METABOLIC COSTS OF EGG PRODUCTION IN BIRDS: INTERACTION OF PHYSIOLOGICAL STATE, ANNUAL VARIATION, AND DAILY ENERGY BUDGET.

by

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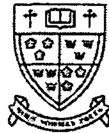

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ABSTRACT

Most estimates of the metabolic costs of egg production in birds are based on theoretical models constructed from the chemical energy content of eggs, reproductive tissue and data on the pattern and duration of yolk and albumen formation. However, several conceptual problems are associated with these models and their predictions are unreliable. This thesis presents empirical measurements of the change in metabolic rate associated with the process of egg formation in two species, the European starling (*Sturnus vulgaris*) studied in the field and the zebra finch (*Taeniopygia guttata*) studied in laboratory conditions. The thesis also presents data on changes in resting metabolic rate (RMR), body composition, and organ aerobic capacity between physiological stages and years in starlings and demonstrates that stage and year variation may introduce biases not usually considered when comparing groups or treatment effects on RMR. In terms of metabolic cost of egg production, the formation of eggs is associated with a 22% increase in RMR in both species, and data on zebra finches demonstrate that non-breeding and egg-production RMR are both repeatable. A proportion of the increase in RMR is related to the growth and function of the oviduct as the mass of this organ explains 18% of the variation in RMR in starlings during the laying period. A 22% increase in RMR in zebra finches appears to be enough to induce energy reallocation through behavioral changes. Indeed, female zebra finches reduce locomotor activity by 56% from non-breeding to the one-egg stage without changing food consumption suggesting that the decrease in activity is an energy reallocation strategy to support the increased energy expenditure required for

egg production. However, the efficiency of this strategy is individually variable and seems to be related to the overall reproductive effort: birds that undergo large increases in RMR show a positive change in daily energy expenditure (DEE) and produce large clutches. Conversely, birds that have a minimal increase in RMR show negative changes in DEE and smaller clutches which suggests that these individuals actually over-compensate for the metabolic increase and this results in decreased DEE at the one egg stage.

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CHAPTER ONE:
GENERAL INTRODUCTION

This thesis is arranged as a series of separate journal article manuscripts presented as independent, stand-alone chapters. The layout of the thesis therefore differs from the traditional approach. Each chapter has its own introduction and discussion sections, as well as a list of references and figures. The format of chapters two through four differs somewhat from the rest of the thesis because they have been formatted for publication in different journals. The General Introduction presents each research paper and gives the rationale behind the work. Some results are described to allow the reader to understand how the findings in one experiment led to the research presented in the following chapter. Therefore, there is some overlap between the information presented in the General Introduction and that presented in the separate section introductions, allowing the reader of the General Introduction to decide whether a specific chapter will be of interest.

The fitness cost of reproduction is a central concept in life history theory (Stearns 1992). In avian research, experimentally modifying reproductive effort via clutch and brood manipulations or food supplementation has shown effects in various life history traits such as timing of laying, parent and chick survival (Heaney and Monaghan 1995; Daan et al. 1996; Monaghan and Nager 1997; Meijer and Drent 1999; Visser and Lessells 2001), and either immune function or parasite levels (Gustafsson et al. 1994; Norris et al. 1994; Oppliger et al. 1996; Ots and Horak 1996). It is often believed that high energy demand during a single breeding attempt may constrain reproduction and future reproductive

success through limited availability of energy (Williams and Vézina 2001). Indeed, since Drent and Daan's seminal paper (Drent and Daan 1980) highlighting a potential maximal sustainable metabolic rate of 4 X basal metabolic rate (BMR) in breeding birds, several studies have extended this view by measuring sustained metabolic scopes (maximum sustained metabolic rate / BMR). It is now known that there are large interspecific differences, with scopes ranging from 1.3 to 6.6 (Peterson et al. 1990; Hammond and Diamond 1997). However, in birds, these scope estimates are based almost exclusively on measurements in incubating and chick-rearing individuals. The lack of information relative to energy expenditure in egg-laying birds may explain why incubation and chick-rearing stages are generally seen as the periods of highest energy demand in the life cycle of birds (Drent and Daan 1980;Williams 1996; Williams and Vézina 2001). In many passerine species living in temperate climates, the timing of laying is adjusted in order to rear chicks during the peak of food availability, meaning that egg-formation often occurs during a much less energetically favorable period (Williams 1998). This timing of breeding suggests that the egg formation period may also involve high energy demands relative to available resources because of the extra foraging requirements that are needed to fuel the potentially demanding physiological process of egg production. Nevertheless, the question remains: what is the actual metabolic cost of egg production and how does it relate to the overall energy budget?

In 1999, when the research for this thesis began, surprisingly little information was available on the actual energetic cost of egg production in wild birds. A study on Barn swallows (*Hirundo rustica*) suggested that daily energy expenditure (DEE) during egg formation was comparable to incubating and chick-rearing values (Ward 1996).

Similarly, Stevenson and Bryant (2000) later reported that DEE in egg-laying great tits (*Parus major*) was about 4 X BMR in individuals producing the largest eggs, but this result was found only in one of two years of research. Unfortunately, DEE provides little information on the actual metabolic cost of producing eggs because it represents the total energy expended in a day (total daily energy budget), including such physiological functions as activity, thermoregulation, and digestion.

In 1999, the only estimates of egg production costs available were based on theoretical models constructed from data on the chemical energy content of reproductive tissues and eggs, as well as the pattern and duration of yolk and albumen formation (see Vézina and Williams 2002). These models typically predicted that the energy investment into egg production was between 45% and 60% of BMR in passerine birds. However, there are several conceptual problems associated with these estimates. For example, models do not take into account the energetic costs of physiological processes such as yolk precursor production in the liver, transport and uptake at the ovary, and they often rely on energy values estimated from allometric equations. Therefore, given its importance in the concept of the cost of reproduction, actual empirical measurements were needed to provide an accurate estimate of the metabolic investment that birds allocate to egg production.

The general goals of this thesis were: 1) to accurately measure the metabolic cost of egg production in birds using wild (European starling; *Sturnus vulgaris*) and captive (zebra finch; *Taeniopygia guttata)* model species; 2) to investigate the potential mechanisms responsible for the metabolic increase associated with egg production and; 3) to evaluate the impact of the energetic cost of egg formation on female daily energy

budget. This research focused on small passerine species because they typically produce a large clutch mass relative to their body mass (starlings: 44%; zebra finch: 40%; F. Vézina unpublished data) and these species tend to lie towards the "income" end of the capital-income breeder spectrum (Meijer and Drent 1999).

Before we can begin to examine the metabolic cost of egg production, it is important to have a general understanding of the physiological processes involved in egg formation in birds. Egg production is complex and under hormonal control through the hypothalamic-pituitary-gonadal axis (Carey 1996; Johnson 1998; Williams 1998, Figure 1). Carey (1996), Johnson (1998) and Williams (1998) describe the egg production cycle as follows. In response to photo-stimulation resulting from the seasonal change in photoperiod, the hypothalamus releases gonadotropin-releasing-hormone (GnRH) which in turn stimulates the pituitary gland to synthesize and release follicle stimulating hormone (FSH) and luteinizing hormone (LH). These two hormones are responsible for the growth and development of the ovarian follicles (see below). At the beginning of the reproductive cycle (fall, winter), follicular growth is slow because GnRH levels are kept low in response to short day length. In the spring, increasing day length induces enhanced GnRH release and thus elevated levels of LH and FSH which will in turn regulate the rapid phase of follicular development ultimately leading to accumulation of yolk in the growing follicles. Yolky follicles are arranged in a size hierarchy prior to ovulation (F_1 to F_4 with F_1 being the largest follicle, Figure 1) and, in response to LH stimulation, they produce progesterone (P_4). Progesterone induces a positive feedback that will lead to increased levels of LH and further accelerate follicular development. As the follicles grow, they also convert P_4 into an estrogen called 17β-estradiol (E_2) which stimulates the

liver to produce yolk precursor molecules vitellogenin (VTG) and yolk-targeted very-low density lipoproteins ($VLDL_y$). Vitellogenin and $VLDL_y$ are taken up by the growing follicles through receptor-mediated endocytosis and provide energy and nutrients for the growing embryo. Progesterone and E_2, acting in tandem, also stimulate the growth and development of the oviduct that will be responsible for albumen and shell deposition in the egg. When the F_1 follicle reaches its maximal size, ovulation is triggered by the rupture of the surrounding membrane and liberation of the oocyte into the distal section of the oviduct. As the oocyte is transported through the oviduct via peristaltic movements, it still produces P_4 which stimulates the oviduct to secrete albumen and to produce the shell. Albumen deposition typically lasts 2-3 hours and then the egg stays in the shell gland for 18-24 hours depending on the species (Williams 1998). The number of growing follicles in the ovary during the laying period and the length of the rapid yolk development are species-specific. In the starling population studied, mean clutch size is 5.0 ± 1.0 (Vézina and Williams 2002), the period of rapid yolk development lasts 4 day per follicle (Challenger et al. 2001) and oviposition usually occurs between 08h00 and 10h00 (Feare et al. 1982). At the one-egg stage, females have 4 - 5 growing yolky follicles in the ovary (Vézina and Williams 2002; 2003). Similarly, in the captive zebra finch, mean clutch size is 6 ± 0.2 (F. Vézina, unpublished results), rapid yolk development lasts 4 days (Haywood 1993) and oviposition occurs within 2 hours after lights are turned on (Christians and Williams 2001). One-egg stage females have a maximum of four yolky follicles growing in the ovary (Haywood 1993). A major difference between the two species relative to the egg production process is that zebra finches are opportunistic breeders, with reproduction being triggered by short and

unpredictable periods of improved environmental conditions (Zann 1996) rather than by photoperiod.

During the laying period, the physiological processes involved in rapid yolk development, albumen and shell deposition occur simultaneously and final clutch size is a function of the number of yolky follicles recruited for development. Therefore, by inspecting the gonads of a dissected bird, it is possible to determinate at exactly which stage of laying a given female is (see Figure 2).

In the six papers that form this thesis, females where classified according to four physiolgical stages that are based in part on the sequence of follicular development, ovulation and egg laying:

1. *Non-breeding* (NB): females that were either caught at the end of the wintering season (starlings) or kept in single-sex cages (zebra finches); they have fully regressed reproductive organs and no yolky follicles present in the ovary.
2. *Pre-laying* (PL): females measured in the period of rapid yolk development from one yolky follicle to full follicular hierarchy with an egg in the oviduct (Figure 2). These individual have not begun laying.
3. *Laying* (LY): females caught and measured during the laying period of reproduction, from the first egg to clutch completion (Figure 2). It should be noted here that this definition describes a *time period* and not the *action* of oviposition. By definition these birds are also producing eggs.
4. *Chick-rearing* (CK): females measured during the period of nestling provisioning. These individuals have fully regressed reproductive organs.

Using the European starling as a model, the study presented in **Chapter two** reports on the metabolic cost of egg production by comparing resting metabolic rate (RMR; defined here as the minimal oxygen consumption by a resting, post-absorptive animal at thermoneutrality, during the quiet phase of the circadian cycle) of females captured at the non-breeding, laying and chick rearing stages. The three-year study also reports RMR changes throughout the complete follicle development and the laying sequence from the beginning of rapid yolk development to clutch completion. Problems associated with theoretical models of egg-production costs are addressed by presenting a model constructed from body composition data taken form the birds that were used for the RMR measurements. This allowed for the comparison of the model's predictions with actual empirically-measured values. It was hypothesized that RMR should be higher in egg-producing compared to non-breeders and chick-rearing individuals, and that predictions from the theoretical model should be inaccurate relative to empirical observations.

At the time Chapter two was written for publication, there were two studies already published regarding changes in either BMR or RMR between non-breeding (Chappell et al. 1999) or wintering (Nisson and Raberg 2001) stages and egg-producing, laying stage. However, as demonstrated in Chapter two, and as should become clear during the rest of this thesis, there are some important issues that must be considered when comparing BMR or RMR between physiological stages.

One of the key findings in Chapter two is that egg formation induces a 22% increase in RMR in egg-producing females. Therefore, an obvious question raised by this finding is: what specific physiological change required for egg production is responsible

for the measured increase? What explains the metabolic cost of egg production? To answer these questions, a correlational approach can be used to investigate whether changes in metabolic rate are associated with known specific physiological transformations occurring during egg production. A likely explanation for the increase in RMR in egg producing starlings is the development and function of the reproductive organs. In fact, variations in RMR are often associated with changes in organ mass (the metabolic "machinery"; Daan et al. 1990, Piersma 2002); changes that can be quite rapid but also reversible (Piersma and Lindstrom 1997). Therefore, it was likely that the growth and maintenance of the reproductive machinery, perhaps one of the fastest reversible organ transformations known (Williams and Ames 2004), was partly responsible for the measured increase in RMR in laying birds.

In an effort to break down the metabolic cost of egg production into its contributing components, **Chapter three** documents the phenotypic flexibility (Piersma and Drent 2003) in body composition in breeding female starlings used in the previous RMR experiment. Organs are known to vary in size and function in response to such things as diet and food intake (Dykstra and Karasov 1992; Piersma et al. 1993; Geluso and Hayes 1999), migration (Piersma et al. 1996; Biebach 1998; Karasov and Pinshow 1998; Piersma et al. 1999; Battley et al. 2000, 2001), altitude (Hammond et al. 1999; 2001) stress (Rogers et al. 1993) or geographic location (Hilton et al. 2000). However, before the research presented here was completed, specific changes in organs in relation to follicular development and laying sequences had never been fully investigated in wild passerine birds. Furthermore, although several papers reported variations in BMR and RMR associated with transformation in organ morphology (see Piersma 2002), body

composition effects on metabolic rate variations in breeding birds remained unexplored. Therefore, egg-producing birds appeared to be an excellent model to investigate the effects of organ mass plasticity on resting or basal metabolism variations (Piersma 2002). More specifically, the study presented in Chapter three asked the question: Is the mass of non-reproductive organs adjusted (potentially changing their maintenance costs) in response to the energy demand of egg production? We hypothesized that changes in non-reproductive organs could be adjusted to either counter-balance or accommodate the increased energy demand of egg production. The three-year study also documented year effects on organ mass variation and investigated the recrudescence and regression of the reproductive system in detail. Finally, in order to relate variations in RMR with changes in body composition, a correlation analysis of the relationships between organ mass and RMR was performed for the three breeding stages studied, with the expectation that the "reproductive machinery" would be responsible for the increase in RMR in the egg-laying group.

One of the major findings reported in Chapter three is the apparent role of the oviduct in explaining variations in RMR in egg producing birds. In fact, oviduct mass explained 18% of laying RMR variation in female starlings and therefore the oviduct seems to be an energetically expensive organ. Indeed, the growth of this organ is very fast and occurs during rapid yolky follicles development (Vézina and Williams 2003; Williams and Ames 2004). Furthermore, the oviduct starts to regress as soon as the last follicle is ovulated even while still processing an egg (Vézina and Williams 2003; Williams and Ames 2004). Although this finding is a likely explanation for the increased RMR in egg-producing birds, more than 80% of the variation in laying RMR remains

unexplained. Therefore, what other physiological systems are responsible for the metabolic cost of egg production?

The study presented in **Chapter four** was designed to evaluate the effect of another significant system, hepatic yolk precursor production, on RMR variation in egg producing birds. In contrast to the other physiological functions involved in egg formation, it is fairly easy to stimulate VTG and VLDL production in non-reproductive birds through administration of exogenous estradiol (see Figure 1). Chapter four presents a study in which the metabolic cost (measured as changes in RMR throughout the treatment) of synthesizing yolk precursors in the liver was investigated in captive zebra finches that were treated with doses of E_2 adjusted to stimulate precursor production to levels comparable to natural breeding values. Because E_2 administration triggers partial growth of the oviduct (Williams and Martyniuk 2000, Figure 1), and because of the known positive relationship between oviduct mass and RMR (Vézina and Williams 2003: Chapter three), the study was performed using male subjects. The prediction was that the metabolic cost of yolk precursor production would result in an elevation in RMR in E_2 treated individuals but the experiment revealed no changes in RMR despite a 80-fold increase in plasma VTG levels and 2-fold increase in VLDL levels. Although this may be interpreted as a low energetic cost of yolk precursor production, there is an alternative explanation that needs to be considered. It is possible that under a certain metabolic demand, the physiological machinery that composes the animal readjusts itself. In other words, up-regulation of certain functions may be counterbalanced by down-regulation of others. This would potentially result in no detectable changes in resting metabolic rate if energy reallocation occurs between physiological systems, and is in fact a major problem

for studies interested in the impact of morphological changes on metabolic rate (Vézina and Williams 2003: Chapter three).

Explaining variation in BMR or RMR by variation in organ mass assumes a constant organ metabolic intensity (energy consumed per unit mass). In other words, these analyses assume that the larger the organ, the higher its energy consumption and its potential impact on overall resting energy expenditure. However, mass-specific organ energy consumption may change independently from organ mass variation and this may confound results of correlations between body composition and metabolic rate. Although this problem has been recognized in some studies (Weber and Piersma 1996; Kvist and Lindström 2001; Piersma 2002), currently there is no information available that document to what extent this may affect correlational studies. That was one of the main purposes of the study presented in **Chapter five**.

Using the activity of the enzyme citrate synthase as an indicator of tissue maximal oxidative capacity, the relationship between organ mass and aerobic activity was investigated in European starlings. The two-year study compared variations in organ mass and enzymatic activity between non-breeding, one-egg and chick-rearing stages. Enzyme analysis was performed on samples of organs considered to be some of the most energy consuming within the body: pectoral muscle, heart and kidney (Krebs 1950; Emmet and Hochachka 1981; Schmidt-Nielsen 1984; Hochachka et al. 1988; Daan et al. 1990). The liver was also important because of its role in egg formation. It was hypothesized that mass-specific organ metabolism could vary independently from organ mass and that in the liver, an increase in citrate synthase maximal activity would be found in egg-producing birds in response to the metabolic cost of yolk precursor production.

Chapter five clearly demonstrates that organ oxidative capacity is indeed not a fixed trait and that changes in organ mass and potentially intensity, between physiological stages, can even occur in opposite directions, thereby canceling each other out.

The metabolic cost of egg production measured as changes in BMR or RMR has been documented in three species of wild birds; house sparrows (*Passer domesticus*; Chappell et al. 1999), great tits (*Parus major*; Nilsson and Raberg 2001) and European starlings (Vézina and Williams 2002: Chapter two). However, non-reproductive organ mass and organ metabolic intensity may vary independently from reproductive stage and therefore influence estimates of metabolic rate in wild animals (Vézina and Williams 2003 and Chapter five). Furthermore, two of the studies cited above are based on one year of data and directly compare RMR between non-breeding and laying stages, a practice that may potentially provide different results between years (Vézina and Williams 2002: Chapter two). Replicating these measurements using captive birds in controlled conditions can therefore provide a good system to accurately measure the metabolic cost of egg production without the confounding effects of natural variability due to ecological factors. Furthermore, captive laboratory conditions also provide another important advantage over field studies: measurements can easily be obtained repeatedly. Repeatability is defined as the amount of variation in a given trait among, rather that within, individuals (Lessells and Boag 1987) and reflects the stability in the inter-individual variation for that phenotype (Bennett 1987). For natural selection to act on a trait, it has to be heritable but also repeatable (Bennett 1987; Dohm 2002). Two studies reported significant repeatability of BMR in both wild and captive birds (Bech et al. 1999; Horak et al. 2002). However, knowing that increased investment in egg production

may have significant costs in terms of fitness (Heaney and Monaghan 1995; Monaghan et al. 1995; Visser and Lessells 2001), it is imperative to investigate the individual stability in the metabolic cost of egg production.

Therefore, in the context of the metabolic cost of egg production, **Chapter six** presents a study undertaken in controlled conditions using captive zebra finches where changes in RMR within females were monitored during non-breeding, one-egg and chick-rearing stages. The study also investigated repeatability of RMR within non-breeding and laying stages by comparing values between reproductive attempts. The effect of time on RMR repeatability was also examined in a separate group of non-breeding males by comparing short (8 days) and long-term (10 months) repeatability estimates. It was hypothesized that, like in starlings, egg-production would induce an increase in RMR compared to non-breeding and chick-rearing stages and that both non-breeding and egg-laying RMR would be repeatable over time.

Chapter six revealed that, as in starlings, the physiological mechanisms involved in egg production induce a 22% increase in resting metabolic rate in zebra finches. Furthermore, the individual variability in RMR between breeding attempts is maintained. An important question to answer is therefore: how do reproductive females cope with a 22% increase in basal energy demands in terms of the overall energy budget? Is this increased demand additive to the other components of the budget resulting in increased daily energy intake? Or is it simply buffered by an energy reallocation mechanisms resulting in a constant daily energy expenditure? The study in **Chapter seven** was designed to answer these specific questions.

A previous study by Williams and Ternan (1999) showed that breeding female zebra finches reduce locomotor activity by 46% during egg development. However, these same birds only show a slight reduction in food intake (8%), suggesting that egg-laying females use a behavioral energy reallocation strategy to sustain the increased energy demand resulting from egg formation. Unfortunately, the study lacked energy consumption measurements. Chapter seven uses an experimental approach to test the reallocation hypothesis suggested by Williams and Ternan (1999). Their original protocol was replicated with the addition of measurements of daily energy expenditure (DEE) at all physiological stages using the doubly labeled water method (Speakman 1997). This experiment was performed using birds whose RMR had been previously measured at all stages (Chapter six). Therefore, individual estimates of RMR and DEE, measured on two consecutive breeding attempts, were available from non-breeding to laying to chick-rearing stages, allowing for a within-bird analysis of energy consumption throughout a breeding sequence. It was predicted that if the energy cost of egg-production had an additive effect, the consequence on the energy budget would be visible in an increase in DEE at the one egg-stage. Alternatively, a reallocation strategy would lead to a constant level of DEE throughout the reproductive event from non-breeding to chick-rearing. These data, coupled with locomotor activity measurements and daily food intake, revealed that zebra finches do, in fact, reallocate energy during egg production by reducing the amount of energy expended for locomotion. Furthermore, it appears that the reallocation efficiency is variable between individuals and is related to the energetic investment (change in RMR from non-breeding to one-egg stage). This study presents a

very flexible, individually-variable system of energy reallocation to meet the short term increased energy demand of egg production.

Chapter eight highlights the main results presented in this thesis and discusses their overall significance and contribution to our understanding of the physiological ecology of birds.

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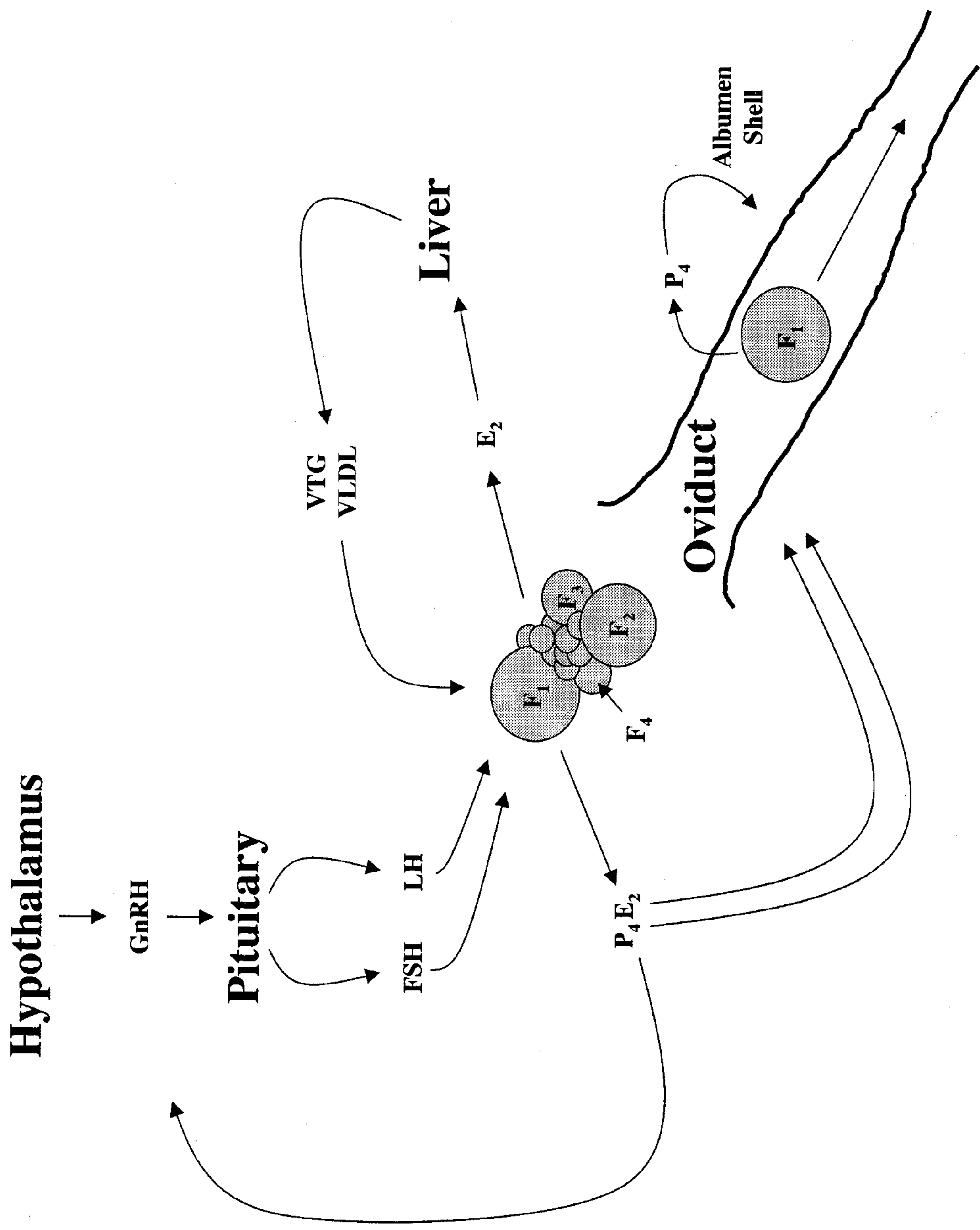
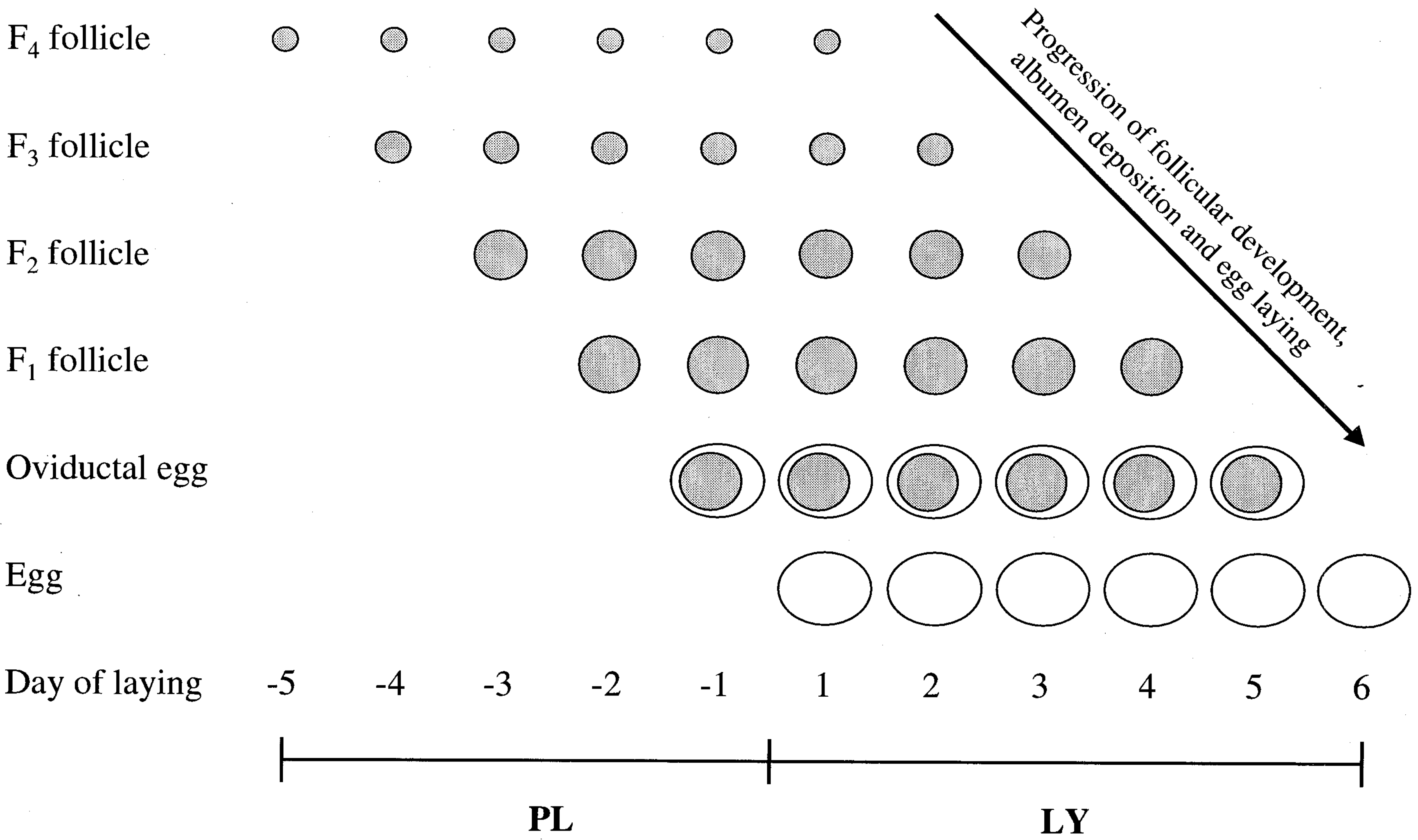
FIGURE LEGENDS

Figure 1: Hormonal control of follicular growth and ovulation in egg producing birds.

Figure 2: Pattern of yolky follicle development in a 6-egg clutch from 5 days before beginning of laying to clutch completion. PL = pre-laying stage, LY = laying stage.



Hypothalamus
GnRH
Pituitary
LH
FSH
Liver
VTG
VLDL
E_2
F_1
F_2
F_3
F_4
$P_4 E_2$
Oviduct
P_4
Albumen
Shell



F_4 follicle
F_3 follicle
F_2 follicle
F_1 follicle
Oviductal egg
Egg
Day of laying
-5
-4
-3
-2
-1
1
2
3
4
5
6
PL
LY
Progression of follicular development, albumen deposition and egg laying

CHAPTER TWO:
METABOLIC COSTS OF EGG PRODUCTION IN THE EUROPEAN STARLING (*STURNUS VULGARIS*).

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ABSTRACT

The energy cost of egg production in passerine birds has typically been estimated to be 45-60% of basal metabolic rate but this is based on theoretical models using data on energy content of eggs and reproductive tissue; there are still very few empirical data on egg production costs. In this study we directly measured resting metabolic rate (RMR) in egg-laying female European starlings (*Sturnus vulgaris*) over three years. We compared these data with RMR of non-breeding and chick-rearing birds, and with estimated energy expenditure generated from a typical "energy content" model but using empirically-derived data from body composition analysis for this species. We found marked variation in RMR between years and between reproductive stages which complicates comparisons among breeding stages for the assessment of relative egg production costs. Based on this method RMR during egg-laying varied from +74% to -13% of non-breeding RMR, and from +20% to -7% of chick-rearing RMR. We therefore used an alternate approach: measuring changes in RMR through the complete cycle of follicle development and ovulation. The increase in RMR from the beginning of pre-laying to the six follicle stage (prior to first ovulation), when birds have a complete developing follicle hierarchy was 22.4%. This value is still much lower than that estimated from our energy content model. We discuss conceptual problems associated with the theoretical energy content approach but also suggest, based on earlier work done in our lab, that the measured increase in RMR might still under-estimate the actual cost of egg production if birds reallocate energy between different physiological systems.

INTRODUCTION

Several recent studies have highlighted the importance of processes involved in actual production of young – that is, egg formation (cf. post-hatch rearing of young) - to our understanding of the evolution of clutch size and fitness costs of reproduction in birds (e.g. Heaney & Monaghan 1995; Monaghan et al. 1998; Nager et al. 2000; see also Partridge & Harvey 1985). These studies suggest that egg production can be costly, in an evolutionary sense, with increased demand during egg production being associated with decreased chick rearing performance (Monaghan et al. 1998) or a decrease in the female's future fitness (Nager et al. 2001; Visser and Lessells 2001). Ward (1996) and Stevenson and Bryant (2000) reported daily energy expenditure (DEE) measurements using the doubly labeled technique during egg production. They showed that DEE during egg-laying can be comparable to that during incubation and chick rearing (Ward 1996) or close to four time BMR in birds laying large eggs (Stevenson and Bryant 2000). However, these DEE measurements represent the total energy budget for egg production including foraging activities and thermoregulation costs (see Stevenson and Bryant 2000). Thus, these studies provide very little information about the actual energy expended in the physiological process(es) of producing eggs (e.g. yolk precursor production, ovary and oviduct growth and maintenance). These "direct" energy costs that an egg-producing female incurs during egg formation remains obscure and are still under debate (Carey 1996; Monaghan and Nager 1997; Nilsson and Raberg 2001).

One of the main reasons for this is that there are very few empirical measurements of metabolic rate during egg formation. Most estimates of egg production costs in birds have been based on theoretical models using chemical energy content of egg components

and reproductive organs, and information on the pattern and duration of yolk and albumen formation. For passerine birds these models typically predict energy investment values during laying of about 45-60% of basal metabolic rate (BMR; King 1973; Ricklefs 1974; Ojanen 1983; Walsberg 1983; Rahn et al. 1985; Krementz and Ankney 1986; Perrins 1996; Carey 1996; Monaghan and Nager 1997; but see Discussion). However, these models make numerous assumptions, for example, that energy costs are additive to routine maintenance costs, they do not include costs of physiological processes such as yolk precursor production, transport and uptake, and they often use values for egg and organ energy content and BMR derived from allometric equations rather than empirically derived values. Recently, Nilsson and Raberg (2001) measured resting metabolic rates (RMR) of great tits (*Parus major*) at different breeding stages and observed a 27% increase over wintering RMR in egg-laying females, a value which is much lower than that predicted by most energy content models.

In this article we report on variation in RMR associated with egg production in free-living female European starlings (*Sturnus vulgaris*). In particular, we compare RMR at different breeding stages, including non-breeding, egg-laying and chick-rearing, over three years and highlight high levels of variation both between years and between breeding stages. Secondly, in two years we investigated changes in RMR during the egg production cycle in relation to specific stages of reproductive development (ovarian follicle size and number) from pre-laying to clutch completion. Finally, we generate a typical "energy content" model of energy expenditure during egg formation for this species, but one based on empirically-derived data from body composition analysis, and compare our measured RMR values with predicted RMR generated by the model.

MATERIAL AND METHODS

Field site

This research was carried out at the Pacific Agri-Food Research Center (P.A.R.C.) in Agassiz B.C., Canada (49°14`N, 121°46`W) under a Simon Fraser University animal care permit (499B), following guidelines of the Canadian Council on Animal Care. The site consists of approximately 175 nest-boxes on farm buildings and telephone poles that were used each year by breeding starlings. In each year all boxes were checked daily to determine dates of clutch initiation and clutch completion and the laying sequence of eggs. During laying and early chick-rearing, females were taken from their nest boxes during night-time (generally between 20:00h and 24:00h); during late chick-rearing, provisioning females were trap-caught (always within an hour before sun set). Non-breeding and pre-laying females were mist-netted at two barns that were used as roosting sites. Eggs were collected when present at the time females were collected, for mass and size measurements.

Collection of birds

In all three years 1999-2001, we measured RMR in birds at the end of the wintering period (non-breeding, NB), at the one egg stage of laying (LY-1) and during chick provisioning (CK). We measured birds at the one-egg stage of laying since this represents the day of peak energy investment in egg formation based on theoretical models (e.g. Ojanen 1983; Krementz and Ankney 1986; Williams and Ternan 1999) and patterns of yolk precursor production (Challenger et al. 2001). At that point all birds had laid an egg, the second egg of the clutch is in the oviduct and all the remaining follicles are

simultaneously developing in the ovary. Final sample sizes were as follows: 1999, NB = 17, LY-1 = 12, CK = 5; 2000, NB = 19, LY-1 = 20, CK = 12; 2001, NB = 17, LY-1 = 14, CK = 19.

In 2000 and 2001, we also measured RMR in random-caught females during the ten days before the first egg appeared in the colony (pre-layers, PL; n = 9 in 2000 and n = 25 in 2001). This pre-laying group contained individuals at different levels of ovarian follicle development, and oviduct growth, but included no females that had laid their first egg. After recording the first clutch initiation in the colony we started measuring RMR in birds at all stages of egg laying (eggs 1 to 6) through to clutch completion (n = 57 in 2000 and n = 55 in 2001). For all birds we measured resting metabolic rate and completed dissections for body composition analysis as described in the section "Body composition analysis".

Measurement of resting metabolic rate (RMR)

We define RMR as the energy consumed (measured as VO_2) by a post-absorptive bird during the resting phase of the circadian cycle at a temperature within the thermoneutral range for the animal. Note that this is what is usually defined as *basal* metabolic rate (Blem 2000). By definition BMR is the lowest measurable VO_2 and because laying birds in this study are producing eggs, they have to be considered in an "active physiological state" which should induce elevated levels of energy consumption. Therefore we consider the term *resting* metabolic rate more appropriate in the present case. All RMR measurements were completed using a flow-trough respirometry system (Sable Systems International). After capture birds were brought to the laboratory, body mass was

measured (± 0.1 g), then birds were placed in metabolic chambers (3.5L) for one hour prior to the beginning of RMR measurements. In some cases females laid an egg in the chamber before the end of the recordings; data collected from these birds were discarded. All birds received about 500ml/min of dry CO_2-free air (using Drierite™ and ascarite™ as scrubbers) and were kept in the dark at 25°C which is within the thermoneutral zone for this species (Lustick and Adams 1977). RMR measurements were always carried out between 23:00h and 5:00h. Our setup consisted of four metabolic chambers fitted with a perch and 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 form 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 (model CA-1 Sable systems) and oxygen analyzers (model FC-1 Sable systems; air water scrubbed before CO2 analyzer and water and CO_2 scrubbed before O_2 analyzer). All measurement sequences started by recording ten minutes of baseline air. After baseline recording the multiplexer switched and the out-coming air from the first chamber was sampled for 55 minutes. Then the system switched back to baseline for 10 minutes before changing again to the second, third, and fourth chamber. On average the birds stayed in their chambers for about 5.5 hours. Preliminary data showed that measuring RMR in a sequence like that did not generate a time effect (see Hayes et al. 1992). Thus having one or four hours to rest did not affect RMR of the birds (F. Vézina and T.D. Williams, unpublished data). After RMR measurements, the birds were weighed for a second time and the average of first and second mass was used in subsequent calculations. To calculate RMR a running

mean representing ten minutes of recording was passed through the data for each bird, with the lowest average taken as RMR.

Body composition analysis

After RMR measurements, birds were sacrificed by exsanguination under anesthesia (ketamine:xylasine at doses of 20mg/kg and 4mg/kg respectively), their feathers were plucked and they were dissected. We recorded the fresh mass of the reproductive organs (oviduct and follicle-free ovary), the individual weights of all ovarian follicles, and the weight of the oviductal egg (± 0.001 g). The number of follicles and the presence of post-ovulatory follicles allowed us to confirm the breeding status of every bird. All samples were kept frozen at -20°C until the end of the field season for further processing. Adipose tissue in starlings is known to have a very low energy consumption (Scott and Evans 1992). Therefore in order to avoid any dilution effect when investigating body mass vs RMR relationships all organs and carcasses were freeze-dried (Virtis^{TM} Freezemobile model 8ES) and fat-extracted for eight hours in a Soxthlet apparatus using petroleum ether (Dobush et al. 1985; eight hours was enough in all cases to have several distillation cycles with samples soaking in completely clear ether indicating complete fat extraction). Here we report lean dry body mass (LDBM) as being lean dry carcass mass plus lean dry organ mass (excluding the oviductal egg and feather mass).

Energy investment model

Energy investment models of egg production are typically constructed from the energetic content of the growing oviduct, which is assumed to grow at a constant rate, the

developing follicles (calculated from the energy content of yolk taken from eggs), and albumen deposition (also calculated from egg content). This type of model generally divides investment over a species-specific period of rapid yolk development (RYD) and a 24 hours period of albumen and shell deposition per egg. Body composition data collected in 2000 and 2001 allowed us to generate such a model based on empirical data (Figure 1) for a European starling with a full six-follicle hierarchy. We present both the model and empirical data based on the pattern of follicular growth and ovulation (x axis in Figures 1 and 3); although this will broadly equate to number of eggs laid they will not correspond exactly (e.g. because of follicular atresia; Challenger et al. 2001). Thus, the x-axis ranges from zero yolky follicles present (just prior to RYD, beginning of PL) to six yolky follicles (the maximum number of yolky follicles found in our birds) just before the first ovulation (PL part of x-axis Figure 1 and 3). The number of yolky follicles then decreases as the bird ovulate one per day (LY part of x-axis Figure 1 and 3) until all follicles are ovulated and the bird reaches the clutch completion stage (CC). Average clutch size in our colony was 5 ± 1 with clutches of 4, 5, 6, and > 6 representing 17.6, 56.5, 17.6 and 3.5% of all the clutches measured for the three years. We used energy conversion values of 39 kJ/g for lipids, 18 kJ/g for tissue proteins (Blem 1990) and 23.5 kJ/g for follicle and egg proteins (Sotherland and Rahn 1987) and evaluated the actual daily energy added to the reproductive system prior to and during egg production. Energy content of the oviduct, follicles and follicle-free ovary was calculated based on their fat and lean dry content and the average lean dry albumen content of the first egg laid in the clutch was used to determine energy transferred in that form. Egg biosynthesis efficiency in wild birds is poorly known (Perrins 1996). El-Wailly (1966) estimated a production

efficiency of 42% for the zebra finch (*Taeniopygia guttata*) but estimates for domestic hens selected for increased level of production were reported to be as low as 30-37.5% (Van Es 1980) and as high as 65-68% (Blaxter 1989). We therefore used a value of 50% for egg production efficiency, i.e. all energy content values were multiplied by a factor of two (see Discussion).

RESULTS

Annual variation in RMR with reproductive stage

Comparing RMR among NB, LY-1 and CK birds, we found a significant effect of year ($F_{2,125}$ = 13.0, P < 0.001), breeding stage ($F_{2,125}$ = 10.6, P < 0.001) and lean dry body mass ($F_{1,125}$ = 15.4, P < 0.001). However, there was also a significant interaction between breeding stage and year ($F_{4,125}$ = 30.1, P < 0.001; see Figure 2) so we reanalyzed variation in RMR for each year separately (no other interaction terms were significant in the full model). For each year, there was a significant effect of breeding stage on RMR: 1999 ($F_{2,30}$ = 75.0, P < 0.001), 2000 ($F_{2,47}$ = 4.1, P < 0.05), 2001 ($F_{2,46}$ = 3.8, P < 0.05; controlling for lean dry body mass in ANCOVA, no stage x LDBM interaction in any case). In 1999, LY-1 birds showed the highest RMR of the three groups with a value 73.8% and 20.3% higher than NB and CK birds respectively (Figure 2). However, in 2000, the only significant difference in RMR was between NB and CK females, with NB individuals showing RMR values on average 16.6% higher than CK birds, and RMR in LY-1 birds was not significantly different form NB or CK birds. In 2001, NB individuals had the lowest RMR of the three groups (as in 1999). However LY-1 and CK birds had mean RMR only 4.8% and 12.9% respectively higher than NB birds.

Variation in RMR during the egg production cycle

RMR increased with the number of growing follicles in PL birds and then decreased in laying females as the number of follicles decreased ($F_{13,130} = 2.79$, $P < 0.005$, ANCOVA controlling for lean dry body mass and year; no significant interaction terms; Figure 3). At the 6-follicle stage, peak RMR was 22.4% higher than at the pre-laying 0-follicle stage (multiple contrast analysis $P < 0.005$), and 13.9% higher than at clutch completion (multiple contrast analysis $P < 0.05$; Figure 3).

