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Individual variation in avian reproductive physiology does not reliably predict variation in laying date

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Abstract

Most animals reproduce seasonally. They time their reproduction in response to environmental cues, like increasing photoperiod and temperature, which are predictive for the time of high food availability. Although individuals of a population use the same cues, they vary in their onset of reproduction, with some animals reproducing consistently early or late. In avian research, timing of reproduction often refers to the laying date of the first egg, which is a key determinant of fitness. Experiments measuring temporal patterns of reproductive hormone concentrations or gonadal size under controlled conditions in response to a cue commonly assume that these proxies are indicative of the timing of egg laying. This assumption often remains untested, with few studies reporting both reproductive development and the onset of laying. We kept in total 144 pairs of great tits (*Parus major*) in separate climate-controlled aviaries over 4 years to correlate pre-breeding plasma luteinizing hormone (LH), prolactin (PRL) and gonadal growth with the timing of laying. Individuals varied consistently in hormone concentrations over spring, but this was not directly related to the timing of gonadal growth, nor with the laying date of the first egg. The timing of gonadal development in both sexes was similarly not correlated with the timing of laying. This demonstrates the female's ability to adjust the onset of laying to environmental conditions irrespective of substantial differences in pre-laying development. We conclude that stages of reproductive development are regulated by different cues, and therefore egg laying dates need to be studied to measure the influences of environmental cues on timing of seasonal reproduction.

Keywords

seasonal timing of reproduction, laying date, *Parus major*, luteinizing hormone, prolactin, gonadal development

1. Introduction

Seasonal timing of reproduction is a key life-history trait with a large impact on reproductive output. A mismatch between reproduction and seasonal high food abundance leads to fewer surviving, and lower quality offspring, or lower winter survival of the parents [7, 24, 32, 39, 40, 42]. In avian research, timing of reproduction often refers to the laying date of the first egg in spring [41]. However, the initiation of gonadal growth and the underlying activation of the reproductive endocrine system is also part of the timing mechanism [5, 8, 13, 14, 17, 20, 23, 34, 37]. This dual vision originates from the fact that evolutionary ecologists are more concerned with behavioral decisions and their fitness consequences, while physiologists are by definition more interested in the proximate mechanisms underlying a certain phenotype, such as gonadal growth and ovulation. Experimental studies combining ecological and physiological approaches to the timing of reproduction have increased understanding of this life history trait [e.g. 4, 5, 27, 43, 46] and are thus especially valuable.

In temperate zone birds, the actual process of egg laying is preceded by a physiological cascade mediated by neuroendocrine responses to environmental cues. Egg laying is preceded by the (re-) activation of the hypothalamic pituitary-gonadal axis by short photoperiods during fall causing the dissipation of photorefractoriness and increased GnRH-I gene expression [36]. During winter and early spring the increase in day length stimulates increased secretion of GnRH-I, leading to a release of luteinizing and follicle stimulating hormone (LH and FSH) from the pituitary and a period of gonadal development that lasts several weeks. LH and FSH act synergistically to facilitate gonadal maturation and spermatogenesis: at the level of the gonads FSH affects Sertoli cell function in males and granulosa cell function in females and stimulates growth of immature follicles in the ovary. LH affects Leydig cell function and stimulates the secretion of androgens in males, while an acute surge in LH triggers ovulation in females. These photoinduced processes, culminating in the laying of the first egg, are fine-tuned by supplementary cues, including temperature, and possibly other climatic and phenological cues, including the seasonality of prey items [9, 47, 51].

