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

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Summary Statement

Absence of food in morning compromised reproductive fecundity but not the quality of offspring, while similar food-absence in the evening compromised quality (skeletal growth and overall size) of offspring but not the reproductive fecundity in continuously breeding diurnal zebra finches.

Abstract

Abundant food supply is crucial to reproductive performance, as shown by restricted food availability experiments, in small-sized vertebrates including birds. However, whether daily times of feeding would affect the reproduction is largely unknown. 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*). To randomly paired birds kept under 12 h light per day for about 52 weeks, food availability period was restricted to 4 h in morning (FA-M) or evening (FA-E), with controls on food *ad libitum*; thus, daily food deprivation period began after 4 h of food in FA-M and was continuous with nighttime starvation in FA-E. Both food restrictions adversely affected reproductive health as shown by reduced sex steroids and mesotocin levels, but not general metabolism as indicated by no-difference in thyroxin and triiodothyronine levels. Food for 4 h negatively affected the reproductive performance, although with differences between FA-M and FA-E pairs. Particularly, there was delayed onset of reproduction and compromised reproductive success in FA-E, but not in FA-M pairs; conversely, the offspring health was severely compromised in FA-M, but not in FA-E pairs. Furthermore, FA-M females were in better health, implicating sex-biasness in parental food provisioning. Overall, we demonstrate trade-off of 'quantity' (offspring produced and/ or survived) for 'quality' (how good offspring were in health) in response to daily food availability times in zebra finches that much like humans are diurnal and retain the ability to reproduce throughout the year.

Introduction

Abundant food supply is crucial for the optimization of breeding schedules and reproductive performance of small-sized vertebrates, which cannot store large food amounts. Increasing evidences have 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 photoinduced gonadal development in European starlings (*Sturnus vulgaris*; Meijer, 1991) and Abert's Towhees (*Melozone aberti*; Davies et al., 2015). Both, the resident and migratory songbirds on a supplementary diet showed an advanced egg laying dates and enhanced breeding success, as measured in egg laying frequency, clutch size, hatchling success, brood size and fledglings (Reynolds et al., 2003; Rutstein et al., 2004; Robb et al., 2008; Kaiser et al., 2014). Similarly, food shortage even during 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). Redheaded buntings (*E. bruniceps*) exposed to non-stimulatory photoperiod and fed for 6 h showed an attenuated testicular growth when they were later subjected to 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 evening (hour 11 – 16, hour 0 = light on; FA-E), but not in morning (hour 0 – 5, FA-M), during 16 h light per day depressed almost by 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, twice-a-day fed broiler hens 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 resource by parents to eggs and hatchling-feeding in order to selectively optimize offsprings' fitness. Particularly, the mother's nutritional state affects size, number and macronutrient composition (e.g. protein and lipid content) of eggs laid (Reynolds et al., 2003). For example, zebra finches fed on high-quality diet laid eggs that were more in number, heavier and larger in size, hence had greater hatching success, compared to birds fed on the low-quality diet (Rutstein et al., 2004); however, the lipid supplement to diet did not affect the egg quality (Williams, 1996). Similarly, there was a significant increase in mass, volume and protein, but not lipid, content of eggs that Floria scrub-jays (*Aphelocom coerulescens*) laid when fed on a high quality diet (Reynolds et al., 2003). There are also evidences

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 maternal allocation of nutrients to egg is described as the primary investment to the offspring health; the parental feeding that can vary with parents' nutritional state is 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 amount of hormones in laid eggs could also influence the development and behaviour of resultant chicks (Groothuis et al., 2005). For example, yolk testosterone levels may affect begging calls and efficiency of the nutrient utilization in hatchlings (Schwabl, 1996; Groothuis et al., 2005).

At the mechanistic level, it remains poorly understood how restricted feeding regimen effects the reproduction. The overall evidence, nonetheless, suggests both, the direct and indirect effects, of food deprivation. In the former, food deprivation during the day (i.e. starvation) could trigger a cascading effect on 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 food availability induced alteration in the plasma levels of estradiol in broiler hens (Onagbesan et al., 2006), testosterone in male house finches (*Haemorhous mexicanus*; Valle et al., 2015) and Abert's toehees (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 the reproductive success, as demonstrated in zebra finches (Naguib and Gil, 2005; Naguib et al., 2006). In contrast, the indirect effects of food could be through the changes in the relationship of closely coupled daily light and food cues and synchronize 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 due to photosensory limitations, food and light cues remain relatively well integrated during the whole day. Compared to this, a feeding regimen in which food is absent during the major part of the day might disrupt the coupling between light and food cues and subsequently affect the 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 the breeding success have not been investigated. We hypothesized that food availability limited to only few hours would negatively affect the reproduction and growth of offsprings, and that the effects would vary dependent on food availability times during the day. To test these, 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 the 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 to longitudinally trace the effects of experimental manipulation on offspring survivors. Here, to adult zebra finches kept for 12 months under 12 h day and constant temperature, we restricted food availability for 4 h in the times of high (morning) and low (evening) food intake (our unpubl. obs.). The predictions were: (1) The limited food availability would negatively affect reproductive physiology at multiple levels, including the circulating sex steroids and mesotocin levels (hormones associated with reproduction and affiliation), egg laying onset, reproductive fecundity, and quality of eggs (size of eggs, percent protein, percent lipid and yolk testosterone) and offsprings (body mass and size). (2) The reproductive effects would vary depending on whether food deprivation period lied before (in evening-fed birds) or after (in morning-fed birds) the food availability period. (3) The limited feeding time available might enforce a sex-biased parental primary investment, with offspring sex having better survival prospect sharing greater investment.

Materials and Methods

Animals and experiment

The experiments were done on adult (12-18 mo. old) zebra finches (*Taeniopygia guttata*) that were born and raised in captivity in our indoor aviary at 24 ± 2 ^{o}C temperature under 12 h light: 12h darkness (12L: 12D; L = ~ 200 lux; D = 0 lux), with *ad libitum* food and water. The compact florescent lamps (5W, 220-240 V CFL lamps; Phillips India) provided the light period, as controlled by automatic timers (Mueller, Badienungsanleitung SC 88).

