Cloacal gland, endocrine testicular, and adrenocortical photoresponsiveness in male Japanese quail exposed to short days

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ARTICLE INFO

ABSTRACT

Photoperiod is the most important "noise-free" seasonal environmental cue for synchronizing physiological states (such as reproductive activity) in birds. However, in photoperiodic birds such as Japanese quail, the effect of photoperiod on adrenocortical activity remains unclear, particularly in males with differences in cloacal gland photoresponsiveness. At 8 wk of age, birds (n = 55) were either assigned to a short photoperiod (8L:16D; SD) or maintained under long photoperiod (16L:8D; LD; control). After 5 wk of SD exposure, males were classified as nonresponsive (SD-NR; with foam production) or responsive (SD-R; with no foam production) to short days, depending on the cloacal gland volume was above or below 1,000 mm^3. At 14 wk of age, droppings were collected during 3 consecutive days to determine corticosterone (CMs) and androgen metabolites (AMs) by enzyme immunoassays. Male Japanese quail under LD showed significantly higher concentrations of CMs (300 ± 10 ng/g) and AMs (1,257 ± 115 ng/g) than birds kept under SD. Under short days, SD-NR and SD-R showed differences ($P < 0.0001$), both in CM (153 ± 8 ng/g and 98 ± 6 ng/g, respectively) and AM concentrations (1,294 ± 309 ng/g and 275 ± 53 ng/g, respectively). Interestingly, although SD-NR males exhibited no cloacal gland arrest (according to cloacal gland volume and foam production) and similar testicular activity (AM values) to LD males, they showed lower concentrations of CMs compared with males that remained on LD ($P < 0.05$). Our findings suggest length of photoperiod affected hypothalamic-pituitary-adrenal activity; however, that was not the only factor involved, because birds subjected to shorter days but whose hypothalamic-pituitary-gonadal axis failed to respond had intermediate CM values. Further research is required to elucidate the underlying mechanisms of this interesting finding.

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1. Introduction

Free-living bird species can seasonally modulate glucocorticoid release, which is commonly elevated during the breeding season [1], such as wild quail (*Perdicula* sp)

[2]. The hypothalamic-pituitary-adrenocortical (HPA) axis through its interaction with other neuroendocrine axes, like the hypothalamic-pituitary-gonadal (HPG) axis, plays an essential role supporting mechanisms by which birds adjust their physiological stages in response to environmental cues (such as photoperiod [3]). In the laboratory, it was clearly shown that long photoperiod stimulates gonadal growth in male Japanese quail (*Coturnix* sp). On the contrary, short photoperiod negatively affects gonadal development [4,5]. However, it remains unclear whether

concentrations of corticosterone (and stress responses) are affected by photoperiod in Japanese quail [6] and other bird species [3]. To precisely characterize HPA axis responsiveness to stressors during natural photoperiodic cycles, it is important to determine the corticosterone response to photoperiod. Unfortunately, collecting blood samples can disturb an animal. Thus, noninvasive methods to determine glucocorticoids or their metabolites have become increasingly popular in farm animals [7].

Male Japanese quail are less active on short days after being exposed to long days, but their activity level can be restored with administration of testosterone [4]. However, not all birds fall out of reproductive condition on short days [4,5,8–13]. As mentioned before [2] increasing day length (typically detected during winter and spring) is positively correlated with adrenal and gonadal functions in mid-sized quail (*Perdicula* sp). In Japanese quail (*Coturnix* sp), another genus of the family Phasianidae, we hypothesize that the photoperiod affects HPA activity in the laboratory. We expect a higher testicular endocrine activity in Japanese quail exposed to long days than in birds under short days. However, the relation with corticosterone secretion and whether its concentrations will also be altered by photoperiod remain unclear and need clarification.

In the present study, we used a noninvasive method for glucocorticoid monitoring that has already been reported in other animals to have several advantages [14,15], together with other noninvasive measurements such as cloacal gland development and cloacal foam production. In Japanese quail cloacal gland development is an androgen-dependent phenomenon, highly positively correlated with testes size, and is known to be controlled by photoperiod [16–19]. Thus, our aim was to determine the effects of photoperiod on corticosterone metabolites (CMs) in droppings of male Japanese quail with differences in cloacal gland photoresponsiveness. We also analyzed whether cloacal gland photoresponsiveness of males exposed to short days and androgen changes are correlated with concentrations of CMs.

