

Specific Immunity and Polymorphism of Breeding Plumage in Pied Flycatcher (*Ficedula hypoleuca*) Males (Aves: Passeriformes)¹

A. B. Kerimov^a, K. A. Rogovin^{a, b}, E. V. Ivankina^c, A. V. Bushuev^a, O. V. Sokolova^d, and T. A. Ilyina^a

^a Department of Vertebrate Zoology, Faculty of Biology, Lomonosov Moscow State University, Leninskie Gory, Moscow, 119991 Russia

^b Severtsov Institute of Ecology and Evolution, Leninsky prospect 33, Moscow, 119071 Russia

^c Zvenigorod Biological Station of Lomonosov Moscow State University, P.O. Box Shikhovo, Odintsovo district, Moscow, 143092 Russia

^d Kovalenko Research Institute of the Experimental Veterinaria, Moscow, Ryazansky prosp. 24-1, 109428 Russia
e-mails: anvar_kerimov@mail.ru, bushuev@mail.bio.msu.ru, ilyina@mail.bio.msu.ru; krogovin@yandex.ru; ivankinalena@yandex.ru; ovsokolova@mail.ru

Received April 30, 2012

Abstract—The relationship between the type of melanin-based plumage colouration and the strength of experimentally induced immune response was studied using as an example a pied flycatcher population from the Moscow region. The breeding plumage of pied flycatcher males exhibits the full spectrum of transitions from contrasting black-and-white to cryptic brownish, the latter being very similar to the colouration of females. In spite of numerous studies, the nature of this polymorphism still remains vague. Unlike many other avian species with monocyclic breeding, a considerable fraction of pied flycatchers overlaps two energy-consuming productive processes, breeding and moult, over the whole species range. During the main experimental treatment we activated the humoral immunity of free-living males in chick-rearing period by injection of nonpathogenic multifactorial antigen (sheep red blood cells, SRBC) and estimated the strength of the immune responses after repeated captures in 6–8 days. In addition, after each capture we evaluated the numbers of leucocytes (WBC), heterophil to lymphocyte ratios (H/L) and measured night time basal metabolic rates (BMR). Non-moulting males of different colour types showed the same immune responses. Among moulting birds, the strength of the immune response was significantly higher in pale males (morphs 4–7 by Drost's scale) than in bright males with rich melanin-based colouration (morphs 2–3). This difference resulted from two opposite processes. During moulting, pale males heightened the antibody titer after immunization, while bright males tended to reduce the strength of immune response. Possibly such an asymmetry in immunocompetence at the first stage of moult reflects the different life strategies of pied flycatcher males—conspicuous birds less commonly overlap breeding with moult than cryptic ones.

DOI: 10.1134/S2079086413030067

INTRODUCTION

When an organism experiences a deficit of some external or internal resources, the high cost of a trait leads to the development of trade-offs between the strength of its expression and other essential functions. Such trade-offs constrain the evolution of a trait and lead to the formation of different intrapopulation life strategies (Moshkin et al., 2000; Artzy-Randrup and Kondrashov, 2006). An example of such a trade-off is the relationship between pigment saturation and some indicators of an organism's health, such as resistance towards invasions and infections (Hamilton and Zuk, 1982) and immunocompetence. It has been shown in experiments on fish and birds that infections have a much stronger influence upon ornamentation than upon other morphological traits (Zuk et al., 1990; Houde and Torio, 1992). Partly such an effect is

related to the fact that pigments possess anti-oxidative properties and are able to regulate the immune response to an antigen (Criffith et al., 2006).

The relationship between plumage colouration and the defending properties of an organism has been demonstrated mainly on birds with carotenoid-based ornamentation (Zuk, 1991; Olson and Owens, 1998; Walther et al., 1999; Fitze and Richner, 2002; Fitze et al., 2007; Saks et al., 2003; Hõrak et al., 2004). Due to strong effects of the environment on the expression of this trait, carotenoid-based colouration is considered to be more costly than a melanin-based one. In such context, the cost of a trait is determined not so much by the physiological and particularly energetic expenditures associated with trait formation, but more by the extent of the individual's access to food resources, its social status, predation risk etc. Since the only source of carotenoids is food, the formation of fully developed ornamentation is to a considerable

¹ The article was translated by the authors.

degree related to the competitive ability of an individual. Thus, it can act as a reliable indicator of an individual's quality as a potential mate. The relatively low heritability of a trait, combined with its high cost, is the essential condition for the formation of evolutionary stable diversity (Maynard Smith and Harper, 1988; Wilson, 1992). However, recent studies cast doubt on the need to distinguish between the two main pigmentation systems (carotenoid-based and melanin-based), both in respect to the role of the environmental components of a trait's expression and the cost of ornament formation (Griffith et al., 2006). Our own results, as well as studies of other researchers, contradict the conception of "cheapness" of development of melanin-based ornamentation and demonstrate the trade-off between the extent of plumage melanisation and the reaction to environmental factors (Ivankina et al., 2007; Slagsvold and Lifjeld, 1992; Fargallo et al., 2007). Such results assume the existence of inconsistent interaction between the formation of rich melanin-based colouration and other essential characteristics of an organism. It is likely that, as in the case of carotenoid-based colouration, such interactions cause changes in physiological and defending properties of an organism, since melanin (as are carotenoids) is an antioxidant (McGraw, 2005) and the α -MSH hormone, which stimulates its synthesis, exhibits anti-inflammatory action (Harris and Bird, 2000).

This problem (the trade-off between the immune status and the ability of an individual to form the rich melanin-based plumage, which can play a role in the context of sexual and intrasexual selection) was studied on the example of a pied flycatcher, *Ficedula hypoleuca* (Passeriformes, Aves). The studied species is a unique example of polymorphism in the male's breeding plumage among European passerines. It is particularly well suited for studying the possible trade-off between the expression of eumelanin colouration (which is one of the indicators used by female when choosing a mate) and the defending properties of an organism. The breeding plumage of pied flycatcher males exhibits the full spectrum of transitions from contrasting black-and-white to cryptic brownish, the latter being very similar to the colouration of females (Drost, 1936). In spite of numerous studies, the nature of this polymorphism still remains vague.