Due to the difficulties in measuring laying dates in captivity in response to a likely cue, manipulative experiments make use of proxies that are presumed to indicate the timing of egg laying, but are also studied for their own sake [e.g. 11, 15, 18, 30, 33,

38, 48-50]. Ideally for getting independent data points, pairs of birds would be kept in isolated aviaries, in which environmental variables can be individually regulated. However, this is often not feasible and in many manipulative experiments, the shortcut of examining reproductive physiology instead of a laying date allows for a larger sample size, e.g. many animals (of only one sex) per room or cage, as well as for shorter and less complex experimental designs, as the laying stage does not have to be reached. The most widely used proxies in avian research are, on one hand, gonadal growth, which means the increase in volume of the male left testis, or, more rarely [2] the development of the largest follicle in the female ovary, as well as plasma concentrations of gonadotropins, prolactin, or sex steroids, measured either in the blood or in feces. These measures can be taken at regular intervals during different reproductive stages. More recently, also processes higher upstream in the hypothalamo-pituitary-gonadal (HPG) axis have been added to the physiologist's toolbox, including the release of GnRH-I [20, 36], or even gene expression [19, 22]. Emphasis has been placed on photic cues, which determine a broad window for egg laying [10, 29], whereas the influence of supplementary cues has been largely neglected. Conversely, interest in processes closely associated with late reproductive stages, such as the exponential growth phase of the follicle, is increasing, using yolk precursors such as vitellogenin or very low density lipoproteins as proxies [6, 26]. This avenue also investigates supplementary cues that might be taken into account in the last days before the actual egg laying takes place.

In studies concentrating on the regulation of the reproductive development by its own means, observations should be made in the context of their adaptive value, most importantly relating to the optimal timing of laying. The way in which an individual female responds to environmental cues affects selection pressures acting on both reproductive physiology, as well as timing of laying [41]. Evolution therefore optimizes both the systems of physiological regulation themselves, as well as the behavioural traits that they precede. For example, birds presumably regress their gonads outside the breeding season, because flying with heavier body weights year-round is costly and thus selected against. This makes a phase of gonadal growth in early spring necessary. Also, even though early laying is generally advantageous, as it results in more surviving offspring in that particular year, advancing the physiological development early in spring when food availability is low may impede fitness costs that counterweight these advantages.

The responsiveness to cues might change over developmental stages. It is convenient to assume that a cue, like temperature, which advances the underlying hormonal and gonadal development would also advance egg laying. Indeed, it has often been postulated that temperature influences the timing of reproduction because of an effect on the gonadal development [8, 48]. However, Schaper et al. [27] showed that in climate-controlled aviaries, moderate spring temperature patterns influenced laying dates of great tits (*Parus major*) without affecting the timing of gonadal growth or increase in LH concentration.

The assumption that an early rise in gonadotropins would directly translate to early gonadal development, which again would lead to an early onset of laying, has, to our knowledge, never been explicitly tested under controlled conditions. This is basically due to the fact that few experimental studies that report laying dates also measure reproductive physiology, and studies that evaluate reproductive development seldom keep pairs of birds to obtain independent laying dates. In addition, individual variation in physiological measurements is seldom explored in detail, as physiologists mostly report mean values per treatment group in response to environmental stimuli [45].

The aim of this study was to use breeding pairs of great tits to investigate if the relationship between the timing of individual early reproductive development and egg laying is as tight as assumed, or alternatively regulated by different processes, resulting in substantial variation in the interval between, for instance, full gonadal development and laying date. Although the prime objective of the experiments presented here was to show the influence of temperature cues on avian physiology and the onset of laying, the setup allows us to relate the timing of the individual rise in LH, PRL, as well as the growth of testes and ovarian follicles to laying date. This study does not include measurements of late stages of the reproductive maturation, such as yolk precursors. These changes, which are connected to an increase in estradiol following gonadal maturation, are tightly correlated with the laying date decision and most likely happen during the last days pre-laying. In the current setup we cannot comment on the feasability of using these measures as proxies. We were interested in physiological mechanisms that determine the individual variation in the onset of laying in response to environmental cues perceived well in advance of the laying date, which in wild great tits varies by up to one month between individual females and can therefore not be significantly regulated by differences in late reproductive maturation.

If predictive supplementary cues affect reproductive physiology, and consequently egg laying via early reproductive development in early spring, we expect a relationship between the timing of a rise in LH, gonadal development and laying date. In contrast, if physiological processes are fine-tuned by different cues, we expect only a loose relationship between these reproductive components and the timing of laying. In addtion, it was suggested that variation in pre-laying PRL titers was associated with laying dates in house *sparrows, Passer domesticus* [21] and with egg laying rate in chicken*, Gallus gallus domesticus*, and thus would play a stimulatory role in gonadal development [16], We therefore tested for a correlation between plasma PRL concentrations pre-laying and laying dates.

