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What comes first, the zebra finch or the egg: temperature-dependent reproductive, physiological and behavioural plasticity in egg-laying zebra finches

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Summary

Avian reproduction is generally timed to synchronize chick-rearing with periods of increased food abundance. Consequently, the energetically demanding period of egg production may coincide with periods of lower food availability, fluctuating temperature and more unstable weather. Little is known about the physiological mechanisms underlying temperature-induced variation in egg production. We therefore examined the influence of low ambient temperature (7°C *vs* 21°C) on reproductive output (e.g. egg mass, clutch size, laying interval, laying rate), daily food consumption and lipid variables in zebra finches *Taeniopygia guttata*. When faced with egg production at 7°C, laying zebra finches increased energy intake by 12.67 kJ day^{-1}, and were thus able to maintain body condition (e.g. body mass, fat and muscle score) and circulating triacylglyceride at levels comparable to those at 21°C. However, when producing eggs at 7°C, females took longer to initiate egg laying (6.5 *vs* 6.1 days at 21°C), and ultimately laid fewer eggs (5.5 *vs* 6.0 eggs) at a slower rate (0.90 eggs day^{-1} *vs* 0.95 eggs day^{-1}). These temperature-related declines in reproductive output were accompanied by decreases in modal (from 36.6 at 21°C to 24.3 nm at 7°C) and median very low density lipoprotein (VLDL) particle diameter (from 29.6 to 26.4 nm) and in the proportion of VLDL particles that were capable of passing through the pores in the ovary to access the developing ovarian follicles (i.e. particles with diameters between 25 and 44 nm; from 45.90% to 32.55%). However, variation in reproductive output was not related to any static concentration or structural measure of VLDL. Therefore, other temperature-dependent mechanisms must be involved in the physiological processes that regulate reproductive output of passerine birds at low ambient temperatures.

Key words: temperature, egg production, lipid allocation, very low density lipoprotein (VLDL), food intake, zebrafinch, chicken.

Introduction

Reproduction in seasonally breeding animals is generally timed such that the period of offspring care coincides with seasonal peaks in food availability and quality (Perrins, 1970). However, the seasonal recrudescence of the reproductive axis and early offspring development (i.e. egg production in oviparous animals and gestation in mammals) often occurs well in advance of the period of offspring care. Therefore, these energetically demanding processes (Vézina and Williams, 2002; Zenuto et al., 2002; Korine et al., 2004) often occur prior to the seasonal peak in food availability, at a time when environmental conditions may be sub-optimal. During this time, breeding females must ensure that they can find enough energy and nutrients to produce their offspring while still meeting their own energetic requirements (Perrins, 1970; Scott, 1973). Thus, breeding females will face a trade-off between allocation of resources to offspring *versus* their own energetic needs (Bernardo, 1996), and this trade-off will be most acute when reproduction coincides with periods of increased energy demand due to poor environmental conditions, such as exposure to inclement weather, extreme low temperatures or low food availability, which can be common during the early stages of breeding (e.g. during egg production) (Perrins, 1970). Little is known about the physiological mechanisms underlying temperature-induced variation in egg production.

Differential allocation of energy-rich lipids during avian egg production provides a model for studying the physiological basis of a temperature-dependent trade-off between current reproduction and maternal survival, as mediated through female body condition. The estrogen-dependent increase in circulating levels of the egg yolk lipid precursor, yolk-targeted very low density lipoprotein (VLDLy), represents a dramatic shift in lipid metabolism as the primary function of plasma VLDL particles changes from general lipid transport to

maternal tissues (e.g. muscle, adipose), to selective lipid transport to developing egg yolks (Neilson and Simpson, 1973; Chan et al., 1976; Walzem, 1996; Walzem et al., 1999; Williams, 1998). Lipid is the primary energy source for the growing avian embryo (Walzem, 1996). Thus, generic VLDL fuels maternal maintenance activities, including thermoregulation and digestion, while the structurally distinct VLDLy predominates in egg-laying domestic fowl (Hermier et al., 1989; Walzem et al., 1994; Walzem, 1996). The near-total shift in hepatic synthesis towards this relatively non-maternally metabolizable form of VLDL during egg production may compromise the condition of laying females during periods of high energetic demand. Consequently, modulation of the trade-off between reproductive output and maternal survival may be achieved by altering maternal VLDL–VLDLy metabolism, e.g. modulation of plasma concentrations of generic and yolk-targeted VLDL, VLDL particle diameter distribution, efficiency of generic VLDL and VLDLy utilization for maternal energetic needs (cf. the energy requirements of their developing offspring), or efficiency of switching from the exclusive synthesis of non-laying, generic VLDL to an increased synthesis of VLDLy (Lin and Chan, 1981; Griffin et al., 1982; Lin et al., 1986; Nimpf et al., 1988; Walzem, 1996; Williams and Christians, 1997).

The goals of this study were to (1) increase total maternal energy requirements during egg-laying in zebra finches *Taeniopygia guttata* by exposing laying females to low ambient temperatures, (2) determine the effects of exposure to low ambient temperature on maternal body condition and reproductive output (e.g. egg mass, clutch size, egg composition, laying rate) and (3) examine the extent to which low ambient temperatures influence lipid allocation to egg production and self-maintenance in egg-laying birds as indicated by changes in VLDL structure and fractional egg lipid content. We hypothesized that increasing the energy demands of laying females would result in either (i) a shift in VLDL particle diameter distribution away from smaller VLDL particles (i.e. VLDLy) in favor of more larger, potentially maternally required, VLDL particles, thereby compromising reproductive output while maintaining female body condition, or (ii) maintenance of the production of yolk-targeted VLDL particles in concert with other energetic compensation strategies by laying females, such as increased energy intake, reduced locomotor activity or reduced immune function (Deerenberg et al., 1997). A potential outcome of the latter strategy is the maintenance of reproductive output with compromised female survival through increased depredation or compromised female body condition and other aspects of non-reproductive physiology.

