The consequences of early- and adult-life nutrition for the colour and conservation of hihi *Notiomystis cincta*

Leila Kathleen Walker

Girton College
July 2013

A thesis submitted to the University of Cambridge for the degree of Doctor of Philosophy
Declaration

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text. The text does not exceed 60,000 words and no part has been submitted elsewhere in application for a higher degree or diploma.

Leila Walker
Summary

Nutrition is profoundly important for practically all aspects of life. Getting enough of the right kind of food affects cellular function and energy acquisition, disease avoidance, mate attraction, trophic interactions and hence ecosystem structure. When nutritional needs are inadequately met, fecundity and survival can be adversely affected. However, the severity of these effects, and the importance of the particular life history stage when they were experienced, is incompletely understood. Understanding how and when nutritional conditions affect fitness is particularly important for the conservation of endangered species, especially when food supplementation is a key part of managing the few populations that remain. In this thesis, I explore the importance of nutrition during early- and adult-life for the hihi (*Notiomystis cincta*), a colourful, sexually dichromatic endangered New Zealand passerine.

I begin by investigating the importance of nestling nutritional environment for the expression of colourful plumage in adulthood. By experimentally supplementing nestlings with alternative dietary treatments, I demonstrate that early life nutritional conditions have long-term consequences for the expression of both carotenoid-based and structurally produced plumage features. Next I investigate whether these colourful plumage traits are sexually selected. I show that different components of a male’s colourful plumage, including yellow carotenoid-based, black melanin-based and white structurally-based colour, are relevant for different aspects of reproductive success. Using the dietary supplementation experiment, I also consider the impact of alternative supplementary foods on the growth and subsequent survival of hihi. I show that directly supplementing nestlings with protein has a negligible long-term survival benefit over supplementing carbohydrates, and present evidence that males and females have divergent nutritional needs during development – an important consideration for any supplementary feeding program. Finally, I consider whether moulting male hihi selectively forage for carotenoid-rich foods in the wild, as would be expected in a species that requires these pigments for sexual display. I present evidence that adult male hihi do indeed target carotenoid-rich foods during moult, which is consistent with the suggestion that dietary carotenoid access maintains signal honesty.

In short, by addressing evolutionary questions from a nutritional perspective, I discover how and when investment in key life history traits is prioritised, how this differs between the sexes and show how this knowledge might be used in supplementary feeding programs to the benefit of endangered species.
Acknowledgements

I have had the privilege and good-fortune of sharing this PhD experience with many wonderful people. It has been a challenging and rewarding past four years, that would not have been possible without the support, expertise and fun provided by those around me.

My supervisors Becky Kilner and John Ewen have been with me every step of the way, full of enthusiasm, encouragement and good scientific sense. Becky has taught me so much about so many things, and seemed to have a belief in me that I have never quite fathomed. Thanks must go to John for introducing me to hihi in the first place (I think the first hihi he showed me was a dead one!), and has continued to motivate me with his limitless interest in, and time for, my work.

I have received much-appreciated help and advice from a number of knowledgeable and patient individuals. Nick Davies and Martin Stevens have been my advisors, reading early reports and providing stimulating discussion. I am grateful to Martin for guiding me through the world of avian vision and for supplying me with invaluable Matlab code. Doug Armstrong, having set me on a path involving MARK and WinBUGS, remotely and (relatively!) painlessly led me through a crash-course in both. In Turkey, Filiz Karadaş, was a wonderful host during two stints of labwork, being my guide to both HPLC and the sights of Van. Closer to home, fellow hihi devotees have provided me with all manner of support and guidance. Rose Thorogood has always been on hand to ease my statistical woes, read countless drafts and lend a listening ear. At the IoZ, Patricia Brekke has shown a bottomless generosity with her time and genetics know-how. Aliénor Chauvenet and Kate Richardson have similarly been generous in their responses to my endless queries and requests.

In the field, data collection was made possible by the kind cooperation of the New Zealand Department of Conservation (DoC) and the Supporters of Tiritiri Matangi (SoTM). The DoC rangers Dave Jenkins and Daryl Stephens were logistical heroes and accommodated my fieldwork habits without complaint. Many members of SoTM made me feel most welcome during my long stints in New Zealand. They are too many to name, but invariably flattered me with their vast curiosity in my work and offers of assistance (including the un-enviable task of ferrying dead birds to the mainland). An army of volunteers, sent forth by Sue Cameron, made light work of the daily hihi duties. On the mainland, Ady Ewen kindly offered her home as a peaceful haven at the beginning and end of each of my visits. My field assistants Matt Gribble and Annette Fayet were simply fantastic. Matt brought a composed
calm to the frequently chaotic Tiritiri bunkhouse, and his enviable tree-climbing agility came into its own during fruit sampling. Annette embraced the gruelling schedule of the feeding experiment without complaint, and seemed to undertake her own feeding experiment with the bunkhouse occupants as subjects, producing daily baked miracles in the kitchen.

In Cambridge, members of the Behavioural Ecology group have inspired and entertained during tea breaks, lunch meetings and pub outings. In particular, a big thank you to my office mates over the years – Sue Aspinall, Amy Backhouse, Lucy Browning, Ana Duarte, Ornella De Gasperin, Carita Lindstedt and Claire Spottiswoode. Thanks also to Line María Arenas, Alfredo Attisano, Giuseppe Boncoraglio, Mike Brooke, Eleanor Caves, Sheena Cotter, Will Feeney, Mike Finnie, Tom Flower, Thanh-Lan Gluckman, Camilla Hinde, Anna Hughes, Nick Horrocks, Ben Jarrett, Kate Marshall, Carmen Panayi, Hannah Rowland, Marj Sorensen, Cassie Stoddard, Alex Török, Jolyen Troscianko and Jo Venables – you’ve all made our group a fantastically friendly place to be.

I owe a heartfelt thank you to my flatmates, who have made life a great laugh. In Cambridge thanks go to Melanie for all the cake, to Rachel for joining me in the Triathlon madness, and to Emma for providing a home. In Norwich, Hugh provided blissful order, Miranda a wonderful chaos, and Alicia stories that money can’t buy. A big thank you also to Jenny for welcoming me as an honorary cabbage. Likewise to Vero, José, Cat and Kelly who have adopted this shelduck/cockler like one of their own.

Finally, of course, thanks to my family. I owe my mum and dad everything; in particular, thanks for driving me back up Huntingdon Road that January. Thanks to my brother, Frank, for paying attention long enough to realise that no, I don’t work on kiwi! And thanks to Becky, for being there through it all.
## Contents

Declaration .......................................................................................................................... i
Summary ........................................................................................................................... ii
Acknowledgements ......................................................................................................... iii

**Chapter 1: General introduction** .................................................................................. 1

How do nutritional conditions in early life impact on key life history traits? ..........2
How do nutritional conditions in adult life impact on key life history traits? ....4
Can knowledge of nutritional needs in early- and adult-life focus conservation efforts to the appropriate life history stage? .......................................................... 5
Hihi – the ideal study species ......................................................................................... 7
Thesis format .................................................................................................................... 9

**Chapter 2: A window on the past: male ornamental plumage reveals the quality of their early life environment** ................................................................. 10

Introduction ................................................................................................................... 11
Methods .......................................................................................................................... 13
Results ............................................................................................................................. 18
Discussion ....................................................................................................................... 21

**Chapter 3: Sexually selected dichromatism in the hihi Notiomystis cincta: multiple colours for multiple receivers** ................................................................. 24

Introduction ................................................................................................................... 25
Methods .......................................................................................................................... 28
Results ............................................................................................................................. 35
Discussion ....................................................................................................................... 47

**Chapter 4: Giving hihi a helping hand: assessment of alternative rearing diets in food-supplemented populations of an endangered bird** ........................................... 53

Introduction ................................................................................................................... 55
Methods .......................................................................................................................... 57
Results ............................................................................................................................. 62
Discussion ....................................................................................................................... 66

**Chapter 5: Foraging for carotenoids: do colourful male hihi target carotenoid-rich foods in the wild?** ................................................................................. 70

Introduction ................................................................................................................... 71
Methods .......................................................................................................................... 73
Chapter 6: General discussion

How do nutritional conditions in early life impact on key life history traits?..90
How do nutritional conditions in adult life impact on key life history traits?..92
Can knowledge of nutritional needs in early- and adult-life focus conservation efforts to the appropriate life history stage?.................................................94
Future directions.................................................................97

References...............................................................................99
Appendix 1: Chapter 2 supplementary material.................................121
Appendix 2: Chapter 5 supplementary material.................................131
Appendix 3.............................................................................135
Chapter 1

General introduction

Nutrition lies at the heart of biology. It is relevant for almost every aspect of life, from aging (Fontana et al. 2010) and cancer prevention (Block et al. 1992) to the growth of populations and the structure of ecosystems (Simpson and Raubenheimer 2011). The consequences when nutritional needs are not met can be dramatic, as is evidenced by a global human population simultaneously battling food shortages and an obesity epidemic (WHO 2009). How well nutritional supply meets demand depends on the nutritional interactions between properties of the ecological environment and properties of the organism itself – the realm of nutritional ecology (Raubenheimer and Simpson 2009).

Nutritional ecology addresses a diverse range of questions, and awareness of an organism’s nutritional needs can enhance understanding of a broad range of topics. Whether an individual invests more in survival or reproduction, for example, may be best understood from the perspective of nutritional constraints. An individual’s foraging behaviour might be explained in terms of their own nutritional needs, or those of their offspring, or of their mate. Physiology, meanwhile, might be constrained by previous developmental nutrition, or conversely optimised to suit predicted nutritional conditions. And so on for a host of other areas in which nutrition may have some bearing. In short, considering evolutionary problems from a nutritional perspective can give insights that may otherwise remain obscured.

In this thesis I take a nutritional approach to evaluate how investment into key life history traits is prioritized. In particular, I consider how the nutritional requirements of an individual may differ according to life history stage, and ask what the consequences of nutritional environment are for key life history traits such as growth, survival and reproduction. I begin by focusing on nutritional conditions during early life development, given the pertinence of early life environment for subsequent fitness. I then move on to consider the importance of nutritional conditions during adulthood. Finally, I combine these two threads to consider whether knowledge of
early- and adult-life nutritional requirements can inform nutritionally focused conservation measures.

**How do nutritional conditions in early life impact on key life history traits?**

A single genotype has the capacity to produce a range of phenotypes given a range of environmental conditions (Pfennig et al. 2010). This phenotypic plasticity is not only responsible for the production of discrete phenotypes (i.e. polyphenisms), such as the alternative omnivore and carnivore morphs of spadefoot toad tadpoles (*Spea* spp.; Pfennig & Murphy, 2002), but also plays a role in the expression of continuous phenotypes, such as degree of coat thickness in meadow voles (*Microtus pennsylvanicus*; Gluckman et al. 2005; Lee & Zucker, 1988). Typically, the early developmental environment plays a fundamental role in determining the phenotypic path available to an organism (Monaghan 2008). In spadefoot toads, for example, early life diet determines whether a tadpole develops into an omnivorous or carnivorous form (Pfennig and Murphy 2002), while coat thickness in meadow voles depends on day-length immediately before birth (Lee and Zucker 1988; Gluckman et al. 2005).

The importance of early life conditions for subsequent phenotypes is seemingly ubiquitous. The range of environmental factors that influence subsequent phenotypes is diverse, and includes, but is not limited to, temperature (Lee et al. 2012), stress exposure (Costantini et al. 2012; Goodrich et al. 2013) immunological conditions (Butler and McGraw 2011), hormone levels (Rubolini et al. 2006), and nutritional environment (Birkhead et al. 1999; Blount et al. 2003; Scheuber et al. 2003; Taborsky 2006; Barrett et al. 2009; Krause et al. 2009). The phenotypic consequences are equally broad; effects of developmental environment have been found on, for example, growth (Birkhead et al. 1999; Searcy et al. 2004; Barrett et al. 2009; Hector et al. 2012; Lee et al. 2012), morphological traits (de Kogel and Prijs 1996; Searcy et al. 2004; Orledge et al. 2011; Hector et al. 2012), behavioural traits (Barrett et al. 2009; Krause et al. 2009; Hector et al. 2012), antioxidant defences (Blount et al. 2003; Costantini et al. 2012; Kim et al. 2013), immunity (Birkhead et al. 1999; Butler and McGraw 2011), telomere attrition (Hall et al. 2004), secondary sexual characters (Gustafsson et al. 1995; de Kogel and Prijs 1996; David et al. 2000; Scheuber et al. 2003).
Limited resources during development will generate subsequent trade-offs in key life history traits, such as growth, survival and reproduction. The trade-off between survival and reproduction has been well explored in the dietary restriction literature. In adult life, it is recognised that a reduced dietary intake often specifically of protein, extends lifespan while reducing reproductive output (Lee et al. 2008; Maklakov et al. 2008; Nakagawa et al. 2012). There is emerging evidence that a similar trade-off occurs during early life; in particular, that increased protein intake increases expression of sexually selected traits but reduces survival (Sentinella et al. 2013). Meanwhile, a trade-off between growth and lifespan has long been suspected, although difficult to prove (Metcalfe and Monaghan 2003). Recently, however, it has been demonstrated that experimentally induced catch-up growth reduces lifespan independently of final body size (Lee et al. 2012), lending robust support to the existence of such a trade-off.

In this thesis, I focus on the consequences of developmental nutrition for growth, survival and sexual display. Studies frequently consider general aspects of nutrition, usually defined broadly in terms of quality (Blount et al. 2003; Barrett et al. 2009; Krause et al. 2009) or quantity (Scheuber et al. 2003; Searcy et al. 2004; Taborsky 2006). Fewer studies have focused on more specific components of the nutritional environment, such as absolute protein content (Ohlsson et al. 2002; Gorman and Nager 2004), protein : carbohydrate ratio (Sentinella et al. 2013), and carotenoid and other dietary antioxidant content (McGraw et al. 2005a; Butler and McGraw 2012; Orledge et al. 2012; Kim et al. 2013). Most work investigating the impact of specific developmental nutrients on key life history traits typically uses laboratory or captive animals (e.g. Gorman and Nager 2004; Butler and McGraw 2012; Sentinella et al.
2013). However, it can be constructive to investigate these topics in wild populations where selection pressures that are absent in a laboratory, such as predation or parasitism, may operate (Tschirren et al. 2009). Therefore one of my aims here is to investigate how subtle shifts in the nutritional environment during development affect life history traits after fledging and in adult life.

**How do nutritional conditions in adult life impact on key life history traits?**

Although early life conditions evidently have considerable importance for the expression of subsequent phenotypes, the more immediate conditions experienced during adulthood will also be important for phenotype expression. This has been particularly well documented in the case of sexually selected traits. A fundamental component of Darwinian fitness is fecundity. To maximise fecundity, individuals must win the zero-sum game that is competition for access to mates, achieved through a combination of out-competing rivals and attracting partners (Andersson 1994). Secondary sexual traits play an important role in achieving these goals, and the condition of the bearer when it expresses these traits, in its adult life, is often of considerable importance in determining trait quality (Zahavi 1975; Grafen 1990; Johnstone 1995a).

One factor that will have a significant bearing on the condition of an individual is nutrition. Adult nutritional condition is important for the expression of a range of secondary sexual characters (Andersson 1994; Johnstone 1995b), and this has been particularly well documented for colourful avian plumage (Hill and Montgomerie 1994; Veiga and Puerta 1996; Hill 2000; McGraw et al. 2002). Feather colour can be a consequence of pigments deposited in the integument or of precise feather nanostructures, and, sometimes, both mechanisms are involved. The extent to which feather colour reflects nutritional condition depends, to some extent, on the mechanism of production. Melanins and carotenoids are two of the most common integumentary pigments; melanins can be produced by animals *de novo* but carotenoids cannot. While there is evidence that both melanin-based (Veiga and Puerta 1996) and carotenoid-based (Hill and Montgomerie 1994; Hill 2000) plumage is constrained by general aspects of nutrition, the latter appears particularly sensitive to dietary carotenoid access (Hill 1992).
Chapter 1: General introduction

An important and currently unresolved debate in the plumage colouration literature concerns identifying the mechanism that maintains the honesty of carotenoid-based signalling (Olson and Owens 1998). One possibility is that carotenoids are scarce in the environment, or at least difficult to obtain, and the quality of carotenoid-based plumage therefore reflects an individual’s foraging ability. In addition, the requirement for dietary carotenoids is potentially magnified by the additional antioxidant and immunostimulatory functions of carotenoids (Svensson and Wong 2011), which dictate that a potentially limiting resource be divided between serving a maintenance role and a signalling role. Central to this debate is establishing whether birds that display carotenoid based plumage selectively forage for carotenoid-rich foods. Evidence is emerging that some species can detect carotenoids in choice tests using artificial foods (Senar et al. 2010; Catoni et al. 2011). Whether this translates into selective foraging for naturally occurring carotenoid rich foods in the wild is unknown. Therefore a second aim of my thesis is to investigate whether wild individuals preferentially forage for foods that will maximize their potential to repel rivals and attract mates through sexual display.

**Can knowledge of nutritional needs in early- and adult-life focus conservation efforts to the appropriate life history stage?**

Conspecifics can have strikingly different dietary requirements according to their life history stage. In the extreme case of many insect species, different stages of the life cycle occupy profoundly different nutritional niches. In butterflies, for example, the larval stage is usually herbivorous and the adult stage usually nectarivorous (Bauerfeind and Fischer 2005). Less extreme, but still of significance, are the different nutritional needs of adults and dependent young in many other taxa, such as birds and mammals. For example, the nutritional demands of growth will be particularly pertinent for developing young, while the nutritional demands of reproduction are relevant only for adults. Degraded and heavily modified habitats will inevitably lack specific nutrients, meaning that many species struggle to meet their nutritional requirements. In endangered species, knowledge of differing nutritional requirements in early- and adult-life, and of the consequences when these are not met,
could be informative for judging which life history stage would gain most from any conservation efforts.

One conservation tool for which this distinction could be particularly relevant is supplementary feeding. Supplementary food is often provided to free-living populations of endangered species. The motivations for this intervention are varied and include increasing breeding success (Gonzalez et al. 2006; Robertson et al. 2006), improving survival (Armstrong and Ewen 2001; Jones and Merton 2012), anchoring transient individuals (López-Bao et al. 2010), providing safe alternatives to contaminated food (Gilbert et al. 2007), and generating viewing opportunities for tourists (Walpole 2001). Supplementary feeding programs have had a considerable degree of success (Jones and Merton 2012) although there are costs and caveats to supplementary feeding that are increasingly recognised (Jones and Reynolds 2008; Blanco et al. 2011). One potentially important consideration, that may enhance the success of supplementary feeding programs, is whether food provision should be tailored according to life history stage. This remains to be investigated.

Identifying the most appropriate nutritional composition of supplementary food has received limited attention in the conservation literature (Raubenheimer and Simpson 2006; Jones and Reynolds 2008). Likewise, how that food might be optimised to suit the needs of the intended recipient, defined in terms of their life history stage, but potentially also in terms of sex, reproductive status and other factors, has received even less attention. One exception is the bearded vulture (Gypaetus barbatus) supplementary feeding program, which recognises the differing dietary requirements of breeding and non-breeding adults (Oro et al. 2008; Margalida et al. 2009; Margalida 2010). A more consistent approach along these lines might be beneficial, targeting efforts where they are most needed. In order to achieve this, more work is required to identify whether, and how, nutritional requirements differ between life history stages, and what the consequences are. This is the final aim of my thesis.
Hihi – the ideal study species

Figure 1: Male (left) and female (right) hihi are sexually dichromatic. Males display carotenoid-pigmented yellow shoulders and breast, a melanin-pigmented black head, and structurally-coloured white ear tufts (Photos: Matt Gribble).

The study species I use to address questions posed throughout this thesis is the hihi (*Notiomystis cincta*), an endangered New Zealand passerine. I study the population on Tiritiri Matangi Island, a 220ha site in the Hauraki Gulf of New Zealand. This population is one of a small number of re-introduced populations established to increase total population size and limit vulnerability to extinction via stochastic events. This study species and site is well suited to answering questions of an evolutionary nature, as well as presenting an exciting opportunity for the immediate transfer of findings to a conservation context.

Hihi are sexually dichromatic. Males express multiple colourful plumage traits, including yellow, black and white plumage features, and females are a less conspicuous olive-brown (Figure 1). They live an average of 3-4 years (Low and Part 2009). Both males and females reproduce in their first year, and females typically show a peak in reproductive output in middle age (Low et al. 2007). Hihi have up to two successful clutches in a season, with up to five eggs per clutch. They are socially monogamous cavity nesters with high levels of extra-pair paternity (on average 68% of offspring within a brood are from extra-pair matings, Brekke et al. 2013). Males
are either territorial and seek a combination of extra-pair fertilizations and within-pair fertilizations, or are non-territorial floaters who rely exclusively on extra-pair fertilizations. Females perform all nest building and incubation, and the majority of provisioning to nestlings (Ewen and Armstrong 2000). The hihi population on Tiritiri Matangi Island breeds almost exclusively in nest boxes, making nestlings amenable to dietary manipulation. All birds at this site are ringed with a unique combination of colour rings, which allows for the identification and tracking of individuals throughout their life. Throughout this thesis, I use the hihi to address the three questions outlined above. Below I restate these central questions, and outline how the relevant chapters contribute to answering them.

How do nutritional conditions in early life impact on key life history traits?
I begin, in chapter 2, by exploring experimentally the importance of nestling protein and carotenoid access for the subsequent expression of adult male breeding plumage. I then establish, in chapter 3, the relevance of colourful plumage traits for the reproductive success of male hihi. In chapter 4 I look at the consequences of nestling dietary manipulations for nestling growth, and for short- and longer-term survival.

How do nutritional conditions in adult life impact on key life history traits?
Having established, in chapter 3, that yellow carotenoid-based plumage in male hihi has some bearing on reproductive success, I consider, in chapter 5, whether carotenoid acquisition is prioritised by moulting male hihi as they forage in the wild.

Can knowledge of nutritional needs in early- and adult- life focus conservation efforts to the appropriate life history stage?
In chapter 4 I target a range of nutritional supplements directly to nestlings and critically evaluate the consequences. Together with chapter 5, which focuses on how foraging for specific nutrients during adult life might be regulated to achieve a colourful sexual display, I contribute a greater understanding of how divergent nutritional needs and their consequences might help conserve both the hihi and other endangered species.
Thesis format

Each data chapter in this thesis has been prepared in manuscript format for publication and a small amount of information is therefore repeated in each chapter. I have included a paper published in collaboration, not directly related to my thesis research, in Appendix 3.
A window on the past: male ornamental plumage reveals the quality of their early life environment


It is well established that the expression of many ornamental traits is dependent on the current condition of the bearer. However, conditions experienced in early life are also known to be important for an individual’s subsequent fitness and therefore, directly or indirectly, for the fitness of their mate. Specifically, a recent hypothesis suggests that sexually selected traits might be sensitive to conditions experienced during early life development and thereby function as honest indicators of developmental history. Whether this applies to colourful male plumage, however, is largely unknown. We tested this idea with a field experiment by manipulating neonatal nutrition in a sexually dichromatic passerine, the hihi (Notiomystis cincta). We found that carotenoid supplementation increased nestling plasma carotenoid concentration, which was in turn correlated with increased yellow saturation in male breeding plumage after moulting. We also found that the post-moult luminance (lightness) of the white ear tufts tended to be reduced in males that had received an all-round nutritional supplement as nestlings. Black breeding plumage was not affected by neonatal nutritional treatment. Although the mechanisms that generate colourful plumage are evidently diverse, our results show that at least some parts of this display are accurate indicators of environmental conditions during development.
INTRODUCTION

Environmental conditions during development are of crucial importance for an individual’s subsequent life history (Metcalfe and Monaghan 2001). In particular, nutritional deficiencies during prenatal and/or early postnatal development can have subtle long-term consequences beyond obvious effects on immediate growth rates. For example, some adult secondary sexual traits, such as song repertoire size in great reed warblers (*Acrocephalus arundinaceus*) (Nowicki et al. 2000) and eye-span in stalk eyed flies (*Cyrtonyssus dalmanni*) (David et al. 2000), are detrimentally affected by a poor nutritional start in life. The developmental stress hypothesis (DSH) was originally proposed to explain how song complexity might honestly indicate male quality by reflecting an individual’s developmental history (Nowicki et al. 1998). More recently the DSH has been used to explain condition dependence of a broader range of sexually selected traits, particularly in terms of the nutritional constraints experienced as neonates (Spencer and MacDougall-Shackleton 2011). By heeding these signals, females stand to receive direct benefits via superior traits correlated with good developmental conditions and/or indirect genetic benefits if developmental stability is heritable.

