What Comes First, the Zebra Finch or the Egg?

Resource Allocation During Avian Egg Production

by

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ABSTRACT

Energy is an essential resource that all living organisms must balance. In vertebrates, very-low density lipoprotein (VLDL) particles play a key role in the transport of energy-rich lipids. During avian egg production, small yolk-targeted VLDL (VLDLy) particles are produced that can pass through the various layers of the ovary and are less susceptible to general metabolism, thereby preserving them for use by the developing ovarian follicles. To investigate how reproductive status and environmental conditions influence the differential allocation of energy-rich lipids to self-maintenance versus egg production in passerine birds, changes in VLDL particle diameter were characterized in relation to the reproductive status and output of Zebra Finches (*Taeniopygia guttata*) exposed to favorable and energetically-challenging conditions. While birds producing eggs in favorable conditions exhibited a higher proportion of circulating VLDLy particles than non-breeding females, variation in VLDLy levels was not related to reproductive performance. When the energetically-demanding processes of thermoregulation and egg production were combined, laying birds consumed more seed and decreased locomotor activity, but took longer to initiate egg laying and laid fewer eggs at a slower rate than under warmer conditions. Cold-acclimated, laying females exhibited a decrease in the proportion of circulating VLDLy particles, but this decrease was not related to changes in reproductive effort. While these results suggest that maintaining a certain proportion of circulating VLDLy may not be an important factor in determining reproductive output in Zebra Finches, they were based on indirect estimates of VLDLy abundance. To develop

an assay to directly quantify circulating VLDLy in passerine birds, two polyclonal antibodies raised against an apolipoprotein component of chicken VLDLy (apoVLDL-II) were tested. Putative Zebra Finch apoVLDL-II was expressed at lower levels in laying Zebra Finches and was less-well recognized by the antisera compared to chicken apoVLDL-II from laying hens, suggesting that inter-specific differences in the expression level or the structure of apoVLDL-II may make quantification of Zebra Finch VLDLy with existing antibodies impossible. Overall, these data suggest that egg-laying birds are able to allocate sufficient energy to fuel self-maintenance and sustain reproduction by increasing energy intake and reallocating energy from other activities.

Keywords

Very-low density lipoprotein (VLDL), egg production, resource allocation, reproduction, life history trade-offs

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CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

Energy fuels all aspects of life. However, when energy is limited, trade-offs arise between energetically-demanding activities, such as thermoregulation, reproduction, locomotion, and general somatic maintenance (Stearns, 1992; Bernardo, 1996; Zera and Harshman, 2001; Ricklefs and Wikelski, 2002). How do animals determine how much energy to allocate to one activity over another? How does differential allocation occur? Is there one pool of resources, and therefore energy, that different physiological systems have access to? Alternatively, are there resources that only some systems can access while others cannot? Do allocation decisions depend on prevailing environmental or physiological conditions, i.e., is there plasticity in decision making, or are they all-or-nothing choices? These were some of the questions that came to mind when I started my Ph.D. research. Answering these questions involves knowing something about the physiological basis of energy allocation to different activities.

One of the most important choices animals face is the decision to allocate limited resources between reproduction and self-maintenance, i.e., the trade-off between current reproduction and survival (Stearns, 1992; Bernardo, 1996; Zera and Harshman, 2001). Allocating too much to reproduction could lead to an increased risk of mortality, while allocating too little can lead to unsuccessful reproduction, which is a waste of precious energy. The main goal of my research was to explore the physiological mechanisms underlying this trade-off in an attempt to answer some of the questions mentioned above. I chose to investigate the differential allocation of energy-rich lipids during avian egg production based on interesting data from domesticated species. In non-laying birds the

function of very-low density lipoprotein (VLDL) particles is to transport lipids throughout the body, where they are either metabolized by tissues to fuel metabolic process or stored in adipose tissue. However, during egg production estrogens stimulate the production of another form of VLDL particle that differs from the generic, non-breeding form in its structure, composition, and function. These estrogen-dependent VLDL particles are "destined" for incorporation into egg yolk, and so have been termed yolk-targeted VLDL, or VLDLy. They are structurally smaller, and can fit into the pores in the ovary, therefore gaining access to the developing ovarian follicles, and are more resistant to lipoprotein lipase (LPL)-dependent metabolism by extra-ovarian tissues, and are thereby preserved for use in egg formation (Chan *et al.*, 1976; Kudzma *et al.*, 1979; Perry and Gilbert, 1979; Griffin, 1981; Dashti *et al.*, 1983; Griffin and Perry, 1985; Lin *et al.*, 1986; Schneider *et al.*, 1990; Walzem *et al.*, 1994; Walzem, 1996; Speake *et al.*, 1998; Walzem *et al.*, 1999; Boyle-Roden and Walzem, 2005). Consequently, examining the changes in generic VLDL-VLDLy dynamics during avian egg production should shed light on the physiological mechanisms underlying the decision to allocate energy to reproduction versus self-maintenance.

Data on changes in VLDL particle diameter distribution in domesticated avian species that have been selected for maximum egg production revealed a near-complete shift in circulating VLDL particles from generic form to the smaller, yolk-targeted VLDL particles (Walzem, 1996). Griffin and Hermier (1988) reported that only about 10% of the lipid associated with VLDLy can be hydrolyzed by LPL. Given this increased resistance of VLDLy to metabolism by maternal tissues for her own energetic needs, I wondered whether this dramatic shift in lipid allocation towards reproduction was

common among all birds. If so, how would small, free-living birds, which often produce eggs early in the spring when environmental conditions are less-than favorable (e.g., spring storm, low ambient temperatures, low food availability) and have relatively high mass-specific metabolic rates, meet their energy demands with so little lipid available for self-maintenance? Surely such a complete shift to VLDLy particles during avian egg production could not be ubiquitous! So, we set out to 1) characterize generic VLDL-VLDLy dynamics in a small, passerine songbird, the Zebra Finch, in order to a) compare its pattern of lipid allocation during egg production with that of domesticated species and b) determine whether variation in VLDL dynamics was related to variation in subsequent reproductive effort; 2) determine how the metabolic costs of egg production compared to the costs of other physiological processes; and 3) increase the energetic demands on laying female Zebra Finches by manipulating the laying environment to see whether energy allocation decisions could be manipulated by changing the energetic requirements of laying females.

AVIAN EGG PRODUCTION

Before we can investigate energy allocation to reproduction in birds, it is important to have a general understanding of the physiological processes involved in avian egg production. Avian egg formation is made up of a series of complex physiological processes that are controlled by the hormones of the hypothalamic-pituitary-gonadal axis (Carey, 1996; Johnson, 1998; Williams, 1998; Figure 1.1). In response to environmental and social cues, including photostimulation resulting from the

seasonal change in photoperiod, increasing temperature, and availability of potential mates, neurosecretory cells of the hypothalamus secrete gonadotropin-releasing hormone (GnRH) at the median eminence, into the hypophyseal portal blood system, through which GnRH travels to the anterior pituitary and stimulates production of the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Williams, 1998; Scanes, 2000). In females the gonadotropins are then secreted into the bloodstream, and progress to the ovary, where they stimulate the synthesis and secretion of estrogens into the general circulation (Williams, 1998). Estrogens stimulate the hepatic production of the egg-yolk precursors, vitellogenin (VTG) and yolk-targeted, very-low density lipoprotein (VLDLy) (Bergink *et al.*, 1974; Deeley *et al.*, 1975; Wallace, 1985; Walzem, 1996; Williams, 1998). Following their synthesis, VTG and VLDLy are secreted into the blood. FSH and various intra-ovarian growth factors also regulate the recruitment and hierarchical development of ovarian follicles (Adashi *et al.*, 1988; Palmer and Bahr, 1992; Adashi, 1994; Onagbesan and Peddie, 1995). Once recruited, the follicles undergo rapid yolk development, during which plasma VTG and VLDLy are taken up by the oocyte through receptor-mediated endocytosis, and are processed within the follicles into yolk, the nutrient and energy source for the developing avian embryo (Bernardi and Cook, 1960; Stifani *et al.*, 1988; Wallace, 1985). In Zebra Finches (*Taeniopygia guttata*), as in other passerine songbirds, rapid yolk development takes three days per follicle, and so is assumed to begin about 4 days prior to the laying of the egg and continue until ovulation of the fully developed follicle, 24 hours prior to oviposition (Haywood, 1993; Williams and Ternan, 1999). Surges in plasma LH induce ovulation of the fully developed follicles into the oviduct (Scanes, 2000), where estrogens

and progesterone regulate the synthesis and deposition of egg albumen and shell (Yu *et al.*, 1971; Burley and Vadehra, 1989). The eggs are then laid approximately 24 hours after ovulation.

VLDL SYNTHESIS AND METABOLISM

Furthermore, to examine the differential allocation of energy-rich lipids to activities such as reproduction and somatic maintenance, it is also important to have a general understanding of the processes involved in VLDL production and metabolism (reviewed in more detail in Walzem, 1996). Dietary lipids travel from the intestine to the liver via the portal vein in the form of portomicrons. Once in the liver, the lipid is incorporated into newly formed VLDL particles. VLDL particle formation begins with the hepatic synthesis of apolipoproteins, amphipathic proteins with a lipophilic, non-polar region that interacts with the lipid within the lipoproteins, and a polar, hydrophilic region that interacts with the aqueous environment of general circulation, which are responsible for aiding in the transport of and metabolism of lipid-rich triglycerides throughout the body. The various apolipoproteins of VLDL have different roles; apolipoprotein B acts as the receptor ligand for metabolism of VLDL by LPL (Nimpf *et al.*, 1988), while apolipoprotein C is an activator of LPL (Breckenridge *et al.*, 1978), and apolipoprotein VLDL-II is believed to be responsible for preserving VLDLy for use in egg production by decreasing its diameter and increasing its resistance to LPL-metabolism (Schneider *et al.*, 1990; Walzem, 1996; Walzem *et al.*, 1999; Boyle-Roden and Walzem, 2005). Synthesis of the apolipoproteins occurs on the rough endoplasmic reticulum (ER) within

hepatocytes, and their combination with lipid-rich triglycerides, cholesterol, and cholesterol esters to form VLDL particles occurs where the rough and smooth ER meet. The assembled VLDL particles then move to the Golgi apparatus where they are packaged into exocytotic vesicles for transport to general circulation (reviewed in Walzem, 1996). Once in the blood, VLDL particles are available for metabolism by tissues that require triglycerides for energy. These tissues synthesize LPL, which then migrates to the capillary endothelium where it has access to free-flowing VLDL particles. VLDL metabolism occurs when apolipoprotein C on the surface of the VLDL particles activates LPL, which then removes and hydrolyses the triglycerides at the core of the VLDL particles, reducing their core size and increasing the abundance of surplus surface lipids and proteins (for reviews see Eisenberg, 1986; Walzem, 1996). The surplus surface lipids (e.g., cholesterol, phospholipids) and proteins (e.g., excess apolipoproteins) are transferred to other lipoprotein particles (i.e., high-density lipoproteins, HDL), further decreasing the size and increasing the density of VLDL particles, transforming them into intermediate-density lipoproteins (IDL), and eventually into low-density lipoproteins (LDL) (for reviews see Eisenberg, 1986; Walzem, 1996).

THE ZEBRA FINCH (*TAENIOPYGIA GUTTATA*)

The Zebra Finch is an ideal model species in which to study the physiological mechanisms underlying the trade-off between current reproduction and survival in passerine songbirds. They breed well in captivity, are amenable to handling and experimental manipulation during breeding, and have a short generation time, as they

reach sexual maturity at only three months of age. There has also been a great deal of research on the breeding biology of both free-living and captive Zebra Finches (see Zann, 1996). They exhibit marked inter-individual variation in egg mass (0.75 – 1.25 g), clutch size (2 to 7 eggs), and initiation of laying (first clutch laid in same season as hatch: 62-162 days after hatching) (Zann, 1996). These birds also display large inter-individual variation in circulating levels of the yolk precursors, VTG and triglyceride, which had been used an index of VLDLy when measured during egg production (Williams, 1996; Williams and Christians, 1997; Salvante and Williams, 2003). Examination of the relationships between variation in VLDL dynamics and reproductive effort in Zebra Finches can be used as a first step in determining the role that differential allocation of lipid resources to reproduction plays in the regulation of reproductive effort in passerine songbirds.

There are limitations of using captive Zebra Finches or other captive species as models for free-living birds. Selective pressures on captive species can differ dramatically from those acting on free-living species, and these differences can influence the decision to allocate resources to self-maintenance or current reproduction. For example, predation risk for the captive Zebra Finches used in this research was non-existent, and therefore the birds' perceptions of life expectancy were likely to be much longer than those of free-living passerine songbirds that face much higher predation risk. Longer perceived life expectancies could potentially shifting resource allocation towards self-maintenance (i.e., survival and potentially future reproduction) and away from current reproduction because individuals are likely to survive to reproduce again. Alternatively, because resources, such as food and water, and environmental conditions,

such as temperature, are generally less variable in captivity, the need for maintaining body reserves to cope with more variable conditions (e.g., decreases in food availability or ambient temperature) may be less important in captive species. Therefore, resource allocation in captive birds may be shifted more towards reproduction and away from self-maintenance. Furthermore, the costs and benefits associated with maintaining immune function may potentially differ between captive and free-living species, as the risk of exposure to diseases or parasites likely differs with environment. If captivity is associated with a lower risk of disease, due to antiseptic husbandry practices, then immune function may require fewer resources, which could then be allocated towards reproduction. However, if captivity is associated with higher risks of infection, due to increased density of conspecifics, then immune function may require more resources, which would limit those available for reproduction. These examples serve to illustrate the complex relationships between varying selective pressures and resource allocation decisions, and should serve as caveats to directly extrapolating findings based on studies on captive species to free-living populations.

SUMMARY OF THESIS CHAPTERS

This thesis consists of five research chapters, each presented in manuscript form with its own summary, discussion and literature cited sections, as well as a list of figures. I have chosen to use the term "we" instead of "I" in these chapters to reflect the important contributions of my collaborators to these studies. The names of the collaborator/co-authors for each study are listed on the first page of each chapter.

The study presented in chapter two describes the validation of an established dynamic laser-light scattering technique to characterize VLDL particle diameter distribution in individual Zebra Finches in relation to reproductive status. We tested the hypothesis that Zebra Finches, and non-domesticated avian species in general, would exhibit less dramatic shifts in VLDL dynamics towards VLDLy particles during egg production than domesticated species that have been selected for maximized egg production. In collaboration with Rosemary Walzem at Texas A&M University, I compared the VLDL particle diameter distributions of laying and non-laying Zebra Finches with previously collected distribution data from laying and non-laying chickens. While we confirmed the previously reported pattern of VLDL dynamics in chickens, a new and unexpected pattern emerged for Zebra Finches.

Chapter three characterizes the magnitude of variation in measures of VLDL particle diameter distribution in laying Zebra Finches and chickens to determine whether variation in lipid allocation to reproduction is related to variation in reproductive effort. This includes comparing the extent of intra-individual repeatability of VLDL particle diameter distribution with repeatability estimates for morphological and reproductive traits (in Zebra Finches), and contrasting the relationships between variation in VLDL particle diameter distribution and measures of reproductive performance (e.g., egg mass, laying rate) in the two species.

In chapter four I examine the metabolic cost of egg production in relation to the cost of another energetically-demanding process, thermoregulation. We measured the metabolic rate of Zebra Finches in a variety of thermal and reproductive states using respirometry to determine how the metabolic costs of egg production, cold-acclimation

and active thermogenesis compared to each other as well as to basal metabolic rate. We also investigated whether the costs associated with these activities were additive when the activities occurred simultaneously.

In chapter five, to determine the influence that decreasing ambient temperature, and therefore increasing the energetic requirements of the laying females, had on lipid allocation to reproduction and self-maintenance, I increased the energetic demands of laying Zebra Finches by exposing them to a low ambient temperature (7°C) prior to and during egg production to determine whether lipid allocation decisions would change based on changes in environmental conditions and the energetic needs of the laying females. Each female Zebra Finch was paired at both 21°C and 7°C, and their VLDL particle diameter distributions under each temperature regime were compared. Furthermore the temperature-dependent changes in reproductive effort and VLDL particle diameter distribution were compared to identify any existing correlations that may suggest that variation in lipid allocation to reproduction, as estimated by VLDL particle diameter, plays a role in regulating variation in reproductive performance in Zebra Finches, and passerines in general.

In chapter six I describe the attempted development of a direct assay for apoVLDL-II for the quantification of circulating levels of VLDLy, including the isolation and purification of apolipoprotein VLDL-II (apoVLDL-II) for the first time from a passerine bird. ApoVLDL-II is an apolipoprotein that is associated with VLDLy particles, but not with generic VLDL (cf. apoB which is present on both generic VLDL and VLDLy). I then assessed whether two antisera that were raised against chicken apoVLDL-II recognized putative Zebra Finch apoVLDL-II.

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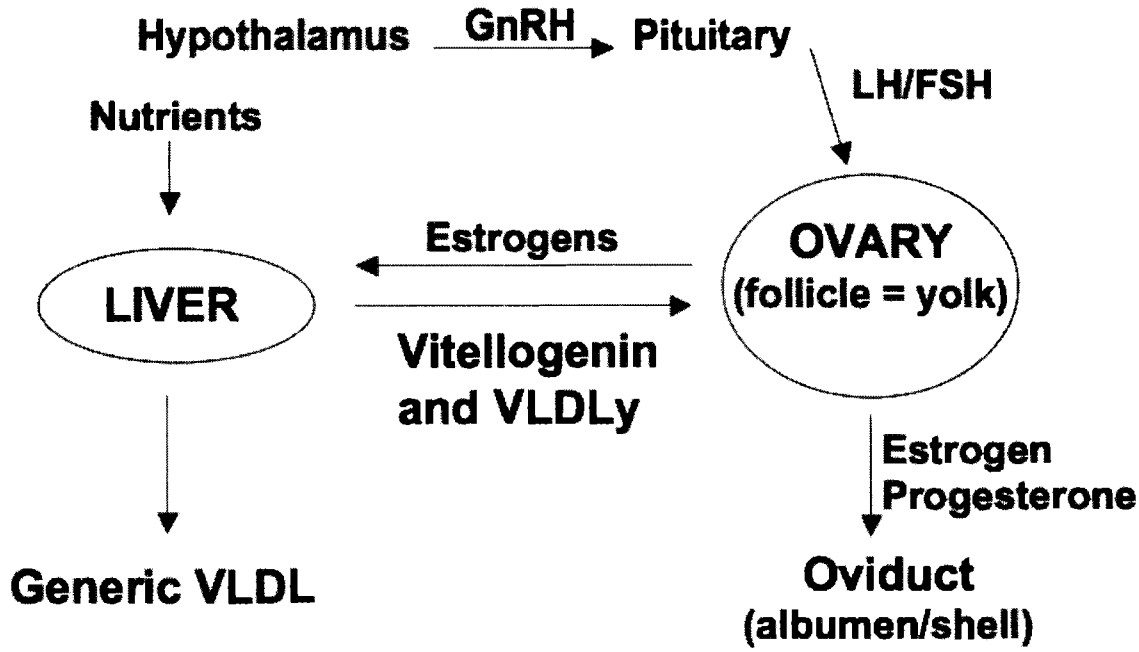
Figure 1.1. Avian egg production, including the hormonal cascade responsible for the up-regulation of reproductive tissues.



CHAPTER 2

CHARACTERIZATION OF VLDL PARTICLE DIAMETER DYNAMICS IN RELATION TO EGG PRODUCTION IN A PASSERINE BIRD

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SUMMARY

During avian egg production, estrogen mediates marked increases in hepatic lipid production and changes in the diameter of assembled very-low density lipoprotein (VLDL). A nearly complete shift from generic VLDL (~70 nm in diameter), which transports lipids to peripheral tissues, to yolk-targeted VLDL (VLDLy) (~30 nm), which supplies the yolk with energy-rich lipid, has been observed in the plasma of laying domestic fowl. We validated an established dynamic laser scattering technique for a passerine songbird *Taeniopygia guttata*, the Zebra Finch, to characterize the dynamics of VLDL particle diameter distribution in relation to egg production. We predicted that non-gallinaceous avian species that have not been selected for maximum egg production would exhibit less dramatic shifts in lipid metabolism during egg production. As predicted, there was considerable overlap between the VLDL particle diameter distributions of laying and non-laying Zebra Finches. But unexpectedly, non-laying Zebra Finches had diameter distributions that peaked at small particles and had relatively few large VLDL particles. As a result, laying Zebra Finches, in comparison, had diameter distributions that were shifted towards larger VLDL particles. Nevertheless, laying Zebra Finches, like laying chickens, had larger proportions of particles within proposed VLDLy particle diameter ranges than non-laying Zebra Finches (e.g., sVLDLy: 50% vs. 37%). Furthermore, Zebra Finches and chickens had similar modal (29.7 nm in both species) and median (32.7 nm vs. 29.6 nm) VLDL particle diameters during egg production. Therefore, although Zebra Finches and chickens exhibited opposing directional shifts in

VLDL particle diameter distribution during egg production, the modifications to VLDL particle structure in both species resulted in the realization of a common goal, i.e., to produce and maintain a large proportion of small VLDL particles of specific diameters that are capable of being incorporated into newly forming egg yolks.

INTRODUCTION

During avian egg production estrogens stimulate the liver to produce the egg-yolk precursors, yolk-targeted, very-low density lipoprotein (VLDLy) and vitellogenin (VTG), which provide embryos with the energy and nutrients required for growth and development (Gruber, 1972; Bergink *et al.*, 1974; Deeley *et al.*, 1975; Neilson and Simpson, 1973; Chan *et al.*, 1976; Wallace, 1985; Walzem, 1996a; Williams, 1998). As a result, total hepatic lipid production increases markedly during this time, from about 3 mg neutral lipid/ml plasma in non-laying turkeys (*Meleagris gallopavo*) to 21 mg in laying turkeys (Bacon *et al.*, 1974) and from 0.5 - 1.5 μmole triacylglycerides/ml plasma in non-laying chickens (*Gallus gallus domesticus*) to 20 – 50 μmole in laying chickens (Griffin and Hermier, 1988). Furthermore, data for egg-laying chickens, turkeys, and quail (*Coturnix coturnix*) show that there is an estrogen-dependent shift in VLDL synthesis from the production of generic VLDL, which ranges in size from 30 to >200 nm, to smaller, yolk-targeted VLDL, which ranges in diameter from 15 to 55 nm in domestic fowl (Griffin, 1981; Walzem *et al.*, 1994; Walzem, 1996a; Speake *et al.*, 1998; Walzem *et al.*, 1999). Furthermore, while generic VLDL has at least six associated apolipoproteins (including apoA-I, apoB and apoC), VLDLy has only two associated apolipoproteins, apoB and apoVLDL-II, the latter of which is thought to be responsible for the decrease in VLDLy diameter (Chan *et al.*, 1976; Kudzma *et al.*, 1979; Griffin, 1981; Dashti *et al.*, 1983; Lin *et al.*, 1986; Schneider *et al.*, 1990; Walzem, 1996a; Speake *et al.*, 1998; Walzem *et al.*, 1999). Consequently, the presence of circulating

VLDLy in egg-producing females represents a dramatic shift in lipid metabolism associated with changes in the composition and structure of VLDL.

The structural changes to circulating VLDL particles directly influence their *in vivo* function during egg production (Walzem, 1996a). While the role of generic VLDL is to transport triacylglycerides throughout the body for tissue utilization or storage in adipose tissue, the function of VLDLy is to deliver triacylglycerides to the oocyte, where they will be used as the energy source for the developing embryo (Walzem, 1996a). The smaller diameter of VLDLy is thought to be critical for enabling the particles to pass through the pores in the granulosa basal lamina of the ovary, allowing them access to the developing ovarian follicles (Griffin and Perry, 1985). In addition, apoVLDL-II also acts as an inhibitor of lipoprotein lipase (LPL), likely by limiting access to the water needed for triacylglycerol hydrolysis (Boyle-Roden and Walzem, 2005). The resistance of VLDLy particles to hydrolysis by extra-ovarian tissues preserves the triacylglycerol-rich VLDLy for uptake by the developing ovarian follicles (Walzem, 1996a). Cross-injection studies on turkeys and chickens using labeled generic VLDL isolated from immature turkeys and labeled VLDLy from laying turkeys and chickens confirm that immature and laying birds utilize generic VLDL and VLDLy differently; a greater proportion of generic VLDL was deposited into tissues, whereas more VLDLy was incorporated into ovarian follicles (Bacon *et al.*, 1978; Bacon, 1981). *In vivo* studies in laying domestic fowl have detected only low circulating levels of intermediate-density and low-density lipoproteins, both by-products of the hydrolysis of VLDL by LPL (Hermier *et al.*, 1989; Walzem *et al.*, 1994; Walzem, 1996a), providing further evidence for the increased *in vivo* resistance of VLDLy to LPL hydrolysis.

Despite the LPL-resistance of VLDLy, chickens and turkeys are able to incorporate radiolabelled VLDLy preparations from laying females into non-ovarian tissues, presumably to metabolize them to meet their energetic needs (Bacon *et al.*, 1978; Bacon, 1981). Griffin and Hermier (1988) noted that some 10% of VLDLy triacylglycerol can be hydrolyzed by LPL; given the high plasma concentrations of VLDLy in laying domestic fowl, this partial hydrolysis may be sufficient to meet the female's own energetic requirements during laying. Others (Chen *et al.*, 1999; Walzem *et al.*, 1999) have proposed that laying domestic fowl could also potentially meet their energetic requirements by metabolizing small amounts of generic VLDL that are synthesized by the avian kidney (Blue *et al.*, 1980; Tarugi *et al.*, 1998).

Chickens have been the target of strong artificial selection for prolonged and consistent egg production, and can maintain high rates of egg production for over a year (Etches, 1996). It is not known whether non-domesticated, non-gallinaceous avian species, which have not been selected for maximum egg production, exhibit such dramatic shifts in lipid metabolism during egg production. Passerine birds have been shown to experience marked increases in the concentration of circulating triacylglycerol during egg production (Christians and Williams, 1999; Challenger *et al.*, 2001). However, the assay used in these studies measured triacylglycerides associated with both generic and yolk-targeted VLDL and was not able to distinguish between the two forms of the lipoprotein. Walzem *et al.* (1994; 1999) used a dynamic laser scattering technique to assess VLDL particle diameter distribution in domestic fowl. This method provides a frequency distribution of VLDL particle diameters (in nm), which has been shown to vary in relation to egg production (Walzem *et al.*, 1994; Walzem, 1996a; Walzem *et al.*,

1999; Peebles *et al.*, 2004). At present there is a paucity of data on VLDL particle diameter distributions for non-domesticated species. In this paper we describe a modification of the dynamic laser scattering technique for use in a passerine songbird, the Zebra Finch (*Taeniopygia guttata*). We used this technique to 1) characterize VLDL particle diameter distributions in non-laying and egg-producing female Zebra Finches, 2) estimate the diameter range of VLDL particles that are available for deposition into the developing eggs of laying Zebra Finches, and 3) compare VLDLy dynamics during egg production in Zebra Finches and chickens. Non-domesticated birds have not been strongly selected for egg production and generally experience much more variable environmental conditions during egg production, including fluctuations in food availability and low ambient temperature. We therefore predicted that laying female passerines should experience greater selection pressures to be able to maintain production of larger, potentially generic, VLDL during egg production in order to meet their unpredictable energetic needs.

MATERIALS AND METHODS

Animal Husbandry

Zebra Finches were housed in the Simon Fraser University Animal Care Facility under controlled environmental conditions (temperature 19-23°C, humidity 35-55%, constant light schedule of 14L: 10D, lights on at 07:00). All birds received a mixed seed diet (Panicum and white millet, 50:50; approximately 12.0% protein, 4.7% lipid; Jameson's Pet Food, Vancouver and Just for Birds, Surrey), water, grit, and cuttlefish

bone (calcium) *ad libitum*. Birds also received a multivitamin supplement in the drinking water once per week. When not paired for breeding, the birds were housed in same-sex cages, but were not visually or acoustically isolated from the opposite sex. Artificial selection for specific traits has never been performed on this breeding colony. However, inadvertent selection on reproductive performance may have occurred over the many generations these birds have spent in captivity. All experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit (no. 692B-94) following the guidelines of the Canadian Committee on Animal Care.

Single comb White leghorn chickens of the W-36 strain (Hy-Line International) were individually housed in light-supplemented, fan-ventilated, open-sided houses at Texas A&M University. Chickens were given *ad libitum* access to water and a corn-soy diet formulated to meet the National Research Council for Poultry's (NRC) requirements for laying hens (15% protein, < 5% lipid, 2900 Kcal/kg; NRC Subcommittee on Poultry Nutrition, 1994), and were provided with 15 hours of light per day. Ambient house temperature varied from 45°F to 85°F. All animal husbandry and experimental procedures were conducted by technicians from the Walzem laboratory in accordance with a protocol approved by the Animal Use and Care Committee of Texas A&M University.

Zebra Finch Breeding and Blood Sampling

Male (n = 36) and female (n = 36) Zebra Finches were weighed (± 0.1 g) at the time of pairing, and tarsus and bill measurements (± 0.1 mm) were taken. Breeding pairs were housed individually in cages (61 x 46 x 41 cm) equipped with an external nest box (15 x 14.5 x 20 cm) and were provided with an egg-food supplement (6 g of a mixture of 62-65 g hard-boiled egg, 13 g cornmeal, 13 g bread crumbs; 30.2% protein and 13.0%

lipid by dry mass) daily between pairing and clutch completion in addition to the normal seed diet (see Williams, 1996). Data on laying interval (days from pairing to initiation of laying) and egg and clutch size were obtained by checking the nest boxes daily between 09:00 and 11:00. All new eggs were weighed (± 0.001 g) and numbered on the day they were laid. Clutches were considered complete if no new eggs were laid over two days. At this time the female was weighed, and the pair was returned to same-sex, non-breeding cages.

Laying females were weighed and blood sampled (200 µl from the brachial vein) on the day their first eggs were laid (laying sample). Randomly chosen female Zebra Finches (n = 27) from the same-sex cages were also weighed and blood sampled (non-laying sample). All blood samples were collected between 09:00 and 11:30 into heparinized capillary tubes, and then expelled into EDTA-coated microcentrifuge tubes containing 0.5 M disodium-EDTA (3 µl; Sigma-Aldrich Canada, Oakville) and centrifuged at 2200 *g* for 10 minutes in a Baxter Canlab Biofuge 13. A sub-sample (5 µl) of each plasma sample was frozen (-20° C) for triacylglyceride analysis, while the remainder of each plasma sample was placed into an EDTA-coated microcentrifuge tube containing 0.5 M disodium-EDTA (5 µl) for VLDL particle diameter distribution analysis. Sodium azide (1% w:v; Sigma-Aldrich Canada, Oakville) was added to each EDTA-coated tube to prevent mold formation (0.01 µl / µl plasma), and the plasma samples were refrigerated (4° C) pending analysis of VLDL particle diameter distribution.

Influence of Feeding, Fasting and Egg-Food Supplementation on VLDL Particle Diameter Distribution in Zebra Finches

Two preliminary studies were performed to assess the potentially confounding effects of feeding versus fasting and diet supplementation (i.e., the egg-food supplement given to breeding pairs) on various measures of VLDL particle diameter distribution and circulating triglyceride levels. For the fasting experiment randomly-selected laying female Zebra Finches were blood sampled twice; once while in the 'fed' state, i.e., *ad libitum* access to seed and egg-food supplement and again while in the 'fasted' state, i.e., 15 to 16 hours without access to food. For the 'fasted' sample, the seed and egg-food supplement containers were removed at 19:00 on the night before blood sample collection. Female Zebra Finches were weighed and blood sampled between 10:00 and 11:00 on the days that their second and third eggs were laid (2-egg and 3-egg stages, respectively). The order in which females were in the fed and fasted states was randomized such that half of the females were in the fasted state at the 2-egg and in the fed state at the 3-egg stage, while the reverse was true for the other half of the females. Previous studies on vitellogenin (VTG), the other estrogen-dependent yolk precursor, have reported comparable levels of plasma VTG levels at the 2- and 3-egg stages (Challenger *et al.*, 2001; Salvante and Williams, 2002). Seed consumption during the 24-hours prior to each blood sample was measured by providing each breeding pair with 30.0 grams of seed on the days the females laid their first and second eggs. Seed was weighed (to the nearest 0.1 g) 24 hours later at the 2- and 3-egg stages.