We used a total of 36 zebra finches of each sex that were almost similar in body mass (12.4 ± 0.1 g). They were housed in same sex cages (size = 54 cm x 42 cm x 30 cm; n = 4 / cage) to avoid visual or acoustic contact with opposite sex, in order to break pair-bond, if it was established when they lived together in the aviary. Birds were kept on 12L:12D and food *ad libitum* (FAL), as before, for 3 weeks during which a female if she laid eggs was replaced to avoid a possible carry-over effect. Thereafter, the food availability period (FAP) to 24/ 36 birds in each sex was restricted to 4 h in the morning (FA-M; beginning at hour 0, light on, of the day) or the evening (FA-E; beginning at hour 8 of the day) by step-wise reduction of FAP for 3 days each to 8 h (FA-M: hour 0 to 8; FA-E: hour 4 to 12), 6 h (FA-M: hour 0 to 6; FA-E: hour 6 to 12) and 4 h (FA-M: hour 0 to 4; FA-E: hour 8 to 12). The remaining 12/ 36 birds in each sex were continued on FAL, and served as controls. Thus, twelve 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 food deprivation period between two restricted feeding groups. We introduced similar food deprivation period but in different directions relative to 4 h food availability to FA-M and FA-E birds. The duration and timing of daily food-restriction period were chosen, based on previous results that food availability restricted to one-third of the long-day in evening, but not in morning, depressed almost by half, the photostimulated testicular maturation in seasonally breeding long-day songbirds (Kumar et al., 2001; Bhardwaj and Anushi, 2004). Each day, we placed a food-filled cup at hour 0 to FAL and FA-M, and at hour 8 to FA-E birds, and the cup was replaced by another food-filled cup next day at hour 0 in FAL, and by an empty food cup same day at hour 4 and hour 12 of the day in FA-M and FA-E cages, respectively.

The seeds of *Setaria italica* (Kakuni; 3.64 Kcal/ g) were served as the primary diet, and hard boiled eggs (1.47 Kcal/ g) mashed with crushed egg shell was given as a daily supplement; water was available at all times to all the birds. After a week of food restriction, separately housed males and females were randomly paired; so, each condition had 12

breeding pairs. Every other day, the nest box in each cage was refreshed with nesting material comprising small twig of leaves, cotton and grass. During this time, with minimal disturbance to breeding pairs, we recorded observations on egg laying latency (time in days from the mate introduction), number of eggs laid and/ or discarded, hatchlings, time taken to fledge, offspring survived, sex ratio, etc. When > 90 days old, the offspring was separated from its parents, housed in same-sex groups (n = 5 per cage) and maintained on the feeding regime, as before. More details are given in the supplementary methods.

Food intake, body mass and morphometric measurements

Food intake and body mass: After 7 days of the 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 post-FAP the unconsumed food was removed and weighed. To avoid food spillage, the cages were lined with an opaque white polythene sheet up to 7 cm from the bottom, and food spillage inside the cage, if any, was collected and accounted for daily food intake. The recorded food intake over two consecutive days was first averaged for a day and then mean (± SE) food intake/ pair/ day was calculated. Faeces were dried and weighed, and faeces production as average g/ pair/ day was calculated. Further, utilization efficiency was calculated from these values using the following formula: [(food intake− faeces produced)/food intake]×100. The observation on body mass of parents and > 120 days old adult offspring was made during the first 15 min of light phase during which food was kept unavailable to all birds to avoid an immediate spike in body mass. They 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 $SMI_i = M_i \, x(L_0/L_i)^b$; where Mi and Li denote the body mass and tarsus length of an individual 'i', respectively, L_0 is the arithmetic mean value of the tarsus length for whole study population, and b is slope estimate of regression of body mass on tarsus length (Peig and Green, 2010).

Morphometric measurements: We measured the length of tarsal, beak, wing and tail to assess restricted feeding-induced irreversible effects on body size, and hence on the offspring quality (Rising and Somers, 1989; Peig and Green, 2010; Andrew et al., 2017), using a vernier calliper to a least count of 0.1 mm for the length of beak and tarsus or using a ruler to an accuracy of 1 mm for the length of flattened wing and tail. We measured the tarsus length from its bottom with toes bent forward to the ankle joint, the wing length from bend of wing to the tip of longest primary feathers, and the tail length from base to tip of 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 parents to account for genetic effect on body size of the offspring.

Measurement of parameters of reproductive health and performance

The egg quality: The quality of eggs laid demonstrated restricted feeding-induced effects on primary maternal investment into offspring quality. It was assessed by morphometric measurement (mass and volume) of the laid eggs and by assay of protein, lipid and yolk testosterone contents in 'discarded' eggs to avoid any influence on the reproductive performance assessed in 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 FAL, 74 of FA-M and 67 of FA-E) were measured for the volume, and 201 eggs (92 of FAL, 63 of FA-M and 46 of FA-E) were weighed. Weight of the 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 the egg protein, lipid and yolk testosterone content. The eggs were weighed to an accuracy of 0.01g and measured for size by a vernier calliper to a least count of 0.1 mm. Discarded eggs were stored at -80 ^{o}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 nearest 0.1 mg. The lipid was removed from dried yolk by diethyl ether extraction, and lipid-free yolk was then reweighed to nearest 0.1 mg to give lean dry mass; this when subtracted from the dry yolk mass, gave yolk lipid content. Dry albumen mass and lean dry yolk mass were assumed to account for approximately 88% egg protein content. Yolk lipid, yolk protein and albumen protein contents were calculated as percent component relative to the total egg mass without the component of interest (e.g., % yolk lipid = yolk lipid mass/ (egg mass - yolk lipid mass) x 100).

The yolk testosterone (T) was assayed by ethanol-extraction method as described by Kozlowski et al. (2009). Briefly, eggs were weighed and carefully broken, and egg yolk and albumin were weighed out. 50 µl of yolk in a 1.5 ml tube added with 200 µl of distilled water and 1mm silica beads was homogenised for 2 min and incubated for 1 h at 37 ^{o}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 the room temperature. Thereafter, 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 ^{o}C. Next day, T

content was assayed using standard kit protocol (Enzo Life Sciences, Ann Arbor, MI; Cat. no. ADI-900-065), as described below for plasma T.

