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Role of Cryophase Temperature and Thermophase Duration in Thermoperiodic Regulation of the Testicular Cycle in the Lizard *Lacerta vivipara*

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ABSTRACT Testicular activity in reptiles is controlled primarily by thermal and thermoperiodic factors; however, little is known about the relative contribution of daily heating and nightly cooling in these processes. This question was addressed in the lizard *Lacerta vivipara* whose cycle is characterized by a 6 month hibernation followed by a single spring spermiogenesis and a summer spermatogenesis. From the autumnal equinox on, lizards were maintained under a constant 12L/12D photoperiod and acclimated to different 24 hour thermoperiodic regimes. Treatments combined a short or a long thermophase (2 or 6 hr basking) in alternation with either a warmer (19–21°C) or a colder cryophase (3–7°C). Testicular activity was monitored by histological examination of the testis and epididymis in late December and in early March. Heat provided daily during either 2 or 6 hour phases advanced spermiogenesis and spermiation by 3–4 months. Cryophase temperatures had no significant effects on the date of onset of spermiogenesis whereas they greatly affected its speed of completion. Thus, the full testicular cycle, from one spermatogenetic wave to the next, was completed within 6 months under warmer cryophases alternating with either long or short thermophases. In contrast, only part of the cycle was completed in lizards experiencing colder cryophases since active spermiogenesis and spermiation were maintained for at least 2 months as under natural conditions. The functional significance of heat and cold per 24 hour cycles in the regulation of the testicular cycle is discussed.

Temperature and thermoperiod are dominant factors controlling the completion of testicular cycles in reptiles (reviewed by Licht, '72a,b, '84). This has been established by comparing effects of different constant temperatures, sometimes with that of 24 hour thermocycles mimicking the warmest conditions during annual activity. Acclimation to such thermoperiodic regimes is at least as efficient in accelerating the spermatogenetic cycle as the preferred high constant temperatures, whereas constant temperatures inferior to the selected range slow down the cycle (Licht, '67, '71, '73; Licht et al., '69; Hawley and Aleksiuk, '76; Weil and Aldridge, '79; Angelini et al., '80; Marion, '82; Ganzhorn and Licht, '83; Mendonça and Licht, '86). Yet knowledge of the nature and flexibility of the behavioral thermoregulation in reptiles prompts us to explore more precisely the relative significance of the daily basking and level of resting temperatures in regulating testicular activity.

Reptiles are capable of behavioral thermoregulation and therefore can exert some control over both their highest and lowest body temperatures (Stebbins, '63; Cowgell and Underwood, '79; Regal, '67; Rismiller and Heldmaier, '88). This depends upon circadian and annual rhythms controlled by photic and thermal factors. Consequently, the well-shaped nycthemeral cycles in body temperatures (T_b) are strongly reinforced by succession of seasons. For instance, during the annual period of activity, the temperature-zone lizard *Lacerta viridis* always maintains its T_b in a high species-specific preferred range for several hours but changes its night T_b (Rismiller and Heldmaier, '88). As a result, the diel amplitude in T_b decreases towards summer and, conversely, increases as winter nears. Thereafter, the lizard stops warming up and voluntary hypothermia (Regal, '67) extends to voluntary hiberation. Reptiles do experience regular seasonality through their cycles in body temperatures (Licht, '72a). In turn, these diel and annual changes in T_b control reproductive activity; however, the rel-

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ative contribution of heat and cold in its thermoperiodic regulation has been poorly explored. Several hours at the selected thermal range are prerequisite for any stimulation (Licht, '67, '71, '73; Licht et al., '69; Weil and Aldridge, '79) but regulation as a result of variable basking opportunities is not documented. Late summer changes in night T_b end thermorefractoriness and induce some testicular development in several species of lizards (Licht et al., '69; Licht, '71, '73; Marion, '82) but the overall significance of annual modifications of night T_b have been little studied.

