Variation in female reproductive tract morphology across the reproductive cycle in the zebra finch

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Background. In seasonally breeding birds, the reproductive tract undergoes a dramatic circannual cycle of recrudescence and regression, with oviduct size increasing 5-220 fold from the non-breeding to the breeding state. Opportunistically breeding birds can produce multiple clutches sequentially across an extended period in response primarily to environmental rather than seasonal cues. In the zebra finch, it has been shown that there is a significant reduction in gonadal morphology in non-breeding females. However, the scale of recrudescence and regression of reproductive tissue within a single breeding cycle is unknown and yet important to understand the cost of breeding, and the physiological readiness to breed in such flexible breeders.

Methods. We examined the reproductive tissue of breeding female zebra finches at six stages in the nesting cycle from pre-breeding, to fledging offspring. We quantified the wet mass of the oviduct, the volume of the largest pre-ovulatory follicle, and the total number of pre-ovulatory follicles present on the ovary.

Results. Measures of the female reproductive tract were highest during nesting and laying stages and declined significantly in the later stages of the breeding cycle. Importantly, we found that the mass of reproductive tissue changes as much across a single reproductive event as that previously characterised between birds categorised as breeding and non-breeding. However, the regression of the ovary is less dramatic than that seen in seasonal breeders. This could reflect low-level maintenance of reproductive tissues in opportunistic breeders, but needs to be confirmed in wild non-breeding birds.

1 **Variation in female reproductive tract morphology across the reproductive cycle in the**

2 **zebra finch**

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19 **Abstract**

20 **Background**. In seasonally breeding birds, the reproductive tract undergoes a dramatic

21 circannual cycle of recrudescence and regression, with oviduct size increasing 5-220 fold from

22 the non-breeding to the breeding state. Opportunistically breeding birds can produce multiple

23 clutches sequentially across an extended period in response primarily to environmental rather

24 than seasonal cues. In the zebra finch, it has been shown that there is a significant reduction in

25 gonadal morphology in non-breeding females. However, the scale of recrudescence and

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31 oviduct, the volume of the largest pre-ovulatory follicle, and the total number of pre-ovulatory

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33 **Results.** Measures of the female reproductive tract were highest during nesting and laying stages

34 and declined significantly in the later stages of the breeding cycle. Importantly, we found that the

35 mass of reproductive tissue changes as much across a single reproductive event as that

36 previously characterised between birds categorised as breeding and non-breeding. However, the

37 regression of the ovary is less dramatic than that seen in seasonal breeders. This could reflect

38 low-level maintenance of reproductive tissues in opportunistic breeders, but needs to be

39 confirmed in wild non-breeding birds.

40

41 **Keywords**: Finch; Opportunistic breeder; Ovarian development; Oviduct; Reproduction

42 **Introduction**

43   The avian reproductive system is typically viewed as a dynamic structure that exhibits

44 dramatic changes in size and development across seasons (Williams 2012). For example, in

45 seasonally breeding birds, the reproductive tract undergoes a circannual cycle of regression and

46 recrudescence (Johnson & Woods 2007; Keck 1934; Wingfield & Farner 1993). In passerines,

47 measurements taken during non-breeding and breeding periods show that oviductal wet mass

48 increases by up to 220-fold during the breeding season, while the linear dimensions of the largest

49 follicle have been shown to increase by up to 60-fold (see Table 1). The reproductive cycle in

50 seasonally breeding birds is primarily regulated by day length and, to a lesser extent, is finely

51 tuned by environmental conditions such as rainfall, temperature, and access to nesting sites

52 (Dawson et al. 2001; Wingfield & Farner 1993; Wingfield et al. 1992). In temperate

53 environments with distinct seasons, day length is a reliable indicator of food availability.

54 Therefore, seasonal environments are thought to exert strong selective pressure on reproductive

55 physiology that allows birds to time reproduction during peak seasonal food availability.

56   However, not all bird species breed during discreet or predictable periods of time. Some

57 species exhibit flexible breeding periods or breed opportunistically when conditions become

58 favourable. In opportunistically breeding bird species, reproduction is timed primarily through

59 non-photic cues such as food availability, temperature, and rainfall, and displays a dampened

60 seasonal peak (Englert Duursma et al. 2019; Hahn et al. 1997; Hahn et al. 2008; Hau et al. 2008).

61 In contrast to seasonally breeding birds, opportunistically breeding species are hypothesised to

62 maintain some degree of gonadal development year-round in order to rapidly initiate

63 reproduction when ecological conditions become optimal (Wingfield 2008).

64   Compared to the well-established literature on seasonally breeding birds, relatively few

65   studies have investigated variation in reproductive morphology in opportunistically breeding

66   birds. The studies that have been conducted suggest that there is annual variation in gonad

67   development, but full regression is not always evident (Hahn 1998; Perfito et al. 2007). This

68   supports the hypothesis that the there is some maintenance of gonads potentially allowing

69   opportunistic breeders to rapidly exploit reproductive opportunities. For example, in wild zebra

70   finches (*Taeniopygia guttata*), Perfito et al. (2007) reported a mean follicle size (for the largest

71   follicle in each female) of $3mm^3$ and $44mm^3$ (early and late within a breeding season,

72   respectively) and a mean follicle size (of the largest follicle) of around $5mm^3$ in a non-breeding

73   population (all values estimated from Fig. 2 in Perfito et al. (2007)). These data suggest that the

74   smallest values found in breeding birds are equivalent to those in non-breeding populations.

