

## Variation in yolk precursor receptor mRNA expression is a key determinant of reproductive phenotype in the zebra finch (*Taeniopygia guttata*)

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### SUMMARY

The vitellogenin/very low density lipoprotein receptor (VTG/VLDL-R), a 95 kDa protein that belongs to the low density lipoprotein receptor gene family, mediates the uptake of yolk precursors by developing follicles during oocyte growth. However, the extent to which variation in VTG/VLDL-R expression plays a role in determining inter-individual variation in reproductive phenotype (e.g. follicle or egg size) is not known. Here we show that the mRNA sequence of the zebra finch (*Taeniopygia guttata*) VTG/VLDL-R shows a high degree of sequence identity (92%) with chicken VTG/VLDL-R mRNA. Using quantitative real-time PCR we measured transcriptional expression of VTG/VLDL-R mRNA in various tissues, and for different stages of oocyte growth, in individual female zebra finches. VTG/VLDL-R mRNA was expressed at high levels in vitellogenic oocytes and in skeletal muscle, and was also detectable in liver, but these tissues expressed different splice variants: the short-form LR8– in oocytes and liver, and the LR8+ form in skeletal muscle. There was significant temporal variation in VTG/VLDL-R expression during follicle growth, with highest levels in ovary and a gradual decrease from pre-F3 to F1 vitellogenic follicles. Variation in ovary mRNA expression was correlated with inter-individual variation in clutch size and laying interval. Furthermore, variation in F3 follicle VTG/VLDL-R mRNA expression was correlated with inter-individual variation in egg mass and F1 follicle mass, suggesting that VTG/VLDL receptor mRNA expression is a key determinant of inter-individual variation in reproductive phenotype.

Key words: VTG/VLDL receptor, yolk uptake, inter-individual variation, egg size, oocyte growth, zebra finch.

### INTRODUCTION

Vitellogenesis and oocyte growth in all oviparous species is characterized by oestrogen-dependent hepatic synthesis of the yolk-targeted lipoproteins vitellogenin (VTG) and yolk-targeted very-low density lipoprotein (VLDL), which provide 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 VTG/VLDL 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 characterized 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 masses of 95 kDa (LR8) and 380 kDa (LRP380) (Stifani et al., 1991; Elkin et al., 1995). 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; Takahashi et al., 1992; Webb et al., 1994). Its regulation and functional significance in humans and 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

VLDL, VTG,  $\alpha_2$ -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 characterized 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 reproductive phenotype remain poorly understood (Christians, 2002; Williams, 2005). Christians and Williams (Christians and Williams, 2001) showed that inter-individual variation in the size of vitellogenic follicles in zebra finches [*Taeniopygia guttata*; a model songbird (Zann, 1996)] was correlated with the rate of incorporation of radio-labelled 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-R mRNA expression in relation to yolk uptake, follicle development and phenotypic variation in female reproductive investment (follicle/egg mass, clutch size) in female zebra finches. Our specific objectives were (a) to identify the sequence of the zebra finch 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/VLDL-R mRNA expression during different stages of ovarian follicle maturation (ovary, and smallest pre-F3 follicle to largest vitellogenic F1 follicle) and (d) to correlate inter-individual variation in VTG/VLDL-R mRNA expression to inter-individual variation in reproductive phenotype (follicle and egg mass, clutch size and laying interval).

## MATERIALS AND METHODS

### Experimental animals and tissue sampling

A captive-breeding population of zebra finches (*Taeniopygia guttata*, Vieillot 1817) was maintained in controlled environmental conditions (temperature 24–28°C; humidity 35–55%; constant light schedule 14h:10h L:D, lights on at 07.00 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 per pair per 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 the onset of egg laying. Female zebra finches were collected between 11.00 and 12.00 h on the day of laying of their first egg [within 2–5 h of oviposition (Christians and Williams, 2001)]. Birds were rapidly killed *via* anaesthesia (Rompun/Ketamine 1:1 v/v) and exsanguination and the following tissues were immediately dissected out: all yellow, vitellogenic follicles, the remaining ovary tissue including all white, non-vitellogenic follicles, any post-ovulatory follicles present, liver and pectoral muscle. Vitellogenic follicles were classified as F1 (the largest), F2 (the second largest), F3 (the third largest) and pre-F3 follicles. Tissues were immediately placed in pre-weighed Eppendorf tubes containing 0.5 ml RNAlater (Qiagen, Mississauga, ON, Canada) and the mass of the three biggest vitellogenic follicles (F1–F3) from each bird was recorded ( $\pm 0.001$  g). Liver and muscle samples were less than 0.5 cm<sup>3</sup> 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 a –80°C freezer until further use. All work was conducted following Canadian Council for Animal Care Guidelines under Simon Fraser University Animal Care permit no. 692B-94.