2. Materials and methods

2.1. Animals and husbandry

Male Japanese quail (*Coturnix coturnix japonica*) were used in the present study. Egg incubation, chick brooding, and lighting procedures were similar to those described elsewhere [20], with the exception that chicks were brooded in wood cages measuring 85 × 45 × 50 cm (length × width × height) from day 1 in mixed-sex groups up to 4 wk of age. Briefly, birds were fed a starter ration (28% CP; 2,800 kcal of ME/kg) and water ad libitum. They were kept under long photoperiod (16L:8D; light on at 6:00 AM; LD) and controlled temperature (brooding temperature was 37.5°C during the first week of life, with a weekly decline of 3.0°C until room temperature 24°C–27°C was achieved). From 4 wk onward birds were switched to a breeder ration (21% CP; 2,750 kcal of ME/kg). At this moment, Japanese quail were sexed by plumage coloration, and only males (n = 55) were randomly and individually housed in cages of two 5-tier cage batteries, each battery comprised 30 cages. Each

cage measured 45 × 20 × 25 cm (length × width × height). The same experimenter weekly measured cloacal gland volume, foam production, and body weight until the end of the study [10,16].

At 8 wk of age, LD was switched to short photoperiod (8L:16D; light on at 6:00 AM; SD) as treatment for 41 male Japanese quail. Another group of males was maintained under LD (n = 14). At 13 wk of age, quail that showed a reduction in the cloacal gland volume below 1,000 mm^3 (according to the classification of Oishi and Konishi [8]) and that did not express any cloacal foam production were classified as short-day quail that undergo regression of gonads (SD-R; n = 20). The remaining males under short days were classified as nonresponsive (cloacal gland volume > 1,000 mm^3; and still expressing some cloacal foam production; SD-NR; n = 21).

2.2. Steroid measurements

2.2.1. Sample collection

At 14 wk of age droppings were individually collected during 3 d. The effect of photoperiod on CM and androgen metabolite (AM) concentrations were studied on pooled droppings collected over 24 h during the first and third day. During the second day, frequent droppings were collected for physiological validation (see 2.2.2) of the cortisone EIA in quail. Briefly, when light automatically turned on at 6:00 AM, we cleaned the cage trays before starting sample collection. Then, individuals were subjected to an ACTH or vehicle injection (time 0), and sample collections were performed every half hour (a 30-min period was needed to ensure enough droppings were collected). All samples were stored immediately at −20°C until hormonal analysis.

2.2.2. Steroid extraction and immunoassays

A total of 0.5 g of each homogenized sample was extracted with 5 mL of 60% aqueous methanol by shaking for 30 min [21]. After centrifugation (2,500× G, 15 min), aliquots of the supernatant fluid (after a 1:10 dilution with assay buffer) were measured with a cortisone EIA and an epiandrosterone EIA. Details of the EIAs (including immunogens, biotinylated steroid labels, and cross-reactions of the antibodies) are given elsewhere [21,22]. Both are group-specific EIAs, the cortisone EIA measured immunoreactive CMs with a 3,11-dione structure and the epiandrosterone EIA measured immunoreactive AMs with 17-oxo structure. The EIA for AMs in male Japanese quail was previously validated by Hirschenhauser et al [23]. Interassay CVs (precision test) of a high and a low concentration pool samples were 6.5% and 6.2% for the cortisone EIA, and 3.7% and 5.0% for the epiandrosterone EIA, respectively; sensitivity was 10 or 25 ng/g, respectively. In addition, to ensure the validity of cortisone EIA in analytical terms, parallelism test was evaluated for the first time in extracts of male Japanese quail, following the approach applied by Busso et al [24]. Parallelism was performed by running serial dilutions of a dropping extract pool (y = 83.24 − 0.31 x for quail samples) and comparing statiscally its slope with that of the standard curve (y = 81.83 − 0.14 x). The standard curve was parallel to the dilution curve (P > 0.05; $F_{1, 11}$ = 3.46).

