

Circannual basis of geographically distinct bird schedules

Barbara Helm*, Ingrid Schwabl and Eberhard Gwinner

Max Planck Institute for Ornithology, 82346 Andechs, Germany

*Author for correspondence (e-mail: helm@orn.mpg.de)

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SUMMARY

To anticipate seasonal change, organisms schedule their annual activities by using calendrical cues like photoperiod. The use of cues must be fitted to local conditions because schedules differ between species and habitats. In complete absence of temporal information, many species show persistent circannual cycles that are synchronised, but not driven, by photoperiod. The contribution of circannual rhythms to timing under natural photoperiodic conditions is still unclear. In a suite of experiments, we examined timing in two closely related songbirds (Siberian and European stonechats) that inhabit similar latitudes but differ in seasonal behaviour. Under a more continental climate, Siberian stonechats breed later, moult faster and migrate further than European stonechats. We tested hypotheses for seasonal timing mechanisms by comparing the birds under constant and naturally changing daylengths. The taxa retained characteristic reproductive and moult schedules and hybrids behaved roughly intermediately. Based on their distinct circannual cycles, we expected European and Siberian stonechats to differ in photoperiodic responses at a given time of year. We found that the taxa responded, as predicted, in opposite ways to photoperiodic simulations as experienced on different migration routes. The findings indicate that circannual rhythms reflect geographically distinct periodic changes in seasonal disposition and cue-response mechanisms. Under natural daylengths, the phase relationship of the underlying circannual rhythm to the external year determines the action of photoperiod. Circannual rhythms are widespread among long-lived species. Accordingly, responses to environmental change, range expansion and novel migration patterns may depend on the particulars of a species' underlying circannual programming.

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INTRODUCTION

Most environments are to some extent seasonal. Their inhabitants time annual activities like reproduction, germination, pupation, moult, hibernation or migration to match the changing seasons and often anticipate conducive conditions well in advance. Seasonal activities must be accurately timed because mismatches with the environment can have severe fitness consequences; however, the particular timing differs considerably with species ecology, environmental seasonality and year-to-year conditions (Lack, 1950; Bradshaw and Holzapfel, 2007; Newton, 2008; Foster and Kreitzman, 2009). Understanding the intricacies of timing has become of urgent interest in view of the disconcerting rate of global change and its implications for seasonality (Bradshaw and Holzapfel, 2006; Bradshaw and Holzapfel, 2007; Visser, 2008). Birds, as a highly visible group, are regarded as sentinels of change and their scheduling is particularly well documented (e.g. Jonzén et al., 2007; McNamara and Houston, 2008; Wingfield et al., 2008).

Avian itineraries differ between species in a given habitat and often also within species, depending on geographical location. With increasing latitude, birds generally have shorter reproductive periods, breed later and moult faster than at lower latitudes. Schedules also differ between habitats at given latitudes, e.g. associated with altitude, urbanisation and climatic gradients (Baker, 1938; Lack, 1950; Curry-Lindhal, 1963; Klein, 1974; Murton and Westwood, 1977; Widmer, 1998; Partecke et al., 2004; Perfito et al., 2004; Moore et al., 2005; Partecke et al., 2005; Newton, 2008). Hence, closely related taxa and populations may show distinct timing.

The basis of these distinct schedules is still largely unclear. In general, timing involves an inherited background mechanism that

provides a temporal framework. It buffers organisms from misleading information, e.g. warm spells in winter, while integrating reliable, predictive, temporal cues – predominantly photoperiod (i.e. the light fraction of the day) (Murton and Westwood, 1977; Gwinner, 1986; Prendergast et al., 2002; Bradshaw and Holzapfel, 2007). Within this framework, schedules may be further modified by environmental conditions, for instance, temperature or food availability (Wingfield, 1980; Hahn et al., 1992; Hahn et al., 1997; Dawson, 2008). The resulting compromise between rigid pre-programmed timing and environmental flexibility should accommodate the requirements of a given species in its given habitat.

Geographical differences could therefore arise from phenotypic plasticity in response to local conditions (Perfito et al., 2004; Dawson, 2008; Hahn and MacDougall-Shackleton, 2008). Yet direct environmental control is an insufficient explanation for the scheduling of most seasonal species studied so far. Timing in captivity approximated timing in the wild (Davies et al., 1969; König and Gwinner, 1995; Lambrechts et al., 1997; Lambrechts et al., 1999; Partecke et al., 2004) [but see Perfito et al. (Perfito et al., 2004; Perfito et al., 2008)], indicating the presence of internal timing programs. These programs involve photoperiod as an accurate external calendar but because seasonal implications of a given daylength depend on locality, timing responses must be tuned to a population's particular situation. Responses to daylength depend on the phase of the annual cycle. Activities are typically accelerated by long days in spring and by short days in autumn (Hahn et al., 1997; Gwinner and Helm, 2003; Sharp, 2005). Birds are able to respond to minute long photoperiodic signals, for instance, enhanced

reproductive growth following a 17-minute increase in daylength (Hau et al., 1998). Yet despite marked daylength effects on timing, many birds and other organisms do not require photoperiodic change to drive their annual cycles, as rhythmicity persists under constant conditions. Marked individual variation, period lengths close to but deviating from 12 months and consequently drift of seasonal timing from the external year reveal the existence of underlying, circannual rhythms (Gwinner, 1986; Holberton and Able, 1992; Gwinner, 1996; Prendergast et al., 2002; Goldman et al., 2004; Helm, 2006; Lincoln et al., 2006; Piersma et al., 2008). Daylength nonetheless plays an important role as a synchronising cue (zeitgeber) for circannual rhythms, entraining the otherwise free-running cycles of individuals to the external photoperiodic calendar. Under natural conditions, cycles are therefore precise and the period is 12 months.

Accordingly, geographical differences in schedules could also be based on modified photoperiodic responses, on different circannual organisation or on interactions between them. Distinct photoperiodic responses, usually interpreted as thresholds, have been described in several species without explicitly addressing circannual programming (Curry-Lindhal, 1963; Murton and Westwood, 1977; Silverin et al., 1993; Lambrechts et al., 1997; Noskov et al., 1999; Helm et al., 2005; Sharp, 2005; Hahn and MacDougall-Shackleton, 2008; Silverin et al., 2008). Likewise, related taxa have differed in circannual rhythms but implications for timing under natural photoperiods remained largely unclear (Gwinner, 1996; Dawson, 2007; Wikelski et al., 2008). The lack of integration of photoperiodic and circannual approaches is regrettable because together both aspects of seasonal timing represent a functional entity (Gwinner, 1996; Bradshaw and Holzapfel, 2007; Paul et al., 2008). Their joint contributions to timing in the 'real world' (Menaker, 2006) can be identified by simultaneous study of circannual rhythms and photoperiodism in species with distinct schedules.