In order to investigate egg size effect on energy investment in LY-1 birds we looked at the relationship between residual RMR (controlling for LDBM) and 1st-egg mass, oviductal egg mass, F1 follicle mass (subsequent follicle to be ovulated) and total follicle mass at the time of RMR measurement. Oviductal egg mass and 1st-egg mass were positively correlated ($r_{104} = 0.64$, $P < 0.001$). In 1999, residual RMR was negatively correlated with oviductal egg mass ($r_{12} = -0.59$, $P < 0.05$) and 1st-egg mass ($r_{12} = -0.78$, $P < 0.005$), but was independent of F1 and total follicle mass ($P > 0.05$ in both cases; Figure 4). In 2000 and 2001 there was no significant relationship between residual RMR and any measure of reproductive output ($P > 0.15$ in all cases).

Comparison of measured versus predicted RMR

Figure 1 shows the estimated energy transferred into reproductive organs and egg components in relation to the number of developing yolky follicles from pre-laying to clutch completion. For this model the energy transferred to the oviduct peaked at pre-laying 4-follicle stage and then started to decrease while energy transferred in the follicle-free ovary was minimal (never more than 1 kJ per day). This model assumes that the

turnover cost of maintaining these organs after the first ovulation is negligible – that is, that there was no new material added after the sixth follicle - and does not consider costs resulting from albumen and shell deposition by the oviduct nor the specific costs of production of yolk precursors by the liver or their uptake by the follicles. However, based on this model the predicted energy transferred into eggs and reproductive organs in our starling population peaks at 47.7 kJ on the day of the second ovulation (at the 4-follicle in the LY group). From Figure 3, measured RMR peaked at the 6-follicle stage with a value 199.1 ± 9.8 mlO_2/h compared with a value of 162.7 ± 5.5 mlO_2/h at the beginning of pre-laying (zero-follicle stage; an increase of 36.4 mlO_2/h). The mean respiratory quotient (RQ) during laying in 2000 and 2001 was 0.77 which complicates energy conversion to kJ/day since an RQ of 0.77 might result from combustion of protein or a mixture of fat and carbohydrates (Schmidt-Nielsen 1990). Using the two extremes of RQ = 0.71 (fat only) and RQ = 1 (carbohydrate only), we calculated the energy equivalent for the 36.4 mlO_2/h increase in LY-1 as 17.2 kJ/day and 18.3 kJ/day, respectively, above PL levels (conversion factors taken from Schmidt-Nielsen 1990). These values represent only 36.1% and 38.4% respectively of the model predictions of peak energy transfer into eggs and reproductive tissues (Figure 1).

DISCUSSION

Female European starlings showed marked inter- and intra-annual variation in RMR over the three years of this study, which has important implications for assessing the cost of egg production relative to other parts of the reproductive cycle. Nevertheless, in each of the three years, absolute mass-corrected RMR was as high during egg-laying as during

the chick-rearing period (cf. Ward 1996), and in one year peak RMR occurred during egg-laying. In birds undergoing egg formation, RMR changed systematically in relation to the number of developing follicles present in the ovary and was 22% higher in birds with a full follicle hierarchy (6-follicle stage) than in pre-layers at the 0-follicle stage. However, controlling for stage of ovarian development, we found that measures of reproductive output (oviductal and 1st-egg mass) were significant predictors of RMR in only one of three years, and then the relationship was negative. Finally, the energy cost of egg production was over-estimated by the model (peak RMR 47.7 kJ/day vs. a maximum of 18 kJ/day), even though this model was based on empirically-derived data for the species.

Variation in RMR between years and reproductive stages

Variation in RMR among years for the same breeding stage was in some cases as large as within years when comparing among breeding stages (Figure 2), but the pattern of variation was not consistent among years. For example, RMR of non-breeders was 31% lower in 1999 than in 2000, whereas the reverse pattern occurred in LY-1 females, with RMR being 37% higher in 1999 than in 2000. In contrast, in 2001 the maximum difference in RMR among stages was only 13% and occurred between non-breeders and chick-rearing birds. This level of variation makes it very difficult to assess the cost of egg production relative to other stages of reproduction. By definition the only difference between non-breeders, 1-egg birds, and chick-rearing birds in our study was the physiological or reproductive state of the birds. The cost of producing eggs should be reflected in the difference in RMR between birds at the peak of investment (1-egg stage)

and birds not engaged in egg formation (the control), but which is the appropriate "control": non-breeders or chick-rearing birds, both of which would have fully regressed ovaries? In a comparable study, Nilsson and Raberg (2001) chose to use wintering RMR in great tits as a control group and reported a 27% increase in RMR associated with egg-laying values compared with wintering values (note that these RMR values were not mass-corrected, which might bias this result; Blem 1984; Packard and Boardman 1988, 1999; Hayes and Shonkwiler 1996; Hayes 2001). It appears from our results that using wintering or non-breeding RMR as a "baseline" level to estimate relative costs of egg production can be misleading. In our study, the relative difference in RMR between non-breeders and laying birds was +74%, -13%, and +4.8% in 1999, 2000, and 2001 respectively. Clearly, future studies will need to obtain data from more than one year (cf. Nilsson and Raberg 2001) to control for annual variation, since data from any one year can lead to very different interpretations. It is well known that metabolism is variable between seasons (Aschoff and Pohl 1970; Daan et al. 1989; Dawson and Marsh 1989; Cooper and Swanson 1994; Piersma et al. 1995) and that wintering acclimatization in birds induce higher levels of RMR (Swanson 1990, 1991a, 1993; Cooper and Swanson 1994; Saarela et al. 1995), therefore it seems most likely that annual variation in temperature and extent of seasonal acclimation might be responsible for the extreme differences in non-breeding RMR in the three years of our study. However, we also found substantial between-year differences in RMR during laying and chick-rearing, and the reasons for this variation are not clear. One possibility is that birds make adjustments to the size of their "metabolic machinery" in relation to variable ecological conditions between years (Daan et al. 1990; Piersma and Lindstrom 1997) and that this may cause

variation in RMR. Indeed, Christians and Williams (1999) and Burness et al. (1998) have reported significant between-year differences in the mass-corrected size of various organs during laying and chick-rearing respectively (we are currently exploring this further in our laboratory). Bech et al. (1999) showed that BMR was repeatable in kittiwakes (*Rissa tridactyla)* provisioning chicks; thus variation in which individuals breed in any given year (i.e. 'high' vs. 'low'-RMR phenotypes) could also contribute to annual variation in mean metabolic rate.

Metabolic cost of egg production

Given the problems of assessing metabolic adjustments for egg production by comparing RMR among breeding stages, we suggest that a better method is to look at changes in RMR throughout the ovarian cycle, as measured by changes in follicle number. In other words, the direct physiological costs of egg production are calculated as the difference (increase) in RMR comparing birds forming eggs and those not (these include production costs of eggs, and production and maintenance costs of reproductive tissues). There was a 22% increase in RMR comparing pre-layers with no yolky follicles to individuals with a full 6-follicle hierarchy (note that RMR values for laying birds with 3-5 follicles are also 190-200 mlO_2/h). Given the frequency of 6-egg clutches in our population clearly not all of these birds would have laid 6-eggs. Therefore we do not think that the estimated 22% increase in RMR is driven solely by high quality birds. The increase in RMR in laying birds that we report is somewhat lower than the 27% increase reported by Nilsson and Raberg (2001) when comparing RMR of wintering and egg-laying great tit's, but higher

than the 15% difference in RMR between their nest-building birds (comparable to our pre-layers) and laying individuals (calculated from data presented in their article).

Nevertheless, this 22% increase in measured energy consumption associated with egg formation is much lower than our model prediction (by 62-64%). Estimated costs of egg production from previous studies, all using the energy content models, are confounded by the fact that these different studies used no or different corrections for biosynthesis efficiency (Perrins 1996; Monaghan and Nager 1997). For example, King (1973), Walsberg (1983) and Krementz and Ankney (1986) used biosynthesis efficiency coefficients of 70%, 75% and 77% respectively. Overall their estimates of egg production costs range between 45-60% of BMR. Recalculating this with a coefficient of 50%, give costs of egg production ranging between 63-83% of BMR. In other words these models appear to totally over-estimate costs of egg production relative to our measured increase in RMR. This difference is unlikely to be due to errors in estimating the nutrient content (lipid or protein) of follicles or reproductive tissue, since in our study we used empirically-derived data to construct our energy content model. Rather, we believe that this discrepancy arises from problems in converting energy-content per se to energy expenditure relative to BMR. As described above, these models typically calculate the energy *content* of eggs and reproductive tissue and express that as a percentage of estimated BMR. First, as Nilsson and Raberg (2001) pointed out, the energy content of the nutrients that form the reproductive organs (follicle free ovary and oviduct) and eggs (follicles and albumen) may be higher than the cost of forming them. In other words, the chemical energy content of the eggs is higher in absolute value than the energy expanded in physiological work to transfer the nutrients from the food to the eggs. Second, many

studies have compared predicted peak energy investment with RMR or BMR values calculated from multi-species allometric relationships (e.g. King 1973; Ricklefs 1974; Ojanen 1983; Walsberg 1983; Krementz and Ankney 1986). However, the relationship between metabolic rate and body mass differ between species and therefore the use of allometric relationships especially when based on multiple species may lead to significant biases (Williams and Vézina 2001). In conclusion, we believe that energy content models do not provide accurate estimates of the metabolic costs of the *processes* of egg formation (they simply assess the result of this process). Thus, comparing estimates based on these models with measured metabolic rates is really invalid, with the latter providing the more accurate method for assessing egg production costs.

Does our empirically derived estimate of a 22% increase in RMR accurately reflect the metabolic costs of egg production? In fact, the cost may still be higher if females also reallocate energy between different demands during egg formation. It is well known that the size and physiological activity of different organs is plastic and can vary depending on such things like diet (Imondi and Bird 1967; Piersma et al.1993; Hammond and Janes 1998; Geluso and Hayes 1999), migration (Biebach 1998; Piersma and Gill 1998; Piersma et al. 1999; Battley et al. 2000; 2001) or season (Swanson 1991b; O'Connor 1995, 1996). Several studies have reported, for example, that thermoregulation costs can be compensated for with heat generated from muscular activity (Webster and Weathers 1990; Bruinzeel and Piersma 1998) or digestion (Masman et al. 1989; Chappell et al. 1997). During egg formation there is a more than 10-fold increase in the size of the reproductive organs, presumably with a concomitant increase in the energy cost of maintaining these organs (thus leading to an increase in RMR). However, Christians and

Williams (1999) reported that laying female starlings have relatively small gizzard, intestine, heart and pancreas mass compared with non-breeding birds. Thus the increase in RMR due to maintenance costs of reproductive tissues (as well as costs of yolk precursor production, follicular development, albumen and shell deposition) might be partly compensated for by a reduction in the size and energy maintenance costs of other organs (e.g. see Geluso and Hayes 1999; Williams and Vézina 2001). Clearly, future studies investigating the metabolic costs of egg production should include body composition (organ mass) analysis in order to highlight possible adjustments in the metabolic machinery associated with egg production and, thus, evidence of energy reallocation between different physiological systems. Thus, simply measuring absolute RMR (summation of all maintenance costs) might be misleading in informing about costs of specific physiological function (Williams and Vezina 2001).

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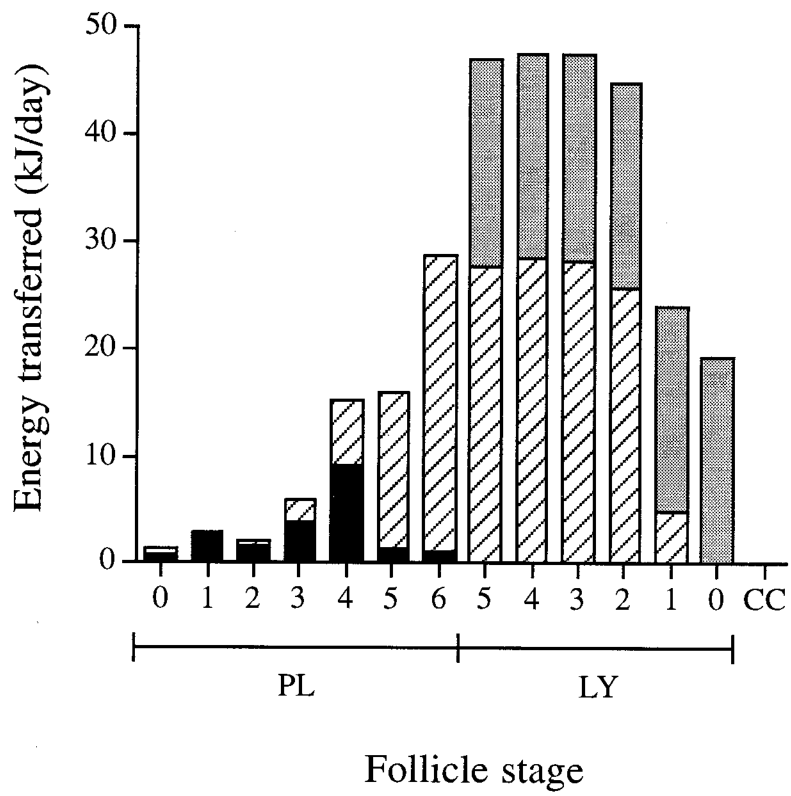
FIGURE LEGENDS

Figure 1: Pattern of energy transfer into eggs and reproductive tissue relative to the number of yolky follicles for a European starlings with full 6-follicle heirarchy (calculated from pooled data for 2000 and 2001). Pre-laying (PL) stage extends from zero yolky follicles (just prior to RYD) to the maximum number of yolky follicles reported in our population (6). During laying (LY) the number of follicles decreases as they are ovulated until clutch completion (CC). Although the energy content of follicle-free ovary is included in this model, it is not visible because it remained under 1kJ in all cases. Energy content was transformed using 50% egg production efficiency (see Methods). Solid = oviduct, hatched = follicles, gray = albumen.

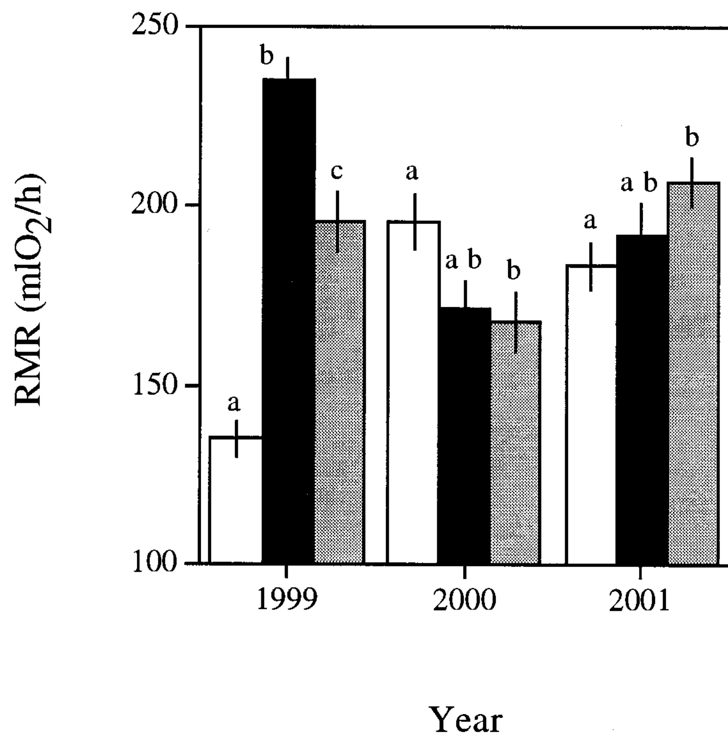
Figure 2: Inter-annual and breeding stage related variation in resting metabolic rate in European starlings. RMR values are least square means ± SE correcting for LDBM. Different letters over the bars indicate significant difference between stages within a given year. White bars = NB, black bars = LY-1, gray bars = CK.

Figure 3: Pattern of variation in resting metabolic rate relative to the number of yolky follicles in development during pre-laying (PL) and laying (LY). RMR values are least square means ± SE correcting for LDBM and year.

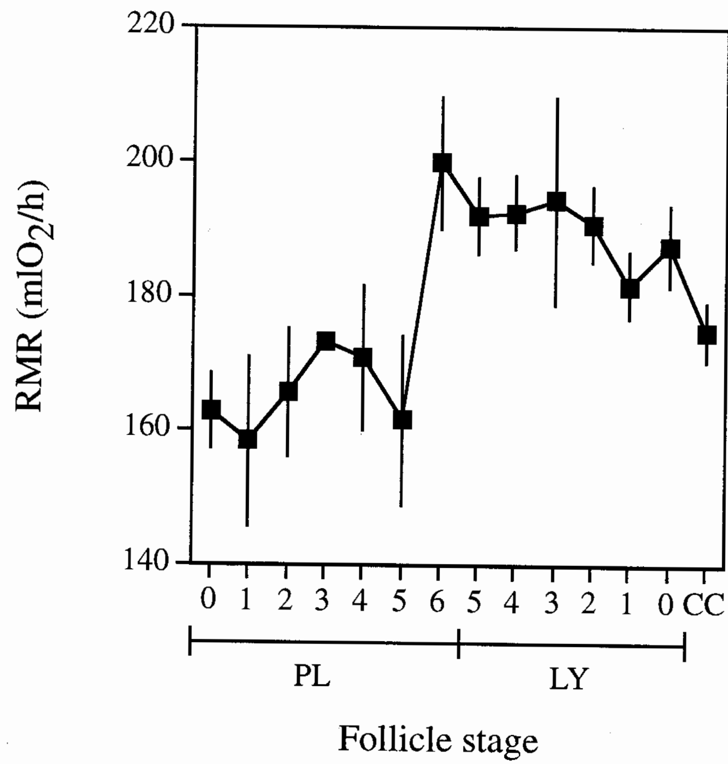
Figure 4: Relationships between residual resting metabolic rate (controlling for LDBM) and oviductal egg mass (top) and first laid egg mass (bottom) in European starlings in 1999.



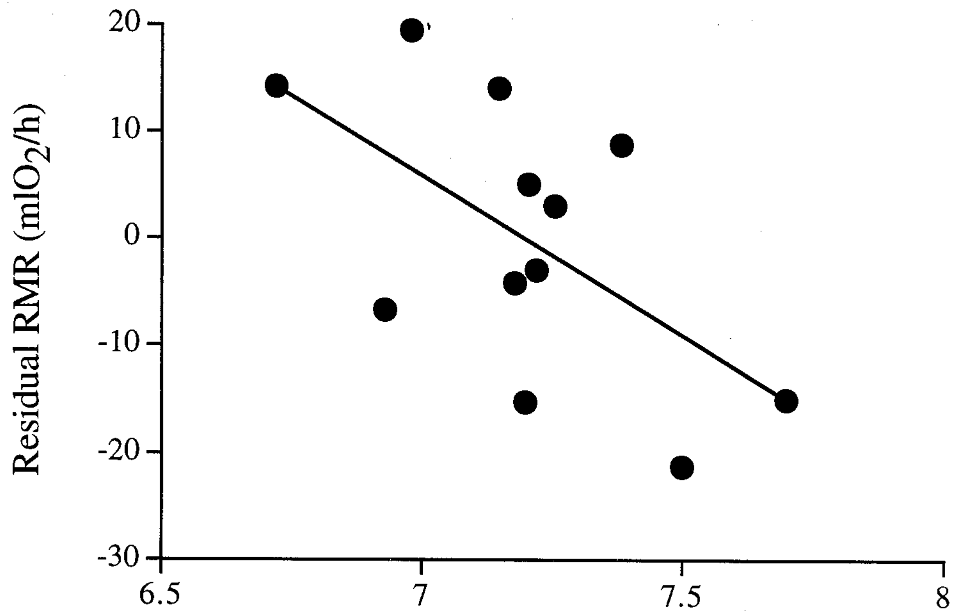
Energy transferred (kJ/day)
50
40
30
20
10
0
0 1 2 3 4 5 6 5 4 3 2 1 0 CC
PL
LY
Follicle stage

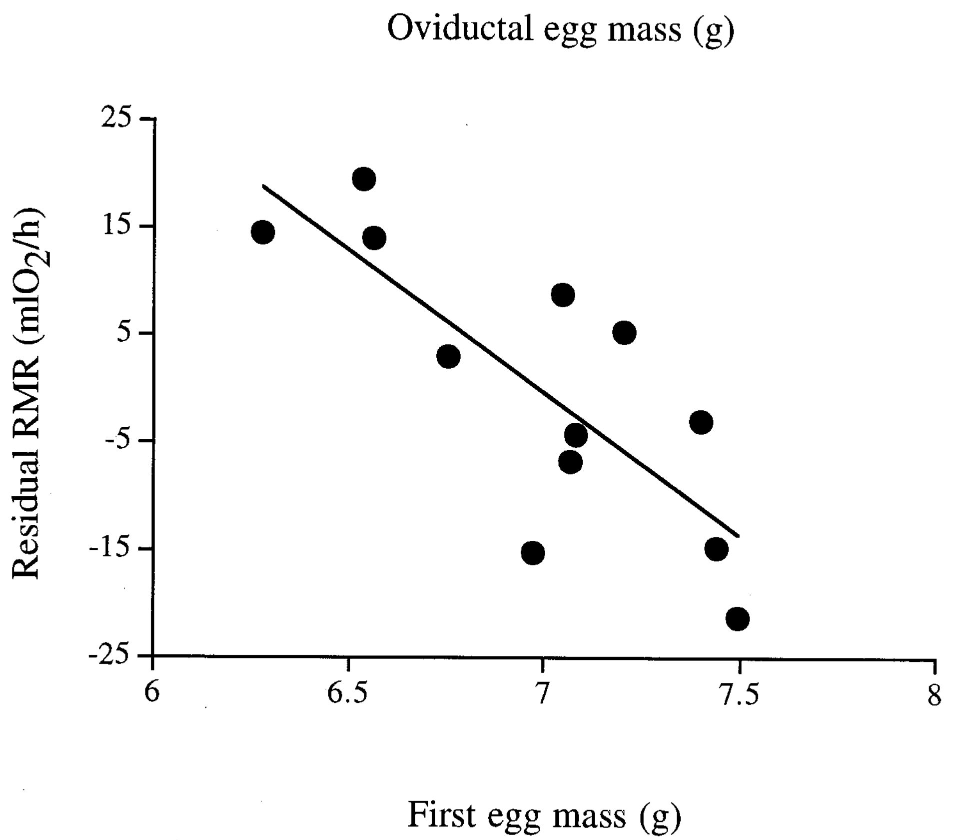


250
200
150
100
RMR (mlO2/h)
b
c
a
a b
b
a
a b
b
a
1999
2000
2001
Year



220
200
180
160
140
RMR (mlO$_2$/h)
0 1 2 3 4 5 6 5 4 3 2 1 0 CC
PL
LY
Follicle stage


Residual RMR (mlO2/h)
20
10
0
-10
-20
-30
6.5
7
7.5
8
Oviductal egg mass (g)


Residual RMR (mlO2/h)
25
15
5
-5
-15
-25
6
6.5
7
7.5
8
First egg mass (g)

CHAPTER THREE:
PLASTICITY IN BODY COMPOSITION IN BREEDING BIRDS: WHAT DRIVES THE METABOLIC COST OF EGG PRODUCTION?

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ABSTRACT

Body composition in vertebrates is known to show phenotypic plasticity, and changes in organ masses are usually rapid and reversible. One of the most rapid and reversible changes is the transformation of the female avian reproductive organs prior to breeding. This provides an excellent system to investigate the effects of plasticity in organ size on basal metabolic rate (BMR) through relationships between organ masses and BMR. We compared body composition of female European starlings (*Sturnus vulgaris*) during various reproductive stages over 3 years and investigated the pattern of changes in reproductive and non-reproductive organ mass during follicular development and ovulation. Furthermore, we analyzed the relationship between organ mass and resting metabolic rate (RMR) in non-breeding, laying and chick rearing females. Our analysis revealed marked variation in organ masses between breeding stages, but no consistent pattern among years except for kidney and pectoralis muscle. Fruthermore, changes in non-reproductive organs did not parallel the cycle of growth and regression of the reproductive organs. The oviduct gained 62% of its 22-fold increase in mass in only three days and oviduct regression was just as rapid, and began even before the final egg of the clutch was laid with 42% of the oviduct mass lost before laying of the final egg. In laying females, 18% of variation in mass-corrected RMR was explained by the mass of the oviduct ($r^2 = 0.18$, $n = 80$, $P < 0.0005$) while pectoralis muscle mass in non-breeding individuals and liver and gizzard mass in chick-rearing females were the only organs significantly related to RMR ($r^2 = 0.31$-0.44). We suggest that the non-reproductive organs are affected more by changes in local ecological conditions than reproductive state

itself, and that the activity and maintenance cost of the oviduct is high enough that selection has lead to a very tight size-function relationship for this organ.

INTRODUCTION

Several recent studies have demonstrated marked phenotypic plasticity in vertebrate anatomy in response to changes in ecological conditions or physiological state. For example, organs vary in size and function in response to such things as diet and food intake (Dykstra and Karasov 1992; Piersma et al.1993; Geluso and Hayes 1999), migration (Piersma et al. 1996; Biebach 1998; Karasov and Pinshow 1998; Piersma et al. 1999b; Battley et al. 2000, 2001), altitude (Hammond et al. 1999; 2001) or stress (Rogers et al. 1993). Moreover, these changes can occur over a short time scale (Gaunt et al. 1990; Secor et al. 1994; Secor and Diamond 1995; Jehl 1997; Piersma et al. 1999b) and are reversible (Piersma and Lindström 1997; Piersma et al. 1999a).

Inter-individual variation in body composition is generally believed to influence basal metabolic rate (BMR) through maintenance costs of organs and tissues (Kersten and Piersma 1987; Daan et al. 1990; Hammond and Diamond 1997; Piersma and Lindström 1997). Indeed, a common approach to investigate the basis of variation in metabolic rate is to examine the relationship between the mass of various body constituents and BMR (Konarzewski and Diamond 1995; Meerlo et al. 1997; Bech and Ostnes 1999, Chappell et al. 1999; Hammond et al. 2000; Piersma 2002). However, such studies have produced very inconsistent results in terms of which organs relate to metabolic rate even within a particular physiological state. For example, Chappell et al. (1999) found that BMR was related to the dry mass of liver, heart, lung and pectoralis muscle in reproductive adult house sparrows (*Passer domesticus*) and to the dry mass of gut, liver, heart and pectoralis muscle in juvenile individuals. However, in juvenile European shags (*Phalacrocorax aristotelis*), Bech and Ostnes (1999) found that RMR

was only related to lean dry liver mass and intestine length while another study by Burness et al. (1998) showed that daytime resting VO_2 in adult reproductive tree swallows (*Tachycineta bicolor*) was related only to fresh kidney and intestinal mass. Conflicting results between studies are not necessarily surprising if 1) the physiological state (e.g. wintering, chick rearing, etc.) of the species under investigation *is not* the primary determinant of organ plasticity, or if 2) changes in organ mass related to the physiological state are of relatively small magnitude. One of the largest and most rapid reversible changes in anatomy is the seasonal recrudescence and regression of the avian reproductive system during breeding. This is most marked in females, where the ovary and oviduct gain a tremendous amount of mass, growing to full functional size generally in a few days. Given this large change in organ size (Christians and Williams 1999) and the increase in RMR associated with egg formation (Nilsson and Raberg 2001; Vézina and Williams 2002), egg producing birds represent an excellent model system to investigate relationships between plasticity of organ mass and metabolic rate.

In a recent study (Vézina and Williams 2002), we investigated changes in resting metabolic rate (RMR, see methods) in female European starlings (*Sturnus vulgaris*) through the complete cycle of follicular development and ovulation during three consecutive breeding seasons. We showed that RMR increases by 22.4% from the beginning of pre-laying to the one-egg stage of laying, when birds have a complete developing follicle hierarchy and an egg in the oviduct. This estimate must reflect the additive energy costs of all the different physiological processes involved in egg formation: yolk precursor production in the liver (vitellogenin; VTG, and very low density lipoprotein; VLDL), follicular growth in the ovary, and albumen and shell

deposition in the oviduct. Here, we investigate the mechanistic basis of this increase in metabolic rate. We predicted that there would be consistent breeding stage-related variation in non-reproductive body composition *if* reproductive state was the prime determinate of organ size and metabolic rate. This would occur either 1) to compensate for the added cost of egg formation to the overall energy budget through resource reallocation, for example, decrease in size of some organs resulting in energy savings through lower maintenance costs (Geluso and Hayes 1999; Vézina and Williams 2002), or 2) to accommodate the increase in energy demand resulting from egg production, for example, increase in size of the food processing organs (Speakman and McQueenie 1996; Hammond and Diamond 1997; Piersma and Lindström 1997). We report data for two levels of analysis by a) comparing three different breeding stages - non breeding, one-egg and chick rearing - and b) by presenting a more detailed analysis of organ mass changes through the complete cycle of follicular development during egg production. Our hypothesis therefore was that organs specifically adjusted to the demands of egg production should be smallest (or biggest) in one-egg birds compared to non-breeders and chick-rearing individuals and that within egg-producing birds, non-reproductive organ mass should show the inverse, (or a parallel) pattern of mass change for gonadal development and regression through the follicle development and ovulation cycle (see Figure 1).

Material and Methods

Field site and collection of birds

Field work was carried out at the Pacific Agri-Food Research Center (P.A.R.C.) in Agassiz B.C., Canada (49°14`N, 121°46`W) under Simon Fraser University animal care permit (499B), following guidelines of the Canadian Council on Animal Care. The site consists of approximately 175 nest-boxes on farm buildings and telephone poles that were used each year by breeding starlings. Each year nest boxes were checked daily to determine dates of clutch initiation and clutch completion and the laying sequence of eggs. During laying and early chick-rearing females were taken from their nest boxes during night-time (generally between 20:00h and 24:00h); during late chick-rearing provisioning females were trap-caught (always within an hour before sun set). Non-breeding and pre-laying females were mist-netted at two barns that were used as roosting sites. Eggs were collected for mass and size measurements at the time females were caught.

Reproductive stages

In all three years (1999-2001), we measured RMR in birds at the end of the wintering period (non-breeding, NB), at the one egg stage of laying (LY-1) and during chick provisioning (CK). We measured birds at the one-egg stage of laying since this represents the day of peak energy investment in egg formation based on theoretical models (e.g. Ojanen 1983; Krementz and Ankney 1986; Williams and Ternan 1999; but see Vézina and Williams 2002) and patterns of yolk precursor production (Challenger et al. 2001).

At this point, all birds have laid an egg, the second egg of the clutch is in the oviduct and all the remaining follicles are sequentially developing in the ovary.

In 2000 and 2001 we also measured RMR in random-caught females during the ten days prior to the appearance of the first egg in the colony (pre-layers; PL). This pre-laying group contained individuals at different levels of ovarian follicle development and oviduct growth, but included no females that had laid their first egg. After recording the first clutch initiation in the colony, we began measuring RMR in birds at all stages of egg laying (eggs 1 to 6) through to clutch completion. For all birds we measured resting metabolic rate and completed dissections for body composition analysis as described below. Sample sizes for RMR measurements are presented in Table 1.

Measurement of resting metabolic rate (RMR)

We define RMR as the energy consumed by a post-absorptive bird during the resting phase of the circadian cycle at a temperature within the thermoneutral range for the animal. Note that this is what is usually defined as *basal* metabolic rate (Blem 2000). By definition BMR is the lowest measurable VO_2, and because laying birds in this study are producing eggs, they have to be considered in an "active physiological state" which induces elevated levels of energy consumption (Vézina and Williams 2002). Therefore we consider the term *resting* metabolic rate more appropriate in the present case. Resting metabolic rate (VO_2) was measured by flow through respirometry (Sable Systems International) following Vézina and Williams (2002). After capture, birds were brought to the laboratory, body mass was measured (± 0.1 g), and birds were then placed in metabolic chambers (3.5L) for one hour prior to the beginning of the measurements. All

birds received about 500ml/min of dry CO_2-free air and were kept in the dark at 25°C, which is within the thermoneutral zone for this species (Lustick and Adams 1977). Each chamber was sampled for oxygen and CO_2 analysis one at a time separated by ten minutes of ambient baseline air readings (starting with baseline). RMR measurements were always carried out between 23:00h and 5:00h. Our setup allowed us to collect RMR data for four birds a night. On average, the birds stayed in their chambers for approximately 5.5 hours. Preliminary data showed that sequentially measuring RMR in this way did not generate a time effect (see Hayes et al. 1992). Thus, having one or four hours to rest did not affect RMR of the birds (F. Vézina, unpublished data). Following RMR measurements, the birds were re-weighed and the average of first and second mass was used in subsequent calculations. To calculate RMR, a running mean representing ten minutes of recording was passed through the data for each bird, with the lowest average VO_2 taken as RMR. In some cases (less than 6%) females laid an egg in the chamber before the end of the recordings; RMR data collected from these birds were therefore discarded.

Body composition analysis

After RMR measurements, birds were sacrificed by exsanguination under anesthesia (ketamine:xylasine at doses of 20mg/kg and 4mg/kg, respectively), their feathers were plucked and they were dissected. We recorded the fresh mass of the reproductive organs (oviduct and follicle-free ovary), the individual weights of all ovarian follicles, and the weight of the oviductal egg (± 0.001 g). The number of follicles and the presence of post-ovulatory follicles allowed us to confirm the breeding status of every bird. We also

dissected out the following organs: pectoralis muscle (left and right reported here together as pectoralis muscle, not including supracoracoideus), heart, kidney, liver, gizzard, small intestine (from the gizzard to the caecae) and pancreas. All samples were kept frozen at -20°C until the end of the field season for further processing. Adipose tissue in starlings is known to have a very low energy consumption (Scott and Evans 1992). Therefore, to avoid any dilution effect when investigating body mass or organ mass vs RMR relationships all organs and carcasses were freeze-dried (Virtis™ Freezemobile model 8ES) and fat-extracted in a Soxhlet apparatus using petroleum ether. Here we report lean dry body mass (LDBM) as being lean dry carcass mass plus lean dry organ mass (excluding the oviductal egg and feather mass). Final sample sizes for body composition data are presented in Table 1.

Yolk precursor analysis

In order to measure plasma levels of VTG and VLDL, blood samples were centrifugated at 5000 rpm for 10 minutes and the plasma portion of each sample was assayed for yolk precursors using vitellogenin zinc (Zinc kit, Wako Chemicals) and total triglycerides (Triglyceride E kit, Wako Chemicals) as indices of VTG and VLDL, respectively (Mitchell and Carlisle 1991; Williams and Christians 1997; Williams and Martiniuk 2000). The overall inter-assay coefficient of variation for the vitellogenic zinc and triglyceride assays (calculated from repeated analyses of the same sample) were 16.3% and 15.3%, respectively.

Statistical analysis

Variations in organ masses between NB, LY-1 and CK groups were investigated on a per year and per organ basis using ANCOVA models. In this particular case we wanted to control for the effect of body mass on organs mass. Because LY-1 birds have fully developed reproductive organs, we used non-reproductive lean dry body mass (NRLDBM) as a covariate, i.e. the total lean dry body mass minus the mass of the reproductive organs. Also, in order to avoid part-whole correlations (Christians 1999) we subtracted the mass of the organ used as the dependent variable from the covariate. Because this procedure generated a substantial amount of post-hoc comparisons (3 reproductive stages and 3 years per organ) we used the Bonferroni procedure (Rice 1989) to correct the *p* level of significance. Organs were compared as functional groups (heart, kidney and muscles being the "metabolic machinery" organs (Daan et al. 1990; Christians and Williams 1999) and liver, small intestine, pancreas and gizzard being the "food processing" organs). Therefore, Bonferroni-corrected *p* values were 0.002 and 0.001 for metabolic machinery and food processing organs, respectively.

Organ mass variation in relation to follicular growth and ovulation was also analyzed for years 2000 and 2001. In this case, we used an ANCOVA model including year and follicle development stage as independent variables, and non-reproductive lean dry body mass (corrected for part-whole correlation) as a covariate. Because we were interested in the pattern of change of non-reproductive organs in relation to growth and regression of the reproductive organs, we subsequently compared differences in organ masses from early rapid yolk development (no yolky follicles) to the peak of development (6 follicles prior to first ovulation) and then from the peak to clutch

completion. Analysis of the relationships between RMR and body composition was performed for the NB and CK birds and on a sub-sample of the LY group (see results). In this case, ANCOVA models with year as an independent variable and NRLDBM (LY birds) or LDBM (NB and CK birds), corrected for part-whole correlation, as a covariate were used to generate residual RMR and residual organ masses when significant. Residual RMR was then compared to residual organ mass in a multiple stepwise regression model. Results are presented as least square means ± SE.

RESULTS

Variation in organ masses between breeding stages and years

There was marked variation in body-mass-corrected organ masses both between reproductive stages (NB, LY-1 and CK) and between years (Figures 2 and 3). In two cases the breeding stage x NRLDBM interaction term in the ANCOVA model was significant: for lean dry intestine in 1999 ($F_{2,52} = 3.28$, $P < 0.05$) and lean dry pancreas in 2001 ($F_{2,46} = 4.01$, $P < 0.05$). For these two cases, least square means presented in Figure 2 were calculated with the interaction left in the model. In all other cases least square means were calculated with only the covariate (NRLDBM corrected for part-whole correlation) left in the model.

Food processing organs.

For the food processing organs, variation in organ mass by breeding stage was not consistent between years (Figure 2). Lean dry liver mass tended to vary significantly between reproductive stages, but the pattern differed from year to year with liver being

11.3% heavier in CK compared to NB birds in 2000 (Bonferroni corrected post-hoc t-test, $P < 0.0005$) and 25.4% heavier in LY-1 compared to NB birds in 2001 ($P < 0.0005$; Figure 2a). In all years lean dry small intestine tended to be heavier in CK birds but this was significant only in 2001 ($P < 0.001$ in all cases) with a maximum mass difference of 18.2% (Figure 2b). Lean dry gizzard mass also showed significant differences between reproductive stages in 1999 ($P < 0.0005$ in all cases; maximum difference of 12.1%) and 2001 ($P < 0.0005$ in all cases; maximum difference of 15.1%) but again the pattern differed between years (Figure 2d). Lean dry pancreas showed no significant difference between stages in all years ($P > 0.02$ in all years, Figure 2c).

Metabolic machinery.

For the metabolic machinery organs, there were more consistent breeding stage-related changes between years. Lean dry kidney mass showed a consistent pattern in 2000 and 2001 being on average 17.2% (Bonferroni corrected post-hoc t-test, $P < 0.0005$) and 16.3% ($P < 0.0005$) heavier in LY-1 and CK birds, respectively, compared to NB birds (Figure 3b) while muscle mass decreased from NB to CK by 10.9%, 13.2% and 9.6% in 1999, 2000 and 2001 ($P < 0.0005$ in all cases; Figure 3c), respectively. In contrast, heart mass did not show any consistent pattern between reproductive stages among year (Figure 3a).

Organ mass variation and yolk precursors during follicular development

Combining data from pre-laying and laying birds for 2000 and 2001 allowed us to look at variation in lean dry organ mass over the complete sequence of egg formation from the

beginning of rapid yolk development (RYD) – that is, no yolky follicles – to clutch completion. Figures 4 to 7 present changes in lean dry organ mass and plasma yolk precursors as the animal is growing the yolky follicles (PL group, follicles 0 to 6 on the x axis), then through egg laying as one follicle is ovulated per day (LY group, follicles 5 to 0 on the x axis) until the clutch is completed (CC).

Reproductive organs.

The reproductive organs varied according to the stage of follicle development (Figure 4; lean dry follicle–free ovary: $F_{13.157} = 14.25$, $P < 0.0001$; lean dry follicles: $F_{13.144} = 129.00$, $P < 0.0005$, lean dry oviduct: $F_{13:144} = 164.46$, $P < 0.0005$). Although there was a significant year x follicle stage interaction both for lean dry follicle mass ($F_{12,144} = 2.76$, $P < 0.005$) and oviduct mass ($F_{12,144} = 2.55$, $P < 0.005$), both organs showed very similar patterns for 2000 and 2001 (Figure 4a and b) with a very rapid growth at two yolky follicles and a similarly rapid loss of mass after the last ovulation. The oviduct increased in lean dry mass 22-fold from follicle 0 to follicle 6 (PL group Figure 4b), achieving 62% of its growth in less than 3 days (between the 2 and 4 follicles stages in PL) and lean dry follicle mass reached 72% of its maximal mass during the same time. The oviduct reached a peak mass just prior to the first ovulation in both years (follicle 6 in PL group, Figure 4b), and maintained a constant mass until the last follicle was ovulated and then began to regress rapidly. In fact, the oviduct started to regress in mass as soon as there were no more yolky follicles in the ovary – that is – the oviduct lost mass while it was *still* producing an egg. Indeed, in LY birds at follicle stage 0 (no remaining follicles, with an oviductal egg), the oviduct had already regressed by an average of 42.5% of peak mass (both years, Figure 4).

Plasma yolk precursor levels.

The pattern of variation in plasma yolk precursor levels in relation to follicular development was similar to that for reproductive organ growth and regression. Plasma VTG increased rapidly during rapid yolk development and stayed high until the last ovulation ($F_{13.154} = 25.54$, $P < 0.0005$, Figure 5a). The year x follicle stage interaction was significant for the VLDL analysis ($F_{12,140} = 3.00$, $P < 0.001$). However, plasma VLDL levels showed similar patterns and mirrored VTG with a sharp decrease after the last ovulation (Figure 5b). This confirms the pattern of plasma yolk precursors production reported earlier by Challenger et al. (2001) for the same population of birds.

Food processing organs.

Lean dry liver, small intestine and pancreas mass varied significantly with stage of follicle development when controlling for year and NRLDBM (liver: $F_{13,143} = 2.82$, $P < 0.005$; small intestine: $F_{13,155} = 2.94$, $P < 0.001$; pancreas: $F_{13,155} = 1.95$, $P < 0.05$, Figure 6). The year x follicle stage interaction was significant for liver mass ($F_{12,143} = 2.17$, $P < 0.05$) so we present data for both years separately (Figure 6a; no other significant interaction terms in any cases). In 2000, lean dry liver mass increased by 28.8% from the beginning of pre-laying (Follicle = 0 in the PL group, Figure 6a) to the day before the first egg was laid (Follicle = 6 in the PL group, Figure 6a; independent contrasts $P < 0.05$). It then remained constant until clutch completion (independent contrast $P = 0.5$). However, this pattern was not detected in 2001 (Figure 6a). Lean dry small intestine mass did not peak at the six follicles stage (independent contrast $P = 0.2$) but instead showed a gradual increase in mass (22.7%) throughout the egg production cycle from the 0 follicle stage to clutch completion (independent contrast $P < 0.0005$, Figure 6b). The pancreas

showed a similar pattern (no peak at six follicles, independent contrast P = 0.2) with a total increase in mass of 14.3% between follicle stage 0 and clutch completion (independent contrasts P < 0.01, Figure 6c). Lean dry gizzard mass did not vary in relation to follicle development stage.

Metabolic machinery.

Among the metabolic machinery organs, only lean dry kidney and pectoralis muscle varied significantly in relation to follicle development stage when controlling for year and NRLDBM (kidney: $F_{13,155} = 5.90$, $P < 0.0005$; muscle: $F_{13,155} = 2.21$, $P < 0.05$). Lean dry kidney mass increased by 17.3% from 0 to 6 follicles in the pre-laying group, but then stayed constant until clutch completion (Figure 7a, independent contrasts 0-6 follicles P < 0.005; 6 follicles to clutch completion P = 0.4). Lean dry pectoralis muscle mass decreased gradually (3.9%) throughout the follicular cycle (Figure 7b, 0 follicle in the PL group to clutch completion; independent contrasts P < 0.05) and showed no distinctive pattern of mass loss (pre-laying 0-6 follicles or 6 follicle to clutch completion in the laying group; independent contrasts P > 0.05).

What drives the metabolic cost of egg production?

The pattern of development of the reproductive organs in addition to the pattern of yolk precursors production in relation to follicle development stage are very similar to changes in RMR in egg producing females we reported in an earlier paper (Vézina and Williams 2002). Therefore, in order to investigate the effect of body composition on metabolism in laying females, we analyzed the potential relationships between residual RMR and

residual organ mass (correcting for follicular stage, year and NRLDBM effect). Since pre-laying birds were caught at different stages of reproductive development, we excluded them from the analysis because they would artificially increase the mass range of the reproductive organs. The same reasoning applies to birds with no remaining follicles (LY at 0 follicles). We therefore restricted the analysis to LY birds having 5 to 1 follicles left to ovulate. At this point all individuals had fully grown oviducts (Figure 4b) and high plasma levels of VTG and VLDL (Figure 5). All the variables to be included in the model were first checked for multicollinearity (Zar 1996; Table 2). Including all the organs in the model, stepwise multiple regression indicated that for the three years of study, 17.6% of the variation in residual LY RMR was explained by residual lean dry oviduct ($r^2 = 0.18$, $n = 80$, $P < 0.0005$, Figure 8). Residual RMR was independent of residual plasma level of VTG or VLDL (correcting for year effect) and residual precursor levels were not related to residual lean dry liver mass (correcting for year and NRLDBM).

