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Signaling function of plumage coloration in Yellowhammer males

Diplomová práce

Vedoucí závěrečné práce:
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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 14. srpna 2017

Abstrakt

Teorie pohlavního výběru se snaží vysvětlit vznik zdánlivě zbytečných znaků, zejména u samců celé řady druhů. K takovým znakům patří i pestré a nápadné zbarvení, tak typické pro celou řadu ptačích druhů. Tyto znaky jsou nějakým způsobem nezfalšovatelné a vypovídají o kvalitách jedince. Karotenoidní zbarvení nese informaci o zdravotním stavu a melaninové o sociálním postavení, i když toto tradiční rozdělení přestává být ve světle nejnovějších poznatků tak striktní. Mimo tyto způsoby udržování nezfalšovatelnosti se v poslední době dostává do popředí zájmu stále více i vliv hormonů, zejména vliv dvou steroidních hormonů: samčího pohlavního hormonu – testosteronu, a „stresového" hormonu – kortikosteronu. Oba dva hormony mohou pozitivně ovlivňovat samčí pohlavní znaky, kterým je i ornamentální zbarvení. Zvýšená hladina těchto hormonů s sebou ale nese i zvýšené riziko pro organismus (vyšší energetické výdaje, chronický stress), tedy i potenciál být pro jedince nákladnými.

Použitím standardizovaných fotografií a spektrofotometrie jsem analyzoval zbarvení opeření samců strnada obecného (*Emberiza citrinella*). Koncentrace testosteronu a kortikosteronu uloženého do opeření byla stanovena pomocí LC-MS/MS. Pro samce z roku 2015 byl také stanoven jejich teritoriální statut a přežití následující zimy. Nejprve jsem zjistil, že při analýze zbarvení spektrofotometrem je nutné použít alespoň deset per k dosažení reprezentativního výsledku. Dále ukazuji, že mladí a staří samci se mezi sebou lišili v odstínu a podílu modrého světla v karotenoidním opeření. Odstín byl také zřejmě pozitivně korelovaný s kortikosteronem, i když tento trend byl pouze marginálně signifikantní. Signifikantní negativní korelace byla nalezena mezi jasem melaninového kostřece a hladinou testosteronu. Mé statistické modely také ukazují tendenci k vyšší úspěšnosti přežití zimy u samců s jasnějším a rozsáhlejším karotenoidním zbarvením a vyšším obsahem kortikosteronu v peří. Vzhledem k použití nové metody stanovení testosteronu z peří, jsou výsledky této práce mezi ostatními studiemi ojedinělé a navíc jsem pravděpodobně jako první, nalezl potenciální vztah mezi obsahem melaninu v peří a uloženým testosteronem.

Klíčová slova

Emberiza citrinella, karotenoidy, melanin, zbarvení, testosteron, kortikosteron, pohlavní výběr

Abstract

Sexual selection theory tries to explain evolution of apparently useless traits which mainly developed in males of numerous species. One such trait is also rich and vibrant coloration, typical for many of the bird species. These traits are difficult to be falsified, and therefore they honestly signal quality of the individual. Carotenoid coloration reflects the health condition and melanin coloration the social status, even though this traditional division might not be as strict according to the latest studies. Apart from these ways of maintaining honesty, recent studies are focusing more also on the effect of hormones, mainly two steroids: male sexual hormone – testosterone –, and the "stress" hormone – corticosterone. Both hormones could positively influence male's sexual traits such as ornamental coloration. On the other hand, elevated levels of these hormones possess risk to the organism (higher energetic expenditure, chronic stress), therefore also might potentially become costly.

Using photographs of birds in standardized conditions as well as spectrophotometry I analyzed the plumage coloration of males of the Yellowhammer (*Emberiza citrinella*). Concentrations of testosterone and corticosterone deposited in feathers were analyzed using the LC-MS/MS. Also, for males in breeding season 2015, their territoriality status and overwinter survival was assessed. I have found that to analyze feather coloration with spectrophotometer, one should measure at least ten feathers to obtain representative results. Then I showed that young and old males differed in hue and blue chroma of their carotenoid coloration. Hue also seems to be positively correlated with feather corticosterone, though this trend was only marginally significant. A significant negative correlation was found between brightness of melanin rump and feather testosterone. My models also suggested a trend that males with brighter and more extensive carotenoid plumage and higher feather corticosterone have higher overwinter survival. Findings of this thesis are unique, as not like other studies, I used a novel method of feather testosterone assessment, and as probably the first one I found potential relationship between melanin and deposited testosterone.

Keywords

Emberiza citrinella, carotenoids, melanin, coloration, testosterone, corticosterone, sexual selection

Acknowledgement

As this thesis is my first real step into the world of science, analyzing data and scientific writing, it is clear that I could not achieve this point alone, or at least not at the level presented here. At this point I would like to thank the people who helped me with various things regarding this thesis. First of all, I would like to thank my supervisor, Tereza Petrusková, not only for being my supervisor, giving me advice and much appreciated feedback, but mostly for introducing me to the topic of evolutionary biology, giving me the chance to work with live animals, and a possibility to present my research at conferences.

Many thanks also belong to my co-supervisor Tomáš Albrecht as he is an experienced scientist in the fields of avian coloration and physiology. I am especially thankful to him for the possibility to work with the spectrophotometer and to analyze the feather hormones, as well as for various comments and advice regarding statistical analysis of the data and the feedback given. I am also grateful for the chance to learn many new methods and experience a different kind of fieldwork.

I am also very grateful to Sharina van Boheemen as this thesis is basically a sub-part of her doctoral studies at the Charles University, a much broader project regarding complex sexual selection analysis in the Yellowhammer for which we collected the data together in the field. That means without her help, the dataset presented here would be much, much smaller.

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Contents

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1 Introduction

Bird coloration is one of the most studied traits when dealing with sexual selection (Andersson 1994). Birds are especially well suited study group as they are diverse in their mating system and show all kinds of parental care. Some of them mate for life, some only for a season, others are polygamous and others do not form a pair bond at all. Most birds, however, are monogamous. But social monogamy does not necessarily mean genetic monogamy as many species exhibit the so called "extra-pair paternity" which causes that single brood can have more than one genetic father (Griffith, Owens & Thuman 2002). In birds, biparental care is the most common type of parental arrangement but both types of uniparental care as well as sex role reversal or no parental care at all occur (Cockburn 2006). This, together with the fact that birds are ubiquitous terrestrial animals, has led to their broad use as model group in sexual selection and mate choice research (for a review see e.g. Andersson & Simmons 2006).

Birds show great variety of colors in their plumage, beaks or wattles suggesting their important role in sexual selection. In this respect I will briefly present the different mechanisms of bird coloration and its role in sexual selection with special emphasis on melanin and carotenoid coloration. Before that, it is important to stress out the differences between avian visual system and ours. Because talking about birds' coloration and not knowing how birds themselves perceive colors makes no sense. I will also shortly introduce the topic of evolutionary endocrinology in birds, mainly the role of two hormones, testosterone and corticosterone, in sexual selection with some examples how they can alter birds' coloration. Last but not least, the Yellowhammer (*Emberiza citrinella*), the key character in this thesis, will be briefly described.

Bird vision

Color vision is mediated by various types of color-sensitive photoreceptors found in the retina called cones. Humans have three types of these cones sensitive to wavelengths in so called "human visible spectrum", i.e. wavelengths from 400 to 700 nm. Apart from three types of cones sensitive in human visible spectrum, birds possess a fourth type, a cone sensitive to near ultraviolet light (UV-A, 300–400 nm, reviewed in Cuthill *et al.* 2000). The ultraviolet sensitive cone, together with UV translucent lens, enable birds to perceive ultraviolet light (Bennett & Cuthill 1994; Lind *et al.* 2013). This means that, unlike humans, birds are tetrachromats. Two distinct types of ultraviolet cones evolved in birds, the one with maximum peak sensitivity in the range 402–426 nm (usually referred to as violet cone, or VS) and the other with this peak in 355–376 nm (ultraviolet cone, or UVS; Ödeen & Håstad 2003; Ödeen, Håstad & Alström 2011; fig. 1).

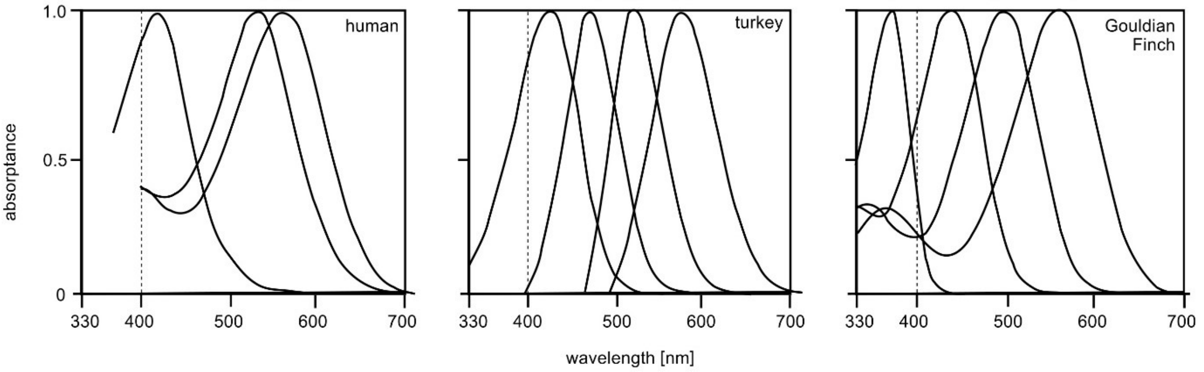

Figure 1: Spectral absorptance of cones in human visual system and two types of avian visual system: the VS system represented by the turkey (*Meleagris gallopavo*) and the UVS system represented by the Gouldian Finch (*Erythrura goudiae*). Dotted line marks the limit of human visible spectrum. Redrawn from Bowmaker & Dartnall (1980); Hart, Partridge & Cuthill (1999); Hart *et al.* (2000).

Unlike human cones, those in birds' retinas contain a small oil droplet which is colored by carotenoids and acts as a cut-off filter and probably further enhances color discrimination in birds (Hart 2001). Birds also possess the "double cone" with broad spectral sensitivity consisting of one smaller and one bigger cone cell located in close proximity. But double cones seem to take part in achromatic, i.e. non–color, vision rather than color discrimination (Bowmaker *et al.* 1997; Hart 2001; Cuthill 2006).

While avian color vision is not yet fully understood, it is more than clear that birds can discriminate between colors better than we do, and besides that they are able to see colors that are beyond our imagination. It is important to bear this in mind when studying bird coloration and its role e.g. in sexual selection, because conclusions based on human vision might be misleading. For example, several studies have shown that birds which appear monochromatic to human vision are dichromatic to each other (Hunt *et al.* 1998; Cuthill *et al.* 1999; Eaton 2005; Burns & Shultz 2012).

Before the broad use of spectrophotometers, bird coloration was assessed by the means of human vision. After the finding that birds are tetrachromats and are able to perceive also near-ultraviolet light, it was evident that human vision cannot compete with that of birds in terms of color discrimination. The use of spectrophotometer produces an "objective" assessment of color, i.e. it measures reflectance for individual wavelengths. But also results obtained by this method and possible differences found, do not necessarily mean we measured what birds can see. That is possible to achieve by modeling bird vision using known sensitiveness of individual cone types, characteristics of the oil droplets found in bird retina and adding information on the ambient light under which the visual model should be computed.

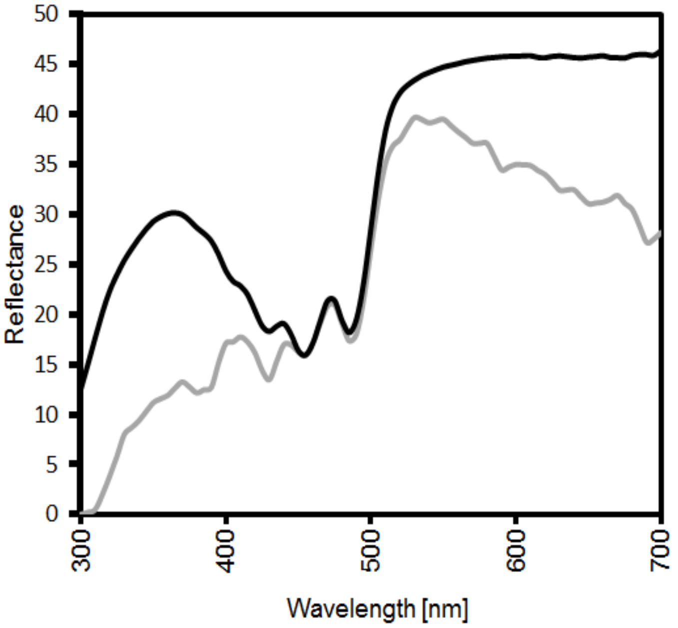

Figure 2: Reflectance of Yellowhammer's yellow carotenoid coloration illuminated by standard light from spectrophotometer (black line) and a simulation of reflectance curve illuminated by daylight (gray line).

Ambient light could have impact on color perceived as e.g. direct sunlight contains slightly more light in the red part of the spectrum than the light in the forest shade (Endler 1993). In any case the direct sunlight is far from the uniform light produced by spectrophotometer[1]. Measuring color under spectrophotometer light and direct sunlight would produce very different curves (fig. 2). Sunlight contains much less light in the ultraviolet range than light from spectrophotometer and the once prominent peak in UV could very well disappear under natural light conditions.

Coloration and its physical nature

However colorful the birds' plumage might be, the mechanisms behind it are much less variable. It is either a pigment based coloration or structural coloration or combination of both (Hill 2007; Pryke 2007; Berg & Bennett 2010). This division is not valid only for feathers. In this way, most other body parts of birds including their beaks or wattles are colored. However, the majority of studies was done on plumage ornamentation, which is also the case of this thesis, and therefore I will mostly focus on feathers too.

Pigment based coloration, as its name suggests, is caused by pigments deposited in the feather itself. This way the color is produced by reflecting only part of the incident light spectrum, while the rest of it is absorbed. These pigments could be of various origin, most

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[1] Also light from spectrophotometer light source is not uniform, but it is corrected to be uniform through the use of "white standards".

frequent are melanins or carotenoids. Other pigments, which seem to be more restricted in avian phylogeny, are porphyrins and psittacofulvins (Toral, Figuerola & Negro 2008).

Structural coloration differs from pigments in the way of the color production. Instead of a simple reflection, the incident light enters a complex system of nano-structures in the feather surface, where it is either interfered or scattered producing different hues of green and blue including metallic ones as well as iridescent colors (plate 1). White is also a structural color (reviewed in Doucet & Meadows 2009).

1.1 Melanin coloration

As stated above, the most common feather colorant is a group of similar pigments, the melanins. There are two types of them involved in coloration, the reddish phaeomelanin and the brown and black eumelanin. Both are polymerized products of the oxidation of the amino acid tyrosine (reviewed in McGraw 2006b). Since melanins are so wide-spread in birds and the melanin ornaments often vary in size or color (plate 2), it is not very surprising that they have been subject of many studies investigating their function in sexual selection, yet sometimes with contradictory results.

The best known role of melanin ornaments in sexual selection and mate choice seems to be their advertisement of the social status of its bearer through so called "melanin badges". These were mostly studied in the House Sparrow (*Passer domesticus*), where the size of the melanin badge signals social status of its bearer (Møller 1987; Gonzalez *et al.* 2002; McGraw, Dale & Mackillop 2003). Similar mechanism is found also in the Harris's Sparrow (*Zonotrichia querula*; Rohwer & Rohwer 1978) and Yellow Warbler (*Dendroica petechia*; Studd & Robertson 1985) and has been proposed for many other species (Rohwer 1975). The honesty of the status signaling could be achieved either by costs of melanin production (see below), or by the time and energy needed to spend by an individual to defend its status (Jawor & Breitwisch 2003).

Melanins as indicators and the cost of melanin ornamentation

One of the possible ways melanin ornaments could be costly – i.e. the higher incidence of stressful interaction with other individuals – has been mentioned before. Another cost could be connected with the diet of birds and its composition. Even though melanins are not usually considered dietary pigments in the same way as carotenoids are (see chapter 1.2 *Carotenoid coloration*), they cannot be synthetized from nothing. Certain studies suggest that either size (McGraw 2007) or color (Poston *et al.* 2005) of the melanin ornament can be influenced by the amount of minerals or amino acid precursors present in the diet of birds. This seems to be true also for House Sparrows as the difference in size of their badges between young and old birds seems to be actually limited by the diet (Veiga & Puerta 1996). In males of Pied Flycatchers (*Ficedula hypoleuca*), the "blackness" of their plumage is predetermined by the diet even sooner – when the birds are still in the nest (Slagsvold & Lifjeld 1992). On the other hand, several other studies failed to find any effect of restricted diet on melanin ornamentation (e.g. McGraw *et al.* 2002; Senar, Figuerola & Domènech 2003; Hill, Hood & Huggins 2009).

Even though melanin ornaments have not been traditionally recognized as costly, it seems that melanogenesis itself may be costly. Not only tyrosine is probably an essential amino acid for birds (Hebert, Shutt & Ball 2002), also the production of phaeomelanin and eumelanins has been suggested to have different costs and their ratio in ornaments could therefore signal condition of individual (reviewed in Jawor & Breitwisch 2003). Apart from that, production of melanins is regulated by the same genes which influence many other phenotypic traits in vertebrates like sexual behavior or aggression (Ducrest, Keller & Roulin 2008; Roulin 2016). Consequences of this so called "pleiotropy in the melanocortin system" will be discussed further in the text.

Melanins have been studied also as indicators of health and condition. So far, studies performed on passerine species failed to show any signs that melanin ornaments could work as such indicators, as evidenced by works studying the effect of various endoparasites on melanin ornamentation (Hill & Brawner III 1998; McGraw & Hill 2000; Hill, Hood & Huggins 2009) or their ability to inform about the immunocompetence of the individual (Poston *et al.* 2005). While studies on passerines found no such indicating quality, there are some indications that in some non-passerines, the opposite could be true (Roulin *et al*. 2001; Gangoso *et al*. 2011; Svobodová *et al*. 2016). It is questionable, whether the studies on passerines could produce any meaningful results as the studied parasite infection does not necessarily need to influence melanin synthesis (see Jawor & Breitwisch 2003).

But melanins can advertise also other attributes potentially interesting to females such as the higher quality of parental care which may lead to higher survival of offspring. Males of Great Tits (*Parus major*) with bigger melanin badge showed higher nest attendance and heavier offspring (Norris 1990) and larger-badged males of House Sparrow and Eastern Bluebirds (*Sialia sialis*) showed more frequent feeding and higher offspring survival than smaller badged males (Voltura, Schwagmeyer & Mock 2002; Siefferman & Hill 2003; Jensen *et al*. 2004), while Yellow Warblers males with larger melanin ornament exhibited lower feeding rates (Studd & Robertson 1985). In sharp contrast, other studies of House Sparrow have found that males with smaller badges had higher reproductive success (Veiga 1993; Grffith, Owens & Burke 1999) and this trend was found also in other species (Lemon, Weary & Norris 1992; Kingma *et al*. 2008). Studies mentioned so far investigated mainly the size of the badge, while their spectral characteristics have been studied much less. Despite this, in other studies it has been found that the melanin plumage coloration might also possess some information about the reproductive success of its bearer, either through brightness (Siefferman & Hill 2003) or ultraviolet chroma (Doucet *et al.* 2005).

Unfortunately, also studies of female preference for melanin badges did not shed more light at these sometimes contradictory results. In House Sparrow, females preferred males with smaller badges (Grffith, Owens & Burke 1999) as well as in American Redstart (*Setophaga ruticilla*; Lemon, Weary & Norris 1992). If females should prefer males with higher parental care, this choice does seem logical which will be discussed in chapter 1.4 *Testosterone and corticosterone in birds*.

Other roles of melanin coloration

In addition, several studies showed that melanin pigment does not serve only as a feather colorant. It provides also a reinforcement of other proteins in feathers, such as keratin, making them less prone to damage or abrasion (Bonser 1994; Riley 1997; Mackinven & Briskie 2014). Producing melanin ornaments could also be an elegant way to dispose of heavy metals as Great Tits show larger melanin ornaments in the environment more polluted by cadmium (Dauwe & Eens 2008).

