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Determination of Testis Temperature Rhythms and Effects of Constant Light on Testicular Function in the Domestic Fowl (*Gallus domesticus*)[1]

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ABSTRACT

There is a wide range of opinions regarding the operating temperature of the testis in the domestic fowl. We used physiological monitoring techniques to investigate testis and body temperature over daily periods and under various light regimes to elucidate body temperature gradients in the fowl. We confirm that the operating temperature of the adult fowl's testes is equivalent to core body temperature (40–41°C). Long-term continuous temperature monitoring showed that there was no difference between the temperature of the testis, liver, and peritoneum during a 24-h period either in a normal light:dark cycle or under constant light conditions. However, there was a slight decrease in all temperatures at subjective night in each case, a decrease that does not appear to be sufficient to influence spermatogenesis. Birds maintained under constant light throughout two cycles of the seminiferous epithelium (28 days) still exhibited normal testis function and structure, even when "nightly" testis temperature decrease was the lowest. Thus, by undergoing spermatogenesis at an elevated temperature, the domestic fowl system is unique among the homeothermic animal systems studied to date.

INTRODUCTION

Spermatogenesis consists of a complex series of germ cell divisions and differentiation events that occur in a coordinated manner and that require controlled programs of stage-specific gene expression (reviewed in [1–3]). The testes, where spermatogenesis occurs, are 5–8°C cooler than core body temperature in mammalian systems [3]. In most cases this decrease in temperature is achieved by placement of the testes outside of the body cavity [3, 4]. As a result, spermatogenesis in mammals has unique testis-specific and temperature-dependent regulatory processes specific to germ cell gene expression [5, 6]. An increase in the temperature of mammalian testes (5–10°C) causes a decrease in sperm production and infertility [7].

In the current literature one finds statements that assume one of several different physiological states of the avian testes, located within the body cavity, in relation to the mechanisms of avian spermatogenesis. As recently as 1995, investigators stated that the avian testes are cooled below the core body temperature by evaporative cooling from the surface of the air sacs (e.g., [8, 9]). One study on the temperature of the lower air sacs found no significant difference between the temperature of these sacs and the core body temperature [10], suggesting that the testes are most likely not cooled by association with an air sac. Alternatively, it has been proposed that the avian testes are situated in a high-temperature environment at core body temperature (e.g., [11–18]). Of the researchers who state that the testes are maintained at body temperature, a subset say that spermatogenesis is occurring at that temperature [11, 12]. Upon review of the early original work, we found a research paper reporting that, as determined by measurements at a defined time point, the temperature of the testis of the domestic fowl is, in fact, the same as body temperature [13]. Others believe that there must be compensatory mechanisms acting to allow for spermatogenesis to occur to completion in the group Aves [14], such as the occurrence of spermatogenesis at night when testis temperature is lower [15, 16] or the occurrence of a temperature-sensitive maturation step in the cooler male sperm storage vesicles [17, 18]. Since mature sperm are produced throughout the day [19], and since fully active mature sperm can be obtained after passage through the epididymis [20], a requirement for exclusively nocturnal spermatogenesis or for maturation in the storage vesicles seems unlikely. Early papers that documented an apparent higher frequency of spermatogenesis at night did so by counting the number of metaphase cells in a testis cross-section at various times of the day [21, 22]. We now know that seminiferous tubules display waves and cycles of spermatogenic cells [19, 23–25]. Because of the differences in the amount of time spent in each stage [19, 23–25] and the small sample sizes used in these studies [21, 22], precise determination of spermatogenic rate was unlikely. Still, each of the previous views of the operating temperature of the avian testis is widely disseminated in textbooks and journals, usually with no direct evidence for any position referred to. The data presented by Williams [13] documenting basic testis temperature seem to have been overlooked in subsequent reviews of avian reproduction. However, that work [13] measured the temperature of the fowl's testis only at discrete times between 0900 and 1100 h. Since temperatures were determined at one time point [13], comparative and integrative understanding of the avian system could not be achieved.