Despite their ability to thermoregulate, reptiles inhabitating temperate zones still face sudden changes in weather conditions and field cycles in T_b may be cooler than if the full choice was always available. This is the case in the lizard *Lacerta vivipara* after arousal from hibernation. Body temperatures during the daily activity are about 10°C lower in the field than when the animal is freely moving in a thermal gradient under laboratory conditions (Van Damme et al., '87). At this time of year males enter an intensive phase of reproductive activity in order to mate as soon as females emerge (Herlant, '33; Gigon-Depeiges and Dufaure, '77; Courty and Dufaure, '79, '80; Van Nuland and Strijbosch, '81; Bauwens and Verheyen, '85). Thus, one may wonder whether the regulation of the testicular cycle is based solely on rather precise thermal ranges and if the unpredictability of weather conditions may impair or accelerate the completion of the cycle.

These questions were addressed in *L. vivipara,* a northern lizard that hibernates continuously between autumn and spring equinoxes (Van Nuland and Strijbosch, '81). Its annual testicular cycle is typical for a temperature-zone lizard (Herlant, '33; Gigon-Depeiges and Dufaure, '77; Courty and Dufaure, '79, '80). Vernal testicular activity resumes with the formation of new spermatids and their transformation into spermatozoa. Plasma testosterone levels increase dramatically and, in response, the epididymis enters its active secretory phase. Spermiogenesis and spermiation develop for about 2 months whereas plasma testosterone levels remain high and the epididymis hypersecretory. Involution occurs in June with the elimination of remaining germ cells, a drop in plasma testosterone levels, and the lysis of epithelial cells in the epididymis. Thereafter a new spermatogenetic wave starts and develops during summer. When hibernation begins, the testes contain few early spermatids. Multiplication and differentiation of epithelial cells in the epididymis are over and cells are ready to be secretory. The present study examines the effects of the duration of daily basking and the level of night temperatures (thermophase duration and cryophase temperature per 24 hour cycle) in the thermoperiodic regulation of the testicular cycle from the autumn equinox on. The results indicate that reduced basking opportunities and cold nights are responsible for the vernal testicular activity in the lizard *L. vivipara.*

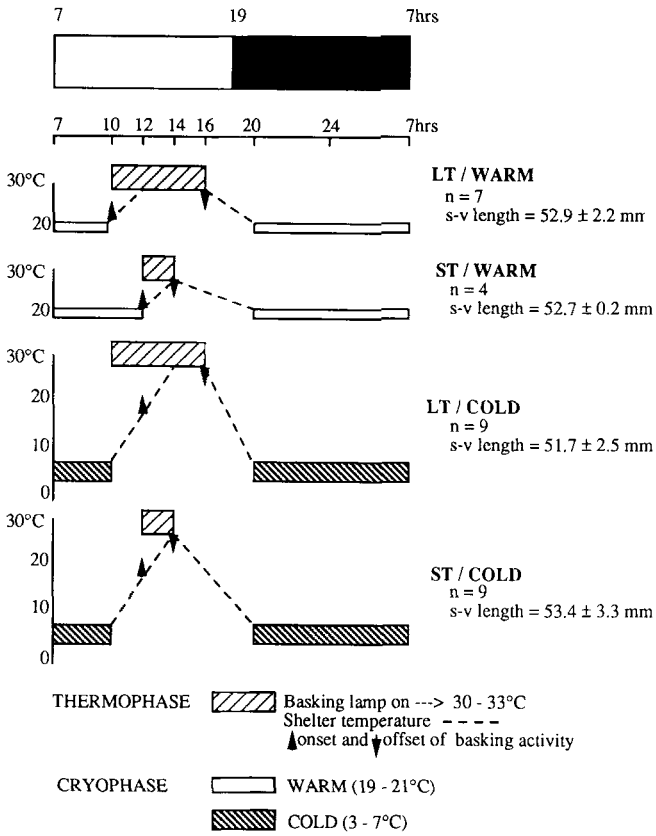


Fig. 1. Schematic representation of the different 24 hour thermoperiodic regimes experienced by the lizard *Lacerta vivipara* from the autumnal equinox until early March. The 4 treatments combine a 6 hour (long, LT) or a 2 hour (short, ST) thermophase in alternation with either a 19–21°C (Warm) or 3–7°C (Cold) cryophase. Arrows indicate the onset and offset of basking activity. Each thermophase is centered within the photophase of the 12L/12D photocycle. The sample size (n) and the mean ± 1 S.E. snout-vent length (s-v length in mm) are given for each group.