RMR and residual organ masses in non-breeders and chick rearing individuals

As for the laying birds, we performed stepwise multiple regressions relating residual RMR to residual organ masses (controlling for effects of year and lean dry body mass) in non-breeding and chick-rearing birds (see Table 3 for multicollinearity). In non-breeders, residual pectoralis muscle mass was the only organ significantly related to residual RMR ($r^2 = 0.31$, $n = 53$, $P < 0.0001$). In chick-rearing birds, variation in residual RMR was explained by two organs: residual liver mass (34%), and residual gizzard mass (10%; overall model $R^2 = 0.44$, $n = 36$, $P < 0.0001$).

DISCUSSION

In the present study we have confirmed that during egg production, female starlings undergo rapid and very large mass changes in reproductive organs (22-fold for the oviduct). This was accompanied by major changes in plasma protein and lipid (yolk precursors) levels and a 22% increase in RMR (Vézina and Williams 2002). This confirms that our egg-producing females were in a very different physiological state compared to non-breeding and chick-rearing individuals. We have also shown that body mass-independent non-reproductive organ masses varied markedly (9-25%) between breeding stages, however, there was no consistent pattern among years in relation to specific breeding stages. Moreover, in egg producing females, the pattern of non-reproductive organ mass change did not reflect the cycle of reproductive development and regression seen for the oviduct, ovary, and yolk precursors. Rather organs either changed linearly through laying, or not at all. On the other hand, the pattern of oviduct recrudescence and regression closely followed that of follicular development with a very rapid mass gain at the two follicles stage and a rapid loss of mass after the last ovulation (even though at this stage the oviduct was still processing an egg). In laying birds, 18% of the variation in residual RMR was explained by residual lean dry oviduct. In contrast, non-breeding residual RMR was correlated to residual lean dry pectoralis muscle mass, while residual lean dry liver and gizzard were the organs that significantly predicted RMR in chick-rearing birds.

Adjustments of non-reproductive organs for egg-production

We hypothesized that if reproductive state is a prime determinant of organ size, in order to adjust to the demand of egg production, non-reproductive organs should either 1) be consistently heavier or lighter in laying birds compared to non-breeding and chick-rearing individuals and/or 2) show a pattern of change in mass mirroring the cycle of gonadal development and regression. We found little support for this hypothesis. In fact, only the gizzard showed one of the predicted patterns: a significant decrease in mass in laying individuals compared to non-breeding and chick-rearing birds. However this was not consistent among years. Conversely, kidney and pectoralis muscle mass did show a consistent pattern between years, but it appears that the laying stage per se was not driving this morphological change since the mass of these organs did not peak or dip at the one egg stage compared to non breeding or chick rearing individuals. Rather, mass tended to stay constant (kidney), or continued to decrease (pectoralis) after clutch completion through to chick-rearing. Furthermore, our more detailed analysis based on the pattern of follicular development and ovulation showed that marked changes in reproductive physiology in terms of ovary, follicles, oviduct and yolk precursors were not accompanied by similar changes in non-reproductive organs. That is, the only organs that showed significant changes in mass related to follicular stages (liver, small intestine, pancreas, kidney and pectoralis muscle) did not vary in a pattern similar or inverse to that of reproductive organs. These results strongly suggest that the physiological state of a bird in itself (i.e. its breeding stage) does not determine the marked organ plasticity that we documented.

Christians and Williams (1999) investigated organ mass changes in breeding female starlings of the same population during two years preceding this study. They also reported an increase in lean dry glycogen-free liver mass from non-breeding to laying, but this was significant for only one year. Similarly, our results for lean dry liver mass showed a significant increase from NB to LY-1 in 2001, but no significant differences were found in 1999 and 2000. Furthermore, liver mass variation showed completely different patterns between the three stages for the three years and the reasons for this between-year difference are not clear. Christians and Williams (1999) also found a correlation between plasma levels of yolk precursors and liver mass but in only one of two years in this population. We did not find such a relationship for the three years of our study and these combined results indicate that increased production of VTG and VLDL is rarely associated with liver hypertrophy.

If plasticity in non-reproductive organ is not directly related to breeding stage, what is responsible for the substantial changes in organ masses reported here? Part of the answer may come from the food processing organs. Lean dry small intestine mass increased by 22.7% from beginning of rapid yolk development to clutch completion and tended to gain even more mass later as shown by a significantly heavier intestine in CK birds in 2001. Changes in small intestine function and mass associated with changes in diet have been reported before for this species (Levey and Karasov 1989; Geluso and Hayes 1999). We do not have information on diet composition for our population, but adjustments in small intestine mass may reflect a gradual seasonal change in diet. Indeed, lean dry kidney and pancreas mass both increased during the same period and these organs are known to gain weight on protein rich diets (Imondi and Bird 1967; Hammond

and Janes 1998; but see Goldstein et al. 2001). It is possible that the diet of our experimental birds included an increasing proportion of protein, possibly coming from insects (Feare 1984).

We also documented a gradual loss in lean dry pectoralis muscle mass from non-breeding to chick-rearing (-10.9%, -13.2% and -9.6% in 1999, 2000 and 2001, respectively) as well as throughout the cycle of follicle growth and egg laying. Many other studies have reported loss of muscle mass during breeding in a variety of avian species (Jones 1990; Houston et al. 1995a,b,c; Cottam et al. 2002). Lean dry muscle contains mostly proteins (Jones 1990) so muscle protein breakdown could also contribute to the reported increase in kidney mass in response to high protein levels in the blood. Some studies have suggested that muscle proteins could be transferred into egg material (Jones 1990; Houston et al. 1995a,b,c; Cottam et al. 2002). Although our experiment was not designed to study this phenomenon in particular, it is of interest to note that the loss of pectoralis muscle mass did not precede onset of follicular growth. Indeed, average NB lean dry pectoralis mass for 2000 and 2001 combined was virtually the same as early PL birds at follicle 0 (4.1 ± 0.05g in NB and 4.2 ± 0.05g in PL-0). At clutch completion, their muscle weighed 3.9 ± 0.3g (7.1% loss during egg production). However, the average CK lean dry muscle mass was 3.7 ± 0.1g indicating that pectoral muscle lost a further 5.1% mass during the incubation and chick rearing periods. Thus it appears that the loss of pectoralis muscle mass was not specifically associated with the egg production phase but was a more general phenomenon associated with all breeding stages. An alternative explanation for muscle mass loss during LY and CK periods is a higher state of physical activity, since activity training has been reported to result in loss of muscle mass in this

species (Swaddle and Biewener 2000). Overall, it appears that kidney and muscle mass changes, even though they show consisteht patterns between years, are not strictly related to egg production, but are affected by factors more or less independent of reproductive state.

Our results suggest that, in European starlings, egg formation has no consistent effect on plasticity of non-reproductive organ masses. Organ masses are dynamic and evidence that they are simply adjusting to local ecological conditions is accumulating (Piersma and Lindström 1997; Summers et al. 1998; Hammond et al. 1999; Hilton et al. 2000). For example, Hilton et al. (2000) showed intra-specific variation in seabird organ masses living in different geographic locations in Iceland and therefore facing different ecological conditions. It is reasonable to assume that varying conditions between years for a given geographic location might have similar effects on body composition as intra-specific differences between different locations. We therefore suggest that yearly differences in ecological conditions at the time of breeding may have more impact on non-reproductive organ mass variation in breeding starlings than the physiological changes associated with egg formation. However, organs may adjust their mass-specific metabolism (Kvist and Lindström 2001) and possibly reduce their energy expenditure without a change in mass, thus still allowing for energy reallocation in breeding females.

What drives the cost of egg production?

We recently showed that RMR in breeding female starlings increases by 22.4% from early RYD (0 follicles in PL) to the day prior to the first ovulation (follicle 6 in PL) (Vézina and Williams 2002). Results of the present study demonstrate that during laying,

when the reproductive organs are fully developed and plasma levels of yolk precursors are maximal, the mass of the active oviduct explains 18% of the variation in mass-corrected RMR. This is consistent with the hypothesis that the maintenance and activity costs of the oviduct are high enough to affect the overall energy consumption of the animal at rest. A positive relationship between metabolic rate and the fresh mass of the oviduct has also been observed in pre-laying zebra finches in our laboratory (F. Vézina, unpublished results). Furthermore, Chappell et al. (1999) reported a correlation between BMR and dry combined ovary and oviduct mass in breeding house sparrows. However, the present study is the first to investigate the full development of the reproductive system in relation to follicular growth and to relate it to breeding metabolic expenditures. It appears that a breeding-size oviduct is consuming enough energy to have an effect on RMR in the only three species for which data are available. This suggests that this organ is probably responsible for part of the 22% increase in breeding RMR in starlings (Vézina and Williams 2002). The very rapid pattern of growth and regression of the oviduct supports the idea that this is an energetically expensive organ. However, an alternative explanation could be that an hypertrophied oviduct adds mass and reduces flight maneuverability, thus potentially increasing the cost of flight.

The pattern of recrudescence and regression of the oviduct occurs very rapidly with most of the mass gain or loss occurring in three days or less. Our most important finding in regards to this organ was that the oviduct begins to lose mass *before* the last egg of the clutch is laid (LY birds at follicle 0). The day before clutch completion, while the last egg is being processed, the oviduct had already lost an average of 43% of its mass. This differs somewhat from the pattern of oviduct regression shown by Houston et

al. (1995b) in the zebra finch (*Taeniopygia guttata*), where the oviduct apparently starts to regress after the first egg is laid. However, their oviduct data were presented relative to the number of days in the laying cycle and since there is a lot of variation in clutch size in this species (Williams 1996) this may result in a significant bias if their sample contains several small clutch individuals.

Since the last egg of the clutch in starlings does not differ in size or quality (Ricklefs 1984; Greig-Smith et al. 1988) compared to previously laid eggs, it seems that a full grown oviduct may not be necessary for proper albumin and shell deposition. However, we think this assumption is unlikely. Why would birds maintain a large oviduct if it can be as efficient when reduced in mass by 42%? Ricklefs (1976) reported a positive correlation between oviduct mass and egg size in starlings and we confirmed this observation by finding a positive relationship between lean dry oviduct mass and oviductal egg mass in our birds across years ($r = 0.49$ $n = 87$ $p < 0.001$; F. Vézina, unpublished data). Similarly, Christians (2001) found that the mass of the oviduct explains approximately 21% of the variation in albumen protein content in starlings. Therefore, because larger eggs generally tend to confer higher early chick survival (Williams 1994), it is reasonable to assume that producing a large oviduct is advantageous. However there is a downside to having a large oviduct. Our results imply that this organ may be energetically expensive to maintain and that it is presumably preferable to shut it down as soon as it has accomplished its function (i.e. after the last ovulation). A possible mechanism explaining the regulation of oviduct recrudescence and regression could be that this organ is sensitive to the same hormonal controls that regulate follicle development and ovulation. Although we did not record data on the pattern of

development and regression of the particular sub-sections within the oviduct itself, we know, from birds that expulsed eggs in the metabolic chambers, that our birds were in the phase of shell formation during measurements. It is therefore reasonable to assume that the oviduct is simply regressing "top-down" as the follicle moves down from the infundibulum to the shell gland. Assuming this is true, the shell gland would still be fully functional at measurement time even if the infundibulum and magnum were starting to regress.

If the oviduct is playing a role in the cost of egg production, its mass nevertheless explains only 18% of the variation in elevated laying RMR. Therefore, there must be other processes, which do not result in significant non-reproductive organ mass variation, that are involved in the increased energy consumption of egg-producing females. Yolk precursor production is estimated to triple the amount of proteins secreted by the liver in laying hens compared to non laying individuals (Gruber 1972). This apparently is not, or very rarely, associated with liver hypertrophy (Christians and Williams 1999 and this study). Laying RMR was not related to VTG and VLDL plasma levels in our study. Nevertheless, because we only measured the amount of yolk precursor present in the blood and not liver metabolic activity, it is still possible that the liver mass-specific metabolism affects RMR in laying females. Liver mass did not correlate with RMR in our birds, but a highly active liver could increase its energy consumption per unit mass without changing its total mass, resulting in no relationship between the mass of this organ and RMR. This could be investigated by comparing liver mass–specific oxidative capacity in non-breeding and laying birds. Clearly, studies investigating effects of organ mass-specific energy consumption on RMR are needed.

Body composition effects on non-breeding and chick-rearing RMR

Comparing the relationship between organ masses and RMR for non-breeding, laying and chick-rearing groups allows us to highlight two important points about our understanding of the basis of variation in RMR or BMR. In our study, 31% of RMR variation was explained by lean dry pectoralis muscle mass in non-breeding birds. These birds where caught at the end of the wintering season (mid-March) and we suggest that this relationship is driven by thermoregulatory demands associated with shivering thermorgenesis (Swanson 1991; O' Connor 1995). This hypothesis is supported by the fact that non-breeding birds had the heaviest pectoralis muscle mass relative to body size of all three breeding stages. In chick-rearing birds, 44% of variation in residual RMR was explained by the combined effects of liver and gizzard mass but, in this case, it is unclear why these organs had the biggest effect in the model. These conflicting observations clearly illustrate that within a single species, analysis relating body composition to RMR variation may yield very different results depending on the animal's physiological state. Moreover, as we pointed out in the introduction, several studies have shown a relationship between RMR and very different organs even in birds with similar physiological states, for example, those functioning at presumed maximum sustained metabolic rates. It is noteworthy that although Daan et al.'s (1990) article reported an interspecific relationship between combined heart and kidney mass and BMR (but not other organs) in chick-provisioning individuals, very few articles have shown consistent results with regard to these organs and resting or basal metabolic rate. For example, Burness et al. (1998) showed that the mass of kidney and intestine was significantly related to resting VO_2 in chick-rearing tree swallows but in our study, RMR was related

to liver and gizzard in chick-rearing starlings. Similarly, in a study on red junglefowl (*Gallus gallus*), Hammond et al. (2000) demonstrated differences between sexes, with males exhibiting a correlation between BMR and the mass of small intestine, proventriculus, large intestine, lung and caecum, while in females only the mass of the spleen was related to BMR. Furthermore, there were no significant correlations between reproductive organ mass and metabolic rates, although according to their data (see Hammond et al. 2000, Table 1) their birds were in a reproductive state. These data therefore conflict with Chappell et al.'s (1999) study as well as the results of our study which showed a significant effect of oviduct mass on RMR. Another important point is the relatively low coefficient of determination generally obtained in this type of analysis for all studies to date. In our study, independent organ masses explained 10-34% of variation in RMR which is comparable to other published results (5-18%, Hammond et al. 2000; 30-52%, Chappell et al. 1999; 39-44%, Bech and Ostnes 1999). This highlights the fact that relative organ masses are often a relatively poor predictor of RMR or BMR variations (c.f. Piersma 2002). Organs show plasticity in response to local ecological conditions, but the direction of the changes may differ between organs and systems resulting in compensatory effects on overall resting energy consumption which truly complicates comparisons between studies. There is no doubt that more research is needed to clarify this problem. However, future studies investigating the physiological basis of variation in BMR or RMR should investigate both the size and the metabolic intensity of specific organs.

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Table 1: Sample sizes for body composition and RMR data collected from 1999 to 2001.

	1999	2000	2001
Body composition			
NB	18	19	17
PL	---	10	25
LY-1	20	20	15
Rest of LY	---	38	63
CK	20	12	20
RMR			
NB	17	19	17
PL	---	9	24
LY-1	12	20	14
Rest of LY	---	37	41
CK	5	12	19

Note. NB = non-breeding, PL = pre-laying, LY = laying, LY-1 = laying birds at the one-egg stage, CK = chick-rearing.

Table 2: Inter-correlation matrix of residual organ masses for laying birds having 5 to 1 yolky follicle left to ovulate for 2000 and 2001.

	Liver	Heart	Kidney	Ovary	Oviduct	Muscle	Intestine	Pancreas
Heart	0.08							
Kidney	0.41*	0.09						
Ovary	0.04	-0.06	0.04					
Oviduct	0.08	0.02	0.16	0.06				
Muscle	-0.13	0.12	0.13	-0.008	-0.14			
Intestine	0.23	0.15	0.21	0.08	0.02	-0.17		
Pancreas	0.15	-0.02	0.17	-0.05	0.16	-0.16	0.38*	
Gizzard	0.08	0.003	0.11	-0.05	0.17	-0.09	0.10	0.11

Note. Residuals correct for effect of follicular stage, year and lean dry non-reproductive body mass. Asterisks denote significant correlation with a level of significance adjusted to $P < 0.0013$ using a Bonferroni correction.

Table 3: Inter-correlation matrix of residual organ masses for non-breeding and chick-rearing birds for years 1999, 2000 and 2001.

	Liver	Heart	Kidney	Ovary	Oviduct	Muscle	Intestine	Pancreas	Gizzard
Liver		0.06	0.59*	-0.12	0.14	0.2	0.22	0.17	0.24
Heart	-0.02		0.02	-0.22	0.05	0.08	-0.01	0.11	0.12
Kidney	0.26	-0.01		-0.1	0.12	0.09	0.05	0.31	0.18
Ovary	0.01	0.18	-0.06		0.3	0.15	-0.13	-0.22	-0.18
Oviduct	-0.08	-0.16	-0.03	0.35		0.05	-0.22	0.17	0.08
Muscle	-0.16	0.42	0.05	-0.07	-0.01		-0.15	-0.15	-0.02
Intestine	0.32	0.08	0.03	0.26	-0.02	-0.13		0.22	-0.06
Pancreas	0.37	-0.06	0.27	-0.07	0.04	-0.19	0.34		0.12
Gizzard	0.18	-0.32	-0.06	0.05	0.12	-0.01	0.18	0.28	

Note. Values below the diagonal are for non-breeding individuals; values above the diagonal are for chick-rearing individuals. Residuals correct for effect of year and lean dry body mass. Asterisks denote significant correlation with a level of significance adjusted to $P < 0.0013$ using a Bonferroni correction.

FIGURE LEGENDS

Figure 1: Examples of organ mass changes that would most strongly support the stated hypothesis. In the first case (a), a decrease in non-reproductive organ mass in birds at the one-egg stage (LY-1) compensate for the added cost of egg production to the overall energy budget through resource reallocation resulting in bigger organs in non-breeding (NB) and chick-rearing (CK) individuals. Within egg producing birds (b), non-reproductive organ mass (circles) shows a pattern of change inverse to that of the reproductive organs (squares). In the second case (c), non-reproductive organs accommodate the increase in energy demand by an increase in their mass. This results in one-egg birds exhibiting heavier non-reproductive organs relative to non-breeding and chick-rearing individuals. In this case, non-reproductive organs show a pattern of mass change that parallels that of reproductive organs (d). Any other pattern of mass change would be inconsistent with our hypothesis. PL = pre-laying, follicular growth phase, LY = laying, follicle ovulation phase, IN = incubation.

Figure 2: Inter-annual and inter-stage variation in lean dry mass of liver (a), small intestine (b), pancreas (c), and gizzard (d) in European starlings in 1999-2001. Values are least square means ± SE controlling for NRLDBM (corrected for part-whole correlation). Bars over the columns indicate within year significant differences between breeding stage. Level of significance is 0.001.

Figure 3: Inter-annual and inter-stage variation in lean dry mass of heart (a), kidney (b), and muscle (c) in European starlings in 1999-2001. Values are least square means ± SE controlling for NRLDBM (corrected for part-whole correlation). Bars over the columns indicate within year significant differences between breeding stage. Level of significance is 0.002.

Figure 4: Changes in lean dry mass of ovary (triangles in a), total yolky follicles (a), and oviduct (b) relative to the number of yolky follicles for a European starling with a

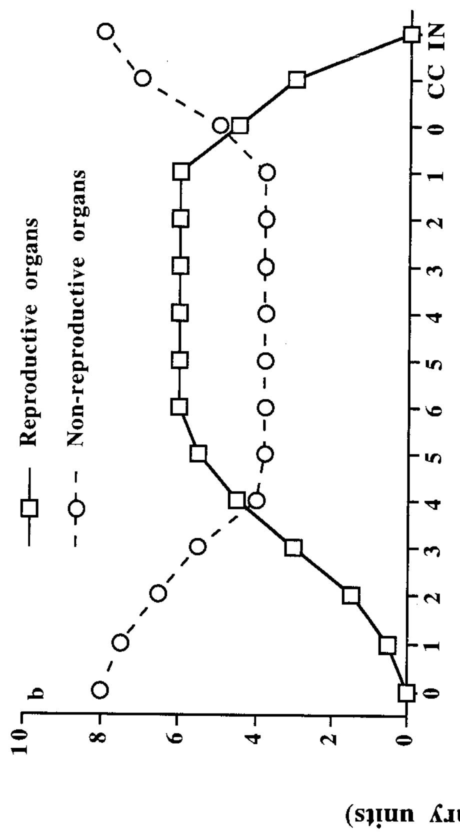
full six-follicle hierarchy in 2000 and 2001 controlling for NRLDBM and year. Pre-laying (PL) stage extends from zero yolky follicles (just before RYD) to the maximum number of yolky follicles reported in our population (six). During laying (LY) the number of follicles decreases as they are ovulated until clutch completion (CC). There was a significant interaction between year and follicle stage in lean dry total follicle and oviduct mass. Therefore both years are presented separately. Squares = 2000, circles = 2001.Values are least square means ± SE.

Figure 5: Changes in yolk precursors vitellogenin (a), and very-low density lipoprotein (b) relative to the number of yolky follicles for a European starling with a full six-follicle hierarchy in 2000 and 2001 controlling for year. There was a significant interaction between year and follicle stage in VLDL. Therefore both years are presented separately. Squares = 2000, circles = 2001. PL = pre-laying, LY = laying, CC = clutch completion. Values are least square means ± SE.

Figure 6: Changes in lean dry mass of liver (a), small intestine (b), and pancreas (c) relative to the number of yolky follicles for a European starling with a full six-follicle hierarchy in 2000 and 2001 controlling for NRLDBM and year. There was a significant interaction between year and follicle stage in lean dry liver mass. Therefore liver mass is represented by year (squares = 2000, circles = 2001). PL = pre-laying, LY = laying, CC = clutch completion Values are least square means ± SE.

Figure 7: Changes in lean dry mass of kidney (a), and muscle (b) relative to the number of yolky follicles for a European starling with a full six-follicle hierarchy in 2000 and 2001 controlling for NRLDBM and year. PL = pre-laying, LY = laying, CC = clutch completion. Values are least square means ± SE.

Figure 8: Relationships between residual RMR and residual oviduct mass. Residuals are correcting for the effect of follicular stage, year and non-reproductive lean dry body mass.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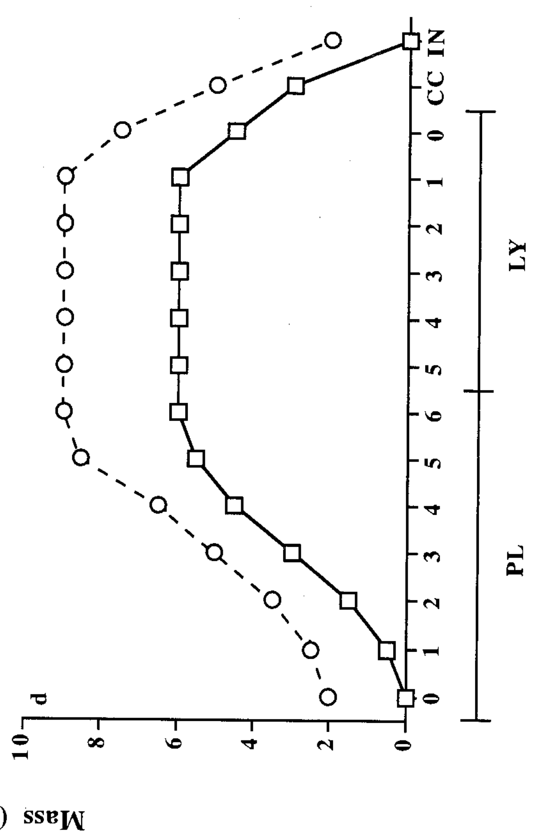
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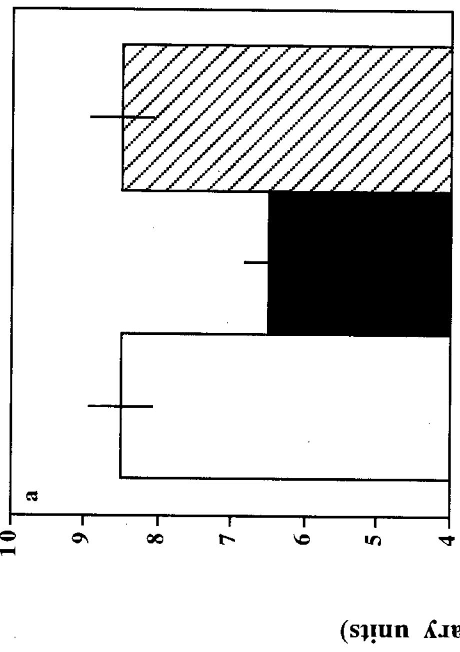
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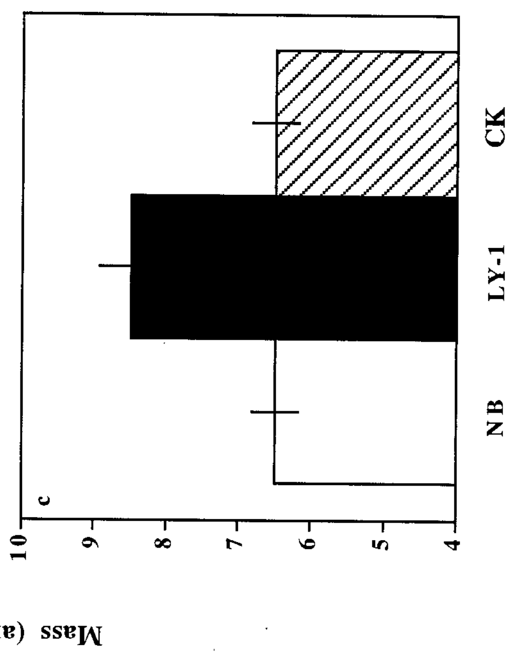
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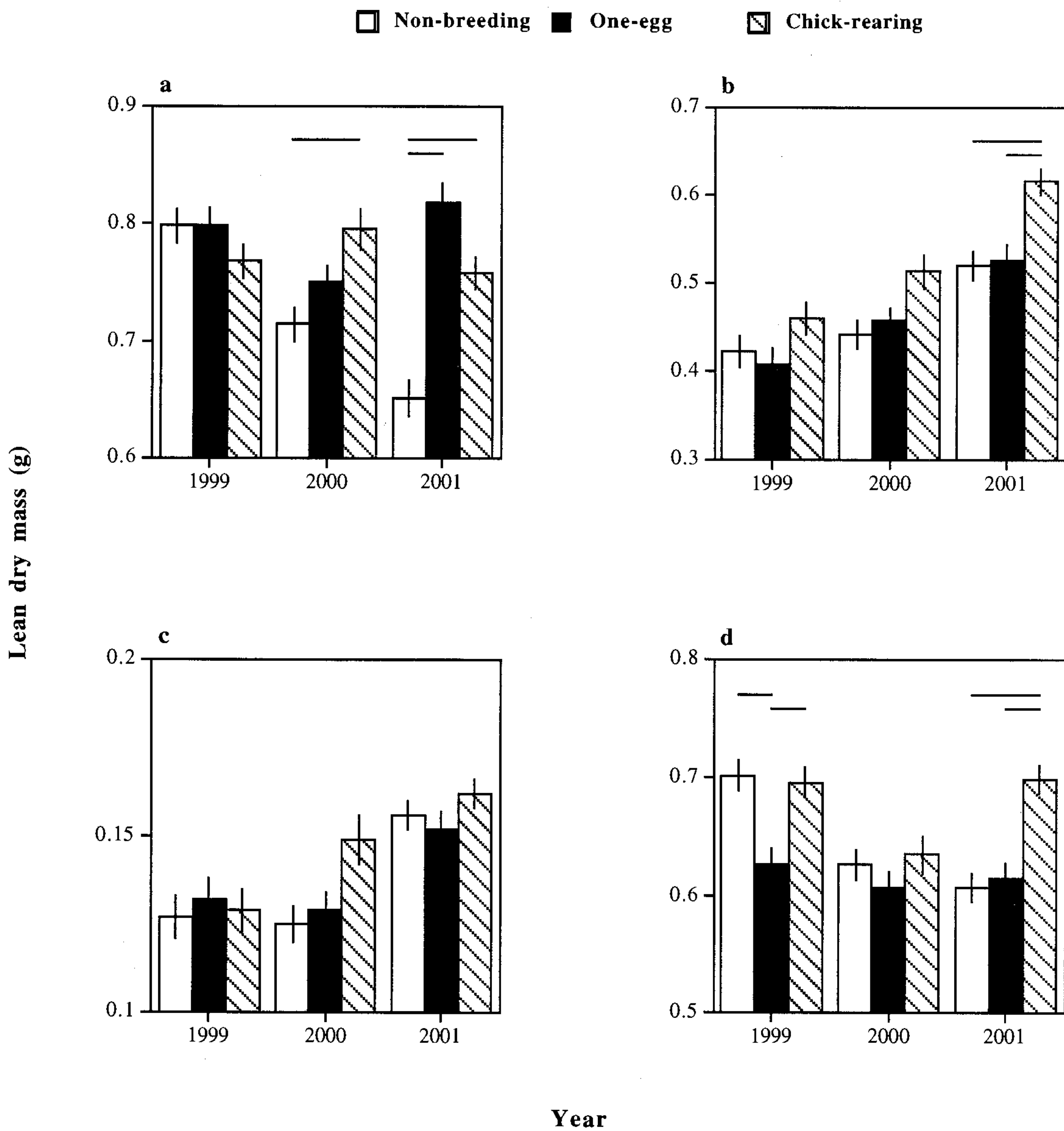
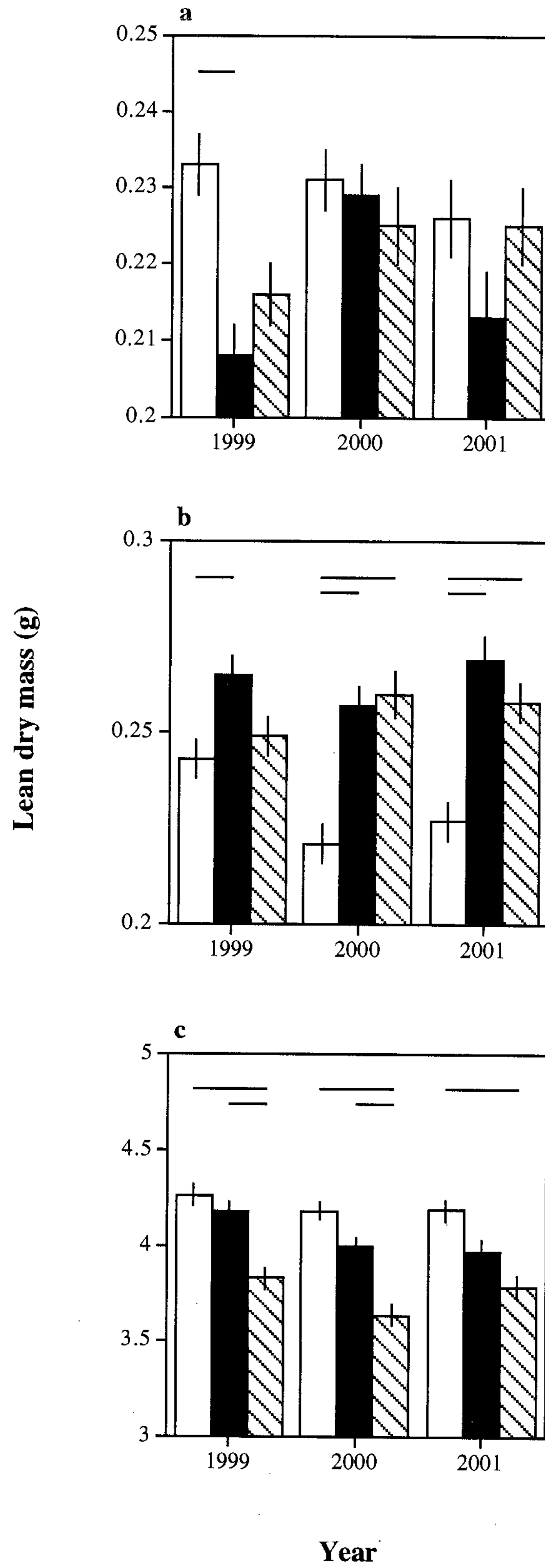
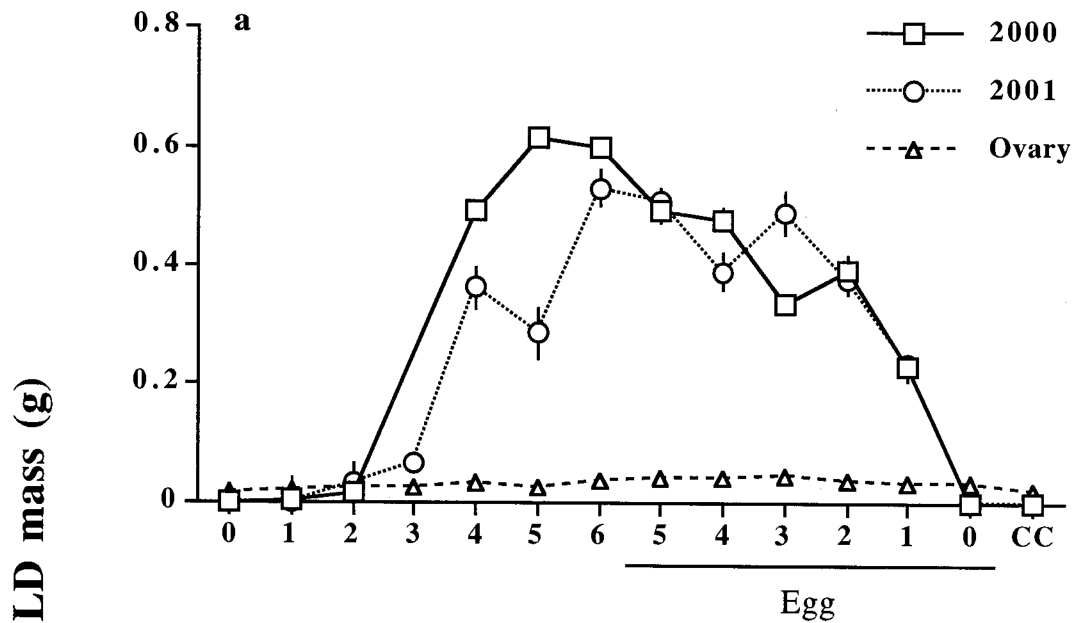
4
NB
LY-1
CK
Breeding stage

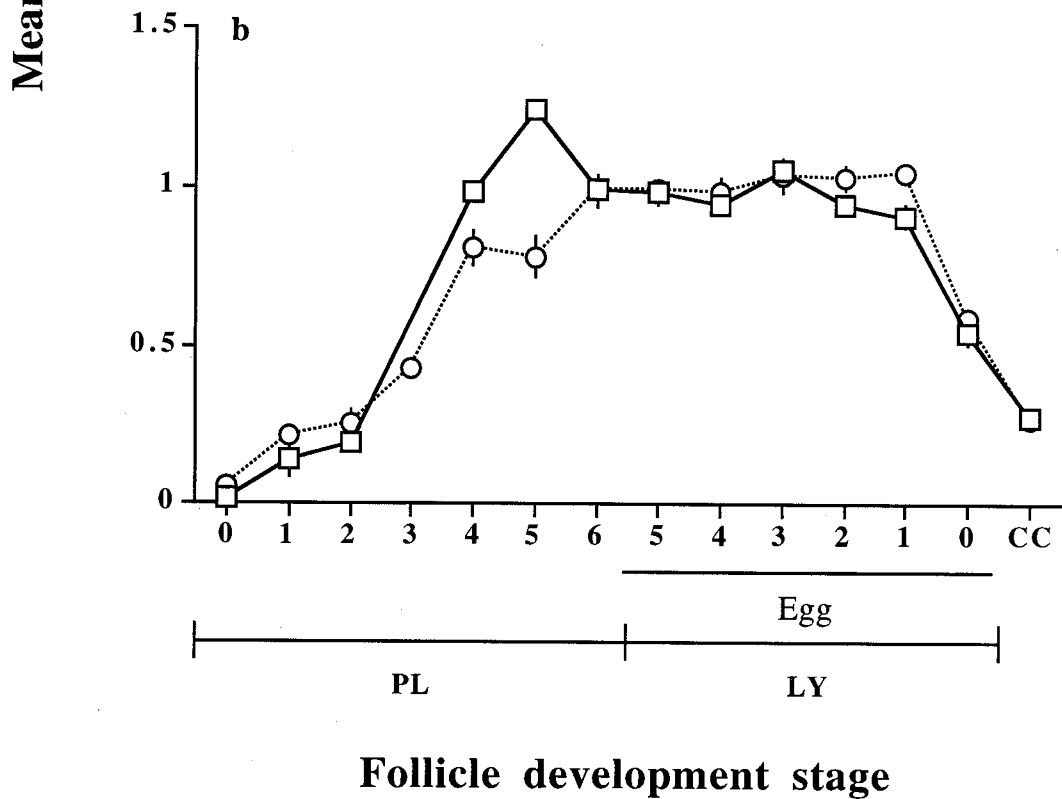


Non-breeding
One-egg
Chick-rearing
a
b
c
d
Lean dry mass (g)
Year
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.15
0.1
1999
2000
2001

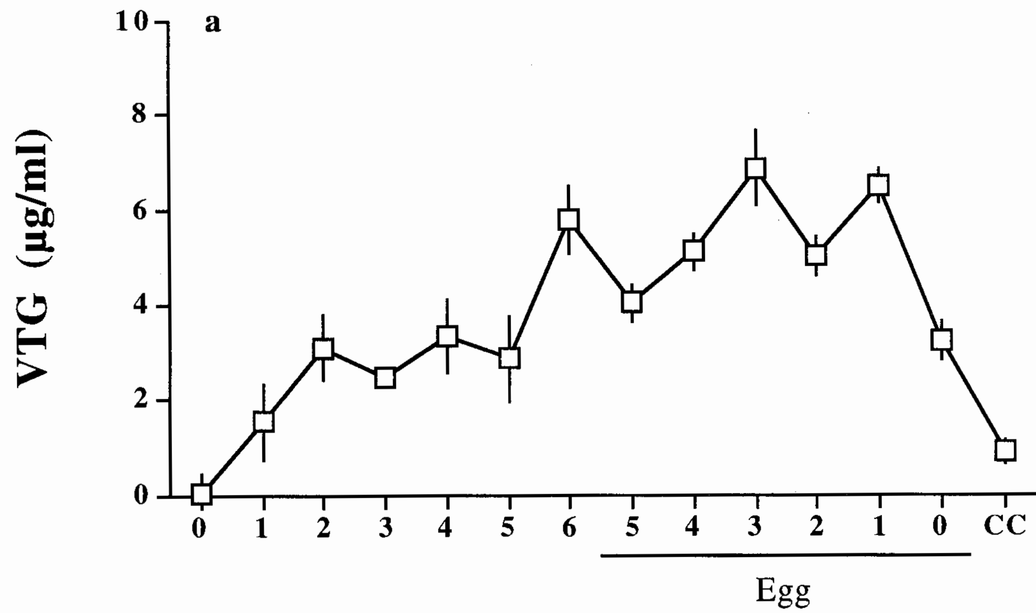

Non-breeding
One-egg
Chick-rearing
a
0.25
0.24
0.23
0.22
0.21
0.2
1999
2000
2001
b
0.3
0.25
0.2
c
5
4.5
4
3.5
3
Lean dry mass (g)
Year

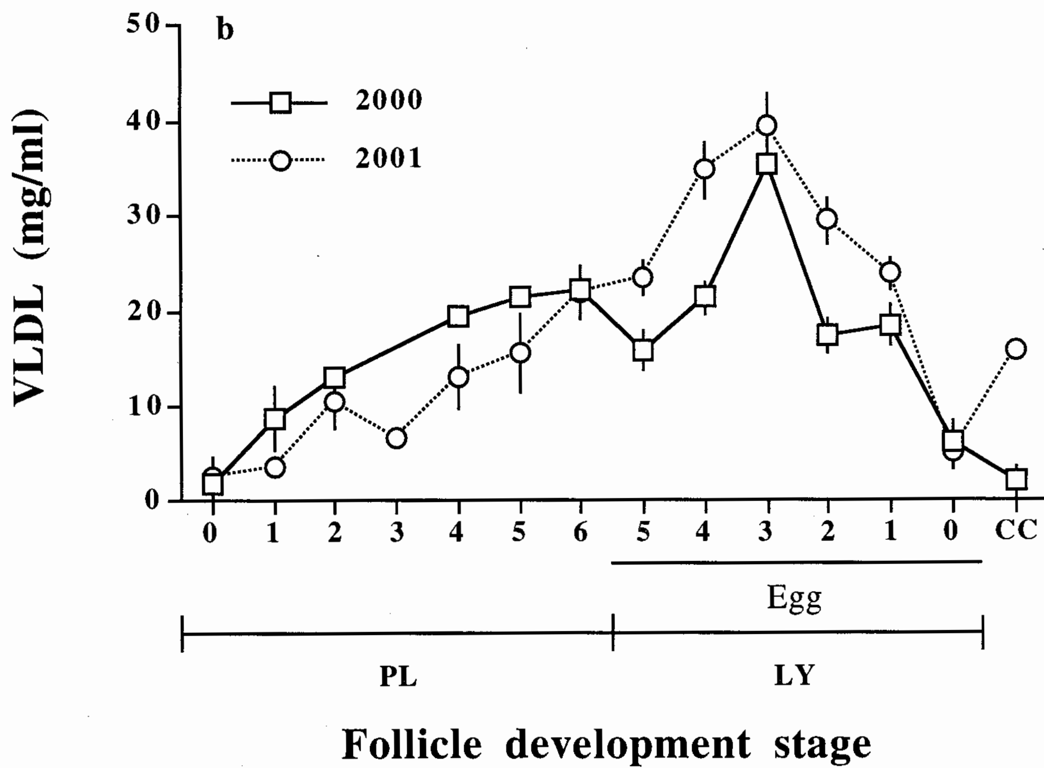


Mean LD mass (g)
a
0.8
0.6
0.4
0.2
0
2000
2001
Ovary
0 1 2 3 4 5 6 5 4 3 2 1 0 CC
Egg

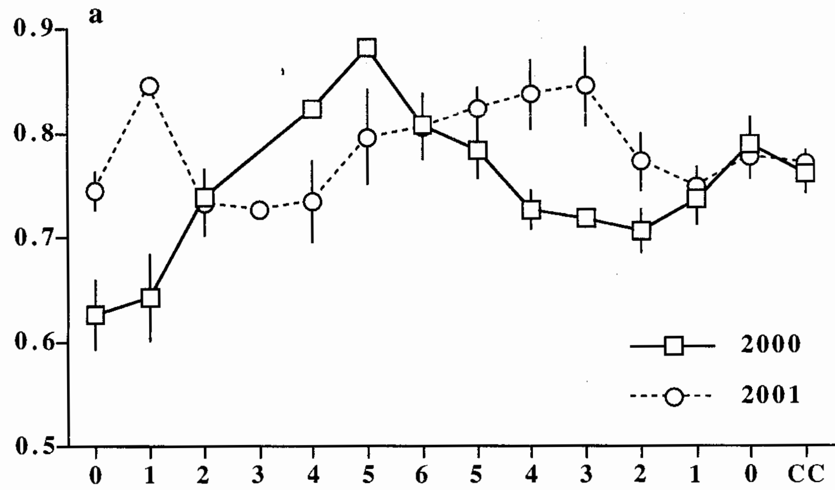


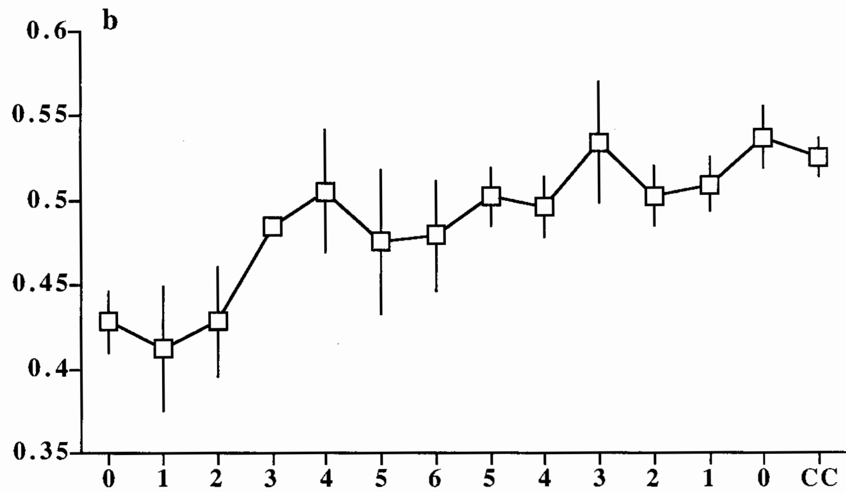
b
1.5
1
0.5
0
0 1 2 3 4 5 6 5 4 3 2 1 0 CC
Egg
PL
LY
Follicle development stage