Colourful avian ornaments are also susceptible to perturbations in developmental environment (Gustafsson et al. 1995; de Kogel and Prijs 1996; Ohlsson et al. 2002) but their potential role as developmental indicators, particularly in natural populations, has received limited attention. It is reasonable to expect a link between developmental environment and colourful traits. The long-term effects of developmental nutrition on morphology, physiology and metabolism can influence adult ability to assimilate the dietary pigments necessary for pigment-based colouration (Blount et al. 2003; McGraw et al. 2005a), and potentially also to form feather nanostructures necessary for structural colouration (Maia et al. 2012). A number of studies have considered the immediate effects of developmental environment on nestling plumage colour (Hõrak et al. 2000; Arriero and Fargallo 2006; Fargallo et al. 2007; Peters et al. 2007), demonstrating, for example, the importance of maternally invested egg yolk carotenoids for yellow plumage in nestling blue tits (Biard et al. 2005). In addition, some studies have tracked the downstream effects of developmental environment on colourful adult traits. However,
these have tended to focus on non-plumage based ornaments, such as the fleshy red wattles of ring-necked pheasants (*Phasianus colchicus*) (Ohlsson et al. 2002; Orledge et al. 2012), and the carotenoid-based bills of zebra finches (*Taeniopygia guttata*) (de Kogel and Prijs 1996; Birkhead et al. 1999; McGraw et al. 2005a; Tschirren et al. 2009) and mallard ducks (*Anas platyrhynchos*) (Butler and McGraw 2012). Despite this important work, the relevance of developmental conditions for colourful adult plumage is largely unknown.

The sensitivity of colourful adult plumage to developmental conditions may depend on the mechanism of colour production, which varies with the particular colour displayed. In adult birds, different mechanisms of colour production are affected by condition during moult to varying extents, and consequently signal different information. For example, colourful plumage resulting from the deposition of carotenoid pigments is often strongly influenced by environmental factors (Hill 2000; McGraw and Ardia 2003; Hõrak et al. 2004). Melanin-pigmented plumage, in contrast, is traditionally considered to be under stronger genetic than environmental control (Hill and Brawner 1998; McGraw and Hill 2000; Senar et al. 2003), and therefore less likely to be influenced by the developmental environment. Meanwhile, there is increasing evidence that structural colours (e.g. blues and whites) show condition dependence (Keyser and Hill 2000; McGraw et al. 2002; Hill et al. 2005; Meadows et al. 2012). In a similar way, colourful ornaments produced by different mechanisms may respond to different aspects of the developmental environment, or show differing degrees of developmental sensitivity, and consequently signal different information about developmental history.

The aim of this study was to investigate experimentally the downstream effects of neonatal nutrition on male breeding plumage in the New Zealand hihi (*Notiomystis cincta*), a sexually dichromatic passerine. Adult males display a black melanin-pigmented head, back and breast, yellow carotenoid-pigmented shoulders and wings (Ewen et al. 2006a), and structurally-coloured white ear tufts. We provided nestlings with nutritional and carotenoid supplements in a balanced and fully crossed experimental design, and subsequently examined the consequences for male breeding plumage. We predicted that properties of the carotenoid-based yellow plumage would be enhanced in nutritionally- and carotenoid-supplemented males, that properties of
the white ear tufts would be enhanced in nutritionally-supplemented males, and that the melanin-pigmented black would not be affected by either nestling treatment.

METHODS

Study species and site

Hihi are an endemic species of New Zealand. They are cavity nesters and lay clutches of 3-5 eggs (Oliver 1955). All nestlings develop female-like plumage initially and fledge at around 30 days. Juveniles then undergo a partial moult of body feathers, starting about six weeks after fledging and finishing about three months after fledging (Walker, unpublished data), during which time males obtain their breeding plumage. All birds reach breeding age in their first year and do not undergo a subsequent pre-breeding moult. Therefore body feathers grown during the post-fledging moult will last until after their first breeding season.

Adult hihi eat nectar, fruit and invertebrates. Nectar provides a source of carbohydrates (Rasch 1985); fruit provides a source of carbohydrates, lipids, fibre and carotenoids (chapter 5); and invertebrates provide a source of protein (Oliver 1998), and probably carotenoids (Eeva et al. 2010). The proportion of each food type in the adult diet varies with season and population. Estimates for summer, when adults are feeding nestlings, range from a high proportion of nectar (83% nectar, 6% fruit, 11% invertebrates; (Gravatt 1971)) to a high proportion of invertebrates (18% nectar, 13% fruit, 70% invertebrates; (Rasch 1985)). Nestling hihi are fed a combination of nectar, fruit and invertebrates by their parents (Lovegrove 1985). The precise proportion of these food types in the nestling diet is unknown, although invertebrates are believed to be the dominant food source up to 8-10 days (Higgins et al. 2001).

We studied a hihi population of around 100 pairs on 220 ha Tiritiri Matangi Island (36°36′ S, 174°53′ E). All adults were ringed, all nesting attempts occurred in nest boxes, and all nestlings were ringed prior to fledging. Supplementary food (sugar water; 20 % by mass) was provided year-round at six feeding stations across the island.
Experimental design

All first clutch nests (n = 84) in the 2010-2011 austral breeding season were randomly assigned to one of two nutritional treatments; a ‘nutritionally-supplemented’ treatment (N+, n = 42) or a ‘control’ treatment (N-, n = 42). Nestlings in N+ nests were hand-fed Wombaroo Lorikeet & Honeyeater Food, a dietary supplement typically used to maintain nectar-eating birds in captivity (nutritional composition: protein 140 g kg\(^{-1}\), fat 60 g kg\(^{-1}\), fibre 6 g kg\(^{-1}\); provided in 30% by mass solution). As a control, nestlings in N- nests were hand-fed a sugar water solution (sucrose 20% by mass, as provided year round at feeding stations and known to be provisioned to nestlings by parents (Thorogood et al. 2008)). Using a graduated plastic syringe, nestlings were fed every second day between four and 20 days of age. Volume fed increased incrementally from 0.2 mL at the age of four days to 3.0 mL at 20 days (Appendix 1 Table A1.1). The resulting amount of supplement (and therefore amount of constituent nutrients) consequently increased as nestlings grew, and was based on Wombaroo manufacturer recommendations (see Appendix 1 for details).

Within all N+ and N- nests, some nestlings had their nutritional treatment enhanced with carotenoids (C+) and some did not (C-). The carotenoids lutein and zeaxanthin were provided in the form of OroGLO® liquid (Kemin Industries) at a final concentration of 100 µg mL\(^{-1}\) (based on recommendations in Biard et al. 2006; see Appendix 1 for details), and at volumes specified previously. Lutein and zeaxanthin are the principle carotenoids used to pigment yellow feathers in hihi, and their ratio in OroGLO® closely matches that found in the circulating plasma of un-supplemented birds (Ewen et al. 2006a).

This design created four treatment groups (N+C+, N+C-, N-C+, N-C-), with a nestling-oriented carotenoid supplement overlaid on a brood-oriented nutritional supplement. Treatment group was randomly assigned to the heaviest nestling in a nest, and carotenoid treatment was then alternated down weight rank while nutritional treatment was held constant. Thus nestlings within a brood shared the same nutritional treatment but could each differ in carotenoid treatment, allowing for within brood comparisons of carotenoid effect. All nestlings within a brood received a treatment
and so in the case of uneven brood sizes the number of C+ and C- nestlings was unbalanced.

Parents continued to provision nestlings throughout the treatment period. Individual nestlings were identified by uniquely trimmed body down when less than or equal to 8 days, and by colour rings when more than 8 days. Nestlings were weighed each time they were hand fed. At 21 days nestlings were ringed with a unique combination of one metal ring and three colour rings, and a blood sample was taken by brachial venipuncture. Blood samples were centrifuged within three hours to separate plasma, which was stored at -20°C for later analysis of plasma carotenoid concentration. A total of 287 nestlings were treated, across four treatment groups (68 N+C+, 76 N+C-, 72 N-C+, 71 N-C-).

**Plasma carotenoid concentration**

Plasma samples were analysed for total carotenoid concentration by high performance liquid chromatography (HPLC), as described previously (Ewen et al. 2006b). Further details are given in Appendix 1.

**Plumage colour measurement**

Treated nestlings from first clutches fledged throughout December 2010. In March and April 2011, at the end of moult, juvenile males were caught for plumage colour measurement. Reflectance spectra were recorded using a USB-2000 spectrometer (Ocean Optics Inc., Dunedin, Florida, USA), DT-Mini Lamp (Deuterium Tungsten Halogen source) and a reflectance probe. The probe was held at 90º to the surface being measured. Each spectrum was an average of four scans and was calculated relative to a diffuse reflectance standard (WS-1, Ocean Optics Inc., Netherlands). The equipment was calibrated immediately prior to each bird being measured. Repeated measurements were taken from the left and right yellow shoulder patches in all males caught (n = 49 males), and from the black head (n = 41 males) and the white ear tufts (n = 35 males) in those males that were advanced enough in moult. The probe was lifted and replaced between repeat measurements within a body region (six repeats each for yellow and white regions, three for black region). Since a minority of birds
had not finished moulting, body moult was scored at the time of colour measurement as ‘finished’ or ‘not finished’. Treated males were captured again for plumage colour measurement in October 2011 (n = 25 males), at the start of their first breeding season. In addition to the measurements taken immediately post-moult, measurements of yellow and white patch size were made (this was not possible until all individuals had moulted the full extent of their yellow shoulders and white ear tufts). Two repeat measurements of white ear tuft length were taken using digital calipers. Digital photographs (Olympus Mju 300) were taken of the ventral surface, the left flank and the right flank of each bird to capture the entire yellow area. Two repeat photographs were taken of each region. A scale rule was included in each image, and yellow patch size was calculated in Image J by calibrating the scale and outlining yellow area using the freehand tool. Measurements of reflectance spectra and patch size were made blind with respect to nestling treatment.

Plumage colour analysis

Reflectance spectra were analysed using models in tetrahedral colour space to extract hue, saturation and luminance variables for each colour patch (Endler and Mielke 2005; Stevens et al. 2009; Stoddard and Prum 2011) (see Appendix 1 for full details, and Appendix 1 Figure A1.1 for mean reflectance spectra). Briefly, we first calculated photon catch values for the four single cones, used in colour vision, and the double cones, used in luminance vision, based on the reflectance spectra and measures of irradiance (Endler and Mielke 2005). Values generated using ‘d65’ irradiance levels and with blue tit spectral sensitivities are presented. Our measure of luminance, the perceived lightness of a patch, was simply the double cone photon catch values. To calculate saturation, the amount of colour compared to white light, we plotted the standardized single cone catch data for each individual in avian tetrahedral colour space (Endler and Mielke 2005) and calculated the distance from the centre of the colour space. To calculate hue, the colour type (e.g. blue versus red), we used ratios based on photon catch outputs that are broadly inspired by the way that opponent colour channels work, based on performing a principal component analysis (PCA) on a covariance matrix of the standardized single cone data (Komdeur et al. 2005; Spottiswoode and Stevens 2011; Stevens 2011).
Data analysis

One-way ANOVAs were used to assess repeatability of the different plumage colour variables (following (Lessells and Boag 1987)). All plumage colour variables demonstrated significantly higher between individual than within individual variation (p<0.0001). Repeatability was relatively high in most cases, with R values >0.73 for patch size measurements (yellow area and white length), and R values ranging from 0.40-0.62 for yellow descriptors (hue, saturation and luminance), 0.25-0.90 for black descriptors, and 0.32-0.64 for white descriptors. These values are consistent with other published repeatability values for colourful traits (Saino et al. 1999; Biard et al. 2005; Budden and Dickinson 2009), justifying the use of these colour variables. Repeated measures were averaged per individual for use in subsequent analyses. We chose to use saturation and luminance (not hue) for each colour patch in subsequent analyses. Yellow hue and saturation were highly correlated (r=0.99, p<0.001), and we chose to use saturation rather than hue as it most consistently reflects feather carotenoid content across species (Saks et al. 2003; McGraw and Gregory 2004). Hue and saturation were also correlated in black (r=-0.29, p=0.07) and white (r=0.48, p=0.004) plumage, and we again used saturation rather than hue. Luminance is encoded independently of colour and is analysed separately by visual systems, justifying its use for yellow, black and white patches.

Statistical analyses were carried out using R v. 2.13.0 (R Development Core Team 2011). The effects of nestling treatment and covariates on nestling plasma carotenoid concentration were investigated by fitting a linear mixed effects model using restricted maximum likelihood. Nest identity was included as a random effect in all models, to control for the fact that multiple nestlings from the same brood were sampled (details provided in Appendix 1), and an induced covariance matrix was used to estimate the similarity among nestlings from the same nest. Nutritional treatment (N+ / N-), carotenoid treatment (C+ / C-), brood size (number hatched), hatch date (centred Julian date), and an interaction between nutritional and carotenoid treatments were included as fixed effects. Carotenoid concentration was Box-Cox transformed to meet the assumptions of normality and homogeneity.
The effects of nestling treatment and covariates on colour variables (saturation and luminance for all patches, plus patch size and ear tuft length for yellow and white patches respectively) were investigated by fitting linear models. Separate models were run with each colour variable as the response. Nutritional treatment, carotenoid treatment, brood size, hatch date, and, where relevant, moult score (finished / not finished) were included as explanatory variables. A random nest effect was not appropriate because most nests were represented by only one nestling by the time of colour measurement. To control for the minority of cases where siblings from the same brood were measured, nests were re-sampled 1000 times (details provided in Appendix 1). The assumptions of normality and homogeneity were checked by the examination of residual plots. In cases where residual plots revealed substantial heterogeneity the linear model was fitted using generalised least squares, which allows the variance structure to be specified (details provided in Appendix 1). In cases of non-normality the response variable was box-cox transformed.

**RESULTS**

*Effect of treatments and covariates on plasma carotenoid concentration*

Our carotenoid treatment significantly increased levels of carotenoids circulating in the blood. Nestlings treated with carotenoids (C+) had significantly higher plasma carotenoid concentration at 21 days than nestlings not treated with carotenoids (C-) (10.21 µg mL$^{-1}$ ± 0.48 SE vs 6.07 µg mL$^{-1}$ ± 0.32 SE, df = 113, t = 4.86, p ≤ 0.0001). Nutritional treatment (N+/N-), brood size and hatch date did not explain a significant amount of variance in plasma carotenoid concentration (nutritional treatment: df = 69, t = 0.12, p = 0.90; brood size: df= 69, t = 0.32, p = 0.75; hatch date: df = 69, t = 0.88, p = 0.38), and there was no interaction between nutritional treatment and carotenoid treatment (df = 113, t = 0.81, p = 0.42). The variance explained by the random nest effect was 0.03 (53.3 %) and the residual variance was 0.02 (46.7 %).

*Effects of treatments and covariates on yellow plumage colour after moult*

Neither nutritional treatment nor carotenoid treatment directly affected properties of yellow plumage, either immediately following juvenile moult or at the start of the
subsequent breeding season (Appendix 1 Table A1.2). We investigated whether individual variation in carotenoid concentration could have masked any treatment effects by searching for a correlation between plasma carotenoid concentration and plumage colour. We found a significant positive correlation between plasma carotenoid concentration at 21 days and yellow saturation immediately following juvenile moult (Kendall’s rank correlation, $r = 0.23$, $p = 0.03$; Figure 1). Nestlings that hatched later in the year moulted breeding plumage that was less intensely yellow, having lower saturation values ($t = -2.34$, $p<0.05$ for 69.1% of re-samples with $n = 35$ nests; Appendix 1 Table A1.2). This effect was however no longer present by the start of the breeding season. The size of the yellow plumage patch at the start of the breeding season was smaller when males developed in larger broods ($t = -2.68$, $p < 0.02$ for 64.7% of re-samples with $n = 22$ nests; Appendix 1 Table A1.2). Moult score did not have a significant effect on yellow plumage properties (Appendix 1 Table A1.2).

**Effects of treatments and covariates on black plumage colour after moult**

Properties of black plumage were not affected by carotenoid or nutritional treatment at either time point that plumage was measured (Appendix 1 Table A1.3). Males that hatched later in the season displayed breeding plumage that was less intensely black (i.e. that had higher luminance; $t = 2.52$, $p<0.03$ for 62% of re-samples with $n = 22$ nests; Appendix 1 Table A1.3). Brood size and moult score did not affect properties of black plumage colour (Appendix 1 Table A1.3).

**Effects of treatments and covariates on white plumage colour after moult**

Supplementation with nutritional treatment influenced the eventual colour of the white ear tufts, but not in the way that we predicted: post-moult white luminance tended to be reduced as a consequence of supplementation ($t = -2.15$, $p<0.05$ for 52.1% of re-samples with $n = 27$ nests; Figure 2; Appendix 1 Table A1.4). This trend was no longer present when plumage was measured pre-breeding ($t = -0.32$, $p<0.05$ for 0% of re-samples with $n = 22$ nests; Appendix 1 Table A1.4). Carotenoid treatment, hatch date and brood size did not influence properties of white plumage at either time point (Appendix 1 Table A1.4).
Figure 1: Relationship between plasma carotenoid concentration at 21 days and yellow saturation immediately after juvenile moult. Plasma carotenoid concentration was significantly elevated in C+ males (closed circles) compared to C- males (open circles).

Figure 2: Mean (±SE) white luminance values immediately after juvenile moult for N- and N+ treated males.
DISCUSSION

Our experiment shows that some (though not all) components of a male’s colourful breeding plumage can accurately indicate the quality of the environment in which he developed, thus providing support for predictions of the developmental stress hypothesis. Previously, the importance of developmental conditions has been demonstrated for some colourful adult integuments (Ohlsson et al. 2002; McGraw et al. 2005a; Butler and McGraw 2012), and for nestling plumage (Hõrak et al. 2000; Peters et al. 2007), but never for adult plumage. The effect was most pronounced for yellow carotenoid-based plumage, which reflected multiple attributes of the nestling environment, such as brood size, hatch date and nutritional conditions. In particular, supplementation of the nestling diet with carotenoids increased plasma carotenoid concentration, and individuals with higher plasma carotenoid concentration, independent of treatment, went on to moult into breeding plumage that was coloured more intensely yellow.

There are at least two mechanistic explanations for how early life carotenoid access might influence carotenoid-based adult plumage. First, carotenoids provided to nestlings may be stored, for example in the liver (Martucci et al. 2004), and later deposited in the integument during moult (in this case 1.5 - 3 months after fledging). Second, individuals with increased access to carotenoids as young birds may have an improved ability to assimilate carotenoids as adults (McGraw et al. 2005a). That we find a correlation between nestling carotenoid concentration and adult plumage colour, but no effect of nestling carotenoid treatment on adult plumage colour, suggests that there is individual variation in nestling response to supplementation. Therefore yellow plumage indicates not only the nature of the nutritional environment in which a male developed, but his intrinsic capacity to exploit those conditions as well.

We also found that the white ear tufts, whose colour is determined structurally, were influenced by conditions experienced during development, although not in a way we expected. Individuals that received the nutritional supplement as nestlings then displayed less bright white feathers after moultling into their breeding plumage. Previous studies have demonstrated positive effects of a high-protein diet on
iridescent (Meadows et al. 2012) and white (McGlothlin et al. 2007) structural plumage and we assumed that our nutritional supplement, which contains proteins, would be beneficial for males. However, our results challenge this assumption. For example, we have found that nestling males whose diet was supplemented with Wombaroo suffered lower survival as a result (chapter 4; Walker et al. 2013a). Here we show that males given Wombaroo also end up producing less brightly white plumage. Perhaps there is an optimal level of protein in the diet for developing males (Raubenheimer et al. 2005), which was exceeded in our treatments, incurring both survival and signalling costs. Alternatively, or perhaps additionally, the lipid component of the nutritional treatment may have had a detrimental impact.

Since white plumage is not pigmented it was traditionally considered relatively cheap to produce (Prum 2006). However, evidence is accumulating that producing white feathers might be costly after all. For example, dark-eyed juncos (Junco hyemalis) maintained on enriched diets during moult grow larger, brighter white tail patches (McGlothlin et al. 2007), and house sparrows that undergo an accelerated moult develop less bright white wing-bars (Vágási et al. 2010). White feather barbs consist of a central air vacuole surrounded by a keratin cortex and typically lack nanostructural organisation (Shawkey and Hill 2005, 2006). The white appearance of feathers is a result of the incoherent scattering of all wavelengths of light by feather keratin (Prum 2006), and it has been suggested that a thicker keratin cortex may reduce the amount of white light reflected (Shawkey and Hill 2006). Potentially, this mechanism might be at work in hihi, with early nutritional stress (such as too much protein) impairing a male’s future ability to produce a keratin cortex of the appropriate thickness.

Finally, and consistent with previous work suggesting melanin-based traits are largely free from nutritional influence (Gonzalez et al. 1999; Hill 2000; Senar et al. 2003), we detected no effect of our nestling feeding treatments on black breeding plumage. Nevertheless, after controlling for any dietary manipulation, we found that earlier-hatched males had darker black plumage by the breeding season than later-hatched males. We cannot tell from our data whether this correlation is driven by the environment or by intrinsic qualities of the parents (because, for example, individuals
that are more melanic might breed earlier in the season and pass on any genetic predisposition to be melanic to their young).

All of the effects of the developmental environment on male breeding plumage found immediately after moult had declined by the time the breeding season began. The most likely explanation is that colour changes occur in the feathers during this 5-6 month period, perhaps as a result of feather degradation, feather soiling and/or pigment degradation (McGraw and Hill 2004). Our data therefore show that it is unwise to assume that the extent of colouration is fixed once feather pigments have been deposited and/or the feather microstructures determining any structural colours are grown. What are the implications for the potential signalling function of this colourful plumage? At this stage we have no definite answers because it is not yet known whether male hihi plumage functions to attract mates, or to repel rival males (e.g. Pryke and Andersson 2003), or both. Nevertheless, during the winter months following the moult, hihi aggregate in social groups providing plenty of opportunity for plumage assessment - whether by rival males or potential mates. In other species, plumage displayed well before the breeding season begins can still influence reproductive behaviours many months later (Mulder and Magrath 1994) and it would be interesting to determine whether the same is true for hihi.

In summary, we have shown that colourful plumage grown after leaving the nest is a window on the developmental history of the male (Spencer and MacDougall-Shackleton 2011), in common with other avian integuments that are known to be affected by neonatal environmental conditions (Ohlsson et al. 2002; McGraw et al. 2005a). Although the mechanisms that link developmental history with colourful plumage have yet to be identified, our study adds to the diversity of secondary sexual traits whose expression is strongly linked to the environment in which they developed.
Sexually selected dichromatism in the hihi *Notiomystis cincta*: multiple colours for multiple receivers

Patricia Brekke is gratefully acknowledged for performing the genetic analysis in this chapter.

Why do some bird species show dramatic sexual dichromatism in their plumage? Although sexual selection is the most common answer to this question, other competing explanations mean that it is unwise to assume that all sexual dichromatism has evolved by this mechanism. Even if sexual selection is involved, further work is necessary to determine whether dichromatism results from competition amongst rival males, or by female choice for attractive traits, or both. Here we test whether sexual selection accounts for the sexually dichromatic plumage displayed by hihi (*Notiomystis cincta*), with detailed behavioural and genetic analyses of a free-living island population. Bateman gradients measured for males and females reveal the potential for sexual selection, whilst selection gradients, relating reproductive success to specific colourful traits, show there is stabilizing selection on white ear tuft length in males. By correlating colourful male plumage with different components of reproductive success, we show that properties of yellow plumage are most likely a product of male-male competition, whilst properties of the black and white plumage are an outcome of both male-male competition and female choice. Male plumage therefore potentially signals to multiple receivers (rival males and potential mates) and this may explain the multi-coloured appearance of one of the most strikingly dichromatic species in New Zealand.
INTRODUCTION

Sexual dichromatism, where one sex is brighter and more colourful than the other, is usually assumed to be the result of sexual selection (Kimball and Ligon 1999). Thus, elevated reproductive success in more colourful males is assumed to be a consequence of more colourful individuals either having improved prospects in male-male contests (intrasexual selection), or having greater success at attracting females (intersexual selection). However, other explanations for sexual dichromatism have been proposed, including that predation pressure has selected for crypsis in the less colourful sex (Götmark 1993; Götmark et al. 1997; Heinsohn et al. 2005), and (conversely) that predation pressure has selected for colour in the more conspicuous sex (Baker and Parker 1979; Shine and Madsen 1994). Indeed, elaborate and colourful plumage does not always improve mating and reproductive success (Ligon and Zwartjes 1995; Van Rooij and Griffith 2012), suggesting that sexual selection need not necessarily be the default explanation for colourful plumage (Tarvin and Murphy 2012).

How, then, can we establish that any sexual dichromatism is the consequence of sexual selection? One approach is to search directly for evidence of sexual selection. The Bateman gradient ($\beta_{\text{sex}}$), for example, quantifies the slope of the relationship between mating and reproductive success for each sex (Bateman 1948; Arnold and Duvall 1994). Such a gradient indicates how strongly offspring production might be constrained by mate availability, a constraint essential (although not necessarily sufficient) for sexual selection to operate (Arnold and Duvall 1994). Unfortunately though, sexual selection is not the only possible explanation for a positive Bateman gradient, which may instead be the result of a number of alternative mechanisms (Gerlach et al. 2012). For example, a positive Bateman gradient may simply be a statistical artefact, given that the chance of detecting multiple mates increases when an individual has more offspring, or may be the result of fecundity selection, whereby the causal relationship is reversed and acquiring multiple mates is instead a consequence of having more offspring (Gerlach et al. 2012). The Bateman gradient, therefore, indicates whether sexual selection might be operating, although falls short of confirming that sexual selection definitely is operating (Klug et al. 2010; Gerlach et al. 2012). Despite these shortcomings, the Bateman gradient is widely acknowledged as having considerable utility in studies of sexual selection (Krakauer et al. 2011;
Jones 2009; Jennions and Kokko 2010). And yet, there is a shortage of high-quality measures from the wild, particularly for females (Bergeron et al. 2012), largely due to the difficulties of genetically assigning parentage to all offspring in a wild population. Another measure that quantifies selection in terms of total reproductive success is the selection gradient (β and γ). This approach relates total reproductive success to specific phenotypic traits, and can therefore identify specific colourful traits that might be sexually selected. Identifying which phenotypic traits contribute to variance in mating success is an important aim of sexual selection research (Gerlach et al. 2012), and can help identify the pattern of selection suggested by a positive Bateman gradient. Indeed, there has been a recent request for studies of sexual selection to quantify multiple aspects of selection, rather than relying on single measures (Klug et al. 2010).