### VTG/VLDL receptor sequence assembly

Sequences representing each of the 18 exons of the chicken VTG/VLDL receptor (gene ID 396154) were blasted against the *Taeniopygia guttata*–WGS database (<http://www.ncbi.nlm.nih.gov/genome/guide/finch/>), using the discontinuous megablast algorithm. To complete the intron sequences and assemble the entire gene, sequence strings upstream and downstream of the exons were used in subsequent searches. The sequence information was used in primer design.

### mRNA extraction and reverse transcription

Total mRNA was extracted from tissue samples using a PickPen and QuickPickM SML mRNA kit (BioNobile, Turku, Finland) following the manufacturer's recommended protocols. Briefly, the tissue sample (up to 15 mg) was homogenized manually using a plastic pestle in an RNase-free Eppendorf tube with 400  $\mu$ l lysis/binding buffer for about 2 min. For vitellogenic follicles, the yolk was removed (the 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 r.p.m. (9860 g) and the supernatant was transferred to another tube with a 21G needle attached to a 1 ml syringe (for homogenization of viscous cell lysates). Oligo (dT<sub>30</sub>)-coated paramagnetic beads (30  $\mu$ l) were added and the samples were incubated with the beads for 5 min. The beads were removed and suspended in 15  $\mu$ l RNase/DNase-free water at room temperature with gentle mixing. The beads were washed twice with 400  $\mu$ l wash buffer A and once with 400  $\mu$ l 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.

Extracted mRNA was reverse transcribed to cDNA with the QuantiTect Reverse Transcription kit (Qiagen) following the manufacturer's recommended protocols. For every sample, 12  $\mu$ l extracted mRNA solution and 2  $\mu$ l of genomic DNA Wipeout Buffer (Qiagen) were incubated for 2 min at 42°C to remove genomic DNA contamination. Quantiscript RT Buffer (4  $\mu$ l), RT Primer Mix (1  $\mu$ l) and Quantiscript Reverse Transcriptase (1  $\mu$ l) 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.

### PCR identification of splice variants

For 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 was used for PCR identification, using the QuantiFast SYBR green PCR kit (Qiagen) on a MiniOpticon real-time PCR system (BioRad, Mississauga, ON, Canada). 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 s; (3) step 3: annealing/extension at 60°C for 30 s; (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, Burlington, ON, Canada) was used to indicate the product size.

### Real-time quantitative PCR

SYBR green-based real-time PCR was used to quantify the transcript abundance of zebra finch VTG/VLDL-R and  $\beta$ -actin (internal control) in the ovary (including white, pre-vitellogenic follicles), vitellogenic follicles (normally 3–4 for each bird), post-ovulatory follicles, liver and pectoral muscle of breeding females, and the ovary of non-breeding females. Primers for real-time PCR were designed using the online software IDT SciTools PrimerQuest and were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA). 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 nmol<sup>-1</sup>. PCR amplifications and fluorescence detection were carried out with the MiniOpticon real-time PCR system (BioRad, iQ<sup>TM</sup> SYBR Green Supermix reaction volume 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 amplification efficiency for the primer pairs was 86% for  $\beta$ -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,  $\beta$ -actin) to variation in fold induction of the control sample was achieved using the equation given by Pfaffl (Pfaffl, 2001). All the measurements in the real-time PCR assay were run in duplicate and some samples were run in triplicate. All analyses were conducted using SAS (SAS Institute 2002–2003 version 9.1; SAS Institute, Cary, NC, USA). We analysed variation in expression with tissue type or follicle stage using mixed models (proc MIXED) with 'bird' as a random effect to control for the fact that multiple tissues were sampled from the same individual. Tukey–Kramer adjustment was used to determine significance for multiple *post-hoc* paired contrasts. Values are presented as means  $\pm$  s.e.m. unless otherwise stated.