The cortisone EIA has been successfully validated and applied to measure adrenocortical activity in other galliformes [21,25–29]. Therefore, this assay was tested in this study for quail too, by demonstrating physiological changes of adrenocortical activity in response to ACTH. An ACTH challenge test was performed to stimulate corticosterone secretion in quail. Adrenocorticotropic hormone gel ($_{1-24}$ Acthelea 40, U.I.; ELEA-Buenos Aires, Argentina) at a dose of 100 μg/kg body weight was injected into the pectoralis major muscle according to Hazard et al [6]. Birds (SD-NR, SD-R, and LD) were randomly assigned to 2 experimental treatments: ACTH (n = 10, 11, and 7, respectively) or vehicle (physiological saline solution; n = 10, 10, and 7, respectively).

2.3. Statistical analyses

All variables were subjected to repeated measures ANOVA. A mixed statistical linear model was performed to evaluate the effects of photoperiodic on CM concentrations, taking into account photoperiodic responses (SD-R, SD-NR, and LD, fixed factor) during 2 d (first and third day). Across all analyses, "male quail" was incorporated as a random effect. Because a repetition factor model was applied, in the case that a bird did not naturally defecate in a particular time interval, an interpolation of concentrations between the previous and next collection time interval was made to get an estimate value and to avoid losing the whole hormonal profile for that bird. Hormonal data were transformed to rank to fit the assumptions of ANOVA. For test validation, the effect of ACTH on adrenocortical activity was also evaluated by repeated measures 2-way ANOVA, incorporating pharmacologic treatment (ACTH and vehicle) as well as time collection as fixed factors. Whenever repeated measures ANOVA reached significance ($P < 0.05$), Di Rienzo, Guzman, Casanove (DGC) post hoc tests were performed [30]. Values are expressed as means ± SEM. All statistical analyses were performed with Infostat (Infostat 2000 version 1.1; Grupo Infostat, Facultad de Ciencias Agropecuaria - Universidad Nacional de Córdoba, Argentina).

3. Results

Photoperiod affected CM concentrations in Japanese quail according to repeated measures ANOVA ($P < 0.0001$; $F_{2, 45} = 33.1$; Fig. 1). Post hoc test showed that males under SD had significantly lower CM values than birds kept under LD, and within the group of birds kept under short days, SD-NR males showed higher CMs than their SD-R counterparts ($P < 0.05$). We detected the same CM pattern during the last sampling day: SD-NR = 288 ± 70 ng/g, SD-R = 101 ± 19 ng/g, and LD = 443 ± 63 ng/g. We also detected the same CM pattern during ACTH challenge (LD > SD-NR > SD-R; $P < 0.0001$; $F_{2, 45} = 38.7$). Besides, the statistical analysis indicated an interaction between collection time and ACTH-gel treatment ($P = 0.0001$; $F_{10, 450} = 3.8$). Post hoc tests (all animals together) indicated that CM concentrations between 60 and 210 min were significantly higher after the ACTH injection than after the vehicle injection ($P < 0.05$). In Figure 2 concentrations of CMs in the different intervals after ACTH injection are shown separately for the
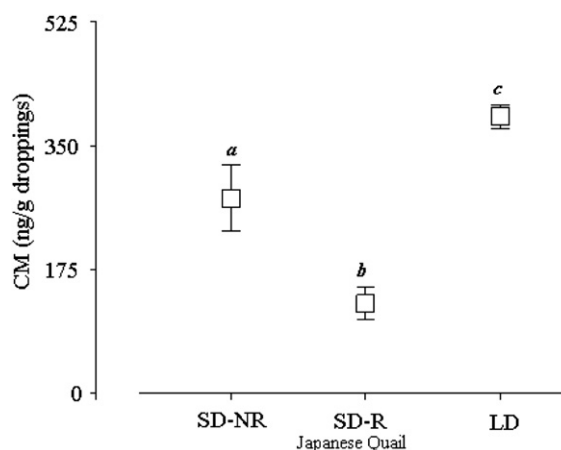

Fig. 1. Corticosterone metabolites (CMs) in droppings of male Japanese quail collected individually over 24 h. At 8 wk of age, birds were assigned to photoperiodic treatment, and males under short photoperiod were classified according to their gonadal photoresponsiveness at 13 wk of age as nonresponsive (SD-NR; n = 20) or responsive (SD-R; n = 21) to short days; long photoperiod (LD) males were kept on long days (n = 14). All values are expressed as mean ± SEM. Post hoc analysis indicates that values in each panel with no common letter differ significantly ($P < 0.05$).

