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Environmental cues and dietary antioxidants affect breeding behavior and testosterone of male European starlings (*Sturnus vulgaris*)

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ABSTRACT

Environmental cues, such as photoperiod, regulate the timing of major life-history events like breeding through direct neuroendocrine control. Less known is how supplementary environmental cues (e.g., nest sites, food availability) interact to influence key hormones and behaviors involved in reproduction, specifically in migratory species with gonadal recrudescence largely occurring at breeding sites. We investigated the behavioral and physiological responses of male European starlings to the sequential addition of nest boxes and nesting material, green herbs, and female conspecifics and how these responses depend on the availability of certain antioxidants (anthocyanins) in the diet. As expected, cloacal protuberance volume and plasma testosterone of males generally increased with photoperiod. More notably, testosterone levels peaked in males fed the high antioxidant diet when both nest box and herbal cues were present, while males fed the low antioxidant diet showed no or only a muted testosterone response to the sequential addition of these environmental cues; thus our results are in agreement with the oxidation handicap hypothesis. Males fed the high antioxidant diet maintained a constant frequency of breeding behaviors over time, whereas those fed the low antioxidant diet decreased breeding behaviors as environmental cues were sequentially added. Overall, sequential addition of the environmental cues modulated physiological and behavioral measures of reproductive condition, and dietary antioxidants were shown to be a key factor in affecting the degree of response to each of these cues. Our results highlight the importance of supplementary environmental cues and key resources such as dietary antioxidants in enhancing breeding condition of males, which conceivably aid in attraction of high quality females and reproductive success.

1. Introduction

Male reproductive success relies heavily upon securing key resources and attracting mates (Emlen and Oring, 1977; Klug, 2011; Reynolds, 1996). Increased availability of breeding resources such as food or nesting sites can directly enhance male reproductive condition (Hau et al., 2000; Nelson et al., 1995). Key resources acquired by males can directly (e.g. via behavior; Godin and Dugatkin, 1996; Reaney and Backwell, 2007) or indirectly (e.g. via territory quality; Dijkstra et al., 2008) indicate the quality of males and thus influence female mate choice and presumably offspring quality. For example, certain types of vocalizations (Dreher and Pröhl, 2014) and aggressive behaviors (Buzzard et al., 2014), and high intensity of behavioral display rates (Kodric-Brown and Nicoletto, 1996), are preferred by females across several distinct taxa including the Neotropical poison frog (*Oophaga pumilio*), wild yak (*Bos mutus*), and the guppy (*Poecilia reticulata*). Furthermore, in birds males may also attract females by adorning nests with attractive items, both natural and anthropogenic (Brouwer and Komdeur, 2004; Gwinner, 1997; Östlund-Nilsson and Holmlund, 2003). For example, male European starlings (*Sturnus vulgaris*) will adorn nests with green plant material (herbs) before females arrive, and males will also display greenery in an eye-catching manner when females are in close proximity to their nest box (Brouwer and Komdeur, 2004; Gwinner, 1997). Less studied is how availability and phenology of these

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key resources directly influences male breeding condition and conspecific interactions associated with breeding.

The ability of male vertebrates to successfully reproduce depends on the interplay between initial predictive information such as photoperiodically-induced physiological changes that are required for reproduction (e.g., stimulation of the hypothalamic-pituitary-gonadal (HPG) axis; Wingfield et al., 1992) and supplementary information (e.g., presence of females, diet quality). For migratory birds, photoperiod is considered the initial predictive cue involved in transitioning individuals from migration to reproduction (reviewed by Dawson, 2008), although supplementary environmental cues, like food, water and social cues, are needed to induce appropriate breeding behavior and displays (reviewed by Visser et al., 2010). Male breeding condition is largely regulated by testosterone including the expression of reproductive behaviors such as aggressive and territorial behaviors (reviewed by Wingfield and Farner, 1993; Wingfield and Silverin, 2002), courtship behaviors (Fusani et al., 2007), and singing (Foerster et al., 2002), and development of secondary sex characteristics, such as cloacal protuberance in passerines (Hegner and Wingfield, 1987). Positive relationships between testosterone levels and indicators of breeding success have been established in several bird species, and include testosterone and the number of nest sites (Gwinner and Gwinner, 1994), the successful rearing of chicks (Moss et al., 1994), and, indirectly, vocalizations (De Ridder et al., 2000; Hunt et al., 1997). However, maintenance of elevated testosterone levels is also known to directly induce oxidative stress in various tissues (Alonso-Alvarez et al., 2007; Chainy et al., 1997) as does increased metabolic rate (Costantini, 2014) that occurs during the energetically demanding breeding season (Drent and Daan, 1980). Vertebrates can defend against such oxidative stress by consuming dietary antioxidants (e.g., Blount, 2004; reviewed by Monaghan et al., 2009) and by up-regulating their endogenous antioxidant system (reviewed by Cooper-Mullin and Mcwilliams, 2016). Insufficient dietary antioxidant consumption is known to impair an individual's ability to repair oxidative damage to tissues (Guarnieri et al., 2008; reviewed by Skrip and Mcwilliams, 2016), which could impede reproductive success. No study to date has determined whether the availability of dietary antioxidants affects the interaction between environmental cues and physiological changes (e.g., circulating testosterone) associated with reproduction in a wild vertebrate.

Many temperate and arctic breeding birds migrate to more benign areas during winter, and for many species, including the European starling, there is an associated sex-biased migration phenology. Typically, males arrive at breeding grounds prior to females (protandry) and then acquire and defend a territory (reviewed by Morbey and Ydenberg, 2001) while preparing nests for female arrival. After females arrive, the males begin to court females with song and displays (Gwinner et al., 1987; Gwinner and Schwabl, 2005; Kessel, 1957). For European starlings, an adequate nest box or natural cavity and the presence of females are among the most important cues for eliciting male courtship behavior and elevating testosterone levels (Gwinner and Gwinner, 1994; Gwinner et al., 2002; Pinxten et al., 2003). For starlings, decorating nests with locally-collected herbs (e.g., *Daccus carota*, *Anthriscus sylvestris*, *Achillea millefolium*) and male displays with herbs positively correlate with female attraction/choice (Brouwer and Komdeur, 2004; Gwinner, 1997; Veiga et al., 2006). The short distance migratory starlings arrive at the breeding grounds many weeks before the first eggs are laid and testicular recrudescence largely occurs during this period (Berthold, 1967). Thus, gonadal recrudescence may in fact interact with the outlined environmental and social cues, as well as resources available through dietary intake.