75   However, an important caveat is that the exact breeding status of the females studied by Perfito

76   et al. (2007) was uncertain. In a laboratory study, Prior et al. (2013) used a water deprivation

77   treatment to significantly inhibit reproduction in females. Specifically, Prior et al. (2013)

78   reported that the largest follicle diameter in each female remained 1.8 mm on average. These

79   data suggest follicle volume in water restricted non-breeding females averaged $3.05 \ mm^3$ (using

80    $V=4/3\pi a^3$ where $V=$ volume and $a =$ radius of follicle), making them somewhat smaller than

81   those reported by Perfito et al. (2007) in a non-breeding wild population in an unpredictable

82   environment, but equivalent to late-breeding in a predictable environment. Importantly, however,

83   these values are greater than the minimum size of the fully regressed follicles reported in

84   seasonal breeders (see Supplemental Table S1).

85   The significant shift in follicle volume and oviduct mass in water restricted zebra finches,

86   did not coincide with a change in estradiol levels (Prior et al. 2013). Further, in wild zebra

87   finches detectable estradiol was found in one of nine non-breeding birds in an unpredictable

88   environment, but in none of the five birds tested when breeding in the predictable environment

89   (Perfito et al 2007). Both of these findings are surprising given that in birds, estradiol aids in

90   regulating reproduction by stimulating the production of yolk precursors from the liver (Wallace

91   1985), and stimulating oviduct development (Pollock & Orosz 2002; Williams 1999). It is

92   possible that changes in estradiol will only be seen during nesting and egg laying when it is

93   stimulating yolk formation and oviduct growth and development (Pollock & Orosz 2002;

94   Wallace 1985; Williams 1999). However, studies conducted thus far have not looked at estradiol

95   production in zebra finches at this fine temporal resolution.

96   Although past studies have established that opportunistically breeding birds broadly

97   maintain a degree of gonadal development when not breeding, no study to date has examined

98   fine scale variation in gonad development within and between reproductive cycles. The average

99   follicle sizes of the non-breeding zebra finches (Perfito et al. 2007; Prior et al. 2013), likely

100   represent the minimal values for the largest follicle in each female in this species. However, they

101   leave two important gaps in our knowledge. Neither study (Perfito et al. 2007; Prior et al. 2013)

102   characterised variation in oviduct tissue or tracked variation in follicle size of birds with known

103   breeding status i.e. across different phases of active breeding such as egg-laying, incubation,

104   offspring rearing etc. As such, the maximal size of both the oviduct and pre-ovulatory follicles is

105   unclear. As a result, and also due to a lack of clarity over the breeding status of birds, it is not

106   clear how quickly, and to what extent reproductive tissues cycle in this model opportunistic

107   breeder. For example, as illustrated in Fig. 1, in comparison to seasonal breeders (Fig. 1A),

108   females could regress their tissues within and between reproductive bouts to the same (Fig. 1B),

109   or lesser extent (Fig. 1C).

110   Studies have suggested that zebra finches consistently maintain some level of gonad

111 functionality, even within a reproductive cycle. For example, female zebra finches can lay eggs

112 within five days of pairing (Haywood 1993; Williams 1996). Additionally, studies show that

113 regression of the oviduct commences as females are laying their last egg (Williams & Ames

114 2004) and that pairs frequently initiate a new clutch before the previous fledglings are fully

115 independent (Griffith et al. 2017). However, no study to date has quantified variation in

116 reproductive tract morphology at different stages throughout the reproductive cycle in an

117 opportunistically breeding bird. Further examination of the extent of variation in reproductive

118 morphology in the zebra finch, as a model opportunistically breeding species, will help us to

119 understand the costs and constraints involved in mounting a reproductive attempt in those birds

120 that have highly flexible breeding times. This is of interest in the context of the rapidly changing

121 ecological conditions that such opportunistically breeding species can face, and the ability of a

122 male and female to physiologically coordinate their reproductive activity and investment

123 (Griffith 2019).

124   Here, we investigated variation in female reproductive tissue morphology and circulating

125 hormone levels (i.e. estradiol) across breeding stages of a single reproductive episode in the

126 zebra finch. Given the ability of zebra finches to rapidly initiate reproduction, we predicted that

127 females would maintain some level of gonadal development across a reproductive cycle.

128 Likewise, we predicted that females would maintain moderate levels of estradiol across most of

129 the reproductive cycle, even when their reproductive development is significantly reduced (Prior

130 et al. 2013).

131

132 **Materials and Methods**

Animals

133

134   Thirty-six pairs of domestic zebra finch were force paired (i.e. we chose partners) into

135   individual cages and allowed to lay at least one clutch of eggs together prior to the

136   commencement of the study. All individuals were bred and maintained at Macquarie University

137   (Sydney, Australia) and were between 18-24 months of age. Between February and May 2015,

138   pairs were moved into one of 12 outdoor aviaries (L x W x H: 4.1 x 1.85 x 2.24 m, 3

139   pairs/aviary) that were physically, but not visually or vocally separated from other pairs. All

140   procedures were conducted according to relevant national and international guidelines and were

141   approved by the Macquarie University Animal Ethics Committee (Animal Research Authority

142   2013/29).