In many species, a male’s route to acquiring reproductive success involves both intra- and inter-sexual selection. Among songbirds, for example, males commonly compete with rivals to acquire a territory (Part and Qvarnström 1997; Marchetti 1998; Andersson et al. 2002), and maintain exclusive mating access to a female (e.g. Delhey et al. 2003; Estep et al. 2005; Lehtonen et al. 2009; Eikenaar et al. 2011). Reproductive success may then be additionally enhanced by extra-pair fertilizations if males are perceived to be particularly attractive by other females (Double and Cockburn 2000; Rubenstein 2007; Chiver et al. 2008) and/or are able to outcompete the territorial male (Akcay et al. 2011). In some populations, some males fail to acquire a territory at all and rely exclusively on extra-pair fertilizations for any reproductive success (Sardell et al. 2010 and references therein). A male’s ability to maximise reproductive success will therefore be a result of intra- and/or inter-sexual selection, and colourful traits associated with total reproductive success may be aligned to one or both of these mechanisms, depending on which component of reproductive success they enhance.
Here we use the approaches outlined above to investigate the evolution of colourful male plumage in a sexually dimorphic and dichromatic passerine, the hihi (*Notiomystis cincta*) (Figure 1). Surprisingly, the relevance of plumage colour for reproductive success in hihi is undetermined, despite it being one of the most strikingly dichromatic bird species in New Zealand. Male hihi weigh *ca.* 40g and display colourful plumage, including carotenoid-based yellow shoulders (Ewen et al. 2006a), melanin-based black heads, and structurally produced white ear tufts. Conversely, females weigh *ca.* 32g and have subtler, olive-brown plumage with a white wing-bar. Hihi are socially monogamous with high levels of extra-pair paternity (on average 68% of offspring within a brood are from extra-pair matings, Brekke et al. 2013). Males are either territorial and seek a combination of extra-pair fertilizations and within-pair fertilizations, or are non-territorial floaters who rely exclusively on extra-pair fertilizations. We began by testing the potential for sexual selection in this species by plotting Bateman gradients ($\beta_{ma}$) for males and females. Next, we calculated selection gradients ($\beta$ and $\gamma$) with respect to various properties of colourful male plumage. Finally, we established the importance of territoriality and within-pair and extra-pair success for achieving total reproductive success. We investigated the relationship between male plumage colour and these components of reproductive success by assessing which properties of colourful plumage were
associated with i) ability to acquire a territory, ii) ability to withstand cuckoldry, and iii) success at gaining extra-pair fertilizations.

METHODS

Study species

Hihi are cavity nesters and females lay up to two successful clutches per season with 3-5 eggs per clutch (Oliver 1955). Females perform all nest building and incubation, and the majority of provisioning to nestlings (Ewen and Armstrong 2000). Both males and females can, and do, reproduce in their first year (Low et al. 2007).

Breeding season sampling

We studied a reintroduced hihi population on 220 ha Tiritiri Matangi Island (36°36’ S, 174°53’ E). This closed population has been intensely monitored since it was established in 1995 such that all breeding pairs and reproductive attempts are recorded (Ewen et al. 2011). In addition, biannual surveys are conducted using resighting of banded individuals to track individual survival accurately. Hence the identity of breeding adults is well known.

This study focuses on the 2010/2011 austral breeding season. Based on the pre-breeding season population survey, and observations of territoriality, nest building, incubation and provisioning behaviour, the identity of territorial males (n=79), floater males (n=17) and resident females (n=98) was recorded. Upon completion of nest building, nests were monitored daily (except during incubation) to retrieve any unhatched eggs and dead nestlings. Tissue samples were collected from dead nestlings (n=180; 81% of all dead nestlings) and from un-hatched eggs that showed obvious signs of embryonic development (n=60; 29% of all un-hatched eggs). Tissue samples could not be collected from dead nestlings that were not found (n=43), obviously, nor from un-hatched eggs that showed no obvious signs of development (n=149). Surviving nestlings were ringed at 21 days with a unique combination of one numbered metal ring and three colour rings, and a blood sample was taken by brachial
venepuncture. Blood and tissue samples were stored in 95% ethanol and refrigerated for subsequent genotyping and paternity assignment. The identity of nestlings that survived to fledging (at about 30 days) was recorded (n=243; 100% of all surviving nestlings sampled). A total of 483 offspring (unhatched embryos + dead nestlings + surviving nestlings) were sampled out of a possible 675 eggs laid.

*Genetic analysis and parentage assignment*

Genomic DNA was extracted from tissue and whole blood using the ammonium acetate precipitation method (Nicholls et al. 2000). All individuals were genotyped at a set of 19 autosomal selectively neutral microsatellite loci (see detailed methods in Brekke et al. 2009, 2013).

The parentage of each sampled offspring was assigned using a maximum-likelihood method in the program COLONY 2.0 (Wang and Santure 2009). This program allows estimation of parentage under a promiscuous mating system and incorporates full and half-sibship relationships to increase the statistical power (Wang 2004). COLONY 2.0 provides a posterior probability value for each maternal and paternal assignment, which usually increases when behavioural information on potential parentage is incorporated. We included maternal information from behavioural observations and there was a high congruence between social and genetic maternity assignment (99%), confirming the power of this method. Candidate fathers (n=91 males) were those males known to be alive and for which genotypes were available (95% were genotyped). The probability of the true parents being in the candidate lists was set at 0.90 for both fathers and mothers. Only parentage assigned with 95% confidence was accepted for use in subsequent analyses. Paternity was confidently assigned to 82% of offspring.

*Plumage colour measurement*

In October 2010, at the commencement of the 2010-2011 breeding season, males were caught in mist-nets or feeding station traps for plumage colour measurement. Reflectance spectra were recorded using a USB-2000 spectrometer (Ocean Optics Inc., Dunedin, Florida, USA) a DT-Mini Lamp (Deuterium Tungsten Halogen source)
and a reflectance probe, following methods described in Walker et al. (2013b) (chapter 2). Repeated measurements were taken from the left and right yellow shoulder patches, the black head and white ear tufts in all males caught (n=89 males; 3 repeats in each region). Repeated measurements of yellow patch size and white ear tuft length were also made, according to Walker et al. (2013b) (chapter 2). Repeatability of these measures is reported below.

*Plumage colour analysis*

To accommodate the fundamental differences between avian and human photoreception (Kelber et al. 2003; Osorio and Vorobyev 2005, 2008; Cuthill 2006), reflectance spectra were analysed using models in tetrahedral colour space (Endler and Mielke 2005; Stevens et al. 2009; Stoddard and Prum 2011). Hue, saturation and luminance variables for each colour patch were extracted according to methods described in Walker et al. (2013b) (chapter 2). In brief, photon catch values for the single and double cones were calculated using ‘d65’ irradiance spectra and blue tit (Cyanistes caeruleus) spectral sensitivities (a species with an ultraviolet shifted shortwave sensitive cone type; (Hart et al. 2000). Our measure of luminance, which describes the perceived lightness of a patch, was the double cone photon catch values. The standardized single cone catch data for each individual was plotted in avian tetrahedral colour space (Endler and Mielke 2005), and saturation, the amount of colour compared to white light, was calculated as the distance from the centre of the colour space. We calculated hue, the colour type (e.g. blue versus red), by performing a principal component analysis (PCA) on a covariance matrix of the standardized single cone data (see Walker et al. 2013b, chapter 2 for full details).

One-way ANOVAs were used to assess repeatability of the different plumage colour variables (following Lessells and Boag 1987). All plumage colour variables demonstrated significantly higher between individual than within individual variation (p<0.0001). Repeatability was relatively high in most cases, with R values >0.94 for patch size measurements (yellow area and white length), and R values ranging from 0.38-0.39 for yellow descriptors (hue, saturation and luminance), 0.25-0.31 for black descriptors, and 0.48-0.60 for white descriptors. These values are consistent with other published repeatability values for colourful traits (Saino et al. 1999; Biard et al.
2005; Budden and Dickinson 2009), justifying their use. Repeated measures per individual were averaged for use in subsequent analyses. Yellow hue and saturation were highly correlated ($r=0.94$, $p<0.001$), and we chose to use saturation rather than hue as it most consistently reflects feather carotenoid content across species (Saks et al. 2003; McGraw and Gregory 2004). Hue and saturation were also correlated in black ($r=-0.22$, $p=0.04$) and white ($r=0.48$, $p=0.004$) plumage, and we again used saturation rather than hue. Luminance is encoded independently of colour and is analysed separately by visual systems, justifying its use for yellow, black and white patches.

Statistical analysis

i) Bateman gradients ($\beta_{sa}$)

A non-parametric mood test was used to test for a difference in variance between male and female mating success (number of mates), and between male and female reproductive success (both number of fertilizations and number of offspring fledged). A mood test is a rank-based test that compares the variances of two samples that are not normally distributed, as is the case for male and female mating and reproductive success. Number of mates was identified as the number of different partners an individual had fertilizations with. Bateman gradients ($\beta_{sa}$) were calculated by performing a linear regression of reproductive success (number of fertilizations or number of offspring fledged) on mating success (number of mates) (Arnold and Duvall 1994). Number of mates, sex and an interaction between number of mates and sex were included as explanatory variables. 91 males and 91 females were included in these analyses.

ii) Linear selection gradients ($\beta$)

Linear (directional) selection gradients ($\beta$) were calculated as the coefficients from a multiple linear regression of relative male fitness on standardized male traits (Lande and Arnold 1983). Three separate measures of male fitness were used – number of mates, number of fertilizations and number of offspring fledged – and all were transformed to relative male fitness by dividing by the population mean. The male
traits considered were yellow saturation, yellow luminance, yellow patch size, black saturation, black luminance, white saturation, white luminance, white ear tuft length and tarsus length, and all were standardized (mean = 0, variance = 1) for selection analyses. Age was not included because this is not a trait on which selection can act. However, first year males had significantly lower values than older males for some traits (yellow saturation, yellow area, black saturation, white saturation, white luminance, and white length), which could obscure any within-age relationships for these traits. For this reason we standardized these traits within age classes (first year and older males) and then combined these two age classes for selection analyses (Sheldon and Ellegren 1999). All males of known age with complete colour measurements, and that were included as candidate fathers in the parentage assignment (i.e. had potential to be assigned as fathers), were included in these analyses (n=79).

Residuals were not normally distributed, and although this assumption is not necessary for estimating selection gradients (Lande and Arnold 1983), it is necessary for significance testing. Splitting selection analysis into parameter estimation and significance testing is commonly implemented (Mitchell-Olds and Shaw 1987; Fairbairn and Preziosi 1996; Sheldon and Ellegren 1999), and we therefore used GLMs with poisson errors (response = count) to test the significance of the regression coefficients. Standardized multivariate selection analysis was devised to estimate selective pressures after accounting for correlations between phenotypic traits (Lande and Arnold 1983). However, severe multicollinearity will affect the results, primarily by elevating standard errors and making it harder to reject the null hypothesis (Mitchell-Olds and Shaw 1987). We therefore assessed the extent of multicollinearity in our models by computing the variance inflation factor (VIF) for each trait. A VIF is the factor by which the standardized unexplained variance is inflated as a result of intercorrelation between explanatory variables (Sokal and Rohlf 1995), such that a large VIF indicates that the explanatory variables are highly correlated. Generally a VIF > 10 indicates harmful multicollinearity (Kennedy 1992). The VIF for a given explanatory variable \( i \) is calculated as \( 1/(1-R_i^2) \) where \( R_i^2 \) is the \( R^2 \) from a regression of \( i \) against all other explanatory variables. The VIFs for our explanatory variables ranged from 1.11 to 1.49, indicating that any correlations between the male traits were not large enough to cause concern.
iii) Decomposing contributions to reproductive success

We decomposed the relative contribution of the different elements of male reproductive behaviour (territory ownership, mate guarding, and extra-pair mating) to reproductive success. For all males, the importance of territoriality for total reproductive success was investigated by fitting generalized linear models (GLMs) with poisson errors and a log link function. The response (total reproductive success) was either total number of fertilizations or total number of offspring fledged. For territorial males only, the importance of within-pair versus extra-pair success for total reproductive success was determined by partitioning the variance in total reproductive success into its component parts (Webster et al. 1995). Since total reproductive success (T) is the sum of within-pair (W) and extra-pair (E) reproductive success, the variance in total reproductive success (var(T)) is the sum of variance in within-pair success (var(W)), variance in extra-pair success (var(E)), and twice the covariance between the two (cov(W,E)), i.e. var(T) = var(W) + var(E) + 2 cov(W,E) (Webster et al. 1995). Dividing by the mean of total reproductive success squared will standardize these variances. The standardized variance in total reproductive success is known as the opportunity for sexual selection (I) and is a measure of the maximum possible strength of selection. Reproductive success was measured as number of fertilizations, and as number of offspring fledged.

iv) Plumage colour and components of reproductive success

Next we investigated the importance of plumage colour for components of reproductive success by asking: i) are males with certain plumage properties more likely to hold a territory?; ii) having acquired a territory, are males with certain plumage properties less likely to be cuckolded?; and iii) are males with certain plumage properties more successful at gaining extra-pair and/or within-pair fertilizations? To answer questions i) and ii) we fitted GLMs with binomial errors and a logit link function. In the territory holding model the binary response variable was whether or not a male was a territory holder (n = 83 males of known age and with complete colour measurements). In the cuckoldry model the two-vector response was composed of the number of fertilizations on a territory that were the territorial male’s and the number that were another male’s (n = 65 territorial males; only territorial
males can be cuckolded). To answer question iii) we fitted GLMs with poisson errors and a log link function, where the response was a count of either the number of extra-pair fertilizations ($n=79$ territorial and floater males; all males eligible for extra-pair fertilizations) or number of within-pair fertilizations ($n=65$ territorial males; only territorial males eligible for within-pair fertilizations). The explanatory variables were as above (in ‘selection gradients’), in addition to age and age$^2$ being included to control for age.

Because the inclusion of the age terms introduced high levels of multicollinearity (age VIF = 22.77 - 25.86 across the three models), we took a multi-model inference approach using the package MuMIn in the program R (Grueber et al. 2011). This allowed us to generate a candidate-set of models that considered all possible combinations of explanatory variables whilst excluding correlated variables. Models were ranked by AICc value and model-averaged coefficients were generated by natural averaging over models with $\Delta$AICc < 2 (Grueber et al. 2011). All explanatory variables were standardized (mean = 0, variance = 1), which is necessary for model averaging (Grueber et al. 2011). Because there was overdispersion in the territory defence model, and in the extra-pair and within-pair models, we specified the dispersion parameters from quasibinomial and quasipoisson models (2.61, 2.92 and 2.33 respectively).

v) Non-linear selection gradients ($\gamma$)

For those traits identified as correlates of components of reproductive success (see (iv) above) we also calculated non-linear selection gradients ($\gamma$). We could not do this for all traits because of limited sample size, and therefore chose to focus on traits that results from (iv) above suggested could be under some form of selection. Non-linear selection gradients were calculated by including squared terms for the traits of interest in regression models (as detailed in (ii) above). Stabilizing selection (where intermediate values of a trait have higher relative fitness) would be indicated by a significant negative coefficient value. Disruptive selection (where extreme values of a trait have higher relative fitness) would be indicated by a significant positive coefficient value.
RESULTS

i) Bateman gradients ($\beta_\text{m}$)

Variance in the number of mates was significantly greater for males than females ($z = 5.19$, $p < 0.001$; Figure 2a), as too was variance in the number of fertilizations ($z = 5.75$, $p < 0.0001$; Figure 2b). Variance in the number of offspring that successfully fledged, however, did not differ between the sexes ($z = 1.33$, $p = 0.18$; Figure 2c). Reproductive success increased with increasing number of mates for both males and females (Figure 3). This was the case when reproductive success was measured as the number of fertilizations, and when it was measured as the number of offspring fledged (Figure 3; Table 1). The rate of increase was greater for males than females in the number of fertilizations model, but in the number of offspring fledged model the rate of increase was equal for males and females (Figure 3, Table 1). Results were qualitatively the same when reproductive success and mating success were relativized by dividing by their respective means.

ii) Linear selection gradients ($\beta$)

There was significant positive directional selection on white ear tuft length, both when fitness was measured as relative number of mates ($\beta = 0.25 \pm 0.10$; Table 2; Figure 4a) and when it was measured as relative number of fertilizations ($\beta = 0.21 \pm 0.10$; Table 2; Figure 4b). There was, however, no evidence for directional selection on ear tuft length when fitness was measured as relative number of offspring fledged ($\beta = 0.04 \pm 0.13$; Table 2). There was also no evidence for directional selection on any of the other plumage traits considered, nor on body size (Table 2).
Figure 2: Frequency histograms of a) mating success, b) fertilization success, and c) fledged offspring success for males (black bars) and females (grey bars). Males showed greater variance than females in mating success and fertilization success, but variance was equal for number of offspring fledged.
Figure 3: Bateman plots of reproductive success against number of mates for males (triangles and dashed lines) and females (circles and solid lines). Reproductive success measured as a) number of fertilizations, and b) number of offspring successfully fledged. Points are means ± SE and lines are predicted from linear regressions: a) $y = 1.88x + 0.32$ (male), $y = 0.93x + 2.49$ (female); b) $y = 0.72x + 0.54$ (male), $y = 0.58x + 1.02$ (female).
Table 1: Bateman gradients (β_m) for males and females (n = 182 birds). Reproductive success is measured as a) number of fertilizations, or b) number of offspring fledged. The significant interaction between reproductive success and mating success in a) indicates that the rate of increase in reproductive success with increasing mating success was greater for males than females. Significant terms highlighted in bold.

<table>
<thead>
<tr>
<th></th>
<th>estimate ± se</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>a) number of fertilizations</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intercept</td>
<td>2.49 ± 0.42</td>
<td>5.98</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>number of mates</td>
<td>0.93 ± 0.14</td>
<td>6.61</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>sex^a</td>
<td>-2.17 ± 0.50</td>
<td>-4.34</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>number of mates : sex</td>
<td>0.95 ± 0.17</td>
<td>5.57</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>b) number of offspring fledged</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intercept</td>
<td>1.02 ± 0.39</td>
<td>2.61</td>
<td>0.01</td>
</tr>
<tr>
<td>number of mates</td>
<td>0.58 ± 0.13</td>
<td>4.37</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>sex^a</td>
<td>-0.47 ± 0.47</td>
<td>-1.01</td>
<td>0.31</td>
</tr>
<tr>
<td>number of mates : sex</td>
<td>0.14 ± 0.16</td>
<td>0.88</td>
<td>0.38</td>
</tr>
</tbody>
</table>

^a Estimate relative to female
Table 2: Standardized linear selection gradients (\(\beta\)) for male plumage traits and body size, where male fitness is measured as relative number of mates, relative number of fertilizations and relative number of offspring fledged (n=79 males). Significant results highlighted in bold.

<table>
<thead>
<tr>
<th>male trait</th>
<th>relative number of mates</th>
<th>relative number of fertilizations</th>
<th>relative number fledglings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\beta \pm SE^a)</td>
<td>t-value^b</td>
<td>p-value^b</td>
</tr>
<tr>
<td>yellow saturation</td>
<td>-0.080 ± 0.116</td>
<td>-0.59</td>
<td>0.56</td>
</tr>
<tr>
<td>yellow luminance</td>
<td>-0.018 ± 0.102</td>
<td>-0.12</td>
<td>0.90</td>
</tr>
<tr>
<td>yellow area</td>
<td>-0.031 ± 0.103</td>
<td>-0.33</td>
<td>0.74</td>
</tr>
<tr>
<td>black saturation</td>
<td>0.043 ± 0.107</td>
<td>0.28</td>
<td>0.78</td>
</tr>
<tr>
<td>black luminance</td>
<td>0.027 ± 0.100</td>
<td>0.23</td>
<td>0.82</td>
</tr>
<tr>
<td>white saturation</td>
<td>0.087 ± 0.102</td>
<td>0.67</td>
<td>0.51</td>
</tr>
<tr>
<td>white luminance</td>
<td>-0.020 ± 0.100</td>
<td>-0.08</td>
<td>0.94</td>
</tr>
<tr>
<td>white length</td>
<td><strong>0.250 ± 0.100</strong></td>
<td><strong>2.46</strong></td>
<td><strong>0.02</strong></td>
</tr>
<tr>
<td>tarsus length</td>
<td>0.124 ± 0.102</td>
<td>1.25</td>
<td>0.22</td>
</tr>
</tbody>
</table>

^a parameter estimation from linear regression

^b significance testing from GLM
Figure 4: Directional and stabilizing selection on white ear tuft length: a) directional selection on white ear tuft length via relative number of mates, b) directional selection on white ear tuft length via relative number of fertilizations, c) stabilizing selection on white ear tuft length via relative number of offspring fledged. Regression lines are plotted with shaded grey area showing 95% confidence interval around the regression line.
iii) Decomposing contributions to reproductive success

Being territorial was important for maximizing total reproductive success. Territorial males had significantly greater fertilization success ($z = 6.48; p < 0.001$), and fledged significantly more offspring ($z = 4.42, p < 0.001$), than non-territorial floater males. Partitioning total variance in reproductive success into its component parts revealed that, for territorial males, variance in extra-pair success was the greatest contributor to male reproductive success (Table 3). Variance in within-pair success explained a smaller percentage of total variance, and covariance a smaller percentage still (Table 3). This was the case both when reproductive success was measured as number of fertilizations, and when it was measured as number of offspring fledged (Table 3). The negative covariance between within-pair and extra-pair success, in both cases, suggests that there is a trade-off between the two, although this is very weak (Table 3).

iv) Plumage colour and components of reproductive success

Males with a larger yellow patch size were significantly more likely to hold a territory than males with a smaller yellow patch size (Table 4a). Intermediate-aged males were also more likely to be territorial, as evidenced by a significant negative age$^2$ term (Table 4a). No other plumage properties influenced the likelihood of a male holding a territory, and neither did tarsus length (Table 4a).

Having acquired a territory, males with lighter yellow plumage (i.e. higher luminance) were more likely to be cuckolded than males with darker yellow plumage (Table 4b). No other terms considered, including age, tarsus length and other colour traits, influenced a male’s likelihood of being cuckolded (Table 4b).
Table 3: Variance in total reproductive success ($\text{var (T)}$) partitioned into within-pair variance ($\text{var (W)}$), extra-pair variance ($\text{var (E)}$), and within-pair extra-pair covariance ($\text{cov (W,E)}$). Reproductive success is measured either as number of fertilizations or as number of offspring fledged. Standardized value is absolute value divided by mean total reproductive success squared.

<table>
<thead>
<tr>
<th></th>
<th>number of fertilizations</th>
<th>number of offspring fledged</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>absolute value</td>
<td>standardized value</td>
</tr>
<tr>
<td>$\text{var (T)}$</td>
<td>16.22</td>
<td>0.50</td>
</tr>
<tr>
<td>$\text{var (W)}$</td>
<td>6.54</td>
<td>0.20</td>
</tr>
<tr>
<td>$\text{var (E)}$</td>
<td>9.76</td>
<td>0.30</td>
</tr>
<tr>
<td>$2 \text{cov (W,E)}$</td>
<td>-0.09</td>
<td>-0.003</td>
</tr>
</tbody>
</table>
Finally, males with lighter black plumage (i.e. higher luminance) and longer white ear tufts had greater extra-pair fertilization success than males with darker black plumage and shorter white ear tufts, respectively (Table 4c). Also, older males had greater extra-pair fertilization success than did younger males (Table 4c). No other plumage traits explained whether a male was likely to be successful at getting extra-pair fertilization success, and neither did tarsus length (Table 4c). None of the variables considered predicted a territorial male’s within-pair fertilization success (Table 4d).

v) Non-linear selection gradients (γ)

There was significant stabilizing selection on white ear tuft length when fitness was measured as relative number of offspring fledged (γ = -0.23 ± 0.10; Table 5; Figure 4c). There was no stabilizing or disruptive selection on white ear tuft length when fitness was measured as either relative number of mates or relative number of fertilizations (Table 5). There was also no evidence of either stabilizing or disruptive selection on yellow area, black luminance or yellow luminance (Table 5).
Table 4: Results of GLMs, following model averaging, investigating whether plumage colour predicts a male’s ability to a) acquire a territory (n=83 males), b) avoid cuckoldry (n=65 territories), c) gain extra-pair fertilizations (n=79 territorial and floater males), and d) gain within-pair fertilizations (n=65 territorial males).

Models a) and b) have binomial errors and estimates are in logits. Models c) and d) have poisson errors and estimates are in logs. All estimates have been standardized. Only terms that appear in model averaged set are listed. Significant terms highlighted in bold.