## RESULTS

### Sequence information for zebra finch VTG/VLDL receptor

Through BLAST searches of the *Taeniopygia guttata* genomic sequence database with chicken VTG/VLDL receptor exon sequences, we identified candidate sequences for all but the first of the 19 exons reported for the chicken VTG/VLDL receptor (Fig. 1). Using an *in silico* 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 (Fig. 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/VLDL receptor sequence, the zebra finch VTG/VLDL receptor showed high sequence identity with the chicken receptor throughout the entire gene (92%), including exon 1 (57% identity, Fig. 1). In a subsequent release of *T. guttata* contigs, the entire sequence was mapped to the Z sex chromosome (accession no. ABQF01022106; reverse complement of 42617-27882).

### Validation of real-time PCR

Specificity of real-time PCR quantification 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  $\beta$ -actin and VTG/VLDL-R produced one PCR product corresponding to the expected lengths of 134 bp and 150 bp (Fig. 1), respectively. The consistent melting temperature and the sequencing results confirmed primer specificity. Inter-assay variability (coefficient of variation), calculated using the control sample, was 1.80% for  $\beta$ -actin and 1.62% for VTG/VLDL-R ( $N=23$ ).

### Tissue distribution of VTG/VLDL-R mRNA expression

VTG/VLDL-R mRNA expression varied significantly with tissue type ( $F_{5,35,7}=9.20$ ,  $P<0.001$ ; Fig. 2A). High levels of expression of VTG/VLDL-R mRNA were observed in the ovary of breeding and non-breeding females, vitellogenic follicles, post-ovulatory follicles and muscle (Fig. 2A). Lower levels of VTG/VLDL-R mRNA expression were also found in liver. VTG/VLDL-R mRNA expression level in ovary tissue of breeding females was significantly higher than in F1 follicles, liver (Tukey–Kramer adjusted  $P<0.001$  in both cases) and post-ovulatory follicles ( $P<0.05$ ; no other differences were significant). 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 the 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 muscle lane showed a higher band (about 400 bp). For each tissue tested, only one splice variant, either LR8– or LR8+, was detected.

### Variation in VTG/VLDL-R mRNA expression during 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

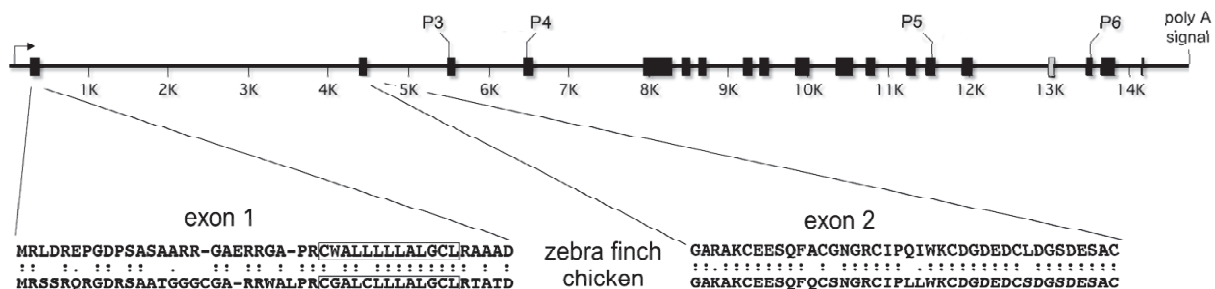


Fig. 1. The zebra finch VTG/VLDL receptor gene. The 14.5 kb gene sequence is found on chromosome Z (contig 19, ABQF01022106; reverse complement of 42617-27882). It contains 19 exons and 18 introns. All exons are translated into the VTG/VLDL receptor, with the exception of exon 16 (shown in lighter grey), which is present only in the LR8+ splice variant. PCR primers P5 and P6 were used to distinguish the splice variants, yielding PCR products of 397 bp (LR8+) or 311 bp (LR8–). The real-time PCR primers for quantification of the mRNA (P3, P4) are located in exon 3 and 4, respectively. Sequence similarity between zebra finch and chicken VTG/VLDL-R is shown for exon 1 and 2. The ER-targeting sequence required for proteins located in the cell membrane is boxed.

