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The influence of thermal environment and food availability on testosterone and gonadal recrudescence in male Chinese skinks [*Plestiodon* (*Eumeces*) *chinensis*]

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ABSTRACT

Most animals show seasonal cycles of reproduction (including cycling of sex steroids). Environmental cues (e.g., temperature) likely play important roles in these seasonal variations but their exact contributions remain unclear. We conducted a two-factor experiment to elucidate the effects of thermal environments and food availability on growth in body mass, testosterone (T) levels and testes morphology in male Chinese skinks [*Plestiodon* (*Eumeces*) *chinensis*]. Skinks in the thermal environment mimicking spring (April) conditions grew slowly but had higher plasma T levels and larger testes with more viable sperms than those in the thermal environment mimicking summer (July) conditions. Skinks exposed to high food treatment grew faster and had higher plasma T levels and more viable sperms than those exposed to low food treatment. Male growth was negatively correlated with reproductive activity as indicated by T levels and testes size. Therefore, both temperature and food availability are important environmental factors that can affect the reproductive cycle of male lizards, and the mechanisms underlying the trade-off between growth and reproduction could involve the regulation of T levels.

1. Introduction

Environmental factors affect many biological processes (e.g., growth and reproduction) via a number of physiological mechanisms that are critical to understanding the adaptation of organisms to their environments [50]. Hormone regulation is one such important mechanism that mediates the influences of environmental factors on growth and reproduction [18]. Correlation analyses have indicated that change in hormone levels of animals are related to variation in environmental factors [5,17,21,37]. Some endocrinologists aim to identify hormone regulation of growth and reproduction using experimental manipulation of hormone levels [20,34,47]. Nonetheless, fewer investigations manipulate environmental factors to see how environmental cues affect hormone regulation.

Seasonal cycles of reproduction accompanied by seasonal variations in sex steroids are widespread in ectothermic vertebrates, including fish [37], amphibians [17] and reptiles [11,22,23,30]. Photoperiod has long been known as the most important environmental cue stimulating the seasonality of reproductive endocrinology and behaviour in endotherms such as birds and mammals [12]. In ectotherms, besides photoperiod, other environmental factors such as temperature and food availability may also provide important cues for seasonal timing of reproduction. For example, temperature plays an important role in regulating the secretion of sex steroids and thereby regulates reproductive physiology and behaviour [14,29,38]; food availability may influence plasma testosterone levels and the reproductive behaviour of lizards [40]. In nature, thermal environment and food resources available to animals vary temporally (from days to years). The effects of such fluctuating natural environments should be especially evident in ectotherms because ambient temperature may determine their body temperature and in turn affect a number of physiological processes as well as behaviour such as food intake and assimilation [10,24]. Therefore, an understanding of the influence of temperature and food availability on sex steroids and gonadal recrudescence in ectotherms would shed significant light on how environmental cues stimulate seasonal timing of reproduction.

Testosterone (T) is the predominant reproductive regulatory hormone in males. Plasma T levels during the mating season are associated with gonadal recrudescence [1,49], the expression of secondary sexual traits [19,36] and reproductive behaviour [26,42,44]. Additionally, T significantly affects energy intake and allocation in organisms. Higher T levels incur increased energy expenditure, decreased energy acquisition [32] and affect energy allocation. Although previous studies have investigated the effect of T on reproductive behaviours and physiological performance

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of organisms by manipulating plasma T levels [7,40], the mechanisms by which environmental factors stimulate the secretion of T and in turn regulate reproduction have not been explicitly explored in many species, despite the fact that such studies would help us understand the proximate mechanisms of environmental regulation of reproduction.

Plestiodon chinensis is a medium-sized skink (adult snout-vent length (SVL) >88 mm) mainly found in southern China and Vietnam [51]. Adult males show seasonal cycles of T levels and reproductive traits [22,23], but the mechanisms underlying this seasonal variation are largely unknown. In this study, we conducted an experiment with a two-factor design to elucidate the effects of thermal environment and food availability on growth, T levels and testes morphology. We hypothesized that both thermal environment and food availability would affect the secretion of T and gonadal recrudescence. Given that male reproductive activity and T levels peak in April (spring) and decrease dramatically thereafter [22], we expected that a thermal environment mimicking spring conditions would facilitate reproduction, whereas a thermal environment mimicking summer conditions would hamper reproduction. In addition, high food supply would meet the extra energy requirement for reproductive individuals and would thus be expected to enhance reproduction. We analysed the relationships between growth and reproductive activity as indicated by T levels to identify the potential role of T regulation in the trade-off between growth and reproduction.