2. Materials and Methods

2.1 Birds

This study used 144 first-year breeding pairs of great tits spread over four years. Birds were offspring of known wild parents at the Hoge Veluwe National Park (the Netherlands), and were taken to captivity as complete broods in 2006 to 2009, respectively. On day 10 post-hatching, chicks were taken to the Netherlands Institute of Ecology (Heteren) for hand-raising [12].

After independence, fledglings were transferred to single-sex groups in open outdoor aviaries (2 x 4 x 2.5 m), where they were housed until December. Breeding pairs were formed randomly, avoiding sib-matings. Due to fatalities in the young birds, we formed some pairs by using 29 additional spare birds over 4 years, which were hand-raised in the same fashion. On the 1st of December the pairs were placed in climate-controlled aviaries to breed in the next year.

2.2 Aviaries

Breeding pairs were housed in 36 separate indoor aviaries (2 x 2 x 2.25 m) under a light regime mimicking the natural photoperiod, which was adapted twice weekly (i.e. for 52°N increasing from 7.45L:16.15D at the winter solstice to 16.30L:7.30D at the summer solstice). Birds were exposed to the same seasonal variation in photoperiod in all four experimental years. Light sources were three high frequency fluorescent light tubes, complemented with an 8 W bulb providing an additional half hour of dawn and dusk. A shaft from the roof (SolaTube), whose opening was synchronized with the light schedule, allowed for supplementary daylight.

The birds were fed *ad libitum* with a constant daily amount of food, consisting of a mixture of minced beef, proteins and vitamin and mineral supplements (Nekton S and Nekton Bio, NEKTON GmbH, Pforzheim), completed by sunflower seeds, fat balls, a mix of dried insects (Carnizoo, Kiezebrink International, Putten), calcium and water for drinking and bathing. Nesting material was provided from March onwards. Birds could choose between two nest boxes, which were inspected for eggs from outside the aviary without disturbance.

2.3 Temperature treatments

Over four experimental years, four times 36 pairs of birds were exposed to varying temperature regimes. Each season, a different experimental setup of four temperature treatments was used, each treatment being replicated in a regular design. For a rationale and thorough description of temperature treatments, see Visser et al. [43] and Schaper et al. [27].

In 2007, the great tits were divided into two groups that differed in the ambient temperature to which they were exposed, with the high temperature treatment set to be always 4°C higher than the cold temperature. From 1st December to the end of February temperatures were kept constant at 4 and 8°C, respectively, after which temperatures gradually increased by 0.65°C per week up to 1st July, reaching 15 and 19°C, respectively (Fig. 1A). This setup was chosen to identify if a difference in mean temperature comparable to the difference between a natural cold and warm year leads to a difference in the onset of egg laying (see [43] for a more detailed rationale).

In 2008, all pairs were exposed to a constant temperature of 15°C from December onwards until summer. In three groups, this temperature was lowered to 7°C in either February, March or April for a month, before it was increased to 15°C again, except for the latest cold period (April), which was maintained until the female initiated laying under cold conditions (Fig. 1B). This setup was chosen to identify if the responsiveness to temperature cues increased over time and thus temperature changes close to the onset of laying has a larger influence on laying dates than changes early in the season (see [27] for a more detailed rationale).

In 2009, there was no seasonal temperature pattern, but a temperature change over the day. Each treatment was composed of a high or low mean with either a high or

low day-night amplitude. The two warm treatments were fluctuating around a mean of 14°C (11-17°C, high amplitude, or 13-15°C, low amplitude), while the two cold treatments were fluctuating around 8°C (5-11°C, high amplitude, or 7-9°C, low amplitude) In all cases the lowest daily temperature was reached at 3 am (Fig. 1C). This setup was chosen to identify if the daily variation in temperature, which seasonally increases in spring, has an influence on the onset of laying (see [27]).