Materials and methods

Animals and husbandry

Zebra finches *Taeniopygia guttata* (Vieillot 1817) with previous breeding experience (i.e. produced at least one previous clutch; N=30 males and 30 females) were randomly chosen from our breeding colony housed in the Simon Fraser University Animal Care Facility. All birds were weighed (±0.1 g), in conjunction with tarsus and bill measurements (±0.1 mm). The birds were then transferred to same-sex cages that were visually isolated from the opposite sex within a Conviron E15 plant growth chamber (Controlled Environments, Winnipeg, Manitoba, Canada). Environmental conditions were maintained at 75% humidity with a constant light schedule of 14 h:10 h L:D, with lights on at 09:00 h, for at least 1 week to acclimate the birds to the environmental conditions, including the specific ambient temperature (7°C or 21°C) of the experiment (described below). All birds received a mixed seed diet (Panicum and white millet, 50:50 w/w; approximately 12.0% protein, 4.7% lipid; Just for Birds, Surrey, BC, Canada), water, grit and cuttlefish bone (calcium) *ad libitum*. All experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit (no. 692B-94) following guidelines of the Canadian Committee on Animal Care.

A repeated-measures design was used; each female zebra finch was acclimated to and paired for breeding at both experimental temperatures (7°C and 21°C). Birds were rested in same-sex cages at 21°C for at least 45 days between the two environmentally defined breeding trials. The pre-pairing acclimation period lasted 1 week during the 21°C trial and 3–4 weeks during the 7°C trial, as temperature was reduced in weekly steps from 14°C to 10°C, and finally to 7°C, and acclimation to 7°C required 1–2 weeks. Acclimation periods were based on the time it took for all birds to return to and maintain their initial experimental, i.e. pre-acclimation body mass. The order in which birds were exposed to the two experimental temperatures was randomized.

Following each acclimation period females were randomly paired with males. Breeding pairs were housed individually in cages (61 cm×46 cm×41 cm) equipped with an external nest box (15 cm×14.5 cm×20 cm). Males and females were weighed (±0.1 g) at the time of pairing, and a subset of birds was inspected for abundance of pectoral muscle and fat stores. We scored pectoral muscle on an arbitrary scale ranging from 0 (representing concave pectoral muscles with a prominent keel) to 3 (indicating convex pectoral muscles that protruded above the keel) [adapted from the 0–2 scale (Gosler, 1991)]. Fat deposits in the furcular fossa and in the abdominal cavity were scored on an arbitrary scale ranging from 0 (representing no visible fat) to 5 (indicating bulging fat deposits) (Wingfield and Farner, 1978).

To obtain a gross estimate of energy intake, seed consumption was measured by providing all pairs with 30.0 g of the mixed seed diet daily in open 946 ml Ziploc™ (Save-On Foods, Burnaby, BC, Canada) containers placed on the cage floor. This method avoided any spillage and allowed for the measurement of daily seed consumption by weighing the remaining seeds in the container after 24 h (±0.1 g). Birds were able to feed *ad libitum* as 30.0 g seed was always in excess of their daily intake. Females eat slightly more food (4.5%) than males, regardless of their breeding status, and this effect does

not change throughout the laying sequence (Williams and Ternan, 1999). Therefore, measuring food intake per pair was a good indicator of female food intake as the proportion of seeds consumed by both sexes remained unchanged throughout the experimental protocol. Pairs were also provided with 6 g of an egg-food supplement (20.3% protein: 6.6% lipid) (see Williams, 1996) daily between pairing and clutch completion, which was completely consumed within 24 h. Water, grit and cuttlefish bone (calcium) were also provided *ad libitum*.

Data on laying interval, egg mass and clutch size were obtained by checking the nest boxes daily between 09.00 h and 11.00 h. All new eggs were weighed (±0.001 g) and numbered on the day they were laid. The second eggs of each clutch were collected for egg composition analysis on the day they were laid and substitute eggs were used as replacement eggs in order to maintain original clutch size. Clutches were considered complete if no new eggs were laid on 3 succeeding days. At this time each female was weighed (±0.1 g), and each pair was returned to same-sex cages in the Animal Care Facility. Females that failed to lay eggs within 15 days were classified as non-breeders and were returned to same-sex cages in the Animal Care Facility.

