



## Original Article

# Do mothers bias offspring sex ratios in carotenoid-rich environments?

Kirsty J. MacLeod,<sup>a</sup> Patricia Brekke,<sup>b</sup> Wenfei Tong,<sup>a,c</sup> John G. Ewen,<sup>b</sup> and Rose Thorogood<sup>a</sup>

<sup>a</sup>Behavioural Ecology Group, Department of Zoology, University of Cambridge, Downing Street CB2 3EJ, Cambridge, UK, <sup>b</sup>Institute of Zoology, Zoological Society of London, Regent's Park, London NW1 4RY, UK and <sup>c</sup>Division of Biological Sciences, University of Montana, 32 Campus Drive, Missoula, MT 59812 USA

Received 2 March 2016; revised 22 July 2016; accepted 17 August 2016; Advance Access publication 14 September 2016.

If environmental or maternal factors favor the fitness of one sex over the other, theory predicts that mothers should produce more offspring of the sex most likely to benefit from prevailing conditions. For species where males depend on carotenoid-based colorful ornaments to secure territory or attract mates, carotenoid availability in the environment could be one such component: mothers experiencing high availability of carotenoids should produce more sons. Here, we test this hypothesis by providing carotenoids to a wild population of a sexually dimorphic passerine, the hihi (stitch bird: *Notiomystis cincta*). Access to carotenoids during early life influences the color of male hihi plumage, which improves territory acquisition as adults. Therefore, carotenoid availability when young may influence male fitness. However, we found no evidence of sex ratio bias in treated or untreated groups, either before or after hatching. First-laid eggs, where carotenoid concentrations are usually highest, were also unbiased. For hihi, access to carotenoids during egg laying does not appear to encourage mothers to alter sex ratios of offspring. Alternatively, the fitness of daughters may also benefit from increased carotenoids during development. Disentangling these alternatives requires further work.

**Key words:** carotenoids, *Notiomystis cincta*, sex ratio, supplementary feeding

## INTRODUCTION

The environment offspring experience during early life can have profound effects on their reproductive productivity (Wilkin and Sheldon 2009; Walker et al. 2013; Kilner et al. 2015), and these effects may differ between the sexes (Trivers 1972; Trivers and Willard 1973). If good environmental conditions increase the reproductive value of one sex more than the other, mothers should bias sex ratios to maximize their fitness returns (Trivers and Willard 1973; Charnov 1982). Some studies suggest that factors such as maternal condition, age, and social rank may influence offspring sex ratios (reviewed in Cameron 2004; West 2009), but experimental evidence for facultative sex ratio manipulation remains mixed, particularly in birds (Sheldon 1998; Ewen et al. 2004). For example, territory quality (Komdeur 1996), social environment (Emlen 1997), and parental quality (Ellegren et al. 1996; Sheldon et al. 1997) sometimes generate bias, whereas other studies have found no evidence of biases according to predictions (Westneat et al. 1995; Sheldon and Ellegren 1996). Females are the heterogametic sex in birds, so bias could be generated before as well as after fertilization. Consequently, a lack of information on pre-hatching sex ratios may lead to an under-estimation of bias (Sheldon 1998).

Alternatively (or additionally), many studies investigating sex allocation in response to resource availability have focused on general food availability (Wiebe and Bortolotti 1992; Appleby et al. 1997), an approach which could overlook fluctuations in key nutrients that may have sex-specific benefits.

