of sensitivity to light (Bünning, 1936). In his pioneering work on runner beans (*Phaseolus coccineus*, **Figure 2B**), he observed the plant's leaf movement and concluded that it was under circadian control because movement continues in the absence of a light-dark cycle and other cyclic environmental cues. Based on this observation, Bünning proposed that the plant's circadian clock is setting a morning and evening phase within the 24-h framework of a day. He defined the morning phase as the period when leaves swell and the evening phase as the period when leaves shrink. In his model on photoperiodism, he argued that these phases can be utilised to detect changes in photoperiod (Bünning, 1936). Under a short photoperiod, light coincides only with the morning phase, while under a long photoperiod light extends into the evening phase. The runner bean is a short-day flowering plant and Bünning reasoned that light during the evening phase is regarded as a signal to suppress flowering. Conversely, under a short photoperiod light does not extend into the evening phase and the suppression on flowering is lifted.

Under the guidance of Colin Pittendrigh, Bünning's initial model has developed into the external and internal coincidence models (Pittendrigh, 1972). The external coincidence model (**Figure 2C**) very much resembles Bünning's initial proposition and describes a dual role of light in photoperiodism. The first role is to entrain an endogenous circadian rhythm, which sets a photosensitive phase in the subjective night. The second role of light is to trigger the photoperiodic response. Under a long photoperiod, light coincides with the photosensitive phase and triggers the response while it does not under a short photoperiod.

Like the external model, the internal coincidence model (**Figure 2D**) is also based on circadian rhythmicity but does not require a direct response to coinciding light. It, instead, describes photoinduction as the result of the phase relationship of two or more factors cycling with differently phased circadian rhythms. These cycling factors shift independently with changing photoperiod, e.g. one factor shifts with dawn and the other with dusk. When photoperiod brings the cycling factors into a certain phase relationship, they trigger a photoperiodic response. A third model (**Figure 2E**), historically advocated by Tony Lees, is emancipated from a circadian rhythm (Lees, 1953). It instead describes photoperiodism as accumulation and depletion of a light-dependent factor. In a long photoperiod, this 'hourglass' model predicts the accumulation of a light-dependent factor above a certain threshold, which leads consequently to a photoperiodic response. In a short photoperiod, the threshold is not crossed and the light-dependent factor is subsequently depleted during the dark phase. The hourglass model can also be inversely described as the accumulation and depletion of a dark-dependent factor.

Various experimental protocols, such as exposure of organisms to skeleton photoperiods and Nanda-Hamner protocols (Hamner, 1964; Nanda and Hamner, 1958), have been employed in order to distinguish between the circadian-based and hourglass model. In skeleton photoperiods, the light phase is replaced by two shorter light blocks marking the beginning and end of the photoperiod they imitate. Hence, a skeleton photoperiod can resemble a long photoperiod in terms of the timing of light and a short photoperiod in terms of amount of light hours within a 24-h period. (Pittendrigh and Minis, 1964). Assuming the external coincidence model, the first light block is setting the photosensitive phase while the second light block coincides with it (**Figure 2C**). According to the internal model, the two light blocks mark dusk and dawn and cause the same phase relationship as the corresponding continuous photoperiod would (**Figure 2D**). The validity of skeleton photoperiods to prove rhythm-based photoperiodism is not unchallenged (Lees, 1973), but between the hourglass model and a rhythm-based model the latter is the more parsimonious explanation (Saunders, 2005).

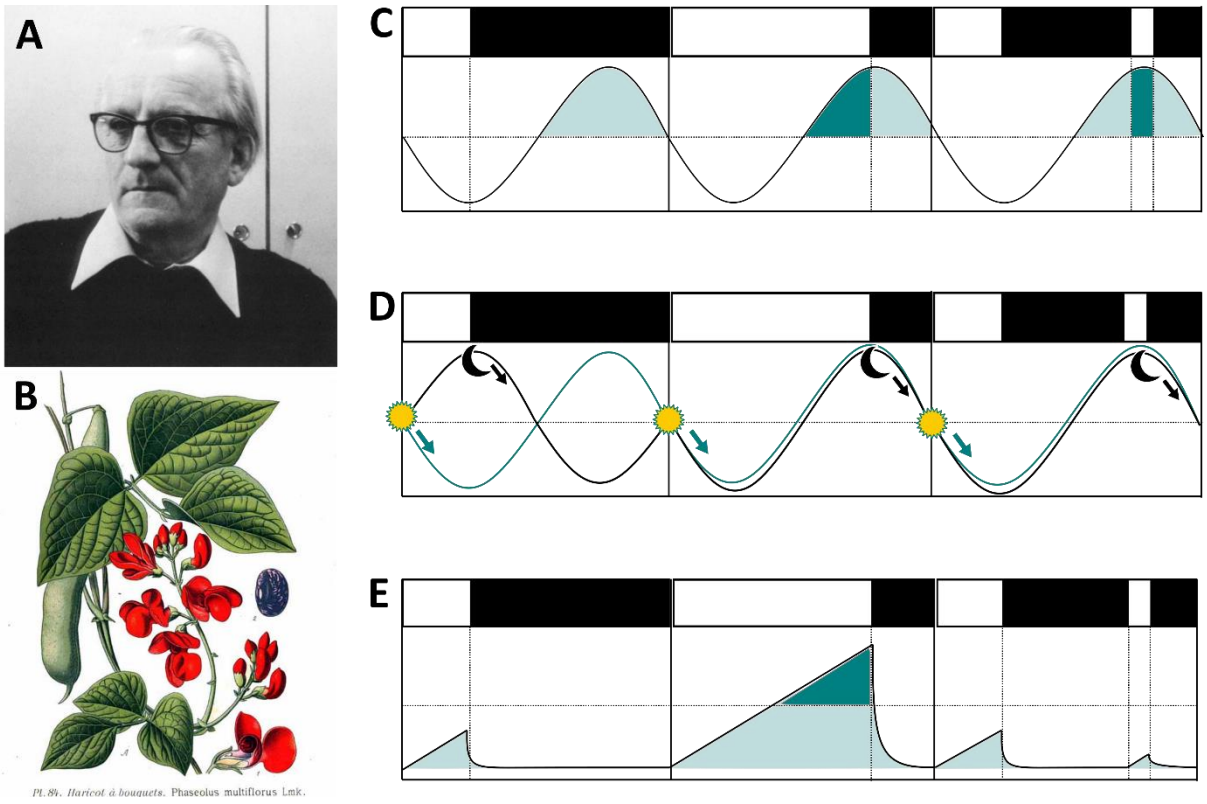


Figure 2. Different models have emerged to explain photoperiodism and the underlying mechanisms.

(A) Erwin Bünning (1906-1990) was the first to propose a circadian basis for photoperiodism. Picture from Bonner (1994).

(B) In his work with red runner beans, he proposed a model resembling the external coincidence model.

(C) The external model is described as an endogenous daily timer that entrains to the light-dark cycle and sets a phase of photosensitivity (light blue). When light coincides with the photosensitive phase (dark blue) a long day response is triggered.

(D) The internal coincidence model also presumes a circadian basis but describes a photoperiodic response as the result of a certain phase relationship of two or more cycling factors, e.g. a dawn and a dusk factor. Both models predict that a photoperiodic response is not triggered through the total amount of light hours but by the timing of light, hence skeleton photoperiods can have the same effect as the long photoperiods they imitate.

(E) In contrast to the coincidence models, the hourglass model describes a circadian-independent system in which the accumulation of a light-dependent (or dark-dependent) factor above a certain threshold (dark blue) leads to a photoperiodic response.

Further experimental distinction between the circadian-based and hourglass model can be achieved by Nanda-Hamner protocols (Hamner, 1964; Nanda and Hamner, 1958). In Nanda-Hamner protocols, short photoperiods are commonly paired with long dark phases, which deviate from the 24-h framework of a day. These experiments assume the circadian rhythm for photoperiodism to be sustained with a near to 24-h period despite the period of the light-dark cycle deviating from 24 h. Photoinduction depends, therefore, at which phase of the circadian rhythm the light phase re-occurs. If the short light and long dark phase equal a multiple of 24 h, the light will always coincide with the same circadian phase and will not trigger a response (**Figure 3A & 3B**). If, however, the light and dark phase derivate from the multiples of 24 h the light phase sequentially coincides with different phases of the circadian rhythm, including the phase which is needed to trigger a photoperiodic response (**Figure 3C**).

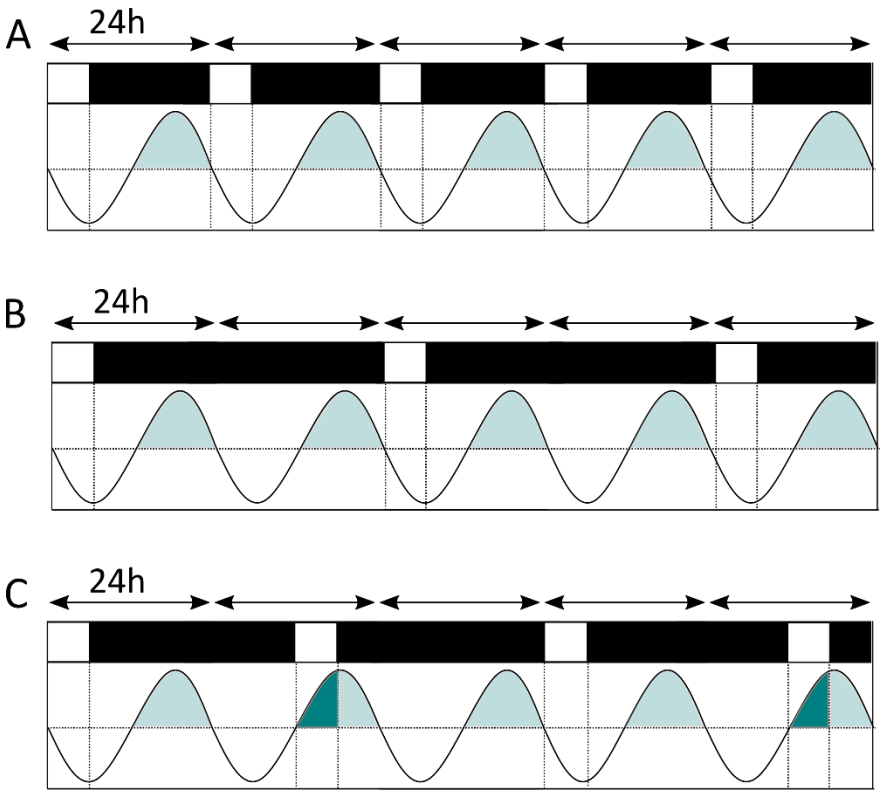


Figure 3. Nanda-Hamner protocols proof circadian-based photoperiodism.

(A) In a normal short photoperiod, the light phase does not coincide with the photosensitive phase.

(B) In a Nanda-Hammer protocol in which light and dark phase equal a multiple of 24 h the light phase is continually not coinciding with the photosensitive phase.

(C) In contrast, if light and dark phase are not equal to a multiple of 24 h the light phase alternately coincides with the photoinducible phase and triggers a photoperiodic response.

The figure illustrates the external coincidence model but is also applicable to the internal coincidence model.

The white-crowned sparrow (*Zonotrichia leucophrys*) is an excellent illustration of the effect of Nanda-Hamner protocols. In white-crowned sparrow, a correctly timed light phase 100 h after the last dusk is sufficient to trigger a photoperiodic response in form of increased plasma concentration of luteinizing hormone (LH) (Follett et al., 1974). This proves that an endogenous rhythm operates in white-crowned sparrows and that this rhythm can persist throughout four days of complete darkness.

Nanda-Hamner protocols presume a self-sustaining circadian oscillator, but circadian clocks are manifold and adapted to their respective environments. Historically, birds belong to one of the most diverse studied class in terms of photoperiodism. Their advantage as photoperiodic research models, e.g. compared to mammals, lies in the fact that a single long day or single correctly timed light pulse is sufficient to trigger an easy measurable photoperiodic response, for example in the plasma concentration of gonadotropins such as LH (Follett et al., 1977; Nicholls et al., 1973). Birds are also a diverse taxonomical class with species inhabiting various habitats, from the tropics to the poles, and displaying different life strategies. The circadian system is naturally adapted to the respective environment and life strategies, which has also implications on circadian-based photoperiodism. Birds like starling (*Sturnus vulgaris*) and white-crowned sparrow express strong self-sustained rhythms and show photoinduction to correctly timed Nanda-Hamner protocols (**Figure 4** white-crowned sparrow depicted as blue bird) (Follett et al., 1974; Gwinner and Eriksson, 1977; King et al., 1997). At the other end of the spectrum, birds like quail (*Coturnix japonica*) often fail to be photoperiodically triggered by Nanda-Hamner experiments (King et al., 1997; Saiovici et al., 1987). Arguably, this is because their circadian rhythm for photoperiodism dampens and re-entrains with every new light phase outside the normal 24-h framework (**Figure 4** quail depicted as yellow bird) (Juss et al., 1995; King et al., 1997). The diverse responses to Nanda-Hamner protocols can be argued against its validity to proof circadian-based photoperiodism. They might only demonstrate presence/ absence of strong sustained circadian-based photoperiodism like in the white-crowned sparrow but often fail to discriminate between organisms reading photoperiod via a damped circadian oscillator (e.g. quail) from those employing a truly hourglass-based system (Saunders, 2005).

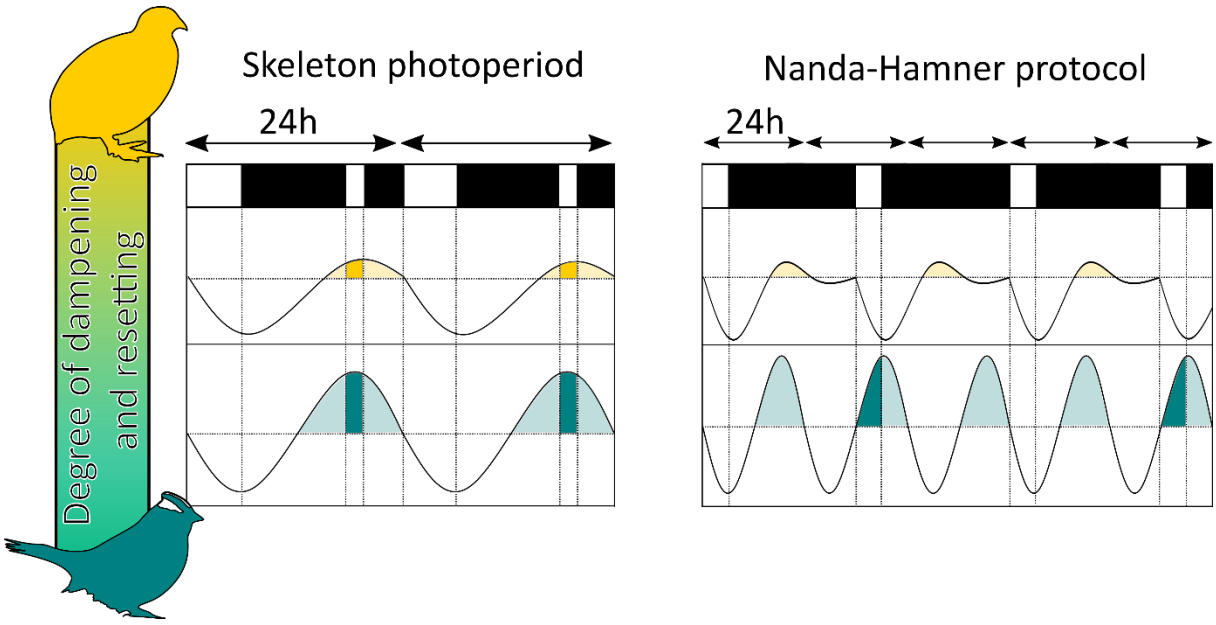


Figure 4. Sustained and dampening circadian rhythms have different implications for photoperiodism. Skeleton photoperiods and Nanda-Hamner protocols can both be used to indicate circadian-based photoperiodism. Skeleton photoperiods lead to a photoperiodic response in a range of species but they have also been interpreted in favour for the hourglass model (Lees, 1973). Nanda-Hamner protocols produce photoperiodic responses based on strong self-sustainable timer, as in white-crowned sparrow (blue bird), but often fail to do so in organisms with a fast dampening and readily re-entrainable circadian system, such as in quail (yellow bird). The figure illustrates the external coincidence model but is also applicable to the internal coincidence model.

1.3 The photo-neuroendocrine cascade in the mediobasal hypothalamus and the pars tuberalis

Support that photoperiodism, especially in mammals and birds, is circadian-based does not only stem from experimental light manipulations but also derives from molecular and genetic research. Many of these studies focus on the supposed centre of photoperiodism: the mediobasal hypothalamus (MBH) and the adjacent pars tuberalis (PT). The most studied seasonal phenotype controlled by this photoperiodic centre is reproduction but the PT/ MBH region has also been linked to seasonal body mass control (Bolborea and Dale, 2013; Ebling and Lewis, 2018; Hanon et al., 2008; Helfer et al., 2019; Helfer and Stevenson, 2020; Nishiwaki-Ohkawa and Yoshimura, 2016; Yoshimura, 2013).

Seasonal reproduction in mammals and birds is controlled through the regulation of gonadotropins secreted by the anterior pituitary gland. The gonadotropins (luteinizing hormone (LH) and follicle-stimulating hormone (FSH)) act on the gonads, regulating gametogenesis and production of sex steroids, leading ultimately to reproduction and the expression of secondary sexual characteristics.

The annual varying synthesis and release of gonadotropins is regulated by a gene cascade transducing the light signal within the mammalian and avian MBH and PT (**Figure 5**). Within the PT, long photoperiod, either transmitted by deep brain photoreceptors in birds (Halford et al., 2009; Nakane et al., 2010) or the melatonin signal in mammals (Hazlerigg et al., 2001; Yasuo et al., 2009), triggers expression of the β subunit of thyroid-stimulating hormone (*Tshβ*) (Nakao et al., 2008). PT-derived TSH leads to increased type II iodothyronine deiodinase (*Dio2*) expression through a cAMP-dependent pathway within specialised ependymal cells lining the third ventricle, so-called tanycytes (Bolborea et al., 2015; Hanon et al., 2008; Nakao et al., 2008; Ono et al., 2008). DIO2 in its turn activates thyroid hormones within the MBH. It catalyses the removal of iodine from the outer ring of prohormone thyroxine (T_4), thereby converting it into bioactive triiodothyronine (T_3). In long day breeding mammals and birds, the increased T_3 concentration leads to increased release of gonadotropin-releasing hormone (GnRH) from the median eminence (ME) into the pars distalis. One proposed mechanism by which T_3 acts on GnRH secretion is by neural remodelling of the ME region, i.e. by changing glial encasement of GnRH nerve terminals (Yamamura et al., 2004; Yamamura et al., 2006). Alternatively, in mammals it has been proposed that neurons expressing Kisspeptin and RFamide-related peptide regulate GnRH secretion and are targets for T_3 (Dardente et al., 2014; Henson et al., 2013; Klosen et al., 2013; Quignon et al., 2020). In either case, in long-day breeding birds and mammals increased hypothalamic T_3 leads to increased GnRH secretion, which ultimately leads to increased release of gonadotropins from the anterior pituitary and to the onset of seasonal reproduction.

Conversely under short photoperiod, increased expression of type III iodothyronine deiodinase (*Dio3*) coincides with decreased *Tshβ* and *Dio2* expression (Milesi et al., 2017; Nakao et al., 2008; Yasuo et al., 2005). DIO3 catalyses inner ring deiodination of T_4 and T_3 to

biologically inactive iodothyronines, causing decreased GnRH release and gonadal inactivity in long-day breeding animals (Yasuo et al., 2005). This reciprocal photoperiod-mediated switch in DIO enzymes and the resulting effects on the hypothalamic concentration of bioactive T_3 has become a central paradigm in photoperiodism.

Besides reproduction, the PT/ MBH region and especially the tanycytes there within have been linked to seasonal body mass control (Bolborea and Dale, 2013; Ebling and Lewis, 2018; Helfer et al., 2019; Helfer and Stevenson, 2020). Hypothalamic T_3 levels not only affect reproduction but also appetite and accumulation and depletion of adipose tissue e.g. in Siberian hamster (*Phodopus sungorus*) (Barrett et al., 2007; Murphy et al., 2012). The mechanisms linking hypothalamic thyroid hormones and seasonal body mass regulation are not well understood but might include downstream targets, such as the retinoic acid pathway. Many elements of the retinoic pathway are located in tanycytes (Helfer et al., 2012; Ross et al., 2004; Shearer et al., 2012) and the expression of *Raldh1*, the rate limiting enzyme for retinoic acid synthesis, is upregulated by T_3 in rats (Stoney et al., 2016). Tanycytes function partly as hypothalamic stem cell source (Lee et al., 2012) and recent models link the T_3/ retinoic acid pathway to seasonal cycles of cellular degeneration, proliferation and differentiation into appetite regulating neurons (Helfer et al., 2019; Lee et al., 2012). Alternatively, T_3 and retinoic acid might affect direct expression of neuropeptides involved in energy regulation such as POMC and VGF (Helfer and Stevenson, 2020).

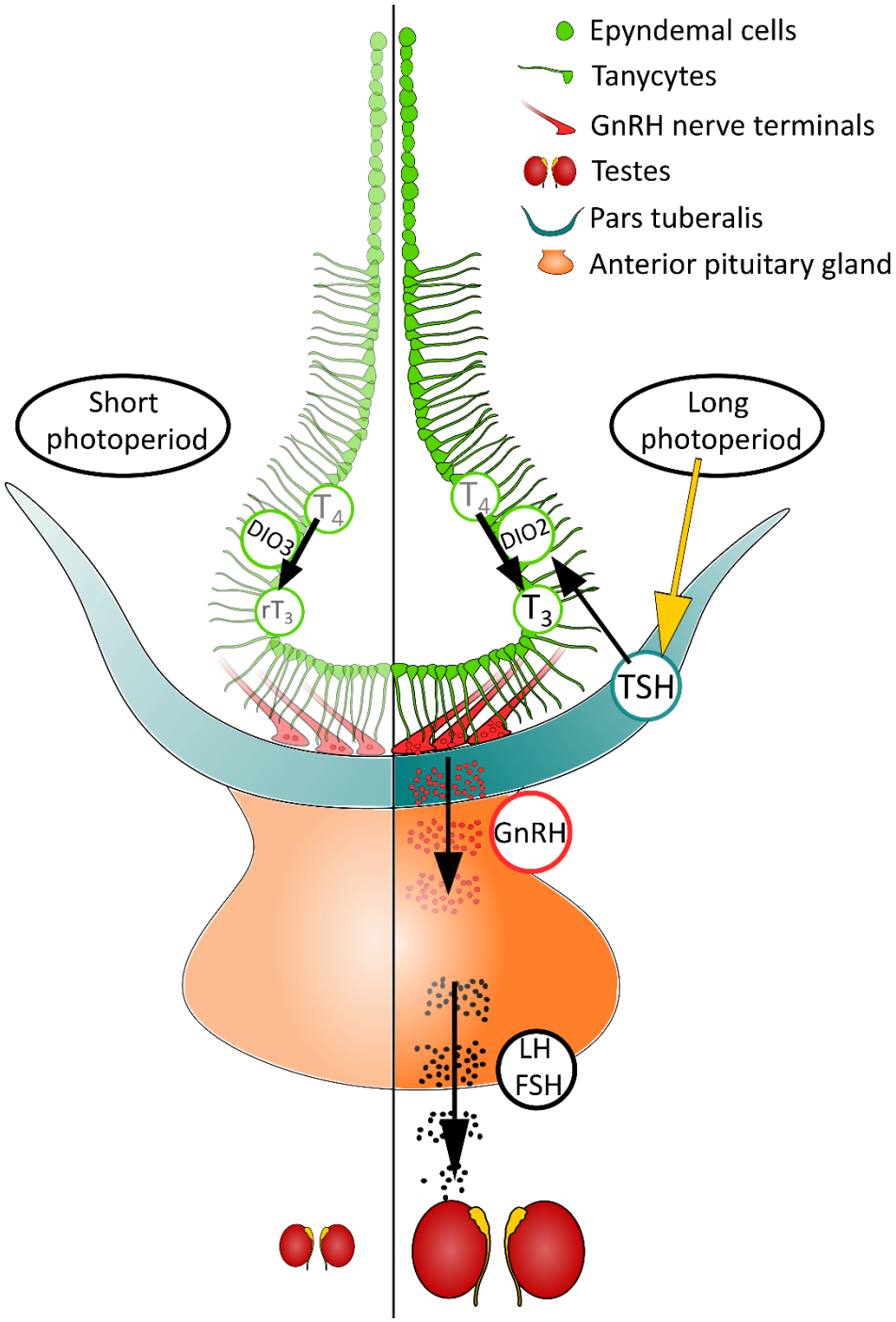


Figure 5. The TSH-DIO-GnRH pathway within the mediobasal hypothalamus and pars tuberalis is at the core of photoperiodic regulation of reproduction in mammals and birds. In seasonal breeding birds and mammals, long photoperiod stimulates *Tshβ* expression within the PT. PT-derived TSH does in its turn leads to increased *Dio2* expression within tanycytes and, as a consequence to elevated hypothalamic T_3 concentration. In long-day breeders, bioactive T_3 does stimulates GnRH secretion into the anterior pituitary. This ultimately increases gonadotropin release from the anterior pituitary gland, to gonadal development and to increased steroid production. Under a short photoperiod, elevated *Dio3* expression coincides with low *Tshβ* and *Dio2* expression resulting in low concentration of bioactive T_3 and to reproductive inactivity. In the case of short-day breeders the TSH/ DIO cascade is preserved but T_3 has an inverted effect on GnRH secretion.

The TSH/ DIO pathway is at the core of photoperiodism in mammals and birds (**Figure 5**) and as discussed earlier there are strong evidence through photoperiodic experiments that photoperiodism is circadian-based in both classes. It is therefore a reasonable assumption that this central regulatory pathway is circadian-based as well and there are several lines of evidence to support this inference.

(1) As described previously, LH can be used as reliable assay to confirm photoperiodic induction through experimental photoperiods, especially in birds. The release of LH and other gonadotropins into the circulation is ultimately controlled by the release of GnRH into the pars distalis and by the gene cascade described above. In line with this, skeleton photoperiods have been shown to not only excite the release of LH but are also able to excite the TSH/ DIO cascade controlling this release (Majumdar et al., 2015; Yoshimura et al., 2003).

(2) Mammals and birds show both rhythmical clock gene expression in the PT/ MBH region. In Japanese quail, this rhythmical expression within the MBH region is unaltered by exposure to different photoperiods which is argued in favour for an external coincidence model (Yasuo et al., 2003). Clock gene expression and therefore the photoinducible phase remains constant relative to the dawn signal and can, as a consequence, be stimulated by a long photoperiod or night-interrupting light pulse (Yasuo et al., 2003). Studies within the PT in quail show shifted expression of *Cry1* (Yasuo et al., 2004) which, relative to a stable *Per2* expression, was argued in favour for an internal coincidence model. Likewise, studies in sheep (*Ovis aries*) (Lincoln et al., 2002) and hamster (*Cricetus cricetus* and *Phodopus sungorus*) (Johnston et al., 2005; Tournier et al., 2007) show characteristics of shifting clock gene expression within the PT.

(3) Clock genes control the activation of the TSH/ DIO pathway at least in mammals. In the PT, long day induced *Tshβ* expression coincides with or is preceded by *Eya3* expression (Dardente et al., 2010; Nakao et al., 2008). EYA3 acts as transcriptional co-activator for *Tshβ* expression (Dardente et al., 2010; Masumoto et al., 2010) and expression of mammalian *Eya3* is dependent on E-box driven activation through the clock genes *Bmal1*, *Bmal2* and *Clock* (Wood et al., 2020).

1.4 Circadian rhythms and photoperiodism in the Arctic

Circadian clocks are theorised to have three adaptive values: external synchronisation with the environment, internal harmonisation of metabolic processes and, as outlined above, forming the basis for photoperiodism at least in mammals and birds (Hut and Beersma, 2011; Vaze and Sharma, 2013). Certain environments, such as the polar region, might pose contradictory selection pressures on these values.

Polar latitudes are characterised by the polar day and the polar night, conditions under which day-night cycles are not present for long periods of the year. Other daily environmental cycles, such as ambient temperature and food availability, are generally diminished as well under these conditions. There is a vast literature concerning daily behaviour and physiology in Arctic animals (**Figure 6A**) (see also review from Williams et al. (2015)). The sum of these studies reveal a broad spectrum on circadian control over behavioural rhythmicity in the Arctic. While some Arctic animals are arrhythmic or predominately ultradian under the constant photic conditions of the polar day and polar night, e.g. Svalbard reindeer, Svalbard ptarmigan and muskox (Lindgård and Stokkan, 1989; Reierth and Stokkan, 1998a; Stokkan et al., 1986a; Stokkan et al., 1994; van Beest et al., 2020; van Oort et al., 2005), others including polar bears, arctic ground squirrels and several migratory birds retain rhythmicity under constant Arctic light conditions (Ashley et al., 2012; Ashley et al., 2014; Steiger et al., 2013; Ware et al., 2020; Williams et al., 2012a; Williams et al., 2017b). The function for retained rhythmicity might range from social synchrony (Steiger et al., 2013) to a continued need for external synchronisation, as many Arctic animals, such as the arctic ground squirrel, are still subjected to pronounced cycles in ambient temperature (Long et al., 2005). In fact, many Arctic animals who retain daily rhythmicity have population boundaries extending south of the Arctic Circle or are migratory and are therefore only partially exposed to Arctic conditions and the selection pressures there within (**Figure 6A**). In contrast, permanent residents of Svalbard are geographically isolated within Arctic conditions all year around. The archipelago of Svalbard reaches from 74° to 81° north latitude and is as such a High Arctic habitat cut off from lower sub-Arctic landmasses (contrary to North America, Greenland, Siberia and Scandinavia). This makes it a fascinating natural laboratory to investigate chronobiological adaptations to the Arctic.

On Svalbard, the Sun remains ≥ 6° below the horizon between mid-November and February but is constantly above the horizon between mid-April and mid-September (**Figure 6B**). Svalbard reindeer and Svalbard ptarmigan (**Box 2**), two permanent residents of this High Arctic archipelago, exhibit arrhythmic behaviour during the extended periods of constant photic conditions, i.e. polar day and polar night (Lindgård and Stokkan, 1989; Reierth and Stokkan, 1998a; Stokkan et al., 1986a; Stokkan et al., 1994; van Oort et al., 2005). It has been proposed that suspending the daily organisation of behaviour and physiology is adaptive to the arrhythmic conditions of Svalbard (Lin et al., 2019; Lu et al., 2010; Stokkan et al., 2007; van Oort et al., 2007). For example: during the polar night on Svalbard, relative mild climate is interchanging with cold spells, causing unpredictable occurring ice covers over the already

sparse vegetation (Pedersen et al., 2006; Pedersen et al., 2005). Under these conditions, herbivores such as the Svalbard ptarmigan must be physiologically permitted to feed anytime conditions are allowing it (Pedersen et al., 2006; Stokkan, 1992).

Conversely, species inhabiting Svalbard are subjected to large annual variations and express strong seasonal rhythms, such as reproduction, moult and pronounced body mass cycles (Arnold et al., 2018; Lindgård et al., 1995; Stokkan, 1992; Stokkan et al., 1995; Stokkan et al., 1988; Stokkan et al., 1986b). These seasonal life traits are energetically costly and the incorrect timing would have negative effects on survival and reproductive success (Feder et al., 2008; Reed et al., 2013). Photoperiodism must be under strong selection pressure in seasonal environments and this selection pressure may be expected to extend to the underlying mechanisms of temporal organisation, including the circadian system.

In conclusion, permanent residents of Svalbard seem to be exposed to opposing selection pressures on the circadian system. The temporal arrhythmic environment would disadvantage strong imposed rhythmicity in behaviour and physiology while the need for correct seasonal timing would favour reliable innate rhythmicity to form the basis for photoperiodism.