Blood sampling and plasma preparation

Regardless of experimental temperature, all females that initiated egg-laying were blood sampled (200 μl from the brachial vein) on the day their first eggs were laid (1-egg stage). All blood samples were collected into heparinized capillary tubes between 09:00 h and 11:30 h. The blood samples were then expelled into EDTA-coated microcentrifuge tubes containing 0.5 mol l^{-1} disodium-EDTA (3 μl; Sigma-Aldrich Canada, Oakville, ON, Canada), and the tubes were centrifuged at 2200 ***g*** for 10 min in a Baxter Canlab Biofuge 13 (VWR International, Edmonton, Alberta, Canada). The plasma from each sample was removed and placed into uncoated microcentrifuge tubes. The new tubes were centrifuged at 2200 ***g*** for 5 min. Sub-samples of each plasma sample were frozen for total triacylglyceride analysis (5 μl) for this study and corticosterone analysis (10 μl) for another study, while the remainder of each plasma sample was placed into an EDTA-coated microcentrifuge tube containing 0.5 mol l^{-1} disodium-EDTA (5 μl) for VLDL particle diameter distribution analysis. Sodium azide (1% w:v; Sigma-Aldrich Canada) was added to each EDTA-coated tube to prevent mold formation (0.01 μl μl^{-1} plasma), and the plasma samples were refrigerated (4°C) pending analysis of VLDL particle diameter distribution.

Triacylglyceride assay

Circulating concentrations of triacylglyceride were measured as an index of total plasma VLDL, i.e. generic VLDL and VLDLy, using previously frozen plasma and an enzymatic test kit (Serum Triglyceride Determination Kit, Sigma-Aldrich Canada). This method was developed for domestic fowl (Mitchell and Carlisle, 1991) and validated for passerines (Williams and Christians, 1997; Williams and Martiniuk, 2000; Challenger et al., 2001). Intra-assay and inter-assay coefficients of variation were 1.85% (N=6 replicates) and 2.13% (N=7 assays), respectively, using a 19-week hen plasma pool. All assays were run using 96-well microplates, and measured using a Biotek Powerwave 340 I microplate reader (BioTek, Winooski, Vermont, USA).

VLDL particle diameter distribution

Plasma VLDL isolation and dynamic laser light scattering

Plasma VLDL was isolated as the fraction of plasma of density d<1.020 g ml^{-1} (Walzem et al., 1994). Briefly, the plasma samples and a blank control sample (NaCl solution, d=1.0063; equivalent to the salt density of undiluted plasma) were combined with NaCl–NaBr solution (d=1.0255) and centrifuged at 148 600 ***g*** for 18 h at 14°C in a Beckman L8-70M ultracentrifuge (Beckman Coulter, Fullerton, CA, USA). Following centrifugation, the supernatant containing the VLDL portion of the plasma was aspirated from each tube using a narrow-bore pipet and refrigerated (at 4°C) until analysis for VLDL particle diameter distribution.

VLDL particle diameter distribution was measured by dynamic laser light scattering using an Ultrafine Particle Analyzer 250 (Microtrac, Montgomery, PA, USA) and 7.02 analysis software (Microtrac) (Walzem et al., 1994; Veniant et al., 2000). This technique utilized the Doppler effect as the basis for diameter distribution determinations by recording light scattering from a directed laser diode as it passed through the lipoprotein particles. The magnitude of Doppler-shifting of light scatter that occurs due to the Brownian motion of the particles was measured as it is proportional to particle velocity, which is in turn a function of particle diameter, fluid temperature and fluid viscosity. As both temperature and viscosity were kept constant, the difference in particle velocity was solely dependent on particle diameter. Sample measurements were made by placement of the flexible probe-tip into the sample and activation of the laser diode (λ=780 mm laser beam). Light scattering from the lipoprotein particles was recorded for 3 min for the blank solution, and for 5 min in triplicate for each VLDL sample. The probe was washed with distilled water and dried between samples.

Estimation of VLDLy and calculation of VLDL particle diameter distribution parameters

The proportion of VLDL particles that were available for incorporation into developing eggs, i.e. yolk-targeted VLDL, was determined by calculating the percentage of particles that fell within the small particle VLDL (sVLDLy) range (25–44 nm in diameter). This diameter range was based on the sieving properties of the ovarian granulosa basal lamina of domestic fowl (Perry and Gilbert, 1979; Griffin and Perry, 1985). Only particles 25–44 nm in diameter have been observed distal to the basal lamina of domesticated fowl (Perry and Gilbert, 1979; Griffin and Perry, 1985; Griffin and Hermier, 1988; Walzem et al., 1999). Egg-laying zebra finches and chickens have been found to maintain a larger proportion of circulating VLDL particles within this diameter range than non-laying females (Salvante et al., 2007). The modal and

median particle diameter and the range (i.e. width, in nm) of each distribution were also determined.

Egg composition analysis

The second egg of each clutch was subjected to protein and lipid fractional content analysis following published methods (Balzer and Williams, 1998). Briefly, eggs were collected within 6 h of being laid, boiled for 3 min, and frozen (at –20°C) until further analysis. Frozen eggs were thawed and separated into shell, albumen and yolk, which were dried to constant mass in a 50°C drying oven, and then weighed to the nearest 0.0001 g (dry mass). Lipid was removed from the dry yolks by soxhlet extraction for 8 h with petroleum ether as the solvent (Dobush et al., 1985). Lipid-free yolks were then weighed to the nearest 0.0001 g (lean dry mass). Lipid content of the yolks was determined by subtracting lean dry yolk mass from dry yolk mass. Dry albumen mass and lean dry yolk mass were assumed to be approximately 88% protein (Burley and Vadehra, 1989). The yolk lipid, yolk protein and albumen protein content of each egg were calculated as the percentage of each component relative to the fresh mass of the egg without the component of interest to control for part-whole correlations [e.g. %yolk lipid=[yolk lipid mass/(fresh egg mass–yolk lipid mass]×100; all masses in g (Christians, 1999)].