It is known that the core body temperature of many animals fluctuates during a 24-h period, decreasing in diurnal species at night, when spermatogenesis has been previously hypothesized to occur in the class Aves. Information regarding the circadian patterns of body temperature of birds, which may affect spermatogenesis, has been documented with respect to core body temperature in quail under normal light:dark conditions [26], and in chickens under constant light regimes [27]. In addition, the circadian rhythm of chicken brain temperatures under light:dark cycles and con-

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stant light has been examined [28]. The data for the domestic fowl indicate that the body temperature does fluctuate over a 24-h period, and in the body and the brain these temperature fluctuations are maintained even under constant light conditions [27, 28]. Likewise, mammals such as humans, tree shrews, and golden hamsters exhibit robust circadian temperature fluctuations under light:dark and constant light conditions [29, 30]. However, no work has documented the circadian or daily rhythms of testis temperature in the domestic fowl or any other bird. We therefore reinvestigated avian testis operating temperature. We expanded the work to include continuous studies of testis temperature over daily periods under various light regimes. Our data allowed us to determine the temperature of the testis and the extent of the circadian temperature change, as well as to ascertain whether this change would be sufficient to permit spermatogenesis to occur at night during periods of significant temperature decrease.

MATERIALS AND METHODS

Experiment 1. Determination of the Temperature of the Testis, Liver, and Peritoneum of the Anesthetized Fowl

Initial characterization of the temperature gradients within the body cavity was carried out to determine the temperature of the testis of the domestic fowl. Individual birds were monitored in experiment 1 while under anesthesia in order to establish the temperature of the domestic fowl's testis in situ. Six adult male birds were used. Each bird was anesthetized with i.v. sodium pentobarbital (100 mg) and immobilized on its back. The dosage of pentobarbital was sufficient to maintain the birds in a surgical plane of anesthesia for the duration of the experiment. A 2-inch incision was made immediately caudal to the last rib. Copper: constantan thermocouples (36 gauge), calibrated for accuracy of ± 0.1°C, were inserted into the testis and liver and then held in place with tissue adhesive (Nexaband; Veterinary Products Laboratories, Phoenix, AZ). A third thermocouple was placed into the visceral mass, and the incision was clamped shut. The bird was allowed a 30-min recovery period to regain temperature homeostasis before temperature readings were recorded while the bird was on the surgical table. The calibrated thermocouple probes used in each experiment were attached to a Campbell Scientific CR-21x Micrologger (Logan, UT) that recorded temperatures every 15 sec, and 5-min averages were recorded for later recovery. In experiment 1, data were logged for each bird over a 1-h period between 0900 and 1600 h. The birds were then killed with an overdose of sodium thiopental, and placement of the thermocouples in the testis and the liver was confirmed. Mean temperature values were calculated for each bird for each probe over the hour period and for all six birds combined. Data were compared by inspection of the approximate 95% confidence intervals of the means.

Experiment 2. Diurnal Rhythms of Temperature of the Testis, Liver, and Peritoneum of the Unanesthetized Fowl

In order to determine the temperature patterns of fully alert birds, and to determine the temperature of the testis during a 24-h period under a light:dark cycle, adult male fowl approximately 2.5 kg were anesthetized i.m. with Telazol (15 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA) and maintained at a surgical plane of anesthesia with isoflurane. The incision and placement of the thermocouple probes were made as described in experiment 1, and the incision was closed. The ends of the probes were tunneled s.c., exiting at the base of the neck. The birds were injected with 0.2 ml of the antimicrobial Baytril (2.27%; Miles, Inc., Shawnee Mission, KS) and allowed to fully recover while being warmed by heating pads. They were then placed in stainless steel cages, 25 × 50 × 50 cm, contained within Research Equipment Company isolation cubicles (CV-800-S; Bryan, TX). Ambient temperature within the cubicles was maintained at 25°C with 10 changes of outside air per hour. Illumination was provided by two strips of 40-W fluorescent lights that resulted in an intensity of 400 lux at the front of the cages. The birds were given food and water ad libitum. After recovering from anesthesia, the tethered birds were maintained in the cages under a 13L:11D cycle and data were collected for 85–110 h. Temperature readings were taken every 15 sec and then recorded as averages over 5 min. Mean temperature values were calculated for each of the six birds for each hour period of the day. The individual hour temperatures for each bird were then averaged for all birds per hour period. Calculations were performed to determine the average low and high temperatures, the amplitude of the wave functions, and the mean temperatures for each bird and all birds over the entire period.