To examine the influence of the high-fat egg-food supplement given to breeding pairs on changes in plasma triglyceride levels and VLDL particle diameter distribution during egg production, randomly-chosen male Zebra Finches were weighed and blood

sampled between 09:00 and 11:30 on two separate occasions: as non-breeding individuals on the seed-only diet (seed sample), and during breeding on the egg-food supplemented seed diet (supplemented sample) on the day that their female breeding partners laid their first eggs.

Chicken Blood Sampling

Blood samples for VLDL particle diameter distribution analysis were collected from two groups of chickens: immature, non-laying females at 17 weeks of age (n = 10) and actively laying females at 29 weeks of age (n = 37). Sampling of the 29-week old layers coincided with the peak of laying for the population (i.e., all females were actively laying eggs and the laying rate for the population was at its peak: 0.9 eggs laid per day). Blood samples were taken from the brachial vein between 09:00 and 11:00 into EDTA-coated Vacutainer tubes (BD Diagnostics, Franklin Lakes). Plasma samples were isolated by centrifugation and refrigerated (4° C) pending VLDL particle diameter distribution analysis.

Triacylglyceride Assay

Circulating concentrations of triacylglyceride in Zebra Finches were measured enzymatically as an index of total plasma VLDL (i.e., generic VLDL and VLDLy) (Triglyceride E kit – Wako Chemicals, Richmond; Serum Triglyceride Determination Kit, Sigma-Aldrich Canada, Oakville) using the method developed for domestic fowl (Mitchell and Carlisle, 1991) and validated for passerines (Williams and Christians, 1997; Williams and Martiniuk, 2000; Challenger *et al.*, 2001). This assay cleaves the fatty-acid chains off of the triglyceride molecules, resulting in unbound glycerol

molecules. The concentration of glycerol in each sample is measured before (free glycerol) and after the cleavage reaction (total glycerol). The difference between total and free glycerol is proportional to the plasma concentration of triglyceride. Intra- and inter-assay coefficients of variation were 1.85% (n = 6 replicates) and 4.79% (n = 13 assay plates), respectively, using a 19-week hen plasma pool. All assays were run using 96-well microplates and were measured at 540 nm using a Biotek 340i microplate reader.

VLDL Particle Diameter Distribution Assay

Whole plasma contains a variety of different lipoprotein classes, e.g., VLDL, low density lipoprotein (LDL), high density lipoprotein (HDL). Therefore, plasma VLDL was isolated as the $d < 1.020$ g/mL fraction of plasma from Zebra finches and chickens. The volume of each Zebra Finch plasma sample (approximately 100 µl) was measured and transferred into Beckmas Ultra-Clear ultracentrifuge tubes (13 x 64 mm, #344088; Beckman Coulter, Fullerton), and NaCl density solution (d = 1.0063; equivalent salt density of undiluted plasma) was added until a final volume of 1 ml was reached. Alternatively, a sub-sample (1 ml) from each chicken plasma sample was transferred into ultracentrifuge tubes. NaCl-NaBr density solution (5 ml; $d = 1.0255$) was then added to each tube. A blank sample was prepared by combining NaCl density solution (1 ml; $d = 1.0063$) with NaCl-NaBr density solution (5 ml; $d = 1.0255$) in an ultracentrifuge tube. The samples were loaded into a Beckman 50.4 fixed-angle rotor and centrifuged at 148600 *g* for 18 hours at 14°C in a Beckman L8-70M ultracentrifuge (Beckman Coulter, Fullerton). Following centrifugation, the supernatant containing the VLDL portion of the plasma was isolated from each tube by aspiration with 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 a UPA 250 and 7.02 analysis software using a UPA 250 and 7.02 analysis software (Microtrac, Clearwater) (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 minutes for the blank solution, and 5 minutes in triplicate for each VLDL sample. The probe was washed with distilled water and dried between samples.

Data Analysis

VLDL Particle Diameter Distribution Measurements

To characterize changes in VLDL particle diameter distribution in the Zebra Finch in comparison to chicken data, the proportion of VLDL particles within three potential VLDLy particle diameter ranges were calculated: 1) a range based on chicken values (hereafter referred to as the cVLDLy range), 2) a range based on the proposed sieving properties of the avian ovary (hereafter referred to as the sVLDLy range), and 3) a range based on Zebra Finch values (hereafter referred to as the mVLDLy range).

Walzem (1996) calculated VLDLy particle diameter range for laying chickens, the cVLDLy range, using the regression of the percentage of VLDL particles within each VLDL particle diameter class against subsequent laying rate, the most common measure of reproductive effort used for domestic fowl. Chickens lay continuously for extended periods. Therefore, laying females were repeatedly sampled at various times throughout the laying period, and these repeated measures were incorporated into the VLDLy calculations (Walzem, 1996a). Each of the resulting correlation coefficients (r) was presented graphically as y-values for each particle diameter class. The different VLDL particle diameter classes vary in their ability to support continuous egg production, and the diameter classes exhibiting positive relationships (i.e., positive correlation coefficients) with laying rate were assumed to have a better ability to support egg production and were therefore selected make up the cVLDLy particle diameter range (21.5 to 51.1 nm for laying chickens; Figure 2.1a; Walzem, 1996b). In contrast, the sVLDLy range was based on the observation in domestic fowl that the only VLDL particles observed distal to the granulosa basal lamina of the ovary during yolk formation, and thus able to reach the plasma membrane of the developing ovarian follicles, ranged from 25 to 44 nm in diameter (Perry and Gilbert, 1979; Griffin and Perry, 1985; Griffin and Hermier, 1988). These studies suggested that pores in the granulosa basal lamina act as selective sieves, allowing only VLDL particles of certain diameters to filter into the ovary (Perry and Gilbert, 1979; Griffin and Perry, 1985; Griffin and Hermier, 1988). Finally, the mVLDLy range (10.7 to 30.4 nm) was calculated similarly to the cVLDLy range described above with the following exceptions. Firstly, because Zebra Finches lay discrete clutches (5 to 7 eggs), laying females were only blood sampled once, on the day

their first eggs were laid. Therefore, only one set of VLDL particle diameter and reproductive output data was used per bird (c.f. the repeated measures of VLDL particle diameter and reproductive performance incorporated into the chicken VLDLy analysis due to their continuous laying). Secondly, because laying rate is not generally informative in Zebra Finches (i.e., there is virtually no variation in laying rate because the majority of females lay one egg per day without skipping a day until the clutch is complete), we used body mass-corrected mean egg mass as a measure of reproductive performance in Zebra Finches (Figure 2.1b). Mean egg mass varies markedly between individual female Zebra Finches (Williams, 1996; Salvante and Williams, 2002), but is highly repeatable within individual females between laying bouts (Williams, 1996), suggesting that mean egg mass is a distinct phenotypic trait of laying Zebra Finches. There is also evidence that egg size reflects a female's "egg laying ability" or "performance"; large-egg females are more capable of laying extended clutches in response to egg removal than small-egg females (Williams and Miller, 2003). VLDLy particle diameter ranges based on other measures of reproductive performance in Zebra Finches (e.g., clutch size, clutch mass) were also determined but were not used because they encompassed a majority of the VLDL particle diameter classes (i.e., 30 to > 200 nm), making them inconsistent with other potential VLDLy diameter range estimates. Finally, the modal and median particle diameter and the range (i.e., width) of each distribution, in nanometers, and the proportion of very small (< 30 nm) and large (> 51 nm) VLDL particles were also determined.

General Statistics

All statistical analyses were performed using SAS (SAS Institute, 1999). All percentage data (e.g., percentage of VLDL particles within the various VLDLy diameter ranges) were arc-sin transformed prior to analysis, however non-transformed percentages were used for graphical purposes. Non-normal variables, as assessed by the Shapiro-Wilk test for normality (Zar, 1996), were normalized through log10 transformation (although some non-transformed values were used for graphical purposes). T-tests were used for intra-specific comparisons of the VLDL particle diameter distributions of laying and non-laying females. When the analyses included variables that were still not normally distributed after log-transformation (e.g., plasma triacylglyceride and VLDL particle diameter distribution range of Zebra Finches, and modal and median VLDL particle diameter and VLDL particle diameter distribution range of chickens), non-parametric Wilcoxon rank-sum tests were performed. The influence of fasting and egg-food supplementation on VLDL particle diameter distribution was assessed using repeated measures ANOVA or ANCOVA (with female body mass as a covariate). If normality of distribution was achieved following data transformation, then the data were analyzed using a mixed model, repeated measures ANOVA or ANCOVA with fed-fasted state for the fasting study or diet for the egg-food supplementation study as a fixed, repeated factor, and individual bird as a random factor (PROC MIXED; SAS Institute, 1999). 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 birds as blocks that received both treatments (i.e., fed and fasted states or seed and egg-food supplemented seed diets) in a

randomized order (PROC FREQ; SAS Institute, 1999). All values are given as means ± standard error, all tests are two-tailed, and the overall significance level is $P < 0.05$ unless otherwise stated.

RESULTS

Influence of Feeding, Fasting and Egg-Food Supplementation on VLDL Particle Diameter Distribution in Zebra Finches

Breeding pairs consumed an average of 37% less seed while being fasted for part of the day than while having *ad libitum* access to seed throughout the day ($F_{1,13} = 119.66$, $p < 0.0001$; Figure 2.2a). This decrease in daily seed intake resulted in significant declines in female body mass (by an average of 0.44 g; $F_{1,13} = 7.21$, $p < 0.025$; Figure 2.2b) and circulating triglyceride levels (by an average of 1.6 mg / ml plasma, i.e., 14%; $F_{1,13} = 7.26$, $p < 0.025$; Figure 2.2c) following fasting. In contrast, fasting for 15-16 hours had no effect on the proportion of VLDL particles within the cVLDLy, sVLDLy (Figure 2.2d), or mVLDLy ranges, modal (Figure 2.2e) and median VLDL particle diameter, VLDL particle diameter distribution range (Figure 2.2f) or the proportion of very small (< 30 nm) or large (> 51 nm) VLDL particles in circulation ($p > 0.1$ in all cases).

When provided with the egg-food supplemented seed diet during breeding, male Zebra Finches actually lost an average of 13% of their body mass compared to when they were maintained on the seed-only diet as non-breeders ($F_{1,16} = 37.24$, $p < 0.0001$; Figure 2.3a). In contrast, egg-food supplementation during egg production did not influence the circulating triglyceride levels (Figure 2.3b), modal (Figure 2.3c) or median VLDL

particle diameter, VLDL particle diameter distribution range (Figure 2.3d), or the proportion of very small (< 30 nm) or large (> 51 nm) VLDL particles in circulation in breeding males ($p > 0.05$ in all cases).

Plasma Triacylglyceride and VLDL Particle Diameter Distribution in Non-Laying and Egg-Laying Zebra Finches

While laying and non-laying female Zebra Finches did not differ in body mass at the time of blood sampling ($p > 0.2$; Table 2.1), laying Zebra Finches had higher plasma triacylglyceride levels than non-laying females (Wilcoxon rank-sum test: $Z = -4.008$, $p < 0.0005$; Table 2.1). In contrast to the results from laying chickens (see Introduction), non-laying Zebra Finches had VLDL particle diameter distributions (Figure 2.4a) that were narrow (177 nm wide, cf. 233 nm in laying Zebra Finches; $Z = -1.980$, $p < 0.05$; Table 2.1) and peaked at very small particle diameters (over 55% of particles had diameters smaller than 30 nm, cf. less than 30% in laying Zebra Finches; $t = 5.867$, $df = 61.0$, $p > 0.0001$; Table 2.1) and contained few large particles (less than 10% of particles had diameters larger than 51 nm, cf., almost 20% in laying Zebra Finches; $t = 3.947$, $df = 59.2$, $p < 0.0005$; Table 2.1). Furthermore, non-laying Zebra Finches also had smaller modal ($t = 4.405$, $df = 61.0$, $p < 0.0001$) and median ($t = 5.332$, $df = 61.0$, $p < 0.0001$) VLDL particle diameters than laying females (Table 2.1). Therefore, in comparison, laying Zebra Finches had VLDL particle diameter distributions that were shifted towards *larger* VLDL particle diameters compared to non-laying females (Figure 2.4a). While there was considerable overlap between the diameter distributions of laying and non-laying Zebra Finches, laying females still had greater proportions of VLDL particles within the cVLDLy ($t = 2.866$, $df = 30.2$, $p < 0.05$) and sVLDLy ranges ($t = 3.058$, $df =$

31.0, $p < 0.005$; gray bar in Figure 2.4a) than non-laying females (Table 2.1). However, laying Zebra Finches had fewer VLDL particles within the mVLDLy range than non-laying birds ($t = 4.581$, $df = 61.0$, $p < 0.0001$; Table 2.1).

VLDL Particle Diameter Distribution in Non-Laying and Egg-Laying Chickens

Non-laying chickens were consistently different from laying chickens in all measures of VLDL particle diameter distribution. On average, VLDL particle diameter distributions of laying chickens at 29-weeks of age were narrow and peaked at small particle diameters (Figure 2.4b), while non-laying chickens possessed wider, less peaked distributions (Figure 2.4b) (range: $Z = 4.816$, $p < 0.0001$; Table 1). Laying chickens had a larger proportion of VLDL particles that fell within the cVLDLy ($t = 9.542$, $df = 10.3$, $p < 0.0001$; gray bar in Figure 2.4b) and sVLDLy ranges ($t = 8.909$, $df = 10.3$, $p < 0.0001$) than non-laying chickens (Table 2.1). Unlike in Zebra Finches, non-laying chickens had and larger modal ($Z = 3.818$, $p < 0.0001$) and median ($Z = 4.797$, $p < 0.0001$) particle diameters, fewer smaller VLDL particles (< 30 nm in diameter; $t = 25.990$, $df = 43.2$, $p < 0.0001$) and more large VLDL particles (> 51 nm in diameter; $t = 11.314$, $df = 9.1$, $p < 0.0001$) than laying chickens (Table 2.1).

DISCUSSION

Influence of Feeding, Fasting, Egg-Food Supplementation and Estradiol Treatment on VLDL Particle Diameter Distribution in Zebra Finches

While all of the chickens and Zebra Finches used in this study had *ad libitum* access to high quality food, the diet that laying Zebra Finches were provided with had a higher lipid content (4.7% lipid from seed and 13% from egg-food supplement) than the non-laying Zebra Finch (4.7% lipid from seed only diet) and the laying chicken diets (< 5% from corn-soy diet). To confirm that any differences observed in VLDL particle diameter distribution parameters between species and between reproductive stages in Zebra Finches were independent of differences in lipid intake or diet quality, we examined the influence of fasting and egg-food supplementation on various measures of VLDL particle diameter distribution in Zebra Finches. The various measures of VLDL particle diameter distribution did not differ with respect to the fed-fasted status of laying Zebra Finches. Likewise, male Zebra Finches exhibited comparable VLDL particle diameter distributions when maintained on the seed-only diet as non-breeders and when actively breeding on the egg-food supplemented seed diet. These findings suggest that these factors did not influence the VLDL particle diameter distribution parameters measured in this study.

Estimating VLDLy particle diameter range in Zebra Finches

Previous studies on egg production in domestic fowl have found that VLDL particles of different diameters vary in the extent to which they contribute to yolk formation, and consequently in their ability to support egg production (Perry and Gilbert,

1979; Griffin and Perry, 1985; Griffin and Hermier, 1988; Walzem, 1994; Walzem, 1996a; Walzem, 1999). To determine the range of VLDL particle diameters involved in egg production in passerine songbirds, we calculated three potential VLDLy particle diameter ranges based on previously reported positive relationships between VLDL particles of particular diameters and parameters associated with egg production in chickens and turkeys (Perry and Gilbert, 1979; Griffin and Perry, 1985; Griffin and Hermier, 1988; Walzem, 1994; Walzem, 1996a; Walzem, 1999), determined the proportion of VLDL particles that fell within each of the potential VLDLy diameter ranges in laying and non-laying Zebra Finches, and then compared these values to similar data from laying and non-laying chickens.

Basing VLDLy particle diameter range on the proposed "sieving" properties of the ovarian granulosa basal laminae of laying chickens and turkeys (Perry and Gilbert, 1979; Griffin and Perry, 1985; Griffin and Hermier, 1988; Walzem *et al.*, 1999) provided a comparable estimate of VLDLy particle diameter for Zebra Finches and chickens, as the proportion of VLDL particles from non-laying females that fell within this range was minimal in both species (only 38% within the sVLDLy range compared to 51% and 59% within the cVLDLy and mVLDLy ranges, respectively, for Zebra Finches, and 19% within the sVLDLy range versus 25% within the cVLDLy range for chickens). The majority of VLDL particles of laying chickens (61%) and half of the particles of laying Zebra Finches fell within the sVLDLy range. This suggests that similarities exist in the suggested sieving properties of the ovaries of different species of birds. The basal lamina of laying chicken follicles has been shown to be approximately 1 μm thick and contain type IV collagen, laminin, and the glycoprotein fibronectin (Griffin and Hermier, 1988;

Conkright and Asem, 1995; Rodgers *et al.*, 1999). Future studies are required to examine the composition, structure and sieving properties of the ovarian granulosa basal laminae of non-domesticated birds to determine whether ovarian sieving of VLDL particles plays a role in selection acting on VLDL particle diameter.

Basing the VLDLy range on Walzem's (1996) original correlation method (i.e., using VLDL particle diameter data from laying chickens and laying rate as the index of reproductive performance) also resulted in a comparable estimate of VLDLy particle diameter for Zebra Finches and chickens, as the majority of VLDL particles from laying females fell within the cVLDLy range (63% in Zebra Finches; 70% in chickens). The difference between laying and non-laying females in the proportion of VLDL particles that fell within a potential VLDLy range was maximized using this range (13% vs. -20% and 12% for the mVLDLy and sVLDLy ranges, respectively in Zebra Finches; 45% vs. 42% for the sVLDLy range in chickens). However, the majority of VLDL particles of non-laying Zebra Finches also fell within this range (51%, cf. only 25% of VLDL particles of non-laying chickens). Our modified version of Walzem's (1996) correlation method to detect associations between selected particle diameter classes and an index of reproductive performance using Zebra Finch particle diameter data and residual mean egg mass resulted in a VLDLy particle diameter range that encompassed a majority of VLDL particles from non-laying Zebra Finches (59% within the mVLDLy range, cf. 25% of VLDL particles from non-laying chickens within the cVLDLy range). Moreover, the mVLDLy range encompassed only 40% of VLDL particles from laying females (cf. 70% of VLDL particles of laying chickens within the cVLDLy range). The discrepancies between the proportion of VLDL particles that fell within the cVLDLy in chickens and

the mVLDLy in Zebra Finches may be due to differences in the way the cVLDLy and mVLDLy ranges were calculated. Many of the VLDL particle diameter classes that were positively associated with laying rate in chickens, and therefore made up the cVLDLy range, had statistically significant correlation coefficients ($p < 0.05$ for r-values greater than 0.444; Walzem, 1996a). In contrast, all of the VLDL particle diameter classes that were positively associated with residual mean egg mass in Zebra Finches, and therefore made up the mVLDLy range, lacked statistically significant correlation coefficients ($p > 0.1$ in all cases). Consequently, the mVLDLy range appears to be the least reliable estimate of VLDLy particle diameter range in Zebra Finches.

Metabolic shifts in VLDL particle diameter distribution

Previous studies on domestic chickens and turkeys have reported dramatic, near total shifts in VLDL particle diameter distribution during egg production from large particles (30 to >200 nm) in non-laying females to small particles (15 to 55 nm) in laying females (Walzem *et al.*, 1994; Walzem, 1996a; Speake *et al.*, 1998; Walzem *et al.*, 1999), resulting in very little overlap between the VLDL particle diameter distributions of non-laying and laying birds. Our study confirmed these differences in VLDL particle diameter distribution between non-laying and laying chickens, with less than10% of the VLDL particles of chickens at the peak of egg laying having diameters larger than 51 nm (cf. nearly 60% of particles in non-laying chickens).

As predicted, female Zebra Finches exhibited less dramatic shifts in lipid metabolism during egg production. However, this was mainly due to the unexpected finding that the majority of VLDL particles of non-laying Zebra Finches were very small in diameter (57% of particles had diameters less than 30 nm). Consequently, the diameter

distributions of laying Zebra Finches actually shifted towards *larger* VLDL particles compared to the distributions of non-laying females. However, the diameter distributions of both laying and non-laying Zebra Finches peaked at small VLDL particles, and therefore overlapped considerably. Similar results have been reported for comparisons between growing (i.e., immature) and egg-producing Tsaiya ducks, *Anas platyrhynchos domestica* (Lien *et al.*, 2005). When provided with *ad libitum* access to food, domesticated Tsaiya ducks had VLDL particle diameter distributions, as assessed by transmission electron microscopy, that included more larger particles (range: 50 to 75 nm) and exhibited larger mean VLDL particle diameters during egg-production at 30 weeks of age (61.57 ± 1.98 nm) than while actively growing at 12 weeks of age (range: 35 to 60 nm; mean diameter: 47.67 ± 2.37 nm) (Lien *et al.*, 2005). Furthermore, similar patterns of VLDL particle diameter distribution have been found in free-living Greater Scaup (*Aythya marila*), wherein egg-laying birds sampled on the breeding grounds in Alaska exhibited larger modal VLDL particle diameters than pre-breeding birds sampled earlier in the breeding season, as assess by dynamic laser light scattering (K. Gorman, D. Esler, R.L. Walzem and T.D. Williams, unpublished data).

Laying Zebra Finches, like laying chickens, had higher circulating triacylglyceride levels and more particles within the sVLDLy and cVLDLy ranges than non-laying females, despite the fact that laying Zebra Finches had fewer very small VLDL particles, more large VLDL particles, and wider diameter distributions than non-laying females. Furthermore, the VLDL particle diameter distributions of Zebra Finches and chickens shifted towards similar modal and median VLDL particle diameters during egg production. These results suggest that, regardless of the direction that VLDL particle

diameter distributions have to shift, specific changes in lipid metabolism (e.g., increased lipid production and maintenance of a large proportion of small VLDL particles of specific diameters) may be essential for egg production in both domesticated and non-domesticated birds. However, data on reproductive status and VLDL particle diameter distribution from more domesticated and free-living avian species are required to confirm the relationship between changes in lipid metabolism and avian egg production.

The differences in VLDL particle diameter distribution between non-laying chickens and Zebra Finches observed in this study may be due to differences in rates of lipid turnover due to variation in metabolic rate. Based on allometric scaling of metabolic rate (for reviews see Calder, 1981; Taylor, 1987), smaller passerine songbirds have higher mass-specific metabolic rates than larger chickens (Lasiewski and Dawson, 1967; Reynolds and Lee, 1996; McKechnie and Wolf, 2004). Consequently, passerine songbirds also have higher rates of lipid turnover. When VLDL particles undergo lipoprotein lipase-mediated metabolism, triacylglycerol is removed by hydrolysis, and surface lipids and apolipoproteins (e.g., apo-A, apo-C, and in mammals, apo-E) are transferred to other lipoprotein particles (e.g., high density lipoproteins, HDL) (for reviews see Eisenberg, 1986; Walzem, 1996a). As their core triacylglyceride and surface lipid and protein content decreases, the proportional weight of other lipids (e.g., cholesterol esters) increases, and VLDL particles decrease in size and increase in density, and are eventually converted to intermediate density lipoproteins (IDL) and then low density lipoproteins (LDL) (for reviews see Eisenberg, 1986; Walzem, 1996a). Therefore, the abundance of very small VLDL particles in non-laying Zebra Finches (57% under 30 nm in diameter, cf. less than 1% in non-laying chickens) may be due to the more rapid

metabolism of larger VLDL particles. Hermier *et al.* (1985) reported that IDL particles from immature chickens had an average diameter of 20.0 nm, as assessed by gradient gel electrophoresis. Therefore, the very small VLDL particles from the non-laying Zebra Finches in this study may have been IDL particles resulting from the metabolism of generic VLDL particles.

Differences in the selective pressures acting on different avian species may contribute to the inter-specific differences in the presence of large VLDL particles in circulation during egg production observed between chickens in this study (less than 10% of particles were > 51 nm in diameter) and both the Zebra Finches in this study (19%), and domesticated Tsaiya ducks (~100%) (Lien *et al.*, 2005). Domesticated fowl, specifically egg-type chickens, have been under heavy artificial selection for continuous and consistent egg production, resulting in birds that can maintain egg production for over a year (Etches, 1996); the laying chickens sampled at week 29 in this study laid continuously until week 86, and in some cases even beyond (cf., ~1 week of laying in Zebra Finches). Walzem (1996) found that laying rate in Single Comb White Leghorn chickens and Nicholas White turkeys was related to the presence of large VLDL particles in circulation during egg production. Laying females of both species that had low rates of laying (i.e., < 7 eggs laid within a 10-day period) had a larger proportion of VLDL particles with diameters greater than 51 nm than laying females with high rates of egg production (i.e., 7 or more eggs laid within 10 days) (Walzem, 1996a). These results suggest that limiting the proportion of larger VLDL particles in circulation during egg production, or maybe more importantly, tightly regulating VLDL particle diameter such

that the majority of particles fall within the cVLDLy or sVLDLy diameter ranges, is critical for continuous egg production in chickens.

Additionally, differences in the environmental conditions that chickens and Zebra Finches are exposed to during egg production may also have an influence on the proportion of large VLDL particles in circulation. Domestic chickens are generally housed under conditions that promote optimal egg production, e.g., light-controlled facilities, a diet regime tailored for egg production, vaccinations against disease, and husbandry practices that eliminate parasites (Etches, 1996). Consequently, they are capable of meeting their own metabolic needs via hydrolysis of the small VLDLy particles (Bacon and Musser, 1977; Bacon *et al.*, 1978; Bacon, 1981) and possibly renal generic VLDL (Walzem *et al.*, 1999) despite the increased resistance of VLDLy to hydrolysis by lipoprotein lipase (Bacon *et al.*, 1978; Bacon, 1981; Griffin *et al.*, 1982; Hermier *et al.*, 1989; Schneider *et al.*, 1990; Walzem *et al.*, 1994; Walzem, 1996a). The extent to which hepatic VLDLy and renal generic VLDL contribute to the levels of utilizable VLDL present in laying, non-gallinaceous birds, such as Zebra Finches, and in particular, in free-living birds faced with far less predictable breeding conditions, remains unknown. Alternatively, previous studies on laying chickens have suggested that individual hepatocytes may vary in their functional capacity to initiate apoVLDL-II, and thus VLDLy, synthesis in response to elevated levels of estrogen (Lin and Chan, 1981; Lin *et al.*, 1986). Concentrations of specific lipoprotein species present in plasma are a function of both secretion and utilization. Therefore, it is possible that the livers of laying chickens continue to make small amounts of larger, generic VLDL in sufficient quantities to meet the laying females' energetic requirements. Rapid and continuous metabolism of

such generic VLDL by highly productive layers would leave minimal concentrations in circulation relative to VLDLy, making their ready detection in laying domestic fowl challenging. Future studies are needed to assess whether the larger VLDL particles observed in laying Zebra Finches contain apoVLDL-II in order to determine whether these particles are generic or yolk-targeted VLDL. Regardless of the source of energy for the laying chickens (i.e., generic vs. yolk-targeted VLDL), dramatically shifting VLDL particle diameter distribution towards smaller particles that are capable of entering the developing ovarian follicles enables chickens to allocate the majority of their lipid resources to egg production and maintain egg production for prolonged periods of time without compromising the their own energetic needs.

In contrast to domesticated birds that have undergone directional selection for specific traits, such as continuous egg production or rapid growth, the selective pressures on laying Zebra Finches, and on non-domesticated birds in general, are generally focused on maintaining traits that maximize the trade-off between current reproductive effort and future fecundity and survival (Williams, 1966; Stearns, 1992; Bernardo, 1996). Reproduction in free-living, non-domesticated birds is generally timed to ensure that the period of chick-rearing coincides with the period of peak food abundance (Perrins, 1970), and egg production often occurs during a period of lower food availability and unpredictable environmental conditions earlier in the breeding season (Williams, 1998). Therefore, the increased LPL-resistance of VLDLy may result in selection for the maintenance of larger, potentially generic, VLDL particles in non-domesticated birds, as observed in laying Zebra Finches in this study and Tsaiya ducks (Lien *et al.*, 2005), to ensure that females have an ample supply of VLDL that can be metabolized in case their

own energetic demands increase during egg production due to rapid onset of inclement weather, declines in food availability, disease, infestation by parasites, or other energetically-costly phenomena. Data on VLDL particle diameter distribution during egg production in many more free-living avian species, including other gallinaceous and passerine birds, are needed to determine whether the differences between chickens and Zebra Finches observed in this study are, in fact, due to differences in selective pressures on these birds, or to phylogenetic differences that are unrelated to inter-specific differences in adaptations to egg production.

Reproduction in non-domesticated species generally involves broody behaviour, i.e., incubation and post-hatching parental care (e.g., provisioning and brooding of young), in addition to egg production. This is in contrast to many breeds of domesticated chickens, whose reproductive activity is limited to egg production as a result of commercial practices (e.g., photoperiod manipulation, egg removal) and a decrease in broodiness, hatchability and fertility as an indirect consequence of selection for increased egg production or, in the case of decreased broodiness, as a result of direct selection for reducing broodiness; Emmerson *et al.*, 1991; Nestor *et al.*, 1996; Sewalem *et al.*, 1998; reviewed in Romanov, 2001). Therefore, given that non-domesticated, laying females must ensure that they have adequate resources to perform post-laying parental behaviors, they may limit lipid allocation to current egg production in exchange for allocating more energy towards self-maintenance (i.e., maintaining larger VLDL particles) to ensure their developing offspring have adequate resources, while also enhancing their chances for survival through the current reproductive period and beyond, thus maximizing current and potentially future reproductive effort. Future studies are needed that assess the

relationships between variation in VLDL particle diameter distribution during egg production in free-living avian species and both current and future reproductive success, and maternal survival and longevity.

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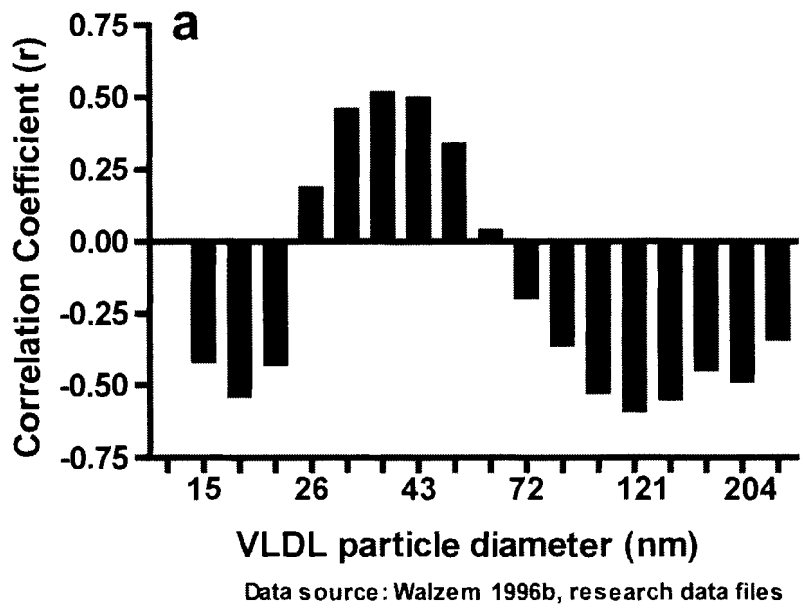
Table 2.1. Total plasma triacylglyceride and measures of VLDL particle diameter distribution for non-laying and laying female Zebra Finches and chickens. Values are means ± SE, with sample size in parentheses. All percentages are arc-sin transformed. * indicates $P < 0.05$, ** $P < 0>005$, *** $P < 0.0005$, and **** $P < 0.0001$ for intra-specific comparisons.

