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The molecular mechanisms and evolutionary significance of plumage colour variation in pied flycatchers

(Ficedula hypoleuca)

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IV. Lehtonen PK, Laaksonen T, Ruuskanen S, Primmer CR, Leder EH. Characterisation of the proteome of developing pied flycatcher (*Ficedula hypoleuca*) feathers. *Manuscript*

ABSTRACT

A common feature of natural populations is that individuals differ in morphology, physiology and behavior (*i.e.* phenotype). A thorough understanding of the molecular mechanisms and evolutionary forces behind this phenotypic variation is a prerequisite for understanding evolution. This thesis examines the molecular mechanism and the roles of the different evolutionary forces in plumage colour variation in pied flycatchers (*Ficedula hypoleuca*). Male pied flycatchers exhibit marked variation in both pigmentary and structural plumage colour and the trait has repeatedly been suggested to be of adaptive significance.

An examination of plumage colour variation on reproductive output revealed that structural colouration, and more specifically the degree of ultraviolet (UV) reflectance had an effect on number of young sired. Paternity analyses of breeding males revealed that males that had been cuckolded by their social mate tended to be less UV reflectant than males that had not been cuckolded. Neither pigment-based nor structural colouration was found to affect the probability of siring young in other nests.

Phenotypic differentiation was found to be markedly greater than differentiation at neutral genetic markers across the pied flycatcher breeding range. Furthermore patterns of differentiation in phenotypes and selectively neutral genes were not uniform. Outlier tests searching for genomic footprints of selection revealed elevated levels of genetic divergence in a gene associated with feather development (and thus potentially structural colouration) and ultraviolet vision. The observed differentiation in allelic frequencies was particularly pronounced in the Spanish pied flycatcher populations.

Examining gene expression during feather development indicated that the TYRP1 gene (known to be involved in the production of black pigment) may be relevant in generating phenotypic variation in pied flycatcher plumage. Also, energy homeostasis related genes featured prominently among the genes found to be expressed in one extreme phenotype but not the other. This is of particular interest in light of what is known about the pleiotropy of the melanocortin system which underlies brown-black pigment production. The melanocortin system is also associated with energy homeostasis (among a number of other physiological functions) and thus the results could be pointing to the signalling function of brown-black plumage.

Plumage colour variation in pied flycatchers, both structural and pigmentary, can thus be concluded to be exhibiting signals of non-neutral evolution. Structural colouration was found to play a role in sexual selection and putative signals of selection were further detected in a candidate gene for this trait. Evidence for non-neutral evolution of pigmentary colouration was also detected. These findings, together with the fact that preliminary evidence for an energy balance associated signalling function for plumage was found, present good starting points for further investigations into the meaning and mechanisms of plumage colour variation in pied flycatchers.

CONTENTS

1. INTRODUCTION

1.1 Evolutionary change

Evolution is change. It is the process that describes change in allele frequencies in time. Biologists have long sought to understand this change. Which factors have produced evolutionary change in a given situation? What defines the potential for change and manner in which it occurs? A thorough understanding of the components behind evolutionary change is of fundamental interest to a wide spectrum of biologists ranging from the conservationist seeking to predict how organisms cope with human-induced habitat perturbation to the naturalist seeking to understand how the species richness we see today came to be. The study of the evolutionary process continues to center on disentangling the contributions of the different evolutionary forces towards generating change and understanding how/why resultant variation is maintained (Mayr 1942, Dobzhansky 1970, Hubbard *et al.* 2010). As the evolutionary trajectory and adaptive potential of a population/species is dependent on the amount of genetic diversity in the population/species gene pool (*e.g.* Frankham 1995, Lande & Shannon 1996, Lynch 1996**,** Reed & Frankham 2003), a crucial first step in the quest to shed light on the evolutionary process is to define the amount and nature of genetic variation in natural populations (Futuyma 1998). In tow comes the evolutionary geneticist's quest for the genotype – phenotype connection; the search for the specific genes and gene regions that produce variation in phenotypes, *i.e.* the molecular mechanisms beneath evolutionary change.

The way in which gene and genotype frequencies change from one generation to the next is influenced by multiple factors at both the population and molecular levels. The genotypes present in any offspring generation are an outcome of the union of the parent gametes, and thus the genotypic frequencies of each generation will depend on the genotypes of the pairs that mated in the previous generation. It follows, that mating systems where mating is non-random, *i.e.* all individuals that belong to the population do not have an equal chance of mating, will result in a change in the gene frequencies in the next generation. Similarly, even in randomly mating populations, inherent inter-individual variation in the viability and fertility (*i.e.* fitness) of the parents that is passed on to their offspring will translate into changes in the population gene pool in subsequent generations. The impact of both of these non-mutually exclusive effects is however dependent on both the amount of gene flow there is into the population and its size. An immigrant-mediated influx of genes that would otherwise be lost from a population has the potential to override the impact of non-random mating and differential fitness on the genetic makeup of the population. Also, as the offspring generation is always a sample of the gene pool of the parent generation, gene and genotype frequencies will, to some degree, vary due to sampling variance (*i.e.* random genetic drift). The magnitude of

the impact of random genetic drift across generations is inversely proportional to population size. Finally, in addition to being determined by one or a combination of the above-described processes, contemporary patterns of genetic variation may also bear the imprint of historical events, such as post-glacial colonization routes (Hewitt 1999).

Evolutionary biology has traditionally highlighted the role natural selection as the primary mechanism driving phenotypic change (Darwin 1859, Fisher 1930). Populations of a species inhabit heterogeneous environments and are thus exposed to differing selection pressures. The differences in the traits that convey the greatest fitness advantage in a given habitat generate alternative fitness optima between the differing environments (Wright 1932, Dobzhansky 1951, Schluter 2000). As ensuing directional positive selection shifts a population's trait mean towards their specific fitness optimum, the different populations begin to diverge in phenotype, the ultimate outcome of which may be speciation.

1.2 Animal colouration – function and mechanisms

The study of animal pigmentation has long served as a model system for gaining insight into molecular, cellular and developmental processes underlying phenotypic diversity and divergence (Hoekstra 2006, Hubbard *et al.* 2010) thus shedding light on processes that may lead to population divergence and speciation. Animal colour patterns have been found to play a role in a wide range of functions which is indicative of the adaptive significance of this phenotypic trait in many species. For example, colour patterns are employed in mate choice and intrasexual communication (see *e.g.* Andersson 1994, Amundsen & Pärn 2006, Hill 2006, Senar 2006 for reviews) they may facilitate the exploitation of alternative food resources (Tso *et al.*2002, Roulin & Wink 2004) and / or serve a role in predator avoidance (Papaj & Newsom 2005, Nachman *et al.* 2003, Linnen *et al.* 2009, Steiner *et al.* 2009, Rosenblum *et al.* 2010). Additionally, the adaptive significance of colouration may stem from its role in thermoregulation (McGraw 2006, Protas & Patel 2008), microbial resistance (Goldstein *et al.* 2004), or as structural support (McGraw 2006).