Experiment 3. Effect of Constant Light on Temperature Rhythms

One argument that has been presented is that birds, like mammals, have a lower temperature requirement for spermatogenesis with spermatogenesis occurring at night when body temperatures are lower [12, 15]. Because body temperature rhythms persist under constant light in the domestic fowl [27], experiment 3 was designed to determine whether the testis temperature rhythm demonstrated in experiment 2 also persists under constant light. If the temperature rhythm was altered, we could then address the issue of spermatogenesis in a new temperature environment. Birds in experiment 3 were implanted with thermocouples as described for experiment 2. The animals were placed in cages with thermal and lighting conditions as described above; however, they were subjected to constant light (400 lux) after 4–14 days of pretreatment at constant light. Temperatures were monitored in these seven birds for at least 80 h. Temperature readings were taken every 15 sec and then recorded as averages every 5 min. As above, all individual birds' hourly temperatures were averaged for all birds per hour period. The overall mean, mean low and high temperatures, and amplitude of the curves were calculated for each bird and for all birds over all times.

Experiment 4. Effect of Constant Light on Testis Function and Structure

Published literature suggests that the domestic fowl is different from the sparrow and the pigeon [31–33] in that its temperature rhythm is dampened, but not extinguished, under constant light ([28]; this study). However, if the temperature differential seen under normal light:dark conditions is required for spermatogenesis, then birds maintained under constant light for 28 days (two complete cycles of the seminiferous epithelium) should exhibit altered morphology of their seminiferous tubules and a reduction in sperm production. To determine whether maintenance of chickens under constant light for an extended period affects testis function and structure, 10 adult male birds were kept under constant light for 28 days while 7 were maintained in a 13L:11D cycle in environmental chambers. Before

TABLE 1. Testis, liver, and body cavity temperatures of anesthetized adult male fowl.

Bird	Mean temperature[a] in °C ± SE		
	Testis	Liver	Peritoneum
1	40.2 ± 0.1	40.2 ± 0.1	40.2 ± 0.04
2	39.8 ± 0.02	39.9 ± 0.01	39.8 ± 0.02
3	39.4 ± 0.04	38.8 ± 0.1	39.5 ± 0.1
4	39.4 ± 0.02	39.5 ± 0.02	38.3 ± 0.1
5	39.8 ± 0.1	40.0 ± 0.1	39.8 ± 0.1
6	38.2 ± 0.01	38.0 ± 0.02	37.8 ± 0.1
Overall mean	39.5 ± 0.3	39.4 ± 0.3	39.2 ± 0.4

[a] Temperature values represent the mean of 13 five-minute intervals obtained over an hour period.

birds were placed into the chamber, a blood sample (5 ml) was obtained from the brachial vein of each bird. The birds were housed for the duration of the experiment in environmental chambers in floor pens of approximately 2.5 × 4.6 m with an ambient temperature of 25°C. The chambers were lit by two 100-W ceiling bulbs resulting in an intensity of 30 lux at floor level. The birds were given a maintenance diet and water ad libitum. After 28 days, a second blood sample was obtained, the birds were killed by CO_2 asphyxiation, and the testes were removed and weighed.

Analysis of testis function and structure. 1. Sperm production. Homogenization-resistant sperm heads were isolated by first blending approximately 2 g of parenchymal tissue from the left testis of each bird in STM buffer (0.5% Triton X-100, 0.15 M NaCl, 0.25 mM merthiolate) and diluting 1:5 in 0.15 M NaCl as described previously [34]. Homogenization-resistant sperm heads were counted (in quadruplicate); the average number for each bird and for all birds in each treatment were calculated as sperm/g per day and sperm/testis per day using 4.5 days as the average time elongated spermatids remain in the testis before passing into the excurrent ducts [35].

2. Testosterone levels. Testosterone levels were determined by RIA as described previously [36, 37]. Again, levels for each bird and means for each group for testosterone levels before and after the experimental period were determined.

