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Vlivy rodičů a prostředí na kvalitu a zbarvení mláďat pěvců

Doktorská disertační práce

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Prohlašuji, že jsem tuto práci vypracovala samostatně, a je mým původním dílem. Výjimku tvoří přiložené články a rukopisy, na nichž se podíleli uvedení spoluautoři. Žádnou část této práce jsem nepředložila k získání dalšího akademického titulu.

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ABSTRAKT

Výsledek hnízdění ptáků závisí na prostředí, v němž mláďata vyrůstají, a na péči rodičů. Jedním z nejdůležitějších aspektů je přitom množství a kvalita potravy, které mohou rozhodujícím způsobem ovlivnit fenotyp jedince v dospělosti a tím i jeho biologickou zdatnost (fitness). V předkládané disertační práci se zabývám vlivem kvality teritoria a prostředí poskytovaného rodiči na kvalitu a zbarvení mláďat volně žijících ptáků. Jako modelové druhy jsem zvolila sýkoru koňadru (*Parus major*) a lejska bělokrkého (*Ficedula albicollis*).

Častým ukazatelem kvality teritoria je v případě sýkory koňadry počet dubů v blízkém okolí hnízda. Nicméně doklady o pozitivním vztahu mezi dostupností dubů a výsledkem hnízdění sýkor nejsou jednoznačné. V našem případě jsme vztah mezi množstvím dubů v teritoriu a hnízdními parametry sýkory koňadry nenalezli. Role dubů jako indikátorů kvality teritoria sýkor patrně není univerzální. Význam dubů pro výživu mláďat sýkor se může lišit mezi různými druhy dubů a i v rámci jednoho druhu může být v čase velmi proměnlivý. Zároveň může záviset na dostupnosti dalších druhů stromů potenciálně bohatých na potravu.

Vliv kvality potravy hodnotíme na příkladu vitamínu E, který má řadu pozitivních funkcí ve fyziologii ptáků. Experimentálním přikrmováním dvou druhů drobných hmyzožravých pěvců jsme doložili, že vitamín E může být pro růst a vývoj ptáků limitující. Míra této limitace se však liší mezi druhy. V rámci jednoho druhu se pak význam vitamínu E mění v čase a liší mezi jedinci. Vitamín E je patrně důležitý zejména pro znevýhodněná mláďata. Pozitivní vliv vitamínu E na karotenoidové zbarvení se v našem případě nepotvrdil.

Rané prostředí a péče rodičů mají vliv i na karotenoidové ornamenty peří mláďat. Na vlastnostech karotenoidového zbarvení se vedle pigmentu podílí také struktura pera. V našem výzkumu jsme experimentálně manipulovali prostředí mláďat, abychom odlišili rodičovské vlivy na strukturální a pigmentární složku karotenoidového zbarvení. Výsledky potvrzují, že každá z těchto složek odráží různé aspekty prostředí a kvality jedince. Karotenoidové ornamenty tak mohou sloužit jako multikomponentní signál.

Klíčová slova

Ficedula albicollis, hnízdění, karotenoidové zbarvení, kvalita teritoria, *Parus major*, *Quercus* sp., rodičovské efekty, signály, vitamín E.

ABSTRACT

In birds, breeding performance depends both on rearing environment and parental care. Namely, food quality and quantity are critical for nestling phenotype in adulthood and fitness. Here, I study the importance of territory quality and parental effects in nestling development, survival and plumage colouration in free living birds. As model species, I chose Great Tit (*Parus major*) and Collared Flycatcher (*Ficedula albicollis*).

In titmice, number of oaks is nest proximity is frequently used as an index of food availability and consequently of territory quality. However, evidence of the positive association between local oak density and breeding performance is equivocal. Here, we investigated the relationship between the number of oaks in nest proximity and breeding performance of Great Tit pairs in a floodplain forest. We found no relationship between oak density and breeding performance in the Great Tit. We suggest that the role of oak as a source of prey may depend on oak species. Moreover, within a given oak species, the density of invertebrate prey may considerably vary in time. Availability of other deciduous trees potentially rich in caterpillars may reduce the importance of oaks as a critical food source.

We examined the effect of food quality on nestling performance and colouration using vitamin E, an important micronutrient with numerous beneficial functions in bird physiology. By supplementation of two species of small insectivorous songbirds, we showed that vitamin E may be limiting for nestling growth and survival. However, the importance of vitamin E differed between species, and within species might vary with season and among individuals. Increased income of vitamin E may be benefical especially for nestlings with a bad start in life. However, our results do not support possitive effect of increased vitamin E availability on carotenoid plumage colouration.

Early environment and parental care may determine the expression of plumage colouration. Carotenoid-based feather colouration has recently been shown to be dependent on both pigment concentration and feather structure. We experimentally manipulated early nestling environment to evaluate parental effects on both the structural and carotenoid-based components of nestling carotenoid colouration. Our results support the hypothesis that pigment-based and structural component of feather colouration reflect different aspects of individual quality and history. Carotenoid colouration thus may potentially form a multicomponent signal.

Keywords

OBSAH

Přílohy

Příspěvek I

Příspěvek II

Příspěvek III

Příspěvek IV

1 SEZNAM PŘILOŽENÝCH PŘÍSPĚVKŮ

Příspěvek I

Matrková J. & Remeš V.: Do oaks matter? Great Tit breeding performance in relation to territory tree composition (rukopis).

Příspěvek II

Matrková J., Mullen W. & Remeš V.: Do Great Tits need vitamíns? Nestling performance and colouration in response to vitamín E supplementation (rukopis).

Příspěvek III

Matrková J. & Remeš V. 2014: Vitamín E improves growth of collared flycatcher *Ficedula albicollis* young: a supplementation experiment. Journal of Avian Biology, 45 (5), 475-483.

Příspěvek IV

Matrková J. & Remeš V. 2012: Environmental and Genetic Effects on Pigment-Based vs. Structural Component of Yellow Feather Colouration. PLOS ONE, 7 (5), e36640.

2 ÚVOD A CÍLE PRÁCE

Výsledek hnízdění ptáků závisí na prostředí, v němž mláďata vyrůstají. V době závislosti mláďat na rodičích vliv prostředí zprostředkovává z rozhodující části péče rodičů. Jedním z nejdůležitějších aspektů, jímž rodiče ovlivňují prostředí mláďat, je jejich výživa (Bernardo 1996, Price 1998). Prostředí, v němž mláďata vyrůstají, přitom zásadně a na celý život ovlivňuje řadu znaků, včetně morfologie, metabolismu, imunity či chování (Lindström 1999, Monaghan 2008, Kasumovic 2013). Potrava v raném věku je proto klíčovým faktorem pro fenotyp i zdatnost dospělého jedince (Metcalfe and Monaghan 2001). V době rozmnožování však bývá pro ptáky potrava limitující. To doložila řada experimentů, v nichž přikrmování nebo redukce snůšky vedly k lepšímu přežívání mláďat a rodičů, zatímco zvýšení počtu mláďat nebo ztráta samce, který by jinak při výchově pomáhal, zhoršily přežívání mláďat i rodičů (Martin 1987, Newton 1990). Rodiče proto musí volit, jak dostupnou energii rozdělit mezi péči o mláďata a vlastní přežití (Martin 1987). Experimentální zvětšení či zmenšení snůšky potvrdilo, že intenzivnější péče o mláďata vede k větší parazitaci, horší imunitní odpovědi a nižšímu přežívání rodičů (Knowles et al. 2009, Santos and Nakagawa 2012).

Dostupnost potravy je často značně proměnlivá v prostoru. Vliv této proměnlivosti na hnízdění ptáků lze hodnotit na několika úrovních. U drobných pěvců to tradičně bývá úroveň biotopu, což odpovídá úrovni populace (Wilkin et al. 2007b). Výsledek hnízdění ptáků se mezi různými biotopy často výrazně liší (Newton 1990). Například hnízdění sýkory koňadry (*Parus major*) a sýkory modřinky (*Cyanistes caeruleus*) bývá úspěšnější v listnatých než jehličnatých lesích. Ve srovnání s jehličnatými jim listnaté lesy poskytují v době hnízdění více potravy, sýkory zde snášejí větší snůšky, mláďata zde rostou rychleji, dosahují vyšší hmotnosti a mají větší šance, že se dožijí opuštění hnízda (van Balen 1973, Massa et al. 2011). Kvalita biotopu zároveň ovlivňuje i nároky na rodičovskou péči: rodiče sýkory modřinky během krmení mláďat létají v horším biotopu pro potravu dále od hnízda. Navíc je-li potrava vzácná, nedokáží vždy zajistit mláďatům dostatečnou výživu (Tremblay et al. 2003, 2005, Stauss et al. 2005).

Na úrovni jedinců, resp. jednotlivých párů, může být vhodnějším ukazatelem dostupnosti potravy spíše než biotop velikost a kvalita konkrétního teritoria (Wilkin et al. 2007b). Rodiče drobných pěvců často sbírají potravu pro mláďata na velmi omezeném území

(Naef-Daenzer and Keller 1999, Naef-Daenzer 2000). Pro výsledek hnízdění tak může být spíše než širší okolí důležitá charakteristika prostředí v těsném sousedství hnízda. V souladu s tím fenologie dubů v okruhu do 20 metrů od hnízda, která určuje dobu nejvyšší dostupnosti potravy, předpovídala časování hnízdění sýkory koňadry lépe, než fenologie dubů v širším okolí (Hinks et al. 2015).

Růst a vývoj mláďat ovlivňuje nejen množství, ale i kvalita potravy poskytované rodiči. Například Johnston (1993) přikrmoval mláďata jiřičky obecné (*Delichion urbica*) tukem, který je bohatým zdrojem energie, ale ve srovnání s hmyzem je chudý na ostatní živiny (zejm. bílkoviny). Na přikrmovaných hnízdech krmili rodiče svá mláďata hmyzem méně než na hnízdech kontrolních, takže při srovnatelném energetickém příjmu dostávala tukem přikrmovaná mláďata méně kvalitní potravu. Tukem přikrmovaná mláďata pak rostla hůře a vylétla z hnízda později než mláďata z kontrolních hnízd. Podobně tukem přikrmované sýkory snášely vejce s menšími žloutky; to ale neplatilo, pokud byl zároveň s tukem podáván také vitamín E, který tedy zřejmě nižší obsah bílkovin kompenzoval (Plummer et al. 2013). Na druhou stranu některé látky mohou mláďatům škodit: například přikrmování taninem brzdilo růst mláďat sýkory modřinky (Perrins 1976). Naopak mláďata sýkory modřinky a koňadry měla delší běhák, pokud jim rodiče nosili potravu s větším podílem pavouků (García-Navas et al. 2013). Oproti ostatním složkám jejich potravy obsahují pavouci vysoké množství aminokyseliny taurinu, který může hrát velmi významnou roli ve vývoji mláďat (Ramsay and Houston 2010). Vztah mezi taurinem a růstem mláďat sýkor však dosud nebyl experimentálně potvrzen a je možné, že pavouci jsou zdrojem také jiných významných látek, například vápníku (García-Navas et al. 2013). Vápník je základním stavebním kamenem skořápek vajec i kostí mláďat a v oblastech chudých na vápník může tento prvek rozmnožování ptáků limitovat. Přikrmování vápníkem v některých případech zlepšilo kvalitu vajec, mláďat i samic (Reynolds et al. 2004).

Vedle základních živin (makronutrienty, tj. sacharidy, tuky a bílkoviny) jsou tedy pro výživu důležité i mikronutrienty - látky, které jsou zastoupeny ve výživě jen v malém množství, ale přesto jsou nutné pro správnou funkci metabolismu (např. vitamíny, minerály). Dostupnost mikronutrientů v raném období má dlouhodobé dopady na morfologii, chování a fyziologii jedince (Blount et al. 2003, Marri and Richner 2014a, Noguera et al. 2015a, b). Prostřednictvím mikronutrientů ve výživě mohou rodiče ovlivnit také zbarvení mláďat (Biard et al. 2009). Zbarvení přitom může mít rozhodující roli

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v účinnosti vizuálních signálů a tím i v komunikaci mezi ptáky (Andersson 2000). Barevné signály mláďat při žadonění pak mohou zpětně ovlivnit intenzitu rodičovské péče, a tím i příjem živin a následně růst a vývoj mláďat (Dugas 2015). Rané prostředí však určuje i zbarvení v pozdějším životě (Evans and Sheldon 2012, 2015, Walker et al. 2013), takže rodiče mohou ovlivnit i expresi signálů mláďat v dospělosti (Biard et al. 2009).

V předkládané disertační práci zkoumám vliv rodičovských efektů a kvality prostředí na fenotyp mláďat volně žijících pěvců. Konkrétně se zabývám vlivy kvality teritoria (**příspěvek I**) a prostředí poskytovaného rodiči (**příspěvek I, II, III, IV**) na kvalitu (**příspěvek I, II, III**) a zbarvení mláďat (**příspěvek IV**).

Jako ukazatel kvality teritoria jsem použila strukturu vegetace, konkrétně počet dubů v blízkém okolí hnízda, který by měl být dobrým indexem dostupnosti potravy. Vliv kvality potravy hodnotím na příkladu vitamínu E. Vedle vlivu prostředí a rodičovské péče na růst a přežívání mláďat jsem hodnotila také jejich vliv na zbarvení peří mláďat založené na karotenoidech. Převažující metodou mého výzkumu byl experiment (manipulace snůšky, přikrmování vitamínem E, výměna mláďat mezi hnízdy; blíže k metodice výzkumu viz jednotlivé příspěvky). Jako hlavní modelový druh jsem pro svůj výzkum zvolila sýkoru koňadru – běžného hmyzožravého pěvce, který je jedním z nejlépe prostudovaných druhů ptáků vůbec (Perrins 1979, Gosler 1993). Sýkora koňadra je spolu se sýkorou modřinkou důležitým modelovým druhem jak v oblasti výzkumu kvality teritoria při rozmnožování, tak pro výzkum funkce a plasticity zbarvení opeření, zejména karotenoidových ornamentů (viz dále). Význam vitamínu E při rozmnožování volně žijících ptáků jsem pak srovnávala u dvou pěvců s rozdílnou potravní strategií ve stejném biotopu: sýkory koňadry a lejska bělokrkého (*Ficedula albicollis*).

3 POČET DUBŮ JAKO UKAZATEL KVALITY TERITORIA

Nabídku potravy v teritoriu lze hodnotit přímo, odhadem množství příslušné potravy (Naef-Daenzer et al. 2004, Mäntylä et al. 2015), nebo pomocí dostupnějších ukazatelů, které s množstvím potravy bezprostředně souvisejí, například trus housenek (Eeva et al. 1998, Seki and Takano 1998). Tyto metody ale bývají velmi pracné, a proto se množství potravy v teritoriu odvozuje často nepřímo ze zastoupení míst typických dobrou potravní nabídkou (Korpimaki 1988, Sergio et al. 2009). V případě drobných pěvců se jako ukazatel dostupnosti potravy v teritoriu často používá charakteristika vegetace, tj. např. výška a pokryvnost porostu, jeho druhové složení, stáří či struktura (Eeva et al. 1998, Hinsley et al. 2002, 2008, 2009, Arriero and Fargallo 2006, Sánchez et al. 2007, Wilkin et al. 2007a, Sanz et al. 2010, Kroll and Haufler 2010, Newell and Rodewald 2011, Mäntylä et al. 2015, Maziarz and Broughton 2015, Amininasab et al. 2016). Současný rozvoj a lepší dostupnost technik, jako jsou např. dálkový průzkum Země (snímkování z letadel či družic; Hinsley et al. 2002, Maícas et al. 2012, Mackenzie et al. 2014) nebo geografické informační systémy (Wilkin et al. 2007a, b, Kroll and Haufler 2010, Hinks et al. 2015) přitom výzkum vlastností prostředí na úrovni teritorií výrazně usnadňují.

Pro úspěšný vývoj mláďat sýkory koňadry a sýkory modřinky je rozhodující dostupnost hlavní složky jejich potravy – housenek motýlů (Lepidoptera; van Balen 1973, Keller and van Noordwijk 1994, Naef-Daenzer and Keller 1999, Naef-Daenzer et al. 2000, Verboven et al. 2001, Wilkin et al. 2009). Distribuce housenek je nerovnoměrná v čase i prostoru. V době rozmnožování sýkor bývají housenky nejpočetnější jen ve velmi krátké časové periodě (tzv. *caterpillar peak*). Hnízdění sýkor je zpravidla výrazně úspěšnější, pokud se období nejvyšších energetických potřeb mláďat překrývá s tímto na potravu bohatým obdobím (Naef-Daenzer and Keller 1999, Visser et al. 2006, Reed et al. 2013). Dostupnost housenek v prostoru byla tradičně hodnocena na úrovni biotopu (viz např. van Balen 1973, Tremblay et al. 2003, Stauss et al. 2005, Massa et al. 2011, García-Navas and Sanz 2012). V rámci stejného biotopu však housenky upřednostňují určité hostitelské rostliny a množství housenek se tak může významně lišit i na úrovni jednotlivých stromů (Naef-Daenzer 2000). Protože duby často hostí více housenek než ostatní druhy stromů (Fischbacher et al. 1998, Naef-Daenzer 2000, Matysioková and Remeš 2010; ale srovnej s Wesołowski and Rowiński 2006), je poslední dobou počet dubů v bezprostředním okolí hnízda často používán jako indikátor kvality teritoria u sýkor (**příspěvek I**, Naef-Daenzer

et al. 2004, Wilkin et al. 2007b, 2009, Hinsley et al. 2008, 2009, Galván et al. 2009, Sanz et al. 2010, Atiénzar et al. 2010, Parker et al. 2011, Maícas et al. 2011, 2012, 2014, Evans and Sheldon 2013, Bell et al. 2014, Mackenzie et al. 2014, Amininasab et al. 2016).

V souladu s očekáváním tyto práce až na výjimky (naše studie – viz **příspěvek I**, Atiénzar et al. 2010) zjistily pozitivní vztah mezi některými ze sledovaných parametrů hnízdění sýkor a množstvím dubů v okolí hnízda (např. dřívější hnízdění, větší snůšky, kvalitnější potrava, lepší kondice mláďat). Celkově jsou ale dosavadní výsledky nejednoznačné. Například samice v teritoriích bohatších na duby často snášely větší snůšky (Naef-Daenzer et al. 2004, Sanz et al. 2010, Parker et al. 2011, Bell et al. 2014, Maícas et al. 2014, Amininasab et al. 2016), někteří autoři však žádný vztah mezi počtem druhů a snůškou nenalezli (**příspěvek I**, Atiénzar et al. 2010, Maícas et al. 2011, 2012). Ve Wythamském lese u Oxfordu opouštěla mláďata v teritoriích bohatších na duby hnízda v lepší kondici. Počet mláďat, která přežila zimu a v následujícím roce zahnízdila, ale s dostupností dubů kolem rodného hnízda nesouvisel (Wilkin et al. 2009). Navzdory řadě pozitivních dílčích asociací mezi dostupností dubů a některými hnízdními parametry sýkor žádná z dosavadních studií nenalezla vztah mezi počtem dubů v teritoriu a celkovou úspěšností hnízdění měřenou jako podíl či počet úspěšně vyvedených mláďat (Sanz et al. 2010, Atiénzar et al. 2010, Maícas et al. 2011, 2012, 2014).

Jedním z možných vysvětlení nejednoznačnosti dosavadních výsledků jsou rozdíly mezi jednotlivými druhy dubů (r. *Quercus*). Severoameričtí pěvci při hnízdění některé druhy dubů upřednostňují před jinými a výsledek jejich hnízdění závisí na zastoupení jednotlivých druhů dubů v porostu (Newell and Rodewald 2011). Různé druhy dubů se mezi sebou liší fenologií, chemickým složením i nutriční hodnotou listů. Tyto rozdíly se odráží v rozdílném růstu housenek krmených listím různých druhů dubů (Foss and Rieske 2003, Milanović et al. 2014) a rozdílný růst housenek může následně ovlivnit hnízdění sýkor. Například stálezelené duby kvůli svým tuhým, pomalu rostoucím listům s vysokým obsahem taninu mohou hostit méně fytofágního hmyzu než duby opadavé (blíže viz Mackenzie et al. 2014). V souladu s tím byla zjištěna pozitivní asociace mezi množstvím dubů v teritoriu a velikostí snůšky, velikostí a kondicí mláďat u několika opadavých druhů dubů (Amininasab et al., 2016; Bell et al., 2014; Mackenzie et al., 2014; Maícas et al., 2014; Naef-Daenzer et al., 2004; Parker et al., 2011; Sanz et al., 2010; Wilkin et al., 2009),

ale jen výjimečně u stálezeleného dubu cesmínovitého (*Quercus ilex*; Atiénzar et al. 2010, Maícas et al. 2011, 2012, 2014).

Přestože doposud většina prací uvádí duby jako nejvýznamnější zdroj potravy sýkor (viz výše), mohou být v některých případech pro hnízdící sýkory významné i jiné druhy listnatých stromů. V Bielověžském pralese byly žírem housenek nejvíce postiženy habry (*Carpinus betulus*), následované javory (*Acer platanoides*), lípami (*Tilia cordata*) a duby (*Quercus robur*; Wesołowski and Rowiński 2006). To je jeden z možných důvodů, proč jsme při našem výzkumu žádný vliv dubů na hnízdění sýkory koňadry nezjistili (**příspěvek I**). V porostech lužního lesa v Grygově, kde průzkum probíhal, totiž převažují lípy a místy jsou hojné i habry – druhy potenciálně významné jako zdroj potravy sýkor. Navíc množství housenek na dubech může mnohonásobně kolísat během sezóny i mezi roky (van Balen 1973, Naef-Daenzer and Keller 1999, Tremblay et al. 2003) a spolu s tím se může v čase měnit i relativní hodnota dubů jako zdroje potravy pro sýkory (Lõhmus 2003, Hinsley et al. 2008). Proto v některých letech a/nebo v některých částech sezóny mohou jiné druhy stromů poskytovat sýkorám potravu v množství srovnatelném nebo dokonce vyšším než duby. Pokud role různých druhů stromů jako zdroje potravy v čase kolísá, nemusí krátkodobé studie vliv dubů odhalit. Pozitivní vztah mezi počtem dubů a výsledkem hnízdění sýkor pak může být patrný až při analýze dlouhodobějších dat na škále několika dekád (Wilkin et al. 2009, Hinks et al. 2015).

V bohatém prostředí nemusí být duby dobrým indikátorem kvality prostředí proto, že dostupnost housenek není pro sýkory limitující (Tremblay et al. 2003). To je patrně důvodem, proč rodiče sýkor vynakládali více energie pro krmení mláďat pouze pokud počet dubů okolo hnízda klesl pod určitou prahovou hodnotu (Hinsley et al. 2008, 2009). Podobně sýkory koňadry v Bělověžském pralese nevybíraly místo pro hnízdění podle počtu na potravu bohatých habrů pravděpodobně proto, že habry mají v porostech bohaté zastoupení a potrava je zde obecně hojná (Maziarz and Broughton 2015). Ptáci navíc přednostně obsazují kvalitnější teritoria, horší teritoria bývají obsazena teprve při vyšších populačních hustotách (Newton 1990). V prostředí, kde mají všichni jedinci přístup k dostatečné výživě, tj. prostředí s hojnými zdroji, v dobrých letech a/nebo při nízkých populačních hustotách, se vliv kvality teritoria nemusí projevit.

Pochopení vlivu dubů na hnízdění sýkor může komplikovat také korelace mezi kvalitou rodičů a kvalitou teritoria. Kvalita rodičů, kvalita teritoria i jejich různorodé interakce mohou mít významný vliv na výsledek hnízdění – vliv jednoho může podle situace vyrovnat, zakrýt, zvětšit či zprostředkovat vliv druhého (Sergio et al. 2009). U teritoriálních druhů obvykle nejkvalitnější jedinci obsadí nejkvalitnější teritoria (Newton 1990, Sergio et al. 2009). Většina studií vlivu kvality teritoria na hnízdění sýkor pro kvalitu jedince nekontrolovala, což jejich interpretaci z tohoto pohledu velmi komplikuje: nelze totiž vyloučit, že za pozorovanou spojitostí mezi počtem dubů a parametry hnízdění sýkor stojí kvalitnější rodičovská péče či "lepší geny". U sýkory koňadry a sýkory modřinky však patrně vztah mezi kvalitou jedince a kvalitou teritoria chybí nebo je slabý (Lambrechts and Dhondt 1988, Lemel 1989, Riddington and Gosler 1995, Przybylo et al. 2001). V souladu s tím u sýkory modřinky kondice rodičů nekorelovala s počtem dubů v teritoriu (Bell et al. 2014). V našem případě jsme vliv kvality rodiče od kvality teritoria částečně odlišili. Výměnou mláďat mezi hnízdy jsme totiž oddělili genetické a rané mateřské efekty (např. kvalita vejce a inkubace) od pozdější rodičovské péče (zejm. krmení; **příspěvek I**), a navíc jsme modely kontrolovali na věk rodičů. To, že jsme nenalezli žádný vztah mezi počtem dubů a hnízděním sýkor pro znaky před výměnou mláďat (časování hnízdění, velikost vejce a snůšky) ani po ní (růst a imunitní reakce mláďat) svědčí přinejmenším proti některým typům interakcím mezi kvalitou teritoria a kvalitou rodičů.

V horších teritoriích mohou také rodiče zvolit odlišnou strategii hnízdění, která může ve výsledku vést k podobné úspěšnosti hnízdění jako v teritoriích kvalitních, i když někdy za cenu vyšších nákladů pro rodiče (Price 1998). V chudém prostředí mohou rodiče například zvětšit své teritorium (Newton 1990, Seki and Takano 1998), zmenšit snůšku a tím i počet mláďat, takže množství potravy na mládě se mezi chudým a bohatými teritorii nemusí lišit (Seki and Takano 1998, Verboven et al. 2001), nebo se může prodloužit doba kterou mláďata stráví na hnízdě i období péče rodičů o mláďata po vylétnutí (Keller and van Noordwijk 1994, Seki and Takano 1998). Rodiče také mohou zvolit odlišnou strategii krmení mláďat. Sýkory modřinky na Korsice létaly v chudém biotopu s převahou stálezelených dubů při krmení mláďat pro potravu dvakrát tak daleko než v bohatším biotopu s převahou dubů opadavých. Celková nalétaná vzdálenost se ale mezi biotopy nelišila, protože v chudším biotopu krmily modřinky mláďata v delších časových intervalech. Zároveň se však mezi biotopy nelišilo ani množství potravy na jedno mládě.

Nižší frekvenci krmení v horším biotopu totiž vyvážili rodiče tím, že nosili mláďatům větší housenky (Tremblay et al. 2005). Také v Německu sýkory v bohatším listnatém lese nosily potravu z menší vzdálenosti než v chudším lese smíšeném, ale průměrná frekvence krmení se v tomto případě mezi biotopy nelišila a nelišila se ani průměrná hmotnost mláďat při opuštění hnízda. Rodiče ve smíšených lesích však při krmení mláďat nalétali celkově větší vzdálenost (Stauss et al. 2005). Pokud rodiče v chudých teritoriích zvýší své úsilí při krmení, mohou škodlivý vliv horšího prostředí na mláďata přinejmenším částečně kompenzovat, může to však být za cenu nižšího přežívání rodičů. Při nižších hustotách housenek je totiž obstarání potravy pro rodiče výrazně nákladnější (Naef-Daenzer and Keller 1999).

4 VLIV VITAMÍNU E NA RŮST A VÝVOJ MLÁĎAT

Ve své práci studuji význam mikronutrientů pro fenotyp mláďat krmivých ptáků na příkladu vitamínu E. Vitamín E totiž patří mezi mikronutrienty s klíčovou rolí v rozmnožování, normálním vývoji a imunitě ptáků (Surai 2002, Catoni et al. 2008). Tradičně je považován zejména za důležitý antioxidant, který brání oxidačnímu poškození buněk tím, že váže volné radikály. Vitamín E zastavuje řetězovou reakci poškození fosfolipidů buněčných membrán (lipoperoxidace; Surai 2002). V současnosti je však význam antioxidačních schopností vitamínu E *in vivo* zpochybňován a větší potenciál je spatřován v jeho dalších funkcích, například v regulaci enzymů, expresi genů, buněčné signalizaci a vnitrobuněčném transportu a membránových procesech (Brigelius-Flohé 2009).

Mláďata jsou na nedostatek vitamínu E a s ním spojené choroby citlivější než dospělí ptáci. Nemají totiž ještě vytvořeny dostatečné zásoby vitamínu E a jsou tak závislá na jeho příjmu z potravy (Surai 2002). Navíc může být u mláďat potřeba vitamínu E vyšší, například proto, že v době rychlého růstu je spotřeba kyslíku a tedy i produkce volných radikálů, které poškozují buňky, vysoká (Mangel and Munch 2005, Alonso-Álvarez and Galván 2008, De Block and Stoks 2008, Kim et al. 2011; srovnej s Monaghan et al. 2009). Zvýšení příjmu vitamínu E u rostoucích mláďat ptáků v některých případech opravdu snížilo míru oxidačního poškození (Sodhi et al. 2008, Noguera et al. 2011, Orledge et al. 2012), jindy však tento pozitivní vliv vitamínu E potvrzen nebyl (Larcombe et al. 2010, Kim et al. 2013, Marri and Richner 2014b, 2015).

