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RESEARCH ARTICLE

The quantity–quality trade-off: differential effects of daily food availability times on reproductive performance and offspring quality in diurnal zebra finches

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ABSTRACT

An abundant food supply is crucial to reproductive performance, as shown by restricted food availability experiments, in small-sized vertebrates including birds. However, whether daily feeding times affect reproduction is largely unknown. The present study investigated the effects of daily food availability times on reproductive performance and quality of eggs and offspring survivors in zebra finches (*Taeniopygia guttata*). In randomly paired birds kept under a 12 h:12 h light:dark cycle for about 52 weeks, food availability period was restricted to 4 h in the morning [morning food availability (FA) group] or evening (evening FA group), with controls provided with food *ad libitum*; thus, the daily food deprivation period began after 4 h of food availability in the morning food access group and was continuous with night-time starvation in the evening food access group. Both food restrictions adversely affected reproductive health, as shown by reduced sex steroid and mesotocin levels, but not general metabolism, as indicated by the absence of a difference in thyroxin and triiodothyronine levels. Restricting food access to a 4 h period negatively affected reproductive performance, although there were differences between pairs in the morning and evening FA groups. In particular, there was delayed onset of reproduction and compromised reproductive success in evening FA but not in morning FA pairs; conversely, offspring health was severely compromised in morning FA but not in evening FA group pairs. Furthermore, morning FA group females were in better health, implying a sex bias in parental food provisioning. Overall, we demonstrate trade-off of 'quantity' (number of offspring produced and/or survived) for 'quality' (how healthy offspring were) in response to daily food availability times in zebra finches, which, much like humans, are diurnal and retain the ability to reproduce throughout the year.

KEY WORDS: Food availability, Hormones, Reproduction, *Taeniopygia guttata*

INTRODUCTION

Abundant food supply is crucial for the optimization of breeding schedules and reproductive performance of small-sized vertebrates, which cannot store large amounts of food. Increasing evidence has shown the effects of food availability on the onset of the breeding, mating and egg-laying times in seasonally reproducing animals including birds. For example, restricted food availability delayed and/or caused sub-optimal photo-induced gonadal development in European starlings (*Sturnus vulgaris*; Meijer, 1991) and Abert's towhees (*Melozone aberti*; Davies et al., 2015). Both resident and migratory songbirds on a supplemented diet showed an advanced in egg-laying dates and enhanced breeding success, measured as egg-laying frequency, clutch size, hatchling success, brood size and number of fledglings (Reynolds et al., 2003; Rutstein et al., 2004; Robb et al., 2008; Kaiser et al., 2014). Similarly, food shortages even during the non-reproductive phase of the annual cycle could have effects on the timing of gonadal growth and regression in the subsequent reproductive phase (Budki et al., 2009). Red-headed buntings (*Emberiza bruniceps*) exposed to a non-stimulatory photoperiod and fed for 6 h per day showed attenuated testicular growth when they were later subjected to a stimulatory 16 h photoperiod with food *ad libitum* (Budki et al., 2009). Furthermore, the times of food availability during the day could have significant effects on reproductive physiology. For example, food availability for 5 h in the evening [hours 11–16, where hour 0=lights on; evening food availability (FA) group], but not in the morning (hours 0–5, morning FA group), during 16 h light per day depressed by almost half the photostimulated testicular recrudescence in migratory blackheaded buntings, *Emberiza melanocephala* (Kumar et al., 2001), and resident subtropical house sparrows, *Passer domesticus* (Bhardwaj and Anushi, 2004). Consistent with these findings, broiler hens fed twice a day laid more eggs than those were fed once a day (Spradley et al., 2008).

In response to nutrient deficit, there can also be differential allocation of available nutritional resources by parents to eggs and hatchlings in order to selectively optimize offspring fitness. In particular, the mother's nutritional state affects the size, number and macronutrient composition (e.g. protein and lipid content) of eggs laid (Reynolds et al., 2003). For example, zebra finches fed on a high-quality diet laid eggs that were greater in number, mass and size, and hence had greater hatching success, compared with birds fed on a low-quality diet (Rutstein et al., 2004); however, lipid supplement to the diet did not affect egg quality (Williams, 1996). Similarly, there was a significant increase in the mass, volume and protein, but not lipid, content of eggs laid by Florida scrub jays (*Aphelocoma coerulescens*) when they were fed on a high-quality diet (Reynolds et al., 2003). There is also evidence suggesting that such diet-induced changes in egg quality can have long-lasting effects on the morphology and health of the offspring survivors (Grigg, 2014). The maternal allocation of nutrients to eggs is described as the primary investment to offspring health; parental feeding, which can vary with the parents' nutritional state, is a secondary investment to offspring health. The nutrition deficit can also have long-term detrimental consequences, including significantly reduced body mass and offspring survival as well as decreased reproductive success of offspring survivors (Naguib and Gil, 2005; Naguib et al., 2006). Varying the amount of hormones in

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laid eggs could also influence the development and behaviour of the resulting chicks (Groothuis et al., 2005). For example, yolk testosterone levels may affect begging calls and the efficiency of nutrient utilization in hatchlings (Schwabl, 1996; Groothuis et al., 2005).

At the mechanistic level, it remains poorly understood how a restricted-feeding regimen affects reproduction. The overall evidence, nonetheless, suggests both direct and indirect effects of food deprivation. Food deprivation during the day (i.e. starvation) could have a direct effect by triggering a cascade of hypothalamic releases which negatively impact the activity of the hypothalamic-pituitary–gonadal axis (Lynn et al., 2015; Davies et al., 2015; Rønning et al., 2009). This is evidenced by a food availability-induced alteration in the plasma levels of oestradiol in broiler hens (Onagbesan et al., 2006), testosterone in male house finches (*Haemorhous mexicanus*; Valle et al., 2015) and Abert's towhees (Davies et al., 2015) and thyroid hormones in Japanese quails, *Coturnix c. japonica* (Rønning et al., 2009). Consistent with this, food deprivation increased gonadotropin inhibiting hormone levels in zebra finches (*Taeniopygia guttata*; Lynn et al., 2015) and Pekin duck (*Anas platyrhynchos domestica*; Fraley et al., 2013). The nutrient deficit-induced stress could also negatively affect reproductive success, as demonstrated in zebra finches (Naguib and Gil, 2005; Naguib et al., 2006). Indirect effects of food restriction could occur through changes in the relationship of closely coupled daily light and food cues and changes in the synchronization of the circadian clock that underlies seasonal gonadal growth and development (Hahn, 1995; Hau and Gwinner, 1996; Perfito et al., 2008). In a diurnal species which forages only during light hours, presumably because of photosensory limitations, food and light cues remain relatively well integrated throughout the day. Thus, a feeding regime in which food is absent during the major part of the day might disrupt the coupling between light and food cues and subsequently affect gonadal growth and development. Notably, an aberrant feeding cycle caused desynchronization of circadian behavioural rhythms and induced concurrently negative effects on reproductive fitness in *Drosophila* (Xu et al., 2011).

The long-term effects of the duration and timing of daily nutrition deficit on reproduction, offspring quality and overall parental investment to breeding success have not been investigated. We hypothesized that food availability limited to only a few hours per day would negatively affect the reproduction and growth of offspring, and that the effects would vary dependent on food availability times during the day. To test this, we found an ideal experimental system in zebra finches (*Taeniopygia guttata*), which much like humans are diurnal and retain the ability to reproduce throughout the year. In zebra finches, gonadal maturation, egg laying and offspring birth are not tied to photoperiod; instead, food can be a potent stimulus for reproduction (Perfito et al., 2008). Furthermore, when held captive under constant photoperiod and temperature, zebra finches continuously reproduce with 3–4 yearly broods; this is useful for longitudinally tracing the effects of experimental manipulation on offspring survivors. Here, in adult zebra finches kept for 12 months under a 12 h:12 h day:night cycle and constant temperature, we restricted food availability to 4 h at the times of high (morning) and low (evening) food intake (I.M. and V.K., unpublished observations). Our predictions were as follows. (1) The limited food availability would negatively affect reproductive physiology at multiple levels, including circulating sex steroid and mesotocin levels (hormones associated with reproduction and affiliation), egg-laying onset, reproductive fecundity and quality of eggs (size of eggs, percentage protein, percentage lipid and yolk testosterone) and offspring (body mass and size). (2) The reproductive effects would vary depending on whether the food deprivation period was before (in evening-fed birds) or after (in morning-fed birds) the food availability period. (3) The limited feeding time would enforce a sex-biased parental primary investment, such that the offspring sex with a better survival prospect have a greater share of the investment.