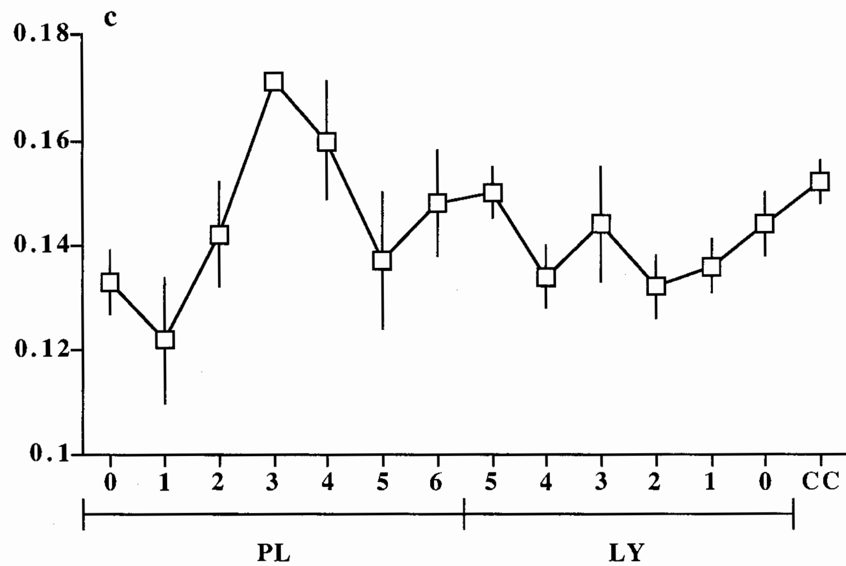

a
VTG (µg/ml)
0
2
4
6
8
10
0 1 2 3 4 5 6 5 4 3 2 1 0 CC
Egg


b
VLDL (mg/ml)
0
10
20
30
40
50
2000
2001
0 1 2 3 4 5 6 5 4 3 2 1 0 CC
Egg
PL
LY
Follicle development stage

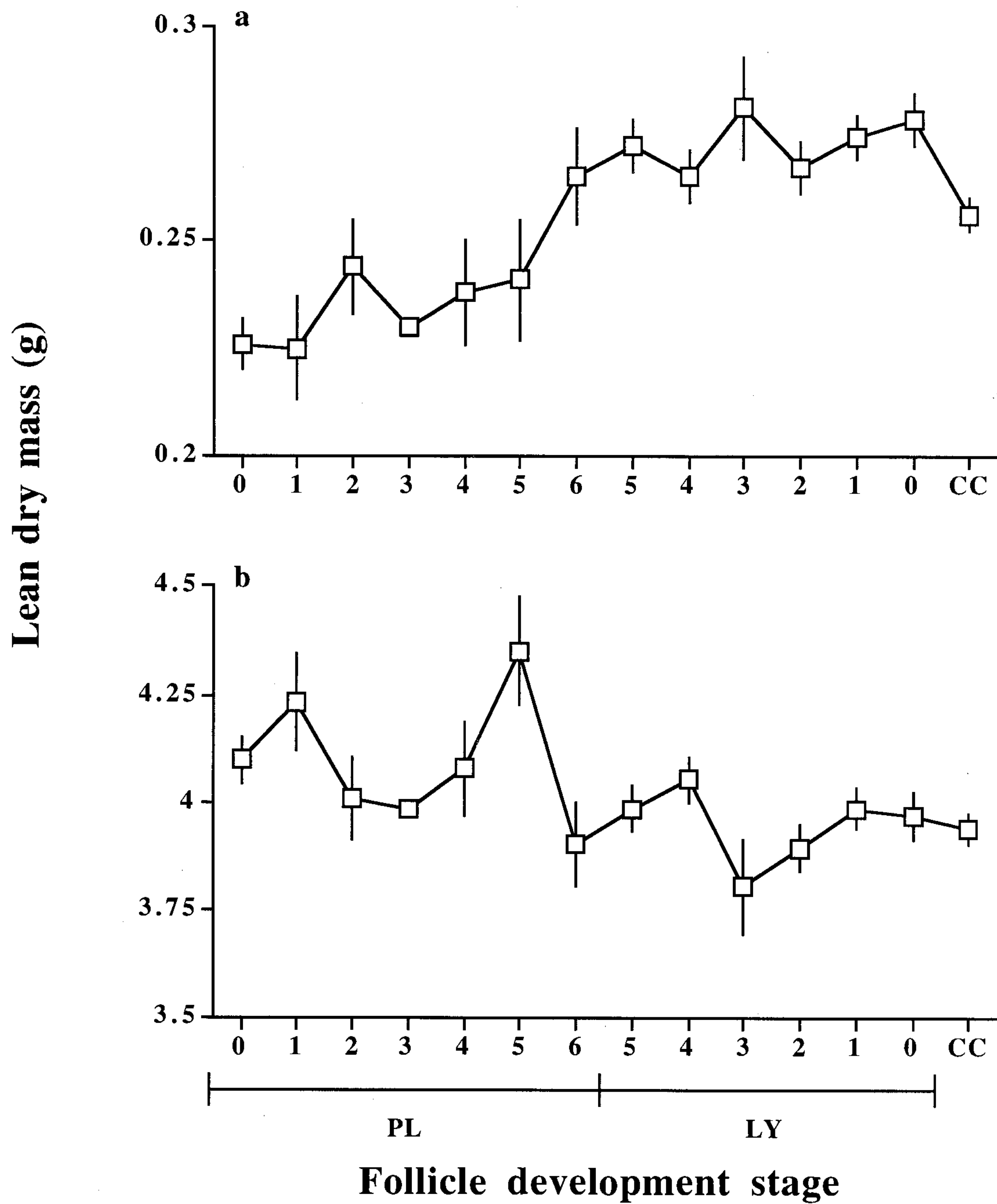
Lean dry mass (g)



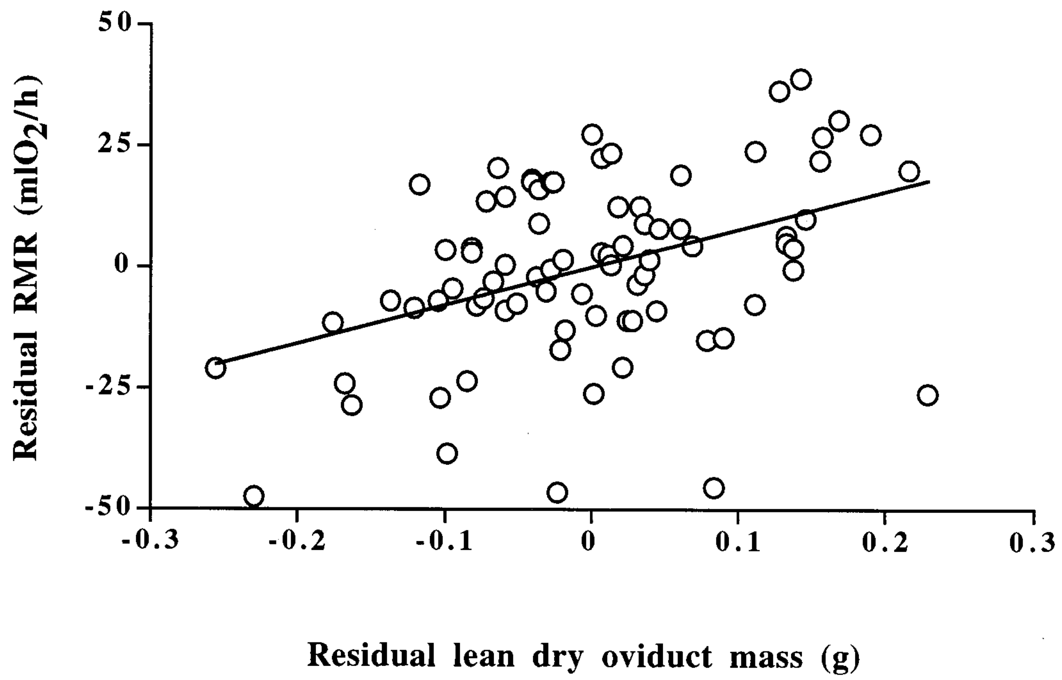




Follicle development stage



a
b
0.3
0.25
0.2
4.5
4.25
4
3.75
3.5
Lean dry mass (g)
0 1 2 3 4 5 6 5 4 3 2 1 0 CC
PL
LY
Follicle development stage


Residual RMR (mlO2/h)
50
25
0
-25
-50
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
Residual lean dry oviduct mass (g)

CHAPTER FOUR: THE METABOLIC COST OF AVIAN EGG FORMATION: POSSIBLE IMPACT OF YOLK PRECURSOR PRODUCTION?

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ABSTRACT

Little is known about the energy costs of egg production in birds. We showed in previous papers that during egg-production, European starlings (*Sturnus vulgaris*) undergo a 22% increase in resting metabolic rate (RMR) and that the maintenance and activity costs of the oviduct are responsible for 18% of the variation in elevated laying RMR. Therefore, other energy-consuming physiological mechanisms must be responsible for the remaining unexplained variation in elevated laying RMR. Yolk precursor (vitellogenin, VTG, and very low density lipoprotein, VLDL) production is likely to be costly because it signifies a marked increase in the biosynthetic activity of the liver. We documented the pattern of yolk precursor production in response to daily injections of 17β-Estradiol (E2) in zebra finches (*Taeniopygia guttata*). Based on this pattern we carried out an experiment in order to evaluate the metabolic costs of producing VTG and VLDL. Our estradiol treatment resulted in a significant increase in plasma VTG and VLDL levels within the natural breeding range for the species. Although RMR was measured during the period of active hepatic yolk precursor production, resting metabolic rate did not differ significantly within individuals in response to the treatment or when comparing E2-treated birds with sham-injected birds. This could mean that yolk precursor production represents low energy investment. However, we discuss these results in light of possible adjustments between organs that could result in energy compensation.

INTRODUCTION

Physiological mechanisms underlying one of the major assumptions of life history theory, namely that an increased effort in current reproduction may have a negative impact on future reproductive success, are not well understood (Stearns, 1992). One reason for this lack of knowledge is that, in avian systems, most of the research attention has been focused on manipulations of reproductive effort at the incubation or chick-provisioning stages (Monaghan and Nager, 1997) and thus the potential physiological costs incurred earlier in breeding i.e. during follicle development and egg production, have received very little attention. Recent research however, has shown that the energy cost of egg production in birds may be significant (Nilsson and Raberg, 2001; Vézina and Williams, 2002). We recently showed that the physiological process of egg formation in female European starlings (*Sturnus vulgaris*) is responsible for a 22% increase in resting metabolic rate (RMR) in laying individuals (Vézina and Williams, 2002). We further demonstrated that 18% of the variation in elevated laying RMR was explained by the maintenance and activity cost of the working oviduct (Vézina and Williams, 2003), and emphasized that this organ is probably costly enough that selection has led to a very tight size-function relationship, explaining its rapid pattern of recrudescence and regression. However, 82% of the variation in laying RMR remains unexplained, suggesting that other energy-consuming physiological mechanisms must be responsible, at least in part, for the metabolic cost of egg production (Vézina and Williams, 2003).

Another component of egg production that is likely to be energetically costly is the increased liver activity involved in protein and lipid production for oogenesis. During the process of egg formation, the hypothalamus initiates a hormonal cascade by releasing

gonadotropin-releasing hormone (GnRH), which induces the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland (Williams, 1998; Scanes, 2000). These hormones stimulate the ovary to produce estrogens (Williams, 1998), which then trigger the production of the egg-yolk precursors, vitellogenin (VTG) and yolk-targeted, very-low density lipoprotein (VLDLy) by the liver (Bergink et al., 1974; Deeley et al., 1975; Wallace, 1985; Walzem, 1996; Williams, 1998), which are then secreted into the blood. During rapid yolk development, plasma VTG and VLDLy are taken up by the ovary 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 laying domestic hens (*Gallus gallus domesticus*), approximately 50% of the liver's daily protein synthesis is attributed to VTG production, potentially tripling the amount of protein in circulation (Gruber, 1972). Hepatic lipid production also increases markedly during this time (from 0.5 - 1.5 μmole triglycerides/ml plasma in non-breeders to 20 – 50 μmole triglycerides/ml plasma in laying hens; Griffin and Hermier, 1988), as VLDL synthesis shifts from the exclusive production of non-laying, generic VLDL to an increase in the hepatic synthesis of estrogen-dependent, yolk-targeted VLDL (Walzem, 1996; Walzem et al., 1999). The presence of circulating VLDLy represents a dramatic shift in lipid metabolism as the structure and function of plasma VLDL particles change from larger, generic VLDL, which are involved in triglyceride (i.e., energy) transport within an individual, to smaller VLDLy, which supply the yolk with energy-rich lipid (Walzem 1996; Walzem et al. 1999). These changes in protein and lipid metabolism are likely to be energetically

costly as they are associated with an increase in the activity of the liver and potentially other organs involved in reproduction, such as the ovary.

However, in female starlings, there is no relationship between laying RMR and lean dry liver mass or plasma levels of the yolk precursors in individuals having 1 - 5 yolky follicles left to ovulate (Vézina and Williams, 2003). Nevertheless, this does not mean that yolk precursor production is not energetically costly. Liver mass *per se* may not be representative of the liver's metabolic intensity i.e. the amount of energy consumed per unit tissue mass. Furthermore, the elevated RMR reported in laying starlings was measured during active laying when yolk precursor levels were already maintained at an elevated level (Challenger et al., 2001; Vézina and Williams, 2003). It is possible that laying female plasma is saturated with yolk precursors, with minimal rate of production by the liver, and therefore no relationship with RMR. Alternatively, the elevated precursor levels may be the result of a balance between high hepatic production and high ovary uptake rate. Consequently, comparing precursor levels with RMR in active layers may be problematic and potentially misleading.

To measure the metabolic cost of yolk precursor production accurately, one has to measure the animal's metabolic rate at a time when the liver is known to be actively involved in VTG and VLDLy synthesis. In the present study, we documented the pattern of yolk precursor production in zebra finches (*Taeniopygia guttata*) in response to daily injections of 17β-Estradiol (E2) and measured the potential metabolic cost of VTG and VLDL production by respirometry. Zebra finches represent a very good model species for this type of study because: 1) ongoing work in our lab has shown that mass-corrected RMR is about 26% higher at the one-egg stage than at the non-breeding stage (F. Vézina,

unpublished) which is very similar to reported values for starlings (Vézina and Williams, 2002) and great tits (*Parus major*) (Nilssón and Raberg, (2001), 2) the pattern of yolk precursor production during the laying cycle is known and similar to the one reported in starlings (Salvante and Williams, 2002; Challenger et al., 2001) and 3) this species responds in a known manner to E2 injections (Williams and Martyniuk 2000). We used E2 doses adjusted to generate plasma yolk precursor levels within the normal range for breeding females at the peak of investment (one-egg stage; Williams and Ternan, 1999). We then measured resting metabolic rate in dosed individuals during the period of known hepatic activity. In order to evaluate the metabolic cost of yolk precursor production, we compared this RMR value to the metabolic rate previously measured in all individuals.

MATERIAL AND METHODS

Animal care

All birds (*Taeniopygia guttata* Gould) were maintained in controlled environmental conditions (temperature 19°-23°C; humidity 35%-55%; constant light schedule, 14L : 10D with lights on at 0700 hours). All individuals were housed in cages (61 X 46 X 41 cm) containing non-breeding, same-sex groups. All finches were maintained on a mixed-seed diet (Panicum and white millet, 50 : 50, approximately 12.0% protein, 4.7% lipid; Jamieson's Pet Food, Vancouver, Canada), water, grit and cuttlefish bone (calcium) *ad lib.* and received 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 (558B), following the guidelines of the Canadian Committee on Animal Care.

Estradiol treatment

We know from previous studies that administration of E2 to female zebra finches triggers partial development of the oviduct (Williams and Martyniuk, 2000). Because the maintenance and activity costs of the oviduct have previously been shown to be related to metabolic rate (Chappell et al., 1999; Vézina and Williams, 2003), we avoided any confounding effects in our results by using males zebra finches as models for this experiment. Males react to E2 injections by producing yolk precursors in the same fashion as females (Bergink et al., 1974; Follett and Redshaw, 1974). Various E2 doses were tested (F. Vézina, K.G. Salvante and T.D. Williams, unpublished results) in order to generate a response in plasma yolk precursor levels comparable to normal female breeding values. The final dose used in this study was 1.5 µg/g (assuming a mean mass of 17 g for all birds) of E2 dissolved in corn oil (No Name™ Pure Corn Oil, Toronto, Canada).

Experiment 1: Pattern of yolk precursor plasma levels in response to daily E2 injections

In order to determine the best possible timing of RMR measurement in relation to the estradiol treatment, we had to document the rise in circulating yolk precursor levels in response to the E2 treatment. Only VTG was monitored since both precursors have been reported to respond similarly to E2 injections in zebra finches (Williams and Martyniuk, 2000). For this experiment 32 male zebra finches were used. The birds were divided into five groups, and all birds received a daily E2 injection (30 µl, IM) over 4 consecutive

days (days 1 - 4, Figure 1a). Starting from the day following the first injection, one group of birds was blood sampled per day until two days following the last injection (sample sizes = 4, 9, 5, 10, 4 for days 2 to 6 respectively, Figure 1a). All birds were blood sampled only once from the brachial vein. We also repeated this experiment using females (n = 23) to confirm that both sexes responded the same way to the estradiol treatment.

Experiment 2: Metabolic costs of yolk precursor production

For this experiment, we used 32 males randomly assigned to one of two groups: E2 (injected with E2 in corn oil; n = 16) and Sham (corn oil only; n = 16). We used a repeated measures design where each bird was used as its own control in order to monitor changes in RMR due to E2 administration within a bird. The experiment lasted 11 days and proceeded as follows (Figure 1b): on the night preceding day 1, all birds had their RMR measured by respirometry (protocol described below) and were blood sampled the following morning (day 1). These data will be referred to as "pre-treatment". Estradiol and sham injections (30 μl, IM) started the morning of day 7 and lasted 4 consecutive days until day 10. Results from experiment 1 led us to measure RMR on the night following the second day of injections, i.e., day 8 (see justification in the Results section), and all birds were blood sampled again the following morning (day 9), from here on referred to as "mid-treatment". The injections were then continued until day 10 to ensure that the liver was actively producing yolk precursors at time of RMR measurement (i.e. no plateau in precursor levels). This was confirmed by a final blood sample on day 11, from here on referred to as "post-treatment".

Measurement of resting metabolic rate (RMR)

Blem (2000) defines basal metabolic rate (BMR) as the energy consumed by a post-absorptive bird during the resting phase of the circadian cycle at a temperature within the thermoneutral range for the animal. By definition BMR is the lowest measurable consumption of oxygen. Because birds in the second part of experiment 2 were artificially stimulated to produce yolk precursors, we considered them to be in an "active physiological state" which may induce elevated levels of energy consumption. Therefore, although the first set of metabolic rate measurements (i.e. pre-treatment) may fall under Blem's (2000) BMR definition (last meal at least 3 hours before beginning of measurement), we use the term *resting* metabolic rate throughout the paper for convenience (note that all of our measurement were taken at thermoneutrality which is often not the case for resting metabolism reported in the literature; Blem 2000). All RMR measurements were completed using a flow-through respirometry system (Sable Systems International, Henderson, NV, USA). Birds were taken from their cages, their body mass was measured (± 0.1 g), and they were placed randomly in one of four metabolic chambers (1.5 L) for one hour prior to the beginning of RMR measurements. All chambers continuously received approximately 500ml/min of dry CO_2-free air (using Drierite™ and Ascarite™ as scrubbers) and were kept in the dark at 35°C, which is within the thermoneutral zone for this species (lower critical temperature = 33°C; Meijer et al., 1996). RMR measurements were always started at 0000 hours. 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, Monterey, CA, USA) for proper air flow reading (STP corrected) and through CO_2 and oxygen analyzers (model CA-1 and FC-1, respectively; Sable Systems International; air water scrubbed before CO2 analyzer and water and CO_2 scrubbed before O_2 analyzer). All measurement sequences started by recording ten minutes of baseline air. After baseline recording the multiplexer switched, and the out-flowing air from the first chamber was sampled for 55 minutes. Then the system switched back to baseline for 10 minutes before changing again to the second, third and fourth chambers. Preliminary analysis showed that measuring RMR using this protocol did not generate a time effect (sensu Hayes et al., 1992) on RMR ($F_{3,15} = 0.48$ $P = 0.7$). The birds stayed in their chambers for approximately 5 hours. After RMR measurement the birds were weighed for a second time and the average of first and second masses was used in subsequent analysis. To calculate RMR, a running mean representing ten minutes of recording was passed through the data for each bird, with the lowest mean taken as RMR.

Yolk precursor analysis

In order to measure circulating levels of VTG and VLDL, blood samples were centrifuged at 2200 g for 10 minutes, and the plasma portion of each sample was isolated. Plasma samples were then assayed for vitellogenic zinc (Zinc kit, Wako Chemicals, Richmond, VA, USA) using the method developed for the domestic hen (Mitchell and Carlisle, 1991) and validated for passerines (Williams and Christians, 1997; Williams and Martyniuk, 2000; Challenger et al., 2001; Salvante and Williams, 2002). The concentration of vitellogenic zinc is proportional to circulating levels of VTG (Mitchell and Carlisle, 1991). The overall inter-assay coefficient of variation for the vitellogenic

zinc assay (calculated from repeated analyses of a reference sample) was 14.2% (n = 9 assays).

Circulating VLDL was assessed by measuring plasma triglyceride levels (Triglyceride E kit, Wako Chemicals) according to the method of Mitchell and Carlisle (1991). Plasma triglyceride has commonly been measured in non-domesticated birds as an index of total plasma VLDL, which consists of both the generic and yolk-targeted forms of VLDL (Williams and Christians, 1997; Williams and Martyniuk, 2000; Challenger et al., 2001). Despite the marked increase in circulating lipid levels reported during egg production (Griffin and Hermier, 1988; Walzem et al., 1994; Walzem, 1996; Walzem et al., 1999), *in vivo* studies on laying poultry hens have detected only low circulating levels of intermediate-density and low-density lipoproteins, both by-products of the metabolism of generic VLDL, suggesting that VLDLy is resistant to metabolism by laying hens (Hermier et al. 1989, Walzem et al. 1994, Walzem 1996). These studies provide evidence that the marked increase in total VLDL during avian egg production or following estrogen administration is the result of increased synthesis of the estrogen-dependent VLDLy component of total VLDL. The overall inter-assay coefficient of variation for the triglyceride assay (calculated from repeated analyses of a reference sample) was 10% (n = 5 assays). All assays were run using 96-well microplates, and measured using a Biotek 340i microplate reader (Winooski, VT, USA).

Statistical analysis

All data were tested to ensure normality (Shapiro-Wilk test; Zar, 1996). Plasma yolk precursor levels violated normality for both the production pattern experiment and

the RMR experiment. Vitellogenin data from the first experiment were log transformed (adding 0.001 to all data to eliminate zeros) to achieve normality. Analysis of the precursor production pattern was therefore carried out on log-transformed data using standard parametric methods (see below). For clarity, yolk precursor production pattern data are presented non-transformed in Figure 2. In contrast, log transformation of VTG and VLDL data for the RMR experiment did not result in normally distributed data. Therefore, non-parametric tests were performed (see below) when the analysis included VTG or VLDL data. For all other analysis, standard parametric statistics were used since all other variables were found to be normally distributed.

Contrary to the results of preliminary testing (see above), there was a significant time effect on RMR in experiment 2 when controlling for body mass (pre-treatment: $F_{3,27} = 2.99$, $P < 0.05$; mid-treatment: $F_{3,26} = 3.57$, $P < 0.05$). This effect translated into a decreasing RMR over time, but with a difference of less than 10% between measurements for either the first or last chamber relative to the mean of all chambers. Overall, pre- and mid-treatment RMR of birds in the first metabolic chamber was, respectively, 3.04 ml O_2/h (7.1%) and 2.10 ml O_2/h (4.7%) higher than the average for the four chambers (42.57 ml O_2/h at pre-treatment and 44.36 at mid-treatment) while RMR of birds in the fourth metabolic chamber was 3.25 ml O_2/h (7.6%) and 4.21 ml O_2/h (9.5%) lower, respectively. The range of maximal differences in the RMR of birds measured first (chamber 1) compared to birds measured last (chamber 4) within the same night spanned from –30.0% to +7.7% of RMR associated with chamber 1 at pre-treatment (mean difference: –15% of mean all-chamber RMR). For mid-treatment this range was –26.7% to +32.6% (mean difference: –7% of mean all-chamber RMR).

However, when considering maximal differences in RMR of birds measured twice in the same chamber throughout the experiment (n =7), the range spanned from –22.7% to 30.0% of the first RMR measurement, indicating that the time effect is within the natural individual variation in RMR measurements. Indeed, this time effect proved to be weak since post-hoc analysis, using Bonferroni correction for multiple comparisons (Rice, 1989), revealed only a marginally significant difference ($P = 0.0074$ with level of significance corrected to $P < 0.008$) between first and last chamber at pre-treatment but failed to detect any significant differences in RMR between birds held in different chambers at mid-treatment. We nevertheless used a conservative approach and included time in the model when correcting RMR for the effect of body mass. In order to evaluate changes in RMR within individual birds throughout the experiment we used repeated measures ANOVA. To control for the effect of body mass in this model we averaged pre- and mid-treatment masses and included it in the model as a covariate (body mass was highly repeatable between pre and mid-treatment; $r^2 = 0.89$ n = 32 $P < 0.0001$). However, because we randomized the position of the birds in their metabolic chambers, we were not able to control for the time effect (i.e. the birds were not consistently put in the same chambers). We do not believe that this introduced a systematic bias. As mentioned earlier, the only detectable time effect was recorded between measurements for chamber 1 and 4 at pre-treatment. However, only 7 out of 32 birds were measured in these 2 chambers over the 2 measurement periods. Out of these 7, only 4 individuals had their RMR measured in chamber 4 at pre-treatment and in chamber 1 at mid-treatment, which would artificially increase their RMR. Data are reported as mean ± S.E.

RESULTS

Experiment 1: Pattern of yolk precursor plasma levels in response to daily E2 injections

Administration of estradiol triggered a significant increase in circulating vitellogenin in male and female zebra finches (two-way ANOVA, treatment effect $F_{4,49}$ = 5.60, $P < 0.005$), and there was no significant difference in the pattern of response to E2 treatment between the sexes (sex effect $F_{1,49}$ = 2.08; $P = 0.2$; no significant interaction term). Therefore we pooled the samples to obtain a more accurate picture of the pattern of VTG production (treatment effect $F_{4,50}$ = 5.31, $P < 0.005$; Figure 2). In response to daily E2 injections, plasma VTG increased to reach a peak of 3.2 ± 0.7 μg/ml on the day following the last injection (day 5 in Figure 2). This vitellogenin level is higher than previously reported values for breeding female zebra finches at the one egg stage (1.68 μg/ml Williams and Christians, 1997), but well within the normal physiological breeding range (0.54 - 5.55μg/ml VTG-Zn in Williams and Christians, 1997; 0.47 – 4.26 μg/ml VTG-Zn in Salvante and Williams, 2002). Bonferroni-corrected, post-hoc contrast analysis revealed significant differences between VTG levels at day 2 and day 5 ($P <$ 0.005) but showed no significant differences between consecutive days from day 2 to day 5, suggesting a gradual increase in plasma VTG throughout the treatment with no significant changes in the rate of precursor production. We compared these data with VTG levels from an independent group of non-breeding individuals from our colony (n = 11). Addition of the non-breeding group did not change the overall analysis (treatment effect $F_{5,60}$ = 17.80, $P < 0.0001$) and showed that our treatment clearly triggered a significant increase in VTG plasma levels even after the very first injection (Figure 2). Indeed, circulating VTG at day 2 was 1,518% higher than non-breeding values

(independent contrast: $P < 0.0001$). By two days following the last injection (day 6 in Figure 2), VTG levels decreased to values comparable with those on the first day of blood sampling (day 2; independent contrast: $P = 0.9$), further demonstrating that hepatic VTG production was clearly dependent on E2 stimulation but also that breakdown of VTG was rapid and comparable in males and females. In fact, 80% of the VTG was already removed from the plasma at day 6 (Figure 2; day 5 vs. day 6 independent contrast; $P < 0.005$). At this rate only 1.25 days is needed to return plasma VTG to non-breeding levels. Therefore our treatment induced a high level of hepatic VTG production in parallel with rapid break down of the protein. Based on these results we chose to perform the RMR measurement in experiment 2 after the second injection in order to measure energy expenditure during active hepatic activity.

Experiment 2: Metabolic costs of yolk precursor production

Based on the results obtained in the first experiment, we measured RMR during the night between the second and third day of E2-treated administration (night of day 8, Figure 1b). At this point VTG levels were still lower than normal one-egg stage values (Mann-Whitney test: $U = 137.00$, $P < 0.005$, Salvante and Williams, 2002), and it is clear that the liver was actively synthesizing yolk precursors as plasma VTG increased for at least two more days (Figure 2).

There was no difference in body mass between E2 and sham birds measured either at pre-treatment or mid-treatment (pre-treatment t-test $t_{30} = 0.21$, $P = 0.8$; mid-treatment t-test $t_{30} = 0.52$, $P = 0.6$). Within groups there was no treatment-related changes in mass in E2 individuals (paired t-test $t_{15} = 1.41$, $P = 0.2$). However sham birds lost 2%

of their mass between the two RMR measurements (paired t-test $t_{15} = 2.52$, $P < 0.05$) decreasing from 15.0 ± 0.4g to 14.6 ± 0.4g.

As in experiment 1, VTG production significantly increased in E2-treated individuals (Friedman test: $\chi^2 = 27.22$, df = 2, $P < 0.001$; Figure 3a) while levels in sham birds remained below the range of VTG for breeding females (Williams and Christians, 1997; Salvante and Williams, 2002) and showed no significant change throughout the experiment ($\chi^2 = 1.97$, df = 2, $P = 0.3$: Figure 3a). Differences between groups were evident at all periods of the experiment. At pre-treatment, sham birds had significantly higher levels of plasma VTG than E2-treated birds (Mann-Whitney test: $U = 53.00$, $P < 0.005$ Figure 3a). However, VTG levels in both groups were low and representative of typical non-breeding birds (Williams and Christians 1997). Therefore, this difference is not biologically relevant. At mid- and post-treatment, E2-treated birds exhibited significantly higher levels of VTG than sham individuals (Mann-Whitney test mid-treatment: $U = 34.00$, $P < 0.001$; post-treatment: $U = 22.00$, $P < 0.0001$ Figure 3a) with VTG levels averaging 1.59 μg/ml at post-treatment. This is lower than the reported maximum for the first experiment, and it is not clear why the same E2 dose resulted in different precursor levels. However, plasma VTG levels reported in experiment 2 are much closer to the natural breeding level of 1.68 μg/ml (Williams and Christians, 1997).

Estradiol administration had a similar effect on the pattern of VLDL production with plasma levels significantly increasing in E2-treated individuals (Friedman test: $\chi^2 = 11.38$, df = 2, $P < 0.005$; Figure 3b). One individual had a surprisingly high VLDL level (106 mg/ml) at pre-treatment. Taking this individual out of the analysis made the increase in plasma VLDL in the E2 group more marked (including outlier: 12.64 ± 6.40 mg/ml at

pre-treatment to 15.71 ± 3.05 mg/ml at post treatment; excluding outlier: 6.42 ± 1.58 mg/ml at pre-treatment to 13.51 ± 2.25 mg/ml at post treatment) and did not change the overall effect of estradiol administration on plasma VLDL levels (Friedman test: χ^2 = 13.73, df = 2, $P < 0.005$). Conversely, sham individuals showed no change in plasma VLDL (Friedman test: $\chi^2 = 4.88$, df = 2, $P = 0.08$; Figure 3b). These results translated into no significant difference between groups at pre-treatment (Mann-Whitney test pre-treatment: $U = 97.00$, $P = 0.4$), 61% higher VLDL levels in E2-treated birds at mid-treatment (Mann-Whitney test mid-treatment: $U = 57.00$, $P < 0.05$) and 109% higher VLDL levels at post-treatment (Mann-Whitney test post-treatment: $U = 45.00$, $P < 0.005$). The maximal VLDL level reported here (post-treatment levels in E2-treated birds: 13.51 mg/ml) is 28% lower than previously reported values for female zebra finches at the one-egg stage (18.87 mg/ml; Williams and Christians, 1997), but still within the natural *wide* range of variation for this lipoprotein (6.4 - 54.5 mg/ml; Williams and Christians, 1997).

Pre-treatment RMR did not differ between the experimental groups ($F_{1,26} = 0.21$, $P = 0.7$) but was positively related to body mass ($F_{1,26} = 8.76$, $P < 0.01$). While mid-treatment RMR was also related to body mass ($F_{1,26} = 10.50$, $P < 0.005$), estradiol injections and increased yolk precursor production did not result in higher RMR in E2-treated birds compared to sham individuals ($F_{1,26} = 0.34$, $P = 0.6$; Figure 4). Similarly, E2-treated and sham groups did not differ in their RMR response to injections over time (repeated measures ANOVA treatment X time interaction: $F_{1,29} = 0.01$, $P = 0.9$) Indeed, repeated measures ANOVA revealed no significant changes in RMR between pre- and mid-treatment measurements when controlling for body mass ($F_{1,29} = 0.24$, $P = 0.6$). Mid-

treatment RMR was not correlated with circulating VTG or VLDL levels at mid- or post-treatment (Spearman rank correlation $P > 0.3$ in all cases) in E2-treated birds.

DISCUSSION

In this experiment, we demonstrated that male zebra finches respond rapidly (i.e. within 24h) to exogenous E2 administration in the form of a gradual increase in plasma vitellogenin and that this response did not differ from that of non-breeding females undergoing the same treatment. This is consistent with previous findings showing that roosters treated with E2 responded by synthesizing VTG as rapidly as 3 to 4 hours after estradiol injection (Bergink et al., 1974). Our birds also responded to the treatment by increasing VLDL production, which is in accordance with previously reported increases in plasma lipoproteins or liver fat content following exogenous estradiol administration (Yu and Maquardt, 1973a, Yu and Maquardt, 1973b; Bergink et al., 1974, Harms et al., 1977; Rosebrough et al., 1982; Williams and Martyniuk, 2000). The rapid decline in VTG after the last injection however, contradicts previous research where VTG synthesis has been shown to increase linearly for several days (Bergink et al., 1974) or even weeks (Robinson and Gibbins, 1984) after E2 treatment. This discrepancy is most likely due to the differences in hormone doses administered between studies. In the Bergink et al. (1974) experiment, E2 was administrated at a dose of 25mg/kg, which was 17 times the dose we used. It is clear that the pattern of yolk precursor production reported here, including the rapid breakdown in VTG plasma levels following E2 administration, is more representative of the "endogenous response". This is supported by the fact that VTG levels in our E2-treated birds remained within the natural range for breeding female

zebra finches even after four consecutive days of injection. Our results clearly demonstrated that the livers of our E2-treated birds were actively synthesizing VTG and VLDL, and that active yolk precursor production was maintained as long as E2 treatment was continued. Similarily, Salvante and Williams (2002) documented a rapid decline in plasma VTG in laying Zebra finches nearing clutch completion when endogenous estrogen production is declining (Sockman and Schwabl, 1999).

Comparing our values of RMR with those from the literature suggest that our respirometry technique provided robust estimates of BMR. Resting metabolic rate in pre-treated birds was 42.6 ml O_2/h, which is equivalent to 18.8 - 21.3 Kj/day for energy substrate going from protein to carbohydrates (Schmidt-Nielsen 1990; mean respiratory quotient was 0.76 which indicate that the birds could be using energy from mixed sources which complicates the energetic conversion). This is almost identical to BMR estimates of 19.7 Kj/day for zebra finches reported by Gavrilov (1997). Our estimate of RMR in pre-treated birds (2.8 ml O2/g/h when using our mean body mass of 15.1 g) is also very similar to Vleck's published results for incubating zebra finches at 35°C (3.0 ml O2/g/h in figure 2 of Vleck, 1981).

However, despite an 80-fold increase in circulating VTG and a 2-fold increase in plasma VLDL in E2-treated birds, resting metabolic rate, when measured during active hepatic yolk precursor production, did not increase significantly in E2-treated individuals compared to pre-treatment measurements or to sham individuals. This is consistent with the findings of Vézina and Williams (2003) which showed no relationships between RMR and plasma VTG and VLDL in laying European starlings. There is no other study

that we are aware of that has specifically investigated the metabolic cost of producing the yolk precursors.

Our results may be interpreted as a low energy investment in VTG and VLDL synthesis. However, this would be surprising given the marked charges in protein and lipid metabolism associated with rapid yolk formation (Gruber, 1972; Griffin and Hermier, 1988). We suggest that there might be an alternative explanation. There is accumulating evidence that birds can adjust their energy consumption in a compensatory manner when challenged by multiple and perhaps competing high-energy demands. For example, a decrease in locomotor activity has been suggested to compensate for the cost of molting (Austin and Fredrickson, 1987), for the reduced energy availability in fasting birds (Cherel et al., 1988), and for the cost of egg formation (Houston et al., 1995; Williams and Ternan, 1999). Similarly, the heat generated by feeding (Masman et al., 1989) or foraging activity (Webster and Weathers, 1990; Bruinzeel and Piersma, 1998) has been shown to partially compensate for thermoregulatory costs. It is clear that changes occurring in physiological systems and organs within an individual may also result in compensatory effects leading to energy reallocation with no net increase in overall energy consumption. For example, Geluso and Hayes (1999) measured BMR and organ composition of starlings under high and low quality diets. They found significant differences in the mass of the gastro-intestinal tract, gizzard, liver and breast muscle, but no differences in BMR indicating that up-regulation of certain organs or systems may be coincident with down-regulation of other systems or organs in order to maintain a constant maintenance energy cost. The question of whether the higher hepatic activity involved in VTG and VLDL production results in the down-regulation of other

physiological systems remains to be resolved. Clearly, studies investigating energy expenditure at the organ level are needed to elucidate this hypothesis. We know from previous work in our laboratory that liver structural size does not systematically increase in association with high levels of endogenous plasma yolk precursor production in European starlings and zebra finches (Christians and Williams, 1999a; Williams and Martyniuk, 2000; Vézina and Williams, 2003) or even in response to administration of exogenous estradiol (Christians and Williams, 1999b; Williams and Martyniuk, 2000). If energy reallocation does take place during precursor production, the metabolic intensity of the liver (i.e., energy consumption per unit mass) may still be high during VTG and VLDL production even though mass-corrected RMR was independent of plasma yolk precursor levels in our study. A possible mechanism for this could be that the regulation of other physiological systems is also triggered by a rise in plasma estradiol. For example, immune function, which has been shown to have a measurable metabolic cost (Demas et al., 1997; Raberg et al., 2001; Martin et al., 2002), can be inhibited by certain doses of estradiol in chickens (al-Afalek and Homeida, 1998; Landsman et al., 2001). Therefore, a reduction in the activity of the immune system could potentially lead to energy savings that can be reallocated to other functions.

This experiment was specifically designed to measure the potential costs of yolk precursor production. However, the influence of other aspects of egg formation on overall energy expenditure should also be examined. For example, egg yolk mass is related to the rate of yolk precursor uptake at the ovary, and is potentially limited by the number of VTG/VLDL receptors and their rate of recycling (Christians and Williams, 2001). Therefore, the very active process of rapid yolk development may also result in

substantial energy investment. These aspects of egg formation cannot be assessed using males or even non-breeding females due to the lack of developing ovarian follicles. Thus, more investigation is needed to explain the remaining variation in elevated laying RMR (Nilsson and Raberg, 2001; Vézina and Williams, 2002) not accounted for by the maintenance and activity costs of the oviduct (Vézina and Williams, 2003)

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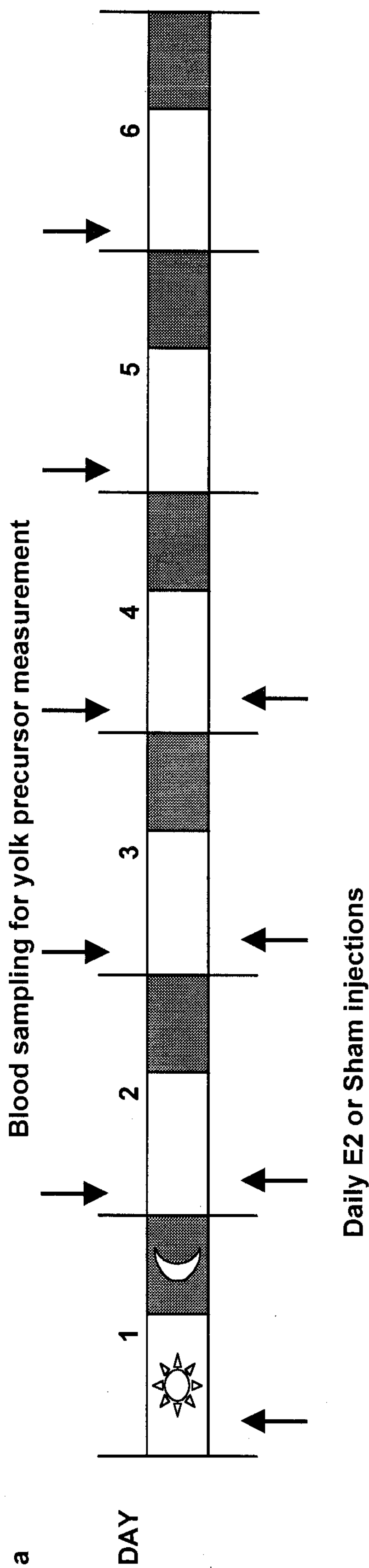
FIGURE LEGENDS

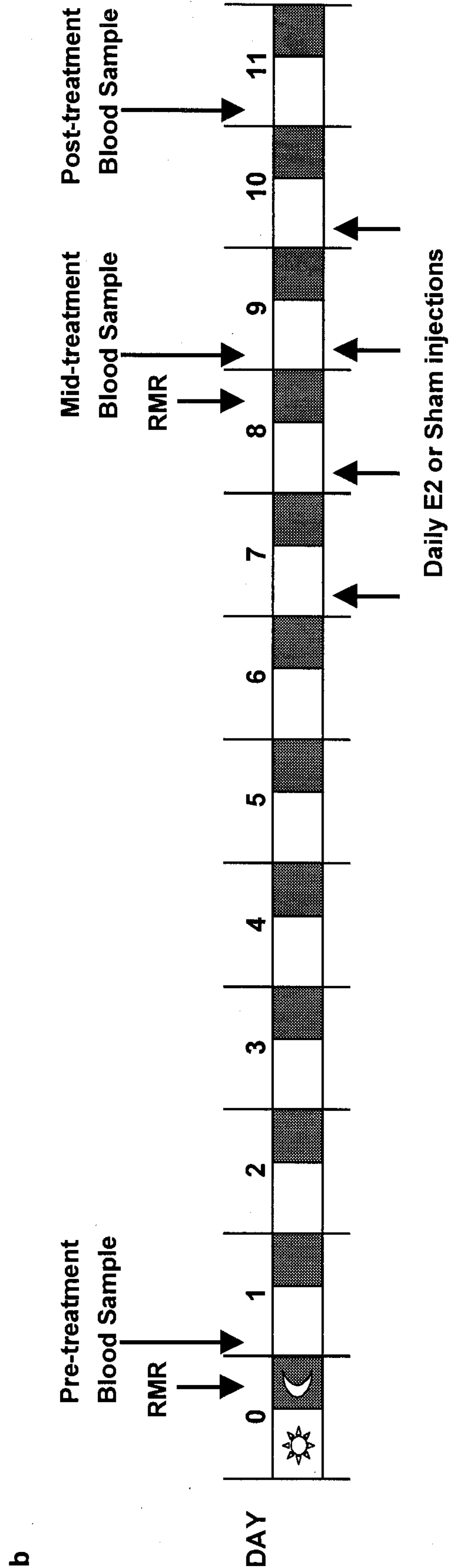
Figure 1. (a) Experimental estradiol injection protocol to determine the pattern of vitellogenin production in response to the treatment; (b) experimental injection and RMR protocol to determine the potential metabolic cost of yolk precursors production. The numbers represent the days into the treatment, with the white sections symbolizing daytime and dark sections symbolizing nighttime. The arrows represent either RMR measurement, injection or blood sampling time.