During the main experimental treatment we activated the humoral immunity of males of different phenotypes by the injection of a nonpathogenic multifactorial antigen and estimated the strength of the immune response. We hypothesized that the effect of colouration type could appear under conditions of increased physiological stress. The latter was estimated by the reproductive impact of male (brood size) and the extent of overlap of two energy-consuming productive processes, last stage of breeding and moult. Such overlap is not common among other avian species with monocyclic breeding, but is typical for the

pied flycatcher over its whole breeding range. In addition to antibody titer we estimated hematological indexes and basal metabolic rate (BMR), changes of which could accompany the formation of an organism's immune response (Ots et al., 2001).

MATERIALS AND METHODS

The study was performed in 2010 and 2011 in Moscow Region at Zvenigorod Biological Station (55°44' N, 36°51' E). The study area (715 ha) included about 600 nestboxes, which were actively occupied by pied flycatchers. Study plots with nestboxes were located in two main types of habitats: in the rich mixed forest of the Moscow River valley and in the mixed forest dominated by conifers at the watershed. Plots with nestboxes were examined in late April, soon after the arrival of pied flycatchers from wintering grounds. Plots were visited with 4–7 day intervals, which allowed us to register the date of first egg with high precision. Birds were captured using automatic traps fastened to the nestbox hole, cages with automatically slammed doors, and "Ecotone" (Poland) mist nets. The moult and breeding dates were taken from our long-term database (1996–2011). For immunological experiments birds were captured twice during chick rearing period: at the chicks' age of 5–7 days and 6–8 days later, on the eve of fledgling from the nest. The choice of timing was based on monitoring of formation of the immune reaction in two captured males, which were kept in the aviary after predation of their broods and mates. Over the period of two weeks the strength of immune response in both males reached its maximum 6 days after immunization and diminished after the 8th day. After banding or old ring number registration, the standard body measurements, body mass, moult stage, and age of birds were recorded. The estimation of age for birds that were captured for the first time was based on the shape of the central rectrices (Vysotsky, 1989; Lundberg and Alatalo, 1992). Colour type of males was estimated using the 7-point Drost's scale (Drost, 1936). According to this scale, the highest number (7) is assigned to the males without black spots in plumage and the lowest is assigned to the males with solid black dorsal colouration (in the studied population only birds with colour type 2–7 are presented). In the morning after each of two captures a drop of blood from the brachial vein was taken from experimental males and smeared on a slide plate. Blood smears were air-dried and fixed in methanol, then imbued with May-Grunwald stain and subsequently with azure-eosine. Microscopy was done using immersion with 10 × 100 magnification. Afterwards, smears were used for estimation of hematological indexes: 1—white blood count (WBC), counted as leukocyte number to 10000 erythrocytes; 2—heterophil/lymphocyte ratio (H/L), based on the count of different cell types in a sample of 100 leukocytes. The increase of the first index indicates that the

inflammatory process induced by microbial or macro-parasitic infection has taken place (Dein, 1986). The second characteristic is used extensively as a stress index in domestic birds (Gross and Siegel, 1983; Maxwell, 1993), but is also useful in wild animals (Hörak et al., 1998; Ots et al., 2001).

In the morning after the first capture, experimental males ($n = 60$) were injected in the pectoral muscle with 30 μL of 40% sheep red blood cell suspension (SRBC) in normal saline solution and control males ($n = 10$) were injected by equivalent volume of normal saline solution. Straight before the injection SRBC was three times rinsed and resuspended in normal saline solution to make the necessary concentration. The strength of the immune response was estimated by a hemagglutination reaction (Wegmann and Smithies, 1966; Lawler, Redig, 1984). After the second capture and BMR measurement, a sample of 80–120 μL blood from brachial vein was taken from experimental and control birds. The blood was centrifuged and used in the hemagglutination reaction in microbiological 96-well plates. Immune response (antibody titer in blood plasma in response to SRBC) was estimated visually by the number of the last well, where after subsequent multiple dilution procedures the amount of antibodies was sufficient for hemagglutination (Ots et al., 2001). Consequently, a higher titer indicated a stronger immune response.

The basal metabolic rate (BMR) was measured after first capture (BMR_1) as well as after second capture (BMR_2) in most experimental and all control birds during the night prior to morning blood sampling and immunization. In 2010 BMR was estimated by oxygen consumption using modified Kalabukhov's closed respirometer systems (see details in Gavrillov et al., 1994; Bushuev et al., 2010; Górecki, 1975; Kerimov and Ivankina, 1999a). The volume of consumed oxygen by birds in small cages in exsiccator under $T = 27^\circ\text{C}$ was recorded every 10 min during one hour and was used for BMR estimation. Temperatures inside chambers and in the laboratory (to 0.1°C), time and atmospheric pressure (to 0.5 mm Hg) were recorded during measurements. The average volume of consumed oxygen from the whole time of BMR measurement was transformed into volume at standard temperature and pressure. Then two measures of energy expenditure were calculated: mass-specific BMR (in $\text{mL O}_2/\text{g hour}$) and whole-organism BMR (in kJ/day), according to the equation $1 \text{ L of O}_2 \approx 20.083 \text{ kJ}$ (Schmidt-Nielsen, 1982).

In 2011 BMR was estimated in an open-flow system using paramagnetic gas analyzer FoxBox-C (Sable Systems International, United States) and a built-in mass flowmeter. Measurements were done from 11:00 p.m. to 5:00 a.m. In order to estimate respiratory metabolism of several birds during one night ($\text{max} = 7$) we used a custom build switching system, which alternately directed to the gas analyzer flows from chambers with birds and from an empty reference chamber.