Data analysis

All statistical analyses were performed using SAS (SAS Institute, 1999). All data were tested for normality of distribution (Shapiro–Wilk test) (Zar, 1996). All non-normal variables were log10 or arc-sin transformed prior to analysis. If normality of distribution was achieved following data transformation, then the data were analyzed using a mixed model, repeated-measures ANOVA or ANCOVA (with female body mass as a covariate) with temperature as a fixed, repeated factor, and individual female as a random factor (PROC MIXED) (SAS Institute, 1989). In contrast, variables that were still not normally distributed following data transformation were analyzed using the non-parametric Friedman's test for treatment differences in a randomized complete block design with individual females as blocks that received both treatments (i.e. experimental temperatures) in a randomized order (PROC FREQ) (SAS Institute, 1999). All data are presented as values at 7°C and at 21°C with a line connecting values for individual females. All tests are two-tailed, and the overall significance level is $P<0.05$.

Results

Maternal condition

Female body mass at pairing and at the 1-egg stage did not differ between breeding bouts at 7°C and 21°C ($F_{1,27.4}=0.92$, $P>0.3$ and $F_{1,25.7}=2.02$, $P>0.1$, respectively). Similarly, the changes in female body mass (temperature with body mass at pairing as a covariate: $F_{1,23.1}=3.23$, $P>0.05$; Fig. 1A), fat score (temperature with body mass at pairing as a covariate: $F_{1,22.2}=0.20$, $P>0.6$; Fig. 1B) and muscle score (temperature: $F_{1,15.8}=0.89$, $P>0.3$; Fig. 1C) from pairing to clutch completion were independent of the temperature at which females were producing eggs.

Seed consumption

On average, breeding pairs consumed 45% more seed at 7°C than at 21°C ($F_{1,13.2}=13.83$, $P<0.0025$; Fig. 1D). The additional 1.9 g seed day^{-1} corresponded to an additional 0.21 g protein, 0.08 g lipid and 1.44 g carbohydrate. This was equivalent of an additional 23.25 kJ day^{-1} for breeding pairs at 7°C, assuming a digestive efficiency of 75% (Shuman et al., 1989). Therefore, based on the observation that females ate slightly more seed (4.5%) than males throughout the laying sequence (Williams and Ternan, 1999), it was calculated that laying females in this study consumed an additional 12.67 kJ day^{-1} at 7°C compared to consumption at 21°C.

Plasma triacylglyceride, VLDL particle diameter distribution, and VLDLy

Circulating triacylglyceride levels ($F_{1,20.3}=0.78$, $P>0.3$) and VLDL particle diameter distribution range ($Q=0.111$, $P>0.7$) were independent of ambient temperature. In contrast, laying females had smaller modal ($F_{1,12.9}=9.50$, $P<0.01$; Fig. 1E) and median VLDL particle diameters ($F_{1,8.96}=6.23$, $P<0.05$), and a smaller proportion of VLDL particles within the sVLDLy range ($F_{1,16.6}=8.01$, $P<0.025$; Fig. 1F) at 7°C than at 21°C.

Reproductive output

Decreasing ambient temperature changed the relationship between female body mass at the 1-egg stage and the average mass of subsequently laid eggs (temperature×female body mass at the 1-egg stage interaction: $F_{1,25.4}=5.56$, $P<0.05$) (Fig. 2). Whereas mean egg mass was positively related to female body mass at 21°C ($F_{1,27}=16.40$, $r^2=0.3779$, $P<0.0005$), it was not related to body mass at 7°C ($F_{1,25}=1.41$, $P>0.2$) (Fig. 2). However, this significant interaction was dependent on two females within the 7°C treatment that produced small eggs despite being heavy. Removal of these two females from the analysis resulted in a similar positive relationship between mean egg mass and female body mass at the 1-egg stage at both temperatures (temperature×female body mass at the 1-egg stage interaction: $F_{1,22.4}=2.04$, $P>0.1$; female body mass at the 1-egg stage: $F_{1,42.6}=13.02$, $P<0.001$). When female body mass was controlled for, mean egg mass was not influenced by ambient temperature ($F_{1,20.6}=3.17$, $P>0.08$; Fig. 3A). In contrast, temperature did have an influence on other measures of reproductive output; when producing eggs at 7°C, females laid an average of 0.4 fewer eggs ($Q=4.765$, $P<0.05$; Fig. 3B), took approximately 0.5 days longer to produce the first egg of the clutch ($Q=5.000$, $P<0.025$; Fig. 3C), decreased laying rate by 5% (i.e. laid 0.90 eggs day^{-1} *vs* 0.95 eggs day^{-1} at 21°C; $Q=4.571$, $P<0.05$; Fig. 3D), and skipped laying an egg on more days during the laying of the clutch (0.7 days *vs* 0.5 days; $Q=4.571$, $P<0.05$).