2 groups of birds that differ in their responsiveness (SD-NR and SD-R) to short days and LD quail. Increases in CM concentrations above vehicle profiles were 2.1-fold in SD-R quail and 1.5-fold in SD-NR or LD quail.

Photoperiod affected AM concentrations in Japanese quail (Fig. 3). Short day nonresponsive quail had also higher AM concentrations than SD-R males. However, their AM concentrations did not differ from those in LD males (LD = SD-NR > SD-R; $P < 0.0001$; $F_{2, 45} = 37.9$; post hoc test $P < 0.05$). As expected, males under short photoperiod showed lower Cloacal volume/body weight (Cvol/bw) (see below) and Cloacal Foam Production (CFP) (data not shown) than LD males ($8.79 ± 0.34$ mm^3/g). Particularly, under short photoperiod, ANOVA also detected statistical differences and post hoc test indicated that SD-NR males exhibited a higher Cvol/bw ($6.82 ± 2.5$ mm^3/g) mean and CFP (data not shown) than the SD-R males ($2.58 ± 0.24$ mm^3/g) ($P < 0.05$).

4. Discussion

At present different aspects of reproductive physiology in birds under photoperiodic cycles have been extensively studied [5,31–35]. However, only a limited number of studies in birds and in mammals deal with the photoperiodic regulation of stress-related functions. For example, Otsuka et al [36] suggested that photoperiod regulates corticosterone rhythms by altered adrenal sensitivity through melatonin-independent mechanisms that may involve the adrenal clock in rats and mice. In birds, it remains controversial whether corticosterone concentrations are affected by photoperiod [6,37–39]. Our study shows the effect of length of day (long > short) on CM concentrations in male Japanese quail exposed to artificial photoperiod, presumably affecting HPA axis activity in this photoperiodic species. As expected, we detected an (long > short) effect of photoperiod on AM concentrations and
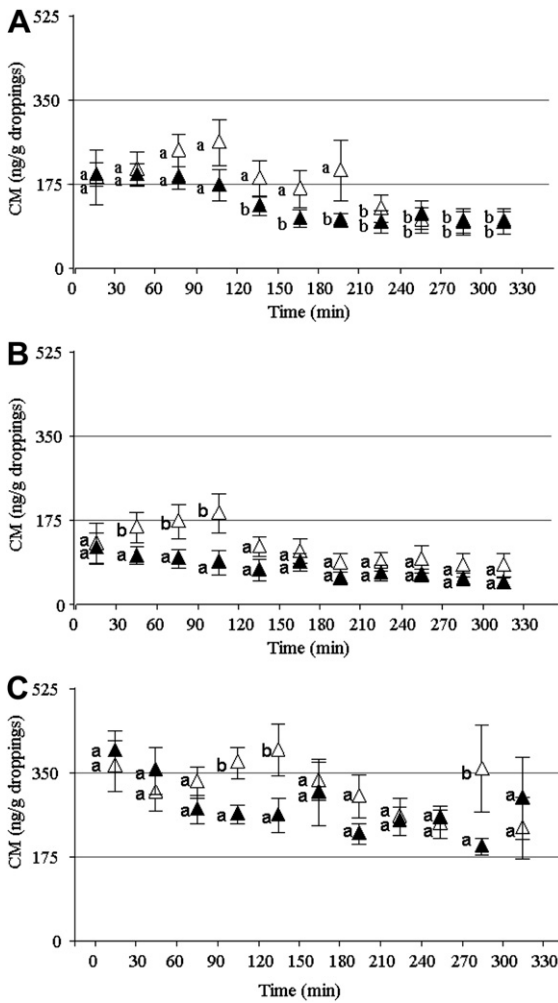
Fig. 2. Concentrations of corticosterone metabolites (CMs; mean ± SEM) in droppings of male Japanese quail subjected to an ACTH (△) or vehicle injection (control; ▲) at time 0 (see details in Materials and methods: 2.2.1). Sample collections were performed every half hour. All samples were stored at −20°C until analysis. (A) SD-NR males, n = 10; (B) SD-R males, n = 10 each group; and (C) LD males, n = 7 each group. Corticosterone metabolite values with no common letters in each panel differ significantly at $P < 0.05$.
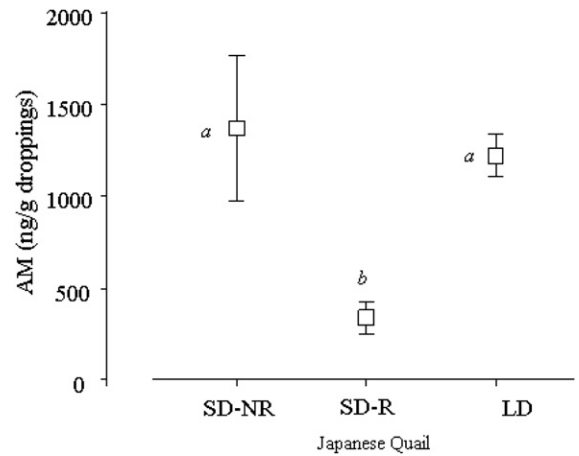