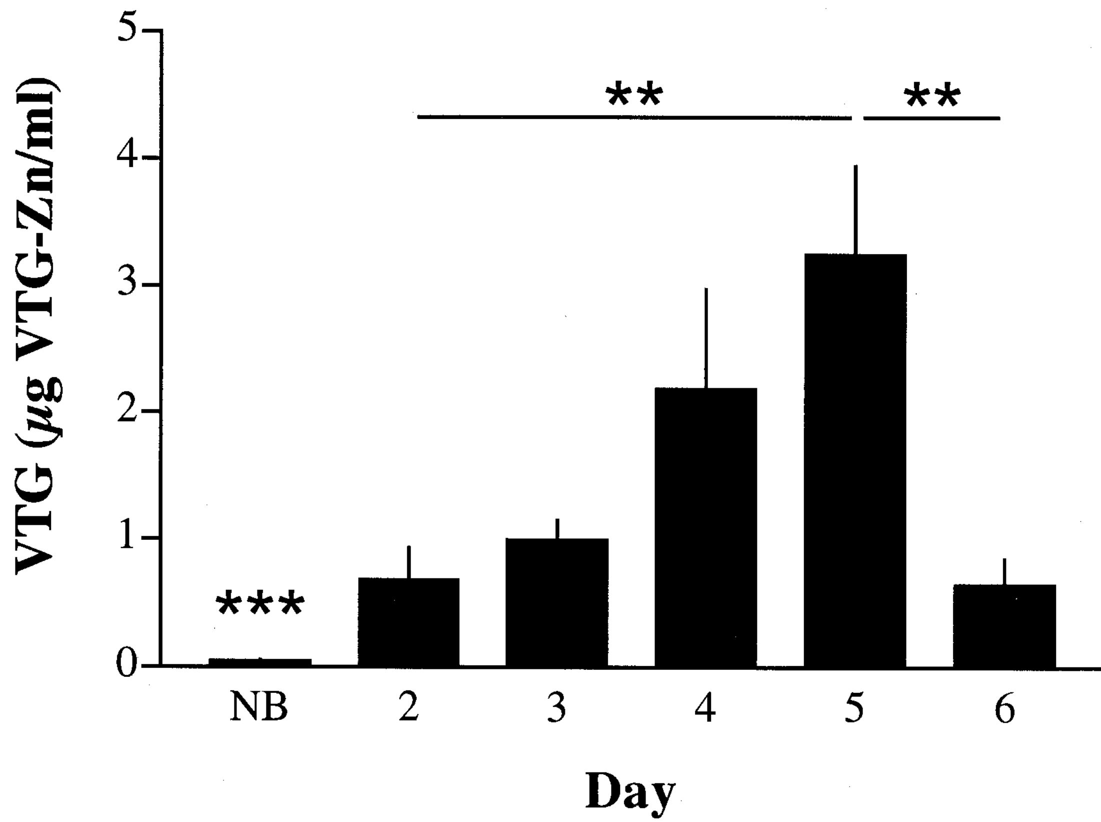
Figure 2. Pattern of circulating vitellogenin measured daily during Experiment 1. Horizontal lines indicate significant differences between VTG levels in birds measured on different days. Values are means ± SE. "Day" corresponds to day in Figure 1a. NB refers to non-breeding values. ** $P < 0.005$, *** $P < 0.0005$. Vitellogenin plasma levels for days 2 to 6 were all significantly different from NB values ($P < 0.0005$ in all cases).

Figure 3. (a) Plasma vitellogenin (VTG) and (b) very-low-density lipoprotein (VLDL) levels measured at pre-, mid- and post-treatment in estradiol (E2)-treated (filled bars) and sham groups (open bars). Values are means ± SE. Comparisons were made between E2 and sham treatment groups at pre-, mid-, and post-treatment. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$. See text for statistics and within treatment group differences.

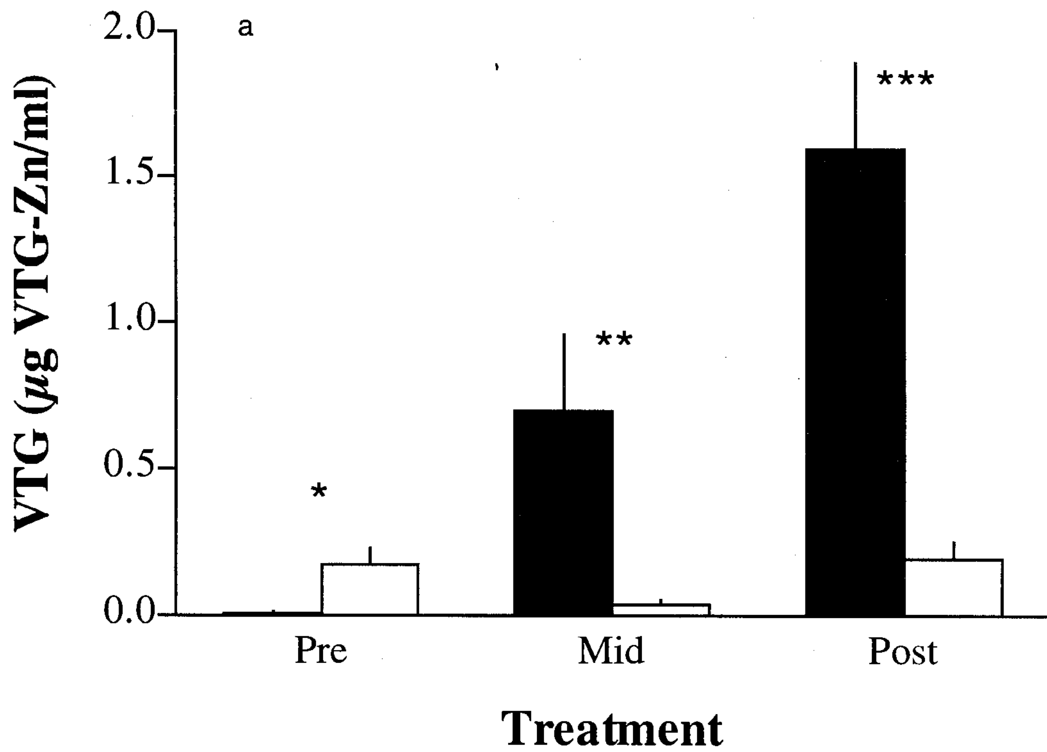
Figure 4. Comparison of least-squared mean resting metabolic rate (RMR; correcting for the effect of body mass and time) between estradio (E2)-treated (filled bars) and sham groups (open bars) at pre- and mid-treatment. Values are means ± SE.

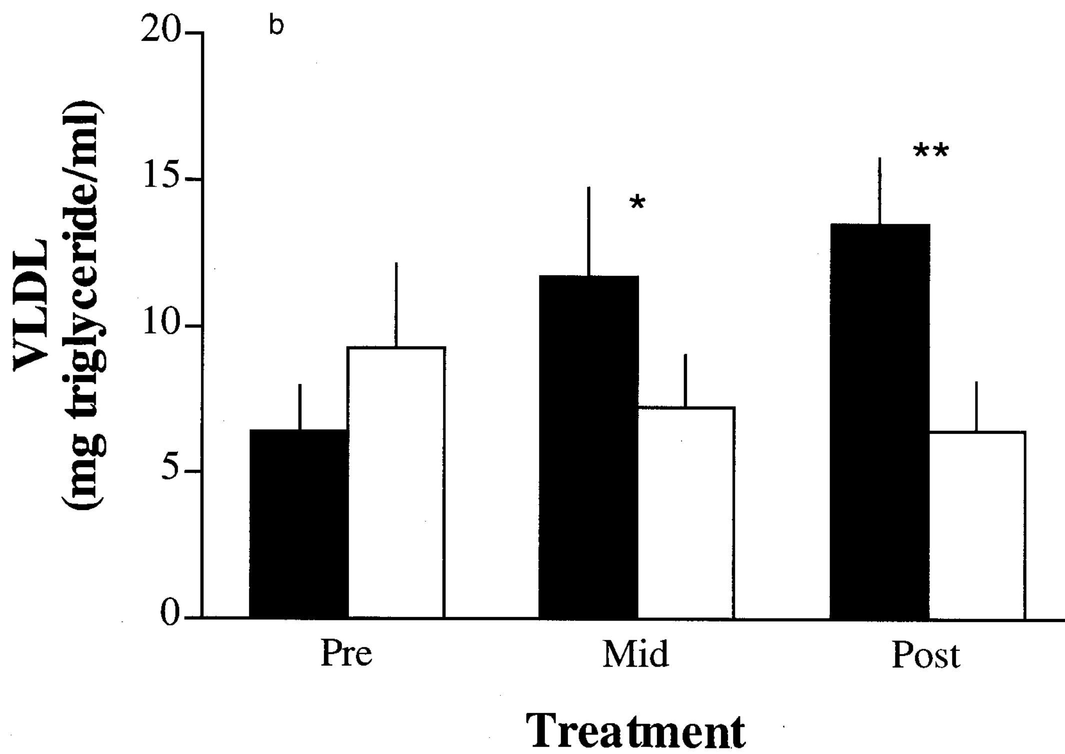

a
DAY
1
2
3
4
5
6
Blood sampling for yolk precursor measurement
Daily E2 or Sham injections

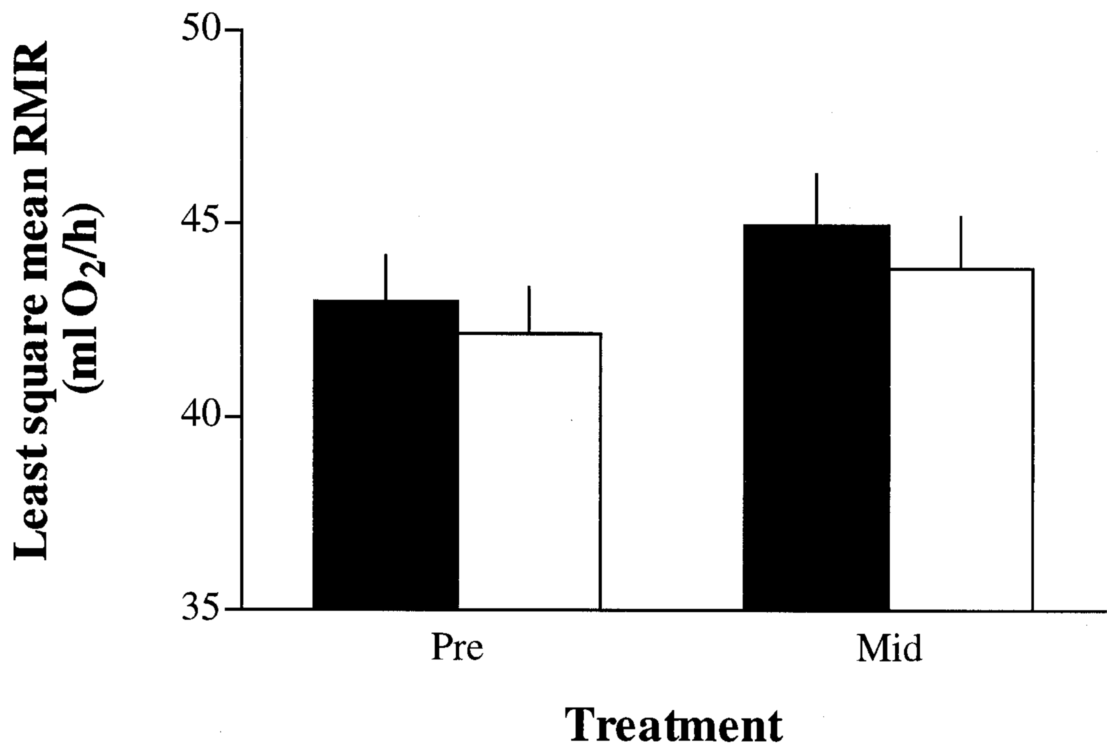

b
DAY
0
1
2
3
4
5
6
7
8
9
10
11
RMR
Pre-treatment
Blood Sample
RMR
Mid-treatment
Blood Sample
Post-treatment
Blood Sample
Daily E2 or Sham injections



VTG (μg VTG-Zn/ml)
5
4
3
2
1
0
**
**
***
NB
2
3
4
5
6
Day


a
VTG (μg VTG-Zn/ml)
2.0
1.5
1.0
0.5
0.0
*
**
***
Pre
Mid
Post
Treatment


b
VLDL
(mg triglyceride/ml)
20
15
10
5
0
*
**
Pre
Mid
Post
Treatment



Least square mean RMR
(ml O2/h)
50
45
40
35
Pre
Mid
Treatment

CHAPTER FIVE: INTERACTION BETWEEN ORGAN MASS AND TISSUE MAXIMAL OXYDATIVE CAPACITY IN BREEDING EUROPEAN STARLINGS: IMPLICATIONS FOR METABOLIC RATE AND ORGAN MASS RELATIONSHIPS.

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ABSTRACT

Basal metabolic rate (BMR) can be defined as the sum of the energy expenditure of all physiological systems and organs at rest; i.e., the maintenance cost of an animal. Studies investigating the basis of variation in BMR often compare it to the mass of internal organs, assuming that elevated sustained daily energy expenditure needs to be supported by larger machinery resulting in an increased maintenance cost. However, organs significantly related to BMR are inconsistent between physiological stages and species. This may not be surprising since an organism under variable conditions may readjust the mass-specific energy use between its various physiological systems without changes in organ mass. In this study, we investigated metabolic adjustments in four key organs (pectoral muscle, heart, kidney and liver), using citrate synthase as an indicator of tissue aerobic potential, in reproductive female European starlings (*Sturnus vulgaris*) throughout the breeding season in order to determine if the cost of egg production results in readjustments in organ mass-specific energy use. Despite an active liver involved in yolk precursor production and significant changes in organ mass between breeding stages and years, metabolic adjustments do not appear to be related to the demand of egg production. Metabolic adjustments at the organ level may occur independently from organ mass changes and this factor may be responsible for part of the inconsistencies found between studies in terms of organs mass – RMR relationships.

INTRODUCTION

Integrated measures of metabolism, such as basal or resting metabolic rate (BMR and RMR respectively) or daily energy expenditure (DEE), are often used as an indicator of the amount of effort animals put into specific activities (Peterson et al. 1990; Hammond and Diamond 1997; Piersma 2002). It is generally accepted that BMR – defined as the overall energy consumption of an animal at rest, in a post-absorptive state and at thermoneutrality – reflects the overall maintenance energy cost of complex and often expensive physiological machinery (Piersma 2002). This concept implies that increased sustained energy demand which results in up-regulation of physiological systems and organ mass will, in turn, induce higher levels of BMR (Hammond and Diamond 1997; Piersma 2002). Based on this idea, there have been an increasing number of studies investigating the basis for variation in BMR and RMR through intercorrelations between these variables and the mass of specific organs (see Piersma 2002 for a review). However, the results of these studies are often conflicting in terms of which organs relate to metabolic rate. Differences may appear within species when comparing different physiological states or between species for a given state. For example, in European starlings (*Sturnus vulgaris*), Vézina and Williams (2003) reported a significant correlation between RMR and pectoralis muscle mass in non-breeding females, between RMR and oviduct mass in egg-laying individuals, and between RMR and both liver and gizzard mass in chick-rearing birds. In contrast, in chick-rearing tree swallows (*Tachycineta bicolor*), resting VO_2 was shown to be significantly related to the mass of the kidney and intestine (Burness et al. 1998). Similar differences have also been reported between sexes in the red junglefowl (*Gallus gallus*) where BMR was significantly

correlated to the mass of the small and large intestine, proventriculus, lungs and caecum in males but only to the mass of the spleen in females (Hammond et al. 2000).

Explaining variations in BMR simply through changes in organ mass makes an assumption of constant organ metabolic intensity, that is, the amount of energy used per unit tissue mass does not vary. However, it is clear that tissue-specific metabolism, and thus tissue energy consumption, can be adjusted with little or no change in mass. For example, the activity of citrate synthase (CS) - an enzyme commonly used as an index of tissue maximal oxidative capacity (Emmett and Hochachka 1981; Hochacka et al. 1988) - was reported to be 42% higher in the leg muscle of dominant compared to subordinate male red junglefowl, with no differences in overall muscle mass (Hammond et al. 2000). Similarly, in blue-winged teal (*Anas discors*) at the end of the molting period, there is a rapid increase in pectoral muscle CS activity that is of greater magnitude than the mass gain in this organ (Saunders and Klemm 1994). Changes in metabolic intensity coupled with rapid and reversible changes in organ mass (Piersma and Lindström, 1997) might therefore occur simultaneously within an organism perhaps even in a compensating manner among different organs. Since BMR measures only the overall energy consumed by the organism, variable adjustments of internal organs and physiological systems may result in reduced or no net changes in BMR when comparing individuals in different physiological states. This important confounding factor in studies investigating effects of specific organs on BMR has been highlighted many times in animals (Weber and Piersma 1996; Kvist and Lindström 2001; Piersma 2002) including humans (Sparti et al. 1997, Hsu et al. 2003) and clearly calls for examination of variation of metabolic intensity at the organ level.

Reproduction in female birds provides a very useful model system for such a study. In our study species, the European Starling, reproductive organs undergo tremendous changes in mass, in parallel with follicle development prior to ovulation, increasing several times over a few days without comparable changes in the non-reproductive organs (Vézina and Williams 2003). In addition, the liver actively produces yolk precursors with plasma levels of vitellogenin (VTG) and very low density lipoprotein (VLDL) increasing 245 and 7 fold respectively to support follicle development in the ovary (Challenger et al. 2001), but this increased hepatic activity does not result in consistent increases in liver mass associated with egg production between years (Vézina and Williams 2003). Production of eggs also induces a 22% increase in mass-corrected RMR compared to pre-breeding values (Vézina and Williams, 2002). However, the single organ significantly related to laying-RMR, the oviduct, explains only 18% of its variation (Vézina and Williams, 2003). Finally, as stated above, the organs specifically related to RMR in starlings differ depending on whether the individuals are at the non-breeding, one-egg or chick-rearing stage (Vézina and Williams 2003). Thus in this system, it is possible that the metabolic intensity of some organs is adjusted to sustain the demands of egg production, confounding the relationship between metabolic rate and organ mass.

This paper investigates metabolic adjustments at the organ level throughout reproduction in European starlings by comparing individuals at the non-breeding, one-egg and chick-rearing stages, using the enzyme citrate synthase as an indicator of maximal tissue aerobic potential. We examine the changes in oxidative capacity of the pectoral muscle, heart, kidney and liver. Pectoral muscle contributes substantially to

energy expenditure under active conditions (Emmet and Hochachka 1981; Hochachka et al. 1988) and represents an important proportion of overall body mass (19% in non-breeding starlings; F. Vézina unpublished data). Heart and kidneys are thought to be among the most metabolically active tissues and are correlated with BMR inter-specifically (Krebs 1950, Schmidt-Nielsen 1984; Daan et al. 1990). Finally, we include the liver in our analysis because of its important contribution to the egg formation process through yolk precursor production. Despite a high level of hepatic activity directed toward yolk precursors (Gruber 1972; Griffin and Hermier 1988), liver mass is not related to RMR in egg laying starlings (Vézina and Williams, 2003). Furthermore, RMR is not affected by hormonally-induced artificial yolk precursor production in zebra finches (*Taenyopigia guttata*), potentially indicating that elevated hepatic activity is counterbalanced by down-regulation of other systems resulting in no changes in RMR (Vézina et al. 2003). This paper does not aim to highlight reallocation of energy between organs per se, but rather investigates whether egg formation results in mass-independent changes in metabolic activity of organs known for their high aerobic energy use.

MATERIAL AND METHODS

Data presented in this paper were collected in 1999 and 2000 as part of a wider study on the metabolic costs of egg production. Results regarding RMR and organ mass variation in breeding starlings have been extensively described in previous papers (Vézina and Williams 2002; 2003). Therefore, in this article we restricted our analysis to birds for which we had CS activity and body composition data for the four organs as indicated in the introduction.

Field site and collection of birds

Field work was carried out at the Pacific Agri-Food Research Center (P.A.R.C.) in Agassiz B.C., Canada (49°14`N, 121°46`W) under Simon Fraser University animal care permit (499B), following guidelines of the Canadian Council on Animal Care. The site consists of c.175 nest-boxes on farm buildings and telephone poles that were used each year by breeding starlings. Each year nest boxes were checked daily to determine dates of clutch initiation and clutch completion and the laying sequence of eggs. Females caught at the one-egg and chick-rearing stage were taken from their nest boxes during night-time (generally between 20:00h and 24:00h); during late chick-rearing, provisioning females were trap-caught (always within an hour before sunset). Non-breeding females were mist-netted at a barn used as a roosting site.

Reproductive stages

For both years, we measured organ CS activity in birds caught at the end of the wintering period (non-breeding, NB; n = 19 in 1999 and n = 19 in 2000), and the one egg stage of laying (LY-1; n = 20 in 1999 except for kidney due to the loss of a sample and n = 23 in 2000 except in heart due to the loss of 3 samples; LY-1 sample also includes four individuals caught after laying the second egg and one caught after laying the fourth egg, no difference in CS activity compared to LY-1 individuals for all organs P > 0.1 in all cases). We measured birds at the one-egg stage of laying since this represents peak investments in egg-production including fully developed reproductive organs, yolky follicle hierarchy and elevated levels of yolk precursors (Challenger et al. 2001; Vézina and Williams 2003). At this point, all birds have laid one egg, the second egg of the

clutch is in the oviduct and all the remaining follicles are sequentially developing in the ovary. We only obtained data for chick-rearing (CK) birds in 1999 (n = 20; due to a liquid nitrogen leak and loss of year 2000 samples). However preliminary analysis of the NB and LY-1 groups revealed no significant year effect in CS activity in all organs; we therefore consider our 1999 CK data as adequately representing this group for both years.

Measurement of resting metabolic rate (RMR)

Basal metabolic rate is defined as the energy consumed by a resting post-absorptive animal during the inactive phase of the circadian cycle at a temperature within the thermoneutral range for the animal (IUPS Thermal Commission 2001). These are the conditions we tried to achieve during our respirometry measurements. However, because laying birds in this study are producing eggs, they have to be considered in an "active physiological state" which induces elevated levels of energy consumption (Vézina and Williams 2002). Therefore we consider the term *resting* metabolic rate more appropriate in the present case. We measured RMR as described in Vézina and Williams (2002). Briefly, after capture, birds were brought to the laboratory, body mass was measured (± 0.1 g), and they were then placed in metabolic chambers (3.5 L) for one hour prior to the beginning of the measurements. All birds received 500 ml/min of dry CO_2-free air and were kept in the dark at 25°C (within the thermoneutral zone for this species; Johnson and Cowan 1975; Lustick and Adams 1977). Each chamber was sampled for oxygen and CO_2 analysis one at a time, and each sample was separated by ten minutes of ambient baseline air readings (starting with baseline). RMR measurements were always carried out between 23:00 h and 5:00 h. Our setup allowed us to collect RMR data for four birds

per night. On average, the birds stayed in their chambers for approximately 5.5 h. Preliminary data showed that sequentially measuring RMR in this way did not generate a time effect (see Hayes et al. 1992). Thus, having one or four hours to rest did not affect RMR (F. Vézina, unpublished data). Following RMR measurements, the birds were re-weighed and the average of first and second mass was used in subsequent calculations. To calculate RMR, a running mean representing ten minutes of recording was passed through the data for each bird, with the lowest average VO_2 taken as RMR.

Body composition analysis and yolk precursor assay

After RMR measurements, birds were sacrificed by exsanguination under anesthesia (ketamine:xylasine at doses of 20 mg/kg and 4 mg/kg, respectively), and were dissected. Tissues samples (ca. 300-700 mg) from the pectoral muscle, liver, heart (left and right ventricle) and kidneys were dissected out in this specific order, weighed in a cryovial and deep-frozen in liquid nitrogen within 10 minutes of the bird's death. We then completed the dissection of these organs to obtain total fresh mass. We also recorded the fresh mass of the reproductive organs (oviduct, follicles and follicle-free ovary) as well as non-reproductive organs (see Vézina and Williams 2003). Tissue samples were stored at –80°C. Plasma vitellogenin and VLDL levels were measured as previously reported in Vézina and Williams (2003) and Vézina et al. (2003) using vitellogenin zinc and triglyceride kits (Mitchell and Carlisle 1991; Zinc kit, Wako Chemicals; inter-assay CV = 16.3%; Triglyceride E kit, Wako Chemicals; inter-assay CV = 15.3%).

Enzyme assays

Sub-samples (ca. 40-90 mg) of pectoral muscle, heart, kidneys and liver where weighed frozen (± 0.0001g), minced on ice and homogenized in 9 volumes of ice-cold buffer (20 mM Na_2HPO_4, 0.5 mM EDTA, 0.2% BSA (defatted), 50% glycerol, 0.1% Triton X-100, pH 7.4). Homogenization was carried out on ice at moderate speed using a Virtis-shear Tempest homogenizer (3 x 10 s, 30 s rest) and further sonicated (model Virtis 60; 3 x 10 s, 30 s rest). This homogenization buffer allows samples to be stored for an extended period of time without a loss of enzyme activity (Mommsen and Hochachka 1994). Citrate synthase (EC 4.1.3.7) maximal activity was measured in a 1 ml reaction volume in a Perkin Elmer UV/Visible spectrophotometer (model Lambda 2) using glass cuvettes. The reaction temperature was maintained at 39°C by a cuvette holder connected to a Lauda K-2/R circulating water bath. The reaction was initiated by adding oxaloacetate (0.5 mM, omitted for control) to a solution of Tris (50 mM pH 8.4), Acetyl CoA (0.15 mM), DTNB (0.15 mM) and 10 µl crude homogenate (re-dilution 1:20 muscle, 1:50 heart, 1:10 liver and 1:10 kidney). Enzyme activity is presented as international units (µmoles of substrate converted to product per minute) per gram of wet tissue, per gram of protein (Bradford protein assay using BSA standard; Biorad) and as overall organ CS activity calculated based on organ wet mass. In some cases there was not enough homogenate left for the protein assay (4 samples total). These samples were omitted from the analysis on protein corrected CS activity.

Statistical analysis

The data on organ mass and organ enzymatic activity was analyzed using ANCOVA models with year and breeding stage as independent variables and non-reproductive body mass (NRBM) as covariate. We used non-reproductive body mass - which is the fresh body mass minus the mass of the reproductive organs, yolky follicles and oviductal egg – in order to control for the extra weight in LY-1 females (see Vézina and Williams 2003). This allows the comparison of birds within the same range of body masses. In order to identify specific differences between breeding stages, we then performed post-hoc multiple comparisons with Bonferroni correction (Rice 1989; P value corrected to 0.016). To examine potential relationships between yolk precursor plasma levels and liver citrate synthase activity in LY-1 birds, we used unpaired t-test to confirm the absence of a year effect in the restricted data set, and then correlations to evaluate the relationship between the variables. To investigate covariance between organ mass and the mass-specific enzymatic activity within groups, we used ANCOVA models relating protein-corrected mass-specific CS activity to fresh organ mass controlling for year. Finally ANCOVA models were used to study the correlations between RMR and CS activity in all organs controlling for year and NRBM effects. Data are presented as both mean ± SE and least squares means ± SE when controlling for year or NRBM.

RESULTS

Variation in organ mass and citrate synthase activity with breeding stage

Pectoral muscle

Fresh pectoral muscle varied in mass between breeding stages ($F_{2,107} = 44.2$, $P < 0.0001$) and this effect was independent of year (no significant year x breeding stage interaction term). However, year and NRBM were significantly related to muscle mass (year: $F_{1,107} = 8.4$, $P < 0.005$; NRBM: $F_{1,107} = 139.5$, $P < 0.0001$; interaction year x NRBM: $F_{1,107} = 9.3$, $P < 0.005$). Controlling for these variables, the breeding stage difference translated into non-breeding individuals having a 7.5% heavier pectoral muscle compared to the average for LY-1 and CK groups (post-hoc comparison $P < 0.0001$ in both cases; Figure 1a). Mass-specific CS activity was also affected by breeding stages ($F_{2,98} = 10.2$, $P < 0.0001$; no significant effect of year, NRBM or interaction; see Figure 1b) with post-hoc comparison revealing a significant difference between NB and LY-1 individuals ($P < 0.0005$) with the latter showing 10.8% lower CS activity. However, this stage effect was lost when reporting CS activity per gram of tissue proteins ($P = 0.2$, no significant effect of year, NRBM or interaction; Figure 1b). Protein-corrected CS activity was independent of fresh muscle mass for all breeding stages when controlling for year ($P \geq 0.3$ in all cases). Total pectoral muscle CS activity varied with stage ($F_{2,97} = 22.7$, $P < 0.0001$; no significant year effect) and was affected by NRBM ($F_{1,97} = 18.8$, $P < 0.0001$, no significant interaction). Controlling for the latter, NB birds had total muscle enzymatic activity 14.4% higher than mean activity level for LY-1 and CK birds (post-hoc comparison $P < 0.0001$ in both cases Figure 1c). There was a significant interaction between year and breeding stage in pectoral muscle protein content ($F_{1,96} = 5.9$, $P < 0.05$).

However, analyzing both years separately revealed that muscle protein content was independent of breeding stage ($P \geq 0.1$ in all cases).

Heart

Fresh heart mass varied with breeding stage and year (year x breeding stage interaction term; $F_{2,102} = 11.8$, $P < 0.0001$; Figure 2a). We therefore investigated changes in heart mass separately for both years. Controlling for NRBM, heart mass was 16.3% and 9.4% heavier in NB birds compared to LY-1 and CK individuals respectively in 1999 ($F_{2.55} = 24.5$, $P < 0.001$; Post-hoc comparison $P < 0.005$ in all cases). In 2000, heart mass was independent of breeding stage or NRBM ($P = 0.1$, no significant interaction). Citrate synthase activity calculated as both mass-specific or corrected for protein content was not affected by year, NRBM or breeding stage ($P > 0.5$ in all cases, no significant interaction; Figure 2b) and protein-corrected CS activity was independent of fresh heart mass within all breeding stages when controlling for year ($P \geq 0.2$ in all cases). Total heart CS activity was not affected by year or breeding stage when controlling for the effect of NRBM ($P = 0.06$; Figure 2c). Heart protein content did not change significantly with either year or breeding stages ($P \geq 0.07$ in both cases).

Kidney

As for the heart, differences in kidney fresh mass between breeding stage was dependent on year (year x breeding stage interaction term; $F_{2,105} = 7.3$, $P < 0.05$; see Figure 3a). In 1999, when controlling for the effect of NRBM, LY-1 kidney mass was 11.2% heavier than in NB birds, while CK individuals were intermediate (post-hoc comparison $P < 0.0005$, no significant interaction). In 2000 however, kidney mass was significantly

different at all stages and heaviest in LY-1 individuals with a maximal mass difference of 22.8% between this group and NB birds (post-hoc comparison $P < 0.01$ in all cases, no significant effect of NRBM or interaction). Mass specific citrate synthase activity showed a reversed pattern with the highest level of activity being recorded in NB individuals and lowest in LY-1 (18.8% difference; post-hoc comparison $P < 0.0001$; overall model $F_{2,97} = 10.55$, $P < 0.0001$, no effect of year, NRBM or interaction, Figure 3b). When performing this analysis on protein-corrected CS activity, the breeding stage effect was still present ($F_{2,96} = 10.05$, $P < 0.0001$, no effect of year, NRBM or interaction) and showed the same pattern (post-hoc comparison $P < 0.01$; Figure 3b). Protein-corrected CS activity was independent of fresh kidney mass in NB and CK birds when controlling for year ($P \geq 0.2$ in both cases). However, there was a positive but weak relationship between the two variables in LY-1 individuals (r = 0.3 n = 41 $P < 0.05$; no year effect) indicating that increasing kidney mass was associated with higher CS activity per unit mass protein than what would be predicted from the simple increase in the overall mass of the organ. Total kidney enzyme activity was not affected by year, NRBM or breeding stage ($P = 0.7$, no significant interaction; Figure 3c) as the increased kidney mass in LY-1 and CK birds was counterbalanced by a reduced CS activity. Protein content in the kidney did not change significantly with either year or breeding stages ($P \geq 0.4$ in both cases).

Liver

Fresh liver mass changed between breeding-stages and the difference varied between years (year x breeding stage interaction term; $F_{2,106} = 10.0$, $P < 0.0001$; Figure 4a). In 1999, when controlling for the effect of NRBM, liver mass was independent of breeding stage ($P = 0.09$, no significant interaction term) but in 2000, LY-1 and CK individuals

had comparable liver masses that were heavier than that of NB birds (14.5% difference, post-hoc comparison $P < 0.0001$ in both cases; overall model $F_{2,51} = 15.3$, $P < 0.0001$, no NRBM effect or interaction). Mass-specific and protein-specific citrate synthase activity were dependent on breeding stage (tissue : $F_{2,95} = 4.9$, $P < 0.01$, protein $F_{2,95} = 4.0$, $P < 0.05$, no year effect in both cases).We also found a significant breeding stage by NRBM interaction (tissue : $F_{2,95} = 5.8$, $P < 0.005$, protein $F_{2,95} = 4.2$, $P < 0.05$) for both measurements. Post-hoc multiple comparison on least square means revealed that mass-specific CS activity decreased by 33.2% from the NB stage to LY-1 and CK ($P < 0.0001$ in both cases, Figure 4b). The same pattern was found for protein-corrected activity, but in this case differences were significant for all groups ($P < 0.01$ in all cases, Figure 4b). This translated into a 40.8% difference between NB and CK birds. Within breeding stage, protein-corrected CS activity was independent of liver mass in NB and LY-1 individuals ($P \geq 0.1$ in both cases), but the variables were positively correlated in CK ($r = 0.6$ $n = 20$ $P < 0.005$). Total liver CS activity was also affected by breeding stage ($F_{2,95} = 3.8$, $P < 0.05$) and an interaction between stage and NRBM ($F_{2,95} = 4.1$, $P < 0.05$). Post-hoc multiple comparison on least square means revealed a significant difference between all stages ($P < 0.01$ in all cases) with a maximal difference of 41.5% in total liver activity between NB and CK individuals (Figure 4c). Liver protein content was also significantly affected by breeding stage ($F_{2,98} = 3.7$, $P < 0.05$; no significant year effect of interaction term), but this effect was weak as post-hoc multiple comparison failed to find any significant differences ($P \geq 0.02$ in all cases)

Restricting the analysis to the LY-1 group allowed for investigation of potential liver metabolic adjustments for yolk precursor production within this group of birds.

There was no year effect on fresh liver mass or liver CS activity presented as mass-specific, protein corrected or total (unpaired t-test $P > 0.3$ in all cases) within one-egg birds. Combining both years, no significant correlations were found between VTG or VLDL plasma levels and organ mass-specific, protein corrected or total liver CS activity ($P > 0.6$ in all cases).

Citrate synthase and RMR

When controlling for the effect of year and non-reproductive body mass, total organ CS activity was independent of RMR for all organs in all groups ($P \geq 0.2$ in all cases). Pooling the NB, LY-1 and CK data and including breeding stage in the model did not change this result ($P \geq 0.3$ in all cases). Investigating a possible relationship between RMR and the summated total citrate synthase activity for all organs revealed a significant interaction between year and total CS activity when controlling for breeding stage and NRBM ($F_{1,66} = 4.3$, $P < 0.05$). However, splitting the analysis by year revealed no significant correlations between summated CS activity and RMR when controlling for stage and NRBM ($P > 0.2$ in both years). Redoing this analysis within breeding stage and controlling for year and NRBM did not reveal significant relationships between RMR and summated organ CS activity either ($P > 0.2$ in all cases).

DISCUSSION

This study shows that organ maximal oxidative capacity is not a fixed phenotypic trait. Indeed, citrate synthase activity measured as either mass-specific, protein-corrected or total can vary more or less independently from actual organ mass variation. Changes in

aerobic potential can even occur in the opposite direction from change in organ mass variation. Indeed, an increase in kidney mass was associated with a decrease in mass-specific citrate synthase activity, resulting in no significant change among breeding stages in total organ oxidative capacity. In the liver, this effect resulted in a net decrease in total organ CS activity between stages regardless of an increase in organ mass in one of two years. The organ mass-independent variation in enzyme activity reported here is not simply an effect of variation in tissue protein content acting on the amount of enzyme present in the organ. Indeed, we found stable levels of proteins per unit mass across breeding stages in all organs. Despite an active liver involved in yolk precursor production and significant changes in organ mass between breeding stages and years (see Vézina and Williams 2003), the oxidative capacity of the organs measured in this study do not appear to be adjusted to the demand of egg production. Undeniably, egg production does not induce up-regulation of the liver maximal oxidative capacity compared to the non-breeding and chick-rearing physiological stages as citrate synthase activity actually decreases from non-breeding to chick-rearing.

High intensity organs

Total pectoral muscle CS activity was 14% higher in non-breeding birds relative to the average for one-egg and chick-rearing stages, but it appears that this difference was mainly driven by the decrease in muscle mass from NB to CK stages. We did find a significant decrease in mass-specific CS activity from non-breeding to laying but the difference was lost when controlling for the homogenate protein content, suggesting that the change in CS activity per gram of wet tissue had more to do with metabolically

inactive cell content than with enzymatic activity per se (Price 1996). This general effect of organ mass affecting total oxidative capacity was also shown in the kidney and to a lesser extent in the heart. In these particular cases, a year effect was detected in organ mass change between stages but not in the enzymatic activity calculated as mass or protein specific. When converted to total organ CS activity, the year interaction was lost. The change in organ mass between breeding stages had an obvious effect in the kidney by buffering the decrease in mass-specific CS activity. In the heart, although not significant, a trend for a decreasing total heart CS activity was probably driven by the strong differences in heart mass between breeding stages in 1999.

Vézina and Williams (2003) reported a correlation between RMR and pectoral muscle mass in non-breeding individuals using a larger sample size from the same starling population. It was suggested that the relationship may have to do with shivering thermoregulation needs as these birds were caught at the end of their wintering period. However, the constant level of protein-specific CS activity across breeding stages reported here suggests that shivering thermoregulation does not involve up-regulation of the pectoral muscle oxidative capacity. Accordingly, citrate synthase activity was shown to be constant in the pectoral muscle of both summer- and winter-acclimatized American goldfinches (*Carduelis tristis*; Marsh and Dawson 1982) and house finches (*Carpodacus mexicanus*; Carey et al. 1988). Higher mass-specific levels of muscle CS activity have been documented in trained rats (Garrido et al. 1996; Matsuo et al. 1999) and active lizards (John-Alder and Joos 1991) and also in migrating birds where it has been attributed to the high energy consumption of the muscle during flight (Lundgren and Kiessling 1985, 1986; Lundgrend 1988). Our protein-specific CS activity data would

therefore suggest that the locomotor activity level in our birds at the three breeding stage was similar in terms of muscle aerobic demands. We do not have time budget data for these birds, but the continuous decrease in pectoral muscle mass from non-breeding to chick-rearing (Vézina and Williams 2003) suggests, in contrast, an increase in activity since exercise training has been reported to result in the loss of muscle tissue in this species (Swaddle and Biewener 2000). Clearly, more research is needed to better understand muscle metabolic adjustments in breeding birds.

Mass or protein-specific heart CS activity did not change between breeding stages and there was no significant differences in total heart oxidative capacity even though the mass of this organ varied significantly among breeding stages in 1999. The physiological changes associated with the passage from non-breeding to egg-producing and to chick-rearing stages, therefore, does not seems to require large metabolic adjustments in the heart in starlings. We found a much different response in the kidney, where kidney mass in egg laying and chick-rearing birds increased compared to non-breeding individuals (Vézina and Williams 2003; this study). In addition, a lower mass-specific maximal citrate synthase activity meant that total kidney oxydative capacity did not change between breeding stages. The reported phenomenon is not simply a dilution effect due to a larger proportion of water or metabolically inactive content in the tissue since results on mass-specific citrate synthase activity corrected for protein content showed the same pattern. Furthermore, the change in kidney mass reported by Vézina and Williams (2003) were based on lean dry data. There are no apparent reasons for this phenomenon. Vézina and Williams (2003) suggested that the gain in kidney mass may be a response to a gradual increase in the protein content of the diet during the transition from winter to

summer as the birds may eat an increasing proportion of insects through time (Feare 1984). However, to our knowledge, there is no information on the effect of diet on oxidative capacity of kidney in birds. It seems also plausible that there is a need for a seasonally-associated structural reorganization in this organ and this may occur more or less independently from the cell metabolic adjustment leading to a larger organ with a slower pace.

Egg-production effect on organ oxidative capacity

Vézina and Williams (2003) highlighted the fact that the mass of the non-reproductive organs in laying females starlings does not change in response to the burden of egg production, but may simply be responding to local changes in ecological conditions. They also suggested that non-reproductive organs could be adjusting mass-specific metabolism independently of organ mass in order to sustain the reproductive demand. Our present study clearly showed that metabolic adjustments may happen within organs independently of their mass. However we found no support for adjustments in non-reproductive organs in relation to egg-production, as pectoral muscle, heart and kidney did not show significant reduction or increase in total CS activity during egg production (see Vézina and William 2003). We were interested in liver oxidative capacity due to its role as the yolk precursor biosynthesis organ during egg formation. During follicle development, the liver secretes vitellogenin and very low density lipoprotein, the nutrient and energy sources for the developing avian embryo, respectively (Williams 1998). During this period, plasma levels of VTG and VLDL rise significantly and stay at their maximal level until ovulation of the last follicle (Challenger et al. 2001; Vézina and

Williams 2003). This may represent an important increase in the liver's "physiological work load". Indeed, in domestic hens (*Gallus gallus domesticus*) approximately 50% of the liver's daily protein production is attributed to VTG synthesis, potentially tripling the amount of protein in circulation (Gruber 1972) while hepatic lipid production also increases markedly (Griffin and Hermier 1988). However, yolk precursor production does not induces a systematic increase in liver mass (Christians and Williams 1999; Vézina and Williams 2003) nor does liver mass correlate with plasma levels of VTG and VLDL (Vézina and Williams 2003). Furthermore, in laying female starlings, elevated RMR is not related to yolk precursor plasma levels or lean dry liver mass (Vézina and Williams 2003). Finally, a recent experiment showed that male zebra finches, stimulated with exogenous 17β-estradiol at doses adjusted to generate yolk precursors plasma levels comparable to breeding females, do not exhibit increased RMR in response to the treatment (Vézina et al. 2003). The authors suggested that the increased biosynthesis activity of the liver, and thus its potentially elevated energy consumption, may be counterbalanced by simultaneous down-regulation of other systems or organs leading to reallocation of energy, with no overall changes in RMR. We found little support for this hypothesis in this study. If liver must be up-regulated in terms of aerobic capacity for the process of yolk precursor production, we would have expected a peak in liver's citrate synthase maximal activity at the one egg-stage compared to non-breeding or chick-rearing individuals. Surprisingly, we found a marked decrease in the enzyme activity over the three physiological states. This translated into a 42% decrease in total liver oxidative capacity even while there was a 15% increase in the organ mass in LY-1 and CK individuals in one year. Clearly, yolk precursor production in wild starlings does not

necessitate an up-regulation of the liver's aerobic machinery compared with the other physiological stages. The reason for the overall decrease in liver citrate synthase activity throughout the breeding season is not clear. There are no studies that we are aware of that reported liver citrate synthase activity in relation to breeding. Alternatively and as stated above for the other organs, the liver may be adjusting its physiology to gradual changes in local ecological conditions (Vézina and Williams 2003). However, it should be mentioned that if the observed pattern is simply a seasonal effect, then liver CS activity in LY-1 birds may still be high relative to non-breeding individuals living at the same seasonal period.