Decreasing ambient temperature had little effect on the composition of the second egg of each clutch; fresh egg mass,

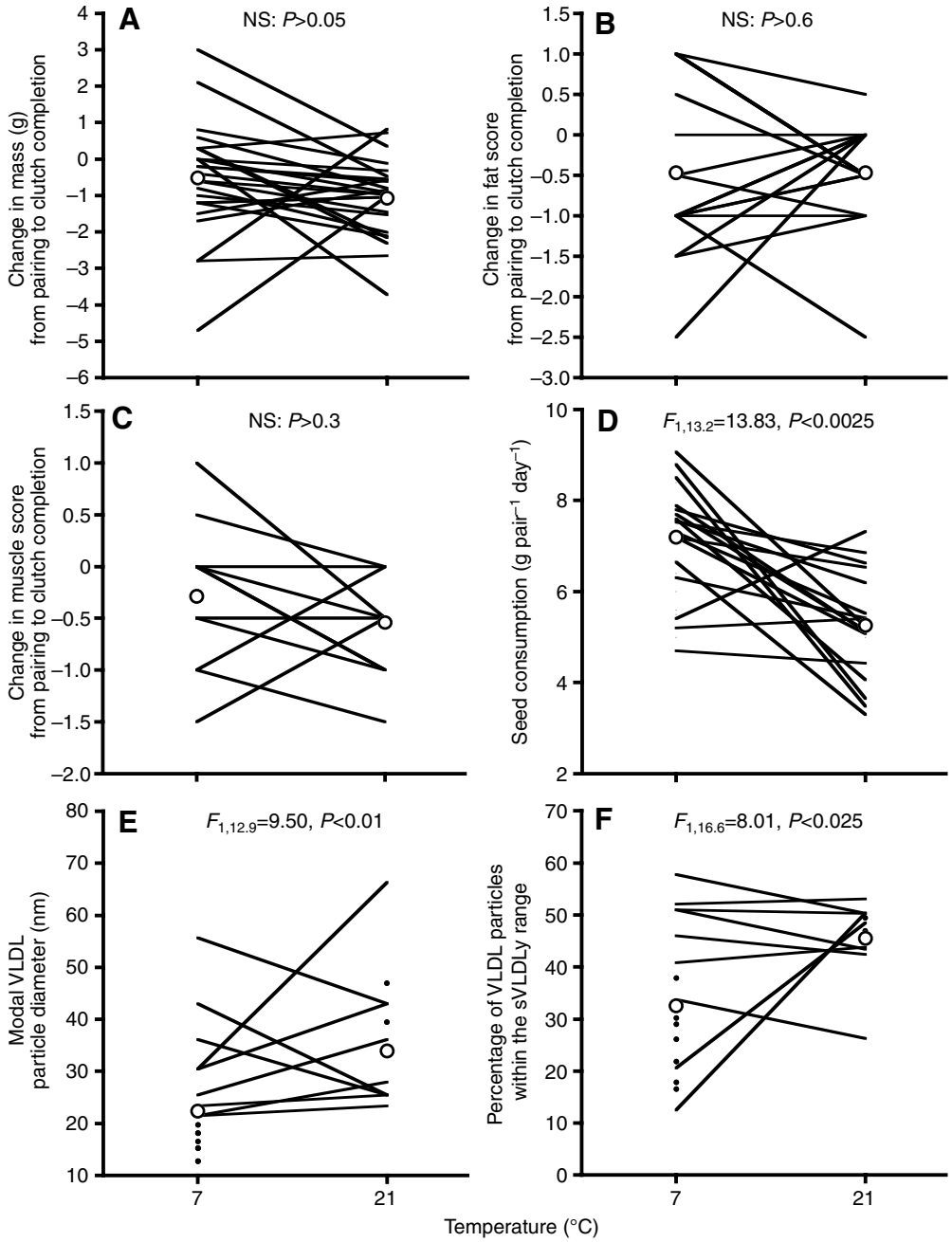

Fig. 1. Comparisons of changes in (A) female body mass, (B) female fat score and (C) female muscle score from pairing to clutch completion, (D) daily seed consumption of breeding pairs throughout laying, (E) modal VLDL particle diameter and (F) the proportion of VLDL particles that fell within the sVLDLy range in females producing eggs at 7°C and 21°C. Lines join values for individual females and open circles represent means.

water content, dry albumen and yolk mass, yolk lipid content (Fig. 3E) and albumen protein content were all independent of ambient temperature (P>0.2 in all cases). However, females laid eggs with 5% more yolk protein, an average of an additional 4.3 mg protein/yolk, at 7°C than at 21°C ($F_{1,23.1}$=4.79, P<0.05; Fig. 3F).

Variation in mean egg mass, clutch size, laying interval and laying rate was not related to variation in circulating triacylglyceride levels, VLDL particle diameter distribution range, modal and median VLDL particle diameter or the proportion of VLDL particles within the sVLDLy range, regardless of ambient temperature during egg production (P>0.09 in all cases).

Discussion

Zebra finches paired and breeding at 7°C consumed 45% more seed per day, the equivalent of an additional

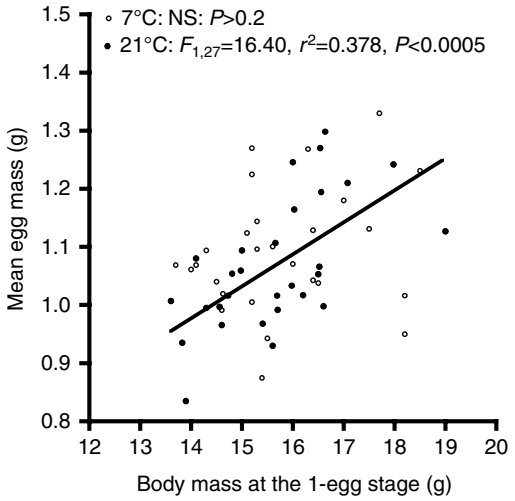

Fig. 2. Relationships between female body mass at the 1-egg stage and the mean mass of subsequently laid eggs at 7°C and 21°C.