In 2010, the setup of the experiment combined two consecutive temperature rises, one during early gonadal development, the other shortly before breeding. All birds were kept at 6°C from December until February. On 8th February, the first two groups experienced a rapid increase in temperature from 6 to 16°C over a course of two weeks, then stayed at 16°C for three or five weeks. On 15th or 29th March, respectively, temperature was increased to 20°C and stayed high during egg laying and molt. Starting on 22nd February, the other two groups were exposed to a more gradual increase in temperature from 6 to 11°C over a course of two weeks, thus experiencing a lower increase rate. These groups then stayed at 11°C for one or three weeks. On 15th or 29th March, respectively, temperatures increased to 15°C for egg laying and molt. Superimposed on the temperature profiles was a day-night rhythm of ± 1°C (Fig. 1D). This setup was chosen to identify if temperature increases close to laying have a higher impact on laying dates than temperature increases in early spring and furthermore if differences in the rate of temperature increase or the timing of the increase in early spring influence laying dates (see [27]).

As shown in Visser et al. [43] and Schaper et al. [27], temperature treatments affected neither the increase in plasma luteinizing hormone, nor the development of female or male gonads, while it affected the onset of laying in 2008 and 2010, but not in 2007 and 2009.

2.4 Data collection

A blood sample of 100 µl was taken monthly from the jugular vein. Samples were kept on ice until centrifugation, plasma was separated from red blood cells and stored at -80°C. In 2007, blood samples were analyzed for prolactin (PRL), in 2008-2010 for luteinizing hormone (LH). No blood sample was taken in January and February 2010 prior to the assessment of gonadal size. Plasma LH concentrations were determined using a chicken LH radioimmunoassay [31] validated for use in blue tits [5]. Plasma PRL concentrations were determined using a recombinant derived starling prolactin radioimmunoassay [3]. The reaction volume was 60 µl comprising

20 µl of plasma sample or standard, 20 µl of primary antibody (rabbit anti-LH or PRL), and 20 µl of ^{125}I-labeled LH or PRL. The primary antibody was precipitated to separate free and bound ^{125}I label using 20 µl of donkey anti-rabbit precipitating serum and 20 µl of non-immune rabbit serum. All samples from each year were measured in a single assay, in duplicate. The intra-assay coefficient of variation for LH was 6.4% for a high value pool and 8.1% for a low value pool, the minimum detectable dose 0.15 ng/ml. The intra-assay coefficient of variation for the prolactin assay was 6.5%, and the minimum detectable dose 1.6 ng/ml.

Alternating in two-week intervals with the blood sampling, a laparotomy was performed monthly to measure gonadal development in 2008-2010. Males were laparotomized from January to July and females up to April in order not to interfere with the laying process. However, in 2009 females were not laparotomized in April, with no apparent effect on the onset of laying, and in 2010 both sexes were not laparotomized in January, as previous years showed little variation in gonad sizes during winter. Birds were unilaterally laparotomized under anesthesia with isoflurane (Forene, Abbott, Hoofddorp, The Netherlands). Left testis dimensions and diameter of the largest developing follicle in the ovary were measured to the nearest 0.1 mm, using a scale engraved in the ocular of a binocular microscope. Testis volume was calculated as: $V = 4/3\ \pi a^2 b$, where a is ½ width and b is ½ length, follicle volume as: $V = 4/3\ \pi a^3$, where a is ½ width. In April 2008, three females with complete nests were not laparotomized in order not to interfere with the laying decision. Assuming a maximum follicle size of 7 mm^3 for them and including them in the dataset did not qualitatively change the results. Data are not available for all individuals each month due to sampling or assay failure. In total, 17 measures of male and female gonads each, and 34 LH values are missing.

After nest building was observed, nest boxes were checked daily for eggs. The day that the first egg was found is referred to as the laying date or date of onset of reproduction.