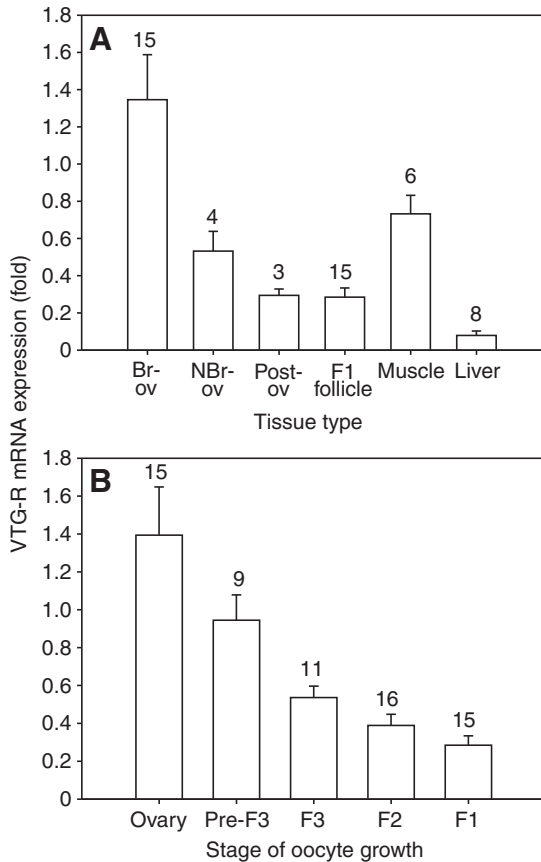


Fig. 2. Expression of VTG/VLDL-R mRNA in (A) various tissues, including ovary of breeding females (BR-ov), ovary of non-breeding females (NBr-ov), post-ovulatory follicle (Post-ov), F1 follicle, skeletal muscle and liver; and (B) in ovary (including white, pre-vitellogenic follicles) and vitellogenic follicles of different stages (from the earliest pre-F3 to the latest F1) in breeding female zebra finch. Relative levels of VTG/VLDL-R mRNA expression were normalized to  $\beta$ -actin mRNA and then compared with that of a control ovarian mRNA preparation (see Materials and methods). Values are means + s.e.m.; sample sizes are given above bars.

mRNA was typical by comparing our data with a larger data set available for zebra finches in our breeding colony (T.D.W., unpublished data; Fig. 4). 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; Fig. 4). We also used this larger data set to test potential errors in the 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 four females which had F3 follicle mass outside of the 99% confidence interval; 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.

VTG/VLDL-R mRNA expression varied highly significantly during oocyte growth in breeding female zebra finches

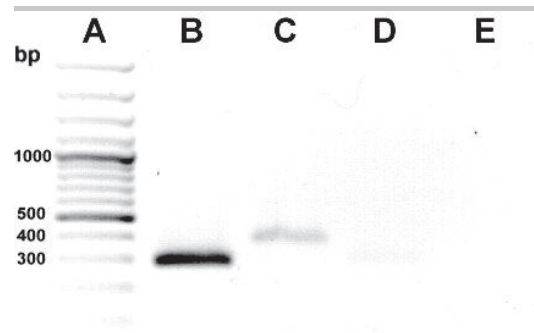


Fig. 3. Tissue distribution of different splice variants of VTG/VLDL-R mRNA in zebra finch. PCR was carried out with primers P5 and P6, yielding PCR products of 400 bp (with exon 16) or 300 bp (without exon 16). Lane A: DNA ladder; lane B: PCR product of oocyte cDNA; lane C: PCR product of muscle cDNA; lane D: PCR product of liver cDNA; lane E: negative control.

( $F_{4,48.1} = 12.55$ ,  $P < 0.001$ ; Fig. 2B). VTG/VLDL-R mRNA expression was highest in the ovary (which potentially included multiple white, pre-vitellogenic follicles, see below), and decreased during follicle development such that mRNA levels were significantly lower in F1 follicles (Tukey–Kramer adjusted  $P < 0.001$ ). F1 follicles also had lower VTG/VLDL-R mRNA expression than pre-F3 follicles ( $P = 0.05$ ). There was a ~5-fold difference in average mRNA levels comparing the ovary and the largest vitellogenic F1 follicle (Fig. 2B).

#### Inter-individual variation of VTG/VLDL-R mRNA expression

VTG/VLDL-R mRNA levels in the ovary were highly variable (c.v. = 69.4%) potentially because ovaries contained multiple pre-vitellogenic follicles (which we did not dissect out). To explore 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 vitellogenic follicles, plus one oviductal egg and one laid egg, her clutch size would be  $\geq 6$  eggs). We designated each female as either (a) a large clutch size female with estimated clutch size  $\geq 6$  eggs ( $N = 7$ ), or (b) a small clutch size female with estimated clutch size  $< 6$  eggs ( $N = 8$ ). Mean VTG/VLDL-R mRNA expression level in the ovary was significantly higher in large clutch size females ( $2.029 \pm 0.342$ ) compared with small clutch size females ( $0.749 \pm 0.148$ ;  $t_{13} = 3.60$ ,  $P < 0.005$ ) but mRNA expression levels of F3 follicles were independent of estimated clutch size ( $P > 0.1$ ). Furthermore, there was a significant positive correlation between the VTG/VLDL-R mRNA expression in ovary and laying interval ( $R = 0.55$ ,  $P = 0.05$ ,  $N = 13$ ; two females were excluded from this analysis because they had laying intervals of 3 days compared with a minimum of 4 days to produce an egg and these two birds must have started egg formation before pairing).