The switching system was based on 8 solenoid valves 20E (ACL S.r.L., Italy) under the control of USBREL8 module (Quancom, Germany) and 8 DEK-REL-24/I/1 relays (Phoenix Contact GmbH&Co. KG, Germany). The USBREL8 module was operated through USB interface by PC using original software. The air was pulled through the chambers in thermostats ($T = 27^\circ\text{C}$) by 8 independent air compressors AC-500 (Resun, China) with flow rate $V = 230 \text{ mL/min}$. The air was dried by self-indicating granulated fine-pored silica gel at the input of each chamber and gas analyzer. To estimate BMR we used the difference between oxygen concentration in reference chamber and minimal (but stable over 5 min) oxygen concentration in each chamber. To calculate BMR from oxygen concentrations we used the Hill equation with fixed respiratory quotient $\text{RQ} = 0.85$ (Hill, 1972). Since in 2011 we used the minimal stable oxygen consumption rate and in 2010 we used the average consumption rate over specific hour, all measures of BMR_1 and BMR_2 were higher in 2010 ($p < 0.001$ based on Mann-Whitney U-test). In 2010 the mean mass-specific $\text{BMR} \pm \text{SD}$ was $\text{BMR}_1 = 4.24 \pm 0.58 \text{ mL O}_2/\text{g hour}$ ($n = 43$) and $\text{BMR}_2 = 5.00 \pm 0.63 \text{ mL O}_2/\text{g hour}$ ($n = 43$); in 2011 it was $\text{BMR} = 3.34 \pm 0.45 \text{ mL O}_2/\text{g hour}$ ($n = 14$) and $\text{BMR}_2 = 3.53 \pm 0.51 \text{ mL O}_2/\text{g hour}$ ($n = 13$). At the same time, the night body mass of males was not different between years ($p > 0.05$). Therefore taken the pooled sample we used year-standardized measures of BMR and the percentage of the change in mass-specific BMR between the two captures.

Just as we had expected, after antigen injection, experimental males differed from control ones in antibody titer (Mann-Whitney U-test: $Z = 4.60$; $p < 0.001$, $n_1 = 60$, $n_2 = 10$). Both leukocyte indexes in every of the two captures and the rate of their change between captures did not differ between experimental and control birds ($p > 0.12$). Also no difference between these groups of birds was found in the day and night body mass after each of the two captures ($p > 0.05$), in the mass change between captures ($p > 0.79$), in both year-standardized measures of BMR_1 and BMR_2 , and in the change in BMR between captures ($p > 0.43$).

The strength of immune response was not affected by small (3 days) variation in the interval between date of immunization and second capture ($p > 0.9$), which corresponds to the results of a detailed test of two single males kept in aviary (see above).

In order to test the significance of the role of different factors which affected the strength of the immune response, we used general linear model (GLM). The strength of immune response in experimental birds was set as a dependent variable. Such categorical and continuous variables as colour type, age, presence of moult, year of experiment, body mass after each capture, tarsus length, broods size, immunization dates, and time interval between first and second captures were treated as independent factors. The interactions

between some factors were also taken into account. The normality of residuals was tested using Shapiro–Wilk W-test. As distributions of most of the variables were not normal, nonparametric Mann–Whitney U-test was used for pair comparisons and Spearman's rank correlation coefficient (R_s) was used as a measure of statistical dependence between continuous variables. To compare differently coloured groups of males in breeding dates and moult overlap, we used Kruskal–Wallis ANOVA and chi-square test.

RESULTS

Immune Response in Males of Different Colour Types

Of all analysed variables, only colour type (in some models) and interaction of colour type with moult (in all models) had significant influence on the strength of immune response (Table 1).

Males of different colour type that did not moult during chicks' rearing period did not differ in the strength of immune response (Mann–Whitney U-test: $n_1 = 25$; $n_2 = 10$; $p = 0.61$). However among moulting birds the strength of immune response in pale males (phenotype 4–7) was higher than in conspicuous males (phenotype 2–3) (Mann–Whitney U-test: $n_1 = 19$; $n_2 = 6$; $Z = 2.13$; $p = 0.033$). Such difference ensues from processes of two different directions. In immunized intermediate and cryptic males (phenotype 4–7) the start of moult was accompanied by a sharp increase in antibody titer after immunization (Mann–Whitney U-test: $n_1 = 19$; $n_2 = 25$; $Z = 2.68$; $p = 0.007$ from the comparison of immune response in moulting and non-moulting males). On the contrary, moulting dark males showed a decrease in immune response, which was, however, statistically non-significant.

The effect of interaction between moult and colour type was most clearly pronounced in the early stages of moult. This was revealed when comparing males that did not start to moult during experiment with males, which started to moult after the first capture (during formation of immune response). In other words, those birds, which already started to moult at the moment of immunization were excluded from analyses. In this case the colour type also affected the strength of immune response directly (Fig. 1; Table 2; R^2 for the whole model = 26.98%; $n = 44$; $p = 0.005$).

The impact of age factor was estimated indirectly, because the group of conspicuous males consisted of only old (≥ 2 years) birds, which is natural enough for the studied region. In many populations (including Moscow region), the dorsal colouration of old (≥ 2 years) males is darker by the average of one point than in year-old males. Among year-old males in Moscow region, dark ones (phenotype 2–3) are very rare. Starting from the second year, colouration of breeding plumage is stabilized in most of males and does not change appreciably throughout the bird's life (Ivankina et al., 2007). If we exclude year-old males

Table 1. Results of the final general linear model (GLM) from the analysis of all immunized and re-caught males ($n = 60$).

Factors	F	<i>p</i>
Intercept	210.07	<0.001
Colour type, 1	2.68	0.108
Moult presence, 2	0.39	0.537
Interaction, 1×2	4.92	0.031

Table 2. Results of the final general linear model (GLM) from the analysis of moulting and non-moulting males in the interval between immunization date and recapture date ($n = 44$).

Factors	F	<i>p</i>
Intercept	151.37	<0.001
Colour type, 1	7.48	0.009
Moult presence, 2	0.30	0.585
Interaction, 1×2	10.42	0.003

from the previous sample and leave thereby only males with definitive plumage colouration ($n = 32$), then the character of the effect of colouration factor and its interaction with moult on immune response is the same ($F = 5.28$; $p = 0.03$ for colouration; $F = 7.31$; $p = 0.01$ for interaction of factors). This signifies that the revealed relationships reflect specifically the effect of colouration, and are not mediated by age.