We investigated seasonal timing in the stonechat (*Saxicola torquata* Linnaeus), a songbird whose vast breeding range from Siberia to southern Africa (Underhill, 1999; Urquhart, 2002) covers diverse climatic and photoperiodic conditions. We have previously shown that stonechats of European, Siberian and African origin differed conspicuously in the timing of postjuvenile moult and migratory restlessness and in the way these behaviours were affected by photoperiod (Helm and Gwinner, 1999; Helm et al., 2005; Helm and Gwinner, 2006). Stonechat seasonal activities are partly under circannual control, and crossbreeding revealed genetic differences in postjuvenile moult (Gwinner and Dittami, 1990; Gwinner, 1996; Helm, 2006). The present study examines reproductive and moult cycles in European and Siberian stonechats. The closely related taxa (Cramp and Simmons, 1988; Illera et al., 2008) breed at similar latitudes but differ in seasonal behaviour and migratory habit (Raess and Gwinner, 2005; Helm et al., 2006a; Flinks et al., 2008; Raess, 2008). Central European stonechats (*S. t. rubicola*) are short-distance migrants. They spend over six months, from March until October, on the breeding grounds where they raise several clutches and undergo postnuptial moult. Siberian stonechats from Kazakhstan (*S. t. maura*), by contrast, are long-distance migrants visiting their breeding grounds only from May until August. Under a more continental climate, they raise a single brood followed by rapid moult. Due to these itineraries, the birds experience different daylengths outside, but not during, the breeding period (Fig. 1). Days are longer for Siberian than for European stonechats on the winter quarters but from the spring equinox onwards (ca. 21 March), days are shorter for Siberian stonechats until both taxa have reached the breeding grounds.

These differences in schedules and daylength during particular phases of the annual cycle can be exploited to test hypotheses for

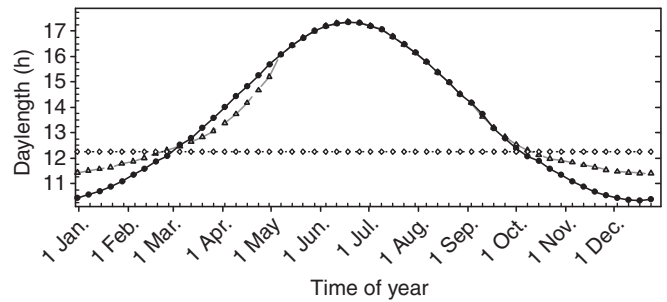


Fig. 1. Photoperiodic simulations used in the present study. Grey triangles and broken line gives daylength simulating the photoperiodic conditions experienced by Siberian stonechats during breeding, migration and wintering. Black closed circles and solid line show conditions of European stonechats, and open diamonds and dotted line indicate constant conditions used for circannual studies.

underlying timing mechanisms. To do so, we investigated cycles of reproductive-organ size, plasma testosterone and moult in first-year stonechats by a suite of experiments. We studied the persistence of schedules under native conditions and compared Siberian and European stonechats and F1 hybrids under identical natural daylength (common-garden conditions). We examined circannual cycles under constant conditions and exposed Siberian and European stonechats to the daylengths they would experience if they adopted each other's migratory behaviour. We tested the following hypotheses and predictions: (1) Siberian and European stonechats have identical timing programs but schedules differ due to phenotypic plasticity in different environments. Under common-garden conditions, both taxa and their hybrids should behave identically. (2) Timing could be based on distinct photoperiodic responses. If the taxa differed in daylength thresholds, characteristic schedules should not persist under constant conditions and timing should be advanced or delayed by different photoperiods. (3) The distinct schedules could be based on circannual rhythms, i.e. different endogenous patterns which, during this phase of the annual cycle, could be largely photoperiod-independent. If so, both taxa should retain their respective time patterns under constant and simulated natural photoperiods. Effects of photoperiod should be negligible and, if present, similar in both taxa. (4) Distinct schedules of the stonechat taxa could result from differences in the way their respective circannual rhythms interact with the zeitgeber. In this case, the taxa should maintain basic time patterns under constant conditions. When exposed to different photoperiods, both taxa should modify their schedules in ways expected from circannual characteristics.

MATERIALS AND METHODS

From 1998 to 2004, we studied seasonal activities of 116 Siberian stonechats, European stonechats and F1 hybrids (Table 1) at the Max Planck Institute for Ornithology in Andechs, Germany. Siberian stonechats were collected as nestlings in 1997 and 1998 near Naursum National Park (51 deg.N, 63 deg.E) south of Kustanaj, Kazakhstan. European stonechats were repeatedly collected as nestlings in Lower Austria (48 deg.N, 16 deg.E). Hybrids and some birds of both parental taxa were offspring of pairs that bred in aviaries. Hybrids were derived from three European mothers (three males, five females) and three Siberian mothers (three males, three females). All were hand-raised from an age of 5 to 8 days as described elsewhere (Gwinner et al., 1987). After independence birds were moved to individual 60×40×40 cm (L×H×W) cages in

Table 1. Sample sizes of male and female stonechats kept under different photoperiods

		European stonechats		Siberian stonechats		F1 hybrids	
		Male	Female	Male	Female	Male	Female
European PP	Gonads and moult	15 (7)	7 (3)	8	4	–	–
	Hormones	11 (7)	–	8	–	–	–
Siberian PP	Gonads and moult	7	–	31 (15)	23 (7)	6	8
	Hormones	7	–	11 (7)	–	6	–
Constant	Gonads	(11)	–	(6)	–	–	–

Values in parentheses indicate the number of birds kept over two years. Hormones were investigated in a subset of the experimental birds. PP, photoperiods.

temperature-controlled chambers at $20\pm 3^\circ\text{C}$. Food and water were provided daily *ad libitum*.

For the simulation of photoperiodic conditions, the time interval between the onset of civil twilight in the morning and the end of civil twilight in the evening was used, assuming that the daylength effective for photoperiodic reactions corresponds closely to this interval. Light during daytime was provided by fluorescent tubes at an intensity of about 300 lx at the level of the perches. At night, incandescent light bulbs produced an intensity of about 0.01 lx at perch level.

Birds were exposed to simulated local daylength (47.5 deg.N) during the breeding season. In autumn, with the onset of natural migration, we split broods and exposed birds to the photoperiods experienced during wintering and migration by either taxon (Fig. 1). Siberian stonechats winter in south-central Asia, at latitudes of *ca.* 15–30 deg.N (Raess and Gwinner, 2005). Thus, the photoperiodic simulation was adjusted at weekly intervals assuming that they reach 45 deg. on 8 Sept., 42.5 deg. on 15 Sept., 40.0 deg. on 22 Sept., 37.5 deg. on 29 Sept., 35 deg. on 6 Oct., 32.5 deg. on 13 Oct., 30 deg. on 20 Oct., 27.5 deg. on 27 Oct. and 25 deg. on 3 Nov. Correspondingly, daylength during spring migration was simulated, assuming that birds move north to 27.5 deg. on 10 March, 30 deg. on 17 March, 32.5 deg. on 24 March, 35 deg. on 31 March, 37.5 deg. on 7 April, 40 deg. on 14 April, 42.5 deg. on 21 April, 45 deg. on 28 April and 47.5 deg. on 5 May. Most Central European stonechats winter in the Mediterranean area at latitudes from *ca.* 32.5–42.5 deg.N (Helm et al., 2006a). Thus, daylength was adjusted assuming that they reach 45 deg. on 4 Oct., 42.5 deg. on 11 Oct. and 40 deg. on 18 Oct. In spring, birds were assumed to reach 42.5 deg. on 28 Feb., 45 deg. on 7 March and 47.5 deg. on 14 March. Persistence of schedules was tested in some birds kept under these conditions for two years. For all others, the experiment ended after a year with the completion of moult. To examine underlying circannual rhythms, an additional group of six Siberian males was exposed to constant daylength (12.25 h:11.75 h L:D) for two years from either before hatching or within the first three weeks of life. For comparison, we used data on European stonechats from a previously published study (Gwinner, 1991). We extracted testes size of the 11 males with complete two-year records from the original protocols. As birds differed in hatching dates (European stonechats: 27 April to 13 July; Siberian stonechats: early June) and received no external time cues subsequently, we plotted all circannual data with reference to age, rounding days of life to months.