Variation observed in animal coloration is a product of variation in the amounts of pigment granules (*i.e.* pigmentary variation) and/or variation in the structural properties (*i.e.* structural variation) of the integuments. The two main pigment-types generating pigmentary variation are carotenoids and melanins. Carotenoid coloration is dependent on pigment supplies obtained from the animal diet and albeit this does not rule out the scope for a genetic component (*e.g.* one that determines foraging efficiency), the link would in any case appear to be an indirect one. In contrast to carotenoids, melanins, the pigment-type focused upon in this thesis, are endogenously produced. The formation and deposition of melanin pigments is known to be under genetic control (Jackson 1994, Rees 2003, Slominski *et al.* 2004, McGraw 2006). Biochemically, the observed variation in melanin-based pigmentation stems from variation in the amounts of the two common tyrosine-derived pigment types

eu- and pheomelanin that are found in the pigmentation cells (melanocytes) of the animal integuments (*e.g.* Boswell & Takeuchi 2005, Griffith *et al.* 2006). To date, more than 150 genes that are associated with melanin-based pigment variation have been identified, cloned and sequenced (Roulin *et al.* 2004, Mundy 2005, Hoekstra 2006, Protas & Patel 2008). The vast majority of these 'pigmentation genes' have been identified in laboratory mice (Bennett & Lamoreux 2003, Hoekstra 2006). It has however become apparent that the melanocortin system, which underlies the production of melanin (melanogenesis) and thus melanin-based pigmentation, has been strongly conserved across species during vertebrate evolution (Boswell & Takeuchi 2005, Schiöth *et al.* 2005). Several attempts to dissect the genetic architecture of pigment variation in the wild have thus succeeded in finding phenotype-genotype associations in non-model organisms by examining sequence variation at genes known to be associated with pigmentation in mice and other model organisms (the so-called candidate gene approach described in more extensive detail below; *e.g.* Robbins *et al.* 1993, Valverde *et al.* 1995, Kijas *et al.* 1998, Våge *et al.* 1999, Newton *et al.* 2000, Theron *et al.* 2001, Nachman *et al.* 2003, Mundy *et al.* 2004, Rosenblum *et al.* 2003, Gratten *et al.* 2008, Kingsley *et al.* 2009, Fontanesi *et al.* 2009). Despite the apparently conserved nature of pigmentation pathways between species it is noteworthy that the obtained results have not painted a uniform picture of the underlying genetic mechanisms. Similar patterns of phenotypic variation have been found to be associated with different mutations among species (*e.g.* Schmutz *et al.* 2003, Mundy *et al.* 2004) and also among populations within a species (*e.g.* Hoekstra & Nachman 2003, Steiner *et al.* 2008, Kingsley *et al.* 2009). Furthermore many studies that have failed to detect an association between sequence variation in pigmentation genes known from model organisms and melanin-based colouration in the wild (*e.g.* MacDougall-Shackleton *et al.* 2003, Hosoda *et al.* 2005).

In contrast to melanin-based colour variation, less is known about the genetic basis of structural variation in animal integuments. In birds, for example, variation in structural coloration (such as ultraviolet; UV) stems from differences in the structural properties of the feather. In melanised feathers ultraviolet reflectance is generated by coherent scattering of light waves from layers of melanin granules found in the keratin of feather barbules (Prum *et al.* 2003, Prum 2006). Unpigmented feathers similarly distort the reflectance spectrum in the UV range, but the mechanism is slightly different as it occurs by the absorption of the feather keratin of the cortex (Finger 1995). Although no suite of 'colour genes' equivalent to this known for melanin-based pigmentation is known for structural colour variation, it seems reasonable to hypothesise that the genetic basis of structural colouration lies in the developmental pathway of the integument.

1.3 Beauty lies in the eye of the beholder – the role of vision

Measurements of colouration based on reflectance spectrometry - as opposed to human perception - have had a phenomenal

impact on studies of animal colouration (Cuthill 1999). It has become apparent that attempting to elucidate the signaling function of colour variation without acknowledging the properties of the perceptual system of the intended recipient is futile (Cuthill 1999, Endler 2005). This is well exemplified in studies on birds. Avian vision is highly developed and in contrast to humans birds have four spectral classes of single cones that appear to underlie their tetrachromatic colour vision (Osorio *et al.* 1999, Hart 2001, Goldsmith & Butler 2005). Of these, the most variable component of 'colour vision sensitivity' has been suggested to lie in the violet or ultraviolet range, a range to which humans are completely blind (Shi & Yokoyama 2003).

The sensory drive hypothesis predicts, that when females favour a male trait that is of adaptive significance, both the male trait and the perceptual system that underlies the preference adapt to local environments (Boughman 2002). This may either stem from both trait and sensor being shaped by the same environment, or because they are in fact co-evolving (Boughman 2002). The specific properties of any habitat that influence visibility are likely to impact communication (Endler 1993). Signal design has indeed been found to be associated with detectability (Scheffer *et al.* 1996, Leal & Fleishman 2004) and it is equally feasible that variable photic environments exert variable selective pressures on the perceptual system of the signal recipient. It follows that studies aiming to describe phenotypic variation in traits with potential signaling function greatly benefit from an understanding of perceptual system of the organisms that the signal is interpreted by.

