1 **Do mothers bias offspring sex ratios in carotenoid-rich**

2 **environments?**

3

4 Abbreviated title: Sex ratio variation and carotenoids in hihi

5

6 **Lay Summary**

7 If carotenoid availability more strongly influences male success, do mothers

8 supplemented with carotenoids produce more sons? We show this is not the case

9 in hihi, a species in which males have carotenoid-based plumage, and so may

10 benefit more from increased carotenoid availability during development. Dietary

11 supplementation can be a valuable conservation tool, but may have unintended

12 consequences. We show carotenoid supplementation is unlikely to alter

13 population dynamics in terms of offspring sex ratio in hihi.

14
15
16 **Abstract**

17 If environmental or maternal factors favour the fitness of one sex over the other,

18 theory predicts that mothers should produce more offspring of the sex most

19 likely to benefit from prevailing conditions. For species where males depend on

20 carotenoid-based colourful ornaments to secure territory or attract mates,

21 carotenoid availability in the environment could be one such component:

22 mothers experiencing high availability of carotenoids should produce more sons.

23   Here, we test this hypothesis by providing carotenoids to a wild population of a

24   sexually dimorphic passerine, the hihi (stitchbird: *Notiomystis cincta*). Access to

25   carotenoids during early life influences the colour of male hihi plumage, which

26   improves territory acquisition as adults. Therefore, carotenoid availability when

27   young may influence male fitness. However, we found no evidence of sex ratio

28   bias in treated or untreated groups, either before or after hatching. First-laid

29   eggs, where carotenoid concentrations are usually highest, were also unbiased.

30   For hihi, access to carotenoids during egg-laying does not appear to encourage

31   mothers to alter sex ratios of offspring. Alternatively, the fitness of daughters

32   may also benefit from increased carotenoids during development. Disentangling

33   these alternatives requires further work.

34

35   **Key words**: *Notiomystis cincta;* carotenoids; sex ratio; supplementary feeding

36

37

38   Introduction

39   The environment offspring experience during early life can have profound

40   effects on their reproductive productivity (Wilkin and Sheldon 2009; Walker et

41   al. 2013; Kilner et al. 2015), and these effects may differ between the sexes

42   (Trivers 1972; Trivers and Willard 1973). If good environmental conditions

43   increase the reproductive value of one sex more so than the other, mothers

44   should bias sex ratios to maximise their fitness returns (Trivers and Willard

45   1973; Charnov 1982). Some studies suggest that factors such as maternal

46 condition, age, and social rank may influence offspring sex ratios (reviewed in

47 Cameron 2004; West 2009), but experimental evidence for facultative sex ratio

48 manipulation remains mixed, particularly in birds (Sheldon 1998; Ewen et al.

49 2004). This may be due to a lack of information on pre-hatching sex ratios, which

50 may lead to an under-estimation of bias (Sheldon 1998). Alternatively (or

51 additionally), many studies investigating sex allocation in response to resource

52 availability have focused on general food availability (Wiebe and Bortolotti 1992;

53 Appleby et al. 1997), an approach which could overlook fluctuations in key

54 nutrients that may have sex-specific benefits.

55   One class of nutrient that may have different consequences for each sex is

56 carotenoids. These antioxidant pigments can only be obtained through diet

57 (Blount et al. 2000; Svensson and Wong 2011), meaning their physiological use

58 is closely tied to environmental availability. Carotenoids are a common pigment

59 used in animal signals, particularly sexual signals (Svensson and Wong 2011),

60 where carotenoid-based pigmentation can be a reliable indicator of quality

61 during mate choice and sexual display (Blount, Metcalfe, Birkhead, et al. 2003;

62 Faivre et al. 2003; Hidalgo-Garcia 2006), and/or in signals used for intrasexual

63 competition and territory acquisition (Walker, Ewen, et al. 2014). This is because

64 carotenoids are important in development and immune system function across

65 all life stages (Blount 2004). Furthermore, carotenoid availability in early life

66 has follow-on effects for an individual's ability to assimilate carotenoids from the

67 diet in later life (Blount, Metcalfe, Arnold, et al. 2003; Butler and McGraw 2012),

68 which may explain a link between early life carotenoid access and adult

69 colouration (Walker et al. 2013). Theoretically then, in species where sons

70 experience high variance in their reproductive success, and carotenoid

71   availability influences their fitness, it may be adaptive for mothers in carotenoid-

72   rich environments to bias their investment towards sons.

73   Despite this putative link between carotenoid availability and sex ratio

74   manipulation, the little evidence that exists is mixed. For example, female barn

75   swallows did not invest carotenoids differentially into egg yolks destined to be

76   sons or daughters (female birds are the heterogametic sex), but instead provided

77   more carotenoids to eggs of both sexes when mated to poorer quality males

78   (Saino et al. 2003). In zebra finches, on the other hand, females experimentally

79   supplemented with carotenoids during laying produced more sons (McGraw et

80   al. 2005). Male sexual signals of both barn swallows (red plumage, (Safran et al.

81   2010)) and zebra finches (carotenoid-pigmented beaks, (McGraw et al. 2002))

82   are influenced by carotenoids, so it remains unclear if, or when, mothers might

83   bias sex ratios to match carotenoid availability.