Implications for BMR versus organ mass relationships.

For the mass of an organ to be significantly related to mass-independent BMR, it must have a disproportionate energy consumption relative to the other organs in order for a relatively small difference in its mass to effect the overall energy consumption of the animal at rest. Organs such as the heart and kidneys that were proposed to explain part of the inter-specific variation in BMR in chick-rearing birds (Daan et al. 1990) typically represent less than 1% of overall body mass in birds and mammals (Schimidt-Nielsen 1984; Daan et al. 1990). Early tissue slice respiration experiments (Krebs 1950) suggested that these tissues were some of the most metabolically active within the body (although these experiments should be viewed with caution, see chapter 8 in Schimdt-Nielsen 1984). Therefore, a small variation in the mass of these organs is likely to have an impact on overall resting energy consumption compared with the large proportion of inactive or low metabolic rate tissues (water, plumage, fat, bone, skin, etc; Schimidt-

Nielsen 1984; Daan et al. 1990). Why then, are we not finding a common pattern among studies in terms of which organ correlate with BMR?

Organ mass-BMR relationships do not take variation in organ metabolic intensity into account. Our study clearly demonstrates that, for a given species, changes in organ mass among physiological stages may also be accompanied by more or less independent variations in organ maximal oxidative capacity. These changes may even occur in opposite directions resulting in no variation in total organ capacity (kidney in the present study) or a completely inversed pattern (1999 liver in this study). It is reasonable to assume that this is also the case when comparisons are made between species. Because citrate synthase activity represents the maximal oxidative capacity of the tissue, correlations with metabolism, if any, should naturally be expected for maximal metabolic rate (VO_2max) rather than BMR or RMR (Emmett and Hochachka 1981). However, it is thought that supporting a high level of maximal metabolism requires more cellular metabolic machinery (including more enzymes; Rolfe and Brown 1997) resulting in elevated levels of energy consumption at rest, mostly due to protein turnover (12-25% of BMR in mammals; Rolfe and Brown 1997). Therefore, if BMR reflects the "physiological maintenances cost", one would assume that maintaining a high level of enzyme activity needed for increased levels of performance should also be associated with higher basal or resting metabolic rate.

We showed that, for a given physiological stage, organ mass does not affect citrate synthase activity per unit mass protein (except in two cases where there was a *positive* relationship). This means that for a given stage, individual variation in organ mass should be accompanied by positively correlated variation in total oxidative capacity

(i.e. larger organs have higher total oxidative capacity). This suggests, that within a physiological stage, an increase in the mass of a high intensity organ may truly result in an elevation of BMR. However, overall organ metabolic activity can be very different from one stage to another and this may or may not be associated with a change in organ mass. Therefore, a given organ may have a very different metabolic rate at different times and thus its relationship with BMR may be apparent only at certain specific points in the life cycle of the animal. Our data may thus explain part the inconsistencies regarding which organ is significantly related to BMR in the literature (see Table 1 in Piersma 2002)

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FIGURE LEGENDS

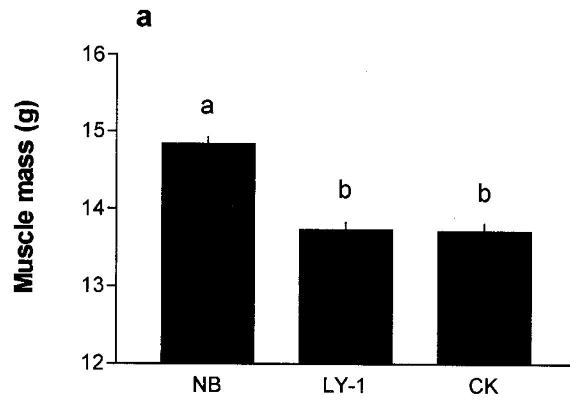
Figure 1: Variations in fresh mass (a), mass-specific citrate synthase activity (b) and total citrate synthase activity (c) for the pectoral muscle in European starlings. Different letters represent significant differences between breeding stages, no letters indicate no differences. Muscle mass is presented as least square means controlling for the effect of year and non-reproductive body mass. Total muscle citrate synthase activity is presented as least square means controlling for the effect of NRBM. NB = non-breeding, LY-1 = one-egg, CK = chick-rearing. Black bars in (b) are for activity per gram of wet tissue and white bars are for activity per gram of protein.

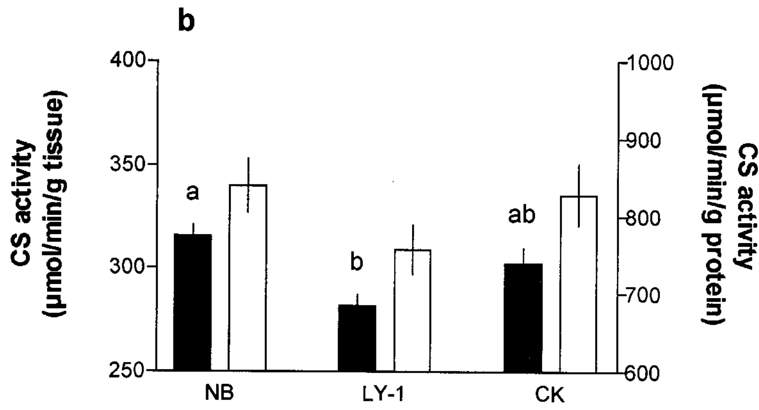
Figure 2: Variations in fresh mass (a), mass-specific citrate synthase activity (b) and total citrate synthase activity (c) for the heart in European starlings. Different letters represent significant differences between breeding stages, no letters indicate no differences. Heart mass for year 1999 is presented as least square means controlling for non-reproductive body mass. Total heart citrate synthase activity is presented as least square means controlling for the effect of NRBM. NB = non-breeding, LY-1 = one-egg, CK = chick-rearing. Black bars in (a) are for year 1999 and white bars indicate year 2000. Black bars in (b) are for activity per gram of wet tissue while white bars indicate activity per gram of protein.

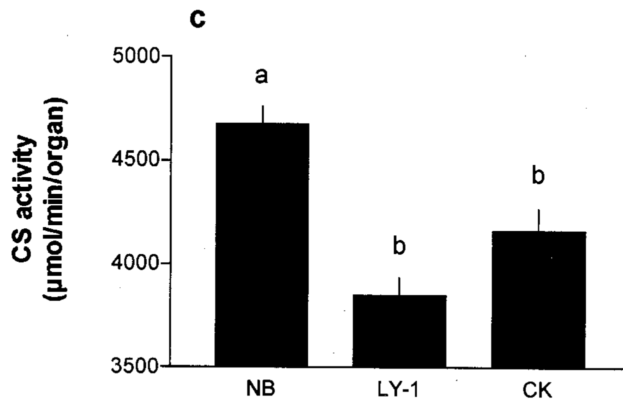
Figure 3: Variations in fresh mass (a), mass-specific citrate synthase activity (b) and total citrate synthase activity (c) for the kidney in European starlings. Different letters and numbers represent significant differences between breeding stages, no letters or numbers indicates no significant differences. Kidney mass for year 1999 is presented as least square means controlling for non-reproductive body mass. NB = non-breeding, LY-1 = one-egg, CK = chick-rearing. Black bars in (a) are for year 1999 and white bars indicate year 2000. Black bars in (b) are for activity per gram of wet tissue while white bars indicate activity per gram of protein.

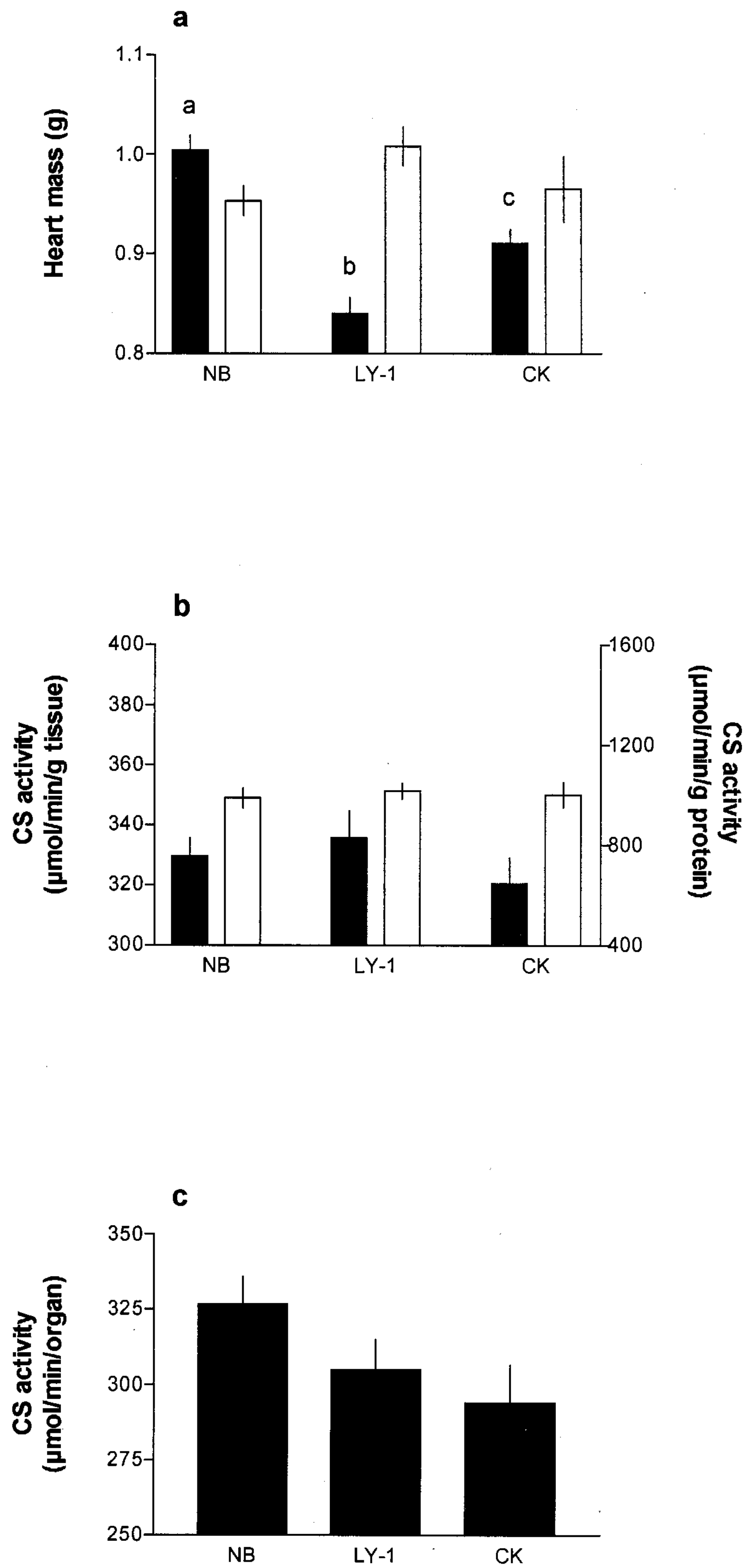
Figure 4: Variations in fresh mass (a), mass-specific citrate synthase activity (b) and total citrate synthase activity (c) for the liver in European starlings. Different letters and numbers represent significant differences between breeding stages, no letters or numbers indicates no significant differences. Liver mass for year 1999 is presented as least square means controlling for non-reproductive body mass. Citrate synthase activity per gram or wet tissue or protein as well as total liver enzymatic activity are presented as least square means controlling for an interaction between breeding stage and non-reproductive body mass. NB = non-breeding, LY-1 = one-egg, CK = chick-rearing. Black bars in (a) are for year 1999 and white bars indicate year 2000. Black bars in (b) are for activity per gram of wet tissue while white bars indicate activity per gram of protein.

Muscle





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CS activity
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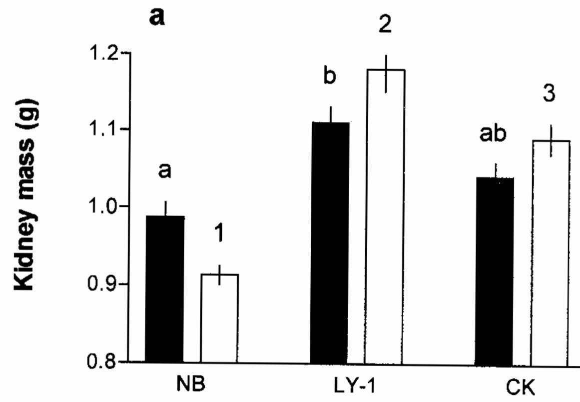
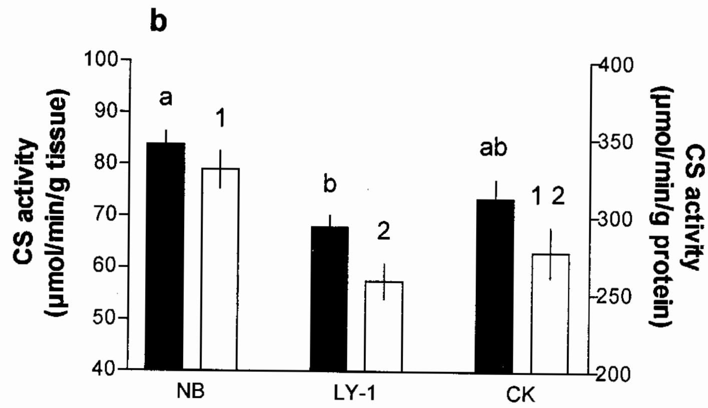
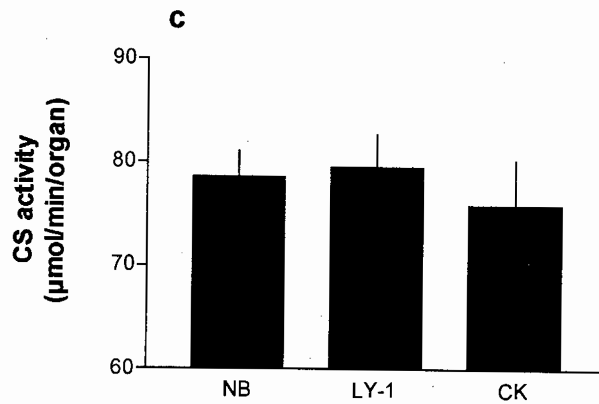
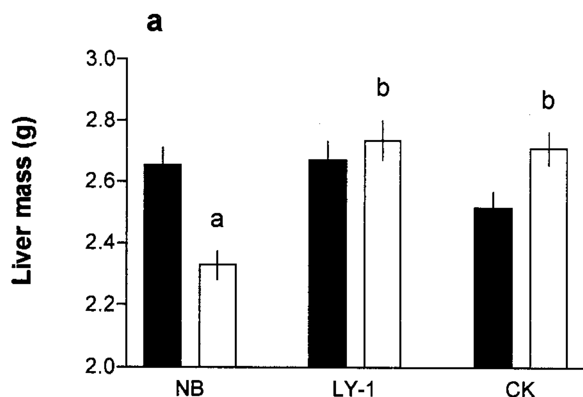
Kidney

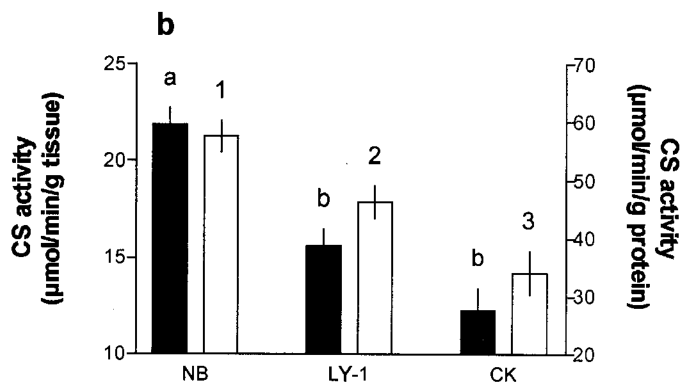


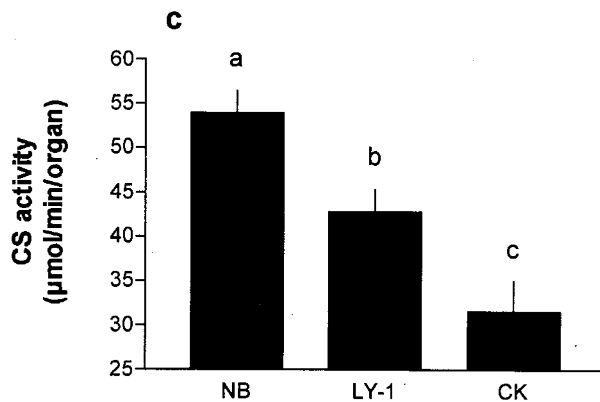




Liver







CHAPTER SIX:
THE METABOLIC COST OF EGG PRODUCTION IS REPEATABLE.

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ABSTRACT

Previous studies on the metabolic costs of egg production in birds reported 16-27% increases in basal or resting metabolic rate (BMR and RMR respectively) when comparing non-breeding values to measures in egg-producing individuals. These data were collected in wild birds. Therefore, variable ecological conditions may influence the non-reproductive physiology and thus obscure the energetic cost of egg-production estimates. The present study show that in captive, controlled conditions, RMR increases by 22% in response to the process of egg formation in zebra finch (*Taniopygia guttata*) and that individual variation in non-breeding and egg-producing RMR is repeatable over time. This, along with some recent findings by other researchers, suggests that the metabolic cost of egg production in birds may be constraining in terms of energy budget and sensitive to selection pressures.

INTRODUCTION

Basal metabolic rate (BMR) is a highly flexible phenotypic trait (Piersma 2002) and represents the overall energy consumed by a post-absorptive animal at rest at thermoneutrality during the inactive phase of the circadian cycle (IUPS Thermal Commission 2001). Because BMR is the sum of the energy consumed by the complete array of organs and physiological systems comprising an animal, it is influenced by variations in the underlying physiological machinery. Thus, changing ecological conditions that have obvious effects on animal body composition (Piersma and Lindström 1997; Piersma and Drent 2003) will influence BMR (Piersma 2002). For example, the mass of various organs has been shown to respond to changes in diet (Drobney 1984; Kehoe et al. 1988; Piersma et al. 1993; Geluso and Hayes 1999), wintering conditions (Heitmeyer 1988; Rogers et al. 1993), altitude and ambient temperature (Hammond et al. 2001) or geographic location (Hilton et al. 2000). Organs are also known to change with physiological stage such as throughout reproduction (Silverin 1981; Ricklefs and Hussell 1984; Christians and Williams 1999; Vézina and Williams 2003) or in preparation for and during migration (Battley et al. 2000, 2001; Piersma 2002; Landys-Ciannelli et al. 2003). Therefore, since ecological conditions and physiological stages may change over time, it is perhaps not surprising to find that repeatability of metabolic rate tends to decrease with time in wild animals (BMR: Bech et al. 1999, VO_2 max: Chappell et al. 1995, 1996). Nevertheless, the extent to which variation in BMR depends on physiological state per se remains unresolved.

We have recently shown that the egg formation process in European starlings (*Sturnus vulgaris*) induces a 22% increase in resting metabolic rate (RMR) over pre-

reproductive values when controlling for the effect of lean dry non-reproductive body mass and variation between years (Vézina and Williams 2002). This value is comparable to those reported for house sparrows (*Passer domesticus*, 16% over non breeding BMR; Chappell et al. 1999) and great tits (*Parus major*, 27% over wintering RMR; Nilsson and Raberg 2001) and suggests that egg production may influence the energy budget of breeding birds. Therefore, assuming that the energetic cost of forming eggs has an impact on fitness (Heaney and Monaghan 1995; Monaghan et al. 1995; Visser and Lessells 2001), it is important to know if the measured change in metabolic rate is stable through time, i.e. repeatable, because for natural selection to act on a particular phenotypic trait, it has to be heritable and repeatable (Bennett 1987; Dohm 2002; see Williams and Vézina 2001 for a discussion on repeatability of metabolic rate in breeding birds). In starlings, although oviduct mass was shown to be significantly correlated with elevated RMR during laying, the mass of non-reproductive organs was found to be changing independently of reproductive stage and inconsistently between years (Vézina and Williams 2003). This means that measured changes in resting or basal metabolic rate in wild egg-producing birds may be affected by variation in non-reproductive physiology. Therefore, the present study had two main objectives: 1) to extend the knowledge of the metabolic costs of egg production to a fourth species, the zebra finch (*Taniopygia guttata*) by comparing measurements of RMR in non-breeding, one-egg stage and chick-rearing individuals kept in controlled laboratory conditions, thus eliminating the confounding effects of natural variations in ecological condition, and 2) to obtain repeatability estimates for non-breeding and laying RMR to evaluate the level of stability of this trait between breeding attempts.

Material and Methods

Animal care

Zebra finches were kept in controlled environmental conditions (temperature 19°-23°C; humidity 35%-55%; constant light schedule, 14L : 10D with lights on at 0800 hours). All birds were maintained on a mixed-seed diet (Panicum and white millet, 50 : 50, approximately 12.0% protein, 4.7% lipid; Jamieson's Pet Food, Vancouver), water, grit and cuttlefish bone *ad libidum*, and received a multivitamin supplement in the drinking water once per week. All non-breeding birds were housed in same-sex cages (61 X 46 X 41 cm). Birds forming breeding pairs were chosen randomly and were housed in the same type of cage and provided with an external nest box (11.5 x 11.5 x 11.5 cm). Nest boxes were checked daily between 1000 and 1200 hours, and all new eggs were weighed (0.001g) and numbered. A clutch was considered complete after two consecutive days with no new eggs. All breeding pairs had access to an egg food supplement replaced daily (20.3% protein, 6.6% lipid). All experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit (692B-94), following the guidelines of the Canadian Committee on Animal Care.

Measurement of resting metabolic rate (RMR)

Basal metabolic rate is defined as the energy consumed by a resting post-absorptive animal during the inactive phase of the circadian cycle at a temperature within the thermoneutral range for the animal (IUPS Thermal Commission 2001). These are the conditions we attempted to achieve during our respirometry measurements. However, because laying birds in this study are producing eggs, they have to be considered in an

"active physiological state" which induces elevated levels of energy consumption (Vézina and Williams 2002). We also noticed that in some cases the bird crop still contained seeds when taken out of the metabolic chambers (captive zebra finches fill their crop before going to roost; Meijer et al. 1996) meaning that the condition of post-absorbtion may have been violated. We therefore consider the term *resting* metabolic rate more appropriate in the present study. All RMR measurements were completed using a flow-through respirometry system (Sable Systems International). Birds were taken from their cages within 10-15 minutes after lights were turned off, their body mass measured (± 0.1 g), and were placed randomly into one of four metabolic chambers (1.5 L) for approximately one hour prior to the beginning of RMR measurements. All chambers continuously received approximately 500 ml/min of dry CO_2-free air (using Drierite™ and ascarite™ as scrubbers) and were kept in the dark at 35°C, which is within the thermoneutral zone for this species (lower critical temperature = 33°C; Meijer et al. 1996). RMR measurements were always started at 23:00 h. 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 water scrubbed before CO2 analyzer and water and CO_2 scrubbed before O_2 analyzer). All measurement sequences started by recording 20 minutes of ambient baseline air. After baseline recording the multiplexer switched, and the out-flowing air from the first chamber was sampled for 33 minutes before switching to the second chamber for 33

minutes. Then the system sampled baseline air for 10 minutes before changing to the third and fourth chambers. This cycle was repeated three times over the night (with ten minutes of baseline in between each set of 2 chambers), giving 99 minutes of recording per chamber over 8 hours. After RMR measurement, the birds were re-weighed and placed back into their cage (approximately 30 minutes to an hour before lights were turned on). To calculate RMR, the average of first and second masses was used and VO_2 was calculated 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. Preliminary analysis showed that this value was always found in one of the last two recordings for a given chamber. Therefore, the first set of measurements was discarded. This means that the RMR value was found in the last 5 hours of the night. Preliminary analysis showed that measuring RMR using this protocol did not generate a time effect (Hayes et al. 1992; ANOVA testing for chamber position in the measurement sequence on mass-corrected RMR: $F_{3,53} = 0.7$ $P = 0.5$).

Study groups

To evaluate the relative increase in resting metabolic rate associated with egg production in zebra finches, we compared RMR values for a given female measured as non-breeders (NB; maintained in single sex group), at the one egg stage (LY-1; day of first egg laid) and at 17 days into the chick-rearing period (CK; approximately 4 days before fledging). To estimate LY-1 RMR repeatability, the females were paired again after a resting period and RMR was measured a second time at the one-egg stage. Because of our respirometry setup, we were limited to a maximum of four birds being measured per night. However,

in some cases, we had more than four birds beginning a clutch for a given day. This forced us to measure RMR the second day of laying in some individuals (7 out of 45 birds; 15%) but preliminary analysis revealed no difference in mass-corrected RMR between the one and two-egg stage (F. Vézina unpublished results). Therefore, all birds are presented here as one-egg stage. For non-breeding RMR repeatability, birds and cage availability prevented us from measuring RMR repeatedly in all females measured as LY-1. We therefore evaluated repeatability of NB RMR using a group of birds composed of 42% of females that were not used in the breeding protocol. As we were interested in the effect of time on RMR repeatability, we measured RMR, in a separate experiment, in a group of non-breeding males twice with measurements 8 days apart and then a third time 127 to 249 days later. Sample sizes for each group are presented in Table 1.

Statistical analysis

All data were tested to ensure normality (Shapiro-Wilk test; Zar, 1996). Metabolic rate is allometrically related to body mass (Schmidt-Nielsen 1984) but using $\log_{10}$ transformed RMR and body mass values for the analysis did not change any of the statistical results compared to when using the untransformed data. Therefore, results for the analysis on untransformed values are presented here (body mass in grams and VO_2 in ml of O_2 consumed per hour). To compare within individual changes in RMR from the non-breeding to one-egg and chick rearing stages we used repeated measures ANCOVA using body mass as a covariate. We used the same method to investigate potential changes in mean RMR measured twice as LY-1 or NB stages. Repeatability of residual RMR (effect of body mass factored out by regression analysis) was calculated following the method

proposed by Lessells and Boag (1987). Therefore, our repeatability index reflects the amount of variation in RMR among rather that within individuals. Post-hoc multiple comparisons between groups where performed using the Bonferroni procedure to reduce the risk of committing type I errors (Rice 1989). Data are reported as mean ± S.E.

RESULTS

Variation in RMR in relation to breeding stages

Repeated measure ANCOVA showed that resting metabolic rate differed significantly between breeding stages when controlling for the effect of body mass ($F_{2,84} = 71.0$, $P < 0.0001$, no significant interaction between stage and body mass; Figure 1). Least square mean comparison (controlling for the mass effect) revealed that LY-1 RMR was on average 22.4% higher compared with NB values (post hoc multiple comparison $P < 0.0001$) and 8.2% higher compared with CK values (post hoc multiple comparison $P < 0.01$; Figure 1).

Repeatability of non-breeding and laying RMR

Non-breeding males

Our data on non-breeding RMR in male zebra finches allows for the investigation of short and long term RMR repeatability. Comparing residual (mass corrected) RMR for the first and second measurements (8 days) resulted in 62.6% of the variation explained by among-individual variability ($F_{26,27} = 4.4$, $P < 0.0001$; Figure 2a). However, repeatability of RMR declined with time (measure 2 vs 3; 127 to 249 days; 44.5%; $F_{23,24} = 2.6$ $P < 0.05$; Figure 2b, measure 1 vs 3; 135 to 257 days; 28.7%; $F_{23,24} = 1.8$, $P = 0.08$;

Figure 2c). Repeatability calculated based on the three sets of measures was 47.0% ($F_{23,48}$ = 3.7, $P < 0.0001$).

Non-Breeding females

In females, we measured NB RMR twice over a period of time spanning 13 to 311 days. Residual RMR repeatability between the two sets of measurements for all birds was 51.6% ($F_{36,37}$ = 3.1, $P < 0.0005$). Including the delay between measurements in a multiple regression model showed that the relationship between first and second RMR measurements was still significant when time was taken into account (time effect $P <$ 0.05, RMR1 effect on RMR2: $P < 0.001$, no significant interaction term; overall model $R^2 = 0.35$ n = 37 $P < 0.001$). Repeated measure ANCOVA showed a 5.9% increase between first and second RMR measurement when controlling for body mass ($F_{1,37}$ = 10.3, $P < 0.005$) (Figure 3).

Non breeding pooled sex

Comparing data on initial NB RMR for males and females, we found that RMR was independent of sex when controlling for body mass (ANCOVA; $P = 0.3$, no significant interaction term between sex and body mass). This allowed for the analysis RMR repeatability using a larger sample size. We included in this data set all females for which we had two NB RMR value and males' first and last RMR measurement (n total = 61). The range of time between measurements spanned 13 to 311 days and repeatability was 51.3% ($F_{60,61}$ = 3.1, $P < 0.0001$) for this set of measurement (Figure 4). Again when controlling for the effect of time in a multiple regression model, the relationship between both RMR measurements remained significant (time effect $P < 0.05$, RMR1 effect on

RMR2: $P < 0.0001$, no significant interaction term; overall model $R^2 = 0.56$ n = 61 $P < 0.0001$).

Laying females

We measured LY-1 RMR twice over a period spanning 38 to 254 days. Residual RMR repeatability was 52.6% ($F_{18,19} = 3.22$, $P < 0.01$; Fig 5) and multiple regression showed no effect of delay between measurements in this particular data set (time effect $P = 0.8$). Repeated measure ANCOVA showed no significant differences between the first and second LY-1 RMR measurement when controlling for the effect of body mass ($P = 0.4$, no significant interaction between body mass and measurement sequence; Fig 3) meaning that absolute mean LY-1 RMR values did not change between breeding attempts. Using a larger sample size based on all females for which we have at least one LY RMR measurement combined with egg data (n = 39), we found no significant relationship between residual LY-1 RMR (correcting the effect of body mass) and mean egg mass, total clutch mass or clutch size ($P \geq 0.4$ in all cases).

DISCUSSION

Our study clearly demonstrates that for female zebra finches living in controlled environmental conditions, the physiological changes associated with egg production induce a significant (22%) increase in resting metabolic rate over non-reproductive value, an increase comparable to earlier reports (Chappell et al. 1999; Nilsson and Raberg 2001; Vézina and Williams 2002). Although previous studies involved wild birds subjected to variations in local ecological conditions potentially impacting non-reproductive

physiology (Vézina and Williams 2002; 2003), the reported increase in metabolic rate associated with egg production are very similar to our measurements in captive birds. This suggests that the effect of egg-production on metabolic rate, reported here and elsewhere, truly reflects physiological changes associated with the reproductive physiological machinery, and is relatively independent of ecological conditions. However, the exact mechanism responsible for the elevated energy consumption is not clearly identified. The mass of female reproductive organs in house sparrows was found to be positively related to reproductive BMR ($r^2 = 0.30$; Figure 3 in Chappell et al. 1999). Similarly, lean dry oviduct mass was found to explain about 18% of the variation in elevated mass-corrected laying RMR in starlings (Vézina and Williams 2003). Furthermore, previous work in our lab revealed that fresh oviduct mass explained 23% of the variation in mass corrected RMR in pre-laying zebra finches (F. Vézina unpublished data: $r^2 = 0.23$, $n = 20$, $P < 0.05$). Nevertheless, this means that at least 70% of the variation in egg-producing RMR remains unexplained and therefore other physiological mechanisms must be responsible for part of the increased energy consumption. Vézina et al. (2003) demonstrated that, in captive zebra finches, producing vitellogenin and yolk-targeted very low density lipoprotein (VTG and VLDLy respectively), the main yolk precursors molecules (Williams 1998), does not have a detectable cost in terms of changing RMR. This observation is in agreement with the recent finding that egg producing female starlings do not up-regulate their liver's oxidative capacity in response to yolk precursor secretion (F. Vézina in prep.). In the present study, we found no relationships between laying RMR and our reproductive effort parameters: egg mass, clutch size and clutch mass. This last finding suggests that the size (F_1 follicle mass and

egg mass are correlated in zebra finches; T.D. Williams unpublished data) and number of yolky follicles in the ovary at measurement time (one-egg stage) has little to do with variations in laying RMR. In our zebra finch population, 7 out of 45 females did not lay the day after their first egg during the laying sequence (i.e. they omitted laying on day 2). Our experimental protocol was not designed to investigate this phenomenon. However, resting metabolic rate at the one-egg stage (the night that the second egg will normally be processed in the oviduct) in these individuals did not differ significantly ($P = 0.6$ controlling for mass) from RMR in normal laying birds, suggesting that shell deposition by the oviduct (occurring during our RMR measurements, F. Vézina personal observations) is also not playing a major role in the increased laying RMR. However, given the sample sizes this result has to be considered with caution. Clearly, more research is needed to identify the exact physiological phenomena responsible for the metabolic cost of egg production.

An interesting finding is that zebra finches at the chick-rearing stage show a 13% higher RMR compared to non-breeding values. This difference is clearly not the result of partially or non-regressed reproductive organs. Vézina and Williams (2003) showed that in European starlings, after the last ovulation, the oviduct is regressed in mass by 42% even while still processing an egg, and almost completely reabsorbed at clutch completion. The same finding was reported more recently by Williams and Ames (2004) in zebra finches where the oviduct was shown to regress top-down following the passage of a developing egg with 44% of its mass reabsorbed the day following the last ovulation but before the last oviposition, and a further 23% at clutch completion. Clearly, resting metabolic rate in chick-rearing individuals is reflecting a physiological state that differs

from non-reproductive or egg–production. The reasons for the increased level of RMR in these females is not clear. Body composition analysis in several bird species at the chick-rearing stage revealed correlations between BMR or RMR and various organ combinations (Daan et al. 1990; Burness et al. 1998; Vézina and Williams 2003). However, organ mass is a highly plastic trait (Piersma 2002; Vézina and Williams 2003) and organ metabolic intensity can change independently of organ mass between physiological stages (Vézina and Williams in prep.), which may render these interspecific comparisons futile. More research is needed in order to comprehend the maternal physiological maintenance costs associated with the need of a growing brood.

In this article, we showed that egg production induces a 22% increase in RMR and that both non-breeding and laying RMR are repeatable, indicating that individual variation in RMR remains constant over time (Bennett 1987; Dohm 2002). This may have significant evolutionary consequences if the increase in RMR associated with egg production is consistently posing a constraint on the animal's energy budget. We do not have data on the actual energy expenditure related to activity in our laying females but Williams and Ternan (1999) showed that egg producing zebra finches reduce their level of locomotor activity by 46% while food intake decreases only by 8%, suggesting that the constraint of egg production forces females to reallocate energy through behavioral changes. This decrease in locomotor activity in egg-producing zebra finches was also reported earlier by Houston et al. (1995). In light of this data, it seems that the process of egg formation imposes an extra energy burden on reproductive female's energy budgets. However these studies need to be replicated and complemented with energy measurements in order to properly test the energy reallocation hypothesis.

In contrast to egg-producing RMR, we found that repeatability of non-breeding RMR decreased with time and even that the average value changed between measurements (5.9%). The time effect was visible in males and females and remained significant when the sample size was enlarged by pooling the sexes. This finding contradicts our expectations. Constant captive laboratory conditions should reduce the intra-individual variability in physiological characteristics. If RMR reflects the overall energy expenditure of all organs and systems at a given time, it is therefore unlikely that birds would undergo significant changes in physiological conditions when kept on constant photoperiod, humidity and diet. The reasons for the loss of repeatability over time is thus not obvious. There is only one other study that we are aware of that reported time effects on repeatability of BMR in captive birds. Horak et al. (2002) found a decreasing level of repeatability in greenfinches (*Carduelis chloris*) going from 0.87 at 8 days between measurements to 0.63 at 4 months between measurements (Table 1 in Horak et al. 2002). In their case however, the birds were maintained in semi-natural conditions between the two sets of measurements (natural changing photoperiod) which could explain part of the decrease in repeatability. A potential explanation for our finding is the fact that our non-breeding individuals were kept in reserve cages forming large groups (10-20 birds). Zebra finches are social birds and will form social hierarchies when maintained in groups (Zann 1996). It is thus possible that social interactions and access to food when kept in non-breeding same-sex groups, affects some aspects of individual physiology like fat content, known for its potential diluting effect on the body mass – metabolic rate relationships (Scott and Evans 1992) or hormonal state which may influence behavior and thus energy expenditure (Ramenofsky 1984; Wikelski et al. 1999

a, b). Therefore, changing group composition (number of birds, social hierarchy, etc) between RMR measurements may be responsible for the reduction in RMR repeatability and the change in mean non-breeding values over time, a condition that was not encountered by breeding females. Another probable explanation could be due to the effects of aging as mass-specific metabolic rate declines in old individuals (Rolfe and Brown 1997). We did not know the age of the birds used in this study. It is thus possible that the proportion of individuals that reached a declining level of RMR changed between repeated measurements, thus adding confounding variation in the repeatability analysis.

In conclusion, this study is the first to demonstrate that 1) egg production in captive female zebra finches induces a 22% elevation in resting metabolic rate over non-breeding levels, an increase comparable to values reported for three other bird species and 2) non-breeding and egg-producing RMR are repeatable over a period of at least 8 to 10 months, suggesting that the metabolic cost of egg formation may be responsive to natural selection.

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Table 1: Sample size at all breeding stages measured for the four analysis treatments.

Experiment	Breeding stage					
	NB_1	LY_1	CK	NB_2	LY_2	NB_3
Repeatability males	27	-	-	27	-	24
Breeding RMR females	57	45	16	-	-	-
Repeatability NB females	37	-	-	37	-	-
Repeatability LY-1 females	-	19	-	-	19	-
Repeatability pooled sexes	61	-	-	61	-	-

Figure Legends

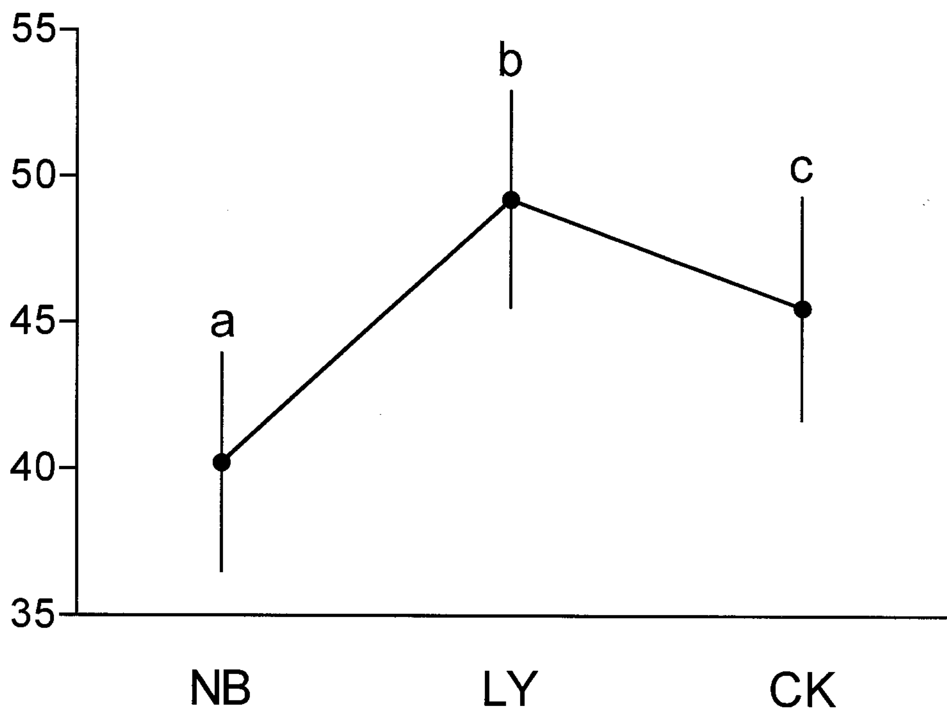
Figure 1: Least square mean resting metabolic rate (RMR) in non-breeding, one-egg stage and chick-rearing female zebra finches. Values are computed from a repeated measures ANCOVA controlling for the effect of body mass. Different letters indicate significant differences between groups.

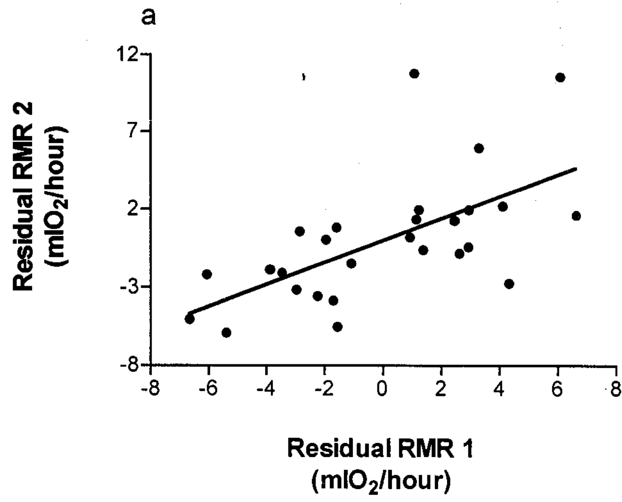
Figure 2: Correlation between residual RMR measured repeatedly in male zebra finches. a) first versus second measurement, b) second versus third measurement, c) first versus third measurement. Residuals are controlling for the effect of body mass.

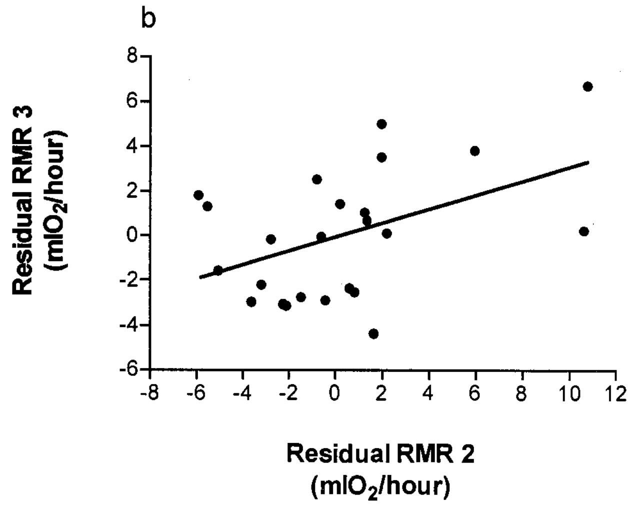
Figure 3: Least square mean RMR measured twice in non-breeding and one-egg stage females. Values are computed from a repeated-measures ANCOVA and control for the effect of body mass. A star indicates significant difference between measurements within stage.

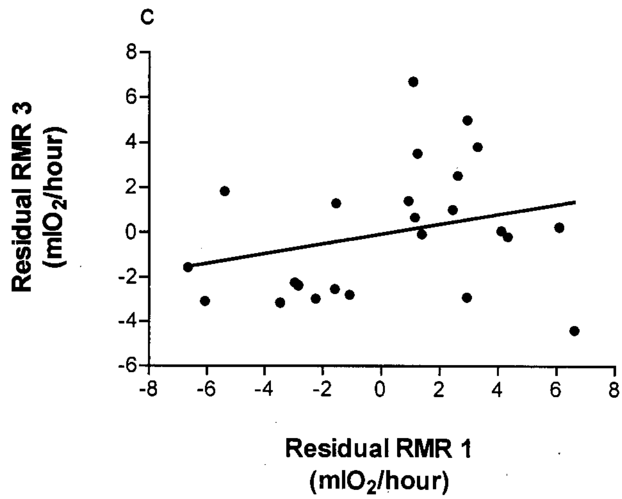
Figure 4: Correlation between first and second RMR measured at the non-breeding stage. Residuals correct for the effect of body mass.