<table>
<thead>
<tr>
<th></th>
<th>estimate ± se</th>
<th>z-value</th>
<th>p-value</th>
<th>relative importance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>a) Territory acquisition</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intercept</td>
<td>2.94 ± 0.63</td>
<td>4.610</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>yellow area</td>
<td>1.36 ± 0.44</td>
<td>3.05</td>
<td>0.002</td>
<td>1.00</td>
</tr>
<tr>
<td>age^2</td>
<td>-0.67 ± 0.24</td>
<td>2.70</td>
<td>0.007</td>
<td>1.00</td>
</tr>
<tr>
<td>white luminance</td>
<td>0.59 ± 0.37</td>
<td>1.57</td>
<td>0.12</td>
<td>0.69</td>
</tr>
<tr>
<td>white saturation</td>
<td>0.61 ± 0.49</td>
<td>1.23</td>
<td>0.22</td>
<td>0.42</td>
</tr>
<tr>
<td>black luminance</td>
<td>-0.26 ± 0.33</td>
<td>0.77</td>
<td>0.44</td>
<td>0.21</td>
</tr>
<tr>
<td>yellow luminance</td>
<td>0.35 ± 0.38</td>
<td>0.90</td>
<td>0.37</td>
<td>0.16</td>
</tr>
<tr>
<td>black saturation</td>
<td>0.21 ± 0.39</td>
<td>0.54</td>
<td>0.59</td>
<td>0.06</td>
</tr>
<tr>
<td><strong>b) Avoiding cuckoldry</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intercept</td>
<td>-0.15 ± 0.16</td>
<td>0.88</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>yellow luminance</td>
<td>-0.39 ± 0.18</td>
<td>2.14</td>
<td>0.03</td>
<td>1.00</td>
</tr>
<tr>
<td>tarsus length</td>
<td>0.16 ± 0.17</td>
<td>0.92</td>
<td>0.35</td>
<td>0.54</td>
</tr>
<tr>
<td>white saturation</td>
<td>0.09 ± 0.15</td>
<td>0.59</td>
<td>0.56</td>
<td>0.17</td>
</tr>
<tr>
<td>white luminance</td>
<td>-0.10 ± 0.18</td>
<td>0.54</td>
<td>0.59</td>
<td>0.16</td>
</tr>
<tr>
<td>black saturation</td>
<td>0.07 ± 0.15</td>
<td>0.47</td>
<td>0.64</td>
<td>0.14</td>
</tr>
<tr>
<td>age^2</td>
<td>-0.04 ± 0.12</td>
<td>0.35</td>
<td>0.73</td>
<td>0.07</td>
</tr>
<tr>
<td>black luminance</td>
<td>-0.08 ± 0.22</td>
<td>0.37</td>
<td>0.72</td>
<td>0.07</td>
</tr>
<tr>
<td>yellow area</td>
<td>0.09 ± 0.17</td>
<td>0.52</td>
<td>0.60</td>
<td>0.07</td>
</tr>
</tbody>
</table>
### Chapter 3: Sexually selected dichromatism

<table>
<thead>
<tr>
<th></th>
<th>estimate ± se</th>
<th>z-value</th>
<th>p-value</th>
<th>relative importance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>c) Extra-pair fertilizations</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intercept</td>
<td>0.99 ± 0.25</td>
<td>4.00</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td><strong>black luminance</strong></td>
<td>0.29 ± 0.12</td>
<td>2.33</td>
<td>0.02</td>
<td>1.00</td>
</tr>
<tr>
<td>age(^2)</td>
<td>-0.35 ± 0.26</td>
<td>1.36</td>
<td>0.18</td>
<td>1.00</td>
</tr>
<tr>
<td><strong>white length</strong></td>
<td>0.48 ± 0.13</td>
<td>3.61</td>
<td>0.0003</td>
<td>0.56</td>
</tr>
<tr>
<td>yellow luminance</td>
<td>-0.13 ± 0.13</td>
<td>0.97</td>
<td>0.33</td>
<td>0.56</td>
</tr>
<tr>
<td><strong>age</strong></td>
<td>0.69 ± 0.20</td>
<td>3.44</td>
<td>0.0006</td>
<td>0.44</td>
</tr>
<tr>
<td>white saturation</td>
<td>0.08 ± 0.12</td>
<td>0.68</td>
<td>0.49</td>
<td>0.30</td>
</tr>
<tr>
<td>tarsus length</td>
<td>0.07 ± 0.14</td>
<td>0.52</td>
<td>0.60</td>
<td>0.20</td>
</tr>
<tr>
<td><strong>d) Within-pair fertilizations</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intercept</td>
<td>1.09 ± 0.11</td>
<td>9.56</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>yellow luminance</td>
<td>-0.19 ± 0.12</td>
<td>1.54</td>
<td>0.12</td>
<td>1.00</td>
</tr>
<tr>
<td>white luminance</td>
<td>-0.11 ± 0.12</td>
<td>0.93</td>
<td>0.35</td>
<td>0.40</td>
</tr>
<tr>
<td>tarsus length</td>
<td>0.12 ± 0.13</td>
<td>0.89</td>
<td>0.37</td>
<td>0.40</td>
</tr>
<tr>
<td>black saturation</td>
<td>0.09 ± 0.10</td>
<td>0.88</td>
<td>0.38</td>
<td>0.40</td>
</tr>
<tr>
<td>white saturation</td>
<td>0.08 ± 0.10</td>
<td>0.79</td>
<td>0.43</td>
<td>0.26</td>
</tr>
<tr>
<td>yellow area</td>
<td>0.08 ± 0.13</td>
<td>0.58</td>
<td>0.56</td>
<td>0.04</td>
</tr>
</tbody>
</table>
Table 5: Standardized non-linear selection gradients ($\gamma$) for yellow area, black luminance, white luminance and white length, where male fitness is measured as relative number of mates, relative number of fertilizations and relative number of offspring fledged (n=79 males). Significant result highlighted in bold.

<table>
<thead>
<tr>
<th>male trait</th>
<th>relative number of mates</th>
<th>relative number of fertilizations</th>
<th>relative number fledglings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\gamma \pm SE^a$</td>
<td>t-value$^b$</td>
<td>p-value$^b$</td>
</tr>
<tr>
<td>yellow luminance</td>
<td>-0.054 ± 0.072</td>
<td>-0.75</td>
<td>0.45</td>
</tr>
<tr>
<td>yellow area</td>
<td>-0.030 ± 0.079</td>
<td>-0.39</td>
<td>0.70</td>
</tr>
<tr>
<td>black luminance</td>
<td>-0.052 ± 0.043</td>
<td>-1.16</td>
<td>0.25</td>
</tr>
<tr>
<td>white length</td>
<td>-0.082 ± 0.087</td>
<td>-1.18</td>
<td>0.24</td>
</tr>
</tbody>
</table>

^a parameter estimation from linear regression

^b significance testing from GLM
vi) Unknown fertilizations

We were not able to assign paternity to every single fertilized egg in the population. The reasons for a fertilized egg not being assigned a father were twofold. Firstly, ~71% of unhatched eggs (149 out of 209) did not show visible signs of development and any developing embryo that may have been present could not be sampled. Secondly, the bodies of ~19% of chicks that hatched but died before fledging (43 out of 223) were not recovered. As a consequence, ~28% of the eggs laid (192 out of 675) were not sampled and could not have paternity assigned (although ~12% of non-developing eggs were likely infertile; Hemmings et al. 2012). If the probability of embryo/chick death is biased according to father phenotype, then these unknown fertilizations could bias our results (for example, if embryos/chicks that die are more likely to be sired by males with short ear tufts then the fertilization success of short-ear tuft males will be underestimated). We therefore tried to establish whether the male traits that we found to be important for mating/reproductive success (ear tuft length, black luminance, yellow luminance) relate to embryo and nestling mortality, because we were able to assign paternity to some dead offspring.

The fathers of offspring that died (both as embryos and chicks) and the fathers of offspring that survived did not differ in either black luminance ($t = -0.39$, $p = 0.70$) or yellow luminance ($t = -0.18$, $p = 0.85$). This suggests that the importance of black luminance for determining extra-pair fertilization success (see above), and the importance of yellow luminance for cuckoldry (see above), should be unaltered if unknown paternity fertilizations (all from dead offspring) were included in the analyses. The fathers of offspring that died had significantly longer white ear tufts than the fathers of offspring that survived ($t = -2.04$, $p = 0.04$). This result is interesting, and might explain why there was directional selection via mating and fertilization success, but stabilizing selection via fledgling success.

**DISCUSSION**

Both male and female hihi exhibit ed positive Bateman gradients, indicating that there is potential for sexual selection to be operating in both sexes. Measuring selection gradients in relation to male plumage traits revealed that there was positive directional
selection for white ear tuft length via mating and fertilization success, but stabilizing selection for white ear tuft length via fledgling success. In addition, we found that different plumage traits were associated with different components of male reproductive success: a larger yellow plumage patch increased likelihood of being territorial, lighter yellow plumage (higher luminance) increased likelihood of being cuckolded, and lighter black plumage (higher luminance) and longer white ear tufts increased extra-pair fertilization success (Figure 5). However, because extra-pair reproductive success was the greatest contributor to total male reproductive success, it was only white ear tuft length, the plumage trait most strongly associated with extra-pair success, which showed evidence of being under selection (Figure 5).

**Figure 5:** A male hihi with distinctive carotenoid-based yellow, melanin-based black, and structurally-produced white plumage. Text indicates how components of reproductive success change with increasing expression of the plumage trait, and whether there was any evidence for selection on these traits.

What do these results tell us about the role of male-male competition and female choice in the evolution of these plumage traits? Territoriality is usually considered to be primarily an outcome of competition amongst males to acquire and defend a territory (Part and Qvarnström 1997; Marchetti 1998; Andersson et al. 2002). Yellow plumage in hihi appears to be a consequence of male-male competition, since it is
associated with both territory acquisition and mate defence (avoiding cuckoldry). For example, the 15 males who failed to acquire a territory had significantly smaller yellow plumage patches than the 68 territorial males. In contrast, extra-pair fertilizations, which often contribute substantial variance to male reproductive success (Vedder et al. 2011), are usually assumed to be under female control (Jennions and Petrie 2000). Female superb fairy-wrens (*Malurus cyaneus*), for example, undertake pre-dawn forays into other territories for the express purpose of seeking out extra-pair mates (Double and Cockburn 2000). However, in some species, females appear to have limited control over extra-pair paternity. If extra-pair copulations occur on her own territory, for example, a female’s choice of extra-pair mate will be restricted to only those males able to evade her social mate, meaning the role of male-male competition will be more prominent (e.g. Akcay et al. 2011). Furthermore, forced extra-pair copulations occur in some species, with males aggressively coercing females into mating with them (Clutton-Brock and Parker 1995). Sexual coercion has been suggested as a third mechanism of sexual selection, and there may be strong selection on traits that improve a male’s chances of successfully forcing copulations (Clutton-Brock and Parker 1995). Aggressive extra-pair copulations are common in waterfowl (Adler 2010) and have also been reported in some passerines (Westneat and Stewart 2003), including hihi (Castro et al. 1996; Ewen et al. 2004; Low 2005).

In hihi, both solicited and forced extra-pair copulations occur, and both are believed to result in fertilizations (Brekke et al. 2013). Given the high frequency of forced extra-pair copulation in hihi (83% of all EPCs observed by Low 2005), the traditional view that extra-pair fertilizations, and therefore traits associated with extra-pair fertilization success, are driven primarily by female choice may not apply. Nevertheless, solicited extra-pair copulations do occur (Low 2005), and perhaps at a greater rate than estimated if they are less conspicuous than the highly visible and vocal forced extra-pair copulations. Furthermore, it is possible that female hihi exert some additional control via post-copulatory female choice, as seen for example in feral fowl where females eject the sperm of subdominant males that have coerced them into mating (Pizzari and Birkhead 2000). Indeed, the positive female Bateman gradient that we present here suggests females do stand to gain from mating multiply, and would therefore benefit from seeking out extra-pair males. Thus, in contrast to traits associated with territoriality, there may be some scope for black luminance and
white ear tuft length, the traits associated with extra-pair fertilization success, to have evolved by both inter- and intra-sexual selection, and potentially also by sexual coercion. Perhaps the black plumage serves to provide a contrasting backdrop for the white ear tufts during their display. Indeed, behavioural observations confirm that white ear tufts are used in displays towards other males, during contests at feeding sites and on territories, and towards females, during courtship and copulation (pers. obs.).

We have demonstrated that male hihi with longer white ear tufts have greater mating and fertilization success, but that their offspring are more likely to die than those of short-ear-tufted males. Stabilizing selection is seen when signals incur costs (e.g. Hinde at al. 2010), so could Zahavian explanations for the evolution of elaborate sexual ornaments account for stabilizing selection on white ear tufts? At first, the Handicap Principle (Zahavi and Zahavi 1997) appears to offer a plausible interpretation of the data because the costs associated with displaying an exaggerated ear tuft potentially convey information we might expect a female to be interested to know about, namely the viability of her future young. A key distinction here, however, is that the costs associated with longer ear tufts are not borne directly by the signaller (the male) but by his offspring (through reduced viability). The Handicap Principle states that the costs of greater signal expression must outweigh the mating advantage (Grafen 1991). Here, males that falsely advertise their qualities with lengthened ear tufts incur no personal costs, in terms of reduced mating advantage, and successfully acquire additional fertilizations. The costs are instead borne later by the offspring and, indirectly, by the female. For these reasons, we conclude that the Handicap Principle does not account for the evolution of white ear tufts in hihi.

Despite yellow and black plumage being correlated with territoriality and extra-pair success, respectively, we did not find evidence that these traits were under either linear or non-linear selection (Figure 5). One possibility is that we had insufficient power to detect selection. However, our sample sizes do fall within the range of those reported in equivalent studies that were generally capable of detecting various forms of selection (Westneat 2006; McGlothlin et al. 2005; Sheldon and Ellegren 1999). Another consideration, is that perhaps with a different combination of ecological factors, as might be generated, for example, in different years, these plumage traits
might become the target of sexual selection. Thus, the persistence of multiple components in the hihi’s sexually dichromatic plumage could simply reflect the spectrum of ecological conditions experienced by the population over several years and, consequently, result from fluctuating selection on a battery of different plumage types (see Brooks and Couldridge 1999). Extrapolating more generally, we might then expect to see more complex sexual ornamentation, involving multiple component parts, in highly variable environments (where the environment includes the sensory system of the potential mate (e.g. Brooks 2002) as well as wider ecological circumstances).

Ecological conditions, and their consequences for evolutionary potential, will also be influenced by management interventions. Despite being a wild population, the hihi on Tiritiri Matangi Island do receive a level of management that has some potential to reduce the intensity of sexual selection on plumage traits. Providing artificial nest boxes and supplementary food may have generated a scenario in which poorer-quality individuals are able to gain territories and/or reproductive success when they might otherwise (i.e. in un-managed habitats) have been unable to do so. This would reduce the degree of variation in reproductive and/or mating success, by elevating the success of individuals that previously had limited success, and therefore reduce the potential for sexual selection. To test this idea would require conducting an equivalent study on a hihi population that receives no management (currently only the Hauturu Island population), which, almost by definition, would be a considerable (but worthwhile) challenge.

A final note of caution is that all of the approaches used here involve correlational analyses and so do not identify cause and effect (Grafen 1987): we cannot tell whether trait expression enhances reproductive success or whether reproductive success enhances trait expression. It is not inconceivable, for example, that reproductive activity could influence the luminance of black plumage. Frequent extra-pair forays, which involve aggressive encounters with other birds and tussles in the abrasive leaf litter (Castro et al. 1996), might cause males to undergo greater feather wear, reducing the melanin content of black feathers and thus making them appear brighter (McGraw et al. 2005b). Likewise with selection gradients, although we measured a number of traits, we cannot exclude the possibility that white ear tuft
In conclusion, we provide a rare quantification of male and female Bateman gradients in a natural population. We have shown that the multiple colourful plumage traits of male hihi are relevant for multiple receivers. Yellow plumage displays are directed primarily towards other males in contests over territories. Black and white plumage displays are directed both towards other males, during disputes for extra-pair fertilizations, and towards females to either to charm them or to force extra-pair success. We show evidence of stabilizing selection on white ear tuft length, during this year of study, but recognise that different ecological pressures during other years may elevate the importance of yellow and black traits such that they come under selection also.
Chapter 4

Giving hihi a helping hand: assessment of alternative rearing diets in food supplemented populations of an endangered bird


Patricia Brekke is gratefully acknowledged for performing the genetic sexing in this chapter

Providing supplementary food to endangered bird species is a common management action. Research has tended to focus on whether or not supplementary food should be provided, and relatively less attention has been paid to the form that food should take. Supplementation is also commonly made directly to adult individuals. However, the potentially long-lasting consequences of developmental diet make it relevant to consider what type of supplementary food is most appropriate for nestling birds. We sought to explore these issues in a wild population of hihi (Notiomystis cincta), an endangered New Zealand passerine in which adults in four out of five extant populations receive supplementary food. We directly provisioned nestling hihi with four alternative dietary treatments (protein- or carbohydrate-based diet with or without carotenoids) and assessed the effect of supplementation on i) nestling growth parameters, ii) survival from hatching to fledging, and iii) survival from fledging to recruitment (breeding age). Firstly, we found a sex-specific effect of protein supplementation on growth inflection point: inflection point was later for females that received a high-protein diet compared to controls, and earlier for males. Secondly, we found an interacting effect of sex and diet on survival from hatching to fledging, such that females benefited from a high-protein diet whilst males suffered. We also found an interacting effect of protein treatment and carotenoid treatment on survival to fledging, such that the positive effects of carotenoid supplementation were negated if carotenoids were provided in combination with the protein supplement. Finally, we
were unable to detect an effect of nestling dietary treatment on survival from fledging to recruitment. Our results suggest the short-term consequences of nestling supplementary feeding are more pronounced than any long-term effects. We also found evidence that dietary supplementation had opposing effects on male and female nestlings, which may be an important, previously overlooked, consideration when evaluating the conservation value of feeding regimes.
INTRODUCTION

Conservation of species often requires supportive management, and there are a number of measures that can be applied to address factors that may be limiting population success (Jones and Merton 2012). Supplementary feeding is one form of intervention that has been used to improve low productivity in a range of bird species (Gonzalez et al. 2006; Robertson et al. 2006; Schoech et al. 2008; Jones and Merton 2012). Conservation management often focuses on whether or not supplementary feeding is required (Armstrong et al. 2007; Oro et al. 2008; Schoech et al. 2008), but there has been little consideration of exactly which nutrients should be provided.

Supplementary feeding is a costly and labour intensive management action, potentially requiring long-term investment of time and resources. It is crucially important that interventionist management on this scale has a net conservation benefit and does not generate undesirable side effects (Blanco et al. 2011). In the critically endangered kakapo (*Strigops habroptila*), for example, supplementary feeding of breeding females inadvertently caused a male-biased offspring sex ratio that effectively increased overall extinction risk of the species (Clout et al. 2002). This was subsequently corrected by changes to the feeding regime (Robertson et al. 2006).

On-going monitoring and evaluation of the outcomes of supplementary feeding is an important part of an adaptive approach to optimising management, in all cases where it is employed (Armstrong et al. 2007).

Here we consider which nutrients should be provided to hihi (*Notiomystis cincta*), an endangered New Zealand bird (IUCN 2011) whose recovery programme is centred around supplementary feeding. Although once found throughout the northern regions of New Zealand, hihi had become restricted to a single small offshore island (Hauturu) by the late 1800s. The remnant population on Hauturu Island exists without supportive management but all four extant translocated populations (Tiritiri Matangi Island, Kapiti Island, Karori Wildlife Sanctuary, and Maungatautari Ecological Island) rely, to varying extents, on the provision of supplementary sugar water. The effects of supplementing sugar water have been assessed previously. On both Tiritiri Matangi and Kapiti Islands there is evidence that provision of sugar water supplement improves adult survival (Armstrong and Ewen 2001; Chauvenet et al. 2012).
Mokoia Island food supplementation (either sugar water or a full supplement) increased reproductive success, although adult survival remained low, resulting in marginal population growth (Armstrong et al. 2007).

Hihi feed on fruit and invertebrates as well as nectar, and whilst sugar water only provides carbohydrates, feeding programs for other endangered species often provide more complete supplements (Jones et al. 1995; Clout et al. 2002). An alternative, full dietary supplement with a high protein content (Wombaroo™ Lorikeet & Honeyeater Food) has been provided to hihi in the past, on both Mokoia and Tiritiri Matangi. On Mokoia, breeding females were estimated to produce 5% more fledglings in years when Wombaroo, rather than sugar water, was supplemented (Armstrong et al. 2007). Given this small effect size, Armstrong et al. (2007) concluded that there was little advantage of supplementing Wombaroo rather than sugar water on Mokoia. Sugar water was instead favoured as a supplement, largely because it is cheaper, easier to mix and clean and does not perish as rapidly in the field. This interpretation of the findings on Mokoia means that sugar water is the supplementation now provided to all translocated hihi populations. Despite this assessment that a full dietary supplement provides little additional benefit, there are continued suggestions for its use (Matt Low, pers. comm.), including field trials at Karori Wildlife Sanctuary (Empson and Robertson 2010).

Besides full dietary supplementation, our recent work has also highlighted the potential benefits of providing more specific nutrients such as carotenoids (colourful pigments with antioxidant and immunostimulant properties) (Ewen et al. 2008, 2009; Thorogood et al. 2011). The health benefits of carotenoids for birds have been well documented (McGraw and Ardia 2003; McGraw et al. 2005a; Biard et al. 2006; Cucco et al. 2006), and in hihi carotenoids are known to offset the costs of ectoparasitism on nestling growth (Ewen et al. 2009). In general, the effects of dietary supplementation at the nestling stage have received relatively little attention. In hihi, for example, the difference in subsequent juvenile survival between nestlings receiving full versus sugar water supplementation has not been reported. Furthermore, the manner in which alternative supplements (such as Wombaroo and carotenoids) interact with each other has not been explored, and therefore the combination of these treatments that is most beneficial for hihi nestlings is unknown.
The aim of this study was therefore to investigate experimentally the effects of neonatal supplementary feeding on nestling growth, nestling survival, and juvenile survival to breeding age (recruitment), in order to identify the most appropriate form of dietary supplement. Few studies have considered the effects, either short- or long-term, of food supplementation on nestlings. Furthermore, we aimed to identify any sex-specific effects of supplementation, given the frequently differing dietary requirements of males and females (Maklakov et al. 2008; Lee 2010), and to investigate how the dietary treatments might interact with each other.

METHODS

Study species and site

Hihi are cavity nesters and lay clutches of 3-5 eggs with nestlings fledging at around 30 days (Oliver 1955). We studied the hihi population on 220 ha Tiritiri Matangi Island (36°36’ S, 174°53’ E). This population is closed to immigration and emigration and supports around 100 breeding pairs that are intensively managed during the breeding season (September to February). Reproductive success is closely monitored; all nesting occurs in boxes, all nestlings are banded prior to fledging, and infestations of the bloodsucking mite (*Ornithonyssus bursa*) are controlled with the miticide Frontline (Merial Ancare New Zealand). In addition, supplementary food (sugar water; 20% by mass) is provided year-round at six feeding stations.

Experimental design

All first-clutch nests in the 2010-2011 breeding season (n = 84) were randomly assigned to one of two nutritional treatments; a ‘nutritionally-supplemented’ treatment (N+) or a ‘control’ treatment (N-). Nestlings in N+ nests received Wombaroo Lorikeet & Honeyeater Food, a high-protein dietary supplement typically used to maintain nectar-eating birds in captivity (protein = 140 g kg\(^{-1}\), fat = 60 g kg\(^{-1}\), fibre = 6 g kg\(^{-1}\), vitamins and minerals; provided in 30% by mass solution), while nestlings in N- nests received a similar-concentration sugar water solution (20% by mass) without
the other nutrients. The sugar water solution is provided year round at feeding stations and is known to be provisioned to nestlings by parents (Thorogood et al. 2008). Within all nutritionally-supplemented and control nests, some nestlings had their nutritional treatment enhanced with carotenoids (C+) and some did not (C-). The carotenoids lutein and zeaxanthin were provided in the form of OroGLO® liquid (Kemin Industries) at a final concentration of 100 µg mL⁻¹ (based on recommendations in Biard et al. 2006). These are the principle carotenoids used to pigment yellow feathers in hihi and their ratio in OroGLO® liquid closely matches that found in the circulating plasma of this species (Ewen et al. 2006a).

This design created four treatment groups (N+C+, N+C-, N-C+, N-C-), with a nestling-oriented carotenoid supplement overlaid on a brood-oriented nutritional supplement. Treatment group was randomly assigned to the heaviest nestling in a nest, and carotenoid treatment was then alternated down the weight ranking while nutritional treatment was held constant. Thus all nestlings within a brood shared the same nutritional treatment but differed in carotenoid treatment (either C+ or C-), allowing for a within-brood comparison of carotenoid supplementation. All nestlings within a brood received a treatment, so the number of C+ and C- nestlings was unbalanced for uneven brood sizes.

Nestlings were directly fed their assigned treatment, using a graduated plastic syringe, every second day between four and 20 days. The volume fed was standardized by age, increasing incrementally from 0.2 ml at four days to 3.0 ml at 20 days, and generally resulted in satiation. Parents continued to feed nestlings throughout the experimental period and hand supplementation represented approximately 5-10% of a nestling’s daily intake (Page 2008). Individual nestlings were identified by uniquely trimmed body down when ≤ 8 days, and by a single colour ring when > 8 days. Nestlings were weighed on each feeding visit, and any dead nestlings were retrieved and a tissue sample taken for subsequent molecular sexing. At 21 days nestlings were ringed with a unique combination of one metal ring and three colour rings, and a blood sample was taken by brachial venipuncture. Blood samples were stored in 95% ethanol and refrigerated for subsequent molecular sexing. Nests were checked daily from 29 days
until fledging to confirm fledging success. A total of 287 nestlings were treated, across four treatment groups (68 N+C+, 76 N+C-, 72 N-C+, 71 N-C-).