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 (estradiol, E2; testosterone, T) and mesotocin (avian homologue of mammalian oxytocin) were assayed in plasma to show restricted feeding -induced effects on reproductive health and associated physiology and behavior. Plasma T and E2 levels correlate with male and female sexual behaviours, respectively (Cain and Ketterson, 2013; Ubuka et al., 2014), and mesotocin levels reflect on interactions and affiliation behaviors 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 days old offspring, beginning 4 weeks after the end of breeding protocol. During this period, the pairs were separated and again housed in the same-sex cage, as before the start of the experiment; however, they were still maintained on respective FAL or restricted feeding regimes. Both, the parent and offspring were bled alternatively at the beginning (hour 0 - 0.5) or middle (hour 6 - 6.5) of the day (hour 0 = light on). After 4 bleeds (2 each in the morning and mid-day) spread over 4 weeks, birds were weighed and the morphometric measurements were recorded. In each bleed, 50 - 100 µl blood was collected by puncturing the wing vein into a heparinised capillary tube 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 T, E2 and cort, 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 the bird capture and handle within 2 minutes to avoid a stress-induced effect on cort levels. We created a cohort of offspring plasma samples so that all breeding pairs had equal contribution in hormone assays. For this, we first included plasma samples from at least a male and a female representative offspring from each breeding pair from the first clutch and thus were similar in age. However, we included plasma samples from 2 males and 1 female offspring from the second clutch of the FA-E since there were only 22 offsprings (10 males and 12 females) in this group at the end of experiment. A few plasma samples were also excluded because an insufficient volume as required for the hormone assay. Thus, for assays, we had a total of 30 samples (10 samples / feeding regime/ generation/ sex) for T and cort, 21 samples (7 samples / feeding regime/ generation/ sex) for E2, T3 and T4, and 18 samples (6 samples / feeding regime/ generation/

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.

***ELISA of corticosterone (cort)*:** The assay (Enzo Life Sciences, Ann Arbor,MI; cat. no. ADI-900-097) used 10 µl plasma samples in 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 out 100 µl each of standards and plasma samples in standard and sample wells, respectively, and 100 µl of assay buffer in the NSB and blank (B0) wells. Followed by an 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) wells. Thereafter, 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 the room temperature. This was followed by 3 washes with 1X wash-buffer. After washes, 5 µl conjugate and 200 µl of pnitrophenyl phosphate in buffer (pNpp) were added to TA wells and to every well, respectively. The plate was incubated at the room temperature for 1 h. At the end, the addition of 50 µl of stop-solution stopped the reaction, and the plate was read at 405 nm by SpectraMax M2e microplate reader (Molecular Devices LLC, USA). The sensitivity and intra-assay variability of the assay were 26.99 pg/ml and 8.4%, respectively.

***ELISA of testosterone (T)*:** An immunoassay kit from Enzo Life Sciences (Ann Arbor, MI; Cat. no. ADI-900-065) measured T in 10 µl bunting plasma in 1:20 dilution with 1% steroid displacement buffer (10 µl plasma + 10 µl steroid displacement buffer + 180 µl assay buffer), according to manufacturer's protocol and as validated and used in our laboratory (Mishra et al., 2017b). First, we pipetted out 100 µl each of standards and plasma samples in the standard and sample wells, respectively. 50 µl antibody was added to each well, except wells designated for blank, total activity (TA) and non-specific binding (NSB), and the plate was incubated on a plate shaker at 500 rpm for 1 h at the room temperature. Then, except for blank and TA, 50 µl conjugate (alkaline phosphate conjugated with testosterone) was added to each well, and reincubated at 500 rpm for 1 h at the room temperature. Thereafter, following 3 buffer washes, first 5 µl conjugate was added to TA wells and then 200 µl of p-nitrophenyl phosphate in buffer (pNpp) was added to every well. The plate was then reincubated for 1 h at 37°C. At the end, the addition of 50 µl of stop-solution stopped the reaction, and the plate was read at 405 nm by SpectraMax M2e microplate reader (Molecular Devices LLC, USA). The sensitivity and intra-assay variability of the assay were 0.08 ng/ml and 5.7%, respectively.

***ELISA of thyroxine (T4)*:** An immunoassay kit from Arbor assays (Ann Arbor, MI; Cat. no. K050-H1) measured T4 in 10 µl plasma in 1:20 dilution with 1 % steroid displacement buffer (10 µl of plasma + 10 µl of dissociation reagent + 180 µl of assay buffer), according to manufacturer's protocol, and as validated and used in our laboratory (Mishra et al., 2017b). Briefly, we pipetted out 100 µl of standards, 100 µl diluted sample,125 µl of assay buffer and 100 µl of assay buffer to wells designated for the 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 the room temperature. Following 4 wash buffer washes, 100 µl of TMB substrate was added to each well, and the plate was reincubated for 30 min without shaking at room temperature. At the end, addition of 50 µl of stop-solution stopped the reaction and the plate was read at 450 nm by Spectra Max M2e microplate reader (Molecular Devices LLC, USA). The sensitivity and intra-assay variability of the assay were 0.29 ng/ml and 3%, respectively.

***Elisa of triiodothyronine (T3)*:** T3 titres were measured in 50 µl samples using an immunoassay kit from Accubind-monobind (Lake Forest, CA; 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, and thereafter incubation for 1 h at the room temperature. Each well was washed thrice with washing buffer and was added with 100 µl of working substrate solution (tetramethylbenzidine, TMB + hydrogen peroxide in buffer). The plate was incubated for 15 min without shaking at the room temperature. The addition of 50 µl stop-solution to every well stopped the reaction, and the plate was read at 405 nm by SpectraMax M2e microplate reader (Molecular Devices LLC, USA). The sensitivity and intra-assay variability of the assay were 0.04 ng/ml and 5.4%, respectively.