143

Experimental design

144

145   Pairs were then randomly assigned to one of six sampling time points (n=6 per time

146   point) across the breeding cycle: pre-breeding (paired, but without a nest for a total of four weeks

147   including first two weeks considered acclimation), nesting (during nest building, once at least the

148   bottom of the nestbox was lined with material), laying (day of laying third egg), incubation, post-

149   hatch (six days post hatching), and fledging (day after first fledgling was observed out of nest).

150   We chose not to sample on day one of egg laying or immediately post-hatch in order to ensure

151   pairs were committed to the breeding stage (i.e. laying a full clutch and rearing nestlings). All

152   birds were given two weeks to acclimate to the new aviary conditions, after which they were

153   provided with nest boxes and nesting material (with exception of those birds in the pre-breeding

154   group). All pairs were checked daily during acclimation and experimental time period to note

155   change in breeding stage, and ensure no nest building or egg laying was occurring in acclimating

156   or pre-breeding pairs.

157

158   **Blood and tissue collection**

159   At the designated sampling time point, a whole blood sample was collected from each

160   female within 5 minutes of initial disturbance, and the sample held on ice (for a maximum period

161   of 1 hour) before plasma was separated from red blood cells and plasma stored at -80 °C until

162   later analysis of estradiol. Females were euthanised using deep anaesthesia with isoflurane. The

163   oviduct was then dissected out (cut off at cloacal juncture) and weighed (after lightly blotting to

164   remove any blood, and if present, eggs were removed prior to weighing). Next, the ovary was

165   dissected out and photographed from two angles, which were then used to later assess the total

166   number of pre-ovulatory follicles present. Finally, on the fresh ovary, the size of the largest pre-

167   ovulatory follicle was measured (three perpendicular measures) using digital callipers (to nearest

168   0.01mm). Follicular volumes were calculated using the formula for an ellipsoid, $V = 4/3\pi abc$,

169   where the axes a, b, and c are equal to half of the value recorded for each of the three

170   perpendicular measures, to account for deviations from a spheroid in large pre-ovulatory

171   follicles. All nestlings and fledglings were successfully fostered to other pairs of zebra finch in

172   the Macquarie University breeding colony.

173

174   **Plasma estradiol analysis**

175   Plasma estradiol levels were quantified using a 17-β estradiol high-sensitivity Enzyme

176   Immunoassay (EIA) kit (Cat No. ADI 901-174, Enzo Life Sciences) following the standard

177   protocol. Briefly, plasma samples (10-30µl) were diluted in assay buffer to a final volume of

178   200µl and, following incubation and washing steps, sample absorbance measured at 405nm

179   using a Varioskan LUX microplate reader (Thermo Scientific). All samples were measured in

180   triplicate, and estradiol levels extrapolated from a ten-point standard curve ranging from 1.95 to

181   3,000 pg/ml, with a minimum detection limit of 0.8 pg/ml. The average intra-plate coefficient of

182   variation was 9.99% and the inter-plate variation 6.55%. Samples were randomly distributed

183   across plates. One pre-breeding sample was not run as it appeared dehydrated.

184

185   **Statistical analyses**

186   All analysis was done with R v. 3.5.3 (R Core Team 2019) in the R package *lme4* (Bates

187   et al. 2015). We used linear models to examine the effect of breeding stage on oviduct mass (log

188   transformed) and volume of the largest pre-ovulatory follicle. Next, we examined the impact of

189   breeding stage on the number of pre-ovulatory follicles using a Bayesian generalized linear

190   model via Stan with negative binomial family function (to control for zero inflation) and

191   breeding stage as a fixed effect using the R package *rstanarm* (Goodrich et al. 2018). In these

192   models, we included scaled mass index (SMI: using tarsus length and body mass, (Peig & Green

193   2009) as a covariate, but when considered alone there was no significant difference in SMI

194   between breeding stages (ANOVA; $F_{5,30} = 0.84$, $P = 0.53$). We did not run a formal statistical

195   analysis on estradiol data due to the low number of detectable samples (see below). Figures were

196   constructed using the R package *yarrr* (Phillips 2017), and modelling assumptions (normality

197   and heterogeneity of variance of residuals) were assessed visually (following Zuur et al. 2009).

198   All tests were two-tailed and considered significant at $\alpha < 0.05$, with significance estimates via

199   *lmerTest* (Kuznetsova et al. 2016) for models, and *emmeans* (Russell 2019) for pairwise

200   comparison of time points corrected for multiple testing using Tukey HSD p-value adjustment.

201   Data presented are mean ± standard deviation unless otherwise noted.

202

203   **Results**

204   **Change in reproductive tissues**

205   Oviduct mass declined significantly between laying (0.43 ± 0.09 g) and incubation (0.06

206   ± 0.03 g), reaching its lowest mass during the post-hatch period (0.03 ± 0.01 g) and remaining

207   low for the remainder of the breeding cycle (Table 1, Fig. 2A). Similarly, following a peak in

208   size during nesting (74.9 ± 75.5 mm^3), follicle volume decreased significantly by incubation

209   (2.48 ± 0.8 mm^3) before reaching its lowest volume during the post-hatch (2.45 ± 1.0 mm^3)

210   period (Table 1, Fig. 2B). As expected, the number of ovarian pre-ovulatory follicles was highest

211   during the pre-breeding and nesting stage. The number of follicles then declined during laying,

212   before reaching zero during both the incubation and post-hatch period. The number of pre-

213   ovulatory follicles increased again during the fledgling period, albeit non-significantly and to a

214   lesser degree than the number observed in the pre-breeding stage (Table 2, Fig. 2C).