2.5 Statistics

The influence of LH concentrations in 2008-2010 on gonadal sizes were analyzed with mixed models [procedure lmer, package lme4 in R 2.10.0, 25]. Data on gonadal maturation and LH concentrations were natural log-transformed and analyzed per month from February to April. Family was fitted as a random effect and LH of the two

previous months as fixed effects. Models also included year as a factor, and a variable indicating if a pair was laying afterwards or not. To correct for body size differences in gonadal sizes, tarsus length was included. In 2010, no LH sample was taken in January and early February, hence the effect of LH on gonadal development in February/March was tested in only two years. An alternative analysis in March including all years, so only LH concentrations in March, did not show significant correlations (data not presented). As LH in January was correlated with follicle growth in February, it was additionally included in the model for March. Follicles were not measured in April 2009, restricting this analysis to two years.

The influences of LH concentrations and gonadal sizes in March and April on the timing of egg laying in 2008-2010 were analyzed in a mixed model, including year, as well as female family as a random effect. The influence of PRL concentrations in March and April 2007 on the timing of laying were analyzed in a mixed model, including female family as a random effect.

We used a stepwise model reduction procedure to eliminate non-significant effects. If more than one fixed factor remained significant, mostly in combination with year, the interaction between the variables was additionally tested in the final model. However, none of these interactions were significant (all $p>0.1$, data not shown). We used Markov Chain Monte Carlo sampling to calculate p-values (function pvals.fnc from package languageR, in R 2.10.0). The results are presented including Bayesian 95% highest posterior density credible intervals, equivalent to 95% confidence intervals. As year is given as a multi-level fixed factor in some analyses, a p-value is created for every level in comparison to the year 2008.

3. Results

3.1 Relationship between individual variation in LH titers and gonadal development

Plasma LH concentrations of females and males increased over spring and peaked around March/April (Fig. 2). Individuals varied consistently in hormone concentrations over spring (assessed via Kendall's coefficient of concordance, females: $W=0.60$, males: $W=0.69$, both $P<0.001$, both $n = 108$ birds over three years). Individual birds showed substantial variation in the timing of the seasonal increase, leading to substantial differences in LH concentrations in April (Fig. 2, unlogged range females: 0.37 to 5.89 ng/ml, males: 0.36 to 5.88 ng/ml). Especially in 2008 and 2009, few

individuals either increased earlier than average, or showed elevated titers in general (Fig. 2). Individual variation in plasma LH concentration in a given month was unrelated to sampling time (P=0.85 for time of day, analyzed with a generalized linear model including year, month and sex as fixed factors and family as a random factor).

The development of the largest follicle in the ovary, which is incorporated into the first egg laid, followed an exponential growth pattern, with a slow maturation phase during January to March, well in advance of laying, and an exponential growth phase in April (Fig. 3). There were large individual differences in follicle volume in April (Fig. 2, unlogged range: 0.03 to 6.37 mm^3). These were probably caused by variation in the timing of the onset of exponential growth, as females differed noticeably in early gonadal development, but the state of maturity did not progress consistently over time across females (Kendall's W=0.37, p=0.042, n = 108).

To investigate the causes of these individual differences, we first explored whether gonadal development was linked to plasma LH concentrations in the same or previous months. Females with high LH concentrations in January had larger follicles in February and March (Table 1, Fig. 4 A,B). Increased LH concentrations in February, March and April did, however, not relate to large follicle sizes in the same or the following month. Non-laying females were characterized by smaller follicles in April compared to females that were going to lay (Table 1).

Testes increased exponentially in volume from January/February onwards, in most cases reaching a fully developed state around April (Fig. 3), before regressing again in May (data not shown). Individual males varied in the timing and speed of testis growth, which led to rather consistent differences in testis volume over time (Kendall's W=0.52, p<0.001, n = 108), and large differences in testis volume in April (Fig. 3, unlogged range: 4.54 to 171.91 mm^3). There was no relationship between plasma LH concentrations and testis volume in February to March (Table 1). In April, males with larger, fully developed testes had lower circulating LH concentrations than males with still growing testes (Table 1, Fig. 5). Testes in April were on average further developed in 2008 than 2009 or 2010 (Table 1, Fig. 3, 5). Testis volume did not differ between males paired to females that were going to lay eggs or not (Table 1).