1.4 The pied flycatcher as a model species

The pied flycatcher (*Ficedula hypoleuca*), together with its sister species the collared flycatcher (*F. albicollis*), has featured prominently in a number of studies examining the evolution of phenotypic diversity from multiple angles (reviewed in Sætre & Sæther 2010). One of the main reasons for this likely lies in the fact that the flycatcher system is one of the few natural vertebrate systems described where spatial variation in phenotypes has convincingly been argued to be an outcome of 'evolution in action'. Pied flycatcher males vary in a number of secondary sexual characteristics, many of which have been found to be affected by selection, making them a good target for studying the evolution of phenotypic diversity and divergence. Perhaps the most striking of these characteristics and also the one that has pinned the evolution in action –idea to the system, is the variation observed in male dorsal breeding plumage. Pied flycatcher males vary in their dorsal breeding plumage along a continuum which ranges from a female-like brown to completely black. The frequencies of differently coloured males vary across the breeding range with brown individuals being very common in Central Europe whereas darker males increase in frequency as the distance from the Central European breeding areas increases (Fig.1; Røskaft *et al.* 1986, Lundberg & Alatalo 1992, Huhta

et al. 1997, Lehtonen *et al.* 2009a). The dorsal plumage colour of pied flycatcher males is a melanin-based (Lundberg & Alatalo 1992) heritable (Alatalo *et al.* 1994) trait that has been indicated to be subjected to different selection regimes depending on the presence or absence of the dominant congeneric black-and-white collared flycatcher (Lundberg & Alatalo 1992, Sætre *et al.* 1997). The high frequency of brown, female-like males in the Central European areas of sympatry is suggested be produced and maintained as it plays a role in species recognition (Sætre *et al.* 1997) and also reduces inter-specific male-male aggression (Sætre *et al.* 1993, Alatalo *et al.* 1994). When the collared flycatcher is not present, the frequency of more darkly coloured males increases and dorsal plumage colour has been suggested to be sexually selected for. The direction of the preference has not, however, been equivocally demonstrated and a female preference for both dark males (Sætre *et al.* 1994) and brown males (Lifjeld *et al.* 1997) and the absence of any obvious preference (Lundberg & Alatalo 1992, Rätti *et al.* 1995, Lifjeld *et al.* 1998, Lehtonen *et al.* 2009b) have been reported.

In addition to variation in melanin-based colouration, pied flycatchers vary in the extent to which their plumage reflects light at ultraviolet wavelengths (UV, 300-400 nm). This trait has similarly been suggested to be shaped by selection, and sexual selection in particular (dorsal plumage: Siitari *et al.* 2002, Siitari & Huhta 2002; white wing patch: Lehtonen *et al.* 2009b, Sirkiä & Laaksonen 2009) and as for melanin-based colouration also appears to exhibit spatial variation in its intensity (Sirkiä *et al.* unpublished data). Thus, it appears that plumage colour variation in pied flycatchers – both pigmentary and structural – is a unique and valuable tool when aiming to examine, not only the evolutionary background and genetic basis of phenotypic divergence, but the evolutionary background and genetic basis of phenotypic divergence in an adaptively significant trait.

Another interesting, relevant but somewhat overlooked characteristic of pied flycatchers is however that they exhibit latitudinal variation in their propensity to disperse (von Haartman 1960, Lundberg & Alatalo 1992). The natal site fidelity in central and southern European populations tends to be higher than that found in northern Europe. Similarly, female return rates decrease with increasing latitude (von Haartman 1960, Lundberg & Alatalo 1992, Sanz 2001). These latitudinal differences in dispersal propensity have been suggested to reflect latitudinal differences in the ecological characteristics of the breeding areas (Lundberg & Alatalo 1992). For example, if patchy forest cover (Lundberg & Alatalo 1992) and/or altitude (Potti & Montalvo 1991) produces isolation or partial isolation between proximate breeding sites the probability of recruiting to the population of origin could increase, if the likelihood of 'straying' to a neighbouring population increases with increasing distance to an appropriate neighbouring site. In the context of the evolution of phenotypic divergence, the fact that pied flycatchers do not freely disperse across the breeding range raises the question of the role of isolation and subsequent drift in generating the observed spatial variation in phenotypes.