MATERIAL AND METHODS

Animals and experimental conditions (Fig. 1)

Adult males (snout-vent length 46–57 mm, 2–4 g in body weight) were captured in early August, in Massif Central (France) at 1,200 m above sea

level. *Lacerta vivipara* is a heliothermic lizard that basks to reach its specific selected range in body temperatures, 30–33°C (Van Damme et al., '87) during 2 to 5 hours depending on availability of direct sunshine (Bauwens and Verheyen, '85). This behavior has been preserved under laboratory conditions by using an incandescent lamp (60W Mazda) to generate the daily thermophase.

Between capture and the beginning of the experiment, 10 to 15 lizards were housed in large terraria (60 × 40 × 45 cm) provided with soil, a shelter, and a basking lamp hanging at one end. Basking lamps were automatically turned on for 6 hours, between 1000 and 1600 hrs (LT for long thermophase) to allow lizards to bask and reach 30–33°C. At the end of each thermophase, air and shelter temperatures decreased to 19–21°C (WARM cryophase). Crickets, mealworms, and water were given ad libitum. In addition, each terrarium was sprayed with water every weekday. Lizards experienced a natural photocycle. Prior to experimentation, males were individually toe-clipped for identification, divided into 3 groups according to their size. Then individuals were randomly assigned to experimental groups so that the size range and mean was as homogenous as possible (Fig. 1).

During the experiment, lizards were fed as described above and maintained under a 12L/12D photocycle using a 20W fluorescent bulb (Phillips Industrial White) as an additional light source (0700–1900 hrs). Four to five lizards were housed in small cages (17 × 30 × 20 cm) with a shelter and the basking lamp positioned centrally 40 cm above each mesh-covered cage. They were acclimated to one of the following thermoperiodic regimes (Fig. 1). Basking lamps were automatically turned on for either 6 hours (1000–1600 hrs; LT for long thermophase) or only 2 hours (1200–1400 hrs; ST for short thermophase). Thereafter, air and shelter temperatures equilibrated within a restricted range prevailing during the cryophase, either WARM (19–21°C) or COLD (3–7°C). In the warmer cryophase groups, the 19–21°C range was maintainted for 14 hours in the LT/WARM group (2000–1000 hrs) and 16 hours in the ST/WARM Group (2000–1200 hrs). All lizards were active for the whole duration of the thermophase. Colder cryophases were obtained by keeping cages inside a 62 × 127 × 25 cm refrigerated tank (Facis) and by turning on the refrigeration between 1600 and 1000 hrs. The 3–7°C gradient was reached at 2000 hrs and maintained during 14 hours, until 1000 hrs. In the LT/COLD group 3 hours were necessary to warm up the cold air temperatures to 30°C; therefore, the shelter gradient ranged between 25 and 30°C. During the thermophase lizards were active from 1200 to 1600 hrs. In the ST/COLD group, the increase of shelter temperatures was slower than under LT/COLD conditions; lizards were active during the full 2 hour thermophase.

Assessment of the testicular cycle

The experiment began by the autumnal equinox (September 26) and ended in early March. Lizards were maintained under the photoperiod of the equinox and acclimated to one of the thermoperiodic regimes described above: LT/WARM, ST/WARM, LT/COLD, or ST/COLD (Fig. 1). Exceptional high mortality occurred in the ST/WARM group, thus reducing the sample size to 4 healthy lizards. In order to study the effects of these thermal acclimations on the testicular cycle, males were sacrificed by decapitation on September 26 (Control), after 13 and 24 weeks acclimation, on December 20, and on March 8, respectively. The right testis and epididymis were separately weighed to the nearest 0.1 mg. The left testis and epididymis were fixed in Bouin's fluid, then embedded in Paraplast. Serial sections of 5 μm were stained by the Azan's trichrome. The completion of the testicular cycle was assessed by histological examination of the testis and the epididymis. The testicular cycle was delineated into 6 stages according to the weight and histological development of both organs (Table 1). This classification is based on studies by Herlant ('33), Gigon-Depeiges and Dufaure ('77), Courty and Dufaure ('79, '80).