Fig. 3. Androgen metabolites (17-oxoandrogen metabolites) in droppings of male Japanese quail collected individually over 24 h. At 8 wk of age, birds were assigned to photoperiodic treatment, and males under short photo-period were classified according to their gonadal photoresponsiveness at 13 wk of age as nonresponsive (SD-NR; n = 20) or responsive (SD-R; n = 21) to short days, and males kept in long days (LD; n = 14). All values are expressed as mean ± SEM. Post hoc analysis indicates that values in each panel with no common letter differ significantly ($P < 0.05$).

cloacal gland development. However, some birds were detected as nonresponsive to short days. Remarkably we found that higher baseline CM concentrations are not only a consequence of longer days but also related to testicular activity. We found that SD-NR Japanese quail had higher (intermediate) values of CMs. These SD-NR males failed to reduce reproductive competence, because their HPG axis did not respond to photoperiod.

The adrenocortical response to different stressors has been extensively studied in the Japanese quail [3,12,40,41]. Activation of the hypothalamic-pituitary-adrenal axis has been linked to depression in the hypothalamic-pituitary-testicular axis, and it has been shown to result in different changes in cloacal gland physiology [16]. Satterlee et al [17] suggested that genetic selection for altered adrenocortical responsiveness to stress does not overcome these natural photoperiodic gonadal responses. We are also interested in

the interaction of HPA axis with HPG axis, although we focused on HPA axis responsiveness during natural photo-periodic gonadal cycles. Among the many environmental interactive factors that control avian reproductive status, perhaps the most powerful one in most birds, is change in day length during the breeding season. In addition, HPA axis plays an essential role to support mechanisms by which birds adjust their physiological stages in response to envi-ronmental cues (such as photoperiod [3]). We hypothesized that the photoperiod also controls adrenocortical function. Hazard et al [6] studied the effect of photoperiod on basal plasma corticosterone concentrations in Japanese quail. However, they could not distinguish between the possibility of a direct effect of photoperiod length and the possibility of an indirect effect that resulted from acquisition of sexual maturity. As a consequence we studied older male Japanese quail, and males kept under LD showed significantly higher CM concentrations than those kept under SD. Thus, photo-period affected HPA activity in this photoperiodic species. Interestingly, under short days, SD-NR and SD-R showed differences both in CM and AM concentrations. On the contrary, a study of Delville et al [9], in which birds were also classified according to their cloacal gland photo-responsiveness during short days, showed no differences in plasma corticosterone concentrations. We think that differences might have been masked in their study because blood samples were only collected from animals that exhibited cloacal gland values lower than those detected in SD-NR (and LD males). In addition, frequent and repeated blood sampling (in contrast to our stress "free" sampling method) might have induced a stress response that affected corticosterone concentrations in the study of Delville et al [9].