We studied the behavioral and hormonal changes of captive male European starlings during spring when presented with sequential additions of the following supplementary environmental cues: nest boxes and nest building material, green herbs, and finally female conspecifics. This sequential addition of environmental cues matches that experienced by free-living males from migratory populations arriving prior to females (Gwinner and Schwabl, 2005). Additionally, we determined the effect of high and low levels of dietary antioxidants (anthocyanin) on male physiology, condition, and behavior. We tested four hypotheses: (1) cloacal protuberance volume and testosterone levels of males increase additively with the addition of nest box, herbs, and female cues; (2) testosterone levels are enhanced in males fed the high antioxidant diet (oxidation handicap hypothesis; Alonso-Alvarez et al., 2007); (3) sequential addition of supplementary environmental cues increases the frequency of breeding behaviors; and (4) male breeding behaviors are enhanced in males fed the high antioxidant diet.

2. Methods

2.1. Starling care, aviaries, and experimental diets

Forty-five male and 33 female 5–8 day-old European starlings were collected from nest boxes in late-April to early-May 2015 from a colony in Upper Bavaria, South Germany (47°58′ N, 11°13′ E). We hand-raised the chicks at the Max Planck Institute for Ornithology (MPIO), Seewiesen, Germany until they were able to feed independently (ca. 35 days old) and then maintained them in separate male and female aviaries until August 2016 (see Supplementary materials for details). Before males were exposed to low or high antioxidant diets, all birds received the standard MPIO maintenance diet (insect powder, produce (apples, oranges, lettuce), dried fruit pellets, and live mealworms) for at least 1.5 months. At the start of the spring breeding experiment beginning on February 9, 2017, males were switched to a semi-synthetic high polyunsaturated fat diet (Table 1) containing either high or low levels of dietary antioxidant (anthocyanin).

All procedures adhered to the ethical guidelines of the North American Ornithological Council (Fair et al., 2010) and were approved by the University of Rhode Island IACUC (Protocol #AN08-02-014) and the Government of Upper Bavaria, Germany (AZ 55.2-1-54-2532-216-2014).

Starting on February 9, 2017, and continuing every 3–9 days, 7–8

Table 1
Composition of the high polyunsaturated fat, high-antioxidant diet fed to European Starlings for the spring 2017 breeding experiment. Half the males ($n = 23$) were fed this semi-synthetic diet without supplemental anthocyanin (low-antioxidant diet) while the other males ($n = 22$) were fed this semi-synthetic diet with supplemental anthocyanin (high-antioxidant diet).

	High polyunsaturated diet	
Ingredients	% wet mass	% dry mass
Glucose[a]	16.84	39.19
Casein[b]	8.21	19.12
Cellulose[c]	2.14	4.97
Salt mixture[d]	2.05	4.78
Canola oil[e]	5.18	12.05
Sunflower oil[f]	3.03	7.06
Anthocyanin[g]	0.18	0.42
Amino acid mix[h]	1.15	2.68
Vitamin mix[i]	0.16	0.38
Ground meal worms	2.65	6.16
Agar[j]	1.37	3.19
Water	57.04	–

[a] Glucose, VWR International GmbH, Darmstadt, Germany.
[b] Casein, Affymetrix UK Ltd., High Wycombe, UK.
[c] Alphacel, MP Biomedicals, Solon, OH, USA.
[d] Brigg's salt mix, MP Biomedicals, Solon, OH, USA.
[e] Canola oil, Jedwards International, Inc., Braintree, MA, USA.
[f] Sunflower oil, Jedwards International, Inc., Braintree, MA, USA.
[g] Standardized Elderberry 6.5% Powder; Artemis International, Inc., Fort Wayne, IN, USA; no anthocyanin was included in the low antioxidant diet.
[h] Amino Acid Mix, Sigma-Aldrich, St. Louis, MO, USA.
[i] AIN vitamin mix, MP Biomedicals, Solon, OH, USA.
[j] Agar, Ombilab-laborenzentrum GmbH, Bremen, Germany.

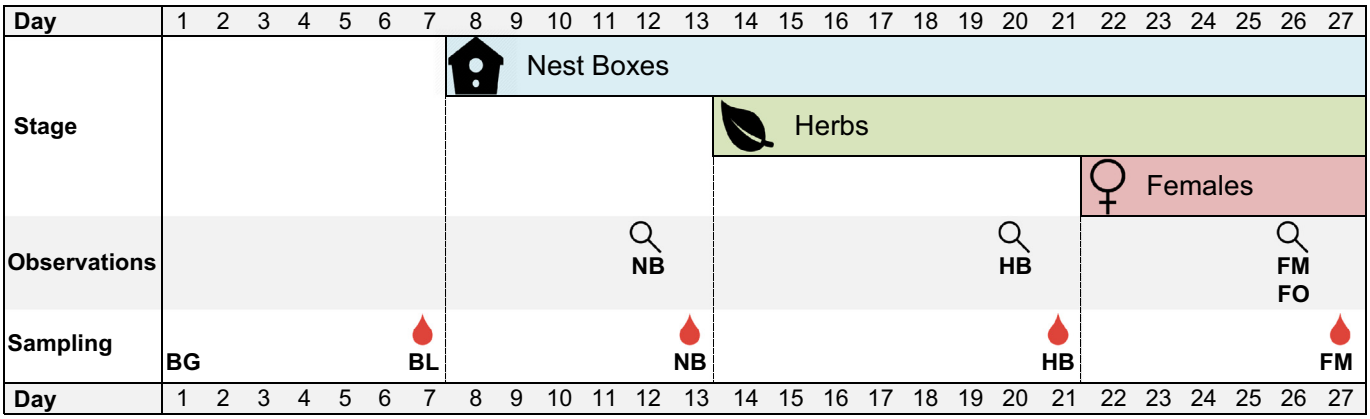

Fig. 1. General timeline of the sequential addition of three breeding stages (nest boxes: NB, herbs: HB, and females: FM), as well as when behavioral observations and blood sampling of the European Starlings was conducted at the Max Planck Institute for Ornithology during February–April 2017. For each of the six breeding aviaries, the males were introduced to the aviaries on background day (BG), which is when they were first fed their semi-synthetic diets, and the three stages were introduced to each aviary at 7, 13 and 21 days, respectively. Plasma testosterone, indicated as blood droplets, was measured for each of the three stages and at one additional point, Baseline (BL), designed to establish baseline testosterone levels once males were acclimated to their semi-synthetic diets. Behavioral observations, indicated as magnifying glasses, were conducted at lights on (0700 h) the last day before the start of the subsequent stage to assess how male breeding behaviors were affected by the sequential addition of nest, herbs, and then females and behavior of females was measured once (Female Observation: FO).