Statistics

Testicular stages and organ weights were compared using a nonparametric one-way analysis of variance by ranks, the Kruskal-Wallis test. This test was followed by a nonparametric multiple-comparison procedure at $P = 0.15$ level of significance (Daniel, '78). Due to its small sample size, the ST/WARM group was not included in this statistical analysis.

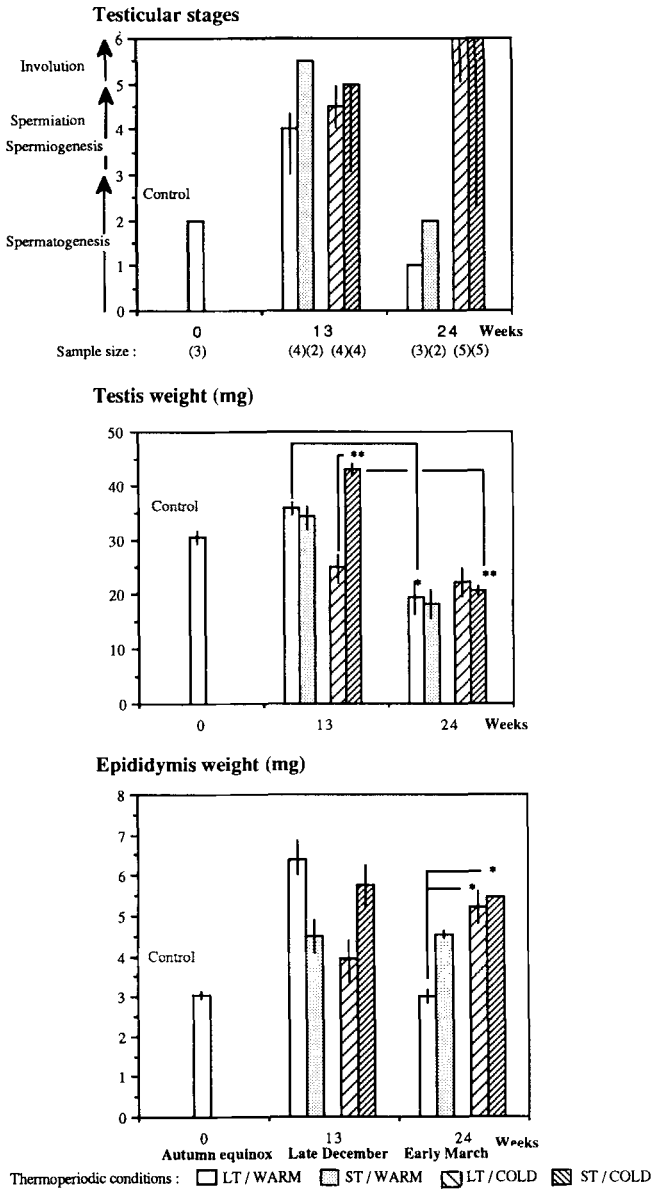
RESULTS

Testicular stages and testis and epididymis weights (mean ± 1 S.E.) are given in Figure 2. When the experiment began, the 3 males sacrificed had only reached mid-spermatogenesis (stage 2). Spermatocytes I and II were present in seminiferous tubules, and epithelial cells in the

TABLE 1. Classification of testicular stages[1] *in the lizard* Lacerta vivipara

Stages	Histological conditions: Testis	Epididymis
1	Compact tubules with spermatogonia and primary spermatocytes	Hyperplasia
2	Lumen appearing, primary and secondary spermacotyes	Hyperplasia and hypertrophy
3	Increasing lumen, early spermatids	Hypertrophy
4	Abundant spermatids, beginning of transformation into spermatozoa	Hypertrophied and beginning of secretory activity
5	Wide lumen, spermatozoa abundant and release into epididymis	Hypertrophied, intense secretory activity and transiting spermatozoa
6	Wide lumen, elimination of germ cells	Lysis