2. Materials and methods

2.1. Animal collection and experimental treatments

Adult male skinks ($n = 69$) were captured from Quzhou (118°44′E, 28°52′N), Zhejiang in late March, 2009. The SVL (SVL ± 1 mm) and body mass (BM ± 1 mg) of the skinks were determined after they were transported to the laboratory. The skinks were kept individually in 300 × 200 × 185 mm plastic cages lined with paper, and PVC pipes cut in half were provided for shelter. The cages were moved into a temperature-controlled room (24 °C) with a light cycle of 12L:12D (0700 h on, 1900 h off). We provided the skinks with the opportunity for thermoregulation by attaching electric heating boards (75 W) to the bottom of the cages; these created thermal gradients from 24 to 45 °C and were switched on for 4 or 8 h per day depending on the treatment.

Skinks are exposed to longer sunshine hours (and thus a warmer environment) in July (average 7.9 h/day) than in April (average 3.9 h/day) in Quzhou (Quzhou Bureau of Meteorology). Food is also more abundant for the skinks in their natural habitat in July than in April, and food intake by the skinks increases with increasing temperatures from 24 to 28 °C [27]. Accordingly, we used two thermal (8 and 4 h heating) and food availability (high and low food intake) treatments to mimic the seasonal variations in thermal environment and food availability for this species. The skinks were randomly assigned to one of the following four groups: (1) 8 h heating (0900 h on, 1700 h off) with high food, (2) 8 h heating with low food, (3) 4 h heating (0900 h on, 1300 h off) with high food and (4) 4 h heating with low food. All the lizards were fed once a day with mealworms (*Tenebrio molitor* larvae) dusted with vitamins and minerals. The mean body temperature of the skinks from 8 h heating groups was 27.5 °C, whereas that of the skinks from 4 h heating groups was 25.5 °C, reflecting mean values of body temperatures that wild skinks could attain in spring and summer [43]. We thus provided the skinks in the high food treatment groups with food intake required by *P. chinensis* at 28 °C, and those in the low food treatment groups with food intake at 25 °C (about 60% of the food intake at 28 °C) in captivity [27]. Water was available *ad libitum*.

2.2. Growth

We measured body mass (BM) of the skinks before and after the experimental treatments (40 days), and used the formula [ln (final BM) − ln (initial BM)]/period of experiment to determine the specific growth rate in body mass of the skinks.

2.3. Testes morphology and sperm quality

At the end of the treatment, the skinks were dissected to determine testes morphology and sperm quality. The mass (±1 mg) of the right testes were measured immediately.

The right spermduct was cut into small pieces within 1 min for the determination of sperm count and motility. The sperms were fully homogenized in 3 ml physiological saline (0.9% NaCl) and residual tissues were excluded using a 200-mesh strainer (200 meshes/in.^{2}). We then collected 1 ml of the homogenate to measure sperm count and motility following the methods described for male rats (for sperm count [15], for sperm motility [39]), with some modifications. Sperm count and motility were measured using a haemocytometer on a microscope (40 objective lens × 10 ocular lens). The number of sperms in five chambers (one at each corner and one in the centre of the haemocytometer) was recorded; the total number of sperms in 1 ml homogenate was calculated and the number of sperms in 100 mg of testes was used as the index for the sperm count [15]. We counted 100 sperms and recorded the percentage of forward motile sperms (FMS) (see [45] for the definition of FMS) to determine sperm motility [39]. Measurements of sperm count and motility for each skink were completed within 5 min. All experimental procedures were approved by the Animal Care and Ethics Committee of Hangzhou Normal University and were conducted in accordance with the NIH *Guide for the Principles of Animal Care*.

2.4. Blood sample collection and radioimmunoassay

Blood samples were collected from skink hearts using heparinized tubes. The samples were centrifuged to separate the plasma from red blood cells after incubation on ice for ⩽1 h. The plasma samples were stored at −80 °C for determination of T levels at a later time.