Trait	Non-laying Zebra Finches	Laying Zebra Finches	Non-laying chickens	Laying chickens
Mass at blood sampling (g)	15.83 ± 0.31 (27)	16.11 ± 0.21 (36)	--	--
Total plasma triacylglyceride (mg / ml plasma)	7.22 ± 1.12 (26)	18.05 ± 3.87 (33) ***	--	--
Percentage of particles within the mVLDLy range (10.7 - 30.4 nm)	59.33 ± 3.48 (27)	39.57 ± 2.67 (36) ****	--	--
Percentage of particles within the cVLDLy range (21.5 - 51.1 nm)	50.77 ± 4.27 (27)	63.47 ± 1.20 (36) *	25.14 ± 4.51 (10)	69.66 ± 1.21 (37) ****
Percentage of particles within the sVLDLy range (25 - 44 nm)	37.96 ± 3.74 (27)	49.95 ± 1.16 (36) **	18.89 ± 4.54 (10)	60.77 ± 1.23 (37) ****
Modal VLDL particle diameter (nm)	22.0 ± 1.4 (27)	29.7 ± 1.1 (36) ****	55.6 ± 9.0 (10)	29.7 ± 0.6 (37) ****
Median VLDL particle diameter (nm)	22.9 ± 1.5 (27)	32.7 ± 1.1 (36) ****	68.0 ± 5.1 (10)	29.6 ± 0.4 (37) ****
VLDL particle diameter distribution range (nm)	177.1 ± 17.0 (27)	232.9 ± 19.0 (36) *	329.5 ± 15.4 (10)	76.1 ± 6.7 (37) ****
Percentage of particles with diameters smaller than 30 nm	57.20 ± 4.17 (27)	27.81 ± 3.01 (36) ****	0.51 ± 0.51 (10)	38.64 ± 1.38 (37) ****
Percentage of particles with diameters larger than 51 nm	9.65 ± 1.33 (27)	18.64 ± 1.85 (36) ***	59.09 ± 4.39 (10)	9.19 ± 0.34 (37) ****

Figure 2.1. Histogram plots of correlation coefficients, r, for specific particle diameter classes of VLDL isolated from the plasma of a) laying chickens (data source: Walzem 1996b), and b) laying Zebra Finches. Correlation coefficients were generated from correlations between the proportion of VLDL particles within each diameter class and subsequent egg production (i.e., laying rate in chickens and mean egg mass in Zebra Finches). VLDL particle diameter classes with $r > 0$ were positively associated with egg production, and were therefore included in estimates of yolk-targeted VLDL (VLDLy) particle diameter range.



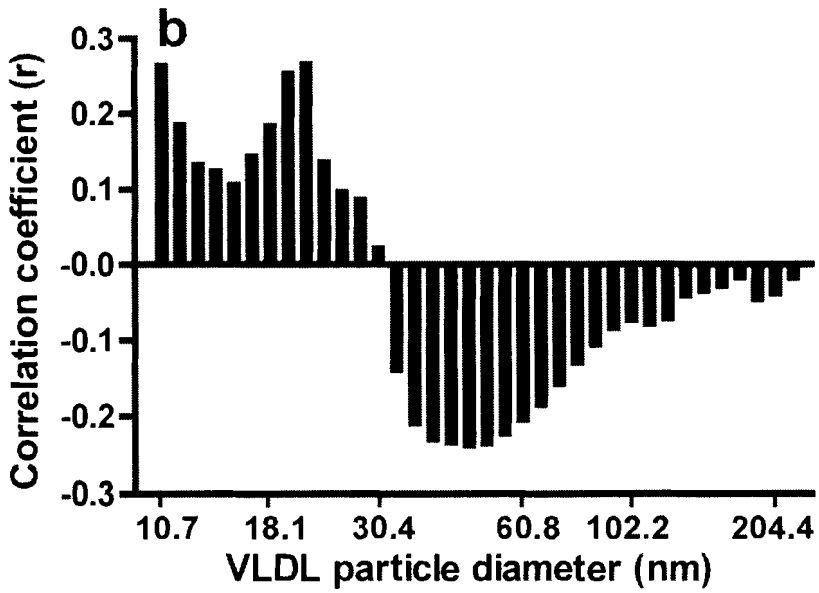


Figure 2.2. Influence of fasting on a) daily seed consumption of breeding pairs of Zebra Finches, and on b) body mass, c) plasma triacylglyceride levels, d) the proportion of VLDL particles available for use by the developing ovarian follicles as defined by the proposed selective sieving properties of the ovary, i.e., the proportion of particles that fell within the sVLDLy diameter range (25 to 44 nm), e) modal VLDL particle diameter, and f) VLDL particle diameter distribution range of laying female Zebra Finches.

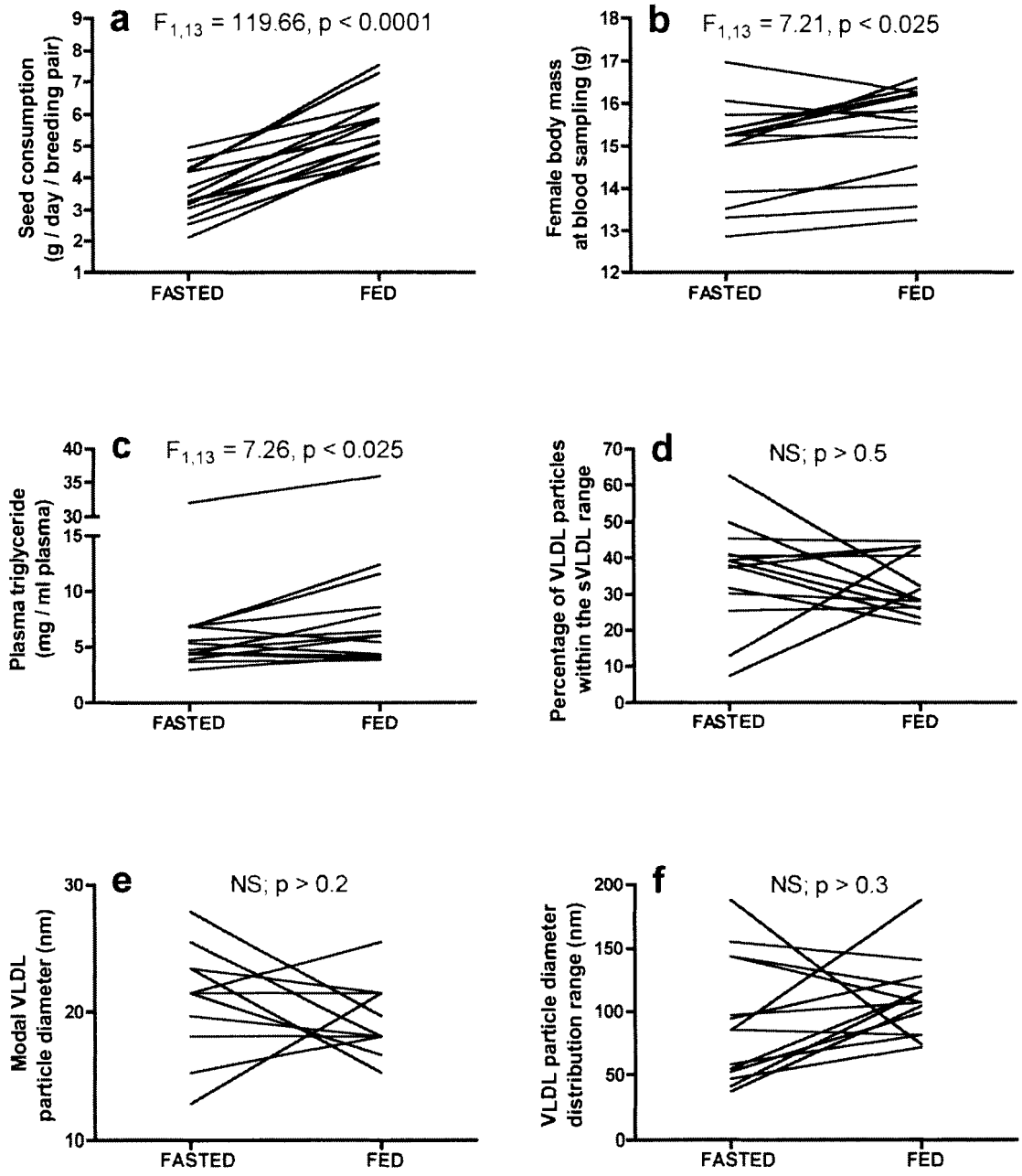


Figure 2.3. The effects of egg-food supplementation and reproductive activity on a) body mass, b) plasma triacylglyceride levels, c) modal VLDL particle diameter and d) VLDL particle diameter distribution range of male Zebra Finches.

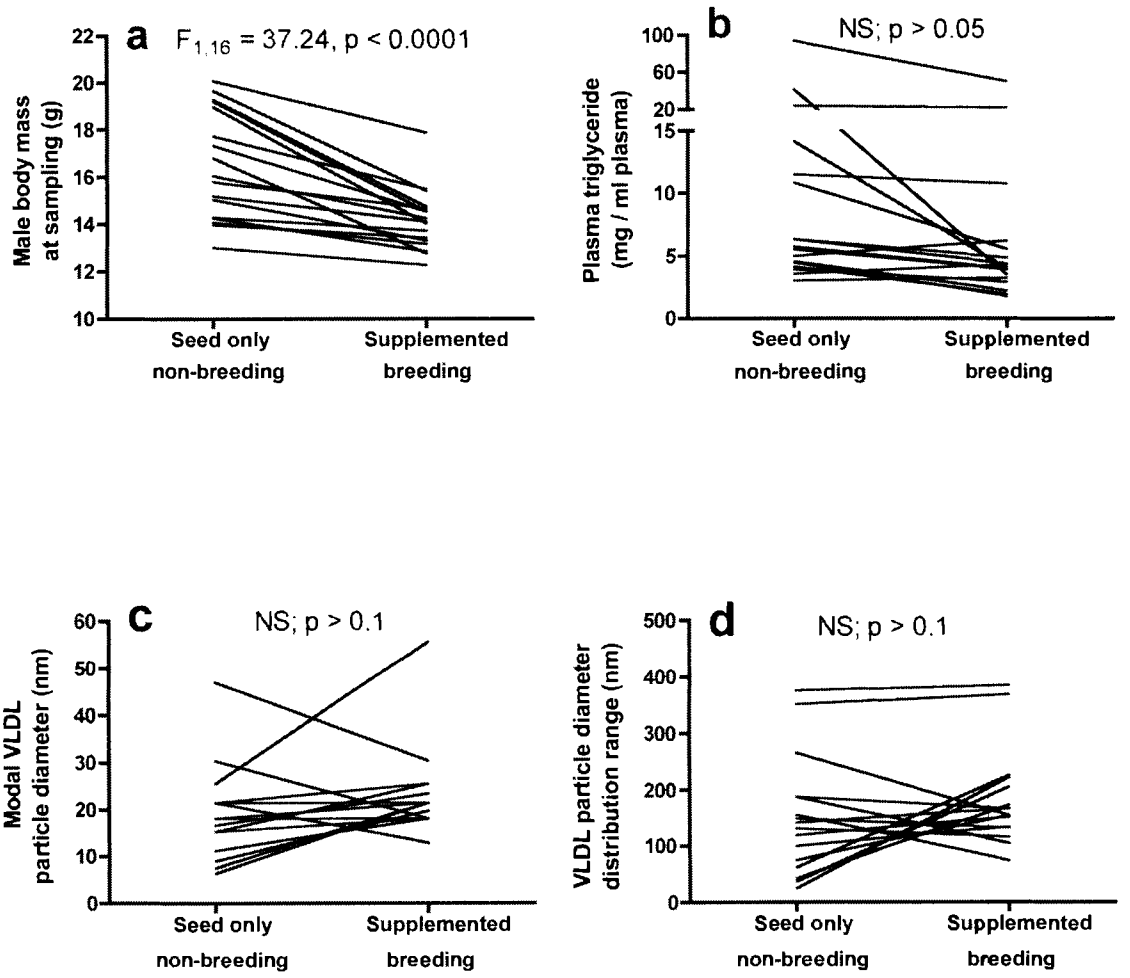
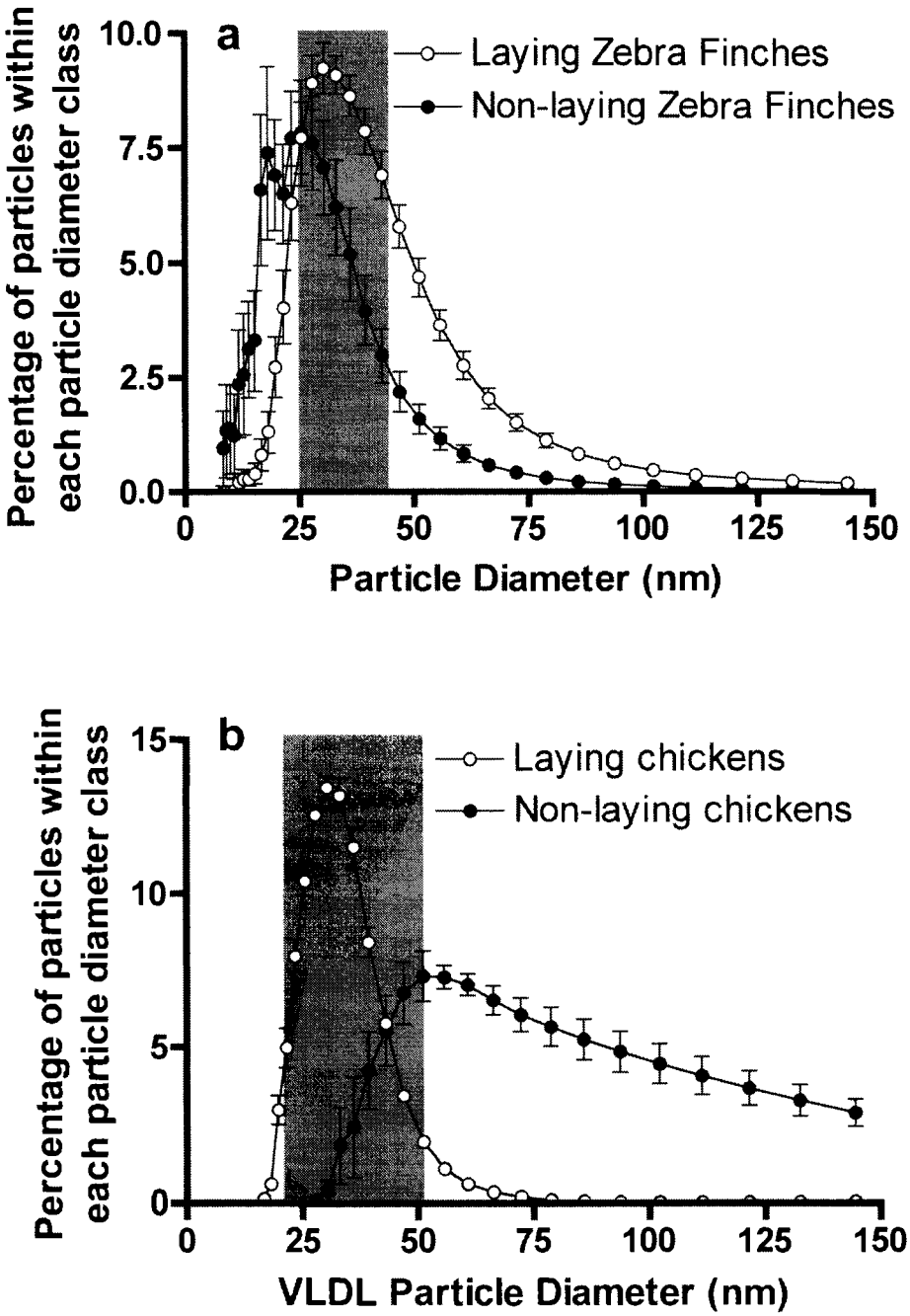


Figure 2.4. VLDL particle diameter distributions of laying and non-laying female a) Zebra Finches and b) chickens. Gray bars indicate estimates of yolk-targeted VLDL (VLDLy) particle diameter range based on a) the proposed sieving properties of the ovary limiting access of VLDL particles to the developing ovarian follicles (sVLDLy range: 25 – 44 nm) or b) the idea that VLDL particle diameter classes exhibiting positive relationships with laying rate in chickens are better able to support continuous egg production (cVLDLy range: 21.5 – 51.1).



CHAPTER 3

INTER-INDIVIDUAL VARIATION AND REPEATABILITY OF VLDL PARTICLE DIAMETER DISTRIBUTION IN EGG-LAYING BIRDS: RELATIONSHIPS WITH REPRODUCTIVE EFFORT

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SUMMARY

Relationships between inter-individual variation in VLDL particle diameter distribution parameters and subsequent reproductive performance were evaluated using the Zebra Finch (*Taeniopygia guttata*), and these findings were compared to those for laying chickens (*Gallus gallus domesticus*). Despite the marked inter-individual variation observed in circulating triglyceride levels in laying Zebra Finches (35-fold), and the less-marked variation in the measures of reproductive effort (ranging from 1.7-fold variation in mean egg mass and laying rate to 4.9-fold variation in total clutch mass) and VLDL particle diameter distribution (ranging from 2-fold variation in the percentage of VLDL particles available for use by the developing ovarian follicles for egg yolk formation as defined by the proposed selective sieving properties of the ovary (i.e., the proportion of particles that fall between 25 and 44 nm in diameter, the sVLDLy diameter range) to 15-fold variation in VLDL particle diameter distribution range), reproductive performance in Zebra Finches was not related to any measure of VLDL, with the exception of the positive relationship between clutch size and circulating triglyceride levels in females that consistently laid large eggs. Laying chickens generally exhibited comparable inter-individual variation in reproductive traits (1.5-fold variation in mean egg mass and 2.5-fold variation in laying rate) and measures of VLDL diameter distribution to laying Zebra Finches (ranging from 1.4-fold variation in the proportion of particles that fell within the sVLDLy range to 10-fold variation in VLDL particle diameter distribution range). However, in contrast to Zebra Finches, variation in reproductive performance in chickens

was related to the proportion of VLDL particles available for use in egg yolk formation. 'Good' layers that laid 7 or more eggs within a 10-day period had a significantly larger proportion of circulating sVLDLy particles than 'poor' layers that laid fewer than 7 eggs in the same time period. Furthermore laying rate, the primary measure of reproductive performance in chickens, was positively related to the proportion of sVLDLy particles in circulation at week 86, when chickens exhibited over 2-fold variation in laying rate. This suggests that the production and maintenance of many circulating small VLDL particles that are capable of being incorporated into developing egg yolks may be important for sustaining continuous laying over prolonged periods in domestic fowl, but may not be important factors in determining reproductive performance in passerine songbirds that lay discreet clutches of eggs like the Zebra Finch.

INTRODUCTION

Marked intra-specific variation in reproductive effort had been widely observed in all vertebrate taxa (e.g., variation in egg and clutch size in oviparous species and litter size and birth weight in mammals). In birds, differences between individuals in laying date, clutch size and egg size have been shown to be related to variation in components of fitness such as survival of parents and offspring and fecundity (laying date: Boyce and Perrins, 1987; clutch size: Rowe *et al.*, 1994; egg size: Williams, 1994). While some of the variation in reproductive effort can be attributed to differential responses of individuals to exogenous factors, like territory quality, temperature, food availability, and social cues (Goodburn, 1991; Yom-Tov and Wright, 1993; Giuliano *et al.*, 1996; Lessells *et al.*, 2002), it is likely that variation in physiological factors, such as the structure and functional capacity of the reproductive system itself, also contribute to inter-individual variation in reproductive effort in addition to providing the mechanism through which exogenous factors influence the reproductive system.

During avian egg production, increasing levels of endogenous estrogen induce the hepatic synthesis of two egg yolk precursors, vitellogenin (VTG) and yolk-targeted, very-low density lipoprotein (VLDLy) (Gruber, 1972; Bergink *et al.*, 1974; Deeley *et al.*, 1975; Neilson and Simpson, 1973; Chan *et al.*, 1976; Wallace, 1985; Walzem, 1996; Williams, 1998). These protein- and lipid-rich compounds provide the nutrients and energy for developing avian embryos. The presence of circulating VLDLy represents a functional shift in lipid dynamics from the allocation of energy-rich lipids towards self-

maintenance to the lipid allocation to reproduction. This occurs through changes in VLDL structure and function from generic very-low density lipoprotein (VLDL), which transports lipids to various tissues (e.g., muscle, adipose) for utilization or storage, to VLDLy, which is chemically and structurally altered to increase its resistance to utilization by peripheral tissues and facilitate its incorporation into egg yolk (Chan *et al.*, 1976; Kudzma *et al.*, 1979; Griffin, 1981; Dashti *et al.*, 1983; Griffin and Perry, 1985; Lin *et al.*, 1986; Schneider *et al.*, 1990; Walzem *et al.*, 1994; Walzem, 1996; Speake *et al.*, 1998; Walzem *et al.*, 1999).

Variation in reproductive performance in domestic fowl has been linked to differences in circulating levels of the yolk precursors. Redshaw and Follett (1976) found that laying chickens (*Gallus gallus domesticus*) with irregular laying patterns had higher and more varied concentrations of plasma VTG than chickens that laid regularly (i.e., one egg per day). The elevated VTG levels observed in these irregular layers on the days when laying was skipped may have been due to slow rates of precursor uptake by the developing follicles since uptake is proportional to follicle size (Christians and Williams, 2001a), resulting in follicles that needed more time (i.e., an extra day) to develop and a larger plasma pool of VTG. In contrast, circulating VTG levels were only weakly, negatively associated with measures of reproductive output in free-living European Starlings (*Sturnus vulgaris*) (Christians and Williams, 1997; Challenger *et al.*, 2001; Christians and Williams, 2001b), and were either not related to reproductive effort in captive Zebra Finches (*Taeniopygia guttata*) or exhibited a weak, diet-dependent relationship with egg size (Christians and Williams, 1997; Salvante and Williams, 2002). These findings suggest that while circulating VTG levels play a role in the regulation of

egg production in passerine songbirds, other physiological factors are still needed to explain the marked inter-individual variation observed in the various measures of reproductive effort. Inter-individual variation in laying rate in chickens and domestic turkeys (*Meleagris gallopavo*) has also been associated with variation in VLDL particle diameter distribution, specifically in the proportion of VLDL particles that are associated with lipid deposition into developing eggs (see Figure 5 in Walzem, 1996). Comparable studies have not been conducted on non-domesticated species, so it is unclear whether the relationship between variation in VLDL particle diameter distribution and reproductive performance is common across avian species. The robust relationships between circulating levels of the yolk precursors and subsequent reproductive performance in domestic fowl are the result of artificial selection on these birds for prolonged, maximized egg production. Therefore, it is possible that species that have not been the targets of such focused selection do not exhibit such clear relationships between reproductive effort and yolk precursors. Consequently, while maintaining a particular proportion of VLDL particles associated with lipid deposition into developing eggs plays a critical role in the ability to maintain continuous egg production in domesticated avian species, it may be of less importance in the regulation of reproduction in non-domesticated species that exhibit marked inter-individual variation in egg size, but little variation in laying rate, and produce discreet clutches of eggs.

Here we assessed the extent of inter-individual variation in VLDL particle diameter distribution in laying Zebra Finches and chickens using a dynamic laser scattering technique that provides a frequency distribution of VLDL particle diameters (in nm) (Walzem *et al.*, 1994; Walzem, 1996; Walzem *et al.*, 1999; Peebles *et al.*, 2004).

We then determined whether the variation observed in VLDL particle diameter distribution was related to reproductive performance by comparing the VLDL particle diameter distributions of "good" and "poor" layers. For the Zebra Finches we compared females that consistently laid large eggs ("good") to females that consistently laid small eggs ("poor"), and for the chickens we compared females that exhibited high laying rates (i.e., 7+ eggs in 10 days; "good") to females that exhibited lower rates of egg laying (i.e., < 7 egg in 10 days; "poor"). Chickens have been the target of strong artificial selection for consistent and continuous egg production over prolonged periods of time (Etches, 1996), and since selection for maximum egg production in domestic fowl has also reduced phenotypic variance in reproductive traits (Williams, 1998; Fulton and Delaney, 2003), we predicted that there would be greater inter-individual variation in the VLDL particle diameter distributions of non-domesticated, egg-laying birds compared to laying chickens. Furthermore, because the reproductive physiology of the domestic chicken has been fine-tuned by generations of selection to maximize egg production, we also predicted chickens would exhibit more robust relationships between reproductive performance and VLDL particle diameter distribution during egg production than non-domesticated birds. In order for a trait to respond to selection it must be repeatable within an individual, have a heritable basis, and exhibit fitness-related variation (Bennett, 1987; Stearns, 1992; Garland and Carter, 1994). Therefore, to investigate whether VLDL particle diameter distribution parameters and circulating triglyceride levels are characteristics of individual females that may be able to respond to selection, we assessed the extent of repeatability (i.e. intra-individual variation between breeding attempts) of these traits.

MATERIALS AND METHODS

Animal Husbandry

Zebra Finches were housed in the Simon Fraser University Animal Care Facility under controlled environmental conditions (temperature 19-23°C, humidity 35-55%, constant light schedule of 14L: 10D, lights on at 07:00). All birds received a mixed seed diet (Panicum and white millet, 50:50; approximately 12.0% protein, 4.7% lipid; Jameson's Pet Food, Vancouver, and Just for Birds, Surrey), water, grit, and cuttlefish bone (calcium) *ad libitum*. Birds also received a multivitamin supplement in the drinking water once per week. When not paired for breeding, the birds were housed in same-sex cages, but were not visually or acoustically isolated from the opposite sex. Artificial selection for specific traits has never been performed on this breeding colony. However, inadvertent selection on reproductive performance may have occurred over the many generations these birds have spent in captivity. All experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit (no. 558B) following the guidelines of the Canadian Committee on Animal Care.

Single comb White leghorn chickens of the W-36 strain (Hy-Line International) were individually housed in light-supplemented, fan-ventilated, open-sided houses at Texas A&M University. Chickens were given *ad libitum* access to water and a corn-soy diet formulated to meet the National Research Council for Poultry's (NRC) requirements for laying hens (15% protein, < 5% lipid, 2900 Kcal/kg; NRC Subcommittee on Poultry Nutrition, 1994), and were provided with 15 hours of light per day. Ambient house temperature varied from 45°F to 85°F. All animal husbandry and experimental procedures

were conducted by technicians from the Walzem laboratory in accordance with a protocol approved by the Animal Use and Care Committee of Texas A&M University.

Reproductive Effort and VLDL Particle Diameter Distribution in Zebra Finches

Six years of breeding data from our captive population of Zebra Finches were used to determine the range of mean egg masses that fell within the first (0.815 g to 1.020 g) and fourth (1.161 g to 1.511 g) quartiles for the population. Small-egg females (mean egg mass < 1.020 g; n = 16) and large-egg females (mean egg mass > 1.161 g; n = 16) were randomly paired twice with an experienced male with at least 28 days between each breeding attempt. Males and females were weighed (± 0.1 g) at the time of pairing. Breeding pairs were housed individually in cages (61 x 46 x 41 cm) equipped with an external nest box (15 x 14.5 x 20 cm) and were provided with an egg-food supplement (6 g of a mixture of 62-65 g hard-boiled egg, 13 g cornmeal, 13 g bread crumbs; 30.2% protein and 13.0% lipid by dry mass) daily between pairing and clutch completion in addition to the normal seed diet (see Williams, 1996a). Data on laying interval (days from pairing to initiation of laying) and egg and clutch size were obtained by checking the nest boxes daily between 09:00 and 11:00. All new eggs were weighed (to 0.001 g) and numbered on the day they were laid. Clutches were considered complete if no new eggs were laid over two days. At this time the female was weighed (± 0.1 g), and the pair was returned to non-breeding cages. Laying rate was calculated as the number of eggs laid divided by the total number of days it took to lay the entire clutch.

Female Zebra Finches were weighed and blood sampled (200 µl from the brachial vein) between 09:30 and 11:30 on the day their first eggs were laid (1-egg stage). All

blood samples were collected into heparinized capillary tubes, expelled into EDTA-coated microcentrifuge tubes containing 0.5 M disodium-EDTA (3 µl; Sigma-Aldrich Canada, Oakville), and centrifuged at 2200 *g* for 10 minutes in a Baxter Canlab Biofuge 13. A sub-sample (5 µl) of each resulting plasma sample was frozen (-20° C) for plasma triglyceride analysis, while the remainder of each plasma sample was placed into an EDTA-coated microcentrifuge tube containing 0.5 M disodium-EDTA (5 µl) for VLDL particle diameter distribution analysis. Sodium azide (1% w:v; Sigma-Aldrich Canada, Oakville) was added to each EDTA-coated tube to prevent mold formation (0.01 µl / µl plasma), and the plasma samples were refrigerated (4° C) pending analysis of VLDL particle diameter distribution.

Reproductive Effort and VLDL Particle Diameter Distribution in Chickens

Laying chickens were checked daily between 09:00 and 11:00, and all new eggs were weighed (to 0.01 g) on the day they were laid. Laying rate was calculated as the number of eggs laid per day during the 10-day period following blood sampling. Two groups of egg-laying chickens were blood sampled: actively laying females at 29 weeks of age (n = 16) and 86 weeks of age (n = 21). Sampling of the 29-week old layers coincided with the peak of laying for the population (i.e., all females were actively laying eggs and the laying rate for the population was at its peak: 0.9 eggs laid per day). In contrast, the blood sample taken at week 86 coincided with a significant decrease in the population's laying rate (0.6 eggs laid per day; Wilcoxon signed rank test comparing population laying rate between weeks 29 and 86: $S = 1899.0$, $n = 87$, $p < 0.0001$). Blood samples were taken from the brachial vein between 09:00 and 11:00 into EDTA-coated Vacutainer tubes (BD Diagnostics, Franklin Lakes). Plasma samples were isolated

following the method described above and refrigerated (4° C) pending analysis of VLDL particle diameter distribution.

Plasma Triglyceride Assay

Circulating concentrations of triacylglyceride were measured enzymatically as an index of total plasma VLDL (i.e., generic VLDL and VLDLy) (Triglyceride E kit – Wako Chemicals, Richmond; Serum Triglyceride Determination Kit, Sigma-Aldrich Canada, Oakville) using the method 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- and inter-assay coefficients of variation were 1.85% (n = 6 replicates) and 4.98% (n = 8 assays), respectively, using a 19-week chicken plasma pool. All assays were run using 96-well microplates and were measured at 540 nm using a Biotek 340i microplate reader.

VLDL Particle Diameter Distribution Assay

Plasma VLDL Isolation and Dynamic Laser Light Scattering

Plasma VLDL was isolated as the $d < 1.020$ g/mL fraction of plasma. The volume of each Zebra Finch plasma sample (approximately 100 µl) was measured and transferred into Beckman Ultra-Clear ultracentrifuge tubes (13 x 64 mm, #344088; Beckman Coulter, Fullerton), and NaCl density solution (d = 1.0063; equivalent salt density of undiluted plasma) was added to each tube until a final volume of 1 ml was reached. Alternatively, a sub-sample (1 ml) from each chicken plasma sample was transferred into ultracentrifuge tubes. NaCl-NaBr density solution (5 ml; $d = 1.0255$) was then added to each tube. A blank sample was prepared by combining NaCl density solution (1 ml; $d =$

1.0063) with NaCl-NaBr density solution (5 ml; d = 1.0255) in an ultracentrifuge tube. The samples were loaded into a Beckman 50.4 fixed-angle rotor and centrifuged at 148600 *g* for 18 hours at 14°C in a Beckman L8-70M ultracentrifuge (Beckman Coulter, Fullerton). Following centrifugation, the supernatant containing the VLDL portion of the plasma was isolated from each tube by aspiration with 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 a UPA 250 and 7.02 analysis software (Microtrac, Clearwater) (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 minutes for the blank solution, and 5 minutes 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 to 44 nm in diameter), which was based on the proposed sieving properties of the ovarian granulosa basal lamina of domestic fowl. To reach the plasma membranes of the developing ovarian follicles, VLDL particles must pass from capillaries within the ovary through pores in the ovarian granulosa basal lamina (Perry and Gilbert, 1979; Griffin and Perry, 1985). Only particles ranging from 25 to 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 (see Chapter 2; K.G. Salvante, unpublished data). Additionally, the modal and median particle diameter and the range (i.e., width) of each distribution, in nanometers, were also determined.

Statistical Analysis

All statistical analyses were performed using SAS (SAS Institute, 1999). All data were tested for normality of distribution (Shapiro-Wilk test; Zar, 1996), and non-normal variables were log10 or arc-sin transformed prior to analysis. All values are given as means ± standard error, all tests are two-tailed, and the overall significance level is $P < 0.05$.

Nested ANOVA was used to determine the repeatability of measures of circulating VLDL and reproductive effort of laying Zebra Finches following Lessells and Boag (1987). In these analyses the two values corresponding to the repeated trait of interest during the two breeding bouts were nested within individual female. Repeatability is reported as the proportion of the variation in the trait of interest that is explained by variation between individual females.