males were randomly selected and transferred from the outdoor maintenance aviary to one of six empty outdoor breeding aviaries, that contained only sawdust, perches, food, and water (see Supplementary materials for size and configuration of the breeding aviaries). On the day males were moved into the outdoor breeding aviaries (background day; see Fig. 1), we measured each male's tarsus and wing chord, which were later used in calculating a body condition index (Peig and Green, 2009). The staggered start times for each of the six aviaries (Aviary 1 introduced on 9 Feb, Aviary 2 on 12 Feb, Aviary 3 on 21 Feb, Aviary 4 on 24 Feb, Aviary 6 on 5 March, and Aviary 5 on 8 March) were designed to accommodate the flight training schedule of females as part of a separate study. Males moved to these breeding aviaries were fed ad libitum the same high or low antioxidant semi-synthetic diets (Table 1) fed to females that would later be introduced into the breeding aviaries on day 21 of their breeding timeline (Fig. 1). Birds in aviaries 1, 3, and 5 were fed the antioxidant low diet ($n = 23$), while those in aviaries 2, 4, and 6 were fed the antioxidant high diet ($n = 22$).

2.2. Light cycles

During January 2017 males in the outdoor maintenance aviaries were maintained on a light cycle (bulbs were Osram LUMILUX T8 58 W/865) typical of their wintering area (civil twilight at Rome, Italy; 10 h 12 min light: 13 h 48 min dark). Starting on January 19, 2017, we photostimulated males by adding 1 h light each week to the outdoor maintenance aviaries so that by 31 January 2017 males had been switched to an early breeding (March 21) local light cycle (civil twilight at Seewiesen, Germany; 13 h 12 min light: 10 h 48 min dark). Outdoor breeding aviaries were kept on a light cycle representative of Rome on December 22 (civil twilight: 10 h 11 min light: 13 h 49 min dark). However, after 21 January, the natural light transmitted through the opaque ceiling and front window of each outdoor breeding aviary was longer than the provided aviary lights so thereafter the birds were exposed to natural photoperiod (see Supplementary materials). In this way, when males were moved from their outdoor maintenance aviaries to the breeding aviaries throughout February and early-March they moved from longer to shorter day length, just as wild starlings that migrate in spring to shorter day length experience as they move north to breed.

2.3. Breeding stages and timeline of blood sampling

To simulate the natural breeding phenology of starlings, we sequentially added nest boxes and nesting material (nest box stage), herbs (herb stage), and then females (female stage) to the breeding aviaries (Fig. 1). Hereafter we refer to each sequential addition of these environmental cues as 'stages' (Fig. 1).

One week after males had been moved to their breeding aviary, we installed eight identical nest boxes (nest box stage; Fig. 1) that were arranged in a given aviary as shown in the supplementary materials. Nest material consisting of dry grass and reeds was also added at this time and thereafter was available ad libitum on the ground of the aviary. Six days later (herbal stage; Fig. 1) we added herbs by supply of two buckets of locally collected elderberry and willow branches with young leaves, eight 3-inch pots of wild carrot (*Daucus carota)*, and a plate of ribbon and birch bark for use as nest ornaments. Nest boxes were checked for male nests six days after herbs were added and, if none were built, we added a mixed handful of reeds and dried grasses to empty nest boxes to promote nest building (according to Gwinner, 1997). As a result, we added nest material to all boxes in the first four aviaries and none in the last two aviaries as birds built such male nests on their own, suggesting that males in aviaries 1–4 were behaviorally and/or physiologically distinct from aviaries 5–6. Hereafter, we refer to birds in aviary 1–4 as "season 1" and those in aviary 5–6 as "season 2". Eight days after herbs were added 5–6 females, who had finished their 15-day flight training (or were sedentary controls as part of separate female experiment), were placed with the 7–8 males already present in the breeding aviary (female stage; Fig. 1). There was always a male biased sex ratio of 1.5:1 males to females so that the males were in excess. The experiment ended for male starlings in a given breeding aviary six days after the females were introduced.

We sampled blood four times from each male starling to determine the separate and sequential effect of the three stages (Nest Boxes: NB, Herbs: HB, and Females: FM) on testosterone (Fig. 1). Aviaries were entered the same time on each of the four blood-sampling days (1500 to 1515 h) and all males were captured and bled within 30 min; we recorded the exact time each blood sample was taken and found no significant effect of bleeding time on plasma testosterone levels ($r = -0.003$, $p = 0.969$). We sampled 75 µl of blood from the brachial vein using heparinized capillary tubes after puncture with a 17 G needle. Blood samples were immediately transferred to 0.5 ml Eppendorf tubes and kept on ice for no longer than 90 min before spinning in

the centrifuge for 5 min at 214*g*. Plasma was separated from red blood cells for the testosterone assay. Samples were kept at −20 °C for no longer than a week and then stored at −80 °C until analysis.

Following blood sampling, the same person (KC) measured cloacal protuberance (width at base, width at opening, height) to 0.01 mm using sliding calipers (for width measurements) and a millimeter ruler to 1.0 mm or height measurements. From these measures, cloacal protuberance volume was calculated assuming a cylindrical shape ($\pi \times \text{radius}^2 \times \text{height}$; Mulder and Cockburn, 1993).

2.4. Behavioral observations of male and female starlings

Three 24-min behavioral observations were conducted to document how males responded to the sequential stages (nest boxes, herbs, and females) across the 27-day breeding period (Fig. 1). In addition, we also conducted a single 24-min behavioral observation of females immediately after we completed the 24-min observation period for males in that same breeding aviary (Female Observation: FO; Fig. 1) to characterize male/female interactions and behaviors indicating pair bonding. Each 24-min behavioral observation began on the specified day within 10 min of lights on (0700 h). Birds were observed from one of the three windows in a given breeding aviary (see Supplementary materials). It was dark outside the aviary and brightly lit inside the aviaries, and this contrast combined with the opaqueness of the window prevented birds from clearly seeing the observer outside the aviaries. Before each observation period began, we waited 10 min to allow the birds to acclimate to any noise disturbance associated with the observer and to allow the observer to identify all birds in sight. In order to reduce variability, a single observer (KC) performed all behavioral observations.