3.2 Relationship between individual variation in PRL titers and the onset of laying

In 2007, plasma PRL concentrations increased over spring (Fig. 6), and peak concentrations were reached in May (data not shown). Similar to LH, there was individual variation in the timing and speed of increase in early spring PRL titers, leading to substantial differences in PRL concentrations in April (Fig. 6, unlogged range females: 6.49 to 70.73 ng/ml, males: 1.82 to 103.77 ng/ml). While males showed rather consistent differences in PRL titers over time (Kendall's W=0.43, p=0.018), this could not be confirmed in females (Kendall's W=0.22, p=0.618). There was no relationship between PRL levels in March or April and the onset of laying (all p>0.1, data not shown).

3.3 Relationship between LH titers, gonadal development and the onset of laying

Egg laying started in mid-April, but was on average later in 2008 (Table 2, Fig. 7), when the variation in laying dates between females was also largest. The onset of laying was not related to plasma LH concentrations in previous months (Table 2). Neither the size of the largest developing follicle in April, nor the development of the partner's testis in April predicted laying date (Table 2). However, females with large follicles in March, quite in advance of the rapid growth phase, laid on average earlier than females with less developed follicles in March (Table 2, Fig. 7 A). In addition, males with larger testes in March had mates that initiated laying early (Table 2, Fig. 7 B). This was true even though females with further developed follicles in March were not paired to males with larger testes (linear model, t=0.33, p=0.7). Yet, especially in 2008, the relationship between gonad size in March and laying dates was not particularly tight (Fig. 7). In a linear model only including follicle volume or testis volume by themselves, gonadal size in March only explained a small amount of the variation in laying dates, 1.4% in case of testes and 2.3% in case of follicles, showing that male and female gonad sizes cannot be indicative of the timing of the laying event.

4. Discussion

We kept pairs of great tits under controlled conditions to investigate if pre-laying endocrine changes and gonadal growth correlated with each other and whether their timing was related to the onset of laying. These physiological measurements, often used as proxies for breeding phenology, showed consistent individual variation, but were at best weakly correlated to each other or to the onset of laying. In

consequence, laying dates could not be predicted by comparing sizes of the largest follicles in late spring.

4.1 Females adjusted their timing of laying independent of gonad development

While some females had already functional gonads in April, they seemed to postpone laying in response to environmental information. Visser et al. [43] and Schaper et al. [27] demonstrated that while in this set of birds the pre-laying physiological development was not influenced by temperature treatments, females adjusted their laying date when the right temperature cues were provided. The disconnection between individual hormone levels, gonad sizes and laying dates presented here further validates that reproductive development is not the factor that constrains laying. The ability to fine-tune the onset of laying to environmental conditions irrespective of large differences in developmental state emphasizes the importance of supplementary cues close to laying. Variation in testis size of their mates predicted female laying dates equally little, which is less surprising, as a laying date is primarily a female-driven trait [4]. In comparison, in one of the few field experiments measuring endocrinology and reproductive physiology in combination with laying dates, Caro et al. [5] showed that two blue tit (*Cyanistes caeruleus*) populations breeding 1 month apart only showed a two-week asynchrony in the seasonal patterns of plasma LH and testosterone, and a comparably small difference in the timing of testis growth. Our standardized aviary setup did specifically not provide the complex of correlated cues that are available for birds in nature, e.g. photoperiod, temperature, visual, olfactory and seasonal food cues, which in combination might result in the closer relationship between the timing of endocrine and gonad development and laying date. Our findings, pointing at the disconnection between the timing of gonadal development and laying dates under standardized conditions, have implications for physiological studies traditionally concentrating on male reproductive development to determine the effect of environmental cues on timing of reproduction. Herewith we emphasize once more the importance of measuring laying dates complementary to reproductive physiology to make inferences about seasonal timing of reproduction.

4.2 Individuals showed unexplained variation in pre-laying physiology

Plasma hormone concentrations, as well as gonadal development of females and males showed phenotypic variation, even under controlled conditions of *ad libitum* food, natural photoperiod and standardized social cues, e.g. keeping birds in individual pairs. Only a small part of the variation in hormone titers could be due to differences in sampling time of day, which is however not responsible for the

consistent individual variation found here. Additional to individual differences within a given year, both hormone titers and gonadal sizes showed between-year variation in some months. Different temperature treatments within a year did not affect reproductive physiology [27, 43] and these between-year differences are more likely the effect of a slight deviation in sampling date between years. It could also reflect that birds from different families were used in different years. For example, the onset of ovarian follicle development showed a heritable component (S.V. Schaper, unpublished data). It is thus possible that family differences, which are taken into account as a random effect in the model, lead to the observed variation between years.