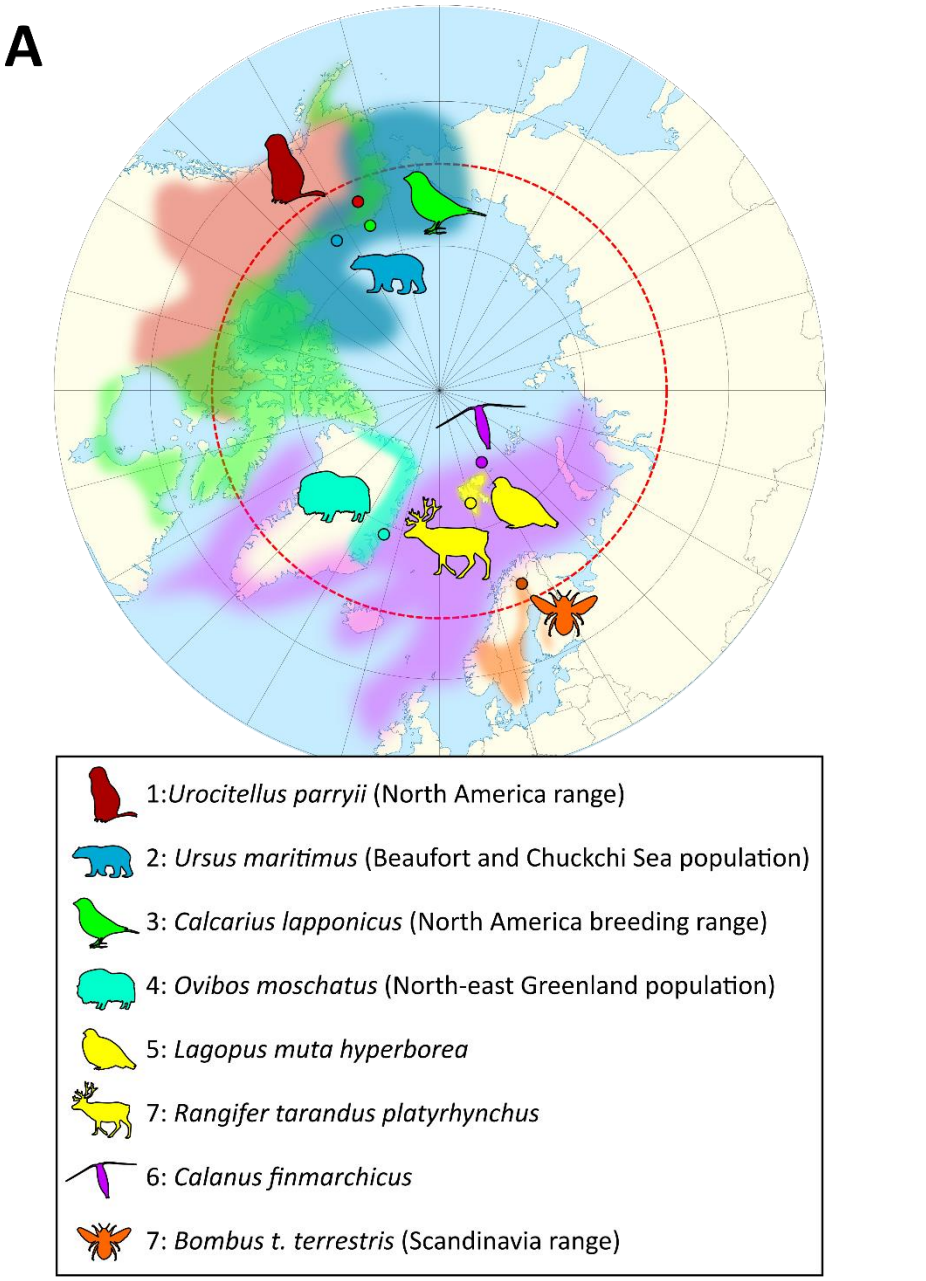


Figure 6. Arctic animals and where to find them. Description on the next page.

Figure 6. Arctic animals and where to find them.

(A) Many organisms have been studied in terms of their chronobiological adaptation to the Arctic but many of them also belong to populations extending south of the Arctic Circle (Arctic Circle indicated by red dotted line). The map shows eight prominent examples of studied Arctic inhabitants (colour coded) with their corresponding population range (transparent colours) and their respective location of the study (coloured dot). The corresponding publications are as follows:

1: Body temperature, activity and clock gene rhythms in arctic ground squirrel (Ikeno et al., 2017; Williams et al., 2012a; Williams et al., 2012b; Williams et al., 2017a; Williams et al., 2017b)
2: Activity rhythms in North American polar bear (Ware et al., 2020)
3: Activity, melatonin and clock gene rhythms in Lapland longspur (North America breeding range) (Ashley et al., 2012; Ashley et al., 2014)
4: Activity rhythms in Greenland muskox (van Beest et al., 2020)
5: Activity and melatonin rhythms in Svalbard ptarmigan (Reierth and Stokkan, 1998a; Reierth et al., 1999; Stokkan et al., 1986a)
6: Activity rhythms in Svalbard reindeer (Arnold et al., 2018; van Oort et al., 2005; van Oort et al., 2007)
7: Clock gene rhythms in *Calanus finmarchicus* (Hüppe et al., 2020)
8: Foraging rhythm in buff-tailed bumblebee in Northern Finland (Stelzer and Chittka, 2010)

(B) Equatorial latitudes are defined by little seasonal variation in photoperiod and other environmental factors such as temperature or food availability (environments with wet and dry seasons excluded). In sharp contrast, the polar regions are defined by drastic changes in day length with extended periods of either constant light (polar day) or constant night (polar night). In addition, the Arctic is extremely seasonal with short growing seasons and harsh winters.

Box 2. The Svalbard ptarmigan (*Lagopus muta hyperborea* Sundevall, 1845)

The Svalbard ptarmigan is a subspecies of the rock ptarmigan, a high altitudinal/ high latitudinal grouse species. It has settled the archipelago of Svalbard (74° to 81° north latitude) after the last Ice Age (10.000 - 12.000 years ago) most likely from Siberia (Sahlman et al., 2009). Svalbard ptarmigan only migrate locally (Fuglei et al., 2017) and were isolated from other rock ptarmigan populations after colonisation (Sahlman et al., 2009). Due to its non-migratory habit, the Svalbard ptarmigan is the northern-most resident bird species and is as such subjected to the polar day and polar night and the climatic and photoperiodic challenges that come with it.

Class: Aves
Order: Galliformes
Family: Phasianidae
Genus: *Lagopus*
Species: *L. muta*

Svalbard winters are long and cold, with limited access to food. Adaptations to these climatic challenges include a high insulated plumage, roosting in the snow and big fat deposits (up to 35% of its body mass) serving as insulation and energy emergency ration over the winter (Mortensen and Blix, 1986; Mortensen and Blix, 1985; Stokkan, 1992).

Captive Svalbard ptarmigan under natural Svalbard light and temperature show no daily rhythms in behaviour under the polar day and polar night, while they are diurnal in the periods in-between (Reierth and Stokkan, 1998a; Stokkan et al., 1986a). Likewise, Svalbard ptarmigan hold in light and temperature controlled rooms show no daily rhythmicity under constant light and constant darkness (Reierth and Stokkan, 1998b). A loss of behavioural rhythmicity is common in birds under constant bright light but uncommon under DD (Gänshirt et al., 1984; McMillan et al., 1975; Simpson and Follett, 1982; Yamada et al., 1988). Plasma melatonin cycles are rhythmic under light-dark cycles but become also attenuated under natural constant light (data is missing for DD) (Reierth et al., 1999). The sum of these studies suggest a lack of imposed circadian rhythmicity on behaviour during periods of constant light and constant darkness in nature and captivity. They further suggest that Svalbard ptarmigan do not entrain to any photic zeitgeber during the polar day and polar night, such as light intensity or spectral composition (Ashley et al., 2012; Krüll, 1976b).

This temporal loss of daily organised behaviour is most likely an adaptation to their High Arctic environment. Svalbard ptarmigan experience long periods of constant light (ca. 5 month without sunset) and constant darkness (ca. 4 month without sunrise) as well as fast changing photoperiods in-between (15 - 40 minutes/ day) (**Figure 6B**). During periods of constant photic conditions, daily cycles in ambient temperature and food availability are attenuated as well. These are conditions under which a strong imposed daily organisation of behaviour and physiology might be maladaptive. For example, Svalbard ptarmigan must be physiologically able to feed whenever the unpredictable weather conditions on Svalbard permit it (Pedersen et al., 2006; Stokkan, 1992).

Box 2 continued

Contrary to their 'weak' circadian rhythmicity, Svalbard ptarmigan are strongly seasonal. Their reproduction, seasonal fattening, food intake, plumage and activity are all under photoperiodic control, i.e. are timed according to the annual change in day length. Under increasing vernal photoperiod, ptarmigan show decrease in body mass, moult from the winter plumage into the brown summer plumage and increased activity through pre-breeding and territorial behaviour (Lindgård and Stokkan, 1989; Lindgård et al., 1995; Stokkan et al., 1995; Stokkan et al., 1986a). Reproduction and chick rearing takes place in the summer, followed by autumnal fattening and moult into the winter plumage (Steen and Unander, 1985; Stokkan et al., 1986b). How much non-photoperiodic cues and circannual rhythmicity (endogenous calendar) contribute to seasonal timing mechanisms is currently unknown but past and ongoing photoperiodic experiments in these birds show clearly that the respective photoperiods are sufficient to trigger and terminate all seasonal life traits.

2. Research aim

Physiologist and Nobel Prize winner August Krogh states in his eponymous principle: "*For a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied*" (Krogh, 1929). The combination of their isolated High Arctic habitat (**Figure 6A**) and the possibility of controlled photoperiodic and molecular experiments makes the Svalbard ptarmigan (**Box 2**) arguably the most convenient model to explore chronobiological adaptations to the Arctic.

Published work indicates that Svalbard ptarmigan are arrhythmic in behaviour under the polar day and the polar night (Reierth and Stokkan, 1998a; Stokkan et al., 1986a). Likewise, melatonin rhythms are attenuated under these conditions (Reierth et al., 1999). While these studies suggest a diminished role for the circadian system in the control of locomotor activity and pineal function, other aspects and outputs of the circadian system remain unexplored. In this thesis, I aimed to address circadian involvement in two aspects of animal physiology of particular relevance in an Arctic setting: thermoregulation and seasonal photoperiodic synchronisation.

Rhythms in core body temperature (T_b) can be sustained despite disruption in activity rhythms (Murakami et al., 2001; Satinoff and Prosser, 1988) but a thorough investigation on T_b cycles and its circadian control is missing in Svalbard ptarmigan. In an attempt to fill the knowledge gap and to further characterise the circadian properties of our Arctic model, we used a combination of implantable temperature data-loggers (iButtons) and passive infrared actimetry to investigate T_b and activity rhythms in Svalbard ptarmigan when entrained to light-dark cycles or acclimated to constant illumination levels (**paper I**). In an additional experiment, we further explored effects of dampening on T_b rhythms when entrained birds are acutely transferred to constant light (LL) or constant darkness (DD).

Studies on activity and T_b rhythms explore the circadian role for daily synchronisation with the external environment but as outlined above, the significance of circadian rhythmicity in the Arctic might rather be found in its role for photoperiodism. For this reason, the second part of this thesis (**paper II** and **paper III**) aimed to study the photo-neuroendocrine cascade in a polar animal and to determine whether photoperiodism in a High Arctic environment has a circadian basis.

Specifically, in **paper II** we tested if circadian entrainment is necessary for photoperiodic responses. Svalbard ptarmigan lose behavioural rhythmicity in DD and LL. If this loss in behavioural rhythmicity reflects loss of endogenous rhythmicity, birds held in constant conditions might not be able to sustain the circadian rhythm necessary for photoperiodic responses. For this purpose, we directly transferred birds from DD to LL and measured gonadal response and expression of photoperiodic key genes within the PT and MBH.

In **paper III** and in additional data, we extended the analysis on circadian basis of photoperiodic responses in Svalbard ptarmigan by a 'first long day' experiment and skeleton

photoperiods. Both photoperiodic treatments were supplemented with analysis looking at overt changes in seasonal physiology and at molecular events within the PT and MBH, as well as locomotor activity to assess entrainment to the lighting regime employed.

3. Results and discussion

Here, I report and discuss the studies that form this thesis. **Paper II** is published in 'Journal of Experimental Biology' (doi: 10.1242/jeb.220699) while **paper I** and **paper III** are under review at the time of writing this thesis. **Paper I** and **paper III** are also supplemented by additional experiments and analysis that will presumably be included in revised manuscripts.

The experiments that make up this thesis study the circadian and photoperiodic system of the Svalbard ptarmigan and especially explore the involvement of circadian rhythmicity in photoperiodic responses in this High Arctic organism. All experiments were conducted on captive Svalbard ptarmigan held in temperature and light controlled rooms.

3.1 Paper I: Body temperature and activity rhythms under different photoperiods in High Arctic Svalbard ptarmigan (*Lagopus muta hyperborea*)

In **paper I**, we further characterised the behavioural and physiological phenotype of Svalbard ptarmigan by studying rhythms of core body temperature (T_b) in different photoperiods. In general, the daily T_b rhythm is defined as high temperature during the active phase and lower temperature during the rest phase. Despite its temporal association with activity, the T_b rhythm is an independently controlled feature in endotherms and is known to be under circadian control (Murakami et al., 2001; Refinetti and Menaker, 1992), yet its function remains uncertain. The lowering of T_b during the rest phase might decrease the cost of heat production but the amplitude of the T_b rhythm is possibly too small to have a significant impact on the energy budget (Menaker, 1959; Refinetti and Menaker, 1992). Alternatively, T_b might serve the master circadian clock to synchronise peripheral clocks (**Box 1**), as temperature changes within the physiological range can sustain rhythmicity in mammalian liver and lung cultures (Brown et al., 2002; Buhr et al., 2010).

In Svalbard ptarmigan, melatonin (Reierth et al., 1999) and activity rhythms (Reierth and Stokkan, 1998a; Stokkan et al., 1986a) are well characterised and with the experiments of **paper I** we aimed to provide a similar characterisation for the T_b rhythm. Through its connection to the circadian clock, being either a simple output or a synchronising avenue, the investigation of the T_b rhythm in Svalbard ptarmigan might give insights into the nature of the circadian system in this High Arctic bird.

For this purpose, we implanted 'iButton' temperature logger into the abdominal cavity and measured T_b alongside activity in different photoperiods while keeping ambient temperature constant. Experimental birds were either held under short photoperiod (SP, L:D 6:18), long photoperiod (LP, L:D 16:8), constant light (LL) or constant darkness (DD). Svalbard ptarmigan in SP and LP showed clear cycles in T_b and activity, with high T_b during the diurnal active phase and low T_b during the nocturnal rest phase. In LL and DD, both activity and T_b rhythm showed no daily rhythmicity for the analysed periods. For activity, however, we observed ultradian rhythmicity in LL. In birds under SP, we further observed a clear rise in T_b before the light-on

signal and before rise in activity. This anticipatory increase in T_b suggests the presence of a functional timekeeping mechanism involving the circadian system.

It must be noted that during the recording under DD, birds often expressed transient peaks in T_b that are reminiscent of sustained rhythmicity. However, we ascribe this observation to regular husbandry and stress related increase in T_b (Nord and Folkow, 2019). In a recent, so far unpublished, experiment we measured T_b in DD and LL again and improved husbandry by minimizing disturbance and varying entry times (see next chapter).

In conclusion, during times with a light-dark cycle, the Svalbard ptarmigan employs a time measuring system, likely the circadian clock. Contrary, under constant photic conditions this system seems either to uncouple from its output or does dampen in rhythmicity (Bloch et al., 2013), allowing arrhythmicity in T_b.

3.2 Extension to paper I: Dampening of body temperature rhythms

Having established that daily rhythmicity in T_b is lost in captive birds under LL and most likely under DD (**paper I**), we asked the question if and how fast this rhythm dampens in light-entrained birds transferred into these constant photic conditions.

In a so far unpublished experiment, we have entrained seven Svalbard ptarmigan to a L:D 12:12 schedule for 25 days and released them directly into DD for 20 days. Thereafter we re-entrained the birds to L:D 12:12 for 20 days and released them into LL for another 20 days. During this photoperiodic treatment we measured T_b with intraperitoneal implanted iButtons at a 30-m interval. Dampening rhythms were analysed by fitting following damped sine wave function (GraphPad 8):

$$Y = Amplitude^{(-K \times X)} \times \sin\left(\frac{2 \times \pi}{Wavelength}\right), \qquad where\ K = decay\ constant$$

Additionally, half-life times ($t_{1/2}$, time by which the rhythm dampened by half) were calculated for each individual bird.

$$t_{1/2} = \frac{\ln(2)}{K}$$

Half-life times between DD and LL were then compared by paired t-test (GraphPad 8).

In both cases, the birds expressed a dampening rhythm (**Figure 7A**). While T_b in LL dampened rapidly (half-life time = 1d 23h ± 1d 12h, mean ± SD), the rhythm dampened slower in DD (5d 12h ± 1d 17h, p = 0.004 by paired t-test) (**Figure 7B**). Exposure to constant bright light disrupts activity rhythms in a range of birds (Gänshirt et al., 1984; McMillan et al., 1975; Simpson and Follett, 1982; Yamada et al., 1988). This disruptive effect of LL might also explain the fast dampening T_b rhythm in Svalbard ptarmigan under LL.

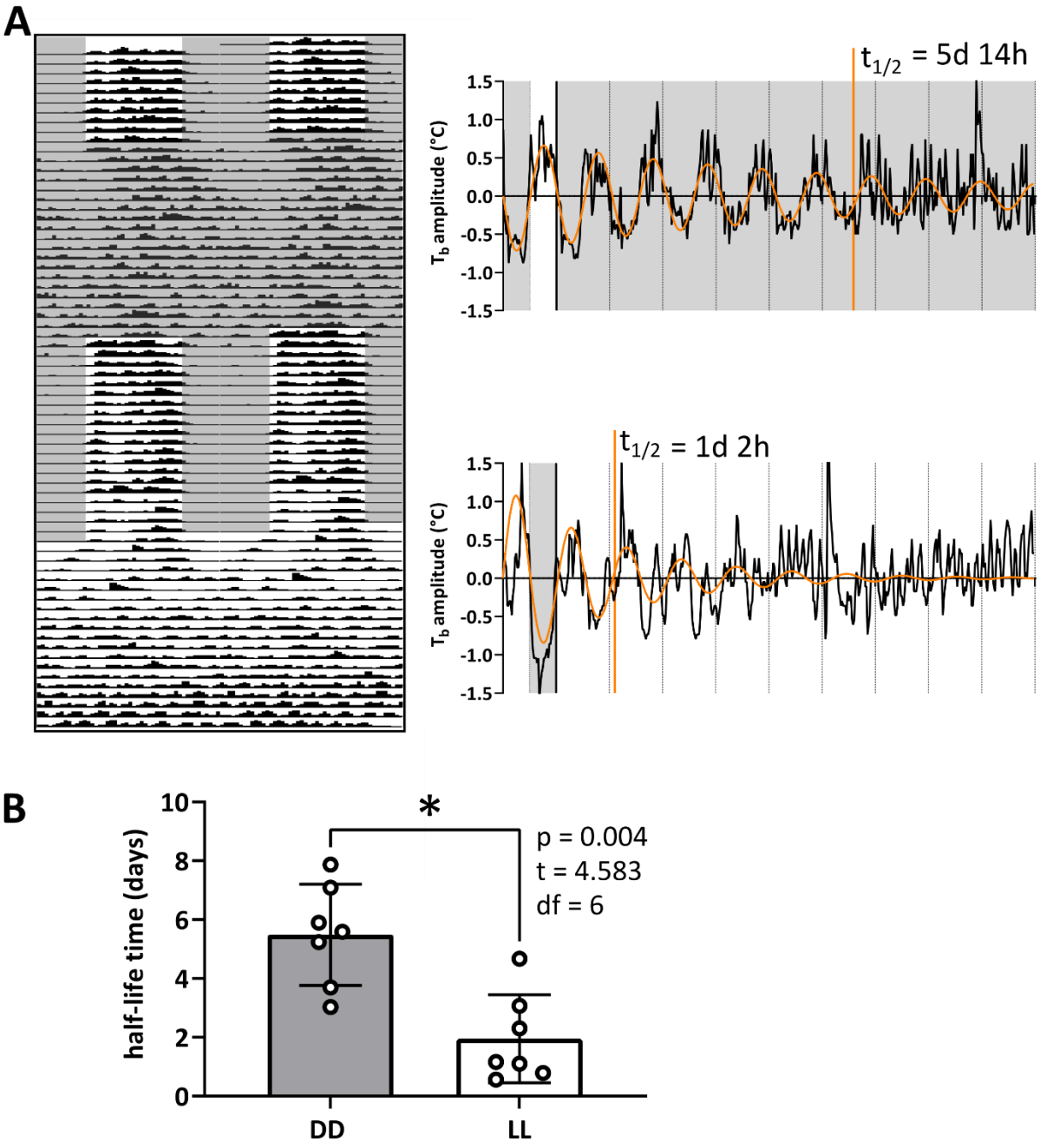


Figure 7. Svalbard ptarmigan show dampening T_b rhythms under DD and LL.

(A) Double plotted T_b-actogram of a representative bird entrained to L:D 12:12 and transferred into DD, re-entrained and released into LL. Next to the T_b-actogram, 10 consecutive days of dampening T_b rhythm are shown for the same representative bird. The first day is L:D 12:12 and the following nine days are either in DD or LL. In each case the fitted sine wave showed a dampening effect (orange wave). Respective half-life times (orange vertical line) for the representative bird are given. The T_b-actogram is plotted between 40 and 42 °C. Grey shadings in T_b-actogram and time series indicate phases of darkness.

(B) Half-life times of all seven birds between different photoperiods were compared by paired t-test. The T_b rhythm dampened significantly slower in DD than in LL. Data is displayed as mean ± SD.

I further analysed free-running periods in the T_b rhythm for all seven birds under DD and LL (**Figure 8**). For this purpose, each 20-day recording in DD and LL was divided into 10 consecutive days and analysed with χ^2 periodograms (Sokolove and Bushell, 1978). For the first 10 days in DD, all birds showed free-running rhythms with periods longer than 24 h (24h 30m – 25h) while for the second 10 days in DD all but one bird lost these rhythms. For the first 10 days in LL, five out of seven birds showed free-running rhythms with significant periods, most of which were shorter than 24 h (22h 30m – 23h 30m). For the second 10 days in LL, all five birds that previously showed free running rhythms lost them. The results from the first 10 days in constant conditions are consistent with Aschoff's rule stating that in a diurnal species the free-running period is shorter in LL than in DD (Aschoff, 1960; Pittendrigh, 1960).

Like in **paper I**, this study further suggests the existence of a functional circadian system in Svalbard ptarmigan, yet one which allows fast dampening of its circadian output, rendering arrhythmicity under LL and DD.

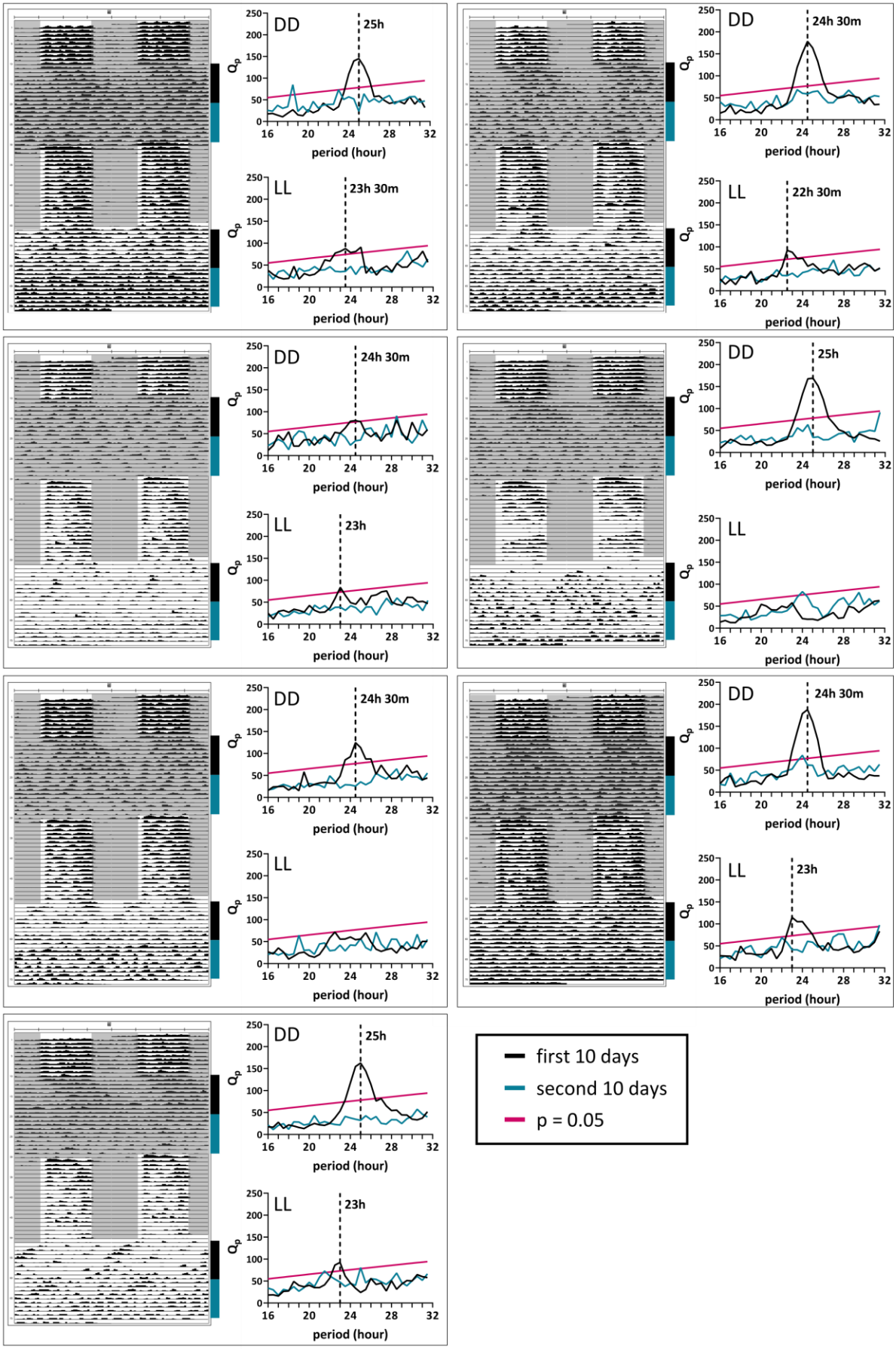


Figure 8. Free-running T_b rhythms in constant darkness and constant light. Description on the next page

Figure 8. Free-running T_b rhythms in constant darkness and constant light. Double plotted T_b-actogram of seven birds entrained to L:D 12:12 and transferred into DD for 20 days, re-entrained and released into LL for another 20 days. Respective χ^2 periodograms are plotted to the right of each T_b-actogram. Each periodogram analyses free-running periods in either DD or LL. The black line in each periodogram represents the first 10 days in constant condition (day 1 to day 10) whereas the blue line represents day 11 to day 20 in constant conditions. Highest periods above the significance level ($p < 0.05$, red line) within the first 10 days in constant conditions are marked by a dotted line. All T_b-actograms are plotted between 40 and 42 °C and grey shadings indicate periods of darkness.

3.3 Paper II: Photoperiodic induction without light-mediated circadian entrainment in a High Arctic resident bird

Knowing that loss of daily rhythmicity in Svalbard ptarmigan occurs in LL as well in DD (**paper I** and extension; Reierth and Stokkan, 1998a; Reierth et al., 1999; Stokkan et al., 1986a); the question arises of whether a circadian-based photoperiodic response still can take place under these conditions.

In the experiment of **paper II**, we used DD-adapted birds and either transferred them to a simulated natural increasing photoperiod (SNP) until they were in LL, retained them in DD or transferred them directly into LL. All experimental photoperiods were conducted over a 10-week period. We measured behavioural responses in form of activity and gonadal response by weighing ovaries and testes *post mortem*. We also measured key genes of the TSH/ DIO pathway within the PT/ MBH region by radioactive *in situ* hybridisation (*Eya3*, *Tshβ*, *Dio2* and *Dio3*). For this purpose, birds were sampled as followed: in DD at week 0 and week 10; in LL 38 h and 10 weeks after the transfer; in SNP at week 5 (photoperiod L:D 12:12) and week 10 (photoperiod LL).

Svalbard ptarmigan under DD did not show any signs of reproductive response to the continuous exposure of DD, while birds under SNP entrained to the light-dark cycle and showed a photoperiodic response in the TSH/ DIO cascade as well as gonadal response at the end of their photoperiodic treatment. Importantly, birds directly transferred from DD into LL showed also clear signs of a photoperiodic response in form of increased expression of *Eya3*, *Tshβ* and *Dio2*, decreased expression of *Dio3* and gonadal response, while being behaviourally arrhythmic in both conditions. This response took place within 38 h after the transfer and was sustained over 10 weeks in LL. This shows that circadian entrainment to a light-dark cycle is not required for a photoperiodic response in Svalbard ptarmigan. For this reason, Svalbard ptarmigan must either employ a circadian rhythm which can be sustained throughout DD but is disconnected from activity, or they must be able to initiate a sufficient rhythm to measure photoperiod through a single transition from DD to LL.

3.4 Paper III: Adaptive value of circadian rhythms in High Arctic Svalbard ptarmigan

In **paper III,** we further addressed the circadian basis of photoperiodism in Svalbard ptarmigan. In the first part, we measured clock gene expression alongside expression of key genes within the PT/ MBH region in birds under LL. We entrained two groups of birds to L:D 6:18 and then released one group into LL while the other group remained under L:D 6:18. In both groups, we sampled birds (N = 4) at 5-h intervals for 24 h and measured expression of the clock genes *Cry1* and *Per2* as well as the expression of *Tshβ*, *Dio2* and *Dio3* within the PT/ MBH region. The rhythm of *Cry1* and *Per2* expression in the PT and MBH was unaltered between the groups. Simultaneously, the expression patterns for *Tshβ*, *Dio2* and *Dio3* were strikingly similar to those seen in quail in response to an acute photoperiod extension (Nakao et al., 2008).

In the second part of the study, we subjected birds to a skeleton photoperiod in which the first light phase of 4 h was fixed and the second light phase of 2 h was shifted away from the first one in increments of 2 h every week. The skeleton photoperiod in which the second light phase occurred 14 to 16 h after the beginning of the first light phase (ZT 14-16) induced a photoperiodic response in terms of increased pre-breeding activity and rapid decline in body mass. This photoperiodic response gradually diminished as the second light phase was shifted further.

In the last part of the study, we exposed birds again to a shifting skeleton photoperiod but halted the second light phase after it reached ZT 14-16. We let birds remain in this photoperiod for an additional two weeks before they were sampled. In these birds we measured increased expression of *Dio2* and decreased expression of *Dio3* compared to SP-control birds (birds held at L:D 6:18).

Taken together, these results demonstrate that the PT/ MBH region of High Arctic Svalbard ptarmigan possesses the circadian elements necessary for a rhythm-based photoperiodic response (first part of the study) and that this system can be triggered by a skeleton photoperiod (second part). This suggests that the circadian clock underpins photoperiodic sensitivity in Svalbard ptarmigan.

3.5 Extension to paper III: Re-entrainment in a shifting skeleton photoperiod

In **paper III**, we subjected Svalbard ptarmigan to a shifting skeleton photoperiod, in which we fixed a 4-h light phase and moved a second 2-h light phase backward by 2 h every week (**Figure 9A**). Birds exposed to this light treatment showed a transient long-day response between week 5 and week 7 of the experiment, and returned to their short-day phenotype thereafter. This observation can be explained through two potential mechanisms. The first, can be described as the shifting light phase moving in and out of the photoinducible phase (external model) or moving oscillating factor in and out of the necessary phase relationship (internal model). Alternatively, the data can be explained as a perceptual shift of the dawn signal (Takahashi and Menaker, 1982b). According to this explanation, at the start of the experiment the light-on switch of the fixed light phase was received as dawn signal, while from week 8 onwards the light-on switch of the moving light phase was perceived as dawn. Consequently, the moving light phase was setting the rhythm necessary for the photoperiodic response from week 8 onwards and the fixed light phase, due to its proximity to the moving light phase, was not received as long-day signal.

In support for the second mechanism, I provide here analysis of pre-dawn anticipatory behaviour (**Figure 9B**). In this analysis, I used activity counts 1 h before the light-on signal of both light phases of the skeleton photoperiod. Initially, Svalbard ptarmigan showed increased activity immediately before the fixed light-on signal but very little change in activity before the moving light-on signal. This changed abruptly in week 8 of the experiment. From week 8 onwards, anticipatory activity was more pronounced before the moving light phase. This

suggests that the light-on signal of the moving light phase was from there onwards considered as dawn. This interpretation is consistent with the view that entrainment of the damped circadian system of the Svalbard ptarmigan has consequences both for daily behaviour patterns (dawn anticipation) and seasonal photoperiodism (return to short day phenotype).