12.67 kJ energy day^{-1} (assuming 75% digestive efficiency) (Shuman et al., 1989), and laying females were thus able to maintain body condition throughout egg production even at this low ambient temperature. The additional seed consumed represents approximately 25% of the daily energy expenditure (DEE) of non-breeding (53.6±1.0 kJ day^{-1}) and egg-laying female zebra finches (51.3±1.3 kJ day^{-1}) maintained at 21°C in our captive breeding colony (Vézina et al., 2006). Despite this substantial increase in energy intake, egg-producing females did not maintain reproductive output at 7°C. Cold-acclimated females took longer to initiate egg laying and ultimately laid fewer eggs at a slower rate. This suggests that, while cold-acclimated birds were able to increase energy intake, the additional energy was used to fuel the energetic cost of increased thermoregulation, and laying females were unable to further increase energy intake to meet the energetic demands associated with maintaining reproductive output at warm-acclimated levels. The amount of food individuals can consume in a day can be limited by the rate at which they can process the food, which in turn is limited by the physical volumetric capacity of the digestive system and by various aspects of enzyme-dependent digestive efficiency, including the rates at which nutrients can be digested, absorbed and transported to general circulation (Kirkwood, 1983; Karasov, 1990; Ricklefs, 1991; Weiner, 1992). Although birds in this study were clearly able to increase total seed consumption, this in itself might have incurred increased energetic costs related to digesting more food, e.g. increasing the size or activity of digestive tissues (Williams and Tieleman, 2000; Nilsson, 2002; Piersma, 2002). In general increased caloric intake has been linked to decreased lifespan, possibly *via* increases in oxidative stress, in many species (Finkel and Holbrook, 2000), though it is not known in birds whether this is specifically associated with processing more food, or what the metabolic costs of this might be (e.g. Alonso-Alvarez et al., 2006). Comparisons of the size and efficiency of the digestive systems of warm- and cold-acclimated, non-breeding and egg-laying females are required to determine whether these factors limit the ability of cold-acclimated, egg-laying females to further increase energy intake to fuel the energetic costs of maintaining reproductive effort.

Interestingly, while decreasing ambient temperature had no effect on fresh egg mass or the fractional lipid content of the second eggs of each clutch, it did result in an increase in yolk protein content. This may be due to the differential influence of low ambient temperature on lipid and protein utilization. While the extra lipid consumed by laying females likely goes towards fueling the combined energetic costs of thermoregulation and egg production, the extra protein may still be allocated to reproduction. Therefore, while the proportion of VLDL particles that have access to the developing ovarian follicles based on particle size was found to decrease with decreasing ambient temperature, it is possible that circulating levels of vitellogenin were independent of or even increased with decreasing ambient temperature. If either scenario were true, the pool of vitellogenin available for uptake during egg formation would actually increase with decreasing ambient temperature because laying females produce fewer eggs over a longer period of time at lower ambient temperatures. Similarly, egg-laying chickens *Gallus gallus domesticus* that exhibited lower rates of egg production due to irregular patterns of laying were shown to have higher and more variable levels of plasma vitellogenin than chickens that laid more regularly and had higher laying rates (Redshaw and Follett, 1976). The potential increase in vitellogenin availability and longer egg formation times, as assumed by the decrease in laying rate of cold-acclimated laying females, may result in eggs with higher yolk protein content. Future studies that assess circulating levels of vitellogenin in laying females acclimated to different temperatures are needed to determine whether this proposed mechanism explains the cold-induced increase in yolk protein content.

Female zebra finches producing eggs at 7°C exhibited concurrent declines in clutch size and laying rate, and an increase in laying interval, but no change in mean egg mass compared to when they were maintained at a warmer and less energetically demanding temperature. To our knowledge, this is the first study to experimentally manipulate the ambient temperature in which female birds were maintained throughout the process of eggs production (cf. studies that experimentally manipulated only night-time nest box temperature) (Nager and van Noordwijk, 1992; Yom-Tov and Wright, 1993). Correlational and experimental studies relating ambient or nest box temperatures to egg production in free-living birds have found similar, but somewhat inconsistent, results. Low ambient temperatures were associated with declines in laying rate, i.e. increases in the number of ‘skipped’ days when no egg was laid, in free-living great tits (Lessells et al., 2002). Similarly, blue tits *Parus caeruleus* laying in experimentally warmed nest boxes had fewer interruptions in laying, i.e. laid at a higher rate, than those in colder control boxes (Yom-Tov and Wright, 1993). Inconsistent relationships have been found

