MOLECULAR APPROCHES TO UNDERSTANDING VARIATION IN REPRODUCTIVE PHENOTYPE OF FEMALE ZEBRA FINCHES (*TAENIOPYGIA GUTTATA*)

by

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ABSTRACT

I investigated inter-individual variation at the cell and molecular level in relation to reproductive phenotype (egg and clutch size, timing of laying) in female zebra finches (*Taeniopygia guttata*). First, I developed an antibody against zebra finch apoVLDL-II. I then developed a dot-blot assay to quantify plasma apoVLDL-II levels. This revealed marked inter-individual variation in apoVLDL-II levels but this was not correlated with differences in reproductive phenotype. Second, I described variation in VTG/VLDL receptor mRNA expression in relation to variation in reproductive phenotype. VTG/VLDL-R mRNA was expressed at high levels in growing oocytes and skeletal muscle and was also detectable in liver, but these tissues contain different splice variants. VTG/VLDL-R mRNA expression decreased during follicle growth. Ovary mRNA expression was correlated with clutch size and laying interval. F3 follicle mRNA expression was correlated with egg size. Thus, VTG/VLDL receptor mRNA expression is a key determinant of inter-individual variation in reproductive phenotype.

Keywords: inter-individual variation, zebra finch, reproductive phenotype, apolipoprotein VLDL-II, VTG/VLDL receptor mRNA

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CHAPTER 1: INTRODUCTION

1.1. Introduction: From genetic inter-individual variation to physiological inter-individual variation

It is widely recognized as a basic biological principle that heritable variation at the genetic level provides the raw material for any evolutionary processes (e.g. Whitehead and Crawford, 2006). Natural selection acts on phenotypic variation within species, and thus studying intraspecific or inter-individual variation is necessary to understand the evolution of phenotypic variation in life-history traits (Zera et al., 2007). However, our knowledge of the mechanisms underlying inter-individual variation in phenotype is relatively poor for many key life-history traits (Williams et al., 2005; Zera et al., 2007), and this is especially true at the cellular and molecular level (Whitehead and Crawford, 2006). In this Introductory chapter I will first, summarize the biological significance of measuring inter-individual variation in RNA expression and protein expression. Second, I will describe some of the technical approaches used to measure inter-individual variation at the transcriptional and post-transcriptional level, highlighting some of the specific problems encountered in measuring true biological variation (cf. technical or measurement error). Finally, I will review some of the few studies to date that have related inter-individual variation at the molecular and cellular level to phenotypic variation.

1.2. Biological significance of measuring variation in mRNA or protein expression

Several studies have shown that inter-individual variation in transcriptional levels, i.e., the mRNA expression level of genes, can vary significantly in relation to the phenotypic differences among populations (reviewed in Crawford and Oleksiak, 2007). On the other hand, inter-individual variation at the transcriptional level may reflect inter-individual variation at the genotypic level, and both genotypic factors and environmental factors can influence the level of mRNA expression. Genotypic variation regulates the variation in transcriptional level of a gene in at least two ways: a) the variable region of the chromosomal DNA can be a region that is tightly linked with the gene and functionally significant for transcriptional regulation (e.g. promoter or upstream enhancer sequences), or b) variation in other relative genes (e.g. the gene's inhibitor or stimulator) can indirectly affect transcription. Similarly, regulation of post-translational processes can provide other mechanisms that play a role in generating phenotypic variation. For example, splicing variation (e.g., expression of extra exons), modification variation (e.g., phosphorylation) or allosteric binding of regulators can all affect protein function without detectable changes in mRNA level (Nikinmaa and Waser, 2007). Thus, measuring the variation in mRNA or protein expression can provide a way to identify the genotypic basis or explain the molecular mechanism of phenotypic differences. As is well known, an important factor that drives the evolutionary process is natural selection acting on heritable, phenotypic variation; thus, by considering the process of natural selection, we can see the role that inter-individual variation in mRNA expression or protein expression – the link

between genotypic variation and phenotypic variation -- has played in evolution. In the following section, I have summarized some recent studies bridging inter-individual variation at the cell or molecular level and inter-individual variation in evolutionarily-significant phenotypic variation.

1.3. Technical approaches to measuring inter-individual variation in mRNA and protein expression

Microarray analysis is currently the most widely used technology in detecting inter-individual variation in mRNA expression (Nikinmaa and Waser, 2007). In one microarray, the expression patterns of hundreds or even thousands of genes can be measured at the same time (Golden et al., 2006; Wu et al., 2004), i.e., the total mRNA expression pattern of one individual can be profiled in one assay. Thus, by comparing the mRNA expression profiles of individuals that show different physiological phenotypes, the candidate genes that contribute the phenotype can be identified (Brown and Botstein, 1999; Cossins et al., 2006). The work by Oleksiak et al. (2005) on *Fundulus*, which I discuss further below, is a successful illustration of this technique, demonstrating correlations between inter-individual variation in mRNA expression levels of certain cardiac metabolic genes (glucose metabolic enzymes, oxidative phosphorylation enzymes, and Krebs cycle enzymes) and inter-individual variation of cardiac metabolic rates. However, the sensitivity and accuracy of DNA microarray techniques for measurement of inter-individual variation is limited by some factors, such as the content of the nucleic acid in the sample (Bier and

Kleinjung, 2001). In other words, to ensure the accuracy of the result, a high DNA concentration in the sample is required. Moreover, microarray analysis is a semi-quantitative technique and the quantitative capability is not very strong, so another key challenge of microarray studies of individual variation is the sample size. In other words, if the sample size is not large enough, then the technical variance can be larger than the biological variance (Golden et al., 2006). Therefore, in some cases, as a more sensitive and quantitative technique, quantitative real-time PCR (qPCR) is used to validate the microarray results (Bier and Kleinjung, 2001; Flores et al., 2007; Ramery et al., 2008). Compared to microarray analysis, quantitative real-time PCR only requires trace amount of nucleic acid. However, a very limited number of genes (normally less than five) can be tested by qPCR at any one time. Many studies have demonstrated that qPCR is a powerful technique to detect inter-individual variation in mRNA expression within species. For example, by using qPCR, Alfonso et al (2002) measured 37 genes in rat hippocampus and showed most of these genes varied 2-3 fold, with some genes showing up to 20-fold variation, among individuals. Moreover, by comparing levels of mRNA expression in replicate samples from the same individual, Alfonso et al (2002) clearly demonstrated that the large inter-individual variation observed reflected true biological variation and was not due to the experimental variability or technical error.

Although many studies have shown a correlation between variation in mRNA expression and physiological rate or phenotype, changes in mRNA expression do not necessarily correlate with, or predict changes in, protein activity or function

(Nikinmaa and Waser, 2007). Therefore, sometimes the measurement of protein expression is necessary for comparison with phenotypic variation. Antibody based technologies, such as protein microarrays, immunohistochemistry or western-blot (Roesner et al., 2006), are potential techniques to measure the variation in protein expression. The common strategy of these kinds of techniques is using specific antibody binding to label the target protein and using the autoradiograph or fluorescence microimaging to quantify the bound antibody. Moreover, the expression of receptor proteins can be measured by isotopic-labeled ligands binding and autoradiography (Olazabal and Young, 2006). In addition, two-dimensional gel electrophoresis and mass spectrometry is also used in measuring protein expression (Gygi et al., 2000). However, the quantitative power of these techniques is not very strong (one limiting factor is that protein cannot be amplified in the same way as nucleic acid), and analysis of variation in protein expression is relatively difficult compared with mRNA analysis. As a consequence of these technical issues there are only a few published studies of inter-individual variation which have measured variation in protein expression level and found correlations with physiological phenotypes.

1.4. Studies of inter-individual variation at the molecular level in relation to phenotypic variation

As described in the Introduction, studying inter-individual variation at the cell/ molecular level is important for explaining the inter-individual variation in

physiological phenotype. Therefore, it is not surprising that there is an increasing interest in the causes and functional significance of inter-individual variation at the molecular level (Crawford and Oleksiak, 2007; Whitehead and Crawford, 2006) involving a wide variety of organisms including yeast, worms, fish, mice, rats and humans (e.g. Erden et al., 2008; Perou et al., 1999).

However, most studies to date have not directly linked inter-individual variation in mRNA or protein expression with inter-individual variation at the physiological level. Instead, they often simply report large-scale variation in the expression of some molecular factor, e.g. tissue-specific mRNA expression levels or protein expression levels in plasma within a population, and simply suggest or propose that this factor is related to some disease or physiological phenotype. Thus, for these studies, a key question is still unanswered, which is: whether the inter-individual variation in mRNA or protein expression not only correlates with, but also provides a causal, mechanistic explanation for, the physiological phenotype and response at the organism level? This question is important for explaining the actual mechanisms that link molecular variation to phenotype at the whole organism level -- I have highlighted the evolutionary significance of this issue in Introduction. These kinds of studies are especially rare for vertebrate species, because of their long generation time and difficulties in identifying the selective forces operating on populations (Nikinmaa and Waser, 2007), so few data are available to address this question although some notable studies have been conducted on several vertebrate taxa.

One important study investigated molecular variation in relation to cardiac physiology in the teleost fish *Fundulus hetroclitus* (Oleksiak et al., 2005). In this study, the researchers found that there is large inter-individual variation in both cardiac metabolism (including the metabolism of glucose, fatty acid and LKA) and the transcriptional expression of related genes in three natural populations (5 or 6 individuals were tested in each population). Microarray analysis showed that expression of 94% of these cardiac genes were significantly different among individuals. Furthermore, variation in metabolic gene expression appeared to be correlated with the variation in metabolic rates. For example, for glucose metabolism rate, the variation in group 1 individuals was positively correlated with the variation in the expression of principal components for glycolytic. In groups 2 and 3, instead, genes of the oxidative-phosphorylation pathway explained the variation in glucose metabolism rates. Thus, the combinations of the gene expression explained the inter-individual variation in metabolism (Oleksiak et al., 2005).

Several similar studies are available in mammals. For example, Olazabal and Young (2006) used receptor autoradiography to show that inter-individual variation in oxytocin receptor (OTR) density in specific brain regions correlates with variation in alloparental care in rats: the juveniles with a higher OTR density in the nucleus accumbens (NA) and caudate putamen (CP), but lower OTR density in the lateral septum (LS), spend more time crouching over the pups. Another study in mammals, similarly, reported that individual differences in estrogen receptor α (Erα) are associated with individual differences in aggression in mice (Trainor et al., 2006).

Specifically, Erα immunoreactivity in the leteral septum (LS), ventral bed nucleus of the astria terminalis (vBNST), and anterior hypothalamus (AHA) of mice was positively correlated with the frequency of a biting behavior (Trainor et al., 2006). Although few in number, these studies clearly demonstrate that variation in expression of particular proteins can explain the inter-individual variation of complex phenotypes such as behavior.

Many studies in humans have related inter-individual variation at the genetic, molecular or cell level to important public health issues. For example, Martin-Fuentes et al. (2007) measured the mRNA expression of Scavenger receptors (SR, a group of receptors that recognize modified low-density lipoprotein by oxidation or acetylation) from 18 volunteers using RT-PCR. They found that the expression of some of these genes correlated (positively or negatively) with the expression of inflammatory molecules under the stimulation of oxLDL (an important promoter of atheroslerosis, an inflammatory disease). This result demonstrated that the variation in SRs expression pattern and level could determine the inflammatory response in atheroslerosis (Martin-Fuentes et al., 2007).

In avian species, there have been very few functional studies on inter-individual variation in mRNA or protein expression to date, most likely because birds have relatively long generation time (compared with fish, amphibians and invertebrates) and are potentially less relevant to human systems (compared with mammals). However, some recent studies in birds confirmed that analyzing variation at the molecular level in the context of phenotypic variation can provide insights into

important questions in evolutionary biology. For example, Abzhanov et al (2004) performed a comparative analysis of mRNA expression levels of various growth factors (shh, Fgf8, Bmp2, Bmp7 and Bmp 4) in six species of the genus *Geospiza* (Darwin's finches). Analysis of *in situ hybridization* images of the developing embryos showed a striking correlation between beak morphology and Bmp4 expression, i.e., the expression of Bmp4 is highest in mesenchyme of the upper beak prominence of *G. magnirostris* (the species with the deepest and broadest beak in the 6 species) and lower in other species with narrower beak. Furthermore, Abzhanov et al., (2004) misexpressed Bmp4 in chicken embryos and demonstrated that the over-expression of Bmp4 in the mesenchyme caused significant increases in the width and depth of the beak, similar to that seen in some Darwin's finches. Thus, by measuring the differences of mRNA expression in closely-related species, this study strongly suggests that Bmp4 may play an important role in the species diversity in bill morphology in birds (Abzhanov et al., 2004). Then in 2006, by using complementary DNA microarray analysis, the same group identified another gene, calmodulin (CaM), which also affected the beak morphological variation. They over expressed this gene in chicken embryo and observed obvious morphological change consistent with those in some species of Dawin's finches (Abzhanov et al., 2006). Other than these studies, to my knowledge, no other studies to date have reported correlations between variation in mRNA or protein expression and phenotypic variation within avian species.