Zatímco role vitamínu E v rozmnožování drůbeže je již dlouho předmětem výzkumu a je dobře známá (Surai 2002), experimenty na volně žijících ptácích započaly teprve nedávno a jejich výsledky nejsou jednoznačné. V některých studiích podávání vitamínu E růst mláďat zlepšilo (vlaštovka obecná: de Ayala et al. 2006; racek středomořský *Larus michahellis*: Noguera et al. 2011, Kim et al. 2013, Parolini et al. 2015; lejsek bělokrký: **příspěvek III**, sýkora koňadra: Marri and Richner 2014b), v jiných však žádný vliv na růst zjištěn nebyl (sýkora modřinka: Larcombe et al. 2010; racek středomořský: Noguera et al. 2010; sýkora koňadra: **příspěvek II**, Marri and Richner 2015, Maronde and Richner 2015). Podobně nejednoznačný je i vliv podávání vitamínu E na přežívání mláďat na hnízdě (pozitivní vliv: Marri and Richner 2014b, Maronde and Richner 2015; vliv neprokázán:

příspěvek II, **příspěvek III**, de Ayala et al. 2006, Noguera et al. 2011, Kim et al. 2013), na koncentraci vitamínu E v krevní plazmě (pozitivní vliv: **příspěvek II**, de Ayala et al. 2006, Pérez et al. 2008, Giraudeau et al. 2013; vliv neprokázán: Larcombe et al. 2010, Maronde and Richner 2015) a na zbarvení (viz následující kapitola) mláďat a dospělců. Pouze v případě imunity jsou dosavadní výsledky konzistentní: žádný z experimentů vliv přikrmování vitamínem E na imunitní reakci nepotvrdil (**příspěvek II**, de Ayala et al. 2006, Hõrak et al. 2007, Losdat et al. 2011, Marri and Richner 2015). Protože nezbytnost vitamínu E v imunitě ptáků je velmi dobře podložená (Surai 2002, Parolini et al. 2015), navrhuji dvě možná vysvětlení. Pro správnou funkci imunitního systému ptáků může postačovat menší množství vitamínu E než např. pro růst či produkci ornamentů, a proto může být v tomto případě jeho příjem z přirozené potravy dostatečný i v situaci, kdy jiný znak limituje (de Ayla et al. 2006). Je také možné, že některé z metod použitých pro vyhodnocení vlivu vitamínu E na imunitní reakce volně žijících ptáků neměřily parametry imunitní reakce ovlivněné vitamínem E (viz diskuse v **příspěvku II**).

Proč jsou výsledky přikrmování vitamínem E mezi studiemi tak protichůdné? Zaprvé, množství vitamínu E a ostatních antioxidantů je velmi variabilní a může se výrazně lišit mezi druhy potravy, v prostoru i v čase (Catoni et al. 2008). Například housenky, hlavní složka potravy mláďat sýkor, jsou na vitamín E mnohonásobně bohatší než létající hmyz, kterým krmí svá mláďata vlaštovky (de Ayala et al. 2006, Arnold et al. 2010). Podobně předpokládáme nižší koncentraci vitamínu E v potravě lejsků než v potravě sýkor, a to i v rámci jedné lokality (blíže viz **příspěvek III**). Zastoupení vitamínem E bohatých housenek v potravě lejsků je totiž nižší než u sýkor (Cholewa and Wesołowski 2011). Lejsci navíc hnízdí později než sýkory, zatímco dostupnost housenek v sezóně klesá (Arnold et al. 2010), takže u pozdních hnízd jsou v potravě mláďat housenky nahrazovány jiným hmyzem (Löhrl 1957, 1976). V souladu s tím jsme potvrdili pozitivní vliv přikrmování vitamínem E na růst lejsků pouze u pozdních hnízd (**příspěvek III**). Vedle horší dostupnosti vitamínu mohou být příčinou i změny nároků mláďat na množství vitamínu E s postupující sezónou, například kvůli rostoucí teplotě či klesající kvalitě potravy (Perrins 1976).

Zadruhé, vliv přikrmování vitamínem E může být nižší, pokud mláďata získají dostatečnou zásobu vitamínu E z vejce. Koncentrace vitamínu E ve žloutku se může významně lišit mezi druhy, lokalitami, mezi roky i v průběhu jedné sezóny (Møller et al. 2005, 2008,

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Biard et al. 2009, Remeš et al. 2011, Plummer et al. 2013) a v rámci snůšky může klesat s rostoucím pořadím kladení vejce (Rubolini et al. 2011). To, že zásoba vitamínu E ve vejcích je pro mláďata důležitá, naznačuje na mezidruhové úrovni pozitivní korelace mezi koncentrací vitamínu E ve žloutcích a rychlostí embryonálního růstu; spolu s vitamínem E však rostla i koncentrace dalších antioxidantů (Deeming and Pike 2013), takže studie svědčí o roli vitamínu E v růstu mláďat jen nepřímo. Na vnitrodruhové úrovni limitaci vitamínem E přesvědčivě dokázal experiment, ve kterém zvýšení koncentrace tohoto vitamínu ve vejcích racků zlepšilo růst mláďat (Parolini et al. 2015).

Zatřetí, přínos zvýšené dostupnosti vitamínu E se může projevit jen v určitém období vývoje mláďat. U vlaštovek byl pozitivní efekt přikrmování vitamínem E na růst a kondici omezen jen na období nejrychlejšího růstu (6 – 10 dní); naopak u racků se projevil až u osmidenních mláďat (de Ayala et al. 2006, Noguera et al. 2010, 2011). Podobně u sýkory koňadry zlepšilo přikrmování směsí vitamínu C a E růst mezi osmým a čtrnáctým dnem věku, ale nikoli dříve (Marri and Richner 2014b). Je tedy možné, že přínos vitamínu E nebyl u některých studií zjištěn proto, že měření mláďat proběhlo mimo období, kdy mohl být vliv přikrmování detekován.

Za čtvrté, zvýšený přísun vitamínu E může být výhodný jen pro některá mláďata. Reakce antioxidačního systému na přikrmování vitamíny se u jednotlivých mláďat může výrazně lišit, takže zvýšený přísun vitamínů zvýší rozptyl tohoto znaku aniž by byl ovlivněn průměrný fenotyp. Malou podobnost v reakci antioxidačního systému na přikrmování vitamíny u mláďat racků ze stejného hnízda vysvětlují Kim et al. (2013) genetickým podmíněním rozdílů mezi mláďaty a vliv prostředí (včetně vlivu rodičovské péče) považují za zanedbatelný. I mláďata ve stejném hnízdě však mohou vyrůstat v rozdílných podmínkách. Opakovaně bylo prokázáno, že z větší dostupnosti vitamínu E těží především znevýhodněná mláďata (nejmladší a/nebo nejmenší), a to jak v případě přikrmování mláďat (**příspěvek III**, Noguera et al. 2011, Kim et al. 2013), tak při zvyšování koncentrace vitamínu ve vejcích (Parolini et al. 2015). Samice racků ukládají do třetího vejce méně vitamínu E, takže jeho dostupností je patrně limitováno pouze mládě vylíhlé ze třetího vejce, a nikoli jeho starší sourozenci (Parolini et al. 2015). Zvýšený příjem vitamínu E vedl u malých mláďat racků k intenzivnějšímu žadonění (Noguera et al. 2010). Autoři to přičítají ochranné funkci vitamínu E před zvýšenou produkcí volných radikálů při žadonění. U sýkory koňadry však přikrmování vitamínem E intenzitu žadonění neovlivnilo

(Maronde and Richner 2015). U znevýhodněných mláďat může být spotřeba vitamínu E vyšší také vinou zvýšeného oxidačního poškození při zrychleném (kompenzačním) růstu (viz výše). V souladu s tím snížilo přikrmování antioxidanty oxidační poškození pouze u mláďat limitovaných ve svém růstu nedostatečnou výživou (Hall et al. 2010, Noguera et al. 2011).

Konečně, efekt vitamínu E na fenotyp může záviset na jeho interakci s dalšími nutrienty. Vitamín E je jen jednou ze součástí antioxidačního systému zahrnujícího bohaté spektrum antioxidantů, vzájemně provázaných řadou interakcí. Antioxidanty lze dělit podle mnoha hledisek. Snaha organismů udržet složitý antioxidační systém v rovnováze s množstvím volných radikálů a tedy vyrovnávat zvýšený či snížený příjem některého z antioxidantů může často vliv přikrmování antioxidanty na měřené znaky zakrývat (Svensson and Wong 2011). Proto je z hlediska vlivu na fenotyp volně žijících ptáků důležitý rozdíl mezi antioxidanty endogenními, které si organismus dokáže vytvářet sám (např. enzymy, kyselina močová) a antioxidanty exogenními, které získává z potravy (např. vitamín E, karotenoidy, polyfenoly; Pérez-Rodríguez 2009). Nedostatek antioxidantů v potravě tak mohou ptáci kompenzovat zvýšenou produkcí endogenních antioxidantů a naopak v reakci na zvýšený přísun antioxidantů z potravy mohou produkci endogenních antioxidantů snížit. Přikrmování antioxidanty se proto nemusí projevit na fenotypu jedince. O vzájemném zastupování endogenních a exogenních antioxidantů u volně žijících ptáků však dosud nemáme dostatek informací, protože pozornost výzkumníků se soustředí téměř výhradně na antioxidanty exogenní (Selman et al. 2006, Monaghan et al. 2009, Pérez-Rodríguez 2009).

Endogenní antioxidanty pak podle jejich rozpustnosti dělíme na hydrofilní (např. vitamín C, polyfenoly) a lipofilní (např. vitamín E, karotenoidy; Catoni et al. 2008). Při vstřebávání antioxidantů ze stejné skupiny převládají interakce negativní (kompetitivní), přímo ve tkáních pak spíše interakce pozitivní, typicky regenerace jednoho antioxidantu druhým (Catoni et al. 2008). Konkrétně vitamín E sdílí podobné absorbční, transportní a metabolické mechanismy s karotenoidy, takže zvýšené dávky jednoho z nich mohou snížit příjem druhého (Furr and Clark 1997, Palozza 1998, Surai 2002, Catoni et al. 2008). Přímo ve tkáních však vitamín E a karotenoidy působí synergicky. Karotenoidy i vitamín E zde mohou účinkem volných radikálů změnit svou formu z antioxidační na prooxidační a samy se tak stát volným radikálem. Protože se však dokáží navzájem před přechodem do

prooxidační formy ochránit a vzájemně regenerovat své radikály zpět na antioxidační formu, poskytují společně větší antioxidační ochranu (Palozza 1998, Surai 2002, Catoni et al. 2008, Svensson and Wong 2011). Podobné synergické interakce byly zjištěny i mezi vitamínem E a C (Surai 2002). V souladu s tím podávání směsi vitamínu C a E zlepšilo růst mláďat volně žijících ptáků (Kim et al. 2013, Marri and Richner 2014b). Stojí přitom za povšimnutí, že u sýkory koňadry byl lepší růst zjištěn právě jen v případě, kdy byl vitamín E podáván spolu s vitamínem C (Marri and Richner 2014b), a nikoli pokud byla mláďata přikrmována pouze vitamínem E (**příspěvek II**, Marri and Richner 2015, Maronde and Richner 2015). Pro pochopení interakcí mezi antioxidanty je nejvhodnější takový design experimentu, kdy někteří jedinci dostávají zkoumané mikronutrienty samostatně a jiní ve směsi. Příkladem může být práce Giraudeau et al. (2013), kde samostatné podávání luteinu zhoršovalo oxidační poškození tkání, kdežto při společném podávání luteinu a vitamínu E bylo toto poškození nižší. Podobně Kopena et al. (2014) zjistili, že přikrmování vitamínem E a karotenidy ovlivňuje barevné ornamenty na pokožce ještěrky Schreiberovy (*Lacerta Schreiberi)* odlišně, když jsou obě látky podávány společně, než když je podávána každá zvlášť.

5 VLIV PROSTŘEDÍ A PŮVODU NA KAROTENOIDOVÉ ORNAMENTY MLÁĎAT

Díky své barevné proměnlivosti a nápadnosti má ptačí opeření vedle ostatních funkcí také důležitou funkci signální. Tvar či zbarvení ornamentů ptačího opeření může vypovídat například o zdravotním stavu a kondici jedince nebo jeho hierarchickém postavení (Hill 2002, Hill et al. 2005, Hill and McGraw 2006a, Blount and McGraw 2008). Tradičně jsou rozlišovány tři hlavní kategorie zbarvení ptačího peří: melaninové, karotenoidové a strukturální (Hill and McGraw 2006b). Melanin, nejrozšířenější ptačí pigment, zbarvuje peří ve škále od černé, šedé a hnědé až po rezavé a okrové odstíny (McGraw 2006a). Největší pozornost je však věnována karotenoidům, druhému nejčastějšímu barvivu, které obvykle způsobuje různé odstíny žluté, oranžové a červené barvy (McGraw 2006b). Plasticita karotenoidového zbarvení peří v reakci na širokou škálu environmentálních faktorů byla potvrzena řadou experimentálních studií: tyto ornamenty např. odrážejí úroveň výživy jedince a obsah karotenoidů v potravě (Olson and Owens 2005, Price 2006, Blount and McGraw 2008), míru parazitace a aktivace imunitní odpovědi (Hill 2002, Blount and McGraw 2008).

Karotenoidy mají velký význam pro fyziologii a zdraví ptáků, zejména v imunitě, antioxidační ochraně a při rozmnožování (Surai 2002, Catoni et al. 2008, Svensson and Wong 2011). Podobně jako u vitamínu E bylo v posledních letech postavení karotenoidů jako antioxidantů *in vivo* u ptáků zpochybňováno (Hartley and Kennedy 2004, Isaksson et al. 2007, Costantini and Møller 2008, Isaksson and Andersson 2008, Cohen and McGraw 2009, Simons et al. 2012). Význam karotenoidů v antioxidačním systému ptáků zůstává zatím nejasný, vzhledem k velké různorodosti ve fyziologii a systémech antioxidační obrany mezi druhy, populacemi i jedinci jsou totiž jakékoli generalizace obtížné. Na základě současných znalostí však antioxidační roli karotenoidů rozhodně nelze obecně vyloučit (Cohen and McGraw 2009, Pérez-Rodríguez 2009, Svensson and Wong 2011, Garratt and Brooks 2012).

Ptáci si nedokáží karotenoidy sami vytvářet a musí je proto získávat z potravy (Surai 2002). Karotenoidové ornamenty mohou být z mnoha důvodů nákladné, např. kvůli omezené dostupnosti, obtížnému získávání a náročnému metabolickému zpracování karotenoidů, kompromisu (*trade-off*) při jejich využití v ornamentech versus využití pro

ostatní fyziologické funkce, kvůli jejich potenciálním toxickým efektům či zvýšenému riziku predace a nákladům sociálních interakcí u výrazněji zbarvených ptáků (Olson and Owens 1998, Hill 2003, Vinkler and Albrecht 2010, Svensson and Wong 2011). Proto mohou karotenoidy sloužit jako tzv. poctivý signál (*honest signal*) kvality jedince, odrážející různé aspekty zdraví a kondice ptáků (Lozano 1994, von Schantz et al. 1999, Hill 2006, Blount and McGraw 2008, Pérez-Rodríguez 2009, Vinkler and Albrecht 2010, Svensson and Wong 2011).

Jedním z mechanismů, který může zajišťovat důvěryhodnost karotenoidových signálů, je právě jejich antioxidační role spolu s faktem, že působením volných radikálů karotenoidy ztrácí barvu (Pérez-Rodríguez 2009). Podle původních představ mělo karotenoidové zbarvení díky této zranitelnosti přímo odrážet dostupnost karotenoidů jako důležitého antioxidantu a tak inzerovat schopnost jedince vypořádat se s oxidačním poškozením (von Schantz et al. 1999). Později bylo navrženo, že karotenoidové ornamenty odráží koncentraci jiných, nebarevných antioxidantů (například vitamínu C a E), které chrání citlivé karotenoidy před oxidací. Tak mohou karotenoidy signalizovat antioxidační stav organismu aniž by samy významně přispívaly k antioxidační obraně (Hartley and Kennedy 2004, Svensson and Wong 2011). Další vysvětlení však s rolí karotenoidů v antioxidační ochraně počítá. Pokud jsou zároveň antioxidanty vzájemně zastupitelné, pak v důsledku udržování homeostatické rovnováhy mezi antioxidanty a oxidanty změna v příjmu nebarevných antioxidantů povede ke změně alokace karotenoidů do zbarvení (viz též předchozí kapitola; Catoni et al. 2008, Monaghan et al. 2009, Svensson and Wong 2011).

Přikrmování nebarevnými antioxidanty způsobilo u ryb (Pike et al. 2007), plazů (Kopena et al. 2014) a ptáků (Bertrand et al. 2006, Pérez et al. 2008, Marri and Richner 2014b) rozvoj výraznějších karotenoidových ornamentů. Přikrmování racků středomořských vitamínem E přitom vedlo jak ke zvětšení červené skvrny na zobáku, tak k vyšší krevní koncentraci karotenoidů odpovědných za tento ornament. To podporuje hypotézu, že karotenoidy skutečně mají roli v antioxidační ochraně a nebarevné antioxidanty je odtud mohou uvolnit pro využití ve zbarvení (Pérez et al. 2008). Zároveň jsou tyto výsledky v souladu s hypotézou, že výrazné karotenoidové ornamenty si mohou dovolit jen jedinci s dobrou dostupností ostatních antioxidantů, které je chrání před škodlivým vlivem nebarevných produktů oxidace karotenoidů (Vinkler and Albrecht 2010).

Je zajímavé, že s jedinou výjimkou (Marri and Richner 2014b) neměla suplementace nebarevných antioxidantů vliv na zbarvení peří ptáků (**příspěvek II**, Karu et al. 2008, Larcombe et al. 2010, Giraudeau et al. 2013). Zdá se tedy, že zbarvení peří je na zvýšený příjem nebarevných antioxidantů méně citlivé než zbarvení neopeřených částí ptačího těla (Giraudeau et al. 2013). Narozdíl od peří mohou být karotenoidy uložené do ornamentů zobáku (Bertrand et al. 2006, Pérez et al. 2008) v případě potřeby znovu využity jinde, takže tyto rozdílné typy ornamentů mohou sdělovat různé informace (Lozano 1994). Karotenoidové zbarvení zobáku čížka žlutého (*Spinus tristis*) se v reakci na stres a imunitní zátěž výrazně měnilo v řádu hodin až dní (Rosenthal et al. 2012) a sezónní změny ve žluté barvě zobáku kosa černého (*Turdus merula*) byly výrazně větší než sezónní proměnlivost ornamentů na opeření (Delhey et al. 2010).

Obvykle jsou vlastnosti karotenoidových ornamentů připisovány pouze karotenoidovým pigmentům, konkrétně jejich molekulární struktuře a koncentraci (viz např. Hill 2002, 2006, McGraw 2006b, Prum 2006). Na výsledné barvě se ale běžně podílí také jemná podkladová struktura pera, která světlo odráží (Shawkey and Hill 2005, Shawkey et al. 2009). Keratinová struktura pera odráží světlo rovnoměrně v celé šířce vlnových délek, čímž vzniká bílé pozadí. Karotenoidy pohlcují světlo specifických vlnových délek v rozmezí přibližně mezi 400 až 500 nm a vytváří tak žlutou až červenou barvu. Protože karotenoidy samy o sobě světlo významně neodráží, bez bílého odrazivého pozadí by barevný ornament nevytvořily. Pro vznik výsledné červené či žluté barvy je tak nutná kombinace odrazu z bílé struktury pera a pohlcení světla o určitých vlnových délkách karotenoidovými pigmenty (Shawkey and Hill 2005, Shawkey et al. 2006). Mechanismus vzájemné interakce pigmentární a strukturální složky a jejich relativní podíl na výsledné barvě pera však dosud zkoumalo jen málo autorů (Shawkey and Hill 2005, Shawkey et al. 2006, Jacot et al. 2010, Galván 2011, D'Alba et al. 2012, Gamero et al. 2015).

Podobně jako karotenoidové ornamenty, také ornamenty založené na struktuře pera mohou být ovlivněny řadou faktorů, např. hormony, parazity, výživou během pelichání či rychlostí pelichání (McGraw et al. 2002, Siefferman and Hill 2005, Hill et al. 2005, Hill 2006, Siitari et al. 2007, McGlothlin et al. 2007, Griggio and Serra 2009; srovnej však s Peters et al. 2011). Z toho vyplývá otázka do jaké míry plasticita karotenoidových signálů závisí na obsahu pigmentů a z jaké části za ni odpovídá struktura pera, a zejména jaké faktory řídí variabilitu těchto dvou komponent. Dosavadní výsledky dokládají, že jak strukturní, tak

pigmentová složka karotenoidového zbarvení jsou značně proměnlivé a že jejich proměnlivost je odlišná. Každá komponenta zbarvení může jinak odrážet věk a pohlaví jedince (Delhey et al. 2010, Evans et al. 2010, 2012, Evans and Sheldon 2013), jinak se mění v prostoru (Evans et al. 2012, Evans and Sheldon 2012, Gamero et al. 2015) a v čase (**příspěvek I**, Delhey et al. 2010, Evans et al. 2012, Evans and Sheldon 2013), jinak odráží kvalitu a množství potravy (Shawkey et al. 2006, Isaksson et al. 2008, Jacot et al. 2010, Romero-Diaz et al. 2013) i další aspekty prostředí (**příspěvek I,** Galván et al. 2009, Jacot et al. 2010, Evans and Sheldon 2012, 2015, Romero-Diaz et al. 2013). Také míra dědivosti strukturní a pigmentové složky je odlišná (Evans and Sheldon 2012, 2015). S výjimkou dvou prací (Shawkey et al. 2006, Delhey et al. 2010) byly však všechny tyto výzkumy omezeny na jediný druh a jediný ornament – žluté peří na hrudi sýkory koňadry. Proměnlivost obou složek se přitom liší nejen v rámci druhu, mezi pohlavími a různými ornamenty, ale především mezi druhy (Delhey et al. 2010). Proto jsou naše dosavadní znalosti velmi limitované.

Vlivy prostředí a původu na pigmentární a strukturální složky zbarvení u mláďat (sýkory koňadry) zkoumaly čtyři z těchto studií. Galván et al. (2009) zjistili, že v teritoriích s lepší potravní nabídkou byla vyšší chromatická složka zbarvení (žlutost) mláďat, ale nikoli strukturální složka. Jacot et al. (2010) doložil, že dostupnost karotenoidů ovlivňuje pigmentární složku zbarvení mláďat, zatímco strukturální složka závisela na manipulaci velikosti snůšky. Podobně v našem vlastním experimentu (**příspěvek IV**) jsme ukázali, že pigmentární složku zbarvení mláďat určují spíše faktory související s kvalitou potravy (stáří matky, sezóna), strukturální složka odráží blíže neurčené faktory prostředí a do určité míry je dána i geneticky. Romero-Diaz et al. (2013) doložili, že přikrmování karotenoidy a stimulace imunity formuje různé složky karotenoidového zbarvení odlišně; pouze přikrmování karotenoidy však ovlivňovalo koncentraci karotenoidů v peří. Obě složky zbarvení mláďat tedy odrážejí jejich původ a prostředí, přičemž chromatická složka patrně více závisí na kvalitě potravy – dostupnosti karotenoidů. Přitom stojí za povšimnutí, že rané vlivy prostředí a kondice mláďat se odlišně odrážejí i v obou komponentách karotenoidového zbarvení dospělých ptáků (Evans and Sheldon 2012, 2015). Sýkory přitom přepelichají juvenilní šat již několik měsíců po opuštění hnízda (Jenni and Winkler 1994) a zbarvení opeření mláďat nekoresponduje se zbarvením dospělců a jde proto o dva na sobě nezávislé znaky (Fitze et al. 2003).

U dospělců i mláďat se tak na výsledném zbarvení karotenoidových ornamentů podílí strukturální a pigmentární mechanismy, které jsou determinovány nezávisle na sobě. Karotenoidový ornament je proto komplexní znak, který může zároveň sdělovat více aspektů historie a kvality jedince (**příspěvek IV**, Grether et al. 2004, Shawkey and Hill 2005, Shawkey et al. 2006, 2009, Jacot et al. 2010, Evans et al. 2010, Evans and Sheldon 2012, 2015, Romero-Diaz et al. 2013). V případě karotenoidového zbarvení však zatím zůstává otázkou, zda a do jaké míry ptáci vnímají obě komponenty dohromady či odděleně. Chromatické a achromatické barvy jsou vnímány různými světločivými buňkami a podle některých studií na ně ptáci mohou reagovat různě (blíže viz Evans et al. 2010). Tyto dvě různé komponenty jedné barvy však mohou být také integrovány do jediného signálu, a tím zvýšit jeho důvěryhodnost (Jacot et al. 2010). Obecně mohou mít vícesložkové signály (*multicomponent signals*) řadu přínosů, například mohou příjemci sdělit více informací o nositeli, nebo mohou zlepšovat přenos, příjem a zpracování signálu či snížit nákladnost signálu (Rowe 1999, Candolin 2003, Hebets and Papaj 2005).

6 ZÁVĚR

V této disertační práci je zabývám vlivem kvality prostředí a rodičovských efektů na fenotyp mláďat volně žijících pěvců. Dokládám zde, že růst, vývoj a zbarvení mláďat jsou ovlivněny jak rodičovskou péčí, tak prostředím, v němž mláďata vyrůstají, a na konkrétních případech studuji mechanismy tohoto ovlivnění.

V našem výzkumu jsme doložili, že role dubů jako indikátorů kvality teritoria sýkor není univerzální (**příspěvek I**). Význam dubů pro výživu mláďat sýkor se může lišit mezi různými druhy dubů a i v rámci jednoho druhu může být velmi proměnlivý v čase i prostoru. Zároveň může být proměnlivý v závislosti na kontextu – význam dubů jako zdrojů potravy pro mláďata může klesat s vyšší dostupností dalších potravou potenciálně bohatých druhů stromů. Obecně je při výzkumu vztahu kvality teritoria a reprodukční úspěšnosti třeba dbát na to, aby zvolený ukazatel kvality teritoria v daném případě odpovídal skutečné dostupnosti potravy. Pro dobré pochopení významu kvality teritoria je také nezbytné odlišit vliv kvality teritoria od vlivu rodičů. Zajímavou oblastí, která dosud nebyla při výzkumu vztahu hnízdění sýkor a počtu dubů příliš zohledněna, je možnost rodičů v různě kvalitních teritoriích volit různé reprodukční strategie a dosahovat tak srovnatelných výsledků hnízdění, aniž by to nutně bylo na úkor jejich vlastního přežití.

Význam kvality potravy na vývoj mláďat dokládám na příkladu vitamínu E (**příspěvek II, III**). Vitamín E může být pro volně žijící ptáky limitující a jeho dostupnost se může odrážet v řadě znaků, ovlivňujících jejich budoucí fitness. Míra limitace se však liší v čase, mezi druhy, populacemi i jedinci. Proto bude přínosné rozšířit spektrum zkoumaných druhů a zahrnout do výzkumu druhy s rozmanitými potravními strategiemi. Nezbytným základem je přitom dobrá znalost zastoupení mikronutrientů v přirozené potravě. Prozatím navíc chybí poznání dlouhodobějších důsledků dostupnosti vitamínu E na zdatnost jedince. Více pozornosti si zaslouží také mateřské vlivy zprostředkované ukládáním nutrientů do vejce. Pro velký potenciál vzájemných interakcí může k lepšímu pochopení mechanismů působení složení potravy na fenotyp jedince významně přispět také současné testování efektu několika mikronutrientů a jejich kombinací, doplněné sledováním fyziologických ukazatelů, zejména v kontextu antioxidační obrany organismu.

Konečně jsem se zabývala také vlivem raného prostředí a péče rodičů na ornamenty mláďat, konkrétně karotenoidové zbarvení peří (**příspěvek II, IV**). Díky velkému potenciálu vzájemných interakcí mohou expresi karotenoidových ornamentů ovlivnit i další, nebarevné mikronutrienty, včetně vitamínu E. V našem experimentu (**příspěvek II**) jsme nepotvrdili vliv vitamínu E na karotenoidové zbarvení ptáků. Jednou z příčin může být odlišný význam a plasticita signálů na opeření a na neopeřených částech ptačího těla. Role karotenoidů i vitamínu E jako antioxidantů *in vivo* je doposud nejasná. Bylo navrženo několik hypotéz, jak mohou vzájemné interakce karotenoidů s dalšími antioxidanty ovlivnit karotenoidové signály. Lepší pochopení role karotenoidů v antioxidační obraně organismu a v signalizaci napomůže současná experimentální manipulace obou těchto složek doplněná sledováním reakce antioxidačního systému. Rozlišení mezi alternativními hypotézami umožní vhodný design experimentů.

Přestože karotenoidové zbarvení peří je tradičně vnímáno jako závislé na množství a koncentraci příslušného karotenoidového pigmentu, podílí se na jeho vlastnostech také strukturální složka. V našem výzkumu (**příspěvek IV**) potvrzujeme, že tyto dvě komponenty stejného ornamentu mohou odrážet různé vlivy prostředí. Tím se otevírá prostor pro lepší poznání determinace obou těchto složek na jedné straně a jejich vlivu na vnímání výsledného signálu ptáky na straně druhé. Multikomponentní charakter karotenoidového zbarvení může usnadňovat signalizaci mezi jedinci, o tom jak ptáci tyto signály vnímají však zatím víme jen málo. Protože se dosavadní výzkum této problematiky soustředil téměř výhradně na sýkoru koňadru, může hodnotné poznatky přinést i rozšíření spektra studovaných druhů.

7 LITERATURA

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PŘÍSPĚVEK I

Matrková J. & Remeš V.: Do oaks matter? Great Tit breeding performance in relation to territory tree composition (rukopis).
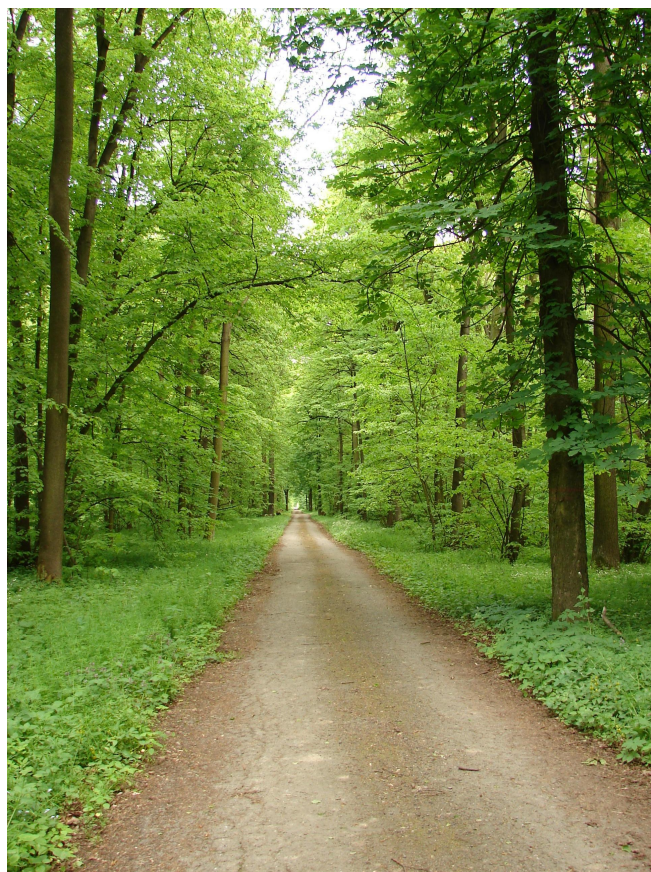
Do oaks matter? Great Tit breeding performance in relation to territory tree composition

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Abstract

Breeding performance of forest songbirds often depends on food availability. As oaks (*Quercus* sp.) usually offer more caterpillars than other tree species, number of oaks is frequently used as an index of territory quality. However, evidence of the positive association between local oak density and breeding performance is equivocal. Moreover, only correlative studies were done so far. Here, we investigated the relationship between the number of oaks in nest proximity and breeding performance of the Great Tit pairs in a floodplain forest. For the first time, we experimentally separated genetic plus pre-natal vs. post-natal effects by cross-fostering of young. We found no relationship between oak density and any of the measured breeding traits, neither before (nest occupancy, timing of egg laying, clutch and egg size), nor after experiment (nestling performance). The density of oaks and other deciduous trees such as limes (*Tilia* sp.), which may provide invertebrate prey in densities similar to oaks, is high in our study site. Thus, our population was probably not limited by oak availability. We suggest that the role of oak as a source of prey may vary in time and may depend on tree species composition of the forest. Evaluating availability of other deciduous trees potentially rich in caterpillars, together with accounting for differences among oak species, will improve use of oaks as an indicator of territory quality.