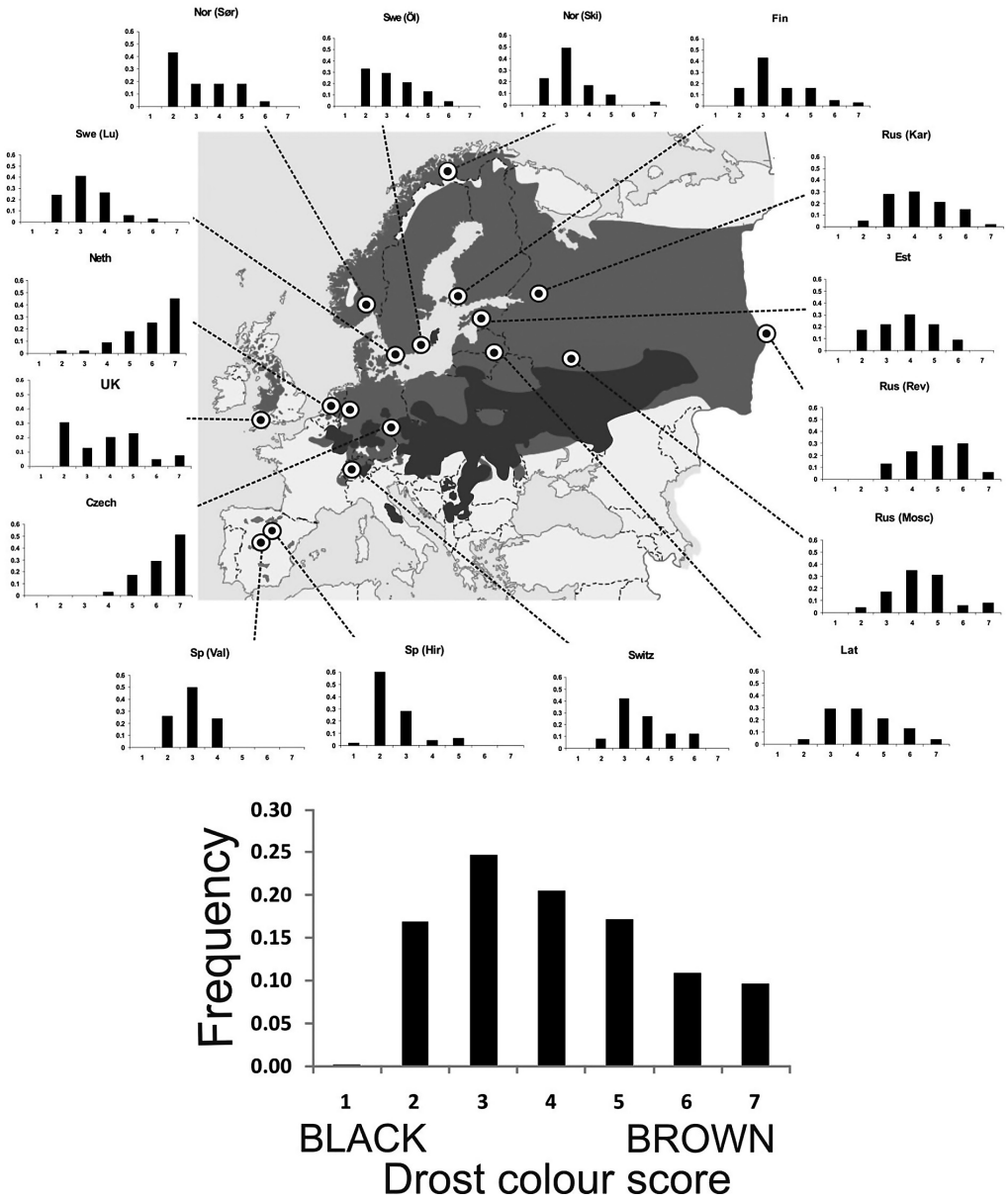

Fig 1. A map of Europe and western Russia indicating the breeding distribution of the pied flycatcher in grey. The darker grey shade in the centre indicates the breeding range of the collared flycatcher which overlaps with that of the pied flycatcher for the most part. The spots indicate the sampling sites of the populations analysed in chapters II and III. Colour was scored according to the Drost scale (Drost 1936) and the histograms represent the Drost score frequencies of each population sample. The large histogram at the bottom is drawn using all sampled individuals. No colour data was available for the German sample.

1.5 Outline of the thesis

This thesis seeks to describe components of evolutionary change using phenotypic divergence in pied flycatchers as the model system. The grand aims are to 1 – determine the relative contributions of neutral and deterministic evolutionary processes in the maintenance of structural and pigment-based colour variation in pied flycatcher plumage (chapters **I**, **II**, **III**) and 2 –elucidate the molecular mechanisms that underlie variation in this trait (chapters **III**, **IV**). To this end, phenotypic and genetic variation at both the individual (**I, IV**) and the population (**II, III**) levels are examined.

2. MATERIALS AND METHODS

2.1 Methodological background

2.1.1 Disentangling the roles of selection and stochasticity in phenotypic variation

Spatial variation in genetically based traits presents compelling evidence for spatial variation in selection regimes (Haldane 1948, Slatkin 1973). Phenotypic variation may however be generated by evolutionary forces other than variable selection pressures. Physical barriers preventing dispersal between populations result in isolation which in turn may result in population phenotypes diverging due to random genetic drift. Apparently connected populations may also be isolated or partially isolated in situations where the maximum capacity for dispersal is not realised (*e.g.* Avise 1996, Piertney *et al.* 1998, 1999). Consequently, spatial variation in the propensity to disperse could lead to isolation or partial isolation resulting in variation in allelic frequencies that is maintained with very little selection or genetic drift alone (Lande 1976, Lynch 1988, Vasemägi 2006). The relative importance of natural selection and random genetic drift in generating phenotypic divergence is a much discussed and one of many unresolved issues in evolutionary biology (*e.g.* Hey 1999, Merilä & Cnokrak 2001, McKay and Latta 2002). Despite the recent leap in our understanding of the genetic basis of phenotypic variation in natural populations (*e.g.* Mundy *et al.* 2004, Hoekstra *et al.* 2006, Chenoweth & Blows 2006, Gratten *et al.* 2008), in the vast majority of cases not enough is known about the genetic details of a trait and the selective factors that influence it to be able to firmly conclude that the trait is evolving non-neutrally (Leinonen *et al.* 2008). One promising platform for examining the contribution of non-neutral evolutionary forces to quantitative trait variation has been to compare differentiation at quantitative traits (Q_{ST} or its phenotypic analog P_{ST}: Spitze 1993) to differentiation at putatively neutral molecular markers (F_{ST}: Wright 1951). The rationale behind this approach lies on the assumption that population differentiation observed in selectively neutral parts of the genome provides the null expectation of the degree of differentiation that can be expected as a result of migration and genetic drift alone (Merilä & Cnokrak 2001, McKay & Latta 2002, Leinonen *et al.* 2008). In situations where differentiation at these two estimators is equal ($P_{ST} = F_{ST}$) the observed degree of differentiation at quantitative traits could have been reached solely by genetic drift (Merilä & Cnokrak 2001, Leinonen *et al.* 2008). One cannot, however, firmly exclude selection as an underlying cause of variation, but merely that it is not possible to quantify the relative contributions of selection and drift (Hendry 2002, Leinonen *et al.* 2008). If differentiation at quantitative traits is more pronounced than that observed for selectively neutral parts of the genome ($P_{ST} > F_{ST}$) this is suggestive of directional selection

influencing the trait and when the opposite holds true and neutral marker variation is found to be greater than that observed for quantitative traits ($P_{ST} < F_{ST}$), balancing selection is inferred (Merilä & Cnokrak 2001, Leinonen *et al.* 2008). A criticism that has been raised concerning the P_{ST} – F_{ST} approach is that the surrogate for the amount of additive genetic variance utilised (P_{ST}), may be confounded by non-genetic (environmental or maternal) or genetic non-additive (epistatic or dominance variance) effects when measured from natural populations (Pujol *et al.* 2008). However, as common garden and reciprocal transplant experiments are not feasible for a wide range of species and morphological traits are known to exhibit substantial additive genetic variance (Cnokrak & Roff 1995, Merilä & Cnokrak 2001) the methodology nevertheless provides a valuable starting point. P_{ST} – F_{ST} comparisons have been increasingly utilised to detect signals of non-neutral evolution in a wide range of taxa (Spitze 1993, Lynch *et al.* 1999, Storz 2002, Saint-Laurent *et al.* 2003, Leinonen *et al.* 2006, Raeymaekers *et al.* 2007, Sæther *et al.* 2007). Also, interestingly, a recent meta-analysis of studies that have employed this approach to examine non-neutral evolution in quantitative traits discovered that studies based on populations from the wild do not, in general, yield higher estimates of Q_{ST} than studies quantifying the parameter in a common garden setting (Leinonen *et al.* 2008). Determining that a trait has indeed been shaped by non-neutral evolution then sets the scene for beginning to unravel the genetics beneath traits of adaptive significance.