As described in the introduction, the quail, in contrast to starling or white-crowned sparrow (Follett et al., 1974; Gwinner and Eriksson, 1977; King et al., 1997), appears to re-entrain to a re-occurring light pulse in Nanda-Hamner protocols rather than to track it (**Figure 4**). Based on our analysis it can be concluded that the photoperiodic system of Svalbard ptarmigan resembles that of a quail in this respect.

Having an easily re-entrainable rather than a strong self-sustained rhythm might be advantageous on the High Arctic archipelago of Svalbard. The periods between polar night and polar day are characterised by fast changes in day length (15-40 minutes/ day). The flexible circadian system of the Svalbard ptarmigan could allow fast re-entrainment to the fast changing photoperiod and can therefore provide precise photoperiodic measurements. Contrarily, birds like starling and sparrows with robust circadian systems might re-entrain slower and might not be able to track the fast changing photoperiod accurately.

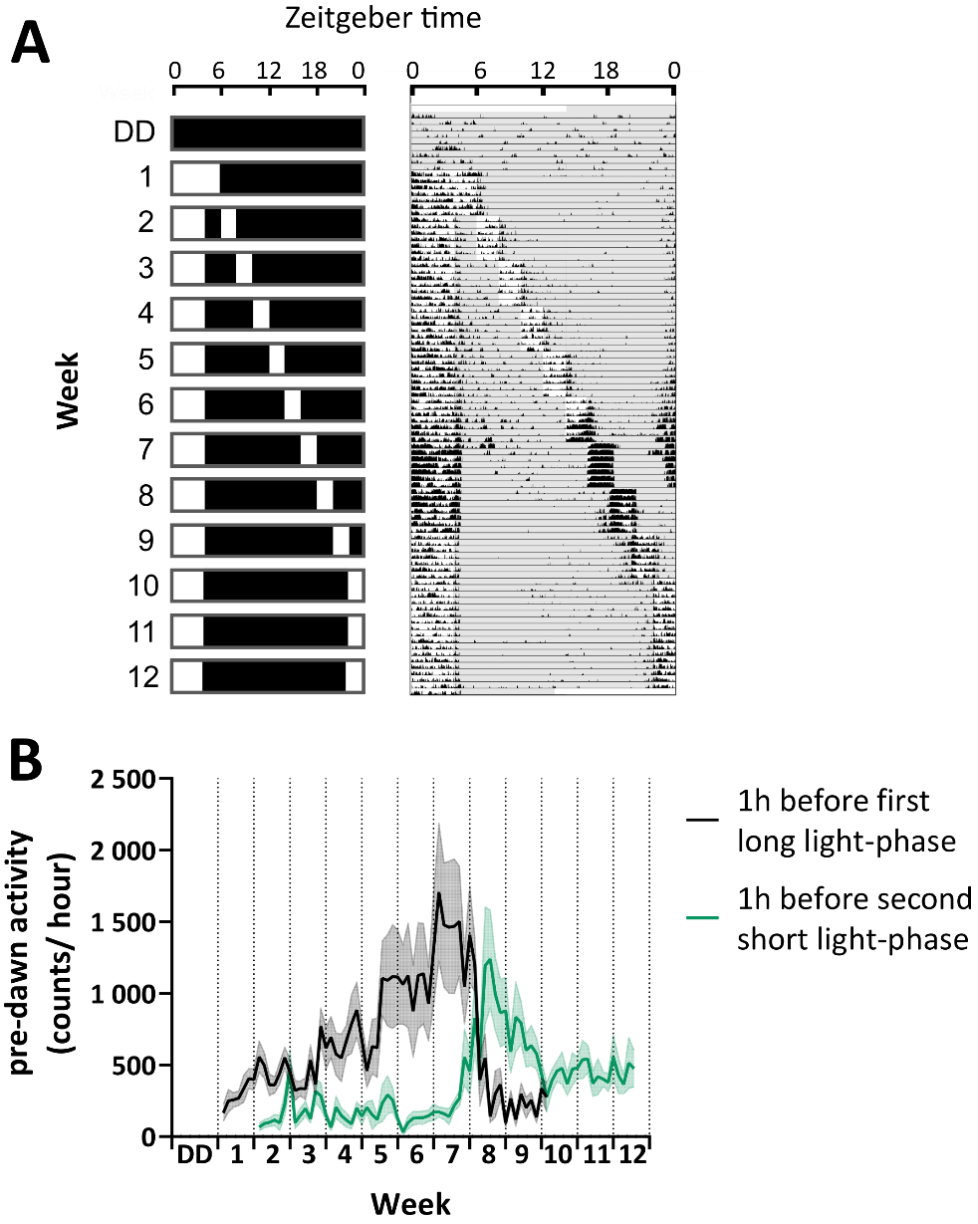


Figure 9. Pre-dawn activity moves from one light phase to the other in a shifting skeleton photoperiod experiment.

(A) In the skeleton photoperiod of **paper III** we fixed a 4-h light phase while moving a second 2-h light phase every week by 2 h. The figure shows the experimental design with a corresponding single plotted actogram from a representative bird. Grey shadings indicate periods of darkness.

(B) 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).

3.6 Extension to paper III: Free-running activity rhythms in Svalbard ptarmigan

Svalbard ptarmigan display normally no daily rhythms under constant photic conditions, but there are exceptions. In **paper III**, we observed several birds expressing free-running daily activity rhythms (**Figure 10**). The birds in which we observed free-running behaviour belonged to the increasing photoperiod group (IP-group), which served as positive control to the shifting skeleton photoperiod (SkP-group). In the IP-group, we fixed the light-on signal and extended the light-off signal by 2 h every week so that it resembled the SkP-group in terms of light timing. Birds of the IP-group, therefore, experienced an increasing photoperiod until they reached LL, in which 5 out of 12 birds showed free-running activity rhythms with periods ranging from 23h 30m to 28h 30m (as measured by χ^2 periodogram (Sokolove and Bushell, 1978)). All birds which showed free running activity were housed together (12 birds in the IP-group divided into two separated room; 6 birds/ room). For this reason the number of birds expressing a true free-running rhythm might be overestimated as one dominant free-running bird could have caused aligned activity in other birds.

Occasional free-running activity rhythms further confirm conclusions made in this thesis: High Arctic Svalbard ptarmigan retain a functional circadian system and this is occasionally reflected by free-running activity patterns under certain experimental light conditions. However, the experimental conditions under which we observed free-running behaviour are not reflecting their natural habitat. Svalbard ptarmigan hold captive in Svalbard under natural light and temperature conditions show no free-running but tonic behaviour during the actual polar day and polar night (Reierth and Stokkan, 1998a; Stokkan et al., 1986a).

Interestingly, 4 out of the 5 birds with free-running activity rhythms had free-running periods longer than 24 h. This contradicts the observations made in chapter '3.2 Extension to paper I'. There, birds under LL displayed free running T_b rhythms with periods shorter than 24 h.

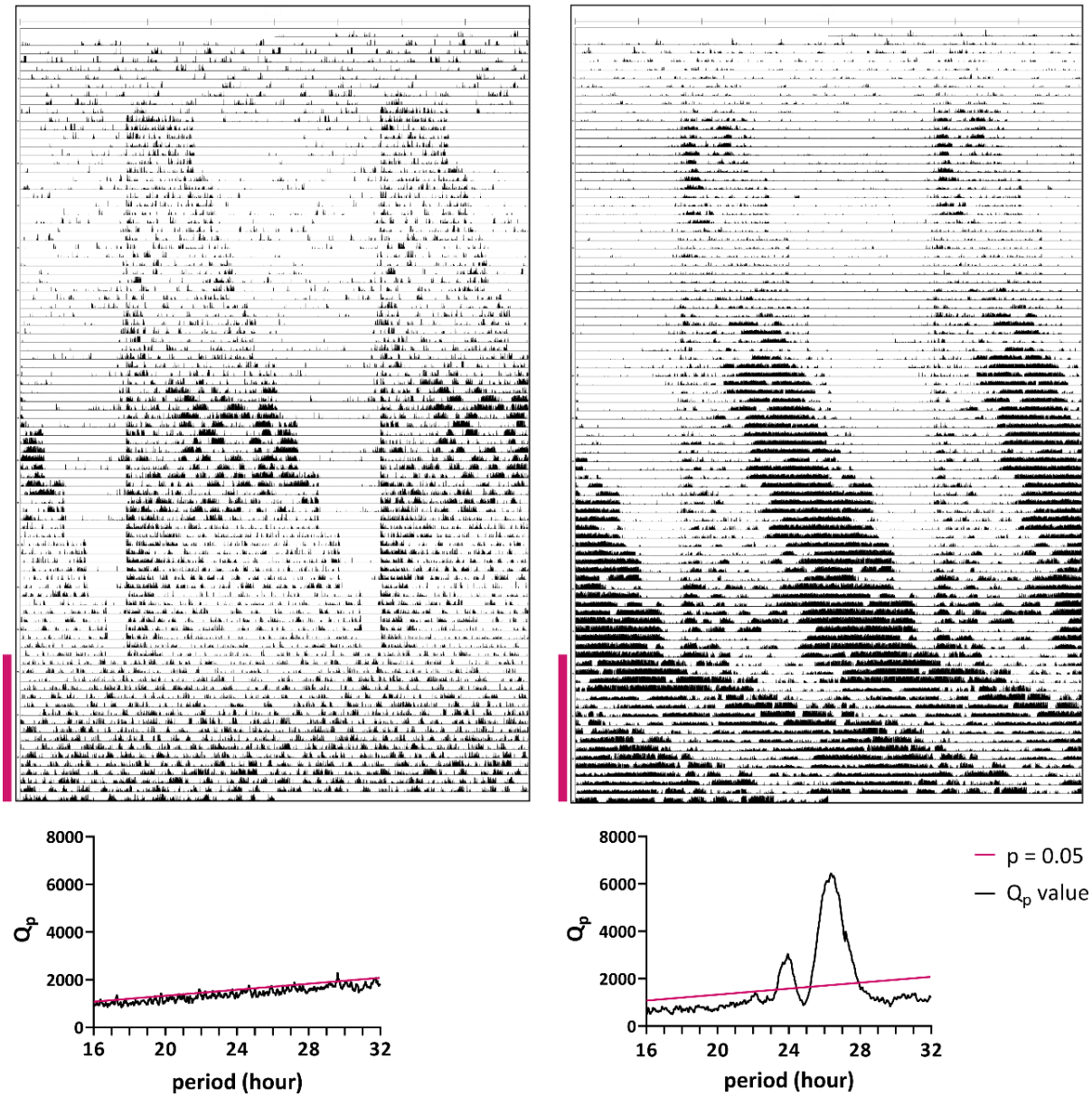


Figure 10. Artificial light experiment followed by constant light can lead to free-running activity rhythms. Both representative birds underwent a photoperiod in which they were transferred from constant darkness to L:D 6:18 and experienced a dusk signal extension by 2 h every week until they reached constant light. The left bird showed no free-running daily activity under LL (red bar), while the right bird showed a free-running activity rhythm with a period of 26h 22m. The activity data is displayed in double-plotted actograms with their respective χ^2 periodograms underneath (the red line indicates a p-value of 0.05 of a χ^2 distribution).

3.7 Discussion

Svalbard ptarmigan show no daily rhythms in activity and T_b under LL or DD. While behavioural arrhythmicity in birds under constant bright light is common, the loss of rhythmicity under DD is not (Gänshirt et al., 1984; McMillan et al., 1975; Simpson and Follett, 1982; Underwood, 1994; Yamada et al., 1988). This suggests that the loss of daily rhythmicity under constant photic conditions is an adaptation to the High Arctic environment of Svalbard. Despite this weak circadian control over behaviour and physiology, Svalbard ptarmigan still seem to utilise circadian rhythmicity for seasonal responses.

It remains unknown how this dichotomy is achieved in this High Arctic bird, but the structure of the avian circadian system might provide some explanation. In contrast to mammals, the avian circadian system contains at least three circadian master clocks: the pineal gland (Deguchi, 1979; Zimmerman and Menaker, 1979), the retina (Underwood et al., 1990) and the avian SCN (Simpson and Follett, 1981; Takahashi and Menaker, 1982a). All of them are able to entrain to a light-dark cycle and produce rhythmicity endogenously (Brandstätter, 2003; Cassone, 2014), but none of them seems to provide the circadian basis for photoperiodism. Neither SCN removal nor enucleation or pinealectomy can abolish photoperiodic responses in birds while having species-dependent effects on activity rhythms (Benoit, 1964; Davies and Follett, 1975; Menaker and Keatts, 1968; Menaker et al., 1970; Siopes, 1983; Siopes and Wilson, 1974; Wilson, 1991). The most reasonable explanation is that the PT/ MBH region acts as an independent oscillator and provides its own circadian basis for photoperiodism. In birds, deep brain photoreceptors, such as neuropsin (Nakane et al., 2010; Stevenson and Ball, 2012) and VA-opsin (García-Fernández et al., 2015; Halford et al., 2009), seem to relay photic information to the PT, allowing direct entrainment to the environment. Furthermore, clock gene expression in the PT/ MBH region can vary from clock gene expression within the SCN and pineal gland in quail, suggesting independence between them (Yasuo et al., 2003; Yasuo et al., 2004). In the case of Svalbard ptarmigan, I propose that rhythmicity must be retained within the PT/ MBH region to provide the rhythm for coincidence timing, while rhythmicity may be lost within the part of the circadian system controlling daily organisation of behaviour, metabolism and physiology (**Figure 11A**).

This loss of rhythmicity could be achieved through different mechanisms as outlined in Bloch et al. (2013). It is possible that the circadian system is endogenously rhythmic even under constant conditions but that this rhythmicity is disconnected from its output. Alternatively, the rhythm produced by the central system might dampen under constant conditions and re-initiates once zeitgebers become available again. The third option is a combination of the former two: individual cells of the circadian master clocks retain rhythmicity but desynchronise from each other in constant conditions leading to arrhythmicity on tissue level and in the output after a period of dampening (**Figure 11B**) (Balsalobre et al., 1998; Nagoshi et al., 2004; Welsh et al., 2004). This alternative has appeal as it would be parsimonious with the retained rhythmicity within the PT/ MBH region. In this scenario, each *Tshβ* expressing cell within the PT would act as an independent coincidence timer, which de-synchronises from its

surrounding cells in constant photic conditions, i.e. without entrainment. Rhythmicity on tissue level would be lost but the rhythm-dependent output would be retained, i.e. increased *Tshβ* expression and the photoperiodic response in reproduction (**Figure 11C**).

Our own data and unpublished results about *Tshβ* expression within the PT is compatible with this scenario. In **paper III**, we observed increased *Tshβ* expression and subsequent decrease 13 h after the transfer from SP to LL. This is arguably because individual PT-thyrotrophs are still synchronised. In **paper II**, however, we were able to measure high *Tshβ* expression after 10 weeks in LL. It is unlikely that all the birds are all in the same circadian phase after 10 weeks, yet we measured consistently high *Tshβ* expression in all of them. Similarly in **paper II**, the SNP-group was exposed to a gradual increasing photoperiod until reaching LL. After four days in LL we sampled the birds, all with a consistently high *Tshβ* expression. Lastly, in another unpublished experiment we transferred birds from a short photoperiod to a gradual increasing photoperiod and sampled them after two weeks in LL with the same result: all birds have consistently high *Tshβ* expression. All these results suggest that Svalbard ptarmigan, show constant high *Tshβ* expression within the PT under prolonged LL.

PT-cells with retained coincidence rhythm, but desynchronised from their neighbouring cells, can explain a constant high *Tshβ* and a sustained photoperiodic response in LL (**Figure 11C**). If the same mechanism is applied to the part of the circadian system controlling behaviour and physiology we can likewise explain the loss of rhythmicity under constant photic conditions as desynchronised cells are unable to produce a rhythmic tissue-dependent output (**Figure 11B**). Hence, the model proposed in **Figure 11** uses the same mechanism to describe how Svalbard ptarmigan can temporarily lose one circadian-dependent output (activity, T_b, melatonin) while retaining another (high *Tshβ* expression in the PT and a consequent photoperiodic response).

Alternatively, the Svalbard ptarmigan's ability to exploit circadian-based photoperiodism but to escape the circadian dictation on behaviour in constant photic conditions can be explained by a dampening endogenous rhythm controlling all outputs in combination with positive feedback loops. In this scenario, photoinduction would be achieved within the first cycles of a dampening rhythm. After photoinduction, this rhythm would break down and a sustained photoperiodic response would be ensured through positive feedback loops. The data presented in this thesis might be very well explained by such a scenario and future research could aim to resolve between the two models described here.

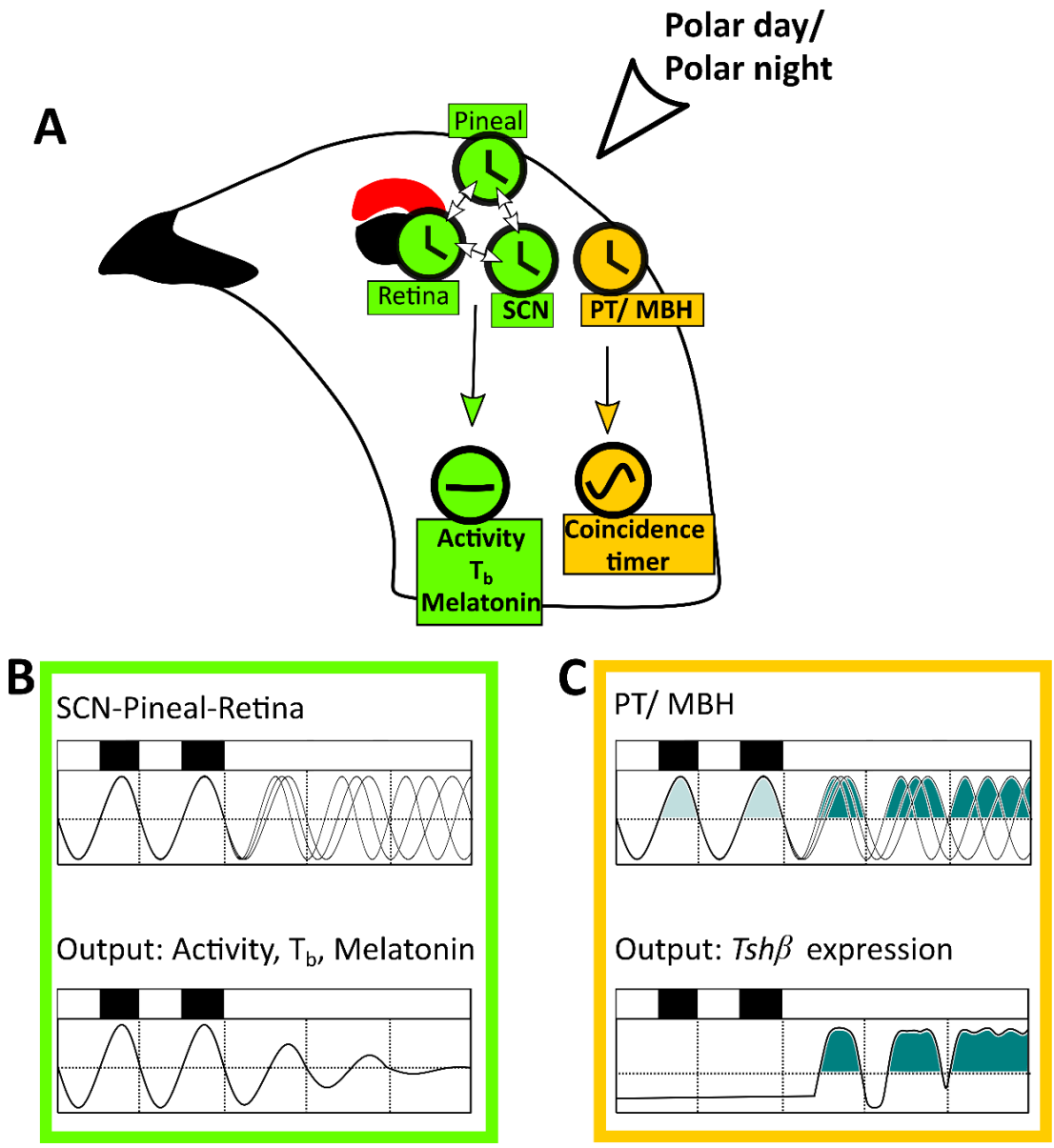


Figure 11. Proposed model of a circadian system adapted to the High Arctic.

(A) The avian circadian system is comprised of three circadian master clocks: the avian SCN, the pineal gland and the retina. All of them are entrainable and produce rhythmicity endogenously. Additionally, the PT/ MBH region might act as another master clock solemnly providing the coincidence rhythm necessary for photoperiodic responses. This structure might allow the Svalbard ptarmigan to lose rhythmicity in behaviour and physiology while retaining rhythm-based photoperiodism.

(B) In the part of the circadian system comprising the avian SCN, pineal and retina, daily rhythmicity in the output might be lost due to cells that retain circadian rhythmicity but desynchronise from one another.

(C) By the same mechanism, PT-thyrotrophs might become desynchronised from each other but continue to act as coincidence timers as individual units. This would result in constant *Tshβ* expression across the PT and would ensure a sustained photoperiodic response in reproduction throughout the polar day.

4. Conclusion

In **paper I** and its extension, we have confirmed that High Arctic Svalbard ptarmigan are behavioural and physiologically arrhythmic in constant conditions. Despite the normally occurring breakdown of daily rhythmicity in the absence of light-mediated entrainment, we were able to trigger a photoperiodic response in these conditions in **paper II**. This suggested that photoperiodic responses in Svalbard ptarmigan are mediated by either a sustained or rapidly initiated coincidence rhythm. In **paper III**, we provide the evidence for circadian rhythmicity in the PT/ MBH region and suggest it to be used as basis for photoperiodic responses in reproduction, reproductive activity and body mass.

The circadian system of Svalbard ptarmigan hence displays a dichotomy perfectly suiting its High Arctic habitat: it retains aspects of circadian organisation necessary to support photoperiodism while allowing temporal organisation of behaviour and physiology to escape circadian hegemony in constant light and constant darkness.

This dichotomy might be explained through the multi-oscillator organisation of the avian circadian system and future research could aim to explore this hypothesis.

5. Ongoing and future research

5.1 Ptarmigan genomics

Based on above described studies, I have concluded that Svalbard ptarmigan have a functional circadian system that forms the basis of their photoperiodic responses. This assertion is further supported by ongoing analysis of clock genes in rock ptarmigan genomes that are available through collaborations with the Höglund group at the University of Uppsala, Sweden and the Magnússon group at University of Akureyri, Iceland (Kozma et al., 2016).

In silico analysis of the circadian gene network can provide valuable insights into chronobiological adaptations to different environments. For example, an *in silico* study on reindeer genomes concluded that a mutation impaired the function of PER2, which might affect the generation of circadian rhythmicity leading to the arrhythmic behaviour that can be observed in reindeer under constant photic conditions (Lin et al., 2019; van Oort et al., 2005; van Oort et al., 2007). I have conducted similar *in silico* analysis on a rock ptarmigan genome, based on samples from Icelandic ptarmigan. More specifically, I have extracted sequences for the clock genes *Arntl*, *Per2*, *Per3*, *Cry1*, *Cry2* and *Clock* and translated them into their respective amino acid sequences. I then aligned them to other bird species and analysed the presence of functional domains. The results of this are that clock genes are most similar to other fowl species and all expected functional domains are present, suggesting intact molecular circadian oscillator in rock ptarmigan.

The genomic work has only focused on an Icelandic rock ptarmigan genome, but the analysis and collaboration is ongoing. Recently, our collaborators have re-sequenced several individuals from distinct ptarmigan populations across a latitudinal cline (**Figure 12**), including individuals from the Svalbard ptarmigan population. By comparing the High Arctic Svalbard ptarmigan to the more southern populations, we hope to further uncover genetic adaptation to the Arctic, especially in a chronobiological context.

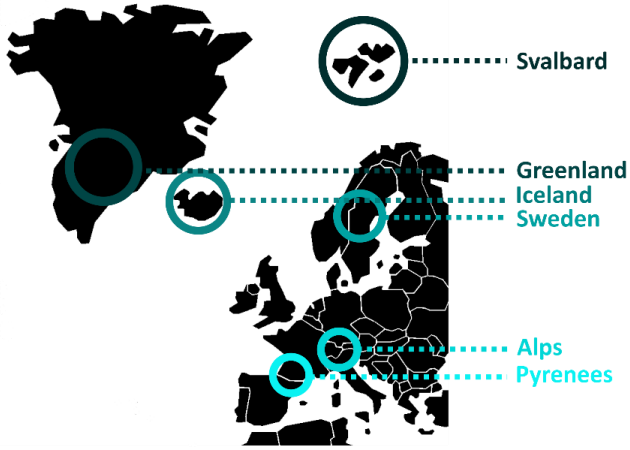


Figure 12. DNA was sampled from different rock ptarmigan populations. In an ongoing collaboration we are studying the genetic variations of rock ptarmigan populations in habitats across different latitudes with highlight on the Arctic and chronobiological adaptations.

5.2 Svalbard ptarmigan fibroblast cultures

The chronobiological adaption of Svalbard ptarmigan excels in the fact that their circadian rhythmicity forms the basis for photoperiodism but that circadian rhythmicity is not inflicted upon behaviour and physiology in constant conditions. The question remains how this dichotomy is achieved mechanistically. Current explanations include the possibility that Svalbard ptarmigan either uncouple central circadian rhythmicity from distinct outputs or that central rhythmicity is not sustained without photic entrainment (Bloch et al., 2013). The breakdown in rhythmicity might either be caused by dampening of molecular rhythmicity or by desynchronised cells (Balsalobre et al., 1998; Nagoshi et al., 2004; Ohta et al., 2005; Welsh et al., 2004).

In order to distinguish between the different explanations we are using fibroblast cultures from Svalbard ptarmigan. In an initial experiment (**Figure 13**), we synchronised fibroblast cultures with a serum shock (Balsalobre et al., 1998) and measured clock gene expression between 24 and 48 h after the serum shock by qPCR. The clock gene expression of *Cry1*, *Cry2*, *Per2*, *Clock* and *Arntl* were all tonic. Only *Per3* expression showed significant changes across the measured period (one-way ANOVA, degree of freedom= 7, F = 4.491, p = 0.0026). These results show that rhythmicity cannot be sustained in fibroblast cultures of Svalbard ptarmigan but at the current point we cannot distinguish how rhythmicity is lost: breakdown of molecular rhythm or desynchronised cells. Ongoing research, including single cell imaging and clock gene reporters, might shed further light on this matter.

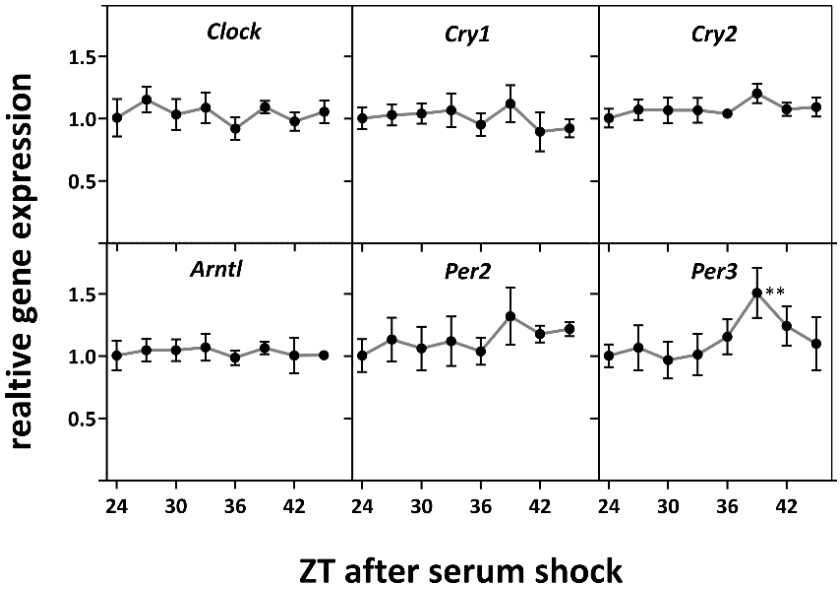


Figure 13. *In vitro* analysis of clock genes in fibroblast cell cultures have revealed tonic expression levels 24 h after a synchronising serum shock (*Per3* excepted). These preliminary results either suggest that fibroblasts cannot sustain rhythmic clock gene expression or that individual cells de-synchronise from each other rapidly. Dots symbolise mean ± SD of 4 wells per sampling point. Asterisk indicates significant changes in expression of *Per3* tested by one-way ANOVA (degree of freedom= 7, F = 4.491, p = 0.0026). Unpublished data.

5.3 Seasonal body mass cycles in Svalbard ptarmigan

Besides their circadian organisation, Svalbard ptarmigan have another outstanding feature that makes them an interesting subject for chronobiological research: their body mass cycle. Svalbard ptarmigan show pronounced seasonal changes in body mass achieved through deposition or depletion of fat pads (Mortensen et al., 1983). As with reproduction, the body mass cycle is under strong photoperiodic control in Svalbard ptarmigan. They increase in fat mass as a result of shortening photoperiod (Lindgård et al., 1995). In captivity, high body mass is retained as long as the photoperiod is short and fat stores are rapidly depleted once the birds are transferred to a long photoperiod (Stokkan et al., 1995). In nature, this deposition of fat ensures emergency energy rations for the long Arctic winter with limited access to food and insulation (Mortensen and Blix, 1986; Stokkan, 1992).

The mechanisms underlying control of seasonal body mass cycles, especially in birds, remain poorly understood. In Svalbard ptarmigan, the energy balance comprised of food intake and activity might play an role, but sliding set-point experiments (Mortensen and Blix, 1985; Stokkan et al., 1995) clearly indicate that body mass follows an endogenous determined value. In order to explore seasonal body mass control in Svalbard ptarmigan, we designed an experiment in which we achieved four different body mass phenotypes (**Figure 14**): fat birds under short photoperiod, birds losing body mass after a transfer to LL, lean birds under LL and photorefractive birds under LL gaining weight again. Among others, we will analyse gene expression patterns within the PT/ MBH region and tanycytes to explore their potential role in the control of energy metabolism (Ebling and Lewis, 2018; Helfer et al., 2019; Langlet, 2019).

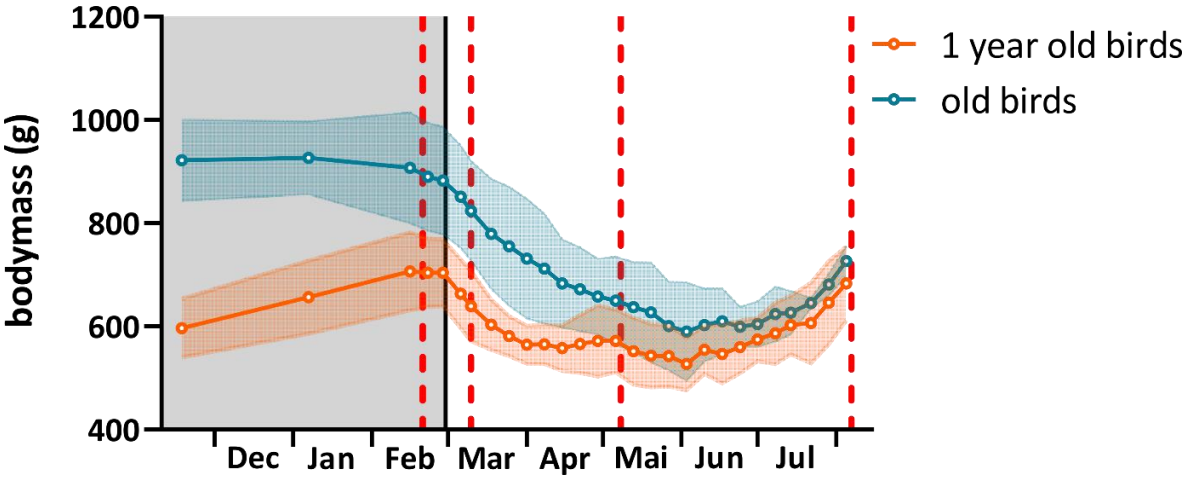


Figure 14. Svalbard ptarmigan exhibit pronounced body mass cycles due to rapid deposition and depletion of fat. There is little knowledge about control over seasonal body mass cycles in birds. In our current research, we aim to shed light on this by studying the state of the PT/ MBH region and tanycytes in birds of different states and different ages. So far, we transferred birds from SP (grey area) into LL (white area) and sampled them at four occasions (red line: fat birds under short photoperiod, birds transferred into LL and losing weight, lean birds, photorefractive birds which gain weight). Data displayed as mean ± SEM.