***Elisa of estradiol (E2)*:** The plasma E2 concentration was measured by using an enzyme immunoassay kit (Estradiol EIA Kit, Cayman Chemical Company, 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. 50 µl of standards and samples were added to respective wells, followed by an addition of 50 µl of AChE (Estradiol- acetylcholinesterase) tracer to all wells, except TA and blank wells. Thereafter, 50 µl of E2 antiserum was added to each well, except the TA, NSB and the blank wells. The 96-well plate was covered with a plastic film and incubated for 1 h on an orbital

shaker (200 rpm) at the room temperature. Each well was washed five times with the 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 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 SpectraMax M2e microplate reader (Molecular Devices LLC, USA). The sensitivity and intra-assay variability of the assay were 20 pg/ml and 7.4%, respectively.

***Elisa of mesotocin*:** We measured mesotocin using an enzyme immunoassay kit from Arbor assay (Ann Arbor, MI; Cat no: K048-H1) with 500 ng/ ml stock mesotocin solution (Cat no: X127-625UL, Ann Arbor, MI), 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 the room temperature, followed by centrifugation for 20 min at 4°C at 1600 g. Thereafter, the supernatant was dried in a speed vacuum centrifuge at 37 °C, followed by its reconstitution in 250 µl of assay buffer. 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. Then, 25 µl each of conjugate and antibody were sequentially added to every well (except antibody to NSB wells). After the incubation of plate at 200 rpm for 15 min at the room temperature, the plate was sealed and stored overnight at 4 °C. Next day, each well was washed 4 times with 300 µl of wash buffer, and this was followed by the addition of 100 µl of TMB substrate to every well. The addition of 50 µl of stop solution stopped the reaction after 0.5 h incubation without shaking. The plate was read at 450 nm by SpectraMax M2e microplate reader (Molecular Devices LLC, USA). The sensitivity and intra-assay variability of the assay were 17 pg/ml 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 per pair record of the egg laying latency (number of days taken to lay the first egg from the 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 offsprings per successful breeding attempt per pair, as described by Murray (2000).

Animal Welfare and Ethics note

This study was done 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, cleaned cages every third day and rooms once a week. There were also frequent veterinarian visits to check on bird's health. The measurement of body mass every alternate week showed that birds maintained good health. All breeding pairs survived restricted-feeding protocol.

Statistics

Statistical analyses used GraphPad prism (versions 5.0 and 7.0) and IBM SPSS statistics (version 20) softwares, 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 posttests 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 annual reproductive efficiency. Two-way ANOVA with Bonferroni posttest tested the effects of the food availability (FA) time, FA (factor 1), sex (factor 2) and factor 1 x 2 interactions on parents' body mass. Furthermore, Wilk's lambda multivariate general linear models (GLM) tested the effects of FA, sex and their interaction on offsprings' morphometric measures (body mass, and the length of tarsal, beak, tail and wing). Similarly, univariate general linear models (GLM) tested the effects of FA, 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, the alpha was set at 0.05.

Results

Body mass and food intake (FI)

At the end of breeding protocol, the mean (± SE) body mass of parent birds was as follows: FAL: male - 12.6 ± 0.4 g, female - 12.7 ± 0.3 g; FA-M: male - 11.5 ± 0.2 g, female - 11.8 ± 0.2 g; FA-E: male -11.9 ± 0.2 g, female - 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 FA, but not of sex or FA x sex interaction ($F_{2,66}$ = 8.304, P = 0.0006, η^2 = 0.201; 2-way ANOVA). Particularly, male parents in FA-M, but not FA-E, were significantly lighter than that FAL ($P < 0.05$; Bonferroni posttest). 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; 1-way ANOVA), with significantly reduced food intake (mean ± SE; kcal/ pair/ day) in FA-M (19.12 ± 1.19 kcal) and FA-E (18.57 ± 2.18 kcal), as compared to FAL (25.97 ± 1.97 kcal) pairs ($P < 0.05$, Tukey's posttest); food intake did not differ between FA-M and FA-E pairs. We also found no significant difference in the utilization efficiency (mean ± SE) between three feeding groups (FAL = 93.0 ± 0.5 %, FA-M = 91.1 ± 0.9 %, FA-E = 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 almost twice longer time to lay the first egg in FA-E, as compared to the FAL ($P < 0.05$, 1-way ANOVA). Over the year, the primary energetic investment in reproduction (eggs laid), reproductive events (egg clutches) and hatchlings were significantly reduced in FA-M and FA-E groups, as compared to the FAL. However, the fledglings that died and so did not contribute to adult offspring population, were significantly higher in FA-E; thus, the annual breeding success rate in FA-E < FA-M < FAL.

***The egg quality*:** The egg quality as the measure of primary maternal investment was assessed in 5 parameters, the egg mass and volume, percent lipid and protein content, and yolk T levels. We found a significant effect of FA on all, except egg protein, parameters (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; % egg lipid: $F_{2,27}$ = 4.215, P = 0.0255, η^2 = 0.238; yolk T: $F_{2,27}$ = 4.039, P = 0.029, η^2 = 0.23; 1-way ANOVA). Eggs were significantly lighter and smaller in size in restricted feeding pairs than in FAL pairs ($P < 0.05$, Scheffe's posttest; Fig. 2). However, eggs from FA-M pairs had significantly higher per cent lipid and lower yolk T contents, as compared to those from FA-E or FAL pairs ($P < 0.05$, Tukey's posttest; Fig. 2).

The offspring quality (body size and condition): Wilk's lambda multivariate GLM tested the effect of FA, sex and their interaction on body mass and the length of tarsal, wing and beak, which we considered as indicators of an overall quality, including body size and condition, of the offspring survivor; tail length did not show restricted feeding-effects and excluded from the statistical model. We found a significant effect of FA on all four parameters, of sex on body mass and wing length, and of FA x sex interaction on the tarsal length (Table 2). Although parents did not differ in body size, we found significant differences between three groups in the body mass and size of offspring when adult. As compared to FAL, offsprings were significantly lighter in weight, and male offsprings, in particular, were significantly smaller in size (short tarsus and beak) in the FA-M ($P < 0.05$; Bonferroni posttest, Table 2).

To show if body mass of offsprings was related to their body size, we calculated 'scaled mass index' (SMI), which is an adjusted value normalized to that of its expected value if all individuals were of the same body size (Peig and Green, 2010). For this, we used tarsal length since 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 (±SE) SMI values for three groups were: FAL = 12.70 ± 0.17; FA-M = 11.38 ± 0.17; FA-E = 12.07 ± 0.13. Overall, there was a significant effect FA ($F_{2,119} = 19.124$, $P < 0.0001$, $\eta^2 = 0.243$; 1-way ANOVA), with overall SMI values in FAL > FA-E > FA-M ($P < 0.05$, Scheffe's posttest).