Reproductive cycles were determined in two ways. For a direct measure of reproductive organs, birds were laparotomised every 3–6 weeks (Gwinner, 1975). Measurements began before birds came into breeding condition and were discontinued after gonadal regression. During the remaining time, only the birds under constant conditions were laparotomised in monthly or bimonthly intervals. We collected far more information on male stonechats than female

stonechats. This is because in captivity, full follicular maturation occurs only in few females and reproductive cycles are accordingly variable. We nonetheless included females to explore whether patterns were generally similar to those observed in males. Testicular width or the diameter of the largest follicle were determined to the nearest 0.1 mm. For a subset of 43 males (Table 1), plasma levels of testosterone were also determined. Blood samples were taken in parallel to gonadal measurements by puncturing the alar vein using a 23-gauge needle. Blood (150–200 μl) was collected into heparinised microcapillaries, immediately centrifuged for 10 min and the plasma was stored at -70°C . Moult was checked at weekly intervals under simulated natural daylengths only. Moult onset and completion refer to the replacement of the nine fully developed primaries and thus to the, presumably, most vulnerable core part of plumage change. Onset was defined as the mean date between the last recording with intact primaries and the first recording with moult, and completion was defined as the date between the last recording with moult and the first recording without moult.

Testosterone concentrations were measured by radioimmunoassay after partial purification on diatomaceous earth/glycol columns following Goymann et al. (Goymann et al., 2006). A total of 480 samples were analysed in seven assays. The lower detection limit of the standard curves was determined as the first value outside the 95% confidence intervals for the zero standard (B_{max}) and was on average 0.7 pg tube^{-1} (range $0.6\text{--}0.8 \text{ pg tube}^{-1}$). Non-detectable values were assumed to be equivalent to the lower detection limits; thus, giving a conservative estimate of hormone levels. The intra-assay variation was 6.1% on average (range: 2.7–12.5%) and the inter-assay variation was 9.1%. Individual recoveries after column separation were calculated as percentage activity eluted from the columns of total activity added prior to extraction and column chromatography. Mean (\pm s.d.) recovery for testosterone was $70\pm 5\%$. Hormone concentrations were log-transformed to match a normal distribution and back-transformed for graphical presentation.

Testosterone levels and size of testes and follicles were analysed by repeated-measure, linear mixed-models, accounting for a first-order autoregressive structure of the data. For moult, we used linear mixed-models over both sexes (Genstat, VSN International, Hemel Hempstead, UK). The main models accounted for effects of age, taxon, sex, photoperiod, time of year and all interactions. In one case, model assumptions were not completely met and analyses were repeated over ranked data for conservative reassessment. Figures show median \pm s.e.median (Sokal and Rohlf, 1995).

All experimental procedures conformed to the relevant regulatory standards under permit by the state of Upper Bavaria.

RESULTS

Birds under native conditions

European and Siberian stonechats showed distinct reproductive and moult cycles under native photoperiodic conditions over two years

(Fig. 2). Testicular cycles of males differed significantly (Wald₆=262.6; $P<0.001$). Similar to that in the field, gonadal growth started much earlier in European than Siberian males. European males had larger testes than Siberian males from January until early April (all $P<0.001$) and, thereafter, testicular sizes were similar, although testicular regression tended to set in earlier in Siberian than European birds (Fig. 2) (late May: $P=0.274$; June: $P=0.080$). Consequently, reproductive condition lasted longer in European than Siberian males. In addition, testes of both taxa were larger (Wald₁=30.0; $P<0.001$) and tended to start to grow earlier in the second year (Wald₆=12.6; $P=0.051$). Similarly, plasma testosterone cycles (Fig. 2) of Siberian and European males were clearly distinct (Wald₆=60.8; $P<0.001$). In February ($P<0.001$) and tentatively in early April ($P=0.082$), testosterone was higher in European males but in early May, was higher in Siberian males ($P<0.001$). Testosterone levels were similarly low in both taxa in January and from late May onward. If judged by testosterone levels, reproductive cycles also started earlier and were possibly slightly more drawn out in European than Siberian males. In contrast to testis size,

testosterone levels were higher in the first year than the second year (Fig. 2) (Wald₁=6.7; $P=0.01$) but were not timed differently (Wald₅=6.4; $P=0.266$).

In females (Fig. 2), follicular cycles showed similar differences between the taxa (Wald₆=19.1; $P=0.004$), with an earlier onset and longer maintenance of breeding condition in European stonechats. European females had larger follicles from January until early May (all $P<0.05$) and, thereafter, follicular sizes were similar. Follicles were larger in second year than first year birds (Wald₁=14.2; $P<0.001$). These differences depended on time of year (Wald₅=35.5; $P<0.001$), as follicles of older females were larger in early and late May only.

Data on moult (Fig. 2) were analysed jointly for the sexes. As in free-living birds, Siberian stonechats initiated moult earlier (Wald₁=21.9; $P<0.001$), moulted more rapidly (Wald₁=244.0; $P<0.001$) and completed moult much before European stonechats (Wald₁=275.6; $P<0.001$). Females started moult after males (Wald₁=30.9; $P<0.001$) but moulted slightly faster (Wald₁=5.9; $P=0.015$) and thus finished at similar times (Wald₁=2.7; $P=0.104$).

