manner, to maintain chronic high plasma levels for later-developing eggs. If yolk E2 levels are associated with circulating levels we therefore predicted that in E2-treated females we would abolish, or even reverse, the sequence-specific pattern of yolk E2 concentrations.

2. MATERIAL AND METHODS

(a) *Animal care and breeding protocol*

Zebra finches were maintained in controlled environmental conditions (temperature 19–23 °C; humidity 35–55%; constant light schedule, 14 L : 10 D, lights on at 07.00). All birds were provided with a mixed seed diet (*Pa*ƒ and white millet, 1 : 3, 11.7% protein, 0.6% lipid and 84.3% carbohydrate by dry mass), water, grit and cuttlefish bone (calcium) *a* , and received a multivitamin supplement in the drinking water once per week. Breeding pairs were also provided with 6 g/pair per day of egg food supplement (20.3% protein, 6.6% lipid) between pairing and clutch completion. All birds used in this experiment were of the 'wild type' plumage morph and were aged 6 months or older. Breeding pairs were housed individually in cages (61 cm × 46 cm × 41 cm), each with an external nest-box (11.5 cm × 11.5 cm × 11.5 cm). Females were weighed (± 0.1 g, initial mass) at the time of pairing, at the one-egg stage, and again at the five-egg stage or at clutch completion (depending on which stage the bird reached first). Nest-boxes were checked daily between 09.00 and 11.00 and all new eggs were weighed (to 0.001 g) and numbered, to obtain data on egg size, clutch size and laying interval (the time between pairing and laying of the first egg). Experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit (no. 558B), in accordance with guidelines from the Canadian Committee on Animal Care (CCAC).

(b) *Experimental protocol*

For any pairs that initiated egg laying, females were alternately assigned to either a control (vehicle-injected) group or an oestradiol-treated (E2) group. The first-laid egg was collected to assay the yolk for oestradiol and was replaced with a zebra finch egg of approximately the same size (to maintain clutch size). Birds that were treated with E2 were given daily intramuscular injections of 17β-estradiol at $1\,\mu g\,g^{-1}$ in 30 µl of canola oil from the one-egg stage until they laid their fifth egg or until clutch completion (for birds that laid only four eggs). Control females received daily injections of 30 µl of canola oil only. The third, fourth and fifth eggs were also collected if laid for oestradiol analysis of yolks, and were again replaced with dummy eggs. At the five-egg stage, or at clutch completion for birds laying four eggs, birds were killed via exsanguination (using the anaesthetic Rompun/Ketamine, 1 : 1 v/v) and blood samples obtained. We dissected out the oviducts, ovaries and liver, which were dried to constant weight (dry mass, ± 0.001 g) in a 60 °C drying oven. Clutch size was recorded as all laid eggs plus the oviductal egg if present (no birds in either group had any remaining pre-ovulatory, yolky follicles in the ovary at the time of collection). All blood samples were centrifuged at 1800 for 10 min and the plasma was separated and frozen at −20 °C until assayed for plasma vitellogenin (VTG) or oestradiol. We therefore obtained plasma samples from birds at the one- and four- or five-egg stages, and yolk samples from eggs 1, 3, 4 and 5 in the laying sequence.

(c) *Hormone determination*

Thecon3 TJ.9(bir524.8(wh(o65.4xx/F1)-582 11.5(a9.2(A524.831qs37(grou2eor)-.4(ande5D (frry)2F1)9(birc)Tj325(.2(A)-26r)-249-296.59TD

CV = 10.3%). Antibody cross-reactivity to testosterone was less than 0.03% and to cholesterol 0.46%.

3. RESULTS

There was marked inter-individual variation in plasma E2 levels in females sampled at the one-egg stage (figure 1*a,* ; range 0.62–3.07 ng ml^{-1}; no effect of body mass, > 0.5), but there was no difference between birds subsequently assigned to control or E2 treatment: control, 1.41 ± 0.30 ng ml^{-1} (s.e.), $\mathcal{F} = 7$, E2-treated, 1.37 ± 0.21 ng ml^{-1} (Wilcoxon rank test, $Z = 0.1$, $>$

egg stages (egg 3, $F_{1,16} = 13.7$, < 0.01; egg 4, $F_{1,16} = 9.27$, < 0.01; egg 5, $F_{1,16} = 5.79$, < 0.05).