MATERIALS AND METHODS

Animals and experimental protocol

The experiments were performed on adult (12–18 month old) zebra finches, *Taeniopygia guttata* (Vieillot 1817), that were born and raised in captivity in our indoor aviary at 24±2°C under a 12 h light:12 h dark cycle (light ∼200 lux, dark 0 lx), with *ad libitum* food and water. Compact florescent lamps (5 W, 220–240 V CFL lamps, Phillips India) provided the light period, as controlled by automatic timers (Mueller, Bedienungsanleitung SC 88).

We used a total of 36 zebra finches of each sex that were similar in body mass (12.4±0.1 g; see Fig. 1). They were housed in same-sex cages (54 cm×42 cm×30 cm; n=4 per cage) to avoid visual or acoustic contact with the opposite sex, in order to break any pair bonds established when they lived together in the aviary. Birds were kept on a 12 h light:12 h dark cycle and given food *ad libitum*, as before, for 3 weeks during which females that laid eggs were replaced to avoid a possible carry-over effect. Thereafter, the food availability period of 24/36 birds in each same-sex group was

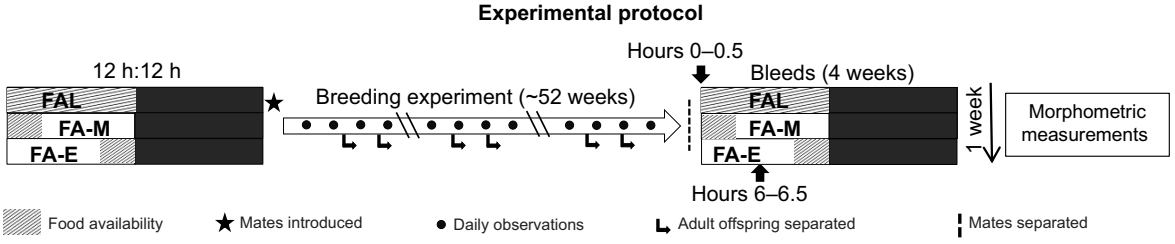

Fig. 1. Experimental protocol. *Taeniopygia guttata* were housed in same-sex cages under a 12 h light:12 h dark photoperiod at 24±2°C. Acclimated males and females were randomly paired and 12 pairs each were subjected to restricted food availability for 4 h in the morning (morning FA group, FA-M; hours 0–4, hour 0=lights on) or evening (evening FA group, FA-E; hours 8–12), with controls provided with food *ad libitum* (FAL) for ∼52 weeks. We made behavioural observations and recorded the number and size of eggs laid, clutch size and other measurements every alternate day with minimal disturbance to the birds. Offspring were separated from their parents when they were ≥90 days old, housed in same-sex cages and kept on the same feeding regime as before. At the termination of the breeding protocol of ∼52 weeks, breeding pairs were separated, housed in same-sex groups of four birds each and maintained on the feeding regimes as before. After 4 weeks of separation, four blood samples (two each in the morning during hours 0–0.5 and in middle of the day during hours 6–6.5) were obtained from both parents and adult offspring over a period of 4 weeks. We also recorded body mass and morphometric measurements at the end of the experiment.

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restricted to 4 h in the morning (morning FA group; beginning at hour 0 of the day: lights on) or the evening (evening FA group; beginning at hour 8 of the day) by step-wise reduction of the food availability period for 3 days each to 8 h (morning FA group: hours 0–8; evening FA group: hours 4–12), 6 h (morning FA group: hours 0–6; evening FA group: hours 6–12) and 4 h (morning FA group: hours 0–4; evening FA group: hours 8–12). The remaining 12/36 birds in each same-sex group were maintained on *ad libitum* food, and served as controls. Thus, 12 breeding pairs constituted the sample size for the breeding experiment in each feeding regime and in spite of an identical daily 4 h feeding:20 h fasting regime, there was a crucial difference in the timing of the food deprivation period between the two restricted-feeding groups. We introduced a similar food deprivation period but in different directions relative to 4 h food availability to morning FA group and evening FA group birds. The duration and timing of the daily food restriction period were chosen based on previous results that food availability restricted to one-third of the long day in the evening, but not in morning, depressed by almost half the photostimulated testicular maturation in seasonally breeding long-day songbirds (Kumar et al., 2001; Bhardwaj and Anushi, 2004). Each day, we provided a food-filled cup at hour 0 to *ad libitum*-fed birds and the morning FA group, and at hour 8 to the evening FA group, and the cup was replaced by an empty food cup at hours 4 and 12 for the morning and evening FA group, respectively, and with another food-filled cup the next day at hour 0 for *ad libitum*-fed birds.

The seeds of *Setaria italica* (Kakuni; 3.64 kcal g^{-1}) were provided as the primary diet, and hard-boiled eggs (1.47 kcal g^{-1}) mashed with crushed egg shell were given as a daily supplement; water was available at all times to all birds. After a week of food restriction, separately housed males and females were randomly paired, so that each condition had 12 breeding pairs. Every other day, the nest box in each cage was refreshed with nesting material comprising small twig leaves, cotton and grass. During this time, with minimal disturbance to breeding pairs, we recorded observations on egg-laying latency (time in days from mate introduction), number of eggs laid and/or discarded, number of hatchlings, time taken to fledge, offspring survival, sex ratio, etc. When >90 days old, the offspring were separated from their parents, housed in same-sex groups (n=5 per cage) and maintained on the feeding regime, as before.

Food intake, body mass and morphometric measurements

Food intake and body mass

At 7 days after mate introduction, 24 h food intake for each pair was measured, for which a weighed quantity of food was dispensed into a food cup and, following the food availability period, the unconsumed food was removed and weighed. To avoid food spillage, the cages were lined with an opaque white polythene sheet positioned 7 cm from the bottom, and food spilt inside the cage was collected and accounted for in daily food intake calculations. The recorded food intake over two consecutive days was first averaged for 1 day and then mean (±s.e.m.) food intake per pair per day was calculated. Faeces were dried and weighed, and mean faeces production (g per pair per day) was calculated. Utilization efficiency was calculated from these values using the following formula: [(food intake−faeces produced)/food intake]×100. Body mass of parents and >120 day old adult offspring was obtained during the first 15 min of the light phase during which food was unavailable to all birds to avoid an immediate spike in body mass. Birds were weighed on a top-pan balance to an accuracy of 0.1 g. We also calculated scaled mass index (SMI) for each bird using the formula $SMI_i=M_i\times(L_0/L_i)^b$; where M_i and L_i denote the body mass and tarsus length of individual 'i', respectively, L_0 is the arithmetic mean value of the tarsus length for the whole study population, and b is the slope estimate of the regression of body mass on tarsus length (Peig and Green, 2010).

Morphometric measurements

We measured the length of the tarsus, beak, wing and tail to assess restricted feeding-induced irreversible effects on body size, and hence on offspring quality (Rising and Somers, 1989; Peig and Green, 2010; Andrew et al., 2017), using a Vernier calliper to an accuracy of 0.1 mm for the length of the beak and tarsus or using a ruler to an accuracy of 1 mm for the length of the flattened wing and tail. We measured the tarsus length from the base with toes bent forward to the ankle joint, the wing length from the bend of the wing to the tip of the longest primary feathers, and the tail length from the base to the tip of the longest feather (Rising and Somers, 1989; Andrew et al., 2017). The tarsus and wing measurements of the left and right sides were averaged for each individual. We also measured these parameters in the parents to account for genetic effects on body size of the offspring.