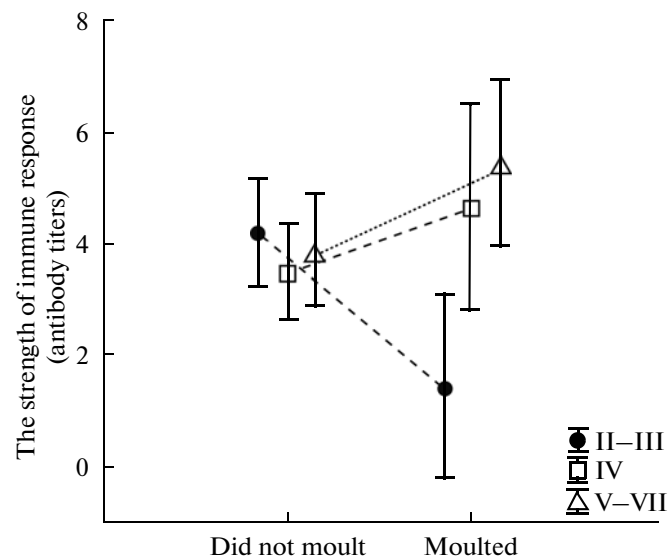


Fig. 1. The strength of immune response in males of different phenotypes, which overlapped or did not overlap breeding with the beginning of moult. Roman numerals indicate the colour type (morphs) of plumage based on R. Drost's scale (Drost, 1936). Vertical lines denote the 95% confidence interval. Statistics for the model with 3 ranks for colour type is $F_{2,39} = 5.22$ ($p < 0.01$).

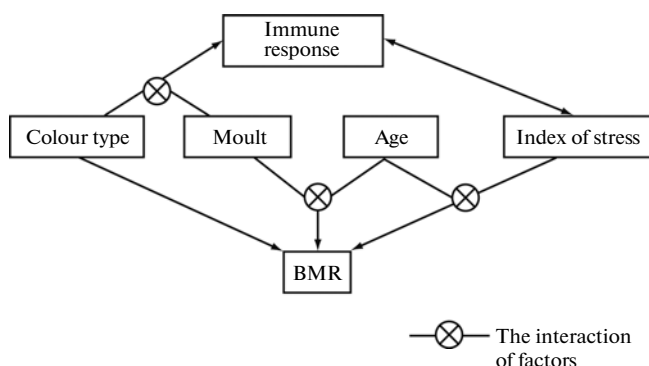


Fig. 2. The scheme of dependence of immune response strength, basal metabolic rate (BMR) and stress level on age, colour type and moult presence in pied flycatcher males.

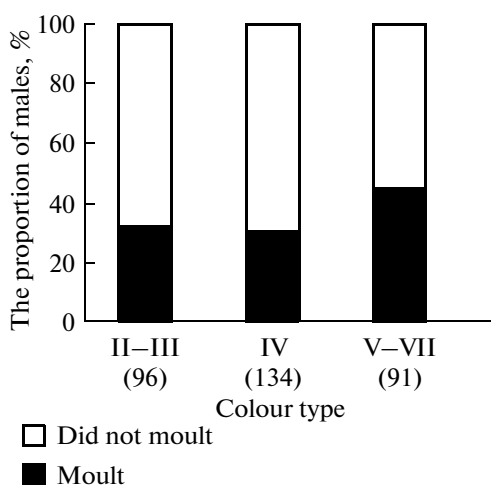


Fig. 3. The ratio of old (≥ 2 years) males of different colour type, which moulted on the eve of chicks' fledgling (at chicks' age of 12–16 days). Roman numerals indicate the colour type (morphs) of plumage based on R. Drost's scale (Drost, 1936); numbers in parenthesis under them indicate sample size.

The brood size, as another indirect index of physiological and energetic load, which defines the intensity of foraging activity and the daily level of workload, did not affect the immune response nor directly, nor through interaction with other factors ($p > 0.46$).

Among experimental birds the intensification of immune response was accompanied by augmentation of H/L index ($R_s = 0.394$; $p < 0.05$), but was not related to the change of WBC index, to mass-specific and whole-organism BMR after the second capture (BMR_2) and to the extent of change of mass-specific BMR during formation of immune response. At the same time these BMR measures were negatively related to H/L after the second capture ($R_s = -0.513$, $R_s = -0.538$, $p < 0.05$ and $R_s = -0.318$, $p < 0.05$ for whole-organism and mass-specific BMR_2 and the extent of change of mass-specific BMR between cap-

tures, respectively). BMR_2 was negatively related to augmentation of H/L ($R_s = -0.391$, $R_s = -0.385$, $p < 0.05$ for whole-organism and mass-specific BMR_2 , respectively). Among old birds, BMR_1 in conspicuous (phenotype 2–3) males was higher than in pale (phenotype 4–7) ones (Mann-Whitney U-test: $n_1 = 20$; $n_2 = 21$; $Z = -2.03$ and $Z = -2.09$; $p < 0.05$ for whole-organism and mass-specific BMR_1 , respectively).

In those GLMs where the strength of the immune response, extent of BMR change, brood size, year of experiment, age, colour type, moult presence and interaction of the last two factors were set as independent variables, the augmentation of H/L index between captures was caused only by the strength of immune response ($F = 8.01$; $p = 0.007$ for the factor “strength of immune response”; $R^2 = 0.170$; $n = 41$; $p = 0.007$ for the whole model).