On top of the individual variation within a given year, a linear relationship between plasma hormone concentrations and effector systems can only be assumed within certain limits [1]. Downstream responses will be modified by individual variation in, for example, the amount of hormone receptors. It would be very worthwhile to further explore causes and mechanisms of the unexplained plasticity in endocrine systems and reproductive physiology, as well as its functional significance, heritability and adaptive value [1, 45].

4.3 Variation in gonadal growth was not related to variation in LH titers in most months

Follicle growth was more closely linked to LH concentrations in January than in subsequent months. LH levels are known to rise after photostimulation in spring [29], and in this setup, day length was increased following a seasonal pattern, leading to a natural increase in LH concentrations. At the time of measurements in January, birds were exposed to ca. 9:15 h of light, including dawn and dusk, likely not enough to fully photostimulate the birds. However, there was remarkable variation in LH levels at this point. We can only speculate that these were (genetic) differences in sensitivity to the seasonal increase in day length, which subsequently affected initial gonadal growth rates. The relationship between increasing LH levels and gonadal growth was less tight in the following months, which could mean that gonadal growth was fine-tuned by other internal or external cues not integrated via the LH pathway. Currently it is impossible to measure avian follicle stimulating hormone (FSH), which could likely form the link between environmental cues and gonadal development. In general, when we assume that cues affect gonadal growth also via different pathways, we expect the relationship between any gonadotropin and gonadal

development to be less tight in the late stages of gonadal development when supplementary cues become more influential.

The negative relationship between LH levels and testis size in males in April exemplifies the difficulty to draw conclusions from punctual or stochastic samples. In this case, high levels of LH were related to small testes, presumably because in males with fully-grown testes LH concentrations decreased already before April due to steroid feedback. Caro et al. [5] found in Corsican blue tits that at the time of laying, when males had fully functional testes, plasma LH levels were similarly decreasing as shown here.It also has to be cautioned here that gonad size does not directly indicate functionality in terms of gametogenesis, again pointing towards the importance of making behavioral observations to complement physiological measures.

4.4 Variation in pre-laying prolactin titers was not related to timing of laying

A stimulatory role of prolactin (PRL) on ovarian follicular development and egg laying was suggested previously, as chicken hens (*Gallus gallus domesticus*) immunized against PRL showed a lower egg laying rate compared to control hens [16]. However, in the present study PRL concentrations were not elevated in female great tits that were closer to laying or in their mates, in contrast to a recent study showing such a correlation in house sparrows [21]. We therefore do not find support for a stimulatory role of PRL on gonadal growth. PRL is generally associated with incubation and parental behavior, and thus exploring the individual variation in PRL levels close to laying in combination with reproductive performance or the timing of incubation behavior would be most interesting, but goes beyond the scope of this paper.

4.5 Approaches to variation in pre-laying reproductive endocrinology and physiology

It is crucial to investigate, under controlled conditions, the variation in endocrine and physiological mechanisms that cause individual variation in the onset of reproduction. For this, there are four complementary avenues that need to be explored.

Firstly, we need to find out in how far non-photic cues regulate reproductive pathways from an early stage onwards. For example, it has been shown that LH levels in male songbirds, even though primarily regulated by photoperiod, increase in response to environmental stimuli, such as the onset of rain [35], or the presence of leafing birch branches [44], but see [28]. Furthermore, if LH plasma concentrations

are only loosely regulating gonadal development, the question remains which external or internal information is reflected in elevated LH concentrations, and which mechanisms might be affected further downstream.

Secondly, we need to acknowledge more the plastic interplay between a hormone signal and its influence on effector sytems. Mechanisms like synergistic or antagonistic effects of hormones acting in concert, but also the role that binding globulines and hormone receptors play in mediating the strength of a hormone signal should be more in the focus of future research. This, however, asks for refined techniques that might not be currently available.