Each 24-min behavioral observation period was subdivided into four 6-min periods, during which observations focused on two of the eight nest boxes. Each pair of next boxes was observed for 6-min in the same sequential order during each 24-min period. During each 6-min sub-period, the observer recorded every 20 s. the behaviors of individual males, identified via unique colored leg bands that were observed at either of the two focal nest boxes. We used the following behavioral categories: perched on nest box (P), perched on nest box and singing (PS), perched on nest box with dry nest material (PD), visual display (VD), aggression (A), and inside nest box (IN; Table 2). In addition to this 20-sec interval sampling of males at focal nest boxes, the observer also recorded the number of times a given individual was observed in aggression (A) or inside nest box (IN) at any of the eight nest boxes throughout the 24-min observations (Table 2). These were behaviors that we considered crucial to assess breeding status but might go unnoticed during focal 6-min observations on a given two nest boxes at a time. For each aviary, total observation time was 72 min for males (24 min for each of the three stages) and 24 min for females.

Table 2
Behaviors recorded during each of the three 24-min observations of males (nest box stage, herbal stage, and female stage) and the single 24-min observation of females (FO) for each of the six breeding aviaries at MPIO.

Behavior	Code	Operational definition
Perched	P	Perched on nest box
Perched singing	PS	Perched on nest box while singing
Perched with dry nest material	PD	Perched on nest box with dry nest material (reeds or dried grass)
Visual display	VD	Perched on roof of nest box while singing and waving wings (Eens and Pinxten, 1990)
Aggression[a]	A	Hostile interaction between two individuals characterized by obvious fighting (i.e., rapid pecking, biting, wing flapping) and usually resulting from displacement of one male from a nest box
Inside nest box[a]	IN	Entered nest box through front facing hole and remained inside

[a] Indicates a rare behavior, which was recorded during each 6-min focal observation of a pair of nest boxes as well as for all males associated with any nest box throughout the entire 24-min observation period conducted in each aviary.

2.5. Testosterone analyses

Testosterone concentration was determined by 3 direct radioimmunoassays (RIA, following Goymann et al., 2006). Plasma samples were extracted with dichloromethane (DCM) after overnight equilibration (4 °C) of the plasma with 1500 dpm of tritiated testosterone (Perkin Elmer, Rodgau, Germany). The organic phase was then separated from the aqueous phase by plunging the extraction tubes into a methanol-dry ice bath and decanting the dichloromethane phase into a new vial. This extraction step was repeated twice to increase extraction efficiency. Then, the DCM phase was dried under a stream of nitrogen at 40 °C, dried samples resuspended in phosphate buffered saline with 1% gelatine (PBSG) and left overnight at 4 °C to equilibrate. An aliquot (80 µl) of the redissolved samples was transferred to scintillation vials, mixed with 4 ml scintillation fluid (Packard Ultima Gold) and counted to an accuracy of 2–3% in a Beckman LS 6000 β-counter to estimate individual extraction recoveries. The remainder was stored at −40 °C until RIA was conducted. Mean ± SD extraction efficiency for plasma testosterone was 90.2 ± 0.4%. For the RIA a standard curve was set up in duplicates by serial dilution of stock standard testosterone ranging from 0.39–200 pg. Testosterone antiserum (T3-125, Esoterix Endocrinology, Calabasas, CA, USA) was added to the standard curve, the controls and to duplicates of each sample (100 µl). Cross reactivities of this antiserum are testosterone (100%), 5a-dihydrotestosterone (44%), d-1-testosterone (41%), d-1-dihydrotestosterone (18%), 5a-androstan-3b, 17b-diol (3%), 4-androsten-3b, 17b-diol (2.5%), d-4-androstenedione (2%), 5b-androstan-3b, 17b-diol (1.5%), estradiol (0.5%), and < 0.2% with 23 other steroids tested. After 30 min testosterone label (13,500 dpm) was added and the assay incubated for 20 h at 4 °C. Then, bound and free fractions were separated at 4 °C by adding 0.5 ml dextran-coated charcoal in PBSG assay buffer. After 14 min incubation with charcoal samples were spun (3600 *g*, 10 min, 4 °C) and supernatants decanted into scintillation vials at 4 °C. After adding 4 ml scintillation liquid (Packard Ultima Gold) vials were counted. Standard curves and sample concentrations were calculated with Immunofit 3.0 (Beckman Inc. Fullerton, CA), using a four-parameter logistic curve fit. The lower detection limits of the standard curves were determined as the first value outside the 95% confidence intervals for the zero standard (Bmax) and ranged from 0.30 to 0.39 pg/tube for the three assays. The intra-assay coefficients of variations of standard testosterone were 10.6%, 7.5% and 4.5%. The intra-extraction coefficients of variation of a chicken plasma pool of the three assays were 6.0%, 6.9% and 16.5%. The inter-assay variation of standard testosterone was 6.9% and the inter-extraction variation of the chicken plasma pool was 7.0%. Because the testosterone antibody used shows significant cross-reactions with 5a-dihydrotestosterone (44%) our measurement may include a fraction of 5a-DHT.

2.6. Statistical analyses

All the analyses were performed in R (R Core Team, 2017). We used linear mixed-effect models (Bates et al., 2014) to infer the effect of stage (4 levels: baseline, nest box, herbal, and female), Diet (2 levels: high or low antioxidant), and Season (3 pairs of aviaries), as well as all interactions on cloacal protuberance volume, body condition index, and testosterone, the latter after log transformation. We included individual identity and aviary as random factors. When possible we removed uninformative interactions to simplify the models, which resulted in having only Stage and Season for cloacal protuberance, Stage and Diet for testosterone, and Stage and Diet for body index. We used generalized linear mixed-effect models with the Poisson link function to

analyze male and female behaviors and rare behaviors. These models included Stage (3 levels: excluding baseline), Diet (2 levels since cloacal protuberance and testosterone did not differ for the first two pairs of aviaries – see Results) and their interaction as fixed effects, and individual identity and aviary nested into Season as random factors. The model used for the analysis of female behavior was similar but did not contain Stage as an explanatory variable.