6. References

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Paper I

Body temperature and activity rhythms under different photoperiods in high Arctic Svalbard ptarmigan (*Lagopus muta hyperborea*)

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Abstract

Organisms use circadian rhythms to anticipate and exploit daily environmental oscillations. While circadian rhythms are of clear importance for inhabitants of tropic and temperate latitudes, its role for permanent residents of the polar regions (where there are constant photic conditions for a large part of the year) is less well understood. The high Arctic Svalbard ptarmigan shows behavioural rhythmicity in presence of light-dark cycles but is arrhythmic in constant photic conditions (i.e., during the polar day and polar night). This has been suggested to be an adaptation to the unique light environment of the Arctic. In this study, we examined regulatory aspects of the circadian control system in the Svalbard ptarmigan by recording core body temperature (T_b) alongside locomotor activity in captive birds under different photoperiods. We show that T_b and activity are rhythmic with a 24-h period under short (L:D 6:18) and long photoperiod (L:D 16:8). Under constant light and constant darkness, T_b is arrhythmic and activity shows signs of ultradian rhythmicity. Birds under short photoperiod also showed a rise in T_b preceding the light-on signal and any rise in activity, which proves that the light-on signal can be anticipated, most likely by a circadian system.

1 Introduction

The Earth's rotation around its own axis causes daily oscillations in environmental factors such as light and ambient temperature. Circadian rhythms have evolved to maintain behavioural, physiological and metabolic synchrony with these ambient cycles, and to anticipate changing conditions within a day. Disruption of this synchrony can consequently affect fitness and survival (Daan et al., 2011; DeCoursey et al., 2000; DeCoursey & Krulas, 1998; DeCoursey et al., 1997; Spoelstra et al., 2016). Biochemical oscillators, so-called circadian clocks, endogenously produce rhythmicity by transcription-translation-feedback loops, in which clock genes are expressed and subsequently inhibited due to the action of their own translated proteins (Darlington et al., 1998; Hardin et al., 1990). In higher vertebrates, circadian rhythmicity is ultimately produced by a single hypothalamic master clock (the suprachiasmatic nucleus in mammals) or a network of clocks (in the pineal gland, eyes and the hypothalamus of bird and reptiles) (Menaker et al., 1997). These master clocks entrain to the environmental cycle primarily through the light-dark signal (Pittendrigh, 1960) and impose rhythmicity onto peripheral tissue, e.g. by circulating hormones such as melatonin produced in the pineal gland (Pevet & Challet, 2011). This ultimately leads to rhythmic physiology and behaviour.

At tropical and temperate latitudes, the light-dark progression and other environmental factors, such as ambient temperature, cycle on a 24-h period throughout the year. This is not the case at polar latitudes which are instead characterized by extended periods of constant light (polar day) and constant darkness (polar night) with short periods of rapidly changing photoperiod in-between. During phases of constant photic conditions, animals inhabiting these latitudes are either free running, arrhythmic or entrained to non-photic or photic cues other than photoperiod, (Arnold et al., 2018; Ashley et al., 2012; Hüppe et al., 2020; Steiger et al., 2013; Stelzer & Chittka, 2010; Swade & Pittendrigh, 1967; van Oort et al., 2007; Ware et al., 2020; Williams et al., 2011). On the Svalbard archipelago, which is amongst the northernmost landmasses in the Arctic (74° to 81°N, **Figure 1B**), the Sun remains ≥ 6° below the horizon between mid-November and February but is constantly above the horizon between mid-April and mid-September. Despite the high latitude, the climate is relatively mild due to warm ocean currents (average temperature in Longyearbyen in December: -6.0 °C) (Norwegian-Meterological-Institute, 2020). This is probably why some cold-hardy species can survive there year-round, including the world's most northerly distributed land bird, the Svalbard ptarmigan (*Lagopus muta hyperborea*) (**Figure 1A**). Svalbard ptarmigan exhibit rhythmic activity during the short periods of light-dark cycles, but become arrhythmic in constant darkness and constant light (Reierth & Stokkan, 1998; Stokkan et al., 1986). Similarly, plasma melatonin levels cycle in a light-dark environment, but are arrhythmic

under constant photic conditions (Reierth et al., 1999). This suggests that either the central circadian clock cannot sustain rhythmicity, or that its molecular output is uncoupled from the peripheral tissue responses in constant photic conditions (Bloch et al., 2013). The above mentioned studies indicate that adaptation to life in the high Arctic has had profound effects on the circadian system. However, little is still known of the physiological parameters that underlie circadian control. For this reason, we explored the implication of Arctic life on the circadian control of core body temperature (T_b).

Most endothermic animals display a daily T_b rhythm which is under circadian control and characterized by lower T_b during the rest phase and higher T_b during the active phase (Aschoff, 1983; Menaker, 1959b; Refinetti & Menaker, 1992). The function of the T_b rhythm is still disputed. The decrease in T_b during the rest phase might reduce energy costs by lowering the need for thermogenesis, though in the case of non-torpid and non-hibernating endotherms this reduction in T_b might be too small to have a significant impact on the energy budget (Menaker, 1959a; Refinetti & Menaker, 1992). Temperature changes within the physiological range have also been shown to sustain rhythmicity in mammalian liver and lung cultures (Brown et al., 2002; Buhr et al., 2010) and it has been proposed that T_b serves the master clock to synchronize peripheral tissue. In a polar animal, T_b might serve the same purpose, especially since melatonin rhythm are often attenuated under constant photic conditions (Miché et al., 1991; Reierth et al., 1999; Stokkan et al., 2007). There is evidence of sustained T_b-rhythmicity through the polar day in Arctic ground squirrels (*Urocitellus parryii*) (Long et al., 2005; Williams et al., 2011), but we are unaware of similar studies in Arctic birds.

In order to characterise the T_b rhythm in a truly Arctic bird and explore its possible circadian control, we implanted abdominal temperature loggers into captive Svalbard ptarmigan and recorded T_b and activity under short photoperiod (SP), long photoperiod (LP), in constant light (LL) and constant darkness (DD) (**Figure 1C**). These photoperiodic treatments were chosen to study expression of the T_b and activity rhythm under entrained conditions (SP and LP) as well as under conditions without entrainable cues, i.e. in free running conditions (LL and DD). We also studied if there were differences in the timing of the rise in activity and T_b before the light-on signal when birds were under SP and LP, because this ‘anticipatory behaviour’ could indicate presence of a functional time-keeping system.

2 Material and Methods

2.1 Housing

All animals were kept at the University of Tromsø in accordance with the EU directive 201/63/EU and licences provided by the Nowegian Food Safety authority (Mattilsynet, permit nos. FOTS 8115 for 2015/2016 and FOTS 7971 for 2017/2018**).** Chicks were hatched from eggs laid by captive females in 2015 and 2017, and were reared either in outdoor cages under natural Tromsø photoperiod (69° 39′ N, 18° 57′ E), or indoors with a photoperiod corresponding to the natural light cycle in Tromsø. When the chicks had reached a body mass of 500 g or more (usually by the end of September in the year of hatching), they were transferred to indoor cages with *ad libitum* access to food (Norgesfor, ref. no. OK2400 070316) and water. Ambient temperature was kept between 3 and 7 °C throughout the experiment, which is within the thermo-neutral zone of physically mature Svalbard ptarmigan (Mortensen & Blix, 1986).

Illumination was provided by fluorescent strip lights (Osram L 58W 830 Lumilux, Osram, Munich, Germany), delivering 1000 lux at floor level. Under constant darkness, illumination was provided by dim red light only (Northlight 36-6557, 15 lm, Clas Ohlson, Insjön, Sweden), which delivered less than 1 lux at floor level.

2.2 Photoperiodic treatment

All experiments were conducted from 30.09.2015 to 04.02.2016 and from 22.12.2017 to 08.04.2018. Birds hatched in the 2015/2016 season were exposed to three different photoperiodic treatments (**Figure 1C**). Initially, all birds were transferred into a photoperiod of L:D 12:12, which was gradually (1 h / day) decreased to L:D 6:18. The birds were subsequently kept in either L:D 6:18 (SP-group, n = 7) or were gradually (1 h / day) transferred into L:D 16:8 (LP), and then to LL (LP/LL-group, n = 8). Birds from the 2017 cohort were directly transferred from L:D 6:18 to DD (DD-group, n = 3). All birds were kept in their respective final light treatments until the end of the experiment. Photoperiodic treatments and exposure times for each bird can be found online at DataverseNO (https://doi.org/10.18710/XLDXQ3).

2.3 Core body temperature recording

T_b was measured at a resolution of ± 0.0625°C using iButton temperature loggers (DS1922L, Maxim Integrated, San Jose, CA, USA; accuracy: ± 0.5°C). All iButtons were calibrated in a high precision water bath (model 6025, Hart Scientific, Pleasant Grove, UT, USA), the temperature of which was monitored by a factory-calibrated (Nordtec, Gothenburg, Sweden) Testo 925 thermometer with a type K thermocouple (Testo, PA, USA) (birds from the 2015 cohort) or a high precision glass thermometer (birds from the 2017 cohort). Calibration was performed in 5°C increments between 35°C and 45°C. This range covered the full range of core T_b shown by Svalbard ptarmigan over the course of the year (Nord & Folkow, 2018).

The calibrated iButtons were implanted into the abdominal cavity under gas anaesthesia. Specifically, the birds were anesthetised with a 4 % isoflurane air mix (Ref. No.: 9623, KDG Baxter, Deerfield, IL, USA) injected through an anaesthetic facemask connected to an Ohmeda vaporizer (Ref. No.: 058294, BOC Health Care, Guildford, UK) and an isoflurane vaporizer (Vapor 2000, Ref. No.: ARXH-1225, Dräger, Lübeck, Germany). Surgery started as soon as the bird showed muscle relaxation and did not respond to a physical stimulus (pinching of the skin).

The place of incision, i.e., ventrocaudal from the sternum, was located, plucked of feathers and disinfected with 2 % iodine (ref. no.: 332452, Sanivo Pharma AS, Oslo, Norway). The skin and muscle tissue were cut along the *linea alba* and the sterilized (70 % EtOH) iButton was then inserted into the abdominal cavity. The muscle tissue was sutured with an absorbable 2-0 Polysorb string (Ref. No.: CL-811, Syneture, Dublin, Ireland) and was disinfected with 2 % iodine. The skin was sutured with an absorbable 0 Dexon string (Ref. No.: 7232-61, Syneture, Dublin, Ireland) and again disinfected with 2 % iodine. After surgery, the facemask was removed, and the bird was observed until it regained full consciousness. The birds were placed into their home cages as soon as they could stand unaided.

The iButtons recorded hourly T_b for the durations outlined online (https://doi.org/10.18710/XLDXQ3). Specifically, in the SP-group, the T_b of seven birds was recorded for 48 days (except for bird SP2 which was measured for 30 days). In the LP/LL-group, eight birds were recorded for 23 days under LP and 14 days under LL. In the DD-group, three birds were recorded for 83 days. All recordings were made at the full hour except for two birds in the SP-group, which recorded at half hour. At the end of the experiment, the implanted iButtons were recovered from euthanized birds and the data were downloaded using the Maxim Integrated software OneWireViewer (version 0.3.19.47).

2.4 Activity recording

Locomotor activity was recorded continuously as movements per minute using passive infrared sensors (HSP 1131, Panasonic, Kadoma, Japan). These were installed on homebuilt circuit boards and mounted on the cage doors. Data were collected for a subset of three birds per photoperiodic group (determined by the number of available recording devices), using an Actimetrics CL200 USB interface coupled to ClockLab data acquisition software Version 2.61 (Actimetrics, Wilmette, IL, USA).

We recorded activity for the experiment during: 9 days in three birds in the LP/LL-group under LP; 14 days in three birds in the LP/LL-group under LL; and 66 days in three birds in the DD-group. In the SP-group we measured activity for 12, 18, and 31 days in three bird. Activity was recorded as counts per minute and normalized from 0 to 1 for each individual bird prior to analysis and plotting.

2.5 Data handling and analysis

All graphs were plotted with GraphPad Prism 8 (Version 8.3.0, San Diego, CA, USA), except for the actograms which were plotted using the ImageJ plugin ActogramJ (Schmid et al., 2011).

We plotted actograms for T_b and normalized activity for each bird over the whole experimental period. Actograms illustrate rhythmicity or the lack of it. All actograms were double-plotted to ease inspection. In a double-plotted actogram, one horizontal line represents two consecutive days (x-axis). Consecutive days are also plotted from top to bottom (y-axis). Normalized activity is displayed as bars of increasing heights between 0 and 1 on each line. This means that the higher the activity, the higher the bar. Low bars or the absence of bars indicate low activity and rest. Patterns or lack of rhythmicity can be observed by reading the actogram from top to button and by observing how phases of high and low activity relate to each other. We also adapted actograms to display T_b between 40 and 42 °C to show T_b rhythmicity. Hence, T_b < 40 °C is blank in the actogram while temperatures > 40 °C were plotted as bars of increasing height up to 42 °C. Rhythmicity in these actograms was tested by calculating a χ^2-periodogram (Sokolove & Bushell, 1978) for ten consecutive days for each bird in each light treatment. The ten-day period was chosen to coincide with reduced frequency of husbandry practices (see 2.6. "Bird husbandry and the effect on T_b"). The χ^2-periodogram algorithm calculated Q_p indices for each period between 1 and 30 h. Q_p follows a χ^2 distribution, and values corresponding to a p value < 0.05 were considered statistically significant.

We also plotted T_b and activity as mean ± SD 24-h profiles for each group and calculated T_b peak and nadir for each respective light treatment. The peak and nadir means are based on maximum and minimum T_b of each day and each individual bird. The difference between daily T_b peak and nadir (henceforth 'amplitude') was compared separately for each photoperiod group using paired *t*-tests (GraphPad 8). We then compared the difference in amplitude between photoperiod groups using mixed effects model fitted with restricted likelihood (lmer function in the lme4 package) (Bates et al., 2014) using R version 4.0.0 (R Core Team 2020) implemented in RStudio (version 1.3.959). Photoperiodic treatment was used as the explanatory variable, and a random intercept for bird ID was included to account for repeated measurements. Group estimates for the amplitude and comparisons between the groups were obtained using the emmeans R package (Lenth et al., 2018). Periods of transition between different photoperiods, and two birds from the SP-group which recorded at half hour, were excluded from this analysis.

T_b and activity were plotted together for a five-day period and for three birds for each photoperiod. In the periods selected for this purpose, the birds were acclimatized to their respective photoperiod since at least a week and were undisturbed apart from normal husbandry.

To analyse effects of photoperiod on dawn anticipation, we calculated mean T_b and activity from the five day-periods for 5 h before light-on when T_b was at its minimum, and for 1 h after light-on. The activity mean was calculated as the mean of the 10 minutes immediately before each T_b measurement. We defined dawn anticipation as nocturnal rise in T_b or activity that preceded the light-on signal. The 6-h period was analysed by fitting a segmented linear regression (GraphPad 8) and we considered the break point of the segmented function (i.e. where the two regression segments meet) as the start of anticipatory rise in activity or T_b. We plotted the data in Zeitgeber time (ZT), in which ZT 0 corresponds to the light-on signal.

2.6 Bird husbandry and the effect on T_b

Birds were monitored daily as part of routine husbandry. This might caused stress, which is known to cause increased T_b in birds (Cabanac & Guillemette, 2001; Nord & Folkow, 2019). This might affected rhythmicity analyses, especially in LL and DD. For this reason, we kept records of the timing of husbandry and tested how these visits affected T_b. We defined three categories (husbandry, 1 hour after husbandry and no husbandry) and assigned the respective T_b reading to each category for birds under LL and DD. The T_b means for each bird and each category were compared using paired *t*-test (Graphpad

8). In addition, we plotted husbandry in LL and DD in form of actograms and conducted χ^2-periodogram analyses on the rhythm of husbandry and on T_b recording of all birds under LL and DD.

3 Results

Svalbard ptarmigan held under SP and LP displayed clear daily rhythms in T_b with a 24-h period ($p < 0.05$ by χ^2-periodogram) while birds under LL and DD showed no significant rhythmicity in T_b for a 10-day period with little interference by husbandry ($p > 0.05$ for all periods by χ^2-periodogram) (**Figure 2 A-C**). The T_b-rhythm under SP and LP is defined by decreased T_b during the dark-phase and increased T_b during the light-phase, which is expressed either as a single peak in SP (**Figure 3A** and **4A**) or as a morning and one or several 'afternoon peaks' in LP (**Figure 3B** and **4B**). Even though we could not measure any significant T_b-period in birds under constant photic conditions, the difference between peak and nadir was significant in all photoperiodic groups, including birds under LL and DD ($p < 0.01$ for peak vs. nadir by paired *t*-test for each group, **Table 1**). Birds under SP and LP displayed T_b amplitudes of 2.52 ± 0.15 °C and 2.27 ± 0.12 °C, respectively (estimate ± SE by mixed model analysis), while birds under LL and DD showed smaller differences between peak and nadir ($p < 0.001$ by mixed model analysis) with 1.46 ± 0.12 °C and 1.30 ± 0.19 °C, respectively. The amplitudes between SP vs. LP ($p = 0.532$) and LL vs. DD ($p = 0.891$) did not differ significantly. The respective peak and nadir for T_b in each photoperiodic group, and the results of the paired *t*-tests, are summarized in **Table 1**. Results from the mixed model analysing the amplitude are presented in **Table 2**.

Svalbard ptarmigan under SP and LP displayed clear 24-h rhythmicity in activity ($p < 0.05$ by χ^2-periodogram) (**Figure 2D-E**) with high activity in the light-phase and low activity in the dark-phase. Similar to T_b, birds under LP displayed also two distinct activity peaks during the light phase (**Figure 3F**). Birds under LL and DD showed various significant periods in activity between 1 and 30 h ($p < 0.05$ by χ^2-periodogram) (**Figure 2E-F**) and LL-birds went through phases of high and low activity within a 24-h period (**Figure 3G**) while birds under DD showed generally low activity (**Figure 3H**).

Birds under SP showed a significant nocturnal increase in T_b preceding the light-on signal and the rise in activity (**Figures 4** and **5**). Under SP, both activity and T_b increased in the 5-h period immediately preceding the light-on signal. However, T_b rose 3 h before light-on (segmented regression breakpoint: ZT 21:04 ± 00:17 (hh:mm ± SD)) whereas activity increased 1 h 40 m before (breakpoint: ZT 22:20 ± 00:26). In LP, birds showed increased T_b and activity starting around half an hour before light-on (breakpoint for T_b: ZT 23:17 ± 00:06; and for activity: ZT 23:26 ± 00:09).

We also observed that SP-birds often expressed a small increase in T_b in the dark-phase around 11 h after the last light-on switch (ZT 11:05 ± 1:00, mean ± SD based on analysis in **Figure 4** and **S4**). The

241 nocturnal peak was also similar to the expression of the 'afternoon peak' in LP-birds that occurred 9 h
242 after the light-on signal (ZT 8:51 ± 1:11).

4 Discussion

In this study, we measured T_b and activity in Svalbard ptarmigan under photoperiods that were representative of those experienced during their annual cycle in the wild. All recorded T_b fell within the reported range of birds (Prinzinger et al., 1991) and were comparable to previous measurements of T_b during subjective daytime in Svalbard ptarmigan (Nord & Folkow, 2018). In SP and LP, birds showed pronounced cycles in T_b with high temperatures during the light-phase and low temperature during the dark-phase. We found clear evidence for 24-h rhythmicity in T_b under light-dark cycles but there was no evidence of sustained or free running circadian T_b-rhythms in LL and DD. Occasionally, birds in LL and DD continued to show transient peaks in T_b, which are reminiscent of sustained rhythmicity. However, these peaks coincided with visits for husbandry and we ascribe the observation to consequences of a stress-related increase in T_b (**Figure S5** and **S6**) (Cabanac & Guillemette, 2001; Nord & Folkow, 2019), which would also explain the significant difference between daily T_b peak and nadir in birds under LL and DD.

Activity was also rhythmic with a 24-h period in SP and LP, which was not sustained in LL and DD. This is in accordance with previous studies on activity rhythms in Svalbard ptarmigan (Reierth & Stokkan, 1998; Stokkan et al., 1986). Instead of a clear 24-h rhythm, birds under LL and DD showed various significant periods between 1 and 30 h, and birds in LL displayed several bouts of low and high activity in a 24-h period. These findings might be explained by the expression of an ultradian rhythm with a period of ca. 4 h (**Figure 2E**) of which subsequent peaks in the periodogram are subharmonics to the fundamental ultradian period. In the absence of an environmental light-dark cycle, the ultradian rhythm might reflect foraging activity and subsequent rest in Svalbard ptarmigan. At this stage, we can only speculate if this activity pattern is under endogenous control of an ultradian oscillator (Bourguignon & Storch, 2017) or if it is produced by the interplay of hunger and satiety.

T_b rose in anticipation to the light-on signal and, in birds under SP, prior to rises in activity. This suggests, firstly, that, as in most other endothermic animals, T_b is a distinct endogenous feature and not only a consequence of activity (Aschoff, 1983; Menaker, 1959b; Refinetti & Menaker, 1992). Secondly, it suggests that the T_b-cycle in Svalbard ptarmigan is controlled by a time-measuring system, which accurately anticipates the light-on signal. In nature, this anticipatory rise in T_b might ensure optimal bodily function at the start of the active phase. Alternatively, cycles in T_b might be utilized by the central system to impose its rhythm on peripheral tissues (Brown et al., 2002; Buhr et al., 2010).

Svalbard ptarmigan might use T_b in the same manner to ensure synchronized physiology in the short periods of light and dark cycles in-between the long stretches of polar day and polar night. Due to the absence of measurable circadian rhythms in T_b and activity in LL and DD, we propose that the central circadian system of Svalbard ptarmigan either uncouples from its output, or dampens in rhythmicity, when there is no periodic environmental synchronization (Bloch et al., 2013). This might ensure around-the-clock foraging without endogenous restraints during the polar day and polar night. However, we cannot exclude the possibility that under natural conditions Svalbard ptarmigan still express rhythms in T_b during the polar day and polar night due to entrainment to other photic or non-photic cues (Ashley et al., 2012; Long et al., 2005).

We also observed transient increases in T_b in the dark-phase of birds under SP. This could reflect nocturnal digestive activity (Rashotte et al., 1997). Alternatively, this observation might be further support for a circadian drive in T_b under light-dark cycles, in which case the transient nocturnal T_b-peak in SP would correspond to the 'afternoon peak' seen in LP birds.

Our findings suggest that Svalbard ptarmigan are using a circadian system under SP and LP to control their T_b but that this rhythm is not sustained in LL and DD. They can, therefore, utilize the benefits of a circadian system during times of a rhythmic environment but are able to escape its restrictions in constant conditions. Instead of a circadian rhythm, Svalbard ptarmigan show signs of ultradian rhythmicity in activity under constant light but we cannot resolve if this rhythm is endogenous or produced by the interaction of hunger and satiety. Future research should aim to elucidate how Svalbard ptarmigan achieve this duality of circadian organization and how ultradian rhythmicity is controlled.

5 Data Availability Statement

The datasets and additional information for this study are deposited online in the DataverseNO repository https://doi.org/10.18710/XLDXQ3.

6 Author Contributions

Conceptualization: DA, DGH, GCW. Data curation: DA. Formal analysis: DA, AN, GCW. Funding acquisition: DGH, GCW. Investigation: DA, AN, GCW. Methodology: DA, AN, DGH, GCW. Project administration: DA, DGH, GCW. Resources: AN, DGH, GCW. Software: AN. Supervision: DGH, GCW. Validation: AN, GCW. Visualization: DA, AN, GCW. Writing – original draft: DA. Writing – review & editing: DA, AN, DGH, GCW.

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8 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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10 Supplementary Material

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Table 1 | Mean ± SD peaks and nadirs of body temperature (°C) over 24 h periods in Svalbard ptarmigan that were kept under different photoperiods representative of their annual cycle in the wild. Results from the paired *t*-tests between peak and nadir (daily amplitude) are given under the respective photoperiod.

	Short photoperiod L:D 6:18 5 birds	**Long photoperiod** L:D 16:8 8 birds	**Constant light** 8 birds	**Constant darkness** 3 birds
T_b peak (°C)	42.03 (0.13)	41.97 (0.33)	41.86 (0.29)	41.31 (0.07)
T_b nadir (°C)	39.51 (0.20)	39.70 (0.40)	40.39 (0.22)	40.01 (0.20)
Paired *t*-test Peak vs. nadir	$p < 0.0001$ $t = 30.88$ df = 4	$p < 0.0001$ $t = 13.78$ df = 7	$p < 0.0001$ $t = 16.00$ df = 7	$p = 0.0090$ $t = 10.46$ df = 2

Table 2 | Differences in body temperature amplitude (i.e., the difference between the daily T_b peak and T_b nadir; °C) under different photoperiods. Estimates (± SE), likelihood ratios (LR) and *p*-values for the differences between photoperiodic treatments. Data were tested using a linear mixed effects model with bird ID as a random intercept. *p*-values and group estimates were obtained and compared using the emmeans package in R.

	Estimate (SE)	**LR**	***p***
Model			
Intercept	1.30 (0.17)		
Treatment		61.584	<0.001
Constant darkness (DD)	1.30 (0.19)		
Constant light (LL)	1.46 (0.12)		
Long photoperiod (LP, L:D 16:8)	2.27 (0.12)		
Short photoperiod (SP, L:D 6:18)	2.52 (0.15)		
Contrast			
DD vs LL	-0.16 (0.22)		0.891
DD vs LP	-0.96 (0.22)		<0.001
DD vs SP	-1.22 (0.24)		<0.001
LL vs LP	-0.81 (0.17)		<0.001
LL vs SP	-1.06 (0.19)		<0.001
LP vs SP	-0.26 (0.19)		0.532

Figure legends

Figure 1 | Svalbard ptarmigan (*Lagopus muta hyperborea*) and experimental design. **(A)** The picture shows a Svalbard ptarmigan male in white winter plumage and a female in the cryptic brown summer plumage (© Ida-Helene Sivertsen). **(B)** The Svalbard ptarmigan is a subspecies of the rock ptarmigan (*Lagopus muta*) but is geographically isolated to the high Arctic archipelago of Svalbard and Franz Josef Land. **(C)** The experimental birds were bred at the University of Tromsø and were separated into three groups: the short photoperiod (SP) group remained under L:D 6:18. The LP/LL-group was gradually transferred from L:D 6:18 to L:D 16:8 (LP), and subsequently into constant light (LL). The constant darkness (DD) group was directly transferred from L:D 6:18 into DD.

Figure 2 | Representative double-plotted actograms for body temperature (T_b) and activity. **(A-C)** T_b was plotted actogram-like between 40 and 42°C for representative birds from each group (bird IDs A: SP3, B: LP/LL11, C: DD3). **(D-F)** Actograms for normalized activity were plotted between 0 and 1 for representative birds from each group (bird IDs D: SP6, E: LP/LL15, F: DD2). χ^2-periodograms were plotted for 10 consecutive days in each light treatment (red shading in actograms) and are displayed next to the respective recordings. Values above the red line indicate that the cycle period was significant ($p < 0.05$). Additional actograms and periodograms can be found in **Figure S1-S3**.

Figure 3 | Diel variation in body temperature (T_b) and activity in different photoperiods. **(A-D)** Mean ± SD T_b over the course of 24 h (01:00 to midnight) in short photoperiod (SP; based on 222 × 24-h recordings from five birds), long photoperiod (LP; based on 184 × 24-h recordings from eight birds), constant light (LL; based on 112 × 24-h recordings from eight birds) and in constant darkness (DD; based on 249 × 24-h recordings from three birds). T_b was measured every hour throughout the experiment. **(E-H)** Mean normalized activity ± SD over the course of 24 h (midnight to midnight) in SP (61 × 24-h recordings from 3 birds), LP (27 × 24-h recordings from 3 birds), LL (42 × 24-h recordings from 3 birds) and DD (198 × 24-h recordings from 3 birds). Light grey shadowing in the panels indicate periods of darkness and dark grey indicates SD.

Figure 4 | Representative time series for body temperature (T_b) and activity. **(A-D)** T_b (red) was plotted together with normalized activity (black) for five consecutive days for one representative bird per

experimental treatment (bird IDs A: SP6, B/C: LP/LL113, D: DD2). Light grey shadings indicate periods of darkness. Additional time series can be found in **Figure S4**.

Figure 5 | Anticipatory rise in body temperature (T_b) based on segmental regression breakpoints. Hourly means of T_b (red) and activity (black) 5 h before and 1 h after the light-on signal, given in Zeitgeber time (ZT). **(A)** T_b in birds under SP was rising 2 h 56 m before the light-on signal while activity only rises 1 h 40 m before. **(B)** In birds under LP T_b increased 43 m before light-on while activity rose 34 m before the light-on signal. The data corresponds to the measurement in **Figure 4** and **S4** and is displayed as mean ± 95% CI. Dotted lines indicate segmented regression breaking points and the shading shows the corresponding SD. Light grey shadings indicate periods of darkness.

(A)


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PHOTOGRAPHY

(B)


Arctic
Ocean
North
Pole
Svalbard

(C)

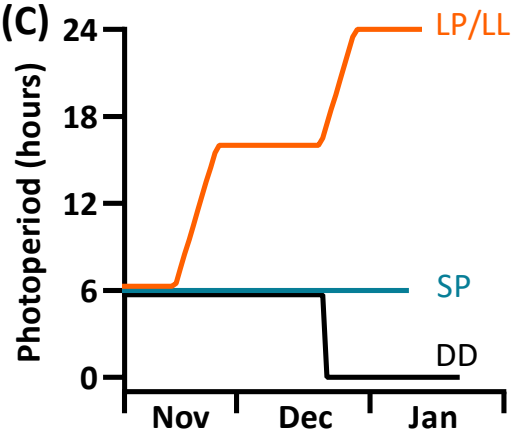
Photoperiod (hours)
24
18
12
6
0
LP/LL
SP
DD
Nov
Dec
Jan

Figure 1.

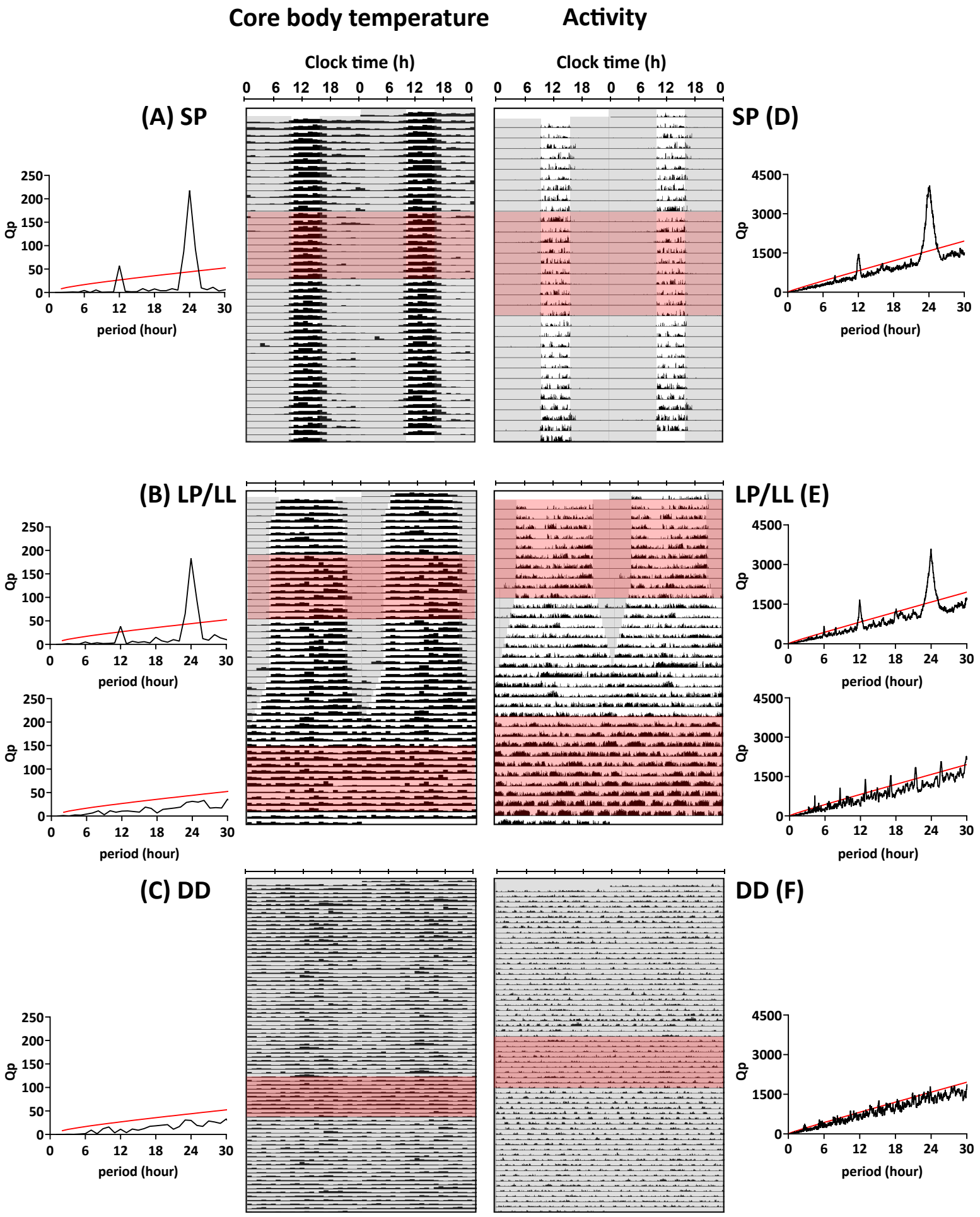
Core body temperature
Activity
Clock time (h)
Clock time (h)
0 6 12 18 0 6 12 18 0
0 6 12 18 0 6 12 18 0
(A) SP
SP (D)
(B) LP/LL
LP/LL (E)
(C) DD
DD (F)
Qp
period (hour)
0 6 12 18 24 30
0 50 100 150 200 250
0 1500 3000 4500

Figure 2.