1.2 Carotenoid coloration

Carotenoids are lipochromes, lipophilic chemical compounds soluble in fats, which are primarily pigments synthetized by plants and algae playing a part in photosynthesis and as a protection from photodamage (Hirschberg 2001). Apart from plant and algae, also certain bacteria and fungi can produce these pigments but vast majority of animals, including birds, is unable to synthetize them and consequently carotenoids have to be obtained from a diet and stored for a later use (McGraw 2006a).

Carotenoids are responsible for the rich yellow, orange and red colors of birds' plumage, beaks or bare skin. Carotenoid coloration is widespread in birds, found in many families, except for parrots, which utilize other pigments, the psittacofulvins (see chapter 1.3 *Less common* *pigments and structural coloration*). The appearance of carotenoid coloration is highly dependent on the quantity and quality of carotenoids in the diet. Birds eating low amount of carotenoids usually produce much drabber plumage in terms of saturation or chroma as in the American Goldfinch (*Spinus tristis*) or the Northern Cardinal (*Cardinalis cardinalis*; McGraw *et al.* 2001). In addition, it was already shown that carotenoid poor diet affects also coloration of other parts of the bird body such as bills of Zebra Finches (*Taenopygia guttata*) in the study of Birkhead, Fletcher & Pellatt (1998). But the plumage coloration might be affected even more dramatically by insufficient carotenoids supply, changing the hue of the color. One of the best studied examples is the House Finch (*Haemorhous mexicanus*) which shows a continuous shift from yellow to red hue according to the amount and type of carotenoids in its diet (Hill 1992, 2000; plate 3). Red carotenoids can also shift the hue of species which normally show yellow carotenoid coloration towards more reddish tones, either in laboratory (McGraw *et al.* 2001) or as a consequence of the introduction of alien plant species (Witmer 1996).

Not all bird species are so helpless in terms of maintaining their typical hue of carotenoid coloration. Many bird species apparently metabolize ingested ubiquitous yellow carotenoids into red ones such as several species of cardueline finches (Fringillidae; Stradi *et al.* 1997) or members of Cardinalidae like *Piranga* tanagers (Hudon 1991) and the Northern Cardinals. In case of Northern Cardinals, their plumage is much less saturated in the absence of red carotenoids in diet, though (McGraw *et al.* 2001). In Crossbills (*Loxia*) or Pine Grosbeak (*Pinicola enucleator*) the metabolization of yellow carotenoids into red ones occurs only in males, while females show yellowish plumage (Stradi *et al.* 1996).

Carotenoids and immunity

Apart from producing vivid colors, carotenoids play role in immune system. Not only they serve as antioxidants protecting tissues from the detrimental effects of oxidative stress (Møller *et al.* 2000; Krinsky & Yeum 2003), carotenoids also regulate and stimulate the immune system through affecting positively T and B lymphocytes and their proliferation and production of cytokines and interleukins (Chew & Park 2004).

The role of carotenoids as antioxidants in birds have been recently questioned by Hartley & Kennedy (2004). Vinkler & Albrecht (2010) even suggest that carotenoids themselves can show pro-oxidative qualities and only individuals in good condition, and having also enough of other non-colorful antioxidants, can afford to grow elaborated carotenoid ornaments together with maintaining high testosterone level associated with it. Costantini & Møller (2008) and

Simons, Cohen & Verhulst (2012) performed a meta-analysis of several studies and found weak or no effect at all of carotenoids as antioxidants in birds. However, a more recent study suggests that most of the studies could find no effect of carotenoids because of using unsuitable methodology. Instead of measuring lipophilic antioxidants (as are carotenoids), most commonly used antioxidant assays measure hydrophilic antioxidant capacity. Furthermore, the antioxidant function was later supported in a laboratory experiment involving Zebra Finches in the same study (Tomášek *et al.* 2016).

Several empirical studies suggest that carotenoids play some role in immunity of birds, indeed. In Barn Swallows (*Hirundo rustica*) lutein plasma concentration was negatively correlated with antibodies and leukocytes indicating that carotenoids are used during an immune challenge (Saino *et al.* 1999). A positive correlation between plasma carotenoids and PHA response, another commonly used measure of immunocompetence, was found in Eurasian Blackbird (*Turdus merula*; Biard *et al.* 2009) and further supported by a meta-analysis (Simons *et al.* 2014).

Carotenoids as indicators of quality

The facts, that carotenoids have to be ingested with diet and that they apparently play an important role in immunity, predict them to be potential sexual indicators, the ornaments which honestly inform potential mate about some attributes of its bearer. Plumage ornaments are especially predisposed to function as honest signals, because carotenoids deposited in feathers are “lost”, i.e. they cannot be used in immune system anymore (Olson & Owens 1998), unlike for example carotenoids deposited in beaks (Tomášek *et al.* 2016).

No matter the exact mechanism of carotenoids in immunity, results of numerous studies suggest that carotenoid coloration might honestly indicate the health status of its bearer, providing females with clues on which male to choose. In several species, elaboration of carotenoid plumage is positively correlated with the overall health condition, e.g. in Red Bishop (*Euplectes orix*; Edler & Friedl 2010) and Cirl Bunting (*Emberiza cirlus*; Figuerola *et al.* 1999). In other species, the carotenoid coloration is correlated with immunocomptence as in European Greenfinch (*Chloris chloris*; Saks, Ots & Hõrak 2003) and Eurasian Blackbird (Faivre *et al.* 2003). Parasite infestation was also shown to negatively influence carotenoid coloration in European Serin (*Serinus serinus*; Figuerola, Domènech & Senar 2003), House Finch (Harper 1999; Brawner III, Hill & Sundermann 2000; Hill 2000), American Goldfinch (McGraw & Hill

2000; Hill, Hood & Huggins 2009), and importantly for this thesis, also in Yellowhammer (Sundberg 1995b).

Carotenoid coloration does not need to reflect only the amount of carotenoids in diet but also overall quality of such diet. As discussed above, carotenoids are often metabolized prior to the deposition into feathers and their utilization is probably costly. House Finches that were fed with restricted diet did not grow as bright plumage irrespective of carotenoid supplementation (Hill 2000). Further support was found in a study by Estep, Shawkey & Hill (2006), as the red carotenoid coloration of Red Fodies (*Foudia madagascariensis*) was positively correlated to the overall body condition of males. Brightly colored males were also paired to females that laid larger clutches suggesting that high quality females preferred such males.

Female preference for elaborated carotenoid ornaments is the key for their role in sexual selection. Without females preferring colorful males, the discussion whether or how carotenoid coloration works in sexual selection, would be meaningless. Indeed, female preference for carotenoid coloration has been found in many studies including the most well studied species, the Zebra Finch (Price & Burley 1994; Simons & Verhulst 2011) and the House Finch (Hill 1990). Laboratory experiment showed female preference for yellower males also in the Yellowhammer (Sundberg 1995a) which was later supported also for wild population (Sundberg & Dixon 1996). Also results of other studies performed on free ranging birds confirm this preference, e.g. in Scarlet Rosefinch (*Carpodacus erythrinus*), a species of finch with similar red carotenoid ornament to House Finch (Albrecht *et al.* 2009) or on a lekking species, the Golden-collared Manakins, (*Manacus vitellinus*; Stein & Uy 2006).

From the evidence brought by numerous studies, it is apparent that carotenoid coloration can signal some qualities of the bird, including the ability to find rich food sources, being able to cope with parasites or the overall health status. Even though the proper control of this signaling might not be fully understood yet, it is important that female birds do take this coloration into account during the mate choice. Especially important for this thesis are the three mentioned studies in Yellowhammer because they suggest a direct benefit of female preference for elaborated carotenoid coloration, i.e. a healthy mate and potential genetic benefits for offspring like a higher resistance to parasites and higher reproductive success of yellower sons (Sundberg 1995a,b; Sundberg & Dixon 1996).

1.3 Less common pigments and structural coloration

Other lipochrome pigments found in birds' plumage are psittacofulvins, found only in parrots (Psittaciformes). They produce yellow, orange or red coloration in these species, basically replacing carotenoids found in other bird taxa (McGraw & Nogare 2005; Berg & Bennett 2010). Unlike carotenoids, psittacofulvins are not dietary pigments but they are synthetized during feather growth (McGraw & Nogare 2005). Another difference between psittacofulvins and carotenoids is that psittacofulvins exhibit fluorescence, i.e. the incident ultraviolet light is absorbed and emitted at longer wavelength (plate 4). This seems to be restricted only to the yellow pigments, not the red ones (McGraw & Nogare 2005; McGraw 2006c). Studies dealing with psittacofulvins and their role in sexual selection are much scarcer than those dealing with carotenoids and melanins. From the few studies performed, it seems that psittacofulvins can work similarly as carotenoids in parrot's mate choice (Pearn, Bennett & Cuthill 2001; Masello *et al.* 2004), but further research is still needed.

Even less studied than psittacofulvins are porphyrins. Porphyrins produce a wide variety of colors, from rusty brown to red, green feathers or even blue in eggshells of some birds (McGraw 2006c). Porphyrins as feather colorants are quite widely distributed across birds clades, e.g. in owls, nightjars, turacos and bustards. Especially turacos possess two pigments, which are found almost exclusively in this family of birds, the red turacin and green turacoverdin, the only green pigment in birds found so far. But the role of porphyrins in sexual selection, or their role in feathers at all, have not yet been understood (McGraw 2006c). Bird ringers have found a way, how these pigments can be informative for them, however. Since also porphyrins show fluorescence under ultraviolet light, the contrasting pattern between freshly molted and old feathers can be used as an aid when assessing age of some owls (Weidensaul *et al.* 2011).

Apart from the mentioned pigments, there are also other pigments found in birds. But these pigments, pterins, flavins or the mentioned bilins, are deposited only in avian irises, yolk or eggshells and therefore I will not discuss them in this thesis. For a review see McGraw (2006c).

Unlike porphyrins or psittacofulvins, structural coloration is much more studied in respect of its role in sexual selection. Similarly to pigment coloration, structural coloration is influenced by the diet of birds, probably because producing the precise microstructures in feathers is costly (McGraw *et al.* 2002; Siefferman & Hill 2005). Likewise, males of some species being in better condition were able to grow more colorful structural plumage (Keyser & Hill 1999, 2000; Doucet 2002). Parasite infections also seem to negatively influence structural coloration in other species (Doucet & Montgomerie 2003; Costa & Macedo 2005). Surprisingly the most common structural color found in the majority of bird species, the white color, is little studied (Pryke 2007). One of the exceptions is the study in Collared Flycatcher (*Ficedulla albicollis*) where the size of the white forehead patch works as badge of status, similarly as in e.g. the House Sparrow (Pärt & Qvarnström 1997).

Although melanins and carotenoids are not the only pigments found in birds, not even the only way to produce color, Yellowhammer shows only melanin and carotenoid coloration, so I have focused in my thesis only on these two types of pigmentary coloration.

1.4 Testosterone and corticosterone in birds

Testosterone

Testosterone, a product primarily of the testes, regulated through the hypothalamic-pituitary-gonadal axis (Adkins-Regan 2008), is a steroid hormone which promotes many traits associated with male behavior in birds (Bicudo *et al.* 2010). One of the most studied species in this respect is the Dark-eyed Junco (*Junco hyemalis*), a distant relative of Yellowhammer. In this species, elevated levels of testosterone were associated e.g. with increased song rate (Ketterson & Nolan 1992) or enhanced attractiveness to females (Enstrom, Ketterson & Nolan, Jr. 1997), which probably resulted in more extra-pair copulations (Raouf *et al.* 1997). Furthermore, elevated level of testosterone in this species comes with a cost because males with higher testosterone levels had lower body mass (Ketterson & Nolan 1992) and overwinter survival (Nolan *et al.* 1992). These studies on a single species are valuable as they show a whole array of effects testosterone has on an individual. Studies on numerous other bird species suggest that these effects might be general patterns in birds (reviewed in Bicudo *et al.* 2010).

Apart from influences described above, testosterone also seems to affect ornamental coloration in some species, another trait associated with males and sexual selection. As shown in previous chapter, melanin badges serves often as indicators of social status as in the case of House Sparrow, where the size of the melanin badge was positively correlated with testosterone (Evans, Goldsmith & Norris 2000; Buchanan *et al.* 2001; Strasser & Schwabl 2004). Results of meta-analysis by Bókony *et al.* (2008) also show a positive correlation between elaboration of melanin plumage and testosterone in several species of birds. Studies on the testosterone and its effect on carotenoid coloration are much scarcer, perhaps because carotenoids are generally viewed as dependent on the environment. Level of testosterone, however, can apparently influence the behavior of the bird and its interactions with environment (e.g. with parasites, Bicudo *et al.* 2010) which could influence carotenoid coloration. Following studies also gives contradictory results. Blas *et al.* (2006) found a positive correlation between testosterone and carotenoid coloration in the Red-legged Partridge (*Alectoris rufa*), while Edler & Friedl (2010) found no correlation at all in the Red Bishop.

According to the evidence, maintaining high testosterone levels could be costly. Testosterone was believed to increase basal metabolic rate in birds, a measure of the lowest cost of living for an endotherm (Hulbert & Else 2004). But basal metabolic rate is probably not directly influenced by testosterone, it rather promotes behavior which increases metabolic rate, such as territorial conflicts with other birds (Bicudo *et al.* 2010). Traditionally, testosterone was also believed to lower the immunity of an individual and therefore only high quality male can afford high levels of circulating testosterone to promote sexual ornaments (Folstad & Karter 1992). But several studies on testosterone immunosuppression abilities have found that testosterone elevation caused also elevation of corticosterone suggesting that the immunosuppressive hormone is rather corticosterone than testosterone (Duffy *et al.* 2000; Evans, Goldsmith & Norris 2000). Nonetheless, Foo *et al.* (2017) found in their meta-analysis a medium sized effect of testosterone as immunosuppressant.

Apparently, females can assess testosterone level of males also through coloration, at least the melanin one. But choosing a male with high testosterone level does not need to be the best choice. Males of several species show negative correlation between testosterone and parental care thus lowering the reproductive success (e.g. Hegner & Wingfield 1987; Oring, Fivizzani & El Halawani 1989; Ketterson *et al.* 1992). If testosterone also enhance attractiveness and the level of testosterone is heritable, the production of attractive offspring can counter weigh this cost.

Corticosterone

Corticosterone, a main glucocorticoid in birds (Romero 2004; but see Koren *et al.* 2012a), is a steroid hormone produced by the adrenal cortex and, similarly as testosterone, regulated by the hypothalamic-pituitary-gonadal axis. As a hormone, corticosterone is associated with the organism's reaction to an acute stressor and promotes behaviors such as increased alertness, or decrease in appetite and reproductive behavior. While these behaviors might be beneficial in short term, chronic stress could have detrimental effects on the organism (Charmandari, Tsigos & Chrousos 2005).

The detrimental effects of higher corticosterone levels have been studied also in birds, and from these studies it seems that corticosterone is correlated with wide array of traits. Higher level of corticosterone was positively correlated with foraging in several bird species (Astheimer, Buttemer & Wingfield 1992). In more recent studies, elevated corticosterone was, surprisingly, negatively correlated with body mass (Hau *et al.* 2010; McGraw, Lee & Lewin 2011). Therefore the higher level of foraging found by Astheimer, Buttemer & Wingfield (1992) could be the result of the overall bad body condition associated with high corticosterone found by the latter studies. This could have also direct effect on survival, as elevated levels of corticosterone also negatively correlated with survival in a wide range of species (Hau *et al.* 2010; Koren *et al.* 2012a; Monclús *et al.* 2017). Elevated level of corticosterone therefore can have important impact on the lives of birds.

Similarly to testosterone, also corticosterone seems to influence the elaboration of ornamental coloration in birds, maintaining its honesty. But again similarly to testosterone, these studies show inconsistent results. Several studies have found a negative correlation between carotenoid coloration and corticosterone (Loiseau *et al.* 2008; Mougeot *et al.* 2010; Martínez-Padilla *et al.* 2013; Grunst *et al.* 2015) while other suggest positive correlation (McGraw, Lee & Lewin 2011; Fairhurst *et al.* 2014; Fairhurst, Damore & Butler 2015). Further, according to other studies, corticosterone apparently positively affects utilization of carotenoids from fat stores and therefore promote expression of carotenoid coloration (Lendvai *et al.* 2013; Fairhurst *et al.* 2014).

In respect to corticosterone and melanin coloration, one of the most studied species is probably the Barn Owl (*Tyto alba*) which shows a slight negative correlation between the degree of elaboration of its melanin ornament and corticosterone, although most of the variation in coloration in this species has genetic basis (Roulin *et al.* 2008; Almasi, Roulin & Jenni 2013). But more studies for other species are lacking at the moment. Also, drawing any conclusions, other than that corticosterone could influence carotenoid coloration in different species differently, is not easy and further research in this field is needed to shed some light onto it. As assessment of hormonal levels, mainly corticosterone, from feathers is being used more often recently (Bortolotti *et al.* 2008), an interesting insight into this topic was brought by Jenni-Eiermann *et al.* (2015). In their study, the more melanized parts of the same feathers showed higher concentration of deposited corticosterone and while circulating plasma corticosterone concentration still accounted for the major part of variability in the data, correlation with color of the feathers was significant.

From studies cited above, it is clear that, also the coloration of birds can be influenced by hormones, either by changes in behavior or influencing the molecular pathway of pigment utilization and its synthetization. As hormones influence also many other important traits of birds, such as parental care or survival, also hormonal levels could be a way to maintain honesty of ornamental coloration and its role in mate choice.

1.5 Yellowhammer

The studied species – the Yellowhammer (*Emberiza citrinella*) – is a small passerine, member of the family of Buntings and New World Sparrows (Emberizidae) which is still common in Czech Republic.

The Yellowhammer breeds across majority of Europe except for the southern part of the Iberian and the Balkan peninsulas and coastal parts of the Apennine peninsula. Its breeding range spans from northern Spain to Central Asia (Cramp, Perrins & Brooks 1994). The Yellowhammer was also deliberately introduced to New Zealand in 19th century where it quickly became naturalized species (Pipek, Pyšek & Blackburn 2015).

As are many other members of the genus *Emberiza*, also the Yellowhammer is an inhabitant of more open habitats such as meadows, open woodland and shrubland, readily breeding also in traditional agricultural landscape (Cramp, Perrins & Brooks 1994).

Males of the Yellowhammer in their summer plumage show extensive bright yellow carotenoid coloration which covers head and lower body. Yellow coloration of females is much less bright and smaller in size. Furthermore there is a difference between young and old birds in both sexes as older birds show more extensive and saturated yellow coloration. Both sexes also display rufous patch of phaeomelanin on rump, some males also a rufous breast band of various size. In winter, the bright coloration is somehow obscured by the darker edges of the feathers and both sexes are much more similar in appearance (Cramp, Perrins & Brooks 1994).

1.6 Aims of the study

In my thesis, I investigated the signaling function of Yellowhammer's plumage coloration, and I focused on following aims, which could be further categorized into three topics.

Methodological approaches:

- To determine the number of feathers that are needed in one sample for spectral analysis to obtain representative results of natural bird coloration.
- To analyze the potential influence of sample composition regarding the melanin-pigmented feathers on feather hormone concentrations.

Pattern in coloration and its association with age, condition and hormone levels:

- To identify any differences between young and old males concerning the area and spectral characteristics of Yellowhammer's coloration as well as feather hormone concentrations among young and old males, and to find their potential differences between these two age groups.
- To describe possible correlation between the concentrations of testosterone and corticosterone deposited into feathers and various attributes of Yellowhammer's coloration.
- To perform an analysis of avian visual model to simulate whether the birds themselves are able to perceive the studied differences.

Territoriality and survival:

- To find out which characteristics of the carotenoid coloration and feather hormones concentrations could predict the ability of Yellowhammer males to acquire and maintain territory and/or their survival to the next season.

