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HEALTH-STATE VARIABLES AND ENZYMATIC BIOMARKERS AS SURVIVAL PREDICTORS IN NESTLING GREAT TITS (*PARUS MAJOR*): EFFECTS OF ENVIRONMENTAL CONDITIONS

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Abstract.—The health state of nestlings can be a useful bioindicator of the quality of the environment in which they are reared, but, to enable detection of responses to environmental change, the variation of health parameters under natural conditions should be evaluated. We describe the variation of morphological, biochemical, and hematological variables in relation to time of sampling, hatching date, brood size and type, and year in nestlings of two populations of Great Tits (*Parus major*) in Choupal, Portugal, and Wytham, United Kingdom. The influence of these health variables on nestlings' survival to first winter and recruitment into the breeding population was assessed in Wytham. Variation in plasma protein, total plasma cholinesterase (ChE), and acetylcholinesterase activities reflected circadian rhythms. Hatching date affected total plasma ChE and butyrylcholinesterase (BuChE) activities, and levels of red-blood-cell hemoglobin (Hb) and hematocrit (HCT). In Choupal, HCT increased with brood size. Nestlings in Choupal had significantly lower protein and Hb levels, and higher glutathione peroxidase (GSH-Px) activity, during a drier year. Second-brood nestlings had significantly lower levels of Hb and HCT. Of the studied variables, only plasma BuChE and red-blood-cell GSH-Px activities were related to nestlings' survival to first winter and recruitment. *Received 13 November 2007, accepted 6 June 2008.*

Key words: blood profile, glutathione peroxidase, Great Tit, hatching date, health state, nestlings, *Parus major,* plasma cholinesterase, survival.

Variables de Condición de Salud y Biomarcadores Enzimáticos como Predictores de la Supervivencia de Polluelos de *Parus major:* Efectos de las Condiciones Ambientales

Resumen.—La condición de salud de los polluelos puede ser un bioindicador útil de la calidad del ambiente en que éstos están siendo criados. Sin embargo, para poder detectar las respuestas a cambios ambientales, la variación de los parámetros de salud debe ser evaluada en condiciones naturales. Describimos la variación en mediciones morfológicas, bioquímicas y hematológicas con relación al período en que fueron tomadas, la fecha de eclosión, el tipo y tamaño de la nidada y el año. Estos datos fueron tomados para polluelos de dos poblaciones de *Parus major* ubicadas en Choupal, Portugal, y en Wytham, Reino Unido. La influencia de estas variables de salud sobre la supervivencia de los polluelos hasta el primer invierno y el reclutamiento a la población reproductiva fue determinada en Wytham. La variación en las actividades de proteínas plasmáticas, colinesterasa plasmática total (ChE, por sus siglas en inglés) y de acetilcolinesterasa reflejó los ritmos circadianos. La fecha de eclosión influenció la actividad de ChE plasmática total y de butirilcolinesterasa (BuChE), y también los niveles de hemoglobina en glóbulos rojos (Hb) y hematocrito (HCT). En Choupal, el HCT aumentó con el tamaño de la nidada. Los polluelos de Choupal tuvieron niveles significativamente menores de proteínas y Hb, y una mayor actividad de la peroxidasa de glutationa (GHS-Px) durante los años más secos. Los polluelos de segundas nidadas tuvieron niveles significativamente menores de Hb y HCT. De las variables estudiadas, sólo las actividades de la BuChE plasmática y la GHS-Px de los glóbulos rojos estuvieron relacionadas con la supervivencia de los polluelos hasta el primer invierno y con el reclutamiento.

The health state of nestling birds has been studied in ecology as an important indicator of their survival and recruitment. Traditionally, it has been assessed using weight or weight corrected for structural size (Tinbergen and Boerlijst 1990, Lindén et al. 1992, Both et al. 1999, Naef-Daenzer et al. 2001, Perrins and McCleery 2001), but recent studies have focused on different health variables that may be correlated with body condition, such as immune function (Christe et al. 2001, Moreno et al. 2005), white-blood-cell counts (Lobato et al. 2005, Hylton et al. 2006, Nadolski et al. 2006), and hematocrit (HCT) and other parameters related to

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red-blood-cell counts and hemoglobin (Hb) (Nadolski et al. 2006, Bańbura et al. 2007) to predict survival of nestlings, either in the nest or after fledging.