84   Here, we experimentally investigate whether increased availability of

85   carotenoids in the environment results in male-biased sex ratios in the hihi

86   (*Notiomystis cincta*). Adult hihi are strongly sexually dimorphic in size and

87   plumage: males have black plumage with white ear tufts as well as carotenoid-

88   based yellow shoulder patches, while females are 25% smaller and cryptic olive-

89   brown in colour (Ewen, Surai, et al. 2006; Walker, Ewen, et al. 2014). Male

90   carotenoid-based colouration is influenced by early life carotenoid availability

91   (Walker et al. 2013), and this colouration is important for various aspects of

92   reproductive success: males with larger yellow shoulder patches are more likely

93   to hold a breeding territory, and those with darker yellow patches are less likely

94   to be cuckolded (Walker, Ewen, et al. 2014). Reproductive success varies among

95   males, and floater males compete with territorial males to gain high rates of

96   extra-pair paternity (Brekke et al. 2015), so factors that influence male success

97   in holding a territory are likely to have substantial effects on their reproductive

98   success. Consequently, carotenoid availability during development may benefit

99   sons more than daughters.

100   We predict that if hihi females can provide abundant carotenoids to

101   offspring, they should favour production of sons. Bias may occur at laying, or via

102   improved survival of male embryos (Alonso-Alvarez 2006). Male hihi are more

103   likely to die as embryos than females (Brekke et al. 2010), so access to

104   carotenoids in the egg yolk may improve this outcome (Pérez et al. 2006). As

105   carotenoid concentrations are commonly highest in the first-laid egg (Royle et al.

106   1999; Blount et al. 2002; Saino et al. 2002; Newbrey et al. 2014), first-laid

107   offspring are particularly likely to receive the benefits of increased availability to

108   mothers (Kilner 1998; Badyaev et al. 2003; Dijkstra et al. 2010) and so we

109   predict these should be male. Previous analyses have found no effect of

110   carotenoid treatment on the sex ratio of hihi fledglings (Ewen et al. 2008).

111   However, egg failure (Brekke et al. 2010; Hemmings et al. 2012) and nestling

112   mortality (Rippon 2010; Rippon et al. 2011) are high in this species, a common

113   problem in testing patterns of avian sex ratios (Sheldon 1998), and one which

114   may have led to under-estimation of bias previously. Here we focus on the sex

115   ratio at laying (including looking specifically at the first egg laid) and the sex

116   ratio at hatching to detect any effects of environmental carotenoids on

117   facultative sex ratio manipulation by mothers.

118

Methods

Study population

The hihi (*Notiomystis cincta*) is a passerine endemic to New Zealand, and is well-suited to studies of sex allocation because they nest in boxes when provided, and take supplementary food readily (Thorogood et al. 2013). We used data collected from a closed breeding population on Tiritiri Matangi Island (36°36'S, 174°53'E), a small (220 ha), low-altitude island where hihi have been reintroduced in two rounds of translocations from a remnant population in 1995 and 1996. Breeding attempts are monitored yearly as part of a conservation effort, and every individual is uniquely identifiable by leg rings. For further details of habitat and climate see Thorogood *et al.* 2013.

Supplementation experiment

We used data from carotenoid supplementation experiments that were carried out during the 2004/2005 and 2005/2006 breeding seasons. These experiments were designed to investigate effects for maternal egg investment (Ewen, Surai, et al. 2006), parental provisioning of chicks (Ewen et al. 2008), and effects of ectoparasites on chick health (Ewen et al. 2009). The supplementation protocol was identical in both years, except that in 2004/2005 pairs were supplemented from first sign of nest building until chicks fledged (Ewen et al. 2008), and in 2005/2006, supplementation continued only until egg-laying was complete (Ewen et al. 2009). Once breeding pairs settled on a nest site, they were allocated to treatment or control groups. Control pairs were provided with a temporary

142   supplementary food source (sugar water) within 10 m of the nest box, while the

143   sugar water provided to treatment pairs was supplemented with carotenoids

144   (lutein and zeaxanthin, dominant carotenoids in yellow plumage in hihi) at a

145   concentration of 100 μg/ml (Ewen, Surai, et al. 2006; Ewen et al. 2008). Birds

146   were familiar with these feeders as food was presented in similar feeding

147   stations across the island throughout the year and used them readily. See Ewen

148   et al. 2008 for more detail.

149

150   *Identification of chick sexes*

151   As part of the long-term monitoring of this population, the sexes of chicks that

152   survived to 21 days of age (standardised age for ringing and sampling, before

153   fledging occurs at 30 days) were determined via molecular sexing of blood (see

154   Dawson 2007; 2015 for details) or feather samples (see Thorogood et al. 2009

155   for details), or via sighting as adults during routine population censuses

156   conducted in Spring and Autumn (Thorogood et al. 2013).

157   For this study, we used similar methodology to sex chicks that died after

158   hatching and any unhatched embryos (116 out of 501 eggs). As nests in our

159   population are closely monitored (every 1 – 2 days), we were able to recover

160   tissue samples for most dead individuals: unhatched eggs were collected several

161   days after the expected hatching date and dead nestlings were recovered soon

162   after death, either from inside the nest box or from the ground outside. Embryos

163   and tissue samples from dead nestlings were then stored in 95% ethanol before

164   we used molecular methods to assign sex.