**Laboratory work**

Nestlings were sexed using a PCR-based molecular sexing technique, with DNA extracted from blood or tissue using the ammonium acetate precipitation method (Nicholls et al. 2000) and sex identified using fluorescently labelled Z002a and Z037b (Operon) primers (Dawson 2007). Products were analysed on an ABI sequencer. We checked error rate by comparing adult individuals sexed morphologically and genetically. The combined error rate for both markers (Z002a and Z037b) is 1.4% (i.e. out of 2066 adults sexed genetically 29 showed discrepancies with the morphological sex assigned).

**Growth analysis**

We modelled the change in nestling weight ($W$) with age ($t$) using a variation of the logistic function (a standard model for avian postnatal growth, Starck and Ricklefs 1998)

$$W = \frac{a}{1 + \exp \left( -k(t - i) \right)}$$

[1]

where $a$ is asymptotic size, $k$ is the rate constant and $i$ is the inflection point of the curve. Preliminary analysis showed that including random effects of ‘individual’ and ‘mother’ on $a$ provided a better fit to the data. We included terms in the model to test for an effect of sex, nutritional treatment, carotenoid treatment, and their two-way interactions on the three growth parameters $a$, $k$, and $i$, giving the starting model:

$$W = \frac{a + \beta_{as}s + \beta_{ac}c + \beta_{aw}w + \beta_{asc}sc + \beta_{awc}wc + \mu_j + \mu_m + \varepsilon}{1 + \exp \left( -(k + \beta_{ks}s + \beta_{kc}c + \beta_{kw}w + \beta_{ksw}sw + \beta_{ksc}sc + \beta_{kwc}wc) \right) + \left( t - (i + \beta_{is}s + \beta_{ic}c + \beta_{iw}w + \beta_{isw}sw + \beta_{isc}sc + \beta_{iwc}wc) \right)}$$

[2]
where \( \mu_j \) is the ‘individual’ random effect, \( \mu_m \) is the random effect of ‘mother’ and \( \epsilon \) is the random error. \( s \) is the nestling’s sex (1 = male, 0 = female), \( c \) is carotenoid treatment (1 = C+, 0 = C-), \( w \) is nutritional treatment (1 = N+, 0 = N-), and the \( \beta \) terms describe the effect of sex, carotenoid treatment, nutritional treatment, and their two-way interactions on the growth parameters. Models were fitted using the nlme function in R v. 2.14.0 (R Development Core Team 2011). We started with the model above, and model selection was performed using a backwards stepwise approach where only significant terms (\( P < 0.05 \)) were retained.

**Survival to fledging**

We modelled the survival of first-clutch (treated) nestlings to the point of fledging. Survival to fledging was known from daily nest checks and the retrieval of dead nestlings. We investigated the effect of sex (female/male), nutritional treatment (N+/N-), carotenoid treatment (C+/C-), and their two-way interactions on the proportion of nestlings surviving to fledging by fitting generalized linear mixed models with binomial errors, in the program WinBUGS (which uses Markov Chain Monte Carlo (MCMC) methods using a Gibbs sampler; Lunn et al. 2000). ‘Mother’ was again included as a random effect to control for the fact that there were multiple nestlings from the same brood. Some dead nestlings (\( n = 28 \)) were not found and therefore the sex of these individuals could not be determined. However, simply excluding individuals of unknown sex from the analysis would have introduced an unknown bias (Nakagawa and Freckleton 2008), since our ability to sex birds was correlated with survival. Instead, we used a data augmentation approach in the program WinBUGS to model the unknown sexes multiple times (for the growth analysis only nestlings that survived the nestling period were included, so this approach was not needed there). This approach is analogous to flipping a coin to assign sex, but with two key modifications. Firstly, our approach assigns sex based on the probability of being male, given the sex ratio amongst known sex birds (whereas flipping a coin gives a 50:50 chance). Secondly, our approach essentially flips a coin many, many times (21,000 times in this case), using MCMC sampling, so that the results are not affected by where the random flips lie. Uninformative priors were used for all parameters, and inference was based on 21 000 iterations once convergence
was achieved after 1000 iterations (checked by examination of trace plots). The sampled values were used to estimate summary statistics for the posterior probability distributions of the parameters of interest. Model selection was performed using a stepwise approach, with terms removed from the model if the 95% credible interval included zero.

Survival to recruitment

Survival from fledging to recruitment (i.e. to breeding age, in September 2011) was analysed using Cormack-Jolly-Seber models in program MARK. Re-sighting surveys were conducted in September 2011 and February 2012, and some treated juveniles were harvested from the population in April 2011 to supplement another hihi population. There were therefore four ‘capture’ occasions: fledging (when individuals were marked), April 2011 (when some individuals were removed from the population for translocation), September 2011 and February 2012. Inclusion of the final capture occasion allowed for the separate estimation of survival probability ($\phi$) and recapture probability ($p$) up to the penultimate capture occasion (i.e. recruitment). Data were coded so that estimation of survival and recruitment probabilities took into account the removal of some individuals from the population in April 2011.

Initial exploration of the data showed that recapture probability was time dependent, so this was included in all models. For survival probability, we started with a model that included sex-nutritional treatment, sex-carotenoid treatment, nutritional treatment-carotenoid treatment, nutritional treatment-time and carotenoid treatment-time interaction terms. This starting model was progressively simplified, first by removing interaction terms and then main effects terms, to find the most parsimonious model. Likelihood ratio tests were performed on pairs of nested models to determine whether each simplification should be accepted (simplification accepted if $p > 0.05$). The best model was that which could not be simplified any further (i.e. $p < 0.05$). A bootstrap goodness-of-fit test of the starting model (with removals treated as deaths) gave an estimated $\hat{c}$ of 1.07, indicating a good fit to the data. For comparison, models were also ranked using the corrected quasi-likelihood Akaike information criterion (QAICc), where the model with the lowest QAICc value is identified as best.
RESULTS

Growth models

The minimum adequate growth model (Table 1) was

\[ W = \frac{a + \beta_{as}s + \mu_j + \mu_m + \varepsilon}{1 + \exp(-k + (t - (i + \beta_{is}s + \beta_{iw}w + \beta_{isw}sw)))} \]  

which gave a good fit to the data (Figure 1). This model includes an effect of sex on asymptotic size \((a)\), such that males had a larger asymptotic size than females (Table 1; Figure 1). It also includes effects of sex, nutritional treatment and a sex:nutritional treatment interaction on inflection point \((i)\); N+ females had a later inflection point than N- females, whilst N+ males had an earlier inflection point than N- males (Table 1; Figure 1). Terms testing for an effect of sex on growth rate \((k)\), carotenoid treatment on \(a, k, \) or \(i\), nutritional treatment on \(a\) or \(k\), and all other interaction terms on \(a, k, \) or \(i\) were non-significant \((P > 0.05)\).

Table 1: Parameter estimates from the minimum adequate logistic model examining nestling growth; includes parameters \(a, k, \) and \(i\), an effect of sex on \(a\), and effects of sex, nutritional treatment and sex:nutritional treatment interaction on \(i\) (see equation 3).

<table>
<thead>
<tr>
<th>parameter</th>
<th>estimate</th>
<th>SE</th>
<th>t-value</th>
<th>p-value</th>
<th>L95%CL</th>
<th>U95%CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>32.85</td>
<td>0.66</td>
<td>50.02</td>
<td>&lt;0.0001</td>
<td>31.56</td>
<td>34.13</td>
</tr>
<tr>
<td>(\beta_{as})</td>
<td>4.72</td>
<td>0.68</td>
<td>6.89</td>
<td>&lt;0.0001</td>
<td>3.38</td>
<td>6.06</td>
</tr>
<tr>
<td>(k)</td>
<td>0.31</td>
<td>0.04</td>
<td>75.31</td>
<td>&lt;0.0001</td>
<td>0.31</td>
<td>0.32</td>
</tr>
<tr>
<td>(i)</td>
<td>8.62</td>
<td>0.09</td>
<td>96.43</td>
<td>&lt;0.0001</td>
<td>8.45</td>
<td>8.80</td>
</tr>
<tr>
<td>(\beta_{is})</td>
<td>0.50</td>
<td>0.11</td>
<td>4.61</td>
<td>&lt;0.0001</td>
<td>0.29</td>
<td>0.71</td>
</tr>
<tr>
<td>(\beta_{iw})</td>
<td>0.24</td>
<td>0.12</td>
<td>2.00</td>
<td>0.05</td>
<td>0.005</td>
<td>0.48</td>
</tr>
<tr>
<td>(\beta_{isw})</td>
<td>-0.36</td>
<td>0.15</td>
<td>-2.34</td>
<td>0.02</td>
<td>-0.66</td>
<td>-0.06</td>
</tr>
</tbody>
</table>
Figure 1: Mean projected growth rates (solid lines) of a) female and b) male hihi under the logistic model. Black lines are for N- individuals and blue lines are for N+ individuals. Dashed lines show the 2.5% and 97.5% percentiles based on the estimated standard deviation in asymptotic size among individuals. Circles are raw data showing the weight of individual nestlings at given ages.
Survival to fledging

The minimum adequate model for survival to fledging retained terms for a sex-nutritional treatment interaction (-2.4, 95% credible interval = -4.2, -1.0) and a nutritional treatment-carotenoid treatment interaction (-1.4, 95% credible interval = -2.8 to -0.03). The survival probabilities estimated by this model are presented in Figure 2, along with the unconstrained estimates of survival probability. Female survival to fledging was enhanced independently by nutritional treatment (73.6% for N-C- vs. 88.3% for N+C-) and carotenoid treatment (73.6% for N-C- vs. 86.6% for N-C+). Combined nutritional and carotenoid supplementation to females did enhance survival (N+C+ 82.3%), but not as much as providing one or the other. For males, survival to fledging is enhanced by carotenoid treatment alone (90.1% for N-C- vs. 95.4% for N-C+), but reduced when providing nutritional treatment alone (90.1% for N-C- vs. 71.5% for N+C-), and reduced even further when both nutritional and carotenoid treatment are provided (N+C+ 60.8%).

Figure 2: Survival to fledging probabilities for all sex-treatment combinations. Estimated using the posterior probability distribution from the best model (dark grey bars), which includes a sex-nutritional treatment interaction and a nutritional treatment-carotenoid treatment interaction (drawn from 21 000 iterations after a burn-in of 1000 iterations). Light grey bars show unconstrained estimates.
Table 2: Results of Cormack-Jolly-Seber survival to recruitment analysis in MARK. Models were compared by likelihood ratio testing in the order listed. A model with fewer parameters was accepted if the increase in deviance was not statistically significant (p > 0.05). Models where sex (‘s’), nutritional treatment (‘n’), carotenoid treatment (‘c’) and/or time (‘t’) affected survival were considered, and factors were combined additively (‘+’) and/or interactively (‘*’). The best model is number 9, where survival is time-dependent only.

<table>
<thead>
<tr>
<th>model</th>
<th>survival $\phi$</th>
<th>re-sighting P</th>
<th>no. of parameters</th>
<th>deviance</th>
<th>comparison</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>s<em>n + s</em>c + n<em>c + n</em>t + c*t</td>
<td>t</td>
<td>15</td>
<td>124.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>s<em>c + n</em>c + n<em>t + c</em>t</td>
<td>t</td>
<td>14</td>
<td>124.74</td>
<td>1-2</td>
<td>0.598</td>
</tr>
<tr>
<td>3</td>
<td>s + n<em>c + n</em>t + c*t</td>
<td>t</td>
<td>13</td>
<td>125.24</td>
<td>2-3</td>
<td>0.481</td>
</tr>
<tr>
<td>4</td>
<td>s + n<em>t + c</em>t</td>
<td>t</td>
<td>12</td>
<td>126.85</td>
<td>3-4</td>
<td>0.204</td>
</tr>
<tr>
<td>5</td>
<td>s + n + c*t</td>
<td>t</td>
<td>10</td>
<td>130.63</td>
<td>4-5</td>
<td>0.151</td>
</tr>
<tr>
<td>6</td>
<td>s + n + c + t</td>
<td>t</td>
<td>8</td>
<td>133.38</td>
<td>5-6</td>
<td>0.253</td>
</tr>
<tr>
<td>7</td>
<td>n + c + t</td>
<td>t</td>
<td>7</td>
<td>133.76</td>
<td>6-7</td>
<td>0.536</td>
</tr>
<tr>
<td>8</td>
<td>c + t</td>
<td>t</td>
<td>6</td>
<td>135.29</td>
<td>7-8</td>
<td>0.216</td>
</tr>
<tr>
<td>9</td>
<td>t</td>
<td>t</td>
<td>5</td>
<td>136.34</td>
<td>8-9</td>
<td>0.306</td>
</tr>
<tr>
<td>10</td>
<td>.</td>
<td>t</td>
<td>4</td>
<td>152.58</td>
<td>9-10</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Survival to recruitment

The best model, following the progressive removal of terms, included an effect of time only on survival probability (model 9, table 2). This model predicted overall survival to recruitment of 46.31% ± 8.30% SE (53% survival between fledging and April, 88% survival between April and September). Terms testing for effects of sex, nutritional treatment, carotenoid treatment, and their two-way interactions with each other and with time, were not significant (models 1-8, table 2). A comparison of models based on QAICc values also identified the time-dependent survival model (model 9) as the best model.

DISCUSSION

To promote the conservation of an endangered species, management interventions are frequently required and implemented. In many cases intensive management has dramatically improved the prospects of species in an otherwise precarious position. The well-known example of the Chatham Island black robin (*Petroica traversi*) is a case in point, where nest-guarding and cross-fostering were instrumental in increasing the population from 5 to 200 birds between 1979 and 2011 (Jones and Merton 2012). However, this success story was not without its problems, with cross-fostered robins imprinting on the wrong species, and careful review and monitoring was central to identifying and rectifying the problem (Butler and Merton 1992). Therefore, given the potentially dramatic ramifications of management interventions there is a crucial responsibility to evaluate management effort critically. We used field experiments to determine which nestling dietary supplementation was most beneficial to young hihi.

Our results suggest that although female nestlings responded positively to the high-protein nutritional supplementation (N+), with increased survival to fledging, male nestlings exposed to this treatment actually suffered reduced survival to fledging. While it is well established that micronutrients can be toxic at high concentrations (Mertz 1981), studies in nutritional ecology demonstrate that a high intake of macronutrients can also be detrimental (Raubenheimer et al. 2005). For example, high-protein diets, relative to optimal protein requirements, result in higher mortality rates in insects (Dussutour and Simpson 2012) and mammals (Simpson and
Raubenheimer 2009). Furthermore, sex differences in optimal dietary protein-carbohydrate ratios have been reported. A high-protein diet is optimal for females rather than males in field crickets (Teleogryllus commodus) (Maklakov et al. 2008) and caterpillars (Spodoptera litura) (Lee 2010), and in song sparrows (Melospiza melodia) there is evidence that early life food restriction could have sex-specific programming effects on energy expenditure (Schmidt et al. 2012). Females are generally considered to have higher protein demands for reproduction than males, given their requirements for egg production (Maklakov et al. 2008; Lee 2010). Thus it is possible that female physiology is programmed to cope better with increased protein supply throughout their lives, even as early as the nestling period.

Our results also suggest that, for females at least, there is an antagonistic relationship between nutritional and carotenoid treatments, whereby carotenoid treatment was beneficial for survival to fledging but only when not received in combination with the high-protein nutritional supplement. Carotenoids have several important health-promoting properties (e.g. antioxidant, anticarcinogen and immunostimulant functions; Surai 2002), and supplementation with carotenoids alone may have improved health, and therefore increased nestling survival, via some or all of these mechanisms. That carotenoid supplementation is not as beneficial when the nutritional supplement was also provided may be because some constituent of Wombaroo impairs carotenoid absorption and assimilation (Castenmiller and West 1998). Fibre and vitamin A are both known to impair carotenoid absorption (Castenmiller and West 1998), and both of these are present in Wombaroo (fibre 6 g kg$^{-1}$; vitamin A 300 µg kg$^{-1}$), albeit alongside constituents that improve carotenoid absorption (e.g. fatty acids; Surai 2002). Raubenheimer and Simpson (2004) have developed the geometrical framework to investigate how different food components interact and identify what the optimal levels of one or more nutrients might be, and suggest that this approach might be used to inform supplementary feeding of endangered species (Raubenheimer and Simpson 2006). Indeed, taking this approach to explore hihi supplementary feeding in the future might help to identify the optimal nutritional context in which carotenoids might be supplemented.
Supplementary feeding is a commonly implemented form of management, and has benefited a range of bird species (Gonzalez et al. 2006; Robertson et al. 2006; Jones 2008; Oro et al. 2008; Schoech et al. 2008). Nonetheless, the implementation of supplementary feeding is not without cost (Blanco et al. 2011). Whilst some costs are relatively self-evident, for example the financial expense of supplements and the time spent maintaining a feeding programme, others may only become evident with targeted assessment. The male-biased offspring sex ratio seen following supplementary feeding to female kakapo (Clout et al. 2002) is one example of the unforeseen consequences of supplementary feeding. The results we report here – a sex-specific survival response to supplementation, and an antagonistic relationship between different supplements – illustrate unforeseen consequences in the hihi.

Although we report effects of supplementary nestling feeding on immediate nestling survival, we did not detect any effects on survival from fledging to recruitment. Supplementation to nestlings during a finite developmental window therefore either has an effect of limited duration, or its long-term effects are masked by other factors that affect survival. Indeed, the Tiritiri Matangi population already has high adult survival, and reasonably high juvenile survival, hence making it hard to find treatment effects on these variables. Potentially, long-term effects may be more evident in some other aspect of life history, for example reproductive success. On Tiritiri Matangi any improvements, or otherwise, in overall survival from hatching to recruitment will evidently be driven by effects seen prior to fledging. For example, N-C- females have an estimated hatching-to-recruitment survival of 35%, which increased to either 41% with carotenoid supplementation (N-C+) or 42% with Wombaroo supplementation (N+C-) (the product of hatching-to-fledging and fledging-to-recruitment survival). Meanwhile, N-C- males have 42% hatching-to-recruitment survival, which either increased to 45% with carotenoid supplementation (N-C+), or decreased to 34% with Wombaroo supplementation (N+C-). Armstrong et al. (2007) reported a 5% increase in the number of hihi fledglings when a full dietary supplement was provided to adults, and this was considered too minimal an improvement to justify implementing a costly management effort. Our effect sizes are of a similar magnitude and likewise do not offer strong support for a change in management on Tiritiri Matangi.
Provisioning adult populations of endangered species with supplementary food is a widespread management action, but targeted supplementation to developing young is less common. The dietary requirements of nestling birds are likely to be different from that of adult birds, and therefore extrapolating treatment effects between the two is difficult. Nonetheless, decisions on precisely what supplement to provide, be it to adults and/or nestlings, should be based on a targeted assessment of the costs and benefits associated with providing a given supplement. Sugar water is provided to adult hihi in all translocated populations because of its proven benefits to survival and/or reproduction, its relative low cost, and the ease of hygienic maintenance at feeding stations. The alternative provision of Wombaroo or carotenoids to adults adds both financial expense and increased effort to the feeding regime (both require specialized attention at feeding stations) without tangible benefits to survival. If these alternatives were provisioned only to nestlings then some of these costs associated with feeding adults would no longer apply. However, in this study, we found no strong evidence that direct supplementation of Wombaroo and/or carotenoids to nestling hihi has long-term survival benefits. Critically assessing supplementary feeding regimes is important for identifying both hidden costs and hidden benefits. The hidden benefit of using carotenoids to offset costly ectoparasitism has previously been shown (Ewen et al. 2009), and here we report a hidden cost of Wombaroo supplementation to nestlings, in the form of a sex-specific survival effect.
Foraging for carotenoids: do colourful male hihi target carotenoid-rich foods in the wild?

Carotenoids are responsible for many of the yellow, orange and red hues that colour the plumage of birds. Because these pigments cannot be produced by animals *de novo*, dietary access to carotenoids is expected to determine the strength of carotenoid-based signal expression and potentially maintain signal honesty. Species that display carotenoid-based plumage are therefore expected to forage selectively for carotenoid-rich foods. This should particularly be the case during moult, when carotenoid pigments are deposited in the integument. However, it is unknown whether species with carotenoid-based plumage target carotenoid-rich foods in the wild, during moult. We set out to answer this question in the hihi (*Notiomystis cincta*), a New Zealand passerine where males, but not females, display yellow carotenoid-based plumage. We determined the nutritional content of common foods in the hihi diet, conducted feeding observations of male and female hihi during moult, and measured circulating carotenoid concentrations over the same period. We found that male hihi have a greater proportion of carotenoid-rich foods in their diet than do females; a consequence of a greater fruit and lower invertebrate intake than females, and an avoidance of low-carotenoid content fruit. Males also showed an increase in circulating carotenoids over the moult period, whilst females did not. We suggest that the form of carotenoids used for pigmentation may determine the importance of dietary carotenoid access, with species that use unmodified dietary carotenoids, like hihi, being most likely to maximise their dietary carotenoid intake.
INTRODUCTION

Carotenoid pigments are responsible for the red, orange and yellow integuments of countless bird, fish and reptile species, and cannot be synthesised by them \textit{de novo} (Goodwin 1984). The perceived importance of dietary carotenoid access for colourful ornamentation has been central in the debate about the honest maintenance of carotenoid-based signals (Olson and Owens 1998; Pérez-Rodríguez 2009; Svensson and Wong 2011). Birds deposit carotenoid pigments into their feathers when they moult, and during this time foraging individuals must acquire an appropriate amount, and balance, of specific nutrients (Murphy 1996). In captive choice-tests, birds have demonstrated an ‘appetite’ for specific nutrients that are potentially important during moult, such as sulphur-containing amino-acids (Murphy and King 1987) and colourful carotenoid pigments (Senar et al. 2010). However, whether moulting birds display carotenoid-specific foraging preferences for naturally occurring foods in the wild, and how this is balanced against other requirements, is unknown (Olson and Owens 1998; Catoni et al. 2008).

It was initially proposed that the scarcity of carotenoids in the environment maintains signal honesty, with only individuals of superior foraging ability able to obtain them (Endler 1983; Kodric-Brown 1989; Hill 1992). Subsequently, greater appreciation of the antioxidant and immunostimulant functions of carotenoids generated the allocation trade-off hypothesis, which suggested that only the healthiest individuals can afford simultaneously to invest carotenoids in both pigmentation and self-maintenance (Lozano 1994; von Schantz et al. 1999). In both cases, carotenoid pigments themselves are the limiting factor in colour expression, either because they are scarce in the environment, or because they are required for multiple functions. In contrast, some authors have questioned whether carotenoids are a limiting resource (Hudon 1994), with the recently proposed vitamin A-redox hypothesis (Hill and Johnson 2012) suggesting that carotenoid-based colouration is linked to various performance functions by shared underlying vital cellular processes. In particular, the hypothesis cites the importance of some carotenoids to act as vitamin-A precursors, and emphasises the implications this has for the uptake, transport and metabolism of all carotenoid pigments. Rather than competing with cellular processes for access to carotenoids, pigmentation instead reflects how well these underlying cellular
Chapter 5: Foraging for carotenoids

processes are operating (Hill 2011). In this context carotenoid limitation appears less important. Indeed, Hill & Johnson (2012) suggest this is the case for species that oxidise dietary pro-vitamin A carotenoids (e.g. β-cryptoxanthin) into red ketolated carotenoids (e.g. 3-hydroxy-echinenone) for deposition into the integument. However, for species that deposit yellow dietary carotenoids unmodified into the integument (e.g. lutein, zeaxanthin; lacking in pro-vitamin A function), the links between colour and environmental carotenoid access are still predicted to be strong.

There are several lines of evidence to suggest that moulting birds might forage preferentially for carotenoids. Firstly, great tits (Parus major) deposit unmodified lutein and zeaxanthin directly from their diet into their feathers (Partali et al. 1987) and within- and between-population differences in carotenoid-based plumage colour have been attributed to dietary carotenoid access in great tits (Slagsvold and Lifjeld 1985; Isaksson 2009) and northern cardinals (Cardinalis cardinalis) (Linville and Breitwisch 1997). Even a species that deposits modified ketolated carotenoids into plumage, the house finch (Carpodacus mexicanus), shows a positive relationship between gut carotenoids content and carotenoid-based plumage colour (Hill et al. 2002). Secondly, birds are capable of detecting small differences in nutrient concentration when feeding (e.g. lipid and sugar content; Schaefer, Schmidt, & Bairlein, 2003), and of self-selecting a nutritionally balanced diet by combining foods that are individually imbalanced (Raubenheimer and Simpson 1997; Köhler et al. 2012). Thirdly, and perhaps most convincingly, great tits have demonstrated a preference for diets that are artificially carotenoid-enriched, both in captive- and field-based choice tests (Senar et al. 2010). Equivalent tests with house finches (Giraudeau et al. 2012) and garden warblers (Sylvia borin) (Catoni et al. 2011) failed to replicate this result possibly because these species use modified ketolated carotenoids in their plumage (house finches), or do not pigment their feathers with carotenoids at all (garden warblers).