We analysed plasma T levels using radioimmunoassay (RIA) [35,44]. Briefly, each sample was extracted twice with diethyl ether, dried under a stream of nitrogen gas and re-suspended in ethyl acetate in isooctane. The separated hormones were collected, dried, and re-suspended again in phosphate buffered saline. The samples were assayed in duplicate with I^{125}-T as a radiolabel and T antiserum developed in rabbits (Ninetripods Life Science Inc., Tianjin, China). We did not separate T from other androgens prior to RIA so the plasma T values reflect the plasma total androgen concentration. However, these values may primarily reflect plasma T levels because the cross-reactivity between other androgens and T antiserum used in our measurements was very weak (5α-dihydrotestosterone $\leqslant 1.6 \times 10^{-2}$%, androstenedione $\leqslant 2.1 \times 10^{-4}$%), and could thus be ignored. The assay's sensitivity was 1.9 ng/dl. The inter-assay and intra-assay variations were 9.8% and 7.4%, respectively. A total of 17 samples (4, 4, 4 and 5 from each of the four treatments) were not included in the subsequent analysis because T values could not be measured by RIA due to the small quantities of blood present in these samples.

2.5. Statistical analysis

We employed STATISTICA 6.0 software (StatSoft Inc.) to analyse the data. The normality of distributions and homogeneity of the variances were tested using the Kolmogorov–Smirnov test and

Levene's test, respectively. The spearman correlation was used to analyse the relationship between specific growth rate in body mass and (1) initial BM, (2) testes mass and (3) T levels of the skinks. Two-way ANCOVAs including initial or final BM as a covariate were used to analyse the effects of thermal environment and food availability on specific growth rate or testes morphology. We analysed the effects of thermal environment and food availability on T levels, sperm count and sperm motility using ANOVAs.

3. Results

3.1. Growth

The initial BM of skinks did not differ among the different treatments ($F_{1,65} = 0.66$, $P = 0.58$). By the end of the experiment, BM increased for most skinks except those exposed to the 4 h heat treatment with low food supply. Specific growth rates in body mass were correlated to initial BM ($R = 0.27$, $F_{1,67} = -2.32$, $P = 0.02$). After the effect of initial BM was removed, specific growth rates were significantly affected by both thermal environment and food availability but not by the interaction between them (thermal environment, $F_{1,64} = 11.28$, $P = 0.001$, food availability, $F_{1,64} = 8.28$, $P = 0.005$, interaction, $F_{1,64} = 0.30$, $P = 0.58$). The specific growth rate was greater for skinks from the 8 h heating groups than the 4 h heating groups, as well as for skinks from high food groups compared with those from low food groups (Fig. 1).

3.2. Testes morphology and sperm quality

Testes mass were significantly affected by the thermal environment but not by food availability or interaction between thermal environment and food availability (thermal environment, $F_{1,64} = 4.59$, $P = 0.04$, food availability, $F_{1,64} = 0.03$, $P = 0.86$, interaction, $F_{1,64} = 0.16$, $P = 0.69$). Skinks from the 4 h heating groups had larger testes than those from the 8 h heating groups (Fig. 2).

Thermal environment and food availability significantly affected sperm count and motility (sperm count: thermal environment, $F_{1,65} = 17.84$, $P < 0.0001$, food availability, $F_{1,65} = 6.47$, $P = 0.01$, interaction, $F_{1,65} = 0.93$, $P = 0.34$; sperm motility: thermal environment, $F_{1,65} = 23.57$, $P < 0.0001$, food availability, $F_{1,65} = 15.82$, $P < 0.001$, interaction, $F_{1,65} = 4.67$, $P = 0.03$). Generally, the sperm count and motility of skinks from the 4 h heating groups and high food treatment groups were significantly higher than those from the 8 h heating groups and low food treatment groups, respectively (Fig. 3). Given the significant interaction between thermal environment and food availability on sperm motility, we further conducted separate ANOVAs for each thermal treatment. These analyses indicated that sperm motility was significantly affected by food availability among skinks from the 4 h heating groups ($F_{1,35} = 17.45$, $P < 0.001$) but not among skinks from the 8 h heating groups ($F_{1,30} = 1.91$, $P = 0.18$).

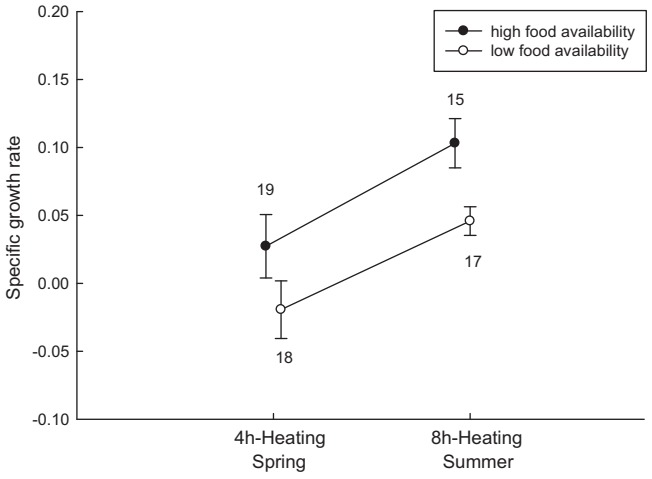

Fig. 1. Specific growth rate of body mass in Chinese skinks [*Plestiodon* (*Eumeces*) *chinensis*] exposed to different thermal environments and different food availability scenarios. Data are expressed as adjusted mean (relative to initial body mass) ± SE. Numbers above the error bars indicate sample size.