165   DNA was extracted from approximately 1 g of each sample using DNeasy

166   Blood and Tissue Kits (Qiagen). Sex chromosome markers were amplified

167   following the protocol of Thorogood & Ewen 2006. PCRs were carried out in GS1

168   Thermal Cyclers (G-Storm), with the following conditions: initial denaturation at

169   94°C for 1 min 30 s, followed by forty cycles at 94°C for 30 s, 48°C for 45 s, and

170   72°C for 45 s before a final annealing step at 48°C for 1 min and an extension at

171   72°C for 5 min, then held at 4°C. Electrophoresis of amplified products was

172   carried out on a 1% agarose gel in tris-borate-EDTA (TBE) buffer at 90V. The

173   products were then stained with ethidium bromide and viewed under UV light,

174   where single and double bands were easily identifiable. Only embryos that were

175   clearly identifiable were sexed, so there was little risk of contamination from

176   parental DNA (Arnold et al. 2003).

177   In total, we knew (through previous blood/feather sampling or adult re-

178   sighting) or assigned (dead material sexed as part of this study) sexes to 363 out

179   of 501 individuals. Inability to assign sexes to the remaining individuals was

180   either because there was insufficient embryonic development to sample (86 out

181   of 199 unhatched eggs across the two seasons; on average 12% of hihi eggs are

182   infertile (Hemmings et al. 2012)), or because we were unable to recover dead

183   material (52 out of 382 hatched chicks). If one sex is over-represented in this

184   unsampled group, we may under- or over-estimate any sex bias in our dataset.

185   As our focus here is on the effect of our carotenoid treatment, this should only be

186   problematic if the number of unknown fertilizations is skewed to one treatment

187   group; however our samples were fairly evenly distributed between the

188   treatment groups (37 of the unsampled eggs were from carotenoid treated nests;

189   49 were from untreated nests).

190

Statistical analyses

We first created two subsets of data: one in which we were able to calculate

clutch sex ratio *at laying* by excluding clutches where the number of sexes

known did not equal the number of eggs laid; and one from which we were able

to calculate sex ratio *at hatching* by excluding clutches where the number of

sexes known did not equal the number of eggs hatched (i.e. this subset included

broods where one or more eggs did not hatch, and we did not know their sex, but

we knew the sex of all eggs that did hatch). The former included 43 clutches; the

latter included a larger sample of 87 clutches.

We first tested the influence of carotenoid treatment on the sex ratios of

a) all eggs laid, and b) all hatched individuals, using binomial GLMMs fit using the

lme4 package (R Core Team 2013), with a bound column of number of males and

number of females set as the dependent variable (sex ratio). Our dependent

variable therefore also inherently accounts for variation in clutch size. We

specified carotenoid treatment (0/1) as an independent variable in each model.

We also included time in the season (days since first clutch hatched) as this has

been shown to influence brood sex ratios in other studies (Dijkstra et al. 2010).

We use the latter as a proxy for time in the season as it provides a comparable

measure of how early or late a brood hatched relative to the commencement of

the population breeding season, which can vary slightly between years. We also

included an interaction term to determine whether carotenoid treatment

influenced sex allocation according to time in the season. We first tested whether

season (2004-2005, 2005-2006) or clutch number within season (first or second

214   clutch) influenced sex ratio in a global model: neither had a significant effect, so

215   we control for them instead as random terms.

216   To test if sex ratios changed from when eggs were laid, to when eggs

217   hatched, we used a paired t-test. Overall bias in sex ratios of eggs laid and

218   hatched was tested using Chi-squared tests of expected frequencies (numbers of

219   male and female chicks).

220   To determine if first-laid eggs were more likely to be male, we tested

221   whether sex ratio of first-laid eggs differed from parity using a Chi-squared test

222   of expected frequencies (number of males). We next tested whether the

223   probability of the first-laid eggs being male differed according to carotenoid

224   treatment, time in the season, or an interaction of both, by fitting these terms in a

225   binomial GLMM with season and clutch as random terms.

226   We tested whether carotenoid treatment influenced embryonic mortality

227   by a) testing the distribution of unhatched eggs in treated and untreated nests

228   using a Chi-squared test of expected frequencies, and b) comparing the

229   proportions of male and female embryos that failed to hatch in treated and

230   untreated nests using Fisher Exact Tests.

231

232   *Compliance with Ethical Standards*

233   Ethical approval for supplementing carotenoids was granted by the Zoological

234   Society of London Ethics Committee (UK). Permissions to conduct research on

235   Department of Conservation Estate and to collect samples as detailed above

236   were also granted from the Auckland Conservancy of the Department of

237 Conservation. These protocols were derived from standard monitoring protocols

238 used for management of hihi by the Department of Conservation.

239

240 **Results**

241 *i) do carotenoid-treated females produce more sons?*

242 We found no evidence that mothers manipulate the sex ratio of their offspring

243 when carotenoids are freely available during laying (Fig 1). Within clutches, sex

244 ratio at laying was not significantly influenced by carotenoid treatment (χ^2_1 =

245 0.42, P = 0.52), date (χ^2_1 = 0.02, P = 0.89), or an interaction between the two

246 (carotenoid treatment*date: χ^2_1 = 0.13, P = 0.72). From our larger dataset of sex

247 ratio at hatching, there were also no effects of treatment (χ^2_1 = 0.01, P = 0.94; Fig.

248 1), date (χ^2_1 = 2.24, P = 0.13), or their interaction (χ^2_1 = 0.01, P = 0.93). Not

249 surprisingly then, across nests in our treatment groups, the number of male

250 embryos produced did not differ (from 20 control nests, 49 males; from 23

251 treated nests, 47 males; Kruskal Wallis χ^2_1 = 0.26, P = 0.61), and nor did the

252 number of males that hatched (from 42 control nests, 76 males; from 45 treated

253 nests, 81 males; Kruskal Wallis χ^2_1 = 0.004, P = 0.95).