2.1.2 Identifying footprints of selection from patterns of DNA polymorphism

Natural selection acts on phenotypes which in turn influences patterns of DNA polymorphism in the genome. On the molecular level selection leaves behind distinct genetic 'signatures' which then signpost the genomic regions that have been its targets (Lewontin & Krakauer 1973, Barton 1999). Where loci that have not been targeted by selection are expected to be uniformly affected by population history and demographic processes, the gene regions that have been shaped by selection should behave differently generating so-called 'outliers' in the genome-wide patterns of DNA polymorphism (Lewontin & Krakauer 1973, Luikart *et al.* 2003). One promising approach to identifying functionally important genes that have been the targets of selection is thus to search different parts of the genome for these signatures of selection (Luikart *et al.* 2003, Schlötterer 2003, Storz 2005). Positive directional selection works to reduce the amount of variation present in its targeted area (Kaplan *et al.* 1989) and also affects the neutral sites linked to the gene ("genetic hitchhiking"; Maynard Smith & Haigh 1974). As an adaptive mutation arises and spreads, the associated (linked) neutral variants spread with it. Among populations, signatures of divergent positive selection are thus expected to be observable as locally increased levels of genetic differentiation at sites that have been differentially affected by selection. Although the strength of the hitch-hiking effect will depend on various parameters such as the initial frequency of the advantageous mutation and the time to fixation (see Sabeti *et al.* 2006), in general

hitch-hiking works to create 'islands' of differentiation around the selected region (Beaumont & Balding 2004). Spatially distinct populations that have been exposed to different selection pressures of varying intensities will thus likely bear distinct signatures of these past selective events – or lack thereof - in their genomes. This idea has recently been harnessed in studies aiming to detect and characterize gene regions affected by selection (*e.g.* Mäkinen *et al.* 2008, Eveno *et al.* 2008, Dayo *et al.* 2009). Not surprisingly, the number and type of tests applicable to detecting signals of selection *via* comparison of among-population genetic diversity and differentiation has increased in concert (see *e.g.* Beaumont & Nichols 1996, Beaumont & Balding 2004, Foll & Gaggiotti 2008, Excoffier *et al.* 2009). As availability of resources often limits the feasibility of examining genome-wide patterns of DNA polymorphism for footprints of selection (particularly on a population level) the ensuing question is then which particular genes or gene regions would it be most fruitful to exert one's efforts on.

2.1.3 The candidate gene approach

The candidate gene approach has proven one prolific means for detecting genes and gene regions that underlie phenotypic variation in adaptively significant traits (Nachman *et al.* 2003, Abzhanov *et al.* 2004, Protas & Patel 2008). The method makes use of the observation that similarities in gene function between different lineages do not necessarily erode during the course of millions of years of evolutionary divergence (Fitzpatrick *et al.* 2005). Thus, a gene that has been demonstrated to be associated with quantitative trait variation in one species may have an equivalent effect on the phenotype of another species. It follows that phenotype-genotype associations discovered in easy-to-rear 'genetic model organisms' function as good starting points for locating the molecular mechanisms of phenotypic divergence in the wild (Fitzpatrick *et al.* 2005).

2.1.4 Gene expression during development

The genetic theory of morphological evolution postulates that form evolves largely by altering the expression of functionally conserved proteins (reviewed in Carroll 2008). Regulatory changes affecting protein abundance have indeed been advocated as being more likely locations of adaptive mutations than protein coding regions of genes (Carroll *et al.* 2001, Hoekstra & Coyne 2007).Thus, a thorough understanding of the factors contributing towards observed variation in phenotypes requires an understanding of the roles of proteins during the development of the phenotype. The protein/peptide identification procedure has recently developed permitting sequence similarity searches against database sequences of closely related organisms (Forné *et al.* 2010). These methodological advances in protein identification together with the substantial increase in publicly available annotated whole-genome sequence data have now made the large scale identification and quantification of proteomes applicable to non-model organisms (see *e.g.* Buggiotti *et al.* 2008, Carpentier *et al.* 2008, Papakostas *et al.* in press). The complete genome

sequences of the chicken (*Gallus gallus*) and zebra finch (*Taeniopygia guttata*) provide an invaluable resource for examining molecular processes in other avian species. In addition to the identification of the proteins present in the tissue of interest, it is also often possible to classify them according to their function and cellular location (Okuda *et al.* 2008, Rhee *et al.* 2008). Despite the fact that the proteomics approach is commonly employed to questions in many fields of biology (Pandey & Mann 2000), relatively few studies in the fields of ecology and evolution have employed it to date (Feder & Walser 2005, Karr 2008).

2.2 Molecular tools

Genetic variation within and between populations has typically been quantified with molecular genetic markers. During the past few decades, advances in molecular methodology, publicly available sequence information and analytical techniques has made it possible to ask (and answer!) questions that require information from each of the levels of organization (*i.e.* DNA – RNA – protein) in non-model organisms. In this thesis I harness the advantages presented by both neutral genetic variation and gene associated (*i.e.* functional) variation to address questions on phenotypic divergence and associated evolutionary change. The approach encompasses two of the three tiers of molecular organization, namely the fixed (bar mutations) DNA level and the dynamic protein level. For further details on each of the molecular markers employed, their inherent properties and potential applications see *e.g.* Jarne and Lagoda (1996), Ellegren (2000) for microsatellites; Brumfield *et al.* (2003), Morin *et al.* (2004), Slate *et al.* (2009) for single nucleotide polymorphisms (SNPs); Karr (2008), Gstaiger and Aebersold (2009), Forné *et al.* (2010) for proteins.

2.3 Sampling scheme

The samples for chapters **I-III** were collected from pied flycatcher males breeding in nest box populations maintained for research purposes across the breeding range. Sample collection was done between 1994 and 2009. The genetic material was obtained via drawing a blood sample or by plucking a single feather from the bird. The samples were then stored at -20 C or room temperature, respectively. Dorsal plumage colour was scored by eye and classified according to the Drost scale (Drost 1936) or by estimating the percentage of black feathers on the back. Additionally, for the initial study (**I**) a tertial feather was plucked and used in measurements of ultraviolet reflectance and the forehead patch of each male was photographed.

The pied flycatcher samples analysed in the final study (**IV**) were taken from males that had been maintained in an aviary (in common garden conditions) over the winter of 2008-09. Developing feathers were collected during the spring moult and frozen in liquid nitrogen on site and subsequently stored in -80 C. Individuals were scored according to the Drost scale as described above once they had acquired their full breeding plumage. As above, a tertial feather was collected from each of the males for UV-measurements once the males had moulted into their full breeding plumage.

2.4 Molecular markers

The microsatellite markers employed (chapters **I**, **II**, **III**) were previously published and had been isolated and developed from the pied flycatcher (Ellegren 1992, Primmer et al. 1996, Leder *et al.* 2008) bar one locus (*SS2-71B*; Rubenstein 2005). The SNP markers utilized (**III**) were specifically developed to address the questions in this study using publicly available sequence information from the zebra finch (*Taeniopygia guttata*), chicken (*Gallus gallus*) and blue tit (*Parus caeruleus*). The identification of the proteins found in developing feathers (**IV**) similarly employed publicly available sequence information for the zebra finch.