Measurement of parameters of reproductive health and performance

Egg quality

Eggs demonstrated restricted feeding-induced effects on primary maternal investment in offspring quality. This was assessed by morphometric measurement (mass and volume) of the laid eggs and by assay of protein, lipid and yolk testosterone content in 'discarded' eggs to avoid any influence on the reproductive performance assessed as the number of hatchlings, fledglings and adult offspring from each zebra finch pair. An egg was considered as discarded by its parents if we found it out of the nest on more than one occasion; the first time an egg was out of the nest, it was returned to it. A total of 239 eggs (98 of the *ad libitum*-fed group, 74 of morning FA group and 67 of evening FA group) were measured for volume and 201 eggs (92 of the *ad libitum*-fed group, 63 of morning FA group and 46 of evening FA group) were weighed. The mass of broken, nicked or dried eggs was excluded from the analysis. From each feeding group, 10 discarded eggs (egg mass=0.80–0.83 g) were analysed for egg protein, lipid and yolk testosterone content. The eggs were weighed to an accuracy of 0.01 g and measured for size by a Vernier calliper to an accuracy of 0.1 mm. Discarded eggs were stored at −80°C for protein, lipid and yolk testosterone assays as per methods described by Salvante et al. (2007). Briefly, the albumen and yolk were separated from each egg, dried to a constant mass in a drying oven at 50°C and weighed to the nearest 0.1 mg. The lipid was removed from the dried yolk by diethyl ether extraction, and lipid-free yolk was then reweighed to the nearest 0.1 mg to obtain lean dry mass; when subtracted from the dry yolk mass, this gave yolk lipid content. Dry albumen mass and lean dry yolk mass were assumed to account for approximately 88% of egg protein content. Yolk lipid, yolk protein and albumen protein content were calculated as a percentage of total egg mass without the component of interest [e.g. % yolk lipid=yolk lipid mass/(egg mass−yolk lipid mass)×100].

The yolk testosterone was assayed by ethanol extraction as described by Kozlowski et al. (2009). Briefly, eggs were weighed and carefully broken, and egg yolk and albumin were weighed. A 50 µl sample of yolk was placed in a 1.5 ml tube containing 200 µl of distilled water and 1 mm silica beads, homogenized for 2 min and then incubated for 1 h at 37°C. Then, 500 µl of absolute alcohol was added to each sample, homogenized for 1 min and reincubated for 5 min at 500 rpm at room temperature. Subsequently, the samples

were centrifuged at 15,871 ***g*** for 10 min, and the supernatant was harvested, dried using a vacuum pump, treated with 50 µl of 100% ethanol and 300 µl of assay buffer, and stored overnight at 4°C. The next day, the testosterone content was assayed using a standard kit protocol (Enzo Life Sciences, Ann Arbor, MI, USA; cat. no. ADI-900-065), as described below for plasma testosterone.

Measurement of circulating hormones

We measured plasma levels of thyroid hormones (T3, triiodothyronine; T4, thyroxine) and corticosterone (CORT) to assess the restricted feeding-induced effects on general health and metabolism. Similarly, sex steroids (oestradiol, E2; testosterone) and mesotocin (avian homologue of mammalian oxytocin) were assayed in plasma to show restricted feeding-induced effects on reproductive health and associated physiology and behaviour. Plasma testosterone and E2 levels correlate with male and female sexual behaviours, respectively (Cain and Ketterson, 2013; Ubuka et al., 2014), and mesotocin levels reflect interactions and affiliation behaviours including bonding and affection between breeding pairs (Carter and Porges, 2013). All hormone assays were done in blood samples that were taken from the parent and >120 day old offspring, beginning 4 weeks after the end of the breeding protocol. During this period, the pairs were separated and again housed in same-sex cages, as before the start of the experiment; however, they were still maintained on their respective *ad libitum* or restricted-feeding regimes. Both the parent and offspring were bled alternately at the beginning (hours 0–0.5) and middle (hours 6–6.5) of the day (hour 0=lights on). After four bleeds (two each in the morning and midday) spread over 4 weeks, birds were weighed and the morphometric measurements were recorded. For each bleed, 50–100 µl blood was collected into a heparinized capillary tube by puncturing the wing vein, and centrifuged immediately at 845 ***g*** for 10 min. The plasma was harvested and stored at −20°C until assayed for hormones. Blood sampling was completed within 2 min to avoid stress-induced changes in hormone levels (Wada et al., 2008). Plasma samples collected early in the day were used for testosterone, E2 and CORT assays, and those collected in the middle of the day were used for T3, T4 and mesotocin assays. Care was taken to complete blood sampling, including bird capture and handling, within 2 min to avoid a stress-induced effect on CORT levels. We created a cohort of offspring plasma samples so that all breeding pairs had an equal contribution in hormone assays. For this, we first included plasma samples from at least one male and one female representative offspring from each breeding pair from the first clutch, which thus were similar in age. However, we included plasma samples from two males and one female offspring from the second clutch of the evening FA group as there were only 22 offspring (10 males and 12 females) in this group at the end of the experiment. A few plasma samples were also excluded because they were of insufficient volume for the hormone assay. Thus, for assays, we had a total of 30 samples (10 samples per feeding regime per generation per sex) for testosterone and CORT, 21 samples (seven samples per feeding regime per generation per sex) for E2, T3 and T4, and 18 samples (six samples per feeding regime per generation per sex) for mesotocin. We used specific ELISA kits for the measurement of hormones that have been validated and used by us and others in other song birds (Wada et al., 2008; Ubuka et al., 2014; Mishra et al., 2017a,b). All assays were run as per the manufacturer's protocols.

CORT ELISA

The CORT assay (Enzo Life Sciences, Ann Arbor, MI, USA; cat. no. ADI-900-097) used 10 µl plasma samples at 1:40 dilution in 1% steroid displacement buffer (10 µl plasma+10 µl steroid displacement buffer+380 µl assay buffer) as standardized and used in our laboratory (Mishra et al., 2017a). First, we pipetted 100 µl each of standards and plasma samples into standard and sample wells, respectively, and 100 µl of assay buffer in the non-specific background (NSB) and blank (B0) wells, followed by addition of 50 µl of assay buffer to NSB wells, and 50 µl blue conjugate (alkaline phosphate conjugated with CORT) to each well, except B0 and TA (total activity (TA) wells. Then, 50 µl of antibody was added to each well, except NSB, B0 and TA wells. The plate was incubated on an orbital shaker at 400 rpm for 2 h at room temperature. This was followed by three washes with 1× wash buffer. After washes, 5 µl conjugate and 200 µl of para-nitrophenyl phosphate in buffer (pNpp) were added to TA wells and to every well, respectively. The plate was incubated at room temperature for 1 h. Addition of 50 µl of stop solution ended the reaction, and the plate was read at 405 nm by a SpectraMax M2e microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA). The sensitivity and intra-assay variability of the assay were 26.99 pg ml^{-1} and 8.4%, respectively.

Testosterone ELISA

An immunoassay kit from Enzo Life Sciences (cat. no. ADI-900-065) measured testosterone in 10 µl plasma at 1:20 dilution with 1% steroid displacement buffer (10 µl plasma+10 µl steroid displacement buffer+180 µl assay buffer), according to the manufacturer's protocol and as validated and used in our laboratory (Mishra et al., 2017b). First, we pipetted 100 µl each of standards and plasma samples into the standard and sample wells, respectively. Then, 50 µl antibody was added to each well, except wells designated blank, TA and NSB, and the plate was incubated on a plate shaker at 500 rpm for 1 h at room temperature. Next, 50 µl conjugate (alkaline phosphate conjugated with testosterone) was added to each well, except for the blank and TA wells, and reincubated at 500 rpm for 1 h at room temperature. Subsequently, following three buffer washes, first 5 µl conjugate was added to TA wells and then 200 µl of pNpp was added to every well. The plate was then reincubated for 1 h at 37°C. Addition of 50 µl of stop solution ended the reaction, and the plate was read at 405 nm by a SpectraMax M2e microplate reader (Molecular Devices LLC). The sensitivity and intra-assay variability of the assay were 0.08 ng ml^{-1} and 5.7%, respectively.

T4 ELISA

An immunoassay kit (Arbor Assays, Ann Arbor, MI, USA; cat. no. K050-H1) was used to measure T4 in 10 µl plasma at 1:20 dilution with 1% steroid displacement buffer (10 µl of plasma+10 µl of dissociation reagent+180 µl of assay buffer), according to the manufacturer's protocol, and as validated and used in our laboratory (Mishra et al., 2017b). Briefly, we pipetted 100 µl of standards, 100 µl diluted sample, 125 µl of assay buffer and 100 µl of assay buffer into wells designated for standards, sample, NSB and Bo (maximum binding), respectively. To each well, 25 µl each of thyroxine conjugate and antibody (except NSB) was added, and the plate was incubated for 1 h on a plate shaker at 200 rpm at room temperature. Following four washes in wash buffer, 100 µl of TMB substrate was added to each well, and the plate was reincubated for 30 min without shaking at room temperature. Addition of 50 µl of stop solution ended the reaction and the plate was read at 450 nm by a Spectra Max M2e microplate reader (Molecular Devices LLC). The sensitivity and intra-assay variability of the assay were 0.29 ng ml^{-1} and 3%, respectively.