254

255 *ii) are first-laid eggs from carotenoid-treated females more likely to be male?*

256 We knew the sex of 43 first-laid eggs (15 were from control nests, and 28 were

257 from carotenoid-treated nests): 23 were female, and 20 were male. Again, sex

258 was not influenced by carotenoid treatment (χ^2 = 0.23, P = 0.63), date in the

259 season (χ^2 = 0.7, P = 0.4), or an interaction of the two (χ^2 = 0.19, P = 0.66), and at

260   the population level, this sex ratio did not differ from an expected equal

261   frequency (χ^2 = 0.21, d.f. = 1, P = 0.65).

262

263   *iii) do carotenoids influence embryonic survival?*
264

265   Of 182 eggs laid in the 43 clutches (22 clutches from carotenoid-treated

266   mothers) for which we knew sex ratio at laying and at hatching, 96 were male,

267   and 86 were female (an overall sex ratio of 1.12 males per each female; no

268   significant bias detected with Chi squared test: $\chi^2{}_1$ = 0.55, P = 0.46). Of these

269   eggs, 20 failed to hatch (11 contained male embryos, and 9 contained female

270   embryos, no significant bias: $\chi^2{}_1$ = 0.2, P = 0.65). This changed the sex ratio

271   overall to 1.09 males to each female; however this change was not statistically

272   significantly different (paired T test T_{41} = 0.71, P = 0.48). The sex ratio at

273   hatching of our larger dataset was very similar: 279 chicks successfully hatched,

274   of which 142 were male, and 137 were female (1.04 males to each female, not

275   significantly different from an equal distribution: $\chi^2{}_1$ = 0.09, P = 0.76), so our

276   inability to detect a change was not likely to be influenced by our inability to sex

277   all embryos.

278   When we looked at every sexed embryo produced across nests (374 eggs

279   from 119 nests), carotenoid treatment did not improve survival of embryos,

280   either overall (53 out of 115 eggs that failed to hatch came from carotenoid

281   treated nests, $\chi^2{}_1$ = 0.70, P = 0.40). Carotenoid treatment did not influence the

282   proportion of male embryos that died before hatching: 8 of 89 male embryos,

283   8.9%, failed to hatch in carotenoid-treated nests, compared to 9 of 94 male

284   embryos in untreated nests, 9.6%, a non-significant difference (Fisher exact test,

12

285   *P* = 0.9). Fewer female embryos died before hatching in untreated nests (6 out of

286   92 female embryos, 6.5%) compared to carotenoid-treated nests (10 out of 99

287   female embryos, 10.1%), but this difference was not significant (Fisher exact

288   test, *P* = 0.44). This meant we did not detect any male bias in the failure of

289   embryos to hatch, as was found in a previous cohort of this population (52% of

290   unhatched embryos were male in our study Vs. 76% in Brekke *et al.* 2010; Fisher

291   exact test, *P* = 0.047); while female mortality rates were similar, mortality rates

292   for males in our study were far lower (Table 1).

293

294

295   **Discussion**

296   According to sex ratio theory, if a maternal or environmental factor more

297   strongly influences the fitness of one sex, mothers with that trait or in an

298   environment with that trait should bias the sex ratio of their offspring to the

299   more affected sex (Trivers and Willard 1973). Although carotenoid availability

300   may be more likely to benefit male hihi (Walker et al. 2013; Walker, Ewen, et al.

301   2014; Walker, Thorogood, et al. 2014), we found that sex ratio at laying and

302   hatching were similar in carotenoid-supplemented and unsupplemented

303   clutches, and regardless of treatment, the sex of first eggs was also equally likely

304   to be male or female. Nor did carotenoids influence embryonic survival, with

305   both sexes enjoying low rates of mortality. This meant that across treatments,

306   there was no change in sex ratio from laying of eggs to their hatching, and so we

307   find no evidence for sex bias at any stage.

308   Since previous work has suggested that carotenoids in early life may lead

309   to differences in reproductive success for male offspring, why did we find no

310   effect of carotenoids on sex ratios? From other analyses of data from this

311   experiment, we know that our manipulation of carotenoids to mothers leads to

312   elevated levels in egg yolk (Ewen, Thorogood, et al. 2006) and influences chicks'

313   growth in response to challenges (Ewen et al. 2009). Therefore, we can be

314   confident that our manipulation had the potential to influence maternal sex

315   allocation decisions. Perhaps carotenoid availability during egg-laying is not a

316   reliable predictor of availability during the nestling provisioning period. This

317   may be especially true in this species, where the provisioning period (chicks

318   fledge at 30 days) is relatively long (Roff et al. 2005), and during which time

319   resource availability may change. Indeed, the study on which we based our

320   predictions for sex ratio adjustment supplemented hihi chicks directly with

321   carotenoids during rearing (Walker et al. 2013), not via their mothers during egg

322   laying. Therefore, our lack of effect here may have arisen because access to

323   carotenoids during chick-rearing is a better cue for sex-biased variance in future

324   reproductive success. Alternatively, adjusting sex ratio according to carotenoid

325   availability at laying, or even during chick-rearing, may be maladaptive.

326   Finally, we may not have detected an effect because improved availability

327   of carotenoids in early life could also benefit the reproductive value of daughters,

328   reducing the difference in likely returns from investing differentially. While

329   increased carotenoids may benefit male offspring in terms of adult plumage

330   (Walker et al. 2013), any role in female colouration has not been explored.