Keywords

Breeding performance, Great Tit, *Parus Major*, *Quercus robur*, Territory quality.

Introduction

In wild birds, reproductive success and survival of both parents and young is often limited by food availability (Martin 1987). In Great Tit (*Parus major*), nestling performance is determined by availability of the main part of nestling diet – caterpillars (van Balen 1973, Keller and van Noordwijk 1994, Naef-Daenzer and Keller 1999, Naef-Daenzer et al. 2000, Verboven et al. 2001, Wilkin et al. 2009). Distribution of caterpillars is uneven both in

time and space. In temperate habitats, caterpillars are often most abundant only in a short time period known as "caterpillar peak". Reproductive output of Great Tit is considerably improved if peak energy demand of nestlings is synchronized with the caterpillar peak (Naef-Daenzer and Keller 1999, Visser et al. 2006, Reed et al. 2013). Much attention has been focused on the fitness consequences of good synchronization of nestling period with food supply, lately especially in connection with climatic changes (van Noordwijk et al. 1995, Visser et al. 1998, 2003, 2006, Cresswell and McCleery 2003, Both et al. 2009, Matthysen et al. 2011). Effect of spatial distribution of food on breeding output in titmice has been traditionally based on between-habitat comparisons (e. g. van Balen 1973, Tremblay et al. 2003, Stauss et al. 2005, Massa et al. 2011, García-Navas and Sanz 2012). However, as recently suggested by Wilkin et al. (Wilkin et al. 2007, 2009), comparing nestling performance on a territory level may be relevant, because caterpillars prefer certain vegetation type and thus are patchily distributed on finer scale than habitat.

Lately, Great Tit breeding performance in relation to environment quality on the territory level has been studied more thoroughly. Oaks were shown to offer more caterpillars than other trees (Fischbacher et al. 1998, Naef-Daenzer 2000, Matysioková and Remeš 2010; but see Wesołowski and Rowiński 2006). Thus, better breeding performance is predicted in oak-rich territories and availability of oaks in nest proximity is often used an indicator of territory quality in titmice (Naef-Daenzer et al. 2004, Wilkin et al. 2007, 2009, Hinsley et al. 2008, 2009, Sanz et al. 2010, Atiénzar et al. 2010, Parker et al. 2011, Maícas et al. 2011, 2012, 2014, Bell et al. 2014, Mackenzie et al. 2014, Amininasab et al. 2016). Most of these authors reported positive associations between some breeding traits and oak density. However, some results are ambiguous so far: for example, in Wytham, fledging raised close to oak had better condition, but not improved recruitment (Wilkin et al. 2009).

Here, we test if the number of oaks in the nest proximity affects Great Tit breeding performance. The studies conducted so far were correlative. However, non-experimental studies cannot tell apart whether the observed effect is due to environmental factors or due to the heritability of "better genes", in case better parents occupy better territories (Riddington and Gosler 1995). We swapped nestlings among broods to separate genetic from environmental causes of variation. We expected: 1) Great Tits breeding more frequently and starting laying earlier in oak-rich areas, and 2) positive associations between the number of oaks close to nestbox and the timing of egg laying, clutch size, egg size, feeding rate, nestling growth, and the intensity of immune response.

Materials and methods

General fieldwork
This work was conducted on three adjacent nestbox plots (188 nestboxes in total) in a deciduous forest near Grygov (49°31′N, 17°19′E, 205 m a.s.l.) in eastern Czech Republic.

The forest is dominated by lime *Tilia* spp. (59.5% of trees within 20 m from occupied nestboxes) and oak *Quercus* spp. (18.6%) with interspersed ash *Fraxinus excelsior*, hornbeam *Carpinus betulus*, and alder *Alnus glutinosa*. Nestboxes are placed about 1.6 m above ground and besides great tits are inhabited by collared flycatchers *Ficedula albicollis*, blue tits *Cyanistes caeruleus*, and nuthatches *Sitta europea*. Their design is described in (Lambrechts et al. 2010).

Fieldwork was carried out in 2006 and 2007 from early April until mid-June. We checked nestboxes daily to record laying of the first egg and final clutch size. When there were between six and seven eggs laid, we collected the 4th egg for other analyses (Remeš 2011, Remeš et al. 2011). After the clutch completion, we weighted the whole clutch (excluding the collected egg) to obtain average egg weight.

We checked nestboxes daily around the expected day of hatching to record hatching day. The day when the first young hatched is day 0. We captured parents in the nestbox during feeding of nestlings (median age of young for females = 7 days, for males = 9 days, range in both cases 6–11 days). Within the subset of nest with data on feeding (n = 94), most females (91) and males (74) were caught. We determined the age of birds based on their plumage as one year old or older (Svensson 2012). To quantify rate of food delivery, we videotaped feeding parents for 90 minutes on day 8 (mean = 8.23 d, range = 8–10 d, n = 93). The method is described in more details in the previous study (Remeš and Matysioková 2013). The number of feeding visits, which was our estimate of parental feeding rates, correlated strongly with prey biomass brought to the nest (Remeš and Matysioková 2013). To control for the effect of weather, we took average ambient temperature and sum of daily rainfall during feeding from a local meteorological station (7 km from the study site).

On day 13, we measured the thickness of left wing web and injected phytohaemagglutinin (PHA). Twenty-four hours later, we re-measured the swollen wing web and calculated the strength of reaction to PHA as the difference in thickness between the two measurements. For details, see (Remeš and Matysioková 2013). On day 14, we measured tarsus length of each young with a digital caliper (nearest 0.01 mm), weighed it on an electronic balance (nearest 0.1 g), and measured its wing length with a ruler (nearest 0.5 mm).

Cross-fostering and brood size manipulation
To separate genetic plus pre-hatching vs. post-hatching effects on offspring performance, we performed a cross-fostering experiment. Two days after the first young in the clutch hatched, we swapped whole clutches between pairs of nests – dyads. The procedure is in detail described in the previous study (Remeš and Matysioková 2013). Briefly, we assigned nests to dyads based on their same hatching day. We created both control nests with unchanged brood size (by exchanging whole broods between nests with the same brood size) and nests with experimentally enlarged or reduced brood size (by exchanging

whole broods between nests differing in brood size; difference of 0–4 nestlings, mean = 1.5, SD = 1.25). There was no difference in brood size in 28 nests, whereas broods differed by at least one nestling in 70 nests (by 1 nestling in 28 cases, 2 nestlings in 22 cases, 3 nestlings in 12 cases, and 4 nestlings in 8 cases). Cross-fostering and brood size treatments were allocated randomly with respect to number of oaks in the nest proximity, as there was no relationship between brood size manipulation (brood size difference from −4 through 0 to +4 chicks, see above) and number of oaks in territory (linear regression; $F_{1,66} = 0.2$, P = 0.625).

Oak number

Great Tits were shown to deliver major proportion of food from near to the nest. The distance where the great majority of their foraging takes place was shown to vary between 25 to over 53 m from the nest (Smith and Sweatman 1974, Naef-Daenzer and Keller 1999, Naef-Daenzer 2000, Stauss et al. 2005, Tremblay et al. 2005). Moreover, within the same region titmice parents in poor habitat collect food significantly further from nest than parents in good habitats (Great Tit: Tremblay et al. 2005); Blue Tit: Stauss et al. 2005). Therefore, we counted number of oaks within 20, 45 and 75 m from each nest. We counted all oaks with a diameter above 10 cm at breast height. Number of oaks within 20 m of nestbox was counted directly in the field, using a laser rangefinder. To obtain the number of oaks within 45 and 75 m, all nest boxes and oak trees in the study area were digitally mapped. We used AshTech ProMark2 geographical position system device and analysed data using ArcGis version 9.3 software.

We compared oak number with another possible indicator of territory quality in titmice, nestbox occupancy (Mänd et al. 2005, Amininasab et al. 2016). We defined nestbox occupancy as the number of years in which the particular nestbox was occupied by Great Tits between 2005 and 2011; it ranged from one to seven (mean = 2.9, SD = 1.31).

Statistical analyses

We used general linear models to identify the effects of oak number on nestbox occupancy, timing of breeding, clutch size, average egg weight, feeding rate and nestling performance. All dependent variables were continuous variables. As a response variable representing nestling performance, we used average values for all nestlings in the nest of the following offspring traits measured at 14d of age: body mass (g), tarsus length (mm), wing length (mm) and wing web swelling as an index of immune response (mm).

In all models except nestbox occupancy, *Year* (2006 and 2007) was entered as a fixed factor. We also controlled these models for the age of males and females (1y old vs. older). Hatching/laying date was entered to control for seasonal changes in dependent variables. As timing of breeding differed between years, we centered it within a given year. Model explaining variation in feeding rate was further controlled for feeding time, temperature, rainfall and brood size at day 6, because feeding rate may depend on daytime, weather and

the number of nestlings (Naef-Daenzer and Keller 1999, García-Navas and Sanz 2012, Pagani-Núñez and Senar 2013). In models testing the effect of oak number on nestling performance, we included brood size manipulation as a fixed factor. Models testing the effect of oak number on nestling growth were also controlled for feeding rate and the model testing the intensity of immune response was also controlled for nestling condition (residuals from the regression of body mass in g on tarsus length in mm at day 14). To check if the effect of oak availability varied among years and depended on season, respectively, we fitted the interactions between the number of oaks and year and between the number of oaks and hatching/laying date. However, none of these interactions were statistically significant and were thus excluded from the models. Sample size of different models varies from 59 to 110 nests; most of this difference is due to limited data on feeding rate and the age of parents.

We conducted all analyses in SAS using GLM function. We checked the models to conform to the requirements of homoscedasticity, normal distribution and linearity of residuals. All tests were two-tailed.

Results

Average number of oaks within 20-m radius from the nestbox was 5.5 (SD = 4.62, n = 125, range 0 to 18), within 45-m radius it was 28.3 (SD = 19.37, n = 120, range 3 to 84), and within 75-m radius it was 69.1 (SD = 34.66, n = 120, range 13 to 150). Numbers of oaks within the three circles were strongly correlated (fig. 1; 20 to 45 m: $r = 0.662$, n = 119, $p < 0.001$; 20 to 75 m: $r = 0.563$, n = 119, $p < 0.001$; 45 to 75 m: $r = 0.871$, n = 120, $p < 0.001$). Consequently, test results for all three radiuses were very similar. Here we present results for the radius of 20 m (fig. 2); results for radiuses of 45 m and 75 m are available in supplementary tables.

1. Timing of egg laying and nestbox occupancy
We found no relationship between oak availability and the number of years when Great Tits nested in the particular nest box out of 7 years. Date of the laying of the first egg was not related to the number of oaks within 20 m from the nest. Older females laid eggs earlier than 1-year old females.

2. Clutch size and egg mass
Neither clutch size nor average egg mass correlated with oak availability. Clutch size and average egg mass also did not differ between years and between young and old females. While clutch size decreased with laying date, no effect of laying date on average egg mass was found.

3. Feeding rate

Feeding rate was independent of the number of oaks around the nestbox. We found no relationship between age of the rearing female and parental feeding rate. On the other hand, nestlings reared by 1-year old males were fed more often than nestling reared by older males. Feeding rate increased with brood size and differed between years. Other measured variables (hatching date, time of feeding, temperature, rainfall) had no statistically significant effect on feeding rate.

4. Nestling performance

Nestling growth and the intensity of immune response to a novel antigen (phytohaemagglutinin) was not related to oak availability. The only exception was a positive relationship between the intensity of immune response and the number of oaks within 45 m from the nestbox (n = 61, estimate = 0.23, SE = 0.096, p = 0.022; Table S2d in supplementary material). However, this relationship was driven by eight territories with the highest numbers of oaks and vanished when these nestboxes were excluded from the analysis (n = 53, estimate = 0.18, SE = 0.762, p = 0.199; full model results not presented). The intensity of immune response differed between years, increased with hatching date and tended to increase with nestling condition. We found no effect of year, age of parents, and brood size manipulation on the intensity of immune response.

All three morphological traits, namely tarsus and wing length and body mass at day 14, differed between years. Nestlings reared by older males and females were heavier and grew longer wings than nestlings reared by 1-year old birds; however, no such effect was found in tarsus length. Similarly, our brood size manipulation affected wing length and body mass of nestlings, but not growth of the tarsus. Nestlings reared in enlarged broods grew shorter wings and reached lower body mass than nestlings reared in reduced brood. Body mass and tarsus and wing length were not related to season or feeding rate.

Discussion

Here, we tested the effect of oak density near the nest on breeding performance of Great Tit. Nestbox occupancy, timing of egg laying, clutch size and average egg mass were not related to number of oaks within 20, 45 and 75 m from nestbox. Moreover, despite we experimentally separated environmental and genetic effects, oak availability had no effect on nestling growth and intensity of immune response to a novel antigen. Thus, in our case, oak availability did not affect any of measured parameter of Great tit breeding performance.

Our results were against our expectations, because so far most studies found a positive association between oak availability and breeding parameters in titmice. Territories with more oaks are occupied more often (Wilkin et al. 2009, Maícas et al. 2011, Amininasab et

al. 2016). Great and Blue Tits in oak-rich territories start breeding earlier and lay larger clutches than those in oak-poor territories (Naef-Daenzer et al. 2004, Wilkin et al. 2007, Sanz et al. 2010, Maícas et al. 2011, 2014, Parker et al. 2011, Bell et al. 2014, Amininasab et al. 2016; but see Atiénzar et al. 2010, Maícas et al. 2011, 2012). Nestlings in nestboxes with higher availability of oaks are provisioned with higher quality prey (Wilkin et al. 2009) and reach better condition and/or higher body mass (Wilkin et al. 2009, Bell et al. 2014, Mackenzie et al. 2014; but see Atiénzar et al. 2010, Maícas et al. 2011). Surprisingly, despite these positive associations between the number of oaks and breeding traits of titmice, no study found an association between small-scale oak availability and overall breeding success, measured as the proportion of fledglings produced or recruitment (Wilkin et al. 2009, Sanz et al. 2010, Atiénzar et al. 2010, Maícas et al. 2011, 2012, 2014). Below, we discuss possible explanations of ambiguous relationship between oak availability and titmice breeding parameters.

First, oak species differ in many aspects, including phenology, leaf chemical composition, and nutritional value. Consequently, caterpillars grow better when fed on some species than on others (Foss and Rieske 2003, Milanović et al. 2014) and these differences may carry over to breeding performance of tits. Indeed, a positive relationship of clutch size, nestling condition and/or body mass to oak availability was demonstrated in several species of deciduous oaks (Amininasab et al., 2016; Bell et al., 2014; Mackenzie et al., 2014; Maícas et al., 2014; Naef-Daenzer et al., 2004; Parker et al., 2011; Sanz et al., 2010; Wilkin et al., 2009). On the contrary, these relationships were absent in evergreen holm oak (*Q. ilex*) in Spain (Atiénzar et al. 2010, Maícas et al. 2011, 2012; but see Maícas et al. 2014). The reason might be that evergreen oaks are likely to host impoverished phytophagous fauna (Mackenzie et al. 2014). Two species of oaks occur in our study site: native English Oak (*Q. robur*) and Red Oak (*Q. rubra*), introduced from North America, which might provide less food for Great Tits. First, introduced tree species usually host less insect prey (Mackenzie et al. 2014). Second, when compared with other oak species, Lepidoptera larvae avoid Red Oak leaves and grow slowly when fed on Red Oak (Foss and Rieske 2003), breeding success of several songbird species is negatively associated with Red Oak abundance, and Red Oaks are avoided as a bird nesting site (Newell and Rodewald 2011). We treated all oak species together; low-quality Red Oaks may thus have concealed positive effects of high-quality English oaks. However, when we repeated our analyses with English Oaks only (n = 59 to 83 nests), our results did not change. Moreover, English Oaks are ten times more abundant than Red Oaks in 20-m diameter circles around Great Tit nests in our study site (English Oak: 83.1%, Red Oak: 9.6%, species not determined: 7.2%, n = 954 trees). Therefore we believe that merging both oak species did not compromise our results.

Second, other trees than oaks may provide invertebrate prey in densities similar to or even higher than oaks. In Białowieża forest in Poland, the load of caterpillars was highest on hornbeams, followed by maples (*Acer*), limes, and oaks (Wesołowski and Rowiński 2006).

In our study site, oaks usually provide more caterpillars than other tree species. However, relative rank of tree species as a source of caterpillars changes in time, so that in some years and some parts of the breeding season limes and hornbeams offer caterpillars in densities similar to or even higher than oaks (Matysioková and Remeš, 2010; J. Matrková, unpublished data). At the same time, high variability within tree species in the amount of caterpillars was observed both by us and in Białowieża (Wesołowski and Rowiński 2006). If variability in food availability among individual trees causes substantial overlap among tree species, the effect of the variability among individual trees may conceal the average effect of tree species. In such case, our two-year study might have not been sufficiently long to find a positive effect of oaks. Accordingly, within the same study site, positive effects of oaks are more apparent when analysing long-term data than in short-term datasets (Wilkin et al. 2009, Hinks et al. 2015).

Compared to most of previous works reporting positive effects of oak density on titmice breeding traits (Naef-Daenzer et al. 2004, Wilkin et al. 2007, 2009, Maícas et al. 2011, 2012, 2014, Parker et al. 2011, Mackenzie et al. 2014, Amininasab et al. 2016), our study site offers more deciduous oaks and limes and hornbeams are also abundant in some territories. Both lime and hornbeam may sometimes provide invertebrate prey in densities comparable to oaks (see above). Titmice parents feeding their young were shown to work harder only if density of tree species with high food supply around their nest decreased below a threshold value of approximately 30 % (oaks, Hinsley et al., 2009, 2008). Thus, availability of oaks may not be limiting in our study site, as they might be functionally replaced by other tree species with high food supply.

Third, using cross-fostering early after hatching, we separated pre-hatching (genetic and early maternal) and post-hatching (mostly environmental) effects. Parents of higher quality may occupy territories with more oaks (but see Bell et all, 2014, who did not find any relationship btw. male and female condition and no. of oaks). In that case, better growth of nestlings in territories with more oaks (Wilkin et al. 2009, Bell et al. 2014, Mackenzie et al. 2014) may be partly attributed to better genes. The absence of association between oak availability and nestling performance in our study may then be explained by our better experimental design. However, we consider this explanation unlikely, as we found no association between oak availability and any of several breeding traits unaffected by our cross-fostering experiment (nestbox occupancy, timing of egg laying, clutch and egg size, and feeding rate).

Conclusion

Contrary to prevailing experience, local oak availability did not affect breeding traits of Great Tit in our study site. We suggest that the role of oaks as an indicator of territory quality may vary in time and depend on species composition of the forest. Other deciduous trees (lime, hornbeam) may provide invertebrate prey in densities similar to oaks. In our study site, both the number of oaks and limes is high and so oak availability may not limit

Great Tit breeding; however, our results are limited to two years. Long-term studies evaluating availability of both oaks and other deciduous trees may help better understand the role of vegetation in local territory on breeding success in titmice. As the value of oaks as a source of food may significantly vary among oak species, considering different oak species may be rewarding in future studies.

Acknowledgement

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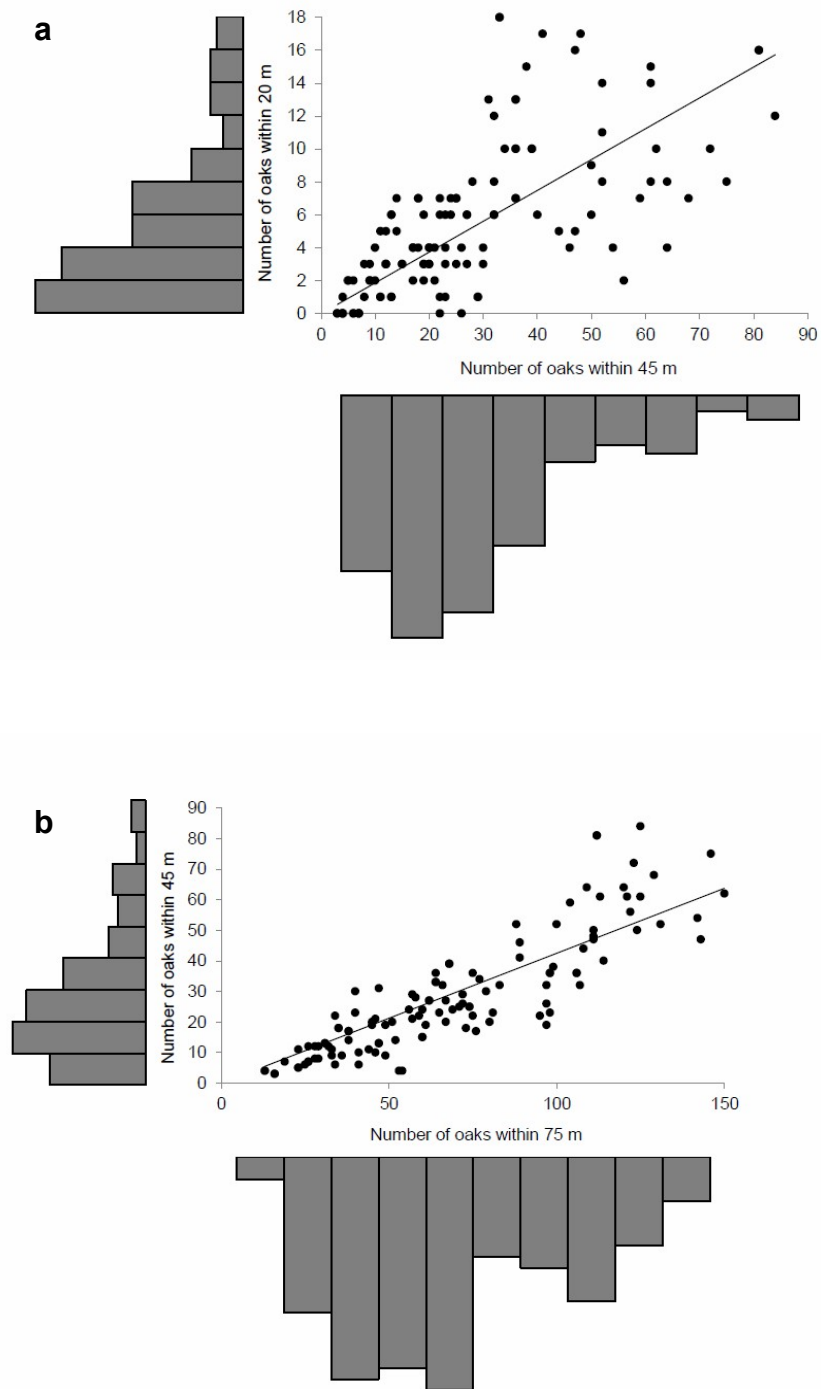
Fig. 1 Relationship between number of oaks within 20 m and 45 m (a) and 45 m and 75 m (b) from nestbox, respectively. Histograms depicture the distribution.
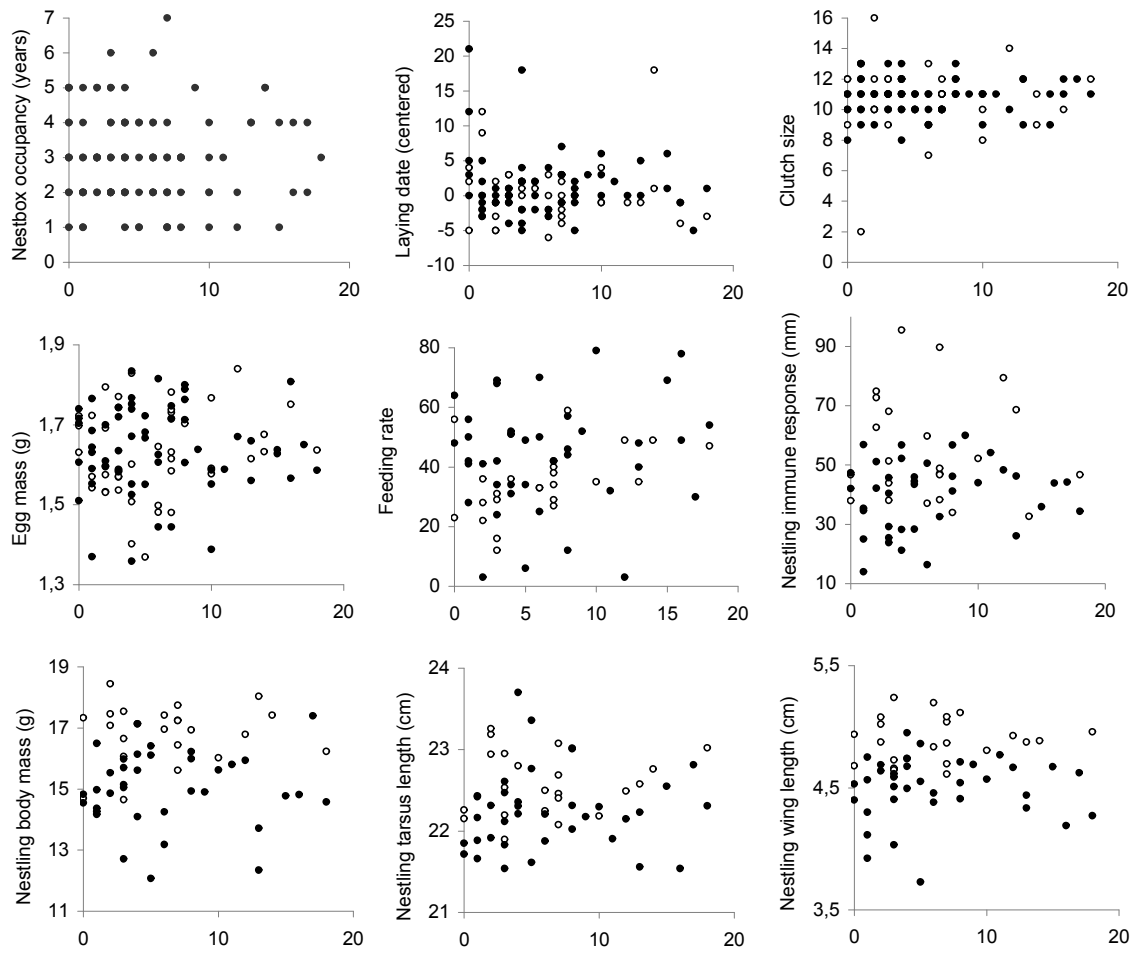
Fig. 2 Relationship between number of oaks within 20 m from nest box (X-axis) and breeding of Great Tit.
White and black circles represent results from years 2006 and 2007, respectively; grey circles represent results from years 2005 - 2011.

Table S1a The effect of the number of oaks within 20 m of nest on nestbox occupancy and the timing of egg laying in Great Tit

Predictor	Nestbox occupancy (n = 110)		Laying date (n = 104)	
	Estimate (SE)	t (P)	Estimate (SE)	t (P)
Intercept	2.95 (0.20)		0.16 (0.83)	
Oak no. within 20 m	>-0.01 (0.03)	-0.1 (0.95)	-0.06 (0.09)	-0.7 (0.51)
Age of female**			2.29 (0.87)	2.6 (**0.01**)

Table S1b The effect of the number of oaks within 20 m of nest on Great Tit clutch size and average egg mass

Predictor	Clutch size (n = 104)		Egg mass (n = 102)	
	Estimate (SE)	t (P)	Estimate (SE)	t (P)
Intercept	11.17 (0.31)		1.63 (0.02)	
Year*	-0.14 (0.29)	-0.5 (0.62)	-0.01 (0.02)	-0.4 (0.72)
Oak no. within 20 m	<0.01 (0.03)	<0.1 (0.99)	<0.01 (<0.01)	0.6 (0.57)
Laying date	-0.15 (0.03)	-4.8 (**<0.01**)	>-0.01 (<0.01)	-0.6 (0.54)
Age of female**	-0.44 (0.29)	-1.5 (0.14)	0.01 (0.02)	0.6 (0.58)

Table S1c The effect of the number of oaks within 20 m of nest on Great Tit feeding rate

Predictor	Feeding rate (n = 65)	
	Estimate (SE)	t (P)
Intercept	-6.28 (24.81)	
Year*	-9.37 (4.68)	-2.0 (**0.05**)
Oak no. within 20 m	0.61 (0.39)	1.6 (0.13)
Hatching date	-0.02 (1.12)	>-0.1 (0.98)
Age of female**	<0.01 (3.91)	<0.1 (<0.99)
Age of male**	12.54 (3.98)	3.2 (**<0.01**)
Time of feeding	0.09 (1.27)	0.1 (0.95)
Temperature	-0.09 (0.67)	-0.1 (0.89)
Rainfall	-0.02 (0.37)	>-0.1 (0.96)
Brood size at day 6	4.56 (1.26)	3.6 (**<0.01**)

Table S1d The effect of the number of oaks within 20 m of nest on the growth and the intensity of immune response of Great Tit nestlings

Predictor	Body mass (n = 59)		Tarsus (n = 59)		Wing (n = 59)		Immune response (n = 61)	
	Estimate (SE)	t (P)	Estimate (SE)	t (P)	Estimate (SE)	t (P)	Estimate (SE)	t (P)
Intercept	15.44 (0.45)		22.18 (0.21)		4.55 (0.09)		40.50 (4.35)	
Year*	1.80 (0.28)	6.5 (**<0.01**)	0.38 (0.13)	3.0 (**<0.01**)	0.39 (0.06)	6.9 (**<0.01**)	14.57 (3.79)	3.9 (**<0.01**)
Oak no. within 20 m	<0.01 (0.03)	0.1 (0.89)	<0.01 (0.01)	0.1 (0.96)	>-0.01 (0.01)	-0.3 (0.81)	0.07 (0.39)	0.2 (0.86)
Hatching date	-0.09 (0.06)	-1.5 (0.15)	-0.01 (0.03)	-0.5 (0.63)	<0.01 (0.01)	0.1 (0.95)	1.91 (0.91)	2.1 (**0.04**)
Age of female**	-0.54 (0.27)	-2.0 (**0.05**)	-0.09 (0.12)	-0.7 (0.46)	-0.15 (0.05)	-2.8 (**0.01**)	0.82 (3.86)	0.2 (0.83)
Age of male**	-0.60 (0.30)	-2.0 (**0.05**)	-0.20 (0.14)	-1.5 (0.15)	-0.18 (0.06)	-2.9 (**<0.01**)	-3.24 (4.03)	-0.8 (0.43)
Brood size manipulation	-0.28 (0.07)	-4.0 (**<0.01**)	-0.04 (0.03)	-1.2 (0.24)	-0.05 (0.09)	-3.3 (**<0.01**)	0.43 (1.15)	0.4 (0.71)
Feeding rate	<0.01(0.01)	-0.1 (0.94)	<0.01 (<0.01)	0.9 (0.37)	<0.01 (<0.01)	1.2 (0.23)		
Condition							4.58 (2.50)	1.8 (0.07)

* Estimate for year 2006 (relative to 2007)
** Estimate for 1y old (relative to older) females/males

General linear models are shown. Type 3 tests are presented, numerator DF = 1. Body mass, wing length, and tarsus length were measured at brood age of 14 days, the intensity of immune response from 13 to 14 days (hatching day = day 0). Hatching date and date of the first egg (laying date) were centred within a given year.