The influence of female body mass on various measures of circulating VLDL (plasma triglyceride levels, percentage of VLDL particles within the sVLDLy range, modal and median VLDL particle diameter, etc.) and reproductive traits (egg mass, clutch size, total clutch mass, etc.) were examined in Zebra Finches by regression of the trait values against female body mass at the 1-egg stage. If normality of distribution was achieved following data transformation, then the data were analyzed using t-tests to compare large-egg with small-egg female Zebra Finches, and laying chickens at week 29 with laying chickens sampled at week 86, and ANOVA to assess the relationships between the various measures of VLDL and reproductive performance. In contrast, non-parametric tests (Spearman rank correlation, Wilcoxon rank-sum test, and Kruskal-Wallis test) were performed when the analyses included variables that were still not normally distributed following data transformation. Laying chickens sampled at week 86 were divided into two groups based on their laying rate: 'good' layers ($n = 14$), which laid 7 or more eggs in the 10-day period following bloos sampling (i.e., laying rate ≥ 0.7 eggs per day), and 'poor' layers ($n = 7$), which laid fewer than 7 eggs in the same 10-day period.

RESULTS

Individual Variation and Repeatability of VLDL Particle Diameter Distribution and Reproductive Effort in Laying Zebra Finches

Circulating levels of plasma triglyceride varied 35-fold from 3.215 to 112.000 mg triglyceride/ml plasma in laying female Zebra Finches at the 1-egg stage. The VLDL particle diameter distributions of individual laying Zebra Finches ranged from very narrow distributions (minimum range = 26.3 nm) to very broad distributions (maximum range = 389.1 nm), a 15-fold difference in particle diameter range (Figure 3.1a). In contrast, other measures of VLDL particle diameter distribution exhibited less marked variation; modal and median VLDL particle diameter varied 2.4-fold from 18 to 43 nm and 22 to 52 nm, respectively, while the proportion of VLDL particles within the sVLDLy range exhibited 2-fold variation among individuals (32.7% to 66.5%). Similarly, laying Zebra Finches varied 1.7-fold in mean egg mass (0.778 to 1.351 g), 4.5-fold in clutch size (2 to 9 eggs), 4.9-fold in total clutch mass (1.9 to 9.4 g) and 1.7-fold in laying rate (0.6 to 1 egg per day).

Female body masses at pairing ($F_{19,20} = 11.01$, $p < 0.0001$) and at the 1-egg stage ($F_{19,20} = 12.47$, $p < 0.0001$; Figure 3.2a) and body-mass corrected mean egg mass ($F_{19,20} = 4.45$, $p < 0.001$; Figure 3.2b) were repeatable, with individual female explaining 83.4%, 85.2% and 63.3% of the total variation in the respective variables. Similarly, plasma triglyceride was marginally repeatable, with individual female explaining 39.8% of the total variation in circulating triglyceride at the 1st-egg stage ($F_{14,15} = 2.32$, $p < 0.06$; Figure 3.2c). In contrast, the proportion of VLDL particles within the sVLDLy range

(Figure 3.2d), modal and median particle diameter, and VLDL particle diameter distribution range were not repeatable between laying bouts ($p > 0.4$ in all cases).

Comparison of Large-egg and Small-egg Female Zebra Finches

Small-egg females had significantly lower body mass than large-egg females at pairing ($t = 3.8807$, $df = 30.0$, $p < 0.0005$) and at the 1-egg stage ($t = 4.6816$, $df = 30.0$, $p < 0.0001$; Table 3.1). Mean egg mass was the only trait that was positively associated with female body mass at the 1-egg stage ($F_{1,30} = 21.53$, $p < 0.0001$, $r^2 = 0.4178$). Body-mass corrected mean egg mass was therefore calculated by taking the residual values from the regression of mean egg mass against female body mass at the 1-egg stage, and was smaller in small-egg females than large-egg females ($t = 3.7478$, $df = 30.0$, $p < 0.001$; Table 3.1). However, the two groups of females did not differ in any of the other measures of reproductive effort, plasma triglyceride levels, VLDL particle diameter distribution range, the percentage of particles within the sVLDLy range, or modal or median particle diameter ($p > 0.05$ in all cases; Table 3.1). Furthermore, the variation observed in all of the measures of reproductive effort in this species was independent of variation in VLDL particle diameter distribution range, modal and median VLDL particle diameter, proportion of VLDL particles within the sVLDLy range, and plasma triglyceride levels regardless of large- or small-egg status ($p > 0.05$ in all cases; Figure 3.3), with one exception. Clutch size was positively related to circulating triglyceride levels in large-egg females ($F_{1,13} = 4.56$, $p < 0.05$; Figure 3.3c).

Individual Variation in VLDL Particle Diameter Distribution and Reproductive Effort in Laying Chickens

The VLDL particle diameter distributions of laying chickens sampled at weeks 29 and 86 ranged from narrow distributions (minimum range = 32.9 nm) to broad distributions (maximum range = 324 nm) (Figure 3.1b and c), a 10-fold difference in particle diameter range. The proportion of VLDL particles within the sVLDLy range exhibited 1.4-fold variation among individual laying chickens (50.1% to 71.0%), while modal and median VLDL particle diameter varied 1.5-fold (25.5 to 39.4 nm and 26.4 to 39.3 nm, respectively). Individual laying chickens also varied 1.5-fold in mean egg mass (58.4 to 79.4 g) and 2.5-fold in laying rate (0.4 to 1 egg per day). The extent of inter-individual variation observed between laying chickens in these traits was comparable to the variation observed between laying Zebra Finches ($p > 0.05$), with the exception of modal and mean VLDL particle diameter, wherein laying Zebra Finches were 1.6 times more variable than laying chickens (modal diameter: $F_{31,36} = 2.79$, $p < 0.005$; median diameter: $F_{31,36} = 3.65$, $p < 0.0005$).

Comparison of 29-week and 86-week Old Laying Chickens

While 86-week old laying chickens laid fewer eggs per day than 29-week old laying females (0.7 eggs per day compared to 0.9 eggs per day; $Z = 4.032$, $P < 0.0001$), the eggs that they did lay were significantly heavier than those laid by 29-week old layers (68.77 ± 0.94 g compared to 55.77 ± 2.07 g; $Z = -3.584$, $p < 0.0005$; Table 3.2). Laying chickens at week 29 also had smaller modal and median VLDL particle diameters than chickens sampled at week 86 ($t = 8.855$, $df = 35.0$, $p < 0.0001$ and $t = 7.085$, $df = 35.0$, $p < 0.0001$, respectively; Table 3.2). In contrast, 29- and 86-week old layers did not differ

in the proportion of VLDL particles within the sVLDLy range or VLDL particle diameter distribution range ($p > 0.1$ in both cases; Table 3.2).

At week 29, when all females were considered 'good' layers (i.e., they laid at least 7 eggs within the 10-day period), there was no relationship between laying rate and any measure of VLDL ($P > 0.4$ in all cases; filled circles in Figure 3.4a, c, and e). Similarly, mean egg mass was not related to VLDL particle diameter distribution range (filled circles in Figure 3.4b), the proportion of VLDL particles within the sVLDLy range (filled circles in Figure 3.4d), or modal VLDL particle diameter ($p > 0.05$ in all cases). However, mean egg mass was positively correlated to median VLDL particle diameter at week 29 ($r_8 = 0.714$, $p < 0.05$; filled circles in Figure 3.4f).

At week 86, 14 of the 21 laying females were considered 'good' layers, and the remaining 7 were considered 'poor' layers (i.e., they laid fewer than 7 eggs within the 10-day period). While 'good' layers laid at a higher rate than 'poor' layers ($Z = -3.790$, $p < 0.0005$), these two groups of chickens did not differ in mean egg mass ($p > 0.9$; Table 3.2). Furthermore, mean egg mass at week 86 was not related to any measure of VLDL ($p > 0.5$ in all cases; open circles in Figure 3.4b, d, and f). However, even though the majority of VLDL particles (over 55%) of all of the laying chickens sampled at week 86 fell within the sVLDLy range, 'good' layers (black lines in Figure 3.1c) still had a significantly larger proportion of VLDL particles within the sVLDLy range than 'poor' layers (red lines in Figure 3.1c) ($t = 2.5921$, $df = 19.0$, $p < 0.025$; Table 3.2). Furthermore, variation in laying rate during week 86 was positively related to the variation in the proportion of particles in the sVLDLy range ($r_{21} = 0.452$, $p < 0.05$; open circles in Figure 3.4c). In contrast, 'good' and 'poor' layers did not differ in modal or

median VLDL particle diameter or VLDL particle diameter distribution range ($p > 0.05$ in all cases; Table 3.2), and there was also no relationship between laying rate at week 86 and any of these measures of VLDL ($P > 0.1$ in all cases; open circles in Figure 3.4a and e).

DISCUSSION

Inter-Individual Variation in VLDL Particle Diameter Distribution

Contrary to our prediction, the extent of inter-individual variation in measures of reproductive effort and VLDL particle diameter distribution was comparable between laying Zebra Finches and chickens, with the exception of laying Zebra Finches exhibiting 1.6 times more inter-individual variability in modal and median VLDL particle diameters than laying chickens (2.4-fold variation compared to 1.5-fold variation). Laying Zebra Finches varied also markedly (35-fold) in circulating triglyceride levels, a measure which had been used previously as an index of circulating VLDL levels in this (Williams and Christians, 1997; Salvante and Williams, 2003) and other species which also show marked inter-individual variation in this trait (e.g., 15-fold variation in Cassin's Auklets, *Ptychoramphus aleuticus*, and 6-fold variation in Marbled Murrelets, *Brachyramphus marmoratus*: Vanderkist *et al.*, 2000; 5-fold variation in European starlings, *Sturnus vulgaris*: Williams and Christians, 1997; Challenger *et al.*, 2001; Christians and Williams, 2001c). In contrast, Griffin and Hermier (1988) reported only 2.5-fold variation (20 to 50 μmoles triglyceride/ml plasma) in circulating triglycerides levels in laying chickens. The inter-specific differences in the extent of variation in modal and

median VLDL particle diameter and circulating triglyceride levels during egg production were consistent with previous findings for circulating levels of VTG. Serum concentrations of VTG varied less than 2-fold in laying chickens (Redshaw and Follett, 1976). In contrast, plasma VTG levels varied 6-fold in egg-producing, free-living Cassin's Auklets and 5- to 10-fold in captive Zebra Finches and free-living European Starlings (Williams and Christians, 1997; Vanderkist *et al.*, 2000; Challenger *et al.*, 2001; Christians and Williams, 2001c; Salvante and Williams, 2002). The differences between laying chickens and non-domesticated avian species in the extent of individual variation in modal and median VLDL particle diameter and circulating VTG and triglyceride levels are consistent with a decrease in phenotypic variance in heavily-selected, domestic fowl species (Williams, 1998; Fulton and Delaney, 2003). However, the similarity between laying chickens and Zebra Finches in the extent of inter-individual variation in reproductive effort and the other measures of VLDL particle diameter distribution implies that this phenomenon is not ubiquitous across reproductive or physiological traits.

The proportion of particles within the sVLDLy range was not repeatable in Zebra Finches, and there was only marginal evidence for low intra-individual repeatability of circulating triglyceride levels between laying bouts, with 'individual' explaining only 40% of the variation in plasma triglyceride. Similarly, a lack of repeatability of plasma triglyceride between successive breeding attempts was reported for free-living European Starlings (Challenger *et al.*, 2001). The marked increase in circulating lipids during egg production is generally attributed to increases in VLDLy (Bacon and Musser, 1977; Bacon *et al.*, 1978; Bacon, 1981). Therefore, these findings suggest that circulating levels of triglyceride, and potentially circulating VLDLy, are not distinct phenotypic traits of

laying Zebra Finches. This is in contrast to the high intra-individual repeatability of other reproductive traits. For example, our study confirmed previous findings that egg size was highly repeatable within individual female birds (van Noordwijk *et al.*, 1980; Williams, 1996b; Zann, 1996). Similarly, circulating levels of vitellogenin are highly repeatable in laying free-living European Starlings ('individual' explained over 70% of the total variation in plasma vitellogenin, Challenger *et al.*, 2001) and captive Zebra Finches ('individual' explained 85% of the total variation, Salvante and Williams, 2002). To be able to respond to selection, a trait must exhibit heritable inter-individual variation that is related to fitness, and be a repeatable characteristic of an individual (Bennett, 1987; Stearns, 1992; Garland and Carter, 1994). Therefore, the lack of intra-individual repeatability in circulating amounts of sVLDLy suggests that this trait will not respond to selection. However, it is possible that because the laying Zebra Finches in this study were held under constant, favorable conditions with *ad libitum* access to food, they had ample resources to meet their own energetic demands for self-maintenance as well as those associated with egg production. In this situation, production of VLDLy could exceed follicular demand for the lipoprotein, and the lack of repeatability of circulating sVLDLy particles calculated in this study may actually reflect the lack of repeatability of overproduction of VLDLy. Therefore, it is possible that repeatability of the levels of circulating VLDLy required to sustain egg production can only be assessed under more energetically-challenging conditions, such as lower ambient temperatures or limited food availability. Under these conditions VLDL dynamics may shift towards increased production of VLDL particles that are more easily metabolized by laying females to meet their own energetic needs, while potentially limiting lipid allocation to egg production to

the minimum required for successful reproduction. Alternatively, since the sVLDLy diameter range was based on the proposed sieving properties of the ovary limiting the access of VLDL particles to the developing ovarian follicles (Perry and Gilbert, 1979; Griffin and Perry, 1985; Griffin and Hermier, 1988; Walzem *et al.*, 1999), classification of VLDL particles as 'yolk-targeted' was based solely on particle diameter. By not taking into account apolipoprotein composition of the VLDL particles, this estimate may include particles that are not destined for lipid deposition into developing eggs or exclude particles that actually contribute to egg formation. Determination of the apolipoprotein composition of VLDL particles of various diameters is needed to directly quantify circulating VLDLy levels.

Relationships Between VLDL Particle Diameter Distribution and Reproductive Effort

Our study confirmed previous reports of consistent differences in VLDL particle diameter distribution in relation to reproductive 'performance' in laying chickens (Walzem, 1996). Laying chickens sampled at week 29 laid at a higher rate than 86-week old chickens, and also had smaller modal and median VLDL particle diameters. Furthermore the variation in median VLDL particle diameter was also positively related to variability in mean egg mass at week 29. However, it is unclear whether the differences observed in modal and median particle diameters are biologically significant, as all of the modal and median diameters in this study fell within the sVLDLy range, and the average difference between chickens sampled at week 29 and week 86 was only 5 nm for both measures (cf. 26 nm difference in modal diameter and 38 nm difference in median diameter between laying and non-laying chickens, see Chapter 2). We also found

that variation in laying rate at week 86 was positively related to the proportion of sVLDLy particles in circulation; 'good' layers sampled at week 86 had a higher proportion of sVLDLy particles (4%) than 'poor' layers sampled at the same time. Walzem (1996) found a similar, but more striking pattern in laying Single Comb White Leghorn chickens and Nicholas White turkeys. 'Good' layers of both species exhibited peaked VLDL particle diameter distributions within the sVLDLy range (Walzem, 1996). However, Walzem's (1996) 'poor' layers had a greater proportion of VLDL particles with diameters larger than 50 nm and broader particle distributions that bore a greater resemblance to distributions from non-laying chickens (see average non-laying chicken distribution, solid circles, in Figure 2.4b of Chapter 2) than to any of the laying chicken particle distributions in our study.

In contrast to laying chickens, the inter-individual variation in VLDL particle diameter distribution parameters in laying Zebra Finches was not related to differences in the mass of the eggs a female laid or any other measure of reproductive performance, and circulating triglyceride levels were only related to clutch size in large-egg females. Previous studies on non-domesticated birds found more complex relationships between circulating yolk precursor levels and reproductive effort. In free-living European Starlings (*Sturnus vulgaris*), plasma vitellogenin was negatively correlated with yolky follicle mass (Challenger *et al.*, 2001), yolk protein and yolk lipid composition (Christians and Williams, 2001b), and an index of reproductive effort that combined ovary mass, oviduct mass, and the mass of the first egg using Principal Components Analysis (Williams and Christians, 1997). In contrast, the same index of reproductive effort was independent of circulating levels of vitellogenin in captive Zebra Finches

(Williams and Christians, 1997). Furthermore, Salvante and Williams (2002) reported a complex diet-dependent relationship between plasma vitellogenin and mean egg mass in Zebra Finches, and no relationship between these traits when the analysis was limited to the wide range of egg sizes (0.913 to 1.154 g) common to both diet groups.

The differences between chickens and Zebra Finches in the relationships between VLDL particle diameter distribution and reproductive performance reported in this paper suggest that the production and maintenance of a large number of small yolk-targeted VLDL particles of specific diameters may be important for sustaining continuous laying over prolonged periods in domestic fowl, but may not be important factors in determining the reproductive performance of Zebra Finches, i.e., there may be greater phenotypic plasticity or flexibility in the VLDLy component of the reproductive system in non-selected, non-domesticated species. In order to test this hypothesis, data on VLDL particle diameter distribution during egg production and subsequent reproductive performance from many more avian species, specifically free-living birds and other domesticated fowl and waterfowl, are needed. Subsequently, analyses that control for phylogenetic relationships can be conducted to determine whether the relationships between VLDL particle diameter distribution and reproductive performance reported in this paper persist.

It is also possible that under more energetically-challenging conditions, variation in the ability to maintain a certain proportion of VLDLy particles in circulation during egg production may be related to reproductive performance in non-domesticated birds. Future studies are needed to assess changes in circulating levels of VLDLy with respect to different environmental conditions to determine whether increasing the energetic

demands of laying females influences the variation and repeatability of VLDLy and its relationships with reproductive performance.

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Table 3.1. Body mass, reproductive effort, plasma triglyceride and VLDL particle diameter distribution for small-egg and large-egg female Zebra Finches. Values are means ± SE, with sample size in parentheses. Mean egg mass was corrected for female body mass at the 1-egg stage and percentage of particles within the sVLDLy range was arc-sin transformed for statistical analyses. ** indicates $P < 0.005$, *** $P < 0.0005$, **** $P < 0.0001$.

Trait	Small-egg females	Large-egg females
Mass at pairing (g)	14.27 ± 0.29 (16)	16.34 ± 0.45 (16) ***
Mass at 1-egg stage (g)	15.36 ± 0.20 (16)	16.87 ± 0.26 (16) ****
Mean egg mass (g)	0.987 ± 0.027 (16)	1.228 ± 0.017 (16) **
Clutch size (number of eggs)	5.6 ± 0.4 (16)	5.3 ± 0.4 (16)
Total clutch mass (g)	5.5 ± 0.5 (16)	6.4 ± 0.5 (16)
Laying rate (eggs laid per day)	0.91 ± 0.04 (16)	0.84 ± 0.04 (16)
Laying interval (days)	7.3 ± 0.6 (16)	6.4 ± 0.5 (16)
Total plasma triglyceride (mg / ml plasma)	11.06 ± 0.96 (15)	21.60 ± 6.8 (15)
Percentage in sVLDLy range (25 - 44 nm)	51.22 ± 1.32 (16)	49.23 ± 2.18 (16)
Modal VLDL particle diameter (nm)	29.4 ± 1.5 (16)	29.1 ± 1.6 (16)
Median VLDL particle diameter (nm)	32.3 ± 1.3 (16)	32.3 ± 1.9 (16)
VLDL particle diameter distribution range (nm)	223.4 ± 28.3 (16)	218.8 ± 29.5 (16)

Table 3.2. VLDL particle diameter distribution parameters and reproductive effort for laying chickens at Week 29 and Week 86. Values are means ± SE, with sample size in parentheses. The percentage of particles within the sVLDLy range was arc-sin transformed for statistical analyses. * indicates P < 0.05, *** P < 0.0005, **** P < 0.0001 for comparisons between weeks 29 and 86, and between 'good' and 'poor' laying chickens at week 86.

Trait	Laying chickens at Week 29	Laying chickens at Week 86	'Good' layers at Week 86	'Poor' layers at Week 86
Percentage in sVLDLy range (25 - 44 nm)	59.91 ± 1.78 (16)	62.66 ± 0.83 (21)	63.99 ± 0.87 (14)	59.99 ± 1.32 (7) *
Modal VLDL particle diameter (nm)	29.6 ± 0.7 (16)	34.3 ± 0.6 (21) ****	35.3 ± 0.6 (14)	32.4 ± 1.0 (7)
Median VLDL particle diameter (nm)	29.0 ± 0.5 (16)	34.2 ± 0.5 (21) ****	34.9 ± 0.5 (14)	32.9 ± 0.9 (7)
VLDL particle diameter distribution range (nm)	72.7 ± 8.3 (16)	124.4 ± 22.7 (21)	116.3 ± 0.5 (14)	140.6 ± 40.7 (7)
Mean egg mass (g)	55.77 ± 2.07 (9)	68.77 ± 0.94 (20) ***	68.66 ± 1.13 (14)	69.03 ± 1.82 (6)
Laying rate (eggs laid / day)	0.9 ± 0.0 (16)	0.7 ± 0.0 (21) ****	0.7 ± 0.0 (14)	0.5 ± 0.0 (7) ***

Figure 3.1. Individual variation in VLDL particle diameter distribution in a) laying Zebra Finches and in laying chickens sampled at b) week 29 and c) week 86 with good layers (7 or more eggs in 10 days) indicated by black lines and poor layers (< 7 eggs in 10 days) represented by red lines. Gray bars indicate the sVLDLy range (25 to 44 nm). VLDL particles of this size have been observed distal to the ovarian granulosa basal lamina, and thus have access to the developing ovarian follicles.

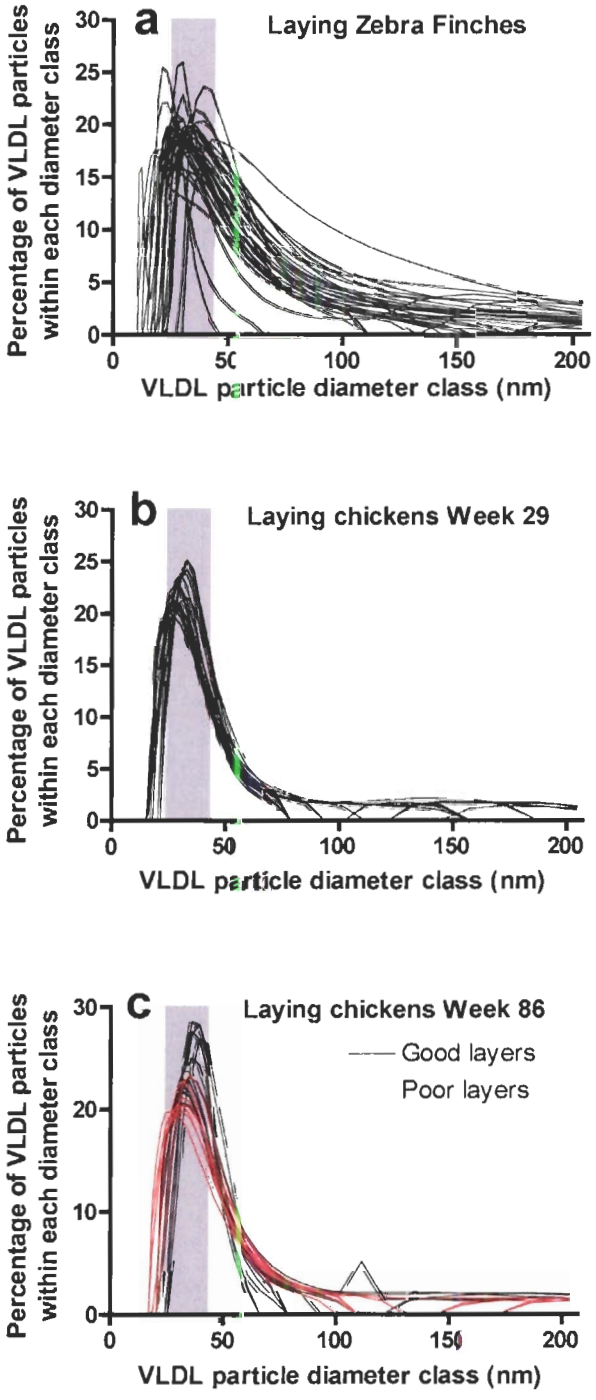


Figure 3.2. Intra-individual repeatability between breeding bouts of a) female body mass at the 1-egg stage, b) mean egg mass, c) plasma triglyceride levels, and d) the proportion of VLDL particles available for use by the developing ovarian follicles as defined by the proposed selective sieving properties of the ovary, i.e., the proportion of particles that fell within the sVLDLy diameter range (25 to 44 nm), in Zebra Finches. Body mass- corrected mean egg mass was used for statistical analyses.

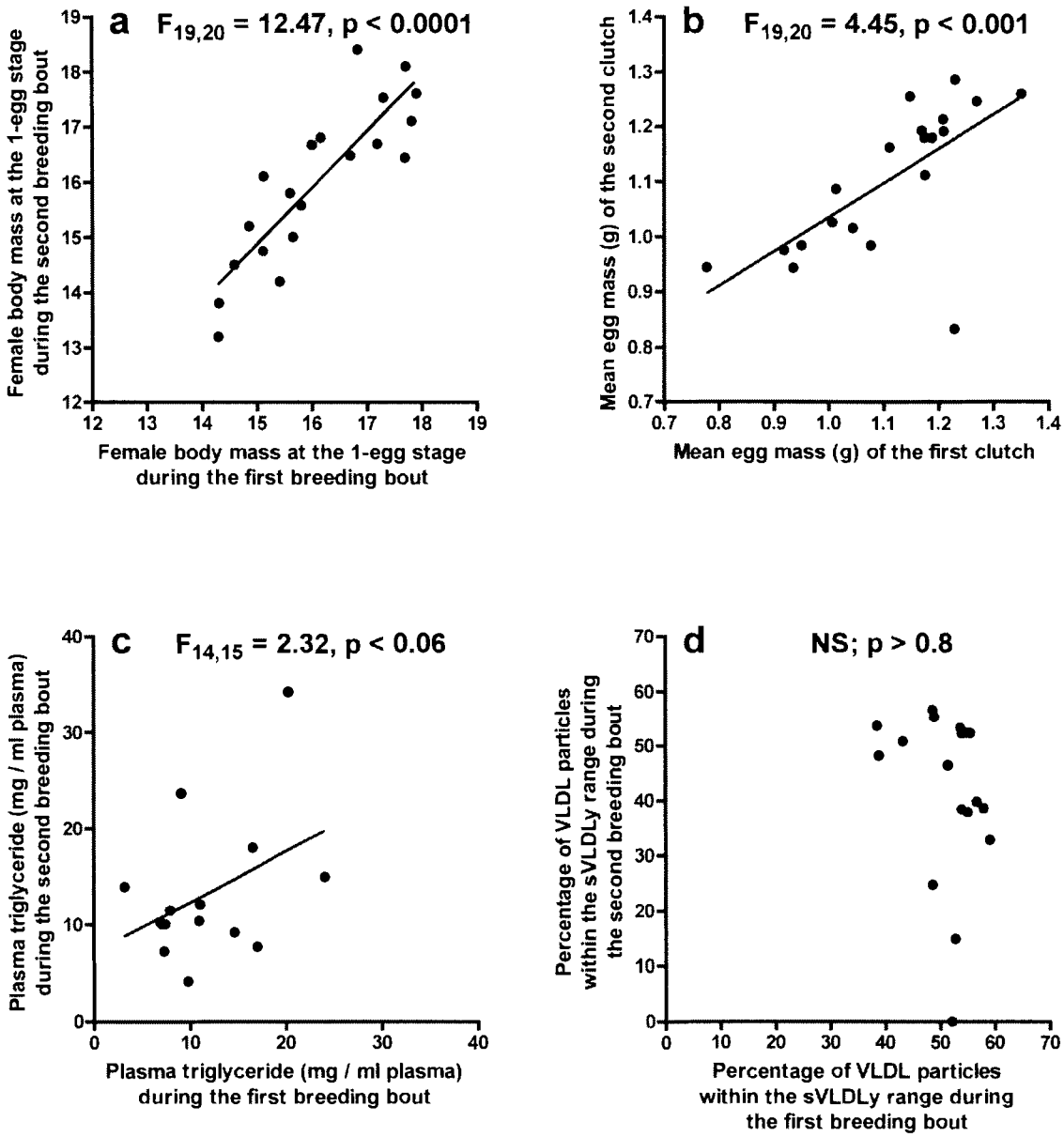


Figure 3.3. Reproductive effort (egg mass, clutch size, and laying rate) of Zebra Finches in relation to circulating triglyceride levels (a, c, and e, respectively) and the proportion of VLDL particles within the sVLDLy diameter range (25 to 44 nm, i.e., particles available for use by the developing ovarian follicles as defined by the proposed selective sieving properties of the ovary) (b, d, and f, respectively). The relationships between the various measures of reproductive effort and circulating triglyceride and sVLDLy particles were assessed separately for large-egg females (filled circles) and small-egg females (open circles). The solid regression line represents a significant relationship within large-egg females.