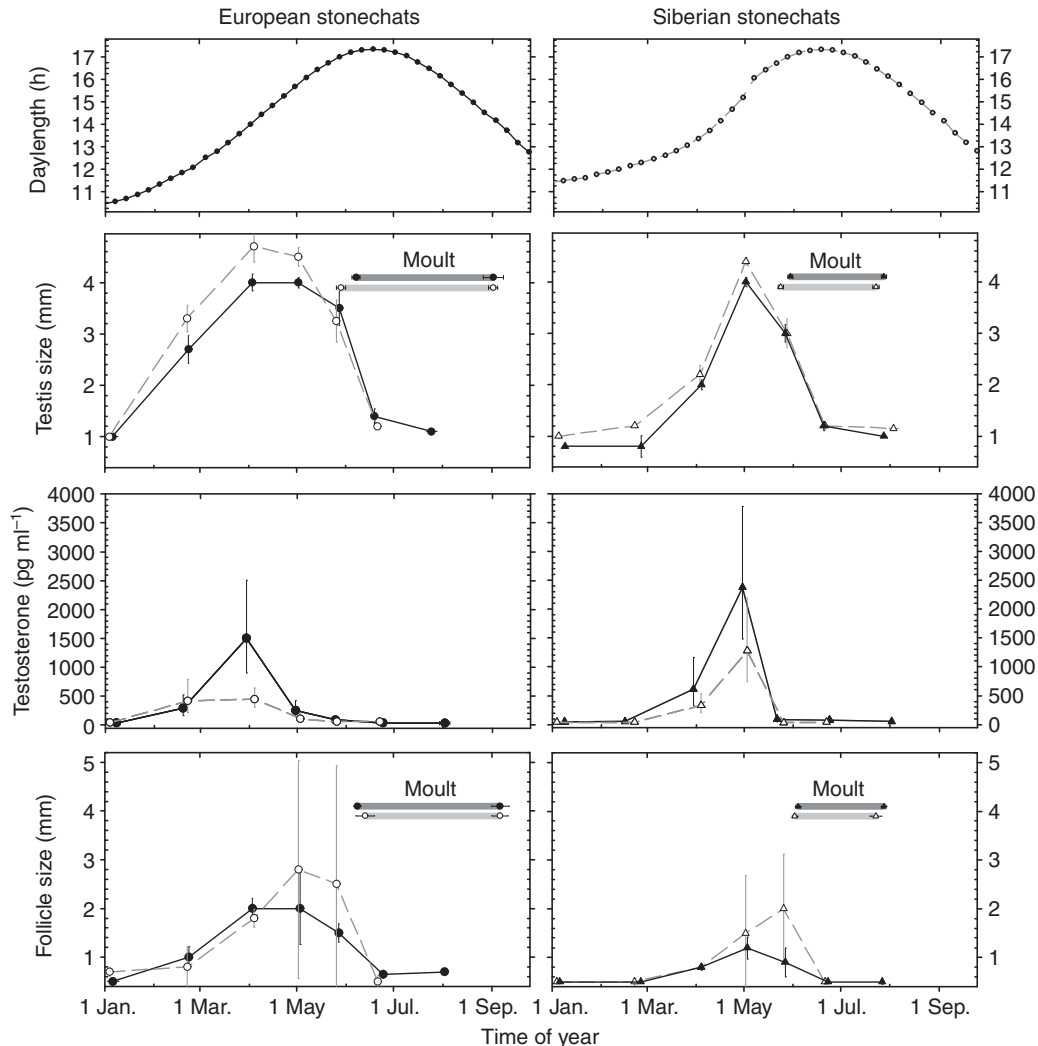


Fig. 2. Reproductive and moult cycles of stonechats under native photoperiodic conditions separated by age class. Age is indicated by black closed symbols and solid lines for yearlings, and white open symbols and broken lines for second-year birds. European stonechats (left column, circles) and Siberian stonechats (right column, triangles) were kept under their respective native photoperiods for two years. The curves show weekly daylength (top panel), monthly medians \pm s.e. median of male testis size and plasma testosterone (middle panels) and follicular size of females (bottom panel). Horizontal bars connect the median \pm s.e. median dates of onset and end of primary moult.

Moult was also somewhat influenced by age. Among birds observed over two years, moult started earlier (Wald₁=4.12; $P=0.042$) and tended to proceed more rapidly (Wald₁=3.0; $P=0.082$) and end earlier (Wald₁=3.7; $P=0.054$) in the second year in both sexes.

Hybrid stonechats and parental taxa in a common-garden setup

To assess genetic determination, we kept F1 hybrids and the parental taxa for one year under common Siberian-type daylength (Fig. 3). Hybrids originating from European and Siberian mothers did not differ in gonadal and moult cycles (Fig. 3) (inlays; gonadal cycles males: Wald₅=3.9; $P=0.56$; gonadal cycles females: Wald₅=7.3; $P=0.199$; moult: all $P>0.46$) and were thus pooled. Hybrid males differed in testicular cycles from those of Siberian (Fig. 3; Wald₅=52.2; $P<0.001$) as well as European stonechats (Wald₅=55.6; $P<0.001$). The differences were not constant over time. Hybrid testes were smaller than those of European males from January until early

April and again in late May and June ($P<0.002$) but larger than those of Siberian males in February and early April ($P<0.001$). Therefore, hybrid testicular cycles were intermediate between the parental taxa during the gonadal growth phase but, during gonadal regression, resembled those of Siberian males. Testosterone concentrations (Fig. 3) increased first in European males, forming a drawn-out shallow peak from late February to early April. Plasma levels peaked in hybrids in early April and in Siberian males in early May. The peak in Siberian males was short and sharp. Testosterone cycles of hybrids and both parental taxa differed significantly (Wald₁₀=26.8; $P=0.003$) but *post-hoc* comparisons were obscured by small sample sizes and striking differences in amplitude. Testosterone cycles differed between hybrid, European (Wald₅=12.7; $P<0.026$) and tentatively Siberian males (Wald₅=10.7; $P=0.058$). Follicular cycles were measured in hybrid and Siberian females only (Fig. 3) and, as in males, differed significantly (Wald=12.3; d.f.=5; $P=0.031$). Follicles developed earlier in hybrids (April: $P=0.002$) but were similar during the remaining period.

Moult onset (Fig. 3) (Wald₂=15.7; $P<0.001$), duration (Wald₂=163.5; $P<0.001$) and completion (Wald₂=353.8; $P<0.001$) differed significantly between hybrids and the parental taxa. Molt completion and duration in hybrids were intermediate and significantly distinct from both parental taxa (all $P<0.001$). Its onset, by contrast, was not intermediate but occurred at the same time as in Siberian stonechats (Wald₁=0.1; $P=0.801$) but much earlier than in European stonechats (Wald₁=7.1; $P=0.003$). This unexpected finding was consistent in both sexes. Overall, females began moult after males (Wald₁=18.8; $P<0.001$) but moulted more rapidly (Wald₁=9.3; $P=0.002$) and therefore finished simultaneously (Wald₁=0.86; $P=0.355$).

Circannual cycles

Testicular cycles of Siberian males kept for two years under constant conditions are shown in Fig. 4 next to replotted data of European males from a previously published study (Gwinner, 1991). Reproductive cycles of the two taxa were clearly distinct (Wald₄=45.5; $P<0.001$). As under synchronising conditions (Figs 2 and 3), gonadal growth occurred earlier in European than Siberian males (Fig. 4). However, median testicular growth of Siberian stonechats was delayed in the first year and partly overlapped with regression in European stonechats. This and the relatively long duration of the first testicular cycle were due to partial gonadal activation prior to full testicular development in several Siberian males but only exceptional European males (Fig. 4). Based on the differences in spontaneous testicular growth, we predicted concomitant differences in photoperiodic response. Since European stonechats entered reproductive condition earlier in the year, we expected them also to respond to photostimulation by long days earlier than Siberian stonechats. Hence, we predicted that the slightly longer days in winter experienced under Siberian-type long-distance migration should stimulate gonadal development of European but not Siberian stonechats.

Photoperiodic contribution: both taxa under reciprocal conditions

Fig. 5 summarises the results of experiments in which both taxa were held under native as well as reciprocal photoperiodic conditions in the first year of life. Testicular cycles depended strongly on taxon (Wald₅=213.8; $P<0.001$; recalculated over ranks: Wald₅=259.8; $P<0.001$) but daylength was also influential (Wald₆=143.0; $P<0.001$; ranks: Wald₆=134.7; $P<0.001$). As predicted, photoperiodic effects (Fig. 5) differed between taxa (Wald₅=21.2; $P<0.001$; ranks: Wald₅=15.8; $P=0.007$). Their daylength responses differed