215

216   **Estradiol detection and levels**

217   Estradiol levels were non-detectable in 22 of the 35 samples assayed (62.9%), although

218   one detectable plasma sample appeared dehydrated and was therefore excluded. Of the

219   remaining detectable samples ($n = 13$), there was a tendency for these samples to be present in

220   the early stages of the breeding cycle and the highest average value was observed during the

221   nesting stage (Fig. 3).

222

223 **Discussion**

224   Across a single reproductive episode, from pre-breeding through to fledging of nestlings,

225 female zebra finches showed dramatic variation in the size and development of their

226 reproductive tract tissue (Fig. 4). Oviduct and ovary weight were highest during nesting and

227 laying and regressed during the later stages of the breeding cycle. These results are generally

228 consistent with other studies in this species (Williams & Ames 2004) and a range of northern

229 hemisphere, temperate zone birds (Dawson 2008; Hurley et al. 2008; Jacobs & Wingfield 2000;

230 Ramenofsky 2011). Similar to the variation in ovary development that we described here, the

231 length of the sperm storage tubules in females vary across a reproductive cycle (Pellatt, 1998)

232 suggesting that the entire female reproductive changes in opportunistically breeding birds. We

233 found that pre-ovulatory follicular development followed a similar pattern, though some females

234 already possessed pre-ovulatory follicles when their first chicks fledged. Importantly, while the

235 observed regression of the female reproductive tract in zebra finches is reminiscent of the

236 regression of these tissues in temperate zone birds, the magnitude of these changes appears to be

237 considerably lower in the zebra finch (Supplemental Table S1). As such, the reproductive tract

238 regression observed in zebra finches can be considered more similar to the resting phase (e.g. the

239 period between first phase differentiation and breeding when development can be suspended:

240 (Sossinka 1980)) of a photosensitive seasonally breeding bird than to that of the fully regressed

241 photorefractory state of such a species.

242   It is widely held that the circannual recrudescence and regression cycle observed in

243 seasonally breeding species mediates the energetic and physiological costs of egg production and

244 the maintenance of reproductive tract tissues. These costs include protein depletion and

245 compromised flight ability, as well as non-resource-based costs such as the negative pleiotropic

246   effects of maintaining high hormone levels, e.g. immunosuppression (reviewed in Williams

247   2005). The idea that such costs influence reproduction is further supported by the fact that

248   seasonally breeding birds typically only engage in one energetically expensive life history stage

249   (e.g. reproduction, moult, migration) at a time (Follett 2015; Wingfield 2008). Furthermore, a

250   number of seasonally breeding species have been shown to shift their metabolic rates with

251   reproductive state (e.g. great tit, *Parus major* (Nilsson & Råberg 2001); European starlings,

252   *Sturnus vulgaris*: (Vézina & Williams 2002)), with this change in metabolism being related to

253   changing energetic demands of reproductive tissue, as well as the liver and gizzard during chick-

254   rearing (Vézina & Williams 2003). Given this, the semi-regressed 'resting phase' state we

255   observed in female zebra finches would likely allow females to rapidly recommence breeding

256   once their current brood is independent, whilst also allowing them to mitigate energetic costs of

257   reproduction via the rerouting of energy from reproductive tissue investment to other activities

258   such as chick rearing. Such rapid turnaround in breeding attempts is supported by our finding

259   that some females exhibited pre-ovulatory follicles as early as the day after the first chick

260   fledged, and is consistent with breeding observations in captive populations (Griffith et al. 2017).

261   The variation between individuals with respect to the presence of pre-ovulatory follicles

262   during the fledgling period could be considered surprising given that all birds were housed under

263   identical conditions. However, zebra finch pairs frequently exhibit considerable variation in the

264   number of days taken to initiate a new clutch, which would likely reflect underlying variation in

265   the presence of pre-ovulatory follicles. In turn, this variation can be attributed to variation in

266   breeding experience as a pair (Hurley et al. 2020) and variation in individual condition and life-

267   history trade-offs (Williams 1996; Williams 2005). The observed variation in follicle

268   development is broadly consistent with findings in wild zebra finches in unpredictable, arid

269   environments. Specifically, Perfito et al. (2007) reported a proportion of female birds were not

270   completely regressed and had small follicles present when sampled despite an apparent absence

271   of breeding within the population (49% in predictable temperate population during winter and

272   58% during a drought in late Spring in an unpredictable arid population).