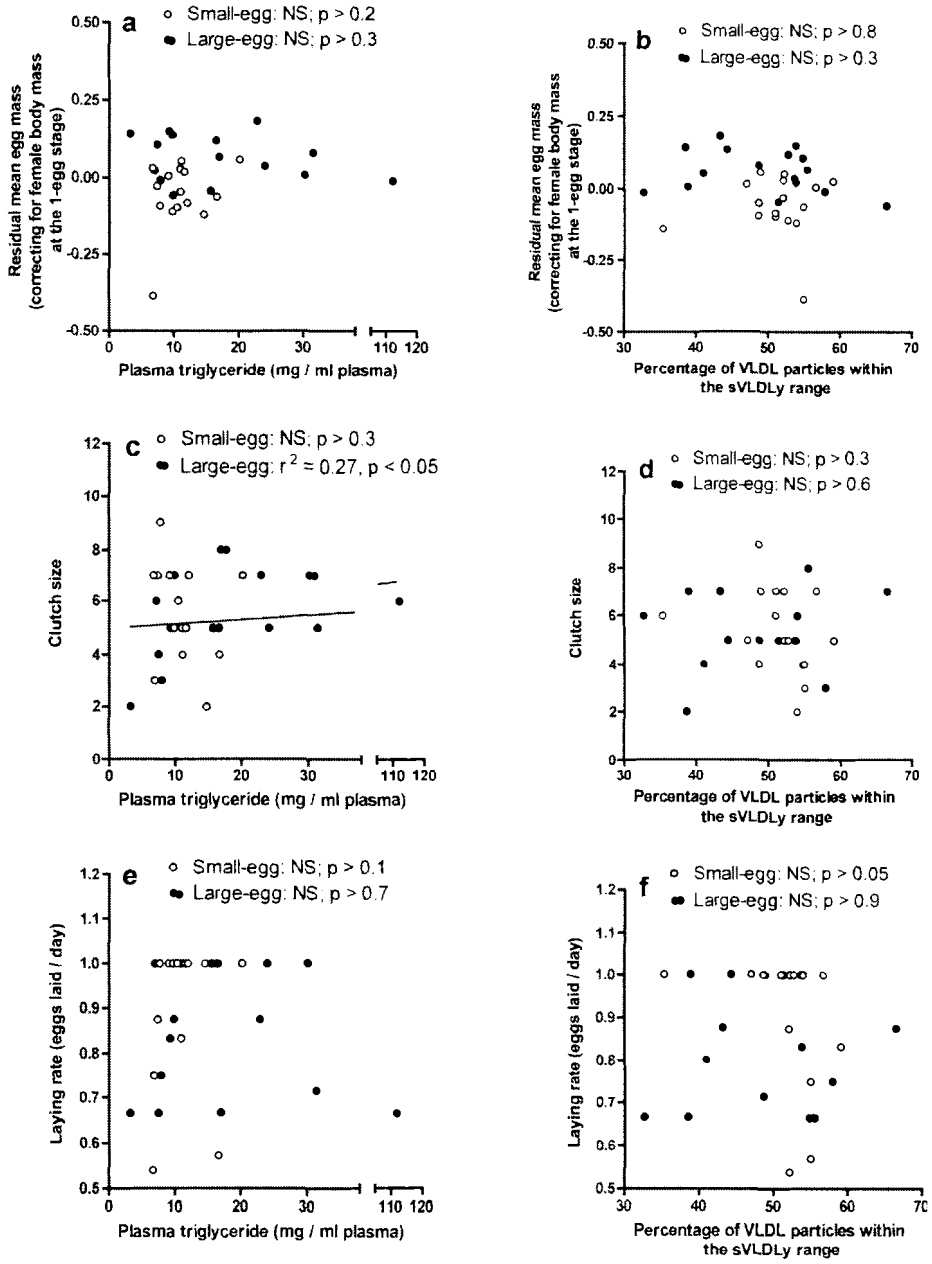
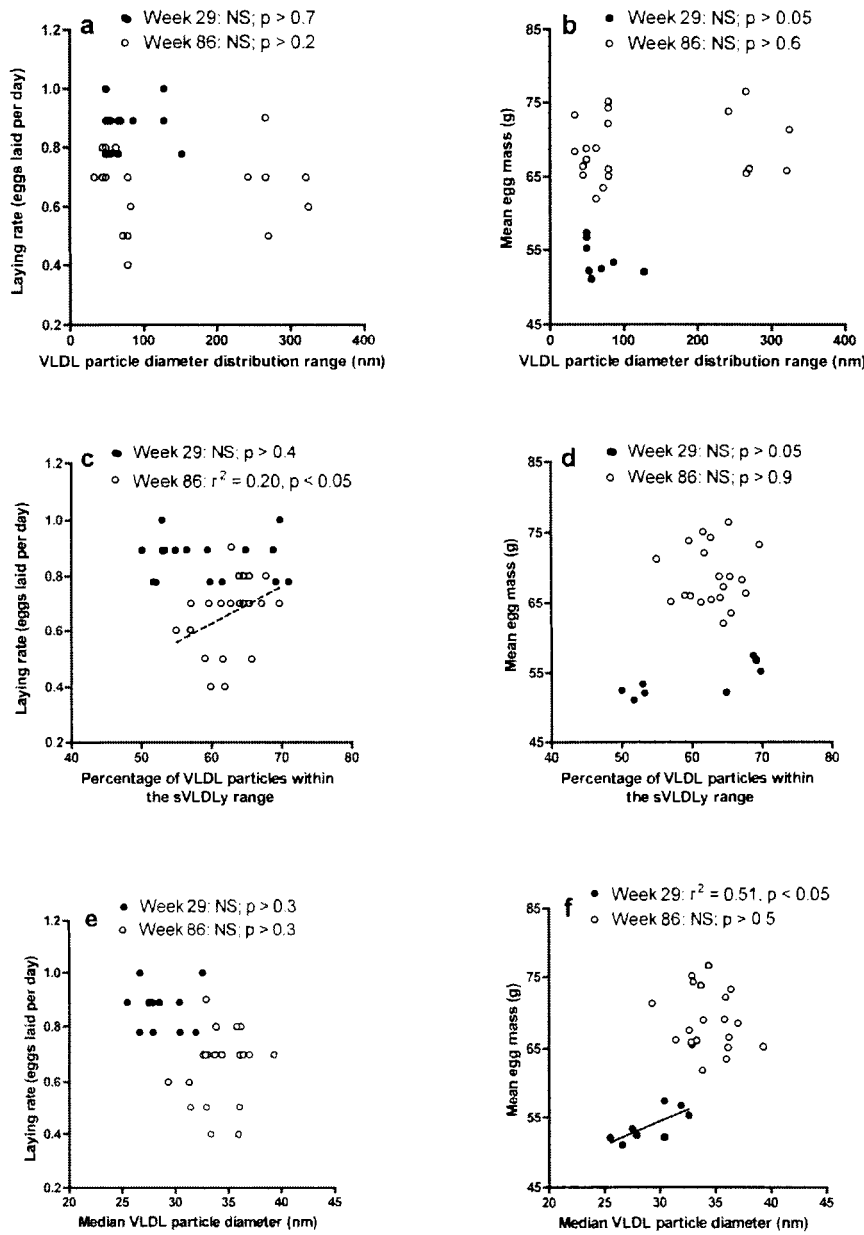


Figure 3.4. Relationships between reproductive effort (laying rate and mean egg mass) and VLDL particle diameter distribution range (a and b, respectively), the proportion of VLDL particles available for use by the developing ovarian follicles as defined by the proposed selective sieving properties of the ovary, i.e., the proportion of particles that fell between 25 and 44 nm in diameter (the sVLDLy diameter range) (c and d, respectively), and median VLDL particle diameter (e and f, respectively). The relationships between the various measures of reproductive effort and VLDL particle diameter distribution were assessed within females sampled at week 29 (filled circles) and within females sampled at week 86 (open circles). The solid and dotted regression lines represent significant relationships within females at week 29 and week 86, respectively.



CHAPTER 4

IS AVIAN EGG PRODUCTION *REALLY* COSTLY? COMPARISON OF THE METABOLIC COSTS OF EGG PRODUCTION, COLD-ACCLIMATION AND THERMOGENESIS

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SUMMARY

Recent studies have shown that the metabolic cost of avian egg production involves a 16-27% increase in basal or resting metabolic rate (BMR and RMR, respectively) above non-reproductive values. To determine how the metabolic cost of egg production compared with the costs of other essential processes (such as cold-acclimation and thermogenesis) and whether these costs were additive, we measured RMR of non-breeding and egg-producing Zebra Finches (*Taeniopygia guttata*) while a) warm-acclimated (to 19-23°C) and measured within their thermoneutral zone (at 35°C), b) cold-acclimated (to 7°C) and measured at thermoneutrality (i.e., not actively producing heat), and c) cold-acclimated and measured below thermoneutrality (at 7°C) (i.e., during active thermogenesis). The metabolic cost of egg production was relatively small (24% above BMR, i.e., 1.2 x BMR) compared to the additive costs of cold-acclimation (82% above BMR; 1.8 x BMR) and thermogenesis (124% over BMR; 2.2 x BMR). Exposure to low ambient temperatures was accompanied by an increase in seed consumption (by 72%) and a decrease in locomotor activity (by 72%) compared to warm-acclimated, non-breeding values, resulting in an increase in the energy available to fuel thermoregulation. In contrast, egg production in thermoregulating females was associated with an 11% decrease in RMR and a 22% decrease in seed consumption compared to non-breeding, thermoregulating values. These data suggest that while the increase in RMR associated with egg production is small in relation to the birds' metabolic capacity to increase RMR in response to other energetically-demanding processes, the addition of egg production to

these metabolically costly activities may be enough to necessitate the use of energy-saving strategies, such as internal energy reallocation and controlled hypothermia, to cope with the additional energetic demands.

INTRODUCTION

Egg production in birds is a complex process that involves up-regulation of a cascade of hormones that initiates the recrudescence and maintenance of reproductive tissues, stimulates the production and mobilization of large amounts of protein and lipid that fuel the reproductive machinery and are incorporated into the developing eggs, and regulates the formation and oviposition of eggs (for reviews see Williams, 1998; Johnson, 2000). The physiological changes associated with egg production in passerine birds have been shown to involve a 16-27% increase in metabolic rate above that of non-breeding females (House Sparrows, *Passer domesticus*, 16% above non-breeding basal metabolic rate (BMR), Chappell *et al.*, 1999; Great Tits, *Parus major*, 27% over wintering BMR, Nilsson and Raberg, 2001; European Starlings, *Sturnus vulgaris*, 22% over pre-reproductive resting metabolic rate (RMR), Vézina and Williams, 2002, 2003; Zebra Finches, *Taeniopygia guttata*, 22% above non-breeding RMR, Vézina and Williams, 2005). The metabolic cost of egg production is comparable to the changes in metabolic rate associated with other reproductive activities; Zebra Finches that were incubating eggs exhibited a 20% increase in RMR above non-breeding values (Vleck, 1981), while chick-rearing Zebra Finches exhibited a 13% increase in RMR (Vézina and Williams, 2005). However, it is unclear whether the changes in RMR associated with reproduction are substantial in the context of the birds' ability and capacity to up-regulate RMR, i.e., how the metabolic costs of reproduction (egg production, inclubation, and chick rearing)

compare with those of other, potentially energetically demanding, physiological processes that occur throughout a bird's lifetime.

The main goal of this study was therefore to determine how the metabolic cost of egg production compared to the metabolic costs of other essential and metabolically demanding activities, specifically thermoregulation. In cold-acclimated birds, the energetic cost of thermoregulation can be divided into two components: a) the costs associated with cold-acclimation itself, i.e., changes in thermoregulatory physiology and maintenance of tissues involved in heat production or fueling heat production, and b) the cost of thermogenesis, i.e., active heat production (Williams and Tieleman, 2000; Vézina, personal communication). In other avian and mammalian species, cold-acclimation is associated with comparable increases in resting metabolic rate (15 to 21%) to avian egg production (Kristan and Hammond, 2000; O'Connor *et al.*, 2000). However, thermogenesis involves even more substantial increases in metabolic rate (50 to 100% increase in RMR above cold-acclimated values; Broggi *et al.*, 2004; Carleton and Martinez del Rio, 2005). Therefore, to compare the metabolic costs of egg production with those of cold-acclimation, thermogenesis, and thermoreguation (i.e., cold-acclimation and heat production combined), we measured and compared the resting metabolic rates of female Zebra Finches (*Taeniopygia guttata*) at a variety of thermal and reproductive stages:

1. To compare the energetics of egg production and cold-acclimation, the resting metabolic rates of non-breeding, warm-acclimated females were compared to the metabolic rates of the same females during egg production, and to the metabolic rates

of non-breeding, cold-acclimated females ('Egg production' and 'Cold-acclimation', respectively, in Figure 4.1a).

2. The RMR of cold-acclimated, non-breeding females measured at thermoneutrality were compared to the metabolic rates of the same females measured below thermoneutrality to determine the metabolic cost of thermogenesis ('Heat production' in Figure 4.1a). The metabolic rates of cold-acclimated, laying females measured at and below thermoneutrality were also compared as a second measure of the metabolic cost of thermogenesis ('Heat production while producing eggs' in Figure 4.1a).

3. To determine the metabolic cost of thermoregulation (the combined costs of cold-acclimation and thermogenesis), the RMR of non-breeding, warm-acclimated birds measured at thermoneutrality were compared to the RMR of non-breeding, cold-acclimated birds measured below thermoneutrality ('Cold-acclimation AND heat production' in Figure 4.1a).

Because daily energy budgets are dependent on the amount of energy taken in and expended, similar comparisons were made to determine how daily seed consumption and locomotor activity were affected by egg production and thermoregulation (Figure 4.1b).

Reproduction in seasonally-breeding birds is generally timed such that the period of chick-rearing coincides with favorable environmental conditions (i.e., warmer weather, increased food availability and quality). Consequently, free-living birds are often faced with the energetically-demanding task of producing and laying eggs while being simultaneously exposed to cold temperatures and unstable weather conditions (Perrins, 1970). Therefore, the second aim of this study was to determine whether the energetic costs of cold-acclimation, thermogenesis, and egg production were additive when these

activities occurred simultaneously. The combined metabolic costs of egg production, cold-acclimation and thermogenesis were assessed and compared to the total cost of these activities when measured separately.

4. To determine the metabolic cost of concurrent egg production and thermogenesis, the RMR of cold-acclimated, non-breeding females measured below thermoneutrality were compared to the RMR of cold-acclimated, laying females, also measured below thermoneutrality ('Egg production while producing heat' in Figure 4.1a).

5. The RMR of warm-acclimated, nonbreeding females measured at thermoneutrality were compared to the metabolic rates of cold-acclimated, laying females measured below thermoneutrality to determine the metabolic cost of simultaneous egg production, cold-acclimation and thermogenesis. ('Egg production, cold-acclimation AND heat production' in Figure 4.1a).

The combined effect of egg production, cold-acclimation, and thermogenesis on daily seed consumption and locomotor activity was also investigated (Figure 4.1b).

MATERIALS AND METHODS

Animals and Husbandry

Zebra Finches with previous breeding experience (i.e., produced at least one previous clutch) were randomly chosen from our breeding colony housed in the Simon Fraser University Animal Care Facility. All birds were housed in cages (61 x 46 x 41 cm), exposed to a constant light schedule of 14L: 10D (lights on at 09:00) and ambient

temperatures between 19 and 21°C, and provided with a mixed seed diet (Panicum and white millet, 50:50; approximately 12.0% protein, 4.7% lipid; Just for Birds, Surrey), 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.

Acclimation Protocol and Non-Breeding RMR Conditions

The male and female Zebra Finches that were chosen for this study were divided into two study groups: a warm-acclimated (to 19-21°C) group and a cold-acclimated (to 7°C) group. Females in the warm-acclimated group (n = 9) were weighed (± 0.1 g) and randomly assigned to same-sex pairs in 'warm' ambient conditions (i.e., temperature 19-23°C) for at least 7 days. Males in the warm-acclimated group (n = 9) were group housed in same-sex cages (n = 4 or 5 birds per cage) under the same environmental conditions as the warm-acclimated females. In contrast, birds in the cold-acclimated group (n = 12 females and 12 males) were weighed (± 0.1 g) and transferred to same-sex group cages (n = 6 birds per cage) within a Conviron E15 plant growth chamber (Controlled Environments, Winnipeg). The temperature within the chamber at the beginning of the acclimation period was 14°C, and was decreased slowly over 4 weeks (i.e., one week at 14°C, one week at 10°C, then two weeks at 7°C). The acclimation period was based on the time it took for all birds to return to and maintain their pre-acclimation body mass at each temperature. Following the 4-week acclimation period, cold-acclimated females were randomly assigned to same-sex pairs for at least 7 days. Cold-acclimated, same-sex pairs were provided with 30 g of the mixed seed diet daily, and seed was weighed (± 0.1 g) and replaced daily to determine daily seed consumption of cold-acclimated, non-

breeding pairs. All same-sex, female pairs were visually, but not acoustically isolated from the opposite sex. The metabolic rate of each female was measured twice, once while the females were in same-sex, non-breeding pairs (NBr sample), and again while the females were paired with males and actively laying eggs (LAY sample) following the protocol described below. The non-breeding metabolic rate measurements (i.e., 'Warm-acclimated NBr-35', 'Cold-acclimated NBr-35' and 'Cold-acclimated NBr-7' in Figure 4.1a) began on the seventh night following same-sex pairing, and continued nightly until all females were measured.

Breeding Protocol and Laying RMR Conditions

Following measurement of non-breeding metabolic rate measurement (protocol described below), all females were randomly paired with similarly thermally acclimated males. Males and females were weighed (± 0.1 g) at the time of pairing, and housed in cages equipped with an external nest box (15 x 14.5 x 20 cm). Breeding pairs were provided with 6 g of an egg-food supplement (20.3% protein: 6.6% lipid; see Williams, 1996) daily between pairing and clutch completion. Data on laying interval, egg mass and clutch size were obtained by checking the nest boxes daily between 09:00 and 11:00. All new eggs were weighed (± 0.001 g) and numbered on the day they were laid. Clutches were considered complete if no new eggs were laid over three 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. The metabolic rate of each female during egg production (i.e., 'Warm-acclimated LAY-35', 'Cold-acclimated LAY-35' and 'Cold-acclimated-7' in Figure 4.1a) was measured during the night following the laying of the second egg.

Energy Intake and Locomotor Activity

To obtain a gross estimate of energy intake by cold-acclimated birds, seed consumption was measured by providing all cold-acclimated pairs with 30.0 g of the mixed seed diet daily in open 946 ml Ziploc^{TM} containers placed on the cage floor. This method avoided any spillage and allowed for the measurement of daily seed consumption by same-sex and breeding pairs by weighing the remaining seeds in the container after 24 hours (± 0.1 g). Birds were still able to feed *ad libitum* as 30.0 grams of seed was always in excess of their daily intake. Williams and Ternan (1999) showed that females ate slightly more food (4.5%) than males, regardless of their breeding status, and that this effect did not change throughout the laying sequence. Therefore, measuring food intake per pair is a good indicator of female food intake as the proportion of seeds consumed by both sexes remains unchanged throughout the experimental protocol. The seed consumption data for cold-acclimated same-sex and breeding pairs were compared to previously collected data for warm-acclimated pairs that were maintained in comparable conditions as the warm-acclimated pairs in this study (Vézina, Speakman and Williams, unpublished data).

The locomotor activity of all same-sex and breeding pairs was monitored using a micro-switch system connected to a cage perch as described by Williams and Ternan (1999). This system does not discriminate potential differences between sexes in locomotor activity, but previous work by Williams and Ternan (1999) involving direct observations of activity showed that activity does not differ between sexes throughout the experimental protocol.

Measurement of Resting Metabolic Rate

Basal metabolic rate (BMR) is defined as the energy consumed by a resting, post-absorptive bird during the inactive phase of the circadian cycle at a temperature within the thermoneutral range for the animal (Blem, 2000; Commission for thermal physiology of the International Union of Physiological Sciences, 2001), and can be applied to the metabolic rate of the warm-acclimated, non-breeding females in this study. However, because laying and thermoregulating birds in this study were producing eggs and heat, respectively, we considered them to be in an "active physiological state." Furthermore, the metabolic rate of thermoregulating birds was measured at 7°C, which is well below the thermoneutral range for Zebra Finches (lower critical temperature = 33°C; Marschall and Prinzinger, 1991; Meijer *et al.*, 1996). We therefore consider the term *resting* metabolic rate (RMR) more appropriate in the present study, but refer to the metabolic rate of the warm-acclimated, non-breeding females in this study as BMR.

All RMR measurements were completed using a flow-through respirometry system (Sable Systems International) as follows. At 21:00, three hours prior to the beginning of RMR measurement, the food was removed from the cages of females undergoing RMR measurement that night. Warm-acclimated females were taken from their cages, their body masses were measured (± 0.1 g), and they were placed randomly in one of four metabolic chambers (1.5L) for one hour prior to the beginning of RMR measurements (at 23:00). All chambers continuously received approximately 500 ml/min of dry CO_2-free air (using Dryrite© and ascarite© as scrubbers), were kept in the dark, and were maintained at 35°C, which is within the thermoneutral zone for this species (lower critical temperature = 33°C; Marschall and Prinzinger, 1991; Meijer *et al.*, 1996). Our

setup consisted of four metabolic chambers connected to a divided air line with a valve multiplexer which allowed us to sample air coming from either ambient baseline air (scrubbed for water and CO_2) or from one metabolic chamber at a time. The air then passed through a mass flow valve (Sierra Instruments) for proper air flow reading (STP corrected) and through CO_2 and oxygen analyzers (model CA-1 and FC-1 Sable systems, respectively; air was scrubbed to remove water before entering the CO2 analyzer and scrubbed to remove water and CO_2 before entering the O_2 analyzer). RMR measurements were always started at 00:00 hours. The measurement sequence was as follows: baseline air was recorded for 10 minutes, then the out-flowing air from the first chamber and then the second chamber, then baseline air again, followed by the out-flowing air from the third chamber and then the fourth chamber, and finally ending with baseline air. This sequence was repeated three times overnight giving 99 minutes of recording per chamber spanning 8 hours. After RMR measurement the birds were weighed for a second time and released back into their cages (approximately 30 minutes to an hour before the lights were turned on), and their food was returned. The average of first and second body masses was used in subsequent analyses. The oxygen consumption (VO_2) of each bird was computed using Equation 4b of Withers (1977). To calculate RMR, VO_2 was computed using a running mean representing ten minutes of recording that was passed through the data for each bird, with the lowest average taken as RMR. The lowest value for RMR was always found during the second or third rounds of RMR measurement (i.e., during the last 5 hours of the night). Preliminary analysis showed that measuring RMR using this protocol did not generate a time effect (sensu Hayes *et al.*, 1992).

The resting metabolic rates of cold-acclimated females were measured using the same protocol as described above for warm-acclimated birds with the following exceptions. Firstly, females were placed randomly in one of two metabolic chambers (1.5L) for one hour prior to the beginning of RMR measurements. The temperature was maintained at 7°C for the first part of the night, and then increased to and maintained at 35°C for the remainder of the measurement period. This measurement protocol was designed such that the air recordings from the test chambers occurred during the last 5.5 hours of the night (cf. the second and third rounds of RMR measurement for the warm-acclimated females). The measurement sequence was as follows: starting at 02:00, baseline air was recorded for 40 minutes, then the out-flowing air from the first chamber for 45 minutes, baseline air again for 15 minutes, and then the out-flowing air from the second chamber for 45 minutes, all at 7°C. Following the first set of measurements, the temperature was increased to 35°C, which took approximately 40 minutes. Once 35°C was maintained, birds were allowed to adjust to the new temperature for approximately 55 minutes. Baseline air was recorded while the temperature was increased to 35°C, and during the adjustment period. The measurement sequence was then repeated (i.e., baseline, chamber 1, baseline, chamber 2), and finally, baseline air was recorded for the last 15 minutes. The RMR at each temperature (i.e., 7°C and 35°C) was calculated using the same protocol described above.

Data Analysis

All statistical analyses were performed using SAS (SAS Institute, 1999). All data were tested for normality of distribution (Shapiro-Wilk test; Zarr, 1996), and all variables with non-normal distributions were log10 transformed prior to analysis (although some

non-transformed values were used for graphical purposes). Comparisons of RMR, locomotor activity, and seed consumption within the acclimation groups, between different reproductive stages (e.g., non-breeding vs. laying) or within the cold-acclimation group, between different reproductive stages measured at different temperatures (e.g., non-breeding RMR measured at 35°C vs. laying RMR measured at 7°C) were assessed using mixed model, repeated measures ANOVA or ANCOVA (with female body mass as a covariate) with stage (i.e., reproductive stage or 'reproductive stage - measurement temperature') as a fixed, repeated factor, and individual female as a random factor (PROC MIXED; SAS Institute, 1989). Comparisons between acclimation groups (i.e., warm vs. cold) were examined using t-tests or ANCOVA (with female body mass as a covariate). Sequential Bonferroni-correction for multiple comparisons, as introduced by Holm (1979) (also described in Rice, 1989), was applied to post-hoc paired contrasts within and between acclimation groups. Briefly, the P-values for each pair-wise comparison were ranked from smallest (P_1) to largest (P_k), and an overall significance level (α) was chosen. The smallest P-value (P_1) was then judged against α / k. If $P_1 > \alpha / k$, then the corresponding test and all other tests with larger P-values (P_2 through P_k) were not significant at the overall significance level of α, taking into account all of the pair-wise comparisons. If $P_1 \leq \alpha / k$, then the pair-wise comparison was significant at an overall significance level of α, taking into account all of the paired contrasts, and the second smallest P-value (P_2) was then judged against $\alpha / (k - 1)$. This continued until $P_i > \alpha / (1 + k - i)$. For example, for comparisons of RMR between acclimation groups, 7 comparisons were made and $\alpha = 0.05$ was chosen. Therefore, an initial P-value of 0.0071, i.e., 0.05 / 7, was used to judge the smallest P-value, followed by 0.0083, i.e., 0.05 / 6, for

the next smallest P-value, and so on until $P_i > 0.05 / (1 + 7 - i)$. All tests were two-tailed, and the overall significance level was $p < 0.05$. ANOVA were used to examine the relationships between reproductive effort (e.g., mean egg mass, laying interval, clutch size) and RMR, seed consumption and locomotor activity within warm-acclimated females and within cold-acclimated females. All data are presented as values at particular stages with a line connecting values for individual females and a gray circle representing the mean at each stage.

RESULTS

Resting Metabolic Rate

RMR varied significantly between the six different thermal-reproductive stages ($F_{5,47.9} = 86.53$, $p < 0.0001$; Table 4.1; Figure 4.2) and was positively related to body mass ($F_{1,22.5} = 23.54$, $p < 0.0001$). Therefore, female body mass was included as a covariate in all RMR analyses, and the percent change in RMR between comparison groups were calculated using least squares mean RMR controlling for body mass at the stages being compared. Egg production involved a 24% increase in RMR of warm-acclimated females above non-breeding values (1.2 x BMR, $t = 2.90$, $df = 4.92$, $p < 0.04$; Table 4.1; Figure 4.2). Cold-acclimation resulted in an 82% increase in mass-corrected RMR above non-breeding, warm-acclimated values (1.8 x BMR, $F_{1,18} = 26.28$, $p < 0.0001$; Table 4.1; Figure 4.2), while heat production alone induced a 65% increase in mass-corrected RMR over cold-acclimated, non-breeding values ($t = 9.66$, $df = 32.9$, $p < 0.0001$; Table 4.1; Figure 4.2), the equivalent of a 124% increase above non-breeding,

warm-acclimated values (2.2 x BMR). Similarly, thermogenesis by egg-producing, cold-acclimated females was associated with a 68% increase in RMR over cold-acclimated, egg-laying values ($t = 8.81$, $df = 32.9$, $p < 0.0001$; Table 4.1; Figure 4.2), the equivalent of a 113% increase in RMR above non-breeding, warm-acclimated values (2.1 x BMR). The increase in metabolic rate due to egg production alone was still significant in the broader context of comparing the metabolic cost of egg production to the costs of cold-acclimation and thermoregulation, as the sequential Bonferroni-adjusted P-value for this pair-wise comparison was 0.05, i.e., 0.05 / (1 + 7 – 7).

When combined, cold-acclimation and heat production resulted in a 204% increase in mass-corrected RMR of non-breeding birds above non-breeding, warm-acclimated values (over 3 x BMR, $F_{1,18} = 234.50$, $p < 0.0001$; Table 4.1; Figure 4.2). This was equal to the predicted increase in RMR of 195% to 206% (based on laying and non-breeding females, respectively) if the metabolic costs of cold-acclimation and heat production were additive. Interestingly, cold-acclimated females that were actively thermoregulating actually exhibited *lower* mass-corrected RMR during egg production than as non-breeders (11% decrease; $t = 2.56$, $df = 35.3$, $p < 0.015$; Table 4.1; Figure 4.2), and this difference was significant when taking into account the other comparisons between groups as the sequential Bonferroni-adjusted P-value for this pair-wise comparison was 0.025, i.e., 0.05 / (1 + 7 – 6). As a result, the combination of cold-acclimation, heat production and egg formation only induced a 165% increase in mass-corrected RMR above non-breeding, warm-acclimated values (2.6 x BMR, $F_{1,18} = 312.42$, $p < 0.0001$; Figure 4.2), well below the 230% increase (3.2 x BMR) predicted if the metabolic costs of all three activities were additive.

Locomotor Activity

Locomotor activity was not related to female body mass ($p > 0.05$), but did differ between stages ($F_{3,6.2} = 14.32$, $p < 0.005$). Because four paired contrasts were made to assess the effects of decreased ambient temperature and egg production on locomotor activity, the sequential Bonferroni-corrected p-values for stages comparisons were 0.0125, 0.018, 0.025 and 0.05. The locomotor activity of warm-acclimated birds decreased from an average of 1852 ± 1418 perch hops per day by non-breeding, same-sex pairs to an average of 662 ± 270 hops by breeding pairs, a 64% decrease ($F_{1,4.53} = 34.07$, $p < 0.005$; Table 4.1; Figure 4.3a). A similar decrease in hopping was observed in cold-acclimated, non-breeding, same-sex pairs, which exhibited an average of 520 ± 264 perch hops per day, a 72% decrease in locomotor activity compared to the warm-acclimated, non-breeding pairs ($t = 3.52$, $df = 12.1$, $p < 0.005$; Table 4.1; Figure 4.3a). In contrast to the warm-acclimated birds, the locomotor activity of cold-acclimated birds was not related to reproductive stage ($F_{1,11} = 3.06$, $p > 0.1$; Figure 4.3a; Table 4.1). However, cold-acclimated, laying pairs hopped an average of 81% less than warm-acclimated, non-breeding pairs ($t = 4.79$, $df = 12$, $p < 0.0005$; Table 4.1; Figure 4.3a).

Seed Consumption

Seed consumption was also not related to female body mass at the various stages ($p > 0.05$), but did differ between stages ($F_{3,18.3} = 64.01$, $p < 0.0001$). As in the locomotor activity analyses, four pair-wise comparisons were made to assess the effects of cold ambient temperature and egg production on daily seed consumption. Gross energy intake of warm-acclimated birds was not affected by reproductive stage ($p > 0.5$; Table 4.1; Figure 4.3b). In contrast, cold-acclimation and thermogenesis induced a 72% increase in

seed consumption in non-breeding birds ($t = 12.63$, $df = 35.8$, $p < 0.0001$; Table 4.1; Figure 4.3b). Interestingly, thermoregulating birds consumed 22% less seed while producing eggs than as non-breeders in same-sex pairs ($F_{1,11} = 8.73$, $p < 0.013$; Table 4.1; Figure 4.3b). As a result, egg production, cold-acclimation and thermogenesis combined did not induce a significant increase in seed intake above warm-acclimated, non-breeding values ($t = 2.49$, $df = 13.5$, $p < 0.03$, which was greater than the sequential Bonferroni-adjusted P-value of 0.025 for this pairwise comparison; Table 4.1; Figure 4.3b). However, post-hoc power analysis (G-Power, University of Trier, Germany; Erdfelder *et al.* 1996; Buchner *et al.* 1997) revealed that the power to detect a significant difference at $p < 0.025$ given the variance observed in seed consumption and the sample size used in this study ($n = 12$ at each reproductive-themal stage) was only 0.64, i.e., there was a 36% chance of committing type II error.

Reproductive Effort

Warm- and cold-acclimated Zebra Finches did not differ in laying interval, i.e., days from pairing until initiation of egg laying (5.4 ± 0.4 vs. 5.3 ± 0.5 day, respectively; $p > 0.8$), mean egg mass (1.048 ± 0.033 vs. 1.068 ± 0.024 g; $p > 0.6$), or clutch size (4.9 ± 0.4 vs. 4.6 ± 0.4 eggs; $p > 0.7$) (Table 4.1). Regardless of acclimation temperature, variation in reproductive effort was not related to variation in RMR as non-breeders, RMR during egg production, locomotor activity or seed consumption ($p > 0.1$ in all cases).

DISCUSSION

Resting metabolic rate of female Zebra Finches increased by 24% above non-breeding values when the birds were acclimated to favorable conditions, i.e., warm ambient temperatures of 19°C to 23°C. Our measure of the metabolic cost of egg production was comparable to previous studies on free-living and captive passerine birds which reported 16-27% increases in metabolic rate above values for non-breeding females (Chappell *et al.*, 1999; Nilsson and Raberg, 2001; Vézina and Williams, 2002, 2003, 2005). However, the metabolic cost of egg production (1.2 x BMR) was small when compared to the additive metabolic costs of cold-acclimation (1.8 x BMR) and active thermogenesis (2.1 to 2.2 x BMR), two very energetically-demanding activities. In contrast, the cost of egg production was comparable to, and sometimes greater than, the metabolic costs of other essential physiological processes. The metabolic cost of mounting a cell-mediated immune response was 4.2 kJ per day, the equivalent of 29% of RMR of House Sparrows (Martin *et al.*, 2002), while mounting a humoral immune response varied between having no effect on the metabolic rates of Blue Tits, *Parus caeruleus*, and Greenfinches, *Carduelis chloris* (Svensson *et al.*, 1998; Hõrak *et al.*, 2003), to inducing 8.5% and 8.6% increases above BMR of Great Tits and Collared Doves, *Streptopelia decaocto*, respectively (Ots *et al.*, 2001; Eraud *et al.*, 2005). The metabolic cost of digestion and assimilation of seed in female Zebra Finches that were not fasted prior to metabolic rate measurement represented a 14% increase in RMR above fasted values (Vézina and Salvante, unpublished data).