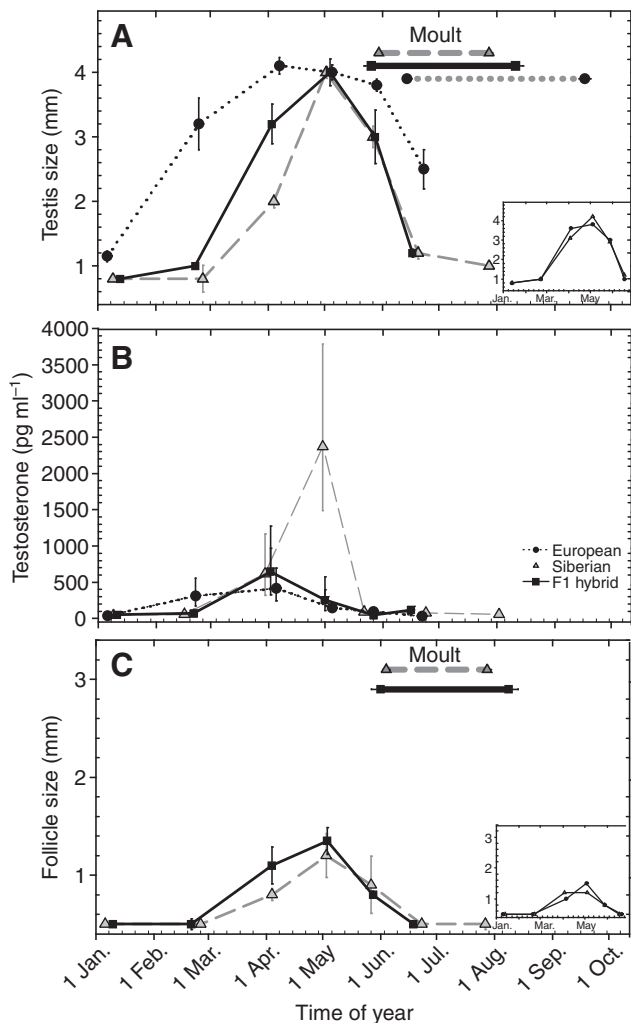


Fig. 3. Breeding and moult schedules of F1 hybrids and parental stonechat taxa under identical conditions. Birds were kept under the photoperiod of Siberian-type long-distance migration. Data as in Fig. 2. (A) Testicular cycle and moult (horizontal bars) in males; (B) male plasma testosterone; (C) follicular cycles and moult in females. Hybrids: solid dark lines and black closed squares; Siberian stonechats: broken grey lines and triangles; European stonechats: dotted lines and black closed circles. Inlays: gonadal cycles of F1 hybrid offspring of European (black closed circles) and Siberian (grey triangles) mothers.

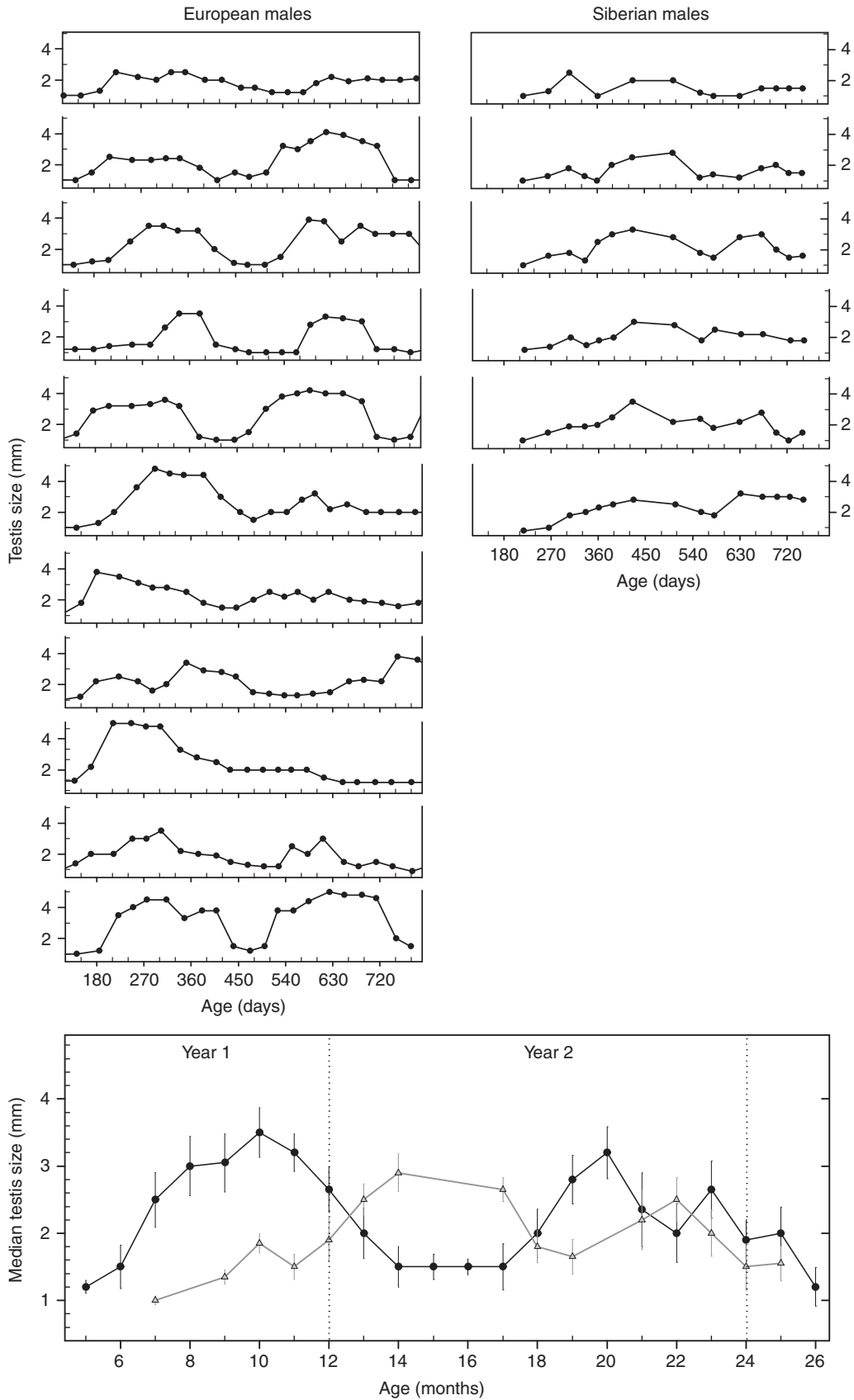


Fig. 4. Circannual reproductive cycles of European and Siberian stonechats. Birds were kept under a constant 12.25 h photoperiod for at least two years. Upper graphs show circannual testicular cycles of individual European males (left) and Siberian (right) males. Lower graph shows median cycles of European (solid black line and black closed circles) and Siberian (grey line and grey triangles) males; years are shown by vertical dotted lines. Data for European stonechats are replotted based on published data (Gwinner, 1991).