Year-standardized whole-organism BMR_2 was related to ranked H/L change (GLM: $F = 15.43$; $p < 0.001$), moult presence ($F = 8.24$; $p = 0.007$) and the interaction between moult and age ($F = 8.14$; $p = 0.007$; $R^2 = 0.382$; $n = 42$; $p = 0.007$ for the final model, which was fitted without such insignificant variables as full-scale colour type, night body mass, brood size, breeding date and interaction between H/L change and age). Increased BMR_2 in moulting males when compared to non-moulting ones was noted for year-old males (Mann-Whitney U-test: $p = 0.033$), but not for older ones ($p = 0.47$). The negative relationship between BMR_2 and the extent of H/L increase also depends significantly on the age factor: a decrease in BMR coupled with an increase in H/L index was attributed primarily to old ($p < 0.001$), but not to young males ($p = 0.36$). The mass-specific BMR_2 demonstrated close relations.

Figure 2 schematized the interrelationship of immune response and BMR with such factors as H/L change, colouration type, age and moult.

The Overlap of Moult with Breeding in Males of Different Phenotypes

The effect of colouration in respect to overlap between moulting and chick-rearing period appeared only in old (≥ 2 years) males with definitive plumage colouration. In that group pale males (phenotype 5–7) started moulting more often in the breeding period comparing to brighter males ($\chi^2 = 5.66$; $d.f. = 2$; $p = 0.017$) (Fig. 3). On the other hand, the dependence of the breeding date on colour type (Kruskal-Wallis ANOVA: $H_{2, 692} = 11.7$; $p = 0.003$) was shown in old males: conspicuous males were characterized by more early breeding dates (Fig. 4). Moult was rather sharply related to calendar date and the delay in breeding increased the probability of overlap between two productive processes.

DISCUSSION

Our results indicate the existence of a trade-off between the formation of rich melanin colouration and immune function of an organism in the pied flycatcher. In this species the increase of immune response was quantified by the augmentation of leukocyte stress index. In contrast to free-living great tits in wintering flocks, which underwent the same procedure (Ots et al., 2001), and house sparrows, which were kept in outdoor aviaries and provoked to form T-cell mediated immunity (Martin et al., 2002), the formation and strength of antibody response in pied flycatcher males was not related to accessory energetic cost, an increase in BMR. The increased immunocompetence of the cryptic phenotype when compared to a conspicuous one is not unconditioned, but is revealed only in a certain physiological state, namely when moult is combined with one of the most energy-consuming stages of the breeding period. The overlap between these two productive processes is not typical for many avian species and is a peculiar “identification card” of pied flycatcher (Lundberg and Alatalo, 1992). Our result is one of the first evidences that demonstrate the apparent advantage of cryptic phenotype over conspicuous phenotype in an essential functional characteristic. Previous studies, which compared pied flycatcher phenotypes either demonstrated the neutrality of the colouration trait or the advantage of conspicuous males over cryptic ones in those traits, which play a role in the context of sexual selection (Järvi et al., 1987; Lampe and Espmark, 1994; Dale and Slagsvold, 1996; Ivankina et al., 1995; Sætre et al., 1995). Such advantages could not explain the phenotypic ratio adequately, because even a small advantage of conspicuous phenotype should over evolutionary times result in a hiatus in colouration and conspicuous sexual dimorphism. Nevertheless, in spite of evident geographic variation in mean colour type, practically each pied flycatcher population contains almost a full spectrum of male phenotypes. Possibly, physiological and biochemical features of this species are the main impediment to formation of rich contrasting melanin colouration under the influence of sexual selection. The indirect evidence of the “physiological” nature of morphogenesis in pied flycatcher is data on energetic differences in males of different phenotypes, specifically in the basal metabolic rate, its seasonal dynamics and change in response to cold stress (Gavrilov et al., 1993; Kerimov and Ivankina, 1999b; Kerimov et al., 2006). The higher energetic metabolism of conspicuous males (especially in the period of maximal reproductive workload) should result in an increased production of free radicals, oxidative stress and, as a consequence, in a stronger load on the reparation system of an organism. It was recently shown in the Spanish subspecies of pied flycatcher that the ability to neutralize the negative effects of oxidative stress is not related to colour type (Moreno et al., 2011), which indicates the absence of specific compensatory mechanism in

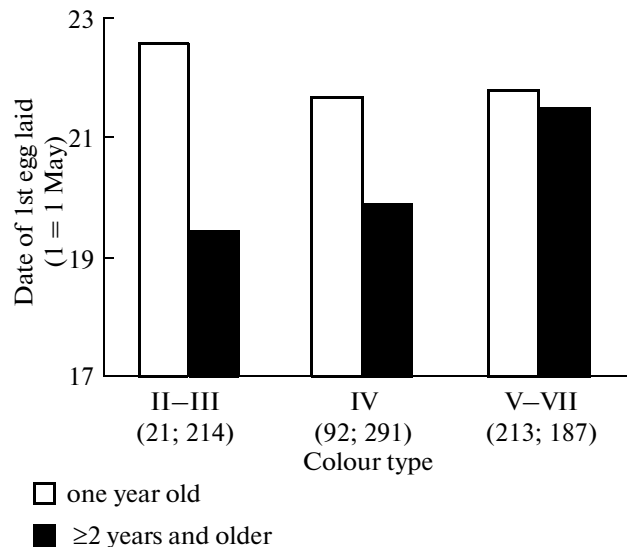


Fig. 4. The breeding dates in males of different colour types and age.

On the *X*-scale legend agrees with Fig. 3 legend. Kruskal-Wallis ANOVA statistics for old (≥ 2 years) males: $H_{2,692} = 11.7$; $p = 0.003$.

conspicuous males. Melanin colouration is controlled genetically to a considerable degree, but both levels of circulatory hormones (steroid and α -MSH) and the value of oxidative stress can have an effect on its formation (Mougeot et al., 2012). It is possible that against a background of a reproductive workload, a relatively negative in physiological terms process leads to a delayed (until the next prenuptial moult) partial depigmentation of dorsal plumage, which exhibits only in males with the richest melanin-based colouration (Ivankina et al., 2007). As the immune response formation is also attended by the increased production of free radicals, competition for limited resources of antioxidants (e.g., glutathione) can arise between immune function and formation of rich melanin-based colouration (Hörak et al., 2010).