331   Studies of carotenoid effects on colouration have mostly focused on male traits

332   (Pryke et al. 2001; Pryke and Griffith 2006; Walker, Ewen, et al. 2014), but

14

333 evidence is growing that carotenoid-based colouration is also important in

334 female status display (Crowley and Magrath 2004; Murphy, Rosenthal, et al.

335 2009; Murphy, Hernández-Muciño, et al. 2009). In hihi, females are at the bottom

336 of both an inter- and con-specific dominance hierarchy for foraging

337 opportunities (Rasch and Craig 1988), so signals of status may play a role for

338 both sexes in mediating differences in condition and eventual ability to breed

339 successfully.   Plumage aside, when a female hihi nestling's access to carotenoids

340 is experimentally increased, their survival to fledge increases by 13% (males

341 enjoy no improvement in survival). And, if carotenoids are provided to mothers

342 when rearing chicks, they are encouraged to attempt a second clutch that season

343 (Thorogood et al. 2011). Whether increased carotenoid availability in early life

344 environments primes daughters' responses to environmental conditions when

345 breeding themselves remains unknown.

346   While we found no evidence that mothers manipulated the sex ratio of

347 their clutches at laying, we did expect to detect sex-biased mortality before

348 hatching. Previous work with hihi has suggested that male embryos are more

349 vulnerable than females, perhaps because they are more susceptible to

350 inbreeding effects (Brekke et al. 2010). We did not find increased male mortality

351 in our cohorts: while female mortality rates were similar to those found by

352 Brekke et al., males enjoyed a much reduced mortality rate (9.5% compared to

353 21.1%). Differences in male mortality could be due to variability in the effects of

354 inbreeding depression, the expression of which is the combination of inbreeding

355 level and (variable) environmental conditions (Keller et al. 2012). Determining

356 what environmental factors (including food supplementation regimes) influence

357 the expression of inbreeding depression in hihi would be a valuable next step.

358   We show that, in hihi, increased access to carotenoids during egg-laying

359   does not result in an increase in male offspring produced. This result did not

360   support our predictions that, as male hihi rely on carotenoid-based signals to

361   maintain territories, they should benefit more from an increase in carotenoid

362   availability, and so should be produced in greater numbers when carotenoids in

363   the environment are plentiful. However, to understand the role that

364   environmental conditions play in sex allocation clearly requires a better

365   knowledge of the consequences of carotenoids for long-term reproductive

366   success in both sexes.

367   **References**

368   Alonso-Alvarez C. 2006. Manipulation of primary sex-ratio: an updated review.
369   Avian Poult. Biol. Rev. 17:1–20.

370   Appleby BM, Petty SJ, Blakey JK, Rainey P, MacDonald DW. 1997. Does variation
371   of sex ratio enhance reproductive success of offspring in tawny owls (Strix
372   aluco). Proc. R. Soc. London B Biol. Sci. 264:1111–1116.

373   Arnold KE, Orr KJ, Griffiths R. 2003. Primary sex ratios in birds : problems with
374   molecular sex identification of undeveloped eggs. Mol. Ecol. 12:3451–3458.

375   Badyaev A V, Hill GE, Beck ML. 2003. Interaction between maternal effects: onset
376   of incubation and offspring sex in two populations of a passerine bird. Oecologia
377   135:386–90. [accessed 2014 Nov 17].
378   http://www.ncbi.nlm.nih.gov/pubmed/12721828

379   Blount JD. 2004. Carotenoids and life-history evolution in animals. Arch.
380   Biochem. Biophys. 430:10–15. [accessed 2014 Oct 26].
381   http://www.sciencedirect.com/science/article/pii/S0003986104001833

382   Blount JD, Houston DC, Moller a. P. 2000. Why egg yolk is yellow. Trends Ecol.
383   Evol. 15:47–49.

384   Blount JD, Metcalfe NB, Arnold KE, Surai PF, Devevey GL, Monaghan P. 2003.
385   Neonatal nutrition, adult antioxidant defences and sexual attractiveness in the
386   zebra finch. Proc. R. Soc. London B Biol. Sci. 270:1691–1696.

387   Blount JD, Metcalfe NB, Birkhead TR, Surai PF. 2003. Carotenoid Modulation of
388   Immune Function and Sexual Attractiveness in Zebra Finches. Sci. 300 :125–127.

389   Blount JD, Surai PF, Nager RG, Houston DC, Møller AP, Trewby ML, Kennedy MW.
390   2002. Carotenoids and egg quality in the lesser blackbacked gull Larus fuscus: a
391   supplemental feeding study of maternal effects. Proc. R. Soc. B Biol. Sci. 269:29–
392   36. [accessed 2014 Oct 27].

393   http://rspb.royalsocietypublishing.org/content/269/1486/29.short

394   Brekke P, Bennett PM, Wang J, Pettorelli N, Ewen JG. 2010. Sensitive males:
395   inbreeding depression in an endangered bird. Proc. Biol. Sci. 277:3677–3684.

396   Brekke P, Ewen JG, Clucas G, Santure AW. 2015. Determinants of male floating
397   behaviour and floater reproduction in a threatened population of the hihi (
398   Notiomystis cincta). Evol. Appl. 8:796–806.

399   Butler MW, McGraw KJ. 2012. Differential Effects of Early- and Late-Life Access
400   to Carotenoids on Adult Immune Function and Ornamentation in Mallard Ducks
401   (Anas platyrhynchos). PLoS One 7:e38043.