Table S2a The effect of the number of oaks within 45 m of nest on nestbox occupancy and the timing of egg laying in Great Tit

Predictor	Nestbox occupancy (n = 104)		Laying date (n = 103)	
	Estimate (SE)	t (P)	Estimate (SE)	t (P)
Intercept	2.96 (0.23)		-0.04 (0.84)	
Oak no. within 45 m	>-0.01 (0.01)	-0.4 (0.68)	-0.01 (0.02)	-0.3 (0.77)
Age of female**			2.20 (0.88)	2.5 (**0.01**)

Table S2b The effect of the number of oaks within 45 m of nest on Great Tit clutch size and average egg mass

Predictor	Clutch size (n = 103)		Egg mass (n = 102)	
	Estimate (SE)	t (P)	Estimate (SE)	t (P)
Intercept	11.00 (0.26)		1.63 (0.02)	
Year*	0.06 (0.25)	0.2 (0.81)	-0.01 (0.02)	-0.4 (0.68)
Oak no. within 45 m	<0.01 (0.01)	0.5 (0.64)	<0.01 (<0.01)	0.7 (0.50)
Laying date	-0.12 (0.03)	-4.5 (**<0.01**)	>-0.01 (<0.01)	-0.6 (0.53)
Age of female**	-0.35 (0.25)	-1.4 (0.17)	0.01 (0.02)	0.5 (0.66)

Table S2c The effect of the number of oaks within 45 m of nest on Great Tit feeding rate

Predictor	Feeding rate (n = 65)	
	Estimate (SE)	t (P)
Intercept	-5.71 (25.61)	
Year*	-9.66 (4.77)	-2.0 (**0.05**)
Oak no. within 45 m	0.07 (0.10)	0.7 (0.46)
Hatching date	-0.20 (1.13)	-0.2 (0.86)
Age of female**	-0.17 (4.02)	>-0.1 (0.97)
Age of male**	11.48 (3.99)	2.9 (**0.01**)
Time of feeding	1.16 (1.32)	0.1 (0.91)
Temperature	-0.15 (0.69)	-0.2 (0.83)
Rainfall	>-0.01 (0.37)	>-0.1 (<0.99)
Brood size at day 6	4.74 (1.28)	3.7 (**<0.01**)

Table S2d The effect of the number of oaks within 45 m of nest on the growth and the intensity of immune response of Great Tit nestlings

Predictor	Body mass (n = 59)		Tarsus (n = 59)		Wing (n = 59)		Immune response (n = 61)	
	Estimate (SE)	t (P)	Estimate (SE)	t (P)	Estimate (SE)	t (P)	Estimate (SE)	t (P)
Intercept	15.36 (0.45)		22.07 (0.20)		4.52 (0.09)		34.82 (4.11)	
Year*	1.79 (0.28)	6.5 (**<0.01**)	0.37 (0.13)	3.0 (**<0.01**)	0.39 (0.06)	6.9 (**<0.01**)	14.21 (3.61)	3.9 (**<0.01**)
Oak no. within 45 m	0.01 (0.01)	0.7 (0.47)	0.01 (<0.01)	1.7 (**0.09**)	<0.01 (<0.01)	0.6 (0.55)	0.23 (0.10)	2.4 (**0.02**)
Hatching date	-0.10 (0.06)	-1.5 (0.13)	-0.02 (0.03)	-0.7 (0.52)	<0.01 (0.01)	<0.1 (0.98)	1.76 (0.87)	2.0 (**0.05**)
Age of female**	-0.57 (0.27)	-2.1 (**0.04**)	-0.13 (0.12)	-1.0 (0.30)	-0.16 (0.06)	-2.9 (**0.01**)	-0.53 (3.72)	-0.1 (0.89)
Age of male**	-0.58 (0.28)	-2.0 (**0.05**)	-0.17 (0.13)	-1.3 (0.19)	-0.17 (0.06)	-2.9 (**0.01**)	-2.23 (3.75)	-0.6 (0.55)
Brood size manipulation	-0.28 (0.07)	-4.0 (**<0.01**)	-0.04 (0.03)	-1.2 (0.23)	-0.05 (0.01)	-3.3 (**<0.01**)	0.46 (1.10)	0.4 (0.68)
Feeding rate	>-0.01 (0.01)	>-0.1 (0.99)	<0.1 (<0.01)	0.7 (0.50)	<0.01 (<0.01)	1.1 (0.28)		
Condition							4.96 (2.38)	2.1 (**0.04**)

* Estimate for year 2006 (relative to 2007)
** Estimate for 1y old (relative to older) females/males

General linear models are shown. Type 3 tests are presented, numerator DF = 1. Body mass, wing length, and tarsus length were measured at brood age of 14 days, the intensity of immune response from 13 to 14 days (hatching day = day 0). Hatching date and date of the first egg (laying date) were centred within a given year.

Table S3a The effect of the number of oaks within 75 m of nest on nestbox occupancy and the timing of egg laying in Great Tit

Predictor	Nestbox occupancy (n = 104)		Laying date (n = 103)	
	Estimate (SE)	t (P)	Estimate (SE)	t (P)
Intercept	3.32 (0.29)		-0.18 (1.03)	
Oak no. within 75 m	-0.01 (<0.01)	-1.7 (0.09)	>-0.01 (0.01)	>-0.1 (0.97)
Age of female**			2.17 (0.87)	2.5 (**0.02**)

Table S3b The effect of the number of oaks within75 m of nest on Great Tit clutch size and average egg mass

Predictor	Clutch size (n = 103)		Egg mass (n = 102)	
	Estimate (SE)	t (P)	Estimate (SE)	t (P)
Intercept	10.98 (0.31)		1.63 (0.03)	
Year*	0.06 (0.25)	0.2 (0.82)	-0.01 (0.02)	-0.4 (0.69)
Oak no. within75 m	<0.01 (<0.01)	0.4 (0.67)	<0.01 (<0.01)	0.3 (0.81)
Laying date	-0.13 (0.03)	-4.5 (**<0.01**)	>-0.01 (<0.01)	-0.7 (0.52)
Age of female**	-0.35 (0.25)	-1.4 (0.17)	0.01 (0.02)	0.5 (0.60)

Table S3c The effect of the number of oaks within 75 m of nest on Great Tit feeding rate

Predictor	Feeding rate (n = 65)	
	Estimate (SE)	t (P)
Intercept	-3.45 (26.24)	
Year*	-9.98 (4.79)	-2.1 (**0.04**)
Oak no. within 75 m	0.01 (0.06)	0.2 (0.82)
Hatching date	-0.18 (1.14)	-0.2 (0.88)
Age of female**	0.25 (3.99)	0.1 (0.95)
Age of male**	11.46 (4.00)	2.9 (**0.01**)
Time of feeding	0.03 (1.33)	<0.1 (0.98)
Temperature	-0.19 (0.69)	-0.3 (0.78)
Rainfall	-0.02 (0.37)	-0.1 (0.95)
Brood size at day 6	4.87 (1.28)	3.8 (**<0.01**)

Table S3d The effect of the number of oaks within75 m of nest on the growth and the intensity of immune response of Great Tit nestlings

Predictor	Body mass (n = 59)		Tarsus (n = 59)		Wing (n = 59)		Immune response (n = 61)	
	Estimate (SE)	t (P)	Estimate (SE)	t (P)	Estimate (SE)	t (P)	Estimate (SE)	t (P)
Intercept	15.49 (0.48)		22.08 (0.22)		4.54 (0.10)		35.86 (4.93)	
Year*	1.81 (0.28)	6.5 (**<0.01**)	0.38 (0.13)	2.9 (**<0.01**)	0.39 (0.06)	6.9 (**<0.01**)	14.28 (3.73)	3.8 (**<0.01**)
Oak no. within 75 m	>-0.01 (<0.01)	-0.2 (0.88)	<0.01 (<0.01)	1.0 (0.31)	<0.01 (<0.01)	<0.1 (0.97)	0.07 (0.05)	1.4 (0.17)
Hatching date	-0.09 (0.06)	-1.5 (0.15)	-0.02 (0.03)	-0.6 (0.57)	<0.01 (0.01)	0.1 (0.94)	1.83 (0.89)	2.0 (**0.05**)
Age of female**	-0.54 (0.27)	-2.0 (**0.05**)	-0.10 (0.12)	-0.8 (0.42)	-0.15 (0.05)	-2.8 (**0.01**)	0.53 (3.80)	0.1 (0.89)
Age of male**	-0.61 (0.28)	-2.2 (**0.04**)	-0.19 (0.13)	-1.5 (0.15)	-0.17 (0.06)	-3.0 (**<0.01**)	-2.86 (3.86)	-0.7 (0.46)
Brood size manipulation	-0.28 (0.07)	-4.0 (**<0.01**)	-0.04 (0.03)	-1.2 (0.24)	-0.05 (0.01)	-3.3 (**<0.01**)	0.51 (1.13)	0.5 (0.65)
Feeding rate	<0.01 (0.01)	0.1 (0.90)	<0.1 (<0.01)	0.8 (0.43)	<0.01 (<0.01)	1.2 (0.24)		
Condition							5.03 (2.48)	2.0 (**0.05**)

* Estimate for year 2006 (relative to 2007)
** Estimate for 1y old (relative to older) females/males

General linear models are shown. Type 3 tests are presented, numerator DF = 1. Body mass, wing length, and tarsus length were measured at brood age of 14 days, the intensity of immune response from 13 to 14 days (hatching day = day 0). Hatching date and date of the first egg (laying date) were centred within a given year.

PŘÍSPĚVEK II

Matrková J., Mullen W. & Remeš V.: Do Great Tits need vitamíns? Nestling performance and colouration in response to vitamín E supplementation (rukopis).

Do Great Tits need vitamins? Effect of vitamin E on nestling performance and coloration.

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Abstract

Parents can strongly affect performance and phenotype, and thus reproductive prospects, of their young. For instance, growth and survival of altricial nestlings critically depend on both the quality and quantity of food provisioned by their parents. Vitamin E is an important micronutrient with numerous functions in bird physiology. Moreover, it may interact with other micronutrients, for example by replacing carotenoids in antioxidant protection of macromolecules or directly protecting carotenoids from oxidation. Thus availability of vitamin E may modify carotenoid availability for signaling and affect carotenoid-based coloration. Yet, despite its potentially strong physiological effects, experimental studies of vitamin E function in wild-living animals are rare. Here, we examined the role of vitamin E in the Great Tit (*Parus major* Linnaeus, 1758) reproduction. We supplemented nestlings with vitamin E and examined their growth, survival, intensity of immune response to a novel antigen (phytohaemagglutinin), characteristics of carotenoid-based plumage coloration, and plasma vitamin E and carotenoid levels. We found no detectable effects of increased vitamin E intake on nestling growth, survival, immune responsiveness, coloration, and plasma lutein and γ-tocopherol concentration. In initially small nestlings, vitamin E supplementation increased plasma   -tocopherol concentration compared to control nestlings. However, this effect was significant only for the lowest dose of vitamin E. We conclude that 1. vitamin E is probably not limiting the performance of Great Tit nestlings in our wild population; 2. the non-linear relationship between vitamin E intake and circulating   -tocopherol suggests an interaction with other antioxidants and/or dose-depended changes in tocopherol distribution among tissues.

Introduction

In animals with parental care, offspring phenotype and performance often strongly depend on the environment provided by parents. One of the most important aspects of this environment is postnatal nutrition (Bernardo 1996; Price 1998). Both quality and quantity of food provisioned by parents is crucial in determining nestling growth and survival (Perrins 1976; Martin 1987; Massias and Becker 1990; Johnston 1993; Saino et al. 1997; Wright et al. 1998; Romano et al. 2006). At the same time, parental effort incurs costs to condition and survival of parents (Knowles et al. 2009; Santos and Nakagawa 2012). Thus, parental strategies in terms of the amount and composition of food supplied to the offspring must be balanced against parental well-being to yield maximum fitness.

Besides energetic content and macronutrients, micronutrient composition is an important aspect of the food provided by parents. Vitamin E is an important micronutrient with various physiological functions. It is considered the most important antioxidant in cell membranes, where it prevents oxidative damage by scavenging free radicals and thus breaks the chain propagation of lipid peroxidation (Surai 2002). This is particularly important during the period of rapid growth, when consumption of oxygen is high and production of cell-damaging free radicals is therefore also high (Rollo 2002; Alonso-Álvarez et al. 2007; Kim et al. 2011). Physiological effects of vitamin E further include its role in regulation of enzymes and gene expression, signaling and membrane processes (Brigelius-Flohé 2009). Sufficient intake of vitamin E has been repeatedly shown to condition normal avian development, functional immune system, and reproduction (Surai 2002; Catoni et al. 2008).

Vitamin E is just one part of a complicated antioxidant system, which includes endogenous antioxidants such as enzymes or uric acid, and dietary antioxidants including carotenoids and vitamin E (Pérez-Rodríguez 2009). They do not act separately but form a complex web of interactions. Competitive interactions between antioxidants may occur during absorption, whereas positive interactions such as regeneration predominate inside tissues (Catoni et al. 2008). Like vitamin E, carotenoids are fat-soluble antioxidants. These two micronutrients thus share similar absorption pathways and, moreover, interact in defense against oxidative damage (Surai 2002; Catoni et al. 2008). Although *in vivo* antioxidant role of carotenoids in birds has recently been questioned, its importance cannot be ruled out based on current evidence (Pérez-Rodríguez 2009; Svensson and Wong 2011; Simons et al. 2012). In addition, positive effects of carotenoids on avian health, especially in reproduction and immunity, have been firmly established (Surai 2002; Catoni et al. 2008; Svensson and Wong 2011).

Besides their physiological functions, carotenoids serve as pigments giving yellow to red hues to various animal structures (McGraw 2006). They cannot be synthesized by animals and thus must be obtained from diet (Surai 2002). Due to the scarcity of carotenoids and/or other

mechanisms of costliness (Olson and Owens 1998), carotenoid-based traits may serve as an honest signal of individual quality (Olson and Owens 1998; Pérez-Rodríguez 2009). Accordingly, carotenoid-based traits have been shown to reflect several aspects of health and condition (Olson and Owens 1998; Hill 2006; Blount and McGraw 2008; Svensson and Wong 2011). Carotenoid coloration has been originally hypothesized to directly reflect the availability of carotenoids as important antioxidants and thus advertise the ability of an individual to deal with oxidative damage (von Schantz et al. 1999). Later, carotenoid-based traits have been suggested to indicate concentration of colorless antioxidants such as vitamin C or E, which protect carotenoids from oxidative bleaching and these may be allocated into coloration. Under this hypothesis, carotenoids themselves do not contribute significantly to antioxidant defense (Hartley and Kennedy 2004; Svensson and Wong 2011). Experimental supplementation with colorless antioxidants affected carotenoid coloration of integument in fish and birds (Bertrand et al. 2006; Pike et al. 2007; Pérez et al. 2008).

The effect of such supplementation on feather carotenoid coloration has produced conflicting results. In three studies (Karu et al. 2008; Larcombe et al. 2010; Giraudeau et al. 2013) supplementation with colorless antioxidant had no effect on feather coloration. However, in a study carried out by Marri and Richner, feather coloration was enhanced (Marri and Richner 2014). Last, the intensity of carotenoid-based traits may signal the availability of carotenoids for their non-antioxidant biological functions, such as immunomodulation or embryonic development (Hartley and Kennedy 2004; Pérez-Rodríguez 2009; Simons et al. 2012).

Unlike in poultry, effects of vitamin E supplementation have rarely been tested in free-living birds and results of the experimental studies published so far are ambiguous (de Ayala et al. 2006; Hõrak et al. 2007; Karu et al. 2008; Larcombe et al. 2010; Noguera et al. 2010; Losdat et al. 2011; Noguera et al. 2011; Giraudeau et al. 2013; Plummer et al. 2013; Maronde and Richner 2014; Matrková and Remeš 2014; Marri and Richner 2015). Here, we studied the effects of vitamin E availability on offspring performance and coloration in an important model bird species, the Great Tit (*Parus major*). We supplemented nestlings in a wild-ranging population and subsequently evaluated the effect of vitamin E on nestling growth, survival, immune responsiveness to a novel antigen, and carotenoid-based coloration. We expected nestlings supplemented with vitamin E to be superior over control nestlings in all these characteristics, i.e. to grow faster, to have higher survival, to mount stronger immune response, and to show more intense yellow plumage than control nestlings. We also expected that supplementation would increase the concentration of vitamin E and carotenoids in plasma of nestlings.

Materials and Methods

General fieldwork

This experiment was conducted on three adjacent nest-box plots (188 nest-boxes in total) in a deciduous forest near Grygov (49°31′N, 17°19′E) in eastern Czech Republic. The forest is dominated by lime (*Tilia* spp.) and oak (*Quercus* spp.) with interspersed ash (*Fraxinus excelsior*), common alder (*Alnus glutinosa*) and common hornbeam (*Carpinus betulus*). Nest-boxes were placed about 1.5 m above ground. Design of the nest-boxes is described in more details in Lambrechts et al. (2010), study site Olomouc. In addition to Great Tit, the nest boxes were inhabited by Blue Tits (*Cyanistes caeruleus*), Collared Flycatchers (*Ficedula albicollis*), and Nuthatches (*Sitta europaea*). Field work was carried out in 2009 and 2010 from early April until mid June. We checked the nest-boxes daily to record laying of the first egg and clutch completion. At the end of incubation, we visited nests once a day in late afternoon to record hatching dates. The day when the first nestling hatched was considered as day 0 of brood age.

At a brood age of two days we marked the nestlings by clipping down feathers on the head and back for their further individual recognition. When the young were eight days old, we ringed them with an aluminium ring. To assess the growth of nestlings, we weighted them at day 2 and 14 of brood age (referred bellow as day 2 and 14, respectively; likewise, day 5, 8, 11, 13 etc. refer to respective day of brood age). We chose the age of 14 days because pre-fledging offspring mass is an important predictor of survival after fledging in the Great Tit (Perrins and McCleery 2001). At day 14, we also measured nestlings´ tarsus length, wing length, and the length of the outermost (in 2010) or the second outermost (in 2009) right rectrix as an indicator of feather development. We measured both rectrices on a subsample of 216 nestlings and their lengths strongly correlated (r = 0.96). At the same time, we took from 10 to 15 yellow feathers from the upper right part of the breast of each bird for later spectrophotometric analysis.

Nestlings were weighed on a digital balance to the nearest 0.05 g, tarsus and rectrix lengths were measured with a digital calliper to the nearest 0.01 mm and wing length with a ruler to the nearest 0.5 mm. All these measurements and blood sampling (see below) were done prior to supplementation. We kept the handling of nestlings to a minimum, average manipulation time ranged from 7 min at day 5 to 26 min at day 14. To avoid body heat loss in cold and wet days in 2010, nestlings were kept on hand warming pads while out of the nest.

At day 13, we measured thickness of the right wing web of nestlings with a pressure-sensitive gauge (model PK-1012E, Mitutoyo, Tokyo, Japan) and then injected it with 0.09 mg of phytohaemagglutinin (L-8754, Sigma-Aldrich, St. Louis, MO, USA) in 25 μL of phosphate-buffered saline. We re-measured the wing web 24 h later (mean time lag: −3 ± 13.6 min, range

–40 to +57 min). We always measured the wing web twice and took the average. The intensity of immune response was quantified as the difference in wing web thickness between day 14 and day 13 of age.

Vitamin E supplementation

We used three dosages of vitamin E (see below) and a control. We distributed all treatments among nestlings within each nest in a stratified random way. First, we chose four heaviest nestlings from a nest and assigned each of them randomly to one of four treatments. We repeated the procedure again always with four (or less) heaviest nestlings left in the nest, unless all nestlings were assigned to a treatment. In this way, we ensured that all four treatments were represented in each brood, and the number of nestlings in each treatment was maximally balanced within a nest. At the same time, within each nest all treatments were distributed among nestlings with different initial body mass.

We supplemented nestlings every third day: at day 2, 5, 8 and 11. To reduce the variability in the age of supplementation and to avoid supplementation of very small nestlings, we performed the first two supplementations in late afternoon. The supplementation started at 4:42 p.m. ± 1h31min and 3:06 p.m. ± 2h15min [mean ± SD] at day 2 and 5, respectively. For logistical reasons, time of supplementation was more variable in older nestlings (start of supplementation at 12:23 p.m. ± 2h58min and 2:22 p.m. ± 3h40min [mean ± SD] at the age of 8 and 11 days, respectively).

Vitamin E includes several forms of tocopherols and tocotrienols. Of these, α-tocopherol was repeatedly shown to have the highest biological activity (Surai 2002) and generally dominates over other forms of vitamin E in avian tissues (Surai and Speake 1998; Karadas et al. 2005; Ewen et al. 2006). However, the physiological role differs between each of the diverse forms of tocopherol. In mammals, other forms of tocopherol and/or a mixture of several tocopherols provide stronger specific health effect than α-tocopherol alone (Jiang et al. 2001; Hensley et al. 2004; Saldeen and Saldeen 2005). Although this problem was mostly omitted in avian studies, the same may be anticipated in birds. For example, in poultry, a high degree of biodiscrimination was found between different forms of vitamin E at transition from the feed to egg and also among embryonic tissues (Surai 2002). Further, in the free-living Hihi (*Notiomystis cincta*) concentrations of α- and γ-tocopherols in egg yolk and nestling plasma responded differently to carotenoid supplementation (Ewen et al. 2006), Therefore, supplementation with different tocopherols may result in different biological effects. Thus, we decided to provide nestlings with a mixture of tocopherols rather than α-tocopherol alone.

Dosage was based on the amount of tocopherols presumably obtained by Great Tit nestlings from natural diet at a given age. Caterpillars are the main food of nestlings in our population,

comprising ca. 70% of food items (n = 68 nests; V. Remeš, unpublished). As the concentration of tocopherols in caterpillars was unknown at the beginning of our study, we used the concentration in insects provided by Barn Swallows (*Hirundo rustica*) to their nestlings, which was at the start of our study the only available estimate for a wild insectivorous bird species: 3.93 μg/g of α-tocopherol and 0.51 μg/g of γ-tocopherol (de Ayala et al. 2006). The weight of food provisioned to Great Tit nestlings was estimated based on a study of Great Tits in an oak wood (van Balen 1973), where young had growth curve similar to nestlings in our population (V. Remeš, unpublished). Food provisioned per nestling was estimated to be 1.10, 3.59, 6.24 and 6.65 g of insects at day 2, 5, 8 and 11, respectively (the value for day 11 is an average of food provisioned in days 9, 10, 12 and 13, because the value stated by van Balen for day 11 is extremely high). We prepared three concentrations of tocopherols. The lowest dose (E1) was equal to the estimated daily amount of α- and γ-tocopherol received in food, i.e. we multiplied the estimate of food provisioned per nestling by the concentrations of tocopherols in Barn Swallow diet. The concentration of the dose E2 was double of the E1 and the concentration of the dose E3 was 8 × E1. The actual average mass-specific dosage for the highest dosage (E3) averaged 14.0 mg total tocopherols per one kg of nestling body mass at day 2 (SE = 0.25, n = 189) and 20.9 mg/kg at day 8 (SE = 0.24, n = 183).

A caveat of this approach is that a recent study indicated that tocopherol content in the diet of titmice nestlings might be higher than we estimated originally. Arnold et al (2010) showed that some caterpillars contain substantially more α-tocopherol than aerial insects fed by Barn Swallow parents to their nestlings (de Ayala et al. 2006, Arnold et al. 2010). However, we believe that at least our highest dose E3 was relevant. First, our knowledge of vitamin E in titmice natural diet is still limited to the one study by Arnold et al. (2010). That single study was performed at different latitude and in different habitat compared to our study site and was limited to five weeks in one year, three groups of invertebrates, and one form of tocopherol. In addition, the concentration of tocopherol greatly varied among the three tested groups of invertebrates and even between the two groups of caterpillars. Second, our dosage is comparable to the doses which improved growth of Flycatcher nestlings (Matrková and Remeš 2014). Third, our dosage was sufficient to alter circulating tocopherols in Great Tit nestlings. Thus, we think that our nestlings received biologically significant amounts of tocopherols.

To prepare the supplement, we used dl-α-Tocopherol (DSM Nutritional Products Ltd., Basel, Switzerland) and Mixed Tocopherols 95 (DSM Nutritional Products, Inc., Parsippany, NJ, USA). The Mixed Tocopherols 95 contains approximately 62% of γ-tocopherol and 7% of α-tocopherol, while the dl-α-Tocopherol consists of pure α-tocopherol. To reach the α:γ tocopherol ratio of 7.71:1 found in the insects eaten by Barn Swallows (de Ayala et al. 2006), we mixed the two sources of tocopherols in a ratio of 4.71 g of dl-α-Tocopherol to 1 g of

Mixed Tocopherols 95. The Mixed Tocopherols 95 further contains approximately 29% of δ-tocopherol and a small amount of β-tocopherol. Thus, the final mixture consisted of 83.7% of α-tocopherol, 10.9% of γ-tocopherol, 5.1% of δ-tocopherol and 0.3% of β-tocopherol.

At the beginning of each breeding season, we prepared stock solutions of each dosage in olive oil, which was shown to have relatively low concentrations of both α- and γ-tocopherols (Herting and Drury 1963). Stock solutions were stored in dark glass bottles. To avoid any unnecessary manipulation with the stock solutions, every 2 to 4 days we poured the working solution into a 10 mL dark glass bottles. Both stock and working solutions were stored in 6 °C. Every morning, the working solutions were shaken and poured into 1.5 mL eppendorf tubes for field supplementation. In the field, the eppendorf tubes were kept in black plastic boxes filled with polystyrene to avoid light and thermal degradation. Moreover, if many broods were to be supplemented within one day, more boxes with a set of tubes had been prepared and a single box was used for max. 3 hours. In this way we minimized the oxidation or soiling of the oil. Feeding supplementation was performed by pipetting one of the three vitamin E dosages or the control into each nestling's mouth. Nestlings received 3.0, 9.8, 17.2 and 18.3 μl of oil at day 2, 5, 8 and 11 respectively. To avoid cross-infection, the micropipette tip was never re-used among broods.

Feather coloration
We wanted to test the effect of vitamin E supplementation on lutein deposition into feathers of Great Tit young. Thus we performed a spectrophotometric measurement of the amount of carotenoids in breast feathers. We quantified reflectance spectra of yellow feathers sampled from the breast as in Matrková and Remeš (2012), except we took and averaged five readings per nestling. As a measure of carotenoid content of feathers, we used carotenoid chroma. We calculated it as $(R_{700} - R_{450}) / R_{700}$, where R_{700} is reflectance at 700 nm and R_{450} reflectance at 450 nm. Carotenoids present in Great Tit breast feathers (lutein, zeaxanthin) absorb most light at around 450 nm and theoretical modelling showed that carotenoid chroma directly reflected the amount of carotenoids in feathers (Andersson and Prager 2006). Carotenoid chroma correlated positively with feather carotenoids also in field studies of the Great Tit (Isaksson et al. 2008; Isaksson and Andersson 2008). We also calculated brightness, as this characteristics derived from reflectance spectra was often used in previous studies. Brightness (R_{avg}) was calculated according to Andersson and Prager (2006) as the reflectance averaged over the interval from 320 to 700 nm.

Analysis of antioxidants
Blood sampling was conducted in 2010. We sampled nestlings at day 11 in the afternoon (at 2:52 pm ± 3.5 h [mean ± SD]). On cold and rainy days, larger broods were sampled in two rounds to reduce the time nestlings spent out of nest. Blood samples of 20−50 μl were taken

from the brachial vein into heparinized glass capillaries and immediately stored on ice. Within less than 10 hours, we centrifuged the samples at 2800 g for 5 min, separated the plasma and froze it at −20 °C. At the end of the season (4 to 41 days after sampling) the samples were frozen at −70 °C.

To determine the effect of supplementation on antioxidant levels in plasma, we measured plasma concentrations of α-tocopherol, γ-tocopherol and lutein. Antioxidant extraction was carried out according to Rupérez et al. (2004) with slight adjustments. Acetone was added to plasma (5-10 µl) in ratio 5:1 (v/v), vortexted for 30 s, sonicated in ice for 5 min, and centrifuged for 5 min at 5 ºC at 2300 g. The supernatant was then analyzed by HPLC with absorbance and fluorescence detection immediately after extraction. Samples were extracted and analyzed in series of 9 to 11 and the order in the series did not affect the antioxidant concentration (results not shown). Recovery of compounds was measured using both human and Zebra Finch pooled plasma samples. These samples were spiked with known amount of test compound and the extraction efficiency calculated. Extraction efficiency of tocopherols and lutein was 82.4±2.25% and 81.5±4.31% respectively (mean ± SE). Within-run precision was assessed on 5 µl pooled Great Tit plasma samples (n = 5) and was 2.9% for α-tocopherol, 9.7% for γ-tocopherol, and 5.5% for lutein. Between-run precision was assessed on 10 µl human plasma samples (n = 8) and was 9.76% for α-tocopherol, 15.0% for γ-tocopherol, and 12.8% for lutein.