We set out to test whether moulting birds with carotenoid-based plumage selectively forage in the wild for naturally occurring carotenoid-rich foods. We also aimed to evaluate whether other components of the diet are regulated to facilitate maximal availability of carotenoids for pigmentation. The hihi (Notiomystis cincta) is an ideal
species in which to do this. Males display carotenoid-based yellow shoulders and breast, and the pigments used to colour their feathers are predominantly the unmodified dietary carotenoids lutein and zeaxanthin (Ewen et al. 2006a). Females, in contrast, lack an equivalent carotenoid-pigmented plumage patch. We compared the foraging behaviour, during moult, of males and females. We also quantified the nutritional value, including carotenoid, vitamin E and fat content, of foods most commonly occurring in the diet. Finally, we measured plasma carotenoid concentration at multiple points during the moult period, to determine whether foraging behaviour was reflected at a physiological level. We predicted that males would maximise their carotenoid intake, compared to females, by targeting carotenoid-rich foods, and that this would be reflected in circulating carotenoid levels. We also predicted that males would have a greater fat intake than females, to facilitate carotenoid absorption. Finally, we predicted that males would differ in their vitamin E intake compared to females. They may either have a greater vitamin E intake than females, in order to compensate the antioxidant function of carotenoids being lost to pigmentation, or a reduced vitamin E intake compared to females, to minimize competition with carotenoids during absorption.

METHODS

Study site and species

Hihi are a sexually dimorphic and dichromatic passerine endemic to New Zealand. Males display yellow carotenoid-based (Ewen et al. 2006a) shoulders and breast, a melanin-based black head and structurally-produced white ear tufts. Females are a less conspicuous olive-brown all over, save from a white wing bar. Adult hihi eat nectar, fruits and invertebrates, and the proportion of each food type in the diet appears to vary with season and population (Gravatt 1969, 1971; Angehr 1984; Lovegrove 1985; Rasch 1985). Hihi moult once a year, immediately after the breeding season, at which time adults replace all of their feathers and first-year birds replace their body feathers only (remiges and rectrices, grown in the nest, are retained until the next year’s moult). At this time carotenoid-pigments (lutein and zeaxanthin; Ewen et al. 2006a) are deposited in the feather follicles. Adults typically moult between late December/early January and early April.
We studied the hihi on 220 ha Tiritiri Matangi Island (36°36’ S, 174°53’ E), which supports a population of ~180 colour ringed, individually identifiable, adult birds. The island was originally covered in coastal broadleaf forest, but following clearing for cultivation and grazing in the 19th century (Drey et al. 1982), and the subsequent re-planting of native plants between 1983 and 1995 (Mitchell 1985), the island’s vegetation now comprises ~60% remnant and regenerating forest and ~40% grassland. Hihi inhabit the forested areas of the island, which contain diverse vegetation typical of the region. As part of a conservation management program, adults have access to supplementary sugar water feeding stations throughout the year (Armstrong and Ewen 2001). Data was not collected on feeder use during the study because sugar water feeders are used minimally at this time of year (pers. obs.).

Feeding observations and blood sampling

Feeding observations were collected from 18th January to 22nd March 2010, when birds were moulting. During the hours of peak bird activity (06:00 - 10:00 and 16:00 - 19:00) we walked the network of trails through forest patches and located and followed individual birds until they ate a natural food item. All forest patches were surveyed for approximately equivalent durations. The identity (colour ring combination) and sex of the bird and the category of the first food item eaten (fruit / nectar / invertebrate) were recorded. Fruit and nectar were categorized according to species, and invertebrates according to order where possible. Observations of the same individual were separated by at least one hour, and only one food item was recorded per individual per sampling event. This sampling method has been used previously to estimate the proportions of different food categories in primate diets (Struhsaker 1975; Simmen and Sabatier 1996), and is appropriate when it is not practical to follow focal individuals for extended periods of time (e.g. Felton et al., 2009; Rothman, Dierenfeld, Hintz, & Pell, 2008). A total of 975 feeding observations from 233 different individual birds (males and females, including first-years) were made.

Concurrently (25th January to 21st March 2010), individuals were captured at mist nets and feeding stations and a blood sample was taken by brachial venipuncture. Blood samples were centrifuged within three hours to separate plasma, which was stored at -
20°C for subsequent analysis of plasma carotenoid concentration. A total of 124 samples were taken from 93 different individuals (males and females).

**Fruit sampling**

Individual plants that hihi were observed taking fruit from were marked with flagging tape and returned to, usually later the same day but occasionally after a few days, for fruit sampling. Cabbage tree (*Cordyline australis*), hangehange (*Geniostoma ligustrifolium*), mahoe (*Melicytus ramiflorus*), mapou (*Myrsine australis*), five-finger (*Pseudopanax arboreus*) and *Coprosma robusta* each had ten different individual plants flagged for sampling, and *Coprosma macrocarpa* and small-leaved *Coprosma spp.* (*Coprosma areolata* and *Coprosma rhamnoides*) each had three different individual plants flagged for sampling. 20 ripe fruit were collected by hand from each flagged plant, and within two hours were vacuum packed and stored at -20°C. Due to time constraints fruit were not sampled from kohekohe (*Dysoxylum spectabile*), kawakawa (*Macropiper excelsa*), puriri (*Vitex lucens*) and *Coprosma repens*. Hihi were only seen feeding on them very infrequently (collectively they account for just 4% and 6% of fruit in male and female diets, respectively), and this was probably due to in-accessibility rather than for nutritional reasons (kohekohe, kawakawa and puriri are all too large for hihi to consume whole).

**Laboratory analyses**

Half of the fruit samples collected were analysed for individual and total carotenoid concentration, and for individual and total vitamin E concentration. 200-300 mg fruit sample (excluding seeds) was saponified with ethanolic KOH in the presence of pyrogallol for 30 min at 70°C (Surai et al. 1996). After cooling, carotenoids were twice extracted by homogenization with hexane. Hexane extracts were pooled and evaporated under nitrogen and then re-dissolved in dichloromethane / methanol (1:1 v/v). 10 µl aliquots were injected into HPLC for analysis. Individual carotenoids were detected using a Spherisorb S30DS2, 3 µ C18 reverse-phase HPLC column (25 cm x 4.6 mm, Phenomex) with a mobile phase of acetonitrile / methanol (85:15 v/v) and acetonitrile / dichloromethane / methanol (70:20:10, v/v/v) in gradient elution and using detection at 445 nm (Surai et al. 2001a,b,c). Total carotenoids were detected
with a Spherisorb S5N2 DS2, 5 µ C₁₈ reverse-phase HPLC column (25 cm x 4.6 mm, Phase Separations, Clwyd, UK) with a mobile phase of methanol / water (97:3 v/v) at a flow rate of 1.5 ml min⁻¹. The HPLC was calibrated using carotenoid standards obtained from various sources. Vitamin E was determined using the same HPLC system (Shimadzu Liquid Chromatograph, LC-20AD, Japan Spectroscopic Co. Ltd with Florosance Spectrofluorometer) fitted with a Spherisorb ODS2 3 µ C₁₈ reverse-phase column (15 cm x 4.6 mm; Phase Separations), and using a mobile phase of methanol / water (97:3 v/v) at a flow rate of 1.05 ml min⁻¹. The excitation and emission wavelengths were 295 and 330 nm. A standard solution of α-tocopherol in methanol was used for instrumentation (HPLC) calibration. Carotenoid and vitamin E content was determined for all fruit species collected.

The remaining fruit samples were sent to Massey University’s Nutrition Laboratory (Palmerston North, New Zealand), where moisture, ash, crude protein and fat content were determined according to the procedures of the Association of Official Analytical Chemists (AOAC 1990). In most cases there was insufficient sample to perform repeat analyses, so samples from multiple individual plants were pooled to guarantee sufficient sample. In brief, samples were dried in a convection oven at 105°C to determine moisture content (AOAC 930.15, 925.10). Total nitrogen was estimated using a Leco FP-528 combustion analyser (AOAC 968.06), and crude protein was then calculated by multiplying total nitrogen by 6.25 (nitrogen to protein conversion factor). Fat content was determined by cold extraction using chloroform / methanol (AOAC 969.24). Finally, samples were placed in a 550°C furnace for ~3hrs to obtain ash content (AOAC 942.05). Total carbohydrate content was determined as 100% minus percent protein, fat, ash and moisture. There was sufficient sample to determine the nutritional content of cabbage tree (Cordyline australis), hangehange (Geniostoma ligustrifolium), mahoe (Melicytus ramiflorus), mapou (Myrsine australis), five-finger (Pseudopanax arboreus), Coprosma robusta, and Coprosma macrocarpa. There was insufficient sample remaining to determine the nutritional content of small-leaved Coprosma spp. (Coprosma areolata and Coprosma rhamnoides).

Plasma samples were analysed for total carotenoid concentration by high performance liquid chromatography (HPLC), as described previously (Walker et al. 2013b).
**Statistical analyses**

Feeding observations were used to estimate the proportional make-up of fruit, nectar and invertebrates in the diets of males and females, and a chi-squared test was used to test for sex differences in proportions of fruit, nectar and invertebrates taken. The test was performed on a single contingency table since proportions of dietary intake are non-independent of each other. The same approach was used to summarize the contribution of different fruit species to male and female diets. Because there were multiple observations from the same individuals, and there were a range of observations per individual (between 1 and 18 observations), it is possible that some individuals are ‘over-represented’ and may bias the results. To check for this, we used a re-sampling approach where we randomly sampled one observation per individual, used these sampled observations to estimate proportional make-up, and repeated this multiple times (n=100). We then calculated the mean proportional make-up from this distribution, and compared this to our original estimate (which used all observations at once).

Next, we re-cast feeding observations according to the carotenoid-content of different fruit species. The 25th and 75th percentiles of fruit carotenoid concentration were calculated, and each sampled fruit species was then categorised according to these percentiles. Fruit species falling below the 25th percentile were categorized as low carotenoid-content, fruit species falling between the 25th and 75th percentiles were categorized as medium carotenoid-content, and fruit species falling above the 75th percentile were categorized as high carotenoid-content (category assignment detailed in Table 1). The proportion of feeding observations that were on fruit of each of these carotenoid categories was then calculated for males and females, and a chi-squared test was performed (on a single contingency table) to detect any sex differences in the proportions of high-, medium-, and low-carotenoid content fruits consumed (as above). The same approach was also taken for the vitamin E, and fat content of fruits (category assignment detailed in Table 1).

In order to visualise the nutrient content of sampled fruit species in a broader context, we used right-angled mixture triangles (RMTs; Raubenheimer 2011) to compare the nutritional content of sampled fruit species with published values of invertebrate
Chapter 5: Foraging for carotenoids

orders that hihi potentially feed on (Diptera, Hemiptera, Lepidoptera; Banjo, Lawal, & Songonuga, 2006; Eeva, Helle, Salminen, & Hakkarainen, 2010; Finke, 2002, 2013; O’Malley & Power, 2012; Oonincx & Dierenfeld, 2012; Raksakantong, Meeso, Kubola, & Siriamornpun, 2010; Ramos-Elorduy et al., 1997). Given the comparatively simple composition of nectar (mainly water and sugars; Nicolson et al. 2007), nectar is not represented in these plots. The RMT approach is well-suited to field-based nutritional ecology studies where food items and dietary intake are described in terms of their proportional composition, rather than amounts (Raubenheimer 2011).

Finally, we investigated whether any sex differences in foraging behaviour during the moult period were reflected in differences in circulating carotenoid levels during this time. Since a large number of individuals had plasma carotenoid concentration measured multiple times, we fitted a linear mixed effects model using restricted maximum likelihood and included individual as a random effect. The response variable was plasma carotenoid concentration (box cox transformed, to meet the assumptions of normality and homogeneity), and the explanatory variables were sex, date of measurement (centred Julian date), and an interaction between sex and date of measurement. This model allows us to investigate whether the sexes had different plasma carotenoid concentrations, whether carotenoid concentration changed over the course of the moult period, and whether this change differed between the sexes.

RESULTS

Feeding observations

Our feeding observations revealed that males and females differed significantly in their proportional intake of fruit, nectar and invertebrates ($\chi^2 = 17.33$, df = 2, p < 0.001). Males had a greater proportion of fruit in their diet than did females (0.77 vs. 0.63; Figure 1a), a lesser proportion of invertebrates (0.19 vs. 0.31; Figure 1a), and a similar proportion of nectar (0.04 vs. 0.07; Figure 1a). The estimated proportional make-up did not differ markedly from this when a re-sampling approach was taken to account for some individuals being represented by multiple observations (Appendix 2 Table A2.1).
Males were observed feeding on 12 different species of fruit, and females on 11 different species of fruit (Figure 1b; Appendix 2 Table A2.1). Overall, there was a trend for the proportional intake of different fruit species to differ for males and females ($\chi^2 = 20.02$, df = 2, $p = 0.05$). This is largely driven by males feeding on a lesser proportion of mahoe than did females (0.36 vs. 0.47; Figure 1b; Appendix 2 Table A2.1). The proportion of all other fruit species in the diet, based on feeding observations, was similar for males and females (Figure 1b; Appendix 2 Table A2.1). The estimated proportional make-up of different fruit species did not differ markedly from these values when a re-sampling approach was taken (Appendix 2 Table A2.1).

_Nutritional content_

The macronutrient (i.e. protein, fat and carbohydrate), carotenoid and vitamin E wet weight content of fruit species commonly fed on by hihi are summarized in Table 1. In most fruit species, lutein and α-tocopherol were the predominant forms of carotenoid and vitamin E, respectively (Table 1). Additional forms of carotenoids and vitamin E generally occurred at low concentrations (Appendix 2 Tables A2.2 and A2.3). Hangehange had a notably higher total carotenoid concentration than any other fruit species sampled, followed by _C. robusta_ (Table 1). _C. macrocarpa_, five-finger, _C. areolata_, mapou and cabbage tree all had intermediate total carotenoid concentrations, whilst _C. rhamnoides_ and mahoe had the lowest total carotenoid concentrations (Table 1). Hangehange also had the highest total vitamin E concentration of all fruit species sampled, followed by _C. macrocarpa_. Cabbage tree, _C. areolata_, _C. robusta_, five-finger, and mahoe all had intermediate vitamin E concentrations, whilst mapou and _C. rhamnoides_ had low vitamin E concentrations (Table 1). Figure 2 compares the carotenoid and vitamin E concentrations ($\mu$g/g dry weight) of sampled fruit species with invertebrate orders potentially fed on by hihi. In general, invertebrates had a lower carotenoid and vitamin E content than hihi fruit (Figure 2).
Figure 1: a) The proportion of all feeding observations that were on fruit, invertebrates and nectar, and b) the proportion of all fruit feeding observations that were on different fruit species, for males and females. The number of observations and number of different individuals are indicated.
Table 1: Macronutrient content (% wet weight) and carotenoid and vitamin E content (mean ± SE µg/g wet weight) of ripe fruit species fed on by hihi. H, M, and L indicate which of high-, medium- and low- nutritional content categories each fruit species falls into. See Tables A2.2 and A2.3 in Appendix 2 for concentrations of additional forms of carotenoids and vitamin E.

<table>
<thead>
<tr>
<th>species</th>
<th>macronutrient content (% wet weight)</th>
<th>carotenoid content (µg/g wet weight)</th>
<th>vitamin E content (µg/g wet weight)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>moisture</td>
<td>ash</td>
<td>protein</td>
<td>fat</td>
</tr>
<tr>
<td>cabbage tree</td>
<td>78.2</td>
<td>0.9</td>
<td>2.9</td>
<td>4.1 (M)</td>
</tr>
<tr>
<td>C. areolata</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C. macrocarpa</td>
<td>73.3</td>
<td>0.4</td>
<td>0.9</td>
<td>2.2 (L)</td>
</tr>
<tr>
<td>C. rhamnoides</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C. robusta</td>
<td>72.6</td>
<td>0.6</td>
<td>1.4</td>
<td>2.5 (L)</td>
</tr>
<tr>
<td>five-finger</td>
<td>72.2</td>
<td>1.2</td>
<td>2.0</td>
<td>4.4 (M)</td>
</tr>
<tr>
<td>hangehange</td>
<td>72.0</td>
<td>1.2</td>
<td>2.4</td>
<td>5.9 (H)</td>
</tr>
<tr>
<td>mahoe</td>
<td>75.3</td>
<td>1.1</td>
<td>3.6</td>
<td>5.8 (H)</td>
</tr>
<tr>
<td>mapou</td>
<td>74.8</td>
<td>0.8</td>
<td>1.4</td>
<td>2.7 (L)</td>
</tr>
</tbody>
</table>

*a* insufficient sample to determine nutritional content; *b* value from unripe *C. macrocarpa*
Re-casting feeding observations according to the carotenoid content of fruits (see Table 1 for category assignment), revealed that males and females differed significantly in their proportional intake of high-, medium- and low-carotenoid content fruit ($\chi^2 = 6.67$, df = 2, p = 0.04; Figure 3a). This is driven by males feeding on a lesser proportion of low-carotenoid content fruit than did females (0.38 vs. 0.51; Figure 3a). Males and females did not differ in their consumption of fruit based on vitamin E content ($\chi^2 = 1.22$, df = 2, p = 0.54; Figure 3b), but did differ in their consumption of fruit based on fat content ($\chi^2 = 6.55$, df = 2, p = 0.04; Figure 3c). The latter result was driven by males feeding on a lesser proportion of high-fat content fruit than did females (0.44 vs. 0.57; Figure 3c). Both of these relationships (carotenoid and fat) appear to be driven by the relative avoidance of mahoe by males and preference by females. As shown above, this fruit is both low in carotenoid content and high in fat content.

An RMT plot of proportional protein, fat and carbohydrate composition revealed hihi fruit to be of lower protein content, lower fat content and higher carbohydrate content than potential invertebrate food items (Figure 4a). An RMT plot of proportional carotenoid, vitamin E and fat composition reveals that fruit with a proportionally high carotenoid content tend to also have a proportionally high vitamin E content and a proportionally low fat content (Figure 4b). The positive relationship between proportional carotenoid and vitamin E content is, however, not significant (Kendall’s rank correlation, $r = 0.07$, p = 0.62), suggesting that carotenoid and vitamin E intake can vary independently of each other. These plots also emphasise that mahoe, of all fruit species measured, is most similar in composition to invertebrates, having the highest protein and fat content, and lowest carotenoid content (Figure 4).

**Plasma carotenoids**

Males and females did not differ significantly in plasma carotenoid concentration during the moult period (males 14.04 µg mL$^{-1}$ ± 0.83 SE, females 15.96 µg mL$^{-1}$ ± 1.49 SE; $t = -0.90$, p = 0.37). However, there was a significant interaction between sex and date of measurement ($t = 2.35$, p = 0.03), indicating that males, but not females, showed a significant increase in plasma carotenoid concentration over the course of the moult.
DISCUSSION

Male hihi appear to target carotenoid-rich foods during moult. Firstly, males eat proportionally more fruit than females, and fruit have a high carotenoid content compared to other components of the diet. Secondly, within the fruit component of the diet, males are selecting the most carotenoid rich fruits. In contrast, females eat proportionally more invertebrates than do males, and the fruit they prefer is the most invertebrate-like in its composition. Although a greater carotenoid intake for males does not translate into higher mean circulating carotenoids during moult, males do show a significant increase in circulating carotenoids over the moult period, a change that is not evident in females. This is the first study of which we are aware to support a dietary carotenoid preference in the wild during moult.

Our findings are consistent with recent studies suggesting that birds are able to detect carotenoid presence in artificially manipulated food, and adjust their consumption in response (Senar et al. 2010; Catoni et al. 2011). Great tits maximise their carotenoid intake by preferentially choosing carotenoid-enriched mealworms (Senar et al. 2010), whilst garden warblers are able to detect carotenoid presence and maintain a consistent (although not necessarily maximal) carotenoid intake (Catoni et al. 2011). In contrast, house finches do not display an ability to detect or maximize dietary carotenoids, at least based on a specific olfactory cue (Giraudeau et al. 2012). We suggest that the nature of the carotenoids used for pigmentation may explain the discrepancies between these studies. Species that pigment their feathers with unmodified dietary carotenoids are those most likely to maximise their carotenoid intake. Species that use endogenously modified carotenoids are less likely to maximise carotenoid intake, because of the relatively greater importance of physiological processes compared to dietary access in determining colour production (Hill and Johnson 2012). Finally, species without carotenoid pigmentation only require dietary carotenoids for the remaining properties, such as antioxidant-function, egg production and vision (roles which can often be fulfilled by other compounds; Svensson & Wong, 2011), and are therefore the least likely to maximise intake.
Chapter 5: Foraging for carotenoids

![Graph a) showing carotenoids ug/g dry weight]

![Graph b) showing vitamin E ug/g dry weight]

84
Figure 2 (overleaf): a) Mean (± 95% confidence interval) total carotenoid (triangles) and lutein (circles) concentration (µg/g dry weight), and b) mean (± 95% confidence interval) total vitamin E (triangles) and α-tocopherol (circles) concentration (µg/g dry weight) of different fruit species commonly fed on by hihi, and invertebrate orders potentially fed on by hihi (invertebrate data in a) from Eeva et al., 2010 and Finke, 2013; invertebrate data in b) from Oonincx & Dierenfeld, 2012, Finke, 2002 and Finke, 2013. Numbers in brackets correspond to fruit/invertebrate codes in legend of Figure 4.

As well as predicting male carotenoid intake to be greater than for females, we also predicted that male intake of fats and vitamin E, dietary components relevant for carotenoid absorption and function, would be different than for females. Our results suggest that vitamin E intake was similar for males and females, and that fat intake was greater for females. The vitamin E result is not consistent with either of our predictions; males seem to neither elevate nor reduce their vitamin E intake compared to females, as might be expected with elevated carotenoid intake. The fat result is also contrary to our prediction. It is important to remember that requirements for both of these dietary components will not be solely determined by carotenoid intake. Fats, for example, inevitably have a vast range of functions besides aiding carotenoid absorption; for example, they are a source of energy, are important cell membrane components, and serve as nerve ‘insulators’ (Cheeke and Dierenfeld 2010). Consequently, the driver behind differing male and female requirements for fat, and also for vitamin E, may be unrelated to their interaction with carotenoids. Interestingly, our finding that carotenoid and vitamin E intakes are not simultaneously maximized is inconsistent with the influential prediction that carotenoid-based signals may in fact indicate the availability of other antioxidant resources such as vitamin E (Hartley and Kennedy 2004). Nevertheless, we have only considered one alternative antioxidant, and the intake of other relevant resources, such as vitamin C and antioxidant enzymes, may still be tied to carotenoid intake in a manner consistent with Hartley and Kennedy’s (2004) hypothesis.
Figure 3: The proportion of male and female fruit feeding observations that were on fruit of low- (white), medium- (grey), and high- (black) carotenoid (a), vitamin E (b), and fat (c) content. Based on 529 observations of 111 different males, and 131 observations of 72 different females. Fruits in each low-, medium- and high-content category are detailed in Table 1.
Figure 4: Right-angled mixture triangles (RMTs) showing a) the proportional protein, fat and carbohydrate composition, and b) the proportional carotenoid, vitamin E and fat composition of fruit species fed on by hihi (circles), and invertebrate orders potentially fed on by hihi (triangles). In a three-component RMT plot, two components are represented as standard $x$ and $y$ axes (protein and fat in a), carotenoid and vitamin E in b)), and the third component (the ‘implicit’ axis, carbohydrate in a) and fat in b)) varies inversely with the distance from the origin. The value of the implicit axis is read by following the dashed lines from the point of interest to the $x$-axis and reading the value off the $y$-axis legend (Raubenheimer 2011). Percentages are based on dry mass in grams. Fruit: 1 = cabbage tree, 2 = *Coprosma macrocarpa*, 3 = *Coprosma robusta*, 4 = five-finger, 5 = hangehange, 6 = mahoe, 7 = mapou. Invertebrates: 8 = Diptera, 9 = Hemiptera, 10 = Lepidoptera. Invertebrate data is averaged from references cited in text.
Chapter 5: Foraging for carotenoids

We have not made any attempt to measure the availability of the alternative food items that appear in the hihi diet. Instead we control for availability by comparing between the sexes, under the assumption that availability is the same for males and females. However, we cannot fully discount the possibility that food availability does differ between the sexes, and that it is this, instead, that explains the different foraging behaviours we report. For example, hihi are sexually dimorphic and this may generate sex differences in food profitability (e.g. via different handling times) and/or foraging locations (e.g. via aggressive intra-sexual exclusion). Future work that seeks to quantify the availability of carotenoids in the diet, rather the source of carotenoids alone, would add strength to the findings we report here.

Our results would be further strengthened by a demonstration that the discrepancy in carotenoid intake between males and females is different during moult than outside of moult. Indeed, females might be expected to have a higher carotenoid intake than males during the breeding season, when they will be depositing carotenoids into egg yolk. However, we are aware of only one hihi study that compares male and female proportional intake throughout the year (Angehr 1984). This suggests that males also eat more fruit than females when they are not moultting, implying that carotenoid intake is greater for males outside of, as well as during, moult. Interestingly, the carotenoid preference in great tits was also demonstrated outside of moult (Senar et al. 2010). Perhaps in those cases male physiology is primed to maintain a high carotenoid content throughout the year. Within species, the more colourful sex may require a year-round higher carotenoid intake than the less colourful sex. Alternatively, there might not be a year-round higher requirement for carotenoids by males, but sustained foraging for carotenoid-rich foods might provide the information necessary for obtaining the required levels during moulting. Which, if either, of these explanations apply would be an interesting direction for future research.