There is a number of data in favour of two different versions of the immunomodulative role of melanin-based colouration. The first states that the intensive pigmentation is positively correlated with immunocompetence because α -MSH hormone not only regulates the formation of colouration, but also intensifies the functionality of components of innate and acquired immunity (Ducrest et al., 2008; Gasparini et al., 2009; Jacquin et al., 2011). Alternative hypotheses are based on the idea that an increased level of steroid hormone (Gonzalez et al., 1999) or α -MSH (Moreno and Møller, 2006) has immunosuppressive effect. In accordance with those hypotheses, which postulate the handicap principle in immunocompetence, only individuals of high quality can have rich colouration and simultaneously resist its negative effects. Those effects are the cost of a trait, which in

the present case can reliably indicate the quality of an individual.

Our result is an evidence of the immunosuppressive effect of colouration in pied flycatcher with the adjustment that additional physiologic load modulates this effect, namely moult combined with breeding. The existence of a contradictory trade-off between immunocompetence and moult was recently shown in a number of birds (Martin, 2005; Moreno-Rueda, 2010; Männiste and Hõrak, 2011) including the pied flycatcher in conditions when moult overlapped breeding (Moreno et al., 2001; Sanz et al., 2004). In the annual cycle of birds moult can play the role of a peculiar bottleneck. Besides the additional energetic expenditures (Dolnik, Gavrilov, 1979), moult is accompanied by multiple small ruptures of the integument, which enhances the risk of contagion. This increases demands for the defensive systems of an organism. In this respect the cryptic males have an evident advantage over conspicuous ones and show an intensification of immune function against a background of moult, which is a rare but documented case in birds (Silverin et al., 1999; Nava et al., 2001). The effect of intensification of immune function contradicts the consequences of the hypothesis of competition for limited resources of an organism. The clarification of that effect requires further investigations.

It is possible that physiological peculiarities of males of different phenotypes have an influence on the divergence of their life strategies, which, in particular, become apparent in the different structure of their annual cycle: conspicuous birds less commonly overlap breeding with moult than cryptic ones.

ACKNOWLEDGMENTS

Financial support was provided by RFBR (grants no. 09-04-01690-a and 10-04-00278a) and Russian Ministry of Education and Science (R&D project no. 6-09/10). We thank I.S. Litvinov and A.P. Baranik from the Institute of Bioorganic Chemistry of the Russian Academy of Sciences (IBCh RAS) for consultations and practical help in hematological handling. We are grateful to A.B. Savinetsky for the development of software for automatic channel switching in measurements of gas concentration.

REFERENCES

- Artzy-Randrup, Y. and Kondrashov, A.S., Sympatric speciation under incompatibility selection, *Proc. Natl. Acad. Sci. U.S.A.*, 2006, vol. 103, no. 31, pp. 11619–11624.
- Bushuev, A.V., Kerimov, A.B., and Ivankina, E.V., Estimation of heritability and repeatability of resting metabolic rate in birds, with free-living Pied Flycatchers *Ficedula hypoleuca* (Aves: Passeriformes) as an example, *Zh. Obshch. Biol.*, 2010, vol. 71, no. 5, pp. 403–425.
- Dale, S. and Slagsvold, T., Mate choice on multiple cues, decision rules and sampling strategies in female pied flycatchers, *Behaviour*, 1996, vol. 133, nos. 11/12, pp. 903–944.
- Dein, J., Hematology, in *Clinical Avian Medicine*, Harrison, G.J., and Harrison, W.R., Eds., London: Saunders, 1986, pp. 174–191.
- Dolnik, V.R. and Gavrilov, V.M., Bioenergetics of molt in the chaffinch (*Fringilla coelebs*), *Auk*, 1979, vol. 96, no. 2, pp. 253–264.
- Drost, R., Über das Brutkleid männlicher Trauerfliegenfänger, *Muscicapa hypoleuca*, *Vogelzug*, 1936, vol. 6, pp. 179–186.
- Ducrest, A.-L., Keller, L., and Roulin, A., Pleiotropy in the melanocortin system, coloration and behavioural syndromes, *Trends Ecol. Evol.*, 2008, vol. 23, no. 9, pp. 502–510.
- Fargallo, J.A., Padilla, J.M., Díaz, A.D., Moreno, J.S., and Dávila, J.A., Sex and testosterone effects on growth, immunity and melanin coloration of nestling Eurasian kestrels, *J. Anim. Ecol.*, 2007, vol. 76, no. 1, pp. 201–209.
- Fitze, P.S. and Richner, H., Differential effects of a parasite on ornamental structures based on melanins and carotenoids, *Behav. Ecol.*, 2002, vol. 13, no. 3, pp. 401–407.
- Fitze, P.S., Tschirren, B., Gasparini, J., and Richner, H., Carotenoid-based plumage colors and immune function: is there a trade-off for rare carotenoids?, *Am. Nat.*, 2007, vol. 169, suppl. 1, pp. 137–144.
- Gasparini, J., Bize, P., Piau, R., Wakamatsu, K., Blount, J.D., Ducrest, A.-L., and Roulin, A., Strength and cost of an induced immune response are associated with a heritable melanin-based colour trait in female tawny owls, *J. Anim. Ecol.*, 2009, vol. 78, no. 3, pp. 608–616.
- Gavrilov, V.M., Kerimov, A.B., and Ivankina, E.V., Population and geographic variation of plumage color and metabolism in males of different color type in The Pied Flycatcher *Ficedula hypoleuca*, *Dokl. Akad. Nauk*, 1993, vol. 333, no. 6, pp. 807–810.
- Gavrilov, V.M., Kerimov, A.B., Golubeva, T.B., Ivankina, E.V., and Ilyina, T.A., Ecological energetics and population ecology of the great tit, in *Kol'tsevanie v izuchenii migratsii ptits v Rossii i soprodel'nykh gosudarstvakh. 1986–1987gg.* (Banding in the Study of Bird Migration in Russia and Neighboring Countries: 1986–1987), Moscow: Nauka, 1994, pp. 110–158.
- Gonzalez, G., Sorci, G., and de Lope, F., Seasonal variation in the relationship between cellular immune response and badge size in male house sparrows (*Passer domesticus*), *Behav. Ecol. Sociobiol.*, 1999, vol. 46, no. 2, pp. 117–122.
- Górecki, A., Kalabukhov-Skvortsov respirometer and resting metabolic rate measurement, in *IBP Handbook no. 24: Methods for Ecological Energetics*, Grodziński, W., Klekowski, R.Z., and Duncan, A., Eds., Oxford, UK: Blackwell, 1975, pp. 309–313.
- Griffith, S.C., Parker, T.H., and Olson, V.A., Melanin- versus carotenoid-based sexual signals: is the difference really so black and red?, *Anim. Behav.*, 2006, vol. 71, no. 4, pp. 749–763.
- Gross, W.B. and Siegel, H.S., Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens, *Avian Dis.*, 1983, vol. 27, no. 4, pp. 972–979.