Before interpreting the results, we checked whether model assumptions were met by inspecting the residuals for normality, homoscedasticity, and lack of remaining pattern. For the generalized linear mixed-effect model we further controlled for overdispersion using the function dispersion_glmer (Korner-Nievergelt et al., 2015). In order to obtain parameter estimates, we used Maximum Likelihood (ML) estimation because we were most interested in fixed effects (Zuur et al., 2009). To infer the explanatory power of the different fixed factor and relative interaction we ran an ANOVA on the mixed model output and used the F-test to calculate *p*-values with the package "lmerTest" (Kuznetsova et al., 2014). When necessary, we used a post hoc test using the function lsmeans of the homonym package (Lenth, 2016) setting a Bonferroni correction for multiple testing. We calculated the effect size as eta squared (η2) for ANOVAs and Cohen's d (d) for post-hoc pair-wise comparisons.

To investigate the correlations between testosterone level, body condition, and behaviors within stages we used linear mixed models with diet, season and aviary as random factors and extrapolated marginal and conditional r^2 values (Barton, 2017; Nakagawa and Schielzeth, 2013; i.e. how much of the variance is explained by fixed effects alone and by the combination of fixed and random effects). We used contingency table analysis, specifically a Model II G-test of independence (Sokal and Rohlf, 2012), to investigate whether the frequency of reproductively active males changed with stage, diet, or season.

3. Results

3.1. Effect of time and stage on male physiology

The cloacal protuberance volume of early season birds remained constant during all stages, from baseline until after females were added (x ± SE = 232.94 ± 10.74 mm^3 to 237.80 ± 22.14 mm^3, $n = 29$, $t = -0.242$, $p = 1$, d = 0.052), whereas the cloacal protuberance volume increased in late season birds from baseline to after females were added (300.78 ± 30.06 mm^3 to 506.95 ± 47.24 mm^3, $n = 16$, $t = -7.631$, $p \leq 0.0001$, d = 0.516; Fig. 2). Diet antioxidant content did not affect these trends (low antioxidant diet: 271.92 ± 29.44 mm^3, $n = 23$; high antioxidant diet: 299.70 ± 30.20 mm^3, $n = 22$, $t = 0.987$, $p = 0.3290$, d = 0.196; Fig. 2).

There was also an effect of season on testosterone levels, as early season birds (aviaries 1–4) had significantly lower testosterone than late season birds (aviaries 5–6; 304.26 ± 116.63 pg/ml, $n = 29$, vs. 1424.635 ± 457.95 pg/ml, $n = 15$, $t = -6.585$, $p < 0.0001$, d = 0.842; Fig. 2). Just as with cloacal protuberance, the seasonal change in testosterone was best explained by the simpler two season model (AIC values were 530.7 and 531.6 for the two-season and three-season, respectively). Both diet and stage significantly affected testosterone levels (Diet: F = 21.806, $p < 0.0001$, η2 = 0.152; Stage: F = 17.051, $p < 0.0001$, η2 = 0.356); moreover, there was also a strong interaction between the two factors that resulted in a significant effect on testosterone levels (Diet × Stage: F = 8.046, $p < 0.0001$, η2 = 0.168). Specifically, testosterone levels of birds within a high antioxidant aviary were always greater than testosterone levels of their paired low antioxidant aviary, and testosterone levels peaked before (at the herbal stage) cloacal protuberance volume did (baseline: high antioxidant diet 493.68 ± 240.31 pg/ml, $n = 22$ vs. low antioxidant diet 159.802 ± 65.73 pg/ml, $n = 23$, $t = 1.082$, $p = 0.2813$, d = 0.403; nest box stage: high antioxidant diet 916.44 ± 370.72 pg/ml vs. low antioxidant diet 417.97 ± 209.50 pg/ml, $t = 2.238$, $p = 0.0269$, d = 0.351; herbal stage: high antioxidant diet 1643.05 ± 240.19 pg/ml vs. low antioxidant diet 702.77 ± 344.9 pg/ml, $t = 6.439$, $p < 0.0001$, d = 0.664; female stage: high antioxidant diet 864.68 ± 279.74 pg/ml vs. low antioxidant diet 427.30 ± 216.98 pg/ml, $n = 22$, $t = 3.130$, $p = 0.0021$, d = 0.372; Fig. 2). Given that testosterone levels peaked earlier than cloacal protuberance volumes, we wanted to determine if these physiological measures of breeding condition were strongly related. Notably, at the herbal stage there was a strong positive correlation between cloacal protuberance volume and testosterone levels across the six aviaries ($r^2m = 0.40$, $r^2c = 0.51$, $t = 5.238$, $p < 0.0001$). At the nest box and female stages there was a moderate positive correlation between cloacal protuberance volume and testosterone levels across the aviaries (NB: $r^2m = 0.08$, $r^2c = 0.375$, $t = 3.960$, $p = 0.0002$; FM: $r^2m = 0.08$, $r^2c = 0.49$, $t = 3.803$, $p = 0.0004$).

We detected a small increase in body condition index between the baseline and the female stage during the early season (80.87 ± 2.15 to 82.66 ± 2.06, $t = -3.592$, $p = 0.0027$, d = 0.157), but not the late season (82.95 ± 1.85 to 81.49 ± 2.00, $t = 2.231$, $p = 0.1641$, d = 0.189). Moreover, there was no significant effect of diet alone on body condition (F = 2.5212, $p = 0.1624$, η2 = 0.051), but a significant interaction between stage and diet (F = 4.9400, $p = 0.0027$, η2 = 0.311) that did not result in significant difference between the two diets at each single stage ($t < 2$, $p > 0.09$). We found no correlation between testosterone levels and body condition index at any of the three stages and considering antioxidant diet type ($r^2m < 0.07$, $r^2c < 0.18$, $p > 0.10$).

3.2. Effect of time and stage on male activity

The frequency of males actively engaged in at least one of six behavior(s) on a nest box (Table 2) significantly increased over the season (G = 22.96, $p < 0.001$), and significantly decreased as nest boxes, herbs, and females were sequential added (G = 1116.72, $p < 0.001$; Table 3). Frequency of active males was independent of dietary antioxidants (G = 0.02, $p > 0.05$).