The differences we observed in macronutrient intake may be biased by ignoring feeds on supplementary sugar water. However, since sugar water provides only carbohydrates, it is likely that we will underestimate only this component of the diet. We do not believe that this would have a significant bearing on our results. Firstly, hihi use feeding stations minimally at this time of year (pers. obs.). Secondly, there is evidence that males use feeders to a greater extent than do females (Roper 2012),
which, if accounted for, would only accentuate the current observed difference of males having a greater carbohydrate intake. An additional consideration is that, although consistent with a male foraging strategy that maximises carotenoid intake, our results are also consistent with a female foraging strategy that maximises protein and fat intake. The differing reproductive strategies of males and females can result in differing nutritional requirements (Maklakov et al. 2008; Morehouse et al. 2010), and protein and fat demands might be higher for females during moult because, for example, they have a greater need to compensate following the demands of breeding. Nevertheless, our results are robust to both of these strategies co-occurring, and a female fat-maximising strategy may explain why the fat result is contrary to our prediction for males.

In conclusion, our results provide support for the hypothesis that colourful male hihi target carotenoid-rich foods during moult. We do not find any evidence that other components of the diet are regulated in a manner consistent with maximising carotenoid availability. Further work is needed to establish whether such patterns are maintained outside of moult, and to clarify the role that female requirements have in shaping these relationships.
Chapter 6

General discussion

In this thesis I assess the effect of the nutritional environment on key life history traits, and apply these findings in a conservation context. I found that colourful sexually selected plumage in male hihi is influenced by the diet experienced both as a dependent nestling and as a self-sufficient adult. I also show that the growth and subsequent survival of nestling hihi is sensitive to the combination of dietary supplements they receive in the nest, and that the consequences of dietary supplementation are sex specific. I now draw on these results to answer the questions I posed in the introduction and to suggest directions for future research.

**How do nutritional conditions in early life impact on key life history traits?**

Some aspects of the hihi male’s colourful plumage display have a profound effect on their reproductive success (chapter 3), and some of these elements additionally indicate environmental conditions experienced during development (chapter 2). Firstly, I show that males with larger carotenoid-based yellow patches are more likely to be territorial, whilst males with lighter yellow plumage are more likely to be cuckolded. Yellow patch size is influenced by the size of the brood that a male developed in, with those males from larger broods, a more competitive environment with a possibly reduced per nestling food supply from parents, having smaller yellow patches. Secondly, I show that males with lighter black plumage have greater extra-pair success, and that this property of black plumage is influenced by hatch date; males that hatch later in the season, typically associated with limited food supply relative to earlier in the season, have lighter black plumage. Thirdly, I show that males with longer white ear tufts have greater extra-pair success. Interestingly, though, I could not find any evidence that aspects of the early life environment influence ear tuft length specifically.

Some properties of hihi plumage appear to be influenced by early life nutrition, without any current evidence of being under sexual selection. For example, I found no
evidence that the luminance of white ear tufts is relevant for reproductive success (chapter 3) but it is reduced in males that have an enhanced protein intake as nestlings (chapter 2). Perhaps ear tuft luminance buffers ear tuft length against the detrimental effects of elevated early life protein intake, in the same manner that nutritionally limited rats prioritise investment into essential organs over investment into other organs (Desai and Hales 1997). Male hihi would do better to sacrifice the brightness of ear tufts than their length, since it is the latter that is important for gaining reproductive success. I also found no evidence that yellow saturation is sexually selected (chapter 3), and yet it is enhanced by greater carotenoid access during nestling development (chapter 2). Why would hihi apparently invest an increased carotenoid resource into making yellow plumage more saturated, rather than, for example, increasing yellow area? Potentially this result is simply a non-adaptive consequence of having a surplus carotenoid supply. Alternatively, yellow saturation may be under selection from other forces not related to reproductive success. For example, the intensity of yellow plumage may influence detectability to predators, and therefore have implications for survival.

In this thesis I also show that specific components of the neonatal diet influence growth and survival, and in a sex-specific way (chapter 4). Females receiving a protein supplement during development reach the point of maximum growth later in the nestling period than control females, and, possibly as a consequence of this, are more likely to survive to fledging. In contrast, males receiving the same protein treatment reach the point of maximum growth earlier in the nestling period, and are less likely to survive to fledging. These results suggest that male and female nestlings have differing dietary requirements. It is generally accepted that the larger sex is more susceptible to a nutritional shortfall because of a faster growth rate and consequently greater nutritional requirement (Clutton-Brock et al. 1985). Why the larger sex should apparently be susceptible to an increased supply of a specific nutrient, as I show here, is less intuitively easy to explain. However, in-depth consideration of the nutritional balance sought by each sex may provide insight into which life history traits are prioritised.

For example, many animals regulate their nutritional intake to maximise fitness (Raubenheimer and Simpson 1997; Simpson et al. 2004; Lee et al. 2008; Maklakov et
al. 2008; Jensen et al. 2012). In some animals it has also been shown that males and females have different nutritional optima (Maklakov et al. 2008; Lee 2010), as a consequence of divergent life history strategies for maximising fitness. Although I have not identified the nutritional optima of nestling hihi, by restricting birds to a certain intake and measuring the response, my results hint at the respective life history priorities of males and females. While males capitalize on an imposed nutritional environment by maximizing investment into colourful secondary sexual traits, females take the opportunity to maximize survival. This is analogous to the situation described in field crickets (*Teleogryllus commodus*) (Hunt et al. 2004), where males gain most reproductive success by investing in attractiveness and females by investing in lifespan. In hihi, understanding which foods help males to maximise plumage attractiveness and females to maximise survival, their apparent paths to maximum fitness, will have direct relevance for promoting the species’ persistence. Previous work has focused on identifying nutritional optima in species amenable to dietary manipulation and subsequent monitoring, generally invertebrates in a laboratory setting (e.g. Jensen et al., 2012; Lee et al., 2008; Maklakov et al., 2008). Identifying the sex-specific balance of nutritional components that maximises reproduction and survival in wild vertebrates, including birds, would be a challenging but worthwhile extension of this work.

**How do nutritional conditions in adult life impact on key life history traits?**

In chapter 5 I show that moulting male hihi prioritise carotenoid acquisition whilst foraging in the wild. Adult nutritional condition has generally been considered important for carotenoid-based plumage, particularly in comparison to melanin-based and structurally-produced plumage (Hill 2006). There is evidence that both general nutritional state (Hill and Montgomerie 1994; Hill 2000) and specific carotenoid access (Hill 1992) during moult have some bearing on the quality of carotenoid-based plumage that is grown. However, some authors have remained sceptical over whether dietary access to carotenoid pigments is really a limiting factor for ornamentation (Hudon 1994; Bortolotti et al. 1996; Hill and Johnson 2012), and therefore whether this can be a mechanism for the honest maintenance of carotenoid-based signals. Demonstrating that a species displaying carotenoid-based plumage targets carotenoid-
rich foods (chapter 5) lends considerable support to the assumption that carotenoid access is limiting in some way.

The manner in which carotenoid pigments are limiting remains to be determined. They may be scarce in the environment (Olson and Owens 1998), either because foods with a high carotenoid-content are a rare resource or perhaps because certain individuals are able to exclude such foods from other foraging individuals. Moulting occurs at the end of the breeding season when some territorial behaviour is still exhibited, and carotenoid-rich food sources might be monopolised by territorial individuals, for example. Alternatively, or additionally, carotenoids may be limiting because they are required for different functions in the body (Olson and Owens 1998), forcing a trade-off between allocation to different roles. We do not know which of these explanations is most likely in the hihi. Knowing which foods have the highest carotenoid concentration (chapter 5) is a first step towards assessing environmental carotenoid availability. The next step would involve quantifying the abundance and distribution of these foods in the environment to produce a ‘carotenoid-map’ of pigment availability. It would be equally interesting to determine whether females are seeking out food that prolongs life, assuming this is the female route for maximising fitness, and what the availability of these foods is.

For what purpose would adult male hihi seek to maximise their carotenoid intake? In terms of ornamentation, they are presumably seeking dietary carotenoid pigments to enlarge the area of their yellow plumage, since this has the capacity to enhance reproductive success (chapter 3). Hue and saturation are the properties of carotenoid-based colour that most consistently reflect the carotenoid content of feathers (Saks et al. 2003; McGraw and Gregory 2004, although plumage brightness has also been linked to feather carotenoid content: Mays Jr et al. 2004), but a larger area of pigmented plumage will inevitably increase pigment demand too. Increasing the size of a carotenoid-based patch will require that pigment deposition occurs in a greater number of feathers, but is presumably also limited by the underlying genetic and hormonal restrictions on which feathers can be yellow (Kimball 2006; Mundy 2006). Given a finite carotenoid resource, an individual bird will face allocation decisions over whether to deposit more pigment into fewer feathers or less pigment into a greater number of feathers. For the hihi the optimal strategy would appear to be
depositing the hard-won carotenoid resource across a greater number of feathers, therefore increasing the size of the yellow patch and increasing a male’s chances of acquiring a territory.

On the issue of maximizing fitness, findings in my thesis are relevant to the long-standing and unresolved debate of why socially monogamous females mate multiply. Two non-mutually exclusive hypotheses are typically put forward to explain the evolution of extra-pair reproduction in females. Females may stand to gain directly from mating with extra-pair males, for example by gaining access to foraging areas (Griffith et al. 2002; Griffith 2007), and they may also stand to gain indirectly, in terms of additive or non-additive genetic benefits to their offspring (Akçay and Roughgarden 2007; Jennions and Petrie 2000). A fundamental prediction of the hypothesis that extra-pair reproduction evolved via indirect genetic benefits to females is that extra-pair young are fitter than within-pair young. However, there has been mixed support for this prediction (Arnqvist and Kirkpatrick 2005; Akçay and Roughgarden 2007). In fact, recent evidence from song sparrows (Melospiza melodia) shows that extra-pair offspring have both lower survival (Sardell et al. 2011) and lower additive genetic breeding value (Reid and Sardell 2011) than within-pair offspring. This not only casts doubt on the idea that extra-pair paternity confers indirect fitness benefits for females, but even suggests it may impose a genetic cost. In chapter 3, I show that males with longer white ear tufts both have greater extra-pair reproductive success and produce offspring with a lower probability of survival. Although not tested directly, this would imply that extra-pair offspring have reduced survival compared to within-pair offspring, in line with the song sparrow results. A more focussed study comparing fitness of within-pair and extra-pair hihi offspring, and therefore exploring the relationship reported in chapter 3, would be very worthwhile.

**Can knowledge of nutritional needs in early- and adult-life focus conservation efforts to the appropriate life history stage?**

Nestling and adult birds are predicted to have different dietary requirements, and in this thesis I have identified components of the diet that are important during the
nestling (chapters 2 and 4) and adult (chapter 5) phases of a hihi’s life. Some patterns emerge that confirm nutritional differences between life history stages. Carotenoids, for example, enhance both male and female survival during the nestling period (chapter 4), and males, more so than females, prioritise carotenoid intake in adult-life (chapter 5). Equally, some patterns emerge that suggest consistency between life history stages. For example, an elevated protein intake during development is beneficial for female survival (chapter 4) and, as adults, females appear to have a greater protein intake than do males (chapter 5). Importantly, understanding these nutritional requirements, and the consequences of any divergence from them, can help inform where conservation efforts are best directed.

Previous work on hihi has already demonstrated the utility of supplementary feeding. Providing a carbohydrate supplement to adults throughout the year, in the form of sugar water, enhances adult survival in at least two populations (Armstrong and Ewen 2001; Chauvenet et al. 2012), although is not always successful at limiting population decline (Armstrong et al. 2007). Providing a more complete nutritional supplement to adults throughout the year, in the form of Wombaroo, increases fledging success (Armstrong et al. 2007), but not to an extent that justifies a change in management. My findings confirm the marginal gains that complete nutritional supplementation provides, and also suggest that in some contexts, when provided to male nestlings and when combined with carotenoid supplementation, the effects may in fact be detrimental (chapter 4). Carotenoids are not, to my knowledge, provided routinely as part of any supplementary feeding program. However, previous work with hihi suggests their provision to breeding females can offset the costs of ecotoparasitism on nestling growth (Ewen et al. 2009). Here I show that carotenoid provision directly to nestlings enhances fledging success to the same small extent as does wombaroo (chapter 4). Again this probably does not justify a change in management, at least in populations with low parasite prevalence. Chapter 4 contributes to the existing body of work by examining the sex-specific longer-term consequences of direct supplementation to nestling birds.

One of the potential advantages of providing supplementary food directly to developing young is that it provides them with a good nutritional start in life, the benefits of which may be reaped long into the future (Metcalf and Monaghan 2001).
Although I do not find any evidence for long-term survival effects of nestling supplementation (chapter 4), it remains possible that benefits may be seen elsewhere, for example on reproductive success. Indeed, the proven consequences of developmental nutrition for colourful traits (chapter 2) would suggest this is a possibility. The possibility of long-term benefits also remains for other species, particularly those that have a lower baseline adult survival than do the hihi on Tiritiri Matangi, where the already high adult survival leaves little room for additional improvement.

An important consideration for any supplementary feeding program is whether feeding during early and/or adult life creates a mismatch between developmental and adult environments (Monaghan 2008). Under adaptive phenotypic plasticity, the developmental environment will shape an individual’s phenotype so that it is adapted to live in an adult environment that is expected given the developmental environment (Pigliucci 2005). If the adult environment is in fact not as predicted, fitness will be low. This is well illustrated in human populations, where children from resource-poor environments that go on to live in resource-rich environments as adults are more likely to develop type II diabetes and other health problems (Bateson et al. 2004). Supplementary feeding programs should be wary of creating an equivalent mismatch such that individuals are adapted for an environment they never experience and suffer fitness costs as a consequence. It is therefore important that conservation practitioners are mindful of the nutritional environment that supplementary feeding creates, and make attempts to understand how developmental and adult nutritional environments interact with each other. To help achieve the necessary consistency between developmental and adult environments, particularly in habitats that are degraded (which is invariably the case when supplementary feeding is employed), strategic habitat restoration might be utilised. For example, planting species that will help meet both juvenile and adult nutritional requirements, and allow the latter to be accurately predicted from the former, might ultimately provide a longer term and more sustainable fix than supplementary feeding.

A related consideration is whether management practices create an evolutionary mismatch between modified (i.e. managed) and ancestral environments. The themes discussed in this thesis straddle both evolutionary biology and conservation biology,
and thus represent an opportunity to consider the evolutionary consequences of conservation management. Just as anthropogenic perturbations (overharvesting, habitat fragmentation etc) can influence the strength and mode of selection, so too can well-intentioned conservation interventions (Stockwell et al. 2003). Indeed, the potential for some management interventions to induce evolutionary change in a population is well recognised for some conservation measures. For example, the translocation of individuals to new sites can represent cultural (Parker et al. 2012) and genetic (Brekke et al. 2011) bottlenecks, and considerable effort is therefore spent in maintaining the evolutionary potential of these populations (Keller et al. 2012). The results I present in chapter 3 describe the evidence for sexual selection operating on plumage traits in a population that undergoes intensive management. The hihi in the Tiritiri Matangi population receive supplementary food, are provided with nest boxes, and receive parasite treatments as nestlings; any or all of these measures might be exerting selection pressures that are both different from those experienced by ancestral populations, and that result in traits mal-adapted for ancestral environments. These issues are by no means unique to this study system, and raise the philosophical conundrum of whether the act of conserving is changing the very species we seek to conserve (Ashley et al 2003). One potential way of addressing this issue would be to explicitly define the maintenance of evolutionary potential as a fundamental objective when making decisions about environmental management (Gregory et al. 2012).

**Future directions**

A central theme of this thesis is that early life experiences can shape and determine important future events throughout an individual’s lifetime. But, what are the mechanistic processes that link early life to subsequent fitness? Answering this question will require an expansion of the type of approach used here, whereby early life environment is manipulated and the resulting impact on fitness is measured, to include an assessment of hypothesised mechanistic links. Two likely candidates for this linking role are telomere dynamics and oxidative stress, both of considerable importance in the process of aging.

Telomeres are non-coding regions at the ends of eukaryotic chromosomes that protect coding regions from degradation. Telomeres shorten after each round of cell division,
resulting in cellular senescence, impaired tissue function and, ultimately, organism aging. Telomere length and the rate at which they shorten has been linked to lifespan in a number of long-lived species (Monaghan and Haussmann 2006), but, most significantly, telomere length in early life has been shown to predict lifespan in captive species (Heidinger et al. 2012). Oxidative damage is also intimately linked with aging (Harman 1956). It is caused when reactive oxygen species, by-products of normal metabolism, oxidise biologically important molecules such as lipids, protein and DNA (Halliwell and Gutteridge 2007). A battery of antioxidant defences have evolved to limit the extent of oxidative damage (Monaghan et al. 2009), but some damage inevitably accumulates with age. As I have suggested, both telomere dynamics and oxidative stress might mediate the link between early life nutritional condition and later-life fitness. One possible pathway sees developmental nutrition accelerate growth, which consequently elevates oxidative stress (e.g. Alonso-Alvarez et al. 2007). This in turn reduces telomere length, because telomeric DNA is more vulnerable to oxidative damage (Houben et al. 2008), such that individuals begin life with shortened telomeres. Such individuals can then be expected to have greater vulnerability to future stressful events that will generate further oxidative stress and telomere shortening, exacerbating the aging process. However, although there is sound justification for each step along this pathway, it is unknown whether these relationships will persist in natural populations, where individuals will potentially face environmental hardship and be exposed to extrinsic causes of mortality. Future work to establish whether this is the case will help to bridge the mechanistic gap between early and adult life.

To sum up, I investigated evolutionary questions from a nutritional perspective and applied the findings to a conservation context. I found that male hihi prioritize investment of nutritional resources into colourful plumage, which functions to repel rivals and attract mates, during both development and adulthood. Female hihi, in contrast, invest developmental and adult nutritional resources in a manner consistent with maximizing survival. As a consequence, male and female hihi benefit from different nutritional supplements during development. The challenges for future work include pinpointing the nutritional optima of the two sexes and identifying the mechanistic processes that link early life nutrition to subsequent fitness.


Cheeke, P. R., and E. S. Dierenfeld. 2010. Comparative animal nutrition and metabolism. CABI, Wallingford, UK.


Roper, M. 2012. Resource partitioning between two competitive species, the hihi (Notiomystis cincta) and bellbird (Anthornis melanura), during the non-breeding season on Tiritiri Matangi Island. MSc Thesis. Massey University, New Zealand.


Chapter 2 supplementary material

Experimental design

Detailed information on the composition of hihi nestling diet is lacking, and so the doses of the nutritional treatment were based on the manufacturer’s recommendations. The manufacturers of Wombaroo Lorikeet & Honeyeater Food recommend that wild honeyeaters be fed a daily maximum of 5 mL of a 30% by mass Wombaroo solution. Given the range of honeyeater body mass (8 – 200 g; (Higgins et al. 2001)), and the nutritional composition of Wombaroo (140 g kg\(^{-1}\) protein, 60 g kg\(^{-1}\) fat, 6 g kg\(^{-1}\) fibre), this represents a daily intake of 0.1 - 2.6 % protein, 0.05 - 1.1 % fat, and 0.005 - 0.1 % fibre, expressed as a percentage of body mass. In our experiment, we used a fixed concentration of Wombaroo (0.3 g mL\(^{-1}\)) and increased volume with age to achieve protein, fat and fibre amounts within the recommended intake (Table A1.1).

We followed the recommendations of (Biard et al. 2006) to calculate daily carotenoid doses. Mean hihi fledgling body mass is 36.15 g (± 0.46, n = 192) and mean plasma carotenoid concentration is 6.0 µg mL\(^{-1}\) (Thorogood et al. 2008). Given that blood volume represents about 10% of nestling body mass (Sturkie 1986), total quantity of circulating carotenoids in fledgling hihi plasma can be estimated as 21.69 µg. Assuming the efficiency of carotenoid absorption in birds is about 20% of the used dose (Surai 2002), we aimed to supplement 100 µg (i.e. five times the total quantity of circulating carotenoids) per feeding. Carotenoids were supplemented at a final concentration of 100 µg mL\(^{-1}\) in volumes of 0.2 mL to 3.0 mL (Table A1.1), such that amount supplemented ranged from 20 µg at 4 days old to 300 µg at 20 days old. This allows for the growth of nestlings over the 17-day period of supplementation, and is equivalent to receiving a daily carotenoid supplement of 80 µg.
Table A1.1: the volume of Wombaroo, and the resulting amount of protein, fat and fibre, expressed in absolute values and as a percentage of body mass, supplemented at each age

<table>
<thead>
<tr>
<th>Age</th>
<th>Vol (mL)</th>
<th>Protein (g)</th>
<th>Fat (g)</th>
<th>Fibre (g)</th>
<th>Mean body mass (g)</th>
<th>Protein (as % of body mass)</th>
<th>Fat (as % of body mass)</th>
<th>Fibre (as % of body mass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.2</td>
<td>0.0084</td>
<td>0.0036</td>
<td>0.00036</td>
<td>5.79</td>
<td>0.15</td>
<td>0.06</td>
<td>0.006</td>
</tr>
<tr>
<td>6</td>
<td>0.3</td>
<td>0.0126</td>
<td>0.0054</td>
<td>0.00054</td>
<td>9.39</td>
<td>0.13</td>
<td>0.06</td>
<td>0.006</td>
</tr>
<tr>
<td>8</td>
<td>0.5</td>
<td>0.021</td>
<td>0.009</td>
<td>0.0009</td>
<td>14.24</td>
<td>0.15</td>
<td>0.06</td>
<td>0.006</td>
</tr>
<tr>
<td>10</td>
<td>1.0</td>
<td>0.042</td>
<td>0.018</td>
<td>0.0018</td>
<td>19.62</td>
<td>0.21</td>
<td>0.09</td>
<td>0.009</td>
</tr>
<tr>
<td>12</td>
<td>1.5</td>
<td>0.063</td>
<td>0.027</td>
<td>0.0027</td>
<td>24.28</td>
<td>0.26</td>
<td>0.11</td>
<td>0.011</td>
</tr>
<tr>
<td>14</td>
<td>2.0</td>
<td>0.084</td>
<td>0.036</td>
<td>0.0036</td>
<td>28.15</td>
<td>0.30</td>
<td>0.13</td>
<td>0.013</td>
</tr>
<tr>
<td>16</td>
<td>2.4</td>
<td>0.1008</td>
<td>0.0432</td>
<td>0.00432</td>
<td>30.59</td>
<td>0.33</td>
<td>0.14</td>
<td>0.014</td>
</tr>
<tr>
<td>18</td>
<td>2.8</td>
<td>0.1176</td>
<td>0.0504</td>
<td>0.00504</td>
<td>32.89</td>
<td>0.36</td>
<td>0.15</td>
<td>0.015</td>
</tr>
<tr>
<td>20</td>
<td>3.0</td>
<td>0.126</td>
<td>0.054</td>
<td>0.0054</td>
<td>34.09</td>
<td>0.37</td>
<td>0.16</td>
<td>0.016</td>
</tr>
</tbody>
</table>

**Plasma carotenoid concentration**

20 µL plasma was homogenised with 20 µL NaCl 5% and 40 µL ethanol, and carotenoids were twice extracted by homogenization with 500 µL hexane. Hexane extracts were pooled and evaporated at 70 °C under nitrogen flow, and the residue was dissolved in 50 µL dichloromethane and 50 µL methanol. Total carotenoids were detected with a Spherisorb type S5NH2 reverse-phase column (25 cm x 4.6 mm, Phase Separation Ltd., Clwyd, UK) with a mobile phase of methanol (97:3) at a flow rate of 1.5 mL min⁻¹, using UV detection by absorbance at 445 nm.

**Plumage colour analysis**

Given the differences between avian and human vision, plumage colour analysis should take into account the avian visual system (Kelber et al. 2003; Osorio and Vorobyev 2005, 2008; Cuthill 2006). Therefore, reflectance spectra were analysed.
using models in tetrahedral colour space to extract hue, saturation and luminance variables for each colour patch (Endler and Mielke 2005; Stevens et al. 2009; Stoddard and Prum 2011). Hue, the colour type (e.g. blue versus red), and saturation, the amount of colour compared to white light, can be used to describe the colour of a patch. Luminance captures the achromatic component of the signal and describes the perceived lightness of a patch. We first calculated photon catch values for the four single cones, used in colour vision, and the double cones, used in luminance vision, based on the reflectance spectra and measures of irradiance (Endler and Mielke 2005). Models were run using standard ‘d65’ (full light) and ‘forest shade’ irradiance spectra. In addition, since the spectral sensitivities of hihi are not known, we calculated photon catches using spectral sensitivities from a species with an ultraviolet shifted shortwave sensitive cone type (UVS) (blue tit; *Cyanistes caeruleus*) (Hart et al. 2000) and a species with a violet (VS) system (peafowl; *Pavo cristatus*) (Hart 2002). Results did not differ significantly when using different irradiance levels and spectral sensitivities, therefore only values generated using ‘d65’ irradiance levels and with blue tit spectral sensitivities are presented. Following this, our measure of luminance was simply the double cone photon catch values. To calculate saturation, we plotted the standardized single cone catch data for each individual (using relative cone catches to remove variations in absolute brightness) in avian tetrahedral colour space (Endler and Mielke 2005) and calculated the distance from the centre of the colour space. Larger values indicate greater saturation. To calculate hue, we adopted an approach similar to (Komdeur et al. 2005) and (Spottiswoode and Stevens 2011). We used ratios based on photon catch outputs that are broadly inspired by the way that opponent colour channels work, based on performing a principal component analysis (PCA) on a covariance matrix of the standardized single cone data (see (Stevens 2011)). The PCA enabled us to encode colour in a biologically meaningful way (Komdeur et al. 2005; Spottiswoode and Stevens 2011). The first principal component (PC) explained 97 percent of the variation in yellow plumage colour, with the colour channel being: \((LW+MW)/(SW+UV)\), where LW, MW, SW and UV indicate longwave, mediumwave, shortwave and ultraviolet cone catches, respectively. The same colour channel explained 85 and 84 percent of the variance for the black and white patches respectively.
Data analysis

Variation in plasma carotenoid concentration was explored by fitting a linear mixed effects model using the lme function in R’s nlme package. This function calculates degrees of freedom in such a way that the random effect needn’t be explicitly nested in the nutritional treatment, as some other statistical programs require. A full description of how this function calculates df can be found in (Zuur et al. 2009).