Glucocorticoids after ACTH stimulation are extensively metabolized and excreted into droppings in birds [14]. Particularly, fecal CM measurements in Japanese quail have

been recently reported [42,43]. We used an EIA suitable for measuring CM in droppings of chicken (Gallus domesticus) developed by Rettenbacher et al [21]. In the present study, changes in CM concentrations after ACTH (in gel form, i.m.) challenge clearly indicated ($P = 0.0001$) that the assay is also suited to monitor adrenocortical activity in Japanese quail housed under laboratory conditions, showing elevated CM concentrations between 60 to 210 min after ACTH injection. However, it should be considered that CM baseline and stress-induced concentrations strongly depend on photoperiod (Figs. 1 and 2). In view of that, CM profiles in SD-NR showed 2 peaks after ACTH, the first peak resembling urinary excretion, whereas the second peak probably represented fecal CMs [21,43]. The second peak (180–210 min) in SD-NR was statistically higher than in the vehicle group. Less active Japanese quail classified as SD-R exhibited only high concentrations of CMs in response to ACTH between 30 to 60 and 90 to 120 min (first peak). Both groups of Japanese quail exposed to short days apparently exhibited comparable sensitivity with an ACTH challenge (increases in CM concentrations above vehicle profiles were 2.1-fold in SD-R quail and 1.5-fold in SD-NR); however, differences in HPA photoperiodic reactivity are evident. Because male Japanese quail engage in less activity on short days after being exposed to long days, we speculate that SD males exhibited lower HPA responses to stressors during inhibitory photoperiod. When exploring corticosterone response to ACTH in long days, we detected that Japanese quail injected with ACTH under long days exhibited the same pattern of CM excretion as SD-NR males. Increases of CMs (60 and 210 min) in the ACTH group narrowly failed to reach statistical significance (ACTH vs vehicle). We assume that hormone baseline of the vehicle group could have been disturbed by other stressors; further experiments are necessary to confirm this hypothesis. In fact, different studies conducted in Japanese quail selected for different levels of behavior (and fear) response to stressors (isolation, crush test, restraint, handling) have shown that circulating corticosterone concentrations were increased [6,40,41,44–45], indicating that HPA axis reactivity may also involve differences in psychobiological processes of perception and integration.

Finally, we consider that high androgen and corticosterone concentrations in SD-NR quail, probably with similar general and metabolic activity as LD males, are showing a degree of synergy in the activity of both the HPG and HPA axes (an "adrenal-testicular unit"), as it has been postulated by Carsia and Harvey [3]. Different scenarios are possible to explain results. It should be pointed out that high concentrations of circulating corticosterone, in the appropriate physiological context, augmented the HPG axis response in some avian species [3]; however, this has not been directly tested in our study. Neither was the androgen effect on HPA axis activity directly tested. On the basis of our data, we consider that glucocorticoid would act as a preparative to overcome subsequent stress responses. A similar argument has been proposed by Sapolsky et al [46]. When HPG axis does not respond to photoperiod, endocrine testicular function can be increased under chronic stressor such as inhibitory photoperiod; therefore; HPA axis could support the recovery of cloacal gland development under short days. Studies by our group are in progress to test this hypothesis. Besides, our SD-NR male Japanese quail exhibited comparable cloacal gland volume and foam production with other group of fertile Japanese quail [47]. Interestingly, it had been reported that some female Japanese quail also continue to lay eggs under short days or even in continuous darkness [4]. Therefore, we consider that nonresponsive quail deserve more attention. Considering reproductive competence of SD-NR males, these birds exhibited lower baseline values of CMs (and probably a reduced stress response); these lower values could even suggest that quail under those particular conditions have better welfare than their LD counterparts [7,48].

5. Conclusions

Our findings suggest length of photoperiod affected hypothalamic-pituitary-adrenal activity; however that was not the only factor involved, because birds subjected to shorter days but whose HPG axis failed to respond had intermediate CM values. Further research is required to elucidate the underlying mechanisms of this interesting finding.

Acknowledgments

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