3.3. Effect of diet and stage on behavior

About half the nest boxes ($n = 25/48$) were found to have herbs in them, with nearly all late season nest boxes adorned with herbs ($n = 15/16$ nest boxes) whereas only 10 of the 32 early season nest boxes had herbs. Perching on nest box, inside nest box and perched on nest box singing were the most common behaviors recorded (645, 121, and 106 times, respectively), whereas visual display, perched with dry nest material and aggression were observed less commonly (30, 4, and 2 times, respectively). The three most common behaviors as well as visual display were not strongly correlated with one another (< 0.22 in all cases) and a Principal Component Analysis (PCA) produced PC loadings that confirmed the independence of these four primary behaviors (i.e., each of the four PC axes had loadings > 0.98 for each of the four behaviors). Thus, we used the summed frequency of behaviors as the independent variable to assess the effect of diet and stage on male breeding behavior.

The number of behaviors performed by males per stage was diet dependent (Fig. 3). Males fed the low antioxidant diet decreased the number of behaviors performed as the stages progressed, showing a significant decrease comparing the nest box stage (8.00 ± 1.30, $n = 22$) and female stage (5.68 ± 0.94, $n = 19$, $z = 3.297$, $p = 0.0029$, d = 0.446; Fig. 3), while the number of behaviors performed at the herbal stage (6.00 ± 1.12, $n = 20$) was not significantly different from the nest box stage ($z = 1.900$, $p = 0.172$, d = 0.361) or the female stage ($z = 1.277$, $p = 0.605$, d = 0.071). Conversely, males fed the high antioxidant diet performed a constant number of behaviors across all stages (nest box stage: 9.29 ± 1.20, $n = 21$; herbal stage:

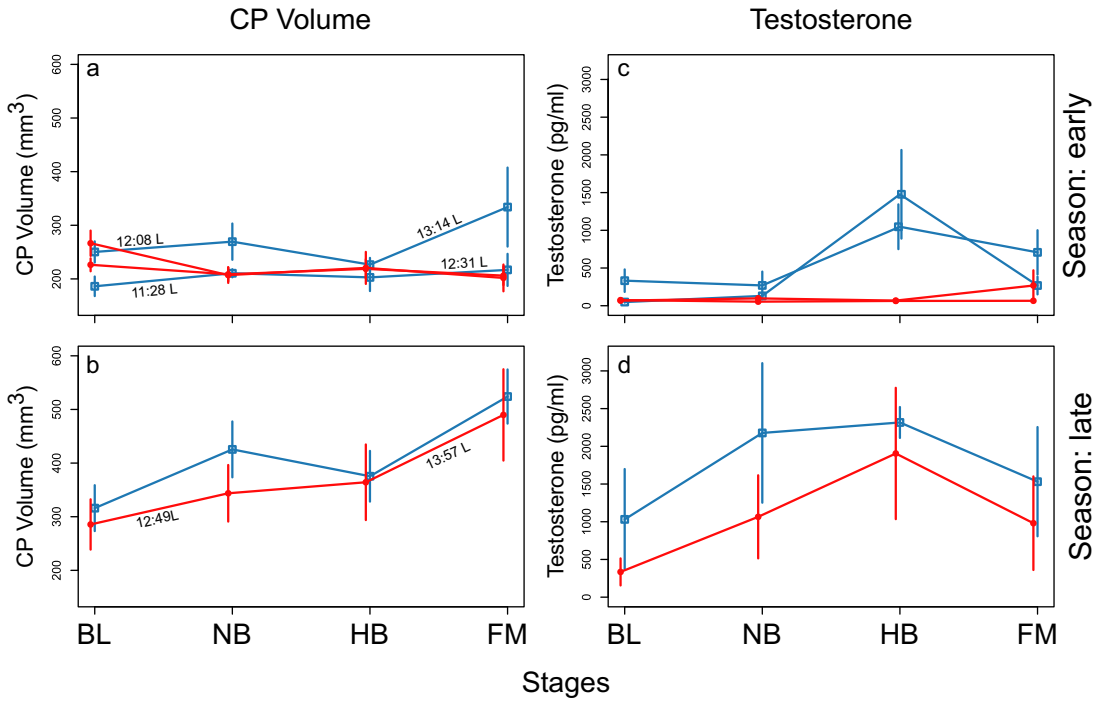

Fig. 2. (a–b) Average (± SE) cloacal protuberance (CP) volume (mm^3) and (c–d) plasma testosterone levels (pg/ml) of male starlings in each experimental aviary at Baseline (BL) and each of three stages (Nest Box: NB, Herbal: HB, and Female: FM) ca. one week apart. The pattern of change in cloacal protuberance and testosterone across stages was different for males tested early in the season (aviaries 1–4) compared to late in the season (aviaries 5–6) – see text for details. From February–April 2017 each aviary housed 7–8 male (n = 45 males) during the BL, NB, and HB stages and an additional 5–6 female European Starlings (n = 33 females) during the FM stage. Males were fed diets either low in antioxidants (red, •; Aviaries 1, 3, 5) or high in antioxidants (blue, □; Aviaries 2, 4, and 6) and both diet types were paired in time. Hours of light experienced by starlings at BL and FM stages (mid-point for each pair of aviaries) are indicated in the CP volume panels.

9.21 ± 1.32, n = 19; female stage: 8.93 ± 1.37, n = 15; nest box to herbal stage: z = 0.32, p = 1, d = 0.014; nest box to female stage: z = 0.743, p = 1, d = 0.066; herbal to female stage: z = 0.439, p = 1, d = 0.050; Fig. 3). This interaction between diet and stage resulted in a significant difference in the number of behaviors displayed at the female stage between the two diets (z = 2.187, p = 0.0287, d = 0.685; Fig. 3). As expected, plasma testosterone concentration and frequency of behaviors across the six aviaries at the three stages were positively correlated (nest box stage: r^2m = 0.16, r^2c = 0.17, t = 2.752, p = 0.009; herbal stage: r^2m = 0.12, r^2c = 0.14, t = 2.217, p = 0.034; female stage: r^2m = 0.19, r^2c = 0.34, t = 2.838, p = 0.008).