2 Material and methods

2.1 Study locality and population

Field work was conducted in the Czech Republic, between villages Vižina and Osov (49.843 N, 14.104 E, approx. 370–450 m above sea level) in Beroun county (fig. 3). Study locality is an agricultural man-made habitat which comprises mostly of agricultural meadows as a source of hay and therefore cut short in late June. The meadows are interspersed with hedgerows with shrubs, mostly various species of Rosaceae (*Rosa, Rubus, Prunus*), and trees (mostly *Fagus, Aesculus hippocastanum, Betula, Alnus*). In the middle of the locality there is a smaller patch of woodland, dividing it in two sections (plate 5). From three sides our locality is enclosed by a mixed forest (composed predominantly of *Picea abies* with intermixed *Fagus*) which acts as a distinct border, the remaining western border is less distinct and is arbitrarily chosen since there is no clear barrier, and Yellowhammers occur there too.

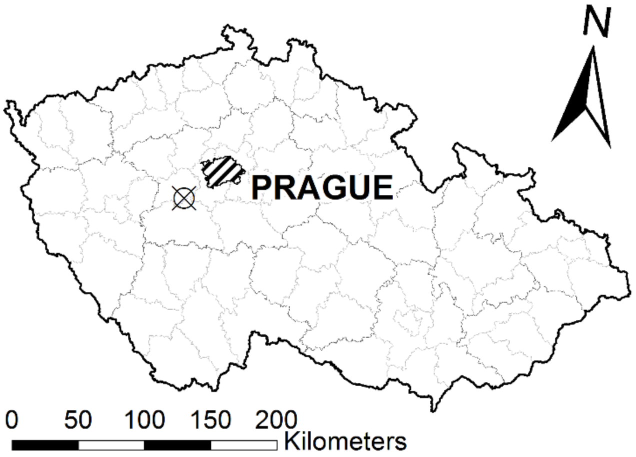

Figure 3: Approximate position of the study locality in Czech Republic (marked with crossed circle).

Data were collected during three consecutive breeding seasons (end of March to end of July) in the years 2015, 2016 and 2017. Numbers of individual male Yellowhammers caught during the study are given in tab. 1. Apart from the birds sampled in our study locality, 14 Yellowhammers also included in this study were trapped outside our study locality throughout 2016 and 2017 (listed in appendix 1). These individuals served to assess the number of feathers needed to get representative measurements using spectrophotometer (see below).

Table 1: Numbers of individual male Yellowhammers in different age categories caught in the study locality.

breeding season	young	old
2015	15	16
2016	8	10
2017	7	8
TOTAL	**30**	**34**

To prevent further confusion I will explain the meaning of the age categories I use in this thesis – the young and old males. *Young* males are males in their first year of life, EURING category 5. These males are the males which survived their first winter and are attempting to breed for the first time in life. *Old* males, EURING category 6, are males older than the young ones, i.e. males which survived at least two winters and are attempting to breed for at least the second time. Unfortunately it is not possible to distinguish between the old males any further so exact age of these *old* males is unknown.

During breeding seasons of 2016 and 2017 nest search was also conducted in our study locality. I used a method of systematically searching the locality with a long pole gently tapping the potential nest places to flush away an incubating female. This way I could locate the nest very precisely during the incubating period. Outside the incubation period I had to rely on acoustical warnings of parent birds or other cues such as birds bringing food items to their chicks. In 2017 my colleague also tried a thermal imaging camera to locate the nests (Galligan, Bakken & Lima 2003) but without any success (Sharina van Boheemen, *pers. comm.*). Because of the very secretive nesting of Yellowhammer, together with its habit of placing the nest in the very dense bramble, only very few nests were found in the end, n = 10 in 2016, n = 0 in 2017. From the 10 nests of 2016 only 4 nests probably lasted long enough to successfully fledge young, giving success rate of only 40 %. Other nests failed either because of torrential rains filling the ditches where the nests were placed (n = 2) or because of predation (n = 4). I witnessed three of these predation events. One nest was predated by a Common Raven (*Corvus corax*), but the two others by a less expected predator – Grass Snake (*Natrix natrix*; *personal observation*).

2.2 Measurements and data collection

Mist netting and morphometric measurements

Birds were captured using mist nets which are generally considered a safe method to capture birds (Spotswood *et al.* 2012). Mist nets were placed in suitable habitats where higher occurrence of Yellowhammers was expected. Usually additional playback recording of the Yellowhammer song was broadcasted to increase the chance of capturing a Yellowhammer male in his territory. Playback recordings including also the contact calls were used when capturing both sexes.

Each captured bird was equipped with the standard aluminum ring from the Czech ringing scheme and with a unique combination of three plastic color rings for easier identification in the field without the need to re-capture the individual[2]. Then, the sex and age of the bird was assessed using the color and pattern of crown feathers, shape of tail feathers and/or finding molt limits in wing feathers (Svensson 1992; Demongin, Lelièvre & Candelin 2016; Blasco-Zumeta & Heinze 2017; Ottenby Bird Observatory 2017). Subsequently, following morphometric measurements were taken: length of tarsometatarsus (so called "tarsus") with digital calipers to the nearest tenth of millimeter, length of flattened wing using a metal ruler with "zero stop" measured to the nearest millimeter and the bird was weighed using Pesola spring balance with the precision of 0.5 grams. Because of the inconsistence between measured values for the length of tarsus, wing length was used as a proxy for body size.

Photographs

After the measurements were taken, the birds were photographed in a standardized way in three standardized positions: so called "back" (photograph of the dorsal surface), "belly" (photograph of the ventral surface) and "side" (photograph of the left lateral surface; fig. 4) that were same through the whole study.

However, pictures from 2015 were taken using a less standardized method than in later years (see below). Bird was held by one person and placed on a pad – a 5 × 5 mm grid printed on white paper with yellow and grey blocks intended as color reference. Second person was needed to operate the camera which was held in a fixed distance determined with a tube made of hard paper. Also the camera used in 2015 was different from the later seasons. It was a Canon IXUS 155 set to "automatic regime" – camera automatically set ISO, white balance, shutter speed and aperture according to the light conditions. The image size was set to 12 Mpix, no flash was used and photographs were taken with no optical zoom (focal length 7.40 mm, equivalent of 34 mm for full frame sensor). Pictures were saved on an SD card in JPEG format, resolution of pictures was 4000 × 3000 pixels. Because of the lack of standardization of light conditions during photographing the birds in 2015 and the fact that the pad used was laminated making its surface reflective, the yellow and grey blocks intended for color standardizations were improper. Consequences of this approach are discussed later in text.

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[2] Color rings served mainly as a tool to identify territorial males and to assess their survival. Moreover, they were also used to identify specific individual when the birds were recorded throughout the season for another part of the project.

After the analysis of photographs from 2015, the need for more standardized pictures was obvious. To be able to standardize photographic conditions in the field, I developed a special tool which will be referred to as "the Apparatus". The Apparatus is a tent-like structure with black cloth to block sunlight and a gray card with a 5 × 5 mm grid at the edges as a size reference. The grey card is used in photography to calibrate color (white balance) and light settings. Inside a camera is mounted in standardized distance from photographed objects (more information is available in appendix 2). The camera used in the Apparatus is a Sony DSC-HX60. To facilitate the comparison of the photographed birds, all photographs were taken using the same camera settings: shutter speed 1/100 s, aperture 3.5, color temperature: 6500 K, ISO 80, image size 20 Mpix, quality: "fine", flash mode: "fill-flash" and with the aid of automatic focus: single-shot AF, flexible spot settings. All the other picture effects were turned off. Optical zoom was not used during photographing making the focal length 4.30 mm (equivalent of 28 mm for full frame sensor). Pictures were saved on an SD card in JPEG format, resolution of pictures was 5184 × 3888 pixels. The camera was operated with a remote shutter release which enables holding the bird in both hands and therefore a single person operation.

Feather samples

At the very end of the process, around ten feathers from the belly region and around ten feathers from the rump were taken. These were then used for the spectral analysis using spectrophotometer and samples from 2015 and 2016 were later also used for feather hormones concentration assessment. During the extraction of the feathers, special emphasis was taken to choose feathers from more than one spot at the area of interest not to alter the bird's coloration. Collected samples were stored until their processing in dark and dry place at room temperature.

The 14 individuals from outside our study locality mentioned above underwent a shorter version of the sampling protocol. This consisted only of fitting a standard aluminum ring and extracting 20 feathers from the belly region.

Territoriality assessment

During the breeding season in 2015, individual Yellowhammer males were regularly checked at the locality to assess their territorial status. Since Yellowhammer's territory is usually very small (Cramp, Perrins & Brooks 1994), it was possible to discriminate between males with territory and those without. Males that showed sign of stable territory, e.g. were found regularly singing in the same area, were considered to have stable territory, those that did not show these signs were considered not. Also, males trapped at the locality and then never re-trapped were considered without stable territory.

Survival status

The survival status of adult Yellowhammer males between the years 2015 and 2016 was assessed by searching the locality multiple times during the breeding season in 2016 for color ringed males or recapturing them. As the Yellowhammer show strong breeding site fidelity (Paradis *et al.* 1998), and since northern winter poses such a survival challenge to birds, males that were absent next breeding season were considered dead.

Both territoriality and survival status strongly depend on our ability to find males all the males and the assumption that they will stay at our study locality. The potential limitations of this approach are discussed.

Ethics Statement

Birds were captured using standard methods (described above) and by persons allowed to capture birds in the wild (i.e. “ringers”): by myself, license number: 1197; Sharina van Boheemen, license number: 1220 and Tereza Petrusková, license number: 1120. Ringers, that captured the birds for the analysis of number of feathers needed for one sample, were: myself; Tereza Králová, license number: 1219; Radek Lučan, license number: 959 and Anna Koukolíková, license number: 1222. Majority of the data were collected by myself and Sharina van Boheemen, hormonal concentrations were measured by Marie Kotasová–Adámková and I performed the statistical analyses used in this thesis. Birds were handled for the shortest time necessary to obtain all the data and to minimize any distress. At the end of the process birds were released close or at the place of capture. Study was approved by the Ethical Committee of Charles University.

2.3 Photography analysis

For every photographed bird I have selected a set of three photographs in the three above mentioned standardized positions. Each photograph was then color calibrated using Adobe Photoshop CS6 (with the gray card used as a color reference). For most of the photographs from the seasons 2016 and 2017 this meant only slight adjustments of the color balance. Photographs from 2015 differed in light conditions to such an extent that color calibration was virtually impossible to achieve and this step was omitted for this reason. This was taken into account later when discussing the results of the analyses using these data.

After the color calibration, measurements of the area of yellow and rufous color were performed using the software ImageJ, v 1.50b (Schneider, Rasband & Eliceiri 2012). First, with the use of the function "Polygon section", an area of "whole body" was selected and its area was measured with the "Measure" function. For each Yellowhammer individual, the same "whole body" regions were selected using following criteria (also see fig. 4):

- **Back (dorsal):** head, whole back including rump feathers, upper wing coverts and tertials, excluded are primaries, secondaries and tail feathers.
- **Belly (ventral):** head and belly up to the point where legs are emerging from feathers, excluded is region "behind" legs: undertail coverts (since these are most often covered with fingers holding the bird), tail and whole underwing.
- **Side (lateral):** head, sides of belly and back including wing and rump feathers, excluded are legs, tail and protruding tips of primaries.

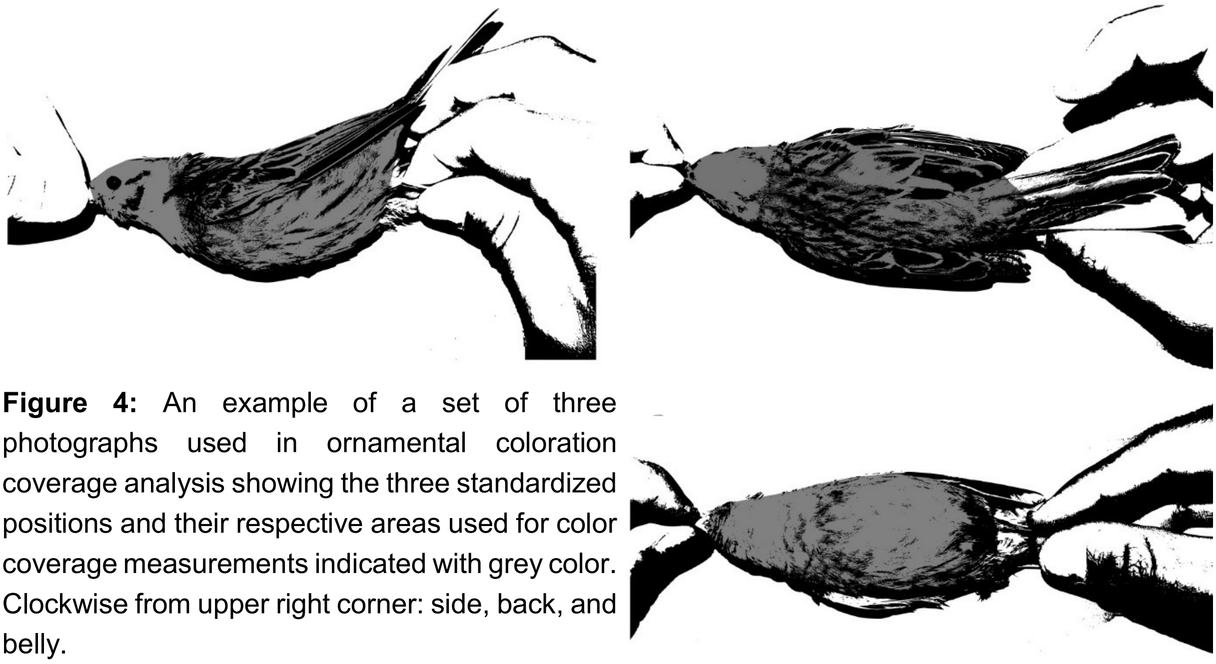

Figure 4: An example of a set of three photographs used in ornamental coloration coverage analysis showing the three standardized positions and their respective areas used for color coverage measurements indicated with grey color. Clockwise from upper right corner: side, back, and belly.

When the "whole body" region area was known, the "Threshold" function was used to measure the areas of yellow and rufous colors within its borders. For the yellow color the function was set to: hue: 30–45, saturation: 80–255, brightness: 120–255; and for rufous color settings were: hue: 10–30, saturation: 80–255, brightness: 120–255. These values were chosen based on personal judgment of color and cover the whole spectrum of Yellowhammer colors found in studied specimens. From these values it is obvious that – in the HSB color space – the border between yellow and rufous color is very narrow and it is arbitrarily chosen as it separated yellow from rufous. Considering the lack of standardization in the pictures from 2015, values of hue, saturation and brightness for yellow and rufous color had to be adjusted for every set of Yellowhammer photographs from this period.

2.4 Feather spectrophotometer analysis

To obtain objective assessment of the coloration of the individual Yellowhammers, a spectrophotometric measurements were made using a spectrophotometer (Andersson & Prager 2006). All samples obtained in this study were measured as described below.

Spectrophotometer analysis

Before measuring feather samples with spectrophotometer feathers were arranged one on top of each other to simulate feather arrangement found in living birds similarly as in Quesada & Senar (2006). Feathers were fixed with adhesive tape on a black cardboard by their feather shafts (calamus) and handled with tweezers to minimize pollution of measured parts. Samples prepared in this fashion were then measured by reflectance spectrophotometer following the methods of Tomášek *et al.* (2016), i.e. spectrophotometer used was AvaSpec 2048 with AvaLight-XE (Avantes, Netherlands) as a light source. A specially modified adapter for sensing head was used to maintain constant distance 3.5 millimeters between sensing head and the measured sample and to exclude ambient light. Following settings of measurements in software AvaSoft, v 7.8 were used: integration time: 100 ms, 15 averages, smoothing: 3, number of flashes: 3. Measurements were performed under standardized light conditions and each sample was measured three times in distal (yellow or rufous) part of the feathers under angle of 90°. Sensing head was lifted from the sample between every measurement. After measuring two individuals, spectrometer was calibrated using standard of white color WS-2 (Avantes, Netherlands) and absolute dark – sensing head was covered and light source turned off.

Number of feathers in a sample

To know how many feathers are sufficient to obtain adequate results, I also prepared samples of another 14 Yellowhammers caught outside the study locality (see chapter 2.1 *Study locality and population*, and appendix 1). Measuring these samples with spectrophotometer followed the same protocol as the samples from the study locality. Number of feathers arranged on the black cardboard was different, though. In the beginning, each sample comprised of 20 feathers, and after each measurement, two feathers were removed and measurement was repeated. This way feathers were removed until only two remained in each sample.

2.5 Feather hormones

To establish the concentration of corticosterone (CORTf) and testosterone (TESTf) in feathers a protocol based on Koren *et al.* (2012a,b) and following methods described in Kotasová-Adámková *et al.* (in prep) were used.

Feathers were weighed and then pulverized with a ball mill to obtain a fine powder. After pulverizing, 1 ml of HPLC-grade methanol was added and samples were shaken with the use of orbital shaker (450 rpm, 24 hours). Then, samples were centrifuged (5 000 rpm, 5 minutes), supernatants were collected and sediment was diluted in 1 ml of methanol. This extraction was performed twice and resulting extracts mixed together. Extracts were then spiked with steroid mix (corticosterone-2,2,4,6,6,17α,21,21-d8, testosterone-16,16,17-d3) and methanol was evaporated by nitrogen steam. Then extracts were resuspended in 2 ml of a mixture of methanol and water (5:95) and purified by solid phase extraction (SPE) using Bond Elut C18 SPE cartridges (3 ml, 100 mg sorbent, end-capped). At first columns were preconditioned with 5 ml of methanol and further then with 5 ml of the mixture of methanol and water. Samples were then placed to SPE columns, washed with 2 ml of de-ionized water and eluted by 2 ml of methanol to vials. Elutes were then dried with nitrogen stream and 50 µl of derivatization reagent working solution was added and kept at 65 °C for 2 hours. Then 10 µl de-ionized water was added and mixed with vortex mixer.

The quantification of hormones with LC/ESI/MS-MS itself was performed on an Agilent 1200 chromatographic system equipped with a vacuum degasser, binary pump, autosampler and column thermostat which was connected online to a ESI/QqQ mass spectrometer Agilent Triple Quad 6410 (Agilent, USA) and controlled by Mass Hunter software. For analytical separation an analytical column ACE 3 C18, 150 mm x 2,1 mm i. d., 3 µm particle size with integrated guard column ACE 3 C18 (2.1 mm × 10 mm, 3 µm particle size), (ACE, Scotland, UK) were used with temperature set to 25 °C. A mobile phase consisted of 0.1 % formic acid in water (solvent A) and 0.1 % formic acid in methanol (solvent B). The mobile phase gradient raised from 30% of B to 90% and then dropped backed to 30 % during a 50 minute cycle. The flow rate was 0.2 ml/min; 10 µl of sample was injected. The ion source were set to following settings: gas temperature: 340 °C, gas flow 8 l/min, nebulizer: 35 psi, capillary voltage: 2000 V and was operated in the ESI-positive mode.

Final concentrations of hormones in feathers were given in picograms of hormones per one gram of feathers.

2.6 Statistical analysis

All statistical analyses were performed in the open source statistical software and programming language **R**, v 3.3.3 (R Core Team 2017) using the IDE RStudio, v 1.0.153 (RStudio Team 2016). To calculate the primary component analysis, I used the R package vegan, v 2.4-3 (Oksanen *et al.* 2017), to assess repeatability I used the package rptR, v 0.9.2 (Stoffel, Nakagawa & Schielzeth 2017). To process the raw data from spectrophotometer as well as to model the bird vision I used the R package pavo, v 1.1.0 (Maia *et al.* 2013).

All linear and generalized linear models were tested and simplified by gradual removing non-significant interactions or factors as described in Pekár & Brabec (2009) and Crawley (2013). When relevant, models were compared by their Second-order Akaike Information Criterion (*AICc*) computed with the use of package MuMIn, v 1.15.6 (Bartoń 2016).

Calculation of the yellow and rufous indices

From the analysis of Yellowhammer standardized photographs, nine values of measured areas were obtained in total per bird: three values of total body area, three of yellow and three of rufous color patch. To control the measurements for varying area of photographed specimens, proportion of the area of color patch to the "whole body" region area was calculated. This produced three relative and unitless values – proportions.

To get a "color index", proportion data on yellow and rufous color were orthogonalized – a PCA was conducted for all proportion values (each color separately). From resulting graphs, it is clear that proportion values for each body part are correlated with each other, aligning with primary component 1 (fig. 5), therefore its scores were selected as such index, explaining 70.6 % of variability in data for yellow color and 77.74 % of variability in the data for rufous color.