[1]Based on studies by Herlant ('33), Gigon-Depeiges and Dufaure ('77), Courty and Dufaure ('79, '80).



epididymis were hypertrophied. Testis and epididymis mean weights were 30.7 ± 2.9 mg and 3.0 ± 0.3 mg, respectively.

Effects of thermal acclimations on the completion of the testicular cycle (Fig. 2, upper panel)

In late December, regardless of thermoperiodic conditions, males had started spermiogenesis and spermiation. Spermatozoa were either present in the testis but not yet released into the secretory epididymis (5 males out of 14; stage 4) or evacuated into the epididymal tractus (7 males out of 14; stage 5). Among the latter, 2 males in the ST/WARM group were already starting involution. Two additional males were very delayed since they were only finishing spermatogenesis (LT/WARM and ST/COLD groups; stage 3). Thus, 13 weeks acclimation to a daily 6 or 2 hour heat input in alternation with either warmer or colder cryophases phase advanced spermiogenesis/spermiation to late December instead of April in natura. Differences among groups were not significant. One may, however, notice that the less advanced males experienced the warmest conditions (LT/WARM; median stage 4).

Fig. 2. Completion of the testicular cycle in the lizard *Lacerta vivipara* after 13 and 24 weeks acclimation to 4 different thermoperiodic regimes, long (LT) or short (ST) thermophases alternating with either a warmer or colder cryophases. The upper panel gives the testicular stages (median and range) as defined in Table 1. The middle and lower panels illustrate changes in the testis and epididymis weight respectively (mean ± 1 S.E. in mg). Levels of significance: $*P = 0.05$; $**P < 0.01$. Sample sizes are indicated under the upper panel.

Two months later, in early March, there was a marked and significant discrepancy between lizards exposed to either warmer or colder cryophases ($0.05 < P < 0.01$). Males in the LT/WARM group were significantly more advanced than males acclimated to colder cryophases. In both warmer cryophase groups, spermiogenesis and spermiation were completed and the new spermatogenetic cycle had started. Spermatogonia and spermatocytes I filled compact seminiferous tubules in all males in LT/WARM group (stage 1). Moreover, hyperplasia was occurring in the epididymis. Two males in the ST/WARM group were slightly more advanced since spermatocytes II were already present and since epithelial cells in the epididymis were hypertrophied (stage 2). Conversely, 8 males out of 10 in cold cryophase groups were only finishing spermiation and germ cells were in the process of being eliminated (stage 5 to 6). The secretory activity in the epididymis was decreasing while the transiting spermatozoa were close to their complete evacuation. In the ST/COLD group, 2 males had reached mid- and late-spermatogenetic stages. Regular assessment of plasma levels in testosterone (data not shown) indicated that the cycle was probably very delayed for one individual and advanced for the other. In conclusion, males in both colder cryophase groups completed spermiogenesis and spermiation within 2 months at least, whereas less than a month was necessary for males in warmer cryophase groups.

Whatever the thermophase duration, only part of the testicular cycle was completed within the 6 months acclimation to colder cryophases. Conversely, warmer cryophases allowed the occurrence of the full cycle, from one spermatogenetic wave to the next. Males in the ST/WARM group were more advanced than in the LT/WARM group. In late December, spermiogenesis and spermiation were finishing in the former and starting in the latter. Later in early March, mid- and early spermatogenesis were reached in ST/WARM and in LT/WARM, respectively.