3. Testis structure. The weight of each bird's left testis was recorded at the termination of the treatment period. Testis morphology was examined microscopically. The right testis from each bird was removed, and a 4-mm slice was placed into Bouin's solution (75% picric acid [v:v], 0.0925% formalin, 5% acetic acid) to fix overnight. The slices were transferred to 10% buffered formalin, embedded in paraffin, and sectioned at 2 μm. All sections were stained with periodic acid-Schiff and hematoxylin as previously described [37]. Histological analysis was performed by visual inspection of the sections under the light microscope to discern the structure of the seminiferous tubules.

Statistical Analyses

We analyzed differences between light treatments over time by using repeated measures analysis of variance on 24-h temperature profiles derived from hourly average temperatures from each individual. Paired sample *t*-tests were used to compare the high and low temperatures for each bird in each treatment, while independent sample *t*-tests were used to judge significance of observed differences in temperatures between the birds in each group. Sperm production, testosterone values, and testis weight for each bird in each group were compared using an independent samples *t*-test. Comparisons within a treatment for testosterone levels were done by paired samples *t*-test. The type one error level was set to the value of $p < 0.05$. The data are presented as the mean ± SE.

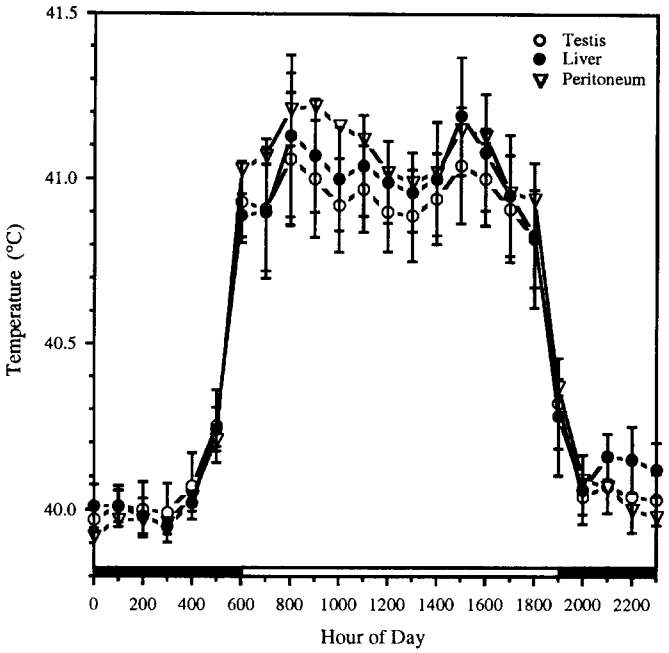

FIG. 1. Testis, liver, and peritoneum exhibit indistinguishable temperature rhythms under a light:dark cycle. Birds were maintained under a 13L: 11D cycle. Bars under the horizontal axis represent periods of dark (closed bars) and light (open bar). Mean temperature values are presented for this group of birds for individual hour periods during the day. Error bars on the vertical axis are 1 SE around the mean.

RESULTS

Testis, Liver, and Peritoneum Have Statistically Indistinguishable Temperatures

The temperature averages for three probes (testis, liver, and peritoneum) for six birds monitored for 1-h periods while under anesthesia in experiment 1 showed that there was no significant difference between the temperatures of the testis, liver, and body cavity with the domestic fowl under anesthesia (Table 1). The temperature of the testis was equivalent to that of the liver (i.e., the core body temperature), given the low level of measurement error.

Testis, Liver, and Peritoneum Exhibit Indistinguishable Circadian Rhythms under a Light:Dark Cycle

Six birds monitored for extended periods with a 13L: 11D cycle in experiment 2 showed clear circadian rhythm in body temperature and no difference between the temperatures of the testis, liver, and peritoneum, as outlined above (Fig. 1). Lights were turned on at 0600 h and were extinguished at 1900 h as indicated by the bars on the horizontal axis. The temperatures had a basic wave form with the amplitude of 1.0°C (SE = 0.1°C). At night the temperatures were at their lowest (40.0°C), and they began to rise just prior to lights-on. The temperatures maintained a plateau during the day (41.0°C) and then decreased again as the lights went off. In fact, the animals appeared to be anticipating the change in lighting conditions, as changes in temperature (increases and decreases) appeared prior to the changes in lighting. The circadian period was 24 h, as ex-