T3 ELISA

T3 titre was measured in 50 µl samples using an immunoassay kit (Accubind, Monobind, Lake Forest, CA, USA; cat. no. 125e300) according to the manufacturer's protocol, and as validated and used in our laboratory (Mishra et al., 2017b). The assay began with the addition of 50 µl of standards and samples to designated wells, followed by the addition of 100 µl of T3 enzyme conjugate, followed by incubation for 1 h at room temperature. Each well was washed thrice with washing buffer and 100 µl of working substrate solution (tetramethylbenzidine, TMB+hydrogen peroxide in buffer) was added. The plate was incubated for 15 min without shaking at room temperature. Addition of 50 µl stop solution to every well ended the reaction, and the plate was read at 405 nm by a SpectraMax M2e microplate reader (Molecular Devices LLC). The sensitivity and intra-assay variability of the assay were 0.04 ng ml^{-1} and 5.4%, respectively.

E2 ELISA

The plasma E2 concentration was measured by using an enzyme immunoassay kit (Estradiol EIA Kit, Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instruction, and as validated and used in other birds (Ubuka et al., 2014). Briefly, 100 µl and 50 µl of EIA buffer were added to NSB and Bo wells, respectively. Then, 50 µl of standards and samples were added to the respective wells, followed by addition of 50 µl of AChE (oestradiol acetylcholinesterase) tracer to all wells, except TA and blank wells. Subsequently, 50 µl of E2 antiserum was added to each well, except the TA, NSB and blank wells. The 96-well plate was covered with a plastic film and incubated for 1 h on an orbital shaker (200 rpm) at room temperature. Each well was washed five times with washing buffer, followed by the addition of 200 µl of Ellman's reagent (substrate for the AChE) to each well and 5 µl of tracer to TA wells. The plate was again covered with a plastic film and reincubated on an orbital shaker (200 rpm) in the dark at room temperature. The assay developed an optimal colour (i.e. Bo wells≥0.3 OD) in 1 h. Next, the plate was read at 405 nm by a SpectraMax M2e microplate reader (Molecular Devices LLC). The sensitivity and intra-assay variability of the assay were 20 pg ml^{-1} and 7.4%, respectively.

Mesotocin ELISA

We measured mesotocin using an enzyme immunoassay kit (Arbor Assay; cat no: K048-H1) with 500 ng ml^{-1} stock mesotocin solution (Arbor Assay; cat no: X127-625UL), as per the manufacturer's protocol. Briefly, plasma mesotocin was extracted using an extraction solution. For this, 20 µl plasma diluted with 30 µl of extraction solution was nutated for 1.5 h at room temperature, followed by centrifugation for 20 min at 4°C at 1600 ***g***. The supernatant was dried in a speed vacuum centrifuge at 37°C, followed by its reconstitution in 250 µl of assay buffer. Then, 100 µl of the standards, reconstituted samples and assay buffer were added to wells designated for the standards, samples and maximum binding (Bo), respectively; 125 µl of assay buffer was added to NSB wells. Next, 25 µl each of conjugate and antibody were sequentially added to every well (no antibody was added to NSB wells). After incubation of the plate at 200 rpm for 15 min at room temperature, the plate was sealed and stored overnight at 4°C. The next day, each well was washed four times with 300 µl of wash buffer, and this was followed by the addition of 100 µl of TMB substrate to every well. Addition of 50 µl stop solution ended the reaction after 0.5 h incubation without shaking. The plate was read at 450 nm by a SpectraMax M2e microplate reader (Molecular Devices LLC). The sensitivity and intra-assay variability of the assay were 17 pg ml^{-1} and 8.8%, respectively.

Reproductive performance

The reproductive performance was defined by taking into account several parameters that we recorded for each breeding pair. This included a per-pair record of the egg-laying latency (number of days taken to lay the first egg from mate introduction), primary energetic investment (eggs), reproductive event (clutches laid in a year), hatchling number, fledgling failure ratio (number of fledgling deaths/total number of fledglings) and adult offspring survivors. We also calculated the annual breeding success rate for each food condition, i.e. the number of adult offspring per successful breeding attempt per pair, as described by Murray (2000).

Animal welfare and ethics note

This study was carried out as per the approval of the Institutional Animal Ethics Committee (IAEC) of the University of Delhi, India (DU/ZOOL/IAEC-R/2015/02). We daily checked and replenished food and water, and cleaned cages every third day and rooms once a week. There were also frequent veterinarian visits to check on the birds' health. The measurement of body mass every alternate week showed that birds maintained good health. All breeding pairs survived the restricted-feeding protocol.

Statistics

Statistical analyses used GraphPad prism (versions 5.0 and 7.0) and IBM SPSS statistics (version 20) software, as appropriate. Data were tested for normality by Shapiro–Wilk normality test and, when necessary, these were log-transformed to approach normality (Zar, 1996). Data that did not approach normality were tested by non-parametric tests. One-way ANOVA followed by Scheffe's and Tukey's *post hoc* tests for comparison of groups with unequal and equal sample sizes, respectively, analysed food availability-induced effects on daily food intake and parameters that defined egg quality and annual reproductive efficiency. Two-way ANOVA with Bonferroni *post hoc* test tested the effects of food availability time, food availability (factor 1), sex (factor 2) and factor 1×2 interactions on parental body mass. Furthermore, Wilk's lambda multivariate general linear models (GLMs) tested the effects of FA, sex and their interaction on offspring morphometric measurements (body mass, and tarsal, beak, tail and wing length). Similarly, univariate GLMs tested the effects of food availability, sex and generation on hormone levels. Pearson's correlation analysis determined relationships of hormones with the parameters of annual reproductive performance and offspring health. For statistical significance, alpha was set at 0.05.

RESULTS

Body mass and food intake

At the end of breeding protocol, the mean (±s.e.m.) body mass of parent birds was as follows: *ad libitum* fed: males – 12.6±0.4 g, females – 12.7±0.3 g; morning FA group: males – 11.5±0.2 g, females – 11.8±0.2 g; evening FA group: males – 11.9±0.2 g, females – 12.5±0.3 g. Although all parent birds maintained good health irrespective of the feeding regime, we found a significant effect on body mass of food availability, but not of sex or food availability×sex interaction ($F_{2,66}$=8.304, P=0.0006, η^2=0.201; two-way ANOVA). In particular, male parents in the morning FA group, but not the evening FA group, were significantly lighter than those given food *ad libitum* (P<0.05; Bonferroni *post hoc* test). There was also a significant group difference in per-pair daily food intake ($F_{2,30}$=6.384, P=0.0049, η^2=0.299; one-way ANOVA), with

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Table 1. Parameters determining annual reproductive performance of zebra finches (*Taeniopygia guttata*) maintained under a 12 h light:12 h dark photoperiod and subjected to restricted food availability for 4 h in the morning (morning FA group) or in the evening (evening FA group), with controls fed *ad libitum*

	Food availability			One-way ANOVA statistics		
Reproduction parameters	*Ad libitum*	Morning FA	Evening FA	$F_{2,33}$	*P*	η^2
Egg-laying latency (days from mate introduction)	33.75±4.22[a]	50.33±7.305[a,b]	62.67±3.57[b]	7.52	0.002	0.31
Reproductive events (no. of clutches laid in a year)	9.58±0.45[a]	4.17±0.51[b]	5.17±0.34[b]	43.15	<0.0001	0.72
Primary energetic investment (no. of eggs per pair)	52.08±2.94[a]	15.58±2.83[b]	16.33±1.89[b]	64.33	<0.0001	0.80
Hatchlings per pair	12.17±1.32[a]	5.17±0.66[b]	5.75±1.10[b]	13.29	<0.0001	0.45
Adult offspring per pair*	11.5±1.37[a]	4.25±0.48[b]	2.83±0.44[c]	30.06	<0.0001	0.65
Fledgling failure ratio (no. of fledgling deaths/total no. of fledglings per pair)	0.05±0.03[a]	0.14±0.05[b]	0.45±0.07[c]	18.36	<0.0001	0.53
Annual breeding success (no. of offspring/no. of successful breeding attempts)	2.79±0.21[a]	1.06±0.11[b]	0.58±0.08[c]	67.11	<0.0001	0.80

Data are means±s.e.m., *n*=12 pairs. Different superscript letters indicate significant differences, as determined by Tukey's *post hoc* test following one-way ANOVA. The effect size estimates are presented as partial eta squared (η^2). For statistical significance, alpha was set at 0.05. *log-normal values used for one-way ANOVA.

significantly reduced food intake in morning FA group (mean±s.e.m. intake per pair per day; 19.12±1.19 kcal) and evening FA group (18.57±2.18 kcal), as compared with *ad libitum*-fed pairs (25.97±1.97 kcal) (P<0.05; Tukey's *post hoc* test); food intake did not differ between morning FA group and evening FA group pairs. We also found no significant difference in the utilization efficiency (mean±s.e.m.) between the three feeding groups (*ad libitum* fed 93.0±0.5%, morning FA group 91.1±0.9%, evening FA group 93.2±0.8%).