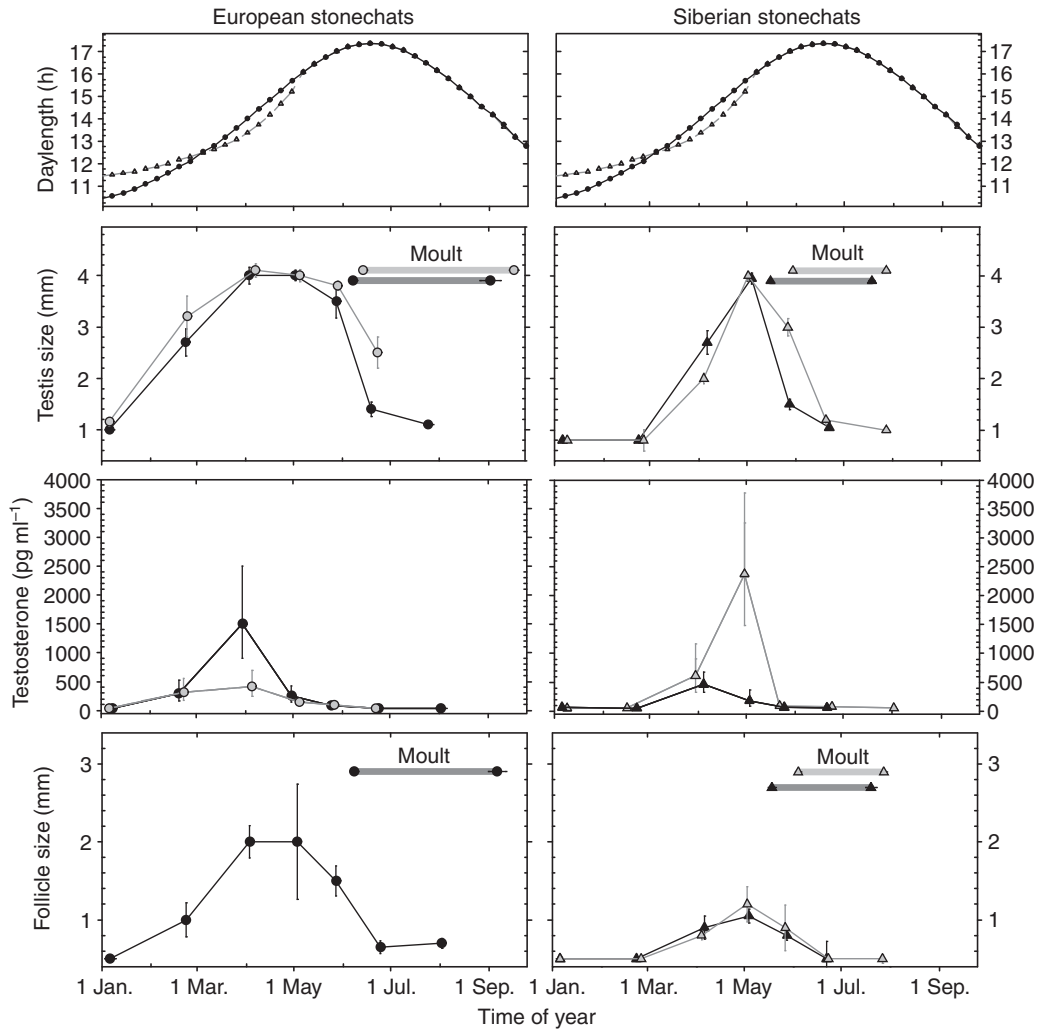


Fig. 5. Schedules of European and Siberian stonechats under native and reciprocal photoperiods. Data as in Fig. 2. Top panel shows the simulation of daylength experienced by European (circles) and Siberian (triangles) stonechats. Middle panels show cycles of testis size, moult and plasma testosterone of males, bottom panel shows follicular and moult cycles of females for European stonechats (left column) and Siberian stonechats (right column); the two photoperiodic treatments to which both taxa were exposed are indicated by different shading; grey shading shows scheduling under Siberian-type conditions and black shading under European-type conditions.

significantly in January ($P < 0.001$), early April ($P = 0.003$) and June ($P = 0.034$). In European males, testes were slightly, but consistently, larger under the conditions of Siberian-type long-distance migration in January. In February, a similar tendency (Fig. 5) was not significant ($P = 0.171$) in view of high inter-individual variation during the maximal growth phase. Testes of European males were fully developed in early April and early May under both photoperiods but in late May and June, were again larger under simulated Siberian-type long-distance migration. Conversely, in Siberian males, photoperiod affected testicular size in early April, at which time testes were larger under European-type short-distance conditions. In early May, testes of Siberian males were fully developed under both photoperiodic treatments, in late May, were larger after Siberian-type long-distance migration and in June, were regressed under both conditions. Thus, testes of Siberian males grew and regressed earlier under European-type short-distance migration whereas testes of European males grew earlier and regressed later under Siberian-type long-distance migration (Fig. 5). Plasma testosterone levels (Fig. 5) were affected by taxon ($\text{Wald}_5 = 29.6$; $P < 0.001$) and photoperiod ($\text{Wald}_6 = 9.5$; $P = 0.002$) but timing in

response to photoperiod did not differ between Siberian and European stonechats ($\text{Wald}_6 = 7.2$; $P = 0.203$). Hormone patterns were dominated by marked differences in testosterone peaks. Males of both taxa had much higher peak testosterone concentrations under native than reciprocal conditions ($\text{Wald}_7 = 9.5$; $P = 0.002$).

In females (Fig. 5), follicular cycles also depended on taxon ($\text{Wald}_5 = 16.0$; $P = 0.007$). As no data were available for European stonechats under Siberian-type photoperiod, daylength effects were examined in Siberian females only and were not significant ($\text{Wald}_5 = 5.3$; $P = 0.381$). Follicles of the taxa differed under identical (European-type) conditions ($\text{Wald}_1 = 16.9$; $P < 0.001$) and were larger in European than Siberian females from February until late May (all $P < 0.01$).

Data on moult timing (Fig. 5) corroborated the patterns observed above. The taxa differed in moult onset ($\text{Wald}_1 = 46.0$; $P < 0.001$), completion ($\text{Wald}_1 = 345.4$; $P < 0.001$) and duration ($\text{Wald}_1 = 254.9$; $P < 0.001$), with earlier and more rapid moult in Siberian than European stonechats. Photoperiod also affected flight feather change. Moult started ($\text{Wald}_1 = 39.2$; $P < 0.001$) and ended ($\text{Wald}_1 = 18.6$; $P < 0.001$) earlier under simulated European-type short-distance

migration but took similarly long ($Wald_1=0.4$; $P=0.527$). The advancement of moult onset under European photoperiod was slightly larger in Siberian than European stonechats ($Wald_1=4.6$; $P=0.033$). Moult under European conditions progressed more slowly in Siberian stonechats but more quickly in European stonechats (interaction taxon and photoperiod: $Wald_1=5.2$; $P=0.023$). Sex influenced moult onset ($Wald_1=12.9$; $P<0.001$) and duration ($Wald_1=6.2$; $P=0.012$) but not completion. Overall, females commenced moult slightly later than males but moulted faster, especially Siberian females under native conditions (Fig. 5).

DISCUSSION

The findings of our present study give clear answers to the initial questions. Under all conditions, including a common-garden setup, Siberian and European stonechats retained distinct schedules. European stonechats initiated reproductive development much earlier and were in moult and breeding condition longer than Siberian stonechats. We can thus reject hypothesis (1), stating that taxon differences are exclusively due to external conditions. Likewise, counter to hypothesis (2), advocating daylength thresholds as the basis of timing differences, distinct schedules persisted under constant conditions and revealed a circannual basis. However, daylength in winter and spring had modifying effects on reproductive and moult timing, contradicting hypothesis (3) that schedules are exclusively determined by circannual rhythms. Responses to daylength differed between the taxa and over the annual cycle, in accordance with expectations based on circannual characteristics and can be explained by phase-specific action of photoperiod. Hence, our data support hypothesis (4), stating that the taxon-specific schedules result from different circannual programs and their interactions with photoperiod.