One class of nutrients that may have different consequences for each sex are carotenoids. These antioxidant pigments can only be obtained through the diet (Blount et al. 2000; Svensson and Wong 2011), meaning their physiological use is closely tied to environmental availability. Carotenoids are a common pigment used in animal signals, particularly sexual signals (Svensson and Wong 2011), where carotenoid-based pigmentation can be a reliable indicator of quality during mate choice and sexual display (Blount et al. 2003; Faivre et al. 2003; Hidalgo-Garcia 2006), and/or in signals used for intrasexual competition and territory acquisition (Walker et al. 2014a). Carotenoids are important in development, and in immune system function across all life stages (Blount 2004). Furthermore, carotenoid availability in early life has follow-on effects for an individual's ability to assimilate carotenoids from the diet in later life (Blount et al. 2003; Butler and McGraw 2012), which may explain a link between early life carotenoid access and adult coloration (Walker et al. 2013). Theoretically then, in species where sons experience high variance in their reproductive success, and carotenoid availability influences their fitness, it may be

Address correspondence to K.J. MacLeod. E-mail: kirstyjmacleod@gmail.com.

adaptive for mothers in carotenoid-rich environments to bias their investment towards sons.

Despite this putative link between carotenoid availability, sexual signals, and sex ratio manipulation, the little evidence that exists is mixed. For example, sexual signals of both male barn swallows [red plumage, (Safran et al. 2010)] and zebra finches [carotenoid-pigmented beaks, (McGraw et al. 2002)] are influenced by carotenoids. While zebra finch females supplemented with carotenoids during laying produce more sons (McGraw et al. 2005), female barn swallows do not invest carotenoids differentially into egg yolks destined to be sons or daughters (Saino et al. 2003). Mothers can however alter deposition of carotenoids in eggs strategically; when mated to poorer quality males, embryos of both sexes developed with yolks containing more carotenoids (Saino et al. 2003). Therefore, while mothers may allocate carotenoids strategically, it remains unclear whether they bias sex ratios if carotenoid availability influences the reproductive value of sons and daughters differently.

Here, we investigate whether increased availability of carotenoids in the environment results in male-biased sex ratios in the hihi (*Notiomystis cincta*). Adult hihi are strongly sexually dimorphic in size and plumage: males have black plumage with white ear tufts as well as carotenoid-based yellow shoulder patches, while females are 25% smaller and cryptic olive-brown in color (Ewen et al. 2006; Walker et al. 2014a). Male carotenoid-based coloration is influenced by early life carotenoid availability: supplementation of egg-laying females with carotenoids raises plasma carotenoid levels of their fledglings (Ewen et al. 2009), and higher carotenoid levels at fledging predict greater saturation of yellow plumage patches after the first moult (Walker et al. 2013). Carotenoid-based coloration is important for reproductive success in male hihi: males with darker yellow patches are less likely to be cuckolded (Walker et al. 2014b). Reproductive success varies among males, which compete with other territorial males and floater males to gain high rates of extra-pair paternity (Brekke et al. 2015), so factors that influence male success in preventing extra-pair fertilizations of their mate are likely to have substantial effects on their reproductive success. Consequently, carotenoid availability during development may benefit sons more than daughters.

We enhanced the availability of carotenoids in the environment of egg-laying mothers to test our prediction that when carotenoids are abundant, hihi females should favor production of sons. Bias may occur at laying, or via improved survival of male eggs (Alonso-Alvarez 2006). Male hihi are more likely to die in the egg than females (Brekke et al. 2010), so access to carotenoids in the egg yolk may improve this outcome (Pérez et al. 2006). As carotenoid concentrations are commonly highest in the first-laid egg (Royle et al. 1999; Blount et al. 2002; Saino et al. 2002; Newbrey et al. 2014), first-laid offspring are particularly likely to receive the benefits of increased availability to mothers (Kilner 1998; Badyaev et al. 2003; Dijkstra et al. 2010) and so we predict these eggs should be more likely to be male. Previous analyses have found no effect of carotenoid treatment on the sex ratio of hihi fledglings (Ewen et al. 2008). However, egg failure (Brekke et al. 2010; Hemmings et al. 2012) and nestling mortality (Brekke et al. 2010; Rippon 2010; Rippon et al. 2011) are high in this species, so may obscure any patterns in sex ratio at egg laying. This is a common problem in testing patterns of avian sex ratios (Sheldon 1998). Therefore, here we focus on the sex ratio at laying (including looking specifically at the first egg laid) and the

sex ratio at hatching to detect any effects of environmental carotenoids on facultative sex ratio manipulation by mothers.