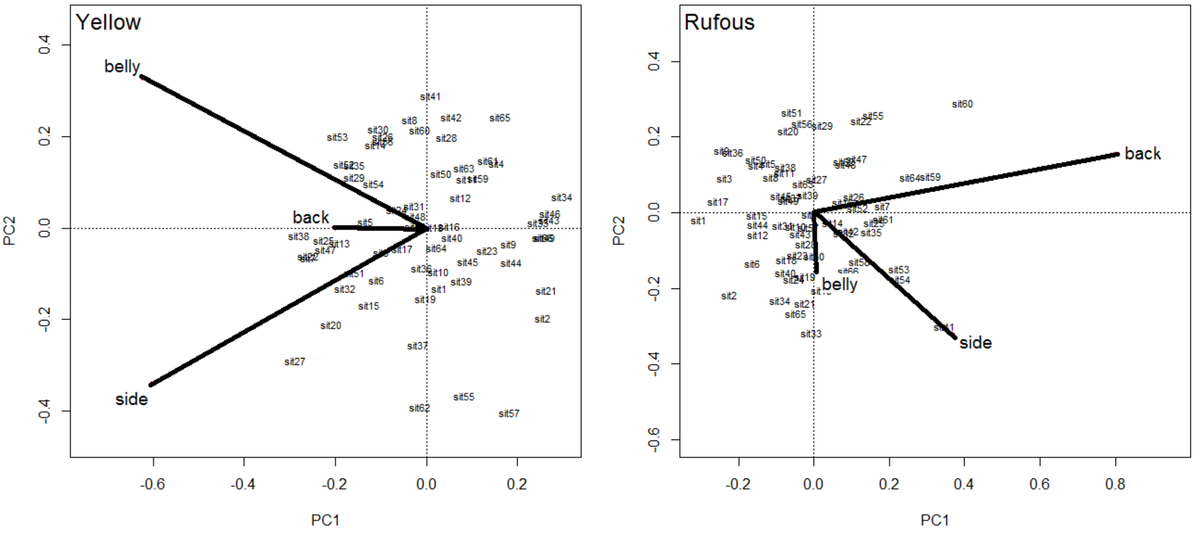

Figure 5: Loadings of body regions coverages on primary components 1 and 2 for yellow and rufous coloration.

Spectral characteristics calculation

When the feather samples were measured with spectrophotometer their measured values were converted to summary table in AvaSoft and exported to a "comma-separated values" file. These were then imported to the R and analyzed with the use of the package Pavo. Only part of the measured spectrum was used – data were trimmed to the range of 300 – 700 nm as this is the part of light spectrum that birds can perceive (Cuthill *et al*. 1999; reviewed in Cuthill 2006; Pryke 2007). Then reflectance equivalent was calculated for integral values of wavelengths and three measurements of the same sample were averaged. This average was then smoothed (span 0.05) to get rid of some of the spectrometer noise. Relatively low value of span of smoothing was selected because increasing the value of span altered the reflectance curve in the carotenoid absorptance part of the spectrum. From these curves following characteristics were

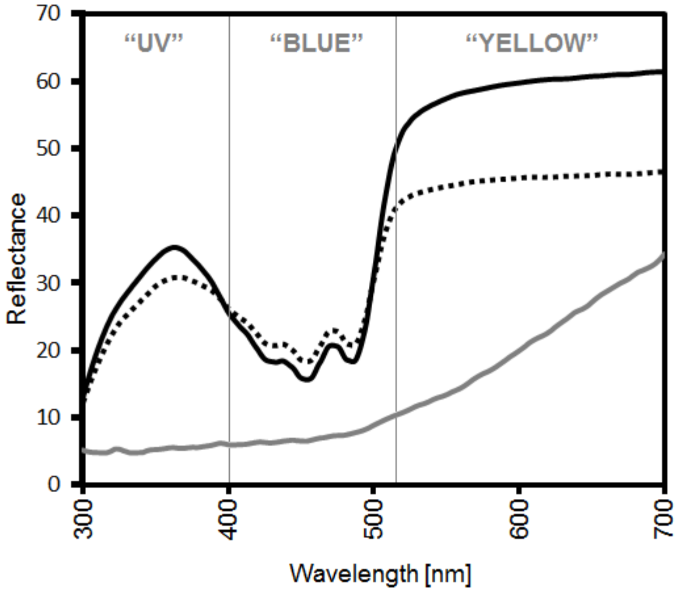

Figure 6: Spectral curves of Yellowhammer belly (black) and rump (gray) coloration and the three respective chroma intervals (described in text). For carotenoid coloration, two examples are presented: an individual with high brightness and carotenoid concentration (solid line) and one with low brightness and carotenoid concentration (dashed line).

calculated using Pavo. Definitions as described in Montgomerie (2006) and Maia *et al.* (2013) or with slight adjustments:

- **Total brightness (B_1):** *sum of the relative reflectance over the entire spectral range.*

$$B_1 = \int_{\lambda_{min}}^{\lambda_{max}} R_i = \sum_{i=\lambda_{min}}^{\lambda_{max}} R_i$$

- **Mean brightness (B_2):** *mean relative reflectance over the entire spectral range.* According to Maia *et al.* (2013) total brightness is difficult to compare across studies and "mean brightness (B_2)" is suggested as a better alternative instead.

$$B_2 = \sum_{i=\lambda_{min}}^{\lambda_{max}} R_i \bigg/ n_i = B_1/n_w$$

- **Chroma (S_1X):** chroma is calculated as *a ratio of brightness of a certain wavelength interval (i.e. "color") and total brightness* (B_1). For this study I have chosen these intervals relevant to the Yellowhammer carotenoid coloration (fig. 6):
    - **UV** (S_1U: $a = \lambda_{min} = 300$ nm, $b = 400$ nm) as Yellowhammer carotenoid coloration shows a clear peak in UV part of the spectrum.
    - **Blue** (S_1B: $a = 400$ nm, $b = 510$ nm) corresponds to the negative peak of the spectral curve, i.e. the concentration of carotenoids in feathers (Kläui & Bauernfeind 1981; MacDougall & Montgomerie 2003; Butler, Toomey & McGraw 2011).
    - **Yellow** (S_1Y: $a = 510$ nm, $b = 700$ nm) represents the second "peak" of the carotenoid coloration in the human visible spectrum responsible for the yellow color of the Yellowhammers we perceive.

$$S_1X = \sum_{i=\lambda_a}^{\lambda_b} R_i \bigg/ \sum_{i=\lambda_{min}}^{\lambda_{max}} R_i$$

- **Hue:** based on the "H_3" hue defined by Maia *et al.* (2013) as *a wavelength at the reflectance midpoint between R_{max} and R_{min}*. Because of the nature of carotenoid data (more than one peak) and the way this value is calculated, minority of the birds had value at wavelengths corresponding to the UV peak, while majority (91 %) at the yellow peak. Therefore, to prevent those nonsense values, hue was calculated from trimmed data ($\lambda_{min} = 450$ nm, $\lambda_{max} = 700$ nm). This way, the UV peak was omitted from the data (as in Grunst *et al.* 2015). For rump rufous coloration, showing a very different spectral curve (fig. 6), non-trimmed data were used as this did not produce nonsense values.

$$H_3 = \lambda_{R\mathrm{mid}}$$

I also analyzed carotenoid and melanin plumage coloration modelling the avian vision, as was done in Stoddard & Prum (2008). From the spectral curve obtained, the relative values of stimulation of the four color cones are calculated under defined light conditions. The light conditions were set to "D65" as the illuminant D_{65} models standard daylight (Ohta & Robertson 2006). I created a visual model of the Atlantic Canary (*Serinus canaria*), because from the available data of Hart & Vorobyev (2005), Atlantic Canary is phylogenetically closest to the Yellowhammer. For the R script of the model see appendix 3. Results of this model are two angles θ and φ in radians and a vector – R achieved. Angles θ and φ correspond to relative stimulation of the photoreceptors and therefore the hue of the color, while R achieved is the relative distance from the achromatic center in relation to the maximum distance achievable, i.e. saturation of the color perceived (Stoddard & Prum 2008).

Repeatability of measurements

Repeatability of the spectral measurements and photography analysis was overall good and is summarized in table 2 and comparable to other studies (Saks, McGraw & Hõrak 2003; Tomášek *et al.* 2016). Only total brightness proved somehow problematic so using mean brightness is probably a better alternative (total brightness and mean brightness are correlated with coefficient = 1).

Table 2: Repeatability of photographic (up, n = 14) and spectral (bottom, n = 17) variables of carotenoid and melanin coloration.

coloration	ventral	dorsal	lateral	index
carotenoid	0.98	0.96	0.99	0.91
melanin	0.99	0.93	0.99	0.93

coloration	total brightness	mean brightness	UV chroma	blue chroma	yellow chroma	hue
carotenoid	0.43	0.71	0.73	0.78	0.6	0.64
melanin	0.4	0.77	0.66	0.8	0.74	0.64

Repeatability of the hormonal concentrations in feather samples was very good: 0.97 for corticosterone and 0.91 for testosterone (Kotasová-Adámková *et al.*, *in prep*).

Number of feathers in a sample

To verify the reliability of our measurements of feather samples, i.e. that they are representative enough, I performed a similar analysis as Quesada & Senar (2006). Unlike mentioned authors, I did not compare our feather samples with living birds, instead I used the sample with 20 feathers as reference and compared samples with 18, 16, 14, 12, 10, 8, 6, 4, and 2 feathers respectively with reference sample. To determine the effect of the number of feathers in one sample to the spectral characteristics obtained from these samples (brightness, UV and blue chroma and hue) I performed a Repeated-ANOVA where number of feathers was dependent variable. After that I calculated a Dunnet *post-hoc* analysis to test the differences in above mentioned variables with different numbers of feathers in the samples. I also checked whether the values measured on 2 feathers compared to 20 feathers are correlated, because several studies (e.g. Saino *et al.* 2013a; Romano *et al.* 2016; Costanzo *et al.* 2017) used just one feather when measuring the spectral variables. To test this, I performed correlation tests to assess the degree of correlation.

Difference between age groups in coloration and hormone concentrations

For the analysis of differences in studied phenomena between the two age groups I used data from all males caught in our study locality in all three breeding seasons. I checked the normality of the data with Shapiro-Wilk test and then performed several two-sample unpaired Welch's t-test because of the unequal variances and sample sizes in the two age groups. Only the hue of the carotenoid coloration and total brightness of melanin coloration were not normally distributed. I used the nonparametric Mann–Whitney U test to test the difference in hue and log transformed total brightness and then used Welch's t-test as with the rest of variables. I compared the males from all three breeding seasons (with the exclusion of retrapped individuals) together. Only yellow and rufous indices were not compared this way, because of the differences in methodology (a different camera and non-standard conditions) between the breeding season in 2015 and those in 2016 and 2017.

I analyzed the differences in hormones concentrations in feathers between the two age groups also using two-sample unpaired Welch's t-test. This time I used a different dataset – males from our study locality from 2015 and 2016.

Effect of composition of feather samples and hormonal concentrations

Because of different protocol when preparing the feather samples for the males from our study locality (sample prepared from belly and rump feathers) and those outside (samples only from belly feathers) I ran a linear model to see whether the presence of rump feathers in the sample influences the hormone concentration. I corrected this linear model for the age of the birds in case age would have some effect on the concentration of feather hormones. The feather hormones concentration were log-transformed as residuals of the models showed log-normal distribution. For this analysis the recaptures in 2016 of individuals trapped previous year were left out from the data to avoid pseudoreplications (n = 3).

Territoriality and Survival

To learn which attributes of the carotenoid coloration or hormonal concentration could predict the ability of individual male Yellowhammers to obtain a territory and their survival of winter, I performed four generalized linear models (GLM) with binomial errors to test following:

1. territoriality and plumage characteristics
2. survival and plumage characteristics
3. territoriality and feather hormone concentration
4. survival and feather hormone concentration

Effect of feather hormone concentrations and size on coloration

I also decided to test the effect of feather corticosterone and testosterone on melanin coloration. The effect was tested with a linear model with carotenoid (yellow index, brightness, hue and blue and UV chroma) or melanin plumage characteristics (rufous index, brightness and hue) as a dependent variable and feather corticosterone, testosterone, age and wing length as independent variables.

3 Results

Judging the type of pigment in feathers by its color only can be risky (McGraw *et al.* 2004b), therefore I analyzed the curves obtained with spectral analysis first. The curve of the rufous rump feathers of the Yellowhammer produced a reflectance curve typical for phaeomelanin (McGraw *et al.* 2004a; Hofmann *et al.* 2007) and showed no peak reflectance in ultraviolet part of the spectrum that could indicate presence of carotenoids (as in some New World Orioles, genus *Icterus*; Hofmann *et al.* 2007). On the other hand, the spectral curve of yellow feathers was a typical reflectance curve of yellow carotenoids as found in other studies (Peters *et al.* 2004; Butler, Toomey & McGraw 2011; Trigo & Mota 2016; fig. 6).

Because of the nature carotenoid coloration is produced (i.e. subtracting "blue" part of the spectrum from otherwise "white" feathers – from human perspective), I performed three correlation tests to see if I can reduce the number of chromatic variables used. All three chromatic variables (UV, blue and yellow) are correlated with each other (fig. 7). UV and blue are correlated positively ($t = 2.89$, df = 68, Pearson's correlation coefficient (PCC) = 0.33, $p < 0.01$), UV and yellow negatively ($t = -9.03$, df = 68, $PCC = -0.74$, $p < 0.001$) as well as yellow and blue ($t = -15.33$, df = 68, $PCC = -0.88$, $p < 0.001$). Relationship between blue and ultraviolet chroma seems to be weakest therefore I decided to use only these two variables in further analysis and omit the yellow chroma.

From the spectral curve of melanin coloration it is evident that the chromatic variables gave no extra information and therefore only total brightness, mean brightness and hue were used (fig. 6).

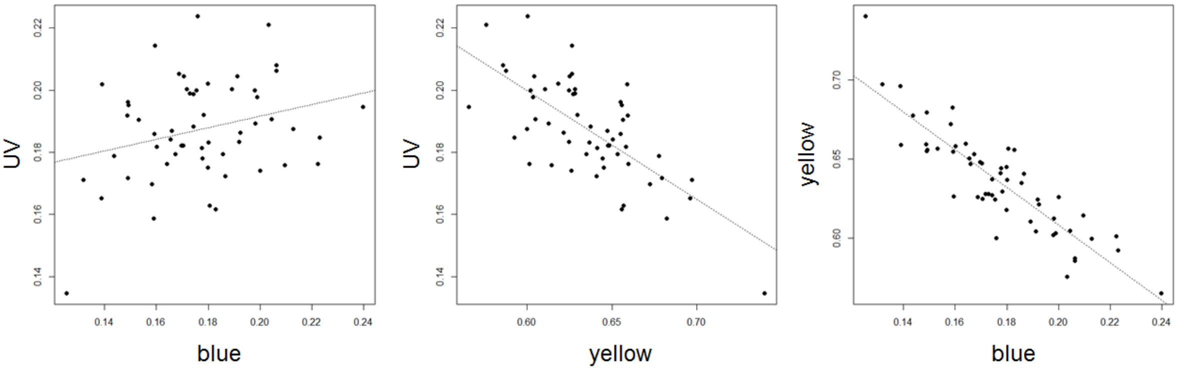

Figure 7: Scatterplots showing relationship between the three chromatic variables (ultraviolet, blue and yellow).

3.1 Number of feathers in a sample

The number of feathers in one sample influenced the measured spectral variables quite dramatically. From fig. 8 it is evident that brightness increased substantially with addition of more feathers up to 10 feathers per sample, since then changes are quite small. By brightness I mean either total or mean brightness. Both variables behaved the same, they differed only in their scale. The ultraviolet and blue chroma both decreased with addition of more feathers but while UV chroma kept lowering with more feathers added, the blue chroma (corresponding to the “carotenoid peak”) stabilized at around 8 feathers in sample. Special case was the hue which seemed to yield representative results in samples with as few as 4 feathers. These numbers of feathers were then confirmed by the *post-hoc* analysis of ANOVA model (summarized in tab. 3): for brightness and blue chroma 8 feathers in sample is enough to produce sample comparable to control sample. For ultraviolet chroma 12 feathers are needed. As evident from fig. 8, representative values of hue were achieved with as few as 4 feathers.

Because of this analysis was conducted late during this study, we could not really take its results into account. But majority of our samples contained 10 feathers (belly: 10.44±1.68 S.D., n = 56; rump = 10.11±1.44 S.D., n = 55) which is sufficient for assessment of brightness and blue chroma. For UV chroma, 12 feathers would be probably optimal and the consequences of this will be discussed later.

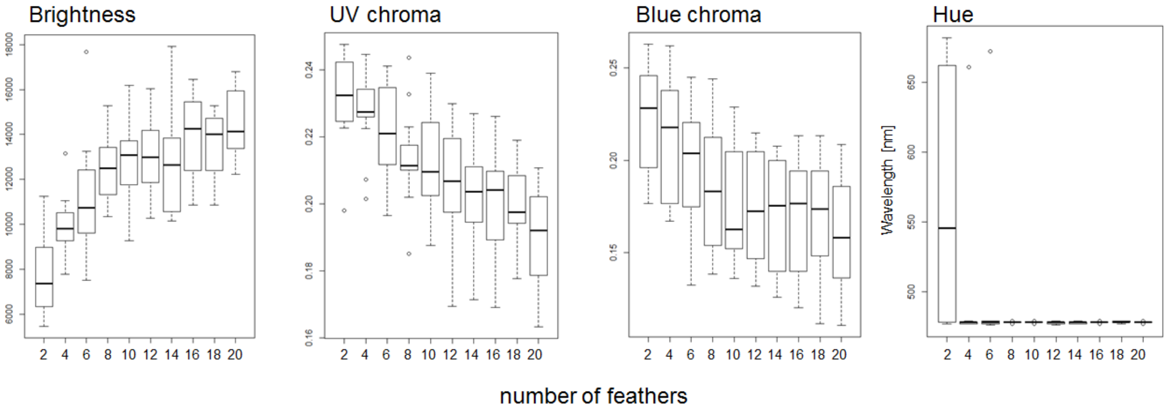

Figure 8: Effect of number of feathers used in one sample on the spectral characteristics

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Table 3: Results of the *post-hoc* analysis comparing measured values of control sample (20 feathers) with tested samples. Samples different from control sample are marked with bold font.

no. of feathers	2	4	6	8	10	12	14	16	18
brightness	**< 0.01**	**< 0.01**	**< 0.01**	0.09	0.25	0.42	0.21	0.99	0.90
UV chroma	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	0.07	0.43	0.63	0.72
blue chroma	**< 0.01**	**< 0.01**	**< 0.05**	0.48	0.81	0.94	0.98	0.99	0.99
hue	**< 0.01**	0.99	0.99	1	1	1	1	1	1

Correlation between 2 and 20 feathers differed for different variables measured. The chromatic variables were all correlated quite strongly but total and mean brightness and hue were not correlated at all (summarized in tab. 4). Even though the spectral curve produced from 2 feathers showed similar positive and negative peaks as the curve from 20 feathers, the two still differed in terms of brightness and hue.

Table 4: Correlation tests of spectral variables measured from 2 and 20 feathers of 14 Yellowhammer individuals. Same results obtained for total and mean brightness.

variable	*t*	df	*PCC*	*p*
brightness	–1.07	12	–0.3	0.31
UV chroma	3.42	12	0.7	< 0.01
blue chroma	4.18	12	0.77	< 0.005
hue	0.1	12	0.03	0.92

3.2 Differences between age groups in coloration

Comparison of the spectral characteristics obtained from the carotenoid feather samples and the yellow index are summarized in tab. 5, fig. 9a and fig, 9b. Statistically significant differences between young and old males were found only in hue and blue chroma, but not in yellow index, brightness or ultraviolet chroma. No statistically significant difference between young and old Yellowhammer males was found in any of the tested characteristics of the melanin coloration, i.e. rufous index, hue and total brightness.

From obtained results, it is apparent, that older males had "deeper" negative peak in the blue part of the spectrum, i.e. more saturated carotenoid coloration. Also, the hue of older males' plumage is higher, i.e. shifted more towards longer wavelengths ($W = 535.5$, $p < 0.05$, n = 26 young and 27 old males).