Effect of thermal acclimations on testis and epididymis weights (Fig. 2)

The testis weight (Fig. 2, middle panel) cycled according to the spermatogenetic development in all groups. Moreover all values were within the weight ranges observed during the normal field cycle (Gigon-Depeiges and Dufaure, '77; Courty and Dufaure, '79, '80). A peak in testis weight occurred in all groups in late December as active spermiogenesis and spermiation were taking place. In early March, the testis weight had decreased in all groups; however, this was correlated with spermatogenetic recrudescence in warm cryophase groups and involution in cold cryophase groups. Variations in weight during the course of the 24 weeks acclimation were significant in the LT/WARM and ST/COLD groups at $P = 0.05$ and $P < 0.01$, respectively. In both groups, the testis weight was significantly higher in late December than in early March: 35.9 ± 1.8 mg versus 19.2 ± 6.0 mg in LT/WARM and 43.1 ± 2.8 mg versus 20.5 ± 4.2 mg in ST/COLD. Differences among groups were significant only in late December ($P < 0.01$). The ST/COLD group was characterized by the highest gonad weight (43.1 ± 2.8 mg) compared to LT/COLD (24.9 ± 5.1 mg) and LT/WARM (35.9 ± 1.8 mg).

Modifications of the epididymis weight over the course of 24 weeks acclimation were not significant in any group (Fig. 2, lower panel). Nevertheless, the epididymis weight decreased in the LT/WARM group from 6.4 ± 1.9 mg in late December during secretory activity to 3.0 ± 0.4 mg in early March following lysis and during restructuration. Conversely, it did not change in both cold cryophase groups (3.0 ± 1.6 mg versus 5.0 ± 0.9 mg in LT/COLD and 5.8 ± 1.5 mg versus 5.4 ± 1.2 mg in ST/COLD). Indeed, the gland remained actively secreting from at least late December until March. At this time, the epididymis weight was significantly different among groups ($P = 0.05$); it was higher in the 2 cold cryophase groups than in LT/WARM.

DISCUSSION

Thermoperiodic conditions imposed on *L. vivipara* from the autumnal equinox on first imply suppression of hibernation. This phase advanced the testicular cycle by 3–4 months regardless of cryophase temperatures; daily heat input during basking was the important factor. Conversely, further acceleration of testicular activity depended primarily upon cryophase temperatures. The warmest conditions speeded up testicular development so that the full annual cycle was completed within 6 months. Colder cryophases slowed down the cycle since spermiogenesis and spermiation lasted about 2 months as in natura. Altogether these data illustrate that both the highest and lowest body temperatures (T_b) reached and controlled by behavioral means display functional significances in the thermoperiodic regulation of a testicular cycle.

Relative significance of heat and cold in thermoperiodic regulation of the testicular cycle

As long as daily basking is possible, cryophase temperatures play a significant role. Warmer cryophases accelerated the formation and release of spermatozoa since this stage lasted about one month. As a result, completion of the full testicular cycle took place within 6 months, from mid-spermatogenesis by the autumn equinox to the next spermatogenetic wave in early March (Fig. 2). As established in other studies, heat likely enhanced the turnover of germ cells at different stages of their differentiation (Joly and Saint Girons, '75, '81; Pearson et al., '76) and the rate of testicular regression (Licht, '73; Marion, '82; Mendonça and Licht, '86). More precisely, the time span between the early spermatid stage and the evacuation of the mature spermatozoa is reduced by half in a related species, *L. muralis,* acclimated to 27.5°C constant compared to 22.5°C constant or to the natural thermoperiodic conditions (Joly and Saint Girons, '75). This explains also that acclimation of *L. vivipara* to cold cryophases increased the duration of spermiogenesis and spermiation to such an exent that it lasted as long as in natura (late March–early June) (Herlant, '33; Gigon-Depeiges and Dufaure, '77; Courty and Dufaure, '79, '80). It is noteworthy that the speed of completion of spermiogenesis and spermiation depends primarily upon cryophase temperatures provided that daily basking is possible.