## 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 2 rounds of translocations 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) and Walker et al. (2014a).

### 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 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 supplementary food source (sugar water) within 10 m of the nest box, while the sugar water provided to treatment pairs was supplemented with carotenoids (lutein and zeaxanthin, dominant carotenoids in yellow plumage in hihi) at a concentration of 100 µg/ml (Ewen et al. 2006; Ewen et al. 2008). This dose was based on previous results investigating dose response of immune activation in zebra finches (Alonso-Alvarez et al. 2004), and has been shown to result in increased concentrations of lutein and zeaxanthin in egg yolk, and subsequently in nestling plasma (Ewen et al. 2006). Birds were familiar with these feeders as food was presented in similar feeding stations across the island throughout the year and used them readily. See Ewen et al. (2008) for more detail.

### Identification of chick sexes

As part of the long-term monitoring of this population, the sexes of chicks that survived to 21 days of age (standardized age for ringing and sampling, before fledging occurs at 30 days) were determined via molecular sexing of blood (see Dawson 2007; Dawson 2015 for details) or feather samples (see Thorogood et al. 2009 for details), or via sighting as adults during routine population censuses conducted in Spring and Autumn (Thorogood et al. 2013).

For this study, we used similar methodology to sex chicks that died after hatching and any unhatched embryos (116 out of 501 eggs). As nests in our population are closely monitored (every 1–2 days), we were able to recover tissue samples for most dead individuals: unhatched eggs were collected several days after the expected hatching date and dead nestlings were recovered soon after death, either from inside the nest box or from the ground outside. Embryos and

tissue samples from dead nestlings were then stored in 95% ethanol before we used molecular methods to assign sex.

DNA was extracted from approximately 1 g of each sample using DNeasy Blood and Tissue Kits (Qiagen). Sex chromosome markers were amplified following the protocol of Thorogood and Ewen 2006. PCRs were carried out in GS1 Thermal Cyclers (G-Storm), with the following conditions: initial denaturation at 94 °C for 1 min 30 s, followed by 40 cycles at 94 °C for 30 s, 48 °C for 45 s, and 72 °C for 45 s before a final annealing step at 48 °C for 1 min and an extension at 72 °C for 5 min, then held at 4 °C. Electrophoresis of amplified products was carried out on a 1% agarose gel in tris-borate-EDTA (TBE) buffer at 90V. The products were then stained with ethidium bromide and viewed under UV light, where single and double bands were easily identifiable. Only embryos that were clearly identifiable were sexed, so there was little risk of contamination from parental DNA (Arnold et al. 2003).

In total, we knew (through previous blood/feather sampling or adult re-sighting) or assigned (dead material sexed as part of this study) sexes to 363 out of 501 individuals. Inability to assign sexes to the remaining individuals was either because there was insufficient embryonic material to sample [86 out of 199 unhatched eggs across the 2 seasons; on average 12% of hihi eggs are infertile (Hemmings et al., 2012)], or because we were unable to recover dead material (52 out of 382 hatched chicks). If one sex is over-represented in this unsampled group, we may under- or over-estimate any sex bias in our dataset. As our focus here is on the effect of our carotenoid treatment, this should only be problematic if the number of unknown fertilizations is skewed to one treatment group; however our samples were fairly evenly distributed between the treatment groups (37 of the unsampled eggs were from carotenoid treated nests; 49 were from untreated nests; 20 of the unsampled nestlings were from carotenoid treated nests; 32 were from untreated nests).