We used a Spectra Model 4000 HPLC pump system with a Phenomenex 250×2 mm ID RP Hydro column (Phenomenex, Macclesfield, UK) maintained at 40º C to determine carotenoid and tocopherol composition of each sample. Analysis was conducted at a flow rate of 0.2 ml/min with a mobile phase of (A) methanol and (B) water/ethyl acetate (2.5:97.5) in a gradient elution of 0 to 90% B over 20 minutes. Tocopherols were detected by Scanning Fluorescence Detector model 474 (Waters, Elstree, UK) with excitation wavelength at 295 nm and emission at 350 nm. Lutein was detected by a PhotoDiode Array (PDA) absorbance detector model UV6000 (Thermo Fisher, Loughborough, UK) at 440 nm. Peaks were identified and quantified by comparison with authentic standards (tocopherols: Sigma-Aldrich, Poole, UK; lutein: Extrasynthése, Genay, France). All standard curves were linear (R^2 > 0.999) and were constructed within a range from 0.25 to 50 ng for γ-tocopherol, from 5 to 250 ng for α-tocopherol, and from 5 to 250 ng for lutein.

Statistical analysis

We used linear mixed models (LMM) to identify the effects of vitamin E supplementation on nestling growth, survival, immune responsiveness, plumage coloration, and plasma antioxidants. The dependent variables were *Body mass*, *Tarsus length, Wing length*, *Immune*

response, *Plumage brightness*, *Plumage chroma*, *Plasma α-tocopherol*, *Plasma γ-tocopherol,* and *Plasma lutein* (continuous variables) and *Survival* (binary variable). *Year* (2009 vs. 2010) and *Nest* (ID of the nest) were entered as random factors.

All models included *Vitamin E* as a fixed factor. *Vitamin E* represented our experimental treatment and was entered as a categorical variable with four levels: the control and doses E1, E2, and E3. To control for the effect of season, all models included *Hatching date* as a covariate. In Great Tits, breeding performance may peak in response to caterpillar availability, being lower in early and late breeders (Naef-Daenzer and Keller 1999; Verboven et al. 2001). Hence, a quadratic effect of season (*Hatching date*Hatching date*) was included in models and was retained if statistically significant. Where relevant, the models also included *Initial body mass* to control for size differences among nestlings at the start of the experiment. Further, we tested the interaction of *Vitamin E* with these two covariates. The interaction *Vitamin E*Hatching date* may be relevant, as α-tocopherol content in caterpillars increases over the course of the nesting season, but overall caterpillar availability decreases over season (Arnold et al., 2010, J. Matrková and V. Remeš, unpublished). Thus, we expected that the effect of supplementation might change over the season. We included the interaction *Vitamin E*Initial body mass*, because nestling body mass at the first supplementation varied (mean 3.0 g, SE ± 0.03, range 0.97 to 4.8 g, n = 764). The dosage of vitamin E was held constant irrespective of nestling size. Thus, smaller nestlings received relatively larger doses than larger nestlings within the same treatment group. The interaction was fit to account for this difference. We kept an interaction in a model only if statistically significant.

The model testing nestling immune responsiveness was controlled for *Condition* (residuals from the regression of body mass [g] on tarsus length [mm], controlled for year). In the models for nestling chroma and brightness, we included *Feather length* (length of rectix) as a covariate to control for feather development in nestlings. We checked that the length of rectrix correlated with the length of breast feathers used for color measurement on a subset of randomly chosen nestlings (r = 0.67, P < 0.001, n = 50). Both *Condition* and *Feather length* correlated with *Initial body mass* (Condition: r = 0.18, P < 0.001, n = 708; Feather length: r = 0.64, P < 0.001, n = 708). Thus we did not include *Initial body mass* into models with either of these two variables. Models testing the effect of supplementation on plasma tocopherols and lutein further included *Sampling time* as a fixed effect. *Sampling time* represents the time of day of blood sampling and accounts for any possible changes of plasma antioxidant concentrations during the day (Hõrak et al. 2004).

We included only first broods into the study. We started supplementation of the whole Great Tit population in our nest-boxes, with 795 nestlings in 86 broods (447 nestlings in 44 broods in 2009 and 348 nestlings in 42 broods in 2010). Eleven extra nestlings that hatched after the

first supplementation had been done were not included into the study. Sample size was lowered due to nest abandonments (3 nests), mortality, missing data, and technical difficulties. One extremely small nestling was excluded from all the tests on 14 days old young. The final sample size differs among models, ranging from 562 to 764 nestlings in 77 to 83 nests (Table 1). Antioxidant concentration in plasma was measured in a subsample of 115 nestlings in 33 nests.

We fitted LMMs using SAS software. Continuous dependent variables were tested in the procedure MIXED. Covariance parameters were estimated by REML (restricted or residual maximum likelihood). We used the COVTEST statement to produce asymptotic standard errors and Wald Z-tests for covariance parameter estimates of random effects. We tested survival in the GLIMMIX procedure with *Survival* as binary dependent variable. We used WALD statement to produce Wald Z test of estimates and asymptotic standard errors of random effects. If appropriate, variables were checked for normal distribution. Residuals from each model were checked to conform to the requirements of normal distribution, equal variance, and linearity. To compare the size of effects both within and between models, we used standardized regression coefficients. We standardized all continuous input variables prior to statistical analysis by subtracting mean and subsequently dividing by one standard deviation (Grafen and Hails 2002). Descriptive characteristics of non-standardized variables used in our models are given in Table S5 in supplementary material.

We tested the effect of vitamin E supplementation on several traits of the same nestlings. Such multiple testing with constant significance level (here, $\alpha = 0.05$) increases the probability of type I error, i.e. rejecting null hypothesis when it is true. To reduce this error, statistical corrections of p-values such as Bonferroni correction are often recommended. However, we decided to avoid these corrections. As we expected only small treatment effects and performed rather large number of tests, the Bonferroni correction would lead to low statistical power. Instead, we report standardized effect sizes with their standard errors and original p-values. We provide results of full models including nonsignificant variables (except interactions). Based on this information it is possible to interpret biological relevance of our results (Moran 2003; Nakagawa 2004; Forstmeier and Schielzeth 2011).

Results

1. Growth and survival

Initial body mass did not differ significantly between treatment groups ($R^2 < 0.01$, F = 1.34, P = 0.26, n = 764). Vitamin E supplementation did not affect any of the measured morphological traits: tarsus and wing length and body mass at day 14 or survival till fledging (Table 1, Table S1 in supplementary material). The interaction between vitamin E supplementation and initial body mass was statistically significant only for wing length. However, despite statistical significance of this interaction (which was quite weak, p = 0.045), slopes of the four treatments were very similar (Fig. 1). Moreover, none of the slopes of vitamin E supplemented nestlings differed significantly from the slope of control nestlings (Table S1). Without the interaction, the results of the model remained consistent, including the non-significant main effect of vitamin E supplementation (p = 0.91; results not shown).

Nestlings that were heavier at the start of the experiment were also heavier at older age, grew longer tarsi and wings, and had better survival till fledging. Nestling growth was also related to season; nestlings hatched early in the season reached higher weight at day 14. All growth parameters of nestlings and their fledging success also differed among nests (Table 1, Table S1 in supplementary material).

2. Immune responsiveness

The intensity of immune response to a novel antigen (phytohaemagglutinin) was not affected by vitamin E supplementation. The relationship between the immune response and season was quadratic: the intensity of immune response reached maximum in nestlings that hatched in the middle of the season. The intensity of immune response also increased with nestling condition and differed among nests (Table 1, Table S2 in supplementary material).

3. Plumage coloration

Vitamin E supplementation affected neither brightness nor chroma of the carotenoid-based plumage coloration of nestlings. Further, plumage coloration of nestlings did not change over the season. Both brightness and chroma differed among nests and were correlated to feather development. While nestling chroma decreased with growing feather length, nestlings with longer feathers showed higher brightness (Table 1, Table S3 in supplementary material).

4. Plasma antioxidants

Concentrations of α-tocopherol, γ-tocopherol, and lutein in nestling plasma were highly positively correlated (α-tocopherol with γ-tocopherol: r = 0.71, p < 0.001; α-tocopherol with lutein: r = 0.75, p < 0.001; γ-tocopherol with lutein: r = 0.62, p < 0.001; n = 115).

We found statistically significant effect of an interaction of vitamin E supplementation with initial body mass on plasma α-tocopherol concentration. Initially smallest nestlings supplemented with the dose E1 were predicted to have up to 19.7 μg/ml higher concentration of α-tocopherol level compared to control nestlings. However, with growing doses (E2, E3) the predicted slopes tended to get gradually closer to the slope predicted for control nestlings (Fig. 2). Plasma γ-tocopherol and lutein concentrations did not change in response to vitamin E supplementation. However, there was statistically significant positive relationship between nestling body mass at the beginning of supplementation and plasma lutein at day 11, when we sampled blood (Table 1, Table S4 in supplementary material).

Concentrations of plasma antioxidants did not change over the season. Concentration of both tocopherols, but not of lutein, increased during the day. Lutein concentration differed significantly among nests, while concentration of both tocopherols did not (Table S4 in supplementary material).

Discussion

We experimentally tested the effect of increased vitamin E intake on nestling performance in the Great Tit, an important model bird species. Vitamin E supplementation did not affect nestling growth, survival, immune response or carotenoid-based coloration. Our supplementation modified nestling plasma α-tocopherol, but not γ-tocopherol or lutein concentration. The effect on plasma α-tocopherol depended both on dose and nestling initial body mass.

Vitamin E supplementation did not affect growth and survival of Great Tit nestlings in our population. Similarly, experimental vitamin E supplementation did not affect chick growth in previous experiments in Great Tit (Maronde and Richner 2014; Marri and Richner 2014; Marri and Richner 2015), Blue Tit (Larcombe et al., 2010), or in Yellow-legged Gull nestlings (*Larus michahellis*; Noguera et al., 2010; Noguera et al., 2011). In contrast, tocopherol supplementation enhanced growth, condition, and feather development in Barn Swallows (de Ayala et al., 2006), and improved growth in Collared Flycatchers (Matrková and Remeš, 2014).

Species may differ in their response to increased vitamin E intake due to different tocopherol availability in their natural diet (Catoni et al. 2008). Although both Barn Swallows and titmice are insectivorous, Barn Swallows probably feed their nestlings with insects with much lower tocopherol content in comparison with titmice (de Ayala et al. 2006; Arnold et al. 2010; Larcombe et al. 2010). Vitamin E level in the diet of Collared Flycatcher nestlings is also

likely to be lower and more variable than in Great Tits (Matrková and Remeš 2014). Consequently, vitamin E supplementation might be beneficial only in species with low-vitamin E diet (Hõrak et al. 2007; Karu et al. 2008; Larcombe et al. 2010). Due to high α-tocopherol content of caterpillar-rich diet, growth and survival of Great and Blue Tit nestlings in the wild may not be substantially limited by vitamin E availability (Larcombe et al. 2010; Marri and Richner 2015). Accordingly, the higher fledging probability of vitamin E supplemented Great Tit nestlings, reported by Maronde and Richner (2014), might be due to unusually severe weather in the breeding season. Importantly, egg yolk concentration of vitamin E in our population of Great Tits is high in comparison to other Great Tit populations and to other bird species (Biard et al. 2009; Remeš et al. 2011), which might further lower the demand for externally supplied vitamin E.

We did not find any effect of tocopherol supplementation on immune response to a novel antigen in Great Tit nestlings. Similarly, Marri and Richner (2015) challenged immune system of Great Tit nestlings by a lipopolysaccharide from *E. coli*. The swelling response to this treatment did not depend on vitamin E intake. In adult Great Tit males, vitamin E supplementation did not prevent a negative effect of experimental immune challenge on sperm quality (Losdat et al. 2011). These results are in agreement with previous experiments on other wild birds, namely Barn Swallow nestlings (de Ayala et al. 2006) and adult male Greenfinches (*Carduelis chloris*; Hõrak et al., 2007). Thus, additional vitamin E may not play an important role in boosting the immune system in wild-ranging birds, even in species where vitamin E supplementation improves nestling growth (de Ayala et al. 2006). It is possible that even a small amount of vitamin E in the natural diet is sufficient for proper immune functioning. Alternatively, immune response to phytohaemagglutinin, used by us and both de Ayala et al. (2006) and Hõrak et al. (2007), might not be the best way to assess the performance of the immune system as suggested previously (Kennedy and Nager 2006). This seems to be in line with results obtained in Ring-necked Pheasants (*Phasianus colchicus*), where vitamin E supplementation in early development did not improve immune response to phytohaemagglutinin injection but supplemented pheasants had reduced parasite load in adulthood (Orledge et al. 2012).

Plumage carotenoid-based coloration of our Great Tit nestlings was not affected by increased intake of vitamin E. Likewise, plumage coloration was unaffected by vitamin E supplementation in Blue Tits (Larcombe et al. 2010), Greenfinches (Karu et al. 2008) and House Finches (*Haemorhous mexicanus*; Giraudeau et al., 2013). On the other hand, carotenoid coloration of Great Tit nestlings supplemented by a mixture of vitamin E and C in another population increased over season, while the coloration of control nestlings decreased (Marri and Richner 2014). Thus, vitamin E affected carotenoid coloration of feathers only when supplemented together with vitamin C. Carotenoid coloration of feathers may thus have

responded to increased availability of vitamin C, another colorless antioxidant (Hartley and Kennedy 2004). Alternatively, vitamin E may affect coloration only in interaction with vitamin C, which may for example recycle oxidized vitamin E (Barclay et al. 1983; Surai 2002).

Concentration of vitamin E in plasma of Great Tit nestlings responded to our vitamin E manipulation. Initially small nestlings supplemented with the lowest dose of vitamin E showed higher level of plasma α-tocopherol than control nestlings. The need of antioxidants may be higher in initially smaller nestlings, as fast growth later in life may increase oxidative damage (Mangel and Munch 2005; Alonso-Álvarez et al. 2007; De Block and Stoks 2008). Smaller nestlings may then benefit more from supplementation than the larger ones. In our previous study, initially smaller Collared Flycatcher nestlings grew longer tarsi if supplemented by vitamin E (Matrková and Remeš 2014). However, we did not find any improvement in growth in the Great Tit.

Vitamin E supplementation in previous studies had variable effects on circulating tocopherols in wild birds. In one study (Pérez et al., 2008) it increased and in the other (Larcombe et al., 2010) it showed no change in circulating tocopherols, and the effect also depended on season (Giraudeau et al. 2013) or dose (de Ayala et al. 2006). In our experiment, only the lowest dose of vitamin E significantly affected the relationship between initial body mass and plasma α-tocopherol in growing Great Tits. Moreover, with higher doses circulating tocopherol tended to get closer to the levels of control nestlings (Fig. 2). Similarly, in Barn Swallows, vitamin E supplemented nestlings reached higher α-tocopherol concentration than control nestlings (de Ayala et al. 2006). Moreover, circulating tocopherol level tended to be lower in nestlings supplemented with lower vs. higher dose of vitamin E. At the same time, only lower dose of vitamin E enhanced body mass, condition and feather growth in Barn Swallows. De Ayala et al. (2006) suggested that the nonlinear effects migh be due to an interaction between vitamin E and other nutrients and in higher doses migh alter overall antioxidant balance. However, neither our results or those of de Ayala et al. (2006) found any damaging effect of higher vitamin E doses on any measured parameter in nestlings.

Another possible source of these conflicting results might be a deposition of additional vitamin to other tissues such as the liver, which is the main storage organ of vitamin E (Surai 2002). It thus seems that without knowledge of vitamin E levels in the liver and other organs, plasma vitamin E might not be informative enough (Svensson and Wong 2011). However, so far relationships between plasma and liver vitamin E levels in wild birds remain almost unstudied. The only study examining simultaneously both plasma and liver vitamin E to our knowledge was conducted on the Barn Swallow and here plasma and liver concentrations of α-tocopherol were positively correlated (Møller et al. 2005). Overall, our current knowledge

of how plasma vitamin E reflects its concentration in other tissues is incomplete, making the interpretation of the response of plasma vitamin E levels to vitamin E supplementation difficult.

In conclusion, we found no effects of vitamin E supplementation on growth, survival, immune responsiveness and coloration of Great Tit nestlings. Vitamin E supplementation changed the plasma concentration of tocopherol, but not of lutein. Our supplementation increased the concentration of tocopherol in plasma of initially small nestlings. The effect faded with higher doses, suggesting an interaction with other antioxidants and/or dose-depended changes in tocopherol distribution among tissues. Vitamin E is probably not limiting when food contains high levels of this micronutrient (Arnold et al. 2010; Marri and Richner 2015). As the availability (and also probably needs) of vitamin E and other important micronutrients varies greatly during the year (Catoni et al. 2008), it might be interesting to assess the effect of supplementation in other parts of the year, for example during molt or in winter. Further studies in species with low vitamin E availability in natural diet, such as aerial insectivores, may bring more light on the physiological role of vitamin E in birds. To allow better inference, more tissues and types of possibly interacting micronutrients should be analyzed simultaneously (Cohen and McGraw 2009; Svensson and Wong 2011).

Acknowledgments

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Table 1 Summary of the effects of vitamin E supplementation on growth, survival, immune response, coloration and plasma antioxidants in Great Tit nestlings.

Response variable	Explanatory variables										Sample size	
	Vitamin E	Hatching date	Initial body mass	Condition	Feather length	Sampling time	Vitamin E * Initial body mass	Vitamin E * Hatching date	Year (random)	Nest (random)	Nestlings	Nests
Body mass	ns	●	●	-	-	-	ns	ns	ns	●	709	80
Tarsus length	ns	ns	●	-	-	-	ns	ns	ns	●	709	80
Wing length	●	ns	●	-	-	-	●	ns	ns	●	707	80
Survival	ns	ns	●	-	-	-	ns	ns	ns	●	764	83
Immune response	ns	●*	-	●	-	-	-	ns	ns	●	562	77
Plumage brightness	ns	ns	-	-	●	-	-	ns	ns	●	703	80
Plumage chroma	ns	ns	-	-	●	-	-	ns	ns	●	703	80
Plasma α-tocopherol	●	ns	●	-	-	●	●	ns	-	ns	115	33
Plasma γ-tocopherol	ns	ns	ns	-	-	●	ns	ns	-	ns	115	33
Plasma lutein	ns	ns	●	-	-	ns	ns	ns	-	●	115	33

Results are from linear mixed models. Result symbols: ● = statistically significant effect, ●* = significant quadratic relationship, ns = not significant, - = not tested. Plasma antioxidants were measured at age 11 days, other characteristics at age 14 days (hatching day = day 0).
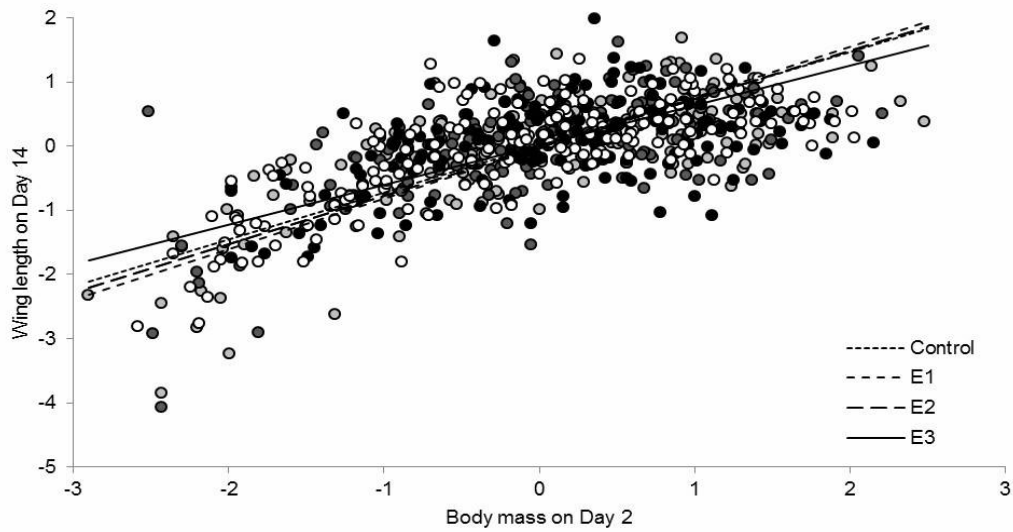
Fig. 1 Effect of vitamin E supplementation in Great Tit nestlings on the relationship between wing length (mm) and initial body mass (g). Depicted are regression lines and residual data points. White, light grey, dark grey and black points represent the control, dose E1, E2 and E3, respectively. Continuous variables were standardized prior to statistical analysis by subtracting mean and subsequently dividing by one standard deviation. Although the interaction was statistically significant ($p = 0.045$), none of the slopes for supplemented nestlings significantly differed from the slope for control nestlings (all $p > 0.076$). E1, E2 and E3 stand for dose 1, 2 and 3 of vitamin E. For detailed results, see Table S1 in supplementary material.
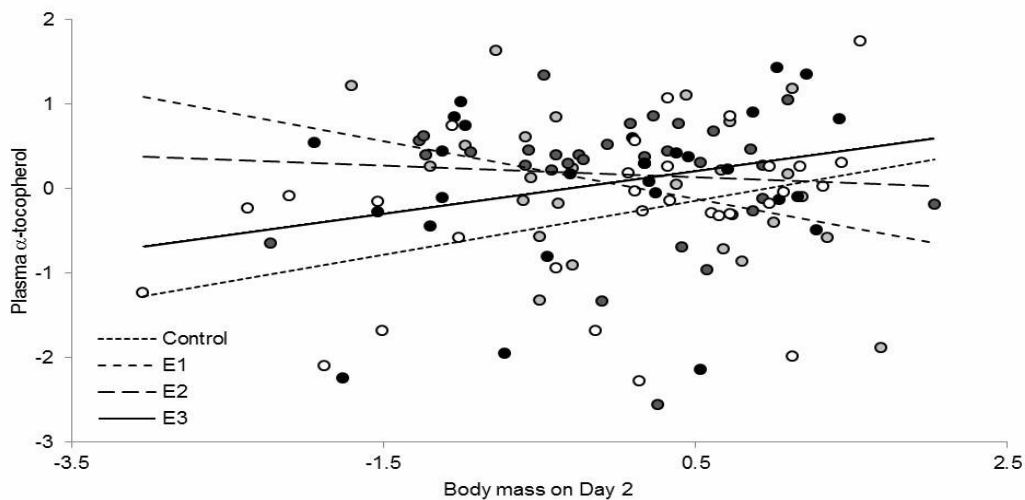
Fig. 2 Effect of vitamin E supplementation in Great Tit nestlings on the relationship between plasma α-tocopherol (μg/ml) and initial body mass (g). Depicted are regression lines and residual data points. White, light grey, dark grey and black points represent the control, dose E1, E2 and E3, respectively. Continuous variables were standardized prior to statistical analysis by subtracting mean and subsequently dividing by one standard deviation. Only the slope for nestlings receiving dose E1 significantly differed from the slope for control nestlings ($p = 0.009$). E1, E2 and E3 stand for dose 1, 2 and 3 of vitamin E. For details, see Table S4 in supplementary material.

Table S1 The effect of vitamin E supplementation on Great Tit nestlings: Growth and survival

| RESPONSE	FIXED EFFECTS							RANDOM EFFECTS					
Body mass	Effect		Estimate	SE	Den. DF	F	P	Effect	Estimate	SE	% Var	Wald´s Z	P
	Intercept		0.08	0.352				Nest	0.43	0.075	46.2	5.7	**<0.001**
	Initial body mass		0.28	0.024	664	137.6	**<0.001**	Year	0.23	0.349	24.7	0.7	0.251
	Hatching date		-0.28	0.080	78	11.9	**<0.001**	Residual	0.27	0.015	29.0	17.7	
	Vitamin E				628	0.2	0.872						
	Effect details for categorical variables	Category	Estimate	SE	Den. DF	t	P						
	Vitamin E	E1	>-0.01	0.056	627	-0.1	0.945						
		E2	0.02	0.056	629	0.3	0.745						
		E3	-0.03	0.056	627	-0.5	0.607						
Tarsus length	Effect		Estimate	SE	Den. DF	F	P	Effect	Estimate	SE	% Var	Wald´s Z	P
	Intercept		0.01	0.080				Nest	0.22	0.048	25.6	4.6	**<0.001**
	Initial body mass		0.33	0.035	702	86.3	**<0.001**	Year	0	-	-	-	-
	Hatching date		-0.11	0.061	77	3.2	0.077	Residual	0.64	0.036	74.4	17.7	
	Vitamin E				634	0.1	0.982						
	Effect details for categorical variables	Category	Estimate	SE	Den. DF	t	P						
	Vitamin E	E1	-0.03	0.085	634	-0.3	0.749						
		E2	<0.01	0.086	635	<0.1	0.969						
		E3	<0.01	0.085	633	<0.1	0.987						

RESPONSE	FIXED EFFECTS							RANDOM EFFECTS					
Wing length	Effect		Estimate	SE	Den. DF	F	P	Effect	Estimate	SE	% Var	Wald´s Z	P
	Intercept		0.01	0.093				Nest	0.22	0.044	42.3	5.1	**<0.001**
	Initial body mass		0.73	0.040	683	826.0	**<0.001**	Year	0.01	0.022	1.9	0.4	0.356
	Hatching date		0.05	0.064	67	0.6	0.451	Residual	0.29	0.016	55.8	17.5	
	Vitamin E				619	0.3	0.851						
	Vitamin E * Initial body mass				631	2.7	**0.045**						
	Effect details for categorical variables	Category	Estimate	SE	Den. DF	t	P						
	Vitamin E	E1	-0.04	0.057	619	-0.6	0.537						
		E2	-0.03	0.058	620	-0.5	0.590						
		E3	0.01	0.057	619	0.1	0.920						
	Vitamin E * Initial body mass	E1	0.06	0.056	631	1.1	0.259						
		E2	0.03	0.060	635	0.4	0.665						
		E3	-0.11	0.061	629	-1.8	0.076						
Survival	Effect		Estimate	SE	Den. DF	F	P	Effect	Estimate	SE		Wald´s Z	P
	Intercept		3.67	0.499				Nest	5.77	1.373		4.2	**<0.001**
	Initial body mass		1.00	0.219	758	21.0	**<0.001**	Year	0	-		-	-
	Hatching date		-0.19	0.343	86	0.3	0.589						
	Vitamin E				758	0.3	0.859						
	Effect details for categorical variables	Category	Estimate	SE	Den. DF	t	P						
	Vitamin E	E1	-0.24	0.513	758	-0.5	0.639						
		E2	0.25	0.564	758	0.4	0.658						
		E3	0.03	0.563	758	0.1	0.952						

Linear mixed model. All data were standardized. For fixed effects, type 3 tests and denominator DF are presented, numerator DF = 3 for Vitamin E and its interactions, numerator DF = 1 in all other cases. For random effects, covariance parameter estimates are presented (REML method), % Var = % of explained variability. Body mass, wing and tarsus length were measured at brood age 14 days (hatching day = day 0), survival at fledging. Response variable Survival is binomial, results for non-survived nestlings are presented. Sample size ranged from 707 to 764 nestlings in 80 to 83 nests (see Table 1).

Table S2 The effect of vitamin E supplementation on Great Tit nestlings: Intensity of immune response to phytohaemagglutinin

RESPONSE	FIXED EFFECTS		Estimate	SE	Den. DF	F	P	RANDOM EFFECTS	Estimate	SE	% Var	Wald´s Z	P
Immune response	Effect		Estimate	SE	Den. DF	F	P	Effect	Estimate	SE	% Var	Wald´s Z	P
	Intercept		0.23	0.234				Nest	0.14	0.041	14.3	3.4	**<0.001**
	Hatching date		0.10	0.061	80	2.7	0.104	Year	0.09	0.137	9.2	0.7	0.257
	Hatching date^2		-0.27	0.053	74	26.9	**<0.001**	Residual	0.75	0.048	76.5	15.5	
	Condition		0.12	0.047	386	6.7	**0.010**						
	Vitamin E				501	<0.1	0.990						
	Effect details for categorical variables	Category	Estimate	SE	Den. DF	t	P						
	Vitamin E	E1	<0.01	0.104	499	<0.1	0.984						
		E2	0.02	0.106	509	0.1	0.886						
		E3	-0.02	0.103	497	-0.2	0.844						

Linear mixed model. All data were standardized. For fixed effects, type 3 tests and denominator DF are presented, numerator DF = 3 for Vitamin E, numerator DF = 1 in all other cases. For random effect, covariance parameter estimate is presented (REML method), % Var = % of explained variability. Immune response and condition were evaluated at brood age 14 days (hatching day = day 0). Sampe size: 562 nestlings in 77 nests.

Table S3 The effect of vitamin E supplementation on Great Tit nestlings: Carotenoid-based plumage colouration

RESPONSE / **FIXED EFFECTS** / **RANDOM EFFECTS**

Plumage brightness

Effect	Estimate	SE	Den. DF	F	P	Effect	Estimate	SE	% Var	Wald´s Z	P
Intercept	-0.05	0.090				Nest	0.18	0.042	18.5	4.2	**<0.001**
Feather length	0.25	0.041	653	35.4	**<0.001**	Year	<0.01	0.014	0.3	0.2	0.429
Hatching date	-0.05	0.068	63	0.6	0.455	Residual	0.77	0.044	81.2	17.7	
Vitamin E			636	1.3	0.269						

Effect details for categorical variables	Category	Estimate	SE	Den. DF	t	P
Vitamin E	E1	0.15	0.094	635	1.6	0.120
	E2	-0.03	0.095	637	-0.3	0.767
	E3	0.03	0.094	634	0.3	0.777

Plumage chroma

Effect	Estimate	SE	Den. DF	F	P	Effect	Estimate	SE	% Var	Wald´s Z	P
Intercept	0.04	0.082				Nest	0.22	0.048	23.7	4.5	**<0.001**
Hatching date	-0.06	0.062	80	1.1	0.304	Year	0	-	-	-	**-**
Feather length	-0.34	0.040	679	70.4	**<0.001**	Residual	0.71	0.040	76.3	17.6	
Vitamin E			631	0.7	0.568						

Effect details for categorical variables	Category	Estimate	SE	Den. DF	t	P
Vitamin E	E1	-0.11	0.090	630	-1.2	0.215
	E2	-0.02	0.091	632	-0.2	0.824
	E3	>-0.01	0.090	639	>-0.1	0.972

Linear mixed model. All data were standardized. For fixed effects, type 3 tests and denominator DF are presented, numerator DF = 3 for Vitamin E, numerator DF = 1 in all other cases. For random effects, covariance parameter estimates is presented (REML method), % Var = % of explained variability. Feathers were sampled at brood age 14 days (hatching day = day 0). Sample size: 703 nestlings in 80 nests.