Table 5: Results of the tests used to compare the yellow index and the spectral characteristics of the carotenoid coloration (for results of hue see text). Sample sizes are given as number of young/number of old birds.

variable	*t*	df	*p*	n
yellow index 2015	–1.03	23.1	0.31	14/13
yellow index 2016/2017	–1.62	15.96	0.12	14/17
total brightness	–0.39	54	0.7	26/27
ultraviolet chroma	–0.61	49.97	0.54	26/27
blue chroma	–3.3	52.44	< 0.01	26/27

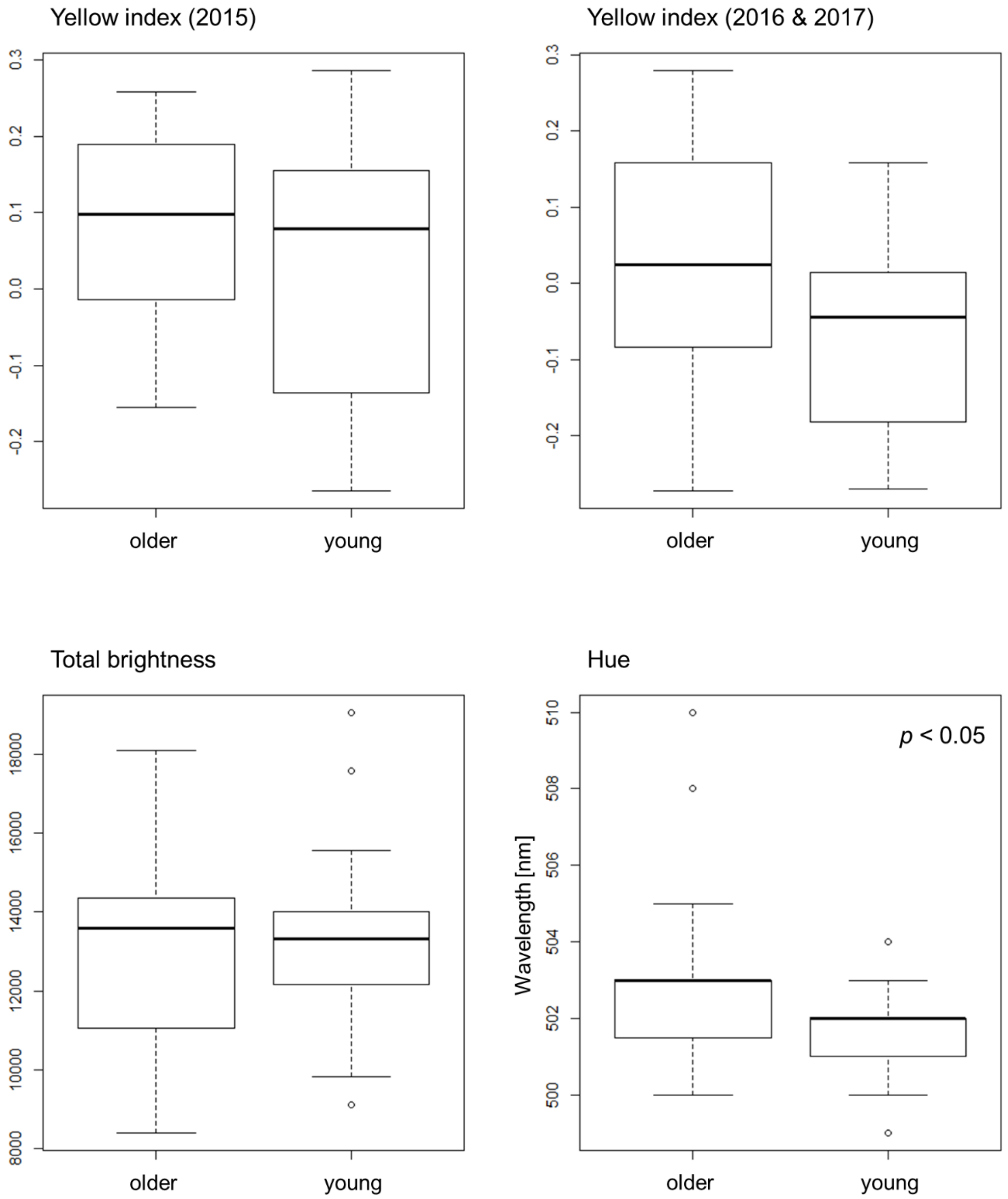

Figure 9a: Boxplots showing differences in the studied characteristics of the yellow plumage between young and old Yellowhammer.

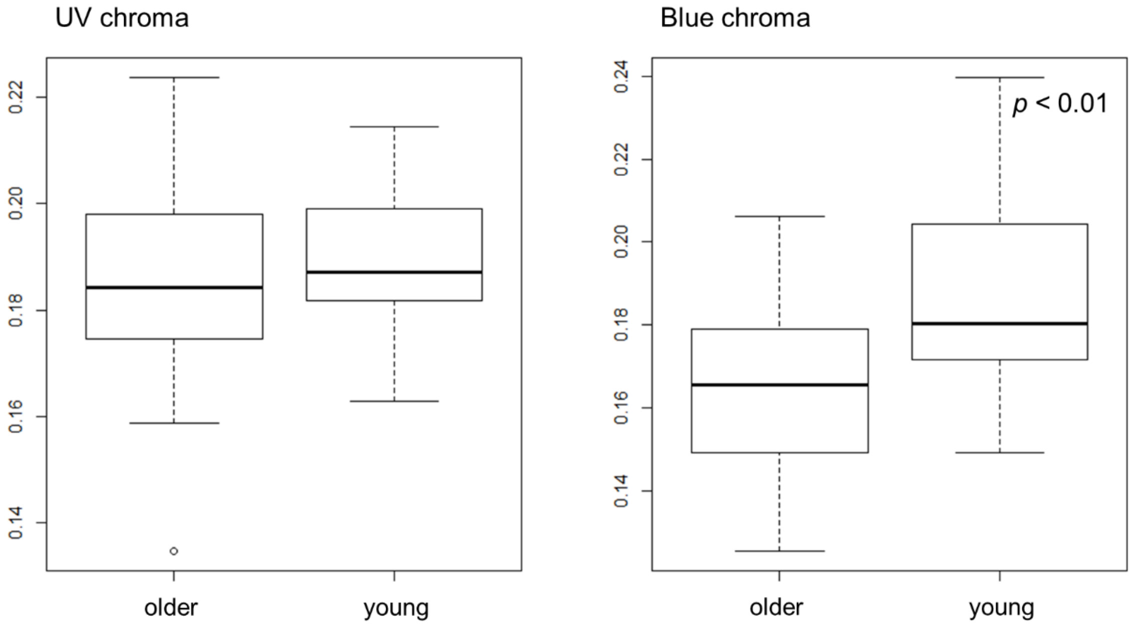

Figure 9b: Boxplots showing differences in the studied characteristics of the yellow plumage between young and old Yellowhammer males.

3.3 Effect of phaeomelanin feathers presence in samples on hormonal concentrations

The effect of the presence of rump feathers on the hormonal concentration in the feather samples was tested in 50 samples for testosterone and 47 samples for corticosterone. For both hormones 13 of the total number of samples were samples without rump feathers. The effect was initially found only in testosterone (adjusted $R^2 = 0.14$, $F_{1,49} = 9.43$, tab. 6). There was no significant effect of rump feathers on corticosterone. But validation of the model for corticosterone showed one highly influential observation. Model refitted without this observation showed better fit (*adj.* $R^2 = 0.06$, $F_{1,46} = 4.25$ compared to *adj.* $R^2 = 0.01$ in original model) and the effect of the presence of rump feathers was significant in this model (tab. 6). For both hormones presence of rump feathers increased the concentration of the hormones. The effect of age or its interaction was non-significant in models for both hormones.

As the samples that contained both feathers from the belly region and feathers from the rump had higher concentrations of both corticosterone and testosterone (fig. 10), it was necessary to analyze them separately from the samples which contained only feathers from the belly region. This was also the case for the 13 males caught outside of our study locality, i.e.

those without rump feathers in hormonal samples, for which I also had fewer data (e.g. no biometrics). Therefore I restrict my analyses to the males caught at our study locality.

Table 6: Parameter estimates and their standard errors of the refitted model of the effect of rump feathers presence in the sample and the feather testosterone (TESTf) and corticosterone (CORTf).

Model	estimate	standard error	*t*	*p*
TESTf ~ rump feathers	0.36	0.12	3.04	< 0.01
CORTf ~ rump feathers	0.22	0.1	2.06	< 0.05

Figure 10: Effect of the presence of rump feathers on the concentrations of feather hormones.

3.4 Effect of hormones and body size on coloration

Before modelling other effects of hormones on plumage coloration I tested whether their concentration in feathers differed between young and old males. I found no statistically significant differences between old and young males in feather hormones concentrations; neither for feather corticosterone ($t = 1.52$, df = 29.32 $p = 0.14$, n = 17 young and 15 old), nor for feather testosterone ($t = -0.2$, df = 22.83, $p = 0.85$, n = 18 young and 16 old; fig. 11). While for feather corticosterone, fig. 11 suggests a trend for older males having higher feather corticosterone, for feather testosterone the situation is very different. Values for feather testosterone of older males showed much lower variance than for young individuals.

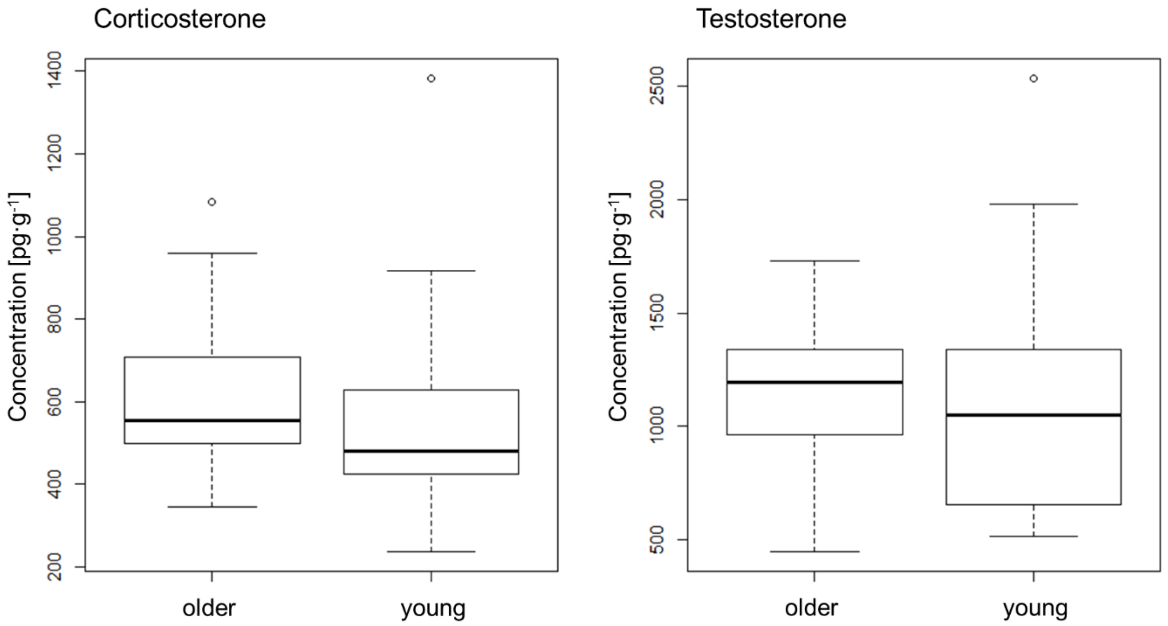

Figure 11: Boxplots showing differences in the concentration of hormones in Yellowhammer's plumage between young and old males caught at our study locality.

To assess the correlation between feather hormones and coloration, initially 29 male Yellowhammers entered the analysis. According to the best selected model, hue of the carotenoid coloration was positively correlated with the feather corticosterone, age and wing length. That means individuals with higher feather corticosterone concentration and longer wing had their yellow coloration slightly shifted towards higher wavelengths (i.e. "red"). Also the hue was higher in older individuals. Model validation showed no patterns in residuals and their homogeneity. However, one of the data points showed disproportionate influence in the model – its Cook's distance was close to 1.0. I decided to exclude this individual, an outlier with unusually high feather corticosterone, and refit the model. Refitted model showed worse fit (adj. R^2 0.30, $F_{3,\,24} = 4.93$, compared to adj. $R^2 = 0.38$ in original model) and the effect of CORTf was only marginally significant in this model (tab. 7, fig. 12). No other effect of feather hormones concentration was found on any other tested spectral characteristics of carotenoid coloration and on the yellow index.

Table 7: Parameter estimates and their standard errors of the model predicting the effect of corticosterone concentration in feathers (CORTf) on hue of the carotenoid coloration.

Predictor	estimate	standard error	*t*	*p*
CORTf	2.04×10^{-3}	1.07×10^{-3}	1.91	0.07
wing length	0.17	0.08	2.16	< 0.05
age	–0.98	0.41	–2.38	< 0.05

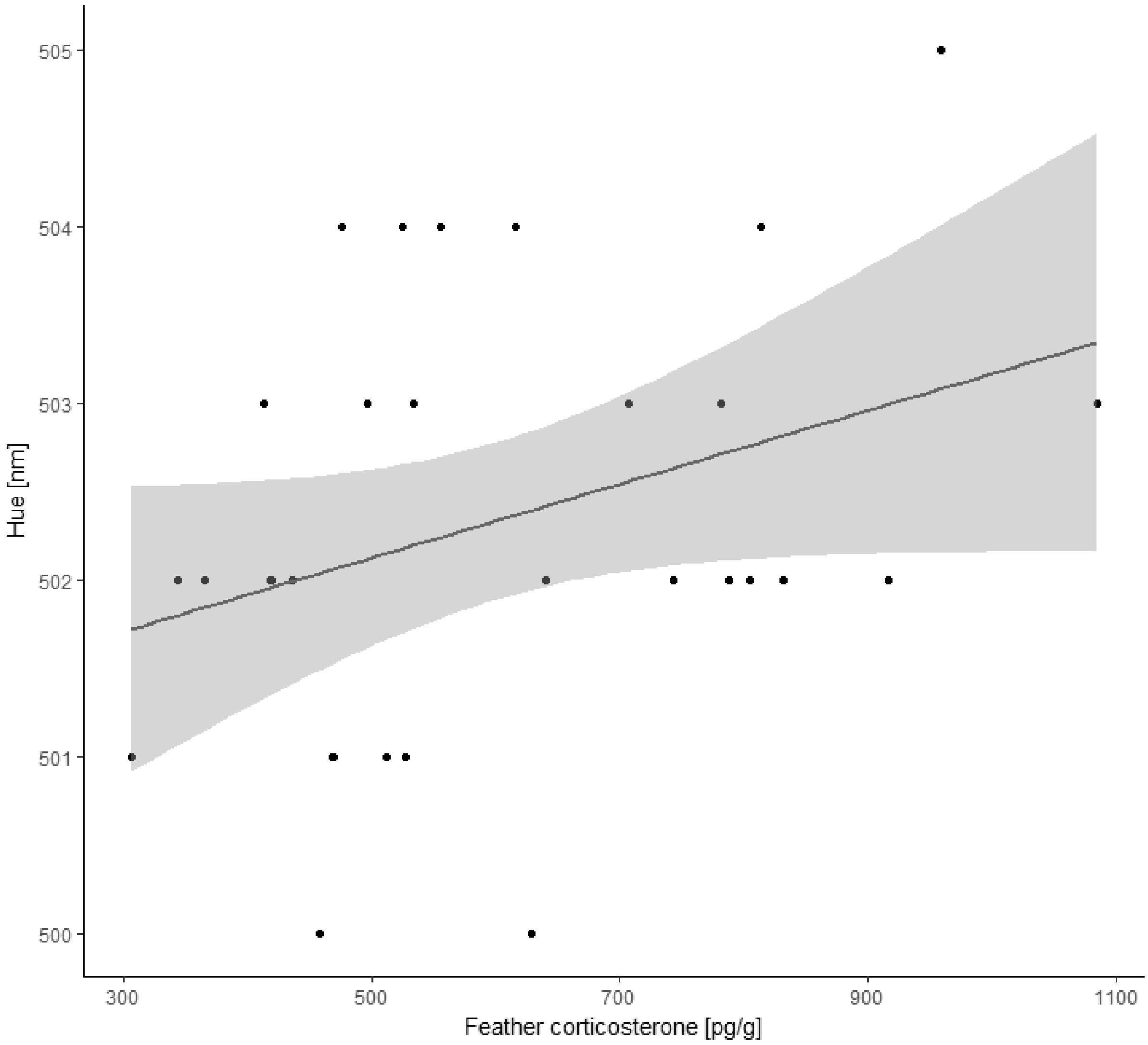

Figure 12: Correlation between hue and feather corticosterone concentration with 95 % confidence interval shown.

For melanin coloration, only its total and mean brightness showed a correlation with feather hormones and more specifically a negative correlation with testosterone concentration (total brightness: adj. $R^2 = 0.12$, $F_{1,\,27} = 4.85$; mean brightness: adj. $R^2 = 0.1$, $F_{1,\,27} = 4.25$, tab. 8, fig. 13). Males with darker rumps had also higher levels of feather corticosterone.

Table 8: Summary of the model predictors of total (B_1) and mean brightness (B_2) of melanin coloration and their correlation with feather testosterone (TESTf).

Model	estimate	standard error	*t*	*p*
B_1 ~ TESTf	–0.79	0.36	–2.2	< 0.05
B_2 ~ TESTf	-1.77×10^{-3}	8.59×10^{-4}	–2.06	< 0.05

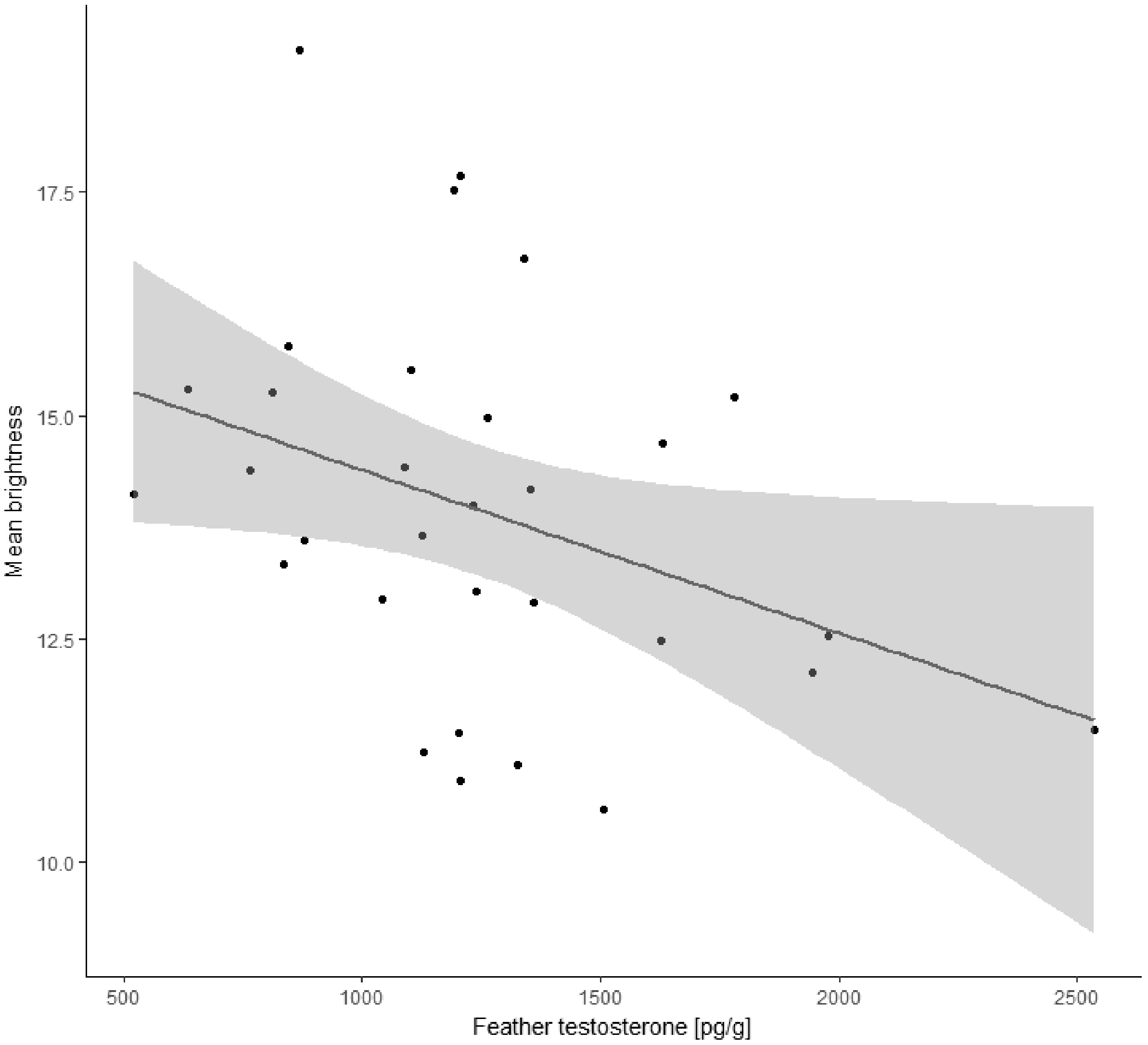

Figure 13: Correlation between mean brightness and feather testosterone concentration with 95 % confidence interval shown.

I have found also other two correlations with wing length and spectral characteristics of carotenoid coloration. There was a marginally significant negative correlation with ultraviolet chroma, although with very low fit (*adj.* $R^2 = 0.07$, $F_{1,\,27} = 3.42$). From this model it seemed that birds with higher peak in ultraviolet part of spectrum had shorter wing. Second correlation, also a negative one, showed that birds with higher mean brightness were shorter winged (*adj.* $R^2 = 0.13$, $F_{1,\,27} = 5.26$). Both models are summarized in tab. 9.

Table 9: Summary of the model predictors of UV chroma and mean brightness (B_2) of carotenoid coloration and their correlation with wing length.

Model	estimate	standard error	*t*	*p*
UV chroma ~ wing length	-1.56×10^{-3}	8.41×10^{-4}	–1.85	0.08
B_2 ~ wing length	–0.77	0.33	–2.29	< 0.05

Visual model

The visual model was calculated using the same dataset of 29 males of Yellowhammer as in analysis above. Results from visual model were largely in accordance with the results obtained with spectral characteristics. I found a negative correlation between the angle φ and the age of the Yellowhammer. Younger males were less colorful in the human visible spectrum than old ones ($adj.\ R^2 = 0.25$, $F_{1,\,27} = 10.56$, tab. 10). The R achieved, i.e. saturation of the color in the eyes of birds, was also negatively correlated with age and positively with wing, only marginally significantly, though ($adj.\ R^2 = 0.18$, $F_{2,\,26} = 4.14$, tab. 10). This saturation was higher in older birds and in birds with longer wing irrespective of age. Surprisingly, no effect of body size or hormones' concentrations was found when modeling the angle θ of carotenoid coloration or any of the visual model characteristics obtained from the melanin coloration.

Table 10: Parameter estimates and their standard errors of the model of the effect of age on the angle φ (upper table) and summary of the model of the effect of age and wing length to the total *R* achieved (bottom) of the carotenoid coloration.

Predictor	estimate	standard error	*t*	*p*
age	–0.05	0.02	–3.25	< 0.005

Predictor	estimate	standard error	*t*	*p*
age	-6.71×10^{-3}	3.21×10^{-3}	–2.09	< 0.05
wing	1.1×10^{-3}	5.72×10^{-4}	1.92	0.07

3.5 Territoriality and Survival

We were able to assess territoriality and survival status for 39 males. When testing the characteristics of the carotenoid coloration I found that none of the interactions or factors entering the GLM (UV and blue chroma, brightness, hue, yellow index and interaction with age) predicted nor the ability of males to acquire and hold stable territory in 2015, or the survival of the winter in 2015/2016. Same was true when I tested the characteristics of melanin coloration as neither its brightness nor hue nor rufous index was correlated with neither ability to acquire territory nor the survival of the males. Similar result was obtained for the characteristics of melanin coloration as there was no correlation of its brightness, hue, or rufous index with male ability to acquire territory or the survival of the males.

Modelling the effect of hormone concentrations also did not show any significant result. There was no effect of testosterone on survival and territoriality and no effect of corticosterone on territoriality. I found only marginal effect of the corticosterone concentration in feathers on the survival of males to the next season (CORTf parameter estimate: $4.53 \times 10^{-3} \pm 2.82 \times 10^{-3}$ S.E., $z = 1.61$, $p = 0.11$)

According to models selected based on *AICc*, males that survived the winter had higher mean brightness and had higher yellow index, the yellow coloration comprised larger area on their body and was brighter in color. These males, in concordance with the model mentioned, had higher concentration of corticosterone in their feathers. But while these models showed lowest value of *AICc*, the difference between these models and the null model were small (less than one, tab. 11), not suggesting strong effect of these predictors.

Table 11: Effects of mean brightness (B_2) and yellow index (left) and concentration of feather corticosterone (CORTf) and testosterone (TESTf; right) on the survival of Yellowhammers (models selected based on AICc).

Model	df	*AICc*
B_2 + yellow index	3	31.09
B_2	2	31.22
yellow index	2	31.53
null	1	31.97
B_2 + yellow index + age	4	33.25

Model	df	*AICc*
CORTf	2	29.01
null	1	29.77
CORTf + age	3	30.19
CORTf × TESTf	4	31.53
CORTf + TESTf	3	31.8
TESTf	2	32

4 Discussion

4.1 One feather may not be enough to estimate coloration

Studying avian coloration with spectrophotometer has become a widely used practice and such studies are numerous. While there is no doubt that measurements taken from living birds or museum skins reflect the plumage characteristics correctly, surprisingly few studies utilizing feathers only (with varying number of them in one sample) compared their measurements with those obtained from living birds. From my results, it is obvious that the decision on how many feathers is enough, depends on the method chosen to test it.

When I compared my measurements in a similar way Quesada & Senar (2006) did, i.e. ANOVA with *post-hoc* comparison, I received almost identical results. At least eight (and in case of ultraviolet chroma twelve) feathers are needed to obtain representative results. However, several studies used just one feather and claimed it is representative (e.g. Saino *et al.* 2013a; Romano *et al.* 2016; Costanzo *et al.* 2017). It is true though, that when I used the same statistical method as in mentioned studies, I found similar results also in this case. Chromatic variables measured from two and twenty feathers were correlated, indeed.

Unfortunately, from the study of Saino *et al*. (2013b), which describes methodology of using just one feather for the analysis followed by later studies mentioned above, it is not clear what kind of background was used under the sample feathers during measuring them. The color and material of the background can influence obtained spectral curve substantially, especially when very low amount of the very fine feathers is used (*personal observation*) like in the discussed studies. Usually either black or white background is used. To exclude the effect of background, I used black cardboard which showed very low reflectance across the whole spectra.

Drawing any conclusions from these results is not straightforward. The decision, whether one feather is enough, depends on the method used to test it, either ANOVA or correlation. While chromatic variables were correlated between samples with two and twenty feathers, yet not that much as in Romano *et al.* (2015), brightness and hue were not. But these are also spectral characteristics very often referred as potential indicators of e.g. health status, body condition or success in obtaining extra-pair copulations (Figuerola *et al*. 1999; Hill 2000; Bitton & Dawson 2008). It is very tempting to measure bird coloration on just one single feather, as the amount of effort needed to prepare such samples, compared to samples made of several feathers, is much lower. However I brought some evidence, in concordance with the study of Quesada & Senar (2006), that such values might not represent the actual coloration of the living bird, therefore I remain skeptical in measuring samples containing just one single feather and strongly suggest using at least ten feathers in such studies.

4.2 Senescence is colorful

From the tests performed, only the blue chroma and hue showed a statistically significant difference between young and old males. Older males had lower values of blue chroma which is reflected in more saturated color of plumage. The blue chroma in many species corresponds to the total carotenoids present in feathers (Kläui & Bauernfeind 1981; MacDougall & Montgomerie 2003; Butler, Toomey & McGraw 2011). Therefore, the differences found between young and old males could be simply result of young males having less time to gather enough carotenoids for plumage coloration than older ones. Young Yellowhammers start their post-juvenile molt, i.e. to molt into adult's plumage, very soon after fledging (Cramp, Perrins & Brooks 1994; *personal observation*). Therefore female can, just by judging the plumage of the male, learn the age of that male.

At first glance the overall differences between measured values of hue might seem very slight: the maximum difference in hue was 11 nm. The exact sensitivities of Yellowhammer's cones are unknown, but for comparison, 25 nm is the difference between pure green and pure blue color for a human. Similarly to blue chroma, hue can reflect the amount of carotenoids deposited into feathers, too. In some birds with red carotenoid coloration, hue varies with the amount of red carotenoid coloration in the diet (Hill 1992, 2000; plate 3). For the European Greenfinch, a species which exhibits yellow carotenoid coloration, a similar positive correlation was found in its hue and carotenoid content (McGraw, Safran & Wakamatsu 2005). Hue of the birds' plumage was calculated by a different method, though.

The differences found between young and old males might not be very surprising yet still biologically relevant. In birds, age is often correlated with survival over the winter. The northern temperate winter poses serious survival challenge to these small passerines and older individuals survive better (Robinson, Baillie & Crick 2007). If the ability to survive was heritable, female could profit by mating with older bird by having offspring with higher probability of survival, too. Also, nesting success generally increases with age (e.g. Nol & Smith 1987; Lozano, Perreault & Lemon 1996; Daunt *et al.* 1999). So females could profit in mating with older and more experienced males, and the hue of the carotenoid coloration might give them a clue who to choose.

4.3 Melanin influences concentration of hormones in feathers

In this thesis, I have found a statistically significant difference in corticosterone and testosterone concentrations in feather samples containing melanin feathers and those that did not. Samples containing feathers pigmented with phaeomelanin had higher concentration of deposited corticosterone and testosterone than samples composed of feathers pigmented only with carotenoids. This could have great effect on results as choosing feathers from differently colored body part can apparently influence the measured concentration of deposited corticosterone.

My findings are to some extent similar to results of Jenni-Eiermann *et al.* (2015), who discovered that in the feral pigeon more melanized parts of the same feather showed higher concentration of deposited corticosterone compared to parts with less eumelanin. However, my samples differed not in somewhat subtle differences in color as in Jenni-Eiermann *et al.* (2015), but rather in presence and absence of melanin, because feathers from belly region lacked any visual sign of deposited melanin.

Because we did not measure the amount of melanin deposited into the feathers, I am unable to conclude how much of the variation in hormones concentration could be explained by the amount of melanins alone and what part is due to other factors as for example the stress encountered during molt. But from the work of Jenni-Eiermann and her team, the effect size of correlation found between color and feather corticosterone concentration was much smaller than the one between circulating hormone levels in blood and those deposited into feathers. That suggest than even though melanin can influence the amount of deposited hormones, their concentrations should still mainly reflect those of circulating in blood. The effect of melanin on hormone levels will be discussed also in next chapters.

Interestingly, no difference in feather corticosterone was found between melanin pigmented tail feathers and carotenoid pigmented breast feathers of male House Finches in a study of Lendvai *et al.* (2013). Possible explanation could be that though carotenoids are the main pigment visible in House Finches’ breast feathers, their lower part is also colored with melanins (this is partly visible in plate 3).

Originally, an inconsistence in the protocol of hormone samples analysis, i.e. having two types of hormone samples (with or without rump feathers) provided me with the opportunity to test whether the findings by Jenni-Eiermann *et al.* (2015) could be valid also for samples obtained from the Yellowhammer. It is important to stress out, that the mentioned study investigated only the possible relationship between melanin and corticosterone. The situation for testosterone is virtually unknown and results from this thesis are among first, if not actually first, to show such difference. But since I had samples mixed from feathers from two different body regions, I cannot exclude the possibility that the difference found could arise also due to differences in blood hormone levels at the time these feathers were growing. These two body regions, belly and rump, could be molted at slightly different time, as the molt of Yellowhammers can be as long as 80 days (Cramp, Perrins & Brooks 1994). Therefore having samples from these two body regions might possibly reflect hormone concentrations of quite long time frame (Bortolotti *et al.* 2009).

4.4 Testosterone makes you darker

My analysis showed a significant negative correlation between the concentration of feather testosterone and the brightness of rump pigmented with phaeomelanin. Males with higher feather testosterone level had less bright, i.e. darker, rumps. My model, however, was based on just 29 individuals and the explained variability in the data was very low. Nonetheless, while this finding is in concordance with other studies that found a correlation between testosterone and elaboration of melanin plumage (e.g. Strasser & Schwabl 2004; Fargallo *et al.* 2007; Bókony *et al.* 2008), it is uneasy to draw any conclusions on the causality of the relationship found.

As I have previously shown, samples containing phaeomelanin pigmented feathers, had higher concentration of corticosterone and testosterone than those without. I did not measure the amount of melanins in feathers directly, but as found by McGraw, Safran & Wakamatsu (2005), feather brightness and its melanin content was strongly negatively correlated in Barn Swallows ($R = -0.88$). If the amount of testosterone in feather depends on feather melanin content similarly as was suggested for corticosterone by Jenni-Eiermann *et al.* (2015), higher concentration of testosterone could be simply a byproduct of testosterone binding abilities of melanin pigment which is deposited in higher concentrations in darker individuals. The solution to this would be to test the correlation between testosterone and rump brightness in individuals for which the feather hormone concentrations were assessed only from belly feathers, i.e. not "contaminated" with melanins. However, the rump feathers from these individuals were not collected at all within this study leaving this question opened for further research.

Concentrations of melanin and testosterone in feathers are probably bound through the pleiotropy in the melanocortin system as many animals show correlation between darkness of their plumage or hair coat and testosterone induced behavior as aggressiveness or sexual behavior (reviewed by Ducrest, Keller & Roulin 2008; Roulin 2016). Production of melanin pigmentation and excretion of sexual hormones (i.e. testosterone) are regulated by the same melanocortin pathway (Ducrest, Keller & Roulin 2008). Therefore, whether higher concentration of testosterone stimulates higher deposition of melanin in feather ornaments or more melanin in feathers binds more testosterone may not be the right question to ask.

Positive correlation between the degree of melanization and testosterone levels have been found in many species (Bókony *et al.* 2008). One of these is the House Sparrow. The size of the melanin ornamental bib, a badge of status in this species (Gonzalez *et al.* 2002; McGraw, Dale & Mackillop 2003) is positively correlated with circulating testosterone (Buchanan *et al.* 2001; Strasser & Schwabl 2004; but see Laucht, Kempenaers & Dale 2010). This might be especially important in species like House Sparrow, i.e. species which are gregarious outside the breeding season. During this time the dominance status could prevent unnecessary expenditures of energy in fights over food sources to which the dominant birds get access first (Møller 1987). Also Yellowhammer shows formation of winter flocks where the birds feed together (Cramp, Perrins & Brooks 1994) and frequent squabbles of males could suggest that birds established some dominance ranks among themselves (*personal observation*). The subordinate status of males with smaller badges, or in the case of Yellowhammer, the lighter colored rump, could lead to lower overwinter survival due to the restricted access to food as it was found in Willow Tits (*Poecile montanus*; Koivula, Orell & Lahti 1997). A meta-analysis by Santos, Scheck & Nakagawa (2011) suggests that signaling the dominance status is important throughout the year, and not only during non-breeding period. It is important to stress out that the status signaling in House Sparrow is based on the size of the melanin ornament. My findings suggest a different relationship, i.e. with brightness. Therefore the mechanisms behind might not be entirely similar.

Choosing male with the highest testosterone level does not need to be that advantageous for female, though. Several studies suggest that males with higher level of circulating testosterone show worse parental effort which lowers their reproductive success (Hegner &

Wingfield 1987; Oring, Fivizzani & El Halawani 1989; Ketterson *et al.* 1992). Testosterone also negatively affects body mass (Ketterson & Nolan 1992) or overwinter survival (Nolan *et al.* 1992; Koren *et al.* 2012a). These negative effects can apparently counter-weigh the positive effect of testosterone to sexual behavior, as attractiveness to females (Enstrom, Ketterson & Nolan, Jr. 1997). Reproductive success was not positively correlated with melanin ornamental plumage in several species, suggesting further link between testosterone and these ornaments (Lemon, Weary & Norris 1992; Veiga 1993; Grffith, Owens & Burke 1999; Kingma *et al.* 2008). Furthermore females seemed to reflect this during mate choice and chose mates with less elaborated melanin ornaments (Lemon, Weary & Norris 1992; Grffith, Owens & Burke 1999).

Unfortunately, we were unable to find a representative amount of nests, therefore I could not test any hypotheses regarding testosterone and parental effort or reproductive success in Yellowhammer. From the results obtained, I can only speculate about the dominance status signaling function of the melanin rump. As mentioned earlier, the melanin itself can influence the level of deposited hormones. On top of that, the explained variability in the data of the model was very low suggesting this correlation is very weak, at best.

At this point, I have to stress out, that it might be questionable whether melanin based rump coloration is actually a sexually selected ornament. There is no obvious difference in coloration of rump between the sexes as it is in yellow carotenoid coloration. Due to the very low number of females trapped and sampled during whole study (n = 6), I could not test this statement statistically. While the absence of sexual dimorphism in a trait does not necessarily mean absence of sexual selection on it (Kraaijeveld *et al.* 2004; Siefferman & Hill 2005), Yellowhammer males do not perform any special courtship display during which the rump feathers could be presented (Cramp, Perrins & Brooks 1994). But Yellowhammer males possess another melanin plumage ornament, possibly a sexually selected one. Individual males differ in the size of rufous breast band. This band was almost complete in some males, but it was virtually absent in others (plate 6). It is also not clear if the findings presented regarding rump feathers could be applied to the breast band. I have found no effect of feather hormone concentrations on the size of the rufous breast ornamentation or overall rufous index. Due to the nature of the melanin breast band, it was impossible to collect enough material to perform any meaningful spectral analysis in the laboratory.

4.5 Being big and yellow is stressful

I have found a trend suggesting positive correlation between the hue of the carotenoid coloration and the feather corticosterone concentration. In accordance with the tests presented earlier, there was also a significant positive correlation of hue with age in this model as well as with wing length (i.e. older males and males with longer wing had higher values of hue). The mechanism of maintaining and signaling function of hue regarding the age have been discussed in chapter 4.2 *Senescence is colorful*. Here I will focus on the other two predictors of this model: feather corticosterone and wing length.

Contrary to my results, in a study conducted on the American Yellow Warbler (*Setophaga petechia*), a species with yellow and rufous ornamental plumage as Yellowhammer, the hue of its carotenoid coloration was negatively correlated with feather corticosterone (Grunst *et al.* 2015). Similar negative correlation was also found in other species, concerning adult birds (Mougeot *et al.* 2010) as well as nestlings (Loiseau *et al.* 2008; Martínez-Padilla *et al.* 2013). As basal level of corticosterone in blood is positively correlated with parasite load (Raouf *et al.* 2006) and individuals with lower basal levels seem to better cope with stress (Saino *et al.* 2002), carotenoid coloration could therefore signal the amount of stress an individual has experienced during its life, in these species. We cannot exclude the effect of parasite load on deposited corticosterone in Yellowhammer, yet. Further analysis of our data could give us some insight as data on ecto- and endo-parasite load are being analyzed at the moment.

Even though Yellowhammer and American Yellow Warbler have very similar plumages, the different results obtained in the study of Grunst *et al.* (2015) and by myself could arose due to the differences in molting between these two species. Unlike Yellowhammers, American Yellow Warblers undergo a much more extensive pre-breeding molt replacing most of the body feathers (Quinlan & Green 2011). This molt takes place on the wintering grounds far away from the place where they breed, because the studied population of the American Yellow Warbler is migratory (Curson 2017). Therefore samples collected by Grunst *et al.* (2015) might reflect different levels of stress, i.e. stress level encountered on wintering grounds after the migration, rather than post-breeding stress level like those collected in my study on Yellowhammer.