402   Cameron EZ. 2004. Facultative adjustment of mammalian sex ratios in support of
403   the Trivers-Willard hypothesis: evidence for a mechanism. Proc. R. Soc. B Biol.
404   Sci. 271:1723 – 1728.

405   Charnov EL. 1982. The theory of sex allocation. Princeton, NJ: Princeton
406   University Press.

407   Crowley CE, Magrath RD. 2004. Shields of offence: signalling competitive ability
408   in the dusky moorhen, Gallinula tenebrosa. Aust. J. Zool. 52:463–474.

409   Dijkstra C, Riedstra B, Dekker A, Goerlich VC, Daan S, Groothuis TGG. 2010. An
410   adaptive annual rhythm in the sex of first pigeon eggs. Behav. Ecol. Sociobiol.
411   64:1393–1402.

412   Ewen JG, Cassey P, Møller AP. 2004. Facultative primary sex ratio variation: a
413   lack of evidence in birds? Proc. R. Soc. B Biol. Sci. 271:1277–1282.

414   Ewen JG, Surai P, Stradi R, Moller AP, Vittorio B, Griffiths R, Armstrong DP. 2006.
415   Carotenoids, colour and conservation in an endangered passerine, the hihi or
416   stitchbird (Notiomystis cincta). Anim. Conserv. 9:229–235. [accessed 2014 Oct
417   22]. http://doi.wiley.com/10.1111/j.1469-1795.2006.00028.x

418   Ewen JG, Thorogood R, Brekke P, Cassey P, Karadas F, Armstrong DP. 2009.
419   Maternally invested carotenoids compensate costly ectoparasitism in the hihi.
420   Proc. Natl. Acad. Sci. U. S. A. 106:12798–802. [accessed 2014 Oct 27].
421   http://www.pnas.org/content/106/31/12798.short

422   Ewen JG, Thorogood R, Karadas F, Cassey P. 2008. Condition dependence of
423   nestling mouth colour and the effect of supplementing carotenoids on parental
424   behaviour in the hihi (Notiomystis cincta). Oecologia 157:361–8. [accessed 2014
425   Oct 27]. http://www.ncbi.nlm.nih.gov/pubmed/18546021

426   Ewen JG, Thorogood R, Karadas F, Pappas AC, Surai PF. 2006. Influences of
427   carotenoid supplementation on the integrated antioxidant system of a free living
428   endangered passerine, the hihi (Notiomystis cincta). Comp. Biochem. Physiol.
429   143:149–154. [accessed 2014 Oct 27].
430   http://www.ncbi.nlm.nih.gov/pubmed/16406271

431   Faivre B, Grégoire A, Préault M, Cézilly F, Sorci G. 2003. Immune Activation
432   Rapidly Mirrored in a Secondary Sexual Trait. Science (80-.). 300:103.

433   Hemmings N, West M, Birkhead TR. 2012. Causes of hatching failure in
434   endangered birds. Biol. Lett. 8:964–967.

435   Hidalgo-Garcia S. 2006. The carotenoid-based plumage coloration of adult Blue
436   Tits Cyanistes caeruleus correlates with the health status of their brood. Ibis

437 (Lond. 1859). 148:727–734.

438 Keller LF, Biebach I, Ewing SR, Hoeck PEA. 2012. The Genetics of
439 Reintroductions: Inbreeding and Genetic Drift. In: Ewen JG, Armstrong DP,
440 Parker KA, Seddon PJ, editors. Reintroduction Biology: Integrating Science and
441 Management. Oxford, UK: Blackwell Publishing Ltd. p. 362–294.

442 Kilner RM. 1998. Primary and secondary sex ratio manipulation by zebra finches.
443 Anim. Behav. 56:155–64. [accessed 2015 Sep 25].
444 http://www.sciencedirect.com/science/article/pii/S0003347298907758

445 Kilner RM, Boncoraglio G, Henshaw JM, Jarrett BJM, De Gasperin O, Attisano A,
446 Kokko H. 2015. Parental effects alter the adaptive value of an adult behavioural
447 trait. Elife 4. [accessed 2015 Sep 25].
448 http://www.ncbi.nlm.nih.gov/pubmed/26393686

449 McGraw K., Adkins-Regan E, Parker R. 2002. Anhydrolutein in the zebra finch: a
450 new, metabolically derived carotenoid in birds. Comp. Biochem. Physiol. Part B
451 Biochem. Mol. Biol. 132:811–818. [accessed 2015 Sep 21].
452 http://www.sciencedirect.com/science/article/pii/S1096495902001008

453 McGraw KJ, Adkins-Regan E, Parker RS. 2005. Maternally derived carotenoid
454 pigments affect offspring survival, sex ratio, and sexual attractiveness in a
455 colorful songbird. Naturwissenschaften 92:375–80. [accessed 2014 Oct 27].
456 http://www.ncbi.nlm.nih.gov/pubmed/16049690

457 Murphy TG, Hernández-Muciño D, Osorio-Beristain M, Montgomerie R, Omland
458 KE. 2009. Carotenoid-based status signaling by females in the tropical streak-
459 backed oriole. Behav. Ecol. 20:1000–1006.

460 Murphy TG, Rosenthal MF, Montgomerie R, Tarvin K. 2009. Female American
461 goldfinches use carotenoid-based bill coloration to signal status. Behav. Ecol.
462 20:1348–1355.

463 Newbrey JL, Paszkowski C a., Mcgraw KJ, Ogle S. 2014. Laying-sequence variation
464 in yolk carotenoids and egg characteristics in the red-winged blackbird Agelaius
465 phoeniceus. J. Avian Biol.:46–54.