1.5. Objectives of this thesis

Previous work in our lab has demonstrated that inter-individual variation in some important reproductive traits of birds, such as egg mass, clutch size, or laying interval may correlate with the expression of some key molecules, such as vitellogenin (VTG), apolipoprotein VLDL-II (apoVLDL-II) and oocyte-expressed VTG/VLDL receptor (Challenger et al., 2001; Christians and Williams, 2001; Salvante & Williams, 2002). However, none of these studies directly measured the mRNA or protein expression; rather, the expressions of these molecules were indexed by some indirect assays (e.g., Zinc-assay for VTG, Triglyceride assay for VLDLy, rate of yolk uptake measured using radio-labelled amino acid for VTG/VLDL-R).

In this thesis I will investigate inter-individual variation at the cell and molecular level (receptor-mediated endocytosis, and apolipoprotein expression) in relation to reproductive phenotype (egg and clutch size, timing of laying) in female zebra finches (*Taeniopygia guttata*) during egg production by directly measuring the mRNA or protein expression of related molecules. Specifically, in Chapter 2, I will test the hypothesis that variation in plasma apolioprotein VLDL-II in female zebra finches can explain variation in reproductive phenotypes. Then in Chapter 3, I will investigate individual variation in molecular mechanisms of receptor-mediated yolk uptake underlying phenotypic variation in egg size. Finally in Chapter 4 I will provide a brief synthesis and integration of these studies in the context of variation in egg size, and will suggest some future directions for further study in this area.

CHAPTER 2: VARIATION IN APOLIPOPROTEIN VLDL-II IN FEMALE ZEBRA FINCHES (*TENIOPYGIA GUTTATA*) IN RELATION TO REPRODUCTIVE PHENOTYPE

2.1 Introduction

Avian embryos develop in shelled eggs, which are abundant in protein and lipids. While the egg proteins are important for tissue growth, yolk lipids are the primary source of energy for developing embryos (Walzem et al. 1999). Egg production, and specifically provisioning of eggs with yolk lipid, involves dramatic changes in lipoprotein metabolism of the laying female: non-laying females synthesise 'generic' very-low density lipoproteins (VLDL) which primarily function to transport lipids to non-reproductive tissues where they are stores or used to fuel metabolism. At the start of egg production in laying females, estrogen from developing follicle cells stimulates hepatic synthesis of a second lipoprotein, yolk-targeted VLDL (VLDLy, Luskey, Brown & Goldstein 1974) which functions specifically in yolk uptake. Intact VLDLy particles bind to a specific receptor on the oocyte surface and are transported across the cell membrane via receptor-mediated endocytosis (Bujo et al. 1997). Yolk-targeted VLDL contains two apolipoproteins: apolipoprotein B100 (apoB) and apolipoprotein VLDL-II (apoVLDL-II) (Chan et al. 1976, Kudzma, Swaney & Ellis 1979, Capony, Williams 1980, Dashti et al. 1983, Lin et al. 1986). ApoB acts as the ligand for the oocyte receptor (Nimpf, George & Schneider 1988), and apoVLDL-II is believed to

increase the resistance of VLDLy to hydrolysis by lipolytic activity of lipoprotein lipase (LPL, Schneider et al. 1990, Griffin, Grant & Perry 1982, Bacon, Leclercq & Blum 1978) In addition, apoVLDL-II is involved in a decrease in particle size of VLDLy facilitating yolk uptake into the developing ovarian follicles by allowing VLDLy particles to fit through pores in the ovarian granulosa basal lamina (Walzem et al. 1999, Schneider et al. 1990, Griffin and Perry 1985, Salvante et al. 2007). Studies on chickens (*Gallus domesticus*) have shown that only VLDLy particles with a certain diameter (25-44 nm) can access oocytic receptors, while the diameter of generic VLDL particle is much larger (~72 nm) (Walzem et al. 1999).

Several previous studies have investigated whether changes in the laying female's lipoprotein metabolism correlate with, or might explain, phenotypic variation in reproductive investment, e.g. rates of egg laying, egg size, etc. For example in chickens, females with higher laying rates had a higher proportion of smaller diameter VLDLy particles which were available to be incorporated into developing yolks (Walzem et al. 1999, Salvante et al. 2007). In contrast in a passerine species, the zebra finch (*Taeniopygia guttata*), variation in reproductive performance (egg and clutch size, timing of laying) was unrelated to variation in VLDLy particle size distribution. Furthermore, Salvante et al. (2007a) found no relationship between egg size and plasma triglyceride levels (an indirect measure of VLDLy) in zebra finches, whereas Challenger et al. (2001) reported a significant negative relationship between plasma triglyceride levels and total mass of developing follicles in the European starling (*Sturnus vulgaris*). In part these inconsistent results might be explained by the fact that

previous studies have relied on indirect assays of VLDLy function or abundance. For example, VLDL particle diameter alone is not sufficient to distinguish between generic and VLDLy particles, especially if there is considerable overlap between particle size distributions of breeding and non-breeding females (e.g., Salvante et al. 2007a). Similarly, use of a triglyceride assay as a simple index of VLDLy (Challenger et al. 2001, Vezina, Salvante & Williams 2003) has limitations in that this assay also measures generic VLDL (Challenger et al. 2001).

Here we developed and tested a novel polyclonal antibody against zebra finch apoVLDL-II. ApoVLDL-II is only expressed on yolk-targeted VLDL particles and therefore should provide a very specific and direct measure of VLDLy abundance. In addition, we tested the cross-species activity and specificity of the apoVLDL-II antibody in several other avian species. We developed a quantitative dot-blot assay and measured inter-individual variation in the plasma apoVLDL-II levels, as a direct measure of VLDLy, in breeding female zebra finches. Finally, we compared variation in VLDLy based on apoVLDL-II measurement with inter-individual variation in body mass and reproductive phenotypes (including egg mass, clutch size and timing of laying).

2.2. Materials and Methods

2.2.1. Experimental Animals and Blood Sample Collections

A captive-breeding population of zebra finches was maintained under controlled environmental conditions (temperature of 24-28°C; humidity of 35-55%; constant light schedule (14L: 10D, lights on at 0700 h), with non-breeding birds kept in single-

sex cages prior to breeding. All birds were provided with mixed seeds (white and panicum millet; 11.7 % protein, 0.6 % lipid, and 84.3 % carbohydrate), water, grit, and cuttlefish bone *ad libitum.* They also received a multi-vitamin supplement in the drinking water once a week. During breeding, birds were housed in single pairs in smaller breeding cages (51 cm × 39 cm × 43 cm), each with an external nest box (14 cm × 14 cm × 20 cm) and were provided with a daily egg-food supplement (20.3 % protein, 6.6 % lipid; 6 g/pair/day). Experienced breeding females were introduced into the breeding cages in the morning and a single male partner was introduced within 1 h. Nest boxes were checked daily at the onset of egg-laying. Female zebra finches were weighed (± 0.01g) and collected between 11.00-12.00 PST on the day when they laid their first egg, within 2-5 hours of oviposition (Christians & Williams, 2001). Birds (breeding and non-breeding females) were rapidly killed via anaesthesia and exsanguination; blood (about 0.5 ml) was collected from the jugular vein using a heparin rinsed pipette and transferred to heparin rinsed Eppendorf tubes. Blood samples were centrifuged at 5000 rmp for 10 min. Plasma (supernatant) was transferred to new tubes and stored in a -80 °C freezer. We obtained plasma samples for non-breeding (n = 7) and breeding (egg producing, n = 22) female zebra finches. In addition, we used plasma samples obtained for starlings, surf scoter, and chicken from other studies in our lab (T.D. Williams, unpub. data).

2.2.2. Preparation of ApoVLDL-II Polyclonal Antibody

ApoVLDL-II for sequencing was purified by Dr. Katrina Salvante (Salvante 2006). Plasma VLDL was isolated from the d<1.020 g/ml fraction of a pooled plasma

sample from E2-treated male zebra finches using the ultra-centrifuge. The apoVLDL-II protein was isolated from the VLDL portion by reconstitution, centrifugation, dialysis, and lyophilization. The molecular weight of the purified protein was confirmed by SDS-Polyacrylamide gel electrophoresis; the protein was blotted onto PVDL membrane. The band corresponding to apoVLDL-II was removed from the membrane and sent for sequencing (Salvante 2006). N-terminal sequencing was carried out by Dr. France Dumas (Institut de Recherche en Biotechnologie, NRC, Montreal). We then used the first 27 residues of the mature zebra finch apoVLDL-II sequence as the antigenic peptide (KSIFDKDRRELLAIPETIASYFYEAVN) for antibody development. This sequence was confirmed by BLAST analysis, in which the sequence of chicken apoVLDL-II (http://wwww.ensembl.org/gallus_gallus) was blasted against the *Taeniopygia guttata*-WGS database (http://www.ncbi.nlm.nih.gov/enome/guide/finch/). Peptide synthesis and apoVLDL-II antibody production and purification were performed by Antibody On Demand ™ custom antibody production service (ABR Affinity BioReagents ™, US).

2.2.3. SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out under reducing conditions in duplicate 0.75mm thick gels (acrylamide concentrations were 15% for the separating gel and 5% for the stacking gel, respectively) in a Bio-Rad Mini Protein III unit (Life Sciences Bio-Rad Canada, Mississauga, ON) at a constant current of 80 mA for 30-40 min. A sample buffer (60 mM Tris-HCL, 25% glycerol, 2% SDS, 14.4 mN M 2-mercaptoethanol, 1% bromophenol blue) was used to dilute the plasma

sample (1: 30), and the sample solution was heated at 99 °C for 15 min. The loading volume was 12 µl per well for sample solutions and 3µl per well for molecular weight markers (10 kDa to 170 kDa; PageRuler™ Prestained Protein Ruler; Fermentas Life Sciences). One gel of each pair was stained with EZblue™ Gel Staining Reagent (Sigma-Aldrich Canada, Oakville) for 1 hr and destained with distilled water for more than 3 hrs. The other gel of each pair was used for the Western blot.

2.2.4. Western Blot

Western blots were performed to test the specificity and the sensitivity of the antibody against zebra finch apoVLDL-II as well as plasma samples from other avian species. Proteins were transferred onto a 0.2 µm pure nitrocellulose membrane (Trans-Blot™ Transfer Medium, Bio-Rad, Hercules, CA, US) using a Trans-BLotTM SD semi-dry electrophoretic transfer cell (Bio-Rad, US) and Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) at 12 V for 15 min. Blots were blocked in the Western Quick Block reagent (GenScript, US) for 5 minutes and washed twice with PBS-T (phosphate-buffered saline, 0.2% Tween 20). The blots were incubated in anti-apoVLDL-II: PBS-T (1: 2500) for 1 hour and then washed with PBS-T four times for 5 minutes each time. Then the blots were incubated in IRDye 800CW Goat Anti-Rabbit IgG secondary antibody(1: 15000 in PBS-T; LI-COR, US), followed by washing with PBS-T four times for 5 minutes each time. Finally, the blots were rinsed in PBS and scanned by Odyssey infrared imaging system (LI-COR, US).

2.2.5. Dot-blot

Plasma sample was diluted with PBS-T plus 0.1% ABS. To generate a standard curve, dilution ratios of 1: 10, 1: 20, 1:40, 1:80, 1: 160, 1:320, 1: 640 and 1: 1280 were used. To test the variations in different samples, a dilution ratio is 1: 60 was chosen. All samples were heated at 99 °C for 15 minutes. Aliquots of 2µl of sample were spotted onto a 0.2 µm pure nitrocellulose membrane (Trans-Blot™ Transfer Medium, Bio-Rad, Hercules, CA, US) and left to air dry completely. Membranes were then analysed as described for the Western blots in the previous section. The quantification of fluorescence signal was performed by Odyssey infrared imaging system (LI-COR, US).

2.2.6. Plasma Triglyceride Assay

Circulating concentrations of triglyceride were measured as an index of total plasma VLDL using a commercially available kit (Serum Triglyceride Determination Kit, Sigma-Aldrich Canada, Oakville) which has been widely used in previous studies (e.g. Challenger et al. 2001, Williams, Christians 1997, Vanderkist et al. 2000, Gorman et al. in press). Inter- and intra-assay coefficients of variation (CV %) were 4% (n=10) and 9.7% (n=6) respectively, determine using a laying hen plasma pool.

2.3. Results

2.3.1. Specificity and Sensitivity of ApoVLDL-II Antibody

Initial analysis of plasma samples from female zebra finches confirmed that our antibody against apoVLDL-II was highly specific and sensitive to apoVLDL-II protein in zebra finch plasma. As shown in Figure 2.1, each of the lanes for breeding female birds (lane C and E) showed one clear band at the expected size for apoVLDL-II (~12 kDa); each of the lanes for non-breeding females showed no band. Thus, we are confident that the apoVLDL-II antibody can be used in quantitative dot-blot analysis for zebra finch plasma samples, which is described below.