2.5 Data analyses

All statistical analyses were conducted with the statistical packages SAS or SPSS. Paternity and population genetic analyses and outlier tests were executed with standard publicly available analysis packages (CERVUS (Marshall *et al.* 1998); Genepop (Raymond & Rousset 1995); FSTAT (Goudet 1995); Arlequin (Excoffier *et al.* 2009); Winbugs (Spiegelhalter *et al.* 2003)). Publicly available sequence and annotation information (*e.g.* Ensembl, NCBI databases) and programs (Cytoscape (Shannon *et al.* 2003); the ClueGO plug-in (Bindea *et al.* 2009)) were employed to the functional analyses of the proteins.

3. MAIN RESULTS

3.1 Plumage colour variation and reproductive output (I)

In the first chapter **I** examine the effect of male characteristics (plumage traits, size and age) on male reproductive output in a Finnish population of pied flycatchers. Like the vast majority of passerines (see Griffith *et al*. 2002 for a review of EPPs), pied flycatchers are socially monogamous but engage in extra-pair copulations (EPCs; Gelter & Tegelström 1992, Lubjuhn *et al*. 2000, **I**). Additionally, after attracting a female to a territory *ca.* 5-10 % of males succeed in attracting a secondary female to a second territory thus siring two clutches (Lundberg & Alatalo 1992). The survival rates of the young in the secondary nests tend to be lower, however, as paternal care is commonly only provided for the primary clutch (Lundberg & Alatalo 1992, pers. obs.). We measured the reproductive output (as quantified by total number of nestlings sired) of all males during two breeding seasons. Paternities were determined using molecular markers. The number of extra-pair young sired and also the number of young 'lost' to other males within a nest were examined in relation to the male characteristics. Our results show that old males (two-year old or older) are the most likely to sire extra-pair young. Also when extra-pair sires could be determined, they were most commonly breeding in neighbouring nestboxes. Age did not, however, have any influence on the probability of losing paternity to a cuckolding male. Instead, the degree of ultraviolet reflectance from the white wing patch of the male appeared to be associated with the probability of losing paternity within a nest. The males that were cuckolded tended to have lower ultraviolet reflectance than the males that were not cuckolded. Age is perhaps the most consistently found trait that influences extra-pair mating success in birds. Age is hypothesized to signal good genes via being an indicator of survival characteristics as a second year male will have survived two migrations to the over-wintering grounds in Africa. Along a similar line, as the ultraviolet reflectance of plumage is dependent on feather structure and consequently a signal of the extent of 'wear and tear' it has sustained. If the quality of a male is associated with the robustness of the plumage this characteristic could signal so called 'good genes' to a female. Thus, females mated with low ultraviolet reflectant males could be more likely to cuckold their social mate. Ultraviolet colouration has also been found to be significant in mate choice decisions in other species (e.g. Bennett *et al*. 1997, Hunt *et al*. 2001, Hausmann *et al*. 2003) and mate choice decisions to be altered in the absence of ultraviolet cues. None of the examined traits appeared to be significant in the context of siring a secondary nest. It is noteworthy, however, that the number of secondary nests identified was low (N=7) consequently reducing the power of the analysis. Our results indicate that both age and the degree of ultraviolet reflectance from the breeding plumage affect the reproductive output of pied flycatcher males.

3.2 Is there selection for plumage colouration in pied flycatchers (I, II, III)?

In chapters **I-III** I address the question of whether there is selection for plumage colouration in pied flycatchers. Chapter **I** (summarized above) revealed that structural plumage colouration of pied flycatcher males impacts the likelihood of losing paternities within the clutch to other males. Thus it appears likely that variation in structural colouration is affected by selection *via* the mating system and more specifically, *via* female choice. In addition to structural colour variation, pied flycatcher males also exhibit marked variation in pigment-based colouration across the breeding range. Chapter **II** seeks to quantify the extent to which spatial variation in phenotypes is governed by non-neutral processes by quantifying the pattern and extent of neutral genetic variation and comparing this to the patterns of phenotypic variation across the pied flycatcher breeding range. To this end, the $F_{ST} - P_{ST}$ (the phenotypic analog of Q_{ST}; Spitze 1993) approach was employed. The answers provided by the methodology come in two varieties, both of which are reliant on the idea that F_{ST} values calculated from neutral genetic markers function as a proxy for the degree of divergence expected in the absence of selection (Leinonen *et al.* 2008). Thus, if (a) the degree of population differentiation observed for phenotypic traits is much greater than that seen at neutral genetic markers and/or (b) the patterns of differentiation seen for phenotype are not concordant with those observed for neutral genetic marker, the trait of interest can be concluded to exhibit sign of non-neutral evolution. In chapter **II** we found both to hold true when comparing patterns of genetic divergence for neutrally evolving microsatellite markers and patterns of phenotypic divergence across the pied flycatcher breeding range. Phenotypic divergence greatly exceeded the amount of genetic divergence observed and overall, the patterns of differentiation were dissimilar (see Fig. 1 for phenotypic divergence and Fig. 2 for distribution of neutral genetic divergence). A sensitivity analysis examining the effect of different degrees of heritability for dorsal plumage colour verified that the result was not an artifact of an over-estimate of the heritability of the trait. In chapter **III** I investigate the gene regions that putatively underlie the trait for signals of selection by examining genetic diversity and differentiation at SNP markers in or near these genes. No signals of selection were detected in any of the candidate genes for melanin-based pigmentation. A candidate gene for structural colouration (ultraviolet reflectance) and a candidate gene for ultraviolet vision, were however found to exhibit levels of inter-population divergence exceeding that expected for neutrally evolving loci (Fig. 3). The signal was found to mainly stem the Spanish pied flycatcher populations being very in allelic frequencies in comparison to the other populations examined. The result in itself does not verify that there is a functional difference in the alleles of the genes associated with the different alleles of SNP marker, but it is tempting to speculate that the signal stems from divergent selection

for these traits across the pied flycatcher breeding range. Thus, based on the findings of chapters **I-III**, it can be concluded that both structural colour variation and pigment-based variation appear to be evolving non-neutrally in pied flycatchers. Additionally, in the former, indications of this were detected on the molecular level.