Thirdly, we need to concentrate our efforts on pathways unrelated to gonadotropins and gonadal growth that can accommodate the transduction of supplementary cues to fine-tune the onset of laying. The disconnection between relatively late stages of gonadal development and the onset of laying shown here exemplifies the scope for such a mechanism, for example accommodating temperature cues.

Fourthly and finally, we need to identify genetic variation underlying both the way in which environmental information is integrated and transduced into a physiological and behavioral phenotype. The genetic mechanisms maintaining plasticity in the physiological phenotype need to be identified if we ultimately want to predict how fast and to what extend animals can adapt their timing of seasonal breeding to changes in their environment, including climate change.

4.6 Summary

Our findings stress that stages of avian reproductive development until egg laying are regulated by different processes and are likely to be responsive to different stimulatory cues. This calls for the investigation of causes of this intriguing individual variation in endocrine systems and reproductive physiology for its own sake. Ultimately, these processes are culminating in egg laying, and acknowledging the paradox of the missing connectivity between early reproductive physiology and the laying decision is essential to fully understand effects of environmental variation on timing of reproduction.

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Fig. 1: Temperature treatments

Temperature treatments to which pairs of great tits breeding in climatized aviaries were exposed in the years 2007 to 2010. For a description of the treatments, see text.

Fig. 2: Individual patterns of plasma luteinizing hormone (LH) for male and female great tits kept in climate controlled aviaries in 2008-2010

Each line represents an individual bird that was sampled once monthly from January until April in 2008 and in 2009 and from March until April in 2010. Each year comprises of 36 pairs, leading to a sample size of 108 birds.

Fig. 3: Individual gonadal growth patterns for male and female great tits kept in climate controlled aviaries in 2008-2010

Each line represents an individual that was sampled once monthly from January until April in 2008, from January until March in 2009 and from February until April in 2010. Each year comprises of 36 pairs, leading to a sample size of 108 birds.

Fig. 4: Relationship between LH levels in January and follicle development in February and March

Concentrations of plasma LH in females in relation to the volume of the largest developing follicle in the ovary in February (A) and March (B). Data were log transformed.

Fig. 5: Relationship between LH levels in April and testis development in April

Concentrations of plasma LH in males in relation to the volume of the left testis in April in the years 2008 (red), 2009 (green) and 2010 (blue). Data were log transformed.

Fig. 6: Individual patterns of plasma prolactin (PRL) for male and female great tits kept in climate controlled aviaries in 2007

Each line represents an individual bird that was sampled once monthly from January until April in 2007. The data consists of 36 pairs.

Fig. 7: Relationship between gonad sizes and laying dates in 2008-2010

Volume of the largest developing follicle in the ovary (A) and volume of the left testis (B) in relation to the pair's laying date in the years 2008 (red), 2009 (green) and 2010 (blue). Gonad data were log transformed. Laying dates are given in April days, where 1 = 1st April.

Table 1: Relationship between plasma luteinizing hormone (LH) concentrations and gonad sizes in 2008 to 2010.

Data on gonadal maturation and LH concentrations were log-transformed and analyzed in mixed models per month with family as a random effect. The results are presented including lower and upper Bayesian 95% highest posterior density credible intervals (L 95% HPD, U 95% HPD). As year is given as a multi-level fixed factor in some analyses, a P-value is created for every level compared to 2008. Significant effects are given in bold. Sample size is given for the final model. A reduction in sample size can be due to missing measurements in either response variable or explanatory variables.

Table 2: Relationship between plasma luteinizing hormone (LH) concentrations, gonad sizes and laying dates in 2008 to 2010.

Data on gonadal maturation and LH concentrations were log-transformed and analyzed in a mixed model with female family as a random effect. The results are presented including lower and upper Bayesian 95% highest posterior density credible intervals (L 95% HPD, U 95% HPD). As year is given as a multi-level fixed factor, a P-value is created for every level compared to 2008. Significant effects are given in bold. Sample size is given for the final model. A reduction in sample size can be due to missing measurements in either response variable or explanatory variables.