In accordance with my data, several other studies also found a positive correlation between carotenoid coloration and blood corticosterone in Zebra Finches (McGraw, Lee &

Lewin 2011) or feather corticosterone in House Finches (Lendvai *et al.* 2013), Common Redpolls (*Acanthis flammea*; Fairhurst *et al.* 2014) and Mallards (*Anas platyrhynchos*; Fairhurst, Damore & Butler 2015). This might indicate that carotenoid ornaments could signal different information in different species. Higher levels of corticosterone can apparently mediate the expression of carotenoid signals and the utilization of carotenoids from fat which can serve as their storage (Lendvai *et al.* 2013; Fairhurst *et al.* 2014). Also, corticosterone seem to increase foraging in birds (Astheimer, Buttemer & Wingfield 1992) which is especially important at the time of molt as the molt itself is energetically demanding (Jenni & Winkler 1994). Therefore birds with higher levels of corticosterone are probably able to find better resources needed for successful feather molt including carotenoids for mate attraction. But maintaining high levels of corticosterone to enhance expression of carotenoid coloration probably comes with a cost. In a study by (McGraw, Lee & Lewin 2011) captive Zebra Finches with higher corticosterone concentrations and better carotenoid coloration were in overall worse condition. That could indicate a trade-off between allocating carotenoids into ornaments and using them as antioxidants during the costly molt.

From my data, this trade-off is not apparent because Yellowhammers with higher hue and corticosterone had also longer wing, a proxy often used for body size. As Yellowhammer can feed on wide variety of diet including insect and grass seeds, it might not be as nutritionally stressed by natural resources as the Zebra Finches in the experimental study of McGraw, Lee & Lewin (2011). It is important to stress out, when interpreting my data, that our sample size was very low and the effect of corticosterone in the model was only marginally significant.

Wing length of Yellowhammers in this study was negatively correlated with mean brightness and marginally negatively with ultraviolet chroma. That further suggests that Yellowhammers with better carotenoid coloration, and therefore possibly with higher corticosterone levels, grew larger, indicating their better condition. Both the lower mean brightness and ultraviolet chroma in larger individuals could indicate coloration more saturated with carotenoids, because in this case low mean brightness does not necessarily mean darker coloration. If the decrease in mean brightness would be on the expanse of UV chroma, as suggested by the other model, this decrease in brightness would also mean higher concentration of carotenoids in feathers. With increasing concentration of carotenoids in feathers, the negative peak in blue part of the spectrum becomes deeper and the peak in ultraviolet light lower ultimately disappearing altogether. That produces much more saturated carotenoid coloration but with proportionally lower mean brightness (Andersson & Prager 2006; plate 7).

In certain passerines, body size expressed as wing length has been found to positively correlate with dominance and longer-winged males are preferred by females (Ketterson 1979; McGlothlin *et al.* 2005; Hasegawa, Soma & Hasegawa 2011). Also, wing length, corrected for body mass, is often used as a proxy for body condition (Jakob, Marshall & Uetz 1996; Schulte-Hostedde *et al.* 2005). Unfortunately, we were unable to weigh all of the birds trapped so doing such correction would lower the sample size even more. Even though I miss such body condition index, I can speculate that larger individuals, and therefore probably also individuals in better body condition, were able to forage on highly nutritious resources. If these resources would be also rich in carotenoids, this could ultimately lead to more carotenoids deposited into feathers which would produce more carotenoid saturated plumage and shift the hue of the plumage towards longer wavelengths as is the case of the House Finch or the European Greenfinch, discussed above (Hill 1992; McGraw, Safran & Wakamatsu 2005). In the European Greenfinch, carotenoid coloration is also positively correlated with the strength of their humoral immune response and with overall health status (Saks, Ots & Hõrak 2003) and in the Yellowhammer, yellower males showed lower parasite loads (Sundberg 1995b). Therefore by mating with a big male with saturated yellow coloration, female can choose a healthy mate and not risk being infected by the male.

Wing length differences could be also associated with other attributes. One of them could be the association with different migratory strategies. Berthold & Querner (1982) found that Eurasian Blackcaps (*Sylvia atricapilla*) with longer wings migrated longer distances. The population of Yellowhammers in Czech Republic is extremely sedentary though (Cepák 2008) and such association is unlikely in the studied population and therefore wing length probably rather reflects the body size of the individual.

4.6 Do birds actually see these things?

Tests performed with the simulated vision of an Atlantic Canary did not bring any additional results to those found when testing the variables calculated from the spectral curve directly without taking into account bird vision or ambient light. It is true though, that any correlations with coloration and feather hormones were non-significant in case of visual model variables. Interpreting these results as simply “birds cannot see these differences” is not that easy, because interpreting the visual model itself correctly is not as straightforward as with the spectral variables. Values obtained from visual models, angles φ and θ and R achieved, represent the relative stimulation of different cone types in the bird retina. (Hart & Vorobyev 2005; Maia *et al.* 2013). But how exactly are the signals from different cone types processed by brain and therefore how color is perceived by birds is still unknown (Cuthill 2006). Also, it might be possible that the visual model based on Canary vision does not need to represent well vision of the Yellowhammer.

Still, modelling avian vision promises much more understanding of how birds see themselves in the future research. The way birds see birds might produce different results than just "simple comparing spectral curves" (Lind & Delhey 2015). For that, more information on spectral sensitivities of different birds are required as well as other information on the fine tuning of their eyes.

4.7 Territoriality and survival

The ability to acquire and hold a territory seems crucial for passerines and birds in general. Males of majority of the species spend a great deal of time defending territories through ritualized contests with other males in a form of either singing (e.g. Krebs, Ashcroft & Webber 1978; Mennill, Ratcliffe & Boag 2002) or displaying colorful plumage (Andersson *et al.* 2002; Stein & Uy 2006). But even if this coloration does not play major role in these contests, female can learn about the quality of the territory through male's plumage anyway as plumage can reflect the richness of food sources in the territory or its size (e.g. Keyser & Hill 2000; Hasegawa *et al.* 2014). In my thesis, however, I did not find any statistically significant correlation of any aspect of Yellowhammer's plumage with their territoriality. Yellowhammer's territory is small in size and basically serves only as a nesting site while gathering food, either for adult or nestlings, takes place outside of it on "neutral" ground (Cramp, Perrins & Brooks 1994). So the effect of food resources, which could influence the coloration, does not need to be tightly linked to the territory quality. Also, in Yellowhammer, song seems to play a major role in territory defense (Hiett & Catchpole 1982).

Basically, same results as for territoriality has been observed also for survival over the winter, i.e. no significant correlations. The best selected model suggested rather a trend than a positive correlation with feather corticosterone. Selecting the models based on *AICc* also suggested a trend between survival and another trend for carotenoid coloration, yet the selected models were not much better than null models (difference in *AICc* was less than 1 in both cases). Therefore, calling the output of these models even a trend might be a bold statement, still I will discuss these trends suggested as the carotenoid coloration was also positively correlated with survival in Zebra Finch (Simons *et al.* 2012). Interestingly, in European Serin, males with intermediate values of brightness of carotenoid coloration had highest survival, suggesting an effect of stabilizing selection (Figuerola & Senar 2007). As survival and age are interconnected, these findings are not surprisingly different from those presented in chapter 4.2 *Senescence is colorful*, and the mechanism is very similar. In studies of hormones and survival in birds, a negative correlation was usually found between the corticosterone and survival (MacDougall-Shackleton *et al.* 2009; Koren *et al.* 2012a; Monclús *et al.* 2017) as higher levels of circulating corticosterone could have detrimental effect on the organism, e.g. a reduction in body weight (McGraw, Lee & Lewin 2011). My results are in sharp contrast as they show a positive relationship similarly as findings made on Tree Swallows (*Tachycineta bicolor*; Harris, Madliger & Love 2017). Interpreting these results is not easy, as the negative correlation of Harris, Madliger & Love (2017) was found only in the experimentally handicapped group and not in the control one. This positive correlation between carotenoid coloration and corticosterone was found also in a non-bird animal, the Common Lizard (*Zootoca vivipara*; Cote *et al.* 2010).

As discussed in previous chapter, higher corticosterone level stimulate foraging (Astheimer, Buttemer & Wingfield 1992). If the high level of corticosterone encountered in the studied population is not high enough to cause damage to the organism, but only high enough to stimulate fat storing, such males would have a higher probability of overwinter survival (Koivula, Orell & Lahti 1997). If such males would also show richer carotenoid coloration, as the other model suggests, the antioxidant capacity of carotenoid could work against the oxidative stress produced by higher corticosterone levels and counter-weigh its negative impact (Lin, Decuypere & Buyse 2004; Cote *et al.* 2010). This way, more colorful males could signal through their coloration not only overwinter survival, but also better ability to cope with stress in general.

5 Conclusion

Our team has rich experience with Yellowhammer as a model species in the fields of bird song dialects (Diblíková 2013; Bílková 2015; Petrusková *et al.* 2015), and invasive ecology (Pipek, Pyšek & Blackburn 2015; Pipek *et al.* 2017), so the choice of Yellowhammer – a still common, non-migratory passerine with obvious carotenoid ornamentation – as a model species for investigating the role of bird coloration and song in sexual selection seemed a logical step. But while other commonly studied European songbirds in this respect, such as Great Tit or Barn Swallow, are very easy to trap with standard methods and one can gain big dataset with comparatively little trapping effort, the opposite is true for Yellowhammer. Even though Yellowhammer males can show quite aggressive reaction to playback, the trapping success using this method is moderate at best. It was very difficult to predict which males will react strongly enough and this reaction was somehow not synchronized with other males at the locality. Retrapping individuals from previous years was nearly impossible and such cases were mere coincidence. This has also lead to apparent reduction of sample size after the first breeding season in 2015. Yellowhammers once caught using playback were reluctant to make the same mistake once again next year even with the use of different recording, similarly as other species (Linhart *et al.* 2012; Petrusková *et al.* 2016)

Trapping individuals during non-breeding season could produce much higher rates of sampled individuals due to the flocking behavior of Yellowhammers outside the breeding season. Unfortunately males captured in winter flocks tend to spread in landscape far away from the place of ringing, i.e. leaving our study locality. Also, as Yellowhammer males show some limited head feathers molt before breeding season, this could influence the assessment of color from photographs. While we had some success with trapping individuals using mist nets in the end, nest search ended in total failure. Due to the nature that Yellowhammers prefer to nest very secretively, locating active nests was a difficult process which consumed considerable amount of time and produced little results. On top of that, nest predation appeared to be very high, which is typical for ground nesting birds (Martin 1993), and torrential rains apparently flooded many nests.

Despite these facts, we were able to collect this unique dataset combining information on coloration and song, blood and feather parasite load, with quite novel method of assessing feather hormones concentration giving us some insight into the avian physiology. Especially the extraction of testosterone concentration from feathers is still a novel method not used by many studies. Even though Yellowhammer does not seem to be a suitable model species for the study of the role of coloration in sexual selection in the field because of above mentioned reasons, it is important to broaden our understanding of mechanisms of carotenoid coloration also on other species than the Great Tit or House Finch. This way we can learn whether results obtained on these species are general patterns or specific characteristics of studied species.

Besides that, my master's thesis was a sub-part of a doctoral project of Sharina van Boheemen. Sharina is also interested in song performance of Yellowhammer, studying the various functions of it and also the way how these sexually selected traits reflect endo- and ecto-parasites loads. The amount of data collected in the field was much larger than presented here as majority of the data are not yet analyzed as in the August 2017, this whole study did not end yet and data will be collected also in subsequent seasons. When this dataset will come together, it will produce a truly unique picture among other studies as especially studies which tried to untangle the different signaling functions of song and coloration in a single species are very rare.

Even though the small sample size which probably negatively affected results of several tests or effects of some predictors, I was able to find some biologically relevant information regarding coloration of Yellowhammer males. From these findings, it seems that the coloration could signal some aspects of the male's quality to female. One of these aspects could be the size or age of the bird which was found to be positively correlated with survival in other species. Other would be the ability to cope with stress or finding resources as corticosterone seems to be positively correlated with carotenoid plumage. Feather hormones showed to be very promising as also corticosterone was negatively correlated with rump brightness suggesting some dominance signaling in melanin coloration of the Yellowhammer. Two very weak models also suggested a trend in that Yellowhammers with higher feather corticosterone and with brighter and bigger carotenoid ornament had higher probability of surviving winter.

Not only small sample size but also the possible hormone binding ability of melanin made making any conclusions much more difficult. There seems to be positive correlation between the feather melanin content and deposited hormones concentration. Unfortunately, I did not measure the melanin content of the feathers directly so I cannot estimate how much melanin influenced the hormonal concentrations, and thus my results. This, together with the test of how many feathers in a sample reflect the birds coloration adequately, were two of my "methodological" findings which were in accordance with other studies. There were also other parts of the methodology used which could be questioned, the territoriality and survival. Both could be influenced by some sort of sampling error and especially the survival could be underestimated. Some of the males may have survived and just move a bit further away from the study locality. Even though Yellowhammer seems to show high fidelity for its breeding site and we made some effort to inspect also neighboring fields, we might have missed some males and incorrectly assigned them as dead. On the other hand, inspecting all potential Yellowhammer territories around our locality was beyond our possibilities due to low personal capacity. However, for assessing territoriality or survival of Yellowhammers, recordings of songs of territorial males are available, and after finishing their analysis we will obtain more precise data about males' presence at the locality and conduct particular test once more with larger sample.

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7 Appendix

Appendix 1: List of Yellowhammers used for the analysis of number of feathers in a sample.

Age is given in years. Age 1 means that at the time of ringing, age of the bird was one year or less (i.e. in the first year of life). Age 1+ means that the bird was more than one year old.

date	ring no.	age	sex	city	ringer
27.06.2016	TS9940	1+	male	Břilice (Třeboň)[1]	Ondřej Kauzál
27.10.2016	TT62133	1	male	Praha (D. Počernice)[2]	Ondřej Kauzál
11.02.2017	TT62149	1+	male	Praha (Dubeč)[3]	Ondřej Kauzál
11.02.2017	TT62150	1+	male	Praha (Dubeč)[3]	Ondřej Kauzál
11.02.2017	TT62151	1	male	Praha (Dubeč)[3]	Ondřej Kauzál
23.02.2017	TT47911	1	male	Studenec[4]	Tereza Králová
25.02.2017	TT47913	1	male	Studenec[4]	Tereza Králová
06.03.2017	TT62155	1	male	Studenec[4]	Ondřej Kauzál
15.03.2017	TT62159	1+	male	Praha (D. Počernice)[2]	Ondřej Kauzál
16.03.2017	N725829	1	female	Lysá n. Labem[5]	Radek Lučan
16.03.2017	N725830	1	male	Lysá n. Labem[5]	Radek Lučan
16.03.2017	N725831	1+	male	Lysá n. Labem[5]	Radek Lučan
20.03.2017	N801449	1+	male	Lysá n. Labem[5]	Anna Koukolíková
21.03.2017	N771596	1	male	Třemblat[6]	Radek Lučan

GPS coordinates for place of ringing:

[1] 49.021 N, 14.737 E

[2] 50.098 N, 14.579 E

[3] 50.067 N, 14.574 E

[4] 49.225 N, 16.056 E

[5] 50.215 N, 14.828 E

[6] 49.941 N, 14.758 E

Appendix 2: The Apparatus

The Apparatus comprises of an aluminum mainframe with black cloth over it (made of modified black shirt with turtle neck collar and long sleeves, fig. 14). On the mainframe a Sony DSC-HX60 camera is mounted to provide standardized distance from photographed objects – approx. 27 cm from the object to the camera image sensor. The bottom of the Apparatus is made of a gray card used in photography to calibrate color (white balance) and light settings. At the edges of the gray card a 5 × 5 mm grid is attached as a size reference.

Bird is inserted through one of the sleeves and held and photographed. The camera is operated with a remote shutter release which enables holding the bird in both hands thus enhancing standardization of pictures. To ease manipulation with the bird inside the Apparatus in the dark environment, an LED light source operating on one 9 V battery can be turned on and then off before the exposure.

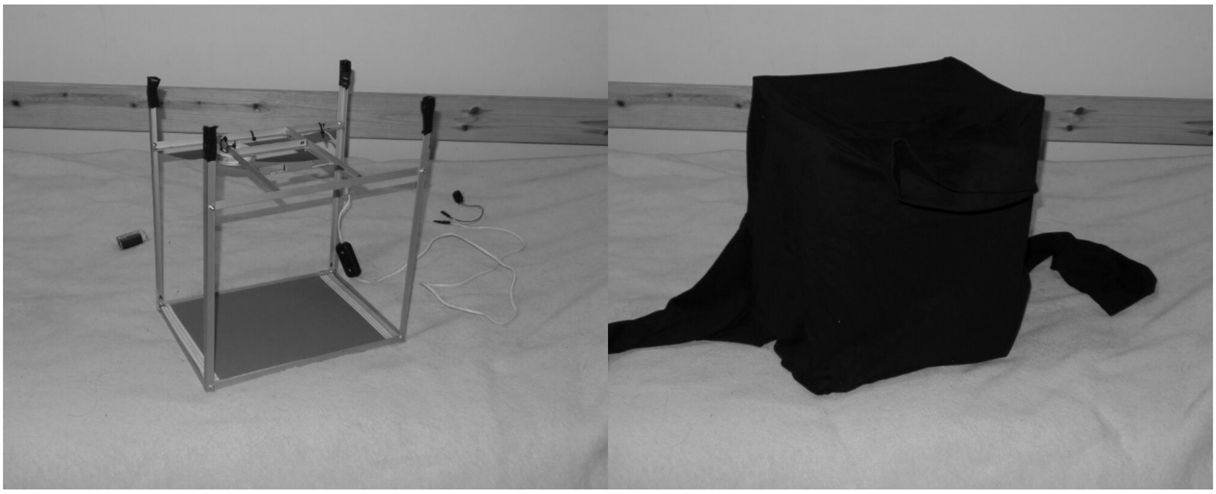

Figure 14: Apparatus used to photograph birds in this study (camera not shown). Left: aluminum mainframe without the black cloth; right: with the black cloth attached and ready to operate.