We analysed the correlation between inter-individual variation in egg mass and the variation in VTG/VLDL-R mRNA expression for different stages of vitellogenic follicle development. Variation in F3 follicle mRNA expression was significantly positively correlated with egg mass ( $R = 0.64$ ,  $P = 0.04$ ,  $N = 11$ ; Fig. 5A) and with F1 follicle mass ( $R = 0.64$ ,  $P = 0.03$ ,  $N = 11$ ; Fig. 5B). No other correlations were significant for other follicle stages with either egg mass or F1 follicle mass ( $P > 0.1$  in all cases).

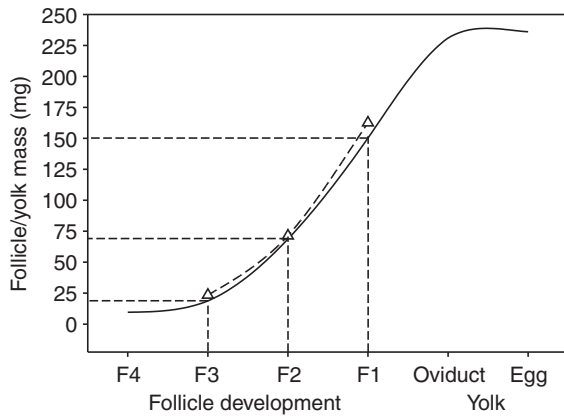


Fig. 4. Follicle growth in zebra finch; growth curve (solid line) based on unpublished data (T.D.W.) compared with data from this study (dashed line, open triangles).

### DISCUSSION

Our main aim in 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 is a key determinant of inter-individual variation in follicle or egg size, i.e. reproductive phenotype [as suggested by Christians and Williams (Christians and Williams, 2001)]. Using an *in silico* approach we were able to identify the sequence for the complete zebra finch VTG/VLDL-R 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 (Bujo et al., 1995a) and fish species, where the receptor has been reported in low amounts in liver (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 a breeding female's ovary. Muscle, however, contains a different splice variant from ovary (LR8+, containing all exons, as opposed to LR8-, which lacks 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) as well as being expressed in germ cells of testes (Lindstedt et al., 1997). Little is known about functional differences between the two receptor forms, but given that the VTG/VLDL-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 mRNA during oocyte growth in zebra finches shows a clear decreasing pattern: ovary tissue, representing the pre-vitellogenic stage, has the highest mRNA expression and expression decreases as follicle development progresses, with lowest levels of VTG/VLDL-R expression seen in the F1 follicle just prior to ovulation. This temporal pattern, reported here for the first time for an avian species, is very similar to that found in fish (Hiramatsu et

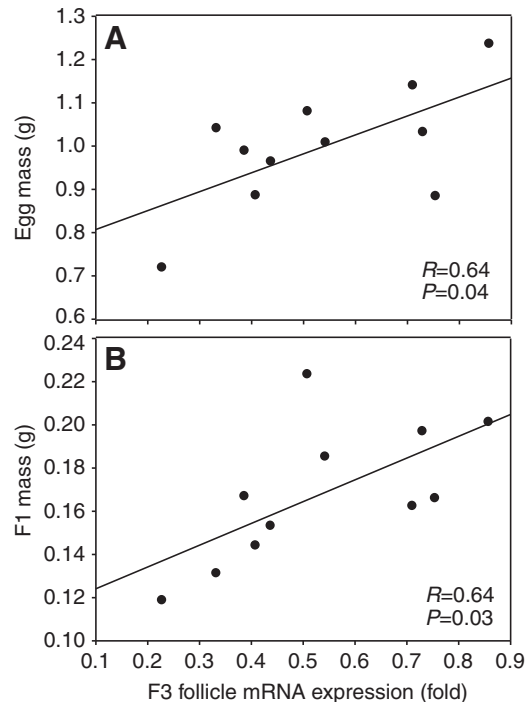


Fig. 5. Relationship between variation in VTG/VLDL-R mRNA levels in F3 follicles and (A) egg size, and (B) F1 follicle mass.