We tested the cross-species activity of our zebra finch apoVLDL-II antibody on other avian species, including the European starling (*Sturnus vulgaris*), chicken (*Gallus domesticus*), and surf scoter (*Melanitta perspicillata)*, as shown in Figure 2.2. In all species except chicken (CK), including zebra finch (ZF), European starling (ST), surf scoter (SS), a band of 12 kDa was present in each breeding individual (Br) and absent in each non-breeding individual (NBr)., Various non-specific binding bands showed in all chicken and scoter lanes also. When we re-tested the starling plasma samples in a seperate western blot (shown in Figure 2.2C) we confirmed that there was a clear specific band at 12 kDa that was present in all breeding individuals and absent in all non-breeding individuals. A weak non-specific band was also present in the lane for the breeding starling sample.

2.3.2. Validation of Quantitative Dot Blot

We validated our dot blot assay by using two methods: 1) measurement of a serially diluted plasma pool sample, to test for linearity of dilution, and 2) comparison of two repeated measurements for a series of zebra finch plasma (n=25). As shown in Fig. 3 A and B (log format), the serial dilution measurement showed a good linear correlation (R>0.99) for dilutions between 1/40 and 1/1280. As seen in Figure 2.3C, repeat measurements of all of our zebra finch plasma samples showed high correlation (R= 0.91, P< 0.0001).

2.3.3. Inter-individual Variation in the Plasma ApoVLDL-II/VLDL Level and Its Relation to Reproductive Output

Our dot-blot assay showed that there was large inter-individual variation in the plasma apoVLDL-II levels of breeding female zebra finches; the highest level was about 5-fold greater than the lowest (CV = 58.4%). However, this inter-individual variation in the plasma apoVLDL-II levels did not correlate with the inter-individual variations in body mass (n=14, p=0.13, Fig. 4B), mean egg mass (n = 20; Figure 2.4D) or laying interval (the time from pairing to laying the first egg, n=16, Figure 2.4F). Similarly, clutch size was also independent of variation in apoVLDL-II (Fig.5B; comparing large-clutch sized females with an estimated clutch size 6 eggs (n = 8) with small-clutch sized female with an estimated clutch size <6 eggs (n = 8).

Total plasma VLDL, measured using the triglyceride assay, also showed large inter-individual variation with a 4-fold difference between lowest and highest values

(CV% = 24.3). Plasma VLDL was not correlated with body mass (Figure 2.4A) and, as with apoVLDL-II measurements, variation in plasma triglyceride levels was not related to inter-individual variations in egg mass (Figure 2.4C), laying interval (Figure 2.4E) or clutch size (Figure 2.5A). Furthermore, when we compared the results of these two methods, we found no correlation between apoVLDL-II levels and plasma triglyceride levels in breeding birds (Figure 2.6). Nevertheless, both the apoVLDL-II and plasma triglycride assays clearly distinguished breeding and non-breeding females, with large up-regulation of plasma levels in egg-producing females (Figure 2.7).

2.4. Discussion

We developed a novel polyclonal antibody against zebra finch apoVLDL-II and investigated its potential utilization for measuring the apoVLDL-II expression level, and hence plasma VLDLy levels, in avian plasma. We demonstrated that this antibody binds specifically and sensitively to apoVLDL-II in zebra finch plasma, as well as several other species including a second passerine, the European starling, and a duck species, the surf scoter (Anatidae). Using this antibody, we developed a dot-blot protocol to rapidly quantify plasma apoVLDL-II levels. This assay revealed marked (5-fold) inter-individual variation in apoVLDL-II levels. However, this variation was not correlated with the inter-individual variation in reproductive phenotypes, including egg size, laying interval and clutch size, in female zebra finches.

We obtained the sequence of the antigenic peptide used for antibody production directly via N-terminal sequencing of purified zebra finch apoVLDL-II protein

(Salvante 2006). A previous study in our laboratory showed that neither a polyclonal rabbit anti-chicken apoVLDL-II antibody (donated by Dr. Wolfgang Schneider, Vienna, Austria) nor an antibody generated using a synthetic, 17-amino acid composite peptide based of NCBI database information was able to detect putative zebra finch apoVLDL-II (Salvante 2006). A comparison of the N-terminal sequence for zebra finch with that of chicken (Table 2.1) provides an explanation for these results: 11 out of the first 27 amino acid residues are different (59.3% identity). Comparison of the first 27 residues shows that the sequences of apoVLDL-II of four other known species (duck, emu, quail, and turkey, as shown in table 2.1) are highly identical to that of the chicken (81.5%, 81.5%, 74.1%, and 81.5%, respectively) but have low identity to zebra finch (63.0%, 59.3%, 51.9%, and 63.0%, respectively). Our data are consistent with these differences in sequence: the anti-zebra finch apoVLDL-II antibody was able to recognize the surf scoter (a duck) apoVLDL-II but did not have cross-activity with chicken apoVLDL-II. However, when using it to test scoter plasma, two non-specific bands were present, at sizes of ~55 kDa and ~34 kDa, respectively, so this antibody did not show the same high specificity for measuring duck apoVLDL-II. The larger 55 kDa band should be albumin based on its size; but the identity of the smaller band is unknown. In the chicken sample, the large ~55 kDa band also appeared in both breeding and non-breeding lanes. We cannot explain the reason for the appearance of this band in plasma of these non-passerine species because albumin is a highly conserved protein (Webb et al. 2005) and yet these non-specific bands were not detected by the antibody in zebra finch and starling plasma. It

is not surprising that the anti-zebra finch apoVLDL-II antibody worked better in starling than other species because starling is much more closely related to zebra finch. This result suggests that this antibody might be used to quantify apoVLDL-II in other song bird or passerine species.

Colorometric triglyceride assays have been widely used in previous studies to measure plasma VLDL levels in relation to egg production and reproductive phenotype (Williams and Christians 1997, Vanderkist et al. 2000, Gorman et al. in press, Mitchell and Carlisle 1991). Although this simple, indirect assay also measures generic VLDL this assay has produced results consistent with it providing biologically meaningful measurement of VLDLy (e.g. Challenger et al. 2001, Peery et al. 2004, Caro et al. in press). It is surprising therefore that we found no correlation between plasma apoVLDL-II and triglyceride levels even though both methods revealed marked inter-individual variation (3-5 fold) and both were clearly able to distinguish breeding and non-breeding birds. One possible reason for this is that total estrogen-induced hepatic lipoprotein synthesis is markedly upregulated during egg production for both generic VLDL and VLDLy in zebra finch but these might be differentially regulated, i.e. some females might show large increases in VLDLy relative to changes in generic VLDL, and vice versa. There is no evidence to support this idea at present, i.e. changes in generic VLDL during egg production has not been measured directly in laying birds in any study to date. However, mean generic VLDL levels in non-breeding birds (normally 1-3 mg/ml) represent only a small proportion of average total VLDL in laying birds (normally 5-28 mg/ml; Challenger et al. 2001) which might

suggest this explanation is unlikely. In addition in the chicken there is actually down-regulation of hepatic generic VLDL synthesis (Walzem et al. 1999) during egg production. An alternative possibility is that there is variation in the number of apoVLDL-II proteins associated with each VLDLy particle. Walzem et al (1999) estimated that in estrogen-treated roosters each VLDLy particle contained apoB and apoVLDL-II in the ratio 1:23, based on mass ratios. However, it is not known if this number is constant and there is evidence that some apolipoproteins associated with generic VLDL (e.g. C, E/A) are not "integral", i.e. they can move from one lipoprotein to another depending on various factors such as triglyceride content, unlike apo B (Walzem et al. 1994). However, this is not known for apoVLDL-II associated with VLDLy.

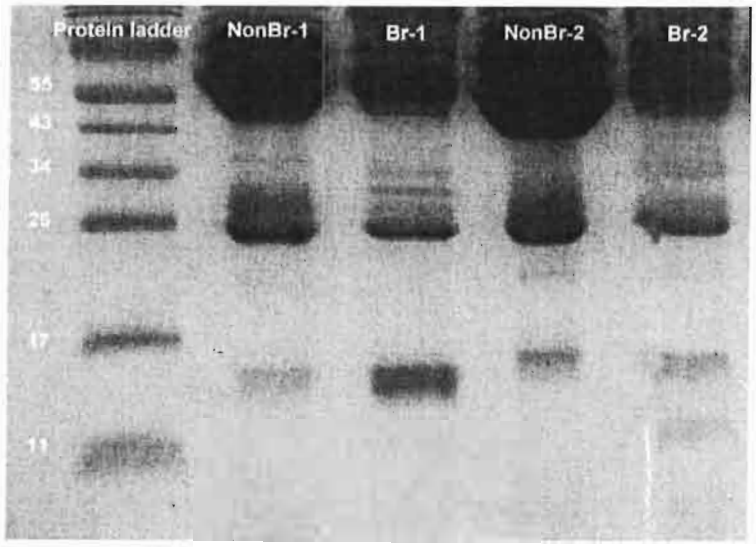
Using a dot-blot assay, we showed that the marked inter-individual variation in plasma apoVLDL-II levels was not correlated with inter-individual variations in reproductive traits (including egg mass, clutch size, and laying interval). Similarly total plasma VLDL, measured using the triglyceride assay, showed no correlation with any reproductive traits consistent with a previous study in female zebra finches (Salvante et al. 2007b). These results suggest that the *circulating* levels of apoVLDL-II are not a key factor in determining inter-individual variation in reproductive output in captive-breeding zebra finches. Interestingly, previous studies on free-living European starlings have shown that both total plasma VLDL levels and plasma levels of the second main yolk precursor vitellogenin (VTG) are negatively correlated with total yolky follicle mass (Challenger et al. 2001, Christians and Williams 2001). These

results strongly suggested that the circulating level of the two different yolk precursors, VLDLy and VTG, are closely co-regulated. Consistent with this idea, both yolk precursors share a common oocytic receptor which functions in uptake of the precursors into the developing yolk (Bujo et al. 1994). Challenger et al. (2001) suggested that the negative relationship between plasma VLDL and follicle mass in starlings might be caused by high rates of yolk uptake in "large follicle" individuals exceeding yolk precursor availability leading to depletion of plasma VLDL levels. It is known that VLDL dynamics in laying birds can be affected by energetic status, resource availability, and ambient temperature (Salvante et al 2007a). Potentially therefore the lack of any relationship between VLDLy and reproductive output that we report in captive zebra finches is due to the fact that females are better able to maintain VLDLy levels in benign conditions of captivity, with ad lib food. c.f. free-living birds under natural conditions. Thus, it would be informative to measure VLDLy using our apoVLDL-II in free-living species, such as the European starling, to confirm previous reports that VLDLy is negatively related to reproductive output.

Table 2.1. The N-terminal peptide sequence of apoVLDL-II in different species.

Zebra finch	K	S	I	F	D	K	D	R	R	E	L	L	A	I	P	E	T	I	A	S	Y	F	Y	E	A	V	N
Chicken				I		R	E		D	W			V			D	A	A		A		I					
Duck					E	R			D	W			V			D	A			A		I			T		
EMU					E	R			D	W			V			D	A	V		A		I			T		
Quail	R					R	G		D	W		M			Y	D	A			A		I					
Turkey					E	R			D	W			V			D	A	V		A		I					

A



B

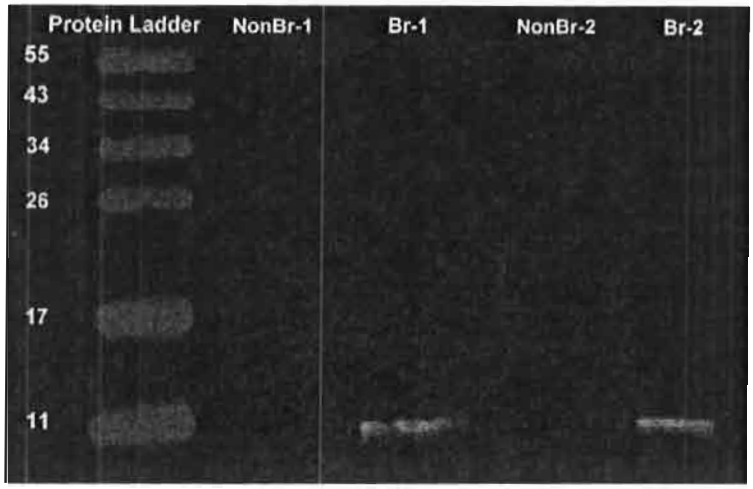
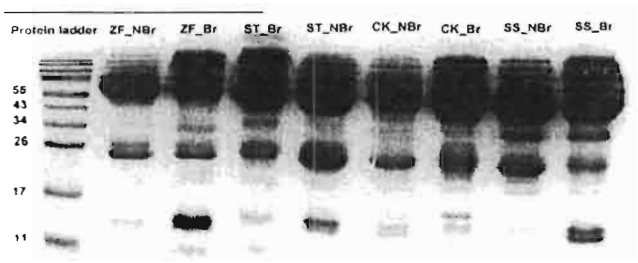
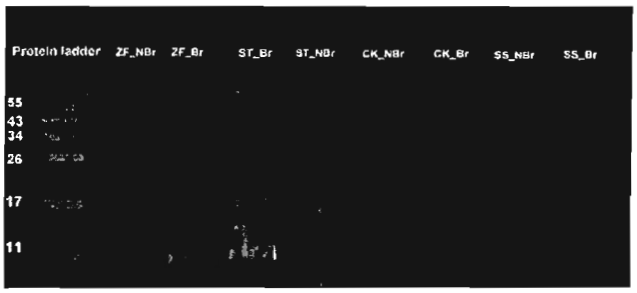


Figure 2.1. Sensitivity and specificity of the apoVLDLII antibody. A) EZblue stained SDS-PAGE gel. Lanes for plasma sample from breeding female zebra finches (Br-1 and Br-2) showed a band at about 12 kDa; lanes for plasma sample from non-breeding female zebra finches (NonBr-1 and NonBr-2) did not show this band. B) Western blot image by Odyssey infrared imaging system. A specific binding band at about 12 kDa can be seen; lanes for plasma sample from non-breeding female zebra finches do not show this band. No non-specific binding bands were detected.