273   Estradiol levels observed in this study were highly variable and a large proportion of the

274   samples were below the detection level. Whilst it was disappointing that we were unable to assay

275   the level of estradiol in so many of our samples, we feel that the data are worth publishing, and

276   that, for the reasons discussed below, they are likely to reflect the underlying biology, and really

277   low values, rather than methodological problems. Past studies have also reported large numbers

278   of undetectable samples. For example, in a study of wild zebra finches using identical methods,

279   estradiol was reported as non-detectable in 38.3% of samples (Crino et al. 2018), and in an

280   earlier study of another passerine, the Western scrub-jay (*Aphelocoma californica*) the authors

281   were unable to detect estradiol levels in the majority of their samples using similar methods

282   (Rensel et al. 2015). The higher percentage of detectable samples in Crino et al. (2018) are likely

283   explained by the use of gonadotropin-releasing hormone challenges to induce maximum

284   estradiol release, whereas as our study examines natural endogenous levels. Finally, the peak in

285   estradiol levels observed during the early stages of the reproductive episode, i.e. nesting and

286   laying, is in line with the essential role estradiol plays in the growth and development of the

287   oviduct (Pollock & Orosz 2002; Williams 1999), as well as egg yolk and eggshell formation

288   (Mishra et al. 2019). This suggests that levels in the zebra finch are generally low, and in many

289   individuals below the threshold of detectability, but that they do show the anticipated pattern of

290   variation across the cycle.

291   Somewhat surprisingly, we found that ovary and oviduct development of females in the

292   pre-breeding stage did not differ from those in the nesting stage. There are at least two plausible

293   explanations for these results. First, domestic zebra finches are highly motivated to breed, and,

294   when paired, females will frequently lay eggs on any suitable surface despite a lack of nest boxes

295   and nesting material. Moreover, as oviduct and follicular development is sensitive to a change in

296   circulating hormones, e.g. estrogen, lutenizing hormone, follicle stimulating hormone (Johnson

297   & Woods 2007; Pollock & Orosz 2002), the still somewhat favourable conditions experienced by

298   these birds (e.g. *ad lib* water and food) may have been sufficient to support elevated levels of

299   hormones linked to reproductive tissue development. Thus it seems likely that females defined as

300   pre-breeding in our study are functionally equivalent to seasonal breeders in a pre-laying state

301   (when predictive cues have primed reproductive tissue), and not a completely non-breeding state.

302   It is unclear how long females will maintain readiness, especially in variable ecological

303   condtions. In normal captive conditions, it can be very difficult to prevent birds from breeding

304   due to the provision of relatively good resources (Griffith et al 2017). Even though the females in

305   our pre-breeding treatment were unable to physically breed due to the lack of a nest site, this is

306   quite an unnatural constraint, that would not be encountered in the wild and it seems likely that

307   physiologically these females were ready to breed. Second, we defined pairs as nesting once

308   males were observed actively building nests. Thus, our distinction between pre-breeding and

309   nesting stage may have been too coarse to detect differences between these stages. Pairs may

310   have already established themselves into reproductive mode in the days before nest building

311   started through the many subtle behaviours and forms of communication that we expect in such

312   closely coordinated partners (Griffith 2019). It is therefore very difficult in captivity to get zebra

313   finches to a state where they can be confidently established to be in a stable and lengthy non-

314   breeding mode. It therefore remains to be checked in a wild population whether the gonadal and

315   endocrine state that we have reported in the pre-breeding birds is truly reflective of non-breeding

316   birds. To an extent however, we are reassured that the wild birds assessed by Perfito et al. (2007)

317   as being in a non-breeding state had a range of folicle size that was similar to those found in our

318   pre-breeding birds (Supplemental Table S1).

319

320   **Conclusion**

321   Detailed examination of the female reproductive tract of zebra finch across a single

322   breeding episode revealed considerable variation in oviduct and ovary development, and a

323   pattern of limited reproductive tract regression during the later stages of the reproductive cycle

324   (i.e. incubation, post-hatch). We suggest the pattern of regression we observed is consistent with

325   the lack of a photorefractory period with less regression than seen in another opportunistically

326   breeding species (Hahn 1998), and stands in stark contrast to observations in seasonally breeding

327   birds. By dampening the degree of regression of the reproductive tract, female zebra finches

328   might maintain a general state of reproductive readiness, whilst also mediating some of the

329   energetic costs associated with reproduction. Future studies could test this hypothesis by

330   examining the metabolic costs associated with partial reproductive regression in

331   opportunistically breeding birds. Furthermore, future studies aimed at determining whether

332   similar patterns of variation in female reproductive tract morphology are observed in the many

333   other species that show a similar or greater extent of reproductive plasticity with respect to

334   phenology (Englert Duursma et al. 2017) would be valuable. To date much of the focus on the

335   extent of female reproductive tract variance has focused on seasonal breeders of the northern

336   hemisphere, and it is likely that quite different patterns will be seen in Australia and other

337   ecologically unpredictable and aseasonal environments (Astheimer & Buttemer 2002).

338

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341   Mitch Francis for assistance in monitoring pairs, as well as photography and labelling during

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344   manuscript; and the staff of Macquarie Animal Research Services for animal care.

345

346   **Author Contributions**

347   LLH, MR, and SCG conceived the study and designed the experiments. LLH conducted

348   the animal and data collection portion of the experiment and wrote the first draft of the article.

349   OLC conducted the estradiol assay. All authors contributed to editing and writing of the final

350   manuscript and approved its submission.

351

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467

Figure 1

Predicted relative change in reproductive tissue mass.