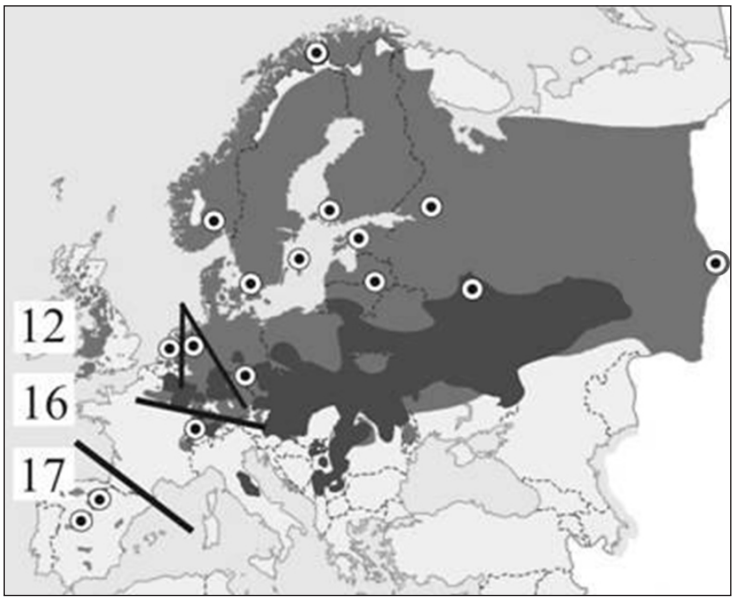

Fig. 2. Areas of genetic discontinuity identified across the pied flycatcher breeding range as quantified by patterns of allele frequency variation at microsatellite markers. The numbers indicate the number of loci (18 total) that partially or fully support the barrier. The figure is reproduced from Lehtonen *et al*. (2009a).

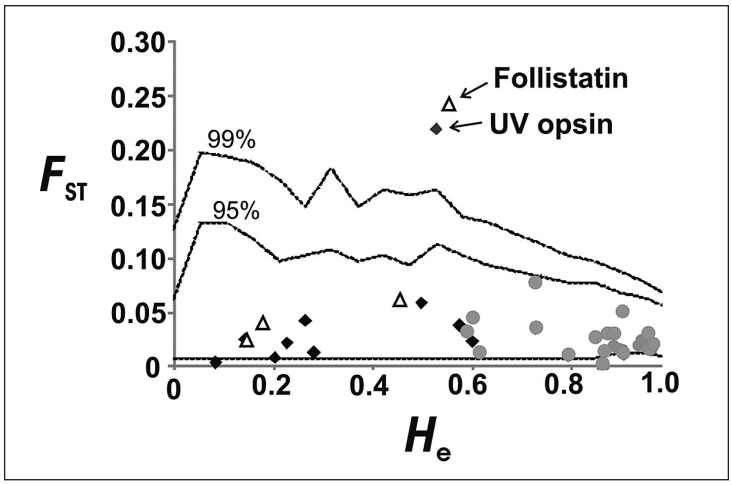

Fig. 3. A plot of the relationship between heterozygosity (H_e) and F_{ST} for the microsatellite and SNP loci. Triangles indicate the sex-linked SNP loci, diamonds the autosomal SNP loci and circles the microsatellite loci. Dashed lines indicate the simulated 95% and 99% confidence limits for the values expected under neutral evolution.

3.3 Population genetic structuring of pied flycatchers across their breeding range (II, III)

In chapter **II** (see chapter **III** for details concerning the British population) the amount of among-population differentiation at loci assumed to be evolving neutrally is quantified. I found that there are four barriers to gene flow (as defined by genetic discontinuity at allelic frequencies; Manni *et al*. 2004; Fig. 2) across the pied flycatcher breeding range. The first and most prominent of these separates the Spanish populations from the other populations. The second and third barriers separate the Swiss and the British populations from all other populations and the final barrier results in a northern Europe – central Europe divide, separating the Dutch, German and Czech populations from the northern and eastern populations. Interestingly, the northern and eastern pied flycatcher populations were wholly genetically undifferentiated even over thousands of kilometers. The pattern of genetic differentiation appeared to be associated with the recruitment and return rates, with the more differentiated populations exhibiting higher recruitment and return rates. However, as the intensity and amount of time that each population had been studied for was quite variable, a formal test of this hypothesis was not possible. Alternatively, or additionally, the patterns of differentiation could be remnants stemming from the post-glacial range expansion of the species. The patterns resemble that seen in the grasshopper (*Chorthippus parallelus*), the great crested newt (*Triturus cristatus*) and the black alder (*Alnus glutinosa*; reviewed in Hewitt 1999).

Comparing patterns of neutral genetic differentiation with patterns of differentiation at genes potentially affected by selection is another approach for getting at the relative contributions of neutral evolutionary forces from those of selection. In chapter **III**, I quantify the extent of genetic divergence between the same pied flycatcher populations at SNP markers situated in or near functional genes. Overall, the patterns of divergence at neutrally evolving microsatellite markers (**II**) and SNP loci that are putatively under selection resembled each other. As was discovered for divergence at microsatellite loci, the Spanish, Swiss and British populations were also most differentiated at the SNP loci (as quantified by F_{ST}). Furthermore, the central European populations had an intermediate level of differentiation falling between the three most highly differentiated populations and the undifferentiated northern and eastern populations.

3.4 Molecular mechanisms underlying plumage colour variation in pied flycatchers (III, IV)

In the final two chapters I seek to find the genetic basis of pigmentation and structural variation (**III, IV**) and also to examine whether candidate genes for colour perception exhibit patterns of non-neutral evolution. Sequence variation at candidate genes for the two types of colour variation and also vision was characterized

by identifying SNP sites using a subset of individuals spanning the sampled range. Two outlier tests were employed to examine these SNP sites for signals of selection. The outlier tests were consistent in their results concerning two loci in the data set. The SNPs near the *follistatin* (FST) gene and the *ultraviolet opsin* gene (UV-opsin) fell above the 95 % confidence limits defined for neutrality (Fig. 3). The signal was generated by the most highly differentiated Spanish populations. No signals of non-neutral evolution were detectable in any of the other SNP sites examined. Although we are not able to rule out the possibility that the observed allele frequency distributions of the *follistatin* locus have been shaped by positive selection, due to the geographic isolation of the Spanish pied flycatcher populations it is feasible that the observed allele frequency distributions have been generated by stochastic evolutionary processes and not natural selection. Overall, the patterns of SNP variability bore similarity to the previously described patterns of microsatellite variation across the pied flycatcher breeding range. Chapter **IV** unveiled the products of *ca*. 200 genes in developing pied flycatcher feathers. The comparison between the two phenotypic classes revealed the vast majority of both the genes and the processes to be the same. This was perhaps expected as it is likely that the majority of the processes that govern feather development are the same between differently pigmented males. Preliminary results of the TYRP1 gene being important for the production of black pigment in the pied flycatcher were obtained. Furthermore, indications of an association between melanin-based pigmentation and energy homeostasis were found.