There is marked variation across mammalian and avian species in the previously reported metabolic costs associated with exposure to cold ambient temperatures. Cold-

acclimation (to 5°C) was associated with a 21% increase in the metabolic rate of virgin, Swiss-Webster laboratory mice, *Mus musculus* (Kristan and Hammond, 2000) and a 15% increase in House Finches, *Carpodacus mexicanus*, above warm-acclimated values (O'Connor *et al.*, 2000). However, there was no measurable metabolic cost of cold-acclimation in House Sparrows, *Passer domesticus*, as warm-acclimated (to 22°C) and cold-acclimated (to 5°C) birds had comparable metabolic rates (Carleton and Martinez del Rio, 2005). In contrast, cold-acclimation (to 15°C) was associated with a 42% increase in the metabolic rate of Hoopoe Larks, *Alaemon alaudipes*, above values for birds maintained at a thermally neutral temperature (36°C) (Williams and Tieleman, 2000). Therefore, the 82% increase in RMR associated with cold-acclimation in this study was much higher than previously reported metabolic costs of cold-acclimation. This may be due to variation in the thermoneutral zones of the different species. The thermoneutral zones of House Sparrows and House Finches range from a lower critical temperature of 20-22°C to an upper critical temperature of 37-38°C (Hudson and Kimsey, 1966; Weathers, 1981; Dawson *et al.*, 1985). In contrast, the lower limit of the Zebra Finch's thermoneutral zone is 33°C (Marschall and Prinzinger, 1991). Attempts to determine the upper limit were unsuccessful as the birds were still at thermoneutrality at 38°C (Marschall and Prinzinger, 1991). The thermoneutral zone of Hoopoe Larks is similarly high, ranging from 32.7°C to 37.5°C (Tieleman *et al.*, 2002). Therefore, the larger increase in RMR associated with cold-acclimation in Zebra Finches may be due to the larger difference between the acclimation temperature and the lower limit of the thermoneutral zone (i.e., 26°C in Zebra Finches vs. 15-18°C in House Sparrows, House Finches and Hoopoe Larks). Thermogenesis has been found to induce much larger

increases in metabolic rate in a wide range of species. For example, in two different free-living populations of wintering Great Tits (*Parus major*) thermogenesis represented a 50% increase in metabolic rate over cold-acclimated values (Broggi *et al.*, 2004). Furthermore, heat production (measured at 5°C) by cold-acclimated captive House Sparrows increased resting metabolic rate by approximately 100% above values measured at thermoneutrality (Carleton and Martinez del Rio, 2005). Therefore, the metabolic cost of thermogenesis found in this study (i.e., 65-68% increase in RMR over cold-acclimated values) was comparable to values for other passerine species.

Locomotor activity of warm-acclimated Zebra Finches decreased by 64% from the non-breeding to egg producing stage, but daily seed consumption remained unchanged, confirming a previous study which found that laying Zebra Finches decrease activity by 57% with no associated change in food intake (Vézina, Speakman and Williams, unpublished data). Vézina and colleagues also found that the daily energy expenditure of warm-acclimated Zebra Finches did not change between the non-breeding and egg-producing stages (unpublished data). Taken together, these findings suggest that the 57-64% decrease in locomotor activity of warm-acclimated, egg-producing birds, along with other potential physiological or behavioural energy reallocation strategies, may conserve sufficient energy to meet the energy demands of egg production. Consequently, it seems as though egg-laying Zebra Finches do not need to increase energy intake under favorable environmental conditions.

Cold-acclimated, non-breeding Zebra Finches also decreased locomotor activity (by 72% of warm-acclimated values), suggesting that Zebra Finches employ a common energy-saving behavioural modification to reallocate energy to fuel the metabolic costs of

reproduction and thermoregulation. These birds also increased energy intake by consuming 72% more seed than their warm-acclimated counterparts, an average of an additional 1.8 grams of seed per female per day, which is the equivalent of an additional 29 kJ per day that likely also goes towards fueling thermoregulation. Similarly, cold-acclimated (15°C) Hoopoe Larks consumed more food than larks maintained at a thermally neutral temperature (36°C) (Williams and Tieleman, 2000). Therefore, while Zebra Finches are capable of increasing food intake above levels generally observed in favorable conditions, they only do so in certain circumstances. Future studies that examine changes in daily energy expenditure due to exposure to cold ambient temperatures are needed to investigate whether the increase in energy intake by cold-acclimated Zebra Finches corresponds to an increase in daily energy expenditure due to exposure to cold ambient temperatures. Furthermore, future studies could also investigate the physiological mechanisms underlying variation in the occurrence of hyperphagia between different environmental conditions.

Interestingly, the RMR of thermoregulating females decreased by 11% with the addition of egg production. Consequently, while the metabolic costs of cold-acclimation and active thermogenesis were additive (over 3 x BMR), the combined metabolic costs of thermoregulation and egg production were not (2.6 x BMR, cf. 3.2 x BMR if the individual costs were additive). The decrease in RMR associated with egg production at low ambient temperatures may be due to the partial substitution of the heat increment of egg production, i.e., the heat produced as a by-product of the metabolic processes involved in egg formation, for thermostatic heat production, i.e., the production of heat to maintain body temperature. It has been shown that the heat increment of feeding, i.e., the

increase in metabolic rate following consumption of a meal (also referred to as specific dynamic action or diet-induced thermogenesis; Ricklefs, 1974; reviewed in Jobling, 1983; Aoyagi *et al.* 1990), can be used to substitute for thermostatic heat production in birds exposed to low ambient temperatures (Biebach, 1984; Meienberger and Dauberschmidt, 1992; Chappell *et al.*, 1997). While the mechanical and biochemical aspects of digestion, such as peristalsis and synthesis of digestive enzymes, are likely contributors to the heat increment of feeding (Carefoot, 1990), previous studies have reported that a large proportion of the increase in metabolic rate following ingestion of a meal is due to the assimilation of food, primarily accelerated rates of protein synthesis (Aoyagi *et al.*, 1990; Brown and Cameron, 1991a, b). Therefore, it is possible that the marked increase in protein synthesis associated with egg production, including the hepatic production of the egg yolk precursors, vitellogenin and yolk-targeted very-low density lipoprotein, and the oviducal production of egg albumen (Yu *et al.*, 1971; Yu and Marquardt, 1973; Gruber, 1972; Bergink, et al., 1974; Deeley, et al., 1975; Chan, 1983; Wallace, 1985; Burley and Vadehra, 1989; Walzem, 1996; Williams, 1998), as well as the additional protein required for the recrudescence and maintenance of reproductive tissues like the ovary and oviduct (Yu and Marquardt, 1973; Williams, 1998), could result in egg production-induced thermogenesis. Just as postprandial birds can partially substitute the heat increment of feeding for thermoregulation (Biebach, 1984; Meienberger and Dauberschmidt, 1992; Chappell *et al.*, 1997), laying females may be able to exploit this metabolic by-product of egg yolk precursor and albumen production to offset the costs of thermogenesis at low temperatures. This could therefore result in a decrease in heat production by cold-acclimated, egg-laying birds, which in turn could

lead to the observed decreases in energy intake (as less nutrients are required to fuel the cost of thermoregulation) and RMR of female Zebra Finches producing eggs at 7°C. However, it is not known whether the energy saved by the substitution of the heat increment of egg production for thermoregulation would be sufficient enough to allow these birds to decrease both energy intake, which itself generates heat through the heat increment of feeding, and metabolic rate while still maintaining egg production and body temperature at such a low ambient temperature.

Another, potentially more plausible, explanation for the decrease in RMR associated with egg production at low ambient temperatures is that the cold-acclimated, egg-producing Zebra Finches in this study were unable to maintain RMR above 3 x BMR. Drent and Daan (1980) suggested that the potential maximum sustainable metabolic rate in breeding birds was 4 x BMR. However, studies that have calculated the sustained metabolic scopes (maximum sustained metabolic rate / BMR) of different species of birds and mammals involved in a variety of activities have found marked inter-specific variation ranging from 1.3 to 6.7, with 'metabolic ceilings' that vary between different activities (Peterson *et al.*, 1990; Hammond and Diamond, 1997). Consequently, the addition of the energetically-demanding process of egg production may limit the energy available for thermoregulation and other concurrent physiological processes (e.g., digestion, immune function), leading to energy reallocation within an individual away from somatic maintenance to save energy (Deerenberg *et al.*, 1997, 1998; Weirsma and Verhulst, 2005). Evidence for this was the 22% decrease in seed consumption by cold-acclimated, laying birds compared to their non-breeding levels. This would suggest that the 11% decrease in RMR observed in thermoregulating, laying birds (cf. their non-

laying RMR measured during thermogenesis) was the result of reduced digestive activity and potentially concurrent decreases in other processes like thermogenesis and maintenance of immune function (Deerenberg *et al.*, 1997). A decrease in thermogenesis by females producing eggs at low ambient temperatures may result in use of facultative, rest-phase hypothermia, the regulated and reversible decrease in metabolic rate and body temperature below normothermic levels, which could potentially result in even more energy savings due to down-regulation of temperature-dependent enzymatic processes in addition to the energy saved by decreasing active heat production (reviewed in Reinertsen, 1996; McKechnie and Lovegrove, 2002). Unfortunately, examination of the differential use of hypothermia by non-breeding and egg-laying birds at low ambient temperatures could not be performed because body temperature was not measured in this study.

The cold-acclimated females in this study were able to maintain egg production at a level comparable to warm-acclimated females based on the reproductive variables measured. However, because of the small sample size used in this study with respect to breeding data (only 9 warm-acclimated and 12 cold-acclimated breeding pairs) and the high degree of inter-individual variation often observed in reproductive traits (e.g. egg size: Manning, 1978; Swennen and Van der Meer, 1992; Williams, 1994, 1996; Christians, 2002; egg composition: Arnold *et al.*, 1991; Williams, 1994; clutch size: Boyce and Perrins, 1987), it remains unclear whether prolonged exposure to cold ambient temperatures during egg production negatively influences reproductive effort in this species. A more in depth breeding study which utilizes a repeated-measures design to control for inter-individual variation and measures a variety of reproductive variables in

addition to those measured in this study, including egg quality (i.e., lipid, protein, and hormone composition) and egg and chick viability, is required to more thoroughly investigate the influence of exposure to cold ambient temperatures on reproductive effort in these birds.

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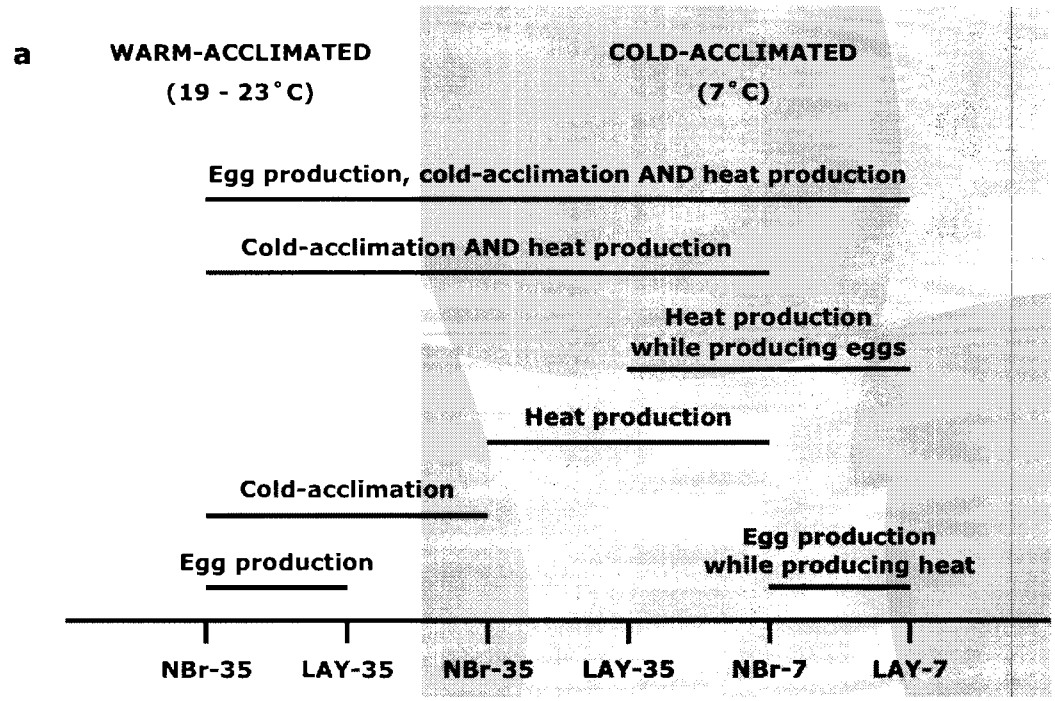
Table 4.1. Resting metabolic rate, seed consumption, locomotor activity and reproductive effort of Zebra Finches in different thermal-reproductive stages. Values are least squares means with sample size in brackets and minimum and maximum values in parentheses. Female body mass at each stage was included as a covariate for statistical analysis of RMR. Seed consumption and locomotor activity were log10 transformed for statistical analyses.

	Warm-acclimated		Cold-acclimated			
	NBr-35	LAY-35	NBr-35	LAY-35	NBr-7	LAY-7
VO_2 (ml O_2 / hour)	35.82 [9] (27.20 - 42.10)	44.59 [9] (40.64 - 58.59)	70.12 [12] (42.90 - 97.20)	61.72 [12] (36.39 - 77.96)	115.92 [12] (87.50 - 152.90)	103.53 [12] (82.18 - 121.95)

	Warm-acclimated		Cold-acclimated	
	NBr	LAY	NBr	LAY
Seed consumption (g / pair / day)	5.1 [23] (2.9 - 7.4)	5.2 [23] (2.5 - 7.2)	8.7 [12] (8.0 - 9.8)	6.8 [12] (2.6 - 10.8)
Locomotor activity (hops / pair / day)	1852 [9] (841 - 3760)	662 [9] (144 - 945)	520 [12] (266 - 975)	346 [12] (145 - 629)
Laying interval (days from pairing to egg laying)	--	5.4 [9] (4 - 7)	--	5.3 [12] (4 - 8)
Mean egg mass (g)	--	1.048 [9] (0.950 - 1.219)	--	1.068 [12] (0.930 - 1.225)
Clutch size	--	4.9 [9] (3 - 6)	--	4.6 [12] (2 - 6)

Figure 4.1. Study groups and the selected metabolic cost comparisons that were made between them for a) analysis of resting metabolic rate and b) analysis of locomotor activity and seed consumption. Horizontal lines connect thermal-reproductive stages for paired contrasts.



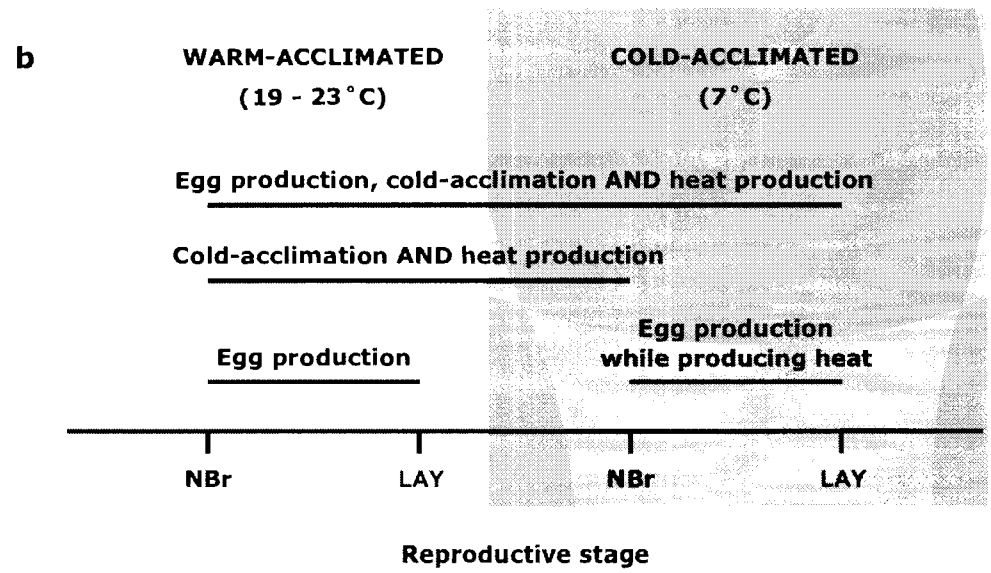


Figure 4.2. Resting metabolic rate (RMR) for warm- and cold-acclimated females as non-breeders and during egg production. RMR measured at 7°C represent values for actively thermoregulating birds. Sequential Bonferroni-correction for multiple comparisons and least squares means of RMR correcting for female body mass at each stage were used for statistical analyses. Lines join values for individual females, and gray circles represent means for each stage. Horizontal lines underneath P-values connect thermal-reproductive stages for paired contrasts.

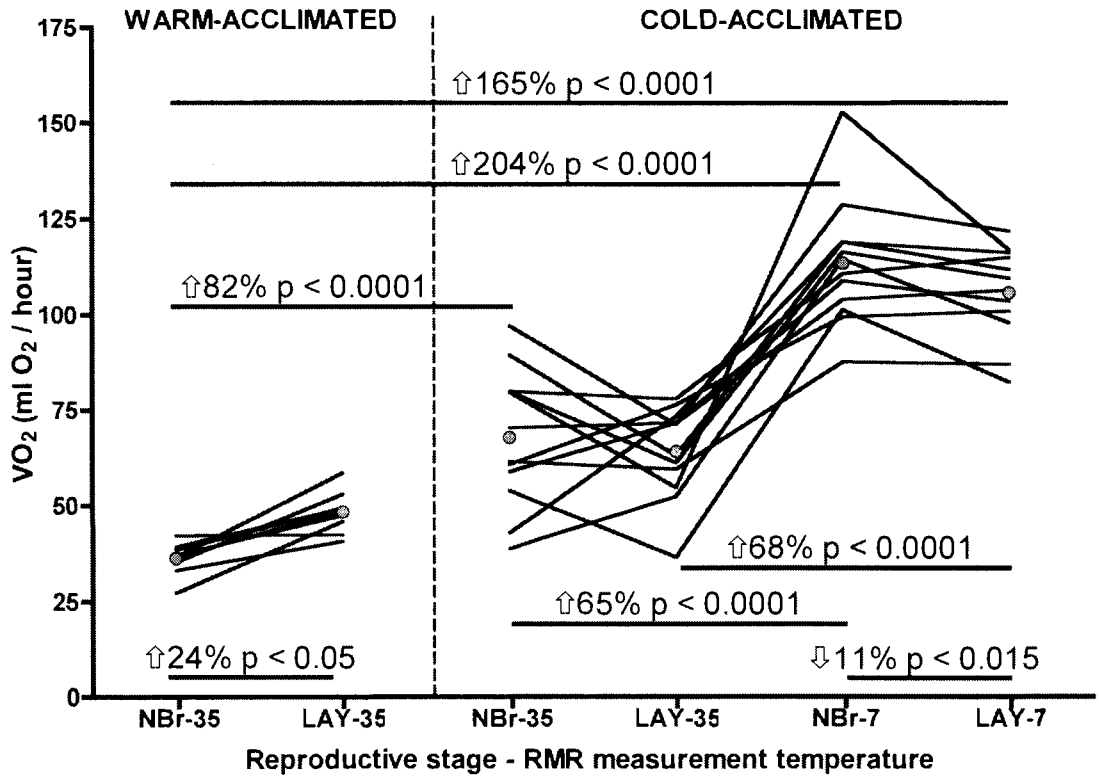
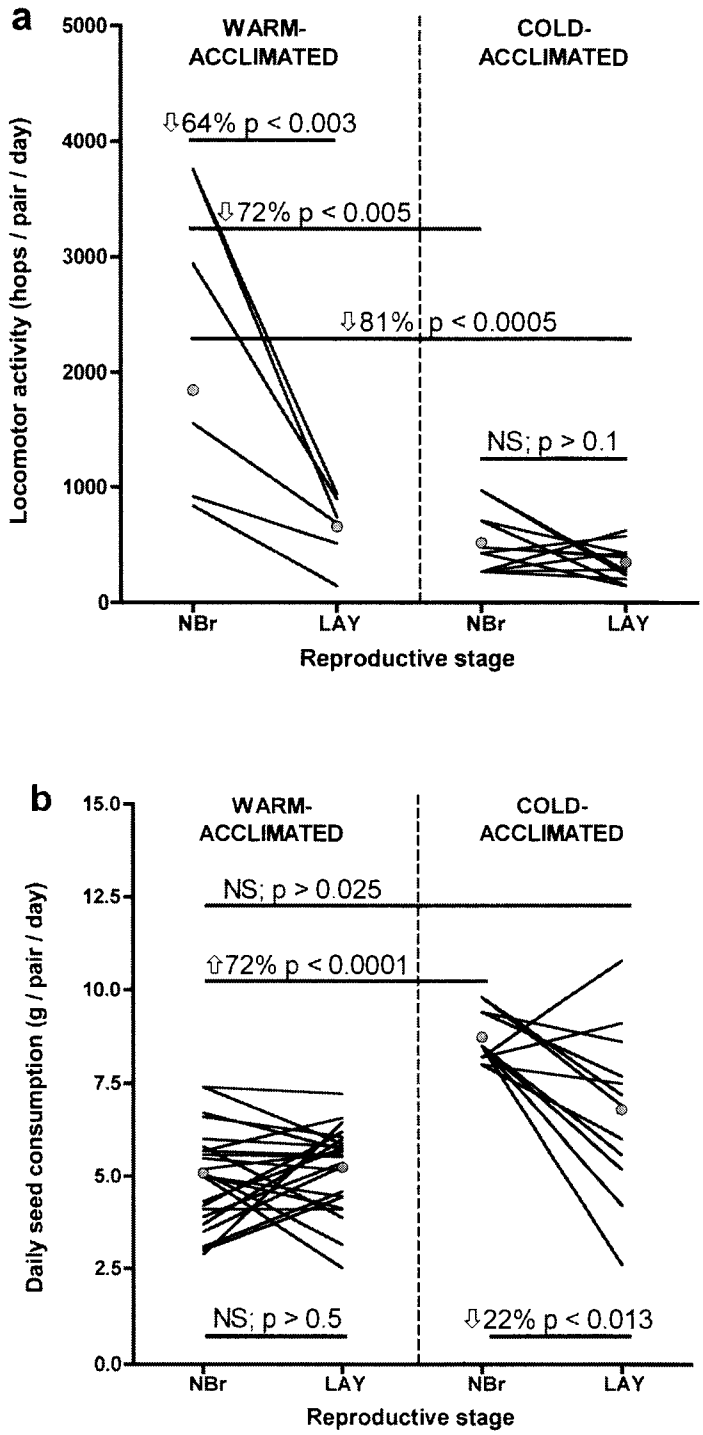


Figure 4.3. a) Locomotor activity and b) seed consumption by warm- and cold-acclimated females as non-breeders and during egg production. Sequential Bonferroni-correction for multiple comparisons was used for statistical analyses. Lines join values for individual females, and gray circles represent means for each stage. Horizontal lines underneath P-values connect thermal-reproductive stages for paired contrasts.



CHAPTER 5

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 dynamics in Zebra Finches (*Taeniopygia guttata*), and investigated the relationships between temperature-induced variation in these traits. When faced with egg production at 7°C, laying Zebra Finches increasing energy intake by 15.85 kJ per day, and were thus able to maintain body condition (e.g., body mass, fat and muscle score) and circulating triglyceride 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 vs. 0.95 eggs/day). These temperature-related declines in reproductive effort were accompanied by decreases in modal (from 36.6 at 21°C to 24.3 nm at 7°C) and median 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 effort was not related to any measure of VLDL particle diameter distribution

or circulating triglyceride levels. Therefore, other physiological traits that vary with temperature must be involved in the physiological mechanisms underlying regulation of reproductive effort of passerine birds producing eggs at low ambient temperatures.

INTRODUCTION

Animals require energy to fuel all aspects of life, including digestion, locomotion, thermoregulation, reproduction, and general maintenance. Trade-offs arise between these activities when resources, and therefore energy, are limited (Williams, 1966; Stearns, 1992; Bernardo, 1996; Zera and Harshman, 2001; Ricklefs and Wikelski, 2002). Bernardo (1996) recognizes three such trade-offs during reproduction: 1) the parental decision to allocate resources to offspring versus their own energetic needs, 2) the decision to allocate resources to many small or fewer large offspring, and 3) parent-offspring conflict over per-offspring investment. The first of these conflicts has implications for maternal survival and future reproduction, and may be of great consequence when periods of reproductive activity coincide with periods of energetic stress, e.g., increased energy demand or decreased energy availability brought about by fluctuations in environmental factors, such as exposure to inclement weather or extreme temperatures, decline in food availability, or human disturbance.

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).

Examination of the differential allocation of energy-rich lipids during avian egg production may give insight into the physiological basis of the 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) (Neilson and Simpson, 1973; Chan et al., 1976; Walzem, 1996; Walzem et al., 1999; Williams, 1998), represents a dramatic shift in lipid metabolism as the primary function of plasma VLDL particles changes from general lipid transport to tissues (e.g., muscle, adipose) for utilization or storage (involving generic VLDL), to supplying the developing egg yolks with energy-rich lipid for use by the growing avian embryo (involving VLDLy) (Walzem, 1996). Given that generic VLDL fuels maintenance activities, including thermoregulation and digestion, the shift to predominantly circulating VLDLy, as seen in egg-laying domestic fowl (Hermier et al., 1989; Walzem et al., 1994; Walzem, 1996), may compromise the condition of laying females during periods of high energetic demand. Consequently, modulation of the trade-off between reproductive effort 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) accentuate the potential trade-off between survival and current reproduction by increasing the energetic demands of egg-laying Zebra Finches (*Taeniopygia guttata*) by exposing them to different ambient temperatures, 2) determine the effects of exposure to low ambient temperatures on maternal body condition and reproductive effort (e.g., egg mass, clutch size, egg composition, laying rate) and 3) examine the physiological mechanisms underlying the temperature-dependent changes in maternal body condition and reproductive effort by investigating how low ambient temperatures influence lipid allocation to egg production and self-maintenance in egg-laying birds. We predicted that increasing the energy demands of laying females would result in either a) a shift in VLDL particle diameter distribution away from smaller VLDL particles (i.e., VLDLy) in favor of more larger, potentially generic, VLDL particles to fuel the added metabolic cost of thermoregulation, thereby potentially compromising current reproduction while maintaining female body condition, or b) maintenance of the production of VLDLy particles to sustain reproductive effort, resulting in other energetic compensation strategies by laying females, e.g., increasing energy intake (see Chapter 4) or reallocating energy away from behavioural activities like locomotor activity (see Chapter 4) or from other physiological processes like immune function (Deerenberg *et al.*, 1997) to fuel the energetic demands of thermoregulation, thus maintaining current reproduction while potentially compromising female survival by increasing the risk of depredation as females increase foraging activity to enhance energy

intake, or compromising female body condition or other aspects of non-reproductive physiology.

MATERIALS AND METHODS

Animals and Husbandry

Zebra Finches (*Taeniopygia guttata*) 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), and tarsus and bill measurements (± 0.1 mm) were taken. 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, Winnepeg), and maintained in controlled environmental conditions (humidity 75%, constant light schedule of 14L: 10D, lights on at 09:00, temperature described below) for at least one week in order to acclimate to the new environmental conditions. All birds received a mixed seed diet (Panicum and white millet, 50:50; approximately 12.0% protein, 4.7% lipid; Just for Birds, Surrey), water, grit, and cuttlefish bone (calcium) *ad lib*. 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 measured design was used; each female Zebra Finch was acclimated to and paired for breeding at both experimental temperatures (7°C and 21°C). The order

in which birds were exposed to the two experimental temperatures was randomized. Birds were rested in same-sex cages at 21°C for at least 45 days between the two acclimation-breeding trials. The pre-pairing acclimation period lasted one week during the 21°C trial and three to four weeks during the 7°C trial (one week at 14°C, one week at 10°C, then one to two weeks at 7°C). Acclimation periods were based on the time it took for all birds to return to and maintain their pre-acclimation body mass.

Following each acclimation period females were randomly paired with males. Breeding pairs were housed individually in cages (61 x 46 x 41 cm) equipped with an external nest box (15 x 14.5 x 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 to 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). Daily seed consumption of breeding pairs was measured as described in Chapter 4. Briefly, pairs were provided with 30.0 g of the mixed seed diet, which was weighed (± 0.1 g) and replaced daily between pairing and clutch completion. 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, and water, grit, and cuttlefish bone (calcium) *ad lib.* Data on laying interval, egg mass and clutch size were obtained by checking the nest boxes daily between 09:00 and 11:00. 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 substituted with replacement eggs to maintain original clutch size. Clutches were considered complete if no new eggs were laid over three 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

During the breeding trials at both experimental temperatures, 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 between 09:00 and 11:30 into heparinized capillary tubes. The blood samples were then expelled into EDTA-coated microcentrifuge tubes containing 0.5 M disodium-EDTA (3 µl; Sigma-Aldrich Canada, Oakville), and the tubes were centrifuged at 2200 g for 10 minutes in a Baxter Canlab Biofuge 13. The plasma from each sample was removed and placed into uncoated microcentrifuge tubes. The new tubes were centrifuged at 2200 g for 5 minutes. Sub-samples of each plasma sample were frozen for total triglyceride analysis (5 µl) and corticosterone (10 µl) analysis for another study, while the remainder of each plasma sample was placed into an EDTA-coated microcentrifuge tube containing 0.5 M disodium-EDTA (5 µl) for VLDL particle diameter distribution analysis. Sodium azide (1% w:v; Sigma-Aldrich Canada, Oakville) was added to each EDTA-coated tube to prevent mold formation (0.01 µl / µl plasma), and the plasma samples were refrigerated (4° C) pending analysis of VLDL particle diameter distribution.

Triglyceride Assay

Circulating concentrations of triglyceride were measured enzymatically as an index of total plasma VLDL (i.e., generic VLDL and VLDLy) (Serum Triglyceride Determination Kit, Sigma-Aldrich Canada, Oakville) using the method developed for domestic fowl (Mitchell & Carlisle 1991) and validated for passerines (Williams & Christians 1997; Williams & 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 340i microplate reader.

VLDL Particle Diameter Distribution

Plasma VLDL Isolation and Dynamic Laser Light Scattering

Plasma VLDL was isolated as the d < 1.020 g/mL fraction of plasma following the method described in Chapters 2 and 3. Briefly, the plasma samples and a blank control sample (NaCl density solution, d = 1.0063; equivalent salt density of undiluted plasma) were combined with NaCl-NaBr density solution (d = 1.0255) and centrifuged at 148600 *g* for 18 hours at 14°C in a Beckman L8-70M ultracentrifuge (Beckman Coulter, Fullerton). Following centrifugation, the supernatant containing the VLDL portion of the plasma was isolated from each tube by aspiration with 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 a UPA 250 and 7.02 analysis software (Microtrac, Clearwater) (described in Chapters 2 and 3; Veniant et al., 2000). 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 minutes for the blank solution, and 5 minutes in triplicate for each of the VLDL samples. 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 to 44 nm in diameter), which was based on the proposed sieving properties of the ovarian granulosa basal lamina of domestic fowl. To reach the plasma membranes of the developing ovarian follicles, VLDL particles must pass from capillaries within the ovary through pores in the ovarian granulosa basal lamina (Perry and Gilbert 1979; Griffin and Perry 1985). Only particles ranging from 25 to 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 (see Chapter 2). Additionally, the modal and median particle diameter and the range (i.e., width) of each distribution, in nanometers, were also determined.

Egg Composition Analysis

The second egg of each clutch was subjected to protein andlipid composition analysis following the method of Balzer and Williams (1998). Briefly, eggs were collected within 6 hours of being laid, boiled for 3 minutes, and frozen (at –20°C) until further analysis. Frozen eggs were thawed and separated into shell, albumen, and yolk, which were dried to constant weight 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 sohxlet extraction for 8 hours 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 composition 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 was calculated as the percentage of each component in relation to the fresh mass of the egg without the component of interest to control for part-whole correlations (e.g., percent yolk lipid = [yolk lipid, g / (fresh egg mass, g – yolk lipid, g)] *100; 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; Zarr, 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: $F_{1,23.1} = 3.23$, $p > 0.05$; Figure 5.1a), fat score (temperature with body mass at pairing as a covariate: $F_{1,22.2} = 0.20$, $p > 0.6$; Figure 5.1b), and muscle score (temperature: $F_{1,15.8} = 0.89$, $p > 0.3$; Figure 5.1c) 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 ($F_{1,13.2} = 13.83$, $p < 0.0025$; Figure 5.1d). The additional 1.9 grams of seed per day corresponded to an additional 0.21 g protein, 0.08 g lipid, and 1.44 g carbohydrate, the equivalent of an

additional 31 kJ per day for breeding pairs at 7°C. Therefore, based on the observation by Williams and Ternan (1999) that females ate slightly more seed (4.5%) than males throughout the laying sequence, laying females consumed an additional 15.85 kJ per day at 7°C.

Plasma Triglyceride, VLDL Particle Diameter Distribution, and VLDLy

Circulating triglyceride 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 ($F_{1,12.9} = 9.50$, $p < 0.01$; Figure 5.1e) and median VLDL particle diameters ($F_{1,8.96} = 6.23$, $p < 0.05$) and a smaller proportion of VLDL particles that fell within the sVLDLy range at 7°C than at 21°C ($F_{1,16.6} = 8.01$, $p < 0.025$; Figure 5.1f),.