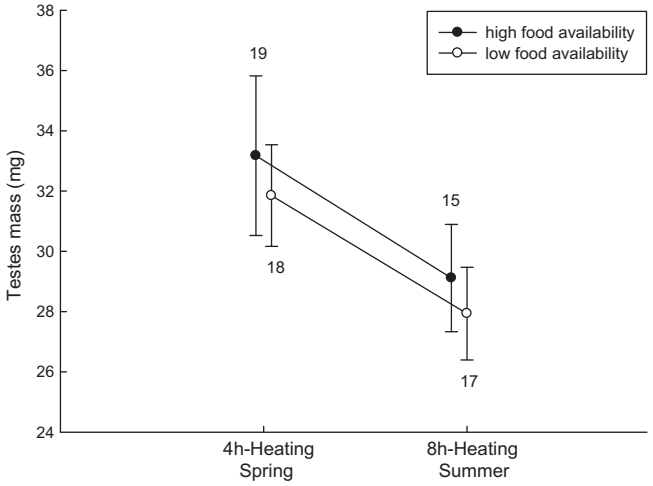

Fig. 2. The effects of thermal environment and food availability on testes mass in the Chinese skink, *Plestiodon* (*Eumeces*) *chinensis*. Data are expressed as adjusted mean (relative to final body mass) ± SE. Numbers above the error bars indicate sample size.

3.3. Testosterone levels

Thermal environment and food availability significantly influenced plasma T levels (thermal environment, $F_{1,48} = 5.74$, $P = 0.02$, food availability, $F_{1,48} = 5.41$, $P = 0.02$, interaction, $F_{1,48} = 0.28$, $P = 0.60$). Plasma T levels were significantly higher among skinks from the 4 h heating groups than among those from the 8 h heating groups and among skinks provided with high food compared with those provided with low food (Fig. 4).

3.4. The relationship between growth and testes mass or T levels

In all the studied skinks, the specific growth rate (SGR) in body mass was negatively correlated with testes mass (TM) ($R = 0.40$, $t_{67} = -3.50$, $P < 0.001$) and T levels ($R = 0.31$, $t_{50} = -2.27$, $P = 0.03$) (Fig. 5). T levels were positively correlated with testes mass ($R = 0.52$, $t_{50} = 4.32$, $P < 0.00001$), suggesting T levels and testes size were closely linked. Except for the group of 4 h heating plus low food ($R = 0.02$, $t_{11} = 0.07$, $P = 0.94$), the specific growth rate was negatively correlated with T levels in each group of treatments (8 h heating plus high food: $R = -0.47$, $t_9 = -1.61$, $P = 0.14$; 4 h heating plus high food: $R = -0.61$, $t_{13} = -2.81$, $P = 0.01$; 8 h heating plus low food: $R = -0.54$, $t_{11} = -2.18$, $P = 0.05$).

4. Discussion

4.1. The effects of thermal environment and food availability on plasma testosterone levels and gonadal recrudescence

As we predicted, thermal environment and food availability can significantly affect plasma T levels, testes morphology and sperm quality. Given the close relationship between T levels and testes recrudescence found in this species as well as other species from