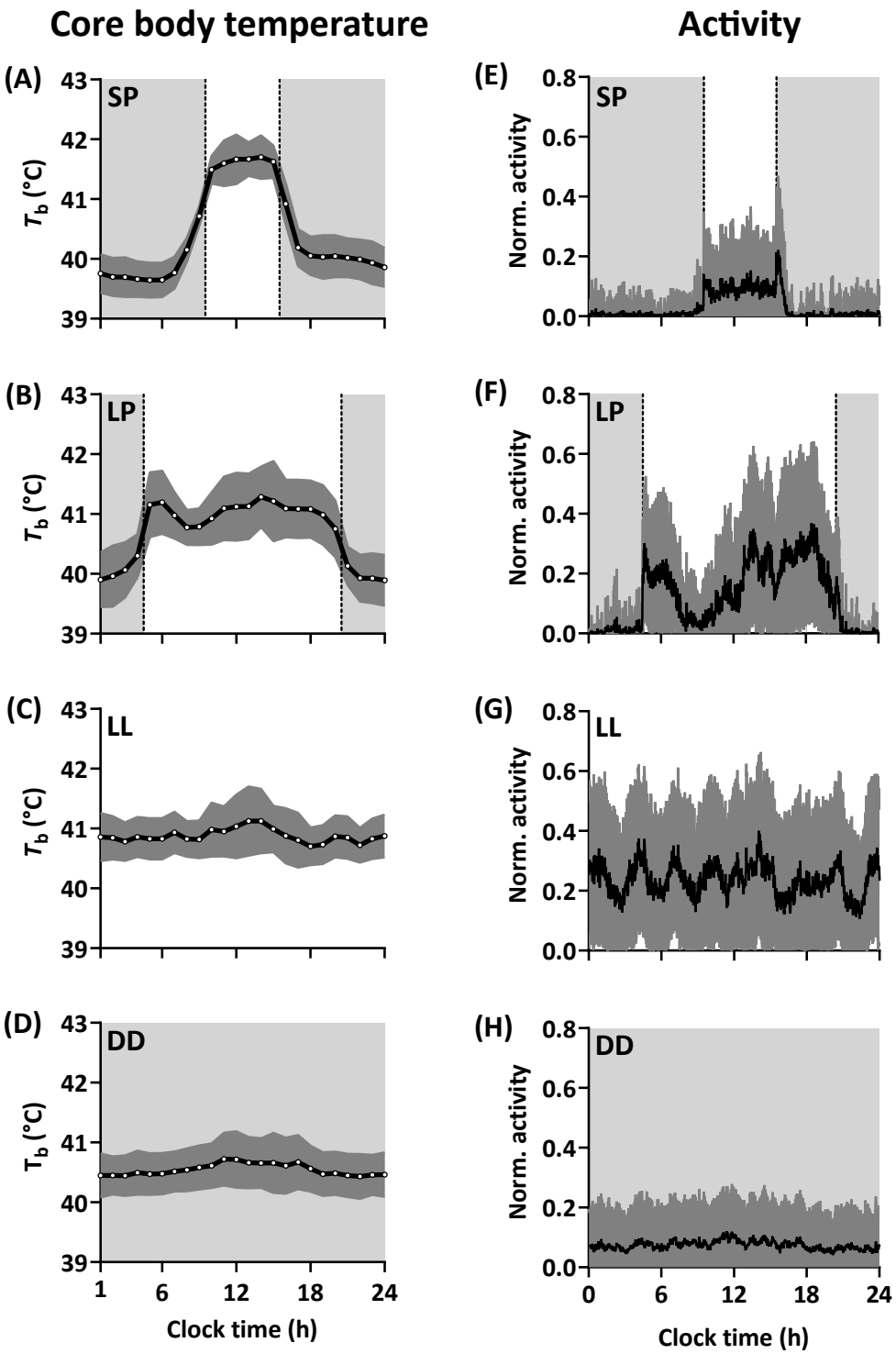
Core body temperature
Activity
(A)
SP
(B)
LP
(C)
LL
(D)
DD
(E)
SP
(F)
LP
(G)
LL
(H)
DD
T_b (°C)
Norm. activity
43
42
41
40
39
0.8
0.6
0.4
0.2
0.0
1
6
12
18
24
0
Clock time (h)

Figure 3.

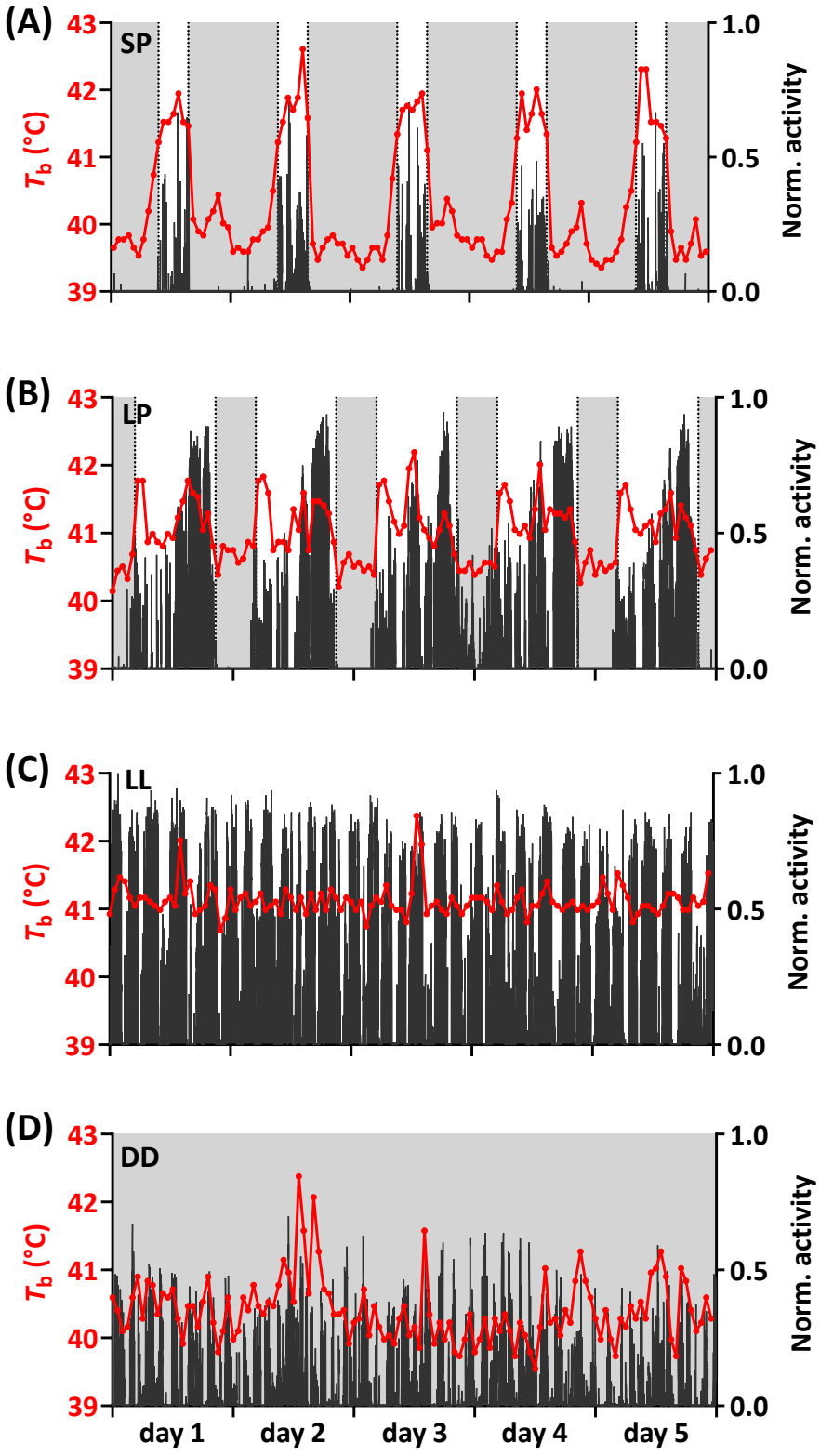
(A)
SP
(B)
LP
(C)
LL
(D)
DD
T_b (°C)
Norm. activity
43
42
41
40
39
1.0
0.5
0.0
day 1
day 2
day 3
day 4
day 5

Figure 4.

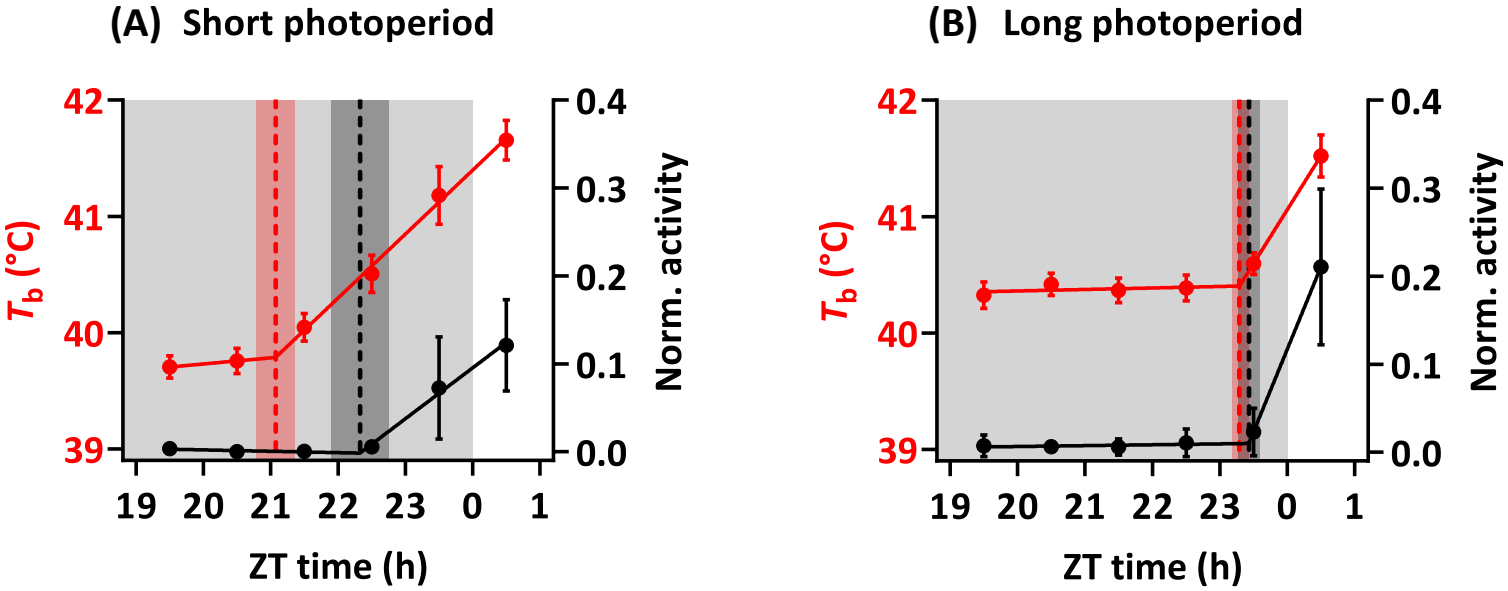
(A) Short photoperiod
(B) Long photoperiod
T_b (°C)
Norm. activity
ZT time (h)
42
41
40
39
0.4
0.3
0.2
0.1
0.0
19
20
21
22
23
0
1

Figure 5.

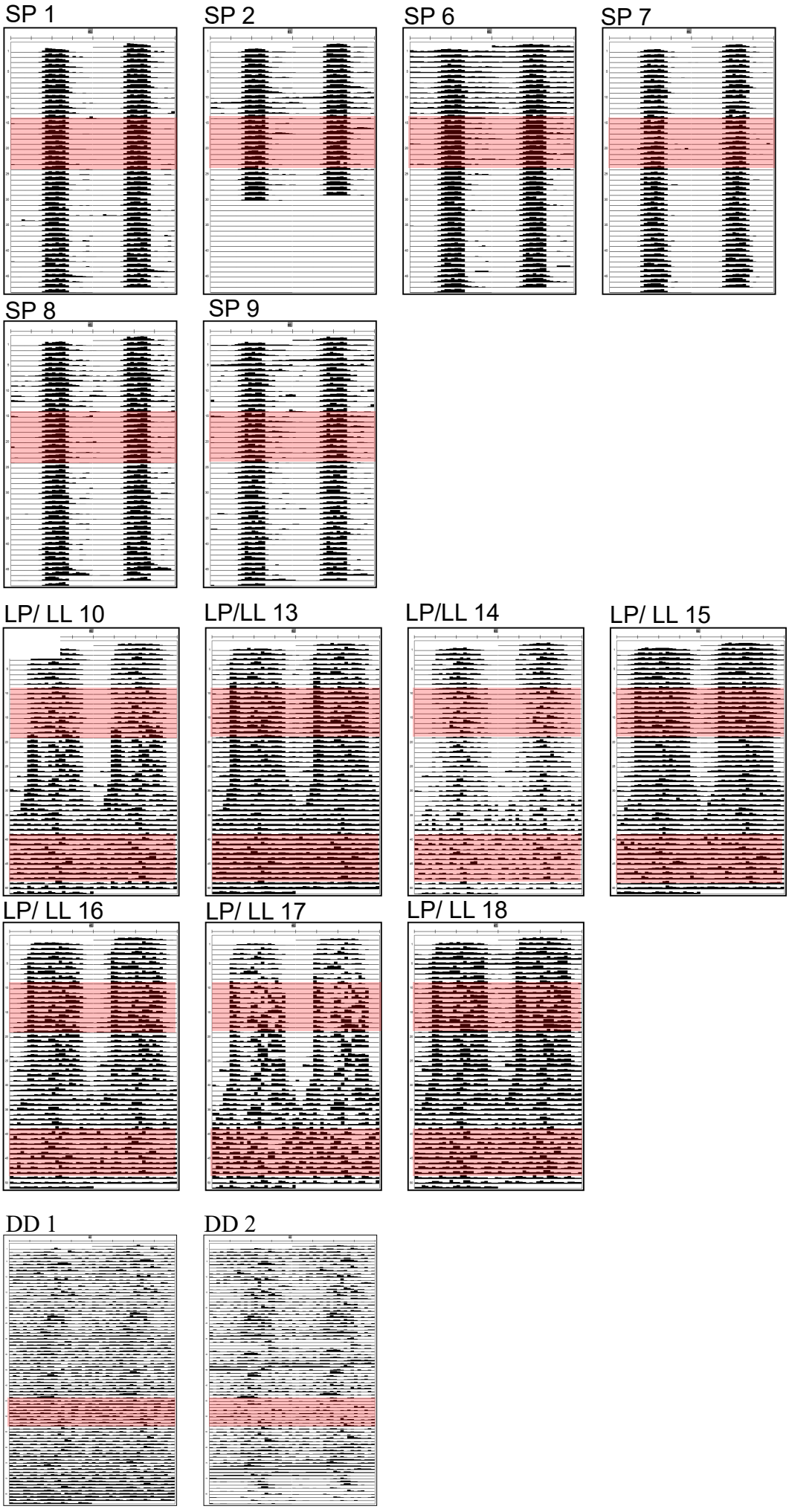

Figure S1 | Additional T_b-actograms. T_b was plotted actogram-like between 40 and 42°C for all birds from each group (representitive birds from the main text exceptd). T_b-actograms are labelled according to the group and bird-ID and red shadings indicate analyses by χ2-periodograms (Figure S2).

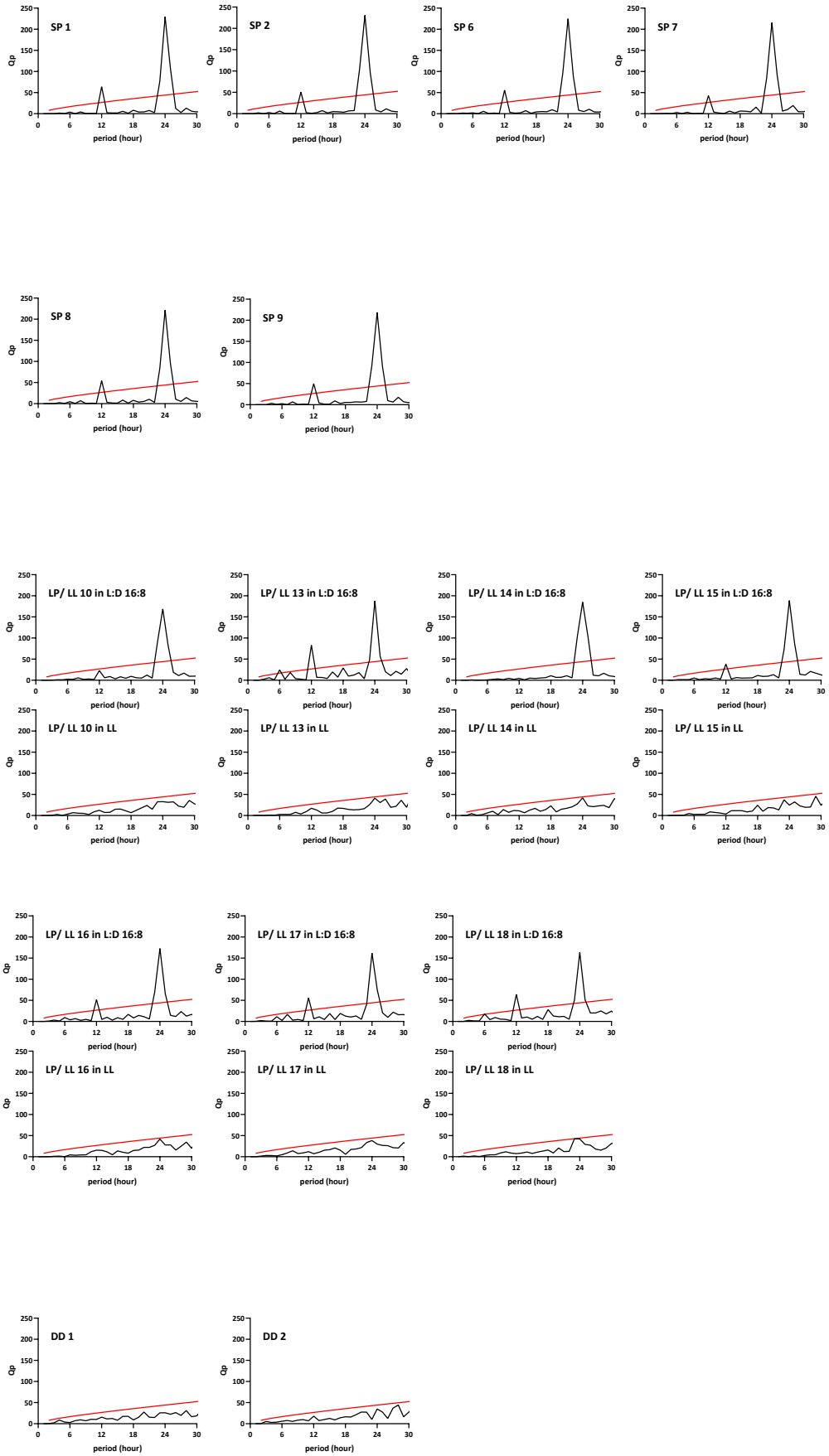

Figure S2 | Additional χ^2-periodograms for T_b-actograms of Figure S1. χ^2-periodograms were plotted for ten consecutive days in each light treatment. Values above the red line indicate significant periods of the cycles ($p < 0.05$). Bird-IDs correspond to Figure S1.

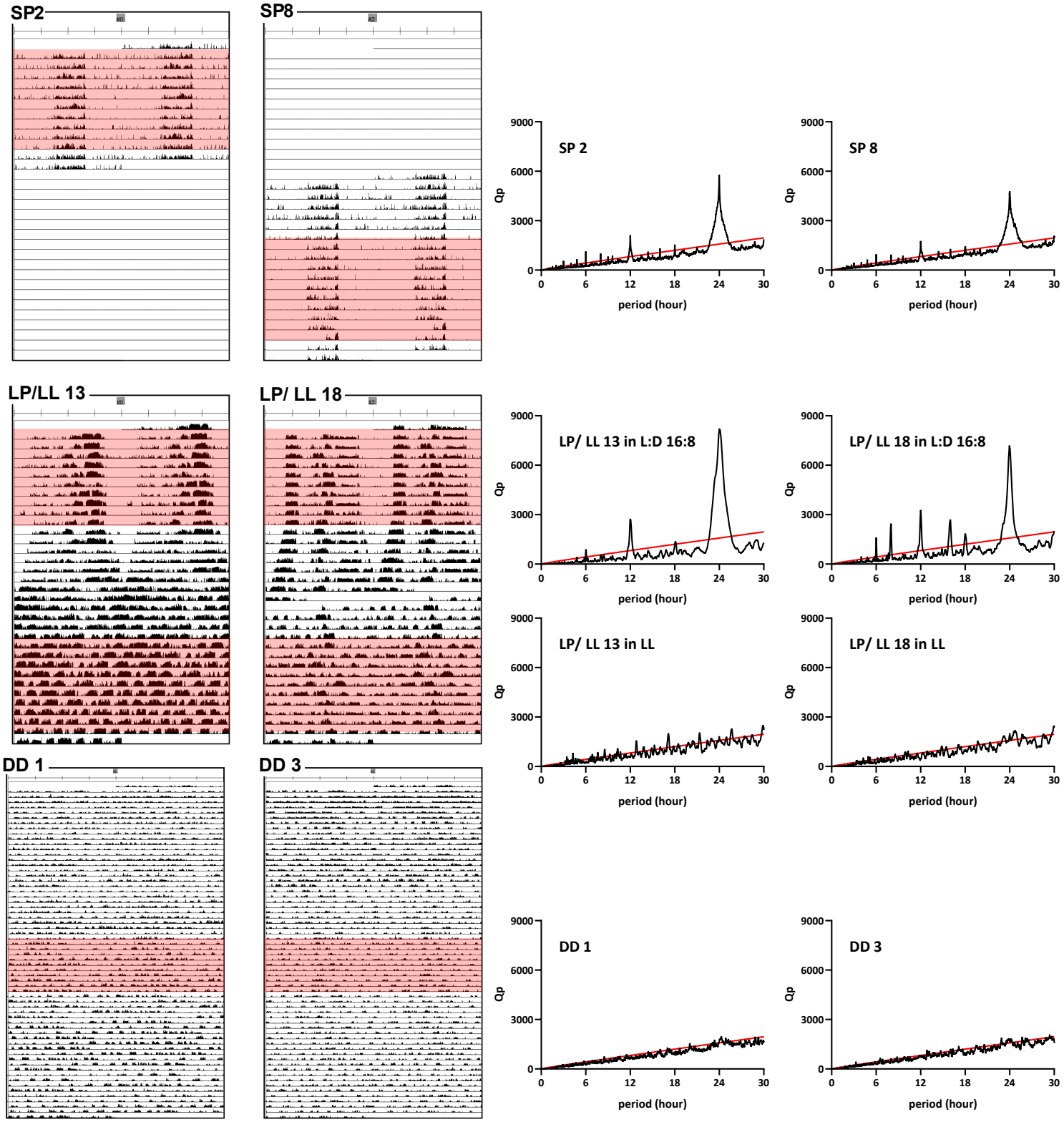

Figure S3 | Additional actograms for normalized activity. All actograms were double plotted for all birds from each group (representitive birds from the main text exceptd). Actograms are labelled according to the group and bird-ID. Red shadings indicate analyses by χ2-periodograms. Corresponding χ2-periodograms were plotted for ten consecutive days in each light treatment (red shading). Values above the red line indicate significant periods of the cycles ($p < 0.05$).

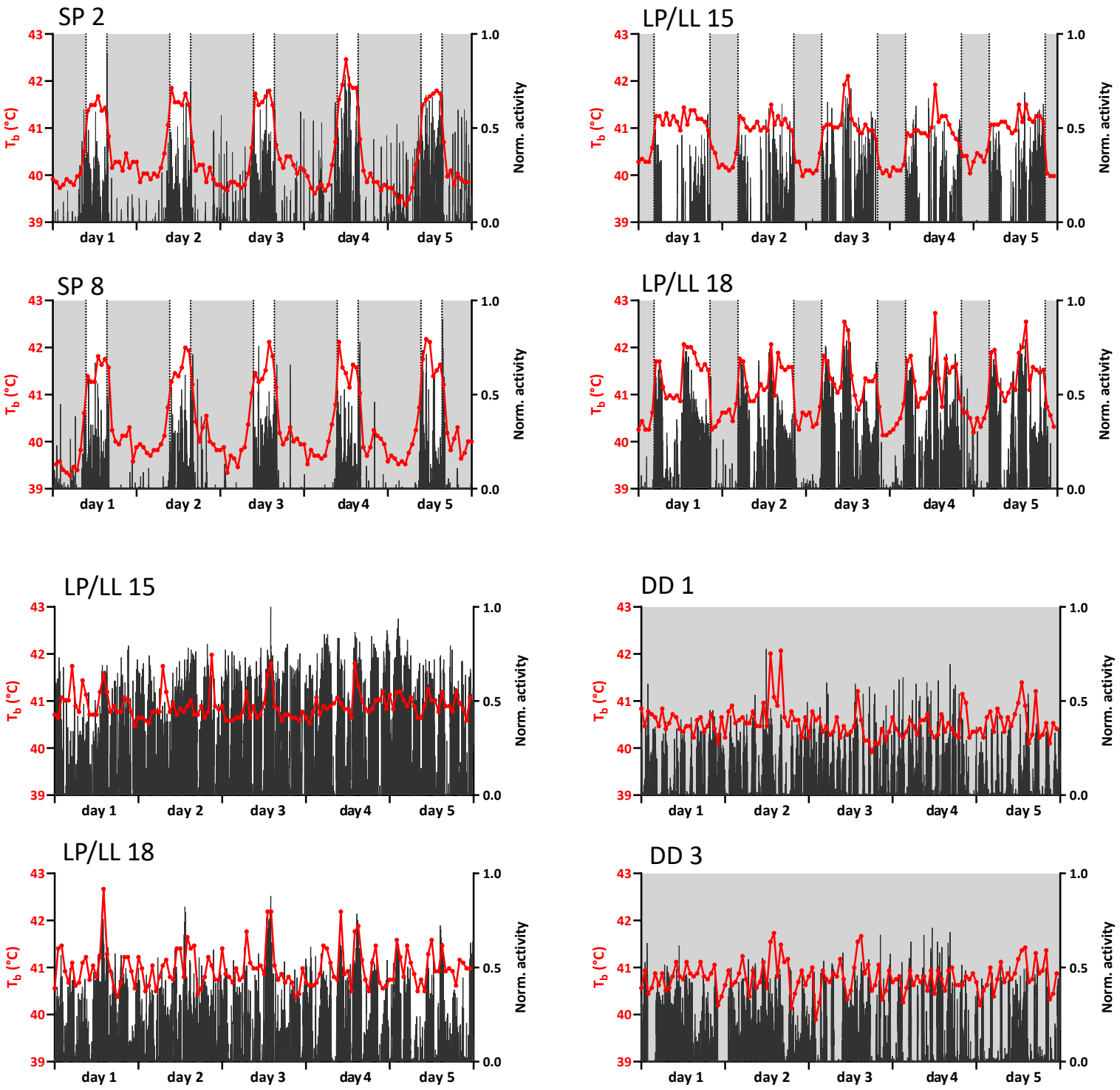

Figure S4 | Additional time series for T_b and activity. T_b (red) was plotted with normalized activity (black) for five consecutive days for two birds from each experimental treatment (bird-IDs are shown in the graph). Light grey shadings indicate periods of darkness.

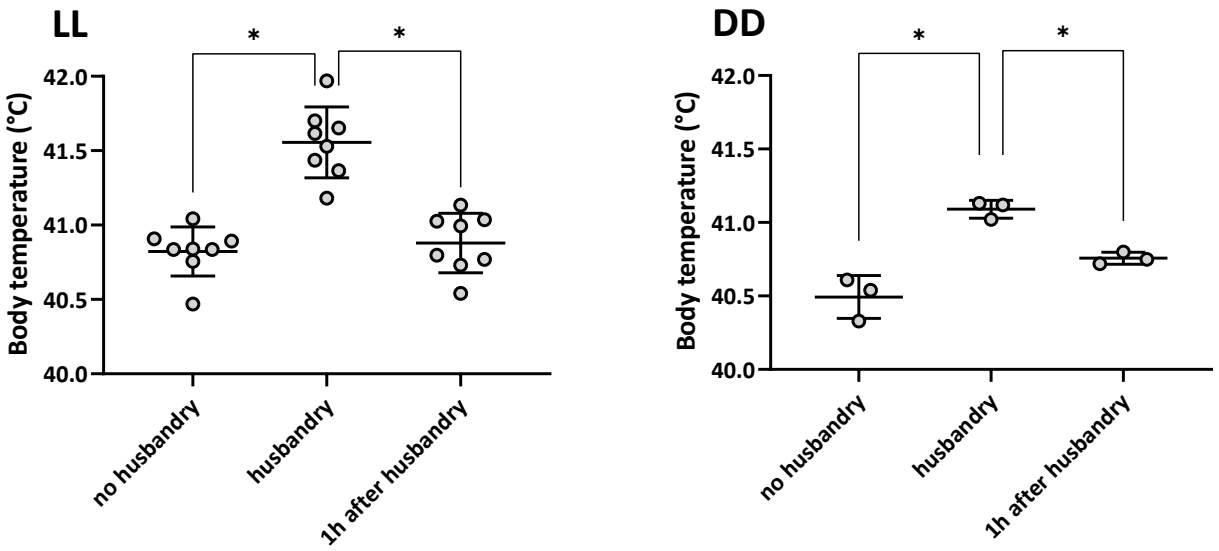

Figure S5 | The effect of husbandry on body temperature (T_b) in constant light (LL) and constant darkness (DD). The mean T_b of each bird under each condition (no husbandry, husbandry and 1 h after husbandry) was compared using paired *t*-tests. T_b was different between 'no husbandry' and 'husbandry' (LL: $p < 0.0001$, $t = 11.86$, df = 7 | DD: $p = 0.0256$, $t = 6.13$, df = 2) and between 'husbandry' and '1 h after husbandry' (LL: $p < 0.0001$, $t = 9.76$, df =7 | DD: $p = 0.0288$, $t = 5.77$, df = 2). There was no significant difference between 'no husbandry' and '1 h after husbandry' (LL: $p = 0.2353$, $t = 1.30$, df = 7 | DD: $p = 0.0987$, $t = 2.94$, df = 2). Data is displayed as mean ± SD.