Reproductive Effort

Decreasing ambient temperature changed the relationship between female body mass at the 1-egg stage and the average mass of subsequently laid eggs (temperature x female body mass at the 1-egg stage interaction: $F_{1,25.4} = 5.56$, $p < 0.05$); mean egg mass was positively related to female body mass at 21°C ($F_{1,27} = 16.40$, $r^2 = 0.3779$, $p < 0.0005$), but was not related to body mass at 7°C ($F_{1,25} = 1.41$, $p > 0.2$) (Figure 5.2). Temperature also influenced other measures of reproductive effort; when producing eggs at 7°C, females laid an average of 0.4 fewer eggs ($Q = 4.765$, $p < 0.05$; Figure 5.3a), took approximately 0.5 days longer to produce the first egg of the clutch ($Q = 5.000$, $P < 0.025$; Figure 5.3b), decreased laying rate by 5% (i.e., laid 0.90 eggs/day vs. 0.95

eggs/day at 21°C; Q = 4.571, $p < 0.05$; Figure 5.3c), 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 (Figure 5.3d), water content, dry albumen and yolk mass, yolk lipid content (Figure 5.3e), and albumen protein content were all independent of the ambient temperature in which the females were producing eggs ($p > 0.2$ in all cases). However, females laid eggs with more yolk protein (an average of 4.3 mg) at 7°C than at 21°C (i.e., a 5% increase in yolk protein content above eggs laid at 21°C; $F_{1,23.1} = 4.79$, $p < 0.05$; Figure 5.3f).

The inter-individual variation in clutch size, mean egg mass, laying interval and laying rate was not related to variation in circulating triglyceride 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

By consuming 45% more seed per day when paired at 7°C, the equivalent of an additional 15.85 kJ of energy per day, laying females were able to maintain body condition throughout egg production. However, despite the increase in energy intake, they did not maintain reproductive effort; females took longer to initiate egg laying and ultimately laid fewer eggs at a slower rate at 7°C. The captive Zebra Finches in this study

were able to increase seed consumption with relatively few additional costs, such as the energetic costs related to digesting more food (e.g., increasing the size or activity of digestive tissues; Williams and Tieleman, 2000; Nilsson, 2002; Peirsma, 2002). This is in contrast to free-living birds, which would face a variety of additional costs associated with increasing the number or duration of foraging bouts in order to increase food intake, including the metabolic costs associated with searching for food and flight (Nudds and Bryant, 2000; Jodice *et al.*, 2003; Wiersma *et al.*, 2005) and the fitness costs associated with longer and/or more foraging bouts, such as the increased risk of depredation (Krebs and Davies, 1987). Therefore, maintaining body condition and sustaining egg production at low ambient temperatures would be even more difficult for free-living birds, and it is likely that these birds would exhibit even more dramatic declines in reproductive effort than those found in this study or deterioration of maternal body condition, or a combination of both.

Interestingly, while decreasing ambient temperature had no effect on the lipid composition of the eggs, 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 is 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 variables levels of plasma vitellogenin than chickens that laid more regularly, and therefore 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. Furthermore, increased vitellogenin availability could also have implication for lipid deposition into egg yolk, as vitellogenin and VLDLy use the same oocyte receptor for uptake into the developing ovarian follicles (George *et al.*, 1987; Stifani *et al.*, 1988; Barber *et al.*, 1991). Future studies that assess circulating levels of vitellogenin in laying females acclimated to different temperatures are required 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 nighttime 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 boxed 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 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 Byant, 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. These results demonstrate the variety of ways in which females producing eggs in sub-optimal conditions can modulate reproductive effort 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 and predicted environmental conditions.

The declines in reproductive effort of Zebra Finches maintained at 7°C were accompanied by a decrease in the proportion of sVLDLy particles in circulation, which 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). During lipoprotein-lipase metabolism of VLDL, particle diameter decreases as the triglycerides 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. These results suggest that the observed declines in the reproductive effort 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. However, there was no relationship between the observed inter-individual variation in the different measures of reproductive effort and the proportion of sVLDLy particles in circulation. Although, the 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, i.e., contained apolipoprotein VLDL-II, which increases the lipoprotein lipase-resistance of VLDLy, thereby protecting it for use in egg production (reviewed in Walzem 1996). The lack of relationship between circulating sVLDLy particles and reproductive effort of females laying at 7°C suggests that other physiological factors, which also differ with temperature, are involved in the mechanisms underlying temperature-dependent variation in the reproductive effort of Zebra Finches.

A possible explanation for the observed decline in the reproductive effort 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 (see Chapter 4). 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 (reviewed

in 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). Furthermore, reproduction in most seasonally-breeding animals is scheduled such that the period of offspring care coincides with seasonal peaks in food availability and quality (Perrins, 1970). In birds, the annual recrudescence, i.e., regrowth, of the reproductive axis occurs well in advance of the period of offspring care. However, 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, 1973). Therefore, if laying females use environmental conditions during egg production such as ambient temperature 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 down-regulation of early reproductive effort. Data on energy budgets of individual females producing eggs at different temperatures are needed to determine whether the observed changes in reproductive performance were due to limited energy availability. 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 (e.g., through egg removal; Williams and Miller, 2003), and then examine whether experimentally increasing clutch size has a detrimental effect on offspring growth and survival in sub-optimal (i.e., cold) conditions.

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Figure 5.1. Comparisons of a) changes in female body mass, b) changes in female fat score, and c) changes in 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.

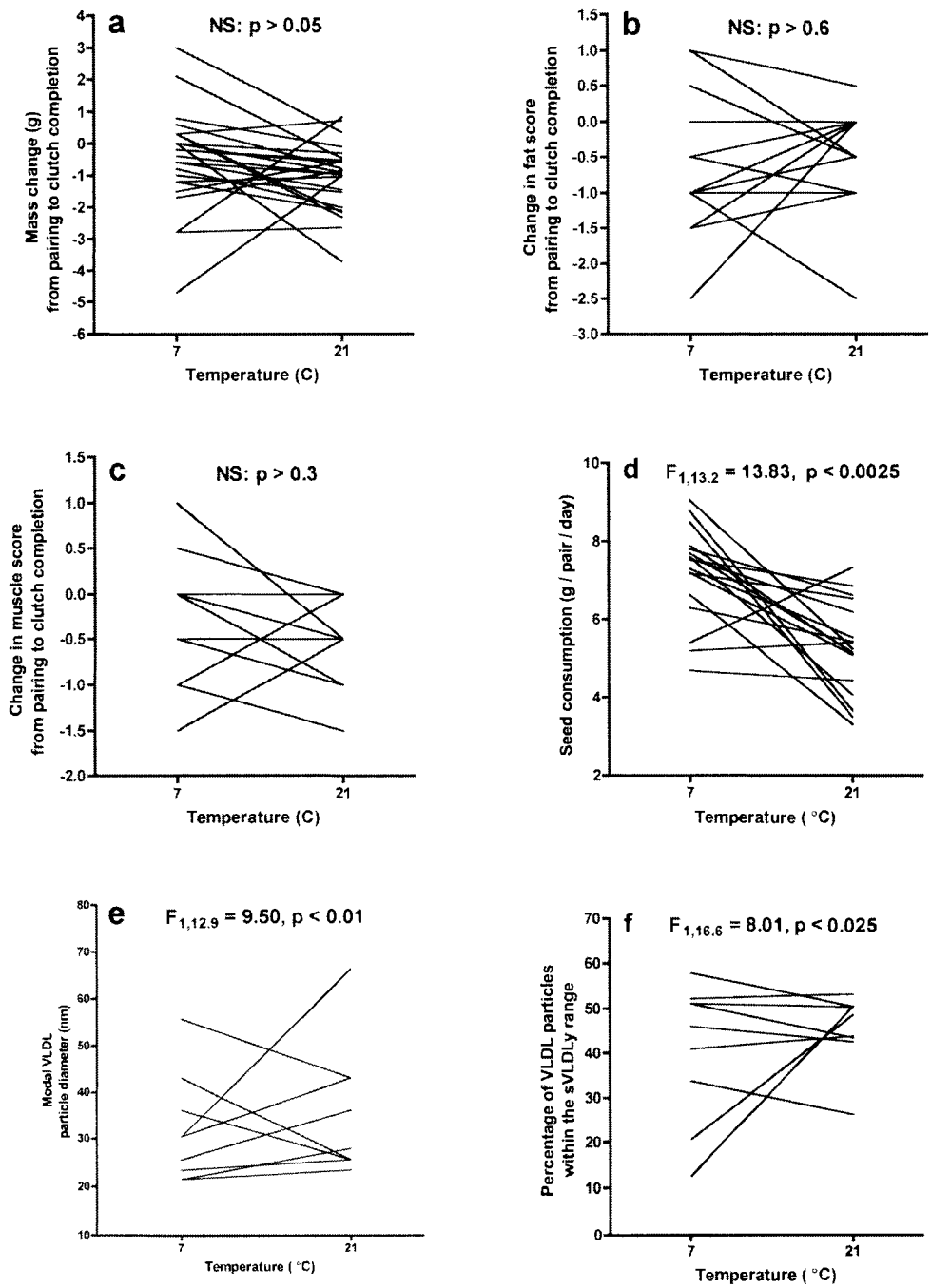


Figure 5.2. Relationships between female body mass at the 1-egg stage and the mean egg mass of subsequently laid eggs at 7°C and 21°C.

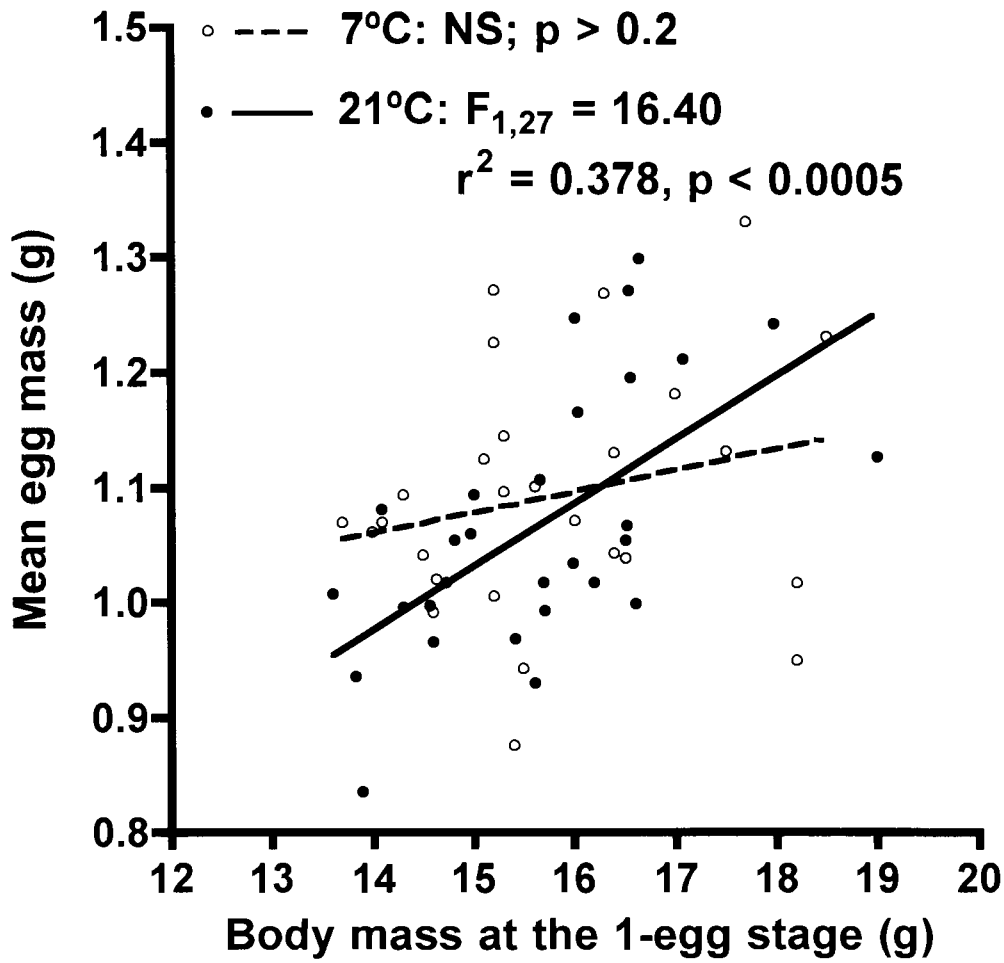
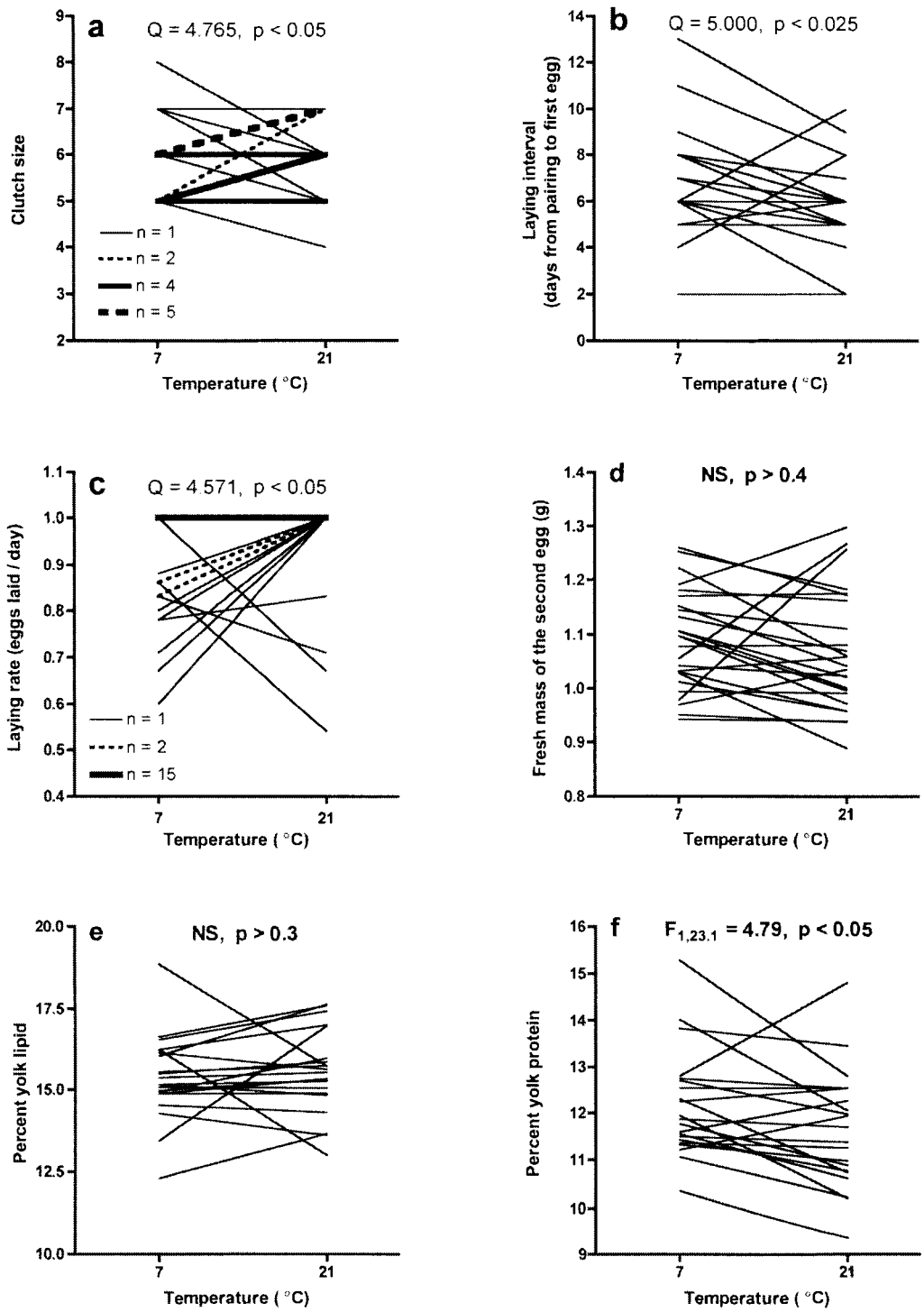


Figure 5.3. Temperature-induced changes in a) clutch size, b) laying interval, c) laying rate, and d) the fresh egg mass, e) yolk lipid content, and f) yolk protein content of the second egg of females producing eggs at 7°C and 21°C. Lines join values for individual females.



CHAPTER 6

ASSESSMENT OF ANTIBODIES FOR THE QUANTIFICATION OF APOLIPROTEIN VLDL-II IN ZEBRA FINCHES AND CHICKENS

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SUMMARY

During avian egg production estrogen induces changes in lipoprotein metabolism, including stimulating the production of the egg yolk precursor, yolk-targeted very-low density lipoprotein (VLDLy). This involves the up-regulation of the synthesis of the two apolipoproteins associated with VLDLy, apolipoprotein B (apoB), which acts as the ligand for the oocyte VLDL receptor, and apolipoprotein VLDL-II (apoVLDL-II), which helps preserve VLDLy for incorporation into the developing ovarian follicles by increasing the resisitance of VLDLy to hydrolysis by extra-ovarian tissues. The apoVLDL-II content of VLDL particles is required to quantify changes in circulating VLDLy due to changes in reproductive status or environmental conditions. Therefore, the aim of this study was to test polyclonal antibodies raised against chicken apoVLDL-II to determine whether they could be used to quantify apoVLDL-II in passerine birds. Expression of chicken apoVLDL-II in laying hens was higher than the expression of proteins of similar electrophoretic mobility from laying Zebra Finches, i.e., putative Zebra Finch apoVLDL-II. The two antisera tested in this study recognized chicken apoVLDL-II, but cross-reacted less strongly with putative Zebra Finch apoVLDL-II, suggesting that there was either very little apoVLDL-II for the antisera to react with, or the structure of Zebra Finch apoVLDL-II may differ from that of chicken apoVLDL-II, or a combination of these two factors. We discuss the implications of the potentially non-conserved nature of apoVLDL-II in the context of avian egg production.

INTRODUCTION

Avian reproduction is regulated by a cascade of neuroendocrine and systemic hormones that up-regulate reproductive physiology, including the recrudescence of reproductive organs and tissues and the production of large amounts of protein and lipid in the form of albumin and the yolk precursors, vitellogenin (VTG) and yolk-targeted, very-low density lipoprotein (VLDLy), which will provide the nutrients and energy required by the developing avian embryos (Gruber, 1972; Bacon *et al.*, 1974; Bergink *et al.*, 1974; Deeley *et al.*, 1975; Neilson and Simpson, 1973; Chan *et al.*, 1976; Wallace, 1985; Griffin and Hermier, 1988; Walzem, 1996; Williams, 1998). With respect to VLDLy, specific changes to the composition and structure of VLDL particles are stimulated by elevated levels of endogenous estrogens. Specifically, estrogen up-regulates the production of the two apolipoproteins that are associated with VLDLy, apolipoprotein B (apoB) and apolipoprotein VLDL-II (apoVLDL-II) (Chan *et al.*, 1976; Kudzma *et al.*, 1979; Capony and Williams, 1980; Lin and Chan, 1981; Dashti *et al.*, 1983; Lin *et al.*, 1986). While apoB acts as the ligand for the oocyte receptor (Nimpf *et al.*, 1988), apoVLDL-II is believed to be responsible for the decrease in VLDLy diameter observed in domestic fowl (Schneider *et al.*, 1990; Walzem, 1996; Walzem *et al.*, 1999), although the mechanism for this is unknown. This change in VLDLy size allows these lipid-rich particles to access the developing ovarian follicles by enabling them to fit through pores in the ovarian granulosa basal lamina that separates the follicles from capillaries within the ovary (Perry and Gilbert, 1979; Griffin and Perry, 1985).

Furthermore, the presence of apoVLDL-II on VLDLy increases the resistance of the lipoprotein to hydrolysis by lipoprotein lipase (LPL) in extra-ovarian tissues (Schneider *et al.*, 1990), likely by limiting access to the water needed for triacylglycerol hydrolysis (Boyle-Roden and Walzem, 2005), thereby preserving VLDLy for incorporation into developing ovarian follicles.

Previous studies have reported positive relationships between both reproductive status (i.e., non-breeding vs. laying) and reproductive output (e.g., laying rate) in birds and estimated VLDLy abundance based on the proportion of VLDL particles within a particular diameter range indicative of small particles that have access to the developing ovarian follicles (reproductive status of chickens, *Gallus gallus domesticus*: Walzem, 1996; Walzem *et al.*, 1999; Chapter 2; reproductive status of Zebra Finches, *Taeniopygia guttata*: Chapter 2; reproductive effort of laying chickens: Walzem, 1996; Chapter 3). However, VLDL particle diameter alone is not sufficient to distinguish between circulating VLDLy and generic VLDL particles. To truly quantify circulating concentrations of VLDLy and to assess the validity of previous estimates of VLDLy abundance, determination of the apoVLDL-II content of VLDL particles of different diameters is required. Therefore, the goal of this study was to test new and existing polyclonal antibodies for the quantification of apoVLDL-II in passerine birds, using the Zebra Finch as a model species.

MATERIALS AND METHODS

ApoVLDL-II Antisera

Two different antisera were used in this study. Rabbit anti-chicken apoVLDL-II was kindly donated by Dr. Wolfgang Schneider (Vienna, Austria) (Nimpf *et al.*, 1988), and will hereafter be referred to as anti-apoVLDL-II. Additionally, polyclonal antibodies were generated against a synthetic composite peptide that was based on a relatively highly conserved 17-amino acid portion of the apoVLDL-II (and apovitellenin I, see below) sequences of chicken (*Gallus gallus domesticus*; National Center for Biotechnology Information (NCBI) accession numbers: AAA48596, P02659, NP_990814, P02659), turkey (*Meleagris gallopavo*; P02660), duck (*Anas platyrhynchos*; AAV65597), common quail (*Coturnix coturnix*; AAB37468), and emu (*Dromaius novaehollandiae*; P02657) that were identified and compared using the algorithm BLASTP (a Basic Local Alignment Search Tool - Peptide) operated by the NCBI (Altschul *et al.*, 1997; Schäffer *et al.*, 2001) (Table 6.1). Dugaiczyk *et al.* (1981) determined that the amino acid sequences of apoVLDL-II from laying hen blood and apovitellenin I from chicken egg yolks were identical, suggesting that a some proportion of circulating apoVLDL-II may be transported intact from the blood to the yolk. Peptide synthesis and antibody production and purification was carried out by ABR Affinity Bioreagents (Golden, CO). The most common amino acids at each of the 17 positions within the most conserved sequence range were chosen to make up the sequence of the synthesized peptide with two exceptions: the leucine at position 11 and the serine at position 13 were selected instead of the more common alanine due to the difficulty in synthesizing peptides with consecutive alanine residues (ABr Affinity Bioreagents

Technical Services, personnal communication; Table 6.1). The polyclonal antibodies to the synthetic peptide (hereafter referred to as anti-peptide) were raised in two New Zealand White rabbits by subcutaneous injection of the synthesized peptide conjugated to glutaraldehyde and attached to keyhole limpet hemocyanin as the carrier protein / antigen. For the first injection the antigen was mixed with Freund's complete adjuvant, and for successive booster injections, with Freund's incomplete adjuvant (days 21, 35, and 49). Serum was collected on days 59 and 63, and the antibodies (IgGs) were isolated and purified peptide affinity purification, yielding IgGs specific for the peptide antigen (i.e., anti-peptide).

Zebra Finch Husbandry and Blood Sample Collection

Zebra Finches (*Taeniopygia guttata*) were housed in same-sex cages (122 x 46 x 41 cm) in the Simon Fraser University Animal Care Facility under controlled environmental conditions (temperature 19-23°C, humidity 35-55%, constant light schedule of 14L: 10D, lights on at 07:00). All birds received a mixed seed diet (Panicum and white millet, 50:50; approximately 12.0% protein, 4.7% lipid; Just for Birds, Surrey), water, grit, and cuttlefish bone (calcium) *ad libitum*, a multivitamin supplement in the drinking water once per week. All experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit (no. 692B-94) following the guidelines of the Canadian Committee on Animal Care.

Randomly chosen non-breeding males (n = 75) were given four daily IM injections (30 µl each) of 17β-estradiol (E2) in 1,2-propanediol (2830 µg/ml; equivalent to 5 mg E2 / kg bird, assuming an average mass of 17 g for all birds; Sigma-Aldrich Canada, Oakville). This dose has been shown to significantly elevate plasma yolk

precursor levels in Zebra Finches (Williams and Martyniuk, 2000). E2-treated birds were anesthetized 24 hours after the last injection via an IM injection of 50µl ketalean and xylazine solution (50:50 by volume; Associated Veterinary Products, Abbotsford). Blood samples were collected between 10:30 and 13:00 by exsanguination through the jugular vein. All blood samples were expelled into heparin-coated microcentrifuge tubes (1000 U/ml; Sigma-Aldrich Canada, Oakville) and centrifuged at 2200 g for 10 minutes in a Baxter Canlab Biofuge 13. The individual plasma samples were then combined into one plasma pool (E2-treated plasma pool) and refrigerated overnight (at 4°C) pending isolation of apoVLDL-II.

Randomly chosen non-breeding (n = 10) and egg-producing (n = 20) females were blood sampled (200 µl from the brachial vein). All blood samples were collected between 09:00 and 11:30 into heparinized capillary tubes, expelled into heparin-coated microcentrifuge tubes, and centrifuged at 2200 g for 10 minutes in a Baxter Canlab Biofuge 13. The individual plasma samples were combined into the appropriate plasma pools, i.e., non-breeding (NBr) and egg-producing (LAY), in microcentrifuge tubes and frozen (at -20°C) pending electrophoresis and Western blot analysis.

ApoVLDL-II Isolation and Purification

Plasma VLDL was isolated as the d < 1.020 g/ml fraction of plasma from E2-treated males. The E2-treated plasma pool (24 ml) was mixed with NaCl-NaBr density solution (93 ml, d = 1.0255), and transferred into Quick-Seal Ultra-Clear tubes (25 x 89 mm, Part #344326; Beckman Coulter, Fullerton). The samples were loaded into a Beckman VTi50 rotor and centrifuged at 109000 g for 18 hours at 14°C in a Beckman L 8-70 ultracentrifuge (Beckman Coulter, Fullerton). Following centrifugation, the

supernatant containing the VLDL portion of the plasma was isolated from the tube using a syringe.

ApoVLDL-II was isolated and purified following the method described by Nimpf *et al.* (1988). Briefly, the VLDL sample was dialyzed against deionized, distilled water (at 4°C) and lyophilized. The remaining dry residue was delipidated by consecutive extractions with ice-cold chloroform-methanol (2:1, v/v), diethylether-ethanol (3:1, v/v), and diethylether alone. The residue was dried under nitrogen, and then lyophilized overnight. Freeze-dried apoVLDL-II was reconstituted by dissolving it in buffer (75 mM NaCl, 50 mM Tris-HCl, and 60 mM N-octylglucoside, pH 7.4) for a final concentration of 2 mg / ml. The solution was refrigerated (at 4°C) for 16 hours, and then centrifuged at 5000 g for 5 minutes. The resulting supernatant was dialyzed against deionized, distilled water overnight at 4°C, and then lyophilized. The reconstitution, centrifugation, dialysis, and lyophilization steps were repeated on the resulting apoVLDL-II residue.

SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out under reducing conditions in duplicate 1 mm thick gels (15% T, 2.6% C) in a Bio-Rad Mini Protean II unit (Life Sciences Bio-Rad Canada, Mississauga, ON) at 50 mA constant current for 50-55 minutes (Laemmli, 1970). Sample buffer (60 mM Tris-HCl, 25% glycerol, 2% SDS, 14.4 mM M 2-mercaptoethanol, 1% bromophenol blue) was used to dilute the freeze-dried apoVLDL-II, laying and non-breeding Zebra Finch plasma, synthetic peptide-spiked laying Zebra Finch plasma and chicken plasma pools (positive controls), and the sample solutions were boiled for 10 minutes. One gel from each pair was stained with Coomassie brilliant blue R 250 and destained with acetic acid: methanol: water

(10:45:45). Molecular weights were determined using full-range molecular weight markers (10000 to 250000 Da; RPN800 Full-range Rainbow molecular weight markers; Amersham Biosciences, GE Healthcare). The second gel from each pair was used for Western blot analysis.

Western Blot

Western blotting was performed to test the specificity of the antisera for Zebra Finch apoVLDL-II. Proteins were transferred onto PVDF membrane using a semi-dry electroblotting unit (LKB Novablot, Bjerrum-Shafer-Nielson buffer: 48 mM Tris, 29 mM glycine, 20% methanol, 0.0375 % SDS) at 30 mA constant current for 6 hours. Detection was carried out at room temperature. Blots were washed with PBS-T (phosphate-buffered saline, 0.1% Tween 20), and blocked for 40 minutes in 5% skim milk powder (Carnation) in PBS-T. The blots were incubated for 40 minutes in either anti-apoVLDL-II:PBS-T (1:5000) or anti-peptide:PBS-T (1:10000) and then washed extensively with PBS-T. The blots were then incubated for 40 minutes with a donkey anti-rabbit IgG secondary antibody conjugated to horse radish peroxidase (Amersham Biosciences, GE Healthcare; 1:5000 in PBS-T for blots previously incubated with anti-apoVLDL-II and 1:10000 in PBS-T for blots previously incubated with anti-peptide) and washed four times with PBS-T. Membranes were developed with ECL Western blotting detection reagents (minimum 0.125 ml / cm2 membrane; Amersham Biosciences, GE Healthcare) and Kodak BioMax Light-2 film (Amersham Biosciences, GE Healthcare).

RESULTS

The putative Zebra Finch apoVLDL-II was associated with the VLDL fraction of Zebra Finch plasma following ultracentrifugation, and had similar electrophoretic mobility to chicken apoVLDL-II under reducing conditions (large band at ~10 kDa in Lane D in Figure 6.1, cf. chicken apoVLDL-II: dark bands at ~10-11 and 14-15 kDa, Figure 6.1 Lane E). When laying Zebra Finch plasma was analyzed under reducing conditions, two faint bands were detected (~14 kDa and ~10 kDa; Lane B in Figure 6.1), while no comparable bands were observed in plasma from non-breeding Zebra Finches (Lane A in Figure 6.1). The faintness of these bands compared to the corresponding darker bands in the laying chicken samples (Lane E in Figure 6.1) suggest that apoVLDL-II expression is lower in laying Zebra Finches than in laying chickens. Many higher molecular weight protein bands, which probably corresponded to apoB-100, its degradation products, and potentially other VLDL apolipoproteins, were apparent in all of the plasma samples (Lanes A, B, D, and E in Figure 6.1).

The anti-apoVLDL-II antiserum clearly recognized two bands (at ~ 10 and ~15 kDa) corresponding to apoVLDL-II from chicken plasma (Lane E in Figure 6.2), but only cross-reacted weakly with proteins of similar molecular weight from laying Zebra Finch plasma (at ~10 kDa in Lanes A and B in Figure 6.2) and purified Zebra Finch apoVLDL-II (at ~10 kDa in Lane D in Figure 6.2). Furthermore anti-apoVLDL-II also recognized and bound to many of the high molecular weight proteins from the VLDL portion of the plasma (Lanes A, B, D, and E in Figure 6.2).

The anti-peptide antiserum weakly cross-reacted with the apoVLDL-II of laying chickens (at ~10 kDa in Lane E in Figure 6.3). However, it did not seem to recognize either the purified Zebra Finch apoVLDL- II (no band at ~10 kDa in Lane D in Figure 6.3) or proteins of similar molecular weight from laying Zebra Finch plasma (no band at ~10 kDa in Lane A or B in Figure 6.3). Like anti-apoVLDL-II, anti-peptide also recognized and bound to some of the high molecular weight proteins from the VLDL portion of the plasma (Lanes A, B, D, and E in Figure 6.3). Interestingly, the anti-peptide antiserum also recognized a protein band in the peptide-spiked Zebra Finch plasma sample (at ~5 kDa in Lane A in Figure 6.3), which did not seem to cross-react with the anti-apoVLDL-II antiserum (no band at ~5 kDa in Lane A in Figure 6.2). The synthetic peptide that was used to spike this sample was only 17 amino acids long, and was therefore much smaller than 5 kDa. Given the strong reaction between the ~5 kDa protein and the anti-peptide antiserum, the "protein" may be an aggregation of many of the small synthetic peptides.