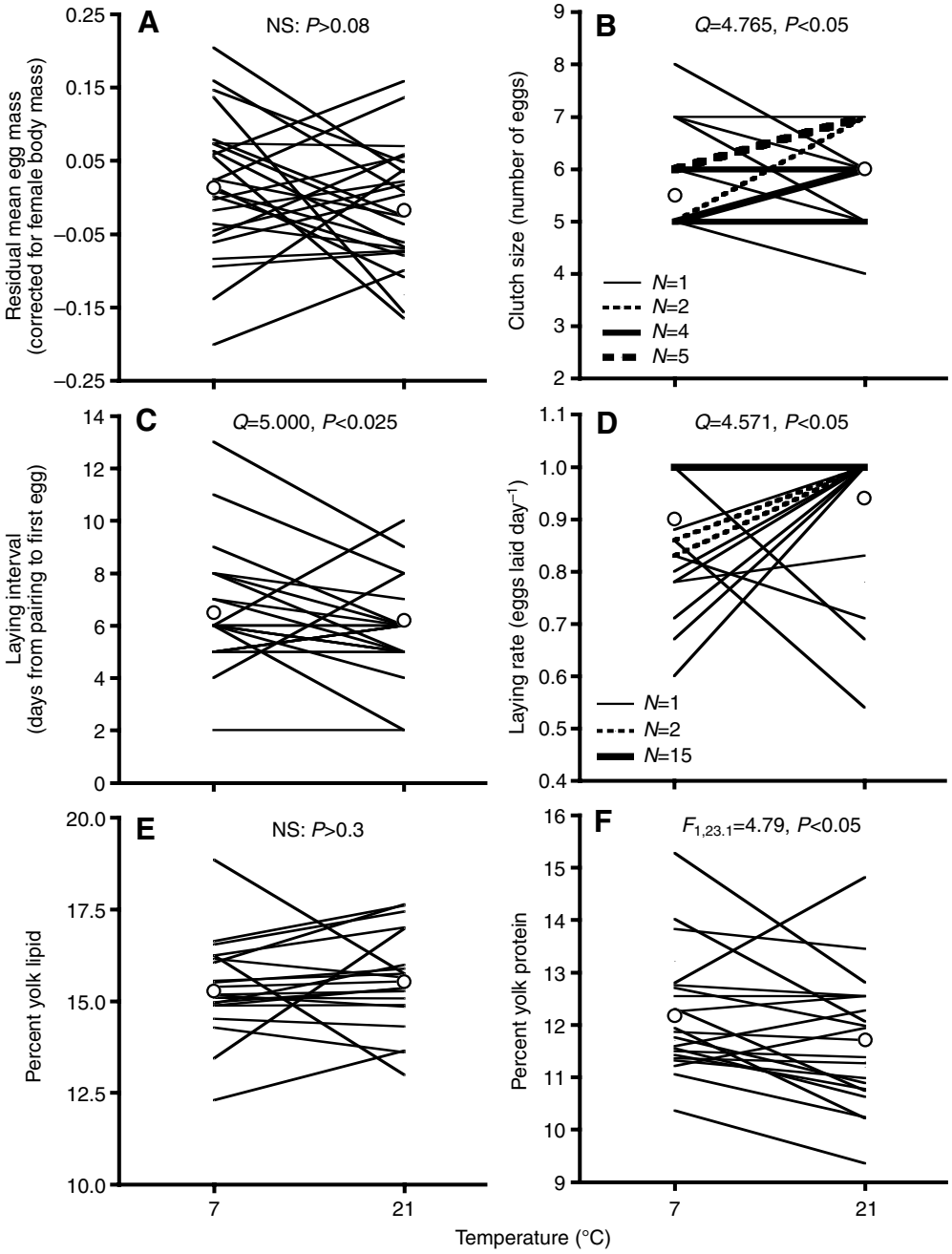

Fig. 3. Temperature-induced changes in (A) residual mean egg mass (corrected for female body mass at the 1-egg stage), (B) clutch size, (C) laying interval, (D) laying rate, (E) yolk lipid content and (F) yolk protein content of the second egg of females producing eggs at 7°C and 21°C. Lines join values for individual females and open circles represent means.

between temperature and both clutch size and laying date, the free-living equivalent to laying interval. While low ambient temperature was associated with decreased clutch size in this study, ambient temperature was not correlated to variation in clutch size in great tits (Pendlebury and Bryant, 2005), and nest box temperature was not related to clutch size in blue tits (Yom-Tov and Wright, 1993). Furthermore, while laying dates of great tits were not related to experimentally manipulated nest box temperature (Nager and van Noordwijk, 1992), ambient temperature was negatively correlated with laying date of European swifts *Apus apus* (O'Connor, 1979), and laying interval in this study. Furthermore, while we found no relationships between the mean egg mass or the masses of the egg components (yolk protein, yolk lipid, albumen protein) and ambient temperature during egg formation, ambient temperature was positively correlated with egg mass in many free-living passerine species [e.g. European starlings *Sturnus vulgaris* (Ojanen et al., 1981); great tits (Ojanen et al., 1981; Pendlebury and Bryant, 2005); pied flycatchers *Ficedula hypoleuca* (Ojanen, 1983); blackbirds *Turdis merula*

(Magrath, 1992); collared flycatcher *Ficedula albicollis* (Hargitai et al., 2005)] and with the energetic content of the egg components of great tits and pied flycatchers (Ojanen, 1983). Similarly, egg volumes of great tits laying in heated nest boxes were greater than those of females laying in experimentally cooled nest boxes (Nager and van Noordwijk, 1992). However, most of these studies did not control for laying date, which could potentially confound the relationship between ambient temperature and egg size through correlations with both variables (Magrath, 1992; Lessells et al., 2002). In contrast to these studies, mean egg mass of blue tits was not related to experimentally manipulated nest box temperatures (Yom-Tov and Wright, 1993). As in this study, the masses of the egg components of free-living great tits were not related to ambient temperature when other factors, such as total egg mass, were controlled for. The results from this study and previous studies examining the influence of ambient temperature on reproductive output demonstrate the variety of ways in which females producing eggs in sub-optimal conditions can modulate reproductive output in order to decrease the energetic demands associated with egg production and increase the energy available for fueling maternal self-maintenance and survival, while still producing offspring that can be raised given the current environmental conditions and those predicted for the future. It is not known whether the decreases in reproductive output observed in this and previous studies are a direct result of energy limitation during egg production (i.e., limited food availability or digestive constraints), or whether females producing eggs in sub-optimal environmental conditions are strategically decreasing current reproductive output in order to maintain their own condition, thereby potentially increasing their own chances of survival and reproductive output during future breeding attempts. Future studies that (1) involve concurrent cold-acclimation and diet supplementation, (2) experimentally increase egg production effort (*via* egg removal) (Williams and Miller, 2003), and (3) examine reproductive output during future breeding attempts are required to test these two hypotheses.