466 Pérez C, Velando A, Domínguez J. 2006. Parental food conditions affect sex-
467 specific embryo mortality in the yellow-legged gull (Larus michahellis). J.
468 Ornithol. 147:513–519.

469 Pryke SR, Griffith SC. 2006. Red dominates black: agonistic signalling among
470 head morphs in the colour polymorphic Gouldian finch. Proc. R. Soc. London B
471 Biol. Sci. 273:949–957.

472 Pryke SR, Lawes MJ, Andersson S. 2001. Agonistic carotenoid signalling in male
473 red-collared widowbirds: aggression related to the colour signal of both the
474 territory owner and model intruder. Anim. Behav. 62:695–704. [accessed 2015
475 Aug 12].
476 http://www.sciencedirect.com/science/article/pii/S0003347201918044

477 R: A language and environment for statistical computing. 2013.

478 Rasch G, Craig JL. 1988. Partitioning of nectar resources by New Zealand
479 honeyeaters. New Zeal. J. Zool. 15:185–190.

480 Rippon R, Alley M, Castro I. 2011. Causes of mortality in a nestling population of

481 free-living hihi (stitchbird— Notiomystis cincta). New Zeal. J. Zool. 38:207–222.
482 [accessed 2014 Oct 30].
483 http://www.tandfonline.com/doi/abs/10.1080/03014223.2011.571266

484 Rippon RJ. 2010. Nestling mortality in a translocated population of hihi /
485 stitchbirds (Notiomystis cincta). Massey University, New Zealand.

486 Roff DA, Remes V, Martin TE. 2005. The evolution of fledging age in songbirds. J.
487 Evol. Biol. 18:1425–1433.

488 Royle NJ, Surai PF, McCartney RJ, Speake BK. 1999. Parental investment and egg
489 yolk lipid composition. Funct. Ecol. 13:298–306.

490 Safran RJ, McGraw KJ, Wilkins MR, Hubbard JK, Marling J. 2010. Positive
491 Carotenoid Balance Correlates with Greater Reproductive Performance in a Wild
492 Bird. PLoS One 5:e9420.

493 Saino N, Bertacche V, Ferrari RP, Martinelli R, Møller AP, Stradi R. 2002.
494 Carotenoid concentration in barn swallow eggs is influenced by laying order,
495 maternal infection and paternal ornamentation. Proc. R. Soc. B Biol. Sci.
496 269:1729–33. [accessed 2014 Oct 27].
497 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1691081&tool=pm
498 centrez&rendertype=abstract

499 Saino N, Ferrari R, Romano M, Martinelli R, Møller AP. 2003. Experimental
500 manipulation of egg carotenoids affects immunity of barn swallow nestlings.
501 Proc. R. Soc. B Biol. Sci. 270:2485–9. [accessed 2014 Oct 27].
502 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1691538&tool=pm
503 centrez&rendertype=abstract

504 Sheldon BC. 1998. Recent studies of avian sex ratios. Heredity (Edinb). 80:397–
505 402.

506 Svensson PA, Wong BBM. 2011. Carotenoid-based signals in behavioural ecology:
507 a review. Behaviour 148:131–189. [accessed 2014 Oct 27].
508 http://georgealozano.com/papers/carotenoids/2010-
509 now/Svensson2011Behaviour.pdf

510 Thorogood R, Armstrong DP, Low M, Brekke P, Ewen JG. 2013. The value of long-
511 term ecological research: Integrating knowledge for conservation of hihi on
512 Tiritiri Matangi Island. N. Z. J. Ecol. 37:298–306.

513 Thorogood R, Ewen JG. 2006. Rare occurrence of embryonic twins in the Hihi (
514 Stitchbird) Notiomystis cincta : an endangered passerine of New Zealand. Ibis
515 (Lond. 1859). 148:828–829.

516 Thorogood R, Ewen JG, Kilner RM. 2011. Sense and sensitivity: responsiveness to
517 offspring signals varies with the parents' potential to breed again. Proc. R. Soc. B
518 Biol. Sci. 278:2638–45. [accessed 2014 Oct 17].
519 http://rspb.royalsocietypublishing.org/content/early/2011/01/24/rspb.2010.2
520 594.full

521 Trivers RL. 1972. Parental Investment and Sexual Selection. In: Campbell B,
522 editor. Sexual Selection and the Descent of Man 1871-1971. Chicago: Aldine-
523 Atherton. p. 136–179.

524 Trivers RL, Willard DE. 1973. Natural Selection of Parental Ability to Vary the Sex

525   Ratio of Offspring. Sci. 179 :90–92.

526   Walker LK, Ewen JG, Brekke P, Kilner RM. 2014. Sexually selected dichromatism
527   in the hihi Notiomystis cincta: multiple colours for multiple receivers. J. Evol.
528   Biol. 27:1522–35. [accessed 2014 Oct 26].
529   http://www.ncbi.nlm.nih.gov/pubmed/24836349

530   Walker LK, Stevens M, Karadaş F, Kilner RM, Ewen JG. 2013. A window on the
531   past: male ornamental plumage reveals the quality of their early-life
532   environment. Proc. R. Soc. London B Biol. Sci. 280:20122852.