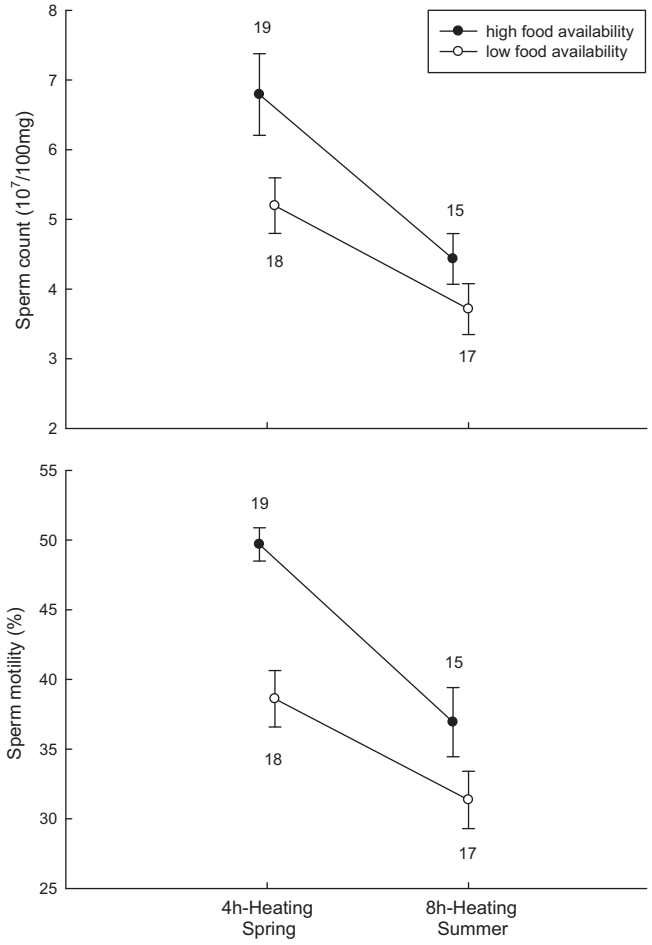

Fig. 3. The effects of thermal environment and food availability on sperm count and motility in the Chinese skink, *Plestiodon* (*Eumeces*) *chinensis*. Data are expressed as adjusted mean ± SE. Numbers above the error bars indicate sample size.

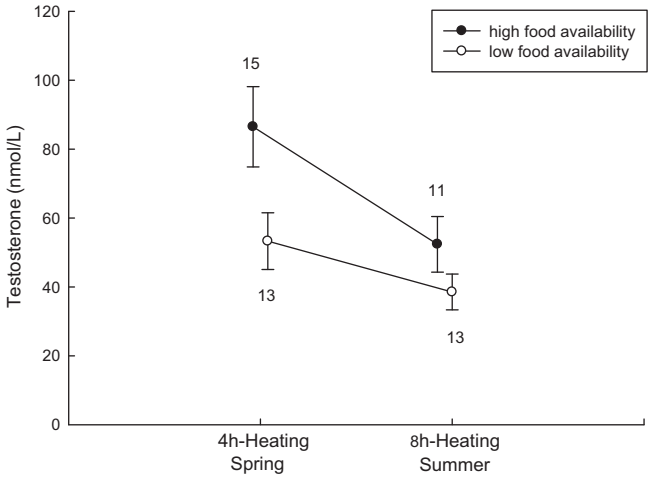

Fig. 4. The effects of thermal environment and food availability on testosterone levels in the Chinese skink, *Plestiodon* (*Eumeces*) *chinensis*. Data are expressed as adjusted mean ± SE. Numbers above the error bars indicate sample size.

different lineages [4,9,48,52], the effects of environmental factors on reproduction are likely mediated by their stimulation of T secretion.

Thermal environments mimicking conditions of different seasons have a significant impact on reproductive physiology. The