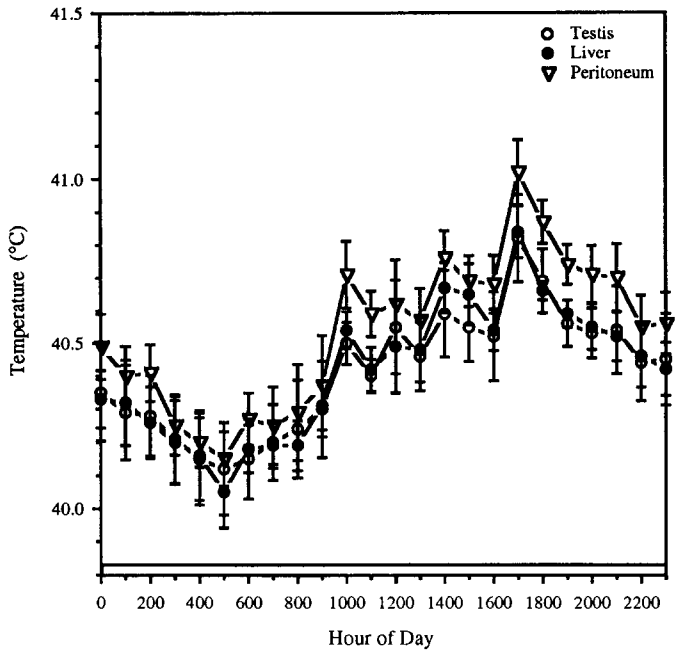

FIG. 2. Testis, liver, and peritoneum maintain indistinguishable circadian rhythms under constant conditions. As in Figure 1, but with seven birds under constant light conditions (400 lux) after 4–14 days of pretreatment at constant light. The bar under the horizontal axis represents the period of light (open bar) for a 24-h light cycle. All temperature readings for each hour period were averaged for all birds and plotted vs. the hour period. Error bars on the vertical axis are 1 SE around the mean.

pected for an entrained state, with the phases set by the light cycle. The mean or mesor temperature was 40.6°C (SE = 0.1°C), slightly above that seen while the birds were under anesthesia.

Testis, Liver, and Peritoneum Maintain Indistinguishable Circadian Rhythms under Constant Conditions

Seven birds maintained for extended periods in constant light in experiment 3 exhibited identical average temperatures for all three probes (testis, liver, and peritoneum) for every hour period (Fig. 2). Although the temperature profiles of these birds are dampened as compared to the light: dark profiles, the temperatures still maintained a cosine wave function throughout a 24-h period (Fig. 2). Interestingly, the acrophase of the wave form was during the subjective day and the period appeared to be 24 h. The amplitude of the constant light curve was 0.4°C (SE = 0.04°C) and therefore was significantly decreased as compared with the light:dark data ($p < 0.0001$, $t = 5.334$, $df = 11$). However, the mean or mesor temperature was not significantly different (40.4°C ± SE = 0.04; repeated measures $p = 0.229$, $f = 1.622$, $df = 1$), with a minimum temperature of 40.2°C and a maximum of 40.6°C. Repeated measures analysis indicated that the changes in temperature over a 24-h period were significantly different ($p < 0.0001$, $f = 25.434$, $df = 23$) within each light treatment. Additionally, the analysis indicated that the temperature wave forms of the two light treatments cycle in a significantly different manner over time ($p < 0.0001$, $f = 12.110$, $df = 23$).

Constant Light Conditions Do Not Affect Sperm Production, Testosterone Production, or Testis Weight or Testis Morphology

After treatment with either constant light or normal light cycles, testes from each treated bird were analyzed for function and structure as determined by sperm production, testosterone level, and weight of the intact left testis (Table 2). Values for each treatment were averaged for all birds and are presented with one standard error around the mean. Neither sperm production ($p = 0.818$, $t = 0.238$, $df = 8.9$) nor left testis weight ($p = 0.274$, $t = 1.174$, $df = 8.1$) was different between treatments. Likewise, testosterone levels between treatments before and after maintenance at the two light regimes ($p = 0.404$, $t = -0.861$, $df = 13.5$, and $p = 0.408$, $t = 0.856$, $df = 12.1$, respectively), and changes of testosterone level within a treatment, showed no significant differences between the determined values (L:D $p = 0.840$, $t = -0.210$, $df = 6$; L:L $p = 0.166$, $t = -1.508$, $df = 9$). The morphology of the right testis from each bird was also examined microscopically for changes in the tubule structure and organization. There were no apparent differences between the samples of a testis from a bird maintained under the light:dark cycle (Fig. 3a), and one from a bird maintained under constant light (Fig. 3b).