Modulation of some of the cryophase effects comes from the duration of basking. Under colder cryophases, the duration of basking modifies testicular growth. In late December, short thermophases had significantly enhanced the testicular growth compared to long thermophases (ST/COLD versus LT/COLD; Fig. 2, middle panel). Similar observations have been made in other species of lizards kept under constant temperatures or thermoperiodic conditions lower than the specific preferred range (Licht et al., '69; Pearson et al., '76; Marion, '82). This is mainly due to an increase in the testis dry weight and lipid content (Pearson et al., '76), but whether the number of spermatozoa is increased has not been established. Further experiments would be needed to confirm that reduced basking possibilities may have a stimulatory role—for instance, to enhance testes growth in spring.

Taken together these data emphasize that optimal or reduced basking possibilities alternating with cold cryophases are responsible for the main aspect of vernal testicular activity in *L. vivipara:* a 2 month duration of spermiogenesis and spermiation. This may be adaptative and linked to the year-to-year unpredictability of the female arousal during April or May. On the other hand, one may not preclude that a long-term spermiogenesis/spermiation means that the number of gametes is increased. The enhanced testicular development under ST/COLD would be another argument in favor of this hypothesis. A quantitative analysis of spermatogenesis is necessary to answer these questions. This could also be interesting in reference to scrotal mammals since temperatures 2–7°C cooler than the body temperatures are required for normal formation of spermatozoa (references in Fowler and Racey, '87).

Different use of thermal ranges by the male and female for the regulation of gonadal cycles

Whatever the climatic conditions, the timing of hibernation in *L. vivipara* is quite similar every year (Bauwens and Verheyen, '85; Van Nuland and Strijbosch, '81). An internal rhythm (reviewed by Gregory, '82) is likely to contribute to the fall entrance and vernal arousal and consequently to schedule the reproductive cycle (Gavaud, '91). Males emerge first and undergo spermiogenesis and spermiation in order to mate as soon as females arouse and start vitellogenesis (Herlant, '33; Panigel, '56; Bauwens and Verheyen, '85). Hibernation precisely times both cycles; however, continuous cold body temperatures exercise quite different influences on males and females. Hibernation interrupts spermiogenesis and is not necessary to the completion of the full cycle (Fig. 2). On the contrary, cold during hibernation is a prerequisite for the induction of vitellogenesis; it entrains an internal rhythm underlying the ovarian cycle (Gavaud, '83, '91). The warmest thermoperiod reduces the duration of the annual testicular cycle to 6 months (Fig. 2) whereas it blocks the ovarian cycle at the quiescent stage (Gavaud, '83, '91). These cold and heat effects are similar in a related species, *L. sicula* (Botte at al., '78; Angelini et al., '80), and in the cold-adapted snake, *T.s. parietalis* (Hawley and Aleksiuk, '76; Whittier et al., '87). This implies that the male and female rely on the same thermal cues, but that cold acts through opposite patterns: "negative" in males since it inhibits the spermiogenetic development and "positive" in fe-

males since it stimulates the ovarian growth. In this respect, the brain-pituitary axis is more likely to be involved than the gonad itself (Gavaud and Yvorra, '86). Although timings, natural history, and thermal ranges are different, it is quite remarkable that similar observations have been made in two different species of turtles (Ganzhorn and Licht, '83; Mendonça, '87; Mendonça and Licht, '86). Females for follicular growth require temperatures about 10°C lower than males for spermiogenesis. In these different species of reptiles, the physiological control of the formation of the female and male gamete requires different thermal ranges and this may mean that the brain-pituitary axis displays opposite ways to process information carried by heat/cold cycles.

Conclusions

After spring arousal in natura, weather conditions still impose cold nights, unpredictable and possibly poor opportunities to bask. The male *L. vivipara* does not switch to thermoconformity but is physically unable to attain its preferred T_b although it devotes considerable amount of time to warming (Van Damme et al., '87). The present study demonstrates that such thermal conditions easily match the heat and cold requirements for the normal completion of the vernal reproductive activity leading to the gamete formation and mating. All together, these data support the idea that ectotherms have come to actively make use of the full variability of their thermal environment and body temperatures rather than merely adapting to heat and surviving cold. This implies that adaptation encompasses the entire range of variability of the thermal conditions, including some of its unpredictability.

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