These findings can be readily integrated with the current understanding of avian timing. The response to any given daylength depends on the precise phase of the annual or circannual cycle. Exposure to lengthening photoperiod prior to breeding can advance reproductive activation but most species do not retain breeding condition indefinitely in long photoperiods. The phenomenon whereby reproductive condition is terminated on long days and usually at first is not even restimulated by constant light (Hahn et al., 1997; Hahn and MacDougall-Shackleton, 2008), has been termed 'photo-refractoriness', but might equally be considered to be the expression of an underlying circannual oscillator. Photoperiod continues to be read by birds and other organisms, as evident from clock gene expression patterns and simultaneous action of photoperiod on moult, for example (Jenni and Winkler, 1994; Noskov et al., 1999; Lincoln et al., 2005). Shortening days during this phase accelerate post-breeding processes. Some species can regain reproductive competence under long days [16 or more hours of light (Hamner and Stocking, 1970; Berthold et al., 1972; Wingfield, 1993)] but others require a period of short days to allow a subsequent response to long days to occur. The requirement for short days can be termed 'the breaking of refractoriness' but equally might be considered to be an advance of the underlying circannual system, which in some species is obligatory (Gwinner, 1986; Sharp, 2005; Dawson, 2007). After moult, short days may still accelerate the dissipation of reproductive inhibition whereas long days become increasingly efficient at reactivating the reproductive system (Murton and Westwood, 1977; Farner, 1985; Gwinner, 1988; Gwinner, 1996; Hahn et al., 1997).

The transition between accelerating and delaying effects of a given daylength is crucial for the timing of the annual cycle. For instance, short days in winter have been associated with advanced breeding schedules in some species. When starlings (*Sturnus*

vulgaris) were kept under different natural photoperiods, those exposed to the shortest winter days (*ca.* 6 h at 67.5 deg.N) were the first to reinitiate reproductive processes in December (Gwinner, 1986). Without exposure to short daylength, starlings remain arrested in the inactive phase of their circannual cycle and do not re-initiate gonadal growth (Gwinner, 1986; Dawson, 2007). Conversely, in long-distance migrants, reproductive inhibition is dissipated gradually and spontaneously under constant photoperiods (Engels, 1969; Gwinner, 1988; Gwinner et al., 1988). Garden warblers (*Sylvia borin*) were kept in 12.8 h days and transferred to 15 h days between November and April. The gonadal response to photostimulation increased gradually and was fully developed in early April. At this time, some birds were just starting spontaneous, circannual reproductive activation. Accordingly, evolutionary modification of reproductive inhibition and of the photoperiodic conditions leading to its termination have been repeatedly invoked as a way in which annual cycles can be adjusted to particular environments (Lofts and Murton, 1968; Hamner and Stocking, 1970; Murton and Westwood, 1977; Gwinner, 1988; Gwinner, 1989; Gwinner, 1996; Hahn et al., 1997; Sharp, 2005). The evolutionary flexibility of such modifications was recently documented in an enlightening review that differentiated between characteristics of 'photo-refractoriness' and found them to vary independently in an extensive dataset on finches and other passerines (Hahn and MacDougall-Shackleton, 2008).

In the stonechats, geographical differences in reproductive timing were hard-wired in distinct circannual programs (Fig. 4). European stonechats started spontaneous, circannual testis growth between November and February, at ages of *ca.* 6 months (Gwinner, 1991). Correspondingly, under natural daylengths, testes developed in early winter (Figs 2 and 5), and responsiveness of the reproductive system to constant light was restored in November (Gwinner and Scheuerlein, 1999). By contrast, in Siberian stonechats, spontaneous, circannual gonadal growth was first recorded in early April (at ages of *ca.* 9–10 months; Fig. 4). The timing coincided with reproductive growth under natural photoperiods (Figs 2 and 5) and occurred at similar times as in other long-distance migrants for which delayed photo-responsiveness has been reported (Engels, 1969; Hamner and Stocking, 1970; Gwinner, 1988; Gwinner, 1989). In the stonechats, the circannual differences in reproductive activation determined whether a given, naturally experienced photoperiod had advancing or delaying effects. In European stonechats, testis growth was slightly advanced under longer-day Siberian-type conditions as early as in January (Fig. 5). Siberian stonechats, by contrast, experienced these winter daylengths during a phase when short, and not long, photoperiods accelerated the annual cycle. Testicular growth was advanced under shorter-day European-type conditions by early April, and testosterone peaked in late March. After the spring equinox (Fig. 1), the progressively longer days may have further accelerated schedules.

Stonechats also modified the termination of breeding in response to photoperiod. Both taxa delayed gonadal regression and moult after simulated Siberian-type long-distance migration (Fig. 5). We have shown in an earlier study that even after identical winter conditions, a slower increase in spring daylength alone (Fig. S1 in supplementary material) delayed post-breeding itineraries of European stonechats (Helm and Gwinner, 2005). After complete gonadal growth, one group of males was continued under native conditions and a second group exposed to the more slowly lengthening spring days of Siberian-type long-distance migration (Fig. S1 in supplementary material). The Siberian-type group delayed all subsequent processes until autumn migration, indicating a shift of the annual cycle and no further synchronisation until autumn (Fig. S1 in supplementary material).

Likewise in the present study, European birds under Siberian long-distance conditions delayed summer and autumn processes but since they had already advanced gonadal growth, their breeding cycle was lengthened (Fig. 5). This finding suggests that a separate photoperiodic response at the time of spring arrival adjusts the termination of the breeding season, possibly securing sufficient time for breeding. Sharp and Blache (Sharp and Blache, 2003) have observed that two hormones, LH (luteinizing hormone) and prolactin, respectively, may differ in their responses to photostimulation and thereby be implicated in defining the opening and closing of the reproductive window (Sharp, 2005). Accordingly, geographical differences in the timing and length of the breeding season, as observed in the stonechats, could be linked to differential photoresponsiveness of LH and prolactin secretion, respectively, and this possibility merits further testing. Siberian stonechats also delayed post-breeding activities under native conditions. Yet due to the timing of their breeding window, we cannot distinguish whether this delay was due to longer winter or shorter spring daylength. Testosterone patterns under the different photoperiods were inconclusive, mostly because of striking attenuation of peak concentrations in both taxa under non-native conditions (Fig. 5).

Non-photoperiodic factors contributed to seasonal timing. Captive stonechats retained distinct schedules but started and ended reproductive cycles earlier than in the field. Advanced breeding in captivity occurs in many, but not all, species and may be related to temperature, food availability and illumination (e.g. Davies et al., 1969; Partecke et al., 2004; Perfito et al., 2004; Silverin et al., 2008). Stonechat moult began earlier in captivity but took longer and thus ended at the same time as in the field (Raess, 2005; Raess and Gwinner, 2005; Flinks et al., 2008). Age had considerable effects on testicular growth (Fig. 2). Testicular cycles started earlier in the second year, a pattern reported in many but not all wild and captive birds (Deville et al., 2000; Dawson, 2003; Partecke et al., 2004). In the wild, additional factors influence reproductive timing, for instance social interactions, temperature, food availability and vegetational change (Wingfield, 1980; Hahn et al., 1997; Perfito et al., 2004; O'Brien and Hau, 2005; Helm et al., 2006b; Voigt et al., 2007; Bauchinger et al., 2008; Dawson, 2008; Perfito et al., 2008; Verhulst and Nilsson, 2008). That the impact of environmental factors on schedules differs between closely related taxa (Ramenofsky and Wingfield, 2006) may in some cases also relate to circannual organisation. Circannual rhythms provide temporal windows for activities like reproduction and migration (Gwinner, 1996; Gwinner, 1999). Under relatively rigid circannual regulation, activities are modified within these windows only whereas under more flexible control, the windows themselves may be modified. This is again illustrated by comparative studies of stonechats. Free-living Siberian and African (*S. t. axillaris*) stonechats both breed once per year (Dittami and Gwinner, 1985; Raess and Gwinner, 2005) but reproductive windows, as defined by active gonads, are much wider in African (Gwinner, 1991; Gwinner, 1996) than Siberian (Figs 2–5) birds. In aviaries, both taxa used their entire respective time window for breeding. Hence, African but not Siberian stonechats boosted reproduction (König and Gwinner, 1995; Helm et al., 2005). Wide breeding windows provide flexibility to take advantage of conducive conditions while ensuring that other processes, e.g. moult, still occur at the right time. Field data from Canary Island stonechats (*S. dacotiae*) indicate similar seasonal organisation at mid-latitudes (Illera and Diaz, 2006). European stonechats, in turn, frequently extended the duration of the breeding window by adding late clutches and consequently delaying moult (Flinks et al., 2008). Thus, differences

in circannual programming may also affect the fine adjustment of rigid *versus* flexible timing (Gwinner, 1996; Gwinner, 1999; Helm et al., 2005).