Appendix 3: source script of canary visual model

```
library(pavo)
canary.vision <- sensmodel(
                           peaksense = c(363, 440, 501, 567),
                           lambdacut = c(0, 411, 506, 587),
                           Bmid = c(0.00, 0.03325, 0.0189, 0.0189),
                           beta = T,
                           om = "bird"
                           )
```

COLOR PLATES

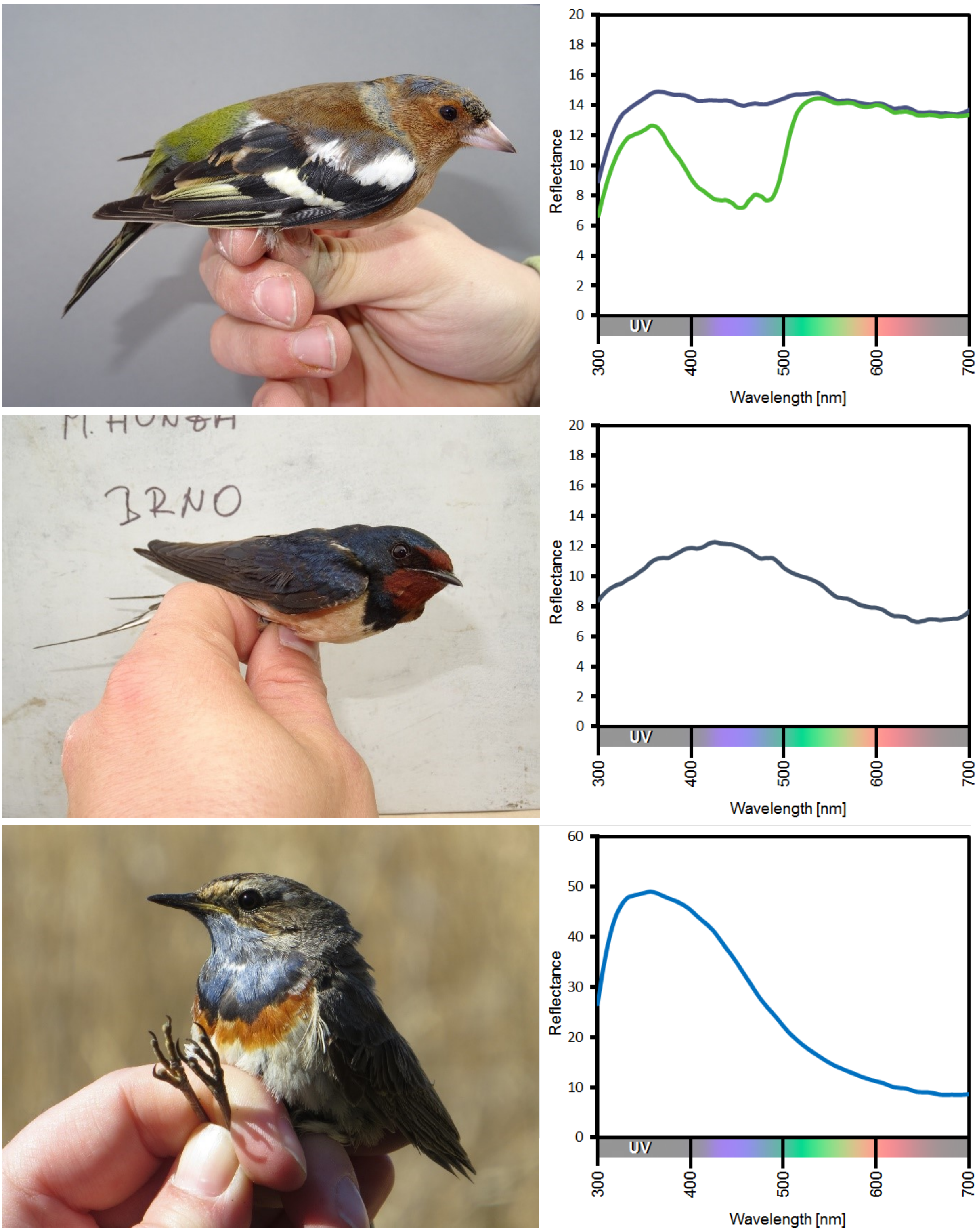

Plate 1: Structural coloration and examples of its respective reflectance spectra in three European passerines. The blue crown and green rump of a Common Chaffinch (*Fringilla coelebs*; top), the metallic blue back of a Barn Swallow (*Hirundo rustica*; middle), and the highly saturated throat of a Bluethroat (*Luscinia svecica*) peaking in the UV wavelengths (bottom, photo courtesy of Tereza Králová)

The green coloration is actually a structural coloration combined with carotenoid pigment as it is nicely illustrated here by the green rump and blue crown of the Common Chaffinch.

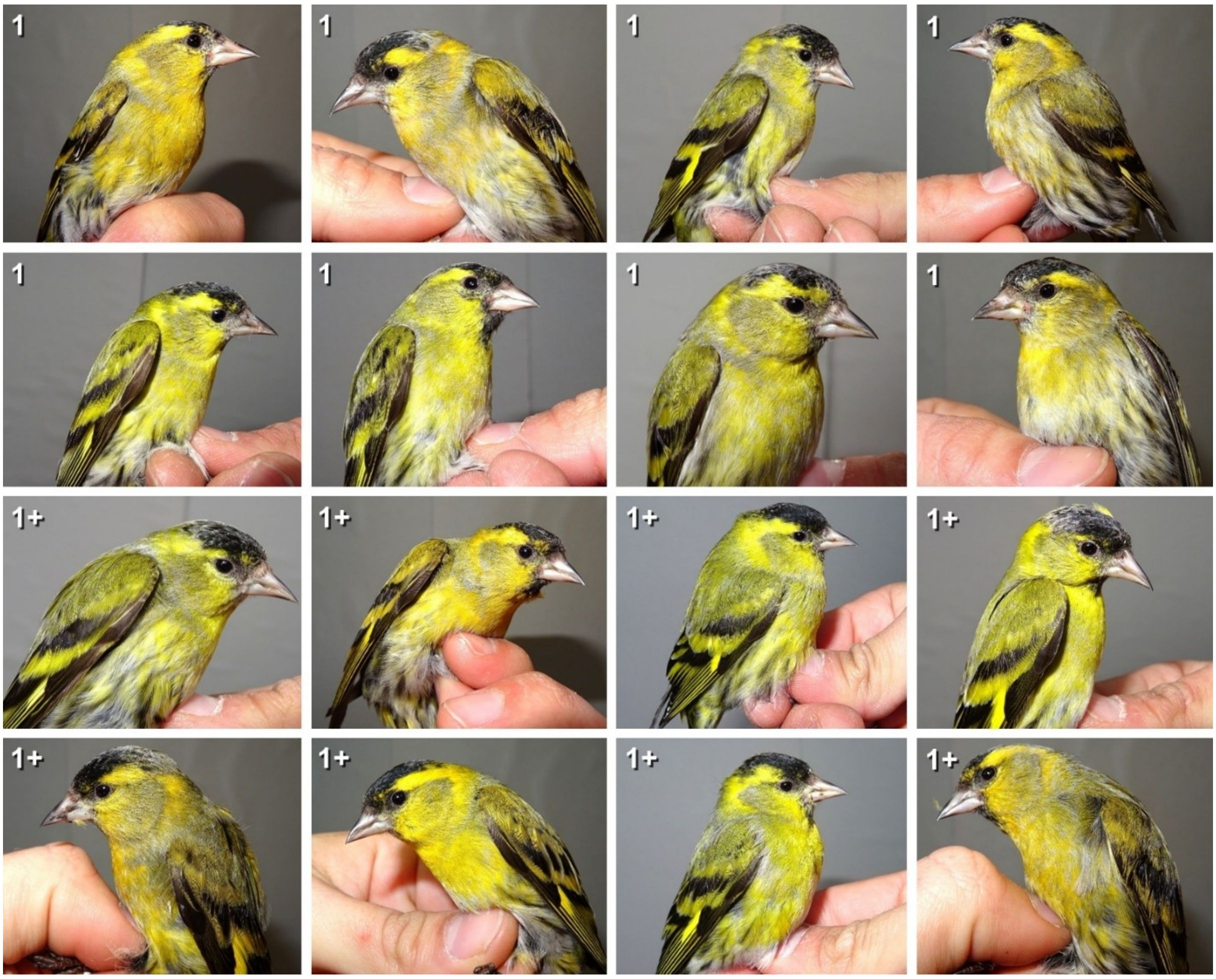

Plate 2: Natural variation in extent of both black melanin and yellow carotenoid ornamental coloration of several males of Eurasian Siskin (*Spinus spinus*), numbers indicate age of the bird; 1: bird in the first year of life, 1+: bird older than one year, exact age unknown. Graph shows examples of reflectance curves of these ornaments: yellow for carotenoid belly, black for melanin crown.

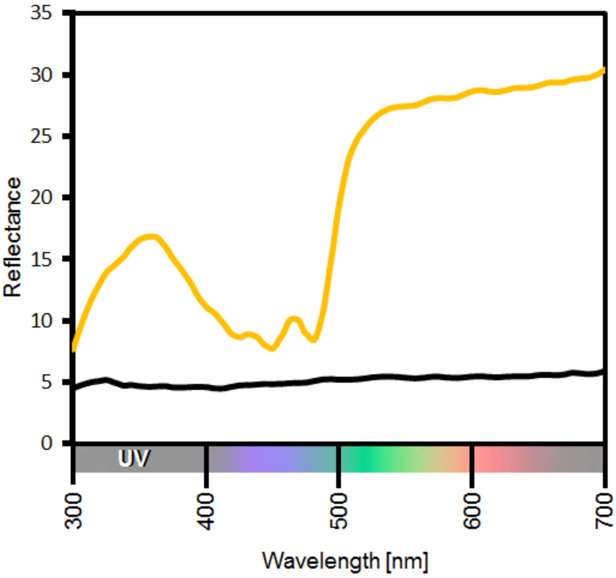

Plate 3: Two pictures of the same individual male House Finch (*Haemorhous mexicanus*) showing how dietary carotenoids can change the color quality of plumage. Picture on the left is from period when the individual was fed with a diet not rich in carotenoids, picture on the right is a half year later after molt when the diet was enhanced with carotenoids.

Plate 4: Fluorescence of psittacofulvins under ultraviolet light. Top: Fischer's Lovebird (*Agapornis fischeri*); bottom: Budgerigar (*Melopsittacus undulatus*)

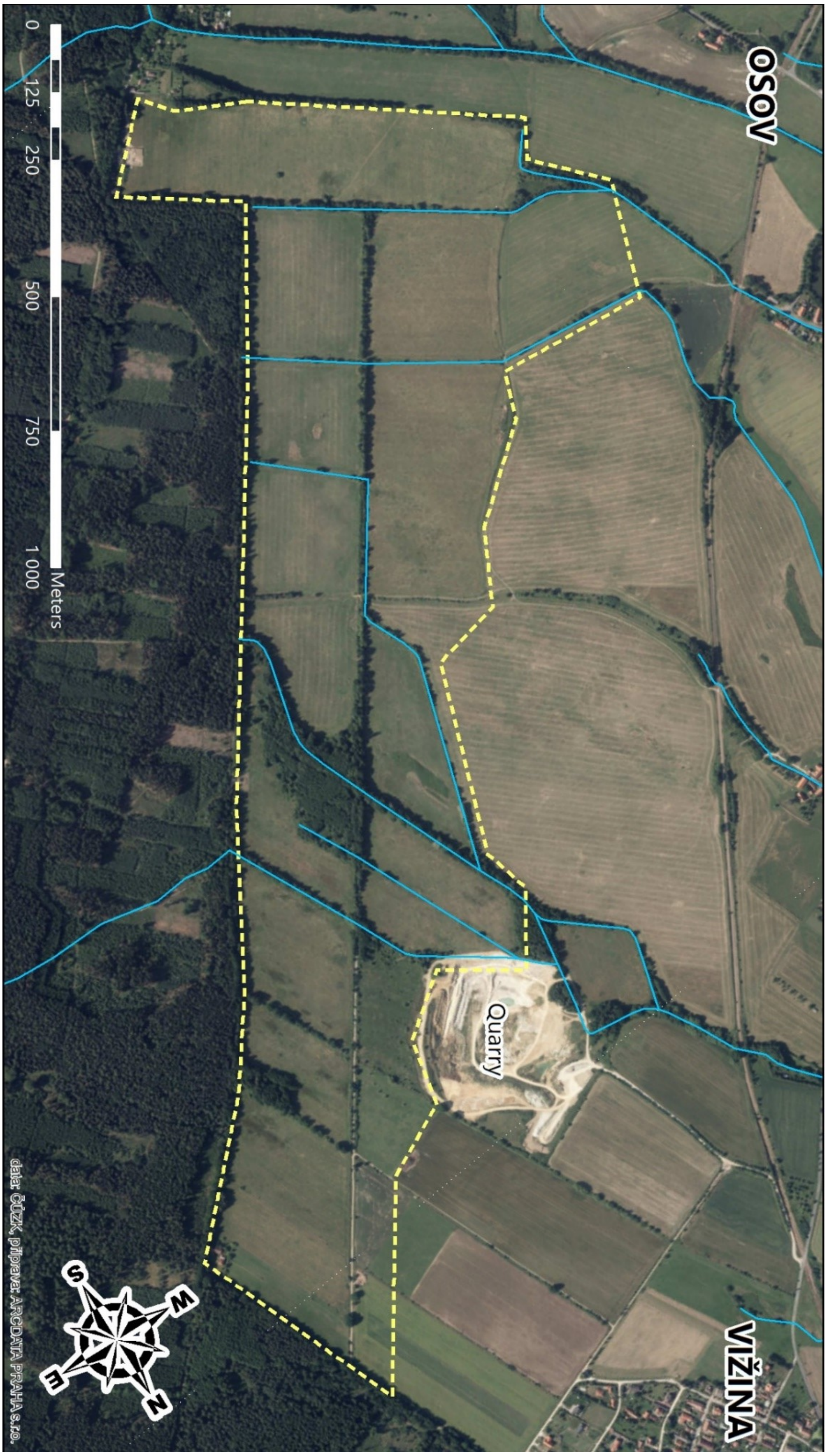

Plate 5: Orthophoto map of the study locality. The approximate border of the study locality is indicated with dashed yellow line and seasonal streams with solid blue lines.

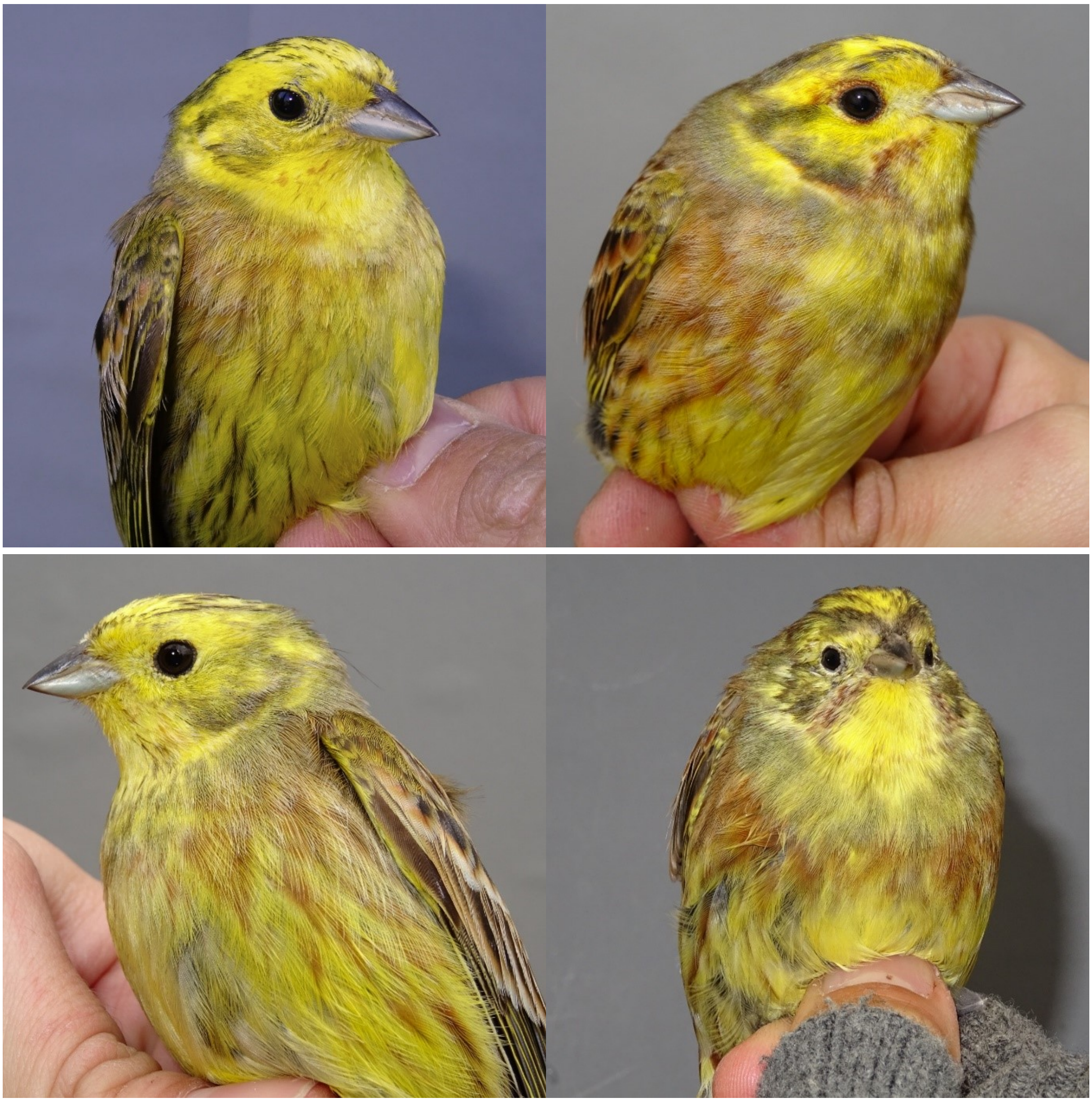

Plate 6: Several Yellowhammer males showing the variation in the extent of the phaeomelanin breast band.

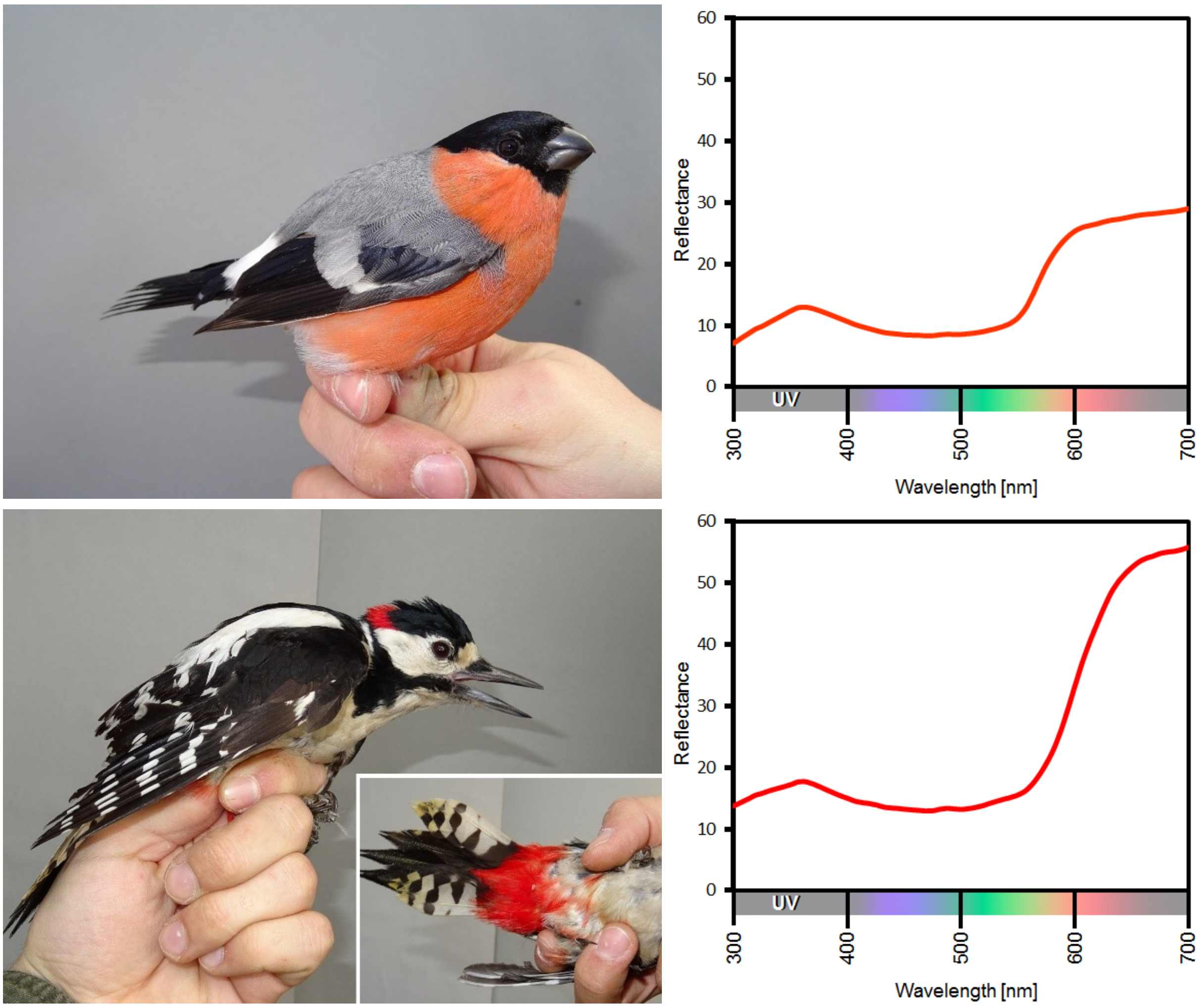

Plate 7: Saturation of carotenoid coloration. Light orange-red belly of a Eurasian Bullfinch (*Pyrrhula pyrrhula*; top) is less saturated than vivid scarlet undertail coverts of a Great Spotted Woodpecker (*Dendrocopos major*; bottom). Graphs show examples of reflectance curves of these carotenoid ornaments. Note the overall lower brightness, shallower negative peak in the blue part of the spectrum and proportionally high peak in the UV in the less saturated Bullfinch plumage and compare it to very deep negative peak in blue part of spectrum with virtually absent UV peak in the Woodpecker.