Reproductive health and performance

Reproductive output

Table 1 provides a summary of results. Overall, we found a significant group difference in the onset of reproduction, with the time to laying the first egg being almost twice as long in evening FA group as compared with the *ad libitum*-fed group (P<0.05; one-way ANOVA). Over the year, the primary energetic investment in reproduction (eggs laid), reproductive events (egg clutches) and hatchlings was significantly reduced in the morning and evening FA groups as compared with the *ad libitum*-fed group. However, the number of fledglings that died, and so did not contribute to the adult offspring population, was significantly higher in the evening FA group; thus, the annual breeding success rate was: evening FA group<morning FA group<*ad libitum*-fed group.

Egg quality

Egg quality as a measure of primary maternal investment was assessed by five parameters: egg mass and volume, percentage lipid and protein content, and yolk testosterone levels. We found a significant effect of food availability on all parameters, except egg protein, (egg mass: $F_{2,198}$=4.928, P=0.008, η^2=0.047; egg volume: $F_{2,236}$=50.524, P<0.0001, η^2=0.30; percentage egg lipid: $F_{2,27}$=4.215, P=0.0255, η^2=0.238; yolk testosterone: $F_{2,27}$=4.039, P=0.029, η^2=0.23; one-way ANOVA). Eggs were significantly lighter and smaller in size in restricted-feeding pairs than in *ad libitum*-fed pairs (P<0.05; Scheffe's *post hoc* test; Fig. 2). However, eggs from morning FA group pairs had a significantly

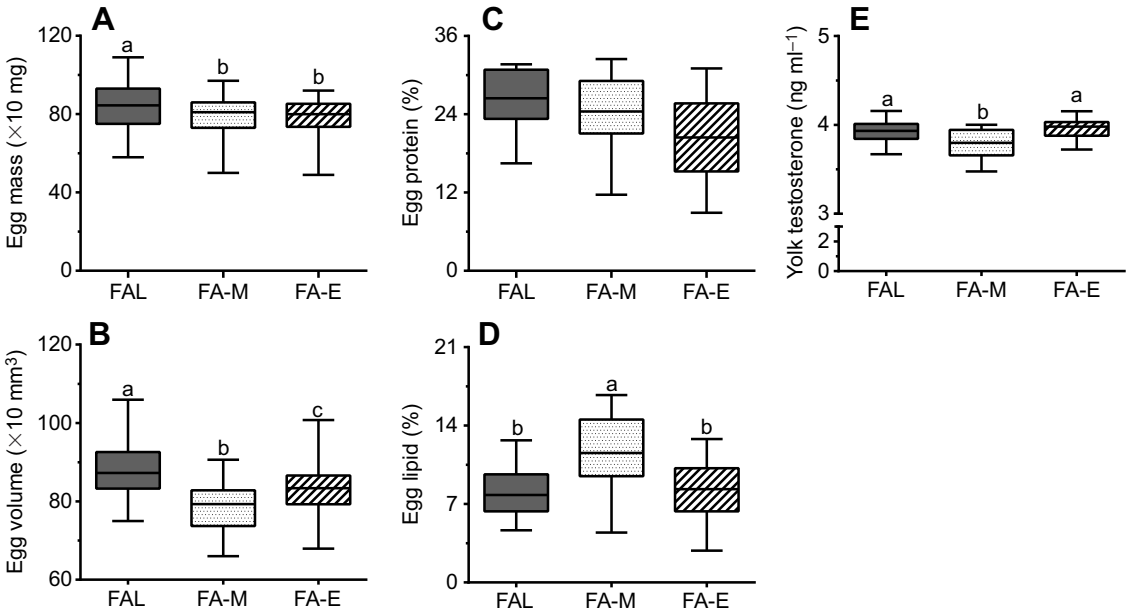

Fig. 2. Effects on egg quality. Whisker plot (minimum to maximum) of egg mass (A), egg volume (B), percentage protein content (C), percentage lipid content (D) and yolk testosterone (E) of eggs of zebra finch breeding pairs that were maintained under a 12 h light:12 h dark photoperiod and subjected to food deprivation (food availability restricted to 4 h in the morning, hours 0–4; morning FA group, FA-M; or in the evening, hours 8–12; evening FA group, FA-E), with controls fed *ad libitum* (FAL). Different letters indicate a significant difference, as determined by one-way ANOVA, followed by Scheffe's *post hoc* test (A,B) or Tukey's *post hoc* test (C–E). P<0.05 was considered statistically significant. Note that percentage protein and lipid content, and yolk testosterone were assessed in the discarded egg. For statistical significance, alpha was set at 0.05.

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Table 2. Results of Wilk's lambda multivariate general linear model (GLM) analysis of morphometric measurements (mean±s.e.m.) of zebra finch offspring in the control and morning and evening FA groups

	Between-subject effect test			Food availability		
Dependent variable	Food availability	Sex	Interaction (food availability×sex)	*Ad libitum*	Morning FA	Evening FA
Tarsal length (cm)	**$F_{2,116}$=11.44, P<0.0001, η^2=0.17**	$F_{1,116}$=1.79, P=0.180, η^2=0.015	**$F_{2,116}$=4.57, P=0.010, η^2=0.07**	♂: 15.01±0.15[a] ♀: 14.6±0.08	♂:13.97±0.19[b] ♀: 14.3±0.13	♂: 14.77±0.16[a,b] ♀: 14.3±0.16
Wing length (cm)	**$F_{2,116}$=6.74, P=0.002, η^2=0.11**	**$F_{1,116}$=10.43, P=0.002, η^2=0.08**	$F_{2,116}$=0.48, P=0.620, η^2=0.008	♂: 5.56±0.02 ♀: 5.46±0.02	♂: 5.51±0.02 ♀: 5.40±0.02	♂: 5.43±0.05 ♀: 5.39±0.04
Beak length (mm)	**$F_{2,116}$=14.78, P<0.0001, η^2=0.20**	$F_{1,116}$=0.001, P=0.990, η^2=0.001	$F_{2,116}$=2.44, P=0.090, η^2=0.04	♂: 9.57±0.05[a] ♀: 9.65±0.08	♂: 9.17±0.07[b] ♀: 9.34±0.07	♂: 9.38±0.09[a,b] ♀: 9.31±0.14
Body mass* (g)	**$F_{2,116}$=21.28, P<0.0001, η^2=0.25**	**$F_{1,116}$=6.701, P=0.010, η^2=0.06**	$F_{2,116}$=1.46, P=0.204, η^2=0.03	♂: 12.25±0.23[a] ♀: 13.18±0.25[a]	♂: 11.07±0.09[b] ♀: 11.67±0.20[b]	♂: 12.07±0.17[a,b] ♀: 12.41±0.22[a,b]

Zebra finch offspring were born and raised under a 12 h light:12 h dark photoperiod with restricted food availability for 4 h in the morning (morning FA group) or in the evening (evening FA group); controls were fed *ad libitum*. The effect size estimates are presented as partial eta squared (η^2). Bold indicates statistical significance. Different superscript letters indicate significant differences, as determined by Bonferroni *post hoc* test. For statistical significance, alpha was set at 0.05. *log-normal values used for GLM.

higher percentage lipid and lower yolk testosterone content, as compared with those from evening FA group or *ad libitum*-fed pairs (P<0.05, Tukey's *post hoc* test; Fig. 2).