The frequency of rare behaviors (IN and A) did not change across the stages (nest box to herbal stage: z = −1.558, p = 0.119; nest box to female stage: z = −0.142, p = 0.887) and there were no differences between antioxidant diets (z = 0.508, p = 0.611). The frequency of female behaviors also did not differ across antioxidant diet type (z = 0.839, p = 0.402).

4. Discussion

By simulating the natural breeding phenology of European starlings, we determined how the sequential availability of nesting opportunities, herbs, and females interacted with dietary antioxidant availability to influence physiological and behavioral traits of male starlings. As anticipated, increased day length in spring induced changes in physiology (i.e., cloacal protuberance volume, testosterone levels) and breeding activity level (number of individuals active on nest boxes at each stage). Sequential addition of nest boxes, herbs and then females progressively increased cloacal protuberance volume of late season birds, while testosterone levels peaked when both nest boxes and herbs were available. Males fed diets with less dietary antioxidants decreased their frequency of breeding behaviors across the sequential stages, whereas males fed

Table 3
Percentage and proportion of male European Starlings (N = 45) active (defined as performing at least 1 behavior on a nest box) at three stages (Nest Boxes: NB, Herbs: HB, and Females: FM) during reproduction within each of the six paired aviaries at Max Planck Institute for Ornithology across the breeding season (February–April 2017). Given that male starlings must acquire and defend nest boxes during the breeding period, the proportion of male starlings in each aviary that were associated with a nest box indicates the level of male breeding activity. The introduction of nest boxes, herbs, and females in each aviary was staggered over time in part to determine the interaction between the effects of photoperiod and the three stages on male behavior and physiology (see Methods).

Date range	Antioxidant diet (aviary number)	Nest box stage		Herbal stage		Female stage		Seasonal average
		Percent	Proportion	Percent	Proportion	Percent	Proportion	
Feb 9–Mar 7	Low (1)	86%	6/7	57%	4/7	71%	5/7	80%
Feb 12–Mar 10	High (2)	86%	6/7	86%	6/7	43%	3/7	
Feb 21–Mar 19	Low (3)	100%	8/8	100%	8/8	75%	6/8	
Feb 24–Mar 22	High (4)	100%	7/7	86%	6/7	71%	5/7	
Mar 5–Mar 31	Low (5)	100%	8/8	100%	8/8	100%	8/8	96%
Mar 8–Apr 3	High (6)	100%	8/8	88%	7/8	88%	7/8	
Stage average		95%	–	86%	–	75%	–	

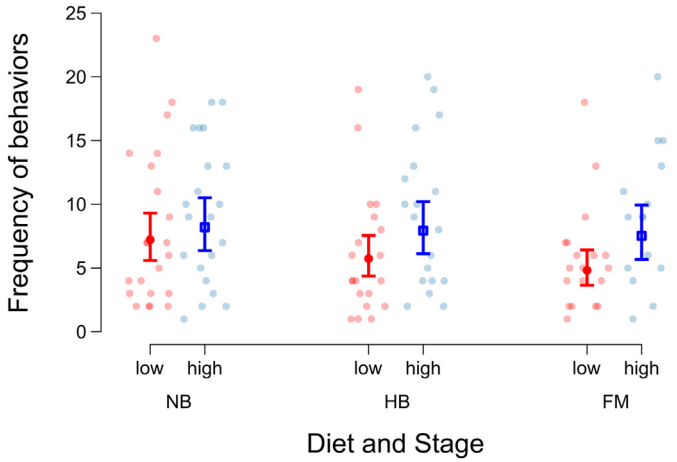

Fig. 3. Frequency of behaviors per individual male (fitted value ± CI) at three consecutive stages (nest box, herbal and females) during the breeding season. Males were fed diets either low in antioxidants (red, •; Aviaries 1, 3, 5) or high in antioxidants (blue, □; Aviaries 2, 4, and 6).

the high antioxidant diet were able to avoid this decrease, maintained a higher frequency of breeding behaviors across the stages, and this enhanced the effects of addition of stages on testosterone level.

4.1. Photoperiod initiates gonadal maturation

Cloacal protuberance is a photoperiod-dependent, copulatory organ that has been shown to increase in size during the breeding season as a result of an accumulation of sperm in the seminal glomeruli (Wolfson, 1952). This increase in size facilitates sperm transfer to females (Sax and Hoi, 1998) and has been used in the past as an indication of a male's readiness to reproduce (e.g. Tonra et al., 2011; Wolfson, 1952). Male starlings in our study that were tested later in spring (aviaries 5 and 6) increased cloacal protuberance volume and testosterone levels to a greater extent than those tested earlier in spring (aviaries 1–4) in support of a strong photoperiodic response (Fig. 2). The link between increasing day length and gonadal maturation has long been established (Rowan, 1925) and other studies have provided evidence that plasma testosterone levels increase when birds are subjected to long light cycles (Donham et al., 1982). We also observed a greater number of individuals active on nest boxes in the late season as compared to the early season, suggesting that photoperiod also contributed to increased levels of breeding activity (Table 3). These findings are consistent with those outlined by Dawson (2008), highlighting that photoperiod is widely understood to be the initial predictive cue stimulating birds to adapt their physiology (e.g., cloacal protuberance as proxy for the amount of fertile sperm, testosterone levels) for breeding.

4.2. Environmental cues (i.e., nest boxes, herbs, and females) affect temporal changes in male behavior and physiology

We were able to uniquely characterize how initial predictive information (i.e., photoperiod) and supplementary environmental cues (i.e., nest boxes, herbs, and females) interact to affect male starling behavior and physiology during the spring breeding period. We found only partial support for hypothesis 1 in that cloacal protuberance volume increased with sequential additions of nest boxes, herbs, and females, but only for late season birds (Fig. 2). This suggests that both photoperiod and environmental cues, such as nest boxes, herbs, and females, contribute to cloacal protuberance growth and potentially development of other secondary sex characteristics. In contrast, we found that testosterone was highest in males when both nest boxes and herbs were available (Fig. 2b) across the spectrum of photoperiods, suggesting that the peak in testosterone levels was directly affected by availability of nest boxes and herbs, not photoperiod alone, which was inconsistent with hypothesis 1. Casto et al. (2001) experimentally increased testosterone in male dark-eyed juncos and found that cloacal protuberance volumes of testosterone-implanted male dark-eyed juncos (*Junco hyemalis*) were significantly larger than those of control males, suggesting that a certain threshold testosterone level was required for upregulation of spermatogenesis and hence increases in cloacal protuberance volume. No previous studies have directly investigated the relationship between testosterone levels and herbs, though Gwinner et al. (2002) showed that the presence of nest boxes was an important factor for the increase in testosterone, which is not surprising since wild starlings occupy nest cavities before acquiring a mate (Feare, 1984). Our results indicate that presence of both nest boxes and herbs may provide a stronger cue for testosterone increase than just nest boxes alone, and that cloacal protuberance volume may increase in response to an initial increase in testosterone and then may be maintained even as testosterone decreases (e.g., after the herbal stage in our experiment).