A



B



C

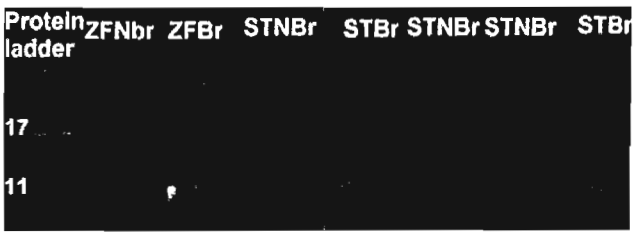


Figure 2.2. Cross-species activity of zebra finch apoVLDL_II antibody. A) EZ-blue stained SDS-PAGE. For all tested species, including zebra finch (ZF), European starling (ST), chicken (CK), surf scoter (SS), a band of 11 kDa detected in each breeding individual (Br) and absented in each non-breeding individual (NBr). B) Western blot image. Specific band of 11 kDa was present in breeding zebra finch (ZF_Br), breeding European starling (ST_Br), and Breeding surf scoter (SS_Br); in contrast; it was absent in all non-breeding birds and breeding chicken (CK_Br). C) Western blot image using fresh starling sample and zebra finch sample as control. Specific band of 11 kDa was present in all breeding individuals and absent in all non-breeding individuals.

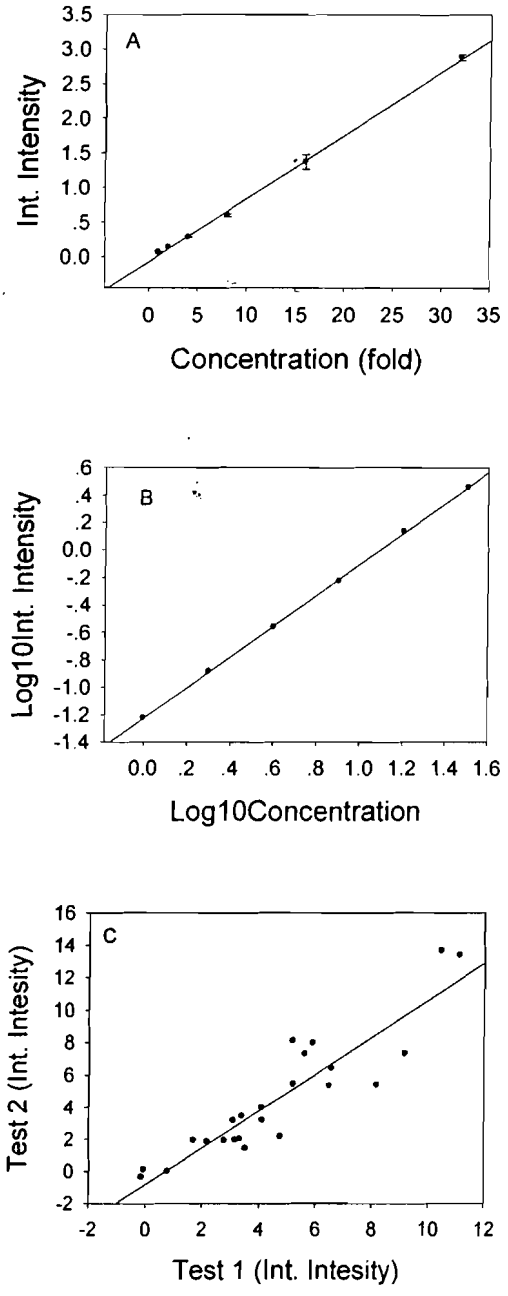


Figure 2.3. Validation of dot-blot assay. A) The measurement of serial dilution from 1/40-1/1280. The error bar indicated the standard deviation (duplications). B) The serial dilution curve after a log10 transformation. C) The repeatability of two measurements of all zebra finch plasma samples.

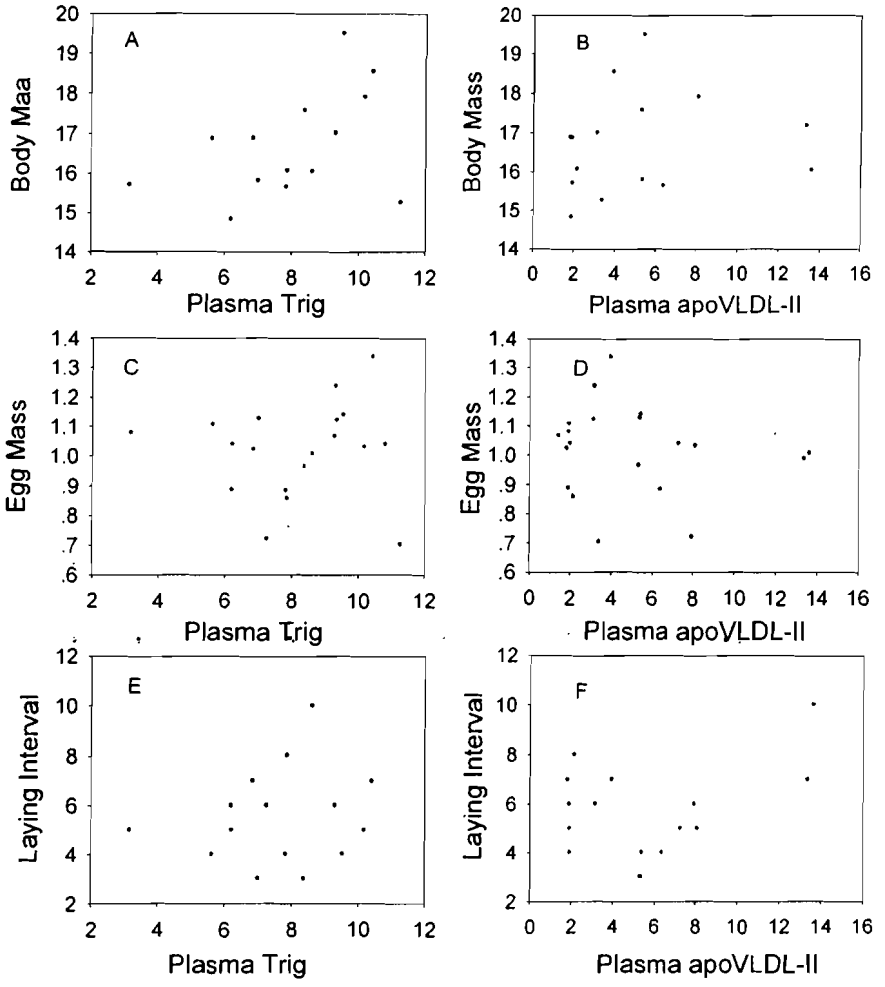


Figure 2.4. Variation in the plasma VLDL (A, C, E) and apoVLDL-II (B, D, F) level in relation to variation in body mass (A, B, measured when sample collecting), mean egg mass (C, D) and laying interval (E, F). No correlation were significant (P > 0.05).

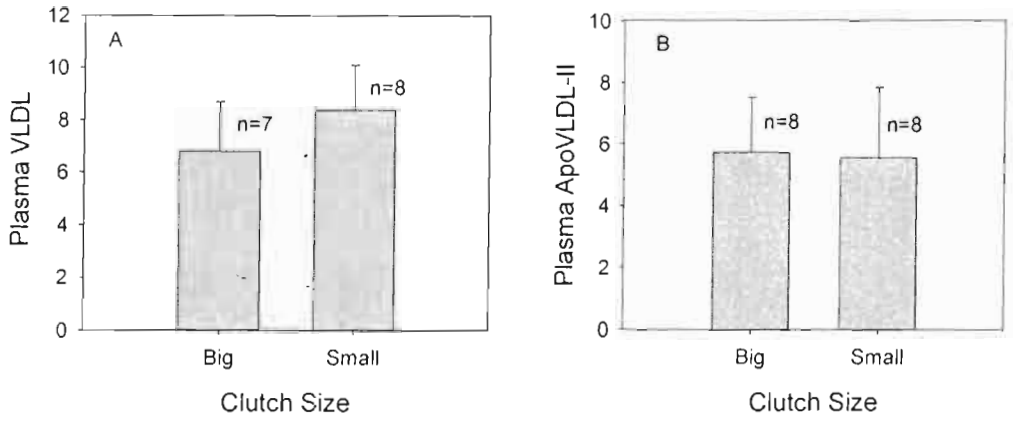


Figure 2.5. Variation in (A) plasma VLDL (A) and (B) apoVLDL-II (B) level in relation to clutch size. Large-clutch sized females have estimated clutch size = 6 eggs, small-clutch sized females have estimated clutch size < 6 eggs. Both plasma measures were independent of clutch size ($P > 0.05$).

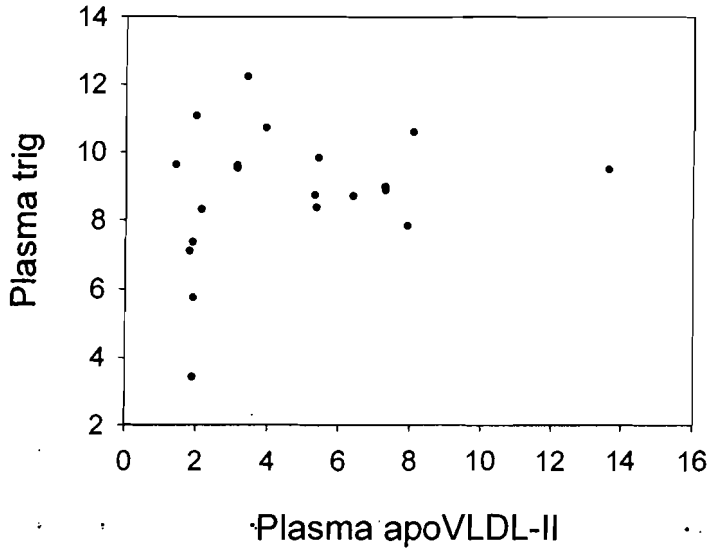


Figure 2.6. Lack of correlation between plasma VLDLy measured using the plasma triglyceride assay and using apoVLDL-II in the dot-blot assay, for the breeding zebra finches blood samples.

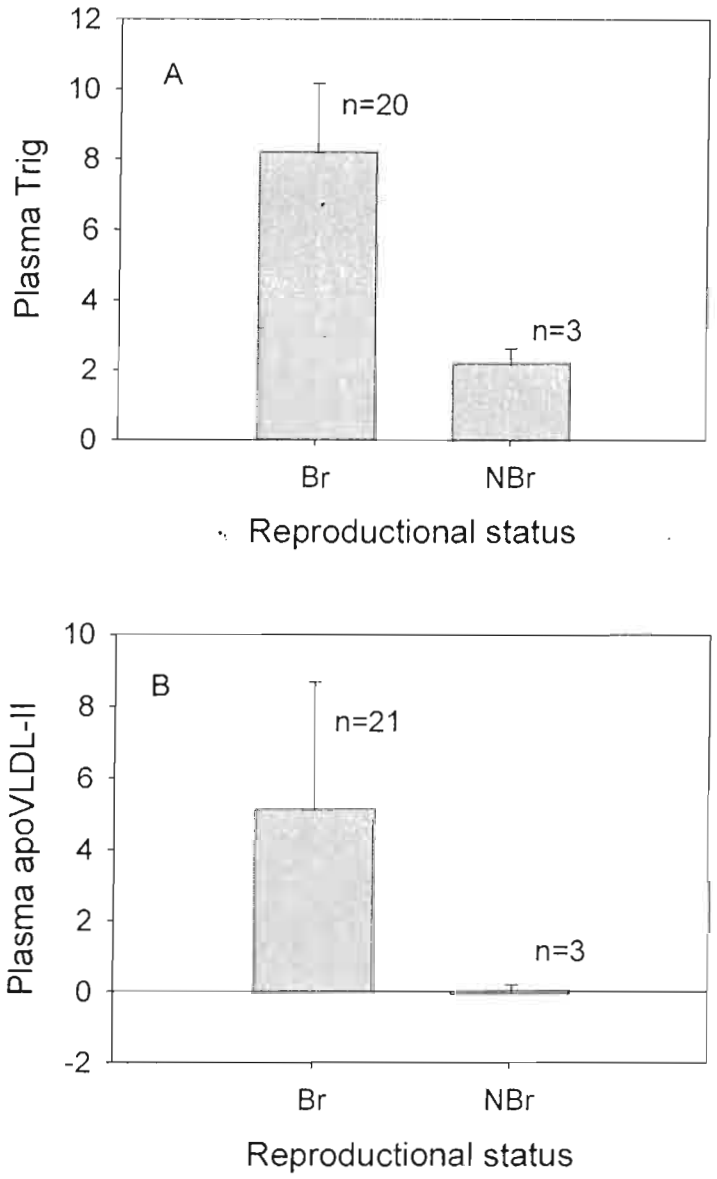


Figure 2.7. Variation in plasma triglyceride (top) and apoVLDL-II (bottom) in relation to breeding status in breeding and non-breeding female zebra finches. Both assays indicate large up-regulation of plasma VLDLy levels in egg-producing females ($p < 0.01$ in both cases).