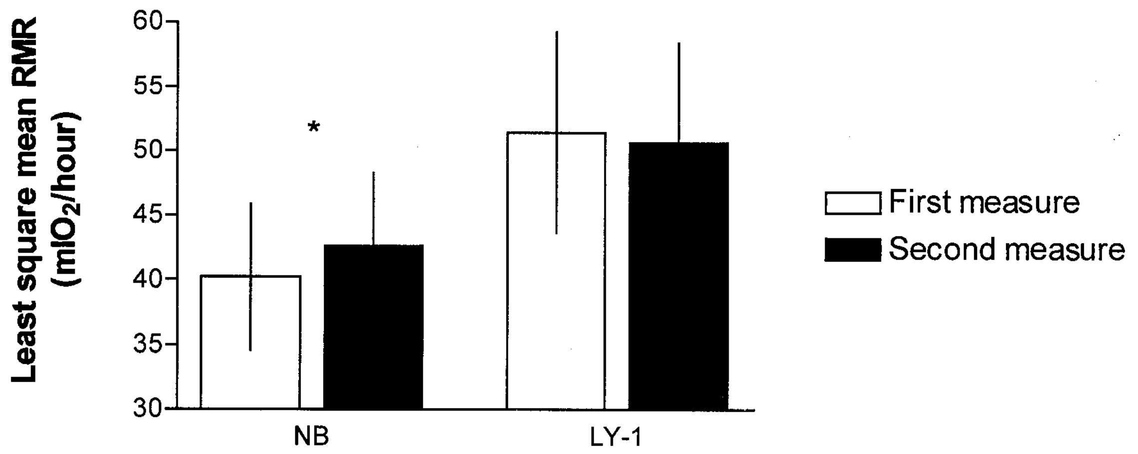
Figure 6: Correlation between first and second RMR measured at the one-egg stage in laying females. Residuals correct for the effect of body mass.

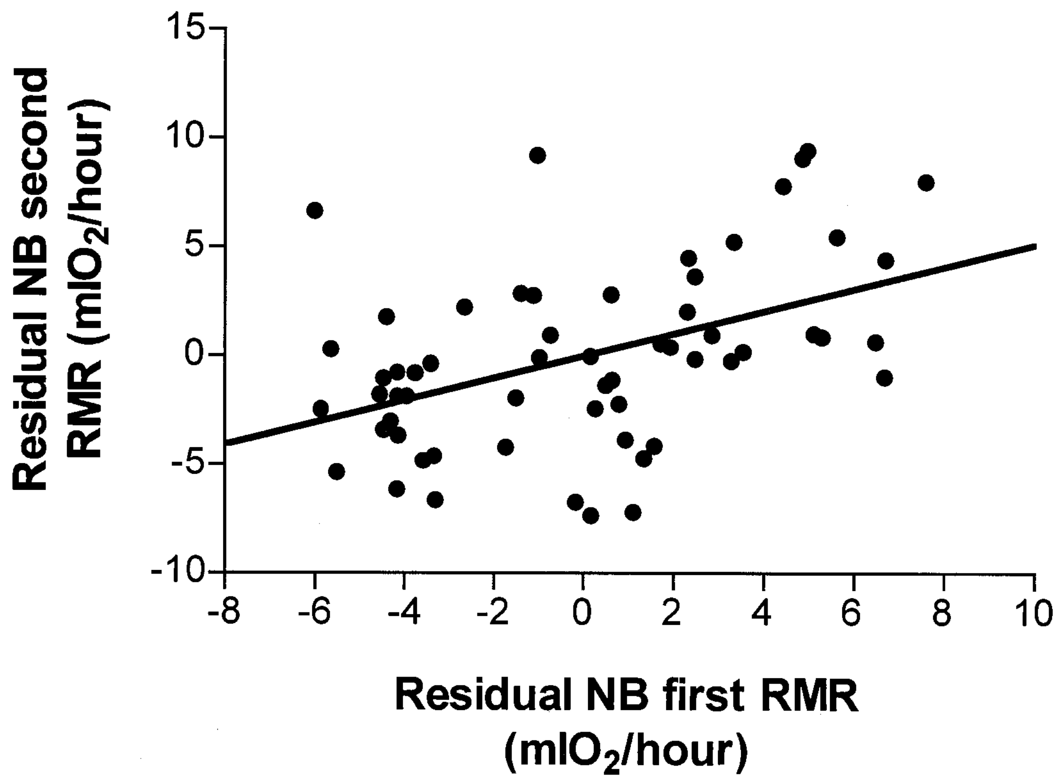

Least square mean RMR
(mlO2/hour)
55
50
45
40
35
a
b
c
NB
LY
CK

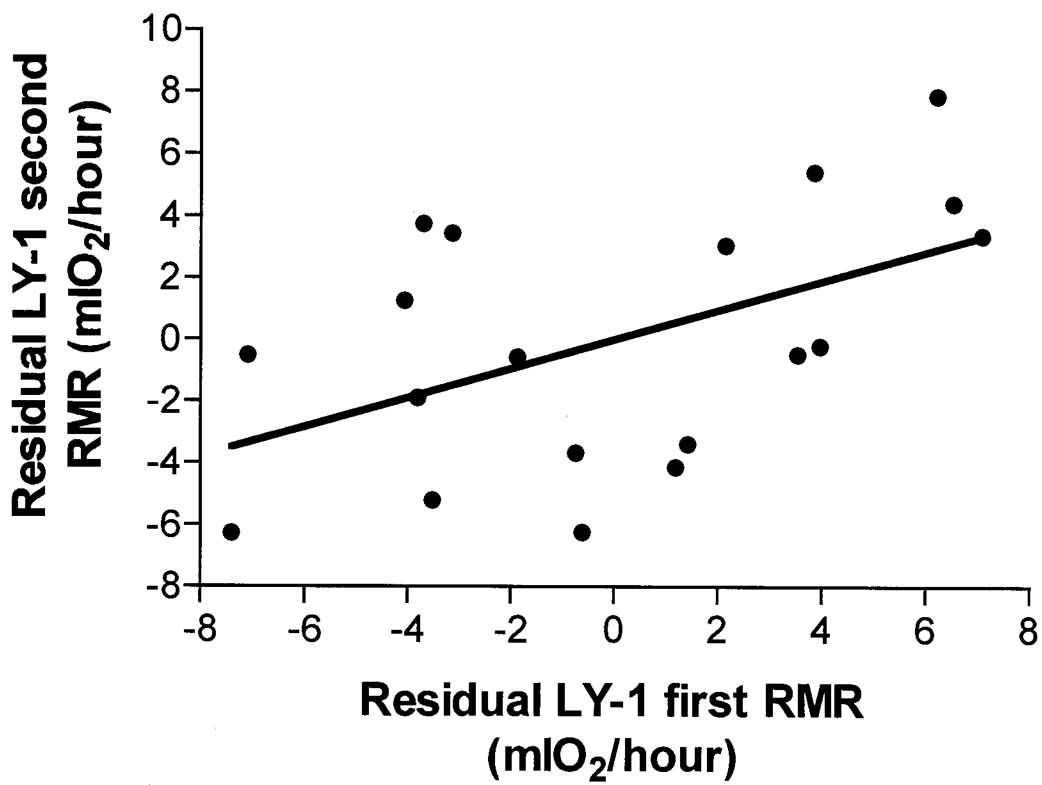

a
Residual RMR 2
(mlO2/hour)
12
7
2
-3
-8
-8
-6
-4
-2
0
2
4
6
8
Residual RMR 1
(mlO2/hour)


b
Residual RMR 3
(mlO2/hour)
8
6
4
2
0
-2
-4
-6
-8
-6
-4
-2
0
2
4
6
8
10
12
Residual RMR 2
(mlO2/hour)


c
Residual RMR 3
(mlO2/hour)
8
6
4
2
0
-2
-4
-6
-8
-6
-4
-2
0
2
4
6
8
Residual RMR 1
(mlO2/hour)


Least square mean RMR
(mlO2/hour)
60
55
50
45
40
35
30
*
NB
LY-1
First measure
Second measure


Residual NB second RMR (mlO2/hour)
15
10
5
0
-5
-10
-8
-6
-4
-2
0
2
4
6
8
10
Residual NB first RMR (mlO2/hour)



10
8
6
4
2
0
-2
-4
-6
-8
Residual LY-1 second RMR (mlO2/hour)
-8
-6
-4
-2
0
2
4
6
8
Residual LY-1 first RMR (mlO2/hour)

CHAPTER SEVEN:
THE METABOLIC COST OF REPRODUCTION IN BIRDS: INDIVIDUALS REALLOCATE ENERGY TO COPE WITH THE DEMANDS OF EGG FORMATION.

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ABSTRACT

Recent empirical studies show that the metabolic cost of egg production represents a 16-27% increase in basal or resting metabolic rate (BMR and RMR respectively), and that this cost is repeatable. However, it is not known how a female copes with this extra energy demand in terms of her overall energy budget. The cost could be additive to daily energy expenditure (DEE), resulting in an increase in DEE in egg producing birds and potentially a need for elevated food intake. Alternatively, the demand could be compensated by reduced expenditure in some other components of the energy budget. We measured RMR and DEE in captive female zebra finches (n = 24) at three stages, non-breeding, one-egg and chick-rearing and found marked variation in the relationship between these traits. Females that showed the largest increase in RMR during egg production were the ones that decreased activity the most but yet still showed an increase in DEE from non-breeding to first-egg stage. Females having high levels of DEE at the one egg stage also produced large clutches. We found a significant correlation between RMR and DEE in females at the non-breeding stage, but these traits were independent in females producing eggs as a result of the individual variation in the level of compensation. Our data support the energy reallocation hypothesis at the population level. However, the extent to which reallocation fully compensates for egg production costs depends on the initial reproductive investment. These results suggest a very flexible, individually-variable system of energy reallocation to meet increased energy demands.

INTRODUCTION

The cost of reproduction is of central importance in life history theory (Stearns 1992). For example, clutch and brood manipulation studies have shown that increased reproductive effort in birds can be associated with elevated parasite infections (Norris et al. 1994; Oppliger et al. 1996; Ots and Horak 1996), decreased immunity (Gustafsson et al. 1994), decreased parental survival (Daan et al. 1996; Visser and Lessells 2001) or reduced offspring quality and future reproductive success (Gustafsson and Sutherland 1988). Although few studies have included the cost of producing eggs in their experimental protocol, recent findings suggest that females having to produce extra eggs do incur fitness costs that are apparent either within the breeding attempt (Heaney and Monaghan 1995; Monaghan et al. 1995; Monaghan et al. 1998; Nager et al. 2000) or in terms of long-term female survival (Visser and Lessells 2001).

The mechanistic link between the cost of egg production and reproductive success or future survival is not clear, but it appears that the energy investment required for the physiological process of egg formation may play a significant role. Producing eggs requires a dramatic transformation of female morphology and physiology. In the few days prior to laying the first egg, a hormonal cascade is triggered that results in secretion of the yolk precursors vitellogenin and very low density lipoprotein by the liver into the blood, yolk precursor uptake and follicular growth in the ovary, development of the oviduct and ultimately ovulation, albumen and shell deposition in the oviduct, and oviposition (Williams 1998). Recent empirical studies have shown that the metabolic cost of egg production results in a 16-27% increase in basal and resting metabolic rate (BMR and RMR respectively; Chappell et al. 1999; Nilsson and Raberg 2001, Vézina and

Williams 2002; Vézina and Williams in prep.) and that RMR in egg-producing birds is repeatable (Vézina and Williams in prep). These findings raise an important question: how does an egg-producing female cope with a 16-27% increase in RMR or BMR in terms of her overall energy budget? There are two possibilities. First, the cost of egg production could be additive, meaning that daily energy expenditure (DEE) would also increase in response to egg production and require increased food intake or withdrawal of stored energy. Alternatively, the additional cost could be compensated for by reducing some other component of the energy budget. This could occur internally (energy reallocation between organs and/or other physiological systems) or externally (behavioral reallocation) and would result in no change in DEE.

Breeding zebra finches (*Taeniopygia guttata*) reduce their level of locomotor activity during egg production (Houston et al. 1995; Williams and Ternan 1999). Indeed, Williams and Ternan (1999) showed a 46% reduction in perch hopping activity during female follicular growth while food intake only decreased by 8% during the same period. The authors suggested that behavioral energy reallocation was taking place with females reducing the level of physical activity to channel more of the ingested energy towards egg production. However, their observations did not include any energy expenditure measurements. If energy reallocation is occurring in egg-producing zebra finches, then, assuming that the compensation is complete, overall daily energy expenditure for a given female should be relatively constant between non-breeding and egg-producing stages. Therefore, to evaluate if the cost of egg production induces a readjustment of the female's energy budget, leading to little or no change in DEE, the metabolic cost of egg formation along with overall energy expenditure need to be measured within individuals.

In this paper, we investigate the impact of the metabolic costs of egg production on female energy budgets. Using zebra finches as a model, our protocol was designed to address two main objectives, 1) to compare DEE estimates of females measured at non-breeding and breeding (laying and chick-rearing) stages in controlled captive conditions, thus reducing any confounding effects of ambient ecological conditions; and 2) to evaluate how egg-producing females adjust their energy budgets by comparing their DEE estimates with previous RMR measurements (made at the same breeding stages for the same individuals), food intake and locomotor activity data. We had a special interest for the intraspecific or interindividual variation as this represents the basis for natural selection and may allow us to better understand how individual birds adjust to the energy constraints of egg production (see Williams and Vézina 2001). We predicted that if female zebra finches used behavioral energy reallocation, DEE would not change between non-breeding to egg producing stages. Alternatively, if egg production costs were additive, then DEE should increase when the birds are forming eggs.

MATERIAL AND METHODS

Animal care

Zebra finches were kept in controlled environmental conditions (temperature 19°-23°C; humidity 35%-55%; constant light schedule, 14L : 10D with lights on at 0800 hours). All birds were maintained on a mixed-seed diet (Panicum and white millet, 50 : 50, approximately 12.0% protein, 4.7% lipid; Jamieson's Pet Food, Vancouver), water, grit and cuttlefish bone (calcium) *ad libitum* and received a multivitamin supplement in the drinking water once per week. During the experiment, all pairs (single-sex and breeding;

see below) were chosen randomly and housed in cages (61 X 46 X 41 cm) provided with an external nest box (11.5 x 11.5 x 11.5 cm). For the single-sex part of the experiment, access to the box was blocked by closing the entrance with a piece of cardboard. During the breeding experiment, nest boxes were checked daily between 10h00 and 12h00, and all new eggs were weighed (0.001 g) and numbered. A clutch was considered complete after two consecutive days with no new eggs. All experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit (692B-94), following the guidelines of the Canadian Committee on Animal Care.

Study groups and protocol

The experimental protocol was as follows. All birds used in the experiment were first paired as single sex non-breeding female pairs (NB). Food consumption and locomotor activity (see below) were measured on day 5, 6 and 7 of the single-sex period, and DEE was measured from day 6 to day 7 using the doubly labeled water (DLW) technique (see below). On day 8, all birds were rearranged to form breeding pairs and they were given access to the nest boxes. Locomotor activity was monitored starting the following day until clutch completion. Food intake data was recorded the first two days after pairing (pre-laying: PL) and again during laying (LY) beginning the day prior to laying of the first egg and during the four following days. All females had their DEE measured at the one egg stage (i.e., on the day they laid their first egg; LY-1) with estimates including a complete ovulation and laying cycle (second egg). The birds were then left to incubate their eggs and raise their chicks. Food intake and locomotor activity were monitored between the 16th and 18th days of the chick-rearing period (CK; approximately 4 days

before fledging). Chick-rearing female DEE was measured from day 17 to day 18. Although zebra finch chicks usually fledge at 21 days, some chicks had already fledged by day 16. Chick-rearing locomotor activity data was discarded for these pairs (n = 2) because of the bias introduced by chicks hopping on the perches.

Our investigation of DEE adjustments associated with egg production in zebra finches used a repeated measures approach to compare DEE values of 24 females measured as non-breeders, at the one egg stage and during chick-rearing (in females that successfully raised chicks, sample sizes NB = 24; LY-1 = 24; CK = 8). All measurements were done within a single breeding attempt. We monitored locomotor activity by 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 validation of the technique using direct observation via video tape recording clearly showed that activity does not differ with sex ($P > 0.5$ reported by Williams and Ternan 1999). Sixty-four female zebra finches were used in the complete experiment, but we could only monitor locomotor activity in 14 cages at a time. Therefore, the complete data set was gathered over 5 identical experimental runs. For each breeding session, there was always a certain proportion of the females that behaved abnormally (laying eggs on the cage floor, laying with significant time gaps between eggs, etc.), therefore all data from these birds were discarded. Ten females paired for breeding did not lay at all. Data from these pairs were used as a separate non-breeding pair control group (see Williams and Ternan 1999).

To obtain a gross estimate of energy input, we measured food intake by giving the birds 25 g/day of seeds in an open 946 ml Ziploc™ food container placed on the cage

floor. This avoided any spillage and allowed us to measure food intake by weighing remaining seeds left in the container after 24 hours. Williams and Ternan (1999) showed that, on average, females eat slightly more food (4.5%) than males and that this sex effect is significant only the two days preceding the first egg laid ($P = 0.016$ and $P = 0.052$ respectively in their Table 1). Therefore, measuring food intake per pair is a good indicator of female food intake as the proportion of seeds eaten by both sexes remains virtually unchanged throughout the experimental protocol. During the experiment, the birds received 6 g of egg food supplement (20.3% protein, 6.6% lipid) daily, which was always completely consumed by the following day.

Measurement of daily energy expenditure (DEE)

We measured DEE using the doubly labeled water technique (Lifson and McClintock 1966; Speakman 1997). The injection solution was made of 4.2182 g $H_2{}^{18}O$ (96.1 atom%), 3.9372 g $H_2{}^{18}O$ (95 atom%) and 3.8825 g ^2H_2O (99.8 atom%). Using a high precision gas tight syringe (Hamilton™), all individuals received 76 µl of labeled water intramuscularly in the right pectoral muscle. To determine the exact amount of water injected, the syringe was weighed (± 0.0001 g) before and after each injection. The birds were then returned to their cages for an hour to allow for isotope equilibration with the body water pool (Williams and Nagy 1984). After the equilibration period, the birds were weighed (± 0.1 g) and blood sampled by puncturing the brachial vein of one wing. About 100 µl of blood was collected per individual into micro-hematocrit tubes that were immediately flame sealed after releasing the birds back in their cages. Twenty-four hours later, a second blood sample was taken from the other wing following the same

procedure. The birds were then re-weighed and released into their cages. Background isotope enrichments can vary significantly over time, and this may result in significant errors in CO_2 production measurements (reviewed in Williams and Vézina 2001). Because background levels are mainly affected by the enrichment of the input sources: water, food, and atmospheric oxygen (Speakman and Racey 1987; Tatner 1988, 1990; Thomas et al. 1994), it is unlikely that our birds would exhibit marked background enrichment variation in controlled conditions, as the only variable input source was drinking tap water. However, we were concerned about changes in body composition between breeding stages and a possible time effect (Williams and Vézina 2001). We therefore took blood samples for background measurements at all breeding stages and all experimental runs (total background sample size NB = 10, LY-1 = 5, CK = 3). All samples were stored at 4°C until distillation and analysis. Samples of blood were vacuum distilled into glass Pasteur pipettes (Nagy 1983) and the water obtained used for isotope-ratio mass spectrometric analysis of ^2H and ^{18}O. The ^2H analysis was performed on hydrogen gas, produced by on-line chromium reduction of water (Morrison et al. 2001). The H_2 gas was carried in the helium stream through the GC column to an open split sampling capillary and into the source of the isotope ratio mass spectrometer (IRMS). We measured the ^2H : ^1H ratios with a single-inlet IRMS (IsoPrime, Micromass UK Ltd) provided with an electrostatic energy filter that separates the helium tail from the ^1H:^2H peak. For analysis of ^{18}O enrichment in blood samples, water distilled from blood was equilibrated with CO_2 gas using the small sample equilibration technique (Speakman et al. 1990). For analysis of ^{18}O : ^{16}O ratios, equilibrated water samples were admitted to an ISOCHROM μGAS system (Micromass UK Ltd), which uses a gas chromatograph

column to separate nitrogen and CO_2 in a stream of helium gas before analysis by IRMS. The total body water estimates were based on the plateau technique (Speakman 1997) and DEE was calculated using Speakman (1997) equation, taking evaporative water loss into account. All sample analyses and calculations were performed blind of the experimental manipulations.

Measurement of resting metabolic rate (RMR)

Changes in RMR associated with egg production in the zebra finch have been extensively described in Vézina and Williams (in prep). In the present study, we retained only the RMR data, measured at the same reproductive stages, from birds for which we had DEE measurements. Basal metabolic rate is the energy consumed by a resting post-absorptive animal during the inactive phase of the circadian cycle at a temperature within the thermoneutral range for the animal (IUPS Thermal Commission 2001). These are the conditions we attempted to achieve during our respirometry measurements. However, because laying birds are producing eggs, they have to be considered in an "active physiological state" which induces elevated levels of energy consumption (Vézina and Williams 2002; Vézina and Williams in prep). In some cases the birds may not have been completely post-absorptive at the end of RMR measurements (captive zebra finches fill their crop before going to roost; Meijer et al. 1996). We therefore consider the term *resting* metabolic rate more appropriate in the present study. Our RMR measurement protocol has been described in Vézina and Williams (in prep). In brief, RMR measurements were completed using a flow-through respirometry system (Sable Systems International). Birds were taken from their cages within 10-15 minutes after the lights

were turned off, weighed (± 0.1 g), and placed randomly in one of four metabolic chambers (1.5L) for approximately one hour prior to the beginning of measurements. All chambers continuously received approximately 500ml/min of dry CO_2-free air (using Drierite™ and Ascarite™ as scrubbers) and were kept in the dark at 35°C, which is within the thermoneutral zone for this species (lower critical temperature = 33°C; Meijer et al. 1996). Measurements were started at 2300 hours. Metabolic chambers were connected to a divided air-line with a valve multiplexer which allowed us to sample air coming from either ambient baseline air (scrubbed of 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 water scrubbed before CO2 analyzer and water and CO_2 scrubbed before O_2 analyzer). Our measurement sequence was as follows: we started with baseline air, then first and second chamber, then baseline air again followed by third and fourth chamber, and finished with baseline air. This sequence was repeated 3 times overnight giving 99 minutes of recording per chamber spanning 8 hours. After RMR measurement, the birds were re-weighed and released back into their cage (approximately 30 minutes to an hour before lights were turned on). To calculate RMR, the average of the first and second masses was used and VO_2 was calculated 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. This lowest value for RMR was always found during the last 5 hours of the night. Preliminary analysis showed that measuring RMR using this protocol did not generate a time effect (Hayes et al. 1992, ANOVA

testing for chamber position in the measurement sequence on mass-corrected RMR: $F_{3,53} = 0.7$ $P = 0.5$).

Our DEE data is presented in kJ per day. Therefore, in order to obtain RMR values using comparable units we converted VO_2 (ml O_2/h) to kJ per day. Average night-time respiratory quotient (RQ) was 0.82 and showed a large degree of variability between individuals (maximal range 0.69 to 0.99 depending on when during the night the lowest VO_2 value was found for an individual), but no differences between breeding stages (repeated measures ANOVA $F_{2,28} = 0.4$, $P = 0.7$; NB = 0.81 ± 0.01, LY-1 = 0.83 ± 0.01, CK = 0.84 ±0.03). Personal observations revealed that day-time RQ in zebra finch had a similar value of 0.85 (range 0.73 to 1.01 n = 14). We cannot consider that individual RQ will stay constant within a 24 h hour period because RQ values are not stable through time (Powers 1991; Walsberg and Wolf 1995). Therefore, we used an energy equivalent of 20.38 kJ/L O_2 considering a mean RQ value of 0.84 (Weir 1949). Gessaman and Nagy (1988) showed that using a RQ of 0.8 to convert VO_2 values into kJ results in less than ± 3% error regardless of the actual protein, fat or carbohydrate mixture being catabolized. Therefore we are confident that our energy conversion is accurate.

Statistical analysis

All data were tested to ensure normality (Shapiro-Wilk test; Zar, 1996). Metabolic rate is allometrically related to body mass (Schmidt-Nielsen 1984) but using $\log_{10}$ transformed values for the analysis did not change any of the statistical results compared to untransformed data. Therefore, we present the results of the analyses using untransformed values (body mass in grams and RMR and DEE in kJ/day). To compare

within-individual changes in DEE and RMR from the non-breeding to one-egg and chick rearing stages, we used repeated measures' ANOVA and ANCOVA using body mass as a covariate when appropriate. Post-hoc comparisons between groups where performed using multiple contrasts and corrected with the Bonferroni procedure to avoid type I errors (Rice 1989). Relationships between continuous variables were investigated using correlations and regression analyses. Data are reported as means ± S.E.

RESULTS

Locomotor activity

Locomotor activity (number of perch hops per day) was independent of female body mass at all breeding stages ($P \geq 0.08$ in all cases). Mean activity was highest at the non-breeding stage compared to all other stages ($F_{3,48} = 10.5$ $P < 0.0001$; Figure 1a), decreasing by 35.3, 56.5, and 54.5% when comparing NB to PL, LY and CK values, respectively (independent contrast $P < 0.001$ in all cases). Mean locomotor activity did not differ significantly between PL, LY and CK stages (Figure 1a) and, during laying, was independent of mean egg mass, clutch mass or clutch size ($P > 0.5$ in all case). Absolute locomotor activity levels varied considerably between breeding pairs, so we standardized these data by calculating the deviation (%) in daily activity from mean activity for the whole laying period for each individual pair (following Williams and Ternan 1999). Locomotor activity in LY pairs decreased rapidly from 8 days before the onset of laying to 4 days before the first egg is laid (Figure 2a), i.e. just before the beginning of rapid yolk development (Haywood 1993). Thereafter the activity level remained low and constant until clutch completion ($F_{15,203} = 6.0$, $P < 0.0001$; independent

contrast day –8 to day –4 $P < 0.0001$; day –4 to day 8 $P = 0.2$). This pattern was not apparent in the male:female pairs that did not subsequently lay eggs ($F_{15,114} = 1.2$, $P = 0.3$; Figure 2b).

Food intake

Food intake was not related to pair or female mass at any of the breeding stages ($P \geq 0.1$ in all cases). Repeated-measures ANOVA indicated that mean food intake of a given female's pair did not change significantly between non-breeding, pre-laying and laying stage (average intake; NB = 5.0 ± 0.3 g/day, PL = 4.8 ± 0.2 g/day, LY = 5.5 ± 0.2 g/day, independent contrast $P \geq 0.1$ in all cases; Figure 1b). During chick-rearing, the amount of food removed from the seed container was much higher than at the other stages (10.4 ± 0.7 g/day; independent contrast $P < 0.0001$ when compared to NB, PL and LY; overall model $F_{3,52} = 52.3$ $P < 0.0001$; Figure 1b). However this value does not represent a pair's actual food intake because part of the food is brought to and consumed by the chicks. None of our reproductive effort measurements (clutch size, clutch mass, mean egg mass) were related to mean (four days) food intake ($P \geq 0.4$ in all cases) in LY pairs or to food intake the day prior to laying of the first egg ($P \geq 0.2$ in all cases).

Daily energy expenditure in relation to locomotor activity and food intake

Daily energy expenditure was not related to body mass for any breeding stage (P ≥ 0.3 in all cases). At the non-breeding stage, DEE was independent of locomotor activity recorded between the two blood samples ($P = 0.5$) and mean food intake over the last 3 days (5, 6 and 7). However, food intake at day 6 (the day of DEE measurement) was

positively correlated to daily energy expenditure (r = 0.49 n = 24 $P < 0.05$; Figure 3). In contrast, LY-1 DEE was positively correlated with locomotor activity recorded between the blood samples (r = 0.48, n = 23, $P < 0.05$; Figure 4a) indicating that during egg production, more active birds spent more energy on a daily basis. Furthermore, average food intake measured either over 4 days or the day of DEE measurement were both significantly correlated with LY-1 DEE (mean food intake: r = 0.51 n = 24 $P < 0.05$, day of LY-1: r = 0.52 n = 24 $P < 0.01$; Figure 4b).

Individual variation in RMR and DEE

Mean resting metabolic rate increased by 3.3 kJ/day, or 16.8% from NB to LY-1 (independent contrast $P < 0.0001$) and remained high in CK individuals (independent contrast between LY-1 and CK $P = 0.5$; repeated-measures ANCOVA with body mass as a covariate; $F_{2,40} = 19.8$, $P < 0.0001$, no significant interaction; least-square mean NB RMR = 19.9 ± 0.4 kJ/day, LY-1 RMR = 23.2 ± 0.4 kJ/day, CK RMR = 22.6 ± 0.8 kJ/day). However, there was marked inter-individual variation in RMR. Indeed, the increase in RMR from NB to LY-1 stage ranged from 4.0% to 41.3% and was independent of both NB and LY-1 body masses ($P \geq 0.3$ in both cases). Considering the change in RMR in relation to activity, only 3 out of 23 pairs showed an *increase* in activity from NB to LY-1. One of these pairs represented a clear outlier with a 97% increase in activity (5.9 times the standard deviation of the mean difference in activity). Excluding this pair, there was a significant negative relationship between the relative change in activity (% difference compared to NB level) and RMR at the one-egg stage (r = - 0.57, n = 22, P < 0.01, Figure 5). Restricting this analysis further to only those pairs

that decreased locomotor activity from NB to LY-1 improved the correlation (r = - 0.70, n = 20, P < 0.001). Thus, individuals that had the highest LY-1 RMR where also the ones showing the largest reduction in locomotor activity from NB to LY-1.

As for RMR, there was marked individual variation in the change in DEE comparing NB and LY-1 values with DEE varying from –33.3% to 46.4%. Change in DEE was independent of body mass (both NB and LY-1, $P \geq 0.1$) and was not related to relative change in locomotor activity (P = 0.7). However, clutch size and mass were both positively correlated to LY-1 DEE (clutch size: r = 0.49, n = 24, $P < 0.05$; clutch mass r = 0.46, n = 24, $P < 0.05$; Figures 6a and b), suggesting that overall energy expenditure at the one-egg stage is related to reproductive effort

Relationship between RMR and DEE

Residual RMR (correcting for body mass) and DEE were positively correlated at the NB stage (r = 0.59 n = 24 P < 0.005; Figure 7a) but not at the LY-1 stage (P = 0.7; Figure 7b). Furthermore, the change in RMR from non-breeding to first egg stage, was positively correlated with the change in DEE for the same period (r = 0.42, n = 24, $P < 0.05$; Figure 8), indicating that the level of energy compensation for the cost of producing eggs (change in DEE) is a function of the initial investment (change in RMR). As a consequence of this individual variation, mean DEE did not change significantly within females across stages (repeated-measures ANOVA; $F_{2,30} = 1.6$, P = 0.2). In other words, birds in which RMR increased relatively little showed a decrease in DEE, whereas birds with a larger elevation in RMR had an increased DEE. Excluding the chick-rearing stage from the analysis did not change this result ($F_{1,23} = 2.6$, P = 0.1).

DISCUSSION

We have documented marked inter-individual variation in RMR and DEE, as well as in the relationship between these two variables, in the context of the energetic cost of egg production. This individual variation obviously confounds the interpretation of average, population level, changes in energy expenditure because mean values mask the particular direction of changes within individuals. Indeed, in our study, although mean RMR increased from non-breeding to one-egg and chick-rearing stages, mean DEE remained constant. This was because, at the one egg stage, mean DEE was based on measurements made on individuals that had either increased or decreased their overall energy expenditure, and this variation in change in DEE cancelled out such that there was no statistical difference in the mean values. We suggest that egg-producing females managed their energy budget in order to minimize potential increases in DEE. They were able to do this by adjusting their behavior, i.e. decreasing locomotor activity, in order to compensate for the increase in RMR. In fact, the individuals showing the largest investment in terms of RMR at the one-egg stage were also the ones decreasing activity the most (down to – 86%). Individual variation in measures of energy expenditure have been reported in earlier studies (Moreno 1989, Williams 1987) but the basis pf this variation has not been investigated (see Williams and Vézina 2001). Our results clearly demonstrate that more attention need to be given to this level of variability as it may explain more precisely the actual energy adjustments that females have to make to balance their energy budget during reproduction.

demonstrated that even if they are potentially capable of sustaining higher levels of DEE by increasing food intake, egg-producing females appears to avoid this strategy. The cost of sustaining high levels of DEE in conditions of food abundance, however, remains unclear.

We found a significant positive correlation between RMR and DEE in non-breeding individuals, but this relationship was lost when the birds were actively producing eggs. A general interpretation for such a correlation is that increased sustained energy expenditure is supported by enlarged organs which results in higher maintenance costs and thus elevated BMR or RMR (Peterson et al. 1990; Hammond and Diamond 1997; Piersma and Lindström 1997; Piersma 2002; but see Ricklefs et al. 1996). Our non-breeding birds showed the highest levels of locomotor activity. However, DEE was not related to activity in this group, which does not provide strong support for this interpretation. If the relationship between RMR and DEE in non-breeding individuals is due to a common effect of the underlying physiological machinery, then its action on energy expenditure is clearly not related to locomotor activity as measured by hopping counts. Speakman et al. (2003) suggested that RMR and DEE follow independent trajectories that may or may not correlate, in response to direct ecological conditions that the organism faces. This may be the case in our laying birds. The relationship between RMR and DEE was lost in egg producing females potentially because these two variables were responding differently to different demands. Mean RMR increased in response to egg production but mean DEE remained relatively unchanged, apparently because females individually adjusted their behavior to maintain a constant energy budget. The birds that showed the largest increase in RMR were also the ones compensating the most.

Therefore, they minimized the impact of an increased RMR on DEE which resulted in a correlation between these two variables showing a slope not significantly different from zero. In other words, reallocation of energy during egg formation uncoupled the relationship between RMR and DEE.

An alternate explanation for the decrease in locomotor activity found in egg-laying females could be that these individuals reduce activity because of the risks of damage to the egg and developing embryo, and saving energy would then be an incidental effect. However, this scenario is unlikely since Schifferli (1976, cited in Houston et al. 1995) showed that, house sparrow (*Passer domesticus*) eggs are not vulnerable to damage from normal activity. Furthermore, this hypothesis would not explain the link at the inidividual level between reduction in locomotor activity and RMR at the one-egg stage.

The fact that locomotor activity was correlated with DEE only at the laying stage suggests that hopping activity in non-breeding individuals does not represent a large component of overall daily energy expenditure or, alternatively, that part of the energy consuming activity is not detectable by monitoring hopping behavior in non breeding birds. This is not simply an effect of the range in the activity data differing between stages rendering the relationship non-significant. In fact, the activity data in non-breeding individuals had a greater range than the same data at the laying stage (data not shown). When females are producing eggs, it is likely that part of their daily energy budget is channeled toward egg production while locomotor activity is limited to the strict minimum. This, in parallel with carrying the extra weight of the reproductive organs, a developing egg (average 1.4 g), and the shifts in the center of gravity due to eggs or

enlarged gonads (Lee et al. 1996), likely means that locomotor activity has a significant impact on a female's daily energy budget during egg production. Therefore, any non-essential activity may result in an energetic cost that is too high relative to the benefit it would provide. Consistent with this idea, Deerenberg (1996) showed that non-breeding zebra finches that were subjected to a high workload (high hopping rate) in order to obtain food reduced the surplus amount of hopping over the required number of hops, thereby becoming more efficient in the use of energy towards locomotor activity.

We are aware of only one other study reporting DEE measurements using the doubly labeled water technique in relation to the cost of egg production in birds. Consistent with the present data, Ward (1996) found no significant differences between DEE of wild barn swallows (*Hirundo rustica*) measured during egg-formation, incubation or chick-rearing. The author suggested that egg production costs are trivial because DEE was not correlated with egg energy content. We strongly disagree with this conclusion; the energy expended in egg formation results from several physiological processes (hormone synthesis, reproductive organ development, function and maintenance, hepatic yolk precursor production and uptake by the ovary, albumen and shell deposition in the oviduct) that should have little to do with the chemical energy content of the eggs (see Vézina and Williams 2002). In fact, our data clearly demonstrate that egg producing females compensate for the extra demand associated with egg formation by decreasing the energy expended in locomotion in function of their change in RMR. Furthermore, this energy compensation strategy appears to be closely related to the period of follicular growth as the reduction in activity occurs just before the onset of rapid yolk development (rapid yolk development begins 4 days prior to laying of the first

egg in zebra finches; Haywood 1993, see Figure 2a). Undoubtedly, egg production involves a considerable metabolic cost (Chappell et al. 1999; Nilsson and Raberg 2001; Vézina and Williams 2002; Vézina and Williams in prep.) and the fact that DEE does not increase significantly during egg production may not mean that this cost is trivial but rather that this physiological stage is as demanding as other life-history stages that are believed to be energetically costly (i.e. chick-rearing, Drent and Daan 1980). In agreement with this idea, Williams and Vézina (2001) compiled absolute values of DEE (kJ/day) in studies where measurements were obtained at more than one physiological stage within species and showed a general tendency for a constant level of DEE during all phases of reproduction and perhaps even throughout the year.

A decrease in locomotor activity in response to an energetically constraining situation has been reported many times before. Birds show decreased activity when facing thermoregulatory challenges (Cherel et al. 1988) or the cost of molting into new feathers (Austin and Fredrickson 1987; Robin et al. 1989). In zebra finches, food limitation also resulted in reduced locomotor activity (Meijer et al. 1996; Dall and Witter 1998), and egg production has now been shown to be associated with reduced activity in three independent studies (Houston et al. 1995; Williams and Ternan 1999; this study). Decreases in activity were also documented in willow flycatchers (*Empiodonax traillii*) during egg production (Ettinger and King 1980) and in mammals such as pregnant female common shrew (*Sorex araneus*; Poppitt et al. 1993) and lactating mice (*Mus musculus*; Speakman et al. 2001). This suggests that reducing locomotor activity whenever possible may be a behavioral mechanism that helps to compensate for temporarily increased demands of other physiological processes. This study shows that individual birds might

differ in their ability to utilize this reallocation strategy, and that this in turn might be related to the absolute level of reproductive investment. Our study therefore suggests a very flexible, individually-variable system of energy reallocation to meet increased energy demands.

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FIGURE LEGENDS

Figure 1: Locomotor activity (a) and food intake (b) in zebra finch pairs at all breeding stages. NB = non-breeding, PL = pre-laying, LY = laying, CK = chick-rearing. Different letters indicate significant differences.

Figure 2: Mean locomotor activity in relation to days of the laying cycle in breeding (a) and non-breeding (b) zebra finches pairs. Values are the mean of the percentage deviation from mean hops per day for each individual pair. Arrow indicate onset of rapid yolk development (RYD).

Figure 3: Relationship between daily energy expenditure in non-breeding zebra finch females and food intake the day of DEE measurement.

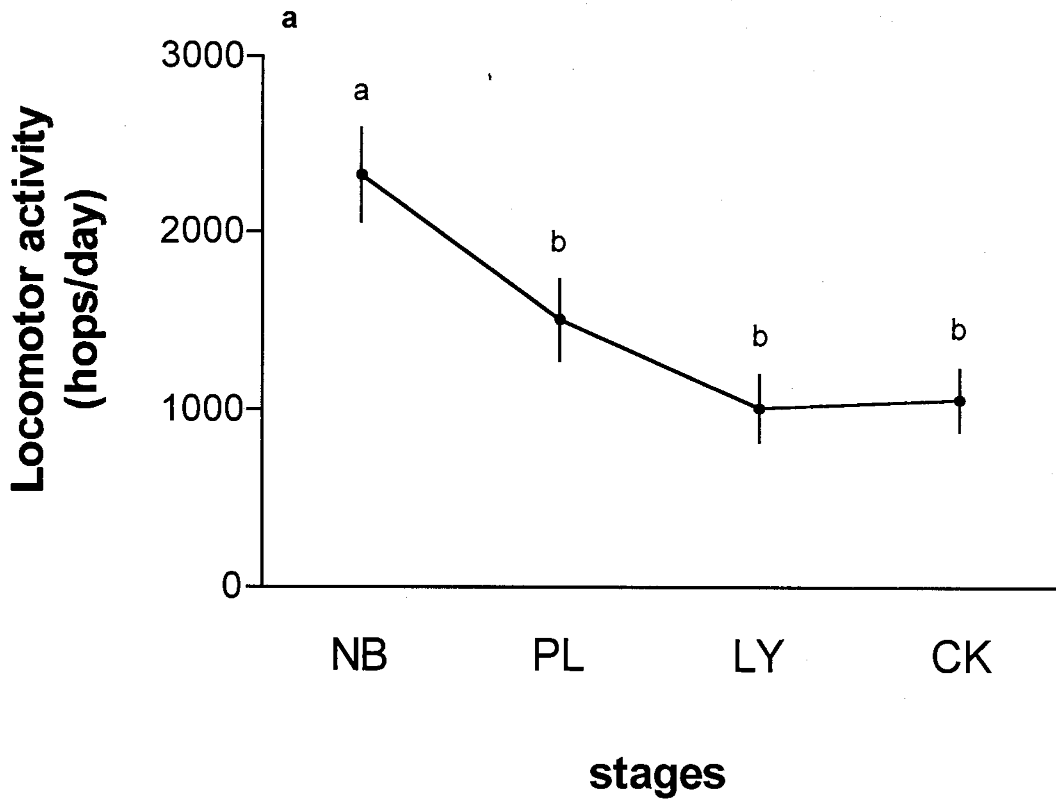
Figure 4: Relationship between daily energy expenditure in one-egg stage zebra finch females and locomotor activity for the 24 hours of DEE measurements (a) and food intake the day of laying the first egg (b).

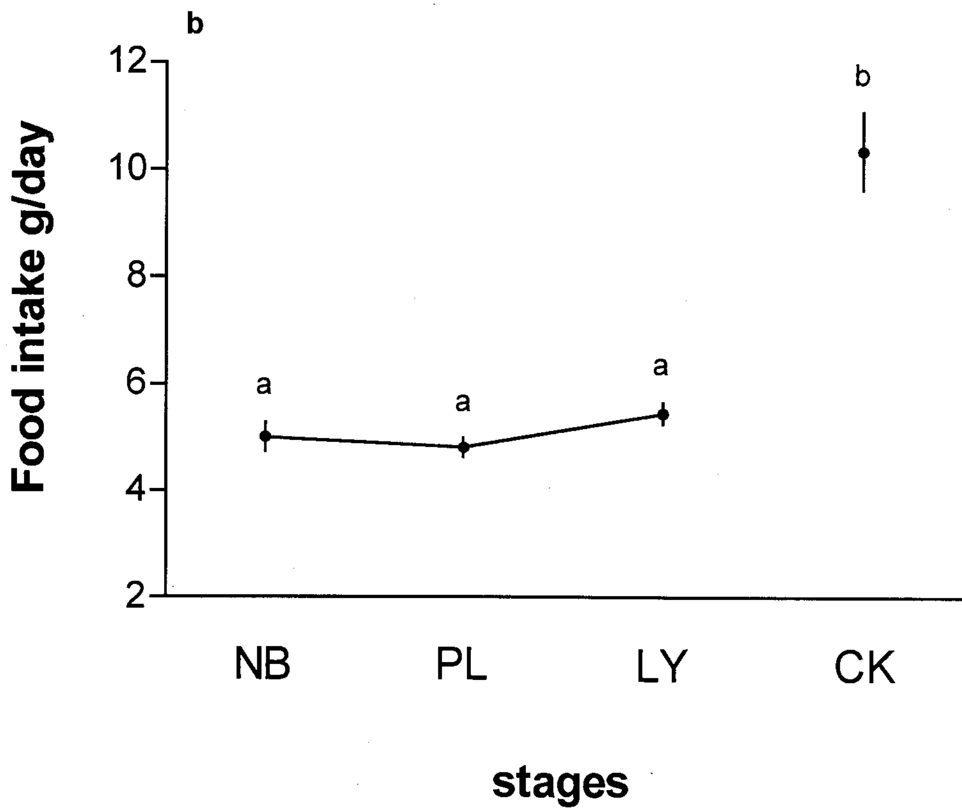
Figure 5: Relationship between the relative change in locomotor activity from non-breeding to one-egg stage and RMR at the one-egg stage. Change is presented relative to non-breeding locomotor activity. The x indicate an outlier that was excluded form the analysis (see text).