Several studies have sought to determine the reason(s) starlings exhibit such distinct behaviors involving herbs (e.g., Gwinner, 1997; Gwinner et al., 2000; Gwinner and Berger, 2005; Veiga et al., 2006), which has resulted in support of several hypotheses, including mate attraction, defense against parasites and pathogens, and/or for stimulating nestling development (Clark, 1991, reviewed by Dubiec et al., 2013). Although our study was not designed to explicitly test these hypotheses, the ability of males to attract mates would be facilitated by the increase in their reproductive condition when both nest boxes and herbs were available. Surprisingly, the introduction of females resulted in a slight decline of testosterone in the four aviaries that were responsive to the stages. It is possible that testosterone levels increased once females were added and subsequently decreased prior to sampling as males had adequate time to interact with and copulate with females. However, during our behavioral observations we did not observe any copulations. Dittami et al. (1986) also found that the presence of female starlings in aviaries had limited effects on male reproductive condition. Thus, it seems testosterone levels were more strongly regulated by nest box and herb cues suggesting that males may pre-emptively increase testosterone levels in anticipation of female arrival.

A consistent decrease in the number of males active on nest boxes was recorded as we subsequently added herbs and then females. Such a decrease in activity of male European starlings was somewhat unexpected since at least in the wild they are highly aggressive during the breeding season and defend their nest sites from intraspecific competitors (Feare, 1984; Eens and Pinxten, 1996). This behavior may be a result of the limited number of nest sites for cavity nesting birds in the wild (reviewed by Newton, 1994), making intraspecific competition between males necessary to secure a mate, which is dependent on acquiring a nest box/cavity. Our experimental design provided at least one nest box per male in an aviary, which likely reduced competition between males. More males were active around the nest boxes soon after they were provided in part because individuals were competing to establish ownership of the nest boxes. Perhaps once males established nest box ownership and were familiar with their aviary residents, the frequency of behaviors and activity decreased, although this response was diet-dependent.

4.3. Dietary antioxidants influence the physiological and behavioral responses of males to environmental cues

In agreement with hypothesis 2, males fed the high antioxidant diet also consistently had higher testosterone than males fed the low antioxidant diet. It is widely accepted that testosterone is primarily responsible for driving breeding behavior in avian species. For example, males with higher testosterone have heightened responses toward aggressors in reproductive contexts (Wingfield et al., 1987), have more elaborate courtship displays (Hill et al., 1999), and are more likely to

defend larger territories (Chandler et al., 1994; Wingfield, 1984) than individuals with lower testosterone. The increase in testosterone in males fed more dietary antioxidants was not associated with an increase in body condition index so this effect may likely be related to the direct influence of such micronutrients on testosterone and its regulation of both reproductive physiology and breeding behavior of starlings. High levels of testosterone in past experiments have resulted in increased oxidative stress in male zebra finches and rats (Chainy et al., 1997; Alonso-Alvarez et al., 2007), potentially via elevated metabolic rate (e.g., Buchanan et al., 2001). However, dietary antioxidants can neutralize the imbalance between reactive oxygen species and antioxidant defenses (oxidative stress). Possibly, the dietary antioxidants protected males from the potential oxidative damage thereby allowing males fed high antioxidant diets to maintain higher levels of testosterone than males fed low antioxidant diets. This is the first study to directly manipulate levels of dietary antioxidants and then document a consistent increase in plasma testosterone levels in response to certain environmental cues somewhat independently of photoperiod alone.

Contrary to hypothesis 3, we found that frequency of behaviors for active males decreased, rather than increased, with the addition of nest boxes, herbs and then females, but interestingly only for those fed the low antioxidant diet. In contrast, males fed the high antioxidant diet maintained the frequency of behavioral displays across the experiment (Fig. 3). This effect of dietary antioxidants on male starling behavior is consistent with hypothesis 4 in that breeding behaviors were enhanced in those fed the high antioxidant diet. Oxidative stress has been identified as a proximate mechanism for the cost of reproduction in captive zebra finch, *Taeniopygia guttata* (Alonso-Alvarez et al., 2004; Wiersma et al., 2004), and it is widely accepted that antioxidants defend against oxidative stress (Monaghan et al., 2009). Performing a high frequency of behaviors may impose energetic/fitness related trade-offs on an individual that a diet rich in antioxidants could conceivably mitigate. It is feasible that males fed the low antioxidant diet were faced with a physiological trade-off: reduced availability of antioxidants made it difficult to defend against oxidative stress, so males reduced behavioral activity, the associated energy demands, and thus also the potential for oxidative stress.

5. Conclusions

By sequentially introducing nest boxes, herbs, and females to captive male European starlings, we investigated how these supplementary environmental cues initiate and maintain male reproductive condition (i.e., cloacal protuberance volume, plasma testosterone) and breeding behavior. Our results confirmed the oft-demonstrated effect of photoperiod on initiating reproductive condition in birds, while also showing that certain supplementary environmental cues (nest boxes, herbs) significantly influenced male physiology (e.g., testosterone) and behavior during the breeding period. We revealed for the first time that the availability of dietary antioxidants consistently enhanced the level of male testosterone response to the sequential addition of nest boxes, herbs, and then females. Dietary antioxidants in natural foods such as fruits are readily available for many songbirds during their fall migration (Smith et al., 2007). Unfortunately, we know little about sources of dietary antioxidants for free-living migratory songbirds in spring as they prepare for breeding. Even less is known about whether these beneficial effects of dietary antioxidants depend on type (i.e., anthocyanins are only one of many antioxidants) and the extent to which availability of these dietary antioxidants may protect migrating birds from oxidative damage associated with long-duration flights almost immediately followed by expectations for breeding.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2018.05.020.

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