Briefly, df for a level 1 variable (which takes a different value for all observations within the levels of the random effect e.g. carotenoid treatment) are calculated as the number of level 1 observations minus the number of level 2 clusters minus the number of level 2 fixed effects. The df for a level 2 variable (which takes the same value for all observations within the levels of the random effect e.g. nutritional treatment) are calculated as the number of level 2 clusters minus the number of level 2 variables minus 1 for the intercept. With 188 individuals and 73 nests in the case of carotenoid concentration, this gives 73-3-1 = 69 df for nutritional treatment (a level 2 variable) and 188-73-2 = 113 df for carotenoid treatment (a level 1 variable) (as reported in the results).

When investigating the effects of treatments and covariates on colour variables, a re-sampling approach was taken to control for the minority of cases where siblings from the same brood were measured. A single nestling was randomly drawn from each nest to generate a sample dataset equal in size to the number of nests (e.g. yellow post-moult models had one nestling drawn from each of the 35 nests that were represented). 1000 sample datasets were generated (with a random nestling being sampled from each nest each time) and linear models were fitted to each sample dataset, thus generating a model distribution for each of the effects. Mean effect sizes, standard errors, and t-values from this re-sampled distribution are reported, along with the 95% confidence intervals around the mean t-value and the percentage of re-sampled p-values that fall below 0.05.

In cases where residual plots of plumage colour models revealed substantial heterogeneity, linear models were fitted using generalised least squares with a specified variance structure (gls function in R). The post-moult yellow plumage saturation model allowed variance to differ by moult score (using varIdent function in
R); the pre-breeding black plumage saturation model allowed variance to differ by nutritional treatment (using varIdent); the post-moult white plumage luminance model allowed variance to differ by hatch date (using varExp); the pre-breeding white plumage saturation model allowed variance to differ by hatch date (using varExp); and the pre-breeding white ear tuft length model allowed variance to differ by nutritional treatment (using varIdent).

Appendix 1 References


Appendix 1


Table A1.2: Results from re-sampled linear models for the yellow plumage patch. Post-moult models based on 49 individuals from 35 different nests, and pre-breeding models based on 25 individuals from 22 different nests. Pre-breeding yellow luminance is Box-Cox transformed. Significant results (where high % of resampled p-values fell below 0.05) are highlighted in bold.

<table>
<thead>
<tr>
<th></th>
<th>mean effect size ± se</th>
<th>mean t-value (95% CI)</th>
<th>% re-sampled p-values &lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>post-moult pre-breeding</td>
<td>post-moult pre-breeding</td>
<td>post-moult pre-breeding</td>
</tr>
</tbody>
</table>

**yellow saturation**

<table>
<thead>
<tr>
<th></th>
<th>intercept</th>
<th>nutritional treatment</th>
<th>carotenoid treatment</th>
<th>brood size</th>
<th>hatch date</th>
<th>moult score</th>
<th>treatment interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.3174 ± 0.0166</td>
<td>-0.0077 ± 0.0091</td>
<td>0.0034 ± 0.0104</td>
<td>0.0018 ± 0.0038</td>
<td>-0.0022 ± 0.0010</td>
<td>0.0004 ± 0.0133</td>
<td>0.0048 ± 0.0132</td>
</tr>
<tr>
<td></td>
<td>0.3394 ± 0.0368</td>
<td>-0.0069 ± 0.0203</td>
<td>-0.0160 ± 0.0160</td>
<td>-0.0018 ± 0.0085</td>
<td>-0.0024 ± 0.0014</td>
<td>na</td>
<td>0.0254 ± 0.0259</td>
</tr>
<tr>
<td></td>
<td>19.40 (19.27 to 19.52)</td>
<td>-0.85 (-0.88 to -0.82)</td>
<td>0.334 (0.29 to 0.38)</td>
<td>0.50 (0.47 to 0.53)</td>
<td>-2.34 (-2.37 to -2.31)</td>
<td>0.03 (0.01 to 0.06)</td>
<td>0.36 (0.33 to 0.39)</td>
</tr>
<tr>
<td></td>
<td>9.23 (9.21 to 9.24)</td>
<td>-0.33 (-0.35 to -0.32)</td>
<td>-0.98 (-1.00 to -0.95)</td>
<td>-0.21 (-0.21 to -0.20)</td>
<td>-1.69 (-1.71 to -1.67)</td>
<td>na</td>
<td>0.97 (0.95 to 0.98)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.3</td>
<td>0.6</td>
<td>0.0</td>
<td>69.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**yellow luminance**

<table>
<thead>
<tr>
<th></th>
<th>intercept</th>
<th>nutritional treatment</th>
<th>carotenoid treatment</th>
<th>brood size</th>
<th>hatch date</th>
<th>moult score</th>
<th>treatment interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.2313 ± 0.0308</td>
<td>-0.0092 ± 0.0171</td>
<td>-0.0178 ± 0.0173</td>
<td>0.0043 ± 0.0071</td>
<td>0.0011 ± 0.0016</td>
<td>-0.0043 ± 0.0149</td>
<td>0.0148 ± 0.0234</td>
</tr>
<tr>
<td></td>
<td>2.4353 ± 0.4256</td>
<td>-0.0267 ± 0.2347</td>
<td>-0.0385 ± 0.1840</td>
<td>0.0804 ± 0.0989</td>
<td>-0.0155 ± 0.0162</td>
<td>-3.38 ± 0.9785</td>
<td>0.0165 ± 0.2988</td>
</tr>
<tr>
<td></td>
<td>7.55 (7.49 - 7.61)</td>
<td>-0.53 (-0.56 - -0.51)</td>
<td>-1.02 (-1.05 to -0.99)</td>
<td>0.61 (0.58 - 0.65)</td>
<td>0.69 (0.65 - 0.73)</td>
<td>na</td>
<td>0.63 (0.61 - 0.66)</td>
</tr>
<tr>
<td></td>
<td>5.72 (5.72 to 5.73)</td>
<td>-0.12 (-0.13 to -0.10)</td>
<td>0.21 (0.19 to 0.22)</td>
<td>0.81 (0.81 to 0.82)</td>
<td>-0.97 (-0.99 to -0.95)</td>
<td>na</td>
<td>0.06 (0.05 to 0.07)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.0</td>
<td>2.8</td>
<td>0.1</td>
<td>0.9</td>
<td>na</td>
<td>0.1</td>
</tr>
</tbody>
</table>

**yellow patch size**

<table>
<thead>
<tr>
<th></th>
<th>intercept</th>
<th>nutritional treatment</th>
<th>carotenoid treatment</th>
<th>brood size</th>
<th>hatch date</th>
<th>moult score</th>
<th>treatment interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>na</td>
<td>na</td>
<td>-3.38 ± 97.85</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>1195.23 ± 226.06</td>
<td>113.72 ± 124.94</td>
<td>-3.38 ± 97.85</td>
<td>-140.16 ± 52.46</td>
<td>0.65 ± 8.61</td>
<td>-40.83 ± 158.89</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>na</td>
<td>na</td>
<td>-0.02 (-0.04 to 0.01)</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>5.29 (5.28 to 5.30)</td>
<td>0.92 (0.90 to 0.93)</td>
<td>-2.68 (-2.68 to -2.67)</td>
<td>0.08 (0.06 to 0.10)</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>100</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
</tbody>
</table>

* Estimate relative to N- treatment, ‡ Estimate relative to C- treatment
Table A1.3: Results from re-sampled linear models for the black plumage patch. Post-moult models based on 41 individuals from 31 different nests, and pre-breeding models based on 24 individuals from 22 different nests. Pre-breeding black saturation is Box-Cox transformed. Significant result (where high % of resampled p-values fell below 0.05) is highlighted in bold.

<table>
<thead>
<tr>
<th></th>
<th>mean effect size ± se</th>
<th>mean t-value (95% CI)</th>
<th>% re-sampled p-values &lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>post-moult</td>
<td>pre-breeding</td>
<td>post-moult</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.0262 ± 0.0128</td>
<td>6.0544 ± 2.5081</td>
<td>2.08 (2.05 to 2.10)</td>
</tr>
<tr>
<td>Nutritional treatment a</td>
<td>0.0033 ± 0.0071</td>
<td>2.1973 ± 1.3542</td>
<td>0.46 (0.43 to 0.48)</td>
</tr>
<tr>
<td>Carotenoid treatment b</td>
<td>0.0067 ± 0.0079</td>
<td>-0.2454 ± 1.2616</td>
<td>0.84 (0.82 to 0.87)</td>
</tr>
<tr>
<td>Brood size</td>
<td>-0.0004 ± 0.0030</td>
<td>0.1247 ± 0.5689</td>
<td>-0.13 (-0.16 to -0.11)</td>
</tr>
<tr>
<td>Hatch date</td>
<td>0.0010 ± 0.0007</td>
<td>0.0671 ± 0.1057</td>
<td>1.32 (1.29 to 1.35)</td>
</tr>
<tr>
<td>Moulting score</td>
<td>-0.0108 ± 0.0069</td>
<td>na</td>
<td>-1.58 (-1.60 to -1.56)</td>
</tr>
<tr>
<td>Treatment interaction</td>
<td>0.00001 ± 0.0098</td>
<td>-1.989 ± 1.7793</td>
<td>0.002 (-0.02 to 0.02)</td>
</tr>
<tr>
<td>Black luminance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>0.0041 ± 0.0040</td>
<td>0.0053 ± 0.0071</td>
<td>1.04 (1.03 to 1.05)</td>
</tr>
<tr>
<td>Nutritional treatment a</td>
<td>0.0007 ± 0.0022</td>
<td>0.0011 ± 0.0039</td>
<td>0.33 (0.31 to 0.34)</td>
</tr>
<tr>
<td>Carotenoid treatment b</td>
<td>0.0018 ± 0.0025</td>
<td>0.0023 ± 0.0031</td>
<td>0.71 (0.68 to 0.74)</td>
</tr>
<tr>
<td>Brood size</td>
<td>0.0013 ± 0.0009</td>
<td>0.0014 ± 0.0017</td>
<td>1.44 (1.43 to 1.46)</td>
</tr>
<tr>
<td>Hatch date</td>
<td>-0.00005 ± 0.0002</td>
<td>0.0007 ± 0.0003</td>
<td>-0.23 (-0.25 to -0.22)</td>
</tr>
<tr>
<td>Moulting score</td>
<td>0.0023 ± 0.0021</td>
<td>na</td>
<td>1.08 (1.07 to 1.10)</td>
</tr>
<tr>
<td>Treatment interaction</td>
<td>-0.0025 ± 0.0030</td>
<td>-0.0041 ± 0.0050</td>
<td>-0.82 (-0.84 to -0.79)</td>
</tr>
</tbody>
</table>

A Estimate relative to N- treatment, B Estimate relative to C- treatment
**Table A1.4**: Results from re-sampled linear model for the white ear tufts. Post-moult models based on 35 individuals from 27 different nests, and pre-breeding models based on 35 individuals from 22 different nests. Post-moult white saturation and white luminance were Box-Cox transformed. Near significant result (where > 50 % of resampled p-values fell below 0.05) is highlighted in bold.

<table>
<thead>
<tr>
<th></th>
<th>mean effect size ± se (post-moult)</th>
<th>mean t-value (post-moult)</th>
<th>mean effect size ± se (pre-breeding)</th>
<th>mean t-value (pre-breeding)</th>
<th>mean p-value (post-moult)</th>
<th>mean p-value (pre-breeding)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>white saturation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>0.3505 ± 0.0464</td>
<td>7.59 (7.55 to 7.62)</td>
<td>3.23 (3.17 to 3.29)</td>
<td>100</td>
<td>77.0</td>
<td></td>
</tr>
<tr>
<td>Nutritional treatment</td>
<td>0.0303 ± 0.0256</td>
<td>1.19 (1.15 to 1.23)</td>
<td>-1.02 (-1.05 to -1.01)</td>
<td>8.3</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Carotenoid treatment</td>
<td>0.0148 ± 0.0284</td>
<td>0.53 (0.49 to 0.57)</td>
<td>-0.17 (-0.21 to -0.13)</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Brood size</td>
<td>0.0055 ± 0.0110</td>
<td>0.31 (0.29 to 0.34)</td>
<td>1.68 (1.68 to 1.69)</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Hatch date</td>
<td>-0.00007 ± 0.0026</td>
<td>-0.03 (-0.04 to -0.01)</td>
<td>-2.03 (-2.07 to -1.99)</td>
<td>0.0</td>
<td>49.1</td>
<td></td>
</tr>
<tr>
<td>Treatment interaction</td>
<td>-0.0368 ± 0.0362</td>
<td>-1.02 (-1.06 to -0.99)</td>
<td>-0.03 (-0.06 to -0.004)</td>
<td>4.2</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td><strong>white luminance</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>0.2767 ± 0.0615</td>
<td>4.70 (4.63 to 4.78)</td>
<td>5.15 (5.14 to 5.16)</td>
<td>100.0</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Nutritional treatment</td>
<td>-0.0796 ± 0.0379</td>
<td>-2.15 (-2.19 to -2.12)</td>
<td>-0.32 (-0.34 to -0.30)</td>
<td>52.1</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Carotenoid treatment</td>
<td>-0.0574 ± 0.0392</td>
<td>-1.48 (-1.49 to -1.47)</td>
<td>1.39 (1.36 to 1.43)</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Brood size</td>
<td>-0.0087 ± 0.0135</td>
<td>-0.71 (-0.74 to -0.68)</td>
<td>-0.40 (-0.41 to -0.40)</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Hatch date</td>
<td>-0.0031 ± 0.0029</td>
<td>-1.11 (-1.14 to -1.08)</td>
<td>1.23 (1.21 to 1.25)</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Treatment interaction</td>
<td>0.0696 ± 0.0474</td>
<td>1.51 (1.49 to 1.54)</td>
<td>-0.74 (-0.76 to -0.72)</td>
<td>6.3</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td><strong>white ear tuft length</strong></td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>100.0</td>
</tr>
<tr>
<td>Intercept</td>
<td>na</td>
<td>10.7591 ± 0.9404</td>
<td>na</td>
<td>11.47 (11.44 to 11.51)</td>
<td>na</td>
<td>100.0</td>
</tr>
<tr>
<td>Nutritional treatment</td>
<td>na</td>
<td>-0.6187 ± 0.7716</td>
<td>na</td>
<td>-0.80 (-0.81 to -0.80)</td>
<td>na</td>
<td>0.0</td>
</tr>
<tr>
<td>Carotenoid treatment</td>
<td>na</td>
<td>-0.6464 ± 0.3358</td>
<td>na</td>
<td>-1.92 (-1.93 to -1.91)</td>
<td>na</td>
<td>11.1</td>
</tr>
<tr>
<td>Brood size</td>
<td>na</td>
<td>0.2131 ± 0.2212</td>
<td>na</td>
<td>0.97 (0.96 to 0.98)</td>
<td>na</td>
<td>0.0</td>
</tr>
<tr>
<td>Hatch date</td>
<td>na</td>
<td>-0.0371 ± 0.0310</td>
<td>na</td>
<td>-1.20 (-1.21 to -1.19)</td>
<td>na</td>
<td>0.0</td>
</tr>
<tr>
<td>Treatment interaction</td>
<td>na</td>
<td>0.5594 ± 0.9038</td>
<td>na</td>
<td>0.62 (0.61 to 0.62)</td>
<td>na</td>
<td>0.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Estimate relative to N- treatment, <sup>b</sup> Estimate relative to C- treatment
Figure A1.1: Mean reflectance spectra for yellow, black and white plumage patches of male hihi. Averaged spectra from control males (N-C-) are plotted (n = 15 for yellow, n = 14 for black, n=14 for white).
Chapter 5 supplementary material

Table A2.1: Observed and mean re-sampled proportions of a) different food categories, and b) different fruit species occurring in the diets of males and females. Mean re-sampled proportion refers to the mean proportion following re-sampling (where one observation per individual was randomly sampled to generate an estimate of proportional make-up, and this was repeated 100 times).

<table>
<thead>
<tr>
<th>food item</th>
<th>observed proportion</th>
<th>mean re-sampled proportion (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>males</td>
<td>females</td>
</tr>
<tr>
<td>a) food category</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fruit</td>
<td>0.77</td>
<td>0.63</td>
</tr>
<tr>
<td>invertebrates</td>
<td>0.19</td>
<td>0.31</td>
</tr>
<tr>
<td>nectar</td>
<td>0.04</td>
<td>0.07</td>
</tr>
<tr>
<td>food item</td>
<td>observed proportion</td>
<td>mean re-sampled proportion (95% CI)</td>
</tr>
<tr>
<td>---------------------------</td>
<td>---------------------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td></td>
<td>males</td>
<td>females</td>
</tr>
<tr>
<td>cabbage tree</td>
<td>0.13</td>
<td>0.08</td>
</tr>
<tr>
<td><em>C. macrocarpa</em></td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td><em>C. repens</em></td>
<td>0.03</td>
<td>0.01</td>
</tr>
<tr>
<td><em>C. robusta</em></td>
<td>0.16</td>
<td>0.13</td>
</tr>
<tr>
<td>five-finger</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>hangehange</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>kawakawa</td>
<td>0.005</td>
<td>0.03</td>
</tr>
<tr>
<td>kohekohe</td>
<td>0.005</td>
<td>0.01</td>
</tr>
<tr>
<td>mahoe</td>
<td>0.36</td>
<td>0.47</td>
</tr>
<tr>
<td>mapou</td>
<td>0.08</td>
<td>0.06</td>
</tr>
<tr>
<td>puriri</td>
<td>0.005</td>
<td>0.00</td>
</tr>
<tr>
<td>small-leaved <em>Coprosma</em></td>
<td>0.03</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* Fruit proportions are expressed as proportion of all fruit species
Table A2.2: Individual and total carotenoid content (mean ± SE µg/g wet weight) of ripe fruit species fed on by hihi. H, M, and L indicate which of high-, medium- and low-carotenoid content categories each fruit species falls into.

<table>
<thead>
<tr>
<th>species</th>
<th>n</th>
<th>lutein (µg/g)</th>
<th>zeaxanthin (µg/g)</th>
<th>cis-lutein (µg/g)</th>
<th>anhydrolutein (µg/g)</th>
<th>echinenone (µg/g)</th>
<th>β-cryptoxanthin (µg/g)</th>
<th>α-carotene (µg/g)</th>
<th>β-carotene (µg/g)</th>
<th>total carotenoids (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cabbage tree</td>
<td>9</td>
<td>3.60 ± 0.63</td>
<td>0.76 ± 0.28</td>
<td>0.09 ± 0.06</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.10 ± 0.10</td>
<td>0.56 ± 0.56</td>
<td>1.14 ± 0.47</td>
</tr>
<tr>
<td>C. areolata</td>
<td>2</td>
<td>7.73 ± 3.23</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.23 ± 0.23</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.58 ± 0.58</td>
</tr>
<tr>
<td>C. macrocarpa</td>
<td>1</td>
<td>4.44</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>3.5</td>
<td>2.46</td>
<td>11.13</td>
</tr>
<tr>
<td>C. rhamnoides</td>
<td>1</td>
<td>1.90</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.54</td>
<td>0.46</td>
<td>2.90</td>
</tr>
<tr>
<td>C. robusta</td>
<td>16</td>
<td>4.25 ± 0.69</td>
<td>0.16 ± 0.07</td>
<td>0.17 ± 0.11</td>
<td>0.07 ± 0.04</td>
<td>0.16 ± 0.11</td>
<td>1.94 ± 0.82</td>
<td>8.46 ± 2.67</td>
<td>3.85 ± 1.05</td>
<td>19.63 ± 5.06</td>
</tr>
<tr>
<td>five-finger</td>
<td>10</td>
<td>8.28 ± 1.30</td>
<td>0.00 ± 0.00</td>
<td>0.19 ± 0.07</td>
<td>0.05 ± 0.05</td>
<td>0.00 ± 0.00</td>
<td>0.03 ± 0.03</td>
<td>0.14 ± 0.14</td>
<td>0.00 ± 0.00</td>
<td>1.55 ± 0.36</td>
</tr>
<tr>
<td>hangehange</td>
<td>8</td>
<td>38.08 ± 3.74</td>
<td>0.00 ± 0.00</td>
<td>1.73 ± 0.57</td>
<td>0.00 ± 0.00</td>
<td>7.76 ± 1.46</td>
<td>0.48 ± 0.15</td>
<td>0.00 ± 0.00</td>
<td>27.82 ± 3.21</td>
<td>81.65 ± 5.45</td>
</tr>
<tr>
<td>mahoe</td>
<td>9</td>
<td>0.55 ± 0.31</td>
<td>0.08 ± 0.04</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.72 ± 0.41</td>
<td>1.36 ± 0.77</td>
</tr>
<tr>
<td>mapou</td>
<td>5</td>
<td>4.06 ± 1.33</td>
<td>0.08 ± 0.08</td>
<td>0.09 ± 0.06</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.94 ± 0.44</td>
<td>5.17 ± 1.82</td>
</tr>
</tbody>
</table>
Table A2.3: Individual and total vitamin E content (mean ± SE µg/g wet weight) of ripe fruit species fed on by hihi. H, M, and L indicate which of high-, medium- and low-vitamin E content categories each fruit species falls into.

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>α-tocopherol</th>
<th>δ-tocopherol</th>
<th>γ-tocopherol</th>
<th>α-tocotrienol</th>
<th>γ-tocotrienol</th>
<th>Total vitamin E</th>
</tr>
</thead>
<tbody>
<tr>
<td>cabbage tree</td>
<td>9</td>
<td>54.19 ± 5.16</td>
<td>0.03 ± 0.02</td>
<td>19.33 ± 2.40</td>
<td>0.81 ± 0.79</td>
<td>0.10 ± 0.06</td>
<td>74.46 ± 5.24 (M)</td>
</tr>
<tr>
<td>C. areolata</td>
<td>2</td>
<td>53.01 ± 33.54</td>
<td>0.49 ± 0.08</td>
<td>12.87 ± 5.33</td>
<td>0.04 ± 0.04</td>
<td>0</td>
<td>66.40 ± 38.76</td>
</tr>
<tr>
<td>C. macrocarpa</td>
<td>1</td>
<td>59.48</td>
<td>2.37</td>
<td>33.59</td>
<td>0.22</td>
<td>0.19</td>
<td>95.86 (H)</td>
</tr>
<tr>
<td>C. rhamnoides</td>
<td>1</td>
<td>20.02</td>
<td>0.16</td>
<td>8.48</td>
<td>0.06</td>
<td>0</td>
<td>28.72</td>
</tr>
<tr>
<td>C. robusta</td>
<td>16</td>
<td>47.55 ± 8.43</td>
<td>0.73 ± 0.34</td>
<td>13.15 ± 3.74</td>
<td>0.17 ± 0.07</td>
<td>0.15 ± 0.07</td>
<td>61.75 ± 9.80 (M)</td>
</tr>
<tr>
<td>five-finger</td>
<td>10</td>
<td>44.60 ± 6.39</td>
<td>3.70 ± 0.79</td>
<td>12.61 ± 2.23</td>
<td>3.62 ± 1.45</td>
<td>0.50 ± 0.16</td>
<td>65.03 ± 9.02 (M)</td>
</tr>
<tr>
<td>hangehange</td>
<td>8</td>
<td>33.45 ± 6.62</td>
<td>4.64 ± 1.08</td>
<td>117.8 ± 9.06</td>
<td>2.08 ± 1.14</td>
<td>1.68 ± 0.36</td>
<td>159.64 ± 7.79 (H)</td>
</tr>
<tr>
<td>mahoe</td>
<td>9</td>
<td>40.30 ± 2.70</td>
<td>0.34 ± 0.11</td>
<td>28.11 ± 4.13</td>
<td>3.30 ± 0.57</td>
<td>0.82 ± 0.25</td>
<td>72.88 ± 5.35 (M)</td>
</tr>
<tr>
<td>mapou</td>
<td>5</td>
<td>19.19 ± 3.18</td>
<td>1.35 ± 0.76</td>
<td>28.28 ± 11.40</td>
<td>1.09 ± 0.88</td>
<td>0.04 ± 0.04</td>
<td>49.95 ± 11.02 (L)</td>
</tr>
</tbody>
</table>
During my PhD, I have published one paper in collaboration that is not directly related to my thesis research: