Environmentally induced variation in DNA methylation and embryonic heart rate in Australian birds

Elizabeth. L. Sheldon, MPhil

Department of Biological Sciences

Macquarie University

MACQUARIE University

This thesis is presented for the degree of Doctor of Philosophy

November 2017

Dedication

To my family, with love

TABLE OF CONTENTS

General Abstract

The environment is profound in its ability to influence phenotypic variation, and this is a necessary condition for integrating the developing organism into its particular habitat. While phenotypic plasticity is well engrained in ecological research, the underlying processes that mediate its effects remain unclear. Recently, epigenetic and metabolic mechanisms have been proposed to provide an avenue through which early environmental conditions can modulate phenotypic variation. However, before these mechanisms can be implicated in translating environmental effects to the phenotype, it must first be clarified whether epigenetic and metabolic processes are sensitive to environmental variation at all. Here, I examined how DNA methylation and metabolic rate are affected by various abiotic and biotic stimuli in two species of Australian birds. I used MS-AFLP to examine population-wide, and condition dependent patterns of DNA methylation in invasive house sparrows (*Passer domesticus*) and zebra finches (*Taeniopygia gu*ttata), and measured embryonic heart rate (a proxy for metabolic rate) in wild, native zebra finches, to examine environmental effects on metabolism, and the behavioural and developmental consequences of this. The patterns of DNA methylation that were observed in my thesis support the potential for epigenetic marks to respond to prevailing environmental conditions (such as habitat, brood size, invasion history and temperature). Embryonic heart rate measures were also found to be extremely sensitive to a range of environmental stimuli, notably changing in response to temperature and conspecific vocalisations. Embryonic heart rate also correlated with pre-natal developmental rate, however was unrelated to variation in nestling activity levels. Together, the environmentally induced changes described in my thesis support a role for epigenetic and metabolic mechanisms to translate environmental effects to ecologically relevant phenotypic variation. Such phenotypic plasticity could be particularly important for wild populations facing unpredictable and rapid environmental changes.

DECLARATION

I certify that this thesis constitutes my original work, and has not been submitted or accepted for the award of a higher degree at another institution. No persons have contributed to this work that have not been given credit, and all the work of previous scholars influencing this work are cited in the appropriate manner. The research presented in this thesis was approved by the Macquarie University Animal Ethics Committee (2015/017-4 & 2015-035).

Elizabeth Louise Sheldon

24th October, 2017

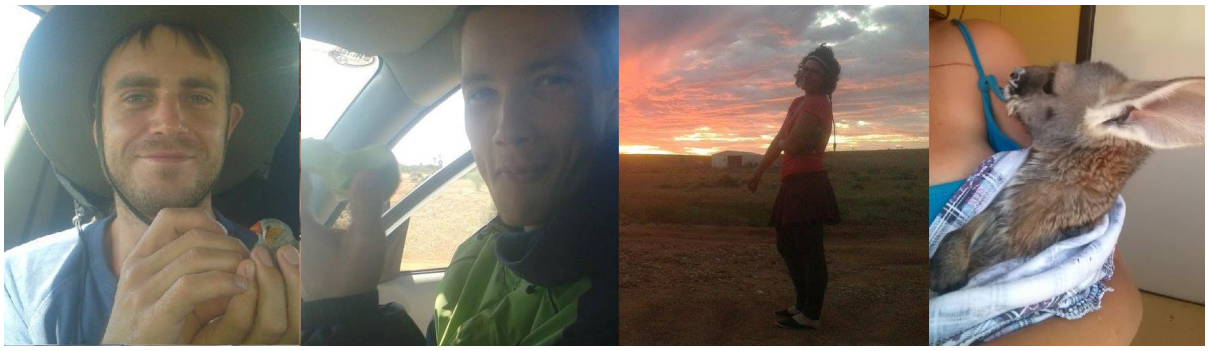
‘I was lucky enough to perceive what the beetle did not.’

Albert Einstein, 1922

Acknowledgements

To the finches and the sparrows, I am grateful that I was able to take a glimpse into their lives, and am ever more appreciative of how beautifully eloquent, peaceful and peculiar the natural world is.

I would like to thank all of those who helped me along the way, Simon Griffith for supervising my work and giving me this amazing opportunity, Aaron Schrey for his much appreciated encouragement and valuable help with the epigenetic analyses, and Sam Andrew for being close by from start to finish. I would like to thank Luke McCowan, Callum McDiarmid and Caterina Funghi for Fowlers Gap laughs and continued friendships. Further thanks to Lori Hurley and Peri Bolton for bird handling and lab work help.



Fowlers Gap team; Luke, Callum and Caterina, a roo and I; whilst doing field work

Thank you James Pay, for having a playful spirit and sharing lots of Tasmanian adventures with me. I would also like to thank Bethan Hockey for being a little bit of Wales in Australia, and of course all of my friends at home for being there always, especially Lauran Hackling, Becky Walker and my springer-Stanley Pancake.

In field trips around Australia, to Cooper Pedy and the likes, I thank Sam and Simon for good company, and I say another thanks to James Pay, and all in Tasmania for making my

fieldwork such fun there. In Savannah, Georgia I also thank Aaron and Alex Ragsdale for being so welcoming in that charismatic town.

Friends and family that helped through my PhD.

Thank you to Matthew Broderick for consistent laughs, tussles, and togetherness. Thank you to those in Sydney who have made this era such fun; Rafael de Fraga, Bethan Hockey, Louis O'Neil, Kiarra Herpinere, Sam Andrews, Brian Entler, Timotime Colins, Malu, Butch, pocket and Ann.

Mostly I thank my Mam and Dad for caring, supporting, helping me all of the time, and having a wide-eyed and bushy tailed outlook on life, thank you!

LIST OF CONTRIBUTORS

The co-authors that contributed to each section of my thesis, and my own percent contribution in brackets. Where ELS=Elizabeth L. Sheldon, SCG= Simon C. Griffith, AWS= Aaron W. Schrey, SCA= Samuel C. Andrew, LSCM= Luke S.C..McCOwan, CM= Callum McDiarmid, LH= Laura Hurley

	Introduction	**Chapter 2**	**Chapter 3**	**Chapter 4**	**Chapter 5**	**Chapter 6**	**Chapter 7**	**Discussion**
Conception and Planning	ELS (100%)	ELS (50%), SCG	ELS (40%), SCG	ELS (60%), SCH	ELS (60%), SCG	ELS (80%) SCG	ELS (90%) SCG	ELS (100%)
Field Data Collection	-	ELS (35%), SCG, SCA	LSCM,	ELS (70%), LH	ELS (70%), LSCM, CD	ELS (80%), LSCM, CD	ELS (65%) LSCM, CD	-
Laboratory Data Collection	-	ELS (70%), SCA, AWS	ELS (80%), AWS	ELS (95%), AWS	-	-	-	-
Data Analysis	-	ELS (60%), SCA, AWS	ELS (80%), AWS	ELS (80%), AWS	ELS (85%), SCA	ELS (90%)	ELS (80%)	-
Interpretation and Writing	ELS (100%)	ELS (80%), SCG, AWS	ELS (95%)	ELS (95%)	ELS (80%), SCG	ELS (90%), SCG	ELS (95%) SCA	ELS (100%)
Review and Editing	ELS (98%), SCG	ELS (80%), SCG, AWS, SCA	ELS (80%), SCG. AWS	ELS (80%), SCG	ELS (70%), SCG	ELS (80%), SCG	ELS (80%) SCA	ELS (98%), SCG

Preface

This thesis by publication is based on 7 research articles (Chapters 2, 3, 4, 5, 6, 7, and Appendix 1), that have been prepared for submission (Chapters 2, 3, 4, 6, 7), or already accepted (Chapter 5 and Appendix 1) to various peer-reviewed scientific journals for publication. Each Chapter has therefore been formatted according to the corresponding journals style.

List of articles that have been prepared for submission or accepted to Scientific Journals.

1. Chapter 2 has been formatted for submission to the journal Integrative and Comparative Biology
2. Chapter 3 has been formatted for submission to the Journal of Avian Biology
3. Chapter 4 has been formatted for submission to the Journal of Experimental Biology
4. Chapter 5 is published as: Sheldon, E.L., McCowan, L.S.C., McDiarmid, C.S., Griffith, S.C. 2017. Measuring the embryonic heart rate of wild birds: An opportunity to take the pulse on early development. *The Auk, Ornithological Advances*, 135. Accepted
5. Chapter 6 has been formatted for submission to the Journal of Animal Biology
6. Chapter 7 has been formatted for submission to the Journal: Royal Society Open Science
7. Appendix 1 has been published as: Sheldon, E.L., Griffith, S.C. 2017. A high incidence of non-cavity nesting in an introduced population of house sparrows suggests that the species should not be constrained by cavity nest site availability, Avian Research. Accepted

General Introduction

Dusk and day at Fowlers Gap Research station, Central Australia

General Introduction

The environment is not merely 'permissive' of development, but to some extent also instructs it (Gilbert, 2001). Developmental plasticity describes the ability of the same genome to produce a range of phenotypes, depending on the context in which the organism develops. As a result, early life conditions can have substantial, long-lasting consequences for individuals. For example, if a young caterpillar feeds on oak flowers it develops into a mimic of an oak catkin, while a second caterpillar from the same egg batch feeds on leaves and becomes a twig mimic (Whitman and Agrawal, 2009); developing at one temperature the snapping turtle (*Chelydra serpentine)* embryo becomes male, at another temperature it becomes female (Rhen and Lang, 1994); and presented with predators, the grey tree frog (*Hyla cryoscelis*) develops a bright deflective tail, and in a predator free environment has no tail colouration at all (Relyea and Werner, 2000a). These examples highlight the profound influence of the developmental environment on individual phenotype. However, while developmental plasticity is well engrained in ecological research, the molecular mechanisms behind these phenomena have only recently been addressed (Feil and Fraga, 2012).

How is the environment translated into the phenotype?

The concept of the reactive genome was first proposed by Waddington, (1956), and posits that the genome not only *acts* to enable phenotypic expression, but also *reacts* to the environment by changing its expression patterns. Epigenetic mechanisms are increasingly recognised as some of the principal mediators of gene expression, and are known to play significant roles in translating environmental effects to phenotypic variation. DNA methylation is the most widely studied epigenetic mechanism (due to its high stability across DNA replication), and involves the covalent attachment of a methyl group to a cytosine that is usually paired with a guanine on the DNA sequence (CpG). The addition of a methyl group

to the genome can alter the transcriptional state of the DNA and can significantly impact the expression of nearby genes (Weber et al, 2007). Thus, different patterns of DNA methylation can result in different phenotypes, without alterations to the underlying DNA sequence (see Figure 1). The profound effects of DNA methylation can be demonstrated within a single individual; every different cell that makes up an organism has the same genotype, but the diverse cell phenotypes are caused (in part) by differences in the patterns of DNA methylation.

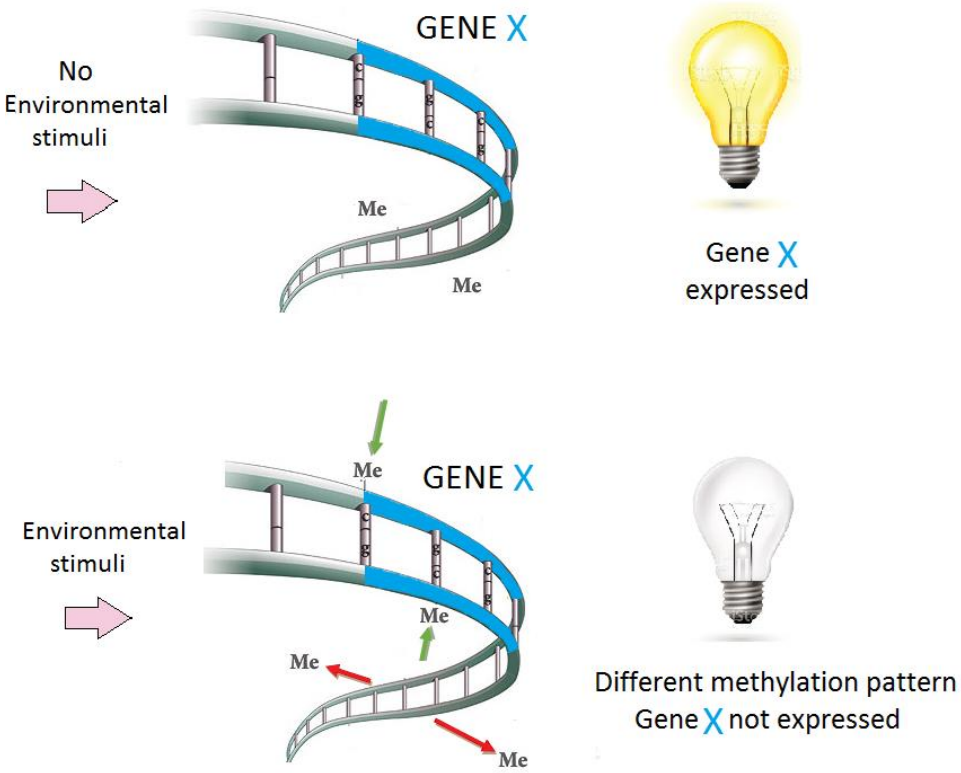


Figure 1. Illustration of how environmentally induced variation of DNA methylation patterns can effect gene expression, which may then effect phenotype. Green and red arrows represent the addition (which represses transcription) and removal of methyl marks to the DNA sequence, respectively.

Genome wide patterns of DNA methylation are established during early development, and are transferred across DNA replication by proteins such as DNA methyltransferases. This process can be interrupted by environmental influences- especially at sensitive phases during

early development, resulting in 'environmentally induced epigenetic variation' (Feil and Fraga, 2011; Heijmans et al, 2009). Environmentally induced changes to DNA methylation patterns have been proposed to provide an avenue through which early developmental conditions can modulate individual phenotypes (Meany and Szyf, 2005). For example, reduced maternal grooming behaviour in rats (*Rattus norvegicus*) increases DNA methylation levels around offspring glucocorticoid receptors, resulting in decreased expression of this gene and offspring with elevated stress responses (Zhang et al, 2013). Laboratory based examples such as this have illustrated the potential for DNA methylation to mediate environmental effects on the phenotype without underlying genetic changes. This has spurred the development of a relatively new field, termed 'ecological epigenetics' which explores the role of epigenetic variation in contributing to ecologically relevant, phenotypic variation.

Recent research in the field of ecological epigenetics has revealed a variety of environmental conditions that can induce changes to DNA methylation patterns (for example resource availability (Lea et al, 2016), and different levels of parental care (McGhee et al 2014)). However, this is still a burgeoning field, and the range of environmental stimuli known to induce epigenetic variation is far from defined, especially in vertebrate species (Snell-Rood et al, 2013; Roth et al, 2014; Weyrich, et al, 2016). For example, developmental temperature and brood size are both known to exert strong effects on fitness related traits in a range of taxa, yet their influence on epigenetic mechanisms- which may be involved in mediating these effects- has received inadequate attention to date.

In addition to specific environmental cues, the effects of genomic stressors (such inbreeding, or population bottlenecks) on epigenetic mechanisms are also unclear. However, given that DNA methylation can contribute to phenotypic variation in the absence of genetic diversity, the significance of epigenetic variation in mediating environmental responses is likely to be particularly important for genetically depauperate populations, such as invasive

populations (Xie et al. 2015, Richards et al. 2012). Local environmental conditions have been shown to elicit persistent epigenetic changes in some invasive species (Massicotte and Angers, 2011; Lea et al, 2016), however the extent to which patterns of epigenetic differentiation arise independently of genetic control (i.e. DNA methylation at CpG sites arising independently of particular genetic polymorphisms) is still unclear (Liu et al, 2012; Baldanzi et al, 2017). Theoretical work has also highlighted a potential for epigenetic diversity to compensate for reduced genetic diversity by acting as an alternative source of variation (Jablonka and Lamb, 2005). Yet, only a handful of studies have assessed whether a relationship between epigenetic and genetic diversity exists in invasive populations. (Liebl et al, 2013). Further work in this field is necessary to elucidate the relationship between genetic and epigenetic variation. This will allow us to realistically interpret the contribution of environmentally induced epigenetic variation to phenotypic variation in populations with low genetic variation.

Responses to the environment during early life

The environment experienced during early life is pivotal in shaping offspring development, and can result in phenotypic changes that persist throughout life (Griffith and Buchanan, 2010; Tschirren et al, 2009; Mariette and Griffith, 2015). Oviparous animals are especially vulnerable to external abiotic and biotic conditions during early life (as they develop outside of the mother's body, in an egg). A suite of adaptive responses exist to mitigate the effects of deleterious environmental conditions on oviparous embryonic development, and many of these adaptive responses involve the behaviour and physiology of the parents. Parents of oviparous embryos can buffer pre-natal developmental conditions of their offspring via egg incubation; nest-site selection; maternal resource allocation into the egg; and parent-embryo communication. The latter occurrence has received considerable

attention lately since the discovery that fairy wren (*Malurus cyaneus*) embryos are able to learn vocal 'passwords' from their mothers (Colombelli-Negrel, et al. 2012). The influence of parent-embryo communication has also recently been explored in the context of climate change, where parental vocalisations made during egg incubation have been purported to program offspring development to suit prevailing temperatures (albeit through an as-yet unknown mechanism) (Mariette and Buchanan, 2016). The potential for parent-embryo communication to mediate climate effects on offspring development is currently unclear. However, insights into this phenomenon could highlight a novel avenue through which acoustically communicating species are able to adapt to changing environments

While the environment can affect embryonic development indirectly via parental effects, less attention has focused on ways in which embryos *themselves* can respond to prevailing external conditions. Du and Shine (2015) propose that this bias reflects the assumption that embryos are 'organisms in process' and have little control over their own destinies until after hatching. Embryos are however, extremely reactive to their environment and are known to adjust their behaviour, physiology, developmental rates and trajectories in order to avoid adverse environments, and maximize the fitness of the post-hatch phenotype. Relatively little information is available on the mechanisms that underlie an embryos response to its environment (Du and Shine, 2015). However, recent studies have highlighted the potential for environmentally induced metabolic variation to effect a range of pre-natal processes that co-ordinate development.

New technologies have provided innovative opportunities to measure embryonic metabolic rate, through the monitoring of embryonic heart rate- a robust correlate of metabolic expenditure. These technologies have had limited use in characterizing variation in avian, embryonic heart rates. Yet, identifying the environmental factors that induce inter-individual variation in heart rate is likely to provide useful insights into the role of

metabolism in mediating environmental effects on pre-natal development (White et al, 2016). Further, because metabolism is intimately linked to a wide range of phenotypes (e.g. activity levels) and life history traits (e.g. growth rates), understanding how metabolism responds to the environment could lend insight into how inter-individual variation is maintained within a population (Biro and Stamps, 2010). Given the importance of inter-individual variation for adaptation, it is increasingly necessary to acknowledge all avenues through which phenotypic variation can emerge. A coherent framework linking environmentally induced metabolic variation with inter-individual phenotypic variation is yet to be achieved (Biro and Stamps, 2010; Careau et al, 2008), however would contribute to a more complete representation of how variation is maintained in wild populations.

Literature cited

Baldanzi, S., Watson, R., McQuaid, C.D., Gouws, G., Porri, F. 2017. Epigenetic variation among natural populations of the South African sandhopper *Talorchestia capensis.* *Evolutionary Ecology*, 31 (1); 77-91

Biro, P.A., Stamps, J.A. 2010. Do consistent individual differences in metabolic rate promote consistent individual differences in behaviour? *Trends in Ecology and Evolution*, 25 (1): 653-659

Careau, V., Thomas, D., Humphries, M., Reale, D. 2008. Energy metabolism and animal personality. *OIKOS, Synthesising Ecology*. 117 (5): 641-653

Colombelli-Negrel, D., M. E. Hauber, J. Robertson, F. J. Sulloway,´ H. Hoi, M. Griggio.,S. Kleindorfer (2012). Embryonic learning of vocal passwords in Superb Fairy-Wrens reveals intruder cuckoo nestlings. Current Biology 22:2155–2160.

Du, W.-G., and R. Shine (2015). The behavioural and physiological strategies of bird and reptile embryos in response to unpredictable variation in nest temperature. . Biological Reviews 90:19–30

Feil, R., Fraga, M.E. 2012. Epigenetics and the environment: emerging patterns and implications. *Nature Reviews Genetics*, 13 (2); 97-109

Gilbert, S. 2001. Ecological developmental biology: Developmental Biology meets the real world. Developmental biology, 233 (1), 1-12

Griffith, S.C., Buchanan K. 2010. Maternal effects in the zebra finch: a model mother reviewed. *Emu*, 110; 251-267

Heijmans, B.T., Tobi, E.W., Lumey, E.W., Slagboom. L.H. 2009. The epigenome, Archive of the prenatal environment. *Epigenetics* 4 (8); 526-531

Jablonka, E., Lamb, M. 2005. Evolution in four dimensions-Genetic, Epigenetic, Behavioural and Symbolic variation in the history of life. MIT Press

Lea.A.J., Altmann, J., Alberts. S.C., Tung.J. 2016. Resource base influences genome-wide DNA methylation levels in wild baboons (*Papio cynocephalus*). *Molecular ecology*, 25; 1681-1696

Liebl, A.L., Schrey, A.W., Richards, C.L., Martin, L.B. 2013. Patterns of DNA Methylation throughout a Range Expansion of an Introduced Songbird. *Integrative and Comparative Biology*, 53 (2); 351–358

Liu, S., Sun, K., Jiang, T., Ho, J.P., Liu, B., Feng, J. 2012. Natural epignetic variation in the female great roundleaf bat (*Hipposideros armiger*) populations. Molecular Genetics, Genomics, 287 (8); 643-650

Mariette, M.M., Buchanan, K.L. 2016. Prenatal acoustic communication programs offspring for high post hatching temperatures in a songbird. *Science*, 353 (6301); 812-814

Mariette, M.M., Griffith. S.C. 2015. The adaptive significance of provisioning and foraging coordination between breeding partners. *The American Naturalist*, 185, 270-280.

Massicotte, R. Whitelaw, E. Angers, B. 2011. DNA Methylation: A source of random variation in natural populations. *Epigenetics*, 6 (4); 421-427

McGhee K.E., Bell, A.M. 2014. Paternal care in fish: epigenetics and fitness enhancing effects on offspring anxiety. *Proceedings of the royal society B.* 281

Meany. M., Szyf. M. 2005. Environmental programming of stress responses through DNA methylation: life at the interface between a dynamic environment and a fixed genome. *Dialogues in clinical neuroscience*, 3; 103-123

Relyea, R.A., Werner, E.E. 2000. Morphological plasticity of four larval anurans distributed along an environmental gradient. Copeia;. 178-190

Rhen, T., Lang. J.W. 1994. Temperature dependent sex determination in the snapping turtle: manipulation of the embryonic sex steroid environment. Gen Comp Endocrinology, 96 (2); 243-254

Richards EJ. 2006. Inherited epigenetic variation—revisiting soft inheritance. *Nature Review Genetics* 7:395–401.

Roth. T.L., Matt, S, Chen, K., Blaze, J. 2014. BDFN DNA methylation modification in the hippocampus and amygdala of male and female rats exposed to different caregiving environments outside the home cage. Developmental psychobiology, 56; 1755-1763

Snell-Rood, E.C., Troth, A., Moczek, A.P. 2013. DNA methylation as a mechanism of nutritional plasticity: Limited support from horned beetles. *Journal of Experimental Biology, Part B, Molecular and developmental evolution*. 320B; 22-34

Tschirren B., Rutstein A. N., Postma E., Mariette M., Griffith S. C. 2009. Short- and long-term consequences of early developmental conditions: a case study on wild and domesticated zebra finches. *Journal of Evolutionary Biology*. 22; 387–395

Valentino, J.B. 2013. Phenotypic plasticity: Molecular Mechanisms, Evolutionary significance and impact on speciation. Nova Science Publishers Inc.

Waddington, C.H. 1956. Genetic Assimilation of the Bithorax Phenotype. Evolution, 10 (1), 1-13

Weber, M., Hellmann, I., Stadler, M., Ramos, L., Paabo. S., Rebhan. M., Schubeler, D. 2007. Distribution, silencing potential and evolutionary impact of promotor DNA methylation in the human genome. *Nature Genetics*. 39 (4); 457-466

Weyrich, A., Lenz, D., Jeschek, M., Hung Chung, T.,Rubensam, K., Goritz, F., Jewgenow, K., Fickel, J. 2016. Paternal intergenerational epigenetic response to heat exposure in male Wild guinea pigs. *Molecular ecology*, 25; 1729-1740

White, S.J., Kells, T.J., Wilson, A.J. 2016. Metabolism, personality and pace of life in the Trinidadian guppy, *Poecilia reticulata. Behaviour*, 153 (13-14); 1517-1543

Whitman, D.W, Agrawal, A.A. 2009. What is phenotypic plasticity and why is it important? In D.W. Whitman and T.N.Ananthakrishna, eds. Phenotypic plasticity of insects: Mechanisms and conseqiences, pp. 1-63. Science publishers, CRC Press, Enfield, NH

Xie H.J, Li, A.H. Liu, A.D. et al. 2015. ICE1 demethylation drives the range expansion on a plant invader though cold tolerance divergence. *Molecular Ecology*. 24; 835-850.

Zhang, Y-Y. Fischer, M., Colot. V., Bossdorf. O. 2013. Epigenetic variation creates potential for evolution of plant phenotypic plasticity. *New Phytology*, 197, 314-322

Thesis Aims

Zebra finches at Fowlers Gap Research station, drinking from the dam

With ever increasing concerns about the effects of anthropogenic change on organismal diversity and population fitness, there is renewed interest in the regulation of phenotypic variation by the environment (Valentino, 2013). Epigenetic and metabolic mechanisms have been proposed to provide an avenue through which early developmental conditions can modulate phenotypic variation. However, before these mechanisms can be implicated in translating environmental effects to the phenotype, it must first be clarified whether epigenetic and metabolic processes are sensitive to environmental variation at all.

For my PhD thesis I sought to examine how DNA methylation and metabolic rate are affected by various abiotic, biotic and genomic (e.g. patterns of genomic differentiation or. levels of heterozygosity) stimuli in two species of Australian birds. I use MS-AFLP

(Methylation Sensitive- Amplification Fragment Length Polymorphism) to examine population-wide, and condition dependent patterns of DNA methylation in invasive house sparrows (*Passer domesticus*) and wild zebra finches (*Taeniopygia gu*ttata), and use embryonic heart rate measures (a proxy for metabolic rate) of wild zebra finches, to examine environmental effects on metabolism, and the behavioural and developmental consequences of this. My thesis can thus be partitioned into two main categories of aims, one pertaining to describe environmentally induced epigenetic variation, and the other to describe environmentally induced metabolic variation:

Aim One:

Question 1. How do patterns of DNA methylation vary within an invasive population?

Chapter 2 aims to broadly define population-wide patterns of epigenetic diversity and differentiation in an invasive population. We aim to describe whether variation in DNA methylation is partitioned across different sites within a population, and how variation in DNA methylation is associated with levels of genomic diversity. In characterizing epigenetic and genetic patterns within multiple sites from three genetically distinct invasion events, we were also able to test the prediction that epigenetic diversity facilities invasive expansion by compensating for low levels of genetic variation within an invasion event, which has been proposed in recent research (Richards et al, 2012; Liebl et al, 2013).

Question 2: Are patterns of DNA methylation associated with developmental conditions?

Site specific patterns of epigenetic differentiation (that were not correlated with genetic differentiation) identified in Chapter 2, suggested that DNA methylation patterns can respond to local habitat conditions. This stimulated us to examine the effects of the early-life environment on epigenetic variation. Consequently, in Chapters 3 and 4 we focused on how

variation in brood size and temperature during perinatal development effect genome-wide levels of DNA methylation, and DNA methylation state changes of specific loci over development.

Aim Two: What are the causes and consequences of metabolic variation in early life?

Question 3: How does embryonic heart rate respond to environmental stimuli?

Environmental effects on heart rate variation have not yet been described in avian embryos. Thus in Chapter 5, we aimed to broadly characterize embryonic heart rate variation in response to a wide range of abiotic and biotic stimuli in wild zebra finches. In doing so we also appraised a new technology that enables the quantification of embryonic heart rate in a non-invasive and logistic manner. Given the practicality of measuring embryonic heart rate in wild birds, we then used embryonic heart rate measures to assess acoustic cognition in wild zebra finch embryos (heart rate declines are indicative of attention and awareness in a range of taxa, including birds (Colombelli-Negrel, 2014) . This allowed us to test predictions of a recent hypothesis suggesting a role for parental vocalizations in programming embryonic development to prevailing climate conditions.

Question 4. Does variation in embryonic heart rate effect post-natal behaviour and development?

Given the sensitivity of embryonic heart rate to a wide range of environmental cues, we then examined whether this variation effects behaviour and developmental rates during the embryonic stage and also later in development. We characterized consistent individual differences in activity levels and growth rates in zebra finch nestlings, and assessed whether these traits could be explained by inter-individual differences in embryonic heart rates.

Study species

I focused my research on two species of Australian birds; the zebra finch (*Taeniopygia guttata*), and the invasive house sparrow (*Passer domesticus*). These species are ideal for researching the effects of the environment on individual variation as they are both exposed to considerable environmental variation at a range of temporal and spatial scales.

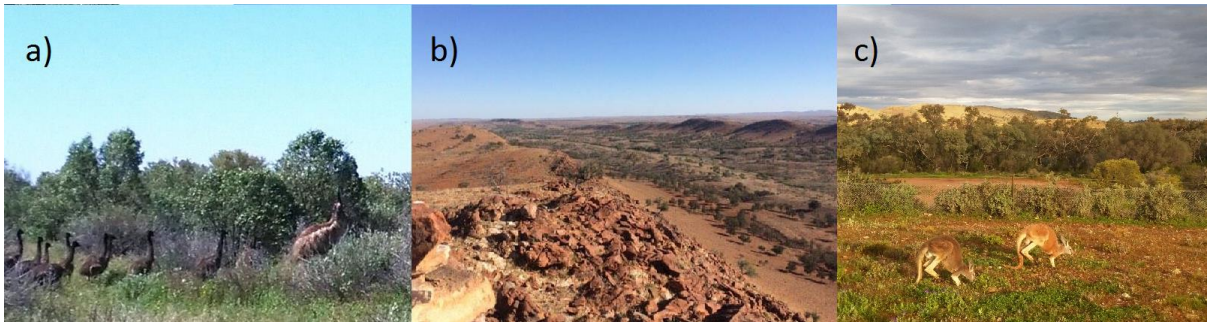
Zebra finch

The zebra finch is predominantly found in arid regions of central Australia, and breeds opportunistically in fluctuating thermal regimes. Ambient temperatures experienced by zebra finch chicks are known to frequently surpass the optimal developmental temperature for passerines of 36^{o}C (Griffiths et al, 2016). Early developmental conditions are also known to be profoundly affected by brood size and resource availability in this species.

Zebra finches at different stages of their life (left to right: a hatchling and its unhatched siblings, day 3 chicks, day 11 chicks and adult birds).

The nature and consequence of environmentally induced change has largely been investigated under laboratory conditions, yet laboratory situations are often unable to imitate the rich and unpredictable milieu that accompany the development of most species (Gilbert, 2001). Consequently, for my thesis, I predominantly studied wild zebra finches that bred in

nest-boxes at Fowlers Gap Arid Zone Research Station, in far-western New South Wales, Australia (31º05'S, '42º42É). The research station is located in the semi-arid zone of southeastern Australia, where atmospheric temperatures vary seasonally (from <5ºC to >45ºC), and rainfall is low (~240mm annually), and unpredictable (Griffith et al. 2016).



Habitat at Fowlers Gap: a) a father emu and his nine chicks, b) the rocky environment surrounding the research station, c) two foraging kangaroos. Zebra finches nest and forage in oases of vegetation (e.g. a, c) amongst the greater, more barren landscape (b).

There are around 300 nest boxes (Figure 2a) situated around Fowlers Gap Station. Zebra finches nest in the majority of these boxes, allowing us to monitor their breeding activity.

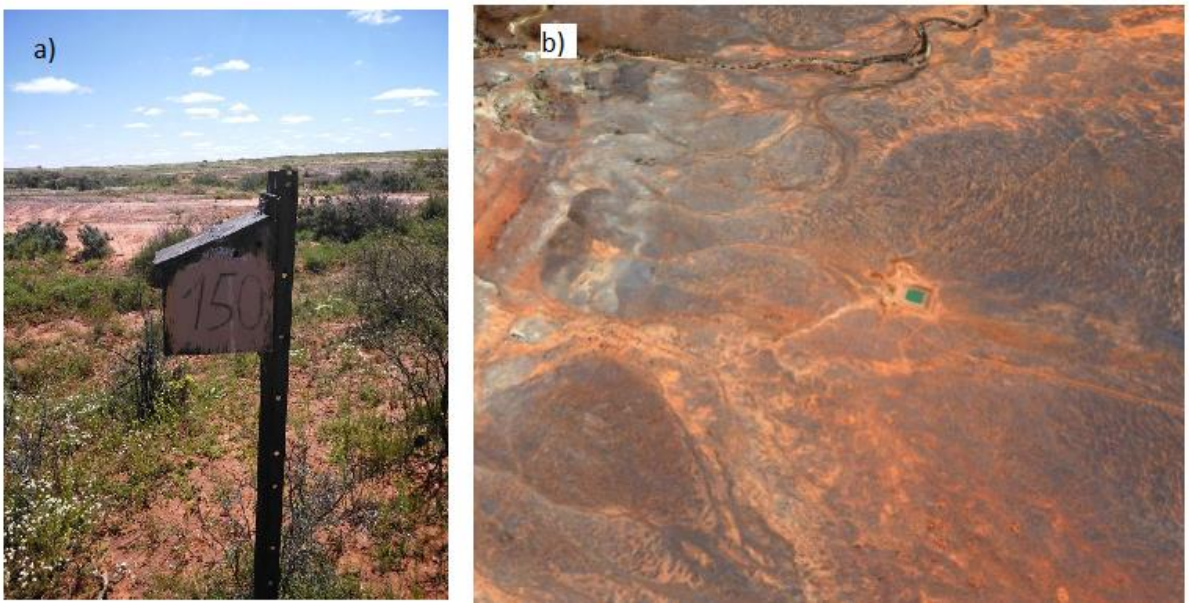


Figure 2. a) A nest box at Gap hills, Fowlers Gap, and b) an aerial view of the field site Gap hills which consists of 5 study sites that are within 1km from the central dam.

In order to manipulate specific temperature conditions of the early life environment, I also used zebra finches that were reared in temperature controlled rooms at Macquarie University (for Chapter 4).

House sparrow

House sparrows were deliberately introduced to Australia, from England, Germany and India, through the efforts of acclimatization societies in the late 1800s. From several, small founding populations, house sparrows have expanded into every climatic zone throughout the Eastern half of Australia, and have adapted to thrive in a variety of different ecosystems.

A fledgling house sparrow in Tasmania, Australia, and the global distribution of the house sparrow (Dark red: Native range, Light red: Introduced/invasive range).

Invasive populations have historically been used as natural experiments to provide insights into the evolutionary potential of populations with low genetic diversity (Liebl et al, 2015; Johnston and Sealander, 1964; Rollins et al, 2013). For my PhD I likewise used the invasive, Australian house sparrow population to examine how epigenetic responses may respond to varying genomic and environmental differences across Australia. For my thesis I

studied the house sparrow at 16 initial sites throughout Australia, which stemmed from three unique introduction events.

Three examples of study sites throughout Australia, right to left: Cooper Pedy, Port Augusta, and a vineyard close to Adelaide. Rural towns, horse stables, and vineyards were generally 'hot spots' for catching adult house sparrows via mist-netting.

Chapter 2

The introduced house sparrow population has expanded across the Eastern half of Australia from several founding events ~160 years ago

Epigenetic and genetic variation among three separate introductions of the house sparrow (*Passer domesticus*) into Australia

E.L. Sheldon[1], A. Schrey[2], S.C. Andrews[1], A. Ragsdale[2], S.C. Griffith[1]

1. Department of Biological Sciences, Macquarie University, Sydney, New South Wales, 2109, Australia
2. Department of Biology, Armstrong State University, Savannah, Georgia, USA

* Corresponding author: elizabeth-louise.sheldon@students.mq.edu.au

Abstract

Invasive populations are often associated with low levels of genetic diversity due to population bottlenecks at the initial stages of invasion. Despite this, the ability of invasive species to adapt rapidly in response to novel environments is well documented. Epigenetic mechanisms have recently been proposed to facilitate the success of invasive species, by compensating for reduced levels of genetic variation. Here, we used MS-AFLP and microsatellite analyses to compare levels of epigenetic and genetic diversity and differentiation across 15 sample sites in the introduced, Australian house sparrow (*Passer domesticus*) population. We found molecular evidence supporting three separate introductions of house sparrows into Australia, which can be categorised into three genetically differentiated 'invasion clusters'. We found that a significant portion of epigenetic differentiation was also partitioned between these three invasion clusters, as well as among specific sites throughout Australia, suggesting that epigenetic variation is affected by founding events, and may also respond to local environmental stimuli. Interestingly, we did not detect any correlations between pairwise site comparisons of epigenetic and genetic differentiation, suggesting that at least some of the observed epigenetic variation has arisen independently of genetic variation. Finally, we failed to detect the potentially compensatory relationship between epigenetic and genetic diversity that has been detected in a more recent house sparrow invasion. We discuss the potential for this relationship to be obscured by recovered genetic diversity in more established populations, and also highlight the importance of incorporating introduction history into population-wide epigenetic analyses in general.

Key words: DNA methylation, invasive species, house sparrow; epigenetic variation, epigenetics, introduction history

Introduction

Invasive species offer an opportunity to investigate rapid evolution in novel environments however, they also challenge our understanding of the process of adaptation. Invasive populations are often associated with low levels of genetic diversity, due to population bottlenecks at the initial stages of invasion (Schrey et al. 2012). Reduced genetic variation is expected to constrain the evolutionary potential of a given population (Dlugosch and Parker, 2008a, b), yet, many introduced populations are successful, and well able to adapt to non-native environments. The expansion of invasive species across novel environments thus presents an *'invasive paradox'* (Allendorf and Lundquist, 2003; Schrieber and Lachmuth 2016).

Epigenetic mechanisms (phenomena that alter gene expression without changing DNA sequences) can contribute to phenotypic variation (Bossdorf et al 2010, Nicotra et al 2015, Zhange et al, 2013), and have recently been proposed to facilitate the success of invasive species in novel environments, by compensating for reduced levels of additive genetic variation (Richards et al. 2012, Liebl et al. 2013). The most widely studied epigenetic mechanism is DNA methylation, which refers to the addition of a methyl group to a cytosine base, most often when the cytosine is immediately followed by a guanine on the DNA sequence (i.e. CpG sites; Schrey et al, 2013). Because CpG sites are enriched in regulatory sequences, variation in DNA methylation can alter gene expression, and potentially affect ecologically relevant phenotypes, without changing the underlying DNA sequence (Bossdorf et al. 2008, Richards 2006; Weaver et al. 2004; Herrera and Bazaga, 2013)

Genome-wide patterns of DNA methylation have substantially higher mutation rates than DNA sequences (Klironomos et al 2013), and can be induced stochastically (via re-patterning errors during DNA replication; Riggs et al. 1998) and in response to local environmental stimuli during an organism's lifetime (Angers et al, 2010, Verhoeven et al,

2010, Massicotte et al. 2011, Herrera et al 2012). Due to their links with phenotypic variation, environmentally induced epigenetic variation and stochastic epimutations have been proposed to be among the potential mechanisms underlying phenotypic plasticity and diversifying bet hedging strategies, respectively (Leung et al. 2016). Such flexibility in phenotypic expression, in the absence of underlying genetic variation could be particularly beneficial to invasive populations exposed to novel environments (Klironomos et al. 2013, Platt et al. 2015).

Relatively little is known about the role of epigenetic variation in mediating adaptation and plasticity in invasive, or introduced vertebrate species. However, the few studies available that focus on epigenetic variation in introduced species indicate that DNA methylation may play an important role in the ability of genetically depauperate populations to adapt to novel environments (Schrey et al. 2012). For example, local environmental conditions have been shown to elicit persistent epigenetic changes in an invasive, clonal fish species (*Chrosomus eos-neogaens*), resulting in a great deal of epigenetic differentiation among different habitats (Massicotte and Angers, 2012). Pairwise comparisons of epigenetic and genetic differentiation between different habitats in an invasive plant species, have also indicated that epigenetic marks can differentiate in response to local conditions, potentially contributing to phenotypic diversity independently of genetic differentiation (Richards et al. 2012). Levels of epigenetic diversity have also been suggested to contribute to invasive expansion, by acting as an alternative source of variation in areas with low genetic diversity. One of the first studies of a terrestrial vertebrate to examine this idea, was the study of the recent house sparrow (*Passer domestics*), introduction into Kenya, Africa by Liebl et al. (2013). In this study of seven populations, from one founding event around half a century ago, a negative relationship between epigenetic and genetic diversity was detected (Liebl et al 2013), suggesting that epigenetic diversity may act as a compensatory source of phenotypic

variation when genetic diversity is reduced in the initial stages of invasion. While these studies have characterised epigenetic diversity and differentiation in invasive populations, it is unclear how epigenetic variation is affected by invasion history (e.g. different founding events that make up one invasive population), and how epigenetic patterns persist across multiple invasion events.

Preceding the later introduction into Kenya by ~100 years, the house sparrow was also introduced into Australia in potentially three independent introduction events in the 1860s (Andrew & Griffith 2016). These multiple introduction events provide an opportunity to examine the extent to which epigenetic patterns observed in other invasive populations (Massicotte and Angers, 2012; Richards et al, 2012), including an earlier study of the same species (Liebl et al 2013) are affected by or are repeatable across multiple and distinct introduction events. In our study, we first characterise the genetic structure of the Australian house sparrow (*Passer domesticus*) population, to examine the idea from historical documents that there were three independent introductions (Andrew & Griffith 2016). We then use methylation-sensitive AFLP to describe epigenetic diversity and epigenetic differentiation across 15 sites within Australia and compare these measures to levels of genetic variation within the same population.

We predict that if epigenetic marks are responsive to different founding events, then epigenetic variation will be structured by the distinct introductions of house sparrows into Australia. Based on previous research (Baldanzi et al, 2017; Massicotte and Angers, 2012), we also predict that house sparrows that have established in different sites throughout Australia will be differentiated at both epigenetic and genetic loci, reflecting potentially adaptive responses to local habitat conditions. Finally, by expanding on the study by Liebl et al. (2013), we also tested whether the negative, potentially compensatory relationship

between genetic and epigenetic diversity also exists across multiple introduction events in a more temporally established population of house sparrows.

Methods

Sampling

As part of a broader study, blood samples were collected from adult house sparrows in multiple populations across the entire range of the house sparrow population in Australia. For this study we initially selected 16 populations to screen for epigenetic variation (see Table 2 for sample sizes); Tolga, Townsville, Charleville, Pittsworth, Dubbo, Cobar, Wentworth, Burrumbuttock, Melbourne, Torquay, Bridport, Mt Gambier, Broken Hill, Adelaide, and Cooper Pedy. A blood sample (<50µl) was taken from each individual at the time of capture and preserved thereafter in 95% ethanol at room temperature. DNA was extracted using the Gentra Puregene tissue kit and stored in 40µl of TE buffer. We aimed to screen variation in DNA methylation for the 24 samples with the most optimal DNA yields from each population selected (above), and attempted to amplify and score microsatellites at around 40 individuals in each population with the exception of two (Coober Pedy and Burrumbuttock in which only 20 and 25 samples were available respectively).

Microsatellite genotyping

Samples were genotyped using two multiplexes developed by Dawson et al. (2012) which included 11 polymorphic loci and a sexing locus (Multiplex 1: Ase18, Pdoµ1, Pdoµ3, Pdoµ6, Pdo10, P2D/P8; Multiplex 2: Pdo16A, Pdo17, Pdo19, Pdo22, Pdo27, Pdo40A). PCRs were carried out using 5 µl reactions. For each reaction 1 µl of genomic DNA (ca. 100 ng/µl) was added to 2.5µl of Master Mix (Qiagen, Valencia, CA, USA), 0.5 µl of primer mix (see concentrations in Dawson et al. 2012) and 1µl of Milli-Q water. Both multiplex reactions

used the same PCR thermal cycle with a hot-start denaturing phase of 10 min at 95 °C followed by 33 cycles of 94 °C for 30 s, 57 °C for 90 s and 72 °C for 90 s, before a final extension at 72 °C for 10 min. The post PCR product was diluted and genotyped on the ABI 3730XL using GS500 (LIZ) as a size standard for Multiplex 1 and GS1200 for Multiplex 2. Microsatellite alleles were scored using the GeneMapper program version 3.7.

DNA methylation using MS-AFLP

We screened samples for variation in DNA methylation using MS-AFLP, which modifies the standard AFLP protocol by substituting the MseI enzyme with the methylation-sensitive isoschizomeric enzymes MspI and HpaII (New England Biolabs). Enzymes MspI and HpaII vary in their sensitivity to cytosine methylation. Cleaving by MspI is blocked when the inner cytosine is methylated, whereas cleaving by HpaII is blocked when either or both cytosines are fully or hemi- methylated (Salmon et al. 2008). Together four types of variation can be scored: Type 1 is when both enzymes cut at the restriction site and indicates no methylation, Type 2 is when MspI does cut and HpaII does not cut, indicating the restriction site has a methylated internal cytosine C; Type 3 is when MspI does not cut and HpaII does cut indicating the restriction site has a methylated outer C; and Type 4 is when neither enzyme cuts indicating either both cytosines are methylated or the restriction site has mutated (Richards et al. 2012). We treated Type 4 as missing because the underlying methylation state cannot be determined. Recently there has been a suggestion that Type 2 and 3 should be analysed as separate states (Schulz et al. 2014); however, the actual source of these types of variation may be more complicated based on nested fragments (Fulnecek and Kovarik 2014). As such, we combined Type 2 and 3 into one methylated category, and treated all other states as not methylated. Throughout, we refer to a MS-AFLP locus to indicate a particular sized band resolved in the selective PCR.

We performed MS-AFLP following the protocol used by Richards et al. (2012), we digested approximately 250ng of genomic DNA at 37º C for 3 h in paired reactions; one with EcoRI and MspI, the other with EcoRI and HpaII. We immediately followed the restriction digest with adaptor ligation with EcoRI and MspI/HpaII adaptors at 16-20 h at 16º C (Supplemental Table 1-all primer and adapter sequences). After adaptor ligation, we conducted pre-selective PCR with EcoRI+1, MspI/HpaII+0 pre-selective primers (Supplemental Table 1) at the following PCR conditions: 75º C for 2 min; 20 cycles of 94º C for 30 s, 56º C for 30s, 75º C for 2 min, final extension at 60º C for 30 min and 4º C hold. Following pre-selective PCR, we conducted selective PCR by multiplexing 6-FAM fluorescently labelled EcoRI+AGC primers with HEX fluorescently labelled EcoRI+ACG primers and unlabelled primers HpaII/MspI+TCAT (Supplemental Table 1) at the following PCR conditions 94º C for 2 min, 8 cycles of 94º C 30 s, 65º C 30 s 72º C 2 min (dropping the annealing temperature 1º each cycle), 31 cycles of 94º C 30 s, 56º C 30 s 72º C 2 min, final extension of 60º C 5 min and a 4º C hold. We sent the selective PCR products to the Georgia Genomics Facility (University of Georgia) for fragment analysis on an ABI 3130XL. We used PEAKSCANNER v 1.0 (Applied Biosystems) to analyse resultant gel files and define fragment sizes and RAWGENO (Arrigo et al. 2012) to define particular bands. We duplicated the entire protocol for at least two individuals from each location to identify bands that consistently occurred and we eliminated bands that inconsistently amplified or occurred at highly variable intensities. We pooled data into two categories: methylated (Type II and Type III) or not methylated (Type I and Type IV).

Statistical Methods

To assess genetic population structure, the R package *adegenet* (Jombart 2008; R Core Team, 2017) was used for a Correspondence Analysis (CoA) of microsatellite data for

all 15 sites; this multivariate approach uses a summary of sample site allele frequencies to create a distance matrix that is used to generate Principal Component (PC) values for each locality, similar to a Principal Coordinate Analysis (PCoA) of individuals. Our CoA used the first five Principal Components because these five were enough to explain almost all the variance in the data (as indicated in results). Visual clusters of sampling localities were identified.

A Discriminant Analysis of Principal Components (DAPC) was used to test if these population clusters fitted with the genotypes of individuals. The population cluster labels that were identified were used in a DAPC in *adegenet* (Jombart et al. 2009, Jombart et al. 2010). This method used the individual data to calculate the percentage of individuals that were correctly assigned to their population clusters identified from the CoA using their genotypes. We choose to use the CoA of sample site (that uses allele frequencies, also accounting for the presence and absence of alleles) to define the main genetic clusters, because we predict founder effects will have had the clearest effect on allele frequency and allelic diversity between localities.

Epigenetic and Genetic differentiation

We estimated epigenetic and genetic differentiation as Φ_{ST} among different sites, and among different introduction events using the AMOVA (Analysis of MOlecular Variance) framework of GENALEX-6. GENALEX6 was also used to produce pairwise Φ_{ST} matrices (Peakall and Smouse 2012), and we performed calculations over all loci and pairwise between sites in both epigenetic and genetic analyses.

We compared pairwise genetic and epigenetic Φ_{ST} using a Mantel's test in R (R core team, 2017) using the function "mantel.randtest()" in the package *ade4* (Dray & Dufour 2007). Finally, we compared epigenetic differentiation to geographic distance among sites

using a Mantel's test. For all AMOVA analyses, statistical significance was estimated after 9999 permutations. We used a sequential Bonferroni correction of α=0.05 for multiple tests.

Epigenetic and Genetic diversity

We calculated epigenetic haplotype diversity (epi-h) and the proportion of polymorphic loci (%Poly) to characterise epigenetic diversity for each site and for each cluster, using GENALEX6 (Peakall and Smouse 2012). We conducted all analyses using a binary haplotype-binding pattern for 41 verified, consistent banding sites between 75 and 450 bp. %Poly represents how much 'raw' epigenetic variation is present in one site/cluster (i.e. the percentage of loci that are polymorphic out of the total 41 loci in our study), whereas epi-h represents haplotype diversity between the epigenetic profiles of all individuals within a site/cluster (i.e. the probability that two individuals in a site have different epigenetic profiles).

For microsatellite data the mean number of alleles (Na) (used for 'among cluster' analyses), mean allelic richness (Ar) (used for 'among site' analyses; Ar is the equivalent of Na, but adjusted for site sample sizes), and mean expected heterozygosity (He) were calculated for each site using FSTAT version 2.9 (Goudet 1995).

We compared the pattern of change between MS-AFLP based estimates of epigenetic diversity (epi-h and %Poly) with genetic characteristics of diversity (He and Ar) based on microsatellite loci genotyped in individuals from the same sample site (Table 1). We initially compared estimates of epi-h/%Poly and He/Ar between all sites in our study, however because our analyses of differentiation identified three separate clusters contingent on introduction history, we then compared levels of diversity separately among sites within the three clusters. All linear regressions conducted in R.

Sample sizes

We attempted to assay the level of DNA methylation in 24 individuals (except Coober Pedy in which only 20 individuals were sampled) of 16 initial populations (Armidale was later excluded from the analysis due to a high failure rate during the MS-AFLP Protocol). From these 380 individuals attempted, we were only successful in genotyping 180 individuals in total (47% of the individuals targeted). This rather high failure rate is due to the difficulty of the AFLP technique used, and the sensitivity of the Restriction digest step to DNA concentration. This failure rate was distributed across all sample populations (sample sizes given in Table 2), except Armidale for which we were only successful in scoring 4 individuals. As a result, we excluded this population from all subsequent analyses.

Results

Epigenetic and genetic differentiation

Microsatellite analyses identified three genetic clusters across our 15 sample sites (Figure 1, Figure 2), consistent with independent introductions and the existence of largely separate clusters since these initial colonisation events. These three clusters are consistent with three independent introductions to Melbourne ("NSW and VIC" cluster), Brisbane ("Queensland" cluster) and Adelaide ("South Australian" cluster). The percentage of individuals that were correctly assigned to each cluster was 95.7%, 95.3% and 76.7% respectively (Figure 2c).

An AMOVA found that a larger portion of genetic differentiation was partitioned between the introduction events compared to between sample sites (among introduction events $\Phi_{ST} = 0.041$, $P < 0.001$ and among sample sites $\Phi_{ST} = 0.025$, $P < 0.001$, respectively).

An AMOVA also detected epigenetic differentiation throughout the Australian house sparrow population. However, a larger portion of epigenetic differentiation was partitioned between sample sites (Φ_{ST}=0.139, P<0.01) compared to between introduction events (Φ_{RT}=0.023, P=0.001).

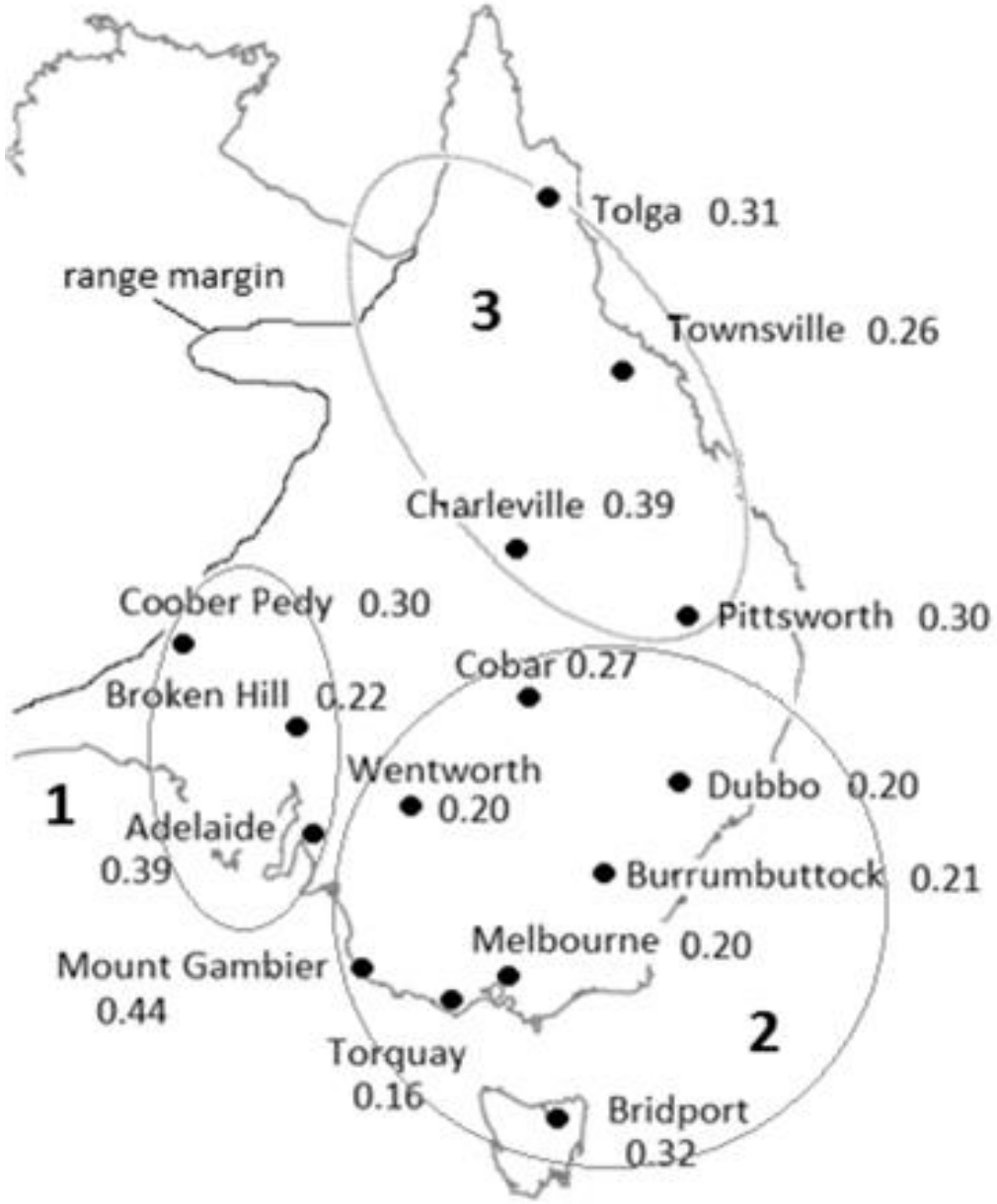


Figure 1. Map of the Eastern half of Australia labelled with the 15 study sites and their corresponding epigenetic diversity (epi-h) values. Sites derived from the same introduction event are grouped within an oval; 1: the South Australia introduction, 2: the Victoria/New South Wales introduction, 3: the Queensland introduction. The house sparrows estimated range edge is also plotted.

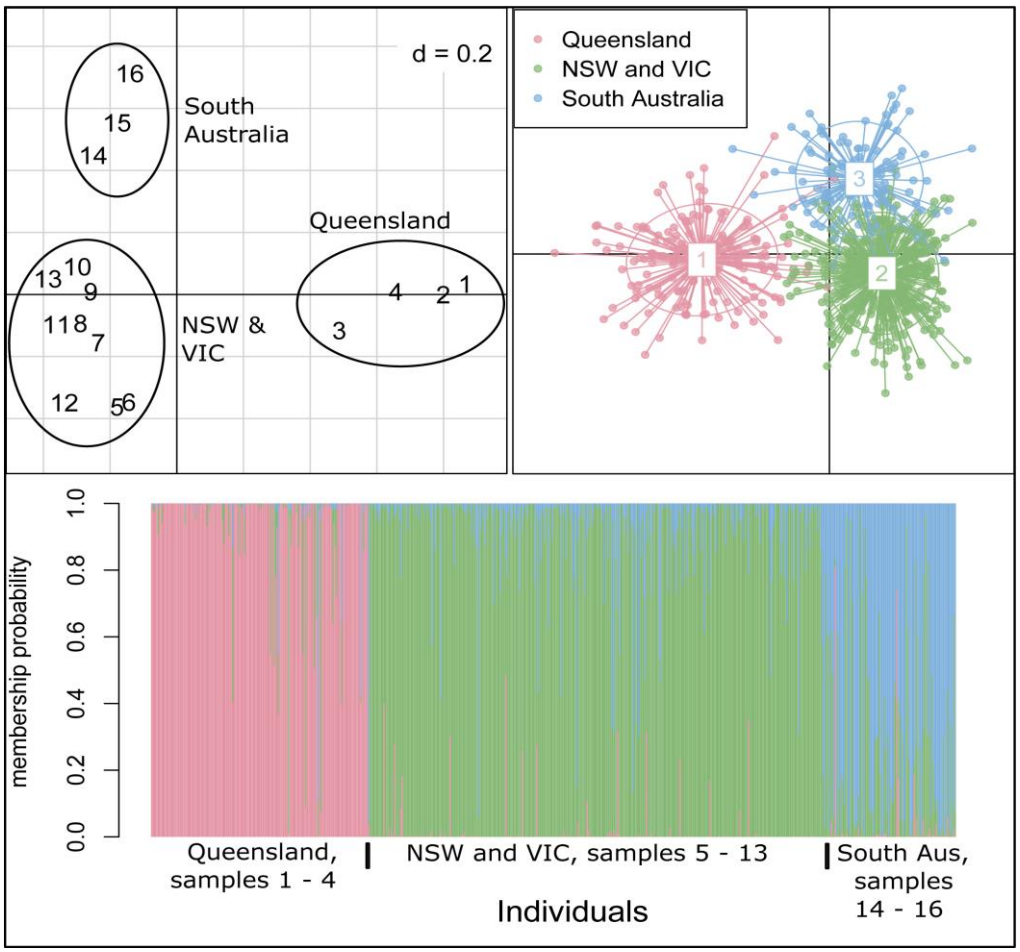


Figure 2. Top left panel shows the scatter plot for the CoA of the 16 sample localities with the three clusters that were identified. Top right shows the scatter plot from the DAPC which used the three population labels with the individual genotypes (n = 623 individuals). Bottom panel shows the membership probabilities for the DAPC in the top right panel. The sample labels 1 – 16 correspond to the sampling localities: Tolga, Townsville, Charleville, Pittsworth, Armidale (removed from epigenetic analyses due to low sample sizes), Dubbo, Cobar, Wentworth, Burrumbuttock, Melbourne, Geelong, Bridport, Mt Gambier, Broken Hill, Adelaide and Coober Pedy respectively (see Table 1).

Pairwise comparisons of Epigenetic (Φ_{ST}) and Genetic (Φ_{ST}) differentiation

Pairwise comparisons of Φ_{ST} for epigenetic data were significant in 89 out of 120 cases (Table 1). Whereas pairwise comparisons of Φ_{ST} for genetic data, were significant in 117 out of 120 cases (Table 1).

Table 1. Pairwise comparisons of epigenetic (below the diagonal) and genetic (above the diagonal) variation across all of our sample sites (n=15). Bold values indicate significant Φ_{ST} values ($\alpha < 0.05$), such that two sites are differentiated by epigenetic and genetic variation. Grey boxes represent pairwise comparisons between sample sites within genetic populations linked to the same introduction event.

Cooper Pedy	Adelaide	Broken Hill	Mt Gambier	Bridport	Torquay	Melbourne	Burrumbuttock	Wentworth	Cobar	Dubbo	Pittsworth	Charleville	Townsville	Tolga	
0.036	**0.161**	0.042	**0.194**	**0.083**	**0.134**	**0.093**	**0.076**	**0.058**	**0.061**	**0.071**	**0.045**	**0.189**	**0.052**	*	Tolga
0.060	**0.181**	0.013	**0.217**	**0.129**	0.048	0.005	0.016	0.010	**0.062**	0.003	**0.088**	**0.258**	*	**0.009**	Townsville
0.197	**0.143**	**0.270**	**0.070**	**0.205**	**0.398**	**0.360**	**0.326**	**0.301**	**0.189**	**0.283**	**0.143**	*	**0.058**	**0.043**	Charleville
0.110	**0.151**	**0.056**	**0.174**	**0.168**	**0.184**	**0.134**	**0.106**	**0.121**	**0.090**	**0.094**	*	**0.034**	**0.018**	**0.012**	Pittsworth
0.048	**0.224**	<0.001	**0.256**	**0.110**	**0.067**	<0.001	<0.001	0.003	0.074	*	**0.061**	**0.075**	**0.085**	**0.080**	Dubbo
0.062	**0.152**	**0.097**	**0.158**	**0.072**	**0.186**	**0.125**	**0.128**	**0.096**	*	**0.017**	**0.061**	**0.077**	**0.082**	**0.080**	Cobar
0.039	**0.239**	<0.001	**0.271**	**0.108**	0.021	<0.001	<0.001	*	**0.015**	**0.022**	**0.053**	**0.074**	**0.077**	**0.072**	Wentworth
0.044	**0.242**	<0.001	**0.294**	**0.118**	0.004	<0.001	*	0.008	**0.013**	**0.025**	**0.067**	**0.086**	**0.098**	**0.088**	Burrumbuttock
0.077	**0.270**	<0.001	**0.305**	**0.160**	0.006	*	**0.015**	**0.014**	**0.024**	**0.028**	**0.063**	**0.073**	**0.093**	**0.085**	Melbourne
0.107	**0.311**	0.055	**0.328**	**0.171**	*	**0.009**	0.007	0.003	**0.013**	**0.023**	**0.062**	**0.086**	**0.089**	**0.087**	Torquay
<0.001	0.077	**0.101**	**0.111**	*	**0.015**	**0.024**	**0.029**	**0.020**	**0.017**	**0.028**	**0.065**	**0.087**	**0.092**	**0.093**	Bridport
0.154	<0.001	**0.239**	*	**0.048**	**0.027**	**0.028**	**0.036**	**0.034**	**0.043**	**0.065**	**0.092**	**0.104**	**0.125**	**0.116**	Mt Gambier
0.031	**0.197**	*	**0.042**	**0.034**	**0.015**	**0.020**	**0.024**	**0.017**	**0.024**	**0.038**	**0.063**	**0.091**	**0.089**	**0.086**	Broken Hill
0.100	*	**0.011**	**0.039**	**0.036**	**0.020**	**0.020**	**0.025**	**0.022**	**0.027**	**0.045**	**0.054**	**0.083**	**0.084**	**0.079**	Adelaide
*	**0.050**	**0.052**	**0.078**	**0.070**	**0.055**	**0.048**	**0.059**	**0.059**	**0.062**	**0.077**	**0.090**	**0.103**	**0.107**	**0.103**	Cooper Pedy

We found no overall relationship between pairwise comparisons of epigenetic and genetic data (Mantels test r = 0.007, n = 15, *P* = 0.434, Figure 4). The overall pattern of epigenetic differentiation was also not related to geographic distance between sites (Mantels test r = -0.081, n = 15, P = 0.737).

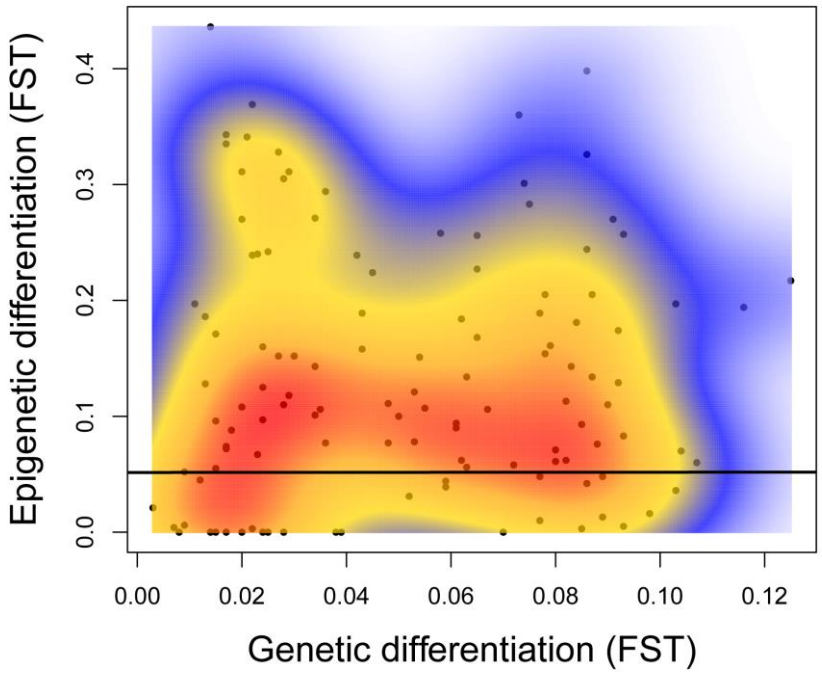


Figure 4. Mantel test to compare genetic and epigenetic pairwise estimates of $\Phi_{ST,}$ across the different populations; there is no relationship (R^2 = 0.007, n = 15, *P* = 0.434).

Relationship between Epigenetic and Genetic diversity

Among the 15 sites surveyed, average epi-h measures for each site ranged from 0.17 to 0.54 and average %Poly for each site ranged from 46.34 to 97.56 (Table 3 in discussion). The two measures of epigenetic diversity (%Poly and epi-h) were strongly related to one another (estimate = 0.004, t_{14} = 5.76, *P* < 0.001, R^2 = 0.70). Measures of genetic diversity (He and Ar) were also strongly related to each other (estimate = 30.93, t_{14} = 4.47, *P* < 0.001, R^2 = 0.59).

Table 2. Introduction clusters and sites where house sparrows were collected and screened for variation in DNA methylation. The number of individuals screened for variation in DNA methylation / genetic variation is represented by N_{epi}/N_G. Epigenetic diversity is shown as haplotype diversity (epi-h) and percentage of polymorphic loci (%Poly), and genetic diversity is shown as expected Introduction cluster & sites heterozygosity (He), the mean number of alleles detected in an introduction cluster (Na)-indicated by a *, and allelic richness within a site (Ar).

INTRODUCTION CLUSTER & SITES	N_{epi} /N_G	epi-h	% Poly	He	Ar/ Na *
QUEENSLAND CLUSTER	**29/103**	**0.36**	**82.11**	**0.814**	**15.00***
TOLGA	23/42	0.31	78.04	0.73	9.39
TOWNSVILLE	16/42	0.26	90.24	0.73	9.78
CHARLEVILLE	11/43	0.39	97.56	0.72	9.55
PITTSWORTH	11/42	0.30	90.24	0.77	10.83
NSW AND VIC CLUSTER	**90/307**	**0.29**	**69.51**	**0.834**	**19.91***
DUBBO	9/39	0.20	60.98	0.79	10.63
COBAR	9/38	0.27	73.17	0.82	12.11
WENTWORTH	10/39	0.20	53.66	0.83	13.62
BURRUMBUTTOCK	16/25	0.21	68.29	0.83	11.20
MELBOURNE	17/42	0.20	75.61	0.82	12.94
TORQUAY	12/40	0.16	46.34	0.85	14.27
BRIDPORT	9/43	0.32	80.49	0.82	11.04
MOUNT GAMBIER	8/41	0.44	97.56	0.79	11.49
SOUTH AUSTRALIAN CLUSTER	**61/169**	**0.37**	**89.02**	**0.750**	**14.46***
BROKEN HILL	9/41	0.22	70.73	0.81	11.84
ADELAIDE	8/42	0.39	90.24	0.81	10.97
COOBER PEDY	12/20	0.30	85.36	0.77	7.69

Although we detected a negative correlation between %Poly and He/Ar across all sites (n=15) (Supplementary Figure 1) is important to note that these 15 sites are not independent and the populations represent three distinct clusters. When we focused on the relationships between epigenetic and genetic diversity within the three introduction clusters,

no correlations were detected. Between sites in the NSW/VIC introduction (n=9), there was no significant correlation between epi-h and He or Ar (Spearman's correlation: r=-0.610, p=0.081, r=-0.475, p=0.197 respectively), or %Poly and He or Ar (r=-0.617, p=0.07, r=-0.317, p=0.406); between sites in the South Australian introduction (n=3), there was no significant correlation between epi h and He or Ar (r=-0.500, p=0.667, r=-0.500, p=0.677), or % Poly and He or Ar (r=-0.500, p=0.667, r=-0.500, p=0.677); and no significant correlation was detected between epi h and He or Ar in sites within the Brisbane introduction (n=4) (r=-0.400, p=0.60, r=-0.60 p=0.40), or % Poly and He and Ar (r=-0632, p=0.368, r=0.316, p=0.684, respectively).

The non-independence of the multiple populations sampled within the three introduction clusters suggests that the most appropriate way to analyse the results is to compare the level of genetic diversity and epigenetic diversity across the three clusters (i.e. reducing the overall sample size to just three clusters, but having each one based on a reasonable sample of individuals (between 29 and 90 individuals (Table 2, Figure 4). Between the three clusters there was a marginally significant negative correlation between Na and epi-h (R^2=0.995, $F_{1, 2}$=217.198, p=0.043), and a negative (but non-significant) trend between: He and epi-h, (R^2=0.586, $F_{1, 2}$=0.965, p=0.506), Na and %Poly (R^2=0.965, $F_{1, 2}$=13.573, p=0.169), and He and % Poly (R^2=0.803, $F_{1, 2}$=4.065, p=0.293), however none of these results were significant after Bonferroni correction for the multiple tests conducted (Figure 4).

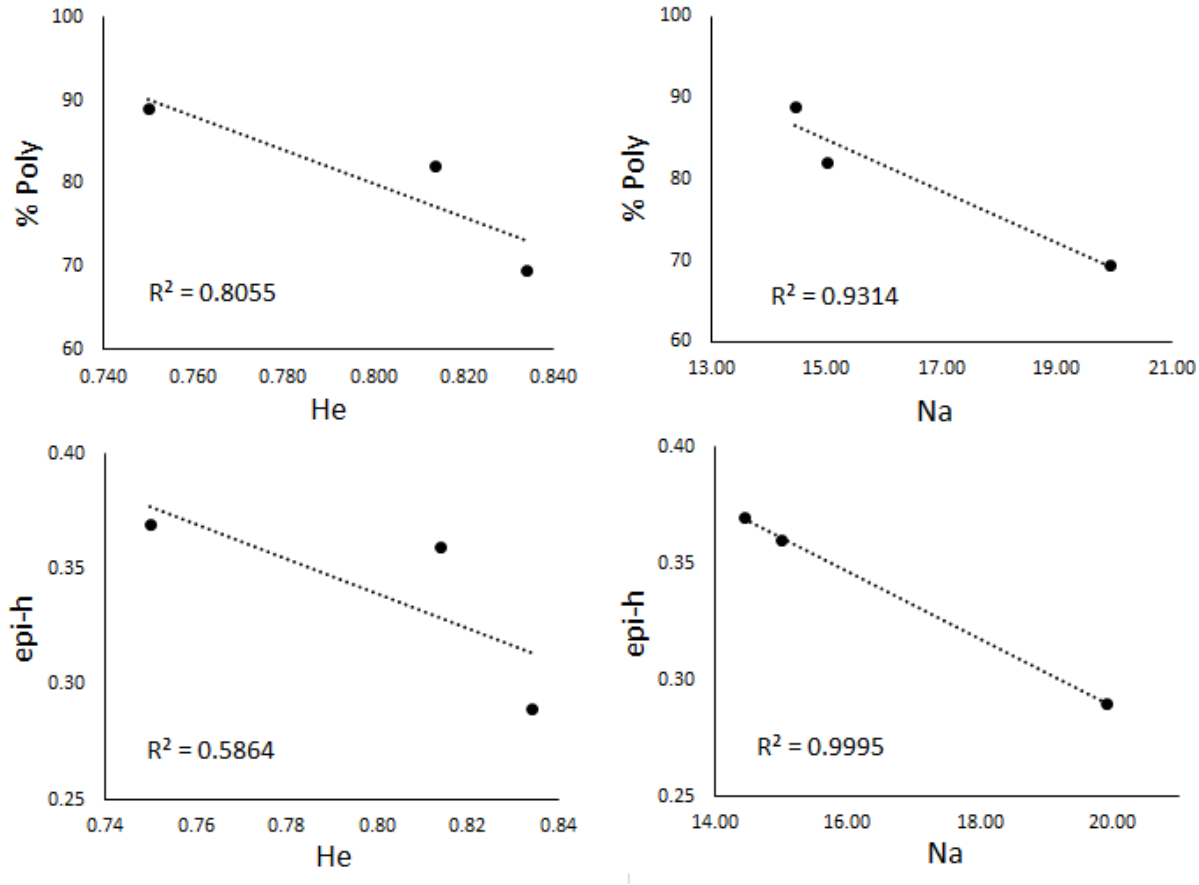


Figure 4. There is a negative trend between epigenetic and genetic measures of diversity between three distinct invasion clusters. However, these correlations were not significant after adjusting for multiple comparisons with a Bonferroni correction.

Discussion

Epigenetic mechanisms have been hypothesised to contribute to the diversity and evolutionary potential of natural populations, however these ideas have rarely been tested in wild populations. Epigenetic variation could be particularly important in introduced populations, which must adjust to novel habitats with relatively low levels of genetic variation (Liebl et al. 2013). To gain insight into how epigenetic mechanisms are associated with different introduction events, we characterised patterns of DNA methylation in multiple, highly successful, introductions of house sparrows into Australia ~160 years ago.

We find that a significant proportion of genetic differentiation is partitioned between three clusters within the invasive Australian house sparrow range. This pattern of genetic

differentiation is consistent with historical records that report that the Australian house sparrow population has derived from three separate introduction events, which can now be categorised into three genetically distinct 'invasion clusters' (Andrew et al, 2016). To a slightly lesser extent, patterns of genetic differentiation were also found to be partitioned between the fifteen different sites across Australia, and nearly every pairwise comparison between these different sites were significantly different. This suggests a reasonable level of isolation between these populations, which is consistent with the ecological barriers that exist between most of these populations. The towns and cities of Australia are largely separated by large patches of native habitat that is difficult for sparrows to disperse over (Andrew et al 2017).

We also found that a significant portion of epigenetic differentiation was partitioned between the three invasion clusters, as well as among specific sites throughout Australia. Finding significant patterns of epigenetic differentiation over such a broad geographic area, may indicate that stable transmission of epigenetic marks is possible, or that epigenetic marks respond reliably to consistently different environments or founding events. It would take a different experimental design to determine the extent of stable transmission of DNA methylation states in house sparrows (and thus the extent to which epigenetic mechanisms may contribute to evolution via natural selection), but stable transmission has been demonstrated in other taxa (i.e. Verhoeven et al 2010; Olsen et al 2012).

Contrary to the patterns of epigenetic differentiation within the Australian house sparrow population, no epigenetic differentiation was detected throughout the more recent introduction of house sparrows across Kenya (Liebl et al, 2013), however epigenetic differentiation has been detected among subspecies of the house sparrow in its native range (Riyahi et al, 2017). These results suggest that during the initial stages of invasion, rates of dispersal and environmental change may operate too rapidly for stable epigenetic marks to

differentiate to location, or selection may exist for the maintenance of labile epigenetic marks. There are two fundamentally different mechanisms by which epigenetic variation can be generated; under stable conditions, environmentally induced epigenetic variation is known to be associated with phenotypic plasticity, and in unpredictable environments stochastic epimutations have been shown to facilitate diversifying bet-hedging strategies (Leung et al, 2016). Further work is necessary to clarify the influence of population age on epigenetic differentiation, however the contrasting patterns of differentiation between the recent Kenyan introduction and more established Australian and native populations of house sparrows may suggest that population age and stability can affect the differentiation of epigenetic marks. Additional samples from these and other locations may identify more subtle patterns in epigenetic differentiation and diversity.

In our study, while overall patterns of epigenetic and genetic differentiation were similar, Mantel tests indicated that no correlation existed between pairwise site comparisons of epigenetic and genetic differentiation. The relationship between epigenetic and genetic differentiation is a key element in epigenetic research, as it determines the extent to which phenotypic variation can be explained by epigenetic effects independently of genetic effects (Richards, 2006, Liu et al, 2012). Significant correlations between epigenetic and genetic differentiation have been reported in a range of plants (Herrera and Bazaga, 2010), and animals (Liu et al, 2012; Bjornsson et al., 2008), however in line with our results, an increasing number of studies also find no correlations between epigenetic and genetic differentiation (Bossdorf and Zhang, 2011; Vaughn et al, 2007; Baldanzi et al, 2017; Richards et al, 2012). Our results suggest that at least some of the observed differentiation in DNA methylation may arise independently or at least partly independently from genetic control, as a potentially alternative source of variation (Richards et al, 2006).

We detected a great deal of epigenetic diversity at each sample site throughout Australia, and this is consistent with reports of high levels of epigenetic diversity across other invasive house sparrow populations (e.g. in Kenya (Liebl et al. 2013), and North America, (Schrey et al, 2012) (see Table 3).

Table 3. Range of epigenetic diversity (epi-h) in different house sparrow populations using MS-AFLP. Epigenetic diversity data was not originally reported in Schrey et al. (2012).

Study	**Location**	**epi-h**
Our study, 2016	Australia	0.17-0.54
Liebl et al. 2013	Kenya	0.28-0.44
Schrey et al. 2012	FL, USA; Kenya	0.35-0.36

While previous research has identified a negative relationship between epigenetic and genetic diversity in introduced house sparrows (Liebl et al, 2013), we failed to detect a relationship between epigenetic and genetic diversity among house sparrow sites within the three separate invasion clusters in Australia. We consequently find no support for the idea that high levels of epigenetic diversity can compensate for low genetic diversity in Australian house sparrows, at least in the contemporary population. Before partitioning the Australian house sparrow population into its three distinct 'invasion clusters', we wrongfully detected a negative correlation across all sites in the population, that possibly represented an artefact of differences between the three clusters, likely arising from founder effects. Our results thus emphasise the need to incorporate invasion history into population-wide analyses of epigenetic patterns, to avoid pseudo-replication in the sample of populations that have derived from multiple introduction events. Elevated epigenetic diversity is likely to only compensate for reduced levels of genetic diversity when genetic diversity is lowest immediately after a bottleneck, and invaders are facing the most novel environments (i.e. before they have established). Consequently, while our results do not indicate that a

relationship between epigenetic and genetic diversity currently exists in Australian house sparrows, such a relationship may have existed in earlier stages of the introduction, and is now obscured by recovered genetic diversity and generally more established population dynamics.

The potential for epigenetic patterns to facilitate invasive success requires a lot more inquiry, and our data highlight the importance of identifying epigenetic patterns in multiple and independent invasive expansions before they can be implicated in facilitating invasive expansion. We conclude that a negative, compensatory relationship between epigenetic and genetic diversity does not currently exist within the Australian house sparrow range. However, future work should assess the extent to which this relationship may exist in populations at more initial stages of an invasion- when genetic diversity is likely to be lowest, and the need to respond to novel environments is likely to be highest. Our detection of epigenetic differentiation among invasion clusters and different sites across Australia indicates that like genomic variation, epigenetic variation is also contingent on founding events, and may also respond to local environmental stimuli. These results provide a foundation for further work to examine the phenotypic and evolutionary relevance of this variation, potentially through combining next-generation sequencing techniques (that elucidate genome-wide patterns of DNA methylation) with RNAseq estimates of gene expression.

Acknowledgments

Our research was supported by Australian Research Council funding to S.C.G. (FT130101253) and a Macquarie University Research Excellence Scholarship (MQRES) to E.L.S. Neither of our funders had input into the content of the manuscript, nor required their approval of the manuscript before submission or publication

Ethics statement: All work was carried out in compliance with the Animal Research Authority (2015/017-4) issued by Macquarie University

Literature cited

Allendorf, F.W., Lundquist LL. 2003. Introduction: population biology, evolution, and control of invasive species. *Conservation Biology* 17:24–30.

Andrew, S.C., Griffith, S.C. 2016. Inaccuracies in the history of a well-known introduction: a case study of the Australian house sparrow (Passer domesticus*). Avian Research* 7 (9)

Andrew SC, Awasthy M, Bolton PE, Rollins LA, Nakagawa S, Griffith SC, in prep. The genetic structure of the introduced house sparrow populations in Australia and New Zealand is consistent with historical descriptions of multiple introductions to each country. Biological Invasions. Accepted

Angers, B., Castonguay, E., Massicotte, R. 2010. Environmentally induced phenotypes and DNA methylation: how to deal with unpredictable conditions until the next generation and after. *Molecular Ecology,* 19: 1283-95.

Arrigo, N., Holderegger, R., and N. Alvarez. 2012. Automated scoring of AFLPs using RawGeno v 2.0, a free R CRAN library. *Methods Mol. Biol*. 888:155-75

Baldanzi, S., Watson, R., McQuaid, C.D., Gouws, G., Porri, F. 2017. Epigenetic variation among natural populations of the South African sandhopper *Talorchestia capensis.* *Evolutionary Ecology*, 31 (1); 77-91

Bjornsson, H. T., Sigurdsson, M. I., Fallin, M. D., Irizarry, R. A., Aspelund, T., Cui, H., Yu, W., Rongione, M. A., Ekström, T. J. and Harris, T. B. (2008). Intraindividual change over time in DNA methylation with familial clustering. JAMA 299, 2877-2883.

Bosdorf o., Richards CL, Pigliocci M (2008). Epigenetics for ecologists. *Ecology Letters* 11; 106-115

Bosdorf, O., Arcuro D, Richards CL, Pigliucci M. 2010. Experimental alteration of DNA methylation affects the phenotypic plasticity of ecologically relevant traits in *Arabidopsis thaliana*. *Evolutionary Ecology,* 24: 541-53

Bossdorf, O. and Zhang, Y. 2011. A truly ecological epigenetics study. Mol. Ecol. 20, 1572-1574.

Dlugosch, K.M. and Parker, I.M. 2008a. Founding effects in species invasions: genetic variation, adaptive evolution and the role of multiple introductions. *Molecular Ecology*, 17; 432-449

Dlugosch, K.M. Parker, I.M., 2008b, Invading populations of an ornamental shrub show rapid life history evolution despite genetic bottlenecks. *Ecology Letters*, 11; 701-709

Dray, S., Dufour A.B. 2007. The ade4 package: implementing the duality diagram for ecologists. *Journal of Statistical Software*, 22; 1–20.

Foust, C.M., Preite, V., Schrey, A.W., Alvarez, M., Robertson, M.H., Verhoeven, K.J.F., Richards, C.L. 2016. Genetic and epigenetic differences associated with environmental gradients in replicate populations of two salt marsh perennials. Molecular Ecology, 25; 1638-165

Herrera, C.M., Pozo, M.I., Bazaga, P. 2012. Jack of all nectars, master of most: DNA methylation and the epigenetic basis of niche width in a flower living yeast. *Molecular Ecology,* 21; 2602-2616.

Fulnecek, J., Kovarik, A. 2014. How to interpret Methylation sensitive amplified polymorphism (MSAP) profiles? *BMC Genetics*, 15 (2)

Goudet, J. 1995. FSTAT (Version 1.2): A computer program to calculate F-statistics. *Journal of Heredity.* 86; 485–486.

Herrera CM, Bazaga P, 2013. Epigenetic correlates of plant phenotypic plasticity: DNA methylation differs between prickly and non-prickly leaves in heterophyllous Ilex aquiforlium (*Aquifoliaceae*) trees. *Botanical journal of the Linnean Society*, 171; 441-452

Jombart, T. 2008. Adegenet: A R package for the multivariate analysis of genetic markers. *Bioinformatics*, 24; 1403–1405.

Jombart T, Devillard S, Balloux F et al. 2010. Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC Genetics*, 11 (94)

Jombart T, Pontier D, Dufour A-B. 2009. Genetic markers in the playground of multivariate analysis. *Heredity*, 102; 330–41.

Klironomos, F. D., Berg, J., & Collins, S. 2013. How epigenetic mutations can affect genetic evolution: model and mechanism. *Bioessays*, *35*(6); 571-578.

Leung, C, Breton, S., Angers, B. 2016. Facing environmental predictability with different sources of epigenetic variation. *Ecology and Evolution*. DOI:10.1002/ece3.2283

Liebl, A.L., Schrey, A.W., Richards, C.L., Martin, L.B. 2013. Patterns of DNA Methylation throughout a Range Expansion of an Introduced Songbird. *Integrative and Comparative Biology*, 53 (2); 351–358

Liebl, AL., Schrey, A., Andrews, SC., Sheldon, E., and SC Griffith. 2015. Invasion genetics: lessons from a ubiquitous bird, the house sparrow (*Passer domesticus*). *Current Zoology.* 61(3): 465–476.

Liu, S., Sun, K., Jiang, T., Ho, J.P., Liu, B., Feng, J. 2012. Natural epignetic variation in the female great roundleaf bat (Hipposideros armiger) populations. Molecular Genetics, Genomics, 287 (8); 643-650

Massicotte, R. Whitelaw, E. Angers, B. 2011. DNA Methylation: A source of random variation in natural populations. *Epigenetics*, 6 (4); 421-427

Nicotra, A.B., Hoyle, G.L., Schrey, A.W., Verhoeven, K.J.F., Richards C.L. 2015. Adaptive plasticity in response to warming in an Australian alpine herb. *Ecology and Evolution*, 5; 634-647

Olsen, A.S., Sarras, M.P., Leontovich, A., Intine, R.V. 2012. Heritable transmission of diabetic metabolic memory in zebrafish correlates with DNA hypomethylation and aberrant gene expression. *Diabetes*, 61; 485-491.

Peakall, R. and Smouse, P.E. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics*. 28:2537-2539.

Platt A, Gugger O, Sork V. 2015. Genome wide signature of local adaptation linked to variable CpG methylation in oak populations. *Molecular Ecology*, 24; 2823-3830.

R Core Team. 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Richards CL, Schrey AW, Pigliucci M. 2012. Invasion of diverse habitats by few Japanese knotweed genotypes is correlated with epigenetic differentiation. *Ecology Letters*. 15:1016–25.

Richards EJ. 2006. Inherited epigenetic variation—revisiting soft inheritance. *Nature Review Genetics* 7:395–401.

Riggs. A.D., Xiong, Z., Wang, L., LeBon. J.M. 1998. Methylation dynamics, epigenetic fidelity and X chromosome structure. J.W.Sons ed. Epigenetics. Novartis Foundation Symposium, Chistester, U.K.

Riyahi, S., Vilatersana, R., Schrey, A.W., Node, G.H., Aliabdian, M., Senar, J.C. 2017. Natural epigentic variation within and among six subspecies of the house sparrow, *Passer domesitcus*. Journal of experimental biology, 220; 4016-4023

Schrey A, Coon CAC, Grispo M, Awad M, Imboma T, Mccoy E, Mushinsky H, Richards CL, Martin LB. 2012. Epigenetic variation may compensate for decreased genetic variation with introductions: a case study using house sparrows (Passer domesticus) on two continents. *Genetic Research International*. DOI.10.1155/2012/979751

Schrey A, Grispo M, Awad M, Cook M, Mccoy E, Mushinsky H, Albayrak T, Bensch S, Burke T, Butler L. 2011. Broad-scale latitudinal patterns of genetic diversity among native European and introduced house sparrow (*Passer domesticus*) populations. *Molecular Ecology,* 20:1133–43.

Schrey, A.W., Alvarez, M., Foust, C.M., Kilvitis, H.J., Lee, J.D., Liebl, A.L., Martin, L.B., Richards, C.L., Robertson, M. 2013. Ecological Epigenetics: Beyond MS-AFLP. *Integrative and Comparative Biology,* DOI:10.1093/icb/ict012

Schrieber, K., Lachmuth, S. 2016. The genetic paradox of invasions revisited: the potential role of inbreeding x environment interactions in invasion success. *Biological Reviews of the Cambridge Philosophy Society.* DOI: 10.1111/brv.12263

Schulz B, Eckstein RL, Durka W. 2014. Epigenetic variation reflects dynamic habitat conditions in a rare floodplain herb. *Molecular Ecology* 23:3523–3537

Sol, D., Sayol, F., Ducatez, S., Lefebvre, L. 2016. The life-history basis of behavioural innovations. *Philosophical Transactions of the Royal Society B,* 371

Vaughn, M. W., Tanurdzić, M., Lippman, Z., Jiang, H., Carrasquillo, R., Rabinowicz, P. D., Dedhia, N., McCombie, W. R., Agier, N., Bulski, A. et al. 2007. Epigenetic natural variation in *Arabidopsis thaliana*. PLoS Biol. 5, e174.

Verhoeven, K. J. F., Jansen, J. J., Van Dijk, P. J., Biere, A. 2010. Stress-induced DNA methylation changes and their heritability in asexual dandelions. *New Phytologist*, *185*(4); 1108-1118

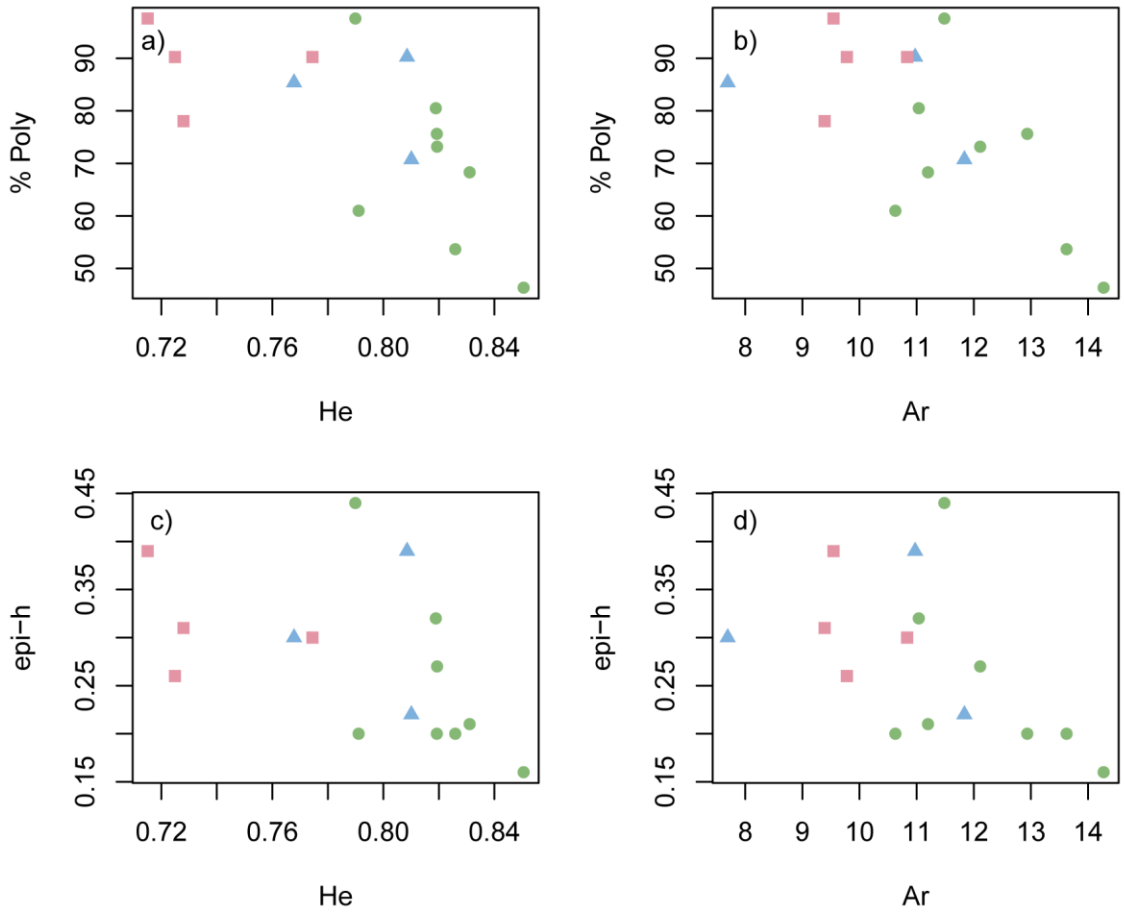
Weaver, I.C.G, Cervoni, N., Champagne, F.A., D'Alessio, A.C., Sharma, S., Seckl, J.R.,

Dymov, S., Szyf, M., & Meaney, M. 2004. Epigenetic programming by maternal behaviour. *Nature Neuroscience*, 7; 847-854

Xie H.J, Li, A.H. Liu, A.D. et al. 2015. ICE1 demethylation drives the range expansion on a plant invader though cold tolerance divergence. *Molecular Ecology*. 24; 835-850.

Zhang Y-Y, Fischer M, Colot V, Bossforf, O. 2013. Epigenetic variation creates potential for evolution of plant phenotypic plasticity. *New Phytologist*, 197: 314-2

Supplementary information



Supplementary-Figure 1. Across all sites (n=15) we detected a significant negative relationship between %Poly and He /Ar. (%Poly and He = -241.77, t_{14} = -3.26, p = 0.006, R^2 = 0.43; %Poly and *for Ar = -5.89, t_{14} = -3.17, P = 0.007, R^2 = 0.42),* epi-h *also had a trend showing a negative relationship (that approached significance) with He and Ar (*epi-h and He = -0.91, t_{14} = -1.94, P = 0.073, R^2 = 0.21; epi-h and Ar = -0.02, t_{14} = -2.11, P = 0.053, R^2 = 0.24. Epigenetic diversity (% Poly =percentage of polymorphic MS-AFLP loci, epi-h=epi-haplotype diversity), genetic diversity (He= expected heterozygosity and Ar= allelic richness). Pink squares: Queensland introduction; Green circles: Melbourne introduction; Blue triangles: South Australian introduction

Supplementary Table 1. All primer and adapter sequences used for the MS-AFLP protocol.

Primer/Adapter Name	Sequence (5’ to 3’)
EcoRI Adapter Forward	CTCGTATACTGCGTACC
EcoRI Adapter Reverse	AATTGGTACGCAGTA
MspI/HpaII Adaptor Forward	GATCATGAGTCCTGCT
MspI/HpaII Adaptor Reverse	CGAGCAGGACTCATGA
EcoRI Pre-selective Primer	TACTGCGTACCAATTCA
MspI/HpaII Pre-selective Primer	ATCATGAGTCCTGCTCGG
EcoRI Selective Primer	6-FAM-TACTGCGTACCAATTCAGC
EcoRI Selective Primer	5HEX-TACTGCGTACCAATTCACG
MspI Selective Primer	ATCATGAGTCCTGCTCGGTCAT

Chapter 3

Field observations: Clutch/Brood size generally ranges from one to eight eggs/chicks. Offspring are cushioned with sheep wool, emu feathers, dried grass and daisies. Eggs are carefully arranged into neat circles that are rotated periodically to evenly distribute incubation warmth. Some nests are used by multiple females to ‘dump’ eggs that are often abandoned.

Brood size influences genome-wide DNA methylation levels in wild zebra finches (*Taeniopygia guttata)*

E.L. Sheldon[1], A. Schrey[2], A.K. Ragsdake[2], S.C. Griffith[1]

1. Department of Biological Sciences, Macquarie University, Sydney, New South Wales, 2109, Australia
2. Department of Biology, Armstrong State University, Savannah, Georgia, USA

* Corresponding author: elizabeth-louise.sheldon@students.mq.edu.au

Abstract

Both natural and experimentally manipulated brood size can influence the competition dynamics amongst siblings in a nest and alter the environment during early development in birds. Brood size affects a variety of life-history and fitness-related traits, however relatively little is known about the mechanisms that might mediate these effects. There is accumulating evidence that early life environments can influence adult phenotypes though epigenetic mechanisms such as variation in DNA methylation. Here, we profile genome-wide DNA methylation patterns in zebra finch nestlings raised in naturally variable and experimentally manipulated brood sizes. We find that i) natal brood size is significantly, positively correlated with the percent of genome-wide DNA methylation, and ii) individuals in manipulated broods experience significantly more demethylation events across early development than individuals from broods that remain the same size. Any manipulation of brood size creates fluctuations in early developmental conditions, potentially explaining why the frequency of demethylation events in these treatments was higher than in offspring in control broods. We also found that the specific loci that lost and gained methylation across early development differed between individuals in enlarged and reduced broods, which may reflect the different developmental pressures imposed by the different manipulations. Although the phenotypic consequences of reduced levels of methylation are yet to be elucidated, our findings support the hypothesis that brood size is associated with the prevalence and pattern of genome-wide DNA methylation in wild birds.

Key words: Brood size; DNA methylation; Zebra Finch; Early development; Epigenetics; MS-AFLP

Introduction

Experiences during early development exert great influence on adult phenotypes in a range of taxa, including birds (Heijmans et al, 2009; Fresard et al. 2013). Brood size imposes long term effects on individual fitness, physiology and behaviour, due to the elevated costs associated with sibling competition (resulting in less food per nestling and more energy expended on begging) and nest crowding (resulting in elevating nest temperature and parasite load) (Wegman, et al. 2015; Naguib and Gil, 2005; Holveck and Verhulst, 2012). Large brood sizes have been linked with a variety of offspring phenotypes including decreased offspring weight (Nettle et al, 2013), reduced reproductive success in adults (Naguib et al, 2006; deKogel and Prijs, 1996), elevated metabolic rates (Verhulst et al, 2006), and shortened telomere lengths (associated with longevity and fitness) (Mizutani et al, 2016). While many phenotypes associated with augmented brood size are deleterious, others may represent adaptive responses (Zimmer et al, 2013; Champane and Meany, 2006). For example, great tits (*Parus major*) raised in large broods are known to be more exploratory and more aggressive than those raised in smaller broods, which could be adaptive in competitive conditions (Carere et al, 2005; Zimmer et al, 2013; Bloxham et al, 2014). Despite a wide range of studies documenting the relationship between brood size and phenotypic variation, we know little about the molecular mechanisms that potentially mediate these effects (Lea et al, 2016). However, insight into these mechanisms would contribute to our understanding of how such widespread and persistent phenotypic variation emerges in response to early developmental conditions.

There is accumulating evidence that early life conditions can influence adult fitness though epigenetic mechanisms, such as DNA methylation (McGhee and Bell, 2011). DNA methylation refers to the addition of a methyl group to a cytosine, which may alter the transcriptional state of the DNA and can significantly impact gene expression (Weber et al, 2007). Genome wide patterns of DNA methylation are established during embryogenesis however, these patterns can be modified by environmental influences at sensitive phases during early development (Feil and Fraga, 2012; Heijmans et al, 2009; Vickaryous and Whitelaw, 2005). Environmentally induced changes to DNA methylation patterns have consequently been proposed to provide an avenue through which early developmental conditions can modulate individual phenotypes (Roth et al, 2009; Meany and Szyf, 2005). A well-studied example involves variation in maternal grooming behaviour in rats (*Rattus norvegicus*), in which reduced grooming increases DNA methylation levels around offspring glucocorticoid receptors, resulting in decreased expression of this gene and elevated stress responses (Zhang et al, 2013).

Changes to DNA methylation patterns have been induced by a variety of developmental conditions including the nutritional environment (Snell-Rood et al, 2013; Lea et al, 2016; Waterland et al, 2010), the level of parental care experienced (McGhee et al 2014; Roth et al, 2014), breeding density (Bentz et al, 2016), thermal regime (Weyrich, et al, 2016; Renaudeau et al, 2011), environmental predictability (Leung et al, 2016), and climate conditions (Rubenstein et al, 2016; Dimond and Roberts, 2016). As brood size exerts such a significant influence on early development in birds, we propose that these effects could be mediated by modifications to genome-wide DNA

methylation patterns. We therefore seek to determine whether there is evidence that DNA methylation is sensitive to brood size.

We compare zebra finch (*Taeniopygia guttata*) nestlings raised in nests of naturally variable brood sizes to determine whether global and site-specific DNA methylation is influenced by natal brood size. We also experimentally manipulate brood size and use repeated blood samples taken across different age to examine whether DNA methylation patterns change with different brood size alterations over developmental time. Previous studies of this species have shown both wild and captive zebra finches to be sensitive to brood manipulations, which have resulted in phenotypic changes at the nestling stage that persist throughout life (Griffith and Buchanan, 2010; Tschirren et al, 2009; Mariette and Griffith, 2015).

Methods

Data collection

Blood was collected from 133 individuals from 48 different zebra finch families at Fowlers Gap Arid Research Station, NSW, Australia, in October 2016. All blood samples were taken from nestlings in nest boxes in their natural habitat (details regarding field site characteristics can be found at Griffith et al, 2008). Blood (<20µl) was sampled from the metatarsal vein of young (3 day old) nestlings, and the brachial vein of older nestlings (10-11days). Blood was preserved in 95% ethanol and stored at room temperature. A brood manipulation experiment was carried out by cross fostering nestlings. The cross-foster experiment involved the pairing of two nests with nestlings at the same developmental age (day 3 after hatching), and cross-fostering a subset of nestlings from

each nest in order to manipulate the brood size. In total, 41 nestlings developed in a brood that was experimentally reduced, 35 nestlings developed in a brood the same size as their natal brood, and 36 developed in a brood larger than their natal brood. Brood sizes naturally ranged from 2 to 8 nestlings, and manipulated broods changed by a minimum of 25%, (SEM: 0.246). We took two blood samples from each nestling during early development to enable a within-individual comparison of methylation states. The first blood sample was taken on day 3 of development, immediately before the brood manipulation, and the second sample was taken ~1 week after brood manipulation (day 10-11 of development). We were unable to take a later sample because the nestlings are prone to leave the nest prematurely if disturbed after ~12 days old.

We attempted to use MS-AFLP (methylation sensitive- amplification fragment length polymorphism), on 96 individuals from 37 families (i.e. 192 blood samples including repeats). We excluded individuals that only had one blood repeat (due to natural mortality), or yielded a blood sample that was too low to extract a sufficient quantity of DNA. The MS-AFLP protocol was only successful for 62 blood samples (32.3% of the total collected) from 43 individuals and 25 families. Logistic constraints (ELS conducted the molecular work whilst visiting the laboratory of AS, and the visit had a fixed duration that did not allow for repeated attempts at the protocol) meant that we were unable to re-run the failed samples; however, it is unlikely that any fundamental differences exist between samples that failed/succeeded, and successful samples were likely to be in the optimal DNA concentration range for the restriction digest step (see MS-AFLP protocol section). In total, we successfully scored methylation in 30 individuals from 18 families at the natal stage (3 days old), and 28 individuals from 13 families at the older time-point (10-11 days old). This allowed us

to compare DNA methylation before and after the brood manipulation for 20 individuals, from 15 different families. For the 20 individuals with successful repeated bleeds repeats, broods were manipulated by an average of 37.5% (SEM: 0.19). We defined three brood manipulation treatments outlined in Table 1. Given our small sample sizes, we took the average level of DNA methylation from the one family in each treatment for which we had data for two siblings.

Tables

Table 1. Brood manipulation treatments (Abbr.) and sample sizes of 20 nestlings from 15 nests, bled at two time points during post-natal development- before and after brood manipulation.

Brood manipulation treatment	**Abbr.**	**Nestlings** *n=*	**Nests** *n=*
Developing brood 1 or 2 individuals greater than natal brood	**+**	7	6
Developing brood 1 or 2 individuals smaller than natal brood	**-**	6	5
Developing brood equal to natal brood	**0**	7	6

MS-AFLP protocol

DNA was extracted using the Gentra Puregene tissue kit and stored in 30µl of DNA Hydration solution. We screened samples for variation in DNA methylation using MS-AFLP, which modifies the standard AFLP protocol by substituting the MseI enzyme with the methylation-sensitive isoschizomeric enzymes MspI and HpaII (New England Biolabs). Enzymes MspI and HpaII have different sensitivities to cytosine methylation of their shared recognition sequence (CCGG), and together result in 4 types of variation that can be scored to indicate methylation status at particular loci (Richards et al, 2012) (Table 2). Type 4 variation may represent epigenetic or

genetic variation (Table 2), however in some cases where individuals had repeated blood samples we were able distinguish between this variation; if one sample had a Type 4 site and the repeated sample did not, we were able to identify this as epigenetic variation. If both repeated samples had Type 4, or in the cases without paired samples, we conservatively treated Type 4 as not methylated. Recently there has been a suggestion that Type 2 and 3 should be analysed as separate states (Schulz et al. 2014); however, the actual source of these types of variation may be more complicated based on nested fragments (Fulnecek and Kovarik 2014). As such, we combined Type 2 and 3 into one methylated category, and treated all other states as not methylated. Throughout, we refer to a MS-AFLP locus to indicate a particular sized band resolved in the selective PCR.

Table 2. Epigenetic variation in DNA methylation scored using MS-AFLP. Y indicates the enzyme has cut at the restriction site, N indicates the enzyme has not cut at the restriction site.

Variation	MspI	HpaII	Methylation status of Restriction site	Methylation?
Type 1	Y	Y	No methylation	No
Type 2	Y	N	A methylated internal cytosine	Yes
Type 3	N	Y	A methylated outer cytosine	Yes
Type 4	N	N	Both cytosines methylated OR a genetic mutation	Dependent

We performed MS-AFLP following the protocol used by Richards et al. (2012), we digested approximately 250ng of genomic DNA at 37º C for 3 h in paired reactions; one with EcoRI and MspI, the other with EcoRI and HpaII. We immediately followed the restriction digest with adaptor ligation with EcoRI and MspI/HpaII adaptors at 16-20 h at 16º C (Supplemental Table 1-all primer and adapter sequences). After adaptor ligation, we conducted pre-selective PCR with EcoRI+1,

MspI/HpaII+0 pre-selective primers (Supplemental Table 1) at the following PCR conditions: 75º C for 2 min; 20 cycles of 94º C for 30 s, 56º C for 30s, 75º C for 2 min, final extension at 60º C for 30 min and 4º C hold. Following pre-selective PCR, we conducted selective PCR by multiplexing 6-FAM fluorescently labelled EcoRI+AGC primers and unlabelled primers HpaII/MspI+TCAT (Supplemental Table 1) at the following PCR conditions 94º C for 2 min, 8 cycles of 94º C 30 s, 65º C 30 s 72º C 2 min (dropping the annealing temperature 1º each cycle), 31 cycles of 94º C 30 s, 56º C 30 s 72º C 2 min, final extension of 60º C 5 min and a 4º C hold. We sent the selective PCR products to Georiga Genomics Facility (USA) for fragment analysis on an ABI 3130XL.

We used PEAKSCANNER v 1.0 (Applied Biosystems) to analyse resultant gel files and define fragment sizes and RAWGENO (Arrigo et al. 2012) to define bands. We eliminated bands that inconsistently amplified or occurred at highly variable intensities among individuals. We pooled data into two categories: methylated (Type II, Type III, and for paired samples appropriate Type IV cases) or not methylated (Type I and Type IV).

Data Analysis

We conducted all analyses using a binary haplotype-binding pattern (methylated 1, not methylated 0) for 107 verified consistent banding sites between 50 and 500 base pairs. Throughout, we used a sequential Bonferroni correction of α=0.05 for multiple tests. We calculated percent methylation as the proportion of the 107 loci that were methylated for each individual. Given our small sample sizes, we controlled for family effects by taking the average level of DNA methylation from the one family in each case that had data for two nestlings.

For the 30 individuals (from 18 families) that had at least the natal bleed, we compared family averages of DNA methylation (%) and natal brood size using a linear regression analysis.

For paired samples (20 individuals, 15 families), we compared the methylation state of the earlier sample to the later sample at the same 107 variable loci. Again, in cases where we had data for more than one chick per rearing family in each brood manipulation treatment, we took family averages to account for familial effects. We calculated the difference in the (%) of genome wide methylation before and after brood manipulation treatment in the three treatments. We used a one-way ANOVA and subsequent Tukey Post-hoc analyses to test whether the difference in the percentage of DNA methylation before and after brood manipulation was different in individuals that experienced a gain +, reduction -, or no change 0, in brood size.

We compared the percentage of individuals that lost, gained or did not change methylation state over development for each individual locus, between the three different brood manipulation treatments. We classified the change in methylation state for each locus in each individual by defining each locus to the three possible states of change; no change = 0; change from methylated to not methylated = -1, change from not methylated to methylated = 1. For each loci, we then summarised the percentage of individuals observed to gain, lose or not change methylation state at any given loci. We then summarised the percentage of individuals at each locus that were observed to gain, lose or not change methylation state across all individuals, and compared these values between the three brood manipulation treatments using a one-way ANOVA and subsequent Tukey Post-hoc analyses.

To highlight differences in loci-specific methylation changes between the + and – brood

manipulations, we selected the six loci that differed most in methylation state change between these treatments. We plotted the percent of individuals that gained or lost methylation at these six loci in a bar chart, and compared these percentages between + and – brood manipulation treatments. This allowed us to illustrate how the methylation state of specific loci respond differently to different (+ or -) brood manipulation treatments.

Results

Natal brood size is positively correlated with genome-wide DNA methylation (%)

We detected a significant, positive, linear regression between the percentage of methylated loci and natal brood size before brood manipulation, such that the larger the natal brood size, the more loci were methylated ($F_{1, 17}$= 10.559, p=0.005; R^2=0.398, family averages from n=30 nestlings, 18 families. (Figure 1)). Percentage of genome methylation differed by an average of 19.04% between natal broods with <four nestlings (average: 27.73%, 15.89-52.34, SEM: 0.19, n=6 families)) and broods with >six nestlings (average: 46.77%; 23.36%-61.37%, SEM: 0.25, n=8 families).

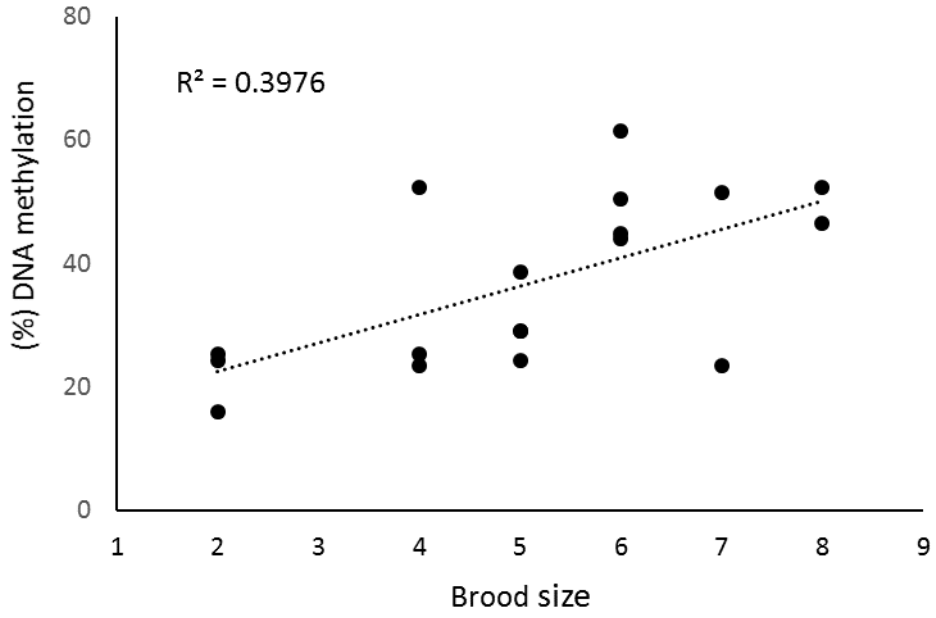


Figure 1. A significant, positive correlation between natal brood size, and family averages of (%) DNA methylation (among 107 loci) for 18 families and 30 nestling zebra finches sampled before cross fostering (y=4.61+13.17).

Brood size manipulations are associated with a loss of DNA methylation

For the 20 individuals with repeated bleeds before and after brood manipulation, the average change in genome-wide methylation was -16.67% for individuals that experienced a reduction in brood size (SEM: 0.22, family averages for n=7 chicks, 6 families); + 7.79% for individuals that experienced no brood size change (SEM: 0.16, family averages for n=6 chicks, 5 families), and -10.37 for individuals in an enlarged brood: + (SEM: 0.33, family averages for n=7 chicks, 6 families) (Figure 2).

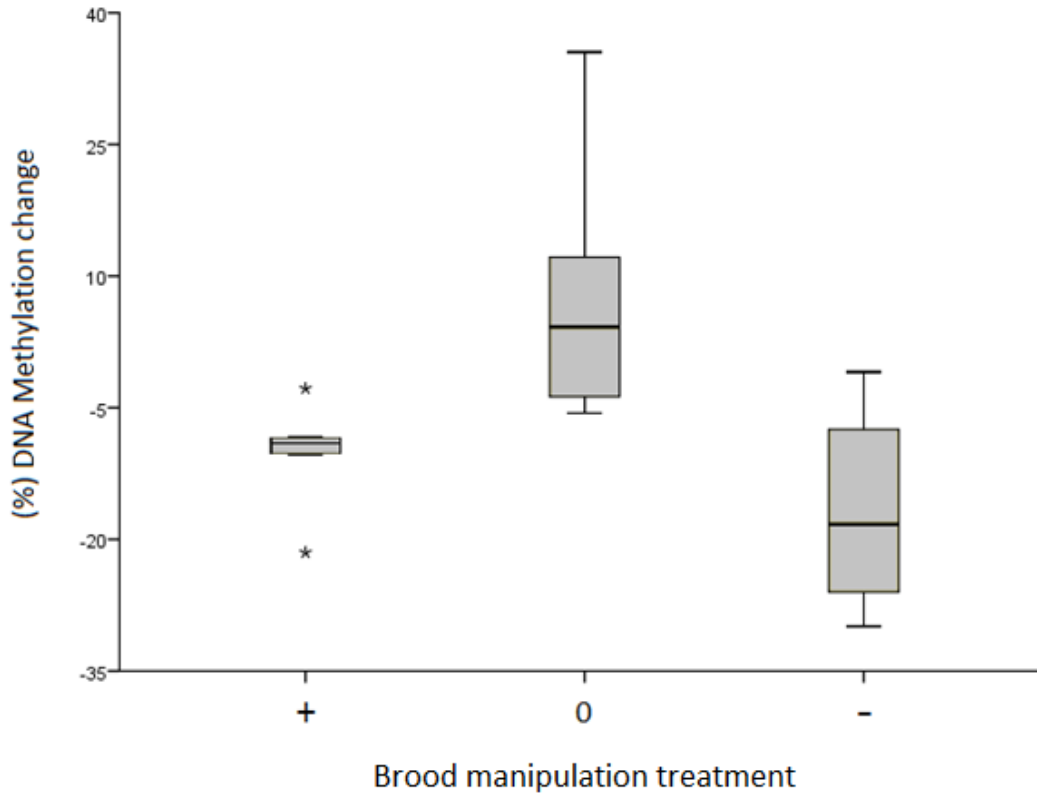


Figure 2. The percentage of genome wide- DNA methylation changed significantly in individuals before and after brood size manipulation based on treatment. Individuals lost more DNA methylation after a brood size reduction and enlargement, than individuals whose broods did not change size. Box plot shows average % DNA methylation change in nestlings that experienced a brood reduction (family averages of n=7 chicks, 6 families), no brood manipulation (family averages of n=6 nestlings, 5 families), and a brood enlargement (family averages of n=7 nestlings, 5 families). Asterisks indicate outliers.

We identified a significant difference in % methylation before and after brood manipulation using a one-way ANOVA among the three brood manipulation treatments: $F_{2, 16} = 6.771$, $p = 0.009$. A Tukey post hoc test showed that there was a significant difference in the % of DNA methylation

change between - and 0 (p=0.008), a marginally significant difference between + and 0 (p=0.060), and no significant difference between + and - (p=0.665) (Figure 2).

We found that 95.3% (102 of the 107) of loci had at least one instance of methylation state change over development. There were significant differences in the percentage of individuals that gained ($F_{2, 320}$=39.48, p<0.001), lost ($F_{2, 320}$=19.085, p<0.001) and did not change ($F_{2,320}$=9.854, p=0.001) methylation state between the three brood manipulation treatments (Figure 3).

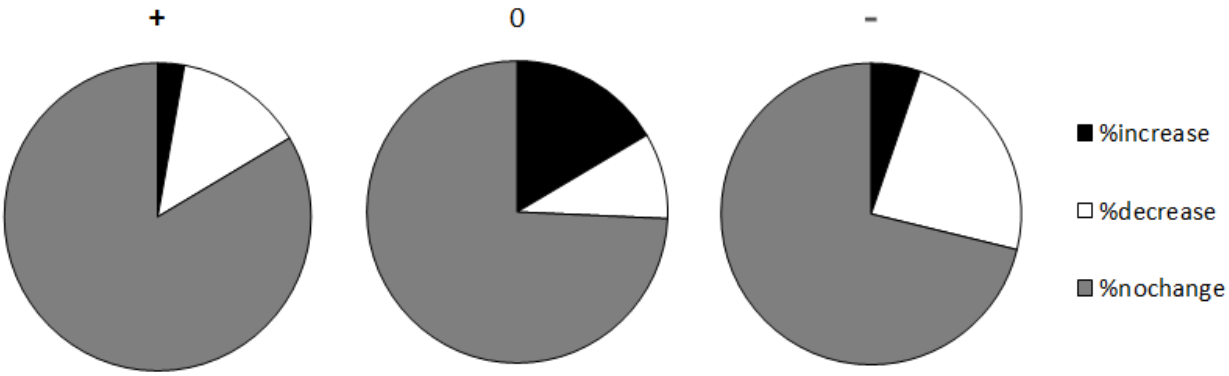


Figure 3. Family averages of (%) DNA methylation state changes among 107 loci for three brood manipulation treatments (see Figure 1). Black: the average percentage of loci that gained methylation, White=the average percentage of loci that lost methylation, and Grey = the average percentage of loci experiencing no overall change in methylation.

The percentage of individuals that changed from methylated to non-methylated (i.e. lost methylation) was significantly higher in the brood manipulation treatment – and + compared to 0 (Table 3). The percentage of loci that changed from a non-methylated to methylated state (i.e. gained methylation) was lower in + and -compared to 0. The percentage of loci that did not change methylation state was higher in + compared to 0 and - (Table 3, Figure 3).

Table 3. A comparison of the percentage of changes leading to a loss or gain of methylated loci between different brood-manipulation treatments. All P-values from the Tukey post-hoc comparisons are given after Bonferroni correction.

	Brood manipulation treatment					
	+		**–**		**0**	
State of change	Mean (%)	95%CI	Mean (%)	95%CI	Mean (%)	95%CI
Loss of Methylation	13.6	9.9-17.4	16.5	13.4-19.6	5.3	3.4-7.2
Gain of Methylation	2.8	1.2-4.4	9.19	6.9-11.4	15.1	19.7-27.3
No Change	83.6	79.3-87.8	74.3	70.5-78.1	71.2	67.0-75.3

Tukey P-values			
State of change	+ vs. 0	- vs. 0	+ vs. -
Loss of Methylation	0.139	<0.001	<0.001
Gain of Methylation	<0.001	<0.001	0.391
No Change	0.005	0.850	<0.001

While the overall pattern of change identified the most common outcome as loci losing methylation after the brood manipulation treatments; + and – compared to 0 (Figure 3), all loci did not respond in the same way in + compared to -. While some changing loci were consistent between

treatments, each brood manipulation treatment also had unique changing loci (e.g. a locus that lost methylation across all individuals from - may have remained unchanged in individuals from +, this is illustrated in Figure 4).

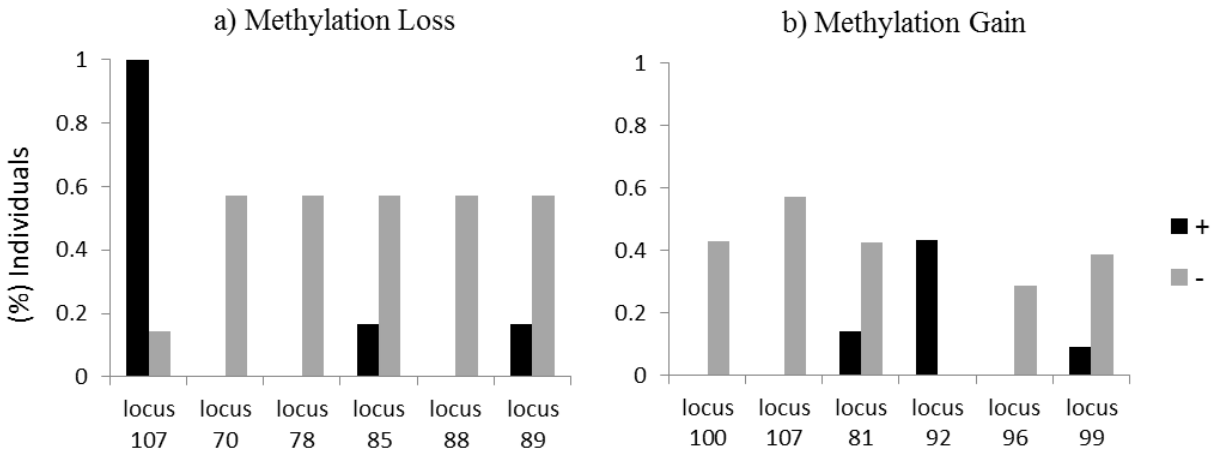


Figure 4. An illustrative column chart demonstrating the (%) individuals that a) lost and b) gained methylation in + and – brood manipulations at the 6 loci that differed most between manipulations.

Discussion

Like many avian species (Wegman et al, 2015, Hegner and Wingfield, 1987) zebra finches are extremely sensitive to brood size manipulations, which induce phenotypic changes that can persist throughout life (De Kogel, 1997; Griffith and Buchanan, 2010; Mariette and Griffith, 2015). Here, we show that i) natal brood size is correlated with the percentage of genome-wide DNA methylation in nestling zebra finches, and ii) individuals in manipulated broods experience significantly more demethylation events across early development than individuals from broods that remain the same size. We also note that the specific loci that lost and gained methylation across

early development tended to be different in each brood manipulation treatment. Our results support the hypothesis that early life conditions associated with brood size can influence the prevalence and pattern of genome-wide DNA methylation in wild zebra finches.

We detect a significant, positive correlation between percentage DNA methylation and natal brood size, such that large natal brood sizes were associated with higher levels of genome-wide DNA methylation. The relationship between percentage DNA methylation at hatching, and natal brood size could occur through pre-natal epigenetic programming, via maternal effects (Champagne et al. 2006; Murgatroyd et al, 2009). For example, females are known to allocate fewer resources to eggs when they produce larger clutches (Williams, 2001), and recently Bentz et al (2016) identified a positive correlation between maternal testosterone allocation to embryos and percentage DNA methylation in wild Eastern Bluebird (*Sialia sialis*) nestlings.

Although most epigenetic programming has been considered to occur prenatally (Vickaryous and Whitelaw, 2005), postnatal experiences have also been shown to influence DNA methylation patterns in a range of taxa (Weaver et al, 2004; Renaudeau et al, 2011). Here, we find a significant trend for individuals that experience a post-natal reduction or increase in brood size to show a greater loss of methylated loci than individuals from broods that remain the same size. Our result suggests that brood size manipulations *in general* induce more de-methylation events than those induced in non-manipulated cases. Leung et al. 2016, find that the rate of stochastic epimutations rises when fish (*Chrosomus.eos-neogaeus*) are exposed to stress imposed by fluctuating environments. Stress is known to induce stochastic variation in DNA methylation as it amplifies the error rate of methyltransferase in the establishment new methylation patterns during

DNA replication (Riggs et al. 1998). Broods that increase and decrease in size *both* create fluctuations in early developmental conditions (brood enlargements may increase competition for food, and brood size reductions may decrease competition for provisions or reduce provisioning if parents suspect reductions to be caused by predation (Martin et al, 2011)). Consequently, the influence of environmental fluctuations on the lability of DNA methylation may explain why methylation patterns were similar between the two experimental treatments in the present study, despite potentially imposing/alleviating different developmental pressures (Herman et al. 2014). Given that stress-induced DNA methylation changes can affect individual phenotypes (Cubas et al, 1999; Miura et al. 2009), further work should examine the potential for stochastically established DNA methylation patterns to facilitate an individual's survival in fluctuating environments via diversified bet-hedging strategies (Piggot, 2010; Herman et al, 2014).

In addition to stochastic epigenetic changes, several studies have shown that environmentally induced DNA methylation changes can be co-ordinated to translate environmental signals to directed phenotypic changes (Rubenstein et al, 2016; Weyrich et al, 2016). For example, baboons (*Papio cynocephalus)* exposed to unpredictable compared with stable food accessibility were found to have different patterns of DNA methylation in loci that differentially affect the expression of genes related to metabolism (Lea et al, 2016). Although we cannot interpret the significance of DNA methylation changes for perceptive gene regulation in the present study, we did identify that the overall direction of change in methylation state for a subset of loci was different in each treatment. This result indicates that DNA methylation changes could be locus specific and context dependent, and that such changes may be identified with larger sample sizes. If

so, this could reflect the different pressures imposed by brood enlargements and reductions, and perhaps indicate that loci specific DNA methylation changes are to some degree tailored to different brood manipulation experiences.

Our findings contribute to a growing body of research that is revealing a pivotal role for early developmental conditions in modulating epigenetic variation (Bentz et al, 2016; Rubenstein et al, 2016; Renaudeau et al, 2011). Other studies that have exploited sequence approach methods, such as bisulfite sequencing (Weyrich et al, 2016; Lea et al, 2016; Bentz et al, 2016), have demonstrated the significance of environmentally induced DNA methylation changes in shaping offspring phenotypes via effects on gene regulation (Szyf and Bick, 2013). The MS-AFLP technique used in our study is limited in its ability to provide information about the sequence and function of methylated loci, thus correlations between the methylation patterns observed in our study, and their phenotypic effects are yet to be elucidated (Shaham et al, 2016). However, the advantage of this MS-AFLP method, is that it can be applied across species as a universal method that will provide a preliminary examination of genome wide DNA methylation patterns in response to different environmental and biological parameters. In a very young field, this is useful to further identify the key parameters that affect epigenetic process at an early stage of development in birds.

Author contributions

ELS and SCG conceived the idea for the project, ELS collected the data in the field, AWS AKR and ELS carried out the MS-AFLP analyses, ELS and AWS analysed the data, ELS wrote the manuscript which was revised by AWS and SCG.

Acknowledgments

Our research was supported by Australian Research Council funding to S.C.G. (FT130101253) and a Macquarie University Research Excellence Scholarship (MQRES) to E.L.S. Neither of our funders had input into the content of the manuscript, nor required their approval of the manuscript before submission or publication. We thank Callum McDiarmid, Luke McCowan, Caterina Funghi and Hanja Brandl for their help monitoring zebra finch nests at Fowlers Gap Arid-zone research station.

Ethics statement: All work was carried out in compliance with the Animal Research Authority (2015/017-4) issued by Macquarie University.

Conflict of interest: No authors have a conflict of interest to declare

Data deposits: Our data will be deposited in the Dryad Digital Repository

Literature cited

Arrigo, N., Holderegger, R., and N. Alvarez. 2012. Automated scoring of AFLPs using RawGeno v 2.0, a free R CRAN library. *Methods in Molecular Biology*. 888:155-75

Bentz, A.B., SIrman, A.E., Wada, H., Navara, K.J., Hood, W.R. 2016. Relationship between maternal environment and DNA methylation patterns of estrogen receptor alpha in wild eastern bluebird (*Sialia sialis*) nestlings: a pilot study. *Ecology and Evolution*. Doi: 10.1002/ece3.2162

Bloxham, L., Bateson, M., Bedford, T., Brilot, B., Nettle, D. 2014. The memory of hunger: developmental plasticity of dietary selectivity in the European starling, *Sturnus vulgaris. Animal Behaviour*, 91: 33-40

Carere, C., Drent, P.J., Koolhaas, J.M., Groothuis, T.G.G. 2005. Epigenetic effects on personality

traits: early food provisioning and sibling competition. *Behaviour,* 142 (9): 1329-1355

Champagne, F.A., Meaney, M.J. 2006. Stress during gestation alters postpartum maternal care and the development of the offspring in a rodent model. *Biological Psychiatry*. 59:1227-35

Champagne, F.A., Weaver, I.C., Diorio, J., Dymov, S., Szyf, M., Meaney, M.J., 2006. Maternal care associated with methylation of the estrogen receptor-alpha1b promoter and estrogen receptor-alpha expression in the receptor-alpha1b promotor and estrogen receptor-alpha expression in the medial preoptic area of female offspring. *Endocrinology,* 147 (6): 2909-2915

De Kogel, C.H. 1997. Long term effects of brood size manipulation on morphological development and sex specific mortality of offspring. *Journal of Animal Ecology,* 66; 167-178

DeKogel, C.H., Prijs. H.J. 1996. Effects of brood size manipulations on sexual attractiveness of offspring in the zebra finch. *Animal Behaviour,* 51 (3); 699-708

Diamond, J.L., Roberts, S. 2016. Germline DNA methylation in reef corals: patterns and potential roles in response to environmental change. *Molecular ecology,* 25; 1895-1904

Feil, R., Fraga, M.E. 2012. Epigenetics and the environment: emerging patterns and implications. *Nature Reviews Genetics*, 13 (2); 97-109

Fresard, L., Morisson, M., Brun, J.m Collin, A., Pain, B., Minvielle, F., Pitel, F. 2013. Epigenetics and phenotypic variability: some interesting insights from birds. *Genetics Selection Evolution*, 45 (16)

Fulnecek, J., Kovarik, A. 2014. How to interpret Methylation sensitive amplified polymorphism (MSAP) profiles. *BMC Genetics* 15 (2)

Griffith, S.C., Buchanan K. 2010. Maternal effects in the zebra finch: a model mother reviewed. *Emu*, 110; 251-267

Hegner, R.E., Wingfield, J.C. 1987. Effects of Brood-size manipulations on parental investment, breeding success, and reproductive endocrinology of house sparrows. *The Auk*, 104 (3): 470-480

Heijmans, B.T., Tobi, E.W., Lumey, E.W., Slagboom. L.H. 2009. The epigenome, Archive of the prenatal environment. *Epigenetics* 4 (8); 526-531

Herman, J.J., Spencer, H.G., Sonohoe, K., Sultan. S.E. 2014. How stable 'should' epigenetic modifications be? Insights from adaptive plasticity and bet hedging. *Evolution*, 68; 632-643

Lea.A.J., Altmann, J., Alberts. S.C., Tung.J. 2016. Resource base influences genome-wide DNA methylation levels in wild baboons (*Papio cynocephalus*). *Molecular ecology*, 25; 1681-1696

Leung, C., Breton, S., Angers, B. 2016. Facing environmental predictability with different sources of epigenetic variation. *Ecology and Evolution*. Doi:10.1002/ece3.2283

Liebl, A.L., Schrey, A.W., Richards, C.L., Martin, L.B. 2013. Patterns of DNA methylation throughout a range expansion of an introduced songbird. *Integrative and Comparative Biology*, 53 (2); 351-358

Martin, T.E., Lloyd, P., Bosque, C., Barton, D.C., Biancucci, A.L., Cheng, Y-R., Ton, R. 2011 Growth rate variation among passerine species in tropical and temperate sites: an antagonistic interaction between parental food provisioning and nest predation risk. Evolution, 65 (6), 1607-1622

Mariette, M.M., Griffith. S.C. 2015. The adaptive significance of provisioning and foraging coordination between breeding partners. *The American Naturalist*, 185, 270-280.

McGhee K.E., Bell, A.M. 2014. Paternal care in fish: epigenetics and fitness enhancing effects on offspring anxiety. *Proceedings of the royal society B.* 281

Meany. M., Szyf. M. 2005. Environmental programming of stress responses through DNA methylation: life at the interface between a dynamic environment and a fixed genome. *Dialogues in clinical neuroscience*, 3; 103-123

Mizutani Y, Niizuma Y, Yoda K. 2016. How Do Growth and Sibling Competition Affect Telomere Dynamics in the First Month of Life of Long-Lived Seabird? *PLoS ONE* 11(11)

Murgatroyd, C., Patchev, A.V., Wu. Y., Micale, V., Bockmuhl, Y., Fischer, D. et al. 2009. Dynamic DNA methylation programs persistent adverse effects of early life-stress. *Nature Neuroscience,* 12; 1559-1566

Naguib, M., Gil, D. 2005. Transgenerational effects on body size caused by early developmental stress in zebra finches. *Biology Letters*, 1; 95-97

Naguib. M., Nemitz,A ., Gil, D. 2006. Maternal developmental stress reduced reproductive success of female offspring in zebra finches. *Proceedings of the Royal Society, Biological Sciences*, 273; 1901-1905

Nettle, D., Monaghan, P., Boner, W., Gillespie, R., Bateson, M. 2013. Bottom of the Heap: Having heavier competitors accelerates early-life telomere loss in the European starling, *Sturnus vulgaris*. PLoS ONE 8(12), e83617

Rapp, R.A., Wendel, J.F. 2005. Epigenetics and plant evolution. *New Phytology.* 168; 81-91

Renaudeau D., Collin, A., Yahav, S., De Basilo, V., Hourdine, J.L., Collier, R.J. 2011. Adaptation to hot climate and strategies to alleviate heat stress in livestock production. *Animal,* 6: 707-728.

Richards, C.L., Schrey, A.W., Pigliucci.M. 2012. Invasion of diverse habitats by few Japanese knotweed genotypes is correlated with epigenetic differentiation. *Ecology Letters*, 15; 1016-1025

Riggs. A.D., Xiong, Z., Wang, L., LeBon. J.M. 1998. Methylation dynamics, epigenetic fidelity and X chromosome structure. J.W.Sons ed. Epigenetics. Novartis Foundation Symposium, Chistester, U.K.

Roth. T.L., Matt, S, Chen, K., Blaze, J. 2014. BDFN DNA methylation modification in the hippocampus and amygdala of male and female rats exposed to different caregiving environments outside the home cage. Developmental psychobiology, 56; 1755-1763

Rubenstein, D.R., Skolnik, H., Alejandro, B., Champagne, F.A., Phelps, S., Solomon, J. 2016. Sex-specific fitness effects of unpredictable early life conditions are associated with DNA methylation in the avian glucocorticoid receptor. *Molecular Ecology*, 25; 1714-1728

Schulz B, Eckstein RL, Durka W. 2014. Epigenetic variation reflects dynamic habitat conditions in a rare floodplain herb. *Molecular Ecology* 23; 3523–3537

Shaham, R., BenShlomo, R., Motro, U., Keasar, T. 2016. Genome methylation patterns across castes and generations in a parasitoid wasp. *Ecology and Evolution*, 6 (22): 7943-7953

Snell-Rood, E.C., Troth, A., Moczek, A.P. 2013. DNA methylation as a mechanism of nutritional plasticity: Limited support from horned beetles. *Journal of Experimental Biology, Part B, Molecular and developmental evolution*. 320B; 22-34

Szyf. M., Bick. J. 2013. DNA methylation: A mechanism for embedding early life experiences in the genome. *Child development*. 84(1); 49-57

Tschirren B., Rutstein A. N., Postma E., Mariette M., Griffith S. C. 2009. Short- and long-term

consequences of early developmental conditions: a case study on wild and domesticated zebra finches. *Journal of Evolutionary Biology*. 22; 387–395

Verhulst, S., Holveck, M., Riebel, K. 2006. Long-term effects of manipulated natal brood size on metabolic rate in zebra finches. *Biology Letters,* 2 (3); 478–480

Vickaryous, N., Whitelaw, E. 2005. The role of the early embryonic environment on epigeotype and phenotype. *Reproductive and Fertility Development*, (17); 335-340

Waterland. R.A., Kellermayer, R., Laritsky, E., et al. 2010. Season of conception in rural Gambia effects DNA methylation at putative human metastable epialleles. *PLoS Genetics,* 6

Weaver, I.C., Cervoni, N., Champagne,F.A., DÁlessio, A.C., Sharma, S., Secki, J.R., Dymov, S., Szyf, M., Meany, M.J. 2004. Epigenetic programming by maternal behaviour. *Natural Neuroscience.* 7 (8); 847-854

Weber, M., Hellmann, I., Stadler, M., Ramos, L., Paabo. S., Rebhan. M., Schubeler, D. 2007. Distribution, silencing potential and evolutionary impact of promotor DNA methylation in the human genome. *Nature Genetics*. 39 (4); 457-466

Wegmann, M., Voegeli, B., Richner, H. 2015. Physiological responses to increased brood size and ectoparastite infestation: Adult great tits favour self-maintenance. *Physiology and Behaviour,* 141; 127-134

Weyrich, A., Lenz, D., Jeschek, M., Hung Chung, T.,Rubensam, K., Goritz, F., Jewgenow, K., Fickel, J. 2016. Paternal intergenerational epigenetic response to heat exposure in male Wild guinea pigs. *Molecular ecology,* 25; 1729-1740

Williams, T.D. 2001. Experimental manipulation of female reproduction reveals an intraspecific egg size clutch size trade-off. *Proceedings of the royal society B. Biological Sciences.* 268 (1465)

Zhang, Y-Y. Fischer, M., Colot. V., Bossdorf. O. 2013. Epigenetic variation creates potential for evolution of plant phenotypic plasticity. *New Phytology*, 197, 314-322

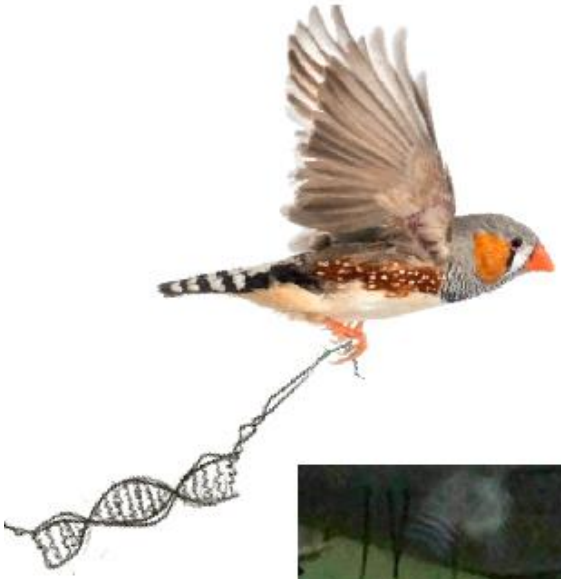
Zimmer C., Boogert N.J., Spencer K.A. 2013. Developmental programming: cumulative effects of increased pre-hatching corticosterone levels and post-hatching unpredictable food availability on physiology and behaviour in adulthood. *Hormone Behaviour.* 64; 494–500

Supplementary material

Table S1. All primer and adapter sequences used for the MS-AFLP protocol.

Primer/Adapter Name	Sequence (5' to 3')
EcoRI Adapter Forward	CTCGTATACTGCGTACC
EcoRI Adapter Reverse	AATTGGTACGCAGTA
MspI/HpaII Adaptor Forward	GATCATGAGTCCTGCT
MspI/HpaII Adaptor Reverse	CGAGCAGGACTCATGA
EcoRI Pre-selective Primer	TACTGCGTACCAATTCA
MspI/HpaII Pre-selective Primer	ATCATGAGTCCTGCTCGG
EcoRI Selective Primer	6-FAM-TACTGCGTACCAATTCAGC
EcoRI Selective Primer	5HEX-TACTGCGTACCAATTCACG
MspI Selective Primer	ATCATGAGTCCTGCTCGGTCAT

Chapter 4



Perched zebra finches at Macquarie University aviaries

The effects of developmental temperature on DNA methylation in captive zebra finches (*Taeniopygia guttata)*

Elizabeth Louise Sheldon[1*], Aaron. W. Schrey[2], Laura L. Hurley[1], Simon Charles Griffith[1]

1. Department of Biological Sciences, Macquarie University, Sydney, New South Wales, 2109, Australia
2. Department of Biology, Armstrong State University, Savannah, Georgia, USA

* Corresponding author: elizabeth-louise.sheldon@students.mq.edu.au

Abstract

DNA methylation is an epigenetic mark that provides an avenue through which early developmental conditions can modulate gene expression and phenotypic variation. Environmentally induced epigenetic variation has been proposed to facilitate acclimation to unpredictable environments, and is likely to be an important response mechanism given the rate of global climate change. Nevertheless, the effects of temperature on DNA methylation remain unclear, especially in vertebrate species. Here, we use methylation-sensitive AFLP to compare patterns of DNA methylation in captive zebra finches that develop under hot (30^{o}C) or cool (18^{o}C) temperature conditions. We find that DNA methylation levels differ among siblings raised at different thermal regimes; however, we find limited effects of temperature on average levels of DNA methylation among groups of unrelated individuals. We also find that temperature has a significant effect on the percentage of loci that change from a non-methylated to a methylated state across all individuals. Our results suggest that ambient temperature effects the ability of methylation states to be maintained over time, and in doing so may nuance baseline levels of genome-wide DNA methylation that have been initially set, or considerably influenced by familial effects. We discuss the potential for temperature to effect the maintenance of methylation marks via its effects on DNA methyltransferases.

Key words: DNA methylation, Temperature, Zebra finch, Ecological epigenetics, development

Introduction

The thermal environment is striking in its ability to influence developmental processes, and can induce phenotypic plasticity in a wide range of morphological, physiological and behavioural traits (Paredes et al, 2016; Moller et al, 2010; Du and Shine, 2015). In birds, temperature has been shown to affect offspring size and shape (Johnston and Selander 1964; Andrew et al. 2017), corticosterone-levels (Lopez-Jimenez et al, 2015), metabolic rate (White et al, 2007) and foraging behaviours (Chatelain et al, 2013) (among others). Wide ranging examples such as these have stimulated interest in the potential for temperature to affect epigenetic mechanisms- which are able to mediate environmental effects on the phenotype through the regulation of gene expression (Schrey et al, 2013). Such response mechanisms are likely to be important for species survival, especially given the increasing pace of global temperature change (Weyrich et al, 2016).

The most widely studied epigenetic mechanism is DNA methylation, which provides a basis for both the switching of gene expression and the maintenance of stable phenotypes (Holiday, 2005). Several studies have demonstrated the capacity for heat and cold stress to affect DNA methylation during early life, making temperature an ideal candidate for studying how developmental stress shapes epigenetic variation (Fresard et al, 2013; Varriale and Bernardi, 2006). For example, heat exposure during development is known to induce immediate modifications to DNA methylation in the promotors of genes related to thermo-tolerance in poultry (Kisliouk et al., 2009; Yossifoff et al., 2008; Tzschentke and Basta, 2002), and wild guinea pigs (Weyrich et al. 2015). Yet, the relationship between DNA methylation and temperature is inconsistent in recent research (genome-wide levels of DNA methylation have been reported to increase (Paredes et al. 2016) decrease

(Naydenov et al, 2015), and remain unchanged (Dorts et al, 2016) in response to heat stress) emphasising the need for further work in this field.

The mechanisms underlying the effects of temperature on DNA methylation are also unclear, however have been proposed to be associated with the temperature dependent expression of genes such as DNA methyl transferases (DNMTs)- which are involved in de novo methylation and its maintenance across cell division (Paredes et al, 2016). The error rate of DNMTs in replicating DNA methylation marks across cell division is known to be amplified in response to stress. Thus, the rate at which the methylation state of specific loci change over time is likely to be higher in individuals experiencing temperature stress, compared to those in optimal thermal conditions. Nevertheless, the consequences of temperature stress on the maintenance of consistent loci specific, and global levels of DNA methylation over time, have received little attention.

Here, we take a first step in exploring the potential for temperature to affect the maintenance of DNA methylation patterns over development in captive zebra finches (*Taeniopygia guttata*). We experimentally test (i) whether ambient temperature influences global DNA methylation levels, and (ii) whether temperature influences the maintenance of site-specific methylation states (i.e. whether a locus loses or gains a methyl mark, or whether its state is consistent) over time. Zebra finches are arid-adapted birds that frequently breed in temperatures that surpass the optimum for development in passerines (Griffith et al, 2016). Strong selection exists for an ability to maintain optimal developmental processes at high temperatures, and a range of adaptive strategies have been

observed in wild zebra finches in response to temperature (such as developmental timing (Griffith et al, 2016) and modulations of body size (Andrew et al, 2017; Mariette et al, 2016), growth and metabolic rates (Wada et al, 2015; Sheldon et al, 2017)). The potential for DNA methylation to mediate the effects of temperature on zebra finch development is thus of great interest, not only for the maintenance of individual-level plasticity, but also for population-level processes, such as climate change adaptation.

Methods

Data collection

Unrelated, captive zebra finches (n = 44 adults) were paired and left to breed in metal cages (~1.5m wide, and 2m high) in 'hot' or 'cool' temperature controlled rooms maintained at 30^{o}C or 18^{o}C, respectively (11 pairs in each temperature regime) . Zebra finches were provided with nest boxes and nesting material in the cages, and fed ad libitum. After the first breeding session, when the offspring were 32-35 days old, the offspring were moved to different cages in a room with no temperature control (at ambient temperature within a shaded shed).

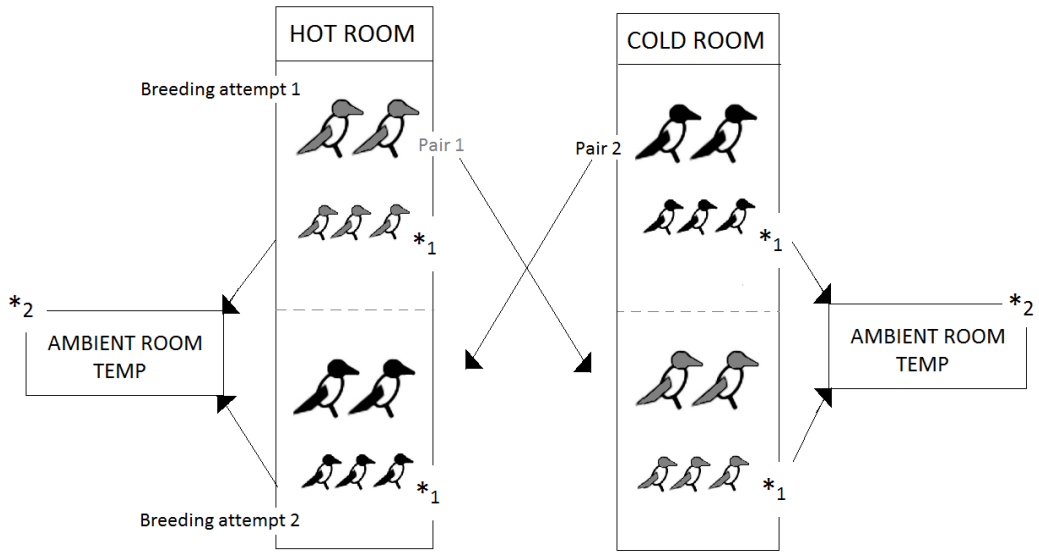


Figure 1. The experimental set up. *1= 1st Blood sample collected from juvenile offspring (5-10 days old). *2= 2nd Blood sample collected from juvenile offspring (50 days old). Different colour birds represent one family, note that each pair have the opportunity to raise offspring in a hot and cold room.

After the first breeding session parental pairs were relocated to the alternate temperature regime, and left to breed a second time, and again, when there second set of offspring reached 32-35 days old they were also relocated to a room with no temperature control (Figure 1). This experimental design means that there were some sets of full siblings that had been reared in the different experimental temperatures.

Each parental pair of zebra finches reared a brood of between 1 and 6 chicks (average 3.7 chicks; SEM 0.14). Across both broods, the 22 zebra finch pairs raised 110 chicks. We bled each of the offspring chicks twice, (i) as a juvenile (between days 5-10 of development) in the hot or cool

temperature regime, and (ii) as an adult (at day 50) in the room-temperature regime (Figure 1). Approximately 0.2ul of blood was extracted with a hypodermic needle and capillary tube. We attempted to use MS-AFLP to assay methylation for 48 individual offspring sampled at the two time points (total of 96 blood samples). 31% of these samples failed during the MS-AFLP procedure (see Table 1 for a break-down of sample sizes).

Table 1. The number of individuals screened for variation in DNA methylation. A total of 66 samples, from 43 individuals and 24 families were successfully screened for variation in DNA methylation using the MS-AFLP technique. The sample sizes highlighted in bold are the final sample sizes used to generate the data for this study.

	HOT		COOL	
Samples	**Chicks**	**Families**	**Chicks**	**Families**
Offspring for which we attempted MS-AFLP assay (juvenile)	22	11	26	13
Successful Juvenile MS-AFLP assays scored	**15**	**9**	**19**	**10**
Offspring for which we attempted MS-AFLP assay (adult)	22	11	26	13
Successful Adult MS-AFLP assays scored	**13**	**9**	**19**	**12**
Individuals assayed at both days	**10**	**8**	**13**	**8**

Due to financial/logistical restraints we were unable to re-run these failed samples, however it is unlikely that any fundamental biological differences exist between the samples that failed/succeeded, and successful samples were likely to have been at the optimal concentration range for the restriction digest step (see MS-AFLP protocol).

The body size (tarsus length; wing length; weight; head and bill length; bill length and bill width) of the offspring when they had matured and moulted into their adult plumage were measured

using digital callipers, a rule, and spring scales.

MS-AFLP protocol

DNA was extracted using the Gentra Puregene tissue kit and stored in 30µl of DNA Hydration solution. We screened samples for variation in DNA methylation using MS-AFLP (methylation sensitive- amplification fragment length polymorphism), which modifies the standard AFLP protocol by substituting the MseI enzyme with the methylation-sensitive isoschizomeric enzymes MspI and HpaII (New England Biolabs). Enzymes MspI and HpaII have different sensitivities to cytosine methylation of their shared recognition sequence (CCGG), and together result in 4 types of variation that can be scored to indicate methylation state at particular loci (Richards et al, 2012). Recently there has been a suggestion that Type 2 and 3 should be analysed as separate states (Schulz et al. 2014; Medrano et al. 2014); however, the actual source of these types of variation may be more complicated based on nested fragments (Fulnecek and Kovarik 2014). As such, we combined Type 2 and 3 into one methylated category, and treated all other states as not methylated. Throughout, we refer to a MS-AFLP locus to indicate a particular sized band resolved in the selective PCR.

We performed MS-AFLP following the protocol used by Richards et al. (2012), we digested approximately 250ng of genomic DNA at 37º C for 3 h in paired reactions; one with EcoRI and MspI, the other with EcoRI and HpaII. We immediately followed the restriction digest with adaptor ligation with EcoRI and MspI/HpaII adaptors at 16-20 h at 16º C (Supplemental Table 1-all primer and adapter sequences). After adaptor ligation, we conducted pre-selective PCR with EcoRI+1,

MspI/HpaII+0 pre-selective primers (Supplemental Table 1) at the following PCR conditions: 75º C for 2 min; 20 cycles of 94º C for 30 s, 56º C for 30s, 75º C for 2 min, final extension at 60º C for 30 min and 4º C hold. Following pre-selective PCR, we conducted selective PCR by multiplexing 6-FAM fluorescently labelled EcoRI+AGC primers and unlabelled primers HpaII/MspI+TCAT (Supplemental Table 1) at the following PCR conditions 94º C for 2 min, 8 cycles of 94º C 30 s, 65º C 30 s 72º C 2 min (dropping the annealing temperature 1º each cycle), 31 cycles of 94º C 30 s, 56º C 30 s 72º C 2 min, final extension of 60º C 5 min and a 4º C hold. We sent the selective PCR products to MACROGEN Genomics Facility (South Korea) for fragment analysis on an ABI 3130XL.

We used PEAKSCANNER v 1.0 (Applied Biosystems) to analyse resultant gel files and define fragment sizes and RAWGENO (Arrigo et al. 2012) to define bands. We eliminated bands that inconsistently amplified or occurred at highly variable intensities among individuals. We pooled data into two categories: methylated (Type II, Type III) or not methylated (Type I and Type IV).

Data Analysis

We conducted all analyses using a binary haplotype-binding pattern for 63 verified consistent banding sites between 50 and 450 base pairs. We calculated epi-haplotype diversity (*epi-h)* and the proportion of polymorphic loci (%P) to characterise epigenetic diversity using GENALEX6 (Peakall and Smouse 2012).

For the families that had >1 chick in the same temperature regime, we quantified within and between family variation in juvenile genome-wide DNA methylation levels (%) We assessed whether DNA methylation levels were repeatable within families using a two-way mixed interclass correlation coefficient (ICC) analysis. The ICC describes how strongly measures from the same family are correlated (a high ICC value indicates high repeatability within a family) (Nakagawa and Schielzeth (2013)). We then tested whether genome-wide DNA methylation levels (%) varied between families using a One-way ANOVA. In all analyses we then took family averages to account for any familial effects (as sample sizes were too low to include family ID as a random effect in these models (Table 1)). Statistics were run in R version 3.3.1 (Core Development Team, 2015), and SPSS Statistics.

Genome wide (%) Methylation state changes over time

We calculated percentage of DNA methylation for each individual as the proportion of the 63 loci that were methylated. To determine the effects of treatment on methylation changes throughout development (i.e. from juvenile to adulthood), we calculated the percentage of loci that gained and lost methylation in hot and cool treatments. We classified the change in methylation state by defining each locus to three possible states of change: no change=0, change from methylated to not-methylated = -1, change form not-methylated to methylated =1. We then summed the number of loci that were observed to gain loose, and not change methylation state for each individual. We carried out two, One-way ANOVAs to test the effects of temperature on the percentage of loci that gained and lost methylation for each individual across development.

Genome-wide (%) and loci specific DNA methylation patterns

To examine how perinatal temperature affects the methylation state of specific loci in juvenile zebra finches, we built a linear mixed model assessing the effects of temperature (fixed effect) on the methylation state of 63 specific loci at a single point in development (juvenile). We tested the 63 loci separately, and controlled for multiple testing via Bonferroni correction. The model was run in R using the package lmer in R (Bates et al. 2015) with the package lmerTest (Kuznetosova et al. 2016) to calculate degrees of freedom and *p*-values. We used a binary error distribution and a logit link, and calculated marginal R^2 and ICC values using the method described in Nakagawa & Schielzeth (2013). We also carried out two one-Way ANOVA to assess whether temperature affected % genome wide DNA methylation as an adult or as a juvenile.

Five sets of parents also reared chicks in both hot and cool rooms, allowing us to compare DNA methylation in five sets of adult siblings raised at different temperatures, but with the same genetic backgrounds. The % DNA methylation was averaged for all siblings ($n \leq 3$) from each parental pair in each temperature regime. A paired t-test was then carried out to test for significant differences between the %DNA methylation of siblings raised in hot and cool temperatures.

Effects of temperature on epigenetic diversity and body condition

We used a Pearson's correlation to test whether the percentage of adult DNA methylation was associated with adult body condition index (calculated as residuals from a least-squares linear regression analysis between adult body mass and adult tarsus length). We also conducted a two-way

MANOVA to assess whether there were differences in the body measurements (tarsus length; wing length; weight; head and bill length; bill length and bill width) of adult chicks based on the temperature condition they were reared, again we used family averages in cases where more than one chick was present per family.

Results

In total we used our MS-AFLP analyses to score variation in DNA methylation in 43 individuals in 24 families. Among all 43 individuals, we verified 63 consistent loci (banding sites), between 50 and 450 base pairs. For 10 families that had >1 chick (n=26 chicks), we detected a reasonable degree of within family repeatability in DNA methylation levels (%); the average ICC was 0.569, with a 95% confidence interval from -0.043-0.709, F_{25} =2.319, p<0.037. Between these 10 families, we also detected a significant difference in DNA methylation levels, One-way ANOVA, $F_{(9, 17)}$ =5.045, p=0.009. Consequently, to account for these familial effects, in cases where more than one chick belonged to one family, we used family averages.

Genome wide (%) Methylation state changes over time

We compared the state of methylation between adult and juvenile in 23 zebra finches from 16 families. An average of 37.2% (SEM: 0.014, range: 30.2-44.4%) loci changed methylation state across development (juvenile to adult). Temperature had a significant effect on the percentage of loci that gained methylation throughout development (family averages of 23 offspring from 16 families) ($F_{1, 15}$ =5.251, p=0.038). On average, 22.22% (SEM: 0.01, range: 14-25%) of loci

changed from non-methylated to methylated (i.e. gained methylation) in the hot treatment, compared to 17.9% (SEM: 0.02, range: 10.3-20.6%) of loci in the cool treatment (Figure 1).

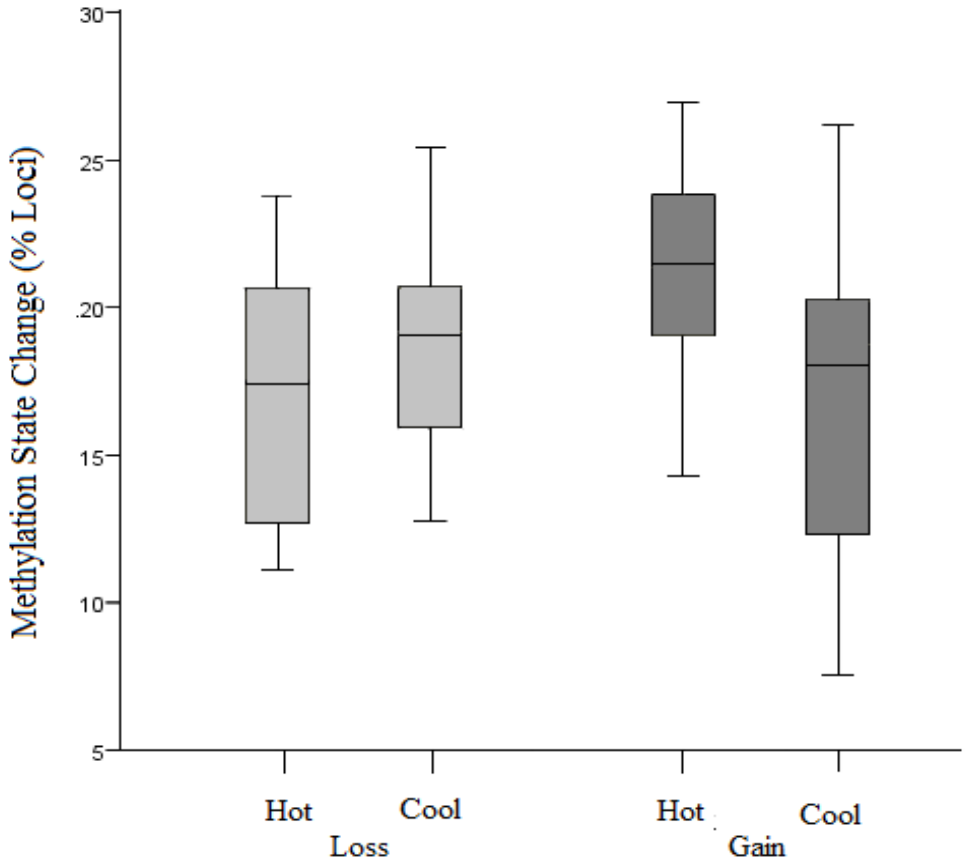


Figure 1. Significantly more loci changed from a non-methylated to methylated state over the development of chicks raised in hot rooms compared to chicks raised in cool rooms. Temperature had no effect on the % of loci that lost methylation over development.

Temperature had no effect on the percentage of loci that lost methylation, $F_{1, 15} = 0.408$, $p = 0.533$). On average, 16.9% (SEM: 0.01), 11-22%) of loci lost DNA methylation in the hot treatment, and 18.2% (SEM: 0.01, 14-25%) lost methylation in the cool treatment (Figure 1).

Genome-wide (%) and loci specific methylation changes at day 5 of development

We calculated genome-wide DNA methylation levels for 34 juvenile (from 19 families), and 32 adult (from 21 families) zebra finches from hot and cool temperature rooms. Temperature did not significantly affect genome-wide levels of DNA methylation in the pooled sample of unrelated juvenile zebra finches (family averages of 34 offspring from 19 families); One-way ANOVA: $F_{1, 18} = 0.716$, $p = 0.404$, or by the time they reached adulthood (family averages of 32 offspring from 21 families); One-way ANOVA, $F_{(1, 20)} = 0.035$, $p = 0.853$. When characterising loci-specific methylation changes, the linear mixed model found that the methylation state of 6.5% (4 out of 63) loci were significantly affected by temperature before Bonferroni correction, however no loci were significantly affected by temperature after Bonferroni correction (see S-Table 1). Temperature explained on average 7% of the variation in loci-specific methylation state between individuals (average marginal R^2: 0.07, SD: 0.14 (S-Table 1).

Sibling Comparisons

5 parental pairs raised chicks in both hot and cold temperature regimes. We compared the DNA methylation levels of these 5 sets of siblings (n=14 chicks (Figure2)). Zebra finches raised in hot temperature rooms had significantly higher levels of genome-wide DNA methylation as adults than their siblings raised at cool temperatures (family averages of 14 offspring from 5 families), Paired t-test: $t_5 = 3.113$, $p = 0.021$, (Figure 2). In this sibling analysis, the average genome-wide DNA methylation level of zebra finches raised in hot temperature room was 48.75% (SEM: 0.02),

compared to adult genome-side DNA methylation levels of their siblings raised in cool temperatures which was 40.14% (SEM: 0.02) (Figure 2).

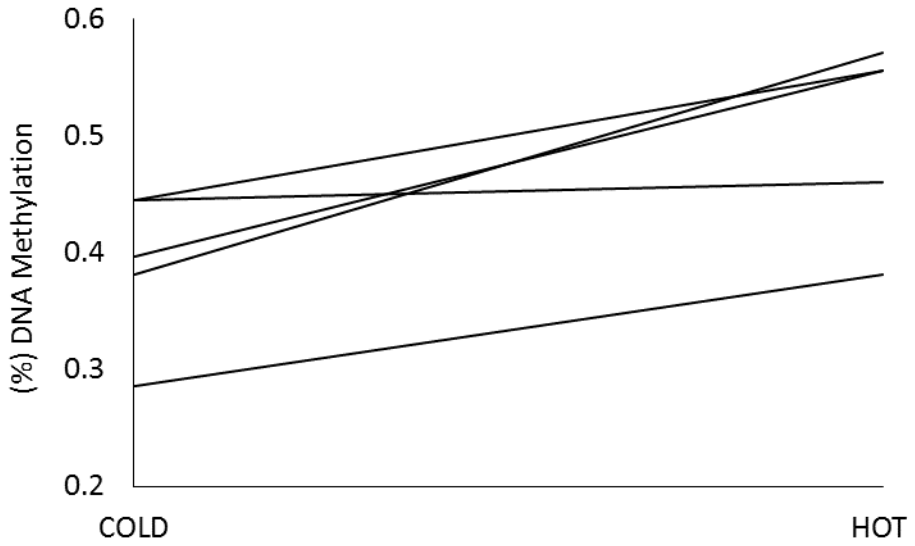


Figure 2. Chicks raised in hot (30ºC) temperatures had significantly higher levels of genome-wide DNA methylation than their siblings raised at cool (18ºC) temperatures (n=5 families, n=14 chicks, family averages used in three cases where one family had >1 chick).

Effects of temperature on epigenetic diversity and body condition

We identified a high level of variation in DNA methylation (epigenetic heterozygosity (epi-h) and %Polymorphism (%Poly)) in zebra finches exposed to both thermal treatments. Levels of DNA variation were consistent between treatments and developmental stage (Table 2). Among hot and cool chicks (at juvenile and adult stage) epi-h ranged from 0.323 to 0.337 and %P ranged from 85.4 to 87.59 (Table 2).

Table 2. Epigenetic diversity (as haplotype diversity (epi-h) and percentage of polymorphic loci (%P)) are shown for day 10 and day 50 chicks reared at different temperature regimes.

Age	Temperature	Uh (epi-h)	SEM	%P	N
Chick	Hot	**0.323**	0.016	**85.4**	16
	Cool	**0.337**	0.016	**85.4**	19
Adult	Hot	**0.345**	0.016	**87.59**	15
	Cool	**0.336**	0.016	**86.86**	18

Percentage of adult genome-wide methylated was not correlated with adult body condition (family averages of 32 offspring from 21 families) ($r=-0.337$, $p=0.136$). We found a trend for adult body size to be smaller in hot temperatures than in cool temperatures, however this trend was not significant (MANOVA: $F_{6, 26} = 2.125$, $p=0.062$, Wilks' $\Lambda = 0.838$).

Discussion

Evidence is accumulating that environmental challenges can affect the normal development of organisms, by altering DNA methylation. However, the effects of temperature on the maintenance of DNA methylation patterns over time are unclear. Here, we characterised DNA methylation patterns in captive zebra finches raised in hot and cool temperature conditions. We find non-significant effects of temperature on average levels of DNA methylation among the pooled sample of unrelated individuals, however DNA methylation levels did differ significantly when we use a conservative test to compare among sets of siblings produced at different thermal

regimes. Elevated temperatures were also found to have a significant effect on the percentage of loci that gained methylation from early development to adulthood. Overall, our results suggest that ambient temperature affects the ability of methylation states to be maintained over time, and in doing so fine-tunes baseline levels of genome-wide DNA methylation that have initially been set by familial effects.

We find that over development, more loci changed from a non-methylated to a methylated state (i.e. gained methylation) in heat stressed zebra finches than in zebra finches reared in cool temperatures- a result that is consistent with recent research on polychaetes (Marsh and Pasqualone, 2014). We also found that more loci changed from a methylated state to a non-methylated state (lost methylation) in zebra finches reared in cool temperatures however, this trend was not significant. These cases of methylation state change did not result in significant changes to genome wide levels of DNA methylation in non-related adult birds because the relative magnitude of methylation gains/losses across loci were inconsistent between individuals in each treatment. These results reflect the findings of Bouwmeester et al. (2016), who showed that the methylation state of specific loci are significantly different in zebra fish exposed to different levels of environmental contaminants, however these changes were not associated with changes in global DNA methylation levels.

Interestingly, our data did show that chicks raised in hot temperatures had significantly higher levels of genome-wide DNA methylation levels as adults than their siblings raised in cooler temperatures- suggesting that temperature may nuance baseline levels of DNA methylation that have been initially set, or considerably influenced by familial effects. Correlations between familial

effects (e.g. genetic makeup) and epigenetic variation are known to exist in several species (Kilionomos et al, 2012; Herrera and Bazaga, 2008, Liebl et al, 2013). Thus a future challenge in understanding the effects of temperature on epigenetic patterns, will be to tease apart the effects of genetic variation from temperature on methylation state change (Baldanzi, et al, 2017) (potentially through the study of individuals with limited genetic variation (Richards et al, 2012), or within family comparisons using a larger sample size than in our study).

Changes to the methylation state of specific loci over time can be caused by modifications to DNMTs. DNMTs maintain DNA methylation patterns across cell division, and their expression and functioning is known to be temperature sensitive (Fang et al. 2013; Naydenov et al, 2015). Cold stress, for example, is known to limit the expression of DNMTs in maize (*Zea mays*), and the downregulation of these genes has been shown to correlate with genome-wide demethylation in this species (Steward et al, 2000). The temperature dependent expression of DNMTs could consequently provide one explanation for the increased number of loci that gain methylation after heat stress in the present study. However, in our study, a large proportion (on average 37.7%) of loci changed methylation state (from methylated to non-methylated and vice versa) throughout development in both hot *and* cool conditions, indicating that the maintenance of labile methylation marks may be a general occurrence in our study sample. Consequently, the significance of heat stress for increasing the frequency of change in DNA methylation state across development requires further investigation (potentially through following the expression levels of DNMT genes, using sequence approach methods such as bisulfite sequencing (Weyrich et al, 2016)).

Recent research has found inconsistent relationships between temperature and DNA methylation, further emphasising the need for further work in this field. For example, cold-stress has been shown to elicit a global reduction in DNA methylation in lizards (Pardes et al.2016), an inverse relationship between global DNA methylation levels and temperature has been found in fish (Naydenov et al, 2015), and heat stress seems to hold no consequence for genome wide levels of DNA methylation in zebrafish (Dorts, et al. 2016; Aluru et al, 2015). In our study, we find that the ambient temperature experienced by perinatal zebra finches affects the state of methylation change over time, and affects within but not between family variations in genome-wide DNA methylation. The insignificant effect of temperature on DNA methylation in our between family analysis, is likely to be a power issue, given the low sample size and fact that there we found significant degree of variation across families that we cannot properly account for. This is further supported by our inability to detect a significant effect of temperature on body size, even though there was a strong trend for body sizes to be smaller in hot temperatures. Because temperature affected DNA methylation in our within family analyses (that remove the confounding variation between families), our results are likely indicative that temperature does indeed influence genome-wide levels of DNA methylation (as in Pardes et al. 2016).

Zebra finches in our study, were raised in fixed temperatures of 18^{o}C or 30^{o}C. In the zebra finches wild, native range, temperatures intermittently reach and surpass 40^{o}C (Griffith et al, 2016). Consequently, while the temperature conditions in our study are ecologically relevant, they likely impose chronic stress. In future, it would be interesting to investigate the effect of an acute heat stress (e.g. for a shorter period at a higher temperature), which could reflect more realistically, the

thermal regime of the zebra finches natural habitat. Additionally, given that the effects of temperature on the denaturation of proteins (such as DNMTs) are buffered by a suite of physiological strategies (such as the upregulation of heat shock proteins (Finger et al, 2017), it could be that the temperature regimes in our study were not stressful enough to elicit more general epigenetic responses. However, Wada et al, 2015, found that a mere 1^{o}C deviation from optimal incubation temperature affected metabolic rates and offspring survival in the zebra finch. Nevertheless, it would be useful for future studies to examine DNA methylation changes in response to more stressful temperature conditions, in species that are more temperature sensitive, and with larger sample sizes. It would also be interesting to examine whether ambient temperature in wild populations of zebra finches affects DNA methylation, which could provide a more realistic insight into the effects of temperature (and its associated effects; e.g. food abundance, energy budgets, parasite load) on DNA methylation.

Ethics statement: All work was carried out in compliance with the Animal Research Authority (2015/017-4) issued by Macquarie University

Acknowledgments

Our research was supported by Australian Research Council funding to S.C.G. (FT130101253) and a Macquarie University Research Excellence Scholarship (MQRES) to E.L.S. Neither of our funders had input into the content of the manuscript, nor required their approval of the manuscript before submission or publication

Literature cited

Aluru, N., Kuo, E., Helfrich, L.W., Karchner, S.I., Linney, E.A., Pais, J.E., Franks, D.G. 2015. Developmental exposure to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin alters DNA methyltransferase (dnmt) expression in zebrafish (*Danio rerio). Toxicology and Applied Pharmacology.* 284(2); 142-151

Andrew, S.C., Hurley, L.L., Mariette, M., Griffith, S.C. 2017. Higher temperatures during development reduce body size in the zebra finch in the laboratory and in the wild. *Journal Evolutionary Biology*; 4

Baldanzi, S., Watson, R., McQuaid, C.D., Gouws, G., Porri, F. 2016. Epigenetic variation among natural populations of the South African sandhopper *Talorchestia capensis. Evolutionary Ecology*, 31 (1), 77-91

Bates, D., Machler, M., Bolker, M., Walker, S.C. 2015. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, 67:1–48

Bouwmeester, M. C. *et al.* 2016. *Zebrafish embryos as a screen for DNA methylation modifications after compound exposure*. *Toxicology and Applied Pharmacology.* 291; 84–96

Dorts, J., Falisse, E., Schoofs, E., Flamion, E., Kestemont, P., Silvestre, F. 2016. DNA methyltransferases and stress-related genes expression in zebrafish larvae after exposure to heat and copper during reprogramming of DNA methylation. *Scientific reports*, 6; 34254

Du, W.G., Shine, R. 2015. The behavioural and physiological strategies of bird and reptile embryos in response to unpredictable variation in nest temperature. *Biological Reviews, Cambridge Philosophy Society.* 90 (1); 19-30

Fang, X., Corrales, J., Thornton, C., Scheffler, B. E., Willett, K. L. 2013. *Global and gene specific DNA methylation changes during zebrafish development*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol, 166;* 99–108

Finger, J.W., Hoffman, A.J., Wada, H. 2017. Temporal variation in constitutive and inducible heat shock proteins in the zebra finch *Taenopygia guttata*. Ibis, 12537

Fresard, L., Morisson, M., Brun, J.m Collin, A., Pain, B., Minvielle, F., Pitel, F. 2013. Epigenetics and phenotypic variability: some interesting insights from birds. *Genetics Selection Evolution,* 45 (16)

Fulnecek, J., Kovarik, A. 2014. How to interpret Methylation sensitive amplified polymorphism (MSAP) profiles. *BMC Genetics* 15 (2)

Griffith, S.C., Mainwaring, M.C., Sorato, E., Beckmann, C. 2016. High atmospheric temperatures and 'ambient incubation' drive embryonic development and lead to earlier hatching in a passerine bird. *Royal Society Open Science,* 3; 1-14

Herrera, C. M., Bazaga, P. 2008. Population-genomic approach reveals adaptive floral divergence in discrete populations of a hawk moth-pollinated violet. *Molecular Ecology* 17; 5378-5390

Holiday, 2005. DNE methylation and epigenotypes. *Biochemistry (Mosc*), 70 (5): 500-504

Johnston, R.F., Selander, R.K., 1964. house sparrows: rapid evolution of races in North America. *Science* 140: 548-550

Kisliouk, T., Ziz. M., Mein, N. 2010. Epigenetic control of translation regulation: alterations in histone H3 lysine 9 post-translation modifications are correlated with the expression of the translation initiation factor 2B (Eif2b5) during thermal control establishment. *Developmental Neurobiology*. 70 (2); 100-113

Kuznetsova, A., P. B. Brockhoff, and R. H. B. Christensen. 2016. lmerTest: Tests in Linear Mixed Effects Models. R package version 2.0-32. http://CRAN.R-project.org/package¼lmerTest

Liebl, A.L., Schrey, A.W., Richards, C.L., Martin, L.B. 2013. Patterns of DNA Methylation throughout a Range Expansion of an Introduced Songbird. *Integrative and Comparative Biology Integrative and Comparative Biology*, 53 (2); 351–358

Lopez-Jimenez, L., Blas, J., Tanferna, A., Cabezas, S., Marchant, T., Hiraldo, F., Sergio, F. 2015. Ambient temperature, body condition and sibling rivalry explain feather corticosterone levels in developing black kites. *Function ecology*, 30 (4); 605-613

Marsh, A.G., Pasqualone, A.A. 2014. DNA methylation and temperature stress in an Antartic polychaete, *Spiophanes tcherniai*. *Frontiers in Physiology*, 5: 173

Moller, A.P., Fiedler. W., Berthold, P. 2010. Effects of Climate change on birds. Oxford University press.

Nakagawa, S., Schielzeth, H. 2013. A general and simple method for obtaining R2 from generalized linear mixed effects models. *Methods in Ecology and Evolution* 4:133–142.

Naydenov, M., Baev. V., Apostolova, E., Gospodinova, N., Sablok, G., Gozmanova, M., Yahubyan, G. 2015. High temperature effects genes engaged in DNA methylation and affected by DNA methylation in Arabidopsis. *Plant Physiological Biochemistry*, 87; 102-108

Paredes, U., Radersma, R., Cannell, N., While, G.M., Uller, T. 2016. Low incubation temperature induces DNA hypomethylation in Lizard *Brains. Journal of Experimental Zoology*. 325A: 390-395

Peakall, R. and Smouse, P.E. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics*. 28:2537-2539.

Richards CL, Schrey AW, Pigliucci M. 2012. Invasion of diverse habitats by few Japanese knotweed genotypes is correlated with epigenetic differentiation. *Ecology Letters*, 15:1016–25.

Schrey, A.W., Alvarez, M., Foust, C.M., Kilvitis, H.J., Lee, J.D., Liebl, A.L., Martin, L.B., Richards, C.L., Robertson, M. 2013. Ecological Epigenetics: Beyond MS-AFLP. Integrative .*Comp.Biol*, doi:10.1093/icb/ict012

Steward, N., Jusano, T., Sano, H., 2000. Expression of ZmMET1, a gene encoding a DNA methyltransferase from maize, is associated not only with DNA replication in actively proliferating cells, but also with altered DNA methylation status in cold-stressed quiescent cells. *Nucleic Acids Research*. 28: 3250-3259.

Tzschentike, B., Basta, D. 2002. Early development of neuronal hypothalamic thermosensitivity in birds: influence of epigenetic temperature adaptation. Comp Biochem Physiol A Mol Integr Physiol 131(4); 825-832

Varriale, A., Bernardi, G. 2006. DNA methylation and body temperature in fishes. *Genetics*, 30 (385); 111-121

Wada, H., Kriengwatana, B., Allen, N., Schmid, K.L., Soma, K.K. MacDougall-Shackleton, S.A. 2015. Transient and permanent effects of suboptimal incubation temperatures on growth, metabolic

rate, immune function and adrenocortical responses in zebra finches. Journal of experimental biology, 218: 2847-2855

Weyrich, A., Lenz, D., Jeschek, M., Hung Chung, T.,Rubensam, K., Goritz, F., Jewgenow, K., Fickel, J. 2016. Paternal intergenerational epigenetic response to heat exposure in male Wild guinea pigs. *Molecular ecology*, 25; 1729-1740

White, C.R., Blackburn, T.M., Martin, G.R., Butler, P.J. 2007. Basal metabolic rate of birds is associated with habitat temperature and precipitation, not primary productivity. *Proceedings of the Royal Society B. Biological Sciences.* 274 (1607)

Yossifoff, M., Kisliouk, T., Meiri, N. 2008. Dynamic changes in DNA methylation during thermal control establishment affect CREB binding to the brain-derived neurotropic factor promoter. The European Journal of Neuroscience. 28 (11); 2267-2277

Supplementary Table 1.

Results of a linear mixed model assessing the effects of temperature (fixed effect) on the methylation state of 63 specific loci at a single point in development (juvenile). Family ID was included as a random effect. No loci were significantly affected by temperature after Bonferroni correction. The conditional (C) R^2 value denoted the amount of variation in DNA methylation explained by family ID and temperature, and the marginal (M) R^2 value is the amount of variation explained by temperature. The ICC value represents the variation in DNA methylation within families (a high ICC value indicates within family variation is low). The β-coefficient represents the effect size of temperature.

Loci No.	β-coefficient	t-value	p-value	MR^2	CR^2	ICC
1	-0.98083	-1.17582	0.239667	0.069142	0.069142	0
2	-0.62861	-0.80049	0.423428	0.029606	0.029606	0
3	0.318663	0.093426	0.925565	0.000214	0.875661	0.875447
4	-0.62861	-0.80049	0.423427	0.029606	0.029606	0
5	2.533695	2.237275	0.025268	0.331398	0.331398	0
6	1.233172	1.045929	0.295594	0.084339	0.281701	0.197362
7	-0.62415	-0.84222	0.399666	0.0292	0.0292	0
8	0.826144	0.760609	0.446891	0.039176	0.256588	0.217412
9	-0.60191	-0.67694	0.498446	0.022871	0.18237	0.159498
10	0.693147	0.9289	0.352941	0.035769	0.035769	0
11	-0.36772	-0.50927	0.61056	0.010333	0.010333	0
12	0.154151	0.212507	0.831711	0.001831	0.001831	0
13	0.916052	0.792747	0.427925	0.038826	0.400736	0.36191
14	0.693147	0.9289	0.352941	0.035769	0.035769	0
15	0.154151	0.212507	0.831711	0.001831	0.001831	0
16	1.280934	1.616679	0.105948	0.112441	0.112441	0
17	-2.36712	-2.83771	0.004544	0.301982	0.301982	0
18	0.90624	1.219736	0.222565	0.059629	0.059629	0
19	0.830307	0.75221	0.451925	0.032064	0.397625	0.365561
20	-0.63966	-0.89443	0.37109	0.030624	0.030624	2.88E-16
21	-0.63966	-0.89443	0.37109	0.030624	0.030624	0
22	0.374693	0.521662	0.601906	0.010724	0.010724	0
23	-0.90672	-1.26787	0.204846	0.059689	0.059689	0
24	-0.41795	-0.55345	0.579955	0.013151	0.02493	0.011779
25	-0.40547	-0.57686	0.564035	0.012534	0.012534	0

26	1.116961	1.503352	0.132748	0.087864	0.087864	0
27	0.553826	0.709185	0.478209	0.021487	0.092685	0.071198
28	0.300105	0.429208	0.667772	0.006906	0.006906	0
29	1.506417	0.989821	0.322262	0.070756	0.596173	0.525417
30	1.334869	1.106974	0.268305	0.081874	0.404879	0.323006
31	0.300105	0.429208	0.667772	0.006906	0.006906	3.75E-16
32	1.330055	1.782431	0.074679	0.120174	0.120174	0
33	-1.19097	-1.57379	0.115535	0.096805	0.116063	0.019258
34	0.510826	0.73058	0.465036	0.019749	0.019749	0
35	-4.00639	-1.01317	0.310978	0.27898	0.774891	0.495912
36	1.070258	1.331776	0.182934	0.076388	0.136294	0.059906
37	-1.00625	-1.191	0.233653	0.070078	0.103611	0.033533
38	-0.94446	-1.3304	0.183386	0.064434	0.064434	2.78E-17
39	-0.94446	-1.3304	0.183386	0.064434	0.064434	0
40	-0.00766	-0.01004	0.99199	4.23E-06	0.066935	0.066931
41	-1.25448	-1.19032	0.233919	0.098019	0.193299	0.095279
42	17.84634	2.947009	0.003209	0.135598	0.994486	0.858888
43	-0.02817	-0.0407	0.967531	6.13E-05	6.13E-05	5.81E-17
44	-0.02817	-0.0407	0.967531	6.13E-05	6.13E-05	0
45	-0.51083	-0.73058	0.465036	0.019749	0.019749	0
46	1.722767	2.279007	0.022667	0.186432	0.186432	0
47	1.178655	1.632357	0.102604	0.096872	0.096872	0
48	0.184922	0.265871	0.790338	0.002633	0.002633	4.34E-19
49	0.213064	0.278475	0.780648	0.003237	0.07659	0.073353
50	-0.9715	-1.05334	0.292187	0.05664	0.22274	0.1661
51	-0.80546	-0.78417	0.432943	0.041764	0.166235	0.124471
52	0.405465	0.576858	0.564035	0.012534	0.012534	0
53	0.405465	0.576858	0.564035	0.012534	0.012534	0
54	-0.90624	-1.21974	0.222565	0.059629	0.059629	0
55	-0.79787	-0.52555	0.599201	0.018877	0.615957	0.597081
56	-0.30443	-0.23567	0.813687	0.004408	0.384087	0.37968
57	-0.37469	-0.52166	0.601906	0.010724	0.010724	0
58	-0.37469	-0.52166	0.601906	0.010724	0.010724	0
59	1.298225	1.27778	0.201327	0.110455	0.151195	0.04074
60	0.961924	1.087135	0.276977	0.060037	0.159634	0.099597
61	0.336472	0.445111	0.65624	0.008665	0.008665	0
62	0.628609	0.800489	0.423427	0.029606	0.029606	0
63	33.88013	2.20E-06	0.999998	0.999841	0.999841	0

Chapter 5

Zebra Finch habitat at Fowlers Gap, and a newly hatched chick, day 0, with an egg tooth on its beak for breaking through its egg shell.

Digital heart rate monitor, eggs ready to go inside the egg monitor, and a regular heart rate on the monitors screen.

Pages 115-149 of this thesis have been removed as they contain published material under copyright. Removed contents published as:

Elizabeth L. Sheldon, Luke S. C. McCowan, Callum S. McDiarmid, and Simon C. Griffith (2017) Measuring the embryonic heart rate of wild birds: An opportunity to take the pulse on early development, *The Auk*, Vol. 135, no.1, pp. 71-82, (8 November 2017). https://doi.org/10.1642/AUK-17-111.1

Chapter 6

Zebra finches weren't the only ones to take respite or breed in a shady nest box. Mother spiders, bats, lizards and moths also frequented our nest checks. Top right- a handful of zebra finch nestlings

Zebra finch (*Taeniopygia guttata)* embryos respond to their parent's calls, but not to the calls of a heterospecific

Elizabeth.L.Sheldon[1.] Simon. C.Griffith[1.]

1. Department of Biological Sciences, Macquarie University, Sydney, New South Wales, 2109, Australia

* Corresponding author: elizabeth-louise.sheldon@students.mq.edu.au

Abstract

Birds are known to vocalise to their eggs during incubation in order to communicate with their offspring during embryonic development. It has recently been reported that zebra finch (*Taeniopygia guttata*) parents emit a specific call during incubation, apparently when ambient temperatures are relatively high. This 'heat call' has been proposed to communicate climatic conditions to embryos, and program the development of offspring for hot temperatures. However, the degree to which zebra finch embryos are acoustically sensitive is currently unclear. Here we explore the ability of wild zebra finch embryos to pay attention to different acoustic cues. We measure embryonic responses to different acoustic stimuli by monitoring embryonic heart rate- a physiological correlate of attention. Zebra finch embryos exhibited a cardiac response when exposed to two different conspecific vocalisations (the contact call and the newly described heat call), suggesting that they are capable of hearing and paying attention to these call types from at least day 8 of incubation. Zebra finch embryos did not however, respond to a heterospecific vocalisation. Given that zebra finch embryos paid attention to two types of calls, future research should assess their potential for in ovo sound discrimination which may help to clarify communicative functions of different conspecific calls.

Key words: Embryonic heart rate, incubation call, zebra finch, acoustic recognition, parent-embryo communication, climate change

Introduction

In several avian species, parents are known to vocalise to their eggs during the later stages of incubation (Dowling et al, 2016; Reed and Clark, 2011). In precocial, non-vocal learning species, these 'incubation calls' are known to mediate parent-embryo communication, and facilitate perceptual learning (Lickliter ad Hellewell, 2005), hatching synchrony (Wolf et al, 1976), and solicit parental incubation (Brua et al, 1996; Bugden and Evans, 1991; Abraham and Evans, 1999). Recently, parent-embryo communication has been discovered in vocal-learning songbirds which are able to learn acoustic elements from their parents in ovo, and imitate them during nestling begging (Colombelli-Negrel et al, 2012). The copy accuracy of these acoustic elements has been shown to predict the frequency of parental provisioning in the red-backed fairy wren (*Malurus melanocephalus*) (Colombelli-Negrel, et al.2016), thus parent-embryo communication has the potential to affect the development and fitness in vocal learning passerines.

A recent study has reported that zebra finch (*Taeniopygia guttata*) parents emit an 'incubation call' exclusively when ambient temperatures exceed 26ºC (Mariette and Buchanan 2016). It has been suggested that these 'heat calls' can influence offspring development and fitness, and program offspring for elevated ambient temperatures (Mariette and Buchanan 2016). However, the degree to which zebra finch embryos are acoustically sensitive is currently unclear. Thus one starting point in the further exploration of this phenomenon is to understand the cognitive capacity of embryonic zebra finches to perceive vocal cues. We propose that if the 'heat call' plays a pivotal role in providing embryos with relevant information about their environment, then we should find some evidence that embryos can hear and pay attention to this call type.

We use a sensory assay first used by Colombelli-Negrel, et al. (2014) to investigate the cognitive abilities of zebra finch embryos to acoustic stimuli – the use of an embryonic heart rate monitor. In many animals including humans and birds, a reduction in heart rate following exposure to a stimulus is considered to be a physiological mechanism for orientating and attention (Wetzel, et al. 2006; Hauber et al, 2002; Courage et al, 2006). If zebra finch embryos have the capacity to respond to auditory cues, we predict that their heart rates will decline in response to the 'heat call'. As a basis for future work to test the capacity for in ovo sound discrimination, we also test whether zebra finch embryos have the capacity to pay attention to a different conspecific call and an unfamiliar, heterospecific call.

Materials and Methods

Field Methods

Fieldwork was conducted on wild zebra finch embryos in nest boxes, in the Gap Hills area of Fowlers Gap Arid Research Station, New South Wales (31º05'S, 142º42'E) between October-December 2016 (further details of the study site are given in (Griffith et al 2008). We monitored nest boxes daily, and labelled each egg (with a fine, soft-tipped permanent pen) on the day that it was laid. In the wild, we have previously shown that parent zebra finches initiate incubation on the day that the last egg is laid (Gilby et al. 2013). Consequently, we considered this day to be the first day of embryonic development, which allowed us to determine the developmental age of each embryo throughout the study (although the earliest-laid eggs may start to develop before this, given the high ambient temperatures; Griffith et al. 2016).

We recorded and isolated three types of vocalisations in this study: i) contact calls of the house sparrow (*Passer domesticus*) (heterospecific calls) ii) zebra finch contact calls (conspecific contact calls), and iii) zebra finch heat call (heat calls) (see Figure 1 for spectrograms of the different calls). We used the house sparrow as a heterospecific stimulus because we already had an archive of sparrow calls available from previous work, and because this represents a species that the embryonic zebra finches would not have heard (given that house sparrows have never been observed in the study area in 14 years of intensive work at the site).

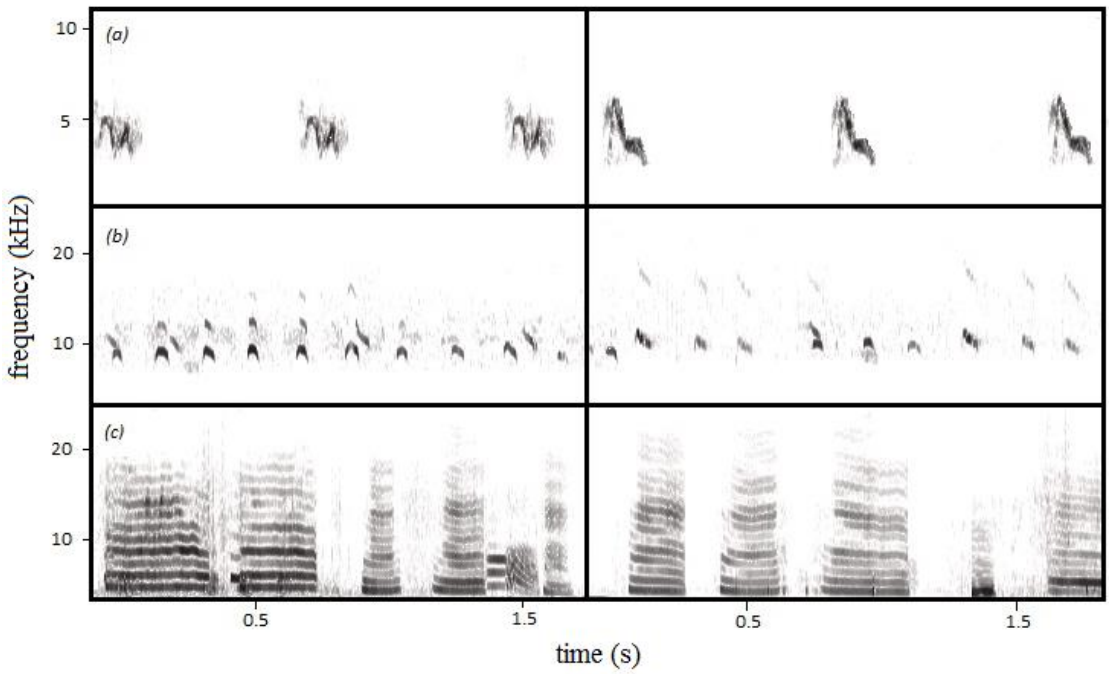


Figure 1. Spectrograms of the three call types used in the experiments. The Figure represents (a) two contact calls from two different house sparrow males, (b) two heat calls from two different zebra finches, and (c) two contact calls from two different zebra finches. (Note: the conspecific calls have a higher minimum frequency than the heterospecific call).

Zebra finch heat calls and conspecific contact calls were recorded from wild birds using time programmable Olympus SM650 and DM 670 recorders. To record the heat call, these recorders were placed inside a nest box during the later stages (>day 8) of egg incubation (as previous reports indicate that the heat call is only produced at these later stages of development (Mariette and Buchanan (2016)). To record the conspecific contact call, the recorders were placed within 1m of an active nest box (but not one of the experimental nests), generally attached to a nearby tree with a Velcro fastener. We recorded close to, and within, 55 nest boxes for 11.5 hours per day from 05:30-10:30am, then 11:30am -12pm then from 1pm-7pm during October-November 2017.

We searched through the sonograms of these recordings using Avisoft SAS Lab Pro v.4.40 until we identified the vocalisation of interest. We identified the 'heat call' using the spectral and temporal parameters described by Mariette and Buchanan (2016), and identified the conspecific (zebra finch) contact call using the spectral and temporal parameters described in Zann 1996, (the latter vocalisation is purported to be involved in communication between the parental pair (Elie et al, 2010)). We isolated 1-minute bouts of the 'heat call' and 'conspecific contact call' (with minimal background noise) from the recordings in Avisoft. We isolated five calls of each vocalisation type, which were recorded from five different nests. These calls were assumed to be from different birds, given the high fidelity of zebra finches to their nests. We refer to each of the five calls for each vocalisation type as having a unique 'recording ID'

For the heterospecific contact calls, we used five sets of contact calls from five different wild house sparrow males at Plasuchaf Farm, Carmarthen, Wales, UK. These birds were identified

with unique colour band combinations. House sparrows were recorded manually within 5m of a calling male, using a Marantz PMD670 digital recorder with a Sennheiser ME67 unidirectional microphone during March 2013.

We generated a separate playback for each recording ID, such that 15 different playbacks were made from 15 different birds, (5x 'heat call', 5x'conspecific contact call' and 5x 'heterospecific call'). Each playback consisted of one continuous minute of pre-playback silence (pre-trial), one continuous minute of playback (trial), and one continuous minute of post playback silence (post-trial); thus were three minutes long in total.

Playback experiment

We targeted 54 nests with embryos that were between 8-12 days through their development for the playback experiments (this age range is consistent with the report by Mariette & Buchanan (2016) that heat calls are only made to embryos in the later stages of incubation, and zebra finch eggs typically hatch after 12 days of incubation). All playback trials were conducted between 13/11/2016 and 9/12/2016 and were conducted between 06:00 and 10:00h local time (as heart rate has been shown to vary with the time of day (Sheldon et al 2017)).

From each nest box, we targeted three eggs at random, and exposed each egg to one of the three different vocalisation types. For the first seven trials, only one egg was targeted from each nest, which were all exposed to the heat call- our main target of inquiry (this did result in a slight bias in the sample sizes across the three treatments, but this did not affect our conclusions). We targeted a total of 148 eggs from 54 nests. Throughout our study, each egg was only presented with

one recording, and different eggs from the same nest would never be exposed to the same vocalisation type. Order of vocalisation type was randomised (i.e. the first egg out of the nest was exposed randomly to either the incubation, contact or heterospecific call). This was carried out to avoid any effects of nest disruption on subsequent heart rate changes. The recording ID (i.e. which of the 5 different birds used to record each for each vocalisation type) was used sequentially, such that they were all used approximately equally.

We broadcast the playbacks as uncompressed files from a laptop, connected to a JBL Clip+ portable speaker placed approximately 5-10cm from the egg, while it was placed in the heart rate monitor. The amplitudes of all playbacks were normalised at -30dB at 1m and saved as uncompressed 16 bit 44.1 kHz wave files using Audacity (version 2.1.3). As described below, we measured the heart rate variation of each embryo in response to the playback to which they were subjected. All data gathering was conducted blind to playback type.

Heart rates were taken close (<10m) to the natal nest box, in a shaded area in the field using a digital egg monitor (Buddy, Vetronic Services; UK) (further details given in Sheldon et al (2017) (data sets from this previous study do not overlap with the present study)). This device generates heart rate data by tracking light absorption changes owing to embryonic blood flow, and is able to detect heart rate from the first week of the incubation period (Sheldon et al 2017). During an experiment, the egg was removed from the nest and placed into a cushioned, shaded box which was used to transport it into the egg monitor device. As temperature can affect embryonic heart rate (Sheldon et al 2017), we measured eggshell temperature immediately before the start of the pre-trial, trial, and post-trial treatments. We measured eggshell temperature at the tip of the egg whilst it

was in the heart rate monitor using a non-contact infrared thermometer (Dual-IR Non-Contact Thermometer). We attempted to keep the eggs at a consistent temperature by keeping them in the shade, conducting our trials in early-mid morning, and minimising handling.

We measured the embryos heart rate once every 10 seconds during the one-minute of pre-playback silence (pre-trial), once every 10 seconds during the one-minute of trial (trial), and once every 10 seconds for one-minute of post-playback silence (post-trial) (therefore a total of 18 heart rate measures were gathered over the 3 minutes, 6 for each phase of the trial).

Embryonic movement in the egg is known to effect (elevate) embryonic heart rate (Pollard et al, 2016), and also disrupts use of the Buddy egg monitor (Sheldon et al, 2017). Because of this, in some cases we were unable to acquire reliable, consecutive heart rate readings for each 10 second time interval during the one-minute playback test. We excluded 23 individuals where >4 readings were acquired during any phase (i.e. the pre-trial, trial or post-trial) of the playback experiment.

Statistical analyses

Mean heart rate readings for each trial 'phase' were calculated from 4-6 heart rate measures over the one-minute of pre-trial, trial and post-trial periods. We expressed each of the 4-6 heart rate values (used to generate the mean for each phase) as a 'percentage of the calculated mean', and excluded 30 eggs from the analysis where the SEM of these 4-6 percentages was greater than 0.03 (which we deemed acceptable variance). This allowed us to only include embryos with relatively steady heart rates, thus any detectable differences in heart rate means between the different trial

phases (e.g. between the pre-trial and trial phase) would be a consequence of consistent heart rate changes within the trial phase, rather than random, momentary drops or spikes in heart rate.

Differences in heart rate were analysed using two paired t-tests, the first t-test compared average heart rates between the 'pre-trial and trial period' and the second t-test compared average heart rates between the 'trial and post-trial period'. Each vocalization type (i.e. heat, conspecific or heterospecific call) was also analysed separately. (We tested for differences in heart rate between the 'trial and pre-trial periods' as previous research has suggested that a prolonged cardiac response after cessation of a stimulus may indicate a greater degree of attention (Colombelli-Negrel, et al. 2014)). All paired t-tests and other statistical test were carried out using SPSS 22 for WINDOWS.

To test if the three different vocalisation types had varied levels of effect on heart rate change (e.g. whether heart rates decline more in response to the heat call than the conspecific contact call), we expressed the average heart rate during the trial as a percentage of the average heart rate during the pre-trial, i.e. 'relative heart rate during the trial' = (trial/pre-trial) x100. This allowed us to calculate a relative change in heart rate after the playback commenced for each individual. We conducted an Analyses of Covariance using the relative heart rate values during the trial to test whether the three different vocalisation types induced any significant changes in heart rate. In this model we controlled for the effects of 'eggshell temperature' and 'embryonic age (days)' on heart rate change by including these variables as a fixed effect. We used a Tukey post-hoc test to determine what playback types induced significantly different heart rate responses.

Results

Overall, heart rate variation in response to playback was successfully quantified in 95 zebra finch embryos from 43 different clutches. Heart rate variation was measured successfully in response to the heterospecific call (n=23eggs, 23 families), the conspecific contact call (n=32 eggs, 32 families), and the heat call (n=40 eggs, 40 families).

Embryonic heart rates declined significantly between the pre-trial period and the trial period in response to conspecific contact calls (paired t-test: $t = 4.51$; d.f. = 31, $P < 0.0005$) and heat calls ($t = 2.93$; d.f. = 39, $P = 0.006$), but not in response to heterospecific calls ($t = 0.846$, d.f. = 22, $P = 0.407$, see Figure 2).

Heart rates during the post-trial period did not differ significantly from the trial period for any playback type (paired t-test; conspecific contact call $t = 0.166$, d.f. = 29, $P = 0.870$; heat call $t = 0.324$, d.f. = 39, $P = 0.747$; and heterospecific call $t = -0.568$, d.f. = 22, $P = 0.5760$) indicating that heart rates are unaffected by the cessation of the playback (Figure 2).

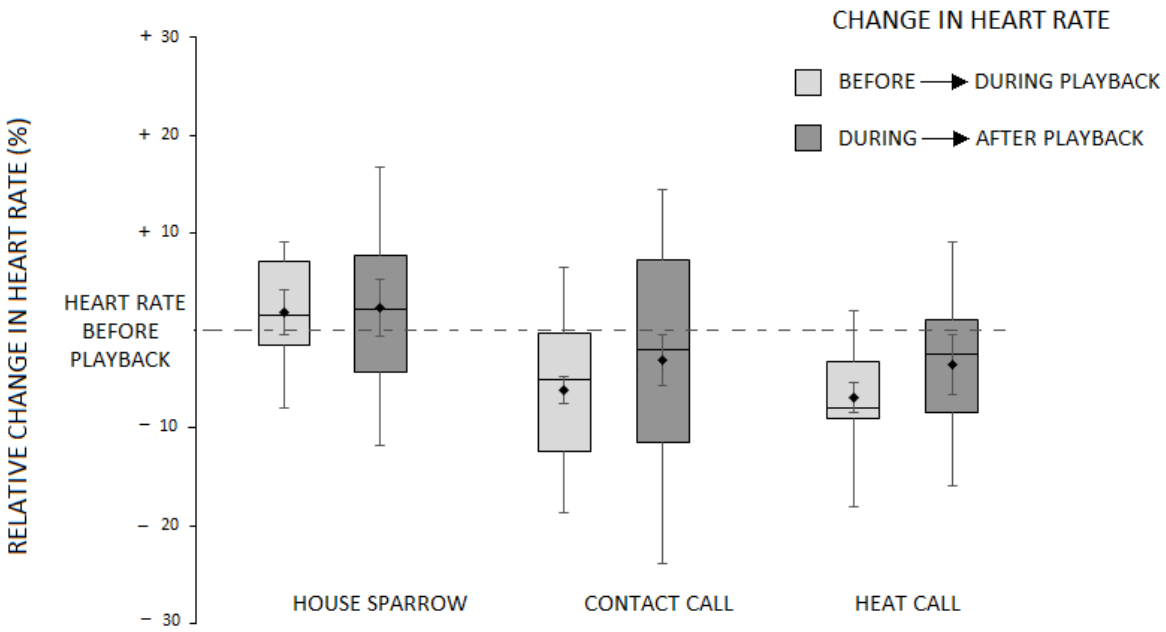


Figure 2. A box and whisker plot showing differences in embryonic heart rate relative to the 'resting heart rate' (taken before exposure to any playback). The light grey boxes show the (%) change in average heart rate between the 60 seconds before and 60 seconds during a playback, and the dark grey boxes show the difference in average heart rate between the 60 seconds before and 60 seconds after the cessation of each playback. The outer error bars around the upper and lower quartiles represent the upper and lower extremes of the dataset. The internal error bars around the mean (diamond symbol) represent the standard error of the mean.

The percent change in embryonic heart rate between the pre-trial and trial was significantly different between the three playback types; ANCOVA: $F_{2, 87} = 9.787$, $P < 0.005$. A Tukey post-hoc test showed that heart rates declined significantly more in response to the heat call, than in response to the heterospecific call ($P = 0.048$), and heart rates also declined significantly more in response to the conspecific contact call than the heterospecific call treatment ($P = 0.068$). There was no

significant difference in the percentage change in heart rate upon exposure to the conspecific contact call or the heat call ($P > 0.999$). We found no significant effect of embryonic age ($F_{1, 87} = 0.523$, $P = 0.472$) or eggshell temperature ($F_{1, 87} = 0.901$, $P = 0.345$) on relative heart rate during the trial. It should be noted that the lack of effect of temperature on heart rate in this study likely reflects our attempt to keep eggshell temperature consistent between different trials (average heart rate temperature was 25.65^{o}C (SEM: 0.217, range: 21.4^{o}C- 28.9^{o}C)), rather than reflecting a 'non-effect' of temperature on heart rate-which we have shown to be significant in a previous study (Sheldon et al, 2017).

Discussion

We find that the heart rates of zebra finch embryos declined in response to two different conspecific vocalizations- the heat call and the contact call, but not in response to the vocalization of an unfamiliar heterospecific- the house sparrow. A reduction in heart rate, upon exposure to a stimulus, has been shown to represent a physiological correlate of attention in a range of taxa (Wetzel, et al. 2006; Hauber et al, 2002; Courage et al, 2006). Therefore, our results demonstrate that zebra finch embryos are physiologically responsive to auditory cues that they are expected to be familiar with, and support the hypothesis that zebra finch embryos have the capacity to be acoustically sensitive to vocalisations from their parents (Mariette & Buchanan 2016). This finding warrants further inquiry into the function of the heat call (and other conspecific calls) in parent-embryo communication.

Although we have shown that zebra finch embryos are acoustically sensitive at an early stage of development (at ~67% of incubation), questions still remain as to the sensitivity of call discrimination in ovo. Given that zebra finch embryos paid attention to two call types in our study (the heat call and the conspecific contact call) it would be useful to use a habituation-dishabituation paradigm (described in Colombelli-Negrel, et al. 2014) to explore whether embryos can learn to discriminate between different conspecific calls they are exposed to. If zebra finch embryos are unable to discriminate between the heat call and other conspecific calls , then it would be difficult to envision how the heat call specifically is able to mediate climatic effects on offspring development (as suggested in Mariette & Buchanan (2016)) without the signal being confounded by other conspecific vocalisations that occur in the nest (some of which might be also directed at embryos, and some of which are believed to be for communication between the parents (Elie et al 2010; Bouchaud et al 2016)). Before purported call type functions can be satisfactorily tested, future research is required to understand zebra finch call variation and response sensitivity to different call types in ovo.

In a broad sense, our findings are consistent with studies on other avian species that have identified the potential for embryos to respond differently to conspecific and unfamiliar (in our case a heterospecific call) acoustic stimuli. For example, Peking duck (*Anas platyrhynchos domestics*) and Muscovy duck (*Cairina moschata*) embryos show sudden heart rate declines in response to maternal calls but rarely to heterospecific calls (Gottlieb, 1979; Hochel et al, 2002); fairy wren embryos show cardiac declines in response to genus specific calls, but not white noise (Colombelli-Negrel, et al, 2014); and chicken (*Gallus gallus domesticus*) embryos show a neurochemical

response following exposure to conspecific, but not artificial sounds (Balaban et al, 2012). The results from our study therefore contribute to a growing body of evidence supporting the ability of avian embryos to pay attention to potentially informative calls of their own species whilst seemingly not perceiving irrelevant artificial or heterospecific calls. Given that the heterospecific/conspecific signals differ in peak frequency in our study (which may affect transmission of the signals), future work could improve on our analyses by accounting for signal degradation of the different playback types over distance.

In conclusion, our study supports the recent work on parent-embryo communication by Mariette & Buchanan (2016), by demonstrating that indeed, as assumed in that study, zebra finch embryos are capable of detecting acoustic calls by the time that they are 8 days old (~ 67% of the way through development). In finding that zebra finch embryos can pay attention to more than one conspecific vocalization, our work also raises questions regarding the sensitivity of call discrimination in ovo and purported call type functions.

Funding statement: Our research was supported by Australian Research Council funding to S.C.G. (FT130101253) and a Macquarie University Research Excellence Scholarship (MQRES) to E.L.S. Neither of our funders had input into the content of the manuscript, nor required their approval of the manuscript before submission or publication.

Ethics statement: All work was carried out in compliance with the Animal Research Authority (2015/017-4) issued by Macquarie University.

Conflict of interest: No authors have a conflict of interest to declare

Author contributions: E.L.S. and S.C.G. formulated the research and wrote the paper, E.L.S. designed the methodology, analyzed the data, and conducted the research in the field.

Data deposits: Our will be deposited in the Dryad Digital Repository

Literature cited

Abraham, C.L., Evans, R.M. 1999. Metabolic costs of heat solicitation calls in relation to thermal need in embryos of American white pelicans, *Animal Behaviour*, 57; 967-975

Anderson, T. 2006. Biology of the ubiquitous House Sparrow, from Genes to populations. Oxford University Press

Bailey, E.D., Ralph, K.M. 1975. The effects of embryonic exposure to pheasant vocalizations in later call identification by chicks. The Condor, 89; 43-49

Balaban, E., Desco, M., Vaquero, J.J. 2012. Waking-like brain function in embryos. *Current Biology*, 22; 852–861

Boucaud, I.C.A., Mariette, M.M., Villain, A.S., Vignal, C. 2016. Vocal negotiation over parental care? Acoustic communication at the nest predicts partners' incubation share. *Biological Journal of the Linnean Society*, 117 (2); 322-336

Brua, R.B., Nuechterlein, G.L., Buitron, D. 1996. Vocal response of eared grebe embryos to egg cooling and egg turning. *Auk,* 113; 525–533

Bugden, S.C., Evans, R.M. 1991. Vocal responsiveness to chilling in embryonic and neonatal American coots, *Wilson Bulletin*, 103; 712-717

Colombelli-Négrel, D., Hauber, M. E., and Kleindorfer, S. 2014. Prenatal learning in an Australian songbird: habituation and individual discrimination in superb fairy-wren embryos. *Proceedings of the Royal Society: Biological Sciences*, 281

Colombelli-Négrel, D., Hauber, M.E., Robertson, J., Sulloway, F.J., Hoi,H., Griggio,M., Kleindorfer,S. 2012. Embryonic learning of vocal passwords in superb fairy-wrens reveals intruder cuckoo nestlings. *Current Biology,* 22; 2155–2160

Colombelli-Négrel, D., Webster, M. S., Dowling, J. L., Hauber, M. E., Kleindorfer, S. 2016. Vocal imitation of mother's calls by begging red-backed fairywren nestlings increases parental provisioning. *Auk,* 133; 273–285

Courage, M.L., Reynolds, G.D., Richards, J.E. 2006. Infants' attention to patterned stimuli: developmental change from 3 to 12 months of age. *Child Development*, 77; 680–695

Dowling, J.L., Colombelli-Negrel, D., Webster, M.S. 2016. Kin signatures learned in the egg? Red-Backed-Fairy wren songs are similar to their mother's in-nest calls and songs. *Behavioural and Evolutionary Ecology*, 4

Elie, J.E., Mariette, M.M., Soula, H.A., Griffith, S.C., Mathevon, N., Vignal, C. 2010. Vocal communication at the nest between mates in wild zebra finches: a private vocal duet? *Animal Behaviour,* 80; 597–605.

Evans, R.M., Whitaker, A., Wiebe, M.O. 1994. Development of vical reglation of temperature by embryos in pipped eggs of ring-billed gulls. The Auk, 111 (3); 596-604

Gilby, A. J., Mainwaring, M.C., Griffith, S.C. 2013. Incubation behaviour and hatching synchrony differ in wild and captive populations of the Zebra Finch. *Animal Behaviour,* 85; 1329–1334

Gottlieb, G. 1979. Development of species identification in ducklings: V. Perceptual differentiation in the embryo. *Journal of Comparative and Physiological Psychology*, 93; 831–854

Griffith, S.C., Pryke, S.R., Mariette, M. 2008. Nest box use by the zebra finch *Taeniopygia guttata:* implications for reproductive success and research. *Emu*, 108; 311-319.

Griffith, S.C., Mainwaring, M. C., Sorato, E., Beckmann, C. 2016. High atmospheric temperatures and ambient incubation drive embryonic development and lead to earlier hatching in a passerine bird. *Royal society Open Science*, 3

Hauber, M.E., Pearson, H.E., Reh, A., Merges, A. 2002. Discrimination between host songs by brood parasitic brown-headed cowbirds (*Molothrus ater*). *Animal Cognition*, 5; 129–137

Höchel J, Pirow R, Nichelmann M. 2002. Development of heart rate responses to acoustic stimuli in Muscovy duck embryos. *Comparative Biochemistry and Physiology Part AL Molecular and Integrative Physiology*, 131; 805–816

Lickliter, R. 2005. Prenatal sensory ecology and experience: implications for perceptual and behavioral development in precocial birds. *Advances in the study of behavior*, 35; 235-274

Mariette, M.M., Buchanan, K.L. 2016. Prenatal acoustic communication programs offspring for high post hatching temperatures in a songbird. *Science,* 353 (6301); 812-814

Pollard, A.S., Pitsillides, A.A. and Portugal, S.J., 2016. Validating a noninvasive technique for monitoring embryo movement in ovo. *Physiological and Biochemical Zoology*, 89(4); 331-339.

Reed, W.L., Clark, M.E. 2011. Beyond Maternal Effects in Birds: Responses of the Embryo to the Environment. *Integrative Comparative Biology,* 51 (1); 73-80.

Sheldon, E.L., McCowan, L.S.C., McDiarmid, C.S., Griffith, S.C. 2017. Measuring the embryonic heart rate of wild birds: An opportunity to take the pulse on early development. *The Auk, Ornithological Advances*, 135

Wetzel, J.M., Quigley, K.S., Morell, J., Eves, E., Backs, R.W. 2006. Cardiovascular measures of attention to illusory and non-illusory visual stimuli. *Journal of Psychophysiology*, 20, 276–285

Wolf, N.K., Bixby, J.L., Capranica, R.R. 1976. Prenatal experience and avian development: brief auditory stimulation accelerates the hatching of Japanese quail. *Science*, 194, 959–960.

Zann, R.A. 1996. The zebra finch, A Synthesis of Field and Laboratory Studies. Oxford Ornithology Series

Chapter 7

It is humbling to think that in a matter of ~12 days, a fully formed zebra finch chick with down feathers develops within a rigid eggshell. Photographed is an adult zebra finch, two hatchlings emerging from an egg just over one cm in length, and an abandoned emu egg up 25cm in length.

Embryonic heart rate is associated with pre-natal development rate but not post-natal activity levels or growth rates in the zebra finch (*Taeniopygia guttata*)

Elizabeth L. Sheldon[1.] Simon. C. Griffith[1.]

1. Department of Biological Sciences, Macquarie University, Sydney, New South Wales, 2109, Australia

* Corresponding author: elizabeth-louise.sheldon@students.mq.edu.au

Abstract

Inter-individual variation in behaviour has been the focus of much recent work, yet the underlying mechanisms that cause and maintain this variation are unclear. It has been proposed that consistent individual differences in metabolism may promote inter-individual variation in behaviour and development throughout life. Here, we tested this idea in the zebra finch (*Taeniopygia guttata*), by investigating whether embryonic heart rate (a proxy for metabolic rate), is associated with pre-natal developmental rates, post-natal activity levels, and post-natal growth rates. Embryonic heart rate and post-natal activity level were significantly repeatable throughout an individual's development, such that consistent individual differences in these traits could be distinguished. We detected a significant, negative relationship between embryonic heart rate and incubation duration. However, we did not detect any relationship between embryonic heart rate, post-natal activity levels or post-natal growth rates. Our findings suggest that although differences in embryonic heart rate are likely to affect inter-individual variation at the embryonic stage, it is unlikely that these affects persist across the key developmental boundary of hatching in birds.

Key words: Metabolic rate, consistent individual differences, behavior, incubation duration, ontogenic boundaries, inter-individual variation.

Introduction

Traits that differ across individuals but are temporally and contextually consistent within individuals are known as 'consistent individual differences' (Biro and Stamps, 2010). The existence of consistent individual differences in behaviour, (commonly referred to as personality), and developmental rates are well established across a wide range of species (Ronning, et al, 2005; McCowan et al, 2015; White et al, 2016). Relatively little is known about how/when these consistent differences emerge during development, or how they change across different life stages (Wuerz and Kruger, 2015; Trillmich et al, 2015). However, in a number of taxa, it has been demonstrated that consistent individual differences can persist across different ontogenic boundaries (Niemela et al, 2012; Sprenger et al, 2012; Wilson and Krause, 2012b; David et al, 2012).

It has recently been proposed that consistent individual differences in energy metabolism could promote and maintain consistent individual differences in behaviour and developmental rate through life (Biro and Stamps, 2010; Bouwhuis et al, 2013). Metabolic rate has the potential to influence a suite of developmental and behavioural traits due to the fact that energy, and the processing of energy, is fundamental in fueling the physiological processes that generate activity and growth (Schmidt-Nielson, 1991). Consistent individual differences in metabolic rate have been identified in a diverse range of animal taxa (Nespolo and Franco, 2007; Moe et al, 2009; Broggi et al, 2009), yet, a coherent framework linking behaviour, developmental rate, and energy metabolism remains unclear (Biro and Stamps, 2010; Careau et al, 2008).

The limited research available that has linked metabolism with behavioural and developmental variation has largely focused on associations within the same life stage (e.g. whether

variation in *adult* metabolism co-varies with variation in *adult* activity level) (Vezina et al, 2006; Mathot et al, 2009; White et al, 2016). However, many taxa have ontogenic boundaries separating life-stages that differ dramatically. Despite this, the extent to which metabolic rate from a previous life stage can influence the phenotype of a later life stage (e.g. whether variation in *embryonic* metabolism co-varies with variation in *adult* activity level) has received relatively little attention (Hall et al, 2016). This idea is worthy of consideration because individual variation in behaviour and developmental rates might be expected to shift between different life stages in association with the often profound changes in environments, physical constraints, and life history priorities across ontogenic boundaries.

Here, we explore the potential for consistent individual differences in embryonic metabolism to affect consistent individual differences in behaviour and developmental rates in the nestling stage in wild zebra finches (*Taeniopygia guttata*). This work extends an earlier study that demonstrated the correlation between individual differences in activity levels across two life stages (nestling and adult) in a captive population of this species (McCowan and Griffith, 2014). We use embryonic heart rate as a proxy for embryonic metabolism due to its strong, positive correlation with metabolic rate in other oviparous species (Du et al. 2010b, Piercy et al, 2015; Ward et al, 2002; Owen, 1969; Butler et al., 2004; Dechmann et al., 2011). We use activity level as a proxy for nestling behaviour, as in the earlier study (McCowan and Griffith, 2014).

Embryonic heart rate can be measured non-invasively using a relatively new device - the Buddy digital egg monitor (Vetronic Services, Devon, UK) (see Sheldon et al. 2017a for more detail). The acquisition of short-term heart rate measurements using the digital egg monitor has

been used to demonstrate plasticity in embryonic heart rate in response to a range of environmental and social cues (Du et al. 2010a; Du and Shine, 2010; Colombelli-Négrel et al. 2014). However, use of the egg monitor for characterizing consistent individual differences in heart rate (i.e. the extent to which some individuals have consistently, relatively low heart rates and others comparatively high heart rates throughout development) is limited to date. Consequently, before we explore the association between embryonic heart rate and activity/development, we first aim to assess whether heart rate measures from the digital egg monitor are repeatable over pre-natal development, such that they can be used to provide a proxy for consistent individual differences in embryonic metabolic rates. The specific goals of our study were (i) to characterize the extent to which both embryonic heart rate and nestling activity level measures are repeatable throughout development and (ii) investigate the extent to which variation in embryonic heart rate is associated with embryonic development rate, nestling growth rate and nestling activity levels.

Methods

Data collection

Fieldwork was conducted at Fowlers Gap Arid Zone Research Station during the main part of the Austral breeding season: September-November 2016. We quantified heart rate variation of zebra finch embryos from eggs that were laid in nest boxes (details on nest boxes and study site given in Griffith et al 2008), and active nests were monitored daily to ascertain the laying date, clutch size and the date on which incubation is likely to have started (further details below). We took heart rate measures at 114 zebra finch nests (for information on sample sizes, see Table 1), at

three time points during pre-natal development (~day 6, 9 and 11 of incubation (zebra finch eggs hatch at day ~12 of incubation)). Embryonic heart rates were taken in a shaded position close to the natal nest box using a digital egg monitor (Buddy, Vetronic Services, UK). This device generates heart rate data by tracking infra-red light absorption changes owing to embryonic blood flow, and is able to detect heart rate in zebra finch embryos at ~ day 5 of embryonic development (Sheldon et al, 2017a). To follow an embryo through development, we labelled each egg with a unique ID (with a fine soft-tipped permanent marker pen). The number of repeated heart rate measurements taken for each embryo across development varied from 2 to 4 (often the monitor was unable to detect a reliable heart rated when the embryo was moving (see Sheldon et al 2017a and Table 1)). On the day of measure, the entire clutch was removed from the nest and placed in a standardised container; a 'soft-box' filled with cotton wool that shaded the eggs from the sun and wind and reduced the risk of eggs cracking. On each day of sampling, we took three repeated measures of each embryos heart rate (where possible) over a ~ 2 minute period and took the average of these measures to characterise heart rate for that day of development. As the time out of the nest has been shown to exert significant effects on the heart rate of zebra finch embryos (Sheldon et al, 2017a), we excluded singular heart rate measures that did not allow us to control for effects of time out of the nest (Table 1).

Short term (<30 seconds) heart rate measures collected with the Buddy heart rate monitor are extremely sensitive to environmental fluctuations, and capture a significant degree of plasticity in response to biotic and abiotic conditions prior to, and during sampling (Sheldon et al, 2017a). 'One-off', short-term heart rate measures thus have limited value for predicting consistent

individual differences in the metabolic rates of embryos measured under different sampling conditions. Use of the egg monitor for long-term heart rate monitoring (10's of minutes) is also problematic, because the infrared energy that the instrument emits heats the egg, and potentially interfere with embryonic development (Sartori et al. 2015). Here we tested a potentially overlooked solution to this problem; we acquired multiple, short-term heart rate measures across an individual's development to test whether these measures were significantly repeatable. If so, we assumed these short-term measures would represent a useful estimate of an individual's 'metabolic rate' (Du et al. 2010b, Piercy et al, 2015).

In the wild, we have previously shown that parent zebra finches only initiate incubation on the day that the last egg was laid (Gilby, et al 2013). Consequently, we used the day of the last laid egg to represent the first day of embryonic development (although the earliest laid eggs may start to develop before this, given the high ambient temperatures (Griffith, et al. 2016)). It has previously been estimated, that zebra finch embryos take ~12 days to hatch after the onset of incubation, and we used this estimation to predict clutch hatch date. Two days prior to a clutch's estimated hatch date, we monitored the nest three times per day between 06.00-17.00hrs; our first nest check was at ~06.00hrs, our second nest check was at ~11.30hrs and our third nest check was at ~17.00hrs. We used these nest check time points to estimate the number of hours the egg had been incubated for before it hatched. We did not include in our sample eggs that hatched between 17.00 and 06.00hrs (unless the chicks were still wet at 06.00 which indicated a very recent hatch at the time of first check in the morning). This allowed us to estimate hatching time to within ~5:30 hours for each individual.

After hatching, each chick had a small, unique patch of down feathers trimmed for post-natal identification within the nest. In cases where multiple eggs hatched synchronously we were unable to identify which chick hatched from which egg (see Table 1). These individuals were not used to investigate the relationship between embryonic heart rate and post-natal growth rates and activity levels, however they were used to investigate the relationship between embryonic heart rate and developmental rate (Table 1). After hatching, we visited the nest at day 3 and day 11 of post-natal development and collected measures of nestling weight and tarsus length using a Pesola spring balance, and digital callipers, respectively. Weight was unable to be accurately measured for a portion of day 3 chicks, as the balances were unstable due to the windy conditions and the low mass of young nestlings (see Table 1).

On days 5 and 7 (after hatching), we visited the nest to conduct our assay of nestling activity. This activity assay involved removing the hatchling from the nest, and placing it in a shaded open-box 'arena' (approximately 15cm x 15cm) that was divided equally into 9 marked squares. A stopwatch was started after the chick had settled in the center square of the arena for 20 seconds. The behaviour of the chick was then monitored every 5 seconds for 60 seconds. We recorded two behaviours - movement and begging (these were binary observations; yes or no at each 5 second time point), and the number of times the whole head of the chick entered a different square in the box. Three proxies for individual activity level were thus attained, referred to hereafter as; 'time spent moving', 'time spent begging' and 'number of squares reached'.

Daily atmospheric temperature data were obtained from the Australian Bureau of Meteorology's Automated Weather Station at Fowlers Gap, located within 20 km of the study sites.

Temperature was averaged over embryonic development (from the first day of incubation to the day of hatch, for each nest in the sample).

Statistical analysis

Consistent individual differences in embryonic heart rate and nestling activity levels

To detect the existence of consistent individual differences in embryonic heart rate, we evaluated whether multiple, short-term, heart rate measures were repeatable over early and late pre-natal development. We averaged the embryonic heart rate measures attained from early (day 5-8 of incubation), and late (day 9 of incubation – hatching day) prenatal development. In our analyses we only included individuals that had more than one heart rate measure during each period of development to attain an average measure for each period (see Table 1).

Table 1. An overview of sample sizes in our study. Heart rate (HR) measures with <2 repeats per sampling session were excluded from the study to mitigate the effects of time out of the nest on HR.

	Eggs/chicks	**Families**
Initial sample size	723	140
Viable clutches (not abandoned)	518	114
>2 HR repeats at 1st ½ detectable development	202	58
>2 HR repeats at 2nd ½ detectable development	288	74
>2 HR repeats at 1st & 2nd ½ detectable development	98	37
Activity assay at day5 & day7	148	51
Chick & egg ID	62	26
Heart Rate & Chick ID & Body condition (at day 3 & 11)	57	23
Heart Rate & Chick ID & Activity Level	61	26

For both the early, and late period of development we ranked every embryo according to its relative heart rate (across all of those measured in that period). We used ranked measures rather than raw data, as an individual's rank better represented its relative population level measure, regardless of differences in developmental age at the two stages of development. For embryos that had an average heart rate rank for both the first and second half of development (total number of individuals for which there was data in the 1st and 2nd half of development, N=98), we assessed whether these averages were repeatable using a two-way mixed interclass correlation coefficient (ICC) analysis. The ICC describes how strongly measures from the same individual are correlated (a high ICC value indicates high repeatability throughout development) (Nakagawa and Schielzeth (2013)).

For each measure of activity: 'time spent moving', 'time spent begging' and 'number of squares reached', we tested whether the data was normally distributed using a Shapiro-Wilk Test. An ICC repeatability analysis was then used to test for repeatability between the activity measures at day 5 and day 7. The measure of activity found to be the most repeatable across development was used as our only representation of activity for the analysis relating activity to metabolism. This was to reduce the number of statistical tests conducted and the likelihood of a type one error.

Post-natal growth rate

A body condition index was calculated as the residuals from a least-squares linear regression analysis between body mass (dependent variable) and tarsus length. Growth rate was considered as

the increase in residual weight per day (i.e. the difference between day 3 and day 11 measures, divided by 8). The change in body condition per day will be referred to as 'growth rate' hereafter.

Relationship between embryonic heart rate, incubation duration, activity and growth rate

Statistics were run in R version 3.3.1 (Core Development Team, 2015), and SPSS Statistics. Linear mixed models (LMM) were run using the package lmer in R (Bates et al. 2015) with the package lmerTest (Kuznetosova et al. 2016) to calculate degrees of freedom and *p*-values. To model the effects of the fixed factor 'embryonic heart rate' on the response variables 'activity level', 'incubation length' (number of hours from first day of incubation to the estimated hour of hatching (see 'data collection' for estimations)), and 'growth rate', we ran three separate linear mixed models. In each model, we accounted for 'ambient incubation' by featuring maximum ambient temperature during an individual's development as a fixed effect. We also included brood size as an additional fixed effect in the two linear models that could be affected by the conditions of the post-natal environment (response variables: growth rate and activity level (brood size was not considered to affect pre-natal response variables, as brood and clutch size often differed due pre-natal mortalities). We also accounted for familial effects by including the identity of the chick's natal nest as a random factor in all models. We calculated marginal R^2 and ICC values for all LMM using the method described in Nakagawa & Schielzeth (2013). We also used a Pearson's correlation analysis to test for a relationship between incubation length (i.e. pre-natal developmental rate) and post-natal growth rate.

Pre-registration with the Open Science Framework

We pre-registered our analyses with the Open Science Framework which facilitates reproducibility and open collaboration in science research (Foster and Deardorff, 2017). Our pre-registration: Sheldon and Griffith (2017b), was carried out to limit the number of analyses conducted and to validate our commitment to testing a priori hypotheses. Our methods are consistent with this pre-registration (Sheldon and Griffith 2017b).

Results

Embryonic heart rate measures were normally distributed: (Shapiro-Wilk=0.955, p=0.071), and demonstrated a reasonable and significant degree of repeatability within an individual from the 'early' and 'late' stages of its pre-natal development. The average measure ICC was 0.643, with a 95% confidence interval from 0.466 to 0.761, F_{96} =2.801, p<0.001 (Figure 1). Given this level of repeatability, we used the heart rate ranks from the second half of detectable development for the final estimation of individual embryonic heart rate (we chose data from the second half of development to increase sample size (Table 1)). Data for the time spent moving was the only measure of activity that was normally distributed (Shapiro-Wilk=0.974, p=0.437) (Table 2), consequently we used the individual average measure of this behavioural parameter to quantify individual activity.

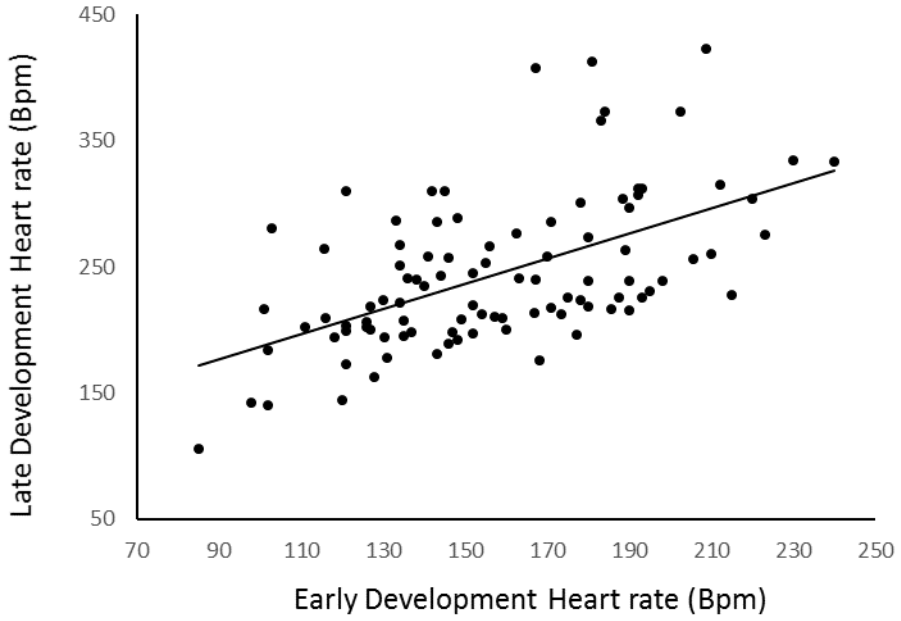


Figure 1. Significant, positive correlation between embryonic heart rate ranks for the early and late stages of prenatal development (r=0.548, p<0.001, n=98, r^2=0.3105)

Table 2. Normal distribution values for the three activity measures, (normally distributed measures are indicated with an (*)).

Activity Measure	**Shapiro-Wilk**	***p***
(%) time spent moving	0.974	0.437*
(%) time spent begging	0.870	<0.001
Number of squares reached	0.531	<0.001

A high, significant degree of repeatability was found between the time spent moving at day 5 and day 7 of 148 zebra finch chicks (Figure 2), with the average measure of ICC being 0.713, with a 95% confidence interval from 0.602 to 0.793, F_{146} =3.482, p<0.001.

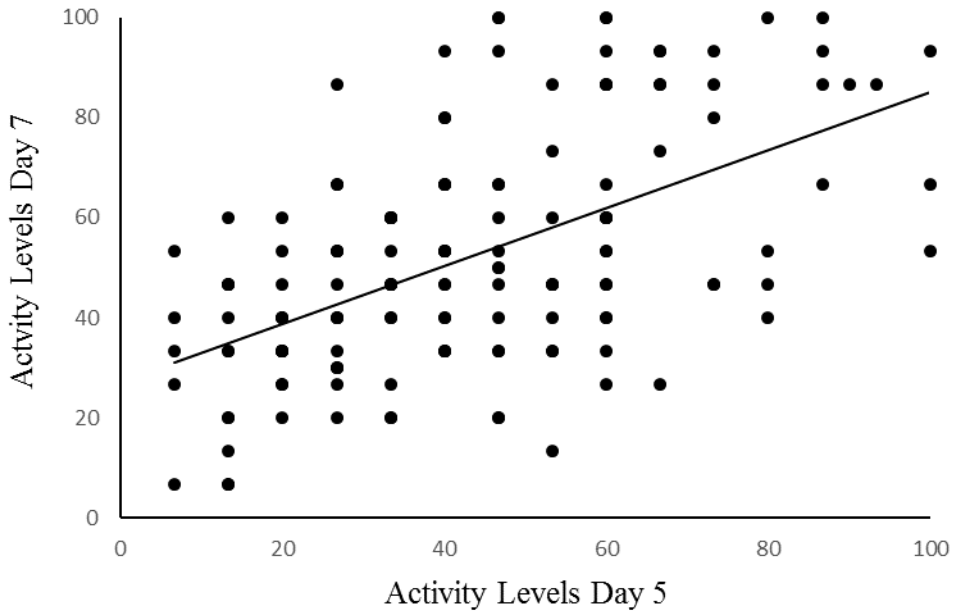


Figure 2. Significant, positive correlation between Activity levels (the percentage of time moving) at day 5 and day 7 of development, (r=0.425, p=0.005, n =148, r^2=0.3072)

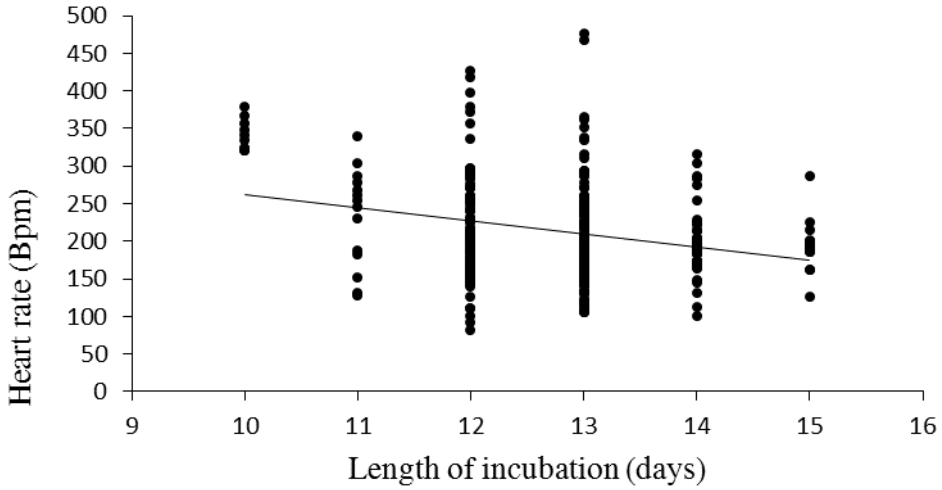


Figure 3. A significant, negative relationship between late-stage embryonic heart rate and length of incubation (days) (r^2=0.07547, *n*=288)

From our analysis using a linear mixed model, we found a significant negative relationship between embryonic heart rate and incubation duration (Table 3c, Figure 3), such that embryos with a lower heart rate took a longer time to develop (before they hatched). Using a linear mixed models we found no effect of embryonic heart rate, ambient temperature, or brood size on nestling growth rates (see Table 3a), although developmental nest ID explained a significant proportion (r^2=56.63%) of variance in nestling growth rates (Table 1a).

Table 3: The effect of heart rate, brood size and temperature on a) growth rate, b) percentage of time spent moving, and c) incubation duration. The conditional R^2 represents the variance explained for the total model, and the marginal R^2 represents the variance explained by the fixed effects. The ICC value represents the degree of similarity between body condition values from the same family (an ICC close to 1 indicates high similarity).

***a)* Growth rate**	**Estimate**	**SE**	**d.f.**	**t-value**	**p-value**
Intercept	0.38	0.05	35.21	7.38	<0.001
Heart rate	-0.01	<0.01	32.07	-0.56	0.578
Temperature	<0.01	<0.01	27.75	1.26	0.217
Brood size	-0.01	<0.01	21.42	-0.98	0.338
	Variance	**SD**	***n***		**ICC**
Marginal R^2	0.07				
Developmental nest	<0.01	0.03	23		0.63
Residual	<0.01	0.03	57	Conditional R^2	0.67

***b)* Activity Level**	**Estimate**	**SE**	**d.f**	**t-value**	**p-value**
Intercept	17.46	27.91	48.52	0.63	0.534
Heart rate	-0.11	0.11	42.17	-1.04	0.306
Temperature	0.99	0.65	51.98	1.53	0.133
Brood size	1.40	3.46	32.80	0.41	0.688
	Variance	**SD**	***n***		**ICC**
Marginal R^2	0.06				
Developmental nest	146.20	12.09	26		0.28
Residual	364.20	19.08	61	Conditional R^2	0.33

c) **Incubation duration**	**Estimate**	**SE**	**d.f**	**t-value**	**p-value**
Intercept	357.90	16.49	57.26	21.71	<0.001*
Heart rate	-0.12	0.04	92.73	-2.75	0.007*
Temperature	-1.02	0.70	62.13	-1.45	0.153
	Variance	**SD**	***n***		**ICC**
Marginal R^2	0.12				
Natal nest	256.90	16.03	74		0.56
Residual	201.10	14.18	288	Conditional R^2	0.62

Using another linear mixed model we found no significant effect of embryonic heart rate, temperature, or brood size on the time spent moving by a nestling during the trial (Table 3b). Again, developmental nest ID explained a significant proportion (r^2=32.56%) of variance in the data from the nestling movement assay (see Table 3b). Finally, there was no correlation between the duration of development in the egg and nestling growth rate (Pearson's correlation, r=0.049, p=0.676, n=81).

Discussion

Behaviour and development are particularly responsive to changes in social and environmental cues throughout life, and indeed in our study we found significant influences of development nest identity on growth rates and nestling activity levels. Whilst we found clear evidence for consistent individual differences in our proxy for embryonic heart rate, we found no evidence that this individual characteristic influenced the behavior or growth of nestlings in their next phase of development (as a nestling after hatching) (a result that differed from our a priori hypotheses (Sheldon and Griffith 2017b)). Interestingly, we also failed to detect any relationship between pre-natal developmental rate and post-natal growth rate. We are reasonably confident that embryonic heart rate is a good proxy for metabolic rate, because those individuals with a high heart rate developed at a faster rate during pre-natal development than those with relatively low heart rates (an organism's metabolic rate limits the rate at which it processes energy, which in turn determines its development rate (Rosenfeld, 2014; Ton and Martin, 2016; McCarthy, 2000)). Together, our results suggest that consistent individual differences in metabolic rate do not persist across ontogenic boundaries, or if they do, their relationship with behaviour and developmental rate

varies between pre and postnatal life stages. This suggestion is consistent with the hypothesis that different selection pressures and priorities at different life stages promote shifts in the regulation of behaviour and development (White et al, 2016).

A clear framework linking metabolism, life history and behaviour remains to be fully established (Biro and Stamps, 2010), and this is reflected by the inconsistent findings of studies that support (Hall et al, 2015; McCowan and Griffith, 2014), and do not support (Petelle et al, 2013; Bell et al, 2004) the maintenance of consistent individual differences across developmental boundaries. A limitation of our study was our failure to measure levels of pre-natal activity levels, and post-natal metabolic rates. Thus the degree to which metabolism affects variation in behaviour in the same ontogenic stage, and the degree to which metabolic variation persists across ontogenic stages remains to be thoroughly examined in the zebra finch. Although more conventional methods for gathering data on metabolic rates could be used for both embryos and nestlings (e.g. respirometric measures (Lierz er al, 2006)), such methods are rather intensive and difficult to achieve with large sample sizes. Assessing whether metabolic rates influence activity level within the same ontogenic stage, and whether metabolic rates persist across hatching will allow us to clarify whether our results indicate that (i) metabolism is not related to activity levels in the zebra finch, or (ii) metabolism is related to activity levels within the same ontogenetic stage (as in other avian species, Ton and Martin (2016)). Addressing these limitations will not only enable us to better interpret the results of our study, but also help to more generally clarify the role of metabolic rate in maintaining consistent individual differences across an individual's life.

Although the link between inter-individual variation and metabolic rate requires further investigation, our study has demonstrated the ability of the Buddy heart rate monitor to detect inter-individual differences in heart rate, and confirmed a degree of individual consistency in embryonic heart rate over time. These findings suggest that multiple, short-term heart rate measures are able to capture inter-individual variation in embryonic metabolism, despite the sensitivity of heart rates to a wide range of environmental and sampling variables (Sheldon et al. 2017a). This finding opens up new opportunities for this device to explore the effects of metabolic variation on animal personality and the pace of life-history traits.

In conclusion, our results suggest that inter-individual variation in metabolism can affect development within the same life stage, but does not seem to persist across the important development milestone of hatching in a developing bird. This finding contrasts with earlier work on the same species across the next ontogenetic boundary (nestling to adult) in which it was shown that variation in activity levels in the nestling stage, persisted through to adulthood (McCowan & Griffith 2014). The contrasting results of this current study and the previous one may reflect differences in resource acquisition between different life stages: development and metabolism in an egg is largely determined by the fixed resources and hormones allocated to the egg by the mother when it is created, but upon hatching, a nestlings' development can dramatically change trajectory dependent on its ability to acquire resources. We did find strong effects of the family ID on pre and post-natal developmental rate, and activity levels. Parental influences such egg resource allocation (Buchanan et al, 2001), nest environment (Sheldon et al, 2017a), and genetic make-up (Sadowska et al, 2009) have previously been shown to effect metabolic rate, which may underlie the familial

effects detected in our study. Further work, clarifying the causes of metabolic variation would thus be useful in understanding the proximate role of metabolic rate in maintaining consistent individual differences across different stages in an individual's life.

Conflict of interest: No authors have a conflict of interest to declare

Funding statement: Our research was supported by Australian Research Council funding to S.C.G. (FT130101253) and a Macquarie University Research Excellence Scholarship (MQRES) to E.L.S. Neither of our funders had input into the content of the manuscript, nor required their approval of the manuscript before submission or publication. Ethics statement: All work was carried out in compliance with the Animal Research Authority (2015/017-4) issued by Macquarie University.

Author contributions: E.L.S. and S.C.G. formulated the research, designed the methodology, and wrote the paper. E.L.S. conducted the research in the field, and analyzed the data.

Literature cited

Bates, D., M. Machler, B. M. Bolker, and S. C. Walker. 2015. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, 67:1–48

Bell AM, Stamps J.A. 2004. Development of behavioural differences between individuals and populations of sticklebacks, *Gasterosteus aculeatus*. *Animal Behaviour*, 68:1339-1348.

Biro, P.A., Stamps, J.A. 2010. Do consistent individual differences in metabolic rate promote consistent individual differences in behaviour? *Trends in Ecology and Evolution*, 25 (1): 653-659Bouwhuis, S., Quinn, J.L., Sheldon, B.C., Verhulst, S. 2013, Personality and basal metabolic rate in a wild bird population. *OIKOS, Synthesising Ecology*, 123 (1): 56-62

Brodin, T. 2009. Behavioural syndrome over the boundaries of life- carryovers from larvae to adult damselfly. *Behavioural Ecology*, 20 (1): 30-37.

Broggi, J. et al. 2009. Long-term repeatability of winter basal metabolic rate and mass in a wild passerine. *Functional Ecology*. 23: 768–773

Buchanan, K.L., Evans, M.R., Goldsmith, A.R., Bryant, D.M., Rowe, L. V. 2001. Testosterone influences basal metabolic rate in male house sparrows: a new cost of dominance signalling? Proceedings of the royal society B, 268 (1474)

Butler, P. J., Green, J. A., Boyd, I. L. and Speakman, J. R. 2004. Measuring metabolic rate in the field: the pros and cons of the doubly labelled water and heart rate methods. *Functional Ecology.* 18: 168-183.

Careau, V., Thomas, D., Humphries, M., Reale, D. 2008. Energy metabolism and animal personality. *OIKOS, Synthesising Ecology*. 117 (5): 641-653

Colombelli-N´egrel, D., Hauber, M.E., Kleindorfer.S. 2014. Prenatal learning in an Australian songbird: Habituation and individual discrimination in Superb Fairy-Wren embryos. *Proceedings of the Royal Society B*, 281:20141154

David, M., Auclair, Y., Cézilly, F. 2012. Assessing short- and long-term repeatability and stability of personality in captive zebra finches using longitudinal data. *Ethology*, 118:932-942.

Dechmann, D. K. N., Ehret, S., Gaub, A., Kranstauber, B. and Wikelski, M. 2011. Low metabolism in a tropical bat from lowland Panama measured using heart rate telemetry: an unexpected life in the slow lane. *Journal of Experimental Biology,*214: 3605-3612.

Du, W.-G., Zhao. B., R. Shine, R. 2010a. Embryos in the fast lane: High-temperature heart rates of turtles decline after hatching. *PLOS One*, 5:e9557.

Du, W.-G., Ye, H., Zhao, B., Warner. D.A., Shine, R. 2010b.Thermal acclimation of heart rates in reptilian embryos. *PLOS One*, 5:e15308.

Gilby, A. J., Mainwaring, M.C., Griffith, S.C. 2013. Incubation behaviour and hatching synchrony differ in wild and captive populations of the Zebra Finch. *Animal Behaviour,* 85:1329–1334

Glazier, D.S. 2015. Is metabolic rate a universal 'pacemaker' for biological processes? *Biological Reviews*, 90: 377-407

Griffith, S.C., Mainwaring, M.C., Sorato, E., Beckmann, C. 2016. High atmospheric temperatures and 'ambient incubation' drive embryonic development and lead to earlier hatching in a passerine bird. *Royal Society Open Science*, 3: 1-14

Griffith, S.C., Pryke, S.R., Mariette, M. 2008. Nest box use by the zebra finch *Taeniopygia guttata:* implications for reproductive success and research. *Emu*, **108:** 311-319.

Hall, M.L., van Asteri, T., Jatsis, A.C., Dingemanse, N.J., Magrath, M.J.L., Mulder, R. 2016. Animal personality and pace-of-life syndromes: do fast exploring fairy wrens die young? *Frontiers in Ecology and Evolution*, 10.3389

Kuznetsova, A., Brockhoff, P.B., Christensen, R.H.B. 2016. lmerTest: Tests in Linear Mixed Effects Models. R package version 2.0-32. http://CRAN.R-project.org/package¼lmerTestLierz, M.,

O. Gooss, and H. M. Hafrez (2006). Noninvasive heart rate measurement using a digital egg monitor in chicken and turkey embryos. Journal of Avian Medicine and Surgery 20: 141–146.

Mathot, K.J., Godde, S., Careau, C., Thomas, D.W., Giraldeau, L-A. 2009. Testing dynamic variance-sensitive foraging using individual differences in basal metabolic rates of zebra finches. *OIKOS Synthesising Ecology*, 118 (4): 545-552

McCarthy, I.D. 2000. Temporal repeatability of relative standard metabolic rate in juvenile Atlantic salmon and its relation to life history variation. *Journal of Fish Biology*, 57: 224–238

McCowan, L.C., Griffith, S.C. 2014. Nestling activity levels during begging behaviour predicts activity level and body mass in adulthood. *Peer- Reviewed Journal*, 2:e566

Metcalfe, N. B., Van Leeuwen, T. E. and Killen, S. S. 2016. Does individual variation in metabolic phenotype predict fish behaviour and performance? *Journal of Fish Biology*, 88: 298–321. doi:10.1111/jfb.12699

Moe, B., Ronning, B, Verhulst, S, Bech, C. 2009. Metabolic ageing in individual zebra finches, Biology Letters, 5:86-89

Nakagawa, S., and H. Schielzeth, H. 2013. A general and simple method for obtaining R2 from generalized linear mixed effects models. *Methods in Ecology and Evolution*, 4:133–142.

Nespolo, R.F., M. Franco.M. 2007. Whole-animal metabolic rate is a repeatable trait: a meta-analysis. *Journal of Experimental Biology*, 210; 2000-2005

Niemela, P.T., Vainkka, A., Forsman, J.T., Loukola O.J., Kortet, R. 2012. How does variation in the environment and individual cognition explain the existence of consistent behavioural differences? *Ecology and Evolution*. 3 2: 457–464

Owen, R.B. 1969. Heart rate, a measure of metabolism in blue winged teal *Comparative Biochemistry and physiology*. 31 (3): 431-436

Petelle, M.B., McCoy, D.E., Alejandro, V., Martin, J.G.A., Blumstein, D.T. 2013. Development of boldness and docility in yellow-bellied marmots. *Animal Behaviour*, 1-8.

Piercy, J., Rogers, K., Reichert. M., Andrade, D.V., Abe, A.S., Tattersall, G.J., Milson, W.K. 2015. The relationship between body temperature, heart arte, breathing rate and rate of oxygen consumption, in the tegu lizard (*Tupinambis merianae*) at various levels of activity. *Journal of Comparitive Physiology B,* 185 (8): 891-903

Rønning, B. *et al*. 2005. Long-term repeatability makes basal metabolic rate a likely heritable trait in the zebra finch *Taeniopygia guttata*. *Journal of Experimental Biology*. 208: 4663–4669.

Rosenfeld, J., Leeuwen, T.V., Richards, J., Allen, D. 2014. Relationship between growth and standard metabolic rate: measurement artefacts and implications for habitat use and life-history adaptation in salmonids. *Journal of animal ecology*, 84 (1): 4-10

Sadowska E.T., et al. 2009. Genetic correlations in a wild rodent: grass-eaters and fast growers evolve high basal metabolic rates. Evolution, 63: 1530-1539

Sartori, M. R., Taylor, E.W., Abe, A.S., Crosley, D.A. 2015. An appraisal of the use of an infrared digital monitoring system for long-term measurement of heart rate in reptilian embryos. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 188:17–21

Schmidt-Nielson, 1991. Animal Physiology, *Cambridge University Press*Sheldon, E.L., McCowan, L.S.C., McDiarmid, C.S., Griffith, S.C. 2017. Measuring the embryonic heart rate of wild birds: An opportunity to take the pulse on early development. *The Auk, Ornithological Advances*, 135

Sprenger, D., Dingemanse, N.J., Dochtermann, N.A., Theobald, J., Walker, S.P.W. 2012. *Aggressive females become aggressive males in a sex-changing reef fish.* Ecology Letters, 15 (9): 986-992

Ton, R., Martin, T. 2016. Metabolism correlates with variation in post-natal growth rate among songbirds at three latitudes. *Functional Ecology*, 30 (5): 743-748

Trillmich, F., Gunther, A., Muller, C., Reinhold, K., Sacher, N. 2015. New perspectives in behavioural development: adaptive shaping of behaviours over a lifetime. *Frontiers in Zoology*, 12

Vezina, F., Speakman, J.R., Williams, T.D. 2006. Individually variable energy management strategies in relation to energetic costs of egg production. *Ecology*, 87 (10): 2447-2458

Ward, S., Biship, C.M., Woakes, A.J., Butler, P.J. 2002. Heart rate and the rate of oxygen consumption of flyinf and walking barnacle geese (*Branta leucopis*) and bar-headed geese (*Anser indicud*). *Journal of experimental biology*, 205 (21): 3347-3356

White, S.J., Kells, T.J., Wilson, A.J. 2016. Metabolism, personality and pace of life in the Trinidadian guppy, *Poecilia reticulata. Behaviour*, 153 (13-14); 1517-1543

Wilson, A.D.M., Krause.J. 2012. Personality and metamorphosis: is behavioural variation consistent across ontogenic niche shifts? *Behavioural Ecology.* 23 (1): 1316-1323

Wuerz, Y., Kruger.O. 2015. Personality over ontogeny in zebra finches: long-term repeatable traits but unstable behavioural syndromes. *Frontiers in Zoology*, 12

General Discussion

Sunrise at Fowlers Gap over a blanket of clouds, and one of our field vehicles.

General discussion

Given the pace of global environmental change, understanding the nature and consequence of environmentally induced variation, is of increasing importance. The research described in my thesis has focused on the abiotic and biotic stimuli that induce and maintain epigenetic and metabolic diversity in wild, Australian birds. The changes reported here may underlie ecologically relevant responses in wild populations, and may be indicative of the proximate mechanisms used to maintain variation in populations exposed to deleterious, anthropogenic change.

My thesis can be partitioned into two major foci; i) the response of DNA methylation to different environmental contexts, and ii) abiotic and biotic stimuli that induce metabolic variation, and the behavioural and developmental consequences of this. Below, I discuss my results in the broader context of evolutionary ecology, recognize the limitations of my work, and highlight future avenues of research.

The response of DNA methylation to environmental context

During my thesis I used MS-AFLP to describe variation in genome-wide DNA methylation patterns in Australian birds. In Chapter 2, I coupled the MS-AFLP technique with microsatellite genetic analyses to assess epigenetic and genetic diversity across different sites in the invasive, Australian house sparrow population. Site specific epigenetic differentiation detected throughout this population highlighted the potential for epigenetic mechanisms to respond to local habitat conditions. However the lack of relationship between epigenetic and genetic diversity within population clusters that derived from different founding events, challenged the hypothesis that high

epigenetic diversity compensates for low levels of genetic diversity (Richards et al, 2012). Our study contrasts with previous findings from an introduction of house sparrows into Kenya (Liebl et al, 2012) and emphasizes the need for invasion history to be considered before epigenetic variation can be implicated in facilitating invasive spread. Further work should focus on different factors within distinct invasion clusters (e.g. founding effects) that could be driving population-wide correlations between epigenetic and genetic diversity.

Few species have been studied in the context of range expansion (Richards et al, 2012), yet finding 'molecular markers', indicative of invasive success, could potentially aid in informing management strategies of high risk populations before ecological (and economic) damage. While this is an exciting prospect, the potential for epigenetic markers to predict invasive success requires a lot more inquiry, and the data from Chapter 2 highlight the necessity to identify epigenetic patterns in multiple and independent invasive expansions before they can be implicated in facilitating invasive success.

In Chapters 3 and 4, I assessed whether brood size and temperature affect DNA methylation in desert dwelling, and laboratory based zebra finches, respectively. Brood size changes were shown to induce genome-wide reductions in DNA methylation, whereas heat exposure significantly increased the proportion of loci that changed from a non-methylated to methylated state, and affected genome-wide levels of DNA methylation within family comparisons. Because zebra finches breed successfully in extremely variable climates with unpredictable levels of resource availability (Griffith et al, 2016), this species may, to a degree, be acclimated to unpredictable levels of thermal stress and intraspecific competition. Future work should thus augment the

environmental cues used to induce epigenetic changes in our study e.g. by exposing developing chicks to acute thermal stress, or larger brood manipulations. This could make the trends observed in our research more apparent, and more realistically depict the stress responses that may occur in wild populations. Additionally, the propensity for familial effects (e.g. genomic factors) to influence DNA methylation levels should also be controlled for in future studies, potentially by comparing DNA methylation levels in siblings raised in different environments (as in Chapter 4).

The biggest limitation in our epigenetic analyses, was the proportion of unexpected sample dropouts during MS-AFLP analyses. In order to assess the generality of our results, future work should repeat our MS-AFLP analyses using larger sample sizes. Additionally, while MS-AFLP proved an adequate technique to investigate DNA methylation changes in response to abiotic, biotic and genetic variation, the technique is limited in its ability to provide information about the sequence and function of methylated loci. Consequently, correlations between the methylation patterns observed in our study, and their phenotypic effects are yet to be elucidated (Shaham et al, 2016). It would thus be useful for future work to focus on next-generation sequence based techniques, such as bisulfite sequencing (Weyrich et al, 2016; Lea et al, 2016; Bentz et al, 2016). These methods will allow us to identify the significance of environmentally induced DNA methylation changes in shaping ecologically relevant phenotypes, via effects on gene regulation (Szyf and Bick, 2013). This line of research could also delve deeper into the proximate mechanisms underlying epigenetic variation by examining environmental effects on the expression of genes involved in de novo methylation and its maintenance across cell division (such as DNMTs (Dorts et al, 2016)).

Epigenetic variation has been proposed to act as a more rapid source of variation than genetic variation alone (Olsen et al, 2012). In line with this, we identified high levels of epigenetic diversity in Chapter 2, and in Chapters 3 and 4 we identified a high rate of methylation state change among all loci (e.g. ~38% of all loci changed methylation state throughout post-natal zebra finch development in Chapter 4). Because some beneficial DNA methylation changes can lead to the fixation of certain phenotypes via genetic assimilation (Hughes et al, 2012), future work should consider (the potentially rapid) epigenetic bases of adaptation, by assessing heritability of environmentally induces methylation changes. This could provide a more nuanced understanding of the timing and drivers of phenotypic variation. That being said, given the high rate of change in DNA methylation patterns, it has been reasoned that adaptation archived via an epigenetic mechanism could be lost again just as quickly as it arose (Kilronomos et al. 2012). Consequently, evolutionary scenarios where pure epigenetic variation would be expected to persist and contribute to adaptation may be limited to those that impose very strong selection. An additional challenge for future work is consequently to determine the scenarios under which changes in DNA methylation can be transmitted to future generations and realistically contribute to adaptation via natural selection.

The consequences of environmentally induced metabolic variation

For the second focus of my PhD, I assessed how abiotic and biotic pressures influence embryonic metabolic rate (heart rate), and the behavioural and developmental consequences of this. To assess metabolic rate in wild zebra finches, we utilized a new machine; the Buddy digital egg

monitor. Because measuring metabolic rate in oviparous embryos has historically been expensive, invasive and impractical (Lierz et al, 2006), I first sought to demonstrate the wide range of research questions that could benefit from use of the Buddy digital egg monitor in Chapter 5. In finding that heart rate is associated with a range of environmental stimuli, we encourage future work to take advantage of the egg monitor to (among others) estimate embryonic age, assess thermal acclimation, and examine embryonic awareness of environmental and social cues. The research possibilities for this monitor are only just beginning to be explored, and the final two chapters of my thesis demonstrated its potential to provide novel insights into an interesting and relatively poorly studied stage of avian life history.

In Chapter 6 of my thesis, I used the egg monitor to explore the ability of wild zebra finch embryos to pay attention to different acoustic cues. This chapter was inspired by a recent study on captive zebra finches which suggested that vocalizations made during incubation can tailor offspring development to prevailing climate conditions (Mariette and Buchanan, 2016). Our data from Chapter 6 have demonstrated acoustic sensitivity in zebra finch embryos, however exactly what elements of vocalizations are important for offspring development, or how an acoustic signal could manifest as a phenotypic adaptation remains unclear. Future studies should focus on how a vocal cue could influence embryonic development, for example, are elements of parent vocalizations incorporated into the begging calls of nestlings? If so could they act as cues for the parent to provision them differently according to environmental conditions? Alternatively could parental vocalizations affect developmental trajectories through neurobiological, hormonal or metabolic processes?

While Chapters 5 and 6 demonstrated that embryonic heart rate is extremely sensitive to a range of biotic and abiotic stimuli, the final chapter of my thesis explored the consequences of this for inter-individual variation in behaviour and developmental rate. Our results preliminarily suggest that inter-individual variation in metabolism can affect development within the same life stage, however the potential for these affects to persist across developmental boundaries requires further investigation. While we successfully quantified consistent inter-individual differences in post-natal activity levels and pre-natal metabolic rates in this study, our main limitation was our inability to measure levels of pre-natal activity levels, and post-natal metabolic rates. Thus the degree to which metabolism effects variation in behaviour and development in the same ontogenic stage, and the degree to which metabolic variation persists across ontogenic stages remains unclear. Addressing these limitations will not only enable us to interpret the results of Chapter 4 more coherently, but also help to more generally clarify the role of metabolic rate in maintaining consistent individual differences across an individual's life.

Literature cited

Bentz, A.B., SIrman, A.E., Wada, H., Navara, K.J., Hood, W.R. 2016. Relationship between maternal environment and DNA methylation patterns of estrogen receptor alpha in wild eastern bluebird (*Sialia sialis*) nestlings: a pilot study. *Ecology and Evolution*. Doi: 10.1002/ece3.2162

Dorts, J., Falisse, E., Schoofs, E., Flamion, E., Kestemont, P., Silvestre, F. 2016. DNA methyltransferases and stress-related genes expression in zebrafish larvae after exposure to heat and copper during reprogramming of DNA methylation. *Scientific reports,* 6; 34254

Griffith, S. C., M. C. Mainwaring, E. Sorato, and C. Beckmann (2016). High atmospheric temperatures and ‘ambient incubation’ drive embryonic development and lead to earlier hatching in a passerine bird. Royal Society Open Science 3: 150371

Hughes, A.L. 2012. Evolution of adaptive phenotypic traits without positive Darwinian selection. Heredity, 108 (4), 347-353

Klironomos, F. D., Berg, J., & Collins, S. 2013. How epigenetic mutations can affect genetic evolution: model and mechanism. *Bioessays*, *35*(6); 571-578.

Liebl, A.L., Schrey, A.W., Richards, C.L., Martin, L.B. 2013. Patterns of DNA Methylation throughout a Range Expansion of an Introduced Songbird. *Integrative and Comparative Biology,* 53 (2); 351–358

Lierz, M., O. Gooss, and H. M. Hafrez (2006). Noninvasive heart rate measurement using a digital egg monitor in chicken and turkey embryos. Journal of Avian Medicine and Surgery 20: 141-146

Mariette, M.M., Buchanan, K.L. 2016. Prenatal acoustic communication programs offspring for high post hatching temperatures in a songbird. *Science,* 353 (6301); 812-814

Olsen, A.S., Sarras, M.P., Leontovich, A., Intine, R.V. 2012. Heritable transmission of diabetic metabolic memory in zebrafish correlates with DNA hypomethylation and aberrant gene expression. *Diabetes*, 61; 485-491

Richards CL, Schrey AW, Pigliucci M. 2012. Invasion of diverse habitats by few Japanese knotweed genotypes is correlated with epigenetic differentiation. *Ecology Letters*. 15:1016–25.