Plasma hormone levels: We measured mesotocin, testosterone (T), estradiol (E2) in females, corticosterone (cort) and thyroid hormones (thyroxin, T4, and triiodothyronine, T3), the markers of reproductive health, general health and metabolism in birds. There was a significant effect of FA and generation, but not of sex, on mesotocin ($P < 0.05$, GLM; Table 3). In parents, the mesotocin levels were significantly higher in FAL than the restricted feeding groups, and the levels in female, not male, offsprings were significantly lower in FA-E than the FAL ($P < 0.05$, Tukey's posttest; Fig. 3). Likewise, there were significant effects of FA, sex and generation on plasma T, with levels significantly higher in males than the females, and in parents than the offspring ($P < 0.05$, GLM; Table 3). Between the three groups, T levels were significantly higher in FA-E parents and FAL male offspring, as compared to the other two groups ($P < 0.05$, Tukey's posttest; Fig. 3). Similarly, we found a significant effect of FA, but not of generation or FA x generation, on female plasma E2 levels ($P < 0.05$, GLM; Table 3). In female parents, E2 levels were significantly lower in FA-M and FA-E than in FAL ($P < 0.05$, Tukey's posttest; Fig. 3). Both, E2 and T levels did not show

group differences in female offsprings (Fig. 3). Furthermore, plasma cort showed a significant effect of FA and sex, and the levels were significantly higher in females than the males ($P < 0.05$, GLM; Table 3, Fig. 3). However, we found no effect on plasma T4 and T3 levels of all the factors tested, except of 2-factorial interaction (T4 - FA x generation, generation x sex - T3; $P < 0.05$, GLM; Table 3).

Relationships: Hormones vs. reproductive performance or offspring quality (SMI)

There was a positive correlation of both sexes' mesotocin levels on average SMI of 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 T or E2 levels, respectively), suggesting FA-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 frequencies, primary energetic investment and annual breeding success rate (with mesotocin only), and both mesotocin and E2 levels were negatively correlated with the reproduction latency, i.e. time taken from mate introduction to lay the first egg ($P < 0.05$, Pearson's correlation; Fig. 4). Similarly, plasma T was negatively correlated with primary energetic investment and annual breeding success rate, and was positively correlated with the 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 FA -induced effects on reproduction in zebra finches.

Discussion

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

Two important conclusions emerged. First, the time when birds were fed selectively affected reproductive physiology and performance in zebra finches. We interpret that nutrition deficit induced by 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 of restricted food availability regimes. We suggest that low mesotocin levels indicated an attenuated affiliability (eagerness to engage in reproduction) and hence delay in the onset of reproduction of FA-M and FA-E breeding pairs. Mesotocin, a homologue of mammalian oxytocin, is indeed 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 FA-M and FA-E female parents are consistent with their reduced reproductive performance and primary maternal investment. Both, positive correlation of E2 with reproductive events and primary energetic investment, and 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 in zebra finches the positive and negative correlations of parent T levels with fledgling mortality ratio and with primary energetic investment and annual breeding success. Intriguingly, plasma T levels were elevated in parents under FA-E in which the survivorship was relatively low. Could then increased hatchling mortality in FA-E pairs be attributed to increased aggression and/or less parental care? This cannot be knows from this study, but the association of elevated T levels with an enhanced aggression and attenuated parental care has been reported in songbirds (McGlothlin et al., 2007; Villavicencio et al., 2014). At the same time, however, no difference in plasma

cort levels between groups suggests that reproductive effects were not due to food deprivation-induced stress to breeding pairs. Although we would not rule out a 'servo-control' of an imposed food availability regime, unlike the previously reported long-lasting effects of developmental stress on reproductive success in zebra finches (Decuypere and Kuhn, 1984). Perhaps, detailed hormone assays across different stages of reproduction (pair formation, copulation, incubation, feeding of fledgling and parenting) could unravel the time-of-feeding induced effects on 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 offsprings, similar to sex differences reported in mountain chickadees, *Poecile gambeli* (Pravosudov et al., 2001). Furthermore, thyroid hormones are the key hormones influencing the 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 negate metabolic effects, although we might have missed out food availability period-induced altered peak times of daily thyroid hormone rhythms, as reported in chicken (Rønning et al., 2009).

Secondly, food availability times dictated the overall reproductive fitness and output, as assessed by the primary maternal investment (quality of laid eggs) and potential offspring recruits to the breeding population (Murray, 2000). Overall, we found higher fecundity but poor quality eggs and offspring (smaller in size and lighter in weight) in FA-M, compared to better quality eggs and offspring which were fewer in number but of larger in size and heavier in weight in FA-E. Thus, as compared to FA-M, FA-E 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 supplement food on 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 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 Floria scrub-jays laid when fed on a high quality diet (Reynolds et al., 2003). There are also evidences 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 FA-M offspring was correlated with low yolk T levels, suggesting that reduced yolk T might have negatively affected hatchlings' food-begging calls and, in turn, the 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 by improved nutrition later in the life (Krause and Naguib, 2014). However, the nutritional stress during development did not affect the relationship between skeletal growth, measured in tarsus length, and body mass in zebra finches (Kriengwatana and MacDougall-Shackleton, 2014). We speculate that nutrition deficit caused selective food provisioning by zebra finch parents to their hatchlings which 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 the offspring SMI; offspring borne to 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 in 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 since with a compromised health they would be most likely to have a reduced reproductive fecundity when adult (Kilner, 1998; Martins, 2004).

Present results showing serious consequences of limited food availability on reproduction and offspring health are consistent with theoretical prediction that energy income from feeding was balanced by its expenditure on growth, survival and reproduction, the latter accounted 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 the offspring number. Enhanced adverse reproductive effects of FA-E further suggested the importance of time-of-feeding (hence the direction of 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 reproductive success of FA-M and FA-E 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 time-of-feeding induced reproductive trade-off cannot be known from this study, we speculate that the reproductive effects were the consequence of two-way interaction between gross energy-deficit and its timing during the day. The delay in reproduction with increased hatchling mortality under FA-E can primarily be recognised as immediate inimical effects of a 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 24-h day, as previously reported in *Drosophila* (Xu et al., 2011). Similarly, the compromised growth and health of offsprings under FA-M can 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 probably can be investigated in a future set of experiment involving reversal from limited *ad libitum* food availability during different stages of reproduction in a long-term experiment.