CHAPTER 3: YOLK PROTEIN RECEPTOR EXPRESSION IS A KEY DETERMINANT OF VARIATION IN REPRODUCTIVE PHENOTYPE [1]

[1] This chapter has been submitted for publication: Han, D.,Haunerland, N.H. and Williams, T.D. (submitted, Nov. 2009) *Journal of Experimental Biology*

3.1. Introduction

Vitellogenesis and oocyte growth in all oviparous species are characterized by estrogen-dependent hepatic synthesis of the yolk-targeted lipoproteins vitellogenin (VTG) and yolk-targeted very-low density lipoprotein (VLDLy), which provide the nutrients and energy required by the developing embryo (Bacon et al., 1974; Bergink et al., 1974; Deeley et al., 1975; Gruber, 1972; Williams, 1998). Uptake of large amounts of yolk lipoproteins into developing follicles involves binding of the yolk precursors to a specific VLDL/VTG receptor on the oocyte surface and transport across the cell membrane via receptor-mediated endocytosis (Griffin and Hermier, 1988; Mommsen and Walsh, 1988; Shen et al., 1993; Wallace, 1985). The VTG/VLDL-R has been isolated and characterised in a wide range of oviparous species including insects (Schneider et al., 1997), amphibians (Okabayashi et al., 1996), fish (Hiramatsu et al., 2004; Perazzolo et al., 1999) and birds (Barber et al., 1991; Elkin and Schneider, 1994; Stifani et al., 1988; Stifani et al., 1990). In chicken

and quail there are two primary receptors with molecular weights of 95 kDa (LR8) and 380 kDa (LRP380) (Stifani et al., 1991). The smaller of these two receptors is a homologue of the mammalian VLDL receptor (VLDL-R), termed LR8 for the eight LDL receptor ligand binding repeats it contains, and previously designated as the OVR receptor (Bujo et al., 1997). The homologous mammalian VLDL receptor protein has been cloned from several species, including humans (Gafvels et al., 1993; Gafvels et al., 1994; Jokinen et al., 1994; Oka et al., 1994; Takahashi et al., 1992; Webb et al., 1994). Its regulation and functional significance in humans or mammalian model animals have been widely studied, mainly in relation to lipid metabolism (Masuzaki et al., 1996; Sato et al., 2002; Van Lenten et al., 1983). In the chicken the VTG/VLDL receptor binds a broad spectrum of ligands including VLDLy, VTG, α_2-macroglobulin and riboflavin-binding protein/VTG complexes (Mac Lachlan et al., 1994). The nucleotide and deduced amino acid sequences of the chicken VLDL/VTG receptor are highly similar to mammalian homologues (e.g., rabbit LDL-receptor, 84% amino acid identity; Bujo et al., 1994) but no sequence data are currently available for other avian species.

The regulation and dynamics of VTG/VLDL receptors in relation to vitellogenesis and follicle development have been characterised in Drosophila (Schonbaum et al., 2000), trout (Nunez Rodriguez et al., 1996; Perazzolo et al., 1999), white perch (Hirayama et al., 2003) and chicken (Shen et al., 1993). These studies have shown that the VTG/VLDL-R is a key component of yolk precursor uptake by developing follicles and may play a role in the regulation of oocyte growth (Hiramatsu

et al., 2004; Shen et al., 1993). These previous studies, however, have not investigated whether variation in VTG/VLDL-R expression plays a role in inter-individual variation in reproductive phenotype (e.g. follicle or egg size). Egg size varies greatly among individuals within avian populations, with the largest-laid eggs being up to 100% larger than the smallest egg, and with high repeatability of egg size (Christians, 2002). Yet, the physiological, cellular or molecular mechanisms underlying such marked inter-individual variation in phenotype remain poorly understood (Christians, 2002). Christians & Williams (2001) showed that inter-individual variation in size of yolky follicles in zebra finches (Taeniopygia guttata; a model songbird (Zann, 1996)) was correlated with the rate of incorporation of radio-labeled amino acid into yolk. This strongly suggests that receptor-mediated yolk uptake, and hence the expression level, or the functional activity of the VTG/VLDL-R, might be a key determinant of phenotypic variation in follicle, yolk or egg size, or other components of reproductive phenotype.

In this paper, we describe variation in VTG/VLDL receptor mRNA expression in relation to yolk uptake and follicle development and to phenotypic variation in female reproductive investment (follicle/egg size) in female zebra finches. Our specific objectives were, a) to identify the sequence of the zebra finch (Taeniopygia guttata) VTG/VLDL-R gene and compare this with the chicken sequence; b) to characterize tissue-specific expression of VTG/VLDL-R mRNA, c) to investigate changes in VTG-R mRNA expression during different stages of ovarian follicle maturation (ovary, and smallest pre-F3 follicle to largest yolky F1 follicle), and d) to correlate

inter-individual variation in VTG-R mRNA expression to inter-individual variation in reproductive phenotype (follicle and egg mass, clutch size, and laying interval).

3.2. Materials and Methods

3.2.1. Experimental Animals and Tissue Sampling

A captive-breeding population of zebra finches was maintained in controlled environmental conditions (temperature 24-28 °C; humidity 35-55%; constant light schedule (14L: 10D, lights on at 0700 h), with non-breeding birds kept in single-sex cages prior to breeding. All birds were provided with mixed seed (white and panicum millet; 11.7% protein, 0.6% lipid, and 84.3% carbohydrate), water, grit, and cuttlefish bone *ad libitum* and they received a multi-vitamin supplement in the drinking water once per week. During breeding birds were housed in single pairs in smaller breeding cages (51 cm × 39 cm × 43 cm), each with an external nest box (14 cm × 14 cm × 20 cm), and were provided with a daily egg-food supplement (20.3% protein, 6.6% lipid; 6 g/pair/day). Experienced females were introduced into the breeding cages in the morning and a single male partner was added within 1 h. Nest boxes were checked daily for onset of egg-laying. Female zebra finches were collected between 1100-1200 h on the day of laying of their first egg (within 2-5 hours of oviposition (Christians and Williams, 2001)). Birds were rapidly killed via anaesthesia and exsanguination and the following tissues were immediately dissected out: any yellow, yolky follicles, the remaining ovary tissue including all white, non-yolky follicles, any post-ovulatory follicles present, liver and pectoral muscle. Tissues were immediately placed in pre-

weighed Eppendorf tubes containing 0.5 ml RNAlater (Qiagen) and the mass of the three biggest yolky follicles from each bird was recorded (± 0.001 g). Liver and muscle samples were less than 0.5 cm^3 in size, to make sure the RNAlater would diffuse into the interior of the sample and prevent RNA degradation. Tissue samples were left in RNAlater at 4 °C overnight, and stored in -80 °C freezer for future use. Yolky follicles from every bird were classified as F1 (the largest), F2 (the second largest), F3 (the third largest) and pre-F3 follicles, which were too small to weigh.

3.2.2. VTG/VLDL Receptor Sequence Assembly

Sequences representing each of the 19 exons of the chicken VTG receptor (gene ID 396154) were blasted against the *Taeniopygia guttata* – WGS database (http://www.ncbi.nlm.nih.gov/genome/guide/finch/), using the discontiguous megablast algorithm. To complete the intron sequences and assemble the entire gene, sequence strings up- and downstream of the exons were used in subsequent searches. The sequence information was used in primer design.

3.2.3. mRNA Extraction

Total mRNA was extracted from tissue samples using PickPen and QuickPickM SML mRNA kit (BioNobile, Turku, Finland) following the manufacturers recommended protocols. Briefly, the tissue sample (up to 15 mg) was homogenized manually in an RNase free Eppendorf tube with 400 µl lysis/binding buffer for about 2 min. For yolky follicles, yolk was removed (follicle membrane can be peeled off when the yolk is in a frozen state) and discarded before homogenizing. Samples were centrifuged for 2 min at 14,000 rpm and the supernatant was transferred to another

tube with a 21G needle attached to 1 ml syringe (for homogenization of viscous cell lysates). Oligo (dT30) coated paramagnetic beads (30 µl) were added and the samples were incubated with the beads for 5 min. The beads were removed and suspended in 15 µl RNase/DNase free water at room temperature with gentle mixing. The beads were washed twice with 400 µl Wash Buffer A and once with 400 µl wash buffer B. After incubation at 70 °C for 5 min, the beads were removed from the mRNA solution, which was kept on ice and used immediately for reverse transcription.

3.2.4. Reverse Transcription

Extracted mRNA was reverse transcribed to cDNA with the QuantiTect Reverse Transcription kit (Qiagen) following the manufacturers recommended protocols. For every sample, 12 µl extracted mRNA solution and 2 µl of genomic DNA Wipeout Buffer (Qiagen) were incubated for 2 min at 42 °C to remove genomic DNA contamination. Quantiscript RT Buffer (4 µl), RT Primer Mix (1 µl) and Quantiscript Reverse Transcriptase (1 µl) were added to the samples, which were held for 25 min at 42 °C and 3 min at 95 °C. The cDNA solutions were stored at -20 °C for future use.

3.2.5. PCR Identification for Splice Variants

For the identification of the splice variants of VTG/VLDL-R mRNA in different tissues, the following primers upstream and downstream of exon 16 were designed: P5 (forward primer), 5'-ACC CTA GTA AAC AAC CTC AAT GAT G-3'; P6 (reverse primer), 5'-AGG AAG AAT GAT CCA AGC TGC TGA T-3'. The cDNA synthesized from breeding zebra finch ovary, muscle and liver were used for PCR identification, respectively, using the QuantiFast SYBR green PCR kit (Qiagen) on a

MiniOption real-time PCR system (BioRad). The thermal cycling protocol was as follows: a) step 1: initial template denaturation/enzyme activation at 95 °C for 5 min; b) step 2: denaturation at 95°C for 10 sec; 3) step 3: annealing/extension at 60 °C for 30 sec; 4) repeat step 2 and step 3 for 29 more cycles; 5) melting curve test from 65 °C to 95 °C, in 0.5 °C steps. The PCR products were subjected to agarose gel (1%) electrophoresis and stained with ethidium bromide. A DNA ladder (GeneRuler 100 bp DNA ladder plus, Fermentas) was used to indicate the product size.

3.2.6. Real-Time Quantitative PCR

SYBR-green-based real-time PCR was used to quantify transcript abundance of zebra finch VTG/VLDL-R and β-actin (internal control) in the ovary (including white, pre-vitellogenic follicles), yolky follicles (normally 3 to 4 for each bird), post-ovulatory follicles, liver, and pectoral muscle of breeding females, and the ovary of non-breeding females using. Primers for real-time PCR were designed using the online software IDT SciTools PrimerQuest and were purchased from Integrated DNA Technologies (IDT, Coralville, IA). The primer sequences were as follow: P1 (forward primer for actin), 5'-TGC CGC GCT CGT TGT TGA CAA TGG TT-3'; P2 (reverse primer for actin), 5'-TCT GAC CCA TAC CGA CCA TCA CAC CCT GA-3'; P3 (forward primer for VTG/VLDL-R), 5'-TTG TGT GCC TCA GTG GTC AAT GTG TGC CTA-3'; P4 (reverse primer for VTG/VLDL-R), 5'-ACT GAG TTG ACT GAG GAC CGC AGC TGA TTT-3'. All primers were used at a concentration of 83 nM. PCR amplifications and fluorescence detection were carried out with the

MiniOpticon real-time PCR system (BioRad, iQ™ SYBR Green Supermix reaction volumes 25 µl). The thermal cycling protocol was as follows: a) step 1: initial template denaturation/enzyme activation at 98 °C for 30 s; b) step 2: denaturation at 92 °C for 1 s; 3) step 3: annealing/extension at 70 °C for 20 s; 4) repeat step 2 and step 3 for 39 more cycles; 5) melting curve test from 65 °C to 95 °C, in 0.5 °C steps. Primer efficiency was calculated by duplicate standard curves, which were generated using a serial dilution of follicle cDNA samples ($r^2 > 0.99$). With this protocol the efficiency of the primer pairs was around 86% for β-actin and 95% for VTG/VLDL-R.