## Statistical analyses

We first created 2 subsets of data: one in which we knew the sexes of all eggs laid ( $N = 37$  clutches), and one in which we knew the sexes of all nestlings that hatched ( $N = 66$  clutches). We calculated clutch sex ratio *at laying* by excluding clutches where the number of sexes known did not equal the number of eggs laid. We calculated 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). We only included first clutches as not all birds attempted second clutches, and for those that did, mothers had been supplemented for much longer compared to first clutches (Thorogood et al. 2011).

We first tested the influence of carotenoid treatment on the sex ratios of 1) all eggs laid, and 2) 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 species (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. It is possible that either the difference in supplementation regime or other variation in environment or food quality between years may alter the effects of carotenoid supplementation. Therefore, we first tested whether season (2004–2005, 2005–2006) influenced sex ratio alone or in interaction with carotenoid treatment in global models: neither had a significant effect (sex ratio at laying: carotenoids\* season  $\chi^2 = 1.02$ ,  $P = 0.31$ ; season  $\chi^2 = 0.57$ ,  $P = 0.45$ ; sex ratio at hatching: carotenoids\* season  $\chi^2 = 0.01$ ,  $P = 0.92$ ; season  $\chi^2 = 0.23$ ,  $P = 0.63$ ). We therefore excluded season from final analyses. Maternal identity was also included as a random term to account for repeated measures across the 2 years ( $N = 60$  mothers). In all models, non-significant terms were removed by stepwise deletion to achieve the most parsimonious model.

To test if sex ratios changed from when eggs were laid, to when eggs hatched, we used a paired *t*-test. Overall bias in sex ratios of eggs laid, hatched, and fledged, was tested using Chi-squared tests of expected frequencies (numbers of male and female chicks).

To determine if first-laid eggs were more likely to be male, we tested whether sex ratio of first-laid eggs differed from parity using a Chi-squared test of expected frequencies (number of males). We next tested whether the probability of the first-laid eggs being male differed according to carotenoid treatment or time in the season by fitting these terms in a binomial GLMM with maternal identity as a random term as before.

We tested whether carotenoid treatment influenced embryonic mortality by 1) testing the distribution of unhatched eggs in treated and untreated nests using a Chi-squared test of expected frequencies, and 2) comparing the proportions of male and female embryos that failed to hatch and fledge in treated and untreated nests using Fisher Exact tests.

## Compliance with ethical standards

Ethical approval for supplementing carotenoids was granted by the Zoological Society of London Ethics Committee (UK). Permissions to conduct research on Department of Conservation Estate and to collect samples as detailed above were also granted from the Auckland Conservancy of the Department of Conservation. These protocols were derived from standard monitoring protocols used for management of hihi by the Department of Conservation.