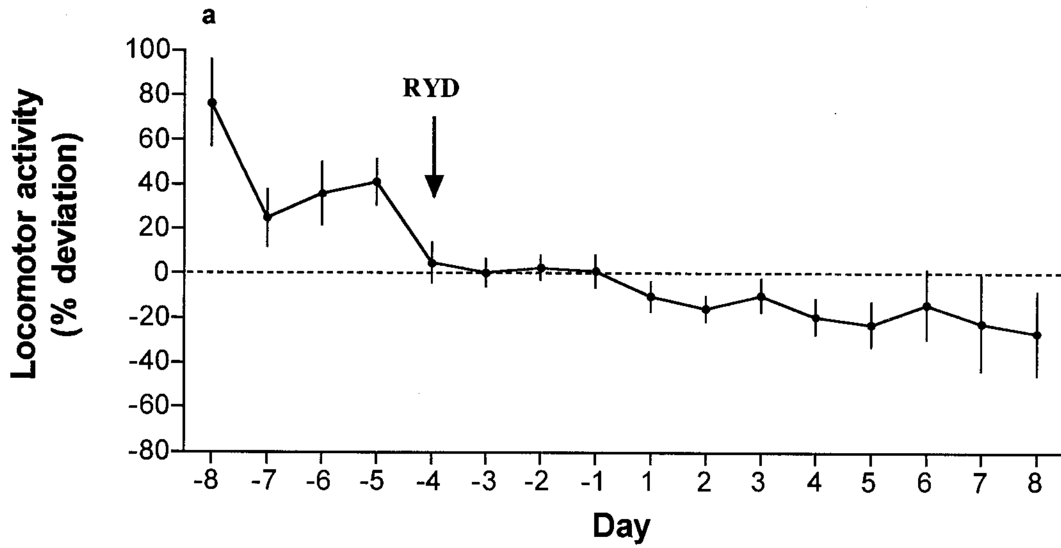
Figure 6: Relationship between daily energy expenditure in one-egg stage zebra finch females and final clutch size (a) and clutch mass (b).

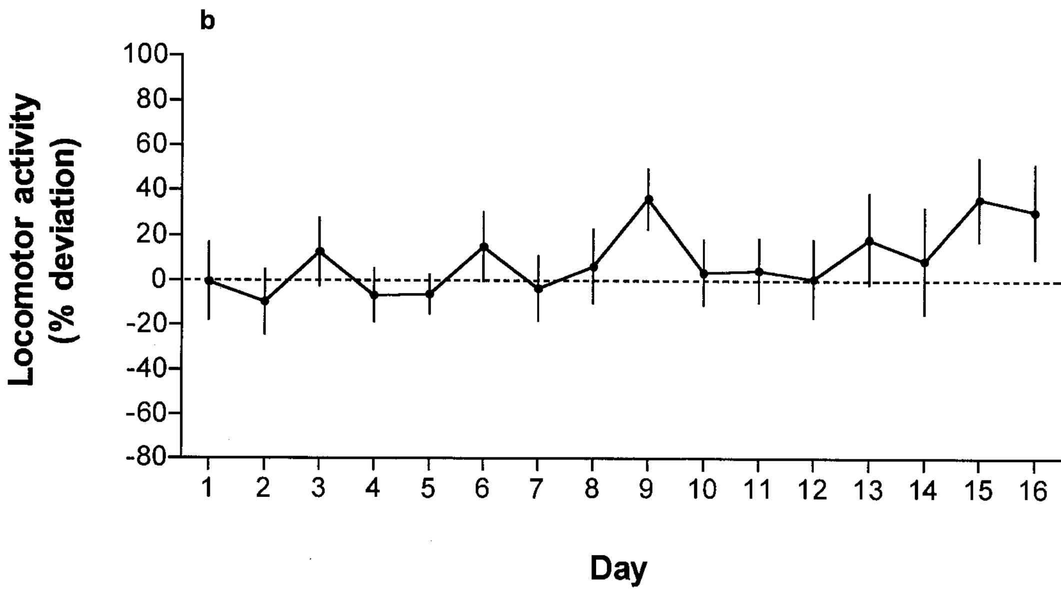
Figure 7: Relationship between daily energy expenditure and residual RMR (correcting for body mass) in female zebra finches at the non-breeding (a) and one-egg (b) stage.

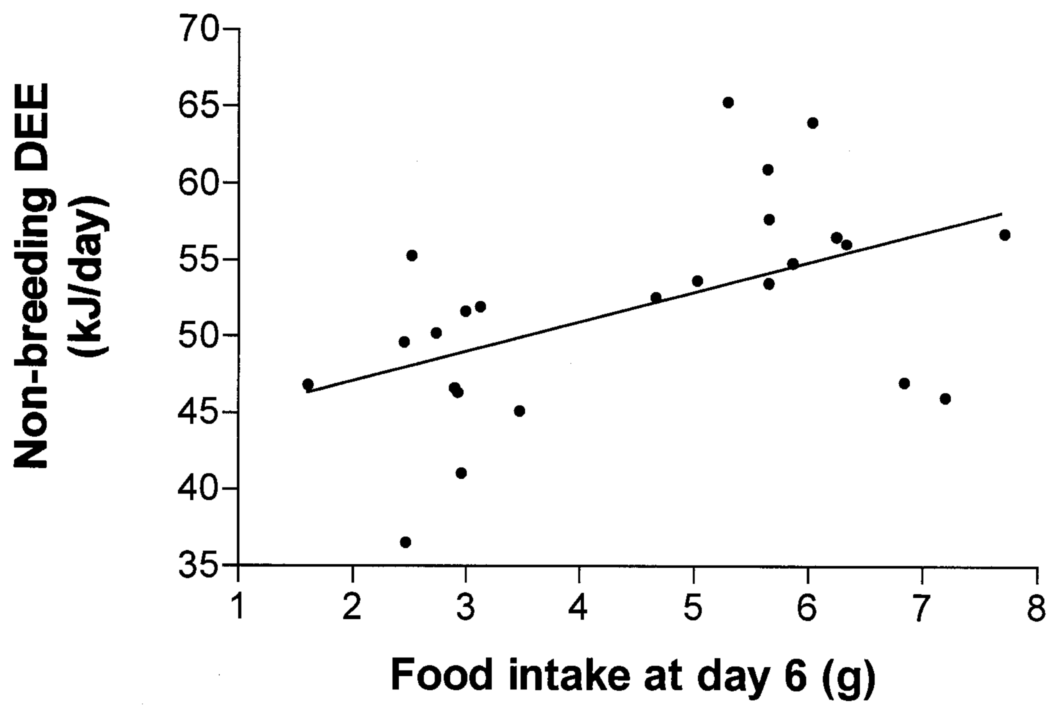
Figure 8: Relationship between change in resting metabolic rate and change in daily energy expenditure between non-breeding and one-egg stage in female zebra finches.

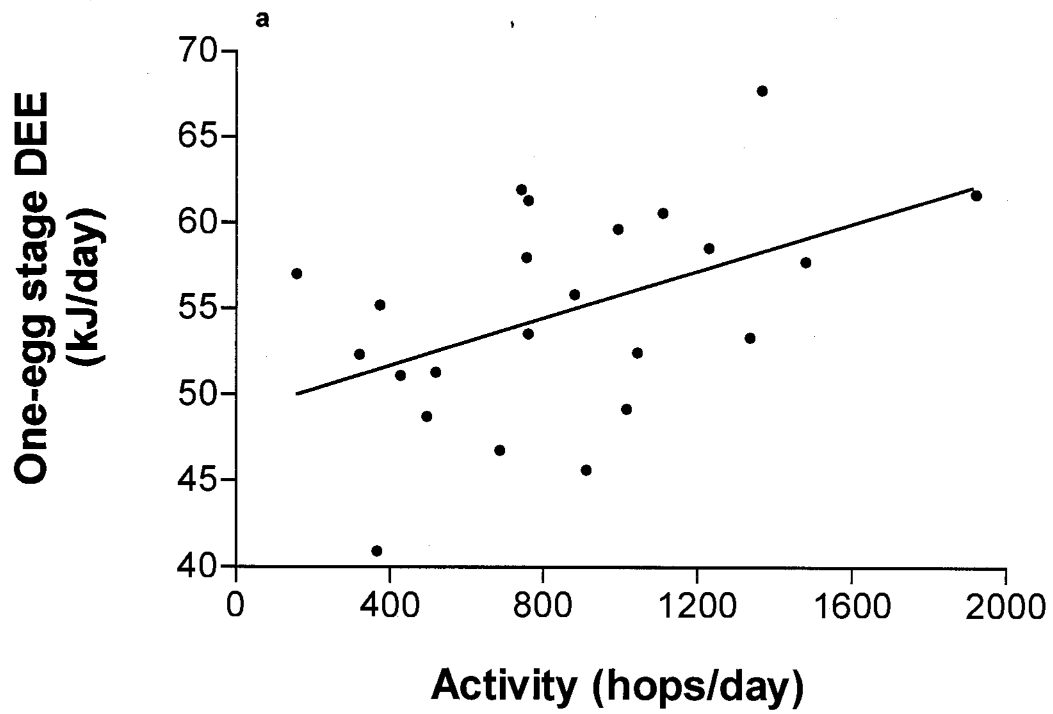

a
Locomotor activity
(hops/day)
3000
2000
1000
0
a
b
b
b
NB
PL
LY
CK
stages

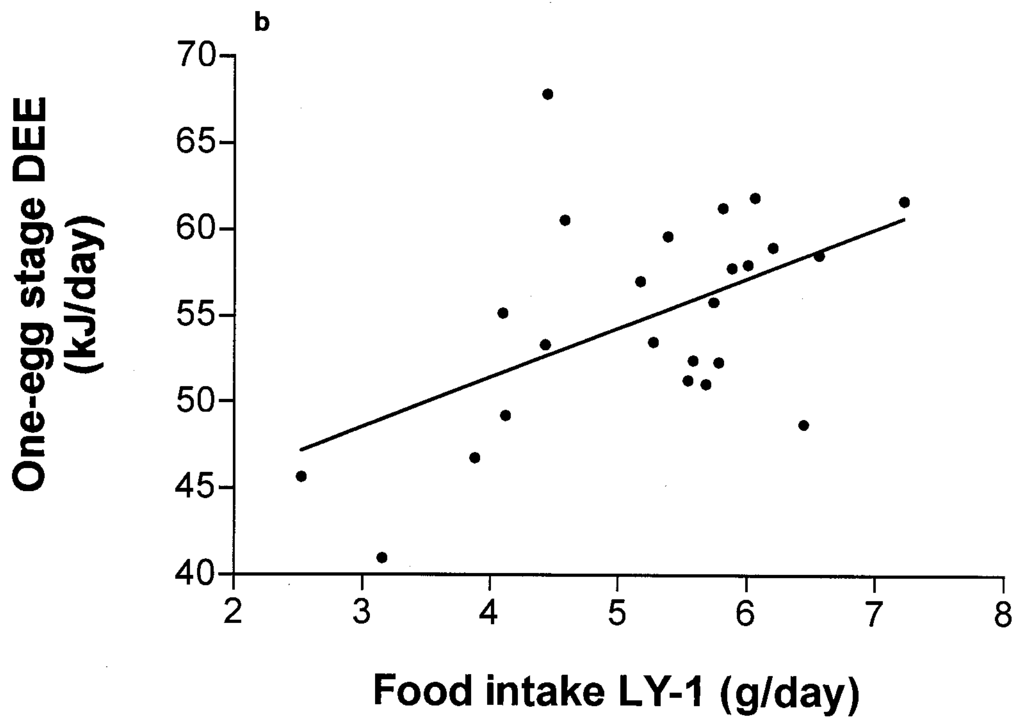

b
Food intake g/day
12
10
8
6
4
2
a
a
a
b
NB
PL
LY
CK
stages

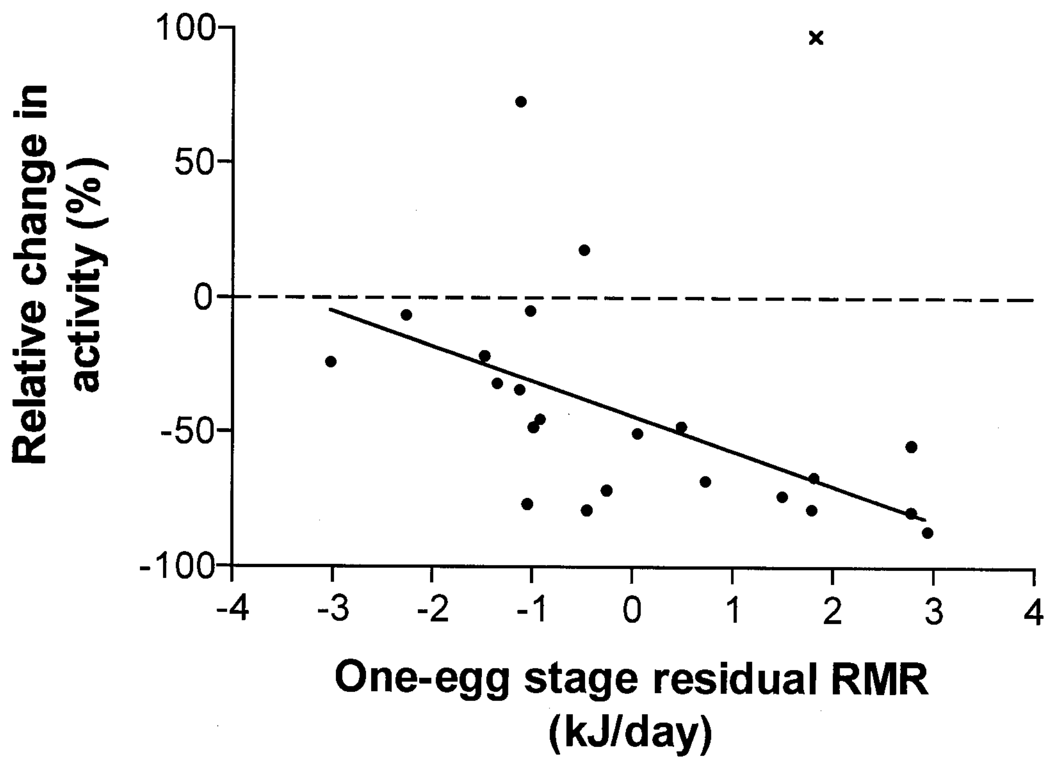

a
RYD
Locomotor activity (% deviation)
100
80
60
40
20
0
-20
-40
-60
-80
-8 -7 -6 -5 -4 -3 -2 -1 1 2 3 4 5 6 7 8
Day

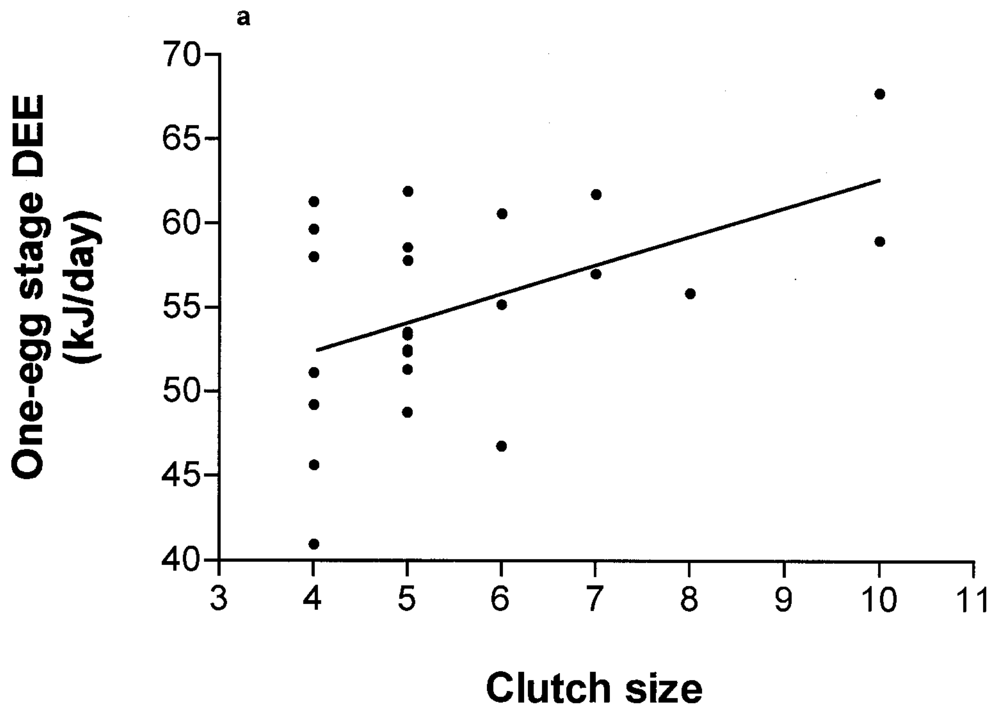

b
Locomotor activity (% deviation)
100
80
60
40
20
0
-20
-40
-60
-80
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Day

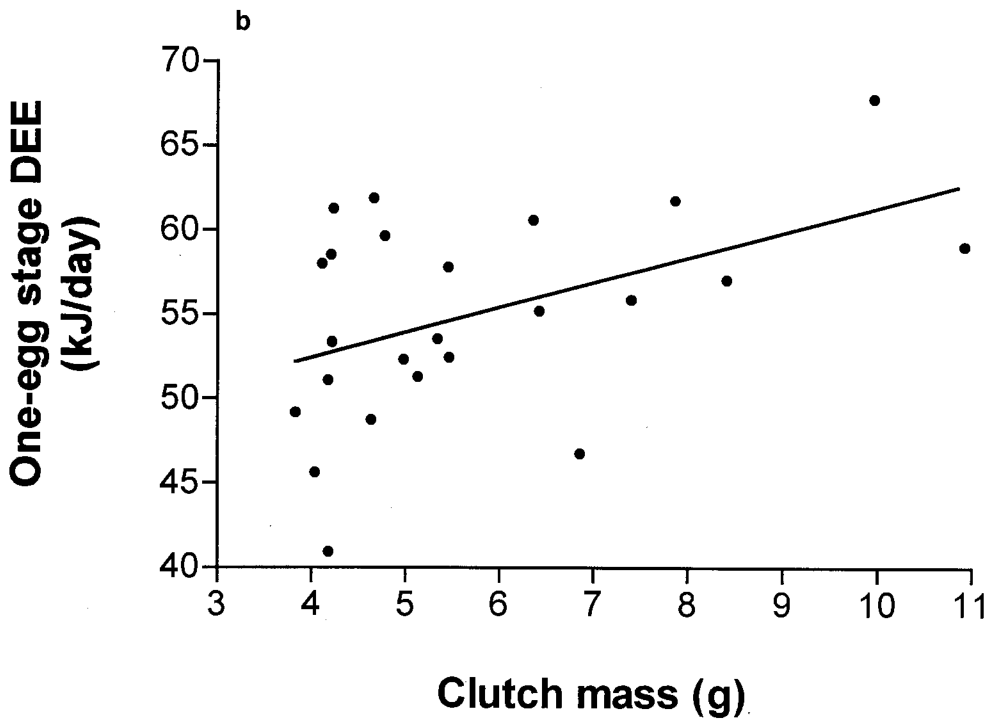

Non-breeding DEE (kJ/day)
70
65
60
55
50
45
40
35
1
2
3
4
5
6
7
8
Food intake at day 6 (g)

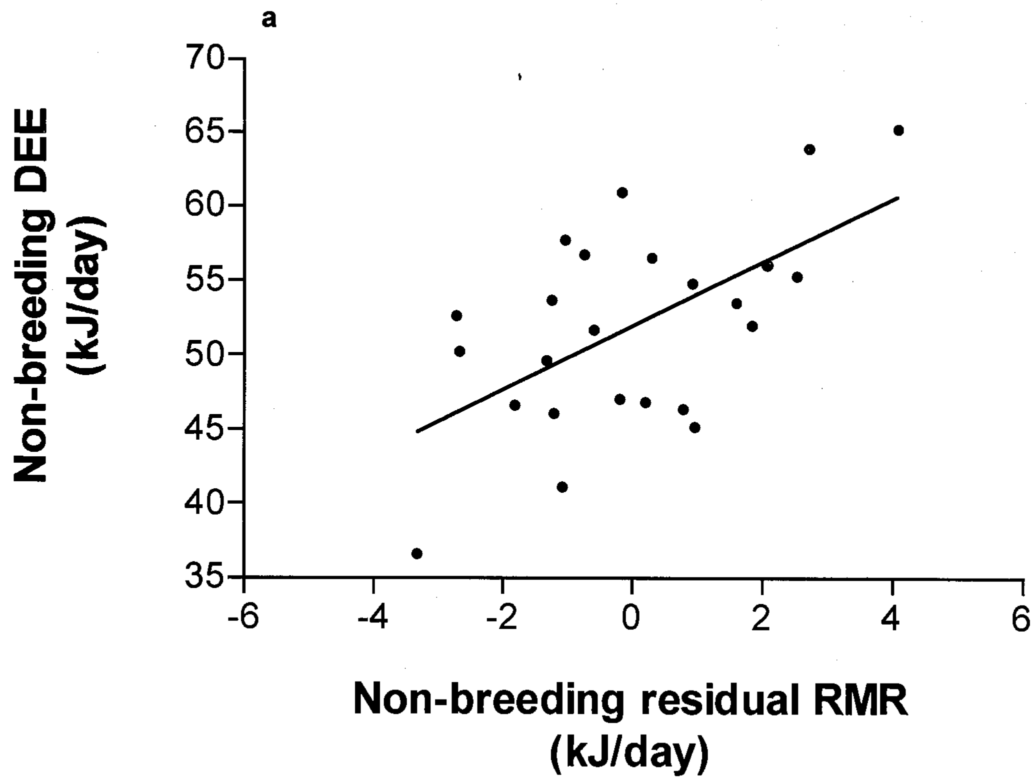

a
One-egg stage DEE (kJ/day)
70
65
60
55
50
45
40
0
400
800
1200
1600
2000
Activity (hops/day)

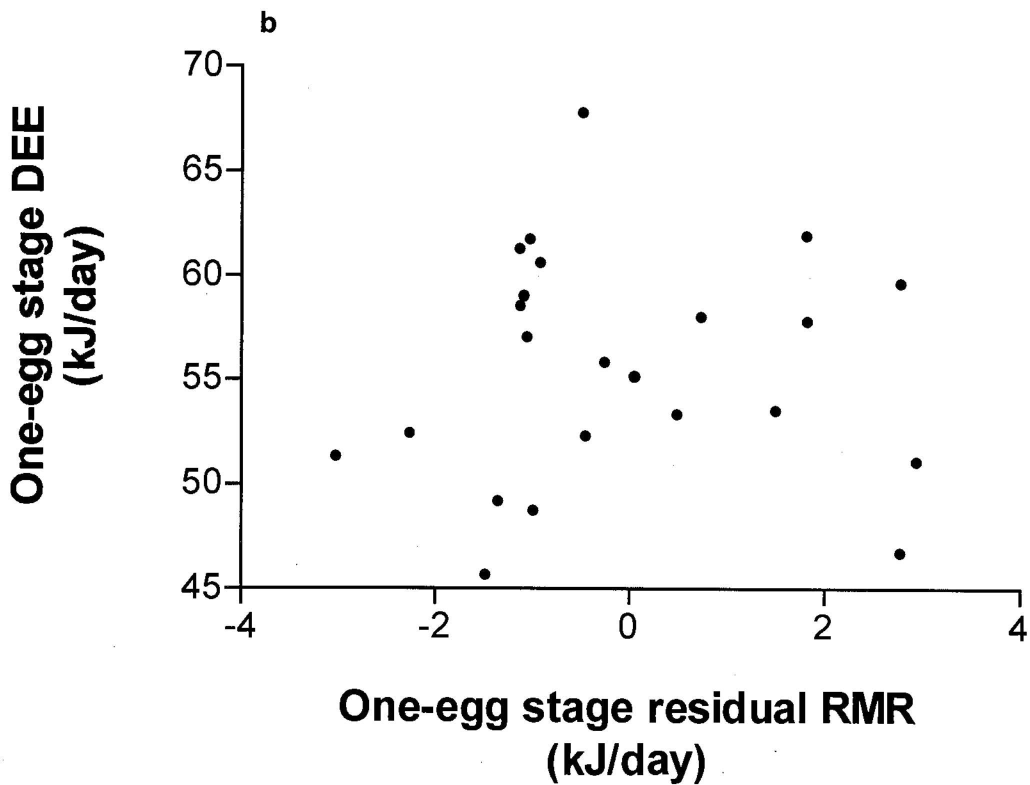

b
One-egg stage DEE (kJ/day)
70
65
60
55
50
45
40
2
3
4
5
6
7
8
Food intake LY-1 (g/day)

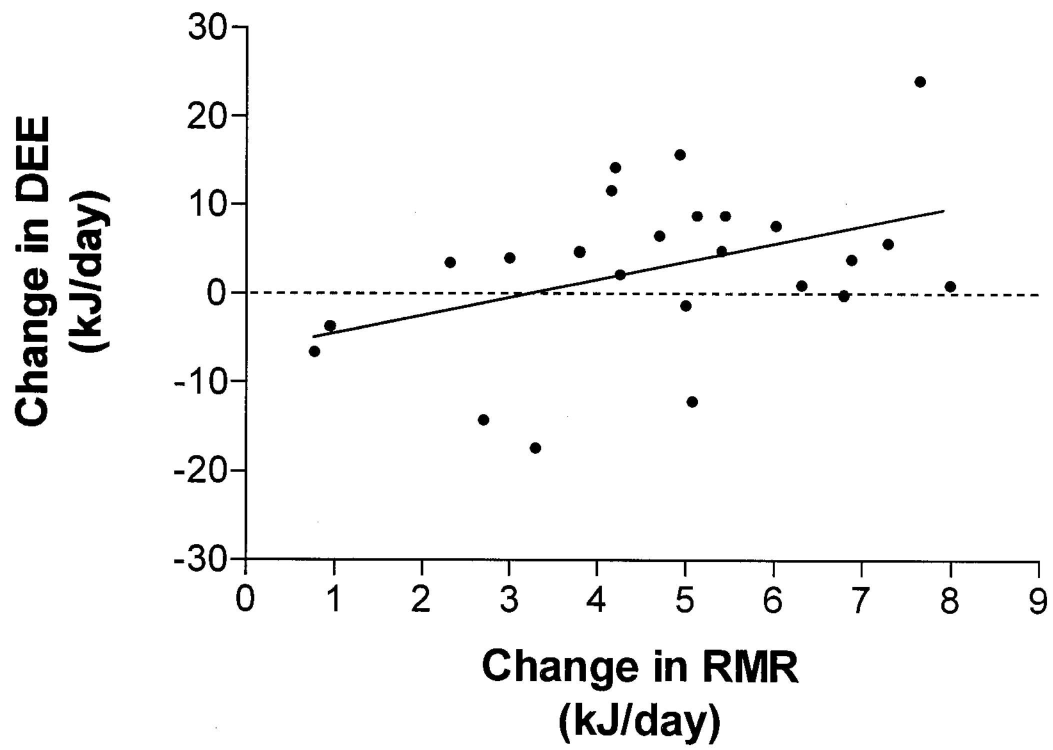

100
50
0
-50
-100
Relative change in activity (%)
-4
-3
-2
-1
0
1
2
3
4
One-egg stage residual RMR (kJ/day)


a
One-egg stage DEE (kJ/day)
70
65
60
55
50
45
40
3
4
5
6
7
8
9
10
11
Clutch size


b
One-egg stage DEE (kJ/day)
70
65
60
55
50
45
40
3
4
5
6
7
8
9
10
11
Clutch mass (g)


a
Non-breeding DEE
(kJ/day)
70
65
60
55
50
45
40
35
-6
-4
-2
0
2
4
6
Non-breeding residual RMR
(kJ/day)


b
One-egg stage DEE
(kJ/day)
70
65
60
55
50
45
-4
-2
0
2
4
One-egg stage residual RMR
(kJ/day)


30
20
10
0
-10
-20
-30
0
1
2
3
4
5
6
7
8
9
Change in DEE
(kJ/day)
Change in RMR
(kJ/day)

CHAPTER EIGHT: GENERAL DISCUSSION

The findings highlighted in this thesis require a re-evaluation of our understanding of some important concepts underlying the variation in resting metabolic rate, DEE and the energetic cost of reproduction. In this final chapter, the results are discussed in terms of their contribution to three main areas: 1) the validity of the notion of "BMR variation being a reflection of organ mass changes" (Kersten and Piersma 1987; Daan et al. 1990) is questioned in light of the high inter-annual and inter-stage variation in both RMR and body composition as well as mass-independent changes in organ aerobic potential; 2) the energetic cost of egg production is addressed in terms of the physiological mechanisms underlying changes in metabolic rates, and their effects on overall energy budget, and 3) the potential consequences of increased daily energy expenditure in breeding birds are discussed in terms of their effects on energy management, physiological trade-offs, and the idea of additive costs of reproduction versus these costs being met by energy reallocation.

BODY COMPOSITION IN RELATION TO BMR

To understand metabolic changes associated with egg production, it is important to first consider the relationships between body composition and metabolism. In the six papers that form the body of this thesis, the term RMR is consistently used. Resting metabolic

rate was defined in each paper as being the lowest energy consumption measured (as VO_2 or kJ) in a resting, post-absorptive animal during the inactive phase of its circadian cycle. This is the definition generally given for BMR (Blem 2000; IUPS Thermal Commission 2001) and this protocol was therefore used in part for the purpose of comparability with other studies. However, the term RMR was used throughout instead of BMR because egg-producing individuals cannot be considered as physiologically similar to non-breeding and chick-rearing individuals since egg-production induces elevated levels of resting energy consumption. In fact, all three breeding-stages may be physiologically different as suggested by the difference in body composition reported in Chapter three. This highlights a very important point: basal or resting metabolic rate are highly flexible traits (Piersma 2002).

It has been known for years that BMR varies daily and yearly (Aschoff and Pohl 1970). Similarly, Vézina and Williams (2002: Chapter two) demonstrated that RMR varies up to 37% for a given, assumed stable, physiological stage between years, even when measured at the same seasonal period. This yearly variation within stage is an unexpected result that has large implications for comparative studies. High inter-year variation in RMR or BMR within and among physiological stages can clearly bias interpretations if year effects are not integrated into the analysis. What caused the between-year RMR variations reported in Chapter two? One common idea is the variation in body composition. Basal metabolic rate is generally seen as reflecting the resting energy consumption of the "metabolic organ machinery" in charge of supporting a certain level of sustained metabolic expenditure (Daan et al. 1990; Peterson et al. 1990; Hammond and Diamond 1997; Piersma 2002). This concept is based in part on the idea

that some organs are assumed to have high levels of mass-specific energy consumption (Krebs 1950; Schmidt-Nielsen 1984) and that variation in the mass of these organs, in response to high daily energy demand, will have a disproportionate effect on the overall resting energy expenditure, thus influencing BMR (Kertsen and Piersma 1987; Daan et al. 1990; Hammon and Diamond 1997; Piersma and Lindstrom 1997). Indeed, several studies showed significant relationships between organ mass and BMR in both birds and mammals at various physiological states (Daan et al. 1990; Konarzewski and Diamond 1995; Burness et al. 1998; Bech and Ostnes 1999; Chappell et al. 1999; Hammond et al. 2000; Piersma 2002; Vézina and Williams 2003). In most cases, functional links exist between the organs related to BMR and the animal's physiological condition (for example: oviduct in laying females; Chapter three). In other cases however, the link is not obvious (liver and gizzard in chick-rearing starlings; Chapter three). The hypothesis also assumes that increased sustained DEE results in increased BMR in response to the maintenance need, resulting in a correlation between these variables (Daan et al. 1990; Peterson et al. 1990; Hammond and Diamond 1997; Piersma 2002). However, although this may be true in mammals, the evidence for such a correlation in birds is not yet convincing (Ricklefs et al. 1996). In some cases, DEE and BMR can be uncoupled, e.g. zebra finches forced to work harder (increased locomotor activity) for a food reward showed increased intra-individual DEE, but a *reduction* in BMR, potentially in order to compensate for the increased demand (Deerenberg et al. 1998). It is possible, as suggested by Speakman et al. (2003) and the data in Chapter seven, that these two variables follow different trajectories in response to different demands. Nevertheless, some studies now begin to assume that changes in BMR are simply reflecting changes in

body composition without reporting organ mass variation data (Nilsson 2002; Wiersma 2003). If this tendency becomes the rule, there will be a very significant problem as this approach may be biased, especially if changes in BMR are assumed to reflect changes in specific organs or groups of organs. Organ mass shows rapid and reversible changes (Piersma and Lindstrom 1997), but organs also vary significantly in their aerobic potential which may occur relatively independently from organ mass variations (Chapter five). This means that large variations in the mass of some "high cost" organs (i.e. kidney in Chapter five) may occur without significant effects on BMR. Indeed, in a recent study, Piersma et al. (2004) found that red knots (*Calidris canutus islandica*), maintained on a trout chow diet (soft texture) for several years and then shifted to a shellfish diet (hard texture), showed increased lean body mass (+12%) and gizzard mass (+213%), yet a reduction in BMR (-8%), suggesting that the increase in metabolically active (lean) tissue mass was accompanied by a decrease in tissue metabolic intensity. Animals are complex, integrated systems and when facing increased daily energy demand, there may be a wide array of physiological adjustments available to respond to the need before having to change the overall "maintenance" energy expenditure (BMR). Furthermore, the value of organ mass for predicting BMR is typically low and several studies show differences in terms of which organs correlate to BMR (see Vézina and Williams 2003: Chapter three). This may not be surprising given the confounding effect of yearly variations and potential changes in organ metabolic intensity. The effect of certain organs in BMR – organ mass relationships may be important at certain times (i.e. heart and kidney in chick-rearing birds; Daan et al. 1990), however the contribution of these organs may be minimal at other times or physiological stages (Chapter three; Chapter five). The research presented

in this thesis strongly suggests that if correlations between organ mass and BMR have to be made to interpret energy expenditure in a wild animal species, the data should encompass year effects and potentially variations in metabolic intensity. Moreover, animals forming different treatment groups should really be seen as being physiologically different and thus the data should be treated accordingly (i.e: extrapolations or application of average values to all groups should be avoided as much as possible).

It is possible, as mentioned by Vézina and Williams (2003: Chapter three), that changing environmental conditions (temperature, climate, diet, etc) between years may play a role in RMR variation through their effects on the animal's physiological state and body composition. Therefore, a promising avenue for future research in this field would be to uncouple the effects of ecological conditions on variation in organ mass and BMR and study their relationships using an experimental rather than a correlational approach. One could study animals in constant controlled conditions while altering one variable (photoperiod, temperature, diet, etc) at a time. For example, as mentioned above, it is possible to manipulate stomach size in shorebirds by changing diet (Piersma et al. 1999). This method, coupled with non-invasive organ size measurements (ultrasonography; Dietz et al. 1999), allows for the study of the interactions between diet, digestive organs and BMR (Piersma et al. 2004) independently of other confounding variables. Another approach would be to manipulate metabolism directly. For example, thyroid hormones play a key role in thermoregulatory heat generation, effect VO_2 (McNabb 2000) and vary seasonally under natural conditions (Jenni-Eiermann et al. 2002). Therefore, manipulating the circulating levels of these hormones could provide a new experimental method for future research. There is no doubt that more studies are needed to fully

understand the basis of variation in resting and/or basal levels of metabolism and their relationships with changing ecological conditions.

THE ENERGETIC COST OF EGG PRODUCTION

What is the energetic cost of egg production in birds? The absolute cost in energy units will probably never be accurately estimated. The physiological processes involved in egg formation are complex and interconnected. Therefore, measuring one part of the system will not provide accurate information on the overall demand that a female must face under natural conditions. Furthermore, because individuals can adjust to the energy demand through compensative measures, estimating the energy costs of each specific physiological process involved in egg production independently will not provide information on the energy challenges that females face when producing a clutch of eggs in the wild.

It is now clear from published work (Chappell et al. 1999; Nilsson and Raberg 2001) and from findings reported in this thesis (Vézina and Williams 2002: Chapter two; Chapter six) that the process of egg formation induces a 16-27% increase in basal or resting metabolic rate in four species of passerine birds (although some of these estimates may be debatable; Vézina and Williams 2002: Chapter two). More specifically, a 22% increase in RMR was reported in starlings and zebra finches (Vézina and Williams 2002: Chapter two; Chapter six) and inter-individual variability in non-breeding and laying RMR was found to be repeatable over 8 to 10 months in the latter species.

Part of the increase in RMR in egg-producing females appeared to be related to the growth and function of the oviduct, as mass variations in this organ explained 18% of

laying RMR in starlings and 23% of pre-laying RMR in zebra finches (Vézina and Williams 2003: Chapter three; Chapter six). This suggests that the oviduct is an energetically expensive organ to run (Vézina and Williams 2003: Chapter three; Williams and Ames 2004). Indeed, the "explosive" growth of the oviduct, documented in Chapter three, occurs over less than 3 days in starlings and is timed with the beginning of ovulation. Oviduct regression in both starlings and zebra finches begins near clutch completion, just after the last yolky follicle is ovulated, even while the organ is still processing an egg (Vézina and Williams 2003: Chapter three; Williams and Ames 2004). In fact, this size-function relationship is so tightly regulated that the organ regresses in a top-down fashion following the passage of the egg to be laid (Williams and Ames 2004). Nevertheless, oviduct function is not the only process involved in energetic cost of egg formation, since nearly 80% of the variation in laying RMR remains unexplained in egg-producing starlings. Looking at the next most obvious potential energy cost revealed that hepatic yolk precursor production had no detectable effect on RMR, assuming no energy compensation between physiological systems (Vézina et al. 2003: Chapter four). Further research is clearly needed to shed light on other processes likely to explain the remaining variation in laying RMR.

Is a 22% increase in RMR an accurate estimation of the metabolic cost of egg production? The only logical answer to this question is that 22% is in fact the *minimal* energetic cost. Although the results presented in this thesis do not provide direct evidence of energy reallocation among physiological systems via changes in non-reproductive organ mass (Vézina and Williams 2003: Chapter three) or in organ maximal oxidative

capacity (Chapter five), it is possible that energy reallocation does occur at other levels (see below).

Even if the increase in RMR represents only 5% of DEE in laying zebra finches, it is evident that egg production costs are constraining enough for females to modify their behavior in order to compensate for the demand (Chapter seven). Indeed, Chapter seven revealed that the energy compensation through reduction of locomotor activity varied individually depending on the level of reproductive investment. Females at the one-egg stage that underwent a large increase in RMR, also showed an increase in DEE, and were birds which laid large clutches. Alternatively, females that ultimately laid smaller clutches were birds that showed smaller changes in RMR and reduced their DEE presumably as a result of overcompensation. However, there was no relationship between clutch size and clutch mass and RMR at the one-egg stage (Chapter six). Therefore, how can changes in RMR and changes in DEE (from non-breeding to one-egg stages) be positively correlated, while clutch size and clutch mass are only related to DEE in laying individuals? The answer to this question is unclear. First, based on the number of yolky follicles at the one-egg stage, we should not expect a relationship between RMR and clutch size. Female zebra finches at the beginning of laying (one-egg stage) show a maximum of four yolky follicles (Haywood 1993; Christians and Williams 2001) and a fully-grown oviduct that does not regress before the last ovulation (Williams and Ames 2004). Furthermore, at this point yolk precursors (VTG and VLDL) are already at their maximal plasma levels and stay high until at least three eggs are laid (Salvante and Williams 2002). All one-egg stage females thus show comparable development of reproductive machinery, independent of future clutch size, and it is therefore unlikely that

the amount of reproductive tissue present at that time would induce higher levels of RMR except for the effect of the oviduct. It appears that the cost of the "future investment", suggested by the relationship between clutch size and DEE in birds at the beginning of laying is instead part of the "non-resting" component of DEE; i.e. the energy cost lies within the active phase of the female's daily energy budget. Female zebra finches reduce locomotor activity during rapid yolk development (Williams and Ternan 1999: Chapter seven) and locomotor activity is not related to clutch size or mass (Chapter seven). However, females may be engaged in other activities, not detectable in perch hopping data, that would ultimately be related to clutch size. In zebra finches, courtship and copulation usually occur on a single perch and involve various types of "dances" and body movements (Zann 1996). Copulations begin up to six days before laying, and peak within the two days prior to the first egg laid followed by a marked decrease after the second egg is laid (Zann 1996). Blazer and Williams (1998) showed that female zebra finches preferentially mate with high quality males that sing longer and more often. These pairs also produce larger clutches (Blazer and Williams 1998). Therefore, if that type of activity can affect the overall energy budget, it is reasonable to assume that females engaged in courtship and mating activities may show increased DEE at the one-egg stage, even if their hopping behavior is reduced, when paired with a high quality male. In this specific case, energy reallocation through reduction of locomotor activity would not be as efficient, resulting in increased DEE, but the pay-off would be a larger clutch.

CONSEQUENCES OF INCREASED ENERGY DEMAND

Chapter seven demonstrated that female zebra finches reduce their locomotor activity to compensate for the energy investment in egg production. This strategy may have been possible because they were able to save energy relatively easily, i.e. decreasing locomotor activity in a food abundant "habitat" may not be very constraining. It should be noted that at least two studies reported decreased activity in zebra finches in response to food *limitation* (Meijer et al. 1996; Dall and Witter 1998). However, DEE was not measured in these specific studies and decreased activity could well be an adjustment to reduce overall DEE in order to match the available energy intake (food intake and DEE are positively correlated in captive zebra finches; Chapter seven). In natural conditions, energy reallocation through decreases in locomotor activity may not always be possible. Small passerine birds often form their eggs before the period of increased seasonal food abundance (Williams 1998) which means that individuals may have to spend a significant amount of energy in foraging activity in order to fulfill the nutritional demands of egg production. What would the best energy saving strategy be in such conditions? Birds forced to work harder often show energy compensation through decreased BMR and night-time metabolic rates (Deerenberg et al. 1998; Nudds and Bryant 2001; Wiersma 2003). However, this may not be possible in egg producing females as egg formation induces an increased level of metabolism at rest. Then, if the energy budget is adjusted to limit variations in DEE, one would expect other forms of energy reallocation, e.g. readjustment in organs and physiological systems (Wikelski and Ricklefs 2001).

A likely candidate for temporary physiological down-regulation is immune function. Evidence is accumulating that building and maintaining immune defense has a detectable metabolic cost (9-29% change in BMR; Demas et al. 1997; Ots et al. 2001;

Martin et al. 2002; Raberg et al. 2002). Furthermore, several studies have reported decreased immunity or elevated parasite lèvels in species forced to work harder due to experimentally enlarged clutches and broods (Gustafsson et al. 1994; Norris et al. 1994; Oppliger et al. 1996; Ots and Horak 1996; Siikamaki et al. 1997 but see Williams et al. 1999). In fact, an experimental increase in reproductive effort (brood enlargement) in captive zebra finches resulted in decreased immune function (Deerenberg et al. 1997; Wiersma 2003) and this response was also triggered by increased non-reproductive workload (locomotor activity) in that same species (Deerenberg et al. 1998).

Why would birds try to limit variations in DEE during reproduction? Artificially increased reproductive effort often leads to decreased residual reproductive value (Dijkstra et al. 1990; Gustafsson and Pärt 1990; Jacobsen et al. 1995; Daan et al. 1996; Verhulst 1998; Reid et al. 2000; Nager et al. 2001). Although the physiological mechanisms by which this may occur are not clear, a recent study on captive zebra finches (Chapter 8 in Wiersma 2003) suggests that females facing the demand of enlarged broods and elevated DEE decrease oxidative protection, as shown by reduced antioxidant enzyme activity. This may lead to increased DNA, protein and lipid damages and accelerated senescence (Wiersma 2003). Therefore, the trade-off between self-maintenance and reproduction (Tuomi et al. 1983; Verhulst and Tinbergen 1997) may be a basis for compensatory energy adjustments in periods of high demand.

Examples of energy reallocation in nature are common and take place at multiple functional levels. For example, energy compensation can be achieved by saving thermoregulatory costs through heat increment of feeding (Masman et al. 1989; Chappell et al. 1997), muscular heat production in active animals (Webster and Weathers 1990;

Bruinzeel and Piersma 1998) or huddling behavior in roosting birds (Chaplin 1982). As mentioned above, energy compensation could also occur via down-regulation of immune function and through metabolic adjustments among organs (see Chapter five). As demonstrated in Chapter seven, energy compensation can result from behavioral adjustments such as a reduction in activity levels (Williams and Ternan 1999; Chapter seven). Finally, reallocation could occur at an even higher level by maintaining a relatively constant DEE throughout the yearly cycle (seasonal reallocation: Weathers and Sullivan 1993; Williams 2001; Bozinovic et al. 2004), although observations may be conflicting (Doherty et al. 2000). More DEE data measured outside of the reproductive season are needed to properly investigate the concept of seasonal reallocation.

It is well understood that birds can raise more chicks than their population average (Monaghan and Nager 1997) at the potential cost of higher levels of DEE (Deerenberg et al. 1998; Wiersma 2003, Chapter seven), and that reproducing mammals can show rather elevated levels of energy expenditure in laboratory conditions (Hammond et al. 1994). The important question may not be "how much higher can DEE be and where is the limitation?" but rather, "what is the cost or benefit of sustaining elevated DEE levels?"

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