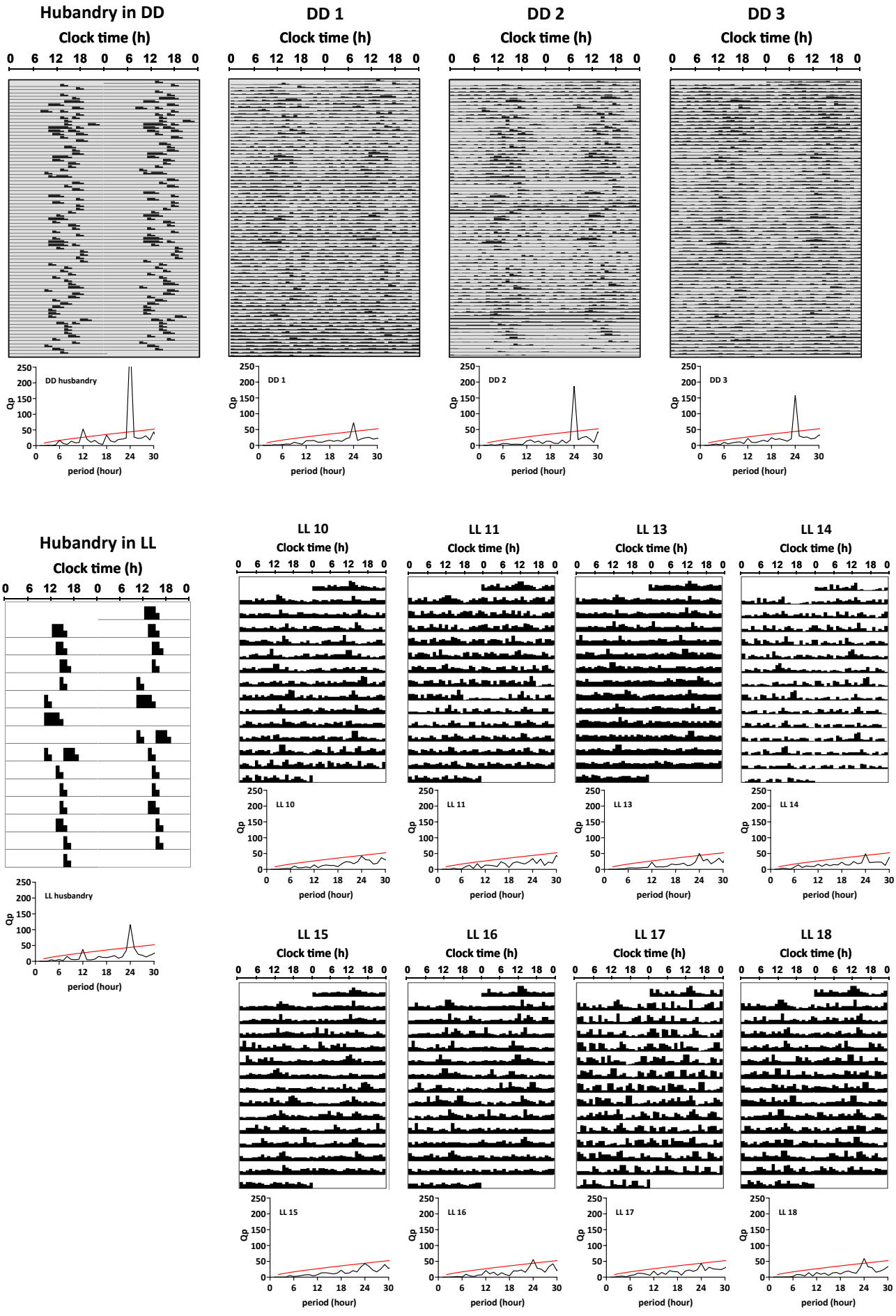

Figure S6 | Actograms of T_b and husbandry in LL and DD and their respective χ^2-periodograms . Husbandry is shown in actogram form with black bars indicating the hour of husbandry (high bar) and 1 h thereafter (low bar). T_b actograms of all birds under LL and DD are displayed with their respective ID next to the husbandry actograms. χ^2-periodograms were calculated for the whole range and values above the red line indicate significant periods of the cycles ($p < 0.05$).

Paper II

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RESEARCH ARTICLE

Photoperiodic induction without light-mediated circadian entrainment in a High Arctic resident bird

Daniel Appenroth[1], Vebjørn J. Melum[1], Alexander C. West[1], Hugues Dardente[2], David G. Hazlerigg[1,‡] and Gabriela C. Wagner*[,1]

ABSTRACT

Organisms use changes in photoperiod to anticipate and exploit favourable conditions in a seasonal environment. While species living at temperate latitudes receive day length information as a year-round input, species living in the Arctic may spend as much as two-thirds of the year without experiencing dawn or dusk. This suggests that specialised mechanisms may be required to maintain seasonal synchrony in polar regions. Svalbard ptarmigan (*Lagopus muta hyperborea*) are resident at 74–81°N latitude. They spend winter in constant darkness (DD) and summer in constant light (LL); extreme photoperiodic conditions under which they do not display overt circadian rhythms. Here, we explored how Arctic adaptation in circadian biology affects photoperiodic time measurement in captive Svalbard ptarmigan. For this purpose, DD-adapted birds, showing no circadian behaviour, either remained in prolonged DD, were transferred into a simulated natural photoperiod (SNP) or were transferred directly into LL. Birds transferred from DD to LL exhibited a strong photoperiodic response in terms of activation of the hypothalamic thyrotropin-mediated photoperiodic response pathway. This was assayed through expression of the *Eya3*, *Tshβ* and deiodinase genes, as well as gonadal development. While transfer to SNP established synchronous diurnal activity patterns, activity in birds transferred from DD to LL showed no evidence of circadian rhythmicity. These data show that the Svalbard ptarmigan does not require circadian entrainment to develop a photoperiodic response involving conserved molecular elements found in temperate species. Further studies are required to define how exactly Arctic adaptation modifies seasonal timer mechanisms.

KEY WORDS: Photoperiodism, Circadian, Seasonal reproduction, Pars tuberalis, Eyes absent, Deiodinase, Svalbard ptarmigan

INTRODUCTION

Animals in temperate and high latitudes use changes in photoperiod (day length) to anticipate upcoming seasons and adjust physiology and behaviour accordingly. The involvement of circadian clocks in this photoperiodic time measurement was first suggested by Erwin Bünning, who proposed a so-called 'external coincidence' mechanism. According to the Bünning hypothesis (Bünning, 1936), organisms express an innate circadian rhythm of photo-inducibility and light exposure coinciding with the photo-inducible phase of this rhythm triggers a photoperiodic response. In order to test the Bünning hypothesis, experimental approaches based on artificial light exposures, such as night break experiments, have been employed (Bünning, 1936; Elliott et al., 1972; Follett and Sharp, 1969; Follett et al., 1992; Gwinner and Eriksson, 1977; Hammer and Enright, 1967; Pittendrigh, 1972). Night break experiments trigger a long day response by combining a short photoperiod with a nocturnal light pulse that occurs in the photo-inducible phase. Positive results of these experiments across diverse taxonomic groups favour a circadian-based photoperiodic readout mechanism.

In birds and mammals, photoperiodic effects on reproduction depend on changes in hypothalamic gonadotrophin releasing hormone (GnRH) secretion at the median eminence, and recent evidence points to a coincidence timer mechanism in the adjacent pars tuberalis (PT) as the key upstream control mechanism (Dardente et al., 2010; Hazlerigg and Loudon, 2008; Lincoln et al., 2002; Masumoto et al., 2010; Nakao et al., 2008; Yasuo et al., 2003; Yoshimura et al., 2003). Within the PT, long photoperiods (LPs) stimulate the expression of the thyroid stimulating hormone (TSH) β subunit gene (*Tshβ*) (Nakao et al., 2008). LP-induced expression of TSH leads to increased *Dio2* expression in the mediobasal hypothalamus (MBH), through a cAMP-dependent pathway in neighbouring ependymal cells known as tanycytes (Bolborea et al., 2015; Hanon et al., 2008; Nakao et al., 2008; Ono et al., 2008). DIO2 locally converts thyroxine (T_4) to the bioactive triiodothyronine (T_3) by outer ring deiodination, thus increasing hypothalamic T_3 concentration under LPs. In long day breeding birds and mammals, this in turn increases the release of GnRH in the median eminence, ultimately leading to gonadal activation (Yamamura et al., 2004, 2006; Yoshimura et al., 2003). Conversely, under short photoperiod, low levels of TSH in the PT coincide with increased type III iodothyronine deiodinase (*Dio3*) expression in tanycytes, keeping the hypothalamic T_3 concentration low and promoting gonadal inactivation (Yasuo et al., 2005). The reciprocal regulation of *Dio2*/*Dio3* expression and the resulting bioactive T_3 concentration in the MBH is at the core of photoperiodic control of seasonal reproduction and has become a central paradigm in photoperiodic time measurement.

Several lines of evidence suggest that this PT-mediated readout system is circadian based. First, in both birds and mammals so-called 'clock genes' show characteristic rhythmical expression in the PT/MBH region, consistent with a possible coincidence timer mechanism (Johnston et al., 2005; Lincoln et al., 2002; Tournier et al., 2007; Yasuo et al., 2003, 2004). Secondly, in the Japanese quail (*Coturnix japonica*) photoperiodic induction of *Dio2* and downstream physiological responses can be triggered by night break experiments (Yoshimura et al., 2003), implying control through a coincidence timer mechanism. Further evidence for the circadian basis on the hypothalamic long day response derives from research on eyes absent 3 (EYA3). In mammals, EYA3 has been proposed to act as a transcriptional co-activator at the *Tshβ* gene promoter and analysis of the ovine *Eya3* promoter demonstrated that its expression

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is controlled by circadian clock genes (Dardente et al., 2010; Masumoto et al., 2010).

Circadian-based models for photoperiodic time measurement place an emphasis on robust circadian cycles of clock gene expression. This raises the question of what happens in species living at Arctic latitudes. Light–dark cycles are absent for extended periods of the year and under such circumstances daily rhythmicity in behaviour and endocrinology breaks down completely (Reierth and Stokkan, 1998; Reierth et al., 1999; Stokkan et al., 1994; van Oort et al., 2005, 2007). Loss of behavioural and endocrine circadian rhythmicity does not necessarily imply loss of circadian-based photoperiodic response circuits, especially in birds where circadian organisation involves multiple circadian oscillators (Cassone, 2014). Moreover, in temperate bird species, lesioning studies resolve behavioural organisation from photoperiodic sensitivity (Binkley et al., 1972; Menaker and Keatts, 1968; Menaker et al., 1970; Rani et al., 2007; Siopes and Wilson, 1974; Wilson, 1991). Nevertheless, adaptation to the Arctic might have had a substantial impact on the entire circadian system, which could also affect circadian-based photoperiodic induction. Fibroblast cultures from reindeer show arrhythmic clock gene expression (Lu et al., 2010) and *in silico* analysis on clock genes revealed mutations that might impact circadian rhythm generation (Lin et al., 2019). If Arctic animals cannot sustain circadian rhythmicity in the polar day and polar night, this might limit photoperiodic responses through coincidence timing to those phases of the year with a robust light–dark cycle.

To investigate this, we have performed photoperiod manipulations in captive Svalbard ptarmigan (*Lagopus muta hyperborea* Sundevall 1845), the northernmost resident herbivorous bird species (Fig. 1). Svalbard ptarmigan are highly seasonal in their breeding physiology (Steen and Unander, 1985; Stokkan et al., 1988, 1986) and become behaviourally arrhythmic around the solstices (i.e. during the polar night and the polar day) (Reierth and Stokkan, 1998). Similar dampening of melatonin rhythmicity has also been observed (Reierth et al., 1999).

In order to test if a light–dark cycle is necessary to induce a long day response in Svalbard ptarmigan, we transferred birds, acclimated to constant darkness (DD), either into a gradually increasing photoperiod or directly into constant light (LL). The former group therefore received a rhythmic light–dark cycle while the latter did not. The control group remained in DD. We measured gonadal mass and behavioural activity as well as *Eya3*, *Tshβ*, *Dio2* and *Dio3* expression in the PT/MBH region.

MATERIALS AND METHODS

Experimental animals and housing

All animals were kept in accordance of the EU directive 201/63/EU under a licence provided by the Norwegian Food Safety authority (Mattilsynet, FOTS 7971). Chicks were hatched from eggs laid by captive adult Svalbard ptarmigan at the University of Tromsø (69°39′N, 18°57′E). Hatching took place between 24 June and 1 August 2017. The chicks were raised either indoors with a photoperiod corresponding to the onset and offset of natural civil twilight in Tromsø or outside on the ground. Upon reaching a body mass of 400–500 g, 29 birds (Table S1) were transferred into individual cages (1.5 m×0.5 m) in light- and temperature-controlled rooms. All birds were transferred at the end of September 2017. Food (standardised protein food; Norgesfor, OK 2400 070316) and water were provided *ad libitum* throughout the study. Female and male birds were housed together.

Controlled lighting was provided by fluorescent strip lights (Osram, L 58 W 830 Lumilux) delivering approximately 1000 lx at floor level. All rooms were further equipped with permanent red illumination (Philips, BR125 IR 250 W). During the initial acclimation phase, the photoperiod was gradually decreased until reaching DD (red light excepted) on 22 December 2017. Birds in DD were held under red light to allow for husbandry. The birds remained in DD for 5 weeks prior to experimental light treatments.

Experimental light treatment and sampling

After 5 weeks of DD, 5 individuals were sampled as an initial control group. This marked the start of the experiment (point 0). Thereafter, the three experimental groups were transferred to their respective light treatments (Fig. 2 and Table S1). Six birds remained in DD until the end of the experiment, 9 birds were directly transferred into LL and 9 birds were exposed to a simulated natural photoperiod (SNP). The SNP treatment reflected an increase in day length following the progression of civil twilight onset and offset of Longyearbyen, Svalbard (78°13′N 15°38′E; Table S2).

Four individuals were sampled after 38 h in LL. This sampling time was chosen to coincide with acute photoperiodic gene induction as previously reported in the quail MBH and PT (Nakao et al., 2008). Subsequent samplings aimed to investigate chronic changes in gene expression, and were undertaken at single time points on the following days: After 5 weeks, 4 individuals were sampled from the SNP group as they reached LD 12:12. This sampling was performed 3.5–4.5 h after lights on. After 10 weeks of light treatment, all remaining birds from all groups were sampled. The SNP group had reached LL through a gradual increase in photoperiod 4 days before the final sampling. All groups were euthanised between 09:00 h and 15:00 h local time. The DD group was euthanised on the day after the LL and SNP group. Sampling of birds in DD was performed under dim red light only. Brains were removed after euthanasia and rapidly transferred onto a cooled metal block until stored at −80°C. Testes and ovaries were removed and measured post mortem.

Activity

Locomotor activity of all experimental birds was continuously recorded as movement per minute by passive infrared sensors,

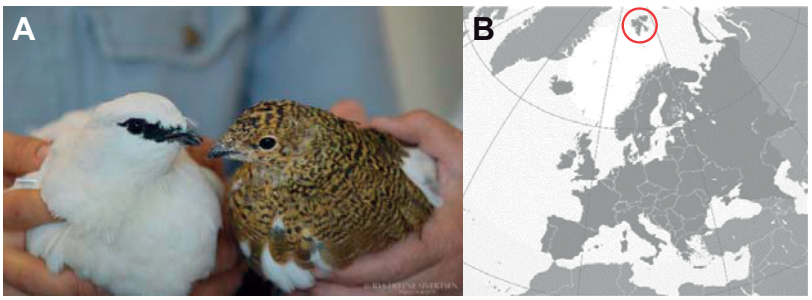

Fig. 1. Svalbard ptarmigan (*Lagopus muta hyperborea*) and where to find them. (A) A male in white winter plumage and a female in brown summer plumage (photo credit: Ida-Helene Sivertsen). (B) The Svalbard ptarmigan is a subspecies of the rock ptarmigan (*Lagopus muta*) and inhabits the High Arctic archipelago of Svalbard (74–81°N latitude).

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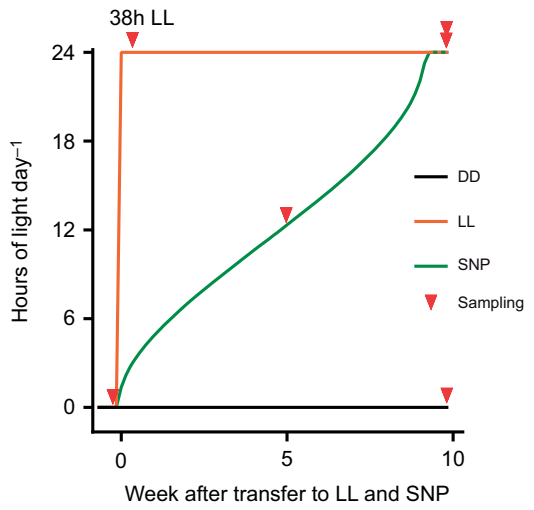

Fig. 2. Experimental design. Constant darkness-adapted (DD) birds were either transferred into constant light (LL), simulated natural photoperiod (SNP) or retained under DD. Red arrowheads indicate sampling points.

mounted on the cage doors. Data were collected by an Actimetrics CL200 USB interface coupled to ClockLab data acquisition software (version 2.61).

cDNA cloning and *in situ* hybridisation

Probe synthesis and *in situ* hybridisation were performed as described in Lomet et al. (2018). RNA was extracted from Svalbard ptarmigan brain tissue using TriReagent (Sigma) and converted into cDNA using Omniscript RT kit (Qiagen). The Icelandic rock ptarmigan genome (Kozma et al., 2016) was used to design PCR primers to amplify cDNA fragments for *Tshβ*, *Eya3*, *Dio2* and *Dio3*. PCR was performed with Taq DNA polymerase (Qiagen). PCR products of correct sizes were extracted and cloned into pGEMT easy vectors (Promega). The inserts (Table S3) were sequenced (Eurofins Sequencing services, Germany) and verified against the reference genome.

Cloned vectors were stored at −20°C until further use. Prior to hybridisation, vectors were linearised and transcribed using a Promega transcription kit in combination with a ^{35}S-UTP isotope (PerkinElmer) to obtain radioactively labelled complementary riboprobes. The riboprobes were purified with illustra MicroSpin G-50 columns (GE healthcare) and incorporation of ^{35}S-UTP was measured by a liquid scintillation counter (Triathler multilabel tester, Hidex).

Frozen brains were cryosectioned at 20 µm and sections containing PT and MBH were mounted to pre-coated adhesion slides (SuperFrost Plus, VWR). Brain sections were fixed in 4% paraformaldehyde (0.1 mol l^{-1} phosphate buffer) for 20 min at 4°C and rinsed twice with 0.1 mol l^{-1} phosphate buffer for 5 min. Fixed sections were acetylated with 3.75% v/v of acetic anhydride in 0.1 mol l^{-1} triethanolamine buffer (0.05 mol l^{-1} NaOH) and rinsed twice with 0.1 mol l^{-1} phosphate buffer for 5 min. Sections were subsequently dehydrated with stepwise increasing ethanol solutions (50%, 70%, 96%, 100% for 3 min each) and dried under vacuum for at least 1 h.

Dried sections were hybridised with 10^6 cpm of riboprobe per slide in hybridisation buffer (50% deionised formamide, 10% dextran sulfate, 1× Denhardt's solution, 300 mmol l^{-1} NaCl, 10 mmol l^{-1} Tris-HCl, 10 mmol l^{-1} DTT, 1 mmol l^{-1} EDTA, 500 µg ml^{-1} tRNA). Hybridisation was performed at 56°C overnight. Hybridised sections were washed with 4× saline sodium citrate (SSC) solutions (3×5 min) and treated with RNase-A solution (500 mmol l^{-1} NaCl, 1 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA, 20 µg ml^{-1}) for 30 min at 37°C. Subsequent stringency washes were performed in SSC (supplemented with 1 mmol l^{-1} DTT) of decreasing concentration: 2× SSC (2×5 min), 1× SSC (1×10 min), 0.5× SSC (1×10 min), 0.1× SSC (30 min at 60°C), 0.1× SSC (rinse).

Slides were dehydrated afterwards in stepwise increasing ethanol solutions (50%, 70%, 96%, 100% for 3 min each) and dried under vacuum. Dried sections were exposed to autoradiographic films (Carestream Kodak BioMax MR film) for 9 to 12 days. Exposed films were developed, fixed and digitalised with an Epson transmission scanner. Optical density (OD) was measured with ImageJ (version 1.51k).

Analysis

Actograms were produced with the ActogramJ plugin for ImageJ (Schmid et al., 2011) and period length of activity was measured by chi-squared periodograms produced by the same program. Graphs of gene expressions in the PT/MBH region and gonadal mass were prepared in GraphPad Prism 8 (version 8.0.2). The results were plotted as each replicate with lines going through the respective mean of each group at each sampling point. Statistical comparisons were made by one-way ANOVA and Tukey's *post hoc* tests, performed on log transformed values to ensure homogeneity of variances; the threshold for significance was $P<0.05$. Individual values for gene expression with the corresponding gender can be found in Table S1.

RESULTS

Activity rhythms

Prior to the experimental treatment, all birds in DD exhibited short episodic bouts of activity with no clear periodicity (Fig. 3, Figs S1 and S2), and for birds continuing on DD the same pattern was maintained. In birds transferred to LL, episodic activity continued, sometimes with ultradian periodicity. Period lengths were typically in the range 3–20 h, and highly variable between individuals. Birds transferred to SNP, based on Svalbard civil twilight progression, showed robust daily rhythms with a period of 24 h ($P<0.05$).

Gonads

Testes and ovaries were initially regressed in all groups (Fig. 4A,B) and subsequent development depended on photoperiodic treatment ($P<0.0001$ by one-way ANOVA in both cases). Exposure to LL strongly stimulated gonadal maturation for both testes and ovaries, so that after 10 weeks masses increased 22-fold and 93-fold, respectively ($P<0.0001$ by Tukey's *post hoc* test in both cases). Gonadal maturation in birds maintained in DD and in female birds under SNP, was negligible (DD, 1.4-fold; SNP, 1.1-fold compared with initial values) while male birds transferred to SNP showed a more modest (3.2-fold) but nonetheless statistically significant increased testicular mass by the end of the study ($P<0.001$ by Tukey's *post hoc* test).

Eya3 and *Tshβ* expression

The expression of *Tshβ* and *Eya3* over the course of the study was dependent on photoperiod ($P<0.0001$ by one- way ANOVA in both cases) (Figs 4C,D and 5). Expression of both genes was below the detection threshold at week 0, and rose dramatically 38 h after the transfer to LL ($P<0.001$ in both cases by Tukey's *post hoc* test). Thereafter expression of both genes was maintained at high levels until the end of the study (week 10). In birds exposed to SNP, levels of both genes remained undetectable 5 weeks after the transfer, when the photoperiod had increased to 12 h of light. Subsequently,

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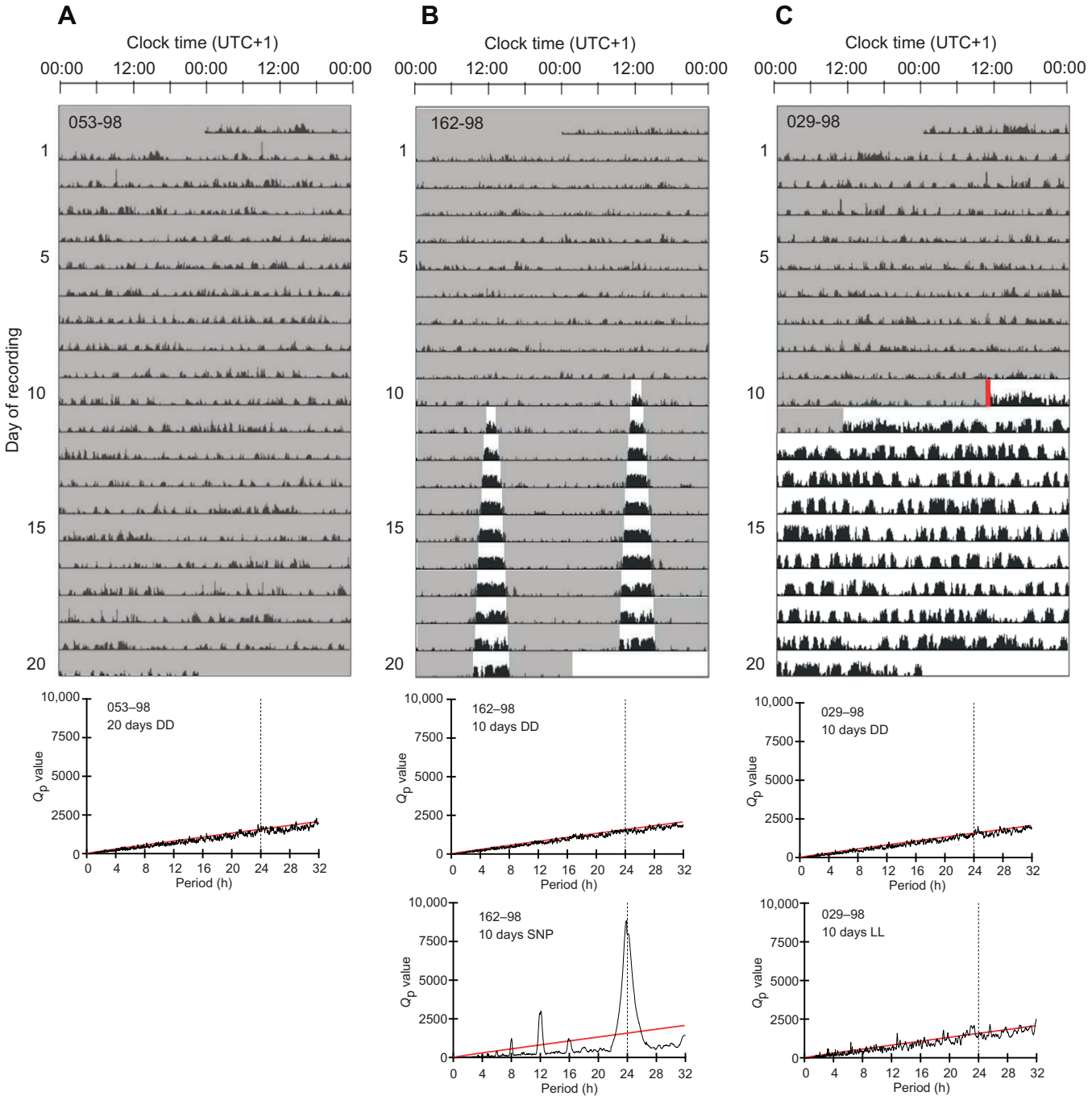

Fig. 3. Representative actograms and their respective chi-squared periodograms. (A–C) Birds adapted to constant darkness (DD) were transferred to their respective light treatments on day 10 of the recording (red line) or retained in DD. Actograms (top) are double plotted and grey shadings indicate periods of darkness. Chi-squared periodograms (bottom) were produced for 20 days for the DD group (A) or 10 days before and within experimental photoperiod for the LL and SNP group [B,C; upper periodogram: 10 days before light treatment (DD), lower periodogram: 10 days in light treatment]. Q_P values above the red line in the periodograms indicate significant periods (P<0.05). IDs of representative birds are given in the respective actograms and periodograms.

after the photoperiod had progressively increased to LL, expression of both genes increased dramatically to values similar to those in the LL treatment group (P<0.001 compared with initial values by *post hoc* Tukey's test). In birds maintained on DD, expression levels of both genes remained at basal levels throughout the experiment.

Dio2 and *Dio3* expression

Dio2 and *Dio3* in the ependymal region of the MBH showed reciprocal changes in expression over the course of the study (P<0.0001 by one-way ANOVA) (Figs 4E,F and 5). Initial *Dio2* expression was relatively weak, while *Dio3* expression was relatively strong (week 0). Transfer to LL increased *Dio2* expression 2.5-fold within 38 h (week 0 vs 38 h LL; P<0.05 by *post hoc* Tukey's test), while over the same period, *Dio3* expression was suppressed to background levels (45-fold decrease; P<0.01 by Tukey's *post hoc* test). Under continued LL exposure, elevated *Dio2* levels and suppressed *Dio3* levels were maintained to the end of the experiment.

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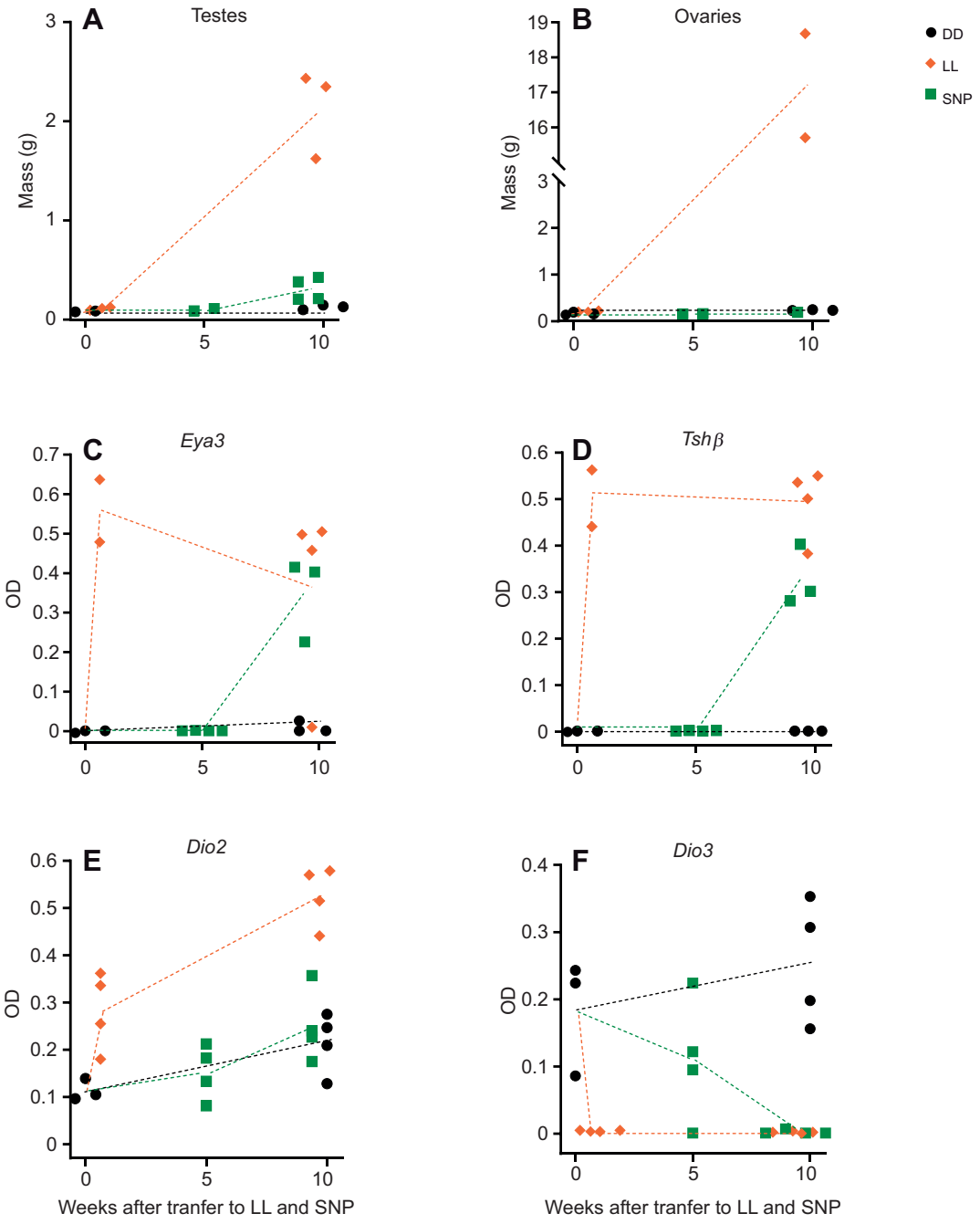

Fig. 4. Gonadal development and gene expression in the MBH measured by *in situ* hybridisation. (A,B) Gonad mass was measured post mortem. (C–F) Hypothalamic gene expression was measured before (point 0) and 10 weeks after the transfer into the respective light regime. Additionally, gene expression was measured after 38 h in LL and 5 weeks after the transfer into the simulated natural photoperiod (LD 12:12). The gene expression is given in optical density (OD) and each replicate is plotted with dotted lines passing through the respective mean.

Expression levels of *Dio2* and *Dio3* from birds under SNP gradually increased and decreased, respectively, over the course of the study. In both cases, expression levels after 5 weeks under SNP did not differ from initial values, while levels at week 10 were increased 2.3-fold for *Dio2* and decreased 60-fold for *Dio3* ($P<0.05$ and 0.01, respectively, by *post hoc* Tukey's test). Under constant darkness, no significant changes in either *Dio2* or *Dio3* expression were observed (Figs 4E,F, 5).