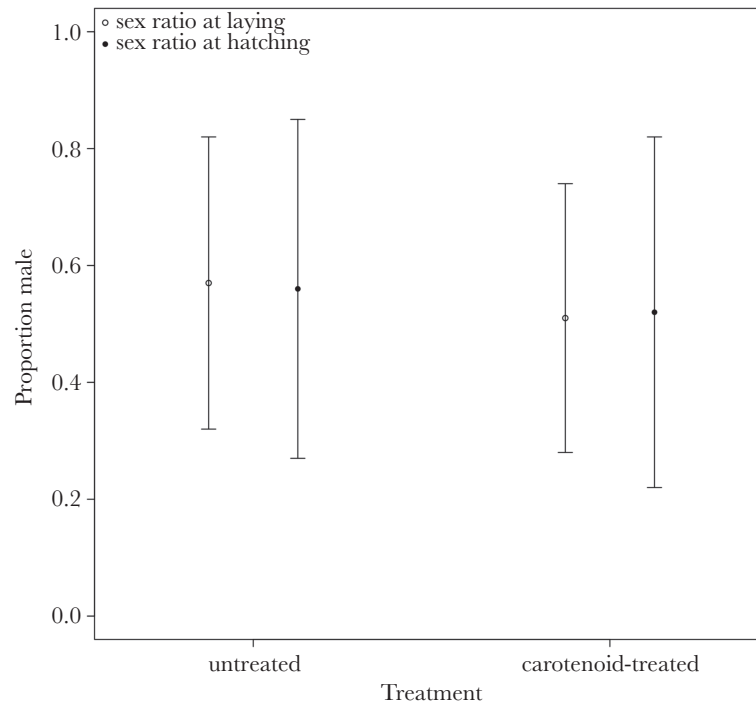
## RESULTS

### Do carotenoid-treated females produce more sons?

We found no evidence that mothers manipulate the sex ratio of their offspring when carotenoids are freely available during laying (Figure 1). Within clutches, sex ratio at laying was not significantly influenced by carotenoid treatment date (Table 1). From our larger dataset of sex ratio at hatching, there were also no effects of treatment, or date (Table 1). For both datasets, there was little variation explained by mother identity (variance ranged from 0 to 0.06).

### Are first-laid eggs from carotenoid-treated females more likely to be male?

We knew the sex of 32 first-laid eggs. Fifteen were from control nests (8 males, 7 females), and 17 were from carotenoid-treated nests (8 males, 9 females). Again, sex was not influenced by carotenoid treatment or date (Table 1).



**Figure 1**

Clutch sex ratios (proportion male) according to carotenoid treatment (mean  $\pm$  SD). Open circles represent sex ratios at laying (where sexes of total brood, including unhatched chicks were known); closed circles represent sex ratios at hatching (where sexes of all hatched chicks were known).

**Table 1**

**Results of GLMMs investigating carotenoid and date effects on sex ratio at laying/hatching and sex of first egg. Terms removed during model selection are reported below the results of the best model**

|                          |  | Estimate | Standard error | $\zeta$ | <i>P</i> |
|--------------------------|--|----------|----------------|---------|----------|
| 1) Sex ratio at laying   | Intercept  | 0.11     | 0.16           | 0.71    | 0.48     |
|                          | Removed from model: carotenoid treatment ( $\chi^2 = 0.42$ , $P = 0.52$ ), date ( $\chi^2 = 0.65$ , $P = 0.42$ ) |          |                |         |          |
| 2) Sex ratio at hatching | Intercept  | 0.14     | 0.13           | 1.04    | 0.3      |
|                          | Removed from model: carotenoid treatment ( $\chi^2 = 0.09$ , $P = 0.76$ ), date ( $\chi^2 = 0.1$ , $P = 0.75$ )  |          |                |         |          |
| 3) Sex of first egg      | Intercept  | -0.12    | 0.49           | -0.24   | 0.81     |
|                          | Removed from model: carotenoid treatment ( $\chi^2 = 0.13$ , $P = 0.72$ ), date ( $\chi^2 = 0.01$ , $P = 0.94$ ) |          |                |         |          |

### Do carotenoids influence embryonic survival?

We found no evidence that carotenoids improved survival of embryos: 49 out of 107 eggs that failed to hatch came from carotenoid treated nests (45.7%, not significantly different from an equal distribution:  $\chi^2 = 0.76$ ,  $P = 0.38$ ) (Table 2). Carotenoid treatment did not influence the proportion of male embryos that died before hatching: 5 of 70 male embryos from carotenoid-treated nests failed to hatch, compared to 6 of 73 male embryos in untreated nests, a non-significant difference (Fisher exact test,  $P = 0.99$ ). Fewer female embryos died before hatching in untreated nests (3 out of 68 female embryos) compared to carotenoid-treated nests (7 out of 72 female embryos), but this difference was not significant (Fisher exact test,  $P = 0.33$ ). This meant we did not detect any male bias in the failure of embryos to hatch, as was found in a previous cohort of this population (52% of unhatched embryos were male in our study vs. 76% in Brekke et al. 2010).