Table S4 The effect of vitamin E supplementation on Great Tit nestlings: Plasma antioxidants

RESPONSE	FIXED EFFECTS							RANDOM EFFECTS					
Plasma α-tocopherol	Effect		Estimate	SE	Den. DF	F	P	Effect	Estimate	SE	% Var	Wald´s Z	P
	Intercept		-0.30	0.178				Nest	0.09	0.090	10.3	1.0	0.156
	Initial body mass		0.32	0.147	100	0.2	0.688	Residual	0.78	0.125	89.7	6.3	
	Hatching date		0.10	0.105	30	1.0	0.328						
	Sampling time		0.26	0.103	30	6.4	**0.017**						
	Vitamin E				97	1.5	0.218						
	Vitamin E * Initial body mass				101	2.9	**0.041**						
	Effect details for categorical variables	Category	Estimate	SE	Den. DF	t	P						
	Vitamin E	E1	0.35	0.246	93	1.4	0.158						
		E2	0.47	0.236	98	2.0	**0.050**						
		E3	0.38	0.246	99	1.6	0.120						
	Vitamin E * Initial body mass	E1	- 0.66	0.248	95	-2.7	**0.009**						
		E2	-0.39	0.244	105	-1.6	0.116						
		E3	-0.07	0.227	100	-0.3	0.769						
Plasma γ-tocopherol	Effect		Estimate	SE	Den. DF	F	P	Effect	Estimate	SE	% Var	Wald´s Z	P
	Intercept		-0.28	0.183				Nest	0.06	0.085	6.6	0.7	0.234
	Initial body mass		0.08	0.099	100	0.7	0.418	Residual	0.88	0.136	93.4	6.5	
	Hatching date		-0.16	0.105	31	2.2	0.146						
	Sampling time		0.22	0.103	32	4.7	**0.037**						
	Vitamin E				102	1.3	0.264						
	Effect details for categorical variables	Category	Estimate	SE	Den. DF	t	P						
	Vitamin E	E1	0.31	0.258	98	1.2	0.231						
		E2	0.49	0.248	103	2.0	0.051						
		E3	0.32	0.257	104	1.2	0.219						

RESPONSE	FIXED EFFECTS							RANDOM EFFECTS					
Plasma lutein	Effect		Estimate	SE	Den. DF	F	P	Effect	Estimate	SE	% Var	Wald´s Z	P
	Intercept		-0.32	0.181				Nest	0.22	0.100	24.6	2.2	**0.013**
	Initial body mass		0.26	0.093	108	7.9	**0.006**	Residual	0.68	0.103	75.4	6.6	
	Hatching date		0.04	0.118	41	0.1	0.760						
	Sampling time		0.18	0.117	39	2.3	0.139						
	Vitamin E				98	1.5	0.213						
	Effect details for categorical variables	Category	Estimate	SE	Den. DF	t	P						
	Vitamin E	E1	0.40	0.231	95	1.7	0.088						
		E2	0.43	0.225	100	1.9	0.056						
		E3	0.27	0.233	100	1.2	0.252						

Linear mixed model. All data were standardized. For fixed effects, type 3 tests and denominator DF are presented, numerator DF = 3 for Vitamin E and its interaction, numerator DF = 1 in all other cases. For random effects, covariance parameter estimates are presented (REML method), % Var = % of explained variability. Plasma was analysed at brood age 11 days (hatching day = day 0). Sample size: 115 nestlings in 33 nests.

Table S5 Descriptive statistics of Great Tit nestlings variables used in the models

Variable		Unit	Mean	SD	n
Response variable	Body mass	g	16.8	1.55	711
	Tarsus length	mm	23.0	0.78	711
	Wing length	mm	51.1	3.15	709
	Immune response	mm × 0.01	70.2	27.4	562
	Plumage brightness		32.8	6.51	706
	Plumage chroma		0.5	0.07	706
	Plasma α-tocopherol	µg/ml	20.6	8.32	115
	Plasma γ-tocopherol	µg/ml	0.7	0.32	115
	Plasma lutein	µg/ml	69.6	23.48	115
Covariate	Initial body mass	g	3.0	0.73	764
	Initial body mass	g	3.2	0.67	115
	Hatching date	order day	128.0	2.78	764
	Hatching date	order day	129.5	2.16	115
	Condition		0.0	1.16	562
	Feather length	mm	19.3	2.77	709
	Sampling time	hh:mm	15:04	2:55	115

Initial body mass was measured at brood age 2 days (hatching day = day 0), plasma variables at day 11 and other variables at day 14.

PŘÍSPĚVEK III

Matrková J. & Remeš V. 2014: Vitamín E improves growth of collared flycatcher *Ficedula albicollis* **young: a supplementation experiment. Journal of Avian Biology, 45 (5), 475-483.**

Articles

Vitamin E improves growth of collared flycatcher *Ficedula albicollis* young: a supplementation experiment

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In altricial birds, the quantity and quality of food provided by parents is a crucial determinant of nestling performance. Vitamin E is an important micronutrient with various physiological functions, including a positive role in the antioxidant system. Sufficient intake of vitamin E has been shown to condition normal avian development in poultry, yet, our knowledge of the role of vitamin E in free-living birds is limited. Thus, we experimentally examined the effects of vitamin E on nestling development in the collared flycatcher *Ficedula albicollis*. We supplemented nestlings with vitamin E and evaluated their growth and survival till fledging. Increased availability of vitamin E did not affect body mass, wing length or survival, but improved tarsus growth. The effect of supplementation on tarsus length changed over season and with initial body mass. Supplemented nestlings that were smaller at hatching and those that hatched later in the season grew longer tarsi compared to the control. Our results suggest that 1) vitamin E may be limiting for the development of collared flycatcher nestlings, 2) seasonal changes of vitamin E availability may affect breeding success of collared flycatchers, and 3) increased income of vitamin E may improve growth of nestlings with bad start in life.

In altricial birds, nestling performance greatly depends on the care provided by parents. One of the most important parts of parental care is food provisioning (Bernardo 1996, Price 1998). Nestling growth is determined by both the quantity and quality of food they receive (Perrins 1976, Martin 1987, Massias and Becker 1990, Johnston 1993, Saino et al. 1997, Wright et al. 1998, Romano et al. 2006). Besides energetic content and macronutrients, micronutrient composition is an important aspect of the quality of food provided by parents.

Vitamin E is an important micronutrient with various physiological functions. It is considered the most important antioxidant in cell membranes, where it prevents oxidative damage by scavenging free radicals and thus breaks the chain propagation of lipid peroxidation (Surai 2002). Apart from its antioxidant role, vitamin E has also been suggested to participate in the regulation of enzymes and gene expression, signalling and membrane processes (Brigelius-Flohé 2009). Sufficient intake of vitamin E conditions normal avian development, functional immune system and reproduction (Surai 2002, Catoni et al. 2008).

Growing chicks are more likely to develop diseases related to vitamin E deficiency than adult birds. When faced with low vitamin E content in food, adult birds can prevent depletion of their existing vitamin E pool by recycling vitamin E molecules (Surai 2002). In contrast, growing birds must gradually build up their vitamin E reserves from food intake, which might be only partly offset by vitamin E

supply in the egg yolk. Moreover, antioxidant function of vitamin E is probably particularly important for chicks during the period of rapid growth, when consumption of oxygen is high and production of cell-damaging free radicals greater (Rollo 2002, Alonso-Álvarez et al. 2007, Kim et al. 2011, but see Garratt and Brooks 2012). This is supported by studies showing that increased intake of vitamin E in growing chicks may lower oxidative damage (Sodhi et al. 2008, Noguera et al. 2011, Orledge et al. 2012) and improve growth performance (de Ayala et al. 2006, Noguera et al. 2011). Hence, vitamin E might be limiting for growing chicks.

The necessity of vitamin E in reproduction and chick development has been firmly established in poultry (Surai 2002). However, studies on free-living birds are rare and their results are ambiguous. Vitamin E supplementation improved nestling growth in some studies (de Ayala et al. 2006, Noguera et al. 2011), but in other studies no such effect was found (Larcombe et al. 2010, Noguera et al. 2010). Vitamin E supply in natural food, and consequently the potential for vitamin E limitation, can differ among species with differing food ecology and foraging behaviour. To broaden the spectrum of species studied, we experimentally studied the effect of vitamin E availability on the performance of collared flycatcher *Ficedula albicollis* nestlings. Collared flycatchers are forest-dwelling songbirds, which hawk aerial prey as one of their primary foraging techniques and thus differ from other

common forest songbirds, (e.g. titmice, *Parus*, *Cyanistes*; Perrins 1979). We supplemented nestlings with vitamin E and evaluated their growth and fledging success. We expected nestlings provided with extra vitamin E to grow faster and survive better than control nestlings.

Material and methods

General fieldwork

This experiment was conducted on three adjacent nest-box plots (188 nest-boxes in total) in a deciduous forest near Grygov (49°31′N, 17°19′E) in eastern Czech Republic. The forest was dominated by lime *Tilia* spp. and oak *Quercus* spp. with interspersed ash *Fraxinus excelsior*, common alder *Alnus glutinosa* and common hornbeam *Carpinus betulus*. Nest-boxes were placed about 1.5 m above ground. Design of nest-boxes is described in more detail in Lambrechts et al. (2010), study site Olomouc. In addition to collared flycatchers, the nest boxes were inhabited by great tits *Parus major*, blue tits *Cyanistes caeruleus*, and nuthatches *Sitta europaea*. Field work was carried out in 2008 from late April until June. We checked the nest-boxes daily to record laying of the first egg and clutch completion. At the end of incubation, we visited nests once a day to record hatching dates. The day when the first nestling hatched was considered as day 0 of brood age.

At a brood age of two days we marked the nestlings by clipping down feathers on their head and back for further individual recognition. When the young were eight days old, we ringed them with aluminium rings. To assess the growth of nestlings, we weighted them at day 2 and 14 of brood age (referred bellow as day 2 and 14, respectively) on a digital balance to the nearest 0.05 g. At day 14, we also measured their wing and tarsus length. Tarsus length was measured with a digital calliper to the nearest 0.01 mm and wing length with a ruler to the nearest 0.5 mm. We measured nestlings at day 14 because pre-fledging offspring size is an important predictor of survival after fledging in collared flycatchers (Merilä et al. 2001) and other bird species (Gebhardt-Henrich and Richner 1998). We kept the handling of nestlings to a minimum. To assess fledging success, we noted each dead nestling found during supplementation and checked nest-boxes for dead nestlings after fledging.

Vitamin E supplementation

We used a control treatment and two dosages of vitamin E (see below). We randomly divided nestlings within each nest into three groups with the same number of nestlings, or, if not possible (i.e. if the number of nestlings was not divisible by 3), kept the difference in the number of nestlings to one nestling only. Each of the groups was then randomly assigned to one of the three treatments. In this way, we ensured that all three treatments were represented in each brood and at the same time the number of nestlings in each treatment was maximally balanced within a nest. At the start of the experiment, the mean (SD) number of nestlings per nest was 1.6 (0.71) for the control and E1, and 1.7 (0.69)

for E2 (mean brood size = 4.9, SD = 1.69, n = 17). We supplemented nestlings every third day: at day 2, 5, 8 and 11. To reduce the variability in the age of supplementation and to avoid supplementation of very small nestling, we performed the first supplementation at day 2 in late afternoon (start of supplementation at 5:17 pm ± 1 h 41 min [mean ± SD]). For logistical reasons, time of supplementation was more variable in older nestlings.

Vitamin E includes several forms of tocopherols and tocotrienols. Of these, α-tocopherol was repeatedly shown to have the highest biological activity (Surai 2002) and generally dominates over other forms of vitamin E in avian tissues (Surai and Speake 1998, Karadas et al. 2005, Ewen et al. 2006). However, the physiological role differs between each of the diverse forms of tocopherol. Other forms of tocopherol and/or a mixture of several tocopherols provide stronger specific health effect than α-tocopherol alone (Jiang et al. 2001, Hensley et al. 2004, Saldeen and Saldeen 2005). Therefore, supplementation with different tocopherols may result in different biological effects. Thus, we decided to provide nestlings with a mixture of tocopherols rather than α-tocopherol alone.

Vitamin E dosage was based on the amount of tocopherols presumably obtained by collared flycatcher nestlings from natural diet at a given age. The concentration of tocopherols in collared flycatcher diet or in the diet of any other passerine with similar ecology was unknown at the beginning of this study. Hence, we used the concentration in insects provided by barn swallows to their nestlings: 3.93 $\mu g\,g^{-1}$ of α-tocopherol and 0.51 $\mu g\,g^{-1}$ of γ-tocopherol (de Ayala et al. 2006). The amount of insects eaten by the nestling was calculated as in de Ayala et al. 2006. As we had no information on nestling body growth in our flycatcher population, we used the growth curve from a nearby population (Krist et al. 2004). As the basis for our calculations, we used original data kindly provided by M. Krist (the data are now available in their full form at Dryad Digital Repository, doi: 10.5061/dryad.1758).

Based on these calculations, we estimated the daily food intake per nestling as 7.1, 13.8, 18.0 and 18.1 g of insects at days 2, 5, 8 and 11, respectively. We prepared two concentrations of tocopherols. The lower dose (E1) was equal to the estimated daily amount of α- and γ-tocopherol received in food, i.e. we multiplied estimated food provisioned per nestling by the concentrations of tocopherols in barn swallow diet. The concentration of the higher dose (E2) was double that of E1. A caveat of this approach is that more recent studies indicated that tocopherol content in the diet of collared flycatcher nestlings might be higher than we estimated originally. Arnold et al. (2010) showed that some caterpillars contain from about 10-fold (*Erannis* larvae) to 30-fold (*Operophtera* larvae) more α-tocopherol than aerial insects fed by barn swallow parents to their nestlings (de Ayala et al. 2006, Arnold et al. 2010). However, for two reasons we still do not know the true vitamin E content of collared flycatcher nestlings' diet.

First, despite decades of research, our knowledge of the collared flycatcher nestlings' diet is limited (for review see Cholewa and Wesołowski 2011). Moreover, this diet may greatly vary among years, habitats, early vs late broods, with the age of chicks, and the actual availability of prey types

(Löhrl 1957, Bureš 1986, 1995, Török 1986, Török and Tóth 1988, Krištín 1992). For example, the proportion of caterpillars in nestling diet in these studies ranges from 3.6 to 47%; most often caterpillars form 20 to 30% of the number of food items. Second, there has been so far only one study of tocopherol content in the diet of nestlings in a forest passerine (Arnold et al. 2010). That unique study was performed in a different latitude and habitat and was limited to five weeks in one year, three groups of invertebrates, and one form of tocopherol. In addition, the concentration of tocopherol greatly varied among the three tested groups and even between two groups of caterpillars.

Based on the limited knowledge, we guessed that the diet of collared flycatcher nestlings could contain about 2- to 13-fold (on average 7-fold) more α-tocopherol than the diet of barn swallow nestlings. Our estimate was based on the following assumptions about nestling diet: 1) the diet contains 4 to 50% of caterpillars (on average 25%); 2) caterpillars are the main source of tocopherols, 3) caterpillars contain about 100 μg g^{-1} α-tocopherol with remaining diet items contain 3.93 μg g^{-1}, and 4) α-tocopherol is the predominant form of tocopherol.

To prepare the supplement, we used Aqua-E (Yasoo Health, Johnson City, TN, USA). Agua-E contains 13.4 mg ml^{-1} of natural form α-tocopherol, d-α-tocopherol, together with 12 mg ml^{-1} of γ-tocopherol and 4.6 mg ml^{-1} of other tocopherols and tocotrienols. To reach the α:γ tocopherol ratio found in the insects eaten by barn swallow nestlings (7.71:1; de Ayala et al. 2006), we further used a microemulsion of dl-α-tocopheryl acetate in water (prepared by DSL Food, Olomouc, Czech Republic). As dl-α-tocopheryl acetate is considered to have lower biological activity than d-α-tocopherol, we multiplied the amount of added dl-α-tocopheryl acetate by 1.49 (based on international units, United States Pharmacopeia 1980). Thus, the final mixture consisted of 79% of dl-α-tocopheryl acetate, 10% of d-α-tocopherol, 8% of γ-tocopherol and 3% of other tocopherols and tocotrienols. Average mass-specific dosage decreased with age. Dose E1 ranged from 25.0 mg of total tocopherols and tocotrienols per one kg of nestling body mass at day 2 (SE = 1.22, n = 25) to 13.4 mg kg^{-1} at day 11 (SE = 1.17, n = 21). Dose E2 was from 53.0 mg kg^{-1} at day 2 (SE = 3.27, n = 27) to 24.7 mg kg^{-1} at day 11 (SE = 0.54, n = 22).

Vitamin E supplements were prepared by dissolving the two sources of vitamin E in water. Because both our sources of tocopherols were water-soluble, we assured the supplement was homogenous. Thus, all nestlings within a treatment received the same concentration of vitamin E, whereas control nestlings received water only. Fresh solution was prepared in the evening prior to supplementation and kept in dark glass bottles at 6°C overnight. Every morning, the solutions were shaken and poured off into 1.5 ml eppendorf tubes for field supplementation. In the field, eppendorf tubes were kept in black plastic boxes filled with polystyrene to avoid light and thermal degradation. Feeding supplementation was performed by pipetting one of the two vitamin E dosages or the control into each nestlings' mouth. Nestlings received 8, 15, 20 and 20 μl of solution at age 2, 5, 8 and 11 d, respectively. Due to extremely slow growth in cold and rainy weather, six nestlings in two nests

were first supplemented at the age 3 d. To avoid cross-infection, the micropipette tip was never reused among broods.

Statistical analysis

To identify potential effects of vitamin E supplementation on nestling growth and survival, we used linear mixed models (LMM). The dependent variables were Body mass, Tarsus and Wing at day 14 (continuous variable) and Survival till fledging (categorical variable with two levels). To control for the effect of common origin, Nest was entered as a random factor. Our experimental treatment was represented by a fixed factor Vitamin E. Vitamin E was entered as a categorical variable with three levels – control, dose E1 and E2. All models included Hatching date to control for the effect of season, and Initial body mass (body mass at the start of the experiment, i.e. day 2) to control for size differences among nestlings at the start of the experiment. Initial body mass did not differ significantly between treatment groups ($R^2 < 0.01$, F = 0.19, p = 0.83, n = 79). All models also included Brood size at day 8 to control for the intensity of sibling competition. In smaller broods, sibling competition is usually reduced (Ricklefs 2002).

Further, we tested the interaction of Vitamin E with these three covariates. The interaction Vitamin E × Hatching date may be relevant, as α-tocopherol content in caterpillars increases over the course of the nesting season, but overall availability of caterpillars decreases over the season (Arnold et al. 2010, our unpublished data). Caterpillars form an important part of collared flycatcher nestlings' diet (Cholewa and Wesołowski 2011). Thus, we expected that the effect of supplementation might change over the season. We included the interaction Vitamin E × Initial body mass because nestling body mass varied (Initial body mass: mean 2.80 g, SE 0.08, range 1.3 to 4.3 g, n = 82). The dosage of vitamin E was held constant irrespective of nestling size. Thus, smaller nestlings received relatively larger doses than larger nestlings within the same treatment group. The interaction was fit to account for this difference. The interaction Vitamin E × Brood size was included to test whether the effect of supplementation depended on the level of sibling competition. We kept an interaction in the model only if it was statistically significant. In the model with Body mass as a dependent variable, we also included Tarsus as a covariate. This approach tested nestling condition (i.e. body mass controlled for tarsus length). However, we note that results of the model without Tarsus (i.e. testing body mass per se) were similar and are not presented here.

We started supplementation of the whole flycatcher population in our nest-boxes in 2008, with 83 nestlings in 17 broods. Sample size was lowered due to nestling mortality, technical problems and missing data. The final sample size was 15 broods with 63 nestlings for models with body size and 74 nestlings for survival model. Descriptive statistics of nestlings included in our study are provided in Supplementary material Appendix 1, Table A1.

We fitted LMMs using SAS software. Continuous dependent variables were tested in the procedure

MIXED. Covariance parameters were estimated by REML (restricted or residual maximum likelihood). We used the COVTEST statement to produce asymptotic standard errors and Wald Z-tests for covariance parameter estimates of random effects. We tested survival in the GLIMMIX procedure with Survival as a binomial dependent variable. We used WALD statement to produce Wald Z tests of estimates and asymptotic standard errors of random effects. Where appropriate, variables were checked for normal distribution. Residuals from each model were checked to conform to the requirements of normal distribution, equal variance, and linearity. In order to be able to interpret tests of main effects in the model with significant interactions (Tarsus length model), we centred all continuous input variables prior to statistical analysis by subtracting the mean.

We tested potential effects of vitamin E supplementation on several traits of the same nestlings. Such multiple testing with a constant significance level (here at $\alpha = 0.05$) increases the probability of type I error, i.e. rejecting the null hypothesis when it is true. To reduce this error, statistical corrections of p-values such as Bonferroni correction are often recommended. However, we decided to avoid these corrections. As we expected only small treatment effects and performed rather large number of tests, the Bonferroni correction would lead to low statistical power. Instead, we report effect sizes with their standard errors and original p-values. We provide results of full models including nonsignificant variables (except nonsignificant interactions). Based on this information it is possible to interpret biological relevance of our results (Moran 2003, Nakagawa 2004, Forstmeier and Schielzeth 2011).

Results

Tarsus length at day 14 was affected by vitamin E supplementation and this effect changed both with the season and with body mass at hatching (Table 1, Fig. 1). Although at the beginning of the season control nestlings grew longer tarsi than nestlings supplemented by the dose E1, towards the end of the season the tarsus of an E1 supplemented nestling was predicted to be 1.6 mm longer than the tarsus of a control nestling (Fig. 1A). At the same time, initially smaller nestlings grew longer tarsi when supplemented compared to control ones. The model predicted that among initially smallest nestlings E1 chicks would grow 1.5 mm longer tarsi than control chicks (Fig. 1B). In both these cases, tarsus length changed in response to vitamin E supplementation similarly irrespective of the dose (E1 vs E2). Besides interactions, overall effect of vitamin E supplementation on tarsus length approached statistical significance, with dose E1 being strongly significant (Table 1). To make sure that our effects were not driven by interactions, we re-ran the Tarsus model without them. Results were similar: overall effect of vitamin E supplementation approached significance (p = 0.054) with dose E1 being strongly significant (p = 0.017, effect size 0.546, SE 0.222). These findings confirm a beneficial effect of vitamin E supplementation on tarsus growth.

Nestling body mass, wing length at day 14 and survival till fledging were unaffected by vitamin E supplementation.

However, nestlings with greater body mass at hatching grew faster and survived better. Neither growth nor survival of nestlings changed over the season (except in the case of tarsus length described above). Growth and survival of nestlings did not depend on brood size either. The only exception was nestling body mass. Here, nestlings in larger broods were in worse condition than nestlings in smaller broods: the model predicted a body mass decrease of 0.57 g per sibling when a positive correlation with tarsus length was controlled for.

Nest identity explained a significant part of variability in wing length and body mass (66.1 and 62.9%, respectively). Tarsus length and survival till fledging were not significantly affected by nest identity.

Discussion

We supplemented collared flycatcher nestlings with vitamin E and evaluated their subsequent growth and fledging success. Our treatment affected tarsus length, but did not alter nestling body mass, wing length or survival. Tarsus length changed with increased vitamin E availability in a season-dependent way and the change depended also on initial nestling body mass. Supplementation was most beneficial (in terms of longer tarsi at the age of 14 d) for initially smaller nestlings and nestlings that hatched later in the season. Thus, we showed that at least in some aspects vitamin E is limiting in the development of collared flycatcher young.

Our supplementation affected tarsus length, but did not affect body mass or wing length. We measured both body mass and tarsus at brood age of 14 d. In the case of body mass, this might have been too late, because nestling collared flycatchers may reach their asymptotic mass earlier (Bureš and Weidinger 2003). Hence we cannot rule out that the increased vitamin E intake affected the body mass of nestlings earlier during growth. For example, we were not able to detect whether nestlings from one of the treatment groups reached their asymptotic mass earlier than nestlings in other groups. This would agree with vitamin E supplementation in barn swallows, where the positive effect of vitamin E supplementation was restricted to a short period of maximal growth (day 6 to day 12; de Ayala et al. 2006).

Inconsistent effects of vitamin E on different phenotypic traits obtained here were found in previous experiments on free-living birds too. In barn swallows, the situation was the opposite of our findings: body mass, condition, and feather length were affected by vitamin E supplementation, while tarsus length was not (de Ayala et al. 2006). In yellow-legged gull chicks, a beneficial effect of vitamin E supplementation on chick body mass was also confirmed, but only at the age of 8 d, not at younger age (Noguera et al. 2010, 2011). In titmice, vitamin E supplementation did not alter nestling growth and survival, regardless of nestling age and morphometric traits in question (Larcombe et al. 2010, Matrková et al. unpubl.). Thus, the effect of increased vitamin E intake differs among species and depends on the trait examined and the age of nestlings.

The positive effect of vitamin E on nestling growth presented here and in the barn swallow (de Ayala et al. 2006) contrasts with the absence of any effect in titmice

Table 1. The effect of vitamin E supplementation on growth and survival of collared flycatcher nestlings.

Tarsus*	Fixed effect	Estimate	SE	DF	F	p
	Intercept	−0.166	0.1540			
	Initial body mass	0.821	0.2141	52.4	4.6	**0.04**
	Hatching date	−0.162	0.0950	10.6	0.8	0.39
	Brood size	−0.072	0.0839	18.1	0.7	0.40
	Vitamin E			43.9	3.0	0.06
	Vitamin E × Hatching date			44.8	6.8	**<0.01**
	Vitamin E × Initial body mass			45.3	4.5	**0.02**
	Category	**Estimate**	**SE**	**DF**	**t**	**p**
	Vitamin E	1	0.459	0.1892	44.9	2.4	**0.02**

	Category	Estimate	SE	DF	t	p
Vitamin E	1	0.459	0.1892	44.9	2.4	**0.02**
	2	0.223	0.1826	43.6	1.2	0.23
Vitamin E × Hatching date	1	0.372	0.1036	45.0	3.6	**<0.01**
	2	0.287	0.1026	47.7	2.8	**<0.01**
Vitamin E × Initial body mass	1	−0.737	0.3091	47.1	−2.4	**0.02**
	2	−0.797	0.2955	44.7	−2.7	**<0.01**

	Random effect	Estimate	SE	% Var	Wald´s Z	p
	Nest	0.081	0.0773	19.8	1.1	0.15
	Residual	0.329	0.0732	80.2	4.5	

Wing	Fixed effect	Estimate	SE	DF	F	p
	Intercept	1.809	4.3862			
	Initial body mass	0.352	0.0366	50.9	92.5	**<0.01**
	Hatching date	0.015	0.0314	11.0	0.2	0.65
	Brood size	0.014	0.0359	13.6	0.1	0.71
	Vitamin E			45.7	0.8	0.46

	Category	Estimate	SE	DF	t	p
Vitamin E	1	0.054	0.0456	46.4	1.2	0.25
	2	0.010	0.0437	45.2	0.2	0.82

	Random effect	Estimate	SE	% Var	Wald´s Z	p
	Nest	0.037	0.018	66.1	2.0	**0.02**
	Residual	0.019	0.004	33.9	4.7	

Body mass	Fixed effect	Estimate	SE	DF	F	p
	Intercept	−13.685	24.2191			
	Initial body mass	0.515	0.2227	51.4	5.4	**0.02**
	Hatching date	0.148	0.1736	12.4	0.7	0.41
	Brood size	−0.575	0.1999	15.5	8.3	**0.01**
	Tarsus	0.437	0.1720	48.2	6.5	**0.01**
	Vitamin E			46.5	0.3	0.75

	Category	Estimate	SE	DF	t	p
Vitamin E	1	−0.202	0.2865	47.4	−0.7	0.48
	2	−0.164	0.2632	45.9	−0.6	0.54

	Random effect	Estimate	SE	% Var	Wald´s Z	p
	Nest	1.095	0.5178	62.9	2.1	**0.02**
	Residual	0.646	0.1369	37.1	4.7	

Survival	Fixed effect	Estimate	SE	DF	F	p
	Intercept	98.316	79.9224			
	Initial body mass	−4.884	2.0638	68.0	5.6	**0.02**
	Hatching date	−0.609	0.5510	33.5	1.2	0.28
	Brood size	−1.041	0.4863	48.7	4.6	**0.04**
	Vitamin E			68.0	1.5	0.23

	Category	Estimate	SE	DF	t	p
Vitamin E	1	3.057	1.8960	68.0	1.6	0.11
	2	2.480	1.6628	68.0	1.5	0.14

	Random effect	Estimate	SE		Wald´s Z	p
	Nest	1.533	2.0476		0.8	0.23

*Centred data (subtracted mean prior to statistical analysis).
Linear mixed model. For fixed effects, type 3 tests and denominator DF are presented, numerator DF = 2 for Vitamin E and its interactions, numerator DF = 1 in all other cases. For random effects, covariance parameter estimates are presented (REML method), % Var = % of explained variability. Body mass [g], tarsus length [mm] and wing length [cm] were measured at the brood age of 14 d (hatching day = day 0). Response variable Survival is binomial, results for non-survived nestlings (13 out of 74) are presented. Sample size was 63 and 74 nestlings from 15 nests, for the models of growth and survival, respectively.
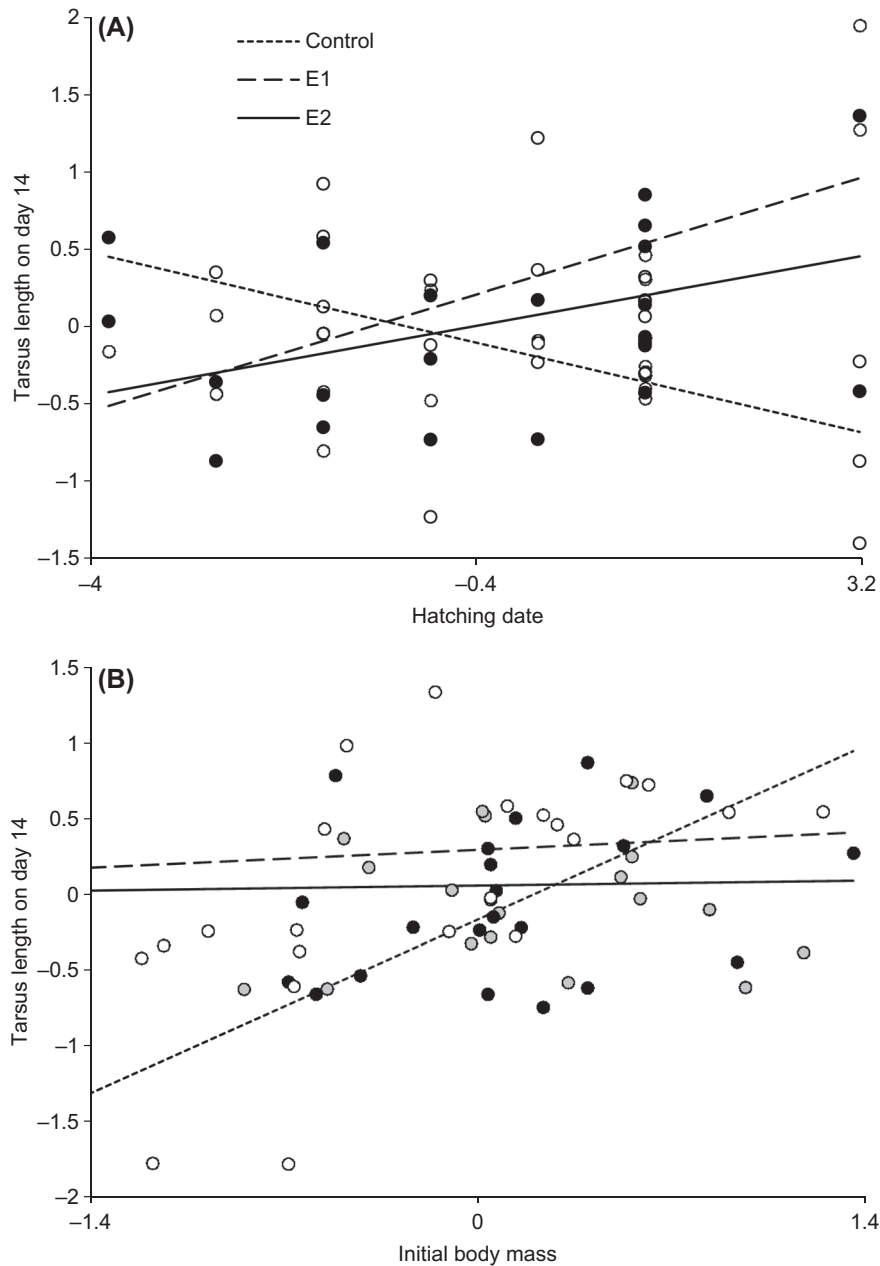
Figure 1. Effect of vitamin E availability on tarsus length. Effect of vitamin E supplementation on collared flycatcher nestling tarsus length at the age of 14 d (in mm) in relation to season (in days, A) and body mass at the start of experiment (in g, B). Depicted are regression lines and residuals; white, grey and black points represents the control, dose E1 and E2, respectively. Continuous variables were centred prior to statistical analysis by subtracting mean. In both cases, the slope of nestlings receiving doses E1 and E2 significantly differed from the slope of control nestlings. For details, see Table 1.