To sum up, we demonstrate 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 a forced eating daily schedule, or an alteration in the habitat structure, environment and life style, which potentially could modify food availability, could have, hitherto unknown, consequences on reproductive fitness and offspring quality in diurnal species including humans with ability of reproducing throughout the year.

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Author contributions

VK and IM designed the study, IM carried out the experiment and analysed data, VK and IM wrote the paper, and VK provided all necessary resources for this study.

Declaration of interests

The authors declare no conflict of interest.

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Figures

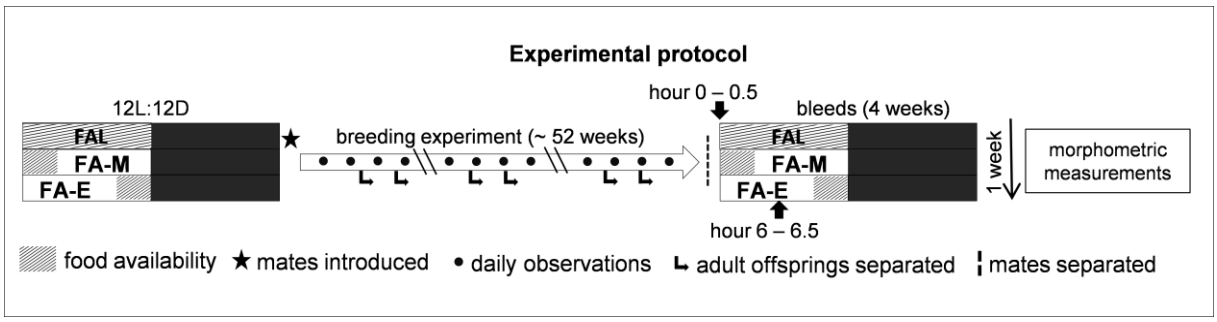

Figure 1. ***Experimental protocol*****.** This experiment investigated the effects of restricted food availability on reproduction in zebra finches (*Taeniopygia guttata*). Birds were housed in same sex cages under 12 h light: 12h darkness (12L: 12D) and 24 ± 2 ^{o}C temperature. Acclimated males and females were randomly paired and 12 pairs each were subjected to restricted food availability for 4 h in morning (FA-M; hour 0 – 4, hour 0 = light on) or evening (FA-E; hour 8 -12), with controls on 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 inmates. The offsprings were separated from 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 breeding protocol of approx. 52 weeks, breeding pairs were separated, housed in same sex groups of 4 birds each and maintained on the feeding regimes, as before. After 4 weeks of separation, both parents and adult offspring were bled over 4 weeks, and 4 blood samples (2 each in the morning during hour 0 - 0.5, and in middle of the day during hour 6 - 6.5) were collected. We also recorded body mass and morphometric measurements at the end of the experiment.

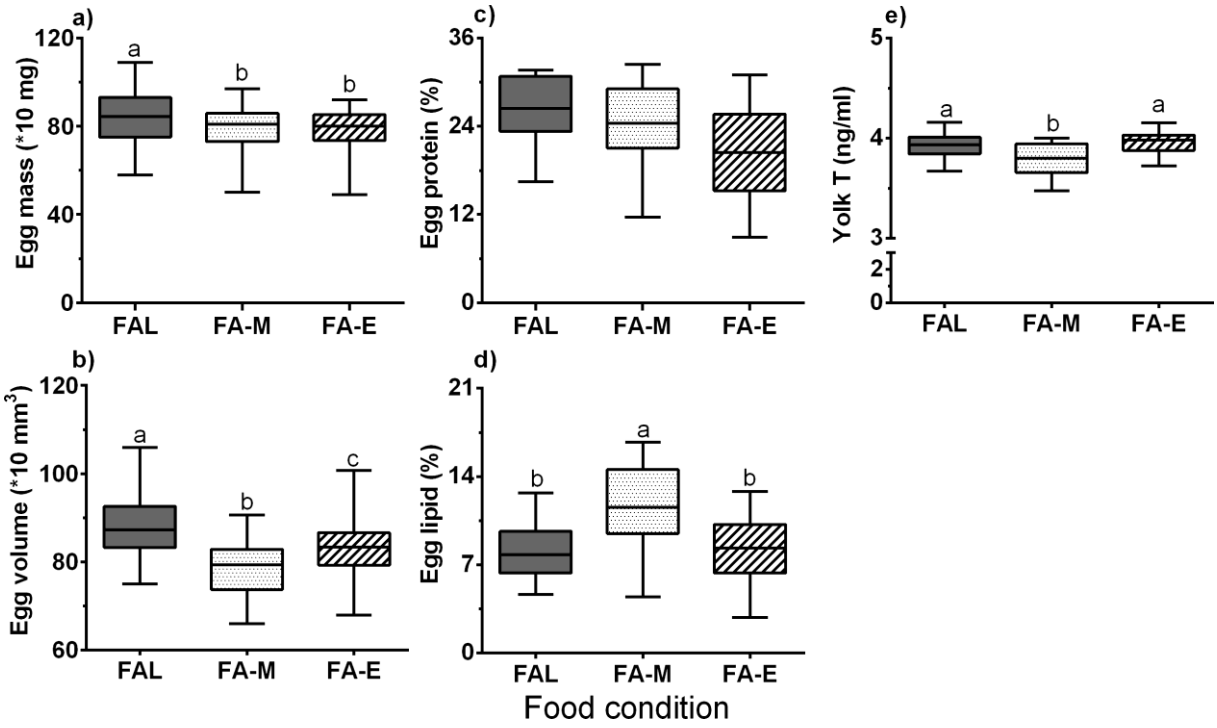

Figure 2. ***Effects on egg quality***. Whisker plot (minimum to maximum) of egg mass (a), egg volume (b), % protein content (c), % lipid content (d) and yolk testosterone (ng/ ml; e) of eggs of zebra finch (*Taeniopygia guttata*) breeding pairs that were maintained under 12h light: 12h darkness and subjected to food for 4 h given in the morning (hour 0 – 4; FA-M) or in the evening (hour 8 – 12; FA-E), with controls on food *ad libitum* (FAL). Different and same alphabets indicate significant and no difference, respectively, as determined by 1-way ANOVA, followed by Scheffe's posttest (a, b) or Tukey's posttest (c-e). $P < 0.05$ was considered statistically significant. Note that % protein and lipid content, and yolk testosterone were assessed in the discarded egg. For statistical significance, the alpha was set at 0.05.