DISCUSSION

ApoVLDL-II from Zebra Finches exhibited similar electrophoretic mobility as apoVLDL-II from chickens, but was expressed at lower levels. However, Western blot analysis revealed that polyclonal antibodies raised against either chicken apoVLDL-II or a peptide sequence closely resembling a relatively highly conserved portion of the chicken apoVLDL-II sequence (88% identity; 94% similarity, where identity indicates the same amino acid in a particular position, and similarity indicates the same amino acid

or an amino acid substitution that is believed to carry out similar functions and occurs more often as an alternative in related sequences than in random sequences; Dayhoff *et al.*, 1978) recognized chicken apoVLDL-II, cross-reacted strongly with higher molecular weight proteins associated with the VLDL portion of plasma, and either did not recognize or only weakly recognized putative apoVLDL-II from Zebra Finches. These results suggest that there may have been too little circulating apoVLDL-II in laying Zebra Finches to elicit binding with the antibodies used in the Western blot analyses (cf. high circulating levels of apoVLDL-II in laying chickens), the structure of Zebra Finch apoVLDL-II may differ sufficiently from that of chickens, or a combination of these factors may be occurring.

Differences in the sequence of apoVLDL-II between chickens and Zebra Finches would not be surprising as the structure of apoVLDL-II differs substantially between the five species of birds for which the sequence is known. For the most conserved region, the 17 amino acid sequence that was chosen for the synthetic peptide, the four non-chicken species varied between 76% (13 out of 17) and 94% (16 out of 17) identity with the chicken sequence (between 88% to 94% similarity) (see Table 6.1 for sequences and literature references). Furthermore, when comparing the mature protein (82 amino acids), chicken apoVLDL-II exhibited 95% identity (78/82 amino acids) with chicken egg (98% similarity), 85% identity (70/82) with turkey (91% similarity), 76% identity (63/82) with duck (84% similarity), 67% identity (55/82) with quail (80% similarity), and only 65% identity (65/82) with emu apoVLDL-II (79% similarity) (see Table 6.1 for sequences and literature references). Despite only 67% identity of the primary structure between chicken and quail apoVLDL-II and differences in protein conformation, i.e., chicken apoVLDL-II

exists as a homodimer while quail apoVLDL-II is incorporated into VLDLy as a monomer, polyclonal antibodies raised against chicken apoVLDL-II cross-reacted with quail apoVLDL-II, and both proteins fulfilled their function of inhibiting lipoprotein lipase (MacLachlan *et al.*, 1996). It is unknown to what extent Zebra Finch apoVLDL-II differs in identity or similarity to apoVLDL-II of other avian species, however an attempt to sequence Zebra Finch apoVLDL-II is currently in progress.

The extent of homology of the structure of apoVLDL-II in birds is similar to that of other apolipoproteins. Collet *et al.* (1997) compared the primary structure and antigenicity of apoA-I in humans, which is believed to be involved in cholesterol transport and activation of lecithin:cholesterol acyltransferase (LCAT) (Fielding *et al.*, 1972; Miller *et al.*, 1985; Fielding and Fielding, 1995), to the sequence in a variety of other mammalian species including cynomolgus monkeys, dogs, pigs, cows, rabbits, and rats. Human apoA-I ranged from being 95% identical (99% similar) to monkey apoA-I to 63% identical (85% similar) to rat apoA-I (Collet *et al.*, 1997). While the antigenicity of some regions of apoA-I was conserved, single amino acid substitutions were enough to prevent some of the monoclonal antibodies from cross-reacting with apoA-I from specific species (Collet *et al.*, 1997). However, in spite of the changes in antigenicity of some regions of this protein, the protein's secondary structure, and therefore likely its functional properties, remained more conserved (Collet *et al.*, 1997). Furthermore, Law and Scott (1990) compared the LDL receptor binding domain of apoB in humans, pigs, rabbits, hamsters, rats, mice and chickens. Homology between mammalian species ranged from 57% between mice and hamsters to 81% between rats and hamsters. The human sequence exhibited 64% to 69% homology with the other species, while the

chicken sequence exhibited the least amount of homology with each of the mammalian species compared to between-mammal comparisons, ranging from 55% with the hamster sequence to 62% with the human structure (Law and Scott, 1990). Despite the inter-specific variation in the primary structure of the LDL receptor binding domain of apoB, the secondary structure of this region was conserved, thereby maintaining its function as a receptor ligand (Law and Scott, 1990).

In general, apolipoproteins are amphipathic components of lipoproteins, meaning they have a lipophilic, non-polar region that interacts with the lipid within the lipoproteins, and a polar, hydrophilic region that interacts with the aqueous environment of general circulation. They play a variety of roles in lipoprotein transport and metabolism including acting as activator or inhibitor proteins and receptor ligands (Breckenridge *et al.*, 1978; Nimpf *et al.*, 1988; Schneider *et al.*, 1990; Boyle-Roden and Walzem, 2005). It has been suggested that homology of the primary structure of apolipoproteins is not of great importance as long as the functional properties of the apolipoproteins, such as their amphipathic nature, as well as their activating or inhibiting properties or ligand conformation, are maintained (Duggan and Callard, 2001). Therefore, if selection only acts on the functional aspects of these proteins, then changes to their primary structure that do not influence overall function will not be the focus of selection. For example, while the amphipathic phospholipid binding region of apoVLDL-II (residues 59 to 75; Chan *et al.*, 1980; Chan, 1983) exhibits less homology than the entire mature protein, ranging from 94% identity between chicken and turkey to a mere 44% identity between chicken and quail, the inter-specific similarity in the sequence of this region is comparatively high, ranging from 94% between chicken and turkey to 83%

between chicken and quail, thereby conserving the functional properties of this critical region. In this study, the female Zebra Finches that were sampled for the laying plasma pool were actively producing and laying viable eggs. Consequently, it is likely that Zebra Finch apoVLDL-II has maintained its ability to transport energy-rich lipoproteins to developing ovarian follicles. Therefore, it is possible that changes to the structure of apoVLDL-II will only be of consequence when it compromises egg production.

The potential lack of homology of apoVLDL-II, combined with the lower expression levels in laying Zebra Finches, and potentially other avian species, compared to laying chickens, and the affinity of the antibodies used in this study for the high molecular weight proteins (> 30 kDa) associated with the VLDL portion of Zebra Finch plasma pose problems for the quantification of VLDLy in the Zebra Finch, and possibly other non-chicken avian species, using antibodies that were raised against chicken apoVLDL-II. Therefore, the development of new antibodies that are raised against apoVLDL-II from species of interest, such as the Zebra Finch, is needed to be able to quantify circulating levels of VLDLy in these species.

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Table 6.1. Amino acid sequences of apoVLDL-II and apovitellenin I from chicken, duck, emu, quail and turkey. The numbering above the sequences is that of whole chicken apoVLDL-II protein (including the signal peptide). Those amino acids which were identical to the first chicken apoVLDL-II sequence have been left blank for clarity. Underlined letters indicate insertions, * indicates deletions, and – indicates no data for that portion of the sequence.

apoVLDL-II signal peptide

					-20										-10									
CHICKEN[a,b]	m	v	q	y	r	a	l	v	i	a	v	i	l	l	l	s	t	t	v	p	e	v	h	s
CHICKEN egg[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DUCK[d]											l													
DUCK egg[e]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EMU egg[c,f]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
QUAIL[g]						e					l													
TURKEY egg[h]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

mature apoVLDL-II / apovitellenin I protein

	1										10										20									
CHICKEN[a,b]	k	s	i	i	d	r	e		r	r	d	w	l	v	i	p	d	a	a	a	a	y	i	y	e	a	v	n	k	v
CHICKEN egg[c]																														
DUCK[d]				f	e		d												i							t				m
DUCK egg[e]				f	e		d												i							t				m
EMU egg[c,f]				f	e		d	n											v				v			t				m
QUAIL[g]	r		l	s			g						m		y				l		s								t	
TURKEY egg[h]				f	e	r	d												v											m
SYNTHESIZED PEPTIDE									r	r	d	w	l	v	i	p	d	a	l	a	s	y	i	y	e					
									1									10							17					

mature apoVLDL-II / apovitellenin I protein

	30											40										50					
CHICKEN[a,b]	s	p	r		a	g	q	f	l	l	d	v	s	q	t	t	v	v	s	g	i	r	n	f	l	i	n
CHICKEN egg[c]																			i		s						
DUCK[d]					v					a		a	a			p			v		t		t				r
DUCK egg[e]					v					a		a	a			p			v		t		t				r
EMU egg[c,f]	f		k		v					a		a	a		i	p		i	v		t						r
QUAIL[g]						a															a			l			r
TURKEY egg[h]										v		i									t						r

					60										70										80		
CHICKEN[a,b]	e	t	a	r	l	t	k	l	a	e	q	l	m	e	k	i	k	n	l	c	y	t	k	v	l	g	y
CHICKEN egg[c]												m	l														
DUCK[d]			t	k		s	l													w							
DUCK egg[e]			s	k			l													w							
EMU egg[c,f]			s	k		s	i					m				v		t		w	n						
QUAIL[g]			t	k	i		i		v			m	v				r	s	i	w	n	g		*			
TURKEY egg[h]							i													w					q		

[a] Dugaiczyk *et al.* 1981; [b] Chan 1983; [c] Dolpheide and Inglis 1976; [d] Yen *et al.* 2005; [e] Inglis and Burley 1977; [f] Dolpheide and Inglis 1974; [g] MacLachlan *et al.* 1996; [h] Inglis *et al.* 1979

Figure 6.1. SDS-polyacrylamide gel electrophoresis of non-breeding and laying Zebra Finch plasma, purified putative Zebra Finch apoVLDL-II, and chicken plasma. Lane A: non-breeding Zebra Finch plasma pool (1:6; sample:ddH_2O and sample buffer, v:v), Lane B: laying Zebra Finch plasma pool (1:25), Lane C: molecular weight markers, Lane D: putative apoVLDL-II purified from VLDL portion of the plasma of estrogen-treated male Zebra Finches (1 mg:100 µl), and Lane E: chicken plasma pool (1:25).

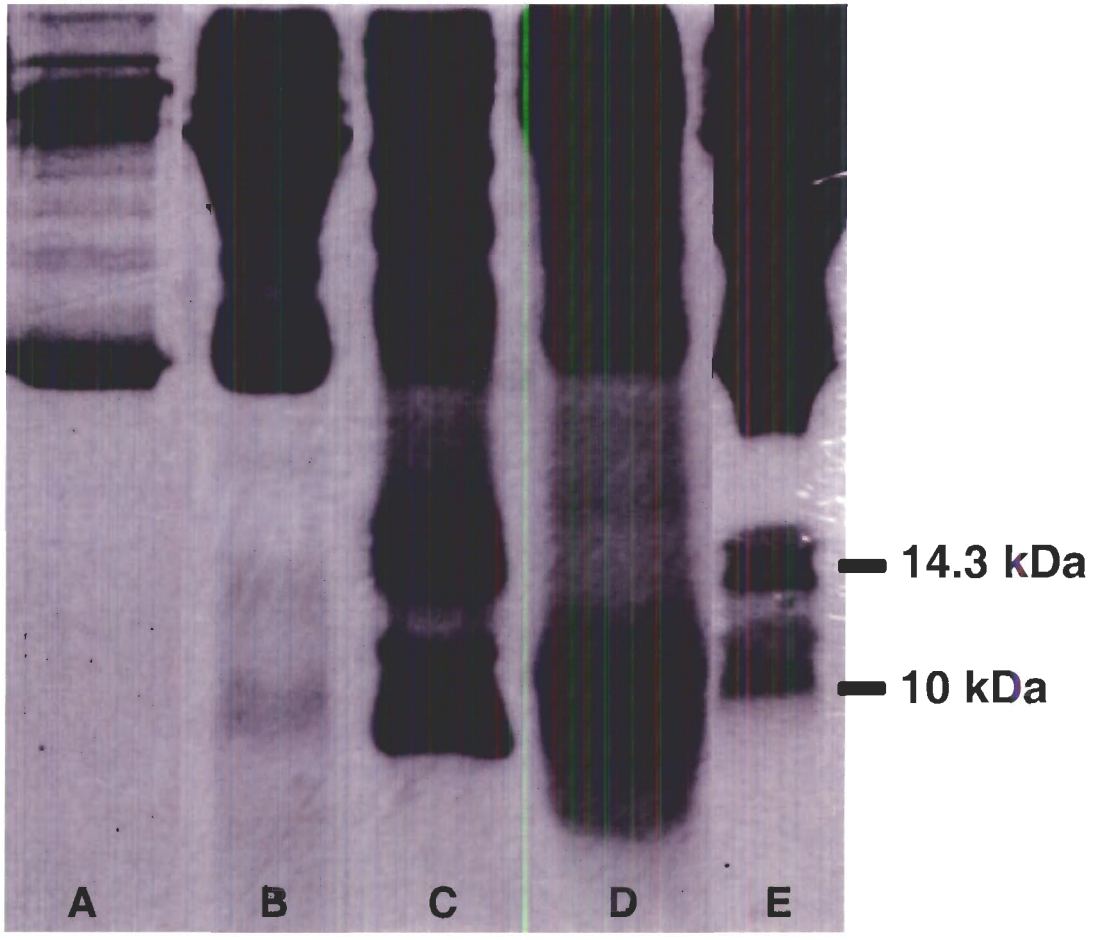


Figure 6.2. Western blot of Zebra Finch and chicken plasma and putative Zebra Finch apoVLDL-II using anti-chicken apoVLDL-II as the primary antibody (1:5000). Lane A: laying Zebra Finch plasma pool (1:25; sample:ddH_2O and sample buffer, v:v) spiked with the synthetic peptide (1 mg:100 µl), Lane B: laying Zebra Finch plasma pool (1:25), Lane C: molecular weight markers, Lane D: putative apoVLDL-II purified from VLDL portion of the plasma of estrogen-treated Zebra Finches (1 mg:100 µl), and Lane E: chicken plasma pool (1:25).

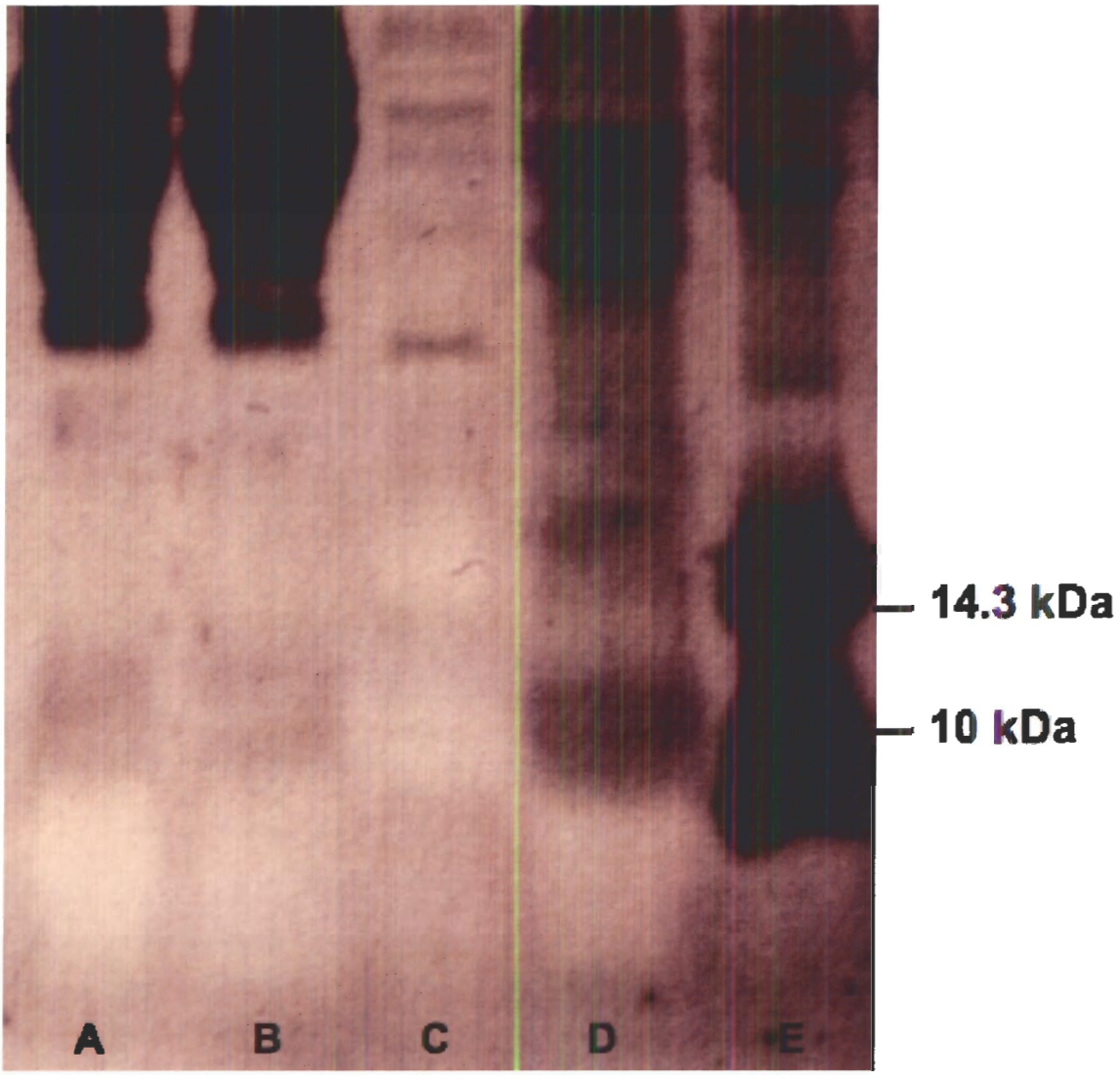
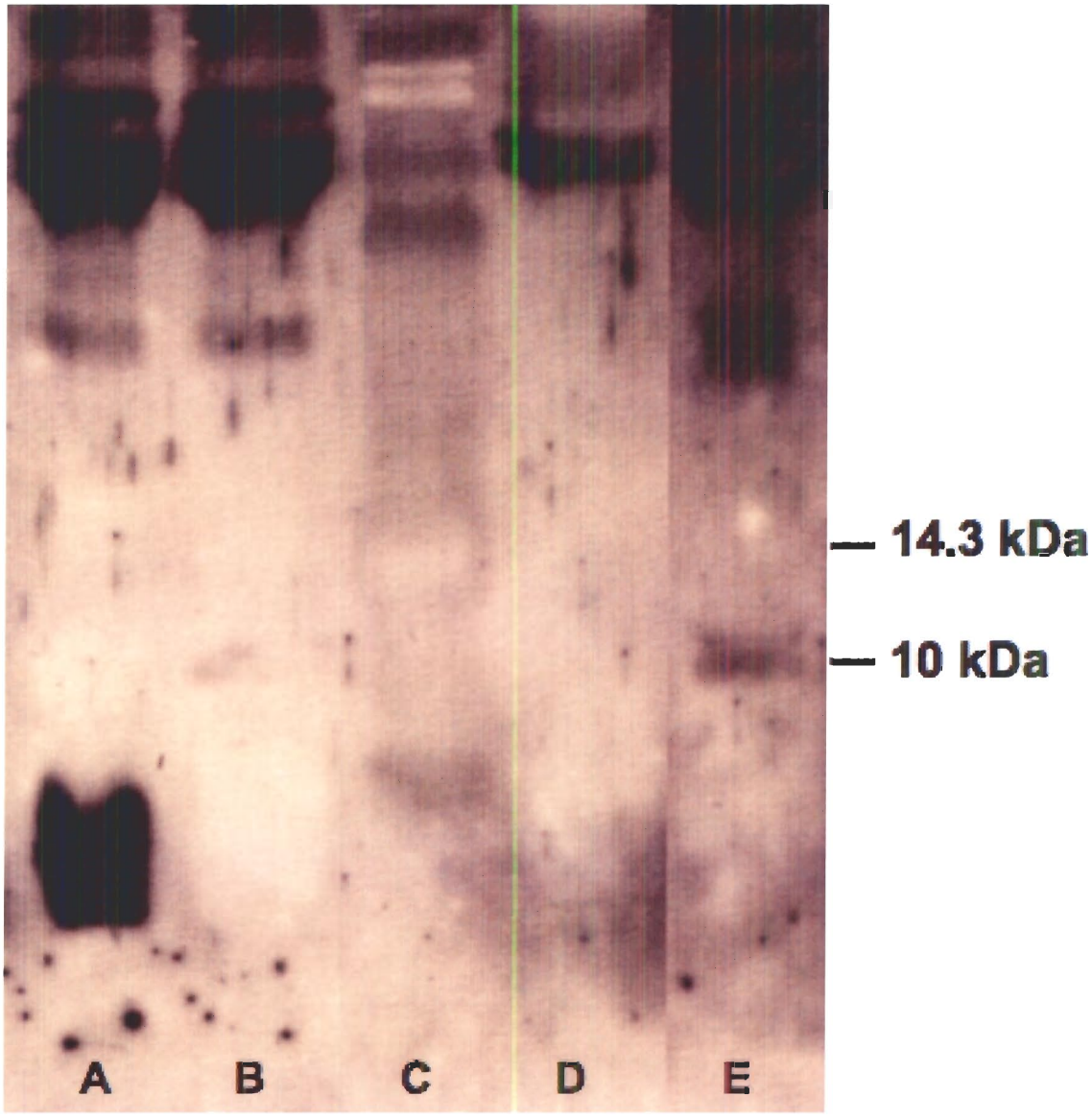


Figure 6.3. Western blot of Zebra Finch and chicken plasma and putative Zebra Finch apoVLDL-II using anti-peptide as the primary antibody (1:10000). Lane A: laying Zebra Finch plasma pool (1:25; sample:ddH_2O and sample buffer, v:v) spiked with the synthetic peptide (1 mg:100 µl), Lane B: laying Zebra Finch plasma pool (1:25), Lane C: molecular weight markers, Lane D: putative apoVLDL-II purified from VLDL portion of the plasma of estrogen-treated Zebra Finches (1 mg:100 µl), and Lane E: chicken plasma pool (1:25).



CHAPTER 7

GENERAL SYNTHESIS AND FUTURE DIRECTIONS

SYNTHESIS

The primary goal of the research described in this thesis was to gain insight into the physiological mechanisms underlying the allocation of energy-rich lipid between egg production in birds and maternal self-maintenance, i.e., the trade-off between current reproduction and survival, as mediated through maternal body condition (Stearns, 1992; Bernardo, 1996). While the results of the individual studies have been discussed in detail in the preceding chapters, in this final chapter they will be synthesized and discussed in terms of some of the questions posed in the beginning of the General Introduction (chapter one).

Is the pattern of lipid allocation to egg production that is observed in domesticated avian species common to all birds?

During egg production, egg-laying females of domesticated and non-domesticated avian species have two very different goals. In domesticated species that have been selected for maximizing egg production, the goal of laying females is to lay constantly and consistently over for a prolonged period of time, i.e., to produce one egg a day for months to over a year. Therefore, it is not surprising that in chickens and turkeys, the allocation of a majority of energy-rich lipids to reproduction, based on changes in VLDL particle diameter distribution during egg production, is related to high laying performance, and any reallocation of lipid resources away from reproduction is associated with declines in laying rate (Walzem 1994; Chapter 3). In contrast, the aim of egg production in birds that have not been selected for continuous and consistent egg production is to maximize the number of offspring that can be raised given the current

condition of the laying female and the prevailing environmental conditions (Drent and Daan, 1980). Given the disparity in these goals, it is not surprising to see that lipid allocation to egg production in Zebra Finches (Chapters 2 and 3), Tsaiya ducks (*Anas platyrhynchos domestica;* Lien *et al.*, 2005), and Greater Scaup (*Aythya marila;* K. Gorman *et al.*, unpublished data), and likely in other birds that have not undergone directional selection for maximizing egg production and often face highly variable environmental conditions, is not as dramatic as in heavily selected, domesticated species that generally do not have to deal with the additional energetic costs associated with incubating the eggs and raising the chicks that subsequently hatch from those eggs (Emmerson *et al.*, 1991; Nestor *et al.*, 1996; reviewed in Romanov, 2001), or with variable environmental conditions (Etches, 1996).

Furthermore, differences between chickens and Zebra Finches in the level of expression of apoVLDL-II during egg production suggest that laying Zebra Finches, which have less circulating apoVLDL-II, may not need to rely on VLDLy for lipid deposition into developing ovarian follicles. As non-breeders, Zebra Finches exhibited large amounts of small lipoprotein particles which we suggested could be the metabolic products, i.e., IDL or very small VLDL particles, of the hydrolysis of larger, generic VLDL particles (see chapter two). During egg production, Zebra Finches also exhibited many larger VLDL particles (> 50 nm in diameter), which may be generic VLDL (see chapters two and three). If this is the case, then the metabolism of these generic VLDL particles by the laying females could generate small VLDL or IDL particles, as in non-breeding females. These small VLDL or IDL particles could easily pass through the pores in the avian ovary and contribute to lipid deposition into the developing egg yolks.

Therefore, while egg-laying Zebra Finches do produce apoVLDL-II (see chapter six), and therefore VLDLy, they may require less of this specialized lipoprotein to maintain adequate lipid allocation to reproduction. This theory emphasizes the need to assess VLDL particle diameter distribution and apolipoprotein composition of circulating VLDL particles of different sizes during egg production in different avian species to determine 1) whether the large VLDL particles observed in laying Zebra Finches are common in other birds during egg production, and 2) whether the large VLDL particles are generic VLDL, VLDLy (i.e., contain apoVLDL-II), or a combination of both types of VLDL.

How does differential resource allocation occur?

Is there one pool of resources, and therefore energy, that different physiological systems have access to? Alternatively, are there resources that only some systems can access while others cannot? In the context of lipid allocation to reproduction in birds, there are potentially two pools of energy, i.e., lipids, available for metabolism: generic VLDL and yolk-targeted VLDL. The structural and functional changes to VLDLy that preserve it for use in egg production (e.g., presence of apoVLDL-II which decreases its size and increases its resistance to LPL metabolism) also make it more difficult to use by the laying female for her own energetic needs (Chan *et al.*, 1976; Kudzma *et al.*, 1979; Griffin, 1981; Dashti *et al.*, 1983; Griffin and Perry, 1985; Lin *et al.*, 1986; Schneider *et al.*, 1990; Walzem, 1996; Speake *et al.*, 1998; Walzem *et al.*, 1999; Boyle-Roden and Walzem, 2005). Based on VLDL particle diameters found in laying Zebra Finches, it seems as though both the generic and yolk-targeted forms of VLDL may be produced during egg production (cf. only the yolk-targeted form in highly productive laying

chickens). However, to determine what these VLDL particles were "destined" for when they were produced, i.e., egg production of self-maintenance, future analysis of the apolipoprotein composition of the VLDL particles of various diameters observed in laying Zebra Finches is required.

Do allocation decisions vary with prevailing environmental or physiological conditions, i.e., is there plasticity in decision making, or are they all or nothing choices?

There is definitely plasticity in the decision to allocate lipid resources to reproduction versus self-maintenance. In chapter 5, birds exposed to low ambient temperatures during egg production exhibited a decline in reproductive effort (laying rate, clutch size and laying interval), and also seemed to decrease their allocation to egg production (fewer particles within the sVLDLy range while still maintaining comparable circulating triglyceride levels as to when they were maintained at 21°C). Therefore, in this case, laying birds are able to use cues regarding environmental conditions or their own energetic state to determine the best activity towards which energetic resources should be allocated.

How do small, free-living birds that produce eggs early in the spring and have relatively high metabolic rates meet their own energy demands along with the energetic costs associated with egg production?

While the dramatic shift in VLDL dynamics that is commonly observed in laying domestic fowl is not apparent in Zebra Finches, small birds with high mass-specific metabolic rates could still have difficulty meeting their energy demands if egg production coincides with other energetically challenging conditions. The experimental temperature manipulations of chapters four and five help to illustrate the costs that are commonly

associated with egg production by free-living birds living in the temperate-zone during the early spring; laying females are cold-acclimated, producing heat to maintain body temperature at normothermic levels, and simlutaneously forming eggs. Given the large metabolic costs of thermoregulation and the additional energetic cost of egg production, some egg-producing birds have no choice but to use energy-saving strategies like increasing energy intake and internal reallocation of energy away from other activities (e.g., locomotor activity, other physiological processes) to sustain egg production while still maintaining body temperature at levels close to normothermy.

FUTURE RESEARCH DIRECTIONS

Electron Microscopy of Ovaries of Egg-Laying Passerines

The estimate of the proportion of circulating VLDLy particles that was used throughout this thesis was based on the proposed sieving properties of laying domestic fowl (Perry and Gilbert, 1979; Griffin and Perry, 1985). While we assumed that there would be little inter-specific variation in the size of the pores in the ovarian granulosa basal lamina of birds, this is still unclear. Therefore, differences in the proposed sieving properties of the ovaries of different bird species should be examined by electron microscopy, specifically pore diameter and the diameters of VLDLy particles observed distal to the granulosa basal lamina (i.e., having already passed through the pores in the ovary).

Quantification of Circulating Levels of VLDLy via Direct Assay for apoVLDL-II

In the studies described in this thesis, the proportion of VLDLy particles in circulation was estimated based on proportion of VLDL particles within a certain diameter range (sVLDLy range: 25-44 nm). However, it is possible that in laying females, some of the VLDL particles that fell within this range were not VLDLy, i.e., there may have been small generic particles that were being metabolized by the laying female. Therefore a direct assay for VLDLy is needed to quantify circulating levels of the yolk precursor. Two such assays exist which quantify circulating levels of apolipoprotein VLDL-II (apoVLDL-II), the apolipoprotein found on yolk-targeted VLDL, but not generic VLDL, using antibodies raised against chicken apoVLDL-II (apoVLDL-II ELISAs) (Pinchasov *et al.*, 1994; Pool *et al.*, 2002). However, attempts to find the developer of one of the assays was unsuccessful, and the other lab in South Africa did not have time to run any validation samples to see whether their assay could be used to quantify apoVLDL-II, and thus VLDLy, in samples from Zebra Finches. Therefore, we attempted to develop our own assay, and in doing so found that apoVLDL-II expression was lower in laying Zebra Finches than in laying chicken hens, and the sequence of apoVLDL-II may not be conserved between avian species. Attempts to isolate and purify Zebra Finch apoVLDL-II resulted in protein samples containing putative Zebra Finche apoVLDL-II, but also what we believe to be other apolipoproteins, including apoB-100. Determination of the primary sequence of Zebra Finch apoVLDL-II is currently underway, and this information will make it possible to synthesize and raise an antibody against apoVLDL-II from a passerine bird in the near future. This antibody could then be used to directly quantify circulating levels of the yolk lipid precursor and investigate the

homologous (or non-homologous) nature of the primary sequence of apoVLDL-II in another family of birds.

Analysis of the ApoVLDL-II Composition of VLDL Particles of Different Diameters

An important question that remains unanswered is whether laying birds can maintain concurrent production of generic and yolk-targeted VLDL particles. Attempts have been made to measure the apoVLDL-II content of VLDL particles of different diameters from laying and non-laying chickens. However, due to contamination of all of the VLDL sub-fractions by small VLDL particles, this work has not yielded useful information. Once an antibody to Zebra Finch apoVLDL-II is produced, future studies could once again attempt to determine whether all of the VLDL particles in laying birds are yolk-targeted, or whether some generic VLDL is produced, but this time in an avian species that maintains some large, potentially generic, VLDL particles in circulation during egg production.

Investigation of Related "Why" Questions

The main questions that were asked when performing the research described in this thesis were "how" questions – questions about the physiological mechanisms underlying differential allocation of energy to reproduction or self-maintenance. However, many "why" questions regarding the differential allocation of energy to reproduction versus self-maintenance also exist. Why did Zebra Finches decrease clutch size in low ambient temperatures? Why does the decision to allocate resources to different activities exhibit plasticity in different environments? Is there variation in the magnitude of plasticity in resource allocation, and does this variation relate to overall

fitness? If so, why does it relate? Why don't energy-limited birds continue to increase seed consumption? The list goes on. The purpose of my thesis was to investigate the mechanisms underlying resource allocation to the competing energy demands of various physiological and behavioural activities an animal must face throughout its lifetime. To be able to answer these "why" questions, we must be able to properly answer the "how" questions. Hopefully the results presented in this thesis will contribute to the answering of some of these "why" questions in the near future.

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