In order to assess inter-assay variation for each PCR run, an aliquot of a mixture of several different follicle cDNA samples was used as control. Normalization of the relative expression levels of VTG/VLDL-R (relative to the reference gene, β-actin) to variation in fold induction of the control sample was achieved with the following formula (Pfaffl, 2001). This ratio calculates the expression of VTG/VLDL-R of a sample relative to the reference sample normalizing for actin:

$$ratio = \frac{E_{target}^{\Delta Ct_{target}(control-sample)}}{E_{ref}^{\Delta Ct_{ref}(control-sample)}}$$

All analyses were conducted using the JMP software program (SAS Institute Inc., Cary, NC). All the measurements in qPCR assay are at least in duplicate and some are in triplicate. Values are presented as means ± S.E.M unless otherwise stated.

3.3. Results

3.3.1. Sequence Information for Zebra Finch VTG/VLDL Receptor

Through BLAST searches of the *Taeniopygia guttata* genomic sequence database with chicken VTG exon sequences, we identified candidate sequences for all but the first of the 19 exons reported for the chicken VTG receptor (Fig. 3.1). Using an *in silicio* genome walking approach, we were able to find overlapping sequences for the entire gene, and to extend the genomic sequence by more than 4000 bp upstream of the second exon. Analysis of the resulting 14.5 kb genomic sequence revealed one likely promoter sequence 4000 bp upstream of exon 2, with an AUG start codon 122 bp downstream of the predicted transcription start site (Figure 3.1.). Splice site prediction suggested an exon-intron boundary 254 bp downstream of the transcription start site; hence, the open reading frame from 122-254 bp may represent exon 1. The mRNA resulted in one open reading frame, coding for a 98 kDa protein. When aligned with the chicken VTG-R sequence, the zebrafinch VTG receptor shows high sequence identity with the chicken receptor throughout the entire gene (92%), including exon 1 (57% identity, Figure 3.1). In a subsequent release of *T. guttata* contigs, the entire sequence was mapped to the z sex chromosome (Accession ABQF01022106; reverse compliment of 42617-27882).

3.3.2. Validation of Real-Time PCR

Specificity of the real-time PCR assay was confirmed with 1) melting curve tests, 2) gel electrophoresis of PCR product, and 3) sequencing. Each primer set designed for amplification of zebra finch β-actin and VTG/VLDL-R produced one PCR product corresponding to the expected length of 134 bp and 150 bp, respectively. The consistent melting temperature and the sequencing result confirmed primer specificity. Inter-assay variability (coefficient of variation), calculated using the control sample, was 1.80% for β-actin and 1.62% for VTG/VLDL-R (n=23).

3.3.3. Tissue Distribution of VTG/VLDL mRNA Expression

As shown in Fig. 2, high levels of expression of VTG/VLDL-R mRNA were observed in oocytes, including ovary of breeding and non-breeding females, yolky follicles, and post-ovulatory follicles, and muscle. Very low levels of VTG/VLDL-R mRNA expression were also found in liver. The expression level in ovary tissue of breeding females was significantly higher than that in other tissues (liver, post-ovulatory follicle, F1 follicle and non-breeder ovary, $p< 0.05$ in each case), but only marginally significantly higher than that in muscle ($t=2.04$, $p=0.056$, Fig. 3.2). Different tissues showed expression of different splice variants of the VTG/VLDL-R mRNA. As shown in Fig. 3, in zebra finch, the VTG/VLDL-R mRNA is LR8- form in oocytes and liver but LR8+ in muscle. The lanes for ovary and liver showed a lower band (about 300 bp) while the lane of muscle showed a upper band (about 400 bp). For each tissue tested, only one splice variant, either LR8- or LR8+, was detected.

3.3.4. Variation in Patterns of Follicle Growth

We confirmed that the pattern of follicle growth and variation in follicle size of the birds for which we measured VTG/VLDL-R mRNA was typical by comparing our data to a larger data set available for zebra finches in our breeding colony (T. D. Williams, unpublished data; Figure 3.4A). For the females used in the present study, mean F1, F2 and F3 follicle masses were all highly significantly different from each other ($P<0.001$ in all cases; Figure 3.4A). We also used this larger data set to test potential errors in assignment of follicles to different stages of development caused by the fact that female birds sometimes skip an egg and resume laying the next day. These "laying skips" are due to follicular atresia and are common in birds especially between the F3 and F4 follicle stages (Challenger et al., 2001). We identified 4/14 females which had F3 follicle mass outside of the 99% confidence interval (Figure 3.4B). For three females the F3 follicle was much smaller than the average for F3 follicles (3.5~4 times smaller than expected), and the other female had a much larger F3 follicle mass than expected (2.8 fold higher than the average F3 mass and only 11% lighter than the average F2 mass). Assuming these reflected laying skips, we reclassified these small F3 follicles as pre-F3 follicles for subsequent analyses, but we retained the large F3 follicle female in subsequent analysis.

3.3.5. Temporal Changes in Ovarian VTG/VLDL-R mRNA Expression during Oocyte Growth

Changes in VTG/VLDL-R mRNA expression during oocyte growth in breeding female zebra finches are shown in Figure 3.5. Transcript abundance of the VTG/VLDL-R was normalized to that of β-actin and reported as a fold change relative to the values obtained for the standard sample. The highest levels of VTG/VLDL-R mRNA were found in ovary (including pre-vitellogenic follicles), and mRNA levels decreased during subsequent follicle development. There was a ~5-fold difference in average mRNA levels between ovary and the biggest yolky, F1 follicle.

3.3.6. Inter-individual Variation of VTG/VLDL-R mRNA Levels

Variation in VTG/VLDL-R mRNA levels in the ovary (CV = 69.4%) was much larger than that for other later stages of follicle development (CV = 42.5% for pre-F3, 37.5% for F3, 39.8% for F2, 48.3%; for F1). To explore potential biological explanations for this large variation we compared ovary mRNA levels with estimated clutch size and laying interval (time from pairing to laying the first egg) of individual birds. We estimated the clutch size of each female based on the dissection results (e.g., if a bird had four developing yolky follicles, plus one oviductal egg and one laid egg, her clutch size would be ≥6 eggs). We assigned each female as either a) large-clutch sized female with estimated clutch size ≥ 6 eggs (n = 9), or b) small-clutch sized female with estimated clutch size < 6 eggs (n = 5). Mean VTG/VLDL-R mRNA expression level in the ovary was significantly higher in large-clutch females compared to small-clutch females ($t = 3.39$, $P < 0.01$; Figure 3.6) but mRNA

expression levels of F3 follicles was independent of estimated clutch size ($t = 0.39$, $P > 0.1$; Figure 3.6). Furthermore, as shown in Figure 3.7, there was a significant positive correlation between the VTG/VLDL-R mRNA expression in ovary and laying interval ($r = 0.60$, $P = 0.04$, $n = 12$; two females were excluded from this analysis because they had laying intervals of 3 days compared with a minimum 4 days to produce an egg and these two birds must have started egg formation before pairing).

We analyzed the correlation between inter-individual variation in egg mass and the variation in VTG/VLDL-R mRNA amounts in different yolky follicle stages. As shown in Figure 3.8, there is significant positive correlation between F3 mRNA expression level and egg mass ($r = 0.64$, $P = 0.04$, $n = 11$), and between F3 mRNA expression and F1 follicle mass ($r = 0.64$, $P = 0.03$, $n = 11$). No other correlations were significant for other follicle stages ($P > 0.1$ in all cases).

3.4. Discussion

The main aim of this study was to investigate temporal and inter-individual variation in VTG/VLDL-R mRNA expression in relation to follicle development in a model songbird, the zebra finch, and to test the hypothesis that VTG/VLDL-R expression level is a key determinant of inter-individual or phenotypic variation in follicle or egg size, i.e. reproductive phenotype. Using an *in silicio* approach we were able to identify the sequence for the complete zebrafinch VTG/VLDL receptor gene, with a predicted promoter ~ 4000 bp upstream of exon 2, and a start codon 122 bp downstream of the predicted transcription start site. As expected for this highly

conserved gene, the gene organization and coding sequence are very similar to the homologous genes from other birds, fish and mammals.

We found that VTG/VLDL-R mRNA is abundantly expressed in ovary tissue but also present in low, but clearly detectable amounts in liver (~6 % of ovary level). While VTG/VLDL-R mRNA could not be found in chicken liver (Bujo et al., 1995a), our findings are in line with various reports from mammalian (Oka et al., 1994) and fish species, where the receptor is found in low amounts as well (Tiebel et al., 1999). We also found high levels of VTG/VLDL-R mRNA in skeletal muscle of zebra finch, up to 50% of the amount present in breeding female's ovary, and higher than in any other tested tissue type. Muscle, however, contains a different splice variant than ovary (LR8+, containing all exons, as opposed to LR8-, which misses exon 16). Tissue-specific expression of these splice variants has also been reported for chicken and other taxa (Bujo et al., 1995b). In chicken, LR8+ is dominant in skeletal muscle and heart, while LR8- is dominant in the ovary (Bujo et al., 1995b). Little is known about functional differences between the two receptor forms, but given that VG/VTG-R can interact with various ligands, it is conceivable that the LR8+ and LR8- forms have different ligand preferences (Bujo et al., 1995b)

Overall, the temporal transcriptional pattern of VTG/VLDL-R during oocyte growth shows a clear decreasing pattern: while the ovary tissue, representing the pre-vitellogenic stage, has the highest mRNA levels expression decreases as vitellogenesis progresses, with lowest levels seen in the F1 follicle just prior to ovulation. This pattern, reported here for the first time for an avian species, is very similar to that

found in fish (Hiramatsu et al., 2004; Perazzolo et al., 1999), even though follicle development proceeds differently in birds and fish ((hierarchical and synchronous, respectively). Thus, our data support the notion that yolk protein receptors must be synthesized at early stages of oocyte development, being stored in the ooplasm for later mobilization to support oocyte growth during vitellogenesis (Hiramatsu et al., 2004; Shen et al, 1993).

We found large inter-individual variation in ovary VTG/VLDL-R mRNA expression (greater than that for yolky follicles) that was correlated with individual variation in clutch size, with larger clutch individuals having higher ovary mRNA expression. This suggests that VTG/VLDL-R mRNA might be functionally related, either directly or indirectly, to clutch size, and presumably higher mRNA levels could support yolk uptake of a greater number of follicles, while lower levels might support only a few follicles. Thus, differences in VTG/VLDL-R mRNA levels in the ovary might therefore be a key component of the mechanism causing variation of clutch size. We also found a positive relationship between ovary VTG/VLDL-R mRNA expression and laying interval. It is likely that this is independent of the relationship with clutch size because clutch size and laying interval are negatively correlated in zebra finches and other birds. Generally, individuals with longer laying intervals lay smaller clutches (Williams, 1996) and one would expect lower mRNA levels in these birds (based on Figure 3.5). However, it is possible that estrogen-induced up-regulation on VTG/VLDL-R mRNA expression is initiated in most females shortly after pairing, and females that delay laying may simply accumulate higher levels of

mRNA in the pre-vitellogenic follicles of the ovary before follicle development is initiated.

While factors such as age, diet quality and mate quality can affect egg size, these are not sufficient to explain the almost two-fold variation in egg size among individual females (Christians, 2002; Williams, 1998), nor do they provide an obvious mechanistic explanation. Recent studies have shown that this marked inter-individual variation in egg size is largely independent of variation in other physiological traits which might be expected to influence egg size: body composition (Vezina and Williams, 2003), circulating plasma levels of the two main yolk precursors, VTG and VLDL, as well as the plasma levels of estradiol, the main hormone that regulates many aspects of egg formation (Williams et al., 2004). However, simple measurements of circulating yolk precursor levels cannot reveal potential differences in rate of yolk precursor synthesis and uptake, which have yet not been directly measured. Yolk protein uptake should depend on the concentration of VTG/VLDL-R on the follicle cells, and thus on its mRNA expression level. In this context, we compared the inter-individual variation in follicle VTG/VLDL mRNA expression and variation in egg and F1 follicle mass and found clear positive correlations, confirming our hypothesis that individual variation in the level of VTG/VLDL-R is an important factor contributing to variation in reproductive phenotype. We suggest that expression of the VTG/VLDL-R in the F3 follicle is functionally significant since the F3 follicle stage is at the start of the most rapid and linear phase of the oocyte growth (Fig. 3.4A). Follicle mass continues to increase rapidly through the period from F2 to F1, although

VTG/VLDL-R mRNA levels are decreased (see Figure 3.5). Thus, it appears that the all the receptor protein needed has already been synthesized, and continues to direct the uptake of VTG/VLDL into the oocyte. Following the uptake of lipoprotein/receptor complexes by receptor mediated endocytosis, the receptors are generally recycled and transported back to the cell membrane, and thus little additional gene transcription is required. Up to now, our work has focused on the mRNA level, as is the case in most studies that simply correlate mRNA and physiological variation in phenotype (Crawford and Oleksiak, 2007). However, changes in mRNA do not necessarily correlate with changes in protein activity and function (Nikinmaa and Waser, 2007), and it will be important to investigate if similar correlations exist for the mature VG/VLDL-R protein. We have recently generated a polyclonal antibody against zebra finch VTG/VLDL-R that will useful for such studies.