Regardless of carotenoid treatment, we also detected no change in sex ratio within clutches. In the 37 clutches for which we knew sex ratio at laying and at hatching, 84 of the 159 eggs laid were male (39 from untreated nests, 45 from carotenoid supplemented

nests), and 75 were female (31 from untreated nests, 44 from carotenoid supplemented nests). Of these eggs, 18 failed to hatch (10 contained male embryos, and 8 contained female embryos, no significant bias:  $\chi^2 = 0.22$ ,  $P = 0.64$ ), but there was no significant change in clutch sex ratio (paired T test  $T_{35} = 0.59$ ,  $P = 0.56$ ). The sex ratio at hatching of our larger dataset was very similar: 223 chicks successfully hatched, of which 119 were male, and 104 were female, so our inability to detect a change was not likely to be influenced by our inability to sex all embryos.

### DISCUSSION

According to sex ratio theory, if a maternal or environmental factor more strongly influences the fitness of one sex, mothers with that trait or in an environment with that trait should bias the sex ratio of their offspring to the more affected sex (Trivers and Willard 1973). Although carotenoid availability may be more likely to benefit male hihi (Walker et al. 2013; Walker et al. 2014a; Walker et al. 2014b), we found that sex ratio at laying and hatching were similar in carotenoid-supplemented and

**Table 2**  
**Hatch failure of male and female embryos in this study**

|                              | Unsexed eggs | Male         |                 |                 | Female       |                 |                 |
|------------------------------|--------------|--------------|-----------------|-----------------|--------------|-----------------|-----------------|
|                              |              | Dead embryos | Total offspring | % hatch failure | Dead embryos | Total offspring | % Hatch failure |
| Food + carotenoid supplement | 37           | 5            | 70              | 7.1             | 7            | 72              | 9.7             |
| Food supplement only         | 49           | 6            | 73              | 8.2             | 3            | 68              | 4.4             |
| Total                        | 86           | 11           | 143             | 7.7             | 10           | 140             | 7.1             |

unsupplemented clutches, and regardless of treatment, the sex of first-laid eggs was also equally likely to be male or female. Nor did carotenoids influence embryonic survival, with both sexes enjoying low rates of mortality in both years of our study. This meant that across treatments, there was no change in sex ratio from laying of eggs to their hatching, and so we find no evidence for sex bias at any stage.

Since previous work has suggested that carotenoids in early life may lead to differences in reproductive success for male offspring, why did we find no effect of carotenoids on sex ratios? It is possible that our supplementation did not provide sufficient cues to mothers of environmental availability of carotenoids. For example, perhaps visual cues from natural carotenoid-rich food sources would promote mothers to manipulate sex ratios. From other analyses of data from this experiment, however, we know that our manipulation of carotenoids to mothers leads to elevated levels in egg yolk (Ewen et al. 2006) and influences chicks' growth in response to the immune challenges of ectoparasitism (Ewen et al. 2009). Furthermore, we know that carotenoids presented in the same way change mothers' behavior; carotenoid supplementation for a relatively short period during chick rearing increases their likelihood of attempting a second clutch during that breeding season (Thorogood et al. 2011). Therefore, we can be confident that our manipulation had the potential to influence maternal sex allocation decisions.

Alternatively, carotenoid availability during egg laying may not be a sufficiently reliable predictor of environmental conditions during the nestling provisioning period. This may be especially true in this species, where the provisioning period (chicks fledge at 30 days) is relatively long (Roff et al. 2005), and during which time resource availability may change. Indeed, the study on which we based our predictions for sex ratio adjustment supplemented hihi chicks directly with carotenoids during rearing (Walker et al. 2013), not via their mothers during egg laying. Therefore, our lack of effect here may have arisen because access to carotenoids during chick rearing is a better cue for sex-biased variance in future reproductive success. Access to carotenoids during nest building and laying also did not influence maternal incubation schedules and timing of egg hatching, possibly for the same reasons (MacLeod et al. 2016). It is also possible that adjusting sex ratio according to carotenoid availability at laying, or even during chick rearing, may not be possible in this species, or may be maladaptive.