Given the timing and pattern of yolk formation in zebra finches (Haywood 1993), most of the yolk for the third-laid egg (*a.* 60%) would have been deposited during the day and night after the female laid her first egg (i.e. within 24 h of the first E2 treatment; note that E2 treatment already affected yolk E2 in the third-laid egg; see above and figure 3). Comparing plasma E2 levels at the one-egg stage with E2 levels in yolk from the third-laid egg, there was a significant negative relationship between these variables in control females ($= -0.87$, $= 7$, < 0.025). However, in E2-treated females yolk E2 levels were independent of plasma E2 concentrations (figure 3).

4. DISCUSSION

We have shown that there can be systematic variation in yolk oestrogen concentrations, in relation to laying sequence, similar to that widely reported for androgenic steroids: in sham-manipulated females, yolk E2 levels decreased with laying sequence. By injecting females with oestradiol-17β from the one-egg stage, we altered the endogenous pattern of plasma E2, such that this was higher during the period of rapid yolk development of later-laid eggs. As a consequence, we reversed the laying-sequence-specific pattern of yolk E2 concentrations: in E2-treated females, yolk E2 concentrations increased with laying sequence. This confirms that the yolks of late-laid eggs developed in a very different maternal hormonal milieu. Overall, yolk E2 levels were a direct reflection of plasma E2 levels. However, at the individual level we found a negative relationship between plasma E2 levels at the egg 1 stage and yolk E2 levels in egg 3. Furthermore, among sham-manipulated females there was considerable inter-individual variation in plasma E2 levels, comparing early- and late-laying birds, with plasma levels decreasing, remaining more or less constant, or even increasing over this period in different individuals.

There have been very few detailed studies of day-to-day, or laying-sequence-specific, patterns of changes in plasma hormone levels during the laying cycle, other than for poultry species (Etches 1996). In free-living female starlings (*S.*) plasma E2 levels increase rapidly from the onset of rapid yolk development to reach maximum levels in birds with a complete follicle hierarchy (four or more yolky follicles). However, levels decrease linearly throughout the later stages of follicle development, returning to pre-breeding values the final yolky follicle is ovulated (Williams *a.* 2004). This pattern is very similar to that reported by Sockman & Schwabl (2000) using faecal oestradiol-17β measurements in captive canaries (*S.* *a.*) during the laying cycle. These data suggest that early- and late-developing yolks are exposed to very different hormonal environments during development and, in the present study, we have shown that this can

ovarian output of E2, and hence plasma levels, should always peak at the onset of ovulation and decline through later stages of laying. Thus, if yolk steroid levels reflect plasma steroid levels, females would be unable to produce yolks in which E2 levels increase through the laying sequence. Data available so far support this scenario for oestrogens: the concentrations of both plasma and yolk oestrogens decrease with egg-laying sequence (Sockman & Schwabl 2000; Elf & Fivizzani 2002; Williams *et al.* 2004) and experimental elevation of plasma E2 levels increases yolk E2 levels (Adkins-Regan *et al.* 1995; this study). Thus, female birds appear to have a limited capacity to uncouple yolk E2 levels from variation in circulating levels (cf. studies in lizards (Painter *et al.* 2002; Lovern & Wade 2003), though these might not be strictly comparable owing to differences in timing and duration of follicle development). However, our results suggest that at the individual level there is some flexibility or variability among females in the endogenous pattern of plasma E2 levels through the laying cycle (see figure 1*a*) that could generate inter-individual variability in yolk hormone levels even if these primarily reflect circulating steroid levels. Given the evidence suggesting that E2 treatment can affect offspring development (Adkins-Regan *et al.* 1995; Millam *et al.* 2001; von Englehardt *et al.* 2004), and considering concerns about environmental contaminants that have oestrogenic effects in wildlife (Ankley *et al.* 1998), individual and ecological factors influencing yolk oestradiol-17β are worthy of future attention.

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