DISCUSSION

Through the use of implanted thermocouples and continuous temperature monitoring of the testis and liver temperatures of the domestic fowl, we have determined that the domestic fowl's testicular temperature is equivalent to core body temperature, confirming previous isolated temperature records [13] and expanding on the study of temperature gradients in the fowl. It is apparent from the data that the testis is not cooled by association with an air sac and, indeed, is not cooled by any mechanism. Therefore, spermatogenesis occurs in the domestic fowl at the core body temperature of 40–41°C. Our results provide evidence for the uniqueness of spermatogenesis in the avian testis as compared to that of the mammals examined thus far, in which spermatogenesis occurs at 33–35°C.

Continuous monitoring for extended periods allowed us

TABLE 2. Testis function and structure analysis after treatment of birds to differing light regimens.

Parameter	Light:Dark* (n = 7)	Light:Light* (n = 10)
Sperm production		
Number/g/day	$129 \times 10^6 \pm 17.0 \times 10^6$	$124 \times 10^6 \pm 8.26 \times 10^6$
Number/testis/day	$2.71 \times 10^9 \pm 0.42 \times 10^9$	$2.23 \times 10^9 \pm 0.31 \times 10^9$
Testosterone level		
Initial (pg/ml)	1261 ± 220	649 ± 393
Final (pg/ml)	1183 ± 369	788 ± 276
Left testis weight (g)	24.71 ± 5.05	18.30 ± 2.09

* Mean value ± SE.

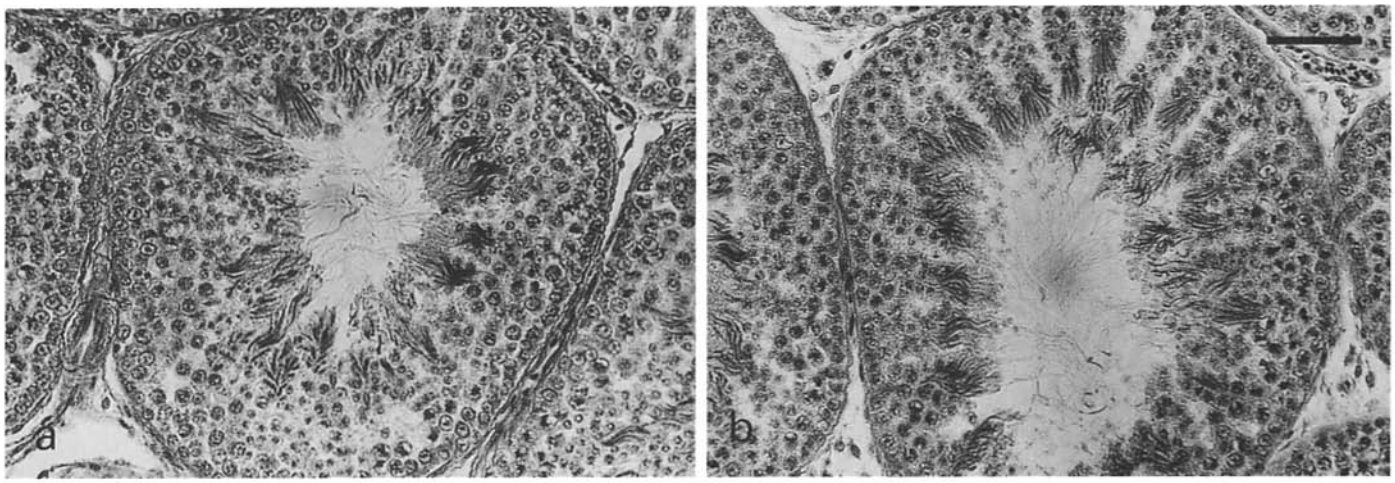

FIG. 3. Testes from birds maintained under a constant light regime exhibit no differences in seminiferous tubule morphology (**b**) as compared with testes from birds maintained under a light:dark cycle (**a**). Ten adult male chickens were maintained under constant light for 28 days, while seven were maintained in a 13L:11D cycle in environmental chambers. Testes were removed and sections were stained with periodic acid-Schiff and hematoxylin. Bar = 50 μm.