533   Walker LK, Thorogood R, Karadas F, Raubenheimer D, Kilner RM, Ewen JG. 2014.
534   Foraging for carotenoids: do colorful male hihi target carotenoid-rich foods in
535   the wild? Behav. Ecol. 25:1048–1057. [accessed 2014 Oct 15].
536   http://beheco.oxfordjournals.org/content/early/2014/05/12/beheco.aru076.s
537   hort

538   West S. 2009. Sex Allocation. Princeton, NJ: Princeton University Press.

539   Wiebe KL, Bortolotti GR. 1992. Facultative sex ratio manipulation in American
540   kestrels. Behav. Ecol. Sociobiol. 30:379–386.

541   Wilkin TA, Sheldon BC. 2009. Sex differences in the persistence of natal
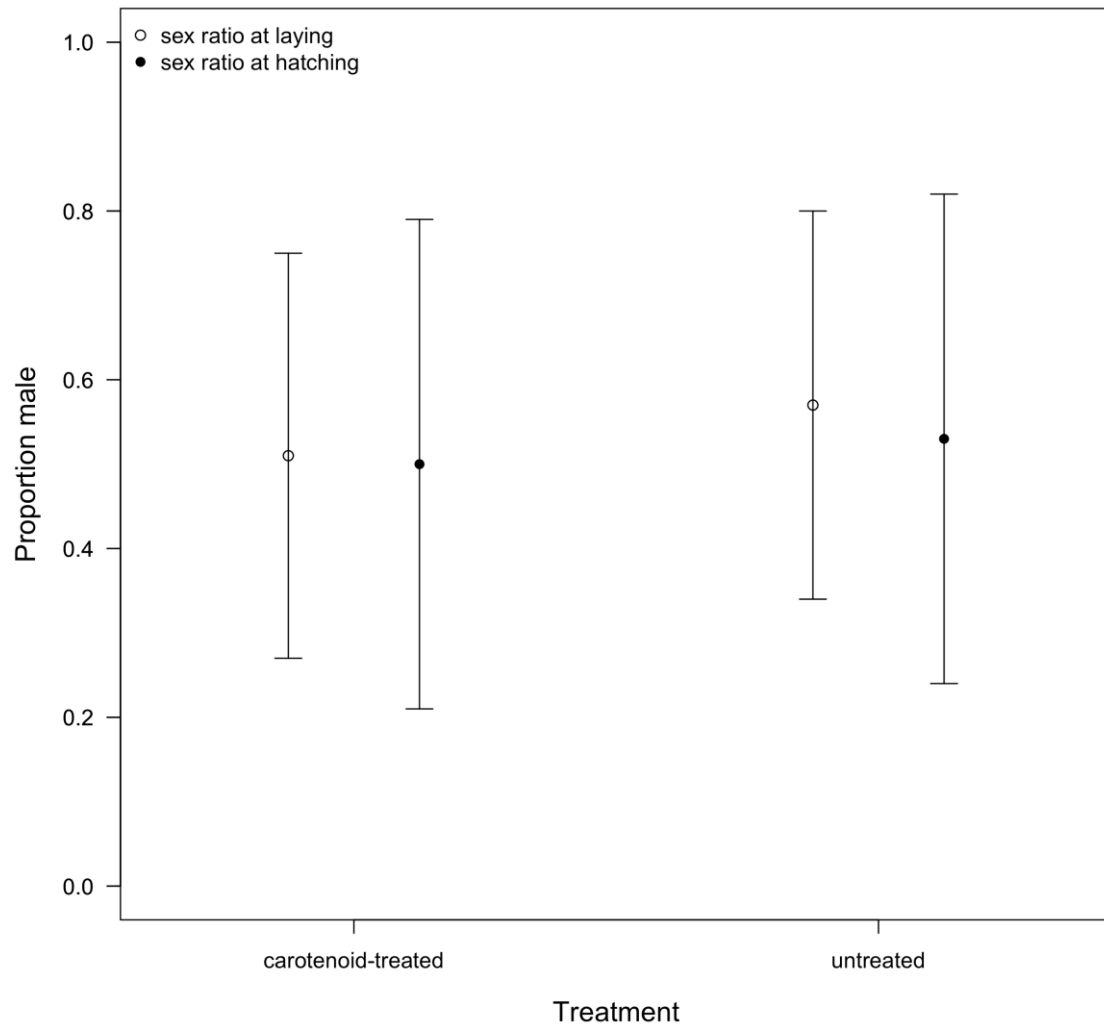542   environmental effects on life histories. Curr. Biol. 19:1998–2002. [accessed 2015
543   Sep 8]. http://www.sciencedirect.com/science/article/pii/S0960982209018466

544

545

546

547

548

549

550 Fig. 1. Clutch sex ratios (proportion male) according to carotenoid treatment
551 (mean ± s.d). Open circles represent sex ratios at laying (where sexes of total
552 brood, including unhatched chicks were known); closed circles represent sex
553 ratios at hatching (where sexes of all hatched chicks were known).

554
555
556
557
558

559 Table 1. Hatch failure of male and female embryos in this study, and in a previous
560 study showing significant male mortality bias (Brekke et al. 2010), which we did
561 not find in this study (% hatch failures of male and female embryos highlighted
562 for comparison).

	MALE			FEMALE		
	Dead embryos	Total offspring	% hatch failure	Dead embryos	Total offspring	% hatch failure
Brekke et al. 2010:						
No supplement	*28*	*133*	*21.1*	*9*	*94*	*9.6*

This study:						
Food + Carotenoid supplement	8	89	8.9	10	99	10.1
Food supplement only	9	94	9.6	6	92	6.5
Total	*17*	*183*	*9.3*	*16*	*191*	*8.4*

563