Finally, we may not have detected an effect because improved availability of carotenoids in early life could also benefit the reproductive value of daughters, reducing the difference in likely returns from investing differentially. While increased carotenoids may benefit male offspring in terms of adult plumage (Walker et al. 2013), any role in female coloration has not been explored. Studies of carotenoid effects on coloration have mostly focused on male traits

(Pryke et al. 2001; Pryke and Griffith 2006; Walker et al. 2014a), but evidence is growing that carotenoid-based coloration is also important in female status display (Crowley and Magrath 2004; Murphy et al. 2009; Murphy et al. 2009). In hihi, females are at the bottom of both an inter- and con-specific dominance hierarchy for foraging opportunities (Rasch and Craig 1988), so signals of female status may play a role in mediating conflict among females as well as males. If carotenoids are provided to mothers when rearing chicks, they are encouraged to attempt a second clutch that season (Thorogood et al. 2011). However, it remains unknown whether increased carotenoid availability in early life environments primes daughters' responses to environmental conditions when breeding themselves.

While we found no evidence that mothers manipulated the sex ratio of their clutches at laying, we did expect to detect sex-biased mortality before hatching. Previous work with hihi has suggested that male embryos are more vulnerable than females, perhaps because they are more susceptible to inbreeding effects (Brekke et al. 2010). We did not find increased male mortality in our cohorts: while female mortality rates were similar to those found by Brekke et al., males enjoyed a much reduced mortality rate (7.7% compared to 21.1%). Perhaps our supplementation of food (regardless of carotenoids content) enabled mothers to incubate their eggs more effectively (Castro et al. 2003). This could have buffered males from the effects of inbreeding depression, as the expression of this is a combination of inbreeding level and (variable) environmental conditions (Keller et al. 2012). Determining what environmental factors (including food supplementation regimes) influence the expression of inbreeding depression in hihi would be a valuable next step, as would cross-fostering eggs between nests to control for potential effects of supplementation on the incubation environment.

We show that, in hihi, increased access to carotenoids during egg laying does not result in an increase in male offspring produced. This result did not support our predictions that, as male hihi rely on carotenoid-based signals to maintain territories, they should benefit more from an increase in carotenoid availability, and so should be produced in greater numbers when carotenoids in the environment are plentiful. However, to understand the role that environmental conditions play in sex allocation clearly requires a better knowledge of the consequences of carotenoids for long-term reproductive success in both sexes.

We thank the New Zealand Department of Conservation and Hihi Recovery Team for supporting this work, and the Supporters of Tiritiri Matangi for accommodating us. The carotenoid supplement OroGlo was supplied by Kemin, and New Zealand Sugar donated the raw sugar base. Thanks also to Nick Mundy for accommodating us in his lab, and to Hanlu Twyman for valuable logistical advice. Naomi Langmore and 2 anonymous reviewers provided helpful comments. K.J.M. was supported by a Society in Science—Branco Weiss fellowship to R.T., and fieldwork was

funded by a post-doctoral research fellowship from the IoZ and a Royal Society Research Grant to J.G.E. W.T. was funded by a BBSRC David Phillips Fellowship to Claire Spottiswoode. Author contributions (following CRediT taxonomy): conceptualization, R.T., J.G.E., K.J.M.; methodology, R.T., J.G.E., K.J.M.; investigation, R.T., J.G.E., and K.J.M.; formal analysis, K.J.M. and R.T.; laboratory analysis, K.J.M. and W.T.; writing—Original draft, K.J.M. and R.T.; writing—review and editing, K.J.M., R.T., J.G.E., and W.T.; data curation, K.J.M. and J.G.E.; supervision, R.T. and J.G.E.; funding acquisition, R.T. and J.G.E.

Data accessibility: Analyses reported in this article can be reproduced using the data provided by MacLeod, Brekke, Tong, Ewen, and Thorogood (2016).

**Handling Editor:** Naomi Langmore

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