to quantify the temperature fluctuation of the testis, liver, and body cavity of the chicken over time under different lighting conditions. Under no condition did the testis temperature differ from that of the liver. In addition, we have shown that the body and testis temperatures exhibit a circadian rhythm with the acrophase occurring during the light periods. The amplitude of the wave function, reflecting the changes in temperature over 24 h, is slightly lower for the liver and the testis than that reported for the chicken brain (1.0°C vs. 1.5°C [28]); but the shape of the curves and the overall mean temperature values are comparable. In fact, the wave form of the testis/liver temperatures from the chicken are quite similar to those temperatures seen after long-term body temperature monitoring of the Japanese quail under normal light:dark periods [26]. Furthermore, as seen in this and previous domestic fowl studies, the animals appear to be anticipating changes in lighting conditions with preparatory increases or decreases in body temperatures [28, 38] possibly due to changes in activity.

The temperature fluctuations observed under standard light:dark conditions continue even under constant light conditions. However, the wave is compressed in animals maintained in constant light conditions compared to light:dark conditions as indicated by a decrease in amplitude of the temperature waves (1.0–0.4°C). Mean daily temperatures, neverless, remain indistinguishable between the two conditions. In the class Aves, this response to constant light conditions varies. Chickens apparently do not lose rhythmic temperature patterns under constant light conditions ([27, 39]; this study). Previous investigators also found that the free-running periods for the domestic fowl were approximately 24 h [27, 28], which is in agreement with observations here. In addition, under constant light conditions there is a compression of the normal wave function of the brain temperatures [28]. However, both of these earlier studies found higher-amplitude values of 0.85°C [27] and 1°C [28] under the light:light regime. In contrast, exposure to high-intensity constant light abolishes circadian behavioral rhythms in both sparrows and pigeons [31–33]. Therefore, the wave form of the chicken data presented above indicates that the temperature profiles for the testis and liver are a true circadian rhythm since the pattern persists, although dampened, in the absence of external time cues.

The amplitude of the temperature rhythm during a typical light:dark cycle is 1°C. In mammals, the decrease in testis temperature as compared to core body temperature ranges from 5 to 8°C. If the mechanisms involved in avian spermatogenesis are analogous to those in mammalian spermatogenesis, then the small fluctuation in testis temperature during a 24-h period should be insufficient to meet any requisite temperature reduction. In fact, male fowl maintained under either normal (light:dark) or constant light conditions, with an amplitude of 0.4°C, undergo spermatogenesis equivalently and have normal testis function and structure. This implies that there must be another compensatory or alternate mechanism that allows for spermatogenesis in the domestic fowl's testis at core body temperature.

Our data raise interesting questions relative to reproductive fitness and evolution. For example, why have most mammals evolved external (and cooler) testes, which makes the testes (and most importantly, the genetic potential they contain) much more vulnerable, while the other predominant homeothermic group, Aves, have evolved testes that function efficiently at elevated core body temperatures? Also, with the evolution of birds and mammals from early reptiles, the fact that birds appear to have spermatogenesis that occurs at body temperature, while mammalian testes do not, is noteworthy. Depending on the qualities of the ancestors of these groups, it is reasonable to question why during evolution of the mammals they either lost the ability to undergo spermatogenesis at high body temperature, or why the germ cells did not adjust to an increase in temperature as somatic cells have done. Alternatively, why were birds able to maintain efficient spermatogenesis at a relatively high body temperature, or why were they able to adapt to the increase in testis temperature during evolution? Regardless of the evolutionary history, it is now apparent that birds and mammals have fundamental differences in the mechanisms of spermatogenesis.

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