- Hamilton, W.D. and Zuk, M., Heritable true fitness and bright birds: a role for parasites?, *Science*, 1982, vol. 218, no. 4570, pp. 384–387.
- Harris, J. and Bird, D.J., Supernatants from leucocytes treated with melanin-concentrating hormone (MCH) and α -melanocyte stimulating hormone (α -MSH) have a stimulatory effect on rainbow trout (*Oncorhynchus mykiss*) head kidney phagocytes in vitro, *Vet. Immunol. Immunopathol.*, 2000, vol. 76, nos. 1/2, pp. 117–124.
- Hill, R.W., Determination of oxygen consumption by use of the paramagnetic oxygen analyzer, *J. Appl. Physiol.*, 1972, vol. 33, no. 2, pp. 261–263.
- Hörak, P., Ots, I., and Murumägi, A., Hematological health state indices of reproducing great tits: a response to brood size manipulation, *Funct. Ecol.*, 1998, vol. 12, no. 5, pp. 750–756.
- Horak, P., Surai, P.F., Ots, I., and Möller, A.P., Fat soluble antioxidants in brood-rearing great tits *Parus major*: relations to health and appearance, *J. Avian Biol.*, 2004, vol. 35, no. 1, pp. 63–70.
- Hörak, P., Sild, E., Soomets, U., Sepp, T., and Kilk, K., Oxidative stress and information content of black and yellow plumage coloration: an experiment with greenfinches, *J. Exp. Biol.*, 2010, vol. 213, no. 13, pp. 2225–2233.
- Houde, A.E. and Torio, A.J., Effect of parasitic infection on male color pattern and female choice in guppies, *Behav. Ecol.*, 1992, vol. 3, no. 4, pp. 346–351.
- Ivankina, E.V., Ilyina, T.A., and Kerimov, A.B., The male plumage variability and the strategy of attracting a female: the estimation of the advertising behaviour's spatial organisation in bright and cryptically coloured Pied Flycatcher males (*Ficedula hypoleuca*, Passeriformes, Aves), *Zh. Obshch. Biol.*, 1995, vol. 56, no. 6, pp. 762–775.
- Ivankina, E.V., Kerimov, A.B., Grinkov, V.G., and Bushuev, A.V., Structural and functional aspects of variation of ornamentation of breeding plumage in pied flycatcher (*Ficedula hypoleuca*) males (Aves: Passeriformes), *Zh. Obshch. Biol.*, 2007, vol. 68, no. 4, pp. 278–295.
- Jacquin, L., Lenouvel, P., Haussy, C., Ducatez, S., and Gasparini, J., Melanin-based coloration is related to parasite intensity and cellular immune response in an urban free living bird: the feral pigeon *Columba livia*, *J. Avian Biol.*, 2011, vol. 42, no. 1, pp. 11–15.
- Järvi, T., Røskft, E., Bakken, M., and Zumsteg, B., Evolution of variation in male secondary sexual characteristics: a test of eight hypotheses applied to pied flycatchers, *Behav. Ecol. Sociobiol.*, 1987, vol. 20, no. 3, pp. 161–169.
- Kerimov, A.B. and Ivankina, E.V., The basal metabolic rate and its relationship to social and reproductive status in individual passerine birds, in *Proc. 22th Int. Ornithol. Congr. Durban*, Adams, N.J. and Slotow, R.H., Eds., Johannesburg: BirdLife South Africa, 1999a, pp. 370–389.
- Kerimov, A.B. and Ivankina, E.V., The relations between resting metabolic rate of fledglings and father's colour type in the pied flycatcher (*Ficedula hypoleuca*), *The Ring*, 1999b, vol. 21, no. 1, p. 203.
- Kerimov, A.B., Ivankina, E.V., Ilyina, T.A., and Bushuev, A.V., Energy heterogeneity of a polymorphic population of the pied flycatcher *Ficedula hypoleuca* in the breeding season, in *Populyatsionnaya ekologiya zhivotnykh* (Animal Population Ecology), Moskvitina, N.S., Ed., Tomsk: Tomsk. Gos. Univ., 2006, pp. 35–36.
- Lampe, H.M. and Espmark, Y.O., Song structure reflects male quality in pied flycatcher, *Ficedula hypoleuca*, *Anim. Behav.*, 1994, vol. 34, no. 4, pp. 869–876.
- Lawler, E.M. and Redig, P.T., The antibody responses to sheep red blood cells of the red-tailed hawk and great-horned owl, *Dev. Comp. Immunol.*, 1984, vol. 8, no. 3, pp. 733–738.
- Lundberg, A. and Alatalo, R.V., *The Pied Flycatcher*, London: T. and A.D. Poyser, 1992.
- Männiste, M. and Hörak, P., Effects of immune activation and glucocorticoid administration on feather growth in greenfinches, *J. Exp. Zool.*, 2011, vol. 315, no. 9, pp. 527–535.
- Martin II, L.B., Scheuerlein, A., and Wikelski, M., Immune activity elevates energy expenditure of house sparrows: a link between direct and indirect costs?, *Proc. Royal Soc. London: B*, 2002, vol. 270, no. 1511, pp. 153–158.
- Martin II, L.B., Trade-offs between molt and immune activity in two populations of house sparrows (*Passer domesticus*), *Can. J. Zool.*, 2005, vol. 83, no. 6, pp. 780–787.
- Maxwell, M.H., Avian blood leucocyte responses to stress, *Worlds Poult. Sci. J.*, 1993, vol. 49, no. 1, pp. 34–43.
- Maynard Smith, J. and Harper, D.G.C., The evolution of aggression: can selection generate variability?, *Phil. Trans. Royal Soc. London: B*, 1988, vol. 319, no. 1196, pp. 557–570.
- McGraw, K.J., The antioxidant function of many animal pigments: are there consistent health benefits of sexually selected colourants?, *Anim. Behav.*, 2005, vol. 69, no. 4, pp. 757–764.
- Moreno-Rueda, G., Experimental test of a trade-off between moult and immune response in house sparrows *Passer domesticus*, *J. Evol. Biol.*, 2010, vol. 23, no. 10, pp. 2229–2237.
- Moreno, J. and Möller, A.P., Are melanin ornaments signals of antioxidant and immune capacity in birds?, *Acta Zool. Sin.*, 2006, vol. 52, no. 1, pp. 202–228.
- Moreno, J., Sanz, J.J., Merino, S., and Arriero, E., Daily energy expenditure and cell-mediated immunity in pied flycatchers while feeding nestlings: interaction with moult, *Oecologia*, 2001, vol. 129, no. 4, pp. 492–497.
- Moreno, J., Velando, A., Ruiz-de-Castañeda, R., Cantarero, A., Gonzáles-Braojos, S., and Redondo, A., Plasma antioxidant capacity and oxidative damage in relation to male plumage ornamental traits in a montane Iberian pied flycatcher *Ficedula hypoleuca* population, *Acta Ornithologica*, 2011, vol. 46, no. 1, pp. 65–70.
- Moshkin, M.P., Gerlinskaya, L.A., and Evsikov, V.I., The role of the immune system in behavioral strategies of reproduction, *J. Reprod. Devel.*, 2000, vol. 46, no. 6, pp. 341–365.
- Mougeot, F., Galván, I., and Alonso-Alvarez, C., Contrasted effects of an oxidative challenge and α -melano-