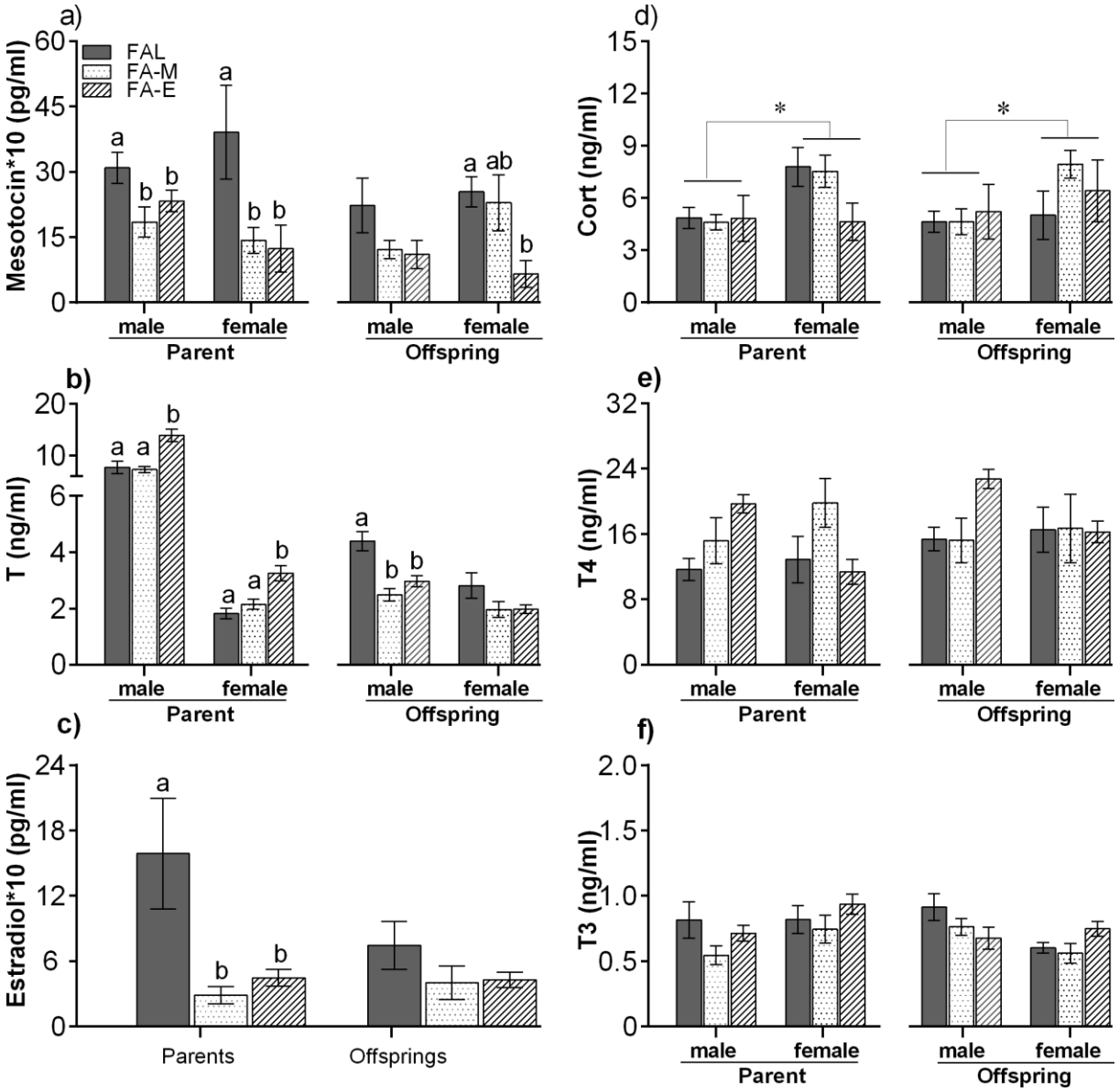

Figure 3. ***Effects on plasma hormone levels*****.** Plasma hormone levels (mean ± SE) of mesotocin (a), testosterone (T, b), estradiol (E2, c), corticosterone (cort, d), thyroxin (T4, e) and triiodothyronine (T3, f) in parent and offspring zebra finches (*Taeniopygia guttata*) maintained under 12h light: 12h darkness and subjected to food for 4 h given in the morning (hour 0 – 4; FA-M) or in the evening (hour 8 – 12; FA-E), with controls on food *ad libitum* (FAL). Different and same alphabets indicate significant and no difference, respectively, between the feeding regimes, as tested for the effects of food availability time, sex and generation by Univariate General Linear Models (GLM) and Tukey's posttest. An asterisk on the bar indicates a significant sex effect. For statistical significance, the alpha was set at 0.05.