DISCUSSION

In our experiment, we transferred DD-acclimated Svalbard ptarmigan either into a simulated natural photoperiod or directly into LL. Both photoperiodic treatments caused increased *Eya3* and *Tshβ* expression and changes in the expression of downstream deiodinases, but birds transferred from DD to LL displayed no circadian behaviour. This absence of circadian rhythmicity, in combination with the lack of an external light–dark cycle, might question the circadian basis of the long day response in Svalbard ptarmigan.

According to theory, a circadian-based rhythm of photoinducibility triggers a photoperiodic response if light exposure occurs during the photoinducible phase (Bünning, 1936). Modern formulations of Bünning's model focus on events in the PT and

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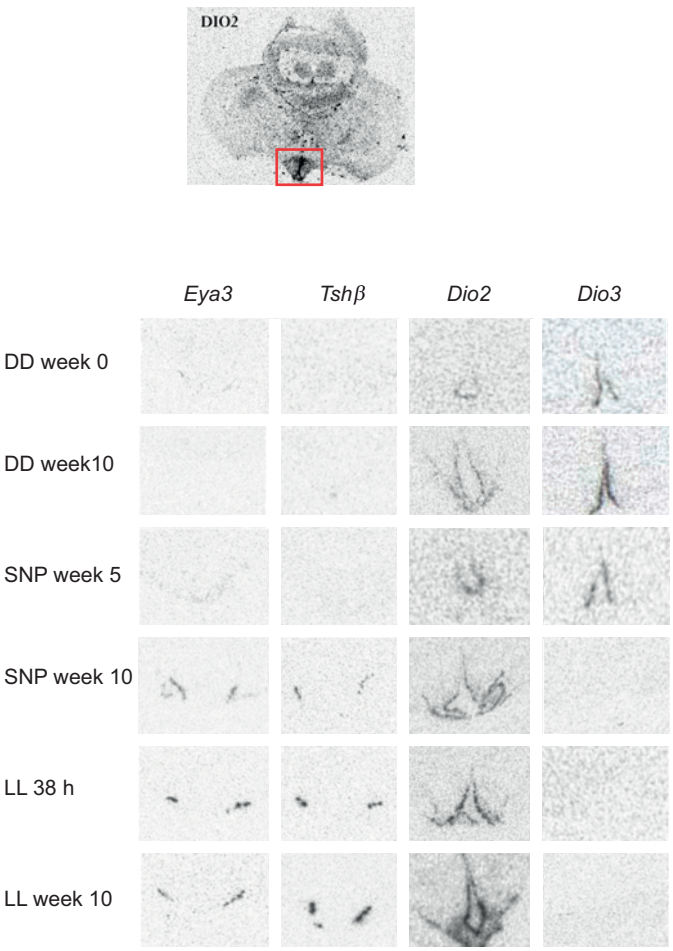

Fig. 5. Representative *in situ* hybridisation radiographs for each gene and each sampling point. Top picture is a whole-brain radiograph for *Dio2* highlighting the region of interest [pars tuberalis (PT) and mediobasal hypothalamus (MBH)]. Radiographs for the respective sampling points show the PT/MBH region (bottom).

MBH, where night-break protocols induce a long day response in local *Tshβ* expression and downstream effects on hypothalamic deiodinase genes (Dardente et al., 2010; Masumoto et al., 2010; Yoshimura et al., 2003). In sheep, promoter analysis of *Eya3*, a co-activator for *Tshβ*, demonstrates transcriptional control through clock genes, further emphasising the circadian basis for photoperiodic time measurement (Dardente et al., 2010).

In contrast, previous studies on Arctic animals report the absence of circadian rhythmicity and suggest this as a possible adaptation to polar latitudes, allowing around the clock foraging in constant Arctic light conditions (Lin et al., 2019; Lu et al., 2010; Reierth and Stokkan, 1998; Reierth et al., 1999; van Oort et al., 2005, 2007). Our study confirms the absence of circadian activity rhythms in DD and LL. In a separate experiment, we further found no evidence of circadian body temperature rhythms in DD and LL (D.A., A. Nord, D.G.H. and G.C.W., unpublished results).

This absence of behavioural and physiological rhythmicity does not exclude the possibility of latent circadian rhythmicity persisting in a coincidence timer mechanism. In non-Arctic bird species, LL can disrupt circadian activity rhythms but still triggers a photoperiodic response in reproduction (Agarwal et al., 2017; Lumineau and Guyomarc'h, 2003; Simpson and Follett, 1982; Wever, 1980). Moreover, Japanese quail show sustained hypothalamic expression of clock genes in LL, despite behavioural arrhythmicity (Lumineau and Guyomarc'h, 2003; Simpson and Follett, 1982; Yasuo et al., 2003). It therefore remains possible that a sustained rhythm of photo-inducibility may also persist within the PT/MBH region of Arctic Svalbard ptarmigan in constant photic conditions. Consequently, the DD-to-LL treatment triggers a long day response as light coincides with the photoinducible phase repeatedly after the transfer (Fig. 6A). Alternatively, the transition from DD to LL might initiate a dampening rhythm of photo-inducibility (Fig. 6B), either by direct induction or by bringing internally desynchronised cellular rhythms into phase (Balsalobre et al., 1998; Nagoshi et al., 2004; Welsh et al., 2004). This scenario would have similar consequences to the persistent rhythmical photo-inducibility described previously and they might prove difficult to resolve from one another.

Finally, we do not formally exclude that an hour-glass type mechanism operates in these birds. Under this scenario, induction relies on the progressive accumulation of a light dependent factor under LL (Fig. 6C). However, we favour a rhythm-based model since our molecular characterization of the photoperiodic response shows broad conservation with species known to rely on

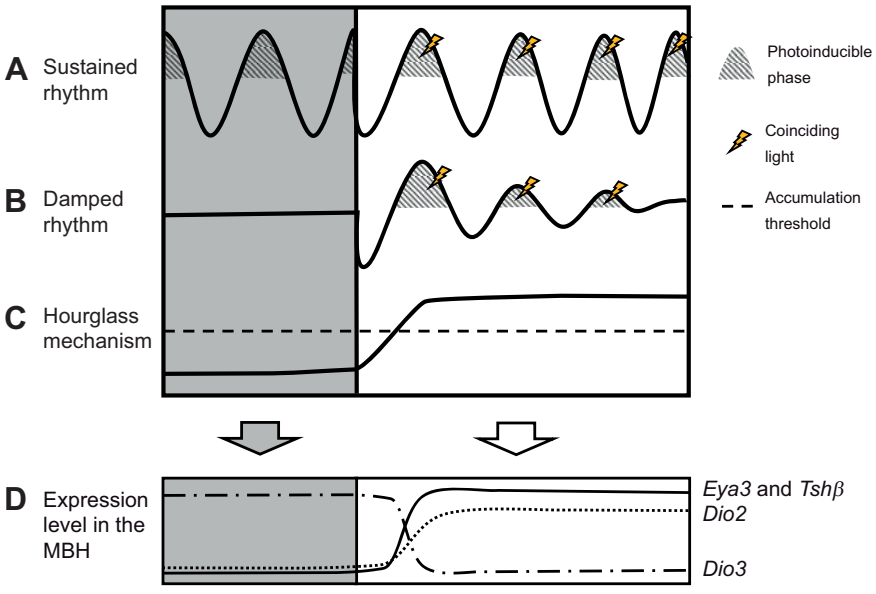

Fig. 6. Proposed mechanisms of photoperiodic time measurement in the Arctic. Svalbard ptarmigan show hypothalamic gene expression characteristic for seasonal reproduction when transferred from DD (grey shading) into LL (white; D). This process has been proposed to consist of a circadian rhythm of photo-inducibility and coinciding light. (A) Despite absent rhythm in activity, a light sensitivity rhythm might be sustained in the PT and MBH throughout constant conditions. (B) The rhythm of photo-inducibility might also be initiated by one dawn either by inducing the rhythm or by synchronising individual cells. (C) Lastly, the photoperiodic response might be circadian independent and instead based on the accumulation of a light-dependent factor.

coincidence timing, such as quail (Nakao et al., 2008; Yasuo et al., 2005; Yoshimura et al., 2003) or sheep (Dardente et al., 2010).

Similarly to Svalbard ptarmigan transferred from DD to LL, birds subjected to a simulated light–dark cycle also showed increased *Eya3* and *Tshβ* expression, and changes in the expression of downstream deiodinases at the final sampling point in LL, but not earlier in the study when the birds were on LD 12:12 (Fig. 4E,F). This is consistent with other mammals and birds which require a photoperiod of between 12.5 and 14 h for acute changes of photoperiodic genes in PT and MBH (Hanon et al., 2010, 2008; Król et al., 2012; Nakao et al., 2008; Ono et al., 2008). By the end of the study, birds in the SNP group showed only limited gonadal development (Fig. 4A,B). This is in line with earlier reports that wild Svalbard ptarmigan undergo a delay of several weeks in gonadal development even after exposure to long days (Stokkan et al., 1986).

In summary, our study showed that a High Arctic bird relies on the same molecular photoperiodic factors in the PT and MBH to initiate reproduction as other seasonal mammals and birds. Similar responses were measured in birds going through a SNP and birds directly transferred from DD to LL. The latter observation can reasonably be explained by a variant form of coincidence timer mechanism similar to that seen in temperate species. Further experiments using night break or Nanda Hamner protocols (Saunders, 2005) provide a route to test this hypothesis.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: D.G.H., D.A., G.C.W.; Methodology: D.A., H.D., G.C.W.; Formal analysis: D.A., G.C.W.; Investigation: D.A., V.J.M., H.D., G.C.W.; Resources: D.G.H., H.D.; Writing - original draft: D.G.H., D.A.; Writing - review & editing: D.G.H., D.A., V.J.M., A.C.W., H.D., G.C.W.; Visualization: D.A.; Supervision: D.G.H., G.C.W.; Project administration: D.G.H., G.C.W.; Funding acquisition: D.G.H.

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Supplementary information

Figure S1. **Actograms from experimental birds.** The DD group was plotted for 20 days. SNP and LL birds were plotted 10 days before and 10 days after the transfer into their respective light treatment. Actograms are double plotted and grey shadings indicate periods of darkness.

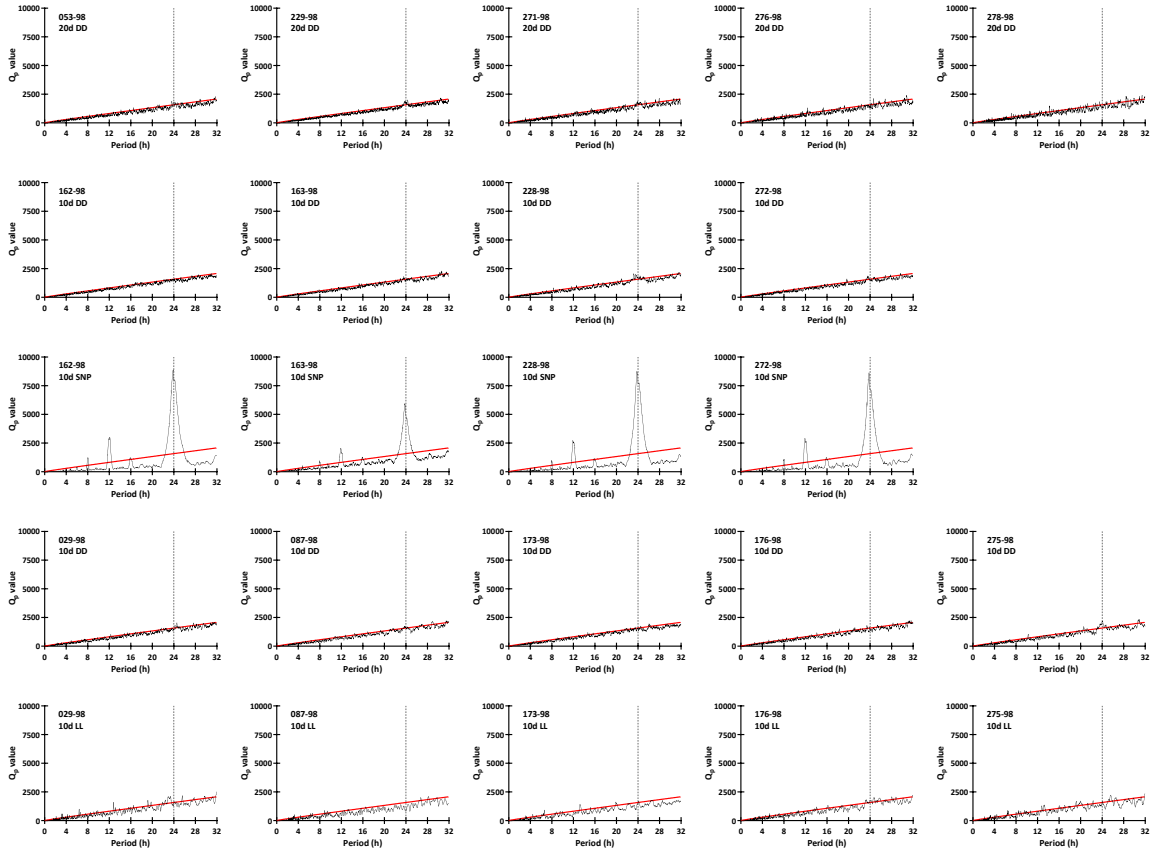

Figure S2. **Chi squared periodogram for actograms (Figure S1).** The DD group was analysed for 20 days. SNP and LL birds were analysed 10 days before and 10 days after the transfer into their respective light treatment. Q_P values above the red line in the periodogram indicate significant periods ($p<0.05$).

Table S1. **Experimental groups.** Bird identifications and their respective sampling point, gonad mass and optical density (OD) values of hypothalamic gene expression as measured by *in situ* hybridisation. The PT became detached in several brain samples during the sampling procedure. Measurement for *Eya3* and *Tsh*β are therefore missing for several individuals.

ID	group	gender	Sampling	testes (g)	ovaries (g)	*Eya3* (OD)	*Tsh*β (OD)	*Dio2* (OD)	*Dio3* (OD)
286-98	DD	female	Week 0		0.159	0.001	0.001	0.105	0.224
056-98	DD	male	Week 0	0.080		0.001	0.001	0.096	0.243
037-98	DD	female	Week 0		0.195	0.001	0.001	0.139	0.086
268-98	DD	female	Week 0		0.141				
057-98	DD	male	Week 0	0.087					
278-98	DD	male	Week 10	0.129		0.001	0.001	0.275	0.198
053-98	DD	male	Week 10	0.146		0.001	0.001	0.247	0.156
229-98	DD	female	Week 10		0.236	0.022	0.001	0.128	0.307
271-98	DD	female	Week 10		0.251	0.026	0.001	0.209	0.353
276-98	DD	male	Week 10	0.100					
267-98	DD	female	Week 10		0.234				
069-98	SNP	male	Week 5	0.113		0.001	0.002	0.081	0.001
060-98	SNP	female	Week 5		0.160	0.001	0.001	0.212	0.095
225-98	SNP	male	Week 5	0.090		0.001	0.001	0.182	0.122
059-98	SNP	female	Week 5		0.165	0.002	0.002	0.133	0.224
066-98	SNP	male	Week 10	0.382		0.403	0.403	0.357	0.007
272-98	SNP	male	Week 10	0.207		0.226	0.302	0.24	0.001
162-98	SNP	male	Week 10	0.428		0.415	0.281	0.227	0.001
163-98	SNP	female	Week 10		0.193	Detached PT	Detached PT	0.175	0.001
228-98	SNP	male	Week 10	0.211					
051-98	LL	female	38h in LL		0.225	0.479	0.441	0.255	0.005
025-98	LL	female	38h in LL		0.200	0.637	0.563	0.362	0.005
064-98	LL	male	38h in LL	0.125		Detached PT	Detached PT	0.336	0.003
070-98	LL	male	38h in LL	0.097		Detached PT	Detached PT	0.18	0.001
176-98	LL	male	Week 10	2.434		0.458	0.501	0.441	0.003
173-98	LL	female	Week 10		18.681	0.498	0.536	0.57	0.002
275-98	LL	female	Week 10		15.704	0.01	0.383	0.515	0.004
029-98	LL	male	Week 10	2.349		0.505	0.55	0.579	0.002
087-98	LL	male	Week 10	1.623					

Table S2. **Simulated natural photoperiod (SNP).** The light schedules follows the progression of civil twilight on- and offset in Longyearbyen Svalbard (78°13'N 15°38'E). Clock times are given as coordinated universal time + 1 (UTC+1).

exp. week	date 2018	light on	light off	minutes of light/ day
	30.jan			0
0	31.jan	11:31	12:53	82
0	01.feb	11:06	13:17	131
0	02.feb	10:48	13:36	168
0	03.feb	10:33	13:51	198
0	04.feb	10:20	14:04	224
0	05.feb	10:08	14:17	249
0	06.feb	09:57	14:28	271
1	07.feb	09:46	14:39	293
1	08.feb	09:36	14:49	313
1	09.feb	09:27	14:59	332
1	10.feb	09:17	15:08	351
1	11.feb	09:08	15:18	370
1	12.feb	08:59	15:26	387
1	13.feb	08:51	15:35	404
2	14.feb	08:42	15:44	422
2	15.feb	08:34	15:52	438
2	16.feb	08:26	16:00	454
2	17.feb	08:18	16:08	470
2	18.feb	08:10	16:16	486
2	19.feb	08:02	16:24	502
2	20.feb	07:54	16:31	517
3	21.feb	07:46	16:39	533
3	22.feb	07:39	16:46	547
3	23.feb	07:31	16:54	563
3	24.feb	07:23	17:01	578
3	25.feb	07:16	17:09	593
3	26.feb	07:08	17:16	608
3	27.feb	07:01	17:23	622
4	28.feb	06:53	17:30	637
4	01.mar	06:46	17:38	652
4	02.mar	06:38	17:45	667
4	03.mar	06:31	17:52	681
4	04.mar	06:23	17:59	696
4	05.mar	06:16	18:06	710
4	06.mar	06:08	18:14	726

exp. week	date 2018	light on	light off	minutes of light/ day
5	07.mar	06:00	18:21	741
5	08.mar	05:53	18:28	755
5	09.mar	05:45	18:36	771
5	10.mar	05:37	18:43	786
5	11.mar	05:30	18:50	800
5	12.mar	05:22	18:58	816
5	13.mar	05:14	19:05	831
6	14.mar	05:06	19:13	847
6	15.mar	04:58	19:20	862
6	16.mar	04:50	19:28	878
6	17.mar	04:41	19:36	895
6	18.mar	04:33	19:44	911
6	19.mar	04:24	19:52	928
6	20.mar	04:16	20:01	945
7	21.mar	04:07	20:09	962
7	22.mar	03:58	20:18	980
7	23.mar	03:48	20:27	999
7	24.mar	03:39	20:36	1017
7	25.mar	03:29	20:46	1037
7	26.mar	03:19	20:56	1057
7	27.mar	03:08	21:06	1078
8	28.mar	02:58	21:17	1099
8	29.mar	02:46	21:28	1122
8	30.mar	02:34	21:41	1147
8	31.mar	02:21	21:54	1173
8	01.apr	02:07	22:09	1202
8	02.apr	01:52	22:26	1234
8	03.apr	01:34	22:47	1273
9	04.apr	01:13	23:16	1323
9	05.apr	00:43	00:00	1397
9	06.apr			1440
9	07.apr			1440
9	08.apr			1440
9	09.apr			1440
9	10.apr			1440
10	11.apr			1440

Table S3. **Nucleotide sequences for riboprobe transcription**. Svalbard ptarmigan specific cDNA was cloned into a pGEMT easy vector and the anti-sense riboprobe was obtained by transcription with either SP6 or T7 RNA polymerase (Promega). Sequence is given in the T7 transcription direction.

	Size of probe (base pairs)	**Anti-sense probe synthesis**	**Nucleotide sequence in pGEMT vector (T7 transcription)**
Eya3	772	SP6	GGAGGATCACAAACCATGCAGACCTTGTGTCCCTTCACCAAGCCCTTGAGTTAGACTTC CTGTAAGAAGCCAGAGCAAAGGTGTGGCTGTGGCTCCTGCAATCTCTCCATCACTGGG GAGGCAAAGATCTCATAAACAACTGGGGACATTTTCAGCTTGATGAAGAAAACATACC TAATGCAAATTGTACAGTAGACTTCAGGTCATTTCCTCAGGAGCACAGACCTGGCCAAG TCCAGAGGTGTCCTATGAGAAGCACTAGACTCAGCAGAGCTAGAGCTTGACTGGTGAA GAGACTCATGGAATCCAGCTGAATTCTTCTGGCAGGTAGTCTCCAGAGGGAGGGGGTA ACACAGCTGAGAAGGCTCTTACTGATACTTCTGCTTTTCTATTCTGCTTAGTTATAGAAA CCCAAGTAAACATAAAACCTTATTTTTATAGAAAAAAACTGATGGCAGAGCTGAACCTC CCTTGTTTTGCAAAGCCAAAAAGAGATATTGGTTGTTTTTTGTTTGTTTTTTTTTCCATGG GAAATATTAATGAAAATGTCAAAAATACCTCTGCTGCTGTGAAAATGTGTCCTCTCTCCT TCTCTGGGTGTTCAAAAGCAGTTAATTTATTATGATATCCTTACATTATTTCTACAACATG GGATTTTTCCCATTCTGGGATAAGTGGTTCTTAGTAGAGGGAGGTGTTGCTGTTGGTTT CTCCCATTGGCTTCTAGGCAGTGTGTGTAGGATGCACGTGGCTGAGCCCTGTTAATGAG CAG
*Tsh*β	404	SP6	TGTCTCTCCTCTTTGGCCTGACCTTTGGTCAAACAGCATCACTTTGTGCTCCTTCAGAGT ACACAATCCACGTGGAGAAACGGGAGTGCGCCTATTGCCTGGCCATCAACACCACCAT CTGCGCCGGATTCTGCATGACTCGGGACAGCAATGGCAAGAAGCTGCTACTCAAAAGT GCTCTGTCCCAAAACGTGTGCACATATAAAGAGATGTTGTATCAAACAGCACTGATTCC GGGCTGTCCTCATCACACCATCCCTTACTATTCCTACCCCGTGGCCATAAGCTGCAAGTG TGGTAAATGTAACACTGACTACAGTGACTGTGTTCACGAGAAGGTTAGGACAAACTAC TGCACTAAGCCACAGAAGCTCTGTAACATGTGAGCTTCCAACAGAACACGG
Dio2	693	T7	CCAAAGACGAACCTCCTGAAGATTGTAGAAAAAGGGGCCTTTACCTCCCAGGTAGGCA ATTTTTTGTCTCTGCACAATGCATACTCGCTCAAATGAAACCCCATAGGCCACATTGGCA TTGTTGTCCATGCAGTCAGCCACTACTTGGCACTGAGGTGGCAAGGAGAAGTGTTCCA GGAGTTGGTAAGCAGCTGCACATCGATCTTCCTGATTTCTGTGCTTCTTAACTTCAAAG GAAGAGGGGGAGATACCAGGAGCAGCCCAGCCATCTGACGGATGAGCCTCATCGATG TAGACCAACAGAAAGTCAGCCACACCAGAGAACTCTTCCACCAGCTTGCTGAAGGCAG ACAGCTGGCTTGTGAACGGTGGTCAAGTAGCTGAGCCGAAATTAACAACCAGTGGCCG CTCAGAGTTGGCAAAATCCAGAAGATGGCACTTGGCCCCACAGTTACCACCAACACTCT TCCAGCTGCTACTGCTACCATCATTGCGCTTGGCTATGTGGATTACACTGGAATTTGGA GCTTCTCCTCCAAGTTTGACCTGCTTGTAGGCGTCCAGGAGGAAGCTGTTCCAGACGCA GCGCAGCCCCTCCGAGGTCAGCATCCTCCGCCACTCACCGCGCGCAGACTTAGAGCGG CTCAGAAACAGCACCATGTGCTTCAGGAGGATCACAGAGTCATACAGCGC
Dio3	556	SP6	ATGTTCACGCTGGAGTCGCTGAAGGCTGTGTGGCACGGGCAGAAGCTGGACTTCTTCA AGTCGGCGCACGTGGGCTCGCCGGCCCCCAACCCCGAGGTGATCCAGCTGGACGGGC AGAAGAGGCTCCGCATCCTCGACTTCGCCCGCGGCAAGAGGCCCCTCATCCTCAACTTC GGCAGCTGCACCTGACCCCCGTTCATGGCCCGCCTGAGGTCCTTCCGGCGCCTGGCCGC GCACTTCGTGGACATTGCCGACTTTCTGCTGGTGTACATCGAAGAAGCGCACCCCTCTG ACGGCTGGGTCAGCTCGGACGCTGCCTACAGCATCCCCAAGCACCAGTGCCTCCAGGA CAGGCTGCGGGCAGCGCAGCTGATGCGGGAAGGGGCGCCCGATTGCCCCCTGGCCGT GGACACCATGGACAACGCTTCCAGCGCTGCCTACGGCGCCTACTTCGAGCGGCTCTAC GTCATCCAGGAGGAGAAGGTGATGTACCAGGGAGGCCGAGGACCGGAGGGTTACAA GATCTCGGAGCTGCGGAGCTGGCTAGACCAGTACA

Paper III

Adaptive value of circadian rhythms in High Arctic Svalbard ptarmigan

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SUMMARY

The arctic archipelago of Svalbard (74 to 81° North) experiences extended periods of uninterrupted daylight in summer and uninterrupted darkness in winter. Species native to Svalbard display no daily rhythms in behaviour or physiology during these seasons, leading to the view that circadian rhythms may be redundant in arctic environments [1, 2]. Nevertheless, seasonal changes in the physiology and behaviour of arctic species rely on photoperiodic synchronisation to the solar year. Since this phenomenon is generally circadian-based in temperate species, we investigated if this might be a preserved aspect of arctic temporal organisation.

Here, we demonstrate the involvement of the circadian clock in the seasonal photoperiodic response of the Svalbard ptarmigan (*Lagopus muta hyperborea*), the world's northernmost resident bird species. First, we show the persistence of rhythmic clock gene expression under constant conditions 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. Our results suggest that the unusual selective pressure of the Arctic relaxes the adaptive value of the circadian clock for organisation of daily activity patterns, whilst preserving its importance for seasonal synchronisation. Thus, our data simultaneously reconnects circadian rhythms to life in the Arctic and establishes a universal principle of evolutionary value for circadian rhythms in seasonal biology.

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 are behaviourally arrhythmic in constant light conditions (Figure 1B) [1, 3]. These data, and similar findings in Svalbard reindeer [2, 4], suggest that circadian rhythms are redundant in the high arctic habitat of Svalbard. The Svalbard ptarmigan, however, uses photoperiod to time seasonal changes in its physiology [3, 5-7], and a vast collection of data supports the role of the circadian rhythm in photoperiodic timekeeping [8-17]. Thus, although the ptarmigan circadian rhythm may not be required for maintaining daily synchrony, it may play an essential role in photoperiodic responses.

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 [18-20]. Our results showed that both genes were strongly rhythmic under short photoperiod (SP, L6:D18) and displayed negligible changes in their expression patterns following 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 [18-23] 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*. 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 (CT13) ($p < 0.0001$ compared to SP control birds by Sidak's *post hoc* test) (Figure 1E) before falling back to SP levels by 23 h after lights-on (CT23). Within the MBH, transfer to LL significantly induced the expression of *Dio2* by CT23 ($p = 0.0011$ by Sidak's *post hoc* test), and suppressed the expression of *Dio3* by CT18 ($p = 0.0085$ by Sidak's *post hoc* test) (Figure 1E). 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*) [18].

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 [24]. 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). 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 [5-7, 25].

We observed a strong diurnal activity preference within all the groups (Figure 2A). The activity profile of the SP-group went unchanged throughout the entire experiment; however, both the IP and SkP groups 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 *post hoc* test). 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 ($p < 0.05$ for all SkP vs SP and SkP vs IP in week 7 by Tukey's *post hoc* test) (Figure 2C), indicating a photoperiodic stimulation of activity.

Associated with these increases in activity, we observed sustained declines in body mass in both the SkP and IP groups continuing until weeks 9 and 11 respectively (Figure 2D). Food intake was similar between all three groups until week 10 (Figure S1B), suggesting that these responses were a consequence of increased activity resulting in a negative energy balance. Longitudinal assessment of plasma testosterone in male birds 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) (Figure 2E), 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 [26, 27]

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 ($p > 0.05$ for SkP vs SP at all points from week 10 onwards by Tukey's *post hoc* test, Figure 2B and 2C). The reversal of the response in SkP- but not IP-birds probably reflects either a

movement of the secondary 2-h block of light beyond the photosensitive phase or 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 [28]. Whichever scenario applies, the birds behaved as if experiencing a declining photoperiod after week 7 even though cumulative daily light exposure remained constant. Overall, these results suggest that the Svalbard ptarmigan use a circadian-based system to mediate spring photoperiodic induction of pre-breeding behaviour, with a photoinducible phase some 14 to 16 h after dawn (ZT 14-16).

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, food intake and testosterone, 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 S2). 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 cements 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 of the birds at ZT 0.5-1.5 which is comparable with the early light-period in our "first long day" experiment, when *Tshβ* expression is low (Figure 1E). This data additionally suggest comparable circadian phase between the SP and SkP groups.

Adaptive value of circadian rhythms in the Arctic

Overall, our results affirm the universal adaptive role of the circadian rhythm for photoperiodic time measurement. We show that an organism in which daily behavioural rhythms disappear under the

midnight sun and during the polar night, maintains a rhythmic molecular clock in tissues essential for seasonal timing and employs a circadian rhythm to set a photoinducible circadian phase.

The unusual phenotypes of the Svalbard ptarmigan and Svalbard reindeer, which shows a similar circadian phenotype [2, 4, 29, 30], reflect the unusual selective pressures of their habitat. Summer and winter on Svalbard provide two wildly contrasting environments for which seasonal synchronisation is essential, particularly of reproductive physiology. Conversely, the daily amplitude of irradiance and temperature are greatly reduced on Svalbard compared to lower latitudes, thus the adaptive value of daily behavioural organisation on Svalbard is likely to be weak, and may even exert counter-adaptive constraints on exploitation of the polar summer (Figure 4). Nevertheless, several reports of sustained circadian behaviour and physiology in other arctic dwellers have emerged, e.g. Arctic ground squirrels [31, 32], bumblebees [33], polar bears [34], copepods [35] and several migrating birds [36-38]. Collectively, these studies suggest that species-specific differences in circadian outputs should be seen as flexible solutions to complex life-history constraints [39]. For the Svalbard ptarmigan, the adaptive solution is to maintain a circadian rhythm to measure photoperiod, but decouple behavioural drive as a circadian output.

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AUTHOR CONTRIBUTION

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, DA, DGH & ACW; Visualization, DA, GCW & ACW; Supervision, DGH, GCW & ACW; Project Administration, all; Funding Acquisition, DGH;

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 right a male in white winter plumage and on the left a female in brown summer plumage (© Ida-Helene Sivertsen).

(B) Double plotted actogram of a Svalbard ptarmigan in Svalbard over one year (March to February). Thick lines indicate on- and offset of civil twilight and thin lines indicate sunrise and sunset. Redrawn using data from Reierth *et al*. (1998) [1].

(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).

(D) Gene expression for *Per2* and *Cry1* in the MBH and PT between the SP- (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 by Sidak's *post hoc* test).

(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 by Sidak's *post hoc* test).

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 remained under SP for 12 weeks. Birds of the IP-group 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 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 forwards 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.

(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.

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

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

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 or experienced a shifting skeleton photoperiod. 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.

(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 ($p < 0.05$ by unpaired t-test). 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) [40, 41]. 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, 19, 42, 43]. We show here that its arctic relative, the Svalbard ptarmigan (right panel), retained its circadian system to sustain 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 of Svalbard we propose that the functional circadian system exhibits weak or no 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 contacts, Alexander West (alexander.west@uit.no) or David Hazlerigg (david.hazlerigg@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 they are isolated from other rock ptarmigan population, as reflected in their low genetic diversity [44] and the specificity of their phenotype compared to other ptarmigan populations, e.g. high amplitude body mass cycles [45].

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 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.

Both sexes were used for the experiments as we have not seen any sex differences in hypothalamic gene expression in our previous study [3].Seasonal rhythm in body mass, activity and food intake is also similar between the sexes [5, 25]. 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 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. 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 once and was used for plotting of both groups as there is 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).

First skeleton-photoperiod experiment (Figure 2)

Photoperiod was gradually decreased from September 2018 until reaching DD (dim red light) 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

304 birds of this group remained in L6:D18 for two more weeks until the end of the experiment in week
305 12.