The declines in reproductive output of zebra finches maintained at 7°C were accompanied by a decrease in the proportion of sVLDLy particles in circulation. However, this decrease resulted from the increase in circulating levels of very small VLDL particles, i.e. the decrease in modal and median particle diameter at 7°C, and not an increase in larger, potentially maternally required, VLDL particles, as we originally hypothesized. During lipoprotein–lipase metabolism of VLDL, particle diameter decreases as the triacylglycerides from the particle's core are removed, and the lipids and proteins from the particle's surface are transferred to other lipoproteins (for reviews, see Eisenberg, 1986; Walzem, 1996). Therefore, the increase in the proportion of very small VLDL particles in laying zebra finches at 7°C was likely the result of an increase in the metabolism of larger VLDL particles by non-ovarian tissues to fuel the cold-acclimated females' own energetic demands. While these results suggest that the observed declines in the reproductive output of laying zebra finches exposed to low ambient temperatures may have been due to a limited supply of VLDLy particles that were capable of being utilized in egg formation, there was actually no relationship between variation in the different measures of reproductive output and the proportion of sVLDLy particles in circulation. Consequently, other temperature-dependent physiological factors must be involved in the mechanisms underlying temperature-dependent variation in the reproductive output of zebra finches. However, analysis of the apolipoprotein composition of the VLDL particles within the sVLDLy range is required to clarify whether all of the VLDL particles within this diameter range were actually yolk-targeted VLDL, that is, whether they contained apolipoprotein VLDL-II, which increases the lipoprotein lipase-resistance of VLDLy, thereby protecting the particles for use in egg production (reviewed in Walzem, 1996).

A possible explanation for the observed decline in the reproductive output of zebra finches exposed to low ambient temperatures is the potential reallocation of energy away from other energetically expensive activities in order to save energy, which could then have an indirect effect on reproductive output. The addition of egg production to the already energetically demanding process of thermoregulation actually resulted in an 11% decrease in RMR compared to the non-laying, actively thermoregulating values of zebra finches (Salvante et al., 2006). A proposed explanation for the decrease in metabolic rate of laying zebra finches maintained at 7°C was the reallocation of energy within individuals away from the energetically demanding process of thermoregulation, which would likely necessitate the use of facultative rest-phase hypothermia, the regulated and reversible decrease in body temperature below normothermic levels, by cold, laying birds (for reviews, see Reinertsen, 1996; McKechnie and Lovegrove, 2002). If any of the processes involved in egg production, such as hormone synthesis or action on target tissues, yolk precursor production or uptake, or albumen or shell deposition, are sensitive to changes in body temperature, then the use of hypothermia could result in slower egg formation, which would explain the longer laying intervals and decrease in laying rate observed in this study.

In addition to potentially limiting the resources available for egg production, experiencing low ambient temperatures during egg production may provide laying females with predictive information about the future environmental conditions in which their young will be raised. Evidence for basing reproductive decisions on 'expected' future conditions that are predicted from current environmental conditions is widespread. For example, parasitic wasps (*Leptopilina heterotoma*) have been shown to increase reproductive effort (e.g. prolonged searching for oviposition sites, oviposition on already parasitized hosts, i.e. superparasitism) if their perceived risk of mortality increased due to changes in barometric pressure or photoperiod (Roitberg et al., 1992; Roitberg et al., 1993). Because many conditions that are favorable for reproduction vary predictably with season every year (Wingfield et al., 1992), birds can use a variety of

environmental cues (e.g. photoperiod, early food availability, rainfall, temperature) that occur early in the breeding season to predict when the peak in essential resources for their offspring will become available later in the season, and thus determine when to initiate the recrudescence of the reproductive axis and subsequent egg production (reviewed in Immelmann, 1971; Immelmann, 1973). Therefore, if laying females use environmental conditions such as ambient temperature during egg production as cues to predict the quality of environmental conditions during later stages of reproduction, the decline in clutch size observed at 7°C in this study may actually result from a facultative decrease in the number of eggs laid by cold zebra finches to match current and future reproductive effort (i.e. incubation activity and brooding and provisioning of chicks) with the sub-optimal conditions predicted during the incubation and chick-rearing stages, based on exposure to low ambient temperatures during egg production. However, it is difficult to determine whether the temperature-related decline in clutch size was due to energy-limitation during egg production or the facultative downregulation of early reproductive output. If the decrease in clutch size resulted from resource limitation, then attempts to induce females to lay more eggs without increasing resource availability (i.e. food supplementation) would likely fail. However, if decreasing clutch size was a facultative 'decision' by the laying female, then future studies may be able to induce zebra finches producing eggs at low ambient temperatures to lay more eggs without increasing resource availability (*via* egg removal) (Williams and Miller, 2003), and then examine whether experimentally increasing clutch size, through replacement of the previously removed eggs, has a detrimental effect on offspring growth and survival in sub-optimal (i.e. cold) conditions.

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