(Larcombe et al. 2010, Matrková et al. unpubl.). The difference in reactions of great tit vs collared flycatcher nestlings was found despite carrying out both studies in the same forest. We think that this difference stems from different nestling diet and different timing of breeding of these species. Caterpillars are estimated to contain more α-tocopherol than aerial insects fed by barn swallow parents to their nestlings (de Ayala et al. 2006, Arnold et al. 2010). Caterpillars dominate both great and blue tit nestling diet (Cholewa and Wesołowski 2011, Remeš and Matysioková 2013), but their share of collared flycatcher nestling diet is lower (Cholewa and Wesołowski 2011; see also Material and

methods). Second, while tocopherol content of caterpillars increases over season, availability of tocopherol-rich caterpillars sharply declines towards the end of season (Arnold et al. 2010). Thus, caterpillars are less available for flycatchers, as flycatchers in our study site breed ca. two weeks later than titmice (our unpublished data). Correspondingly, caterpillars are replaced by other food items in late broods (e.g. ants and flies, Löhrl 1957, 1976). This fits into our results, where only nestlings from late broods benefited from vitamin E supplementation. Thus, we suggest the observed seasonal change in response to vitamin E supplementation is likely to reflect the shift in

nestling diet, which leads to lower vitamin E availability for late broods. Alternatively, but not exclusively, nestling demand for tocopherol may increase over season (e.g. due to increasing temperature or lower food quality – Perrins 1976).

We were surprised to find comparable effects of the two doses of vitamin E (E2 was twice as concentrated as E1). One explanation might be that E1 already provided an optimal level of vitamin E supply and E2 was beyond the saturation threshold (i.e. beyond the threshold where the effect of vitamin E is dose-dependent). However, why would not nestlings then suffer from an excess of vitamin E in the E2-supplemented group? One possible explanation is that the collared flycatcher is a generalist, capable of using different foraging techniques and providing nestlings with a variable diet. Depending on the immediate food supply, parents can switch to different prey types (Bureš 1986, 1995, Török 1986; see also Material and methods). As there is a large difference in tocopherol content among different groups of invertebrates (de Ayala et al. 2006, Arnold et al. 2010), it is likely that the vitamin E content of nestling diet varies considerably even within the same habitat. It is thus possible that collared flycatcher nestlings are adapted for variable tocopherol supply stemming from variable diet supplied by parents. They might thus have evolved a better mechanism to deal with excess supply of vitamin E, which may not be absorbed or may be excreted (Bramley et al. 2000).

Initially smaller nestlings benefited from vitamin E supplementation, while initially larger nestlings did not. At the same time, initial body mass was a strong determinant of body mass in older age. Smaller nestlings may suffer competitive disadvantage from their larger siblings, as parent collared flycatchers do not allocate food according to nestling size or condition, but according to begging intensity (Rosivall et al. 2005). Recently, Moreno-Rueda (2007) suggested that begging may increase oxidative stress. This idea was supported by an experiment in yellow-legged gulls, where vitamin E supplementation increased begging intensity, especially in smaller chicks (Noguera et al. 2010). Noguera et al. (2010) suggest that vitamin E alleviates the oxidative cost of begging, which may enhance solicitation and increase parental feeding effort, resulting in increased growth in supplemented chicks. As at the same level of need smaller chicks are more compromised in survival than larger ones, they should consequently benefit more from more intensive begging (Noguera et al. 2010). The need of antioxidants may be also higher in smaller nestlings, as catch-up growth may increase oxidative damage (Mangel and Munch 2005, Alonso-Álvarez et al. 2007, De Block and Stoks 2008). Consistently with this notion, supplementation of dietary antioxidants in free-living birds reduced oxidative damage only in nestlings limited in their growth by poor nutrition (Hall et al. 2010, Noguera et al. 2011).

Increased availability of vitamin E improved growth of some flycatcher nestlings. Body size at around the time of fledging is an important predictor of future fitness in birds (Gebhardt-Henrich and Richner 1998). In the collared flycatcher, a positive directional selection on tarsus length was found (Kruuk et al. 2001). Thus, although our supplementation did not affect fledging success, higher vitamin E

intake during early life may have resulted in higher post-fledging survival and/or fertility of the young.

To conclude, vitamin E is limiting for collared flycatcher nestlings during development. Experimentally increased intake of vitamin E had a beneficial effect on tarsus growth in late broods and in initially smaller nestlings. Our data thus suggest that increased vitamin E availability may help to compensate for initial disadvantages among nestlings. Future studies would benefit from studying bird species with diverse food preferences and foraging techniques and from including estimates of long-term offspring performance.

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Supplementary material (Appendix JAV-00368 at < www.avianbiology.org/readers/appendix >). Appendix 1.

PŘÍSPĚVEK IV

Matrková J. & Remeš V. 2012: Environmental and Genetic Effects on Pigment-Based vs. Structural Component of Yellow Feather Colouration. PLOS ONE, 7 (5), e36640.

PLoS one

Environmental and Genetic Effects on Pigment-Based vs. Structural Component of Yellow Feather Colouration

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Abstract

Background: Carotenoid plumage is of widespread use in bird communication. Carotenoid-based feather colouration has recently been shown to be dependent on both pigment concentration and feather structure. If these two components are determined differently, one plumage patch may potentially convey different aspects of individual quality.

Methodology/Principal Findings: We evaluated the effects of genetic and environmental factors on carotenoid-based yellow breast colouration of Great Tit (*Parus major*) nestlings. By partial cross-fostering, we separated the genetic and pre-natal vs. post-natal parental effects on both the structural and the pigment-based component of carotenoid-based plumage colouration. We also simultaneously manipulated the post-hatching environment by brood size manipulation. The structural component of nestling colouration reflected features of female colouration. On the other hand, the pigment-based component was more affected by rearing conditions presumably representing food quality. While the structural component was related to both origin- and environment-related factors, the pigment-based component seemed to be environment-dependent only. These results support the notion that pigment-based and structural components of feather colouration are determined differently.

Conclusions/Significance: Chromatic and achromatic components of carotenoid-based feather colouration reflected different aspects of individual quality and history, and thus may potentially form a multicomponent signal.

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Introduction

Signal design is a very important component of animal communication. In visual signals, colouration and overall patterning may be critical for signal efficiency [1]. Thus, factors affecting individual components of a visual signal are of high importance for our understanding of signal function and evolution. In birds, plumage colouration is a conspicuous and versatile trait with important signalling functions [2]. Both feather structure and pigments can determine plumage colouration and are thus important drivers of signal design and efficiency [3]. Structural colours are generated by physical interaction of light waves with tissue matrices, while pigment-based colours are determined by molecular structure of the pigment and its density [4]. Interactions between light-scattering tissue structures and pigment molecules are common in animal colouration, but only one component is typically considered at a time [5]. Accordingly, in behavioural and evolutionary studies, structural and pigment-based plumage colours have usually been treated as distinct. For example, carotenoid-based colouration has been considered to be fully pigment-based (e.g. [6–8]).

Carotenoids are frequently deposited into plumage causing yellow to red hues [9].

However the colour properties of carotenoid-based plumage do not depend solely on the carotenoid content. Recent research has revealed that carotenoid-based feather colouration is produced by the interaction of pigment and feather structure [3,10]. The keratin feather structure uniformly reflects light across all wavelengths, creating the white background. The yellow to red chroma is produced by carotenoids that absorb light in a specific area around 400–500 nm thus eroding reflectance in this wavelength band. Carotenoids cannot produce yellow colour without the white reflective background, because they do not significantly reflect light themselves. A combination of the reflectance of the white feather structure across all wavelengths and the absorbance of specific wavelengths by carotenoid pigments is therefore necessary to produce the yellow to red chroma [3,10].

Experimental studies have firmly established plasticity of carotenoid-based feather colours in relation to a variety of environmental factors. They reflect the nutritional state of a bird, its access to dietary carotenoids [11–13], parasite load and the activation of the immune response [8,13] Carotenoid supplementation has shown that variation in carotenoid content in feathers affects colour properties of these signals [10,14,15]. Similarly to carotenoid-based colouration, colours based on feather structure can be affected by hormones, parasites, nutritional conditions during moult and the moult speed ([16–18,8,19–21]; but see [22]). Thus a critical question arises as to what part of the carotenoid

signal plasticity is due to the variation in pigment content and what part is due to the variation in feather structure. This leads to the further query as to which factors drive variation in these two components. These questions have been rarely investigated [10,23,15,24].

Here we studied carotenoid-based feather colours of nestlings of the Great Tit *Parus major*, which have become an important model system for the investigation of signal function and plasticity. A wide spectrum of environmental and genetic determinants of carotenoid colouration have been evaluated in both Great Tit [25–34,14,35–38] and the Blue Tit *Cyanistes caeruleus* [39–42,31,43–48,33,21,49,50] offspring. However, only a few studies discriminated between the structural and the pigment-based component of carotenoid colouration in titmice [23,51,52,15,24], or in any other species of bird [10]. Consequently, we lack a firm understanding of the relative plasticity of these two components of feather colouration. To our knowledge, no study to date has assessed the genetic determination of these two components separately in juvenal plumage.

Thus, here we evaluated effects of genetic and environmental factors on both the structural and the pigment-based component of yellow carotenoid-based breast colouration of Great Tit nestlings. By partial cross-fostering, we separated the genetic and pre-natal vs. post-natal parental effects on nestling plumage colouration. We also simultaneously manipulated the post-hatching environment by brood size manipulation to expose nestlings to rearing conditions of different quality. Additionally, we directly measured a number of environmental factors (hatching date, egg yolk antioxidants, feeding rate of parents), as well as carotenoid-based breast colouration of females. We analysed possible effects of these factors separately on pigment-based and structural components of nestling carotenoid-based colouration. We show that the structural and the pigment-based component of carotenoid-based colouration reflect different aspects of the rearing environment and the genetic background of nestlings.

Methods

Ethics Statement

Standard methods in capturing and handling birds used in the research of cavity-nesting passerines were used. Adults were captured in the nest-box. They were handled for as short time as possible to minimise any distress. The smallest number of feathers possible to obtain reliable results were plucked, which was based on a previous methodological study [53]. This study complies with the current law of the Czech Republic. We had all necessary permits for this study, and it was approved by the Ethical Committee of Palacký University.

The study was permitted by: Project of experiment according to Section 12, the Decree No. 311/1997 Coll., on the breeding and use of experimental animals (Faculty of Science, Palacký University Olomouc, ID 45979/2001-1020), ringing licence (Vladimír Remeš, ID 1051), the decision on the derogation according to Section 5b (Conditions for Derogations in Protection of Birds), the Act no. 114/1992 Coll., on Nature and landscape protection (Without ID, approved by The Department of Environment, Municipality of Olomouc).

Study Site

This work was conducted on three adjacent nest-box plots (188 nest-boxes in total, their design is described in [54] – study site Olomouc) in a deciduous forest near Grygov (49°31′N, 17°19′E) in the eastern Czech Republic. The forest is dominated by lime *Tilia* spp. and oak *Quercus* spp. with interspersed ash *Fraxinus*

excelsior, common alder *Alnus glutinosa* and common hornbeam *Carpinus betulus*. Nest-boxes were placed about 1.5 m above ground. These nest-boxes were, besides Great Tits, inhabited by Blue Tits, Collared Flycatchers *Ficedula albicollis*, and Nuthatches *Sitta europaea*.

General Fieldwork

Field work was carried out in 2005 from early April until mid-June when the nesting of Great Tits terminated. We checked the nest-boxes every other day to record laying of the first egg. Subsequently, we numbered the eggs with a water proof felt pen. Before birds started the incubation we removed one egg from each clutch. The order of the removed egg in laying sequence was on average 4.2 (ranged 3 to 5). The removed eggs were weighed and stored in –20°C for subsequent analyses.

One day after the clutch was completed we weighed the whole clutch on a digital balance to the nearest 0.01 g. At the end of incubation, we visited the nests daily to find out the day of hatching. The day when the first nestling hatched was considered the day 0 of the brood age. When the young were six days old, we ringed them with an aluminium ring. On day 14, we measured the right tarsus with a digital caliper to the nearest 0.01 mm, the right wing (the longest primary) with a ruler to the nearest 0.5 mm, and weighed the young on a digital balance to the nearest 0.1 g. For each bird, we took from 10 to 15 yellow feathers from the upper right part of the breast for later spectrophotometric analysis.

We captured females in 43 out of 46 nest-boxes during the nestling period (the median age of the young = 7 days). Due to time constraints, we were not able to capture males. We measured the tarsus and wing length of the females, weighed them, and removed breast feathers for the analysis in the same way as in the young. We determined the age of the birds based on their plumage as one year old or older [55].

When nestlings were eight days old (except 5 nests; range 6 to10 days), we placed a video camera about five meters in front of each nest-box on the ground. Parental activity was recorded for 90 minutes in the morning hours (from 7:30 to noon). We discarded the first 15 min of recording and quantified the feeding rate per hour.

Analyses of Yolk Antioxidants and the Feather Colouration

We analysed the concentration of lutein, zeaxanthin, vitamin E and vitamin A in the egg yolks as in [56]. Briefly, we extracted the samples by acetone/methanol method and injected them into the HPLC system. We used an Agilent 1100 Series HPLC system (Agilent Technologies, Waldbronn, Germany). LC separation was carried out on a Zorbax SB-CN rapid resolution (75×4.6 mm, particle size 3.5 μm), reversed-phase column (Agilent Technologies, USA). The mobile phase consisted of 0.05 mM (v/v) ammonium formate and methanol. The flow rate was 0.7 mL/min and typically 10 μL aliquots were injected into the column. The column oven temperature was set at 30°C.

According to the standard procedure [57], we quantified the reflectance spectra of yellow feathers sampled from the breast. We used on average 10 feathers from each bird (10 ± 3.1 feathers; mean ± SE) We used Avantes AvaSpec-2048 fiber optic spectrometer together with AvaLight-XE xenon pulsed light source and WS-2 white reference tile. The probe was used both to provide light and to sample the reflected light stream and was held perpendicular to the feather surface. Feathers were arranged on a black, nonreflective surface so that they overlapped extensively. We took three and five readings per a young and a female, respectively, each from a different part of each set of

feathers. We obtained reflectance (in %) from the wavelength of 320 to 700 nm in 1-nm increments.

We wanted to test the effect of the origin and rearing environment on both the pigment-based and the structural components of the feather colouration of Great Tit young. To assess the structural component of the feather colouration, we calculated **background reflectance** of the feathers as the sum of absolute reflectance between 575 and 700 nm [15]. In this area, the light reflected by the feather structure is unaffected by the carotenoid content, because carotenoids absorb light at lower wavelengths [15,57]. Accordingly, experimental carotenoid extraction from American Godfinch feathers only weakly affected the reflectance above 575 nm [3,10]. The **absolute carotenoid chroma** ($-1 \times (R_{400-515}/R_{575-700})$) represented the pigment-based component of the colouration. Absolute carotenoid chroma correlates with the carotenoid content of feathers [15]. For convenience, we made the values of absolute carotenoid chroma negative (see the multiplication by –1 above) so that the correlation with feather carotenoid content was positive in sign. Accordingly, our absolute carotenoid chroma was strongly positively correlated with the carotenoid chroma ($R_{700}- R_{450})/R_{700}$) of the feathers in both the young (r = 0.97, P<0.001, n = 373) and females (r = 0.99, P<0.001, n = 43). At the same time, carotenoid chroma was shown by theoretical modelling to directly reflect the amount of carotenoids in feathers [57] and correlated positively with feather carotenoids in the Great Tit in previous studies [14,35]. These correlations suggest that our absolute carotenoid chroma was a good indicator of the carotenoid content of yellow breast feathers. Absolute carotenoid chroma correlated only weakly with background reflectance in nestlings (r = 0.21, P<0.001, n = 353) and not at all in females (r = –0.06, P = 0.71, n = 43).

In statistical analyses, we used the average values of colour characteristics calculated from the five readings from each set of feathers for females and from the three readings for nestlings.

Cross-fostering and Brood Size Manipulation

One day after the first young in the clutch hatched, we performed a partial cross-fostering experiment with simultaneous brood size manipulation. Cross-fostering was performed between pairs of nests – dyads. We assigned nest to dyads based on their same hatching day and when possible also their same clutch size. There was no difference in clutch size in 13 dyads, in 8 dyads nests differed by one egg, and in 1 dyad by two eggs.

We weighed all young on a digital balance to the nearest 0.01 g. Within the nest of origin, we ranked them according to their weight from the heaviest to the lightest. Beginning either from the first or the second heaviest nestling of each nest, we swapped every other young between the two nests of the dyad. The rest of the young stayed in their nests of origin. In this way, we exchanged either even- or odd-ranked nestlings (according to the mass hierarchy) within the dyad. The choice of odd- or even-ranked nestlings to be exchanged alternated between subsequent dyads. The mass hierarchy of nestlings after cross-fostering was kept close to the original mass hierarchy before cross-fostering. The weight of the cross-fostered and non-cross-fostered young on the day of cross-fostering did not differ (LMM, nest of origin as a random factor: $F_{1,336}<0.01$, P = 0.94).

We intended to manipulate brood size by two young. Thus, during the cross-fostering, in one nest of each dyad we randomly chose one extra nestling and took it also to the foster nest. In the second nest of the dyad, we additionally randomly chose one nestling not to be cross-fostered and left it in its nest of origin. In this way we increased the brood in the second nest of the dyad by two nestlings, leaving the first nest two nestlings short. The design

of cross-fostering and brood size manipulation is available in supplementary material (see fig S1). Brood size manipulation significantly affected brood size on the day of feather sampling (t test: $t_{40} = 7.67$, p<0.001, $R^2 = 0.60$, enlarged brood: 10.7±0.31 young; reduced brood 7.3±0.31 young; mean ± SE) and nestling size. Nestlings from enlarged broods were on this day lighter than nestlings from reduced broods (16.21±0.169 g vs. 17.04±0.174 g; LMM, nest of origin and nest of rearing as random factors: $F_{1,16.8} = 13.88$, P = 0.002), they had shorter tarsi (22.66±0.073 mm vs. 22.87±0.078 mm; $F_{1,16.0} = 6.21$, P = 0.024) and were in worse condition (–0.24±0.134 vs. 0.39±0.140; $F_{1,8.7} = 18.7$, P = 0.002). On average the whole process of cross-fostering took 13.4 minutes per nest-box (range 9 to 20 minutes). At the beginning of the cross-fostering experiment, we marked the nestlings by clipping down feathers on the head and back for their further individual recognition. In cases with unequal brood size within the dyad, the procedure was adjusted so that the result was the same as in dyads with the same number of the young (i.e., mass hierarchy was kept similar to original nests and brood size was manipulated by two young). Larger broods become enlarged as often as smaller broods in dyads with unequal brood size (4 and 5 cases, respectively).

Statistical Analyses

To identify effects of individual factors on the pigment-based and the structural component of the nestling feather colour, we fitted linear mixed models (LMM) with *background reflectance* and *absolute carotenoid chroma* as dependent variables. *Nest of origin* and *Nest of rearing*, both nested within the *Dyad*, and *Dyad* itself, were entered as random factors. *Dyad* represented a matched pair of cross-fostered nests and accounted for the variability due to the pair of nests. *Nest of origin* accounted for any pre-cross-fostering effects and included genetic effects, prenatal maternal effects and environmental effects early after hatching up to one day of age when cross-fostering took place. *Nest of rearing* accounted for post-cross-fostering effects, i.e. the rearing environment including the parental care.

As fixed factors, our models included *background reflectance* and *absolute carotenoid chroma* of genetic and foster mothers, their *age*, total *feeding rate per capita* (summed feeding rate of both parents per hour per nestling at day 8), *yolk antioxidants*, and *brood size manipulation* (categorical variable, level + or –). Brood size itself was not included in the model, as it tightly correlated with both *nestling condition* (r = –0.49, P<0.001, n = 42) and feeding rate per capita (r = 0.70, P<0.001, n = 43). We wanted to avoid collinearity of our predictors and thus we modelled possible effect of the brood size by including these two factors. Yolk antioxidants were represented by the first principal component (PC1) from a principal component analysis (PCA). PCA was run on concentrations ($\mu g \, g^{-1}$) of vitamin A, vitamin E, lutein and zeaxanthin in egg yolk, and PC1 explained 69.1% of the total variance (factor loadings: vitamin A: 0.77; vitamin E: 0.76; lutein: 0.88; zeaxanthin: 0.89; all factors $\log_{10}$-transformed). The results for models with PC1 did not differ from models where these antioxidants were included separately (results not shown). The model further included *nestling condition* (residuals from the regression of body mass in g on tarsus length in mm at day 14) and *hatching date*. To control for feather development in nestlings, we included the *feather length* (average length of five breast feathers) as a fixed factor. Background reflectance significantly correlated with feather length (r = 0.15, P = 0.008, n = 296), whereas absolute carotenoid chroma did not (r = –0,10, P = 0.083, n = 296).

Nestlings in four nests did not survive until the feather sampling and final measurements. For the delayed feather development, we

excluded from the analyses extremely small (n = 4) nestlings and nestlings hatched after cross-fostering (n = 25). Further nestlings were excluded from the analyses due to missing data (e.g. female was not captured, unsuccessful antioxidant analysis etc.). As a result, 296 nestlings in 40 nests and 21 dyads were included into analyses.

We tested LMM with procedure MIXED in SAS. The covariance parameters were estimated by the REML covariance method. We used the COVTEST statement to produce asymptotic standard errors and Wald Z-tests for the covariance parameter estimates. Variables were checked for normal distribution. Residuals from each model were checked to conform to the requirements of normal distribution, equal variance, and linearity [58]. To compare the size of effects both within and between models, we used standardized regression coefficients. We standardized all continuous input variables by subtracting mean and subsequently dividing by two standard deviations. Due to this standardization, effect sizes for continuous and categorical factors are directly comparable [59]. Finally, to compare mean effect size of specific subsets of factors regardless of the direction of individual effects, we summed absolute values of their standardized regression coefficients. All statistical analyses were conducted in SAS 9.2 (LMM) and JMP 7.0.1 (other tests and data transformations).

Results

Absolute carotenoid chroma of nestlings averaged –0.570 (SD = 0.0681, n = 296) while in adult females it averaged –0.493 (SD = 0.0502, n = 40). Background reflectance of nestlings was on average 4459 (SD = 1196.8, n = 296) whereas that of adult females was on average 4651 (SD = 711.1, n = 40; Fig. 1).

Pigment-based Component of Feather Colouration

The absolute carotenoid chroma of nestlings increased during the season. Nestlings reared by older females also had higher absolute carotenoid chroma, indicating a higher concentration of carotenoids in feathers. Neither the pigment-based nor the structural component of the yellow breast feathers of rearing and genetic mothers predicted absolute carotenoid chroma of the nestlings. Similarly, neither the concentration of antioxidants in yolk nor the brood size manipulation affected absolute carotenoid chroma of nestlings. Only small amount of variation in absolute carotenoid chroma of nestlings was explained by the nest of rearing or the nest of origin (Table 1, Fig 2).

Structural Component of Feather Colouration

Females with lower absolute carotenoid chroma and thus a lower carotenoid content of the yellow breast feathers produced nestlings with higher background reflectance. By contrast, a nearly significant positive relationship was observed between background reflectance of nestlings and absolute carotenoid colouration of the foster female. Background reflectance of nestlings increased with background reflectance of the rearing female, and was significantly explained by the nest of rearing. No other examined factor was significant (Table 2, Fig 2).

Pigment-based vs. Structural Component

Both pigment-based and structural components of nestling carotenoid colouration were related to the environmental conditions. However, the structural component of the feather colouration was also partially genetically determined. The pigment-based component seemed to depend on the rearing conditions related to food quality. By contrast, the structural component seemed to be related to the female colouration and to the general rearing environment which was not represented by the fixed factors included in our study (Tables 1, 2; Figs 2, 3).

Discussion

In this study, we separated origin- vs. environment-related determinants of both the structural and the pigment-based components of carotenoid-based feather colouration. Great Tit nestlings reared by older mothers and those that hatched later in the season were more intensely yellow. Carotenoid chroma of nestlings was independent of the colouration of both the rearing and the genetic mother and of the antioxidant concentration in the egg yolk. On the other hand, the structural component of nestling
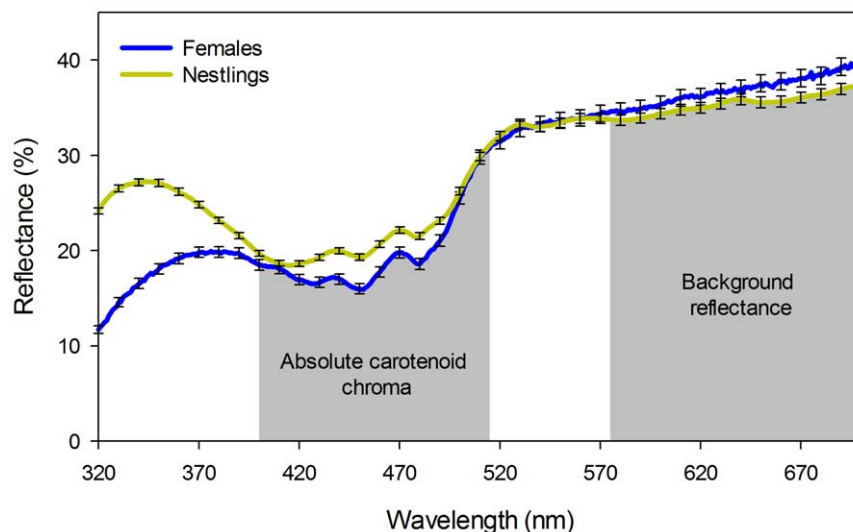

Figure 1. Reflectance curve of Great Tit yellow breast feathers measured by objective spectrophotometry. Mean value (± SE) for females (n = 40) and nestlings (n = 296) is given. The wavelengths used to calculate absolute carotenoid chroma ($-1 \times (R_{400-515}/R_{575-700})$) and background reflectance ($\Sigma R_{575-700}$) are highlighted in grey.
doi:10.1371/journal.pone.0036640.g001

Table 1. Linear mixed model explaining absolute carotenoid chroma of yellow breast feathers in Great Tit nestlings.