4. IMPLICATIONS AND FUTURE DIRECTIONS

In this thesis I examine the roles of the various evolutionary forces in promoting and maintaining phenotypic divergence in pied flycatcher males, which serves to contribute towards our understanding of evolutionary change. It appears that geographic variation in the phenotypic divergence of pied flycatcher males is not a product of spatial variation in their propensity to disperse. The pied flycatcher system thus maintains its status as a true evolution in action -system in a natural setting. It is of interest, that structural colouration, which was not originally the aspect of plumage colour variation we sought to examine repeatedly arose as a significant component of pied flycatcher plumage (chapters **I**, **III**). Clearly, any studies examining plumage colour variation in birds that omit the ultraviolet dimension from their studies are unlikely to be obtaining a full picture.

As is so often the case and also an attractive characteristic of science, the number of questions raised equals, if not outweighs, the number of answers obtained. So where next? One interesting future prospect, inspired by the putative signals of selection detected in chapter **III**, would be to examine whether there actually exists functional variation in the opsin genes of pied flycatchers. The functional properties of these genes, and in the case of birds, short-wavelength sensitive receptor genes (SWS1) in particular, can non-invasively be assessed by examining sequence variation at this gene (Ödeen *et al*. 2009). The spectral tuning sites in the SWS1 sequence are known and nucleotide variation at these sites may then be utilized to estimate the peak absorbance properties of a particular allele.

Secondly, the genetic basis of melanin-based plumage colouration still remains to be resolved. The results from chapter **IV** suggest the involvement of the TYRP1 gene and examining this result to greater detail could prove a fruitful endeavor. The proteomes of a larger number of feathers collected from black and brown birds would need to be characterized to ascertain the role of *TYRP1* in pigmentation in pied flycatchers.

A result produced by comparing genes associated with black-backed individuals and those associated with brown-backed individuals provided indicationso of a signalling function for melanin-based pigmentation (Table 1). The comparison of the black-specific and brown-specific gene lists revealed four functions associated with the redox balance of the cell (cell redox homeostasis and three different functional variants of intramolecular oxidoreductase activity) to be over-represented in the black group. This is of particular interest as in vertebrates, the production of eumelanin (the black pigment) requires that glutathionine levels are low (Galván & Alonso-Alvarez 2008). However, in addition to inhibiting eumelanogenesis, glutathionine functions as a key antioxidant that is employed in neutralizing reactive oxygen species

Table 1. The gene ontology terms that are over-represented in the feather proteome of pied flycatcher males with either a black or a brown dorsum. The oxidative stress associated terms that are consistently over-represented in the black individuals are noteworthy and of particular interest in future work on the evolutionary significance of pied flycatcher pigmentation (see text for details). The results are in part based on protein expression profiles detected in one individual only and are thus very preliminary by nature and not presented in chapter IV.

FUNCTIONAL SUB-GROUP	HIGHEST LEVEL GENE ONTOLOGY	PHENOTYPIC GROUP
Biological Process		
	Cell redox homeostasis	**BLACK**
	Translational elongation	**BROWN**
Cellular component		
	Endoplasmic reticulum lumen	**BLACK**
	ER-Golgi intermediate compartment	**BLACK**
	Pigment granule	**BLACK**
	Melanosome	**BLACK**
	Ribosomal subunit	**BROWN**
	Large ribosomal subunit	**BROWN**
	Cytosolic ribosome	**BROWN**
	Cytosolic part	**BROWN**
	Cytosolic large ribosomal subunit	**BROWN**
	Proteasome core complex	**BOTH**
Molecular function		
	Intramolecular oxidoreductase activity	**BLACK**
	Intramolecular oxidoreductase activity, interconverting keto- and enol-groups	**BLACK**
	Intramolecular oxidoreductase activity, transposing S-S bonds	**BLACK**
	Protein disulfide isomerase activity	**BLACK**
	Threonine-type peptidase activity	**BOTH**
	Threonine-type endopeptidase activity	**BOTH**

that are harmful to the cell. The fact that eumelanogenesis cannot occur when the levels of this antioxidant are high has been hypothesized as one signalling function of melanins, and eumelanins in particular. As eumelanin production leads to increased oxidative stress within a cell, individuals that are capable of producing eumelanic traits must either be able to cope with this increase in intracellular oxidative stress levels and/or sustain and be capable of mobilizing alternative antioxidant sources for reactive oxygen specie neutralization (Galván & Alonso-Alvarez 2008, Galvan & Solano 2009). In light of the fact that gene functions associated with cell redox homeostasis were over-represented in the black individuals, the phenomenon would appear to deserve further research attention in studies on the signaling function of melanin-based plumage colouration in pied flycatchers.

Finally, the energy homeostasis genes found to be expressed more in one phenotypic group than the other, inspire one to also pursue the phenotype-physiology connection in a direction

focused on the pleiotropic effects of the melanocortin system. The melanocortin system is involved in a diverse array of physiological aspects (reviewed in *e.g.* Wikberg 1999, Gantz & Fong 2003, Martin & MacIntyre 2004, Carroll *et al.* 2005) and has commonly been found to be associated with various behavioral and/or physiological features of individuals (*e.g.* Krude *et al.* 1998, Santschi *et al.* 2001, Mau *et al.* 2004, Almasi *et al.* 2010, reviewed in Ducrest *et al.* 2008, Boswell & Takeuchi 2005). Male pied flycatchers of variable phenotypes have indeed been found to be differently affected by ambient temperatures (Ilyina & Ivankina 2001, Kerimov *et al.* 2006, Sirkiä *et al.* unpublished data) supporting the idea of a connection with energy homeostasis and paving yet another interesting new avenue for future research.

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Although there is only one name on the cover of this book this has in many ways been a team effort. The number of people that have helped me in one way or another during these years is both tremendous and humbling. Thus I kick this section off with a collective KIITOS!!!

And then to some specifics.

First and foremost I wish to thank my supervisors. Both of them have been great thus making the following two paragraphs rank amongst the toughest to produce of those found on these pages.

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Finally, there's the next chapter. The long awaited one that nonetheless wholly unexpectedly began to write itself as the previous chapter was coming to a close (in a mega-frenzy). Thank you for putting up with me during the final months. You have changed to colour of my world.

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