306 Body mass and voluntary food intake of all birds was measured weekly with an analytical scale
307 (Mettler). VFI was measured once a week from all birds by measuring food eaten within a 24 hours
308 period. In addition blood was taken weekly from four males per group for plasma testosterone
309 measurements (blood was not taken in week 11 and 12). Locomotor activity of all experimental birds
310 was continuously recorded as movement per minute by passive infrared sensors (home-built),
311 mounted on the cage doors. Data were collected by an Actimetrics CL200 USB interface coupled to a
312 PC with the ClockLab data acquisition software version 2.61 (Actimetrics).

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

315

316 **Second skeleton photoperiod experiment (Figure 3)**

317 The second skeleton photoperiod experiment was conducted with birds from the SP-group (n = 10)
318 from the first skeleton photoperiod experiment. This experiment started on 4th April 2019. The birds
319 were separated into two groups. The SP-group (n = 5) further remained under L6:D18 for eight weeks
320 and where sampled at the end of the experiment. The SkP-group (n = 5) went through a similar shifting
321 skeleton photoperiod as described in the previous experiment. However, the two hour light-period
322 was only shifted until reaching ZT 14-16 in week 6 upon which point birds remained on L4:D10:L2:D8
323 for additional two weeks before they were sampled (ZT 0 corresponds to the lights-on switch from the
324 fixed four hour light-period). All birds of this experiment were euthanised on week 8 between ZT 0.5
325 and ZT 1.5. After the euthanasia brains were removed and rapidly transferred onto a cooled metal
326 block until ultimately stored at -80 °C.

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

330

331 **Hormone measurement**

332 Blood was taken weekly from four male birds of every group. In the first skeleton experiment, four
333 birds were sampled in each group and each week, except in DD and week 1, in which only a total of
334 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 [46]. Brain cryo-sectioning, probe synthesis and *in situ* hybridization were performed as reported previously [3, 47] 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, scintillation cocktail form Gammadata).

Frozen brains, which were cryo-sectioned (Leica Biosystems) and mounted unto pre-coated adhesion microscopic slides (VWR), 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 riboprobe 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 an transmission scanner (Epson). Optical density (OD) was measured with ImageJ (Wayne Rasband).

QUANTIFICATION AND STATISTICAL ANALYSIS

All graphs and statistical tests 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 S1). Activity, body mass, food intake and plasma testosterone of the second skeleton photoperiod experiment was analysed by 2-way ANOVA with *post hoc* Sidak's multiple comparison test (Figure S2). Relative gene expression between the SP-group and SkP-group of the second skeleton photoperiod experiment was tested with unpaired t-tests. Activity was normalized by dividing counts per minute of each bird by its 99^{th} percentile and actograms (Figure 2A, 3A and S3) were plotted with ActogramJ [48], a plugin for ImageJ (Wayne Rasband).

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

SUPPLEMENTAL INFORMATION

Figure S1. Response in body mass and food intake to increasing and skeleton photoperiod.

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

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

Figure S2. 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 S3. 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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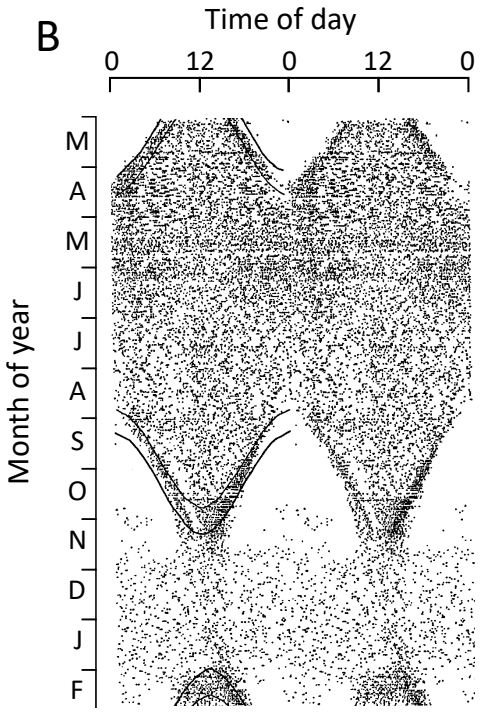
A


B
Time of day
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12
0
12
0
Month of year
M
A
M
J
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O
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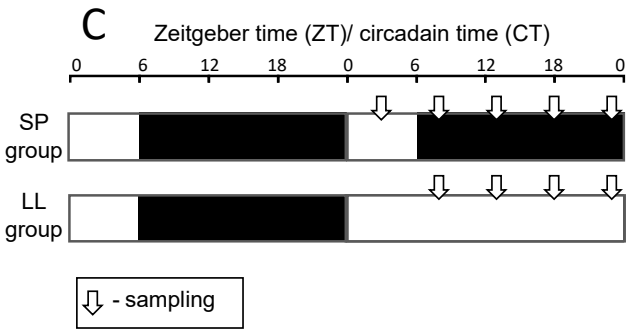
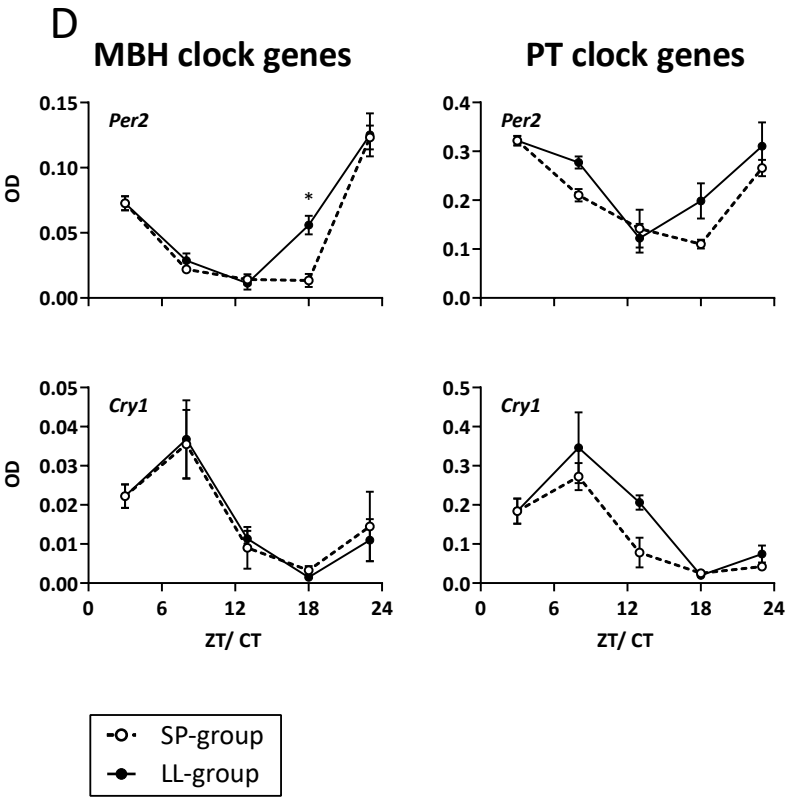

C
Zeitgeber time (ZT)/ circadain time (CT)
0
6
12
18
0
6
12
18
0
SP group
LL group
- sampling


D
MBH clock genes
PT clock genes
Per2
Cry1
OD
ZT/ CT
SP-group
LL-group

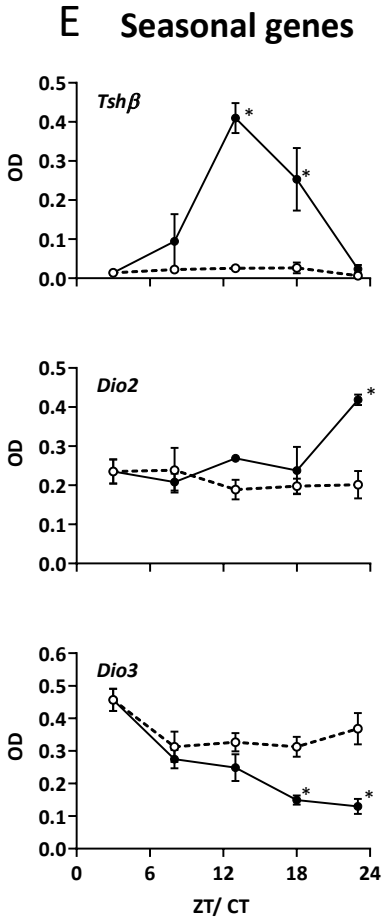

E
Seasonal genes
Tshβ
Dio2
Dio3
OD
ZT/ CT

Figure 1.

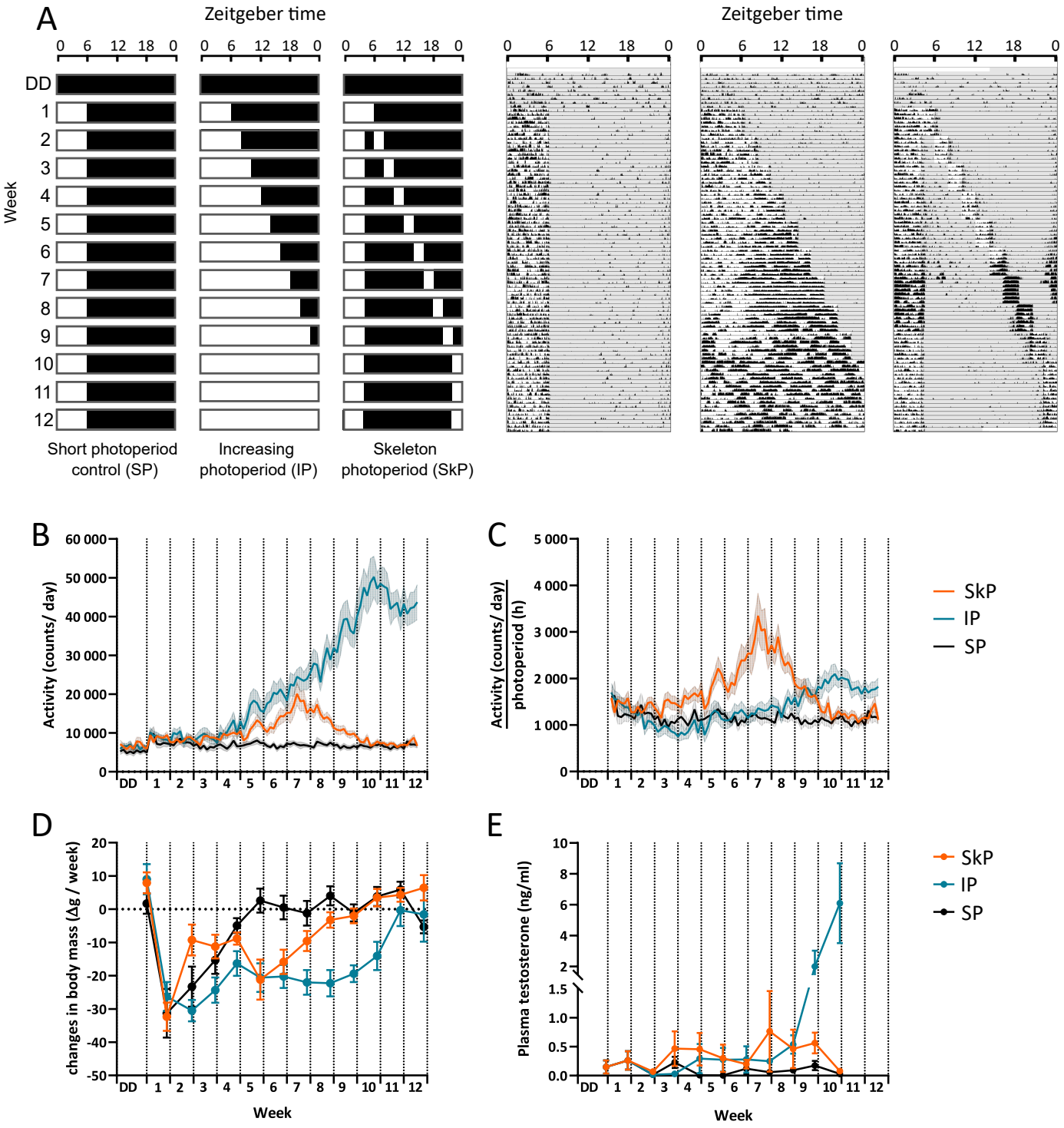
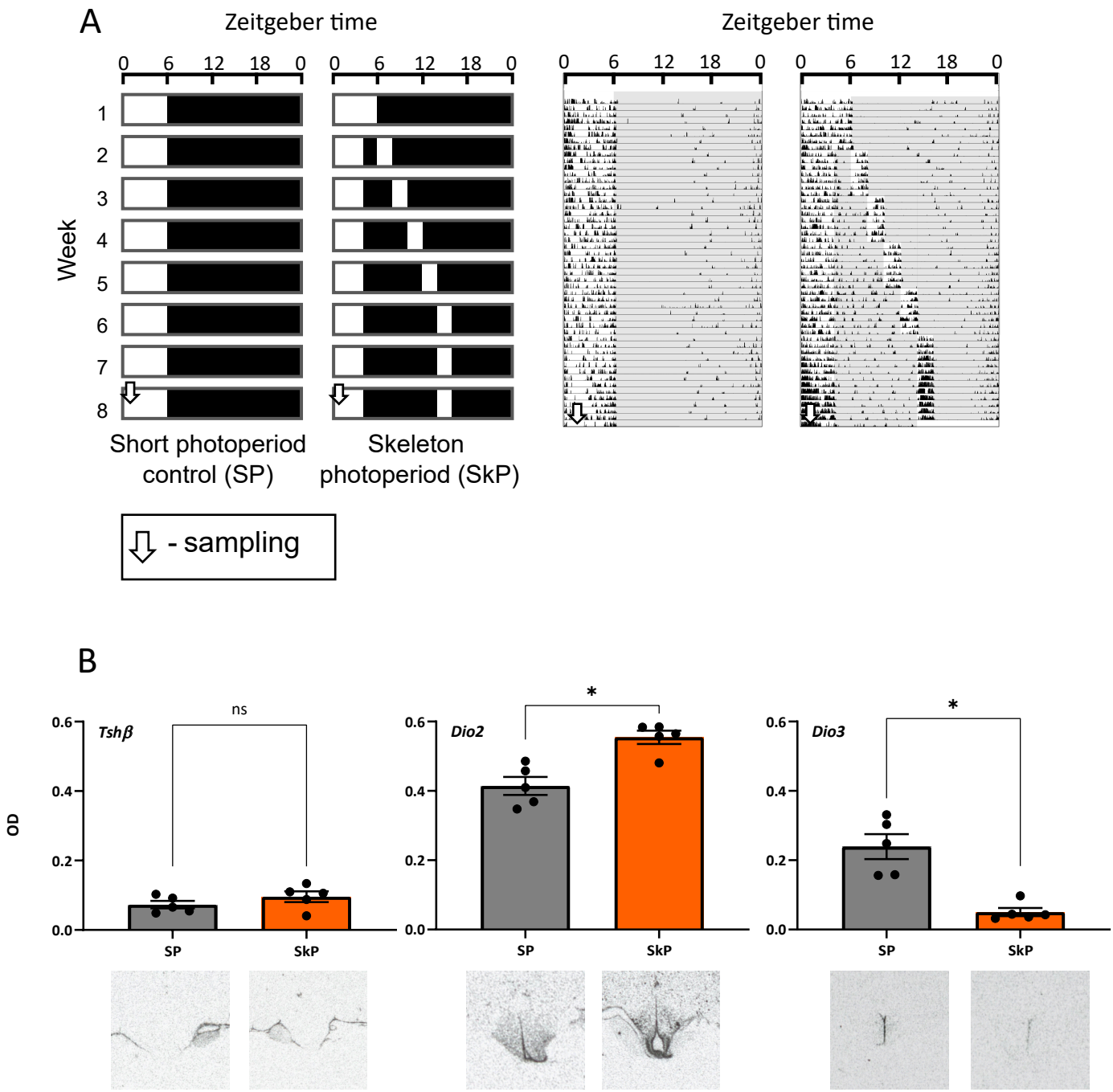

A
Zeitgeber time
Zeitgeber time
0 6 12 18 0
DD
1
2
3
4
5
6
7
8
9
10
11
12
Week
Short photoperiod control (SP)
Increasing photoperiod (IP)
Skeleton photoperiod (SkP)
B
Activity (counts/ day)
C
Activity (counts/ day) / photoperiod (h)
SkP
IP
SP
D
changes in body mass (Δg / week)
Week
E
Plasma testosterone (ng/ml)
Week

Figure 2.


A
Zeitgeber time
Zeitgeber time
0 6 12 18 0
Week
1
2
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6
7
8
Short photoperiod control (SP)
Skeleton photoperiod (SkP)
- sampling
B
OD
Tshβ
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Dio2
*
Dio3
*
0.0
0.2
0.4
0.6
SP
SkP

Figure 3.

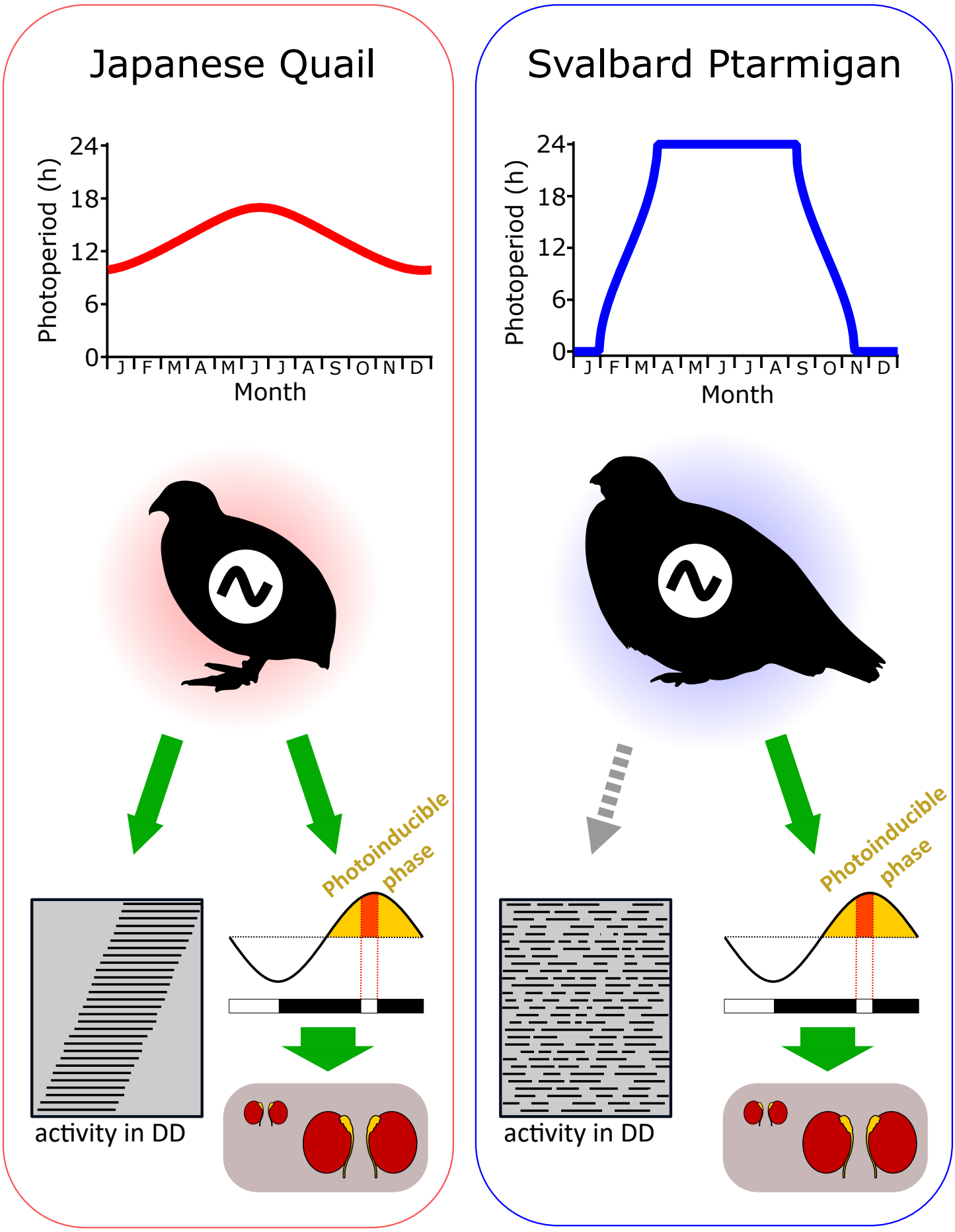

Japanese Quail
Photoperiod (h)
24
18
12
6
0
J F M A M J J A S O N D
Month
Photoinducible phase
activity in DD
Svalbard Ptarmigan
Photoperiod (h)
24
18
12
6
0
J F M A M J J A S O N D
Month
Photoinducible phase
activity in DD

Figure 4.

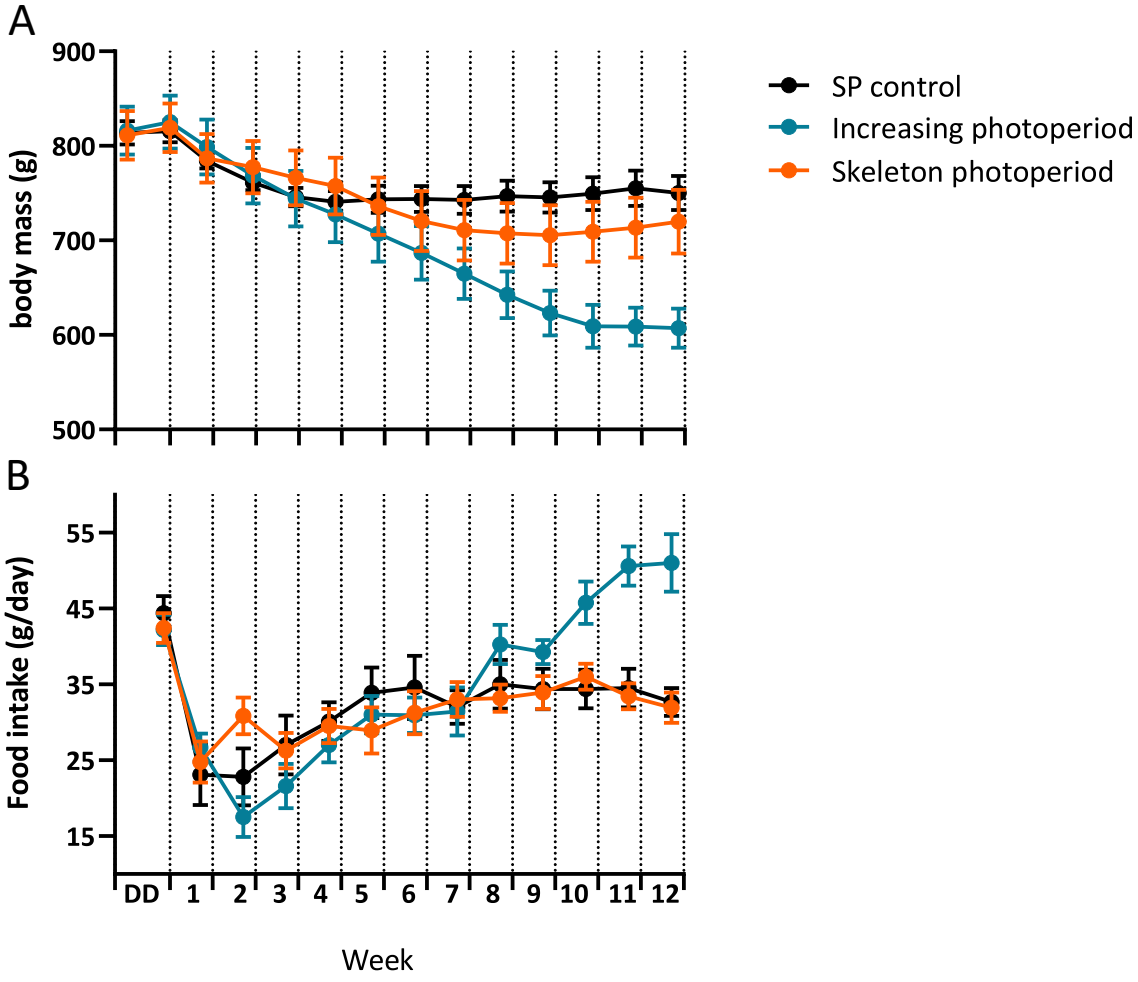

A
900
800
700
600
500
body mass (g)
B
55
45
35
25
15
Food intake (g/day)
DD
1
2
3
4
5
6
7
8
9
10
11
12
Week
SP control
Increasing photoperiod
Skeleton photoperiod

Figure S1.

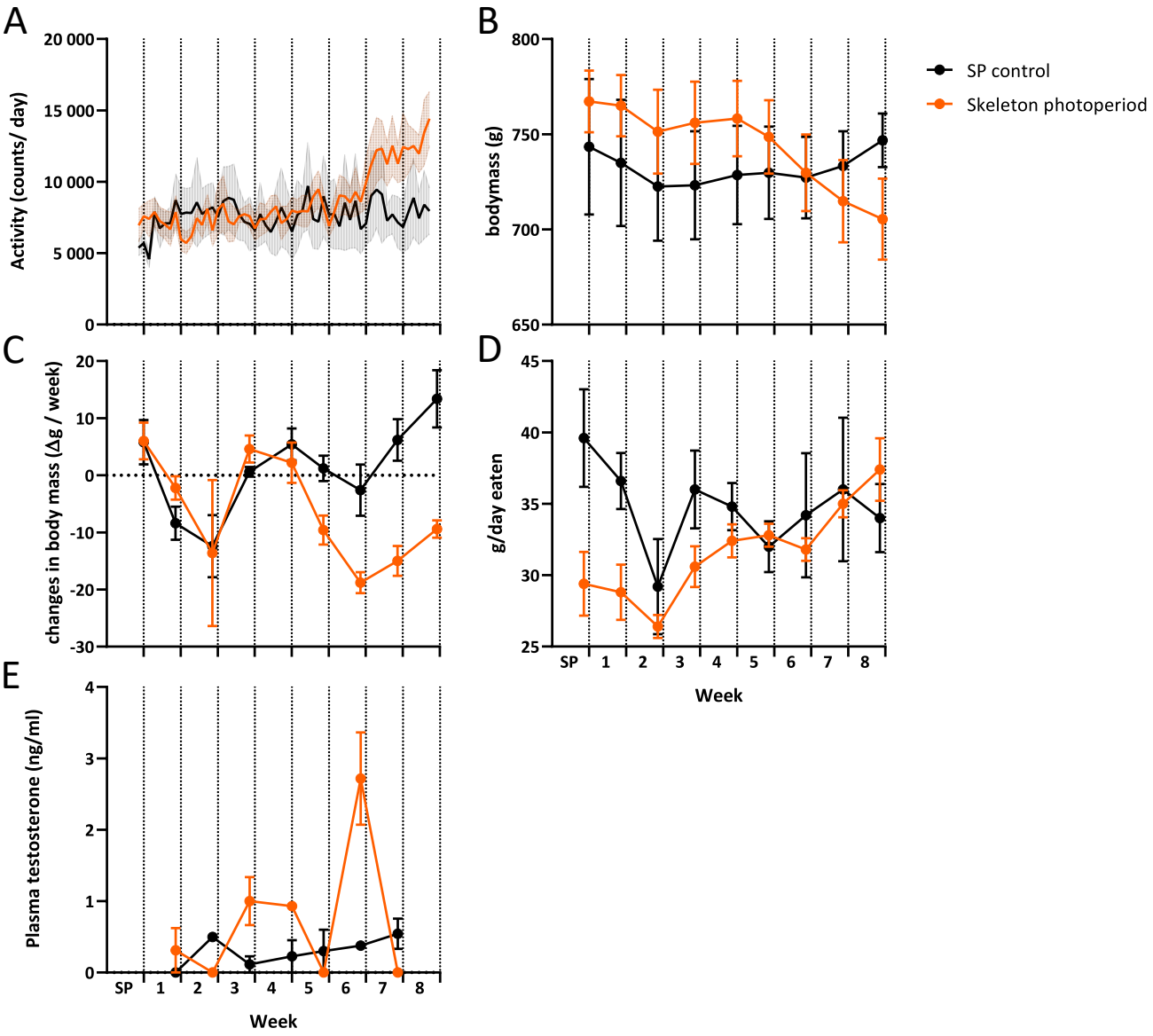
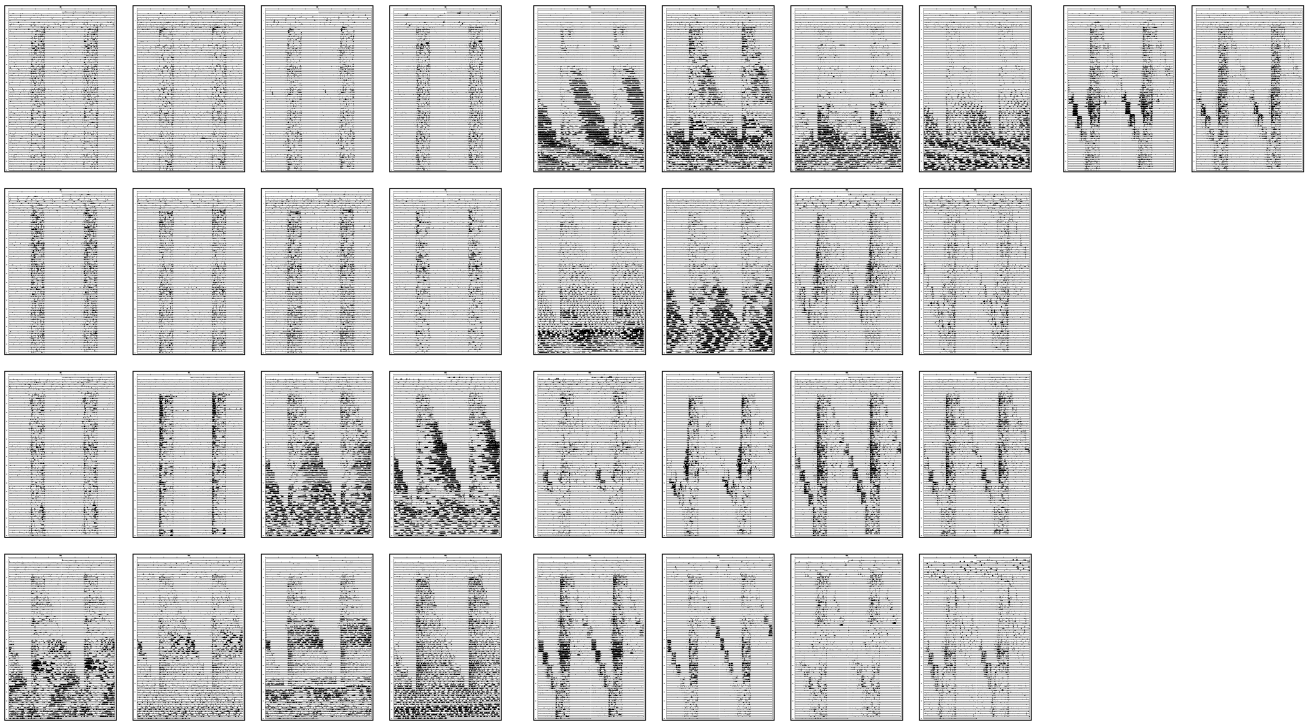

A
Activity (counts/ day)
20 000
15 000
10 000
5 000
0
B
bodymass (g)
800
750
700
650
SP control
Skeleton photoperiod
C
changes in body mass (Δg / week)
20
10
0
-10
-20
-30
D
g/day eaten
45
40
35
30
25
SP 1 2 3 4 5 6 7 8
Week
E
Plasma testosterone (ng/ml)
4
3
2
1
0
SP 1 2 3 4 5 6 7 8
Week

Figure S2.

A



B

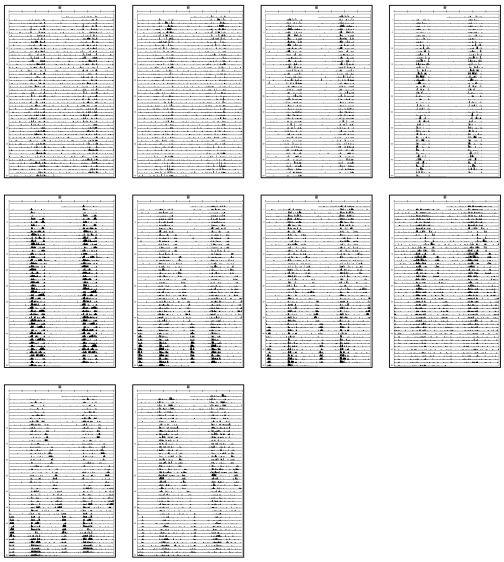


Figure S3.