Change in reproductive tissue mass with breeding events is clearly define in many (A) seasonal breeders with some development prior to rapid increase before breeding (arrow), followed by dramatic decrease after breeding. It is less clear if multiple breeding opportunistic breeders (B) completely regress or (C) partially regress reproductive tissue. Partial regression could be minimal (hatched line) or more significant both with in and out side of active breeding periods.
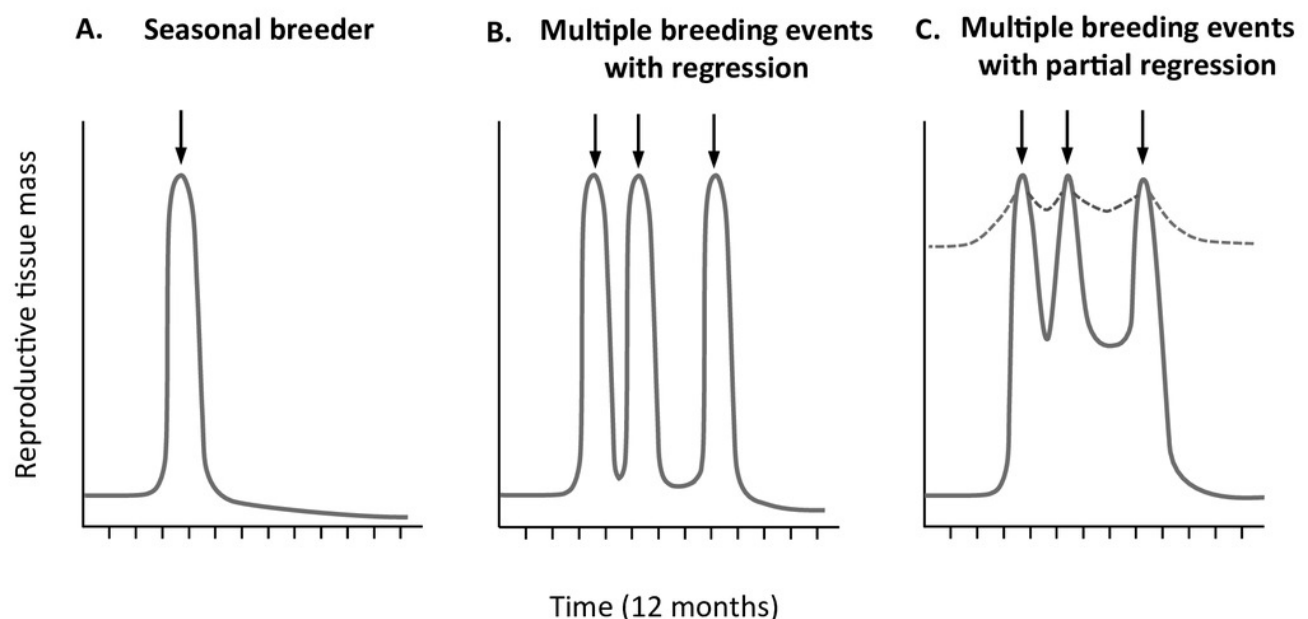
Figure 2

Quantified variation in zebra finch reproductive tract tissue across and within the six stages of the reproductive cycle.

Variation in (A) oviduct mass (g), (B) volume of the largest pre-ovulatory follicle (mm^3), and (C) the number of pre-ovulatory follicles at six reproductive stages: pre-breeding, nesting, laying (day of third egg), incubation, post-hatch, and fledging (day after first fledgling was out of nest). Grey circles represent raw data points, black circle denotes mean with standard error lines.
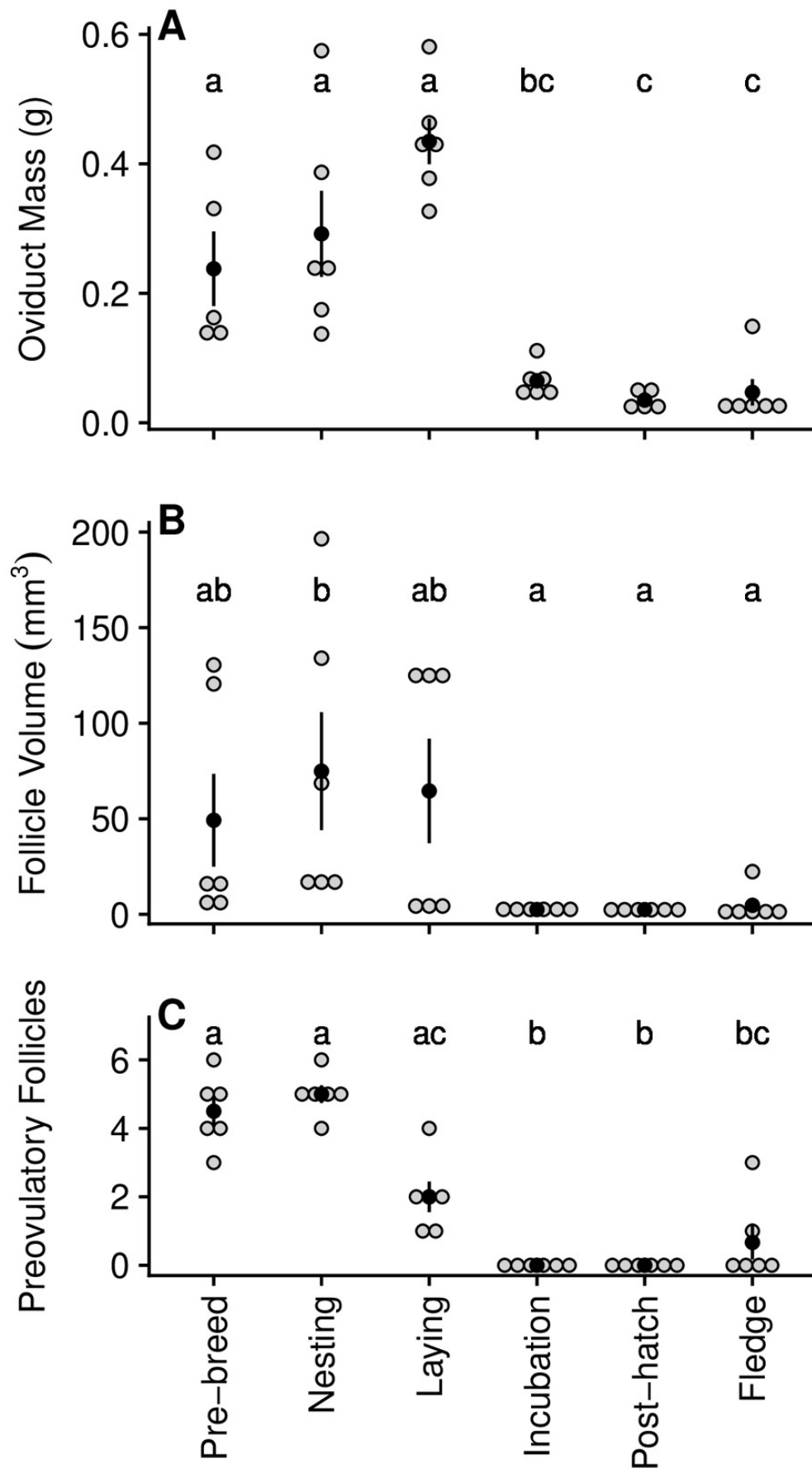
Figure 3