Our study on the relationships between inter-individual variation in VTG/VLDL-R expression and reproductive phenotype (e.g. clutch size, laying interval and egg mass) is the first report of such inter-individual and intra-population differences in gene expression in an avian species, at the whole-organism level. Because natural selection acts on variation within species, assessment of inter-individual variation can give insights into the evolution of phenotypic variation in life-history trait (Whitehead and Crawford, 2006). Hence, our research is an important step to understand the mechanism and evolution of variation in egg mass, a key life-history trait.

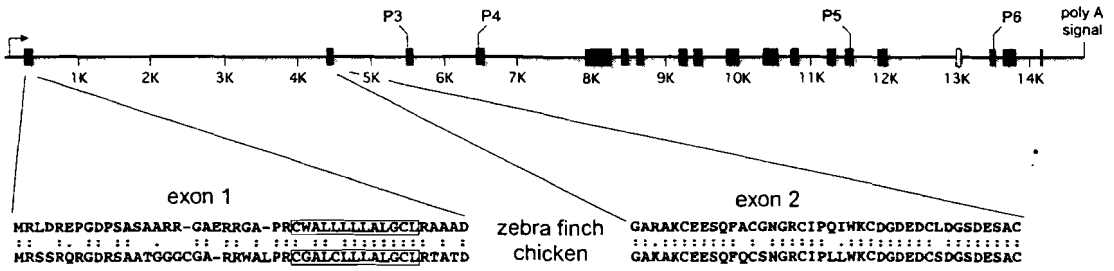


Figure 3.1. The zebra finch VTG/VLDL receptor gene. The 14.5 kb gene sequence is found on chromosome z (contig 19, ABQF01022106; reverse compliment of 42617-27882). It contains 19 exons and 18 introns. All exons are translated to the VTG-R, with the exception of exon 16 (shown in lighter grey) that is present only in the LR8+ splice variant. PCR primers P5 and P6 were used to distinguish the splice variants, yielding PCR products of 397bp (LR8+) or 311 bp (LR8-). The real-time PCR primers for quantification the mRNA (P3, P5) are located in exon 3 and 4, respectively. Sequence similarity between zebra finch and chicken VHDL-R is shown for exon 1 and 2. The ER-targeting sequence required for proteins located in the cell membrane is boxed.

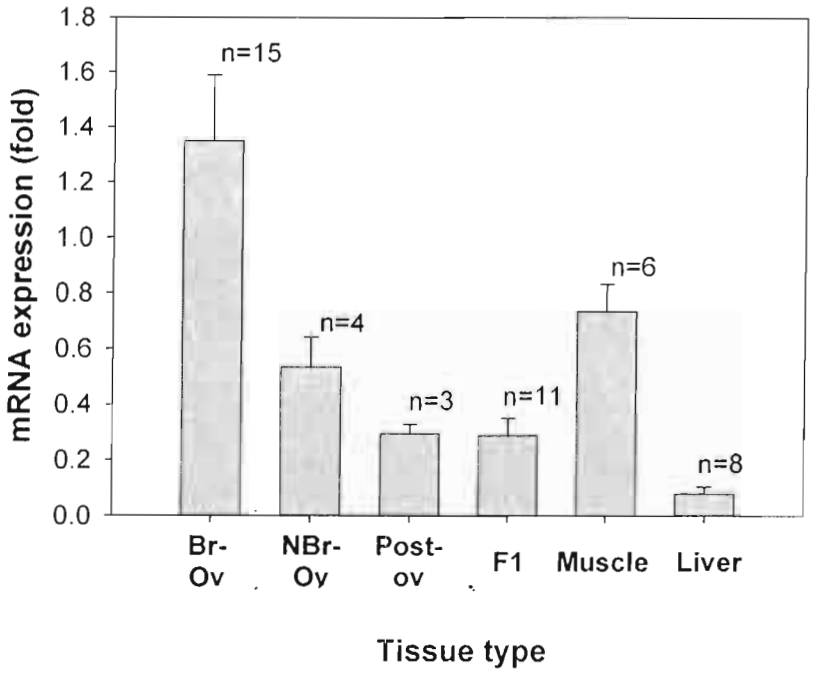


Figure 3.2. Expression of VTG/VLDL-R mRNA in various tissues of female zebra finch. The tissue type includes: ovary of breeding females (BR-ov), ovary of non-breeding females (NBr-ov), post-ovulatory follicle (Post-ov), F1 follicle, skeletal muscle, and liver. Relative levels of VTG/VLDL-R mRNA were normalized to β-actin mRNA and then compared with that of a control ovarian mRNA preparation (see Materials and Methods). Shade bars indicate mean values and vertical brackets indicate SEM.

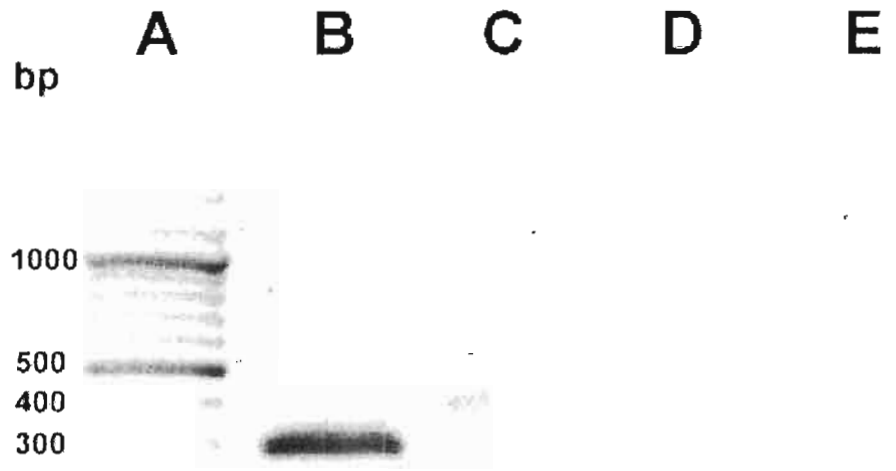


Figure 3.3. Tissue distributions of different splice variants in zebra finch. PCR was carried out with primers P5 and P6, yielding PCR products of 400 bp (with exon 16) or 300 bp (without exon 16) Lane A: DNA ladder. Lane B: PCR product of oocyte cDNA; lane C: PCR product of muscle cDNA; lane D: PCR product of liver cDNA; lane E: negative control.

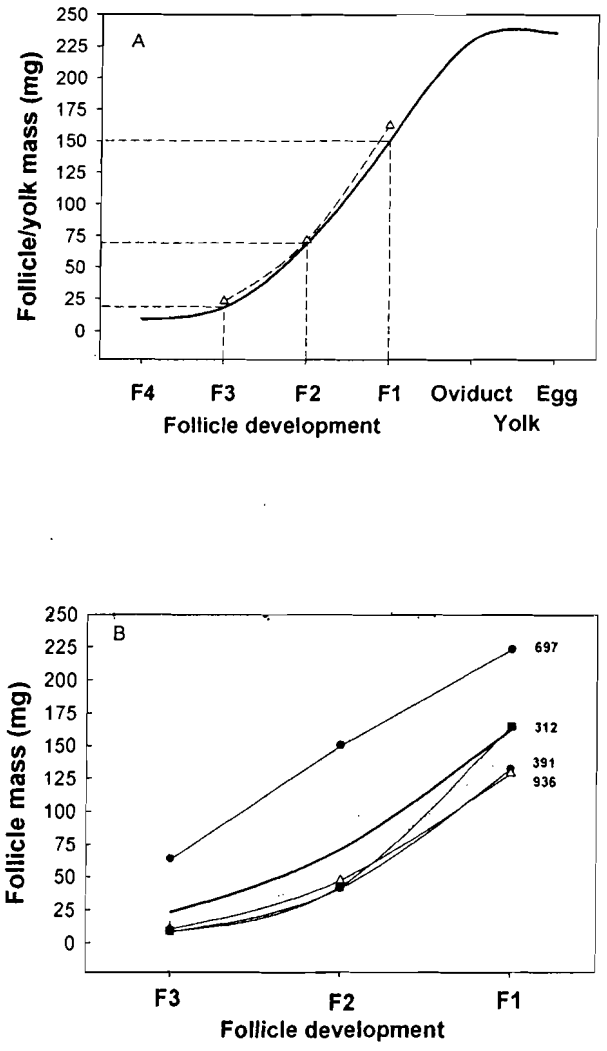


Figure 3.4. The maturation growth of the follicle. (A) The solid curve is the growth curve was drawn based on previous data with large sample size (n > 40 individual; Williams, unpublished data). The dotted-line indicates data from this study. (B) The general growth pattern (solid curve without data points) and growth pattern of abnormal individuals (The solid curve with data points. The numbers are the band numbers of the birds.)

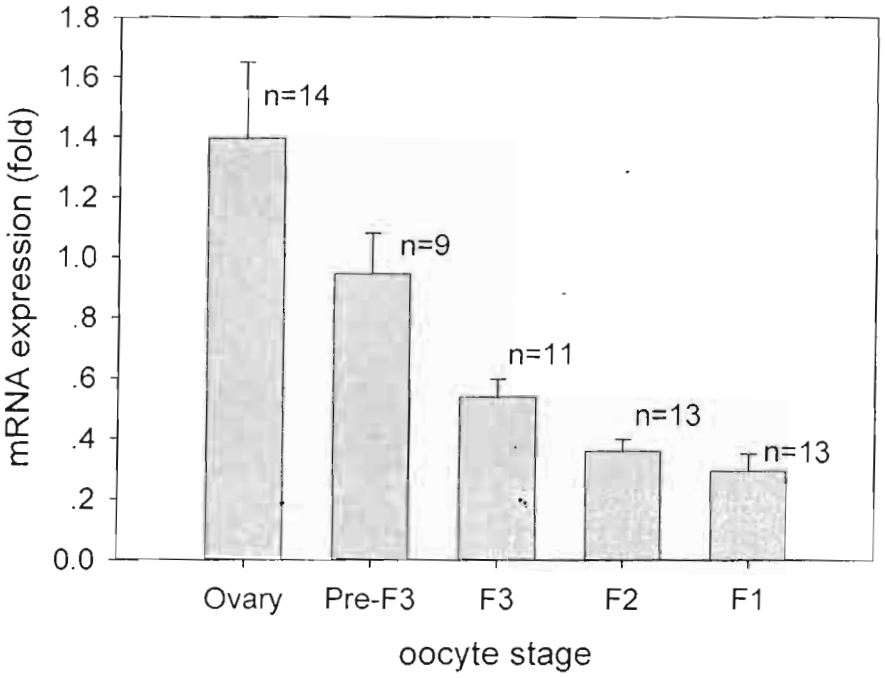


Figure 3.5. Changes in expression of VTG/VLDL receptor (VTG/VLDL-R) mRNA (fold) in ovary (conclude pre-vitellogenic follicles) or yolky follicles of different stages of follicle growth (from the earliest white follicles in ovary to the latest F1) in breeding female zebra finch.

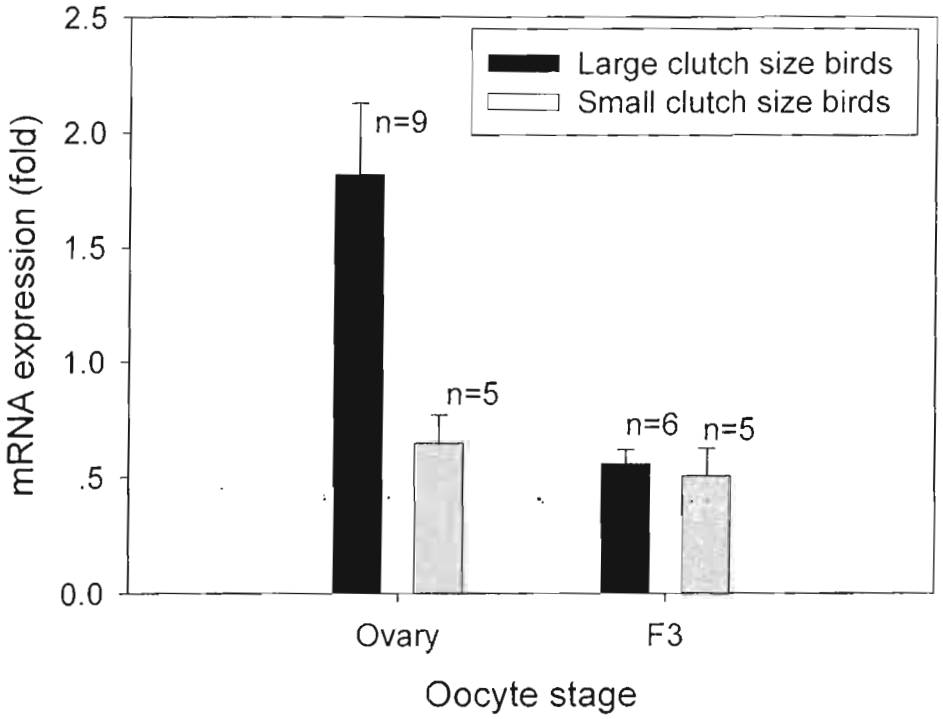


Figure 3.6. Variation in VTG/VLDL-R mRNA expression level in the ovary and F3 follicle in relation to clutch size: large clutch sized birds (clutch size ≥ 6 eggs, n = 9, 3 missing F3 data) vs. small clutch sized birds (estimated clutch size <6 eggs, n=5). Values are means ± SEM.