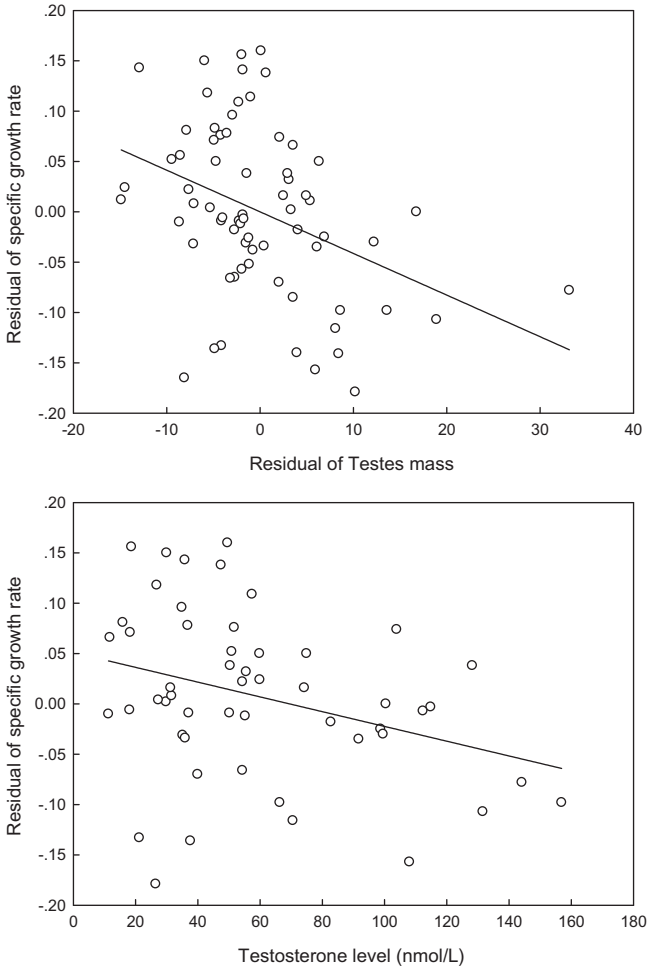

Fig. 5. The relationships between specific growth rate in body mass and testes mass or testosterone levels in the Chinese skink, *Plestiodon* (*Eumeces*) *chinensis*. Growth rates were represented as residuals calculated from the regression against body mass.

skinks from the thermal environment mimicking spring (April) had high plasma T levels close to the maximum level of that known for skinks from the field [22] as well as large testes with more viable sperms. In contrast, skinks from the thermal environment mimicking summer (July) had lower plasma T levels and smaller testes with less viable sperms. These results are consistent with seasonal cycles in T levels and gonadal recrudescence in the natural population of this species, peaking in April and decreasing dramatically in June [22,23]. Therefore, temperature is an important environmental factor that may account for the seasonal cycle in T levels, gonadal recrudescence and spermatogenesis, as shown in lizards [8,29] as well as other ectotherms [14,38]. A previous study suggested that constant low temperatures can maintain the high level of T for a longer period than constant high temperatures [21]. In future studies, it will be of great interest to identify how fluctuating temperature affects the dynamic variation in T and testes morphology, which could provide answers to why T levels differed between the two thermal treatments in the present study.

Food availability significantly affected reproductive endocrinology and spermatogenesis with higher plasma T levels and more viable sperms among male skinks provided with high food compared with those provided with low food. Although the underlying mechanism by which food availability affects T levels and spermatogenesis has not been revealed explicitly, it might be associated with energy allocation to reproduction, which is partly dependent on food supply [5]. When facing energy limitation,

energy allocation to reproduction could be decreased due to the competing requirements of different physiological functions, and hence cause suppression of reproduction. For example, immune activity can suppress reproduction when food is limited in tree lizards (*Urosaurus ornatus*) [13], and calorie restriction may lead to low T levels and suppression of reproduction in rats [41]. In contrast, food supplementation may increase the energy store, immune function and plasma T levels in lizards [40].

Interestingly, the effects of food availability on sperm motility depended on thermal environment; the effects were significant in thermal treatments mimicking spring but not in thermal treatments mimicking summer. In summer, T levels and viable sperms are likely inhibited by high temperatures, and thus were both low despite the food treatments, suggesting the effect of food availability is subject to that of temperature. In spring, temperatures stimulate the secretion of T and spermatogenesis and high food becomes more important because spermatogenesis is an energy-costing process [3,16].

4.2. Trade-offs between reproduction and growth

Trade-offs between reproduction and growth have long been recognized in the life history theory [46]. Such trade-offs between reproduction and growth must involve energy allocation between these two competing functions. Given the finite energy available to an organism, more energy allocated to reproduction means less energy for growth, and *vice versa*. The negative relationship between growth rate and T levels or testes mass of the skinks detected in this study provides a potential mechanistic explanation for this scenario.

Higher plasma T levels may activate a number of physiological and behavioural processes associated with reproduction, and thus, incur more energy expenditure. For example, high plasma T levels may facilitate spermatogenesis [33] and enhance reproductive behaviours including territorial defence, copulation and mating [7,31,32]. High plasma T levels may also incur high energy costs due to decreases in energy acquisition and parasite infestation [6,28]. Therefore, plasma T levels could proximately regulate the reproductive activity of males, and thereby, determine the energy allocation between reproduction and growth.

The present study clearly demonstrated that both temperature and food availability can act as important environmental cues that regulate seasonal variation in reproductive activity in reptiles. In addition to correlation analysis between environmental factors and reproductive cycles (including the sex steroid cycle), factor design experiments like our own may play a significant role in elucidating the determinants of the reproductive cycle [2,22,23,25]. Generally, this study highlights the importance of exploring the relationship between environmental factors and hormone regulation, which is critical for understanding how the environment affects physiological and behavioural functions of organisms.

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