Offspring quality (body size and condition)

Wilk's lambda multivariate GLM tested the effect of FA, sex and their interaction on body mass and tarsal, wing and beak length, which we considered as indicators of overall quality, including body size and condition, of the offspring survivors; tail length did not show restricted-feeding effects and was excluded from the statistical model. We found a significant effect of food availability on all four parameters, of sex on body mass and wing length, and of the food availability×sex interaction on tarsal length (Table 2). Although the parents did not differ in body size, we found significant differences between the three groups in terms of body mass and size of adult offspring. As compared with the *ad libitum*-fed group, offspring were of significantly lower mass, and male offspring in particular were significantly smaller in size (short tarsus and beak) in the morning FA group (P<0.05; Bonferroni *post hoc* test; Table 2).

To show whether body mass of offspring was related to their body size, we calculated the SMI, which is an adjusted value normalized to that of the expected value if all individuals were of the same body size (Peig and Green, 2010). For this, we used tarsal length as it best correlated with body mass (tarsal length: r=0.283, P=0.002). The comparison of body mass/tarsal length correlation coefficients gave a test statistic Z=−0.687 (P=0.492), indicating that the relationship was sex independent. Mean (±s.e.m.) SMI values for three groups were: *ad libitum*-fed group 12.70±0.17; morning FA group 11.38±0.17; evening FA group 12.07±0.13. Overall, there was a significant effect of food availability ($F_{2,119}$=19.124, P<0.0001, η^2=0.243; one-way ANOVA), with overall SMI values of: *ad libitum*-fed group>evening FA group>morning FA group (P<0.05; Scheffe's *post hoc* test).

Plasma hormone levels

We measured mesotocin, testosterone, E2 (in females), CORT and thyroid hormones (T4 and T3), the markers of reproductive health, general health and metabolism in birds. There was a significant effect of food availability and generation, but not of sex, on mesotocin (P<0.05; GLM; Table 3). In parents, the mesotocin levels were significantly higher in the *ad libitum*-fed group than in the restricted-feeding groups, and the levels in female, but not male, offspring were significantly lower in the evening FA group than in the *ad libitum*-fed group (P<0.05; Tukey's *post hoc* test; Fig. 3). Likewise, there were significant effects of food availability, sex and generation on plasma testosterone, with levels significantly higher in males than in females, and in parents than in offspring (P<0.05; GLM; Table 3). Between the three groups, testosterone levels were significantly higher in evening FA group parents and *ad libitum*-fed male offspring, as compared with the other two groups (P<0.05; Tukey's *post hoc* test; Fig. 3). Similarly, we found a significant effect of food availability, but not of generation or food availability×generation interaction, on female plasma E2 levels (P<0.05; GLM; Table 3). In female parents, E2 levels were significantly lower in the morning FA group and evening FA group than in the *ad libitum*-fed group (P<0.05; Tukey's *post hoc* test; Fig. 3). E2 and testosterone levels did not show group differences in female offspring (Fig. 3). Furthermore, plasma CORT showed a significant effect of food availability and sex, and the levels were significantly higher in females than in males (P<0.05; GLM; Table 3, Fig. 3). However, we found no effect on plasma T4 and T3 levels of all factors tested, except for the two-factorial interaction (T4: food availability×generation, T3: generation×sex; P<0.05; GLM; Table 3).

Relationships: hormones versus reproductive performance or offspring quality (SMI)

There was a positive correlation of both sexes' mesotocin levels on average SMI of the offspring/breeding pair, suggesting that parents with higher mesotocin levels produced 'better quality' offspring (P<0.05; Pearson's correlation; Fig. 4). SMI and sex steroid levels were significantly correlated (smaller offspring males and females had lower plasma testosterone or E2 levels, respectively), suggesting evening FA group effects on hormonal secretions (P<0.05; Pearson's correlation; Fig. 4). Plasma mesotocin in both sexes and E2 in females were positively correlated with reproductive frequency, primary energetic investment and annual breeding success rate (mesotocin only), and both mesotocin and E2 levels were negatively correlated with reproduction latency, i.e. time taken from mate introduction to laying the first egg (P<0.05; Pearson's correlation; Fig. 4). Similarly, plasma testosterone was negatively correlated with primary energetic investment and annual breeding success rate, and was positively correlated with fledgling failure ratio (P<0.05; Pearson's correlation; Fig. 4). However, we found no significant correlation of plasma T4, T3 and CORT levels with reproductive performance (data not shown), negating their direct role in food availability-induced effects on reproduction in zebra finches.

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Table 3. Results of univariate GLM analysis of plasma hormone levels in zebra finches in the control and morning and evening FA groups

Hormone	Corrected model	Food availability	Generation	Sex	Food availability×generation	Generation×sex	Food availability×sex	Food availability×generation×sex
T4 (ng ml^{-1}) (n=7 per group)	**$F_{11,72}$=2.01, P=0.039, η^2=0.24**	$F_{2,72}$=2.25, P=0.113, η^2=0.06	$F_{1,72}$=1.63, P=0.21, η^2=0.022	$F_{1,72}$=3.91, P=0.052, η^2=0.051	**$F_{2,72}$=5.41, P=0.006, η^2=0.13**	$F_{1,72}$=0.029, P=0.87, η^2=0.001	$F_{2,72}$=0.48, P=0.62, η^2=0.01	$F_{2,72}$=0.28, P=0.76, η^2=0.008
T3 (ng ml^{-1}) (n=7 per group)	**$F_{11,72}$=2.15, P=0.027, η^2=0.25**	$F_{2,72}$=2.89, P=0.062, η^2=0.74	$F_{1,72}$=0.49, P=0.487, η^2=0.007	$F_{1,72}$=3.24, P=0.076, η^2=0.043	$F_{2,72}$=1.47, P=0.24, η^2=0.023	**$F_{1,72}$=8.48, P=0.005, η^2=0.112**	$F_{2,72}$=0.85, P=0.43, η^2=0.023	$F_{2,72}$=0.54, P=0.59, η^2=0.015
CORT (ng ml^{-1})* (n=10/group)	**$F_{11,108}$=2.02, P=0.033, η^2=0.17**	**$F_{2,108}$=4.02, P=0.021, η^2=0.07**	$F_{1,108}$=0.81, P=0.369, η^2=0.01	**$F_{1,108}$=5.36, P=0.023, η^2=0.05**	$F_{2,108}$=1.26, P=0.288, η^2=0.023	$F_{1,108}$=0.59, P=0.446, η^2=0.005	$F_{2,108}$=1.528, P=0.22, η^2=0.03	$F_{2,108}$=0.91, P=0.41, η^2=0.02
Mesotocin (pg ml^{-1}) (n=6 per group)	**$F_{11,60}$=3.44, P=0.001, η^2=0.39**	**$F_{2,60}$=11.35, P<0.0001, η^2=0.76**	**$F_{1,60}$=4.14, P=0.046, η^2=0.07**	$F_{1,60}$=0.001, P=0.99, η^2=0.00	$F_{2,60}$=1.61, P=0.21, η^2=0.05	$F_{1,60}$=0.88, P=0.35, η^2=0.02	$F_{2,60}$=2.47, P=0.09, η^2=0.08	$F_{2,60}$=1.00, P=0.37, η^2=0.032
Testosterone (ng ml^{-1}) (n=10 per group)	**$F_{11,108}$=40.0, P<0.0001, η^2=0.80**	**$F_{2,108}$=13.40, P<0.0001, η^2=0.87**	**$F_{1,108}$=92.31, P<0.0001, η^2=0.61**	**$F_{1,108}$=165.11, P<0.0001, η^2=0.46**	**$F_{2,108}$=17.78, P<0.0001, η^2=0.25**	**$F_{1,108}$=88.65, P<0.0001, η^2=0.45**	**$F_{2,108}$=8.85, P<0.0001, η^2=0.14**	**$F_{2,108}$=6.95, P=0.001, η^2=0.12**
E2 (pg ml^{-1}) (n=7 per group)	**$F_{5,36}$=4.06, P=0.005, η^2=0.36**	**F=7.03, P=0.003, η^2=0.28**	F=1.60, P=0.21, η^2=0.04	–	F=2.32, P=0.11, η^2=0.11	–	–	–

Zebra finch offspring were born and raised under a 12 h light:12 h dark photoperiod with restricted food availability for 4 h in the morning (morning FA group) or in the evening (evening FA group); controls were fed *ad libitum*. The effect size estimates are presented as partial eta squared (η^2). Bold indicates statistical significance. For statistical significance, alpha was set at 0.05. *log-normal values used for general linear model.