Effect	Estimate	SE	Den. DF	F	P
FIXED EFFECT					
Intercept	−1.583	0.36	22.0		
Absolute chroma of rearing mother	0.071	0.13	25.3	0.3	0,58
Absolute chroma of genetic mother	0.066	0.12	29.4	0.3	0.59
Background reflectance of rearing mother	2.4×10^{-6}	8.2×10^{-6}	24.1	0.1	0.77
Background reflectance of genetic mother	-6.3×10^{-6}	8.0×10^{-6}	31.5	0.6	0.44
Age of rearing mother*	−0.032	0.01	25.5	6.0	**0.02**
Age of genetic mother*	0.016	0.01	32.3	1.5	0.23
Brood size manipulation**	0.005	0.01	17.6	0.3	0.59
Hatching date	0.009	2.5×10^{-3}	21.9	11.7	**<0.01**
Yolk antioxidants, PC1***	-1.8×10^{-4}	3.3×10^{-3}	25.0	<0.1	0.96
Feeding rate per capita	0.007	4.6×10^{-3}	31.4	2.6	0.12
Nestling condition	−0.001	4.7×10^{-3}	223.0	0.1	0.79
Feather length	−0.007	2.4×10^{-3}	245.0	8.0	**<0.01**
RANDOM EFFECT	Estimate	SE	% Var	Walds Z	P
Nest of rearing (Dyad)	2.4×10^{-4}	2.8×10^{-4}	5.3	0.9	0.20
Nest of origin (Dyad)	3.0×10^{-4}	2.7×10^{-4}	6.9	1.1	0.13
Dyad	2.7×10^{-4}	3.7×10^{-4}	6.2	0.7	0.23
Residual	0.004	3.3×10^{-4}	81.4		

For fixed effects, type 3 tests and denominator DF are presented, numerator DF = 1 in all cases. For random effects, covariance parameter estimates are presented (REML method). Likelihood ratio test of the overall significance of random effects: $\chi^2 = 15.09$, DF = 3, P = 0.002. P-values of significant factors are in bold. Least squares means ± SE for nestling reared by 1y old females: −0.58±0.01, nestling reared by older females: −0.55±0.01.
*Estimate for 1y old (relative to older) females.
**Estimate for reduced (relative to enlarged) broods.
***PC1 of yolk antioxidants included the concentrations of vitamin A, vitamin E, lutein and zeaxanthin in egg yolk; all concentrations were $\log_{10}$-transformed.
doi:10.1371/journal.pone.0036640.t001

colouration was related to the feather colouration of both the rearing and the genetic mother and to the nest of rearing, but not to the hatching date, the feeding rate or egg yolk antioxidants. Thus, the two components reflected different aspects of the nestling environment and only the structural component was related to the genetic/pre-natal factors. These results support the notion that the pigment-based and the structural components of feather colouration are determined independently [10,15].

The expression of carotenoid-based signals is likely to be affected by a complex system of physiological trade-offs as well as non-physiological costs and benefits (e.g. predation risk or social interactions; see [60] for a review). However, the intensity of the chromatic component of the carotenoid-based colouration has been repeatedly shown to depend primarily on the carotenoid content of feathers. This in turn depends on the carotenoid access in the diet (see [7,8] for a review). Here we demonstrated that carotenoid chroma of nestlings was related to two factors presumably connected with the quality of the food delivered by parents, namely the season and the age of rearing mother. Breast feathers of Great Tit nestlings hatched later in the season are often more yellow (e.g. [25,27,36]). This occurs despite the fact that the carotenoid content in their main food, caterpillars, tends to be stable or decrease in the season [61,62]. However in our population, the concentration of egg yolk carotenoids increased with the season [56] suggesting that the carotenoid supply may have increased as well. Moreover, older foster mothers raised more chromatic nestlings as compared to 1y-old females, which could suggest that they were able to supply the young with higher-quality food. This is interesting in relation to a recent observation that

Great Tits are able to discriminate food based on its carotenoid content [63]. Despite the experimental evidence that food access is critical for the expression of carotenoid-based colouration [64], but in accordance with a similar study in nestling Great Tits [28], we found no relationship between food quantity represented by feeding rate, and nestling breast colouration. Thus, although the prey biomass delivered to nestlings strongly increased with feeding rates in our population (V. Remeš, unpubl. data), it seems that the amount of food provided by parents was not limiting for the expression of nestling carotenoid colouration in our population. In sum, we were able to identify the specific environmental factors explaining variation in the chromatic component of the carotenoid-based signal, which were presumably related to the food quality. This result agrees with previous studies (see [7,8] for a review).

Carotenoid-removal experiments revealed that the white structural background of carotenoid-coloured feathers is crucial for the production of yellow carotenoid-based displays [3,10,15]. It is produced by an incoherent scattering of all visible wavelengths from a nanostructure of keratin and air vacuoles. Structural colours have been suggested to have a limited condition-dependence [4]. However experimental studies are rare and their results are ambiguous (see [22] for details). Moreover, structural colours are produced by a diverse set of nanostructures, which are likely to differ in developmental mechanisms, so any generalization among different anatomical systems should be made with caution [4]. The reflectance properties of the structural white feather background remained unaffected by the experimentally induced fast moult in the Blue Tit [21] and the manipulation of food and
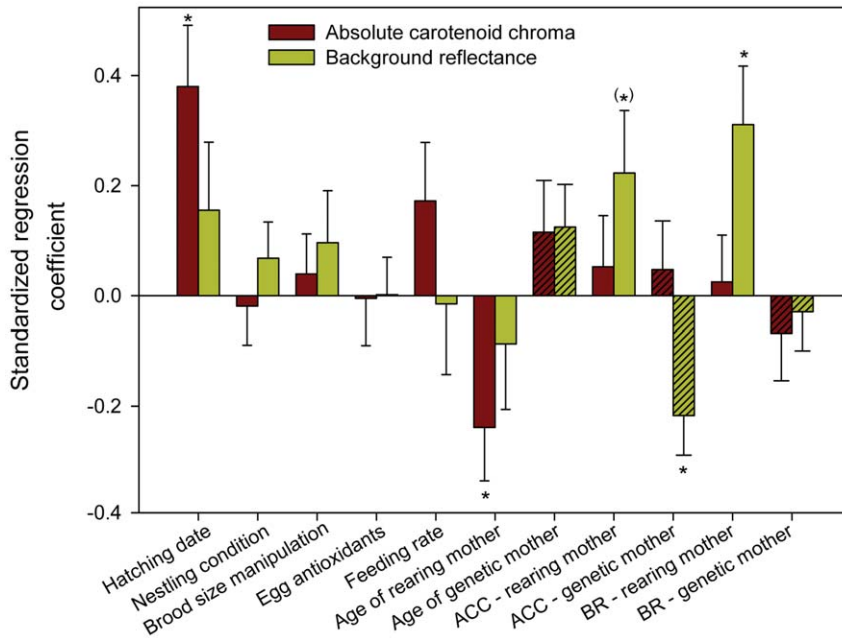
Figure 2. Standardized effects of genetic and environmental factors on yellow breast colouration of Great Tit nestlings. Depicted are regression coefficients (+ SE) of fixed factors from linear mixed models. Hatched bars depict effects of the genetic mother. Asterisks denote significance at *p<0.05, (*) p<0.06. ACC = absolute carotenoid chroma, BR = background reflectance. Parameter estimates are given for 1y old (relative to older) females and reduced (relative to enlarged) broods. Only fixed effect are included (a significant part of background reflectance is explained by nonspecific environmental conditions, represented by the random effect of the nest of rearing).
doi:10.1371/journal.pone.0036640.g002

Table 2. Linear mixed model explaining background reflectance of yellow breast feathers in Great Tit nestlings.

Effect	Estimate	SE	Den. DF	F	P
FIXED EFFECTS					
Intercept	−6258.48	7031.07	23.5		
Absolute chroma of rearing mother	5306.85	2703.22	36.1	3.8	**0.06**
Absolute chroma of genetic mother	−5307.99	1757.94	23.1	9.1	**<0.01**
Background reflectance of rearing mother	0.52	0.18	31.5	8.6	**<0.01**
Background reflectance of genetic mother	−0.05	0.12	25.6	0.2	0.68
Age of rearing mother*	−208.08	284.71	33.3	0.5	0.47
Age of genetic mother*	298.35	184.96	26.6	2.6	0.12
Brood size manipulation**	230.24	226.02	19.8	1.0	0.32
Hatching date	62.26	49.56	23.4	1.6	0.22
Yolk antioxidants, PC1***	1.18	46.69	19.2	<0.1	0.98
Feeding rate per capita	−11.28	96.12	36.0	<0.1	0.91
Nestling condition	79.14	77.00	241.0	1.1	0.30
Feather length	3.29	38.62	261.0	<0.1	0.93
RANDOM EFFECTS	Estimate	SE	% Var	Walds Z	P
Nest of rearing (Dyad)	297099	153221	22.6	1.9	**0.03**
Nest of origin (Dyad)	11262	46395	0.9	0.2	0.40
Dyad	86195	140129	6.5	0.6	0.27
Residual	922179	84174	70.0		

For fixed effects, type 3 tests and denominator DF are presented, numerator DF = 1 in all cases. For random effects, covariance parameter estimates are presented (REML method). Likelihood ratio test of the overall significance of random effects: $\chi^2 = 39.9$, DF = 3, P<0.001. P-values of significant factor are in bold.
*Estimate for 1y old (relative to older) females.
**Estimate for reduced (relative to enlarged) broods.
***PC1 of yolk antioxidants included concentrations of vitamin A, vitamin E, lutein and zeaxanthin in egg yolk; all concentrations were $\log_{10}$-transformed.
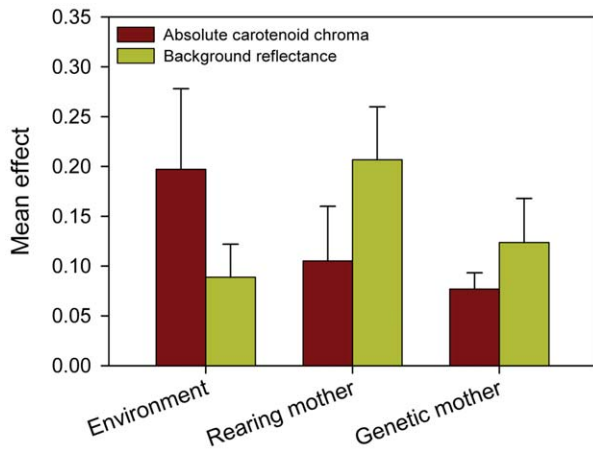doi:10.1371/journal.pone.0036640.t002

Figure 3. Overall effects of the rearing environment and rearing vs. genetic mother on colouration of Great Tit nestlings. Depicted are means (+ SE) of absolute values of standardized regression coefficients (see Fig. 2) to compare overall effect size regardless of effect direction. Environment includes hatching date, brood size manipulation and per-capita feeding rates. Effects of the rearing and the genetic mother include their age and colouration (absolute carotenoid chroma and background reflectance). Only fixed effect are included (a significant part of background reflectance is explained by nonspecific environmental condition, represented by the random effect of the nest of rearing).
doi:10.1371/journal.pone.0036640.g003

carotenoid intake in the American Goldfinch *Carduelis tristis* [10]. By contrast, Great Tit nestlings raised in experimentally reduced broods developed the breast plumage with higher reflectance of the white background structure compared with nestlings from control broods [15]. Dark-eyed Juncos *Junco hyemalis* on a high-protein diet also grew brighter white tail patches than individuals on a low-protein diet [19]. Contradictory findings of experimental studies conducted up to now may reflect interspecific differences in condition-dependence, differences between the juvenal plumage ([15], this study), the first basic plumage [21], the first alternate plumage [10] and the feathers replacing removed feathers [19], or they may reflect sex-specific effects ([10,19] studied only males, whereas [21,15] studied both sexes). We found that the reflectance of the achromatic component of Great Tit nestlings was related to both the rearing environment and attributes of the genetic mother (her carotenoid chroma). Currently, we are not able to explain the mechanism of the antagonistic effect of the rearing vs. the genetic mother chromatic colouration on the nestling achromatic component. A substantial proportion of the variance in the structural component was explained by the random factor of the nest of rearing. Thus, in contrast with the pigment-based component, we were not able to identify specific factors explaining the structural component. This is especially puzzling in the case of brood size manipulation. Although it affected growth of the nestlings (see above), it had no effect on nestling colouration. It seems that structural component of nestling colouration is affected by environmental factors impacting other parameters of nestlings than body size (e.g. certain physiological systems). Overall, our results suggest that in our population the nestling feather colouration is under the control of both environmental and genetic/pre-natal effects.

Pigment-based and structural components of the carotenoid-based colouration are in our population of Great Tits subject to different levels of the environmental vs. the genetic/pre-natal determination. This may be mediated by a single coloured patch

drawing from distinct biochemical pools, e.g. diet-derived carotenoids and synthesized keratins [5]. Hence, a single carotenoid patch may serve as a multicomponent signal, simultaneously conveying different aspects of the individual quality and history [65,3,10,66,52,15,24]. Display of multicomponent signals may provide a variety of benefits, including conveying more information to the receiver or an improvement of efficacy of transmission, reception, and processing of signals (see [67] for a review). However, it is important to stress that the two components can interact in a complex way and that multicomponent signals can be fully understood only by investigating their components simultaneously [68,69]. For example, the brightness of feather structure interacts with its carotenoid content in colour production. High carotenoid concentration may produce intensive chroma only if the underlying feather structure is sufficiently reflective. Alternatively, if the structural component is very bright, this might make it necessary to add more carotenoids to the feather to get the same carotenoid chroma as in a feather with low structural reflectance. Thus, production of the most chromatic yellow signal might require a balance between these two components of colouration. These and similar questions remain virtually unexplored. However, the potential for the two components to interact in producing a visual signal is given by the perception system of birds, i.e., to what extent birds perceive the two components separately versus as one visual signal (e.g. [24]).

Juvenal coloration of Great Tit young became an important model system in the investigation of the expression of carotenoid colouration. However, it is important to note two caveats. First, it is not sure what the current function of the yellow breast coloration in Great Tit nestlings might be. Since breast feathers are moulted in autumn before subsequent breeding season [55], sexual selection cannot play a role. Juvenal breast coloration does not seem to affect parental favouritism when feeding the nestlings in the Great Tit ([70,71], but see [72]). Similarly, there seems to be no natural selection on this trait after leaving the nest, at least in a Swiss population [73]. The possibility of correlated selection through adult plumage was also ruled out by the lack of correlation between juvenal and adult yellow breast coloration in the same population [28]. Second, extrapolation of results from the study of juvenal coloration to adult coloration might be troublesome. Studies of adult yellow coloration in titmice differ in the role they ascribe to genes versus environment in determining the expression of this trait (Great Tit [24,38]; Blue Tit [43,44]), which makes direct comparison with the studies conducted on nestlings difficult. Moreover, the function of yellow breast coloration in adult Great Tits is not clear either. Although some studies suggested that this colour patch might be a signal of individual quality [74], other studies found no functional significance [75,76], and still others suggested that it might even have a function in crypsis [77].

To conclude, our study showed that the pigment-based and the structural components of the carotenoid-based colouration were determined differently. The chromatic component was related to specific environmental factors, whereas the achromatic component was related to both female-related and nonspecific environmental factors as well as genetic/pre-natal factors. Our results reinforce the hypothesis that the carotenoid-based colouration may serve as a multicomponent signal, with the chromatic and the achromatic components reflecting a different aspect of the individual's quality and/or history. We suggest that further experimental studies focus on the effects of specific environmental and genetic factors on both the feather pigment content and the nanostructure in carotenoid-based feather patches. Future studies should also focus on the effects of the nanostructure and the pigment content on the reflectance of feathers [5]. Here, appropriate methods of scoring

the feather reflectance/colouration need to be used, as different methods can generate different results (see [52]). The hypothesis that the variability in both components can be recognized by birds and that both components are used either separately or in interaction in communication needs to be verified by visual modelling and behavioural tests.

Supporting Information

Figure S1   The design of cross-fostering and brood size manipulation. Each box represents a dyad consisting of two synchronously timed nests (points represent hypothetical nestlings). Nestlings within their nest of origin are ranked according to their weight from the heaviest to the lightest. We exchanged either even- or odd-ranked nestlings (exchange of even-ranked nestlings is depicted). The brood size was manipulated by two randomly chosen young: In one nest of the dyad one extra nestling was taken

also to the foster nest. In the other nest of the dyad one extra nestling was left it in its nest of origin.
(TIF)

Author Contributions

Conceived and designed the experiments: VR. Performed the experiments: VR. Analyzed the data: JM. Wrote the paper: JM VR. Measured feather reflectance: JM.

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UNIVERZITA PALACKÉHO V OLOMOUCI

PŘÍRODOVĚDECKÁ FAKULTA

KATEDRA ZOOLOGIE A ORNITOLOGICKÁ LABORATOŘ

Vlivy rodičů a prostředí na kvalitu a zbarvení mláďat pěvců

Autoreferát disertační práce k získání titulu Ph.D.

Jana Matrková

Doktorský studijní program Biologie

Studijní obor Zoologie

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Autoreferát disertační práce

Uchazeč:	**Mgr. Jana Matrková**

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OBSAH

4

ÚVOD

Prostředí, v němž mláďata ptáků vyrůstají, zásadně a na celý život ovlivňuje řadu jejich znaků, včetně morfologie, metabolismu, imunity či chování (Lindström 1999, Monaghan 2008). V době závislosti mláďat na rodičích vliv prostředí zprostředkovává z rozhodující části péče rodičů. Jedním z nejdůležitějších aspektů, jímž rodiče ovlivňují prostředí mláďat, je jejich výživa (Bernardo 1996, Price 1998). Potrava v raném věku je proto klíčovým faktorem pro fenotyp i zdatnost dospělého jedince (Metcalfe and Monaghan 2001), v době rozmnožování však bývá pro ptáky limitující (Martin 1987, Newton 1990).

Dostupnost potravy je často značně proměnlivá v prostoru. Vliv této proměnlivosti na hnízdění ptáků lze hodnotit na několika úrovních. U drobných pěvců to tradičně bývá úroveň biotopu, což odpovídá úrovni populace. Na úrovni jedinců, resp. jednotlivých párů, může být vhodnějším ukazatelem dostupnosti potravy velikost a kvalita konkrétního teritoria (Wilkin et al. 2007). Hlavní potravou sýkor jsou housenky motýlů (Lepidoptera; Cholewa and Wesołowski, 2011, Perrins, 1979). Protože duby často hostí více housenek než ostatní druhy stromů, a protože rodiče drobných pěvců sbírají potravu na velmi omezeném území (Naef-Daenzer a Keller, 1999; Naef-Daenzer 2000), je jako indikátor kvality teritoria u sýkor používán zejména počet dubů v bezprostředním okolí hnízda (viz např. Maícas et al., 2014; Naef-Daenzer et al., 2004; Wilkin et al., 2009). Přestože většina dosavadních prací zjistila pozitivní vztah mezi některými ze sledovaných parametrů hnízdění sýkor a množstvím dubů v okolí hnízda, žádná z nich zatím nepotvrdila souvislost počtu dubů s celkovou úspěšností hnízdění.

Růst a vývoj mláďat ovlivňuje nejen množství, ale i kvalita potravy poskytované rodiči. Dostupnost mikronutrientů v raném období má dlouhodobé dopady na morfologii, chování a fyziologii jedince (Blount et al. 2003, Marri a Richner 2014, Noguera et al. 2015a, b). Ve své práci studuji význam mikronutrientů pro fenotyp mláďat krmivých ptáků na příkladu vitamínu E. Vitamín E má klíčovou roli v rozmnožování, normálním vývoji a imunitě ptáků (Surai 2002, Catoni et al. 2008). Tradičně je považován zejména za důležitý antioxidant, který brání oxidačnímu poškození buněk (Surai 2002). V současnosti je však význam antioxidačních schopností vitamínu E *in vivo* zpochybňován a větší potenciál je spatřován v jeho dalších funkcích (Brigelius-Flohé 2009). Zatímco role vitamínu E v rozmnožování drůbeže je již dlouho předmětem výzkumu a je dobře známá (Surai 2002), experimenty na volně žijících

ptácích započaly teprve nedávno a jejich výsledky nejsou jednoznačné (viz např. de Ayala et al. 2006, Noguera et al. 2011, Larcombe et al. 2010, Marri a Richner 2015).

Prostřednictvím výživy mohou rodiče ovlivnit také zbarvení mláďat (Biard et al. 2009). Zbarvení přitom může rozhodovat o účinnosti vizuálních signálů a tím významně ovlivnit komunikaci mezi ptáky (Andersson 2000). Rané prostředí formuje také zbarvení v pozdějším životě (Evans a Sheldon 2012, 2015, Walker et al. 2013). Tradičně jsou rozlišovány tři hlavní kategorie zbarvení ptačího peří: melaninové, karotenoidové a strukturální (Hill a McGraw 2006). Největší pozornost je věnována karotenoidům, druhému nejčastějšímu barvivu, které obvykle způsobuje různé odstíny žluté, oranžové a červené barvy (McGraw 2006). Plasticita karotenoidového zbarvení peří byla potvrzena řadou experimentálních studií: tyto ornamenty např. odrážejí úroveň výživy jedince a obsah karotenoidů v potravě, míru parazitace a aktivace imunitní odpovědi (Olson a Owens 1998, Hill 2002, Price 2006, Blount a McGraw 2008). Obvykle jsou vlastnosti karotenoidových ornamentů připisovány pouze karotenoidovým pigmentům, konkrétně jejich molekulární struktuře a koncentraci (viz např. Prum 2006). Na výsledné barvě se ale běžně podílí také jemná podkladová struktura pera. Každá z těchto složek přitom může být determinována nezávisle (Shawkey a Hill 2005, Jacot et al. 2010).

CÍLE PRÁCE

V předkládané disertační práci zkoumám vliv rodičovských efektů a kvality prostředí na fenotyp mláďat volně žijících pěvců. Konkrétně se zabývám vlivy kvality teritoria a prostředí poskytovaného rodiči na kvalitu a zbarvení mláďat sýkory koňadry (*Parus major*) a lejska bělokrkého (*Ficedula albicollis*).

Jako ukazatel kvality teritoria jsem použila strukturu vegetace - počet dubů v blízkém okolí hnízda, který by měl být dobrým indexem dostupnosti potravy. Vliv kvality potravy pak hodnotím na příkladu vitamínu E. Vedle vlivu prostředí a rodičovské péče na růst a přežívání mláďat jsem zkoumala také jejich vliv na zbarvení peří mláďat založené na karotenoidech.

PŘEHLED VÝSLEDKŮ

V této práci dokládám, že růst, vývoj a zbarvení mláďat jsou ovlivněny jak rodičovskou péčí, tak prostředím, v němž mláďata vyrůstají.Výsledky mého výzkumu ukazují zejména, že:

1. Role dubů jako indikátorů kvality teritoria sýkor není univerzální. Význam dubů pro výživu mláďat sýkor se může lišit mezi různými druhy dubů a i v rámci jednoho druhu může být velmi proměnlivý v čase i prostoru. Zároveň může záviset na dostupnosti dalších druhů stromů potenciálně bohatých na potravu.

2. Vitamín E může být pro volně žijící ptáky limitující. Míra této limitace se však liší mezi druhy. V rámci druhu se pak význam vitamínu E mění v čase a liší mezi jedinci. Vitamín E je patrně důležitý zejména pro znevýhodněná mláďata. Pozitivní vliv vitamínu E na karotenoidové zbarvení se v našem případě nepotvrdil.

3. Rané prostředí a péče rodičů ovlivňují také karotenoidové ornamenty peří mláďat. Dvě komponenty karotenoidového zbarvení mláďat - strukturální a pigmentární složka - odráží různé aspekty prostředí a kvality jedince. Karotenoidové ornamenty tak mohou sloužit jako multikomponentní signál.

ABSTRAKTY PRACÍ

Matrková J. & Remeš V.: Do oaks matter? Great Tit breeding performance in relation to territory tree composition (rukopis).

Breeding performance of forest songbirds often depends on food availability. As oaks (*Quercus* sp.) usually offer more caterpillars than other tree species, number of oaks is frequently used as an index of territory quality. However, evidence of the positive association between local oak density and breeding performance is equivocal. Moreover, only correlative studies were done so far. Here, we investigated the relationship between the number of oaks in nest proximity and breeding performance of the Great Tit pairs in a floodplain forest. For the first time, we experimentally separated genetic plus pre-natal vs. post-natal effects by cross-fostering of young. We found no relationship between oak density and any of the measured breeding traits, neither before (nest occupancy, timing of egg laying, clutch and egg size), nor after experiment (nestling performance). The density of oaks and other deciduous trees such as limes (*Tilia* sp.), which may provide invertebrate prey in densities similar to oaks, is high in our study site. Thus, our population was probably not limited by oak availability. We suggest that the role of oak as a source of prey may vary in time and may depend on tree species composition of the forest. Evaluating availability of other deciduous trees potentially rich in caterpillars, together with accounting for differences among oak species, will improve use of oaks as an indicator of territory quality.

Matrková J., Mullen W. & Remeš V.: Do Great Tits need vitamíns? Nestling performance and colouration in response to vitamín E supplementation (rukopis).

Parents can strongly affect performance and phenotype, and thus reproductive prospects, of their young. For instance, growth and survival of altricial nestlings critically depend on both the quality and quantity of food provisioned by their parents. Vitamin E is an important micronutrient with numerous functions in bird physiology. Moreover, it may interact with other micronutrients, for example by replacing carotenoids in antioxidant protection of macromolecules or directly protecting carotenoids from oxidation. Thus availability of vitamin E may modify carotenoid availability for signaling and affect carotenoid-based

coloration. Yet, despite its potentially strong physiological effects, experimental studies of vitamin E function in wild-living animals are rare. Here, we examined the role of vitamin E in the Great Tit (*Parus major* Linnaeus, 1758) reproduction. We supplemented nestlings with vitamin E and examined their growth, survival, intensity of immune response to a novel antigen (phytohaemagglutinin), characteristics of carotenoid-based plumage coloration, and plasma vitamin E and carotenoid levels. We found no detectable effects of increased vitamin E intake on nestling growth, survival, immune responsiveness, coloration, and plasma lutein and γ-tocopherol concentration. In initially small nestlings, vitamin E supplementation increased plasma α-tocopherol concentration compared to control nestlings. However, this effect was significant only for the lowest dose of vitamin E. We conclude that 1. vitamin E is probably not limiting the performance of Great Tit nestlings in our wild population; 2. the non-linear relationship between vitamin E intake and circulating α-tocopherol suggests an interaction with other antioxidants and/or dose-depended changes in tocopherol distribution among tissues.

Matrková J. & Remeš V. 2014: Vitamín E improves growth of collared flycatcher Ficedula albicollis young: a supplementation experiment. Journal of Avian Biology, 45 (5), 475-483.

In altricial birds, the quantity and quality of food provided by parents is a crucial determinant of nestling performance. Vitamin E is an important micronutrient with various physiological functions, including a positive role in the antioxidant system. Suffi cient intake of vitamin E has been shown to condition normal avian development in poultry, yet, our knowledge of the role of vitamin E in free-living birds is limited. Th us, we experimentally examined the effects of vitamin E on nestling development in the collared fl ycatcher *Ficedula albicollis*. We supplemented nestlings with vitamin E and evaluated their growth and survival till fledging. Increased availability of vitamin E did not aff ect body mass, wing length or survival, but improved tarsus growth. Th e eff ect of supplementation on tarsus length changed over season and with initial body mass. Supplemented nestlings that were smaller at hatching and those that hatched later in the season grew longer tarsi compared to the control. Our results suggest that 1) vitamin E may be limiting for the development of collared flycatcher nestlings, 2) seasonal changes of vitamin E availability may aff ect breeding success of collared

flycatchers, and 3) increased income of vitamin E may improve growth of nestlings with bad start in life.

Matrková J. & Remeš V. 2012: Environmental and Genetic Effects on Pigment-Based vs. Structural Component of Yellow Feather Colouration. PLOS ONE, 7 (5), e36640.

Background: Carotenoid plumage is of widespread use in bird communication. Carotenoid-based feather colouration has recently been shown to be dependent on both pigment concentration and feather structure. If these two components are determined differently, one plumage patch may potentially convey different aspects of individual quality.

Methodology/Principal Findings: We evaluated the effects of genetic and environmental factors on carotenoid-based yellow breast colouration of Great Tit (Parus major) nestlings. By partial cross-fostering, we separated the genetic and prenatal vs. post-natal parental effects on both the structural and the pigment-based component of carotenoid-based plumage colouration. We also simultaneously manipulated the post-hatching environment by brood size manipulation. The structural component of nestling colouration reflected features of female colouration. On the other hand, the pigment-based component was more affected by rearing conditions presumably representing food quality. While the structural component was related to both origin- and environment-related factors, the pigment-based component seemed to be environmentdependent only. These results support the notion that pigment-based and structural components of feather colouration are determined differently.

Conclusions/Significance: Chromatic and achromatic components of carotenoid-based feather colouration reflected different aspects of individual quality and history, and thus may potentially form a multicomponent signal.

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SUMMARY

In birds, breeding performance depends both on rearing environment and parental care. Namely, food quality and quantity are critical for nestling phenotype in adulthood and fitness. Here, I study the importance of territory quality and parental effects in nestling development, survival and plumage colouration in free living birds. As model species, I chose Great Tit (*Parus major*) and Collared Flycatcher (*Ficedula albicollis*).

In titmice, number of oaks is nest proximity is frequently used as an index of food availability and consequently of territory quality. However, evidence of the positive association between local oak density and breeding performance is equivocal. Here, we investigated the relationship between the number of oaks in nest proximity and breeding performance of Great Tit pairs in a floodplain forest. We found no relationship between oak density and breeding performance in the Great Tit. We suggest that the role of oak as a source of prey may depend on oak species. Moreover, within a given oak species, the density of invertebrate prey may considerably vary in time and space. Availability of other deciduous trees potentially rich in caterpillars may reduce the importance of oaks as a critical food source.

We examined the effect of food quality on nestling performance and colouration using vitamin E, an important micronutrient with numerous beneficial functions in bird physiology. By supplementation of two species of small insectivorous songbirds, we showed that vitamin E may be limiting for nestling growth and survival. However, the importance of vitamin E differed between species, and within species might vary with season and among individuals. Increased income of vitamin E may be benefical especially for nestlings with a bad start in life. However, our results do not support possitive effect of increased vitamin E availability on carotenoid plumage colouration.

Early environment and parental care may determine the expression of plumage colouration. Carotenoid-based feather colouration has recently been shown to be dependent on both pigment concentration and feather structure. We experimentally manipulated early nestling environment to evaluate parental effects on both the structural and carotenoid-based components of nestling carotenoid colouration. Our results support the hypothesis that pigment-based and structural component of feather colouration reflect different aspects of individual quality and history. Carotenoid colouration thus may potentially form a multicomponent signal.

Keywords