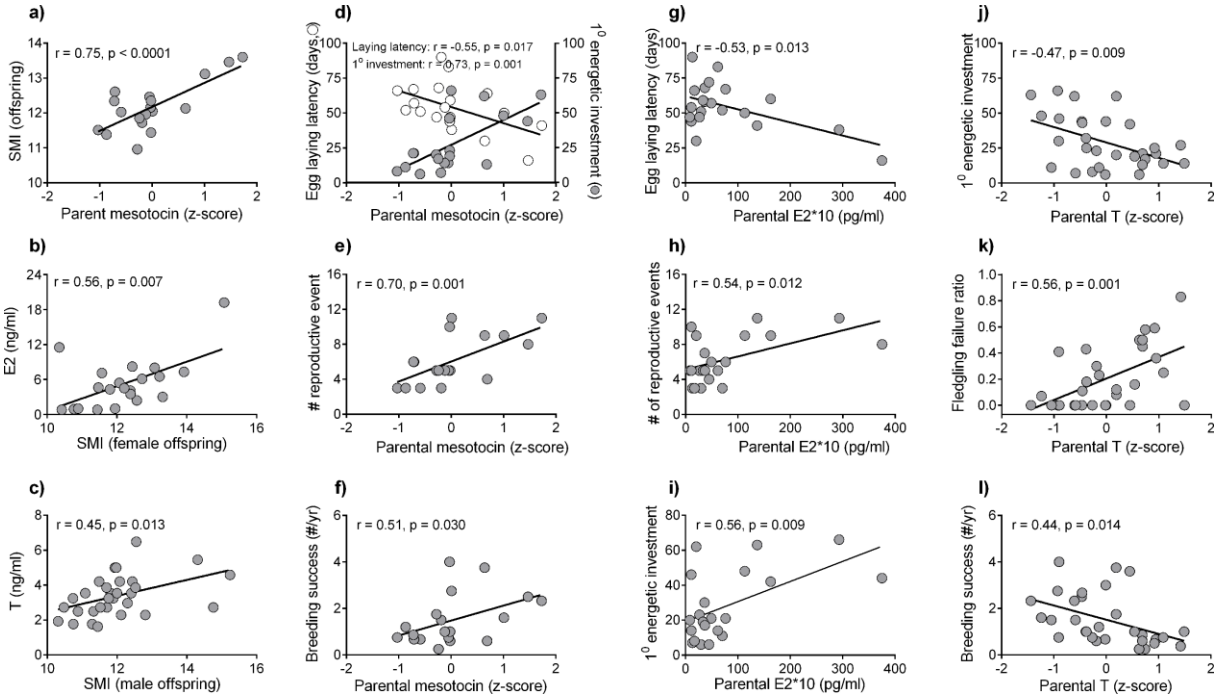

Figure 4. ***Relationships*: *Hormones vs. reproductive performance or offspring body condition.*** a-c): Relationship of parental mesotocin (an affiliation marker) with offspring body condition (SMI, a), and of SMI with sex steroids (b -female offspring: Estradiol, E2; c - male offspring: testosterone, T), which are indicators of the reproductive health. Note: a significant relationship of offspring SMI with plasma mesotocin levels is presented with reference to 'z' score of both male and female parents. d-l): Relationship of parental mesotocin (d-f), female estradiol (E2, g-i) and parent testosterone (T, j-l) with different parameters that defined reproductive health and performance of zebra finches (*Taeniopygia guttata*) maintained under 12h light: 12h darkness and subjected to food ad libitum (FAL), and to food for 4 h given in the morning (hour 0 – 4; FA-M) or in the evening (hour 8 – 12; FA-E). We used 'z' score for a better depiction of the relationship. This was calculated from averaged male and female hormone concentrations, and hence normalized sex- dependent variations, if any, in hormone levels. However, 'z' score was calculated when there was a significant correlation between parents' hormone levels and the reproductive parameter under consideration. For statistical significance, the alpha was set at 0.05.

Table 1: The parameters (mean ± SE) determining annual reproductive performance of zebra finches (*Taeniopygia guttata*; n = 12 pairs) maintained under 12 h light: 12 h darkness and subjected to restricted food availability for 4 h in morning (FA-M) or in evening (FA-E), with controls on food *ad libitum* (FAL). Different and same superscript alphabets indicate significant difference and no-difference, respectively, as determined by Tukey's posttest following 1-way ANOVA. The effect size estimates are presented as partial eta squared (η^2). For statistical significance, the alpha was set at 0.05.

Reproduction Parameters	**Food availability**			**1-way ANOVA statistics**		
	FAL	FA-M	FA-E	$F_{2,33}$	P	η^2
Egg laying latency (*days from mate introduction*)	33.75±4.22^{a}	50.33±7.305^{ab}	62.67±3.57^{b}	7.52	0.002	0.31
Reproductive events (*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 *(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 offsprings 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 *(# fledgling death/#total 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 offsprings /successful breeding attempts*)	2.79±0.21^{a}	1.06±0.11^{b}	0.58±0.08^{c}	67.11	< 0.0001	0.80

* Used log-normal values for 1-way ANOVA.

Table 2: Results of Wilk's Lambda Multivariate General Linear model (GLM) analysis of morphometric measurements (mean ± SE) of zebra finch (*Taeniopygia guttata*) offsprings born and raised under 12 h light: 12 h darkness with restricted food availability for 4 h in morning (FA-M) or in evening (FA-E), or food *ad libitum* (FAL, control). The effect size estimates are presented as partial eta squared (η^2). Bold and unbold values indicate statistical significance and non-significant values, respectively. Different and same superscript alphabets indicate significant difference and no-difference between groups, respectively, as determined by bonferroni-posttest. $P < 0.05$ was considered statistically significant. For statistical significance, the alpha was set at 0.05. * Log-normal values used for General linear model.

Dependent Variable	Between subject effect test			Food availability time		
	Food availability (FA)	Sex (S)	Interaction (FA x S)	FAL	FA-M	FA-E
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^{ab} ♀ : 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^{ab} ♀ : 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^{ab} ♀: 12.41 ± 0.22^{ab}

Table 3: Results of Univariate General Linear model (GLM) analysis of plasma hormone levels in zebra finches (*Taeniopygia guttata*) maintained under 12 h light: 12 h darkness and subjected to restricted food availability for 4 h in morning (FA-M) or in evening (FA-E), with controls on food *ad libitum* (FAL). The effect size estimates are presented as partial eta squared (η^2). Bold and unbold values indicate significant and non-significant values, respectively. For statistical significance, the alpha was set at 0.05

Hormone	Corrected model	Food availability (FA)	Generation (G)	Sex (S)	FA x G	G x S	FA x S	FA x G x S
T4 (ng/ml) (n = 7/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**	$F1_{,72}$ = 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) (n = 7/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)* (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) (n = 6/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

T (ng/ml) (n = 10/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) (n = 7/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	-	-	-

* log-normal values used for General linear model.