Shaham, R., BenShlomo, R., Motro, U., Keasar, T. 2016. Genome methylation patterns across

castes and generations in a parasitoid wasp. *Ecology and Evolution*, 6 (22): 7943-7953

Szyf. M., Bick. J. 2013. DNA methylation: A mechanism for embedding early life experiences in the genome. *Child development*. 84(1); 49-57

Weyrich, A., Lenz, D., Jeschek, M., Hung Chung, T.,Rubensam, K., Goritz, F., Jewgenow, K., Fickel, J. 2016. Paternal intergenerational epigenetic response to heat exposure in male Wild guinea pigs. *Molecular ecology*, 25; 1729-1740

Conclusion

Flying doctor and second opinion; medical services in the Australian outback photographed at Broken Hill airport (artist: Howard Steer)

The phenotype depends to a significant degree on the environment; and this is a necessary condition for integrating the developing organism into its particular habitat. While animals have evolved biological rhythms and movement patterns to survive predictable or slow-paced environmental changes (e.g. day to night, seasonal fluctuations, and climate cycles) they are also increasingly exposed to rapid anthropogenic changes (habitat destruction, species extinction, trophic

cascades, pollution etc.) which are less predictable. The pace of genetic based evolution (via random mutation and natural selection) is often too slow to respond to rapid environmental disturbance. Thus, it is thus becoming increasingly important to understand alternative mechanisms through which environmentally induced variation can emerge in wild populations. Broadly, my PhD thesis has demonstrated that epigenetic and metabolic processes are extremely responsive to prevailing biotic and abiotic conditions. These findings establish foundations for future work to address the potential for environmentally induced epigenetic and metabolic variation to mediate ecologically relevant phenotypic plasticity and rapid adaptation in natural populations.

Appendix 1.

In the initial stages of my PhD, I set up a large number (~300) of house sparrow nest boxes in various locations across rural Tasmania and NSW. I established a wide-spread study site that could be used to monitor house sparrow breeding over different Australian habitats and climates. Whilst I was working on this project, I also systematically searched for natural house sparrow nests, and recorded characteristics of their whereabouts. Although the house sparrows were slow to use these nest boxes, this endeavor allowed me to assess nest site selection in Tasmanian house sparrows, and culminated in a published article, in the Journal of Avian Research (below).

Some of the 100's of house sparrow nest boxes I constructed and placed across rural NSW and Tasmania. A Tasmanian ring tailed possum awoken from a nap in a nest box, and a natural house sparrow nest in Plenty, Tasmania.

A high incidence of non-cavity nesting in an introduced population of House Sparrows suggests that the species should not be constrained by cavity-nest site availability

Elizabeth Louise Sheldon* and Simon Charles Griffith

Department of Biological Sciences, Macquarie University, Sydney, New South Wales 2109, Australia.

* To whom correspondence should be addressed

E-mail: elizabeth-louise.sheldon@students.mq.edu.au

Background The house Sparrow (*Passer domesticus*) has undergone dramatic population declines in many parts of Europe. It has been widely hypothesised that a lack of cavity nest sites has contributed to this decline. However, the idea of the House Sparrow being nest site limited is somewhat incompatible with the long history of nest site plasticity in the species.

Methods The nest-site selection in a population of non-native House Sparrows introduced to Australia from Europe just over 150 years ago was characterised. The prevalence of non-cavity nesting was quantified, and nest-site selection in terms of landscape and nesting structure were described.

Results Flexible nesting behaviors were reported over a range of different landscapes and a surprisingly high rate of nesting in vegetation (43%) was documented. Most nests found in vegetation were not in cavities, but were woven into the foliage and supported by branches and stems.

Conclusion The high rate of vegetation nesting indicates that in this introduced population, the House Sparrow is unlikely to be constrained by cavity-nest site availability. The high degree of nest site plasticity in the Australian population may suggest that European House Sparrows have the potential to shift away from their proclivity for cavity nests. Future work in Europe should examine the incidence of non-cavity nesting in House Sparrows more closely, and perhaps reconsider the idea that House Sparrows are nest-site constrained.

Key words: *Passer domesticus*, Cavity nests, House Sparrow decline, Nesting plasticity

Background

Although the House Sparrow (*Passer domesticus*) has been introduced to many parts of the world, and is now one of the most globally distributed of all birds (Pimentel et al. 2005; Shochat et al. 2010), it has become a species of conservation concern in parts of its native range (Summers-Smith 2003; Robinson et al. 2005). Throughout Northwest Europe, House Sparrow population declines have been abrupt and widespread, with the species currently on the red list of conservation concern in the UK (Crick and Siriwardena 2002). Whilst the cause of the decline remains unclear, one suggestion has been that an increased prevalence of modern or renovated buildings has reduced the availability of crevices for nesting (Shaw et al. 2008). An increasing number of studies have cited nest site limitation as the leading (Dandapat et al. 2010; Balaji 2014; Nath et al. 2016) or contributory cause of House Sparrow population declines (Summers-Smith 2003; Goyal 2005; Anderson 2006; Ghosh et al. 2010; Balaji et al. 2013; Singh et al. 2013; Paul 2015). However, the idea of the House Sparrow being nest site limited is somewhat incompatible with the long history of nest site plasticity in the species.

Whilst nesting behaviour in passerines generally shows a high degree of evolutionary conservatism (Price and Griffith 2017), the House Sparrow has always been recognised as a species that shows flexibility and innovation (Summers-Smith 1963; Martin and Fitzgerald 2005; Anderson 2006). House Sparrows have been reported to excavate cavities for nesting in the ground and in branches (Pitman 1961; Ivanitzky 1996), usurp and modify other birds' nests, nest in the walls of nests of other larger birds (see Anderson 2006 for a review), nest in hornets nests (Bent 1958), hay

bales (Werler and Franks 1975), burrows (Chmielewski et al. 2005), billboards (Burrage 1964), hanging clothing (Sharma 1995), and moving machinery (Tatschl 1968; Weber 1976). In Asia, rocky cliffs have been reported as a common nesting site (Summers-Smith 1963; Schmidt 1966). Further, tree nesting has been considered as an alternative nesting option used when building cavities are limited due to inter-specific competition from the Tree Sparrow (*Passer montanus*) (Morris and Tegetneier 1896; Summers-Smith 1963). Tree-nesting has also been reported following high population densities of invasive House Sparrows after their introduction to the USA (Barrows 1889).

Here, we aim to describe House Sparrow nesting behavior in an introduced, Australian population of House Sparrows. The House Sparrow was introduced into Australia from Europe in 1862 and its population has become established across the eastern half of the continent (Andrew and Griffith 2016). Australian House Sparrows live in heavily human-modified environments in urban and rural settings, however the Australian environment has different climates, predators, and competitors from the natal range. These ecological differences likely presented novel challenges for colonising House Sparrows, however the population's successful establishment and expansion suggests that these sparrows effectively adapted nesting (and other) behaviours to their new environment. In this study, we aim to quantify the frequency of House Sparrow nests in Tasmania (Australia) over rural and urban habitats with different levels of building cover, and describe the height and location of each nest. Describing nesting behaviours of House Sparrows from a recently introduced population such as this can provide insights into either the pre-existing plasticity of House Sparrow nesting behaviour, or its potential to respond to local selection over a relatively

short period of time (~150 years). Understanding how House Sparrows have responded to novel nesting challenges in their introduced range may also provide insights into how House Sparrows may respond to reported nest site alterations (i.e. cavity nest site limitations) in their native range (Wotton et al. 2002).

In addition to describing House Sparrow nesting behaviour in Tasmania, we also aim to provide a summary of research relating to House Sparrow nesting restrictions and population trends. We aim to highlight the difference between studies that infer House Sparrow population trends from nest-site availability/selection surveys, and studies that measure population trends and relate these measures to nest site availability/selection surveys. This may help to clarify a distinction between research that focuses on the availability of preferred nest sites and research that focuses on the availability of nest sites that are a critically limiting resource affecting House Sparrow population size.

Methods

Fieldwork was conducted at a number of locations throughout Tasmania during the Austral spring and early summer (October-December) in 2015. Sites were selected using a stratified sampling technique to ensure that only habitats suitable for House Sparrows were included in the study. We targeted areas where sparrows were known to be living and for which we could gain access by prior communication with landowners and Bird-Life Tasmania members. As a result, we identified and worked at 92 focal sites throughout Tasmania, including farms, horse stables and residential houses in urban, suburban and rural settings. At each site, we systematically searched for nests within the

focal site, and along random transects in the area surrounding the focal site. We had no prior knowledge of House Sparrow presence in the areas adjacent to the focal site, although we had asked landowners whether sparrows were found in the area. Surrounding sites were generally along streets and paths within 500 m of the focal site.

We searched for active House Sparrow nests primarily by observing parental behaviour around the nest, including: males singing from around the nest; construction of the nest; or visits to provision nestlings. Subsequent inspection and the finding of eggs or nestlings confirmed active nests. Although site selection was initially directed by the presence of House Sparrows on the property, at each site our searchers tried to cover all likely possibilities for nesting. We searched for House Sparrow nests by looking in every accessible building, appropriate flora (e.g. trees, bushes, hedges, and shrubs), and other structures (sheds, hollow posts, carports, etc.) around the site. We moved through the area surrounding the focal site at a slow walking pace, and observed buildings and flora for House Sparrow nesting activity by walking along pavements, paths, roads, and along field/park boundaries. We also requested access to private gardens when they restricted our observations (however we did not record the frequency with which we were granted garden access).

Using aerial digital images (Google maps), we categorised the percentage of building cover within 500 m^2 of the 92 sites. To do this we divided the 500 m^2 area around the site into 5 × 5 grids, and visually estimated the percentage of each grid that was covered with buildings rounded to the nearest 5%, which described 20 different levels of building cover over which House Sparrows could

nest. We used the average of these measurements to provide each site with a percentage of building cover, i.e. an estimation of urbanisation.

Once an active nest was identified we estimated a number of nest-site characteristics; nest height from ground, proximity to other nests and buildings, and the height of the highest and average structure within an estimated 50 m diameter of the focal nest. Measures of the nesting vegetation included: the clear bole height (a perpendicular measure of the distance from the base of the tree to the first living branch on the trunk), the canopy height, the percentage of the vegetation canopy missing (estimated in the field from four cardinal directions) and the canopy volume (obtained by multiplying π by the square of the crown radius and canopy height). The trunk diameter at breast height of the majority of hedges with nests could not be estimated. We estimated the highest point of the vegetation or building that a nest was found in, and the height of the nest from the base of the vegetation/building. The distance from the nest to the nearest building and road was also estimated.

We quantified the frequency of nests found at each of the 25 different levels of building cover, and the frequency of nests found in particular locations. We ensured to include vegetation as potential nesting sites, as although trees and hedges are documented to be used by House Sparrows for nesting, their inclusion into nesting surveys is extremely limited (see discussion). We also quantified the frequency of nests found within a particular distance from the nearest conspecific nest.

We used a linear regression analysis to test whether the 'percent of building cover' could predict the number of nests found per km^2. A binomial logistic regression was also performed to ascertain

the effects of ‘percent of building cover’, ‘distance to the nearest road (m)’, and ‘the average structure height in a 50 m^2 area’ on the likelihood that vegetation or a building was used to nest in.

We used an independent *t*-test to test 1) whether there is a significant difference between the number of nests per individual vegetative structure and individual building, 2) whether buildings used to nest in are significantly higher than vegetation, and 3) whether vegetation nesting heights are higher than building nest heights.

Results

A total of 309 active House Sparrow nests, distributed across the 92 study sites, were found. In total, 133 (43%) nests were found in vegetation; of these, 131 (42.4%) were in tree/hedge branches supported by fine, densely entangled twigs, stemming off a main branch, and 2 (0.6%) were found in tree cavities. Overall 171 (55.3%) nests were found in buildings; of these, 93 (30.1%) were found under eaves or in walls and 70 (22.6%) were found under roofing structures such as tiles (generally in the open space between curved tiles and gutters); 4 (1.3%) were found in chimneys, and 4 (1.3%) were found in other structures; a hollow post, two sign poles and an abandoned vehicle engine (Fig. 1). Finally, 5 (1.6%) nests were found in wall/fencing climbers, and we refrained from ascribing these to either buildings or vegetation. The characteristics of nest vegetation varied greatly (Table 1), however dense trees/bushes were a common feature, as indicated by the substantial average canopy volume, average low percentage of canopy missing, and the average low clear bole height (Table 1).

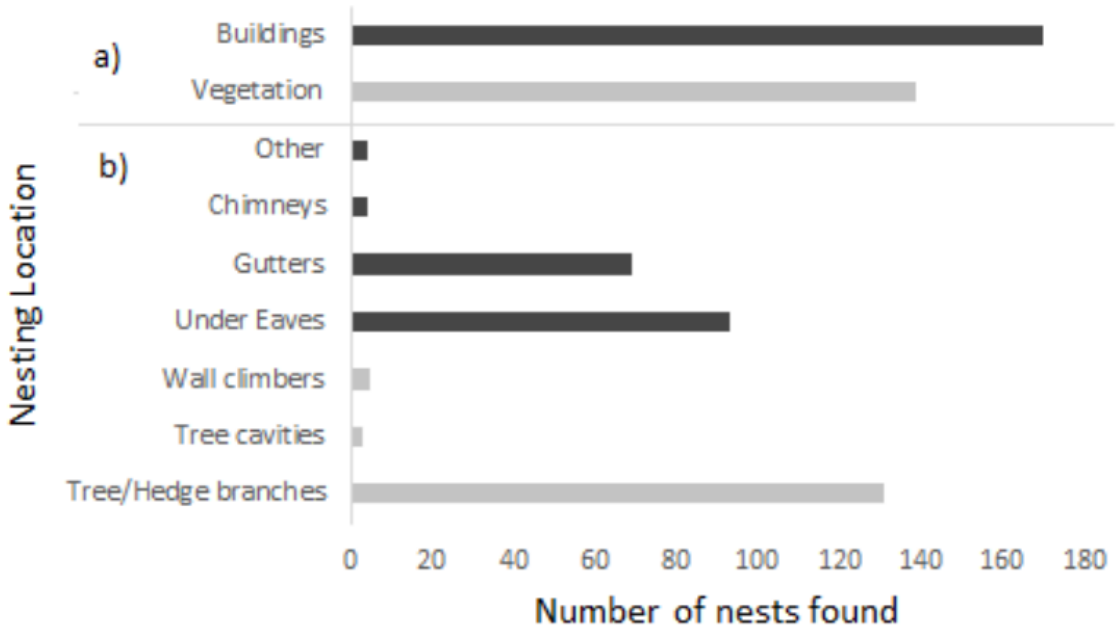


Figure 1. The frequency of nests found at different locations; a) the total number of nests in buildings and vegetation, b) the frequency of nests in different locations within the trees (grey bars) and buildings (black bars).

Table 1. The characteristics of vegetation used by House Sparrows to nest in.

Vegetation characteristics	**Mean**	**SEM**	**Range**	***n***
Trunk diameter at breast height (cm)	3.63	2.68	0.2-17	110
Clear bole height (m)	0.54	0.06	0-3.5	131
Canopy height (m)	4.31	0.35	0.1-23	131
Canopy missing (%)	25	1.56	10-90	131
Canopy volume (m^3)	47.3	6.9	1. 1-263.1	131

A linear regression was calculated to predict the number of nests per km^2 based on the percent building cover. A significant regression equation was found ($F_{1,13}$ = 12.383, p = 0.004, with a R^2 of 0.508, indicating that the reduced building cover was associated with an elevated number of nests found per km^2. The number of nests found per km^2 decreased by 3 nests for each 20% of building cover increase (Fig. 2). We acknowledge that surveillance differences experienced between built up and rural areas (e.g. a potentially reduced detection of nests in vegetation of built up areas due to limited access to some private properties) could affect this result.

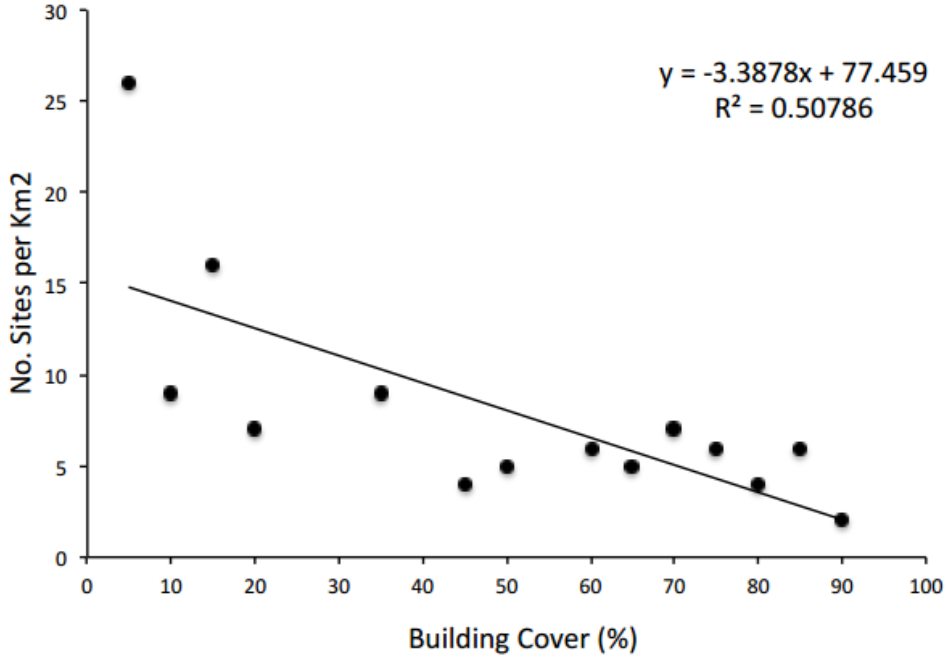


Figure 2: A significant relationship exists between the number of sites per km^2 and the percent building cover.

The binomial logistic regression model explained only 4.5% (Nagelkerke R^2) of the variance in nesting location, and correctly classified 65.5% of cases. The likelihood that a nest was in vegetation instead of a building was not associated with the height of the average surrounding structure; the proximity of the nest to a road; or the level of building ($x^2 = 3.625$, $p = 0.305$). Details of the average nest heights, and surrounding landscape features can be found in Table 2.

Table 2. The height of House Sparrow nests, and the structure they were in and near.

Variable	**Mean (±SEM)**	**Range**
Nest height (m)	3.25 (0.09)	0.75–10
Nest structure height (m)	4.78 (0.17)	1.2–2.4
Nest height as a percentage of structure height (%)	71.9 (0.01)	12.5–100
Average structure height within 50 m^2 (m)	5.5 (0.19)	2–25
Highest structure within 50 m^2 (m)	14.6 (0.4)	3–30

Overall, the majority of nests (54.9%) were between 1–5 m away from the nearest conspecific nest (Table 3). As building cover increased, the distance between nearest nests also increased (Pearson's correlation, $r = 0.359$, $p < 0.001$, $n = 206$), i.e. nests seem to be less aggregated in areas with a higher density of buildings.

Table 3. Proximity of each nest to the nearest conspecific nest (n = 206).

Nearest nest	Frequency of nests (%)
<1 m	12.1
1–5 m	54.9
5–15 m	18
15–30 m	7.8
>30 m	8

Note: Some concealed nests and nests detected indirectly (e.g. through parental provisioning) were not included in this analysis, as the nearest nest could not be detected or accurately estimated.

We identified 71 separate vegetative structures that contained nests, and 83 buildings. Of the 71 trees/bushes in which active nests were identified, on average, 2.25 ± 0.21 (SEM) nests were found in each vegetation structure (range: 1–10). For the 83 buildings in which active nests were located, on average 1.78 ± 0.16 nests were found per building (range 1–8). There was a trend for the mean number of nests per vegetation/bush to be higher than the mean number of nests per building, however this trend was not significant (independent *t*-test; $t_{(152)} = 1.82$, $p = 0.071$) (Table 4). The buildings used for nesting were not significantly higher than the vegetation used for nesting $t_{(307)} = 0.127$, $p = 0.899$ (Table 4). The mean nest height was however significantly higher in buildings than in vegetation $t_{(305)} = -4.64$, $p > 0.0001$ (Table 4).

Table 4. Independent *t*-test results for the effects of nesting in vegetation compared to a building on three dependent variables (significant results indicated by italics).

	Structure		**Independent *t*-test**	
Dependent variables	Vegetation (±SEM)	Building (±SEM)	*t*-value (df)	*p*-value
Mean number of nests per structure	2.25 (0.21)	1.78 (0.17)	1.82 (152)	0.071
Mean nest height in structure	2.76 (0.14)	3.61 (0.12)	–4.64 (305)	*>0.001*
Mean structure height	4.8 (0.35)	4.76 (0.15)	0.127 (307)	0.899

Discussion

Nesting in the branches of trees and bushes (rather than tree or building cavities) has been considered an uncommon behavior by House Sparrows (Summers-Smith 1963; Van der Elst 1981). However, we could only find three 'House Sparrow nesting studies' in the literature that include both buildings and vegetation as potential nesting sites, and quantify House Sparrow nest-sites randomly over different environments (Kulczycki and Mazur-Gierasinska 1968; Indykiewicz 1991; Salek et al. 2015). Other papers that have aimed to characterise House Sparrow nesting only provide descriptive generalisations of nesting locations, isolated examples of rare/unusual nesting sites, the frequencies of nests found in sought after locations, or the frequency of unoccupied, potential nest sites (e.g. Summers-Smith 1958; Heij 1985; Imboma, 2014; Peach et al. 2015; Nath et al. 2016). For example, a study from urban India (Guwahati) found that the majority of House

Sparrow nests were in rolling shutters, and close to walls associated with a pipe (Nath et al. 2016), however the study did not consider or sample vegetation as potential nesting sites at all.

When we compared Tasmanian House Sparrow nesting locations in the present study to the nest locations documented in other studies (e.g. Kulczycki and Mazur-Gierasinska 1968; Indykiewicz 1991; Salek et al. 2015), the most notable difference was the proportion of nests supported by branches/stems in vegetation. Two studies from Poland (Kulczycki and Mazur-Gierasinska 1968; Indykiewicz 1991) found 0 to 19% of nests in vegetation over urban, suburban and rural areas, compared to 43.5% in the present study. The study by Salek et al. (2015) in the Czech Republic similarly found only 2.4% of House Sparrow nests in vegetation, with the large majority being under roof tiles (80%) and in building crevices (17.5%). The lack of vegetation nesting in the Polish studies could indicate temporal differences in the nesting habitats of House Sparrows; the Polish studies are 48 and 25 years older than the present study (Kulczycki and Mazur-Gierasinska 1968; Indykiewicz 1991), and over this time period habitat modifications (e.g. building renovations) may have impacted nesting choices. Ecological differences between introduced Tasmania and native European environments could also have altered selection pressures driving nest site selection differences. Nonetheless, the detection of high levels of vegetation nesting in Tasmania necessitates accurate, and current descriptions of nest-site selection in declining, native populations. This will clarify whether House Sparrows can generally circumvent the effects of cavity limitation by nesting in vegetation, or if this behavior is unique to introduced populations.

We found more House Sparrow nests in areas with lower building cover; however building cover did not affect the probability of finding a nest in vegetation or a building. Further, we found House Sparrow nests to be less aggregated as building cover increased, and more nests were found per site in areas with lower levels of building cover than more built up areas. These results suggest that rural 'hot spots' are supporting much of the nesting in Tasmania. However, given that nesting in vegetation or a building is not dependent on building cover, it is unclear whether the high incidence of rural nesting is due to the availability of suitable nest sites, or the quality of the surrounding habitat for sparrows (e.g. feeding and predator avoidance opportunities) (Chamberlain et al. 2007). We found that House Sparrow nests tend to be higher in buildings compared to nests built in vegetation (however House Sparrows do not tend to nest in the highest available nest site in an area), and House Sparrows tend to nest in dense, bush-like vegetation. These results suggest that protection from predators and nesting support via dense bushes, and building crevices are important criteria for nest site selection in both vegetation and buildings for the House Sparrow.

As House Sparrows were introduced to Tasmania, the potential exists for vegetation nesting in this environment to be a result of the invasion process, or enemy release. Vegetation nesting behaviours may be selected against in Europe, as squirrels, predatory birds and domestic cats are likely to decimate the majority of tree-nesting attempts. However, these pressures are not alleviated in Tasmania where predatory threats from snakes, domestic cats, possums, and predatory birds are common. Additionally, although it is possible, it seems unlikely that the Australian flora has structural traits more conducive to House Sparrow nesting (especially given the sparse foliage of native Eucalypts), and sparrows were found to nest in both native and invasive bushes. Finally,

elevated levels of aggression (Duckworth and Badyaev 2007), and behavioural flexibility (Wright et al. 2010) have been reported to facilitate range expansion in invasive bird populations (Duckworth and Badyaev 2007). Indeed, invasive House Sparrows are known to displace native birds from their nests throughout the USA (Shochat et al. 2010) and are also known to be more exploratory than native House Sparrows (Martin and Fitzgerald, 2005). Such behaviours may have facilitated the establishment of previously rare nesting habits, i.e. nesting in the branches of trees, in the Australian environment. Recent nest site quantifications from the House Sparrow's native environment are necessary to enable comparative studies to clarify whether nesting flexibility in Tasmania is a characteristic of invasive birds, or of House Sparrows in general. However, given the House Sparrow's propensity to nest in varied environments in its native range, we believe the adoption of vegetation-nesting is an additional example of pre-existing nest site plasticity in this species.

We identified several studies in the literature that cite building-cavity limitation as a key parameter in influencing House Sparrow abundance (Table 5). However, on closer examination, most of these studies provide little conclusive proof that the availability of building cavities is a key determinant of reproductive success and population trends (see Table 5a). The few studies available that have focused directly on the effects of nest site availability on the population size of House Sparrows have not been able to find an association (see Table 5b).

Table 5 A summary of a) studies inferring House Sparrow population trends from nest-site availability/selection surveys, and b) studies focusing on population trends and nest site selection/availability

(a) Citation	**Summary**	**Reasoning**	**Weakness of the study in respect to the conclusion drawn**
Sziemer and Holzer 2005; Shaw et al. 2008; Kumar et al. 2015	High incidence of House Sparrow breeding in low socio-economic areas.	Low socio-economic areas have more neglected buildings thus more nesting opportunities.	No evidence of nest site limitation in areas of high socio-economic status. Low socio-economic areas could attract House Sparrows through alternative factors, e.g. invertebrate abundance.
Wotton et al. 2002	House Sparrows are more abundant in older building in rural, but not (sub) urban areas.	Older, rural buildings are not renovated thus have more crevices for nesting.	Public survey data overstates the proportion of older, rural houses available for nesting.
Singh et al. 2013; Balaji et al. 2014	Fewer House Sparrows in urban buildings	Urban buildings are more renovated, thus offer fewer nest sites than rural ones	Studies assume urban areas are more renovated than rural/sub-urban ones without examining the frequency of potential nest-sites in the different settings.
(b) Citation	**Summary**	**Reasoning**	
Von Post and Smith 2015	Although House Sparrows show a preference for nesting under tiles, nest site availability is not a critically limiting resource.	No relationship between the availability and addition of preferred or artificial nest sites affected population numbers.	
Wegrzynowicz 2012	Nest site availability does not affect House Sparrow population trends.	No relationship between the number of available nest sites and House Sparrow population number.	

Consequently, demonstrating that the House Sparrow has a preference for nesting in building cavities does not provide clear evidence that these sites are declining, or a critically limiting resource affecting population sizes. A more comprehensive understanding of House Sparrow nesting is necessary before predictions on the effects of nest-site limitations can be considered to play a role in the population decline of this species.

There are clear challenges involved in measuring the number of small, inconspicuous crevices of all buildings in an area, especially as the number of crevices differs temporally as well as spatially due to the transient nature of building renovations and deteriorations. We have highlighted that the number of crevices should not necessarily relate to the number of suitable nesting sites for the House Sparrow, given its broad nesting niche. Most of the existing studies we have reviewed for example have carried out a directed search for House Sparrows/nests in buildings. This would reduce the possibility of detecting the House Sparrows accurate nesting range e.g. in vegetation and nests of other birds. Differences in small-scale habitat features could also contribute to differences in nesting availability and the impact of cavity reductions. For example, Summers-Smith (2003) suggests that the effect of reduced building cavities is likely to be more severe in areas that do not have alternative nesting opportunities (e.g. in cities with no hedges). As the House Sparrow is such a flexibly nesting species, it is evident that quantifying every potential nesting opportunity would be very difficult. Consequently, it will be difficult to convincingly determine that a decline in House Sparrow numbers is linked with nest site availability. Furthermore, given that housing deterioration is more likely in socially deprived areas it would be especially challenging to detect the effects of a

reduced number of building cavities independently of other ecological factors, such as foraging opportunities and predation avoidance.

Conclusion

In this study, we have characterised nest-site selection of the House Sparrow in a population introduced to Australia just over 150 years ago. We report flexible nest site selection, and document a surprisingly high rate of nesting in vegetation (43% of all nests were found woven into branches and stems rather than in cavities). The high rate of nesting in vegetation in Tasmania suggests that, contrary to numerous suggestions (e.g. Summers-Smith 2003; Shaw et al. 2008; Ghosh et al. 2010; Nath et al. 2016), House Sparrows may not be inherently dependent on cavities in buildings for nesting sites. Further, because the studied Australian House Sparrows have recently descended from European ancestors, our findings may suggest that House Sparrows in Europe are either already capable of constructing free-standing nests in vegetation (and perhaps this is not being examined or detected), or are likely to be able to shift their nesting niche in a relatively short period of time. Our findings emphasise the need for future work to examine the incidence of non-cavity nesting in House Sparrows.

Declarations

ELS and SCG conceived the idea; ELS carried out the fieldwork and analysis, ELS and SCG have both contributed to the writing of the manuscript. Both authors have read and approved the final manuscript.

Acknowledgements

We would like to thank Eric Woehler, James Pay, and Bird Life Tasmania for their guidance throughout the project, Elissa Cameron and the University of Tasmania for their logistical assistance, and the landowners who provided access to their properties during field work. The research was supported by Australian Research Council funding to SCG (FT130101253), and an MQRES PhD scholarship to ELS from Macquarie University.

Competing interests

The authors declare that they have no competing interests

Ethical standards

The Animal Ethics committee at the Department of Biological Sciences, Macquarie University, approved our research

References

Anderson TR. Biology of the ubiquitous House Sparrow: from genes to populations. Oxford (UK): Oxford University Press; 2006.

Andrew SC, Griffith SC. Inaccuracies in the history of a well-known introduction: a case study of the Australian House Sparrow (*Passer domesticus*). Avian Res. 2016;7:9.

Balaji S, Baskaran S, Rajan MK, Pavaraj M. Investigating the cause for the decline and strategies to conserve House Sparrow, *Passer domesticus* in Sivakasi Taluk, Virudhunagar district, Tamil Nadu, India. World J Zool. 2013;8:278-84.

Balaji S. Artificial nest box for House Sparrow: An apt method to save the dwindling species in an urban environment. Int J Biodiver Conserv. 2014;6:194-8.

Barrows WB. The English Sparrow (*Passer domesticus*) in North America: especially in its relations to agriculture. Washington: Government Printing Office; 1889.

Bent AC. Life histories of North American blackbirds, orioles, tanagers, and allies. U.S. National Museum Bulletin. New York: Dover Publications. 1958. p. 1-549.

Burrage BR. A nesting study of the House Sparrow (*Passer domesticus*) in San Diego County, California. Trans Kansas Acad Sci. 1964;67:693-701.

Chamberlain DE, Toms MP, Cleary-McHarg R, Banks AN. House Sparrow (*Passer domesticus*) habitat use in urbanised landscapes. J Ornithol. 2007;148:453-62.

Chmielewski S, Fijewski Z, Nawrocki P, Polak P, Sułek J, Tabor J, Wilniewczyc P. Birds of the Świętokrzyskie Mountains and their vicinity. The Faunistic Monograph. Poznań: Bogucki Wyd Nauk; 2005.

Crick HQP, Siriwardena GM. National trends in the breeding performance of House Sparrows *Passer domesticus*. In: Crick HQP, Robinson RA, Appleton GF, Clark NA, Rickard AD, editors. Investigation into the causes of decline of starlings and House Sparrows in Great Britain. BTO research report 290, DEFRA, Bristol; 2002.

Dandapat A, Banerjee D, Chakraborty DD. The case of the disappearing House Sparrow (*Passer domesticus*). Veterin World. 2010;3:9.

Duckworth RA, Badyaev AV. Coupling of dispersal and aggression facilitates the rapid range expansion of a passerine birds. Proc Natl Acad Sci USA, 2007;104:15017–22.

Ghosh S, Kim K, Bhattacharya R. A survey on House Sparrow population decline at Bandel, West Bengal, India. J Korean Ear Sci Soc, 2010;31:448-53.

Goyal M. Preliminary survey of House Sparrow (*Passer domesticus*) in three different areas of Haridwar, Uttaranchal. Dissertation; 2005.

Heij CJ. Comparative ecology of the House Sparrow *Passer domesticus* in rural, suburban and urban situations. PhD Thesis. Amsterdam: University of Amsterdam. 1985.

Imboma TS. Ecological effects of the invasive House Sparrow (*Passer domesticus*) on sympatric native Sparrows in three urban areas in Kenya. Master's Thesis. Nairobi: University of Nairobi. 2014.

Indykiewicz P. Nests and nest-sites of the House Sparrow *Passer domesticus* (Linnaeus, 1758) in urban, suburban and rural environments. Acta Zool Cracov. 1991;34:475-95.

Ivanitzky VV. 1996. Behavior of males and females of Indian Sparrow *(Passer indicus)* during nesting. J Zool. 1996;75:249–55

Kulczycki A, Mazur-Gierasinska M. Nesting of House Sparrow *Passer domesticus* (Linnaeus, 1758). Acta Zool Cracov. 1968;9:231-50.

Kumar A, Kushwaha S, Kamaujia A. Rejoinder of House Sparrows (*passer domesticus*) to artificial nest boxes in Lucknow District Uttar Pradesh India. Species. 2015;15:1-13.

Martin LB, Fitzgerald L. A taste for novelty in invading House Sparrows, *Passer domesticus*. Behav Ecol. 2005;16:702-7.

Morris FO, Tegetmeier WB. A natural history of the nests and eggs of British birds II. 4th ed. London: John C. Nimmo; 1896.

Nath A, Singha H, Panna D, Dasm AK, Lahkar BP. Nesting in a crowd: response of House Sparrow towards proximity to spatial cues in commercial zones of Guwahati city. Proc Zool Soc. 2016;69:249-54.

Paul RM. A review of House Sparrow population declines in India. Asia Pac J Res. 2015;1 (29):38-40.

Peach WJ, Mallord JW, Ockendon N, Orsman CJ, Haines WG. Invertebrate prey availability limits reproductive success but not breeding population size in suburban House Sparrows *Passer domestics*. Ibis. 2015;157:601-13.

Pimentel D, Zuniga R, Morrison D. Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecol Econ. 2005;52:273-88.

Pitman CR. Unusual nesting behaviour of the House Sparrow, *Passer domesticus* (L.). Bull Brit Ornithol Club. 1961;81:148–9.

Price JJ, Griffith SC. Open Cup nests evolved from roofed nests in the early passerines. Proc Roy Soc B, Biol Sci. 2017: 284

Robinson RA, Siriwardena GM, Crick HQP. Size and trends of the House Sparrow *Passer domesticus* population in Great Britain. Ibis, 2005;147:552–62.

Salek M, Riegert J, Grill S. House Sparrows (*Passer domesticus*) and Tree Sparrows (*Passer montanus*): fine scale distribution, population densities, and habitat selection in a Central European city. Acta Ornithol. 2015;50:221-32.

Schmidt G. Haussperling (*Passer domesticus*) als Felsholenbruter am Nordmeer. Vogelwelt, 1966;67:91–2.

Sharma SK. An unusual nesting site of House Sparrow *Passer domesticus* (Linn.). J Bombay Nat Hist Soc. 1995;92:422.

Shaw LM, Chamberlain D, Evans M. 2008. The House Sparrow, *Passer domesticus* in urban areas: Reviewing a possible link between post decline distribution and human socioeconomic status. J Ornithol. 2008;149:293-9.

Shochat E, Lerman SB, Anderies JM, Warren PS, Faeth SH, Nilon CH. Invasion, competition, and biodiversity loss in urban ecosystems. BioScience, 2010;60:199-208.

Singh R, Kour DN, Ahmad F, Sahi DN. The causes of decline of House Sparrow (*Passer domesticus*, Linnaeus 1758) in urban and suburban areas of Jammu region, J & K. Entomol Zool. 2013;8:803-11.

Summers-Smith D. Nest-site selection, pair formation and territory in the House Sparrow (*Passer domesticus*). Ibis. 1958;100:190-203.

Summers-Smith JD. The decline of the House Sparrow: A review. Brit Birds. 2003;96:439-46.

Summers-Smith JD. The house sparrow. London: Harper Collins Publications; 1963.

Sziemer P, Holzer T. Vienna. In: Kelcey JG, Rheinwald G, editors. Birds in European cities. Germany: Ginster Verlag; 2005. p. 359-88.

Tatschl LJ. Unusual nesting site for House Sparrows. Auk. 1968;85:514.

Van der Elst D. Nidification du moineau danestique *Passer domesticus* dans les arbres en Wallonie. Aves, 1981;18:123-7.

Von Post M, Smith GH. Effects on rural House Sparrow and Tree Sparrow populations by experimental nest-site addition. J Ornithol. 2015;156:231-7.

Weber WC. House Sparrow nest in a revolving sign. The Mississippi Kite, 1976;6:1,7-8.

Wegrzynowicz A. The use of nest-boxes by two species of Sparrows (*Passer domesticus* and *P. montanus*) with opposite trends of abundance the study in Warsaw. Int Stud Sparrows. 2012;36:18–29.Werler E, Franks EC. Some unusual nest sites of the House Sparrow. Wilson Bull. 1975; 87:113.

Wotton SR, Field R, Langston RHW, Gibbons DW. Homes for birds: the use of houses for nesting by birds in the UK. Brit Birds. 2002;95:586-92.

Wright TF, Eberhard JR, Hobson EA, Avery ML, Russello MA. Behavioural flexibility and species invasions: the adaptive flexibility hypothesis. Ethol Ecol Evol. 2010;22:393-404.