DISCUSSION

We have shown here that food availability restricted to the morning did not attenuate reproductive fecundity or offspring survival, although offspring survivors were of poor quality when they reached adulthood. Conversely, food availability restricted to the evening significantly delayed egg laying, reduced clutch size and induced higher offspring mortality. To our knowledge, this is the first evidence of a food availability time-dependent trade-off of 'quantity' (offspring produced and/or survived) for 'quality' (how good offspring survivors were in terms of body condition) in reproduction of a continuously reproducing diurnal species.

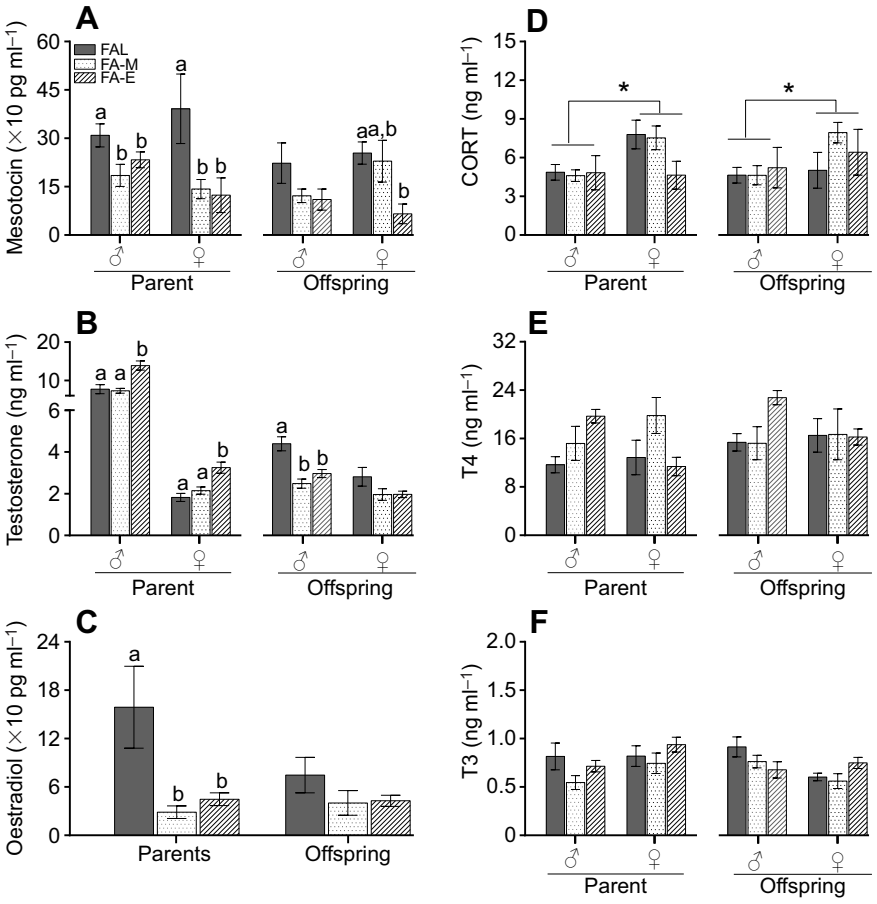

Fig. 3. Effects on plasma hormone levels. Plasma hormone levels (means±s.e.m.) of mesotocin (A), testosterone (B), oestradiol (E2, C), corticosterone (CORT, D), thyroxin (T4, E) and triiodothyronine (T3, F) in parent and offspring zebra finches maintained under a 12 h light:12 h dark photoperiod and subjected to food deprivation (food availability restricted to 4 h in the morning, hours 0–4; morning FA group, FA-M; or in the evening, hours 8–12; evening FA group, FA-E), with controls fed *ad libitum* (FAL). Different letters indicate a significant difference between the feeding regimes in terms of the effects of food availability time, sex and generation by univariate general linear models (GLMs) and Tukey's *post hoc* test. Asterisks indicate a significant sex effect. For statistical significance, alpha was set at 0.05.

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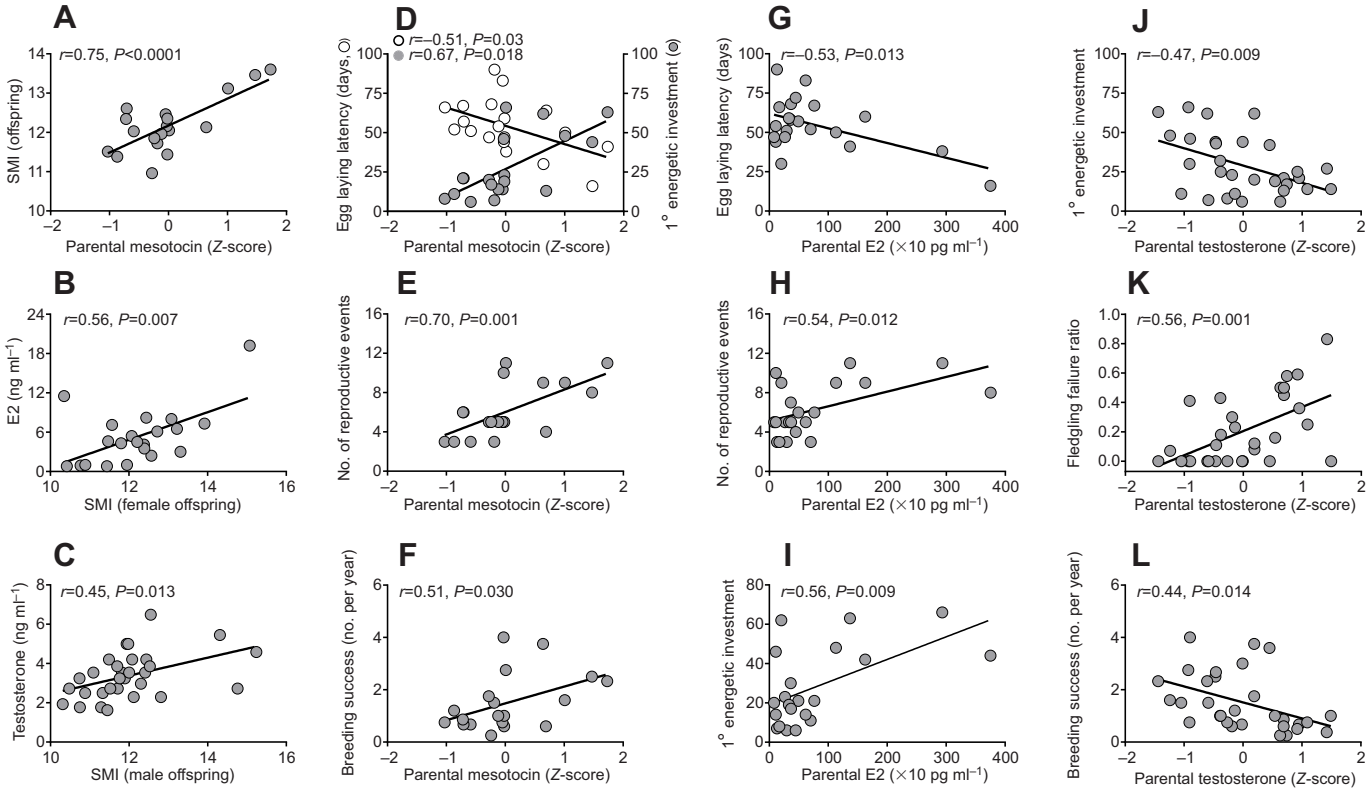

Fig. 4. Relationship between hormones and reproductive performance or offspring body condition. (A–C) Relationship of parental mesotocin (an affiliation marker) with offspring body condition (scaled mass index, SMI; A), and of SMI with sex steroids (B: female offspring: oestradiol, E2; C: male offspring: testosterone), which are indicators of reproductive health. Note: a significant relationship between offspring SMI and plasma mesotocin levels is presented with reference to the *Z*-score of both male and female parents. (D–L) Relationship of parental mesotocin (D–F), female E2 (G–I) and parental testosterone (J–L) with different parameters that defined reproductive health and performance of zebra finches maintained under a 12 h light:12 h dark photoperiod and provided with food *ad libitum* (FAL) or subjected to food deprivation (food availability restricted to 4 h in the morning, hours 0–4; morning FA group, FA-M; or in the evening, hours 8–12; evening FA group, FA-E). We used the *Z*-score for a better depiction of the relationship. This was calculated from averaged male and female hormone concentrations, and hence normalized sex-dependent variation, if any, in hormone levels. However, the *Z*-score was calculated when there was a significant correlation between parental hormone levels and the reproductive parameter under consideration. For statistical significance, alpha was set at 0.05.

Two important conclusions emerged. First, the time when birds were fed selectively affected reproductive physiology and performance in zebra finches. Second, food availability times dictated overall reproductive fitness, as assessed by the primary maternal investment (quality of laid eggs), offspring health and potential offspring recruits to the breeding population (Murray, 2000). Our interpretation is that the nutrition deficit induced by the reduced food availability period affected circulating hormone levels, particularly mesotocin and sex steroids, although with the caveat that hormone levels in this study were measured after, not during, the breeding protocol. Nonetheless, there were reduced mesotocin levels which correlated positively with the measures of reproductive efficiency of parents on the restricted food availability regimes. We suggest that low mesotocin levels indicated an attenuated eagerness to engage in reproduction and hence caused a delay in the onset of reproduction of morning and evening FA group breeding pairs. Mesotocin, a homologue of mammalian oxytocin, is a physiological marker of social interactions, including bonding and affection between breeding pairs (Carter and Porges, 2013; Kelly and Goodson, 2014). Similarly, significantly reduced E2 levels in morning and evening FA group female parents are consistent with their reduced reproductive performance and primary maternal investment. Both the positive correlation of E2 with reproductive events and primary energetic investment and the negative correlation of E2 with egg-laying latency support this. A positive correlation of E2 levels with egg-laying performance was also found in hens (Onagbesan et al., 2006), but not in canaries (*Serinus canaria*), in which there was no association between maternal E2 levels and clutch size variability (Sockman and Schwabl, 1999). We also found a positive correlation of parent testosterone levels with fledgling mortality (failure) ratio and a negative correlation of parent testosterone levels with primary energetic investment and annual breeding success in zebra finches. Intriguingly, plasma testosterone levels were elevated in parents in the evening FA group in which the survivorship was relatively low. Could increased hatchling mortality in evening FA group pairs be attributed to increased aggression and/or less parental care? This cannot be established from this study, but the association of elevated testosterone levels with enhanced aggression and attenuated parental care has been reported in songbirds (McGlothlin et al., 2007; Villavicencio et al., 2014). At the same time, however, the lack of a difference in plasma CORT levels between groups suggests that reproductive effects were not due to food deprivation-induced stress to breeding pairs. However, we would not rule out a ‘servo-control’ of an imposed food availability regime, as, unlike previous restricted feeding reports (Decuypere and Kuhn, 1984), plasma hormone levels were assessed after 1 year of restricted feeding in the present study. Perhaps detailed hormone assays across different stages of reproduction (pair formation, copulation, incubation, feeding of fledglings and parenting) could provide insight into the

effects of time of feeding on the hypothalamus–hypophysis–gonadal axis. Nonetheless, irrespective of feeding regime and age, there were sex differences in the baseline stress response, with significantly lower plasma CORT levels in both male parents and offspring, similar to sex differences reported in mountain chickadees, *Poecile gambeli* (Pravosudov et al., 2001). Furthermore, thyroid hormones are the key hormones influencing basal metabolic rate, and their plasma levels have been shown to be affected by food restriction (Rønning et al., 2009). The absence of differences in food utilization efficiency and plasma T4 and T3 levels negates metabolic effects, although we might have missed the food availability period-induced altered peak times of daily thyroid hormone rhythms, as reported in chickens (Rønning et al., 2009).

Overall, we found higher fecundity but poorer quality eggs and offspring (smaller in size and lower in mass) in the morning FA group, compared with better quality eggs and offspring which were fewer in number but larger in size and heavier in mass in the evening FA group. Thus, as compared with morning FA group pairs, evening FA group pairs showed an enhanced primary maternal investment and improved growth and health, but reduced annual breeding success. The effects of limited food availability and of supplemented food on the quality and size of laid eggs have also been reported in other birds (Clifford and Anderson, 2001; Reynolds et al., 2003; Ruuskanen et al., 2016). Zebra finches fed on a high-quality diet laid eggs that were heavier and larger in size (Rutstein et al., 2004). There was a significant increase in mass, volume and protein, but not lipid, content of eggs that Florida scrub jays laid when fed on a high-quality diet (Reynolds et al., 2003). There is also evidence suggesting that such diet-induced changes in egg quality can have long-lasting effects on morphology and health of the offspring survivors (Grigg, 2014). The overall low SMI of morning FA group offspring was correlated with low yolk testosterone levels, suggesting that reduced yolk testosterone might have negatively affected hatchlings' food-begging calls and, in turn, offspring growth and quality (Groothuis et al., 2005). Importantly, the adverse effects of an early-life nutrition deficit on biometry, especially skeletal growth, were not compensated for by improved nutrition later in life (Krause and Naguib, 2014). However, the nutritional stress during development did not affect the relationship between skeletal growth, measured as tarsus length, and body mass in zebra finches (Kriengwatana and MacDougall-Shackleton, 2014). We speculate that the nutrition deficit caused selective food provisioning by zebra finch parents to those hatchlings that seemingly had better survival prospects (Dijkstra et al., 1990; Riehl, 2010). Reduced food availability-induced suboptimal parenting was further suggested by a significant correlation of parental mesotocin with offspring SMI; offspring of parents with high mesotocin levels had better biometric features when adult. Intriguingly, reduced food availability-induced effects on offspring quality were sex dependent: females were not as adversely affected as males, as shown by the skeletal growth and body size when adult. We suggest *a priori* that an optimal investment was part of the compensatory fitness by parents to female offspring as with a compromised health they would be most likely to have a reduced reproductive fecundity when adult (Kilner, 1998; Martins, 2004).

The present results showing serious consequences of limited food availability on reproduction and offspring health are consistent with the theoretical prediction that energy income from feeding was balanced by its expenditure on growth, survival and reproduction, the last of these measured as the sum of egg laying, incubation and parental care in birds (Sibly, 2012; Sibly et al., 2012). With finite resources, the parental investment per offspring would be inversely related to offspring number. Enhanced adverse reproductive effects of the evening FA group further suggest the importance of time of feeding (hence the direction of the starvation period in relation to food availability) on metabolic costs and energetic relationships during the day (Sibly, 2012; Sibly et al., 2012). Although not shown in this study, restricted food-induced differential synchronization of internal rhythms governing metabolism and physical activity may also have influenced the reproductive success of morning and evening FA group breeding pairs, consistent with the role of daily food availability cycles in the synchronization of circadian behavioural and metabolic rhythms in songbirds (Hau and Gwinner, 1996; Rani et al., 2009). Desynchronized circadian behavioural rhythms and concurrent negative effects on reproductive fitness have been shown in *Drosophila* subjected to an aberrant feeding cycle (Xu et al., 2011). Although the mechanism of the time-of-feeding induced reproductive trade-off cannot be obtained from this study, we speculate that the reproductive effects were the consequence of a two-way interaction between the gross energy deficit and its timing during the day. The delay in reproduction with increased hatchling mortality in the evening FA group can primarily be recognized as an immediate inimical effect of biological asynchrony perhaps due to the alignment of food availability with the time of day that follows lower metabolic activity and physical inactivity of the dark period of a 24 h day, as previously reported in *Drosophila* (Xu et al., 2011). Similarly, the compromised growth and health of offspring in the morning FA group could be the long-term consequence of an imbalance between gross energy intake and expenditure at the times of higher metabolic activity during the day (Sibly et al., 2012). This could be investigated in future studies by reversal from limited to *ad libitum* food availability during different stages of reproduction in a long-term experiment.

To summarize, we have demonstrated for the first time differential effects on reproductive performance and offspring quality of the timing of identical food availability periods during the day in continuously breeding zebra finches. The overall implication of these results is that in the long term, an enforced daily eating schedule, or an alteration in habitat structure, environment and lifestyle, which potentially could modify food availability, could have hitherto unknown consequences on reproductive fitness and offspring quality in diurnal species including humans with the ability to reproduce throughout the year.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: V.K.; Methodology: V.K., I.M.; Validation: I.M.; Formal analysis: V.K., I.M.; Investigation: V.K.; Resources: V.K.; Data curation: I.M.; Writing - original draft: V.K., I.M.; Writing - review & editing: V.K.; Visualization: V.K.; Supervision: V.K.; Project administration: V.K.; Funding acquisition: V.K.

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