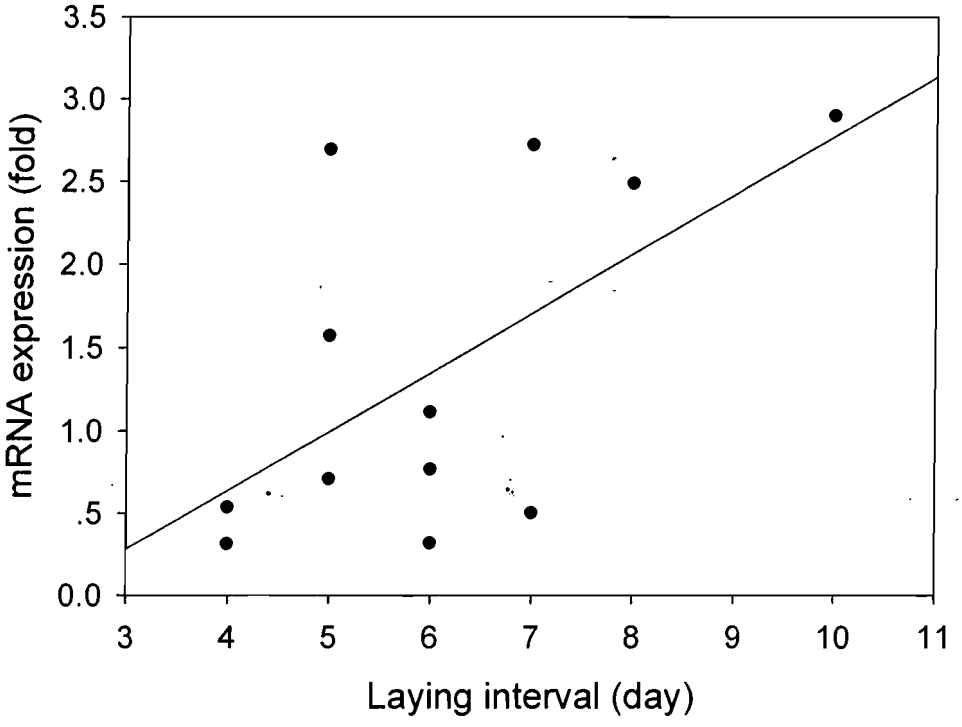


Figure 3.7. Correlation between ovary VTG/VLDL-R mRNA levels and laying interval.

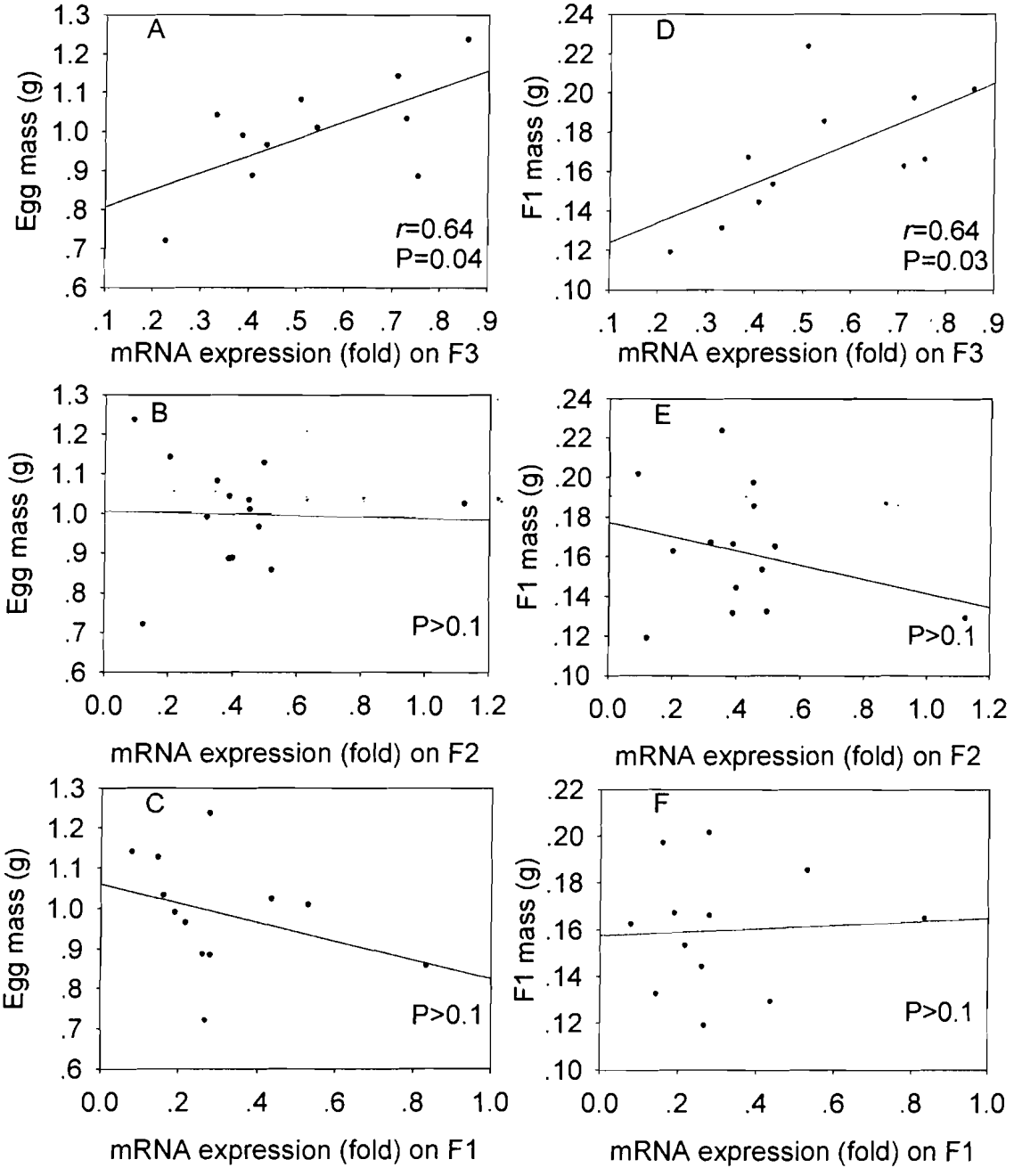


Figure 3.8. Correlation between VTG/VLDL-R mRNA levels level in F3（A, D）, F2 (B, E) and F1 (C, F) and egg size (A, B, C) or F1 follicle mass (D, E, F).

CHAPTER 4: GENERAL SYNTHESIS

The primary goal of the studies presented in this thesis was to investigate inter-individual variation at the cell and molecular level (receptor-mediated endocytosis, and apolipoprotein expression) in relation to avian reproductive phenotype (egg and clutch size, timing of laying). In this study, we used female zebra finches (*Taeniopygia guttata*) during egg production as the experimental animal and directly measured the mRNA or protein expression of two functionally-related molecules by using qPCR technology and dot-blotting. This study included two main projects: 1) investigating the relationship between the variation in plasma apoVLDL-II level and the variation of reproductive phenotypes, and 2) investigating the variation in ovarian VTG/VLDL receptor mRNA expression in relation to follicle development and variation in follicle and egg size. The most important findings of this study include: 1) development of a polyclonal antibody against zebra finch apoVLDL-II which we have demonstrated to be sensitive and specific and, based on this antibody, development of a dot-blot assay which can be used to measure inter-individual variation in plasma apoVLDL-II level; 2) we demonstrated VTG/VLDL receptor mRNA expression is a key determinant of inter-individual variation in reproductive phenotypes.

In Chapter 2, the western blot results have demonstrated that our polyclonal antibody also specifically and sensitively binds European starling apoVLDL-II. Thus, we can develop dot-blot assay for measuring starling plasma apoVLDL-II level in future studies. This assay can be used to confirm results from previous studies on the reproduction physiology of European starlings (e.g., Challenger et al. 2001), in which a

simple triglyceride assay was used as an indirect index of plasma VLDLy levels. For example, Chanllenger et al. (2001) showed the plasma triglyceride levels in breeding female European starlings were negatively correlated the egg mass whereas this relationship is not found in breeding female zebra finch. Thus, a valuable future study would be to use our dot-blot assay to measure the inter-individual variation in the plasma apoVLDL-II level in starlings in order to confirm that this trait is negatively correlated with the variation in egg mass.

Moreover, the specificity and sensitivity of this antibody in measuring both zebra finch and starling apoVLDL-II suggests it might be used to measure plasma apoVLDL-II level in all passerine species. To confirm this assumption, more passerine species should be tested in the future. Furthermore, this antibody also showed across species activity to duck (surf scoter) plasma apoVLDL-II, though some weak non-specific bands were also detected. Currently we are not able to explain the nonspecific bands, but we will try to find a way to eliminate this assay "noise" in the future, e.g., by changing the sample processing method or optimizing the experimental conditions. We will also use this antibody to test variation in plasma apoVLDL-II levels in other species from different taxa to investigate the utilization range of this new antibody.

An unsolved question in Chapter 2 is why the measurement of VLDLy using the triglyceride assay and the apoVLDL-II dot-blot assay are not correlated. As described in Chapter 2, one possible reason is that the apoVLDL-II content of zebra finch VLDLy particles is not constant either among different particles or in different individuals. To test this hypothesis, the exact molecular structure of zebra finch VLDLy is required; thus further biochemical analysis needs to be done. The other possibility is that the increase in

plasma VLDL during egg laying period may include an increase in generic VLDL, so the triglyceride assay cannot correctly or specifically measure VLDLy in zebra finch plasma. We are not aware of any evidence or related literatures that support this hypothesis.

The results in Chapter 3 provide numerous ideas for future research on mechanisms underlying the variation of reproductive phenotypes of zebra finches. In this chapter, we demonstrated the VTG/VLDL-R mRNA expression in the F3 follicle was significantly correlated with follicle and egg mass; however, we did not obtain enough a large sample size to test if the variation in VTG/VLDL-R expression in pre-F3 follicles contributes to the variation in egg and follicle mass. To answer this question, more individuals need to be tested to obtain a larger sample of pre-F3 follicles.

Moreover, due to the important role of VTG/VLDL-R mRNA expression, the transcriptional regulators for this gene might be an important subject for further research. Moreover, as I have described in Chapter 1, although our mRNA expression data have provided significant results in explaining reproductive phenotype, transcript levels are not necessarily correlated with the expression or functional activity of the VTG/VLDL-R protein itself. In future work, we may measure the VTG/VLDL-R protein expression in different follicle stages of different zebra finch individuals. This work would provide an important validation of our current conclusion. Moreover, it will be interesting to determine the temporal pattern of the VTG/VLDL-R protein expression in relation to timing of follicle growth. A comparison of the VTG/VLDL-R mRNA and protein expression pattern during follicle growth may lead to new findings about the regulatory mechanism of the expression of this gene. Currently, we have developed a polyclonal antibody against zebra finch VTG/VLDL-R protein (Genescript, US) which can be used

in immunohistochemistry to measure the inter-individual variation of the localization and density of the VTG/VLDL-R protein as well as the change of the protein expression during the oocyte growth. For detailed techniques, we can refer to the studies by Trainor et al (2006), in which the Erα immunoreactivity in certain brain areas of mice were measured and showed a positive correlation with the frequency of a biting behavior (See Chapter 1). This experiment will also help us to understand changes in the localization of this receptor, which has been suggested to be important in regulating VTG/VLDL-R function (Shen et al. 1993). In this study Shen et al. (1993) used immunogold cytochemistry to assess receptor distribution and showed that in early stage (pre-hierarchical) follicles (~2 mm in diameter), the receptor was localized in vesicles in the central area of the cells but absent in the cortical area. In contrast, in later-stage vitellogenic follicles (~ 1 cm in diameter), the receptors were located in the cortical region suggesting that receptor re-distribution was a key component of rapid follicle growth. By using immunohistochemistry we can also test the expression and localization of this receptor in some other tissues, such as liver and muscle; this will help us further understand the physiological function of this receptor.

Combined with the results of Chapter 2 and Chapter 3, we can see that during follicle development the uptake rate of the yolk precursors have played a dominant role in the variation in the reproductive traits, including egg mass, clutch size and laying interval. However, to complete the whole picture of the molecular mechanisms underlying variation in egg mass, the dynamics of the major precursors, including hepatic production of VLDLy and VTG, must be analysed in relation to receptor dynamics. In Chapter 2, we only tested variation in the plasma apoVLDL-II level and this is not

enough to show the influence of the circulating levels of yolk precursors to the inter-individual variation in egg mass. To solve this issue, further studies should measure mRNA and protein expression of VTG and apoVLDL-II in the liver and the plasma VTG levels in addition to receptor function in individual birds to provide a comprehensive analysis of yolk precursor synthesis, secretion, transport and uptake during follicle development.

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