The new data contribute to growing evidence for distinct circannual rhythms. In stonechats, circannual cycles differed in phase and other more subtle aspects. Siberian males did not regress testes entirely and lengthened the first reproductive cycle (Fig. 4). The long first cycle resulted from early gonadal growth prior to main testicular activation, similar to patterns observed in other long-distance migrants under relatively long winter days (Gwinner, 1988; Gwinner, 1989; Gwinner, 1996; Gwinner and Helm, 2003). Circannual differences had also emerged from comparisons with stonechats from equatorial Africa, which showed particularly robust rhythmicity (Gwinner, 1991; Gwinner, 1996; Helm, 2006). Crossbreeding experiments in several species support inherited schedules, as in F1 hybrids, reproduction, postjuvenile moult and migratory restlessness were generally timed intermediately (Davies et al., 1969; Berthold and Querner, 1993; Gwinner, 1996; Berthold, 2001). In our present study, F1 hybrids generally fit this picture (Fig. 3), with the clear exception of the transition between breeding and moult. Hybrid gonadal regression and moult onset, but not decrease in testosterone, resembled that of Siberian stonechats, possibly indicating that dominance effects acted on the inheritance of this particular phase of the annual cycle.

Our present study shows that geographically distinct schedules of a songbird were primarily based on inherent differences in circannual characteristics. Changes in the duration of reproductive inactivation and activation, respectively, modified the phase of the annual cycle relative to the external year and, consequently, the birds' responses to a given daylength. The results emphasise that photoperiodism and circannual rhythms function together and that findings for both mechanisms need to be integrated. Circannual and photoperiodic studies have converged in documenting geographically distinct time-keeping in birds and other organisms (Joy and Mrosovsky, 1982; Gwinner, 1986; Silverin et al., 1993; Gwinner, 1996; Lambrechts et al., 1997; Heideman et al., 1999; Noskov et al., 1999; Helm et al., 2005; Bradshaw and Holzapfel, 2006; Bradshaw and Holzapfel, 2007; Hahn and MacDougall-Shackleton, 2008; Silverin et al., 2008; Wikelski et al., 2008). Nevertheless, conceptual and experimental differences between the two approaches have left uncertainty about the relative contributions of photoperiodism and circannual rhythms to timing under natural conditions. Organisms differ greatly in persistence of circannual cycles and in the conditions under which they are expressed (Prendergast et al., 2002; Goldman et al., 2004; Paul et al., 2008). The relevance of circannual programs is widely accepted for migration and hibernation given complex photoperiodic conditions (Aschoff, 1955; Hamner and Stocking, 1970; Gwinner, 1989; Gwinner, 1996; Gwinner and Helm, 2003; Sharp, 2005; Helm, 2006) but has been debated for reproduction because photoperiodic cues are usually available (Farner, 1985; Dawson, 2007; Wikelski et al., 2008). Our data offer strong support for the hypothesis that circannual rhythms provide a reference system for phase-specific timing responses, i.e. function as periodically changing dispositions to respond to environmental cues (Gwinner, 1999). Dependence of photoperiodic action on circannual phase has also been documented for other organisms, notably trout, sheep and beetles (Randall et al., 1998; Lincoln et al., 2005; Lincoln et al., 2006; Miyazaki et al., 2007). Together these studies show that daylength acts on circannual rhythms by resetting the phase or by modulating the rate at which seasonal processes run during a given fraction of the annual cycle (i.e. angular velocity) (Gwinner, 1986; Gwinner and Helm, 2003).

Our data add two important points for understanding circannual rhythms in the real world. Firstly, we show that differences in circannual programming matter for responses to naturally experienced conditions, i.e. relatively subtle differences in photoperiod related to migration distance. Secondly, we show that differences in circannual programming can explain geographically distinct schedules of related taxa.

Evolutionary adjustment of circannual programming may be a potent way to fit annual cycles to population-specific conditions. Analogous to circadian clocks, timing could be enhanced by combining precise internal time structuring with flexibility in response to external cues (DeCoursey, 2004). Photoperiod is highly accurate but as a calendar, it is only useful if correctly related to seasonal conditions (Goldman et al., 2004). As the relationship of photoperiod to environmental conditions is variable, a need to readjust daylength responses may be common. For instance, stonechats in most parts of Europe breed under increasing spring daylength whereas closely related Canary Island stonechats breed in winter, sometimes under still decreasing daylength (Illera and Diaz, 2006; Illera et al., 2008). Furthermore, photoperiod times different seasonal functions, e.g. onset and end of reproduction (Fig. 5) or moult and migration, which may need to be independently adjusted (Helm, 2006; Kumar et al., 2006; Piersma et al., 2008). If annual cycles and photoperiodism are separate processes, particular phases and their interactions with daylength could be modified while the overall circannual organisation is maintained. In this way, the fractions of the circannual cycle that regulate various seasonal processes could react to photoperiod such that in each population, particular timing relative to the environment is achieved. The phase-specific action of photoperiod could be thus tuned, in the course of evolution, to the annual cycles of those 'ultimate factors' (Baker, 1938) that are relevant for a bird's fitness (Gwinner, 1986; Helm, 2006; Wikelski et al., 2008). Whether or not circannual cycles persist under a given photoperiod may also follow from adjustment to the conditions to which a population is exposed, e.g. determining the daylength under which reproduction is reactivated (Gwinner, 1988; Gwinner, 1989; Gwinner, 1996). New neuroendocrine studies suggest that such transitions may be achieved by modified action on a common biological substrate (Sharp, 2005; Paul et al., 2008).

Rapid change of scheduling is imperative if organisms are to keep pace with global change. Whether or not this challenge is met depends on underlying mechanisms (Coppack and Pulido, 2004; Nussey et al., 2005; Bradshaw and Holzapfel, 2006; Bradshaw and Holzapfel, 2007; Hedenström et al., 2007; Visser, 2008). Understanding and predicting the changing seasonal behaviour of organisms therefore requires detailed understanding of time-keeping, including possible circannual contributions. For example, earlier studies have reported advanced breeding schedules in long-distance migrants under simulated daylengths of shorter migration routes (Coppack et al., 2003; Coppack and Pulido, 2004). This finding was extrapolated to predict earlier breeding under climate change if migration distances decrease. But as the present study shows, whether or not migrants meet this prediction or, conversely, delay breeding after wintering at higher latitudes (Fig. 5), may depend on their underlying circannual programs.

Circannual rhythms have been documented in various organisms from plants to primates (Pengelley, 1974; Gwinner, 1986). Our data suggest that they encode phase-specific responses to photoperiod in population-specific ways. In species without sustained circannual cycles, photoperiod-dependent timers may be modified to achieve similar purposes. Either way, photoperiodism can only be properly

understood if not only photoperiod but also the underlying programming of photoperiodic responses is taken into account (Gwinner, 1996).

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