- cyte-stimulating hormone on cellular immune responsiveness: an experiment with red-legged partridges *Alectoris rufa*, *Oecologia*, 2012, vol. 169, no. 2, pp. 385–394.
- Nava, M.P., Veiga, J.P., and Puerta, M., White blood cell counts in house sparrows (*Passer domesticus*) before and after moult and after testosterone treatment, *Can. J. Zool.*, 2001, vol. 79, no. 1, pp. 145–148.
- Olson, V.A. and Owens, I.P.F., Costly sexual signals: are carotenoids rare, risky or required?, *Trends Ecol. Evol.*, 1998, vol. 13, no. 12, pp. 510–514.
- Ots, I., Kerimov, A.B., Ivankina, E.V., Ilyina, T.A., and Hõrak, P., Immune challenge affects basal metabolic activity in wintering great tits, *Proc. Royal Soc. London: B*, 2001, vol. 268, no. 1472, pp. 1175–1181.
- Saks, L., Ots, I., and Hõrak, P., Carotenoid-based plumage coloration of male greenfinches reflects health and immunocompetence, *Oecologia*, 2003, vol. 134, no. 3, pp. 301–307.
- Sanz, J.J., Moreno, J., Merino, S., and Tomás, G., Trade-off between two resource-demanding functions: post-nuptial moult and immunity during reproduction in male pied flycatchers, *J. Anim. Ecol.*, 2004, vol. 73, no. 3, pp. 441–447.
- Schmidt-Nielsen, K., *Animal Physiology: Adaptation and Environment*, 4th ed., Cambridge: Cambridge Univ. Press, 1980.
- Silverin, B., Fänge, R., Viebke, P.-A., and Westin, J., Seasonal changes in mass and histology of the spleen in willow tits *Parus montanus*, *J. Avian Biol.*, 1999, vol. 30, no. 3, pp. 255–262.
- Slagsvold, T. and Lifjeld, J.T., Plumage colour is a condition-dependent sexual trait in male pied flycatchers, *Evolution*, 1992, vol. 46, no. 3, pp. 825–828.
- Sætre, G.-P., Fossnes, T., and Slagsvold, T., Food provisioning in the pied flycatcher: do females gain direct benefits from choosing bright-coloured males?, *J. Anim. Ecol.*, 1995, vol. 64, no. 1, pp. 21–30.
- Vysotsky, V.G., Determination of the age of pied flycatchers (*Ficedula hypoleuca*) during the breeding season, *Tr. Zool. Inst. Akad. Nauk SSSR*, 1989, vol. 197, pp. 49–52.
- Walther, B.A., Clayton, D.H., and Gregory, R.D., Showiness of Neotropical birds in relation to ectoparasite abundance and foraging stratum, *Oikos*, 1999, vol. 87, no. 1, pp. 157–165.
- Wegmann, T.G. and Smithies, O., A simple hemagglutination system requiring small amounts of red cells and antibodies, *Transfusion*, 1966, vol. 6, no. 1, pp. 67–73.
- Wilson, J.D., A re-assessment of the significance of status signalling in populations of wild great tits, *Parus major*, *Anim. Behav.*, 1992, vol. 43, no. 6, pp. 999–1009.
- Zuk, M., Parasites and bright birds: new data and a new prediction, in *Bird–Parasite Interactions: Ecology, Evolution, and Behaviour*, Loye, J.E. and Zuk, M., Eds., Oxford, UK: Oxford Univ. Press, 1991, pp. 317–327.
- Zuk, M., Thornhill, R., Ligon, J.D., and Johnson, K., Parasites and mate choice in red jungle fowl, *Am. Zool.*, 1990, vol. 30, no. 2, pp. 235–244.