Variation in and detection of plasma estradiol across the reproductive cycle.

Stages in the reproductive cycle: pre-breeding, nesting, laying (day of third egg), incubation, post-hatch, and fledging (day after first fledgling was out of nest). Left axis: levels of circulating estradiol (pg/ml) in plasma. Black circle denotes mean with standard error lines, grey circles represent raw data points. Right axis: sample detectability. Bar denotes number of samples analysed at each sampling event, dark grey shading denotes samples with detectable levels of estradiol, open bar denotes non-detectable sample.
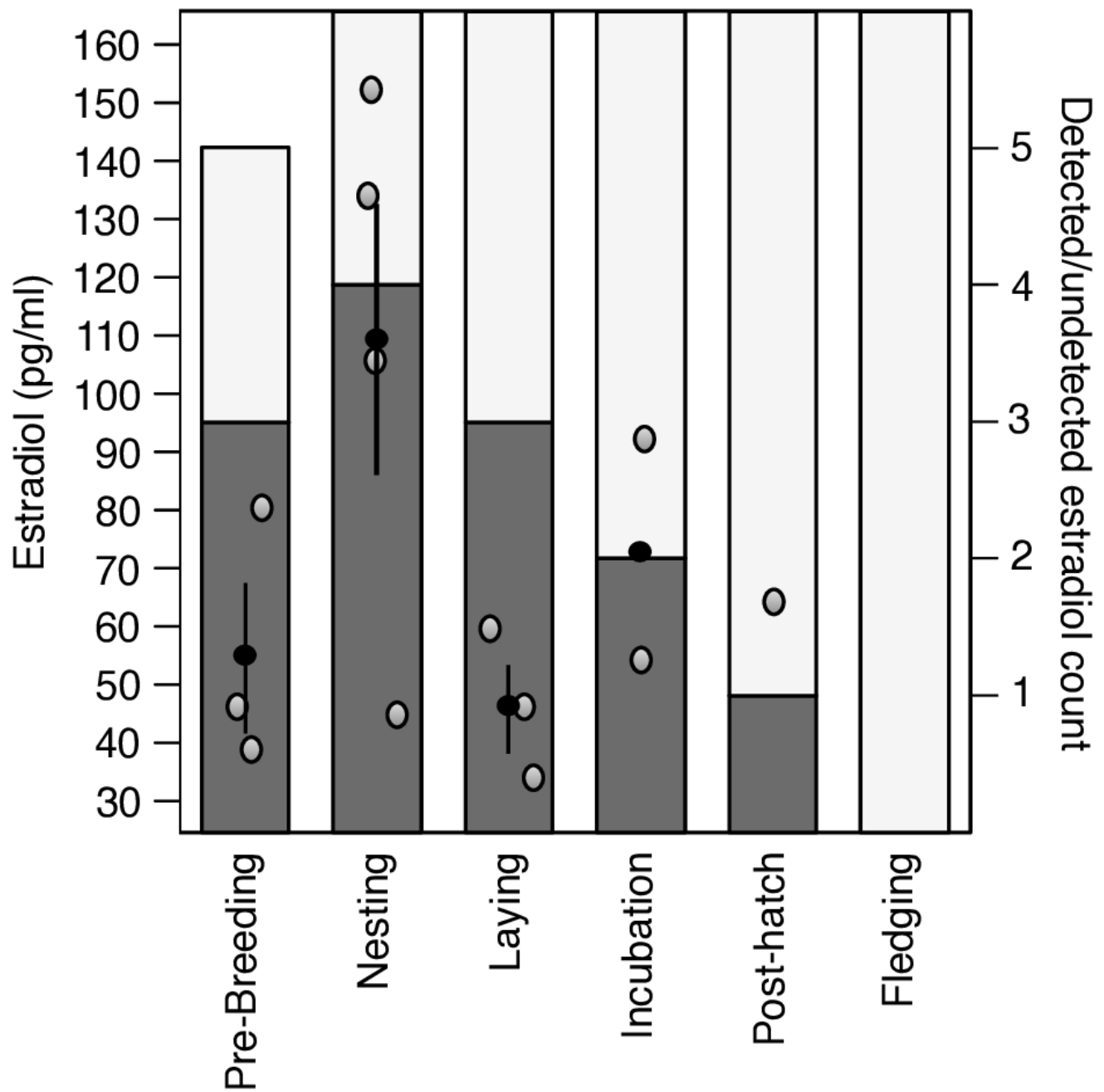
Figure 4