al., 2004; Perazzolo et al., 1999), even though follicle development proceeds differently in birds and fish (hierarchical and synchronous, respectively), highlighting the high degree of conservation of receptor function during oocyte development in oviparous vertebrates. In the domestic hen, receptors of pre-vitellogenic follicles are located centrally not cortically which may explain why there is no yolk precursor uptake (Shen et al., 1993). Shen and colleagues (Shen et al., 1993) proposed that at the onset of rapid yolk development this pre-existing pool of receptors is redistributed to the periphery of the oocyte, with negligible *de novo* synthesis of receptors during the final stage of oocyte growth (see also Hiramatsu et al., 2004). They further suggested that this pattern of receptor expression was due to the fact that the nuclear and biosynthetic machinery necessary for transcriptional, translational and post-translational events would be compromised by mechanical distortion during later stages of rapid oocyte growth when the space not occupied by yolk is less than 0.1% of the oocyte's volume in a layer 2  $\mu\text{m}$  thick (Shen et al., 1993). Our data provide strong support for this hypothesis that yolk protein receptors are synthesized at early stages of oocyte development, when levels of VTG/VLDL-R mRNA expression are high, but that there is negligible *de novo* synthesis of receptors during the final stage of oocyte growth, with low mRNA expression in F1 follicles.

We found large inter-individual variation in ovary VTG/VLDL-R mRNA expression (greater than that for vitellogenic follicles) which was correlated with individual variation in clutch size: larger clutch individuals had higher ovary mRNA expression. This suggests that VTG/VLDL-R mRNA might be functionally related, either directly or indirectly, to clutch size. 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 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. However, it is possible that oestrogen-induced up-regulation of 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 oestradiol, 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 the rate of yolk precursor synthesis and uptake, which have as yet not been directly measured. Yolk precursor uptake should depend on the concentration of VTG/VLDL-R on the oocyte plasma membrane, and thus on its mRNA expression level. In this context, we compared the inter-individual variation in follicle VTG/VLDL-R 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 the 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 oocyte growth (see Fig. 4). Follicle mass continues to increase rapidly through the period from F2 to F1, although VTG/VLDL-R mRNA levels are decreased (see Fig. 2B). Thus, it appears that most of the receptor protein needed has already been synthesized, and continues to direct the uptake of VTG/VLDL into the oocyte (Shen et al., 1993). 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.

In conclusion, our study suggests that VTG/VLDL-R mRNA expression is a key determinant of inter-individual variation in reproductive phenotype, and this provides an important first step in identifying mechanistic links between gene regulation and reproductive effort in oviparous species. Clearly, however, many questions remain unresolved. Firstly, as is the case in most studies that correlate gene expression with physiological variation in phenotype (Crawford and Oleksiak, 2007; Whitehead and Crawford, 2006), our study focused on the variation in mRNA levels. Changes in mRNA, however, do not necessarily correlate with changes in protein activity and function (Nikinmaa and Waser, 2007), and thus it will be necessary to analyse variation in the mature VTG/VLDL-R protein as well. The pattern of oocyte growth, coupled with our data on mRNA expression, would predict that VTG/VLDL-R protein will increase during follicle growth. Secondly, another receptor, the LRP380 receptor, also binds VTG and might function

with the VLDL-R (LR8) in regulating oocyte growth, although the molecular and functional characteristics of this receptor are still poorly known even in the well-studied domestic hen (Schneider, 2007). Maintenance of vitellogenic follicles in the restricted-ovulator (R/O) chicken mutant, which lacks functional VTG/VLDL-R, does imply an alternative system for oocyte uptake (Elkin et al., 2003). However, it is not clear whether this reflects normal LRP380 function typical of 'wild-type' birds, as R/O hens are hyperlipidaemic with 4- to 5-fold elevated plasma levels of VLDL and VTG (Bujo et al., 1995a; Elkin et al., 2003), i.e. the presence of LRP380 receptors does not fully compensate for the lack of VTG/VLDL receptors. Finally, and more generally, it is becoming clear that both yolk precursor receptors bind and transport non-lipoproteins to the yolk (e.g. Mac Lachlan et al., 1994; Mahon et al., 1999). Variation in yolk precursor receptor expression, as we have documented here, might therefore provide an important mechanism mediating inter-individual variation in 'maternal effects', i.e. non-genetic contributions that mother's provide offspring *via* yolk uptake.

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