Variation in zebra finch female reproductive tract across and within the six stages of the reproductive cycle.

Images from across reproductive cycle (A-F) illustrating stage-specific changes in oviduct size, ovary size, pre-ovulatory follicle size, and the number of pre-ovulatory follicles. Left to right: ovary, oviduct orientated infundibulum to cloacal chamber. (A) Pre-breeding, (B) nesting, (C-1) egg laying (variant 1), female with pre-ovulatory follicle present on ovary suggesting additional eggs to be laid in the clutch, (C-2) egg laying (variant 2), female with no obvious pre-ovulatory follicles, suggesting no additional eggs to be laid, but an egg present in the uterus (d) mid-incubation, (E) six days post hatch, (F-1) fledgling (variant 1), female with pre-ovulatory follicle present on ovary suggesting rapid initiation to next breeding episode, (F-2) fledgling (variant 2), female without any observable pre-ovulatory follicles.

Pairwise comparison between breeding stages.

Post hoc comparison between six reproductive time points: pre-breeding (pre), nesting, laying, incubation, post-hatch, and fledging for both oviduct mass and the volume of the largest pre-ovulatory follicle present on the ovary in female zebra finches. Comparisons tested using *emmeans* with p-value adjustment for multiple comparisons using Tukey HSD.

1 **Table 1.**

	Oviduct mass					Follicle volume				
Contrasts	est.	SE	df	t ratio	*P*-value	est.	SE	df	t ratio	*P*-value
Pre-Nesting	-0.22	0.30	27	-0.75	0.97	-0.70	0.76	29	-0.92	0.94
Pre-Laying	-0.79	0.31	27	-2.57	0.14	0.14	0.78	29	0.19	1.00
Pre-Incubation	1.22	0.30	27	4.13	*0.004*	2.24	0.76	29	2.94	0.06
Pre-PostHatch	1.79	0.32	27	5.57	*< 0.001*	2.25	0.78	29	2.88	0.07
Pre-Fledging	1.68	0.32	27	5.31	*< 0.001*	2.44	0.79	29	3.10	0.05
Nesting-Laying	-0.57	0.29	27	-1.97	0.3825	0.85	0.78	29	1.08	0.89
Nesting-Incubation	1.44	0.28	27	5.14	*< 0.001*	2.94	0.76	29	3.86	*0.007*
Nesting-PostHatch	2.01	0.30	27	6.65	*< 0.001*	2.95	0.79	29	3.74	*0.01*
Nesting-Fledging	1.90	0.30	27	6.44	*< 0.001*	3.14	0.80	29	3.94	*0.006*
Laying-Incubation	2.01	0.29	27	6.97	*< 0.001*	2.09	0.78	29	2.68	0.11
Laying-PostHatch	2.58	0.29	27	8.77	*< 0.001*	2.11	0.76	29	2.76	0.09
Laying-Fledging	2.47	0.28	27	8.79	*< 0.001*	2.30	0.77	29	3.00	0.06
Incubation-PostHatch	0.57	0.30	27	1.88	0.43	0.01	0.79	29	0.02	1.00
Incubation-Fledging	0.46	0.30	27	1.57	0.63	0.20	0.80	29	0.25	1.00
PostHatch-Fledging	-0.11	0.30	27	-0.36	1.00	0.19	0.76	29	0.25	1.00

Variation the number of pre-ovulatory follicles across breeding stages in the zebra finch.

Shown are mean ± SD and Bayesian 95% credible intervals (CrI). Letters denote significance differences between given time points, columns with the same letter are not significantly different from one another, columns with different letters are significantly different as CrI boundaries do not overlap.

1   **Table 2.**

Time point	mean ± SD	CrI boundaries		
		2.50%	**97.50%**	
Pre-breeding	4.5±1.05	2.54	7.92	a
Nesting	5±0.63	2.77	8.84	a
Laying	2±1.09	0.94	3.81	ac
Incubation	0±0	0.001	0.42	b
Post-hatch	0±0	0.002	0.39	b
Fledging	0.67±1.20	0.19	1.67	bc