Body condition and health of nestlings can be useful as bioindicators of the state of the environment in which they were reared. Physiological condition (e.g., immunocompetence, Hb levels, and growth) of nestling birds has been reported to reflect environmental quality associated with food availability—related to brood size (Hõrak et al. 1999), hatching date (Christe et al. 2001, Dubiec and Cichoñ 2005), and territory quality (Hoi-Leitner et al. 2001, Bańbura et al. 2007)—and with parasite loads (Słomczyński et al. 2006) and presence of pollutants (Eeva and Lehikoinen 1996, Belskii et al. 2005). More specifically, the presence of pesticides (e.g., organophosphates) can be assessed by the use of biomarkers such as cholinesterase (ChE) enzymatic activities in nestlings, which are inhibited in their presence (Cordi et al. 1997). Knowledge of how environmental conditions affect the health state of nestlings and how health state, in turn, affects their survival provides valuable information about possible effects of environmental change on populations.

The use of nestlings' health state to make inferences about environmental quality and presence of stressors assumes an understanding of natural variation in health state. Here, we focus on the following health-state variables: (1) morphological: body-condition index; (2) biochemical: total plasma protein, plasma ChE activity, and red-blood-cell glutathione peroxidase (GSH-Px) activity; and (3) hematological: HCT, red-blood-cell Hb index, white-blood-cell count, and heterophil-to-lymphocyte ratio. We described the variation of these variables in nestling Great Tits (*Parus major*) in relation to year, hatching date, brood size, and brood type (first or second brood). We also tested whether any of these health-state variables influenced nestlings' survival to the following winter or their recruitment into the breeding population.

Methods

Field work.—Data were collected in the suburban mixed deciduous wood of Choupal, Coimbra, Portugal (40°13′N, 8°27′W), during the breeding seasons of 2003, 2005, and 2006. Additional data were obtained in the seminatural mixed deciduous woodland of Wytham, Oxford, United Kingdom (51°46′N, 1°18′W), during the breeding season of 2004. Nest boxes occupied by breeding pairs of Great Tits were monitored from the start of the breeding season onward to determine the laying and hatching dates (day 1 = 1 January). Nestling birds were banded, measured (body mass and tarsus length), and blood-sampled when they were 14 days old (hatching date = day 0). Asynchronously hatched nestlings were not sampled because hatching asynchrony was low, and underdeveloped nestlings were not included in the analysis. Blood (100–150 μL) was collected from the brachial vein into heparinized capillary tubes, under permission of national authorities. Body-condition index was obtained as the residuals of the linear regression of weight on tarsus length (Choupal: $b = -6.46 \pm 1.74$ [SE], $F = 170$, df = 1 and 286, $P < 0.0001$; Wytham: $b = 3.31 \pm 1.99$, $F = 53.95$, df = 1 and 271, $P < 0.0001$). The blood was used to make a thin film smear, and the remaining blood was separated into plasma and red-blood-cell fractions and frozen for later analysis. Survival to first winter and recruitment into the breeding population were estimated at Wytham, using information from winter mist-net recaptures (January–March 2005) and monitoring of nest boxes during the 2005 and 2006 breeding seasons. If a fledgling was not captured in the following winter (January to March) or caught as a breeder in Wytham nest boxes in the following two years, it was assumed to have died; if a fledgling was not caught as a breeder in Wytham nest boxes in the following two breeding seasons (2005 and 2006), it was assumed not to have recruited. Of the 274 ringed nestlings, 40 were recaptured in their first winter and 19 were recruited into the breeding population in the two following years. We recognize that these estimates of survival and recruitment include both mortality and dispersal, though we assumed that dispersal from Wytham was low and that the fledglings that dispersed were a random sample of individuals, therefore not differing in health-state variables (Perrins 1965, Verhulst et al. 1997). Survival to first winter and recruitment estimates were not possible in Choupal, because only 17 of the 318 banded nestlings were recaptured (A. C. Norte unpubl. data).

Variables studied.—Body-condition index was obtained as the residuals of the linear regression of body weight on tarsus length (Brown 1996), reflecting weight corrected for size and, therefore, energy reserves (e.g., fat) of nestlings. Plasma protein is a result of what the bird ate recently and reflects short-term condition and nutrition (Brown 1996). High values can also result from dehydration (Campbell 1995) or infection (Altman 1978, Lewandowski et al. 1986). Cholinesterase activities are frequently used as bioindicators of pollutants in the environment because they have characteristic biochemical responses (inhibition) to chemicals such as organophosphates and carbamates (Greig-Smith et al. 1992, Peakall 1992). The well-known function of ChE is to hydrolyze cholinesters in nerve synapses (Walker and Thompson 1991). The function of ChE in plasma is still under discussion (Satoh et al. 2002). Acetylcholinesterase (AChE), in particular, may reflect ChE activity in the central (Dell'Omo et al. 1996) and peripheral nervous systems (Cornish 1971), being an indicator of metabolic rate (Westlake et al. 1983) and behavior (Holmes and Boag 1990, Hart 1993). Butyrylcholinesterase (BuChE), because of its protease activity and catalytic activity toward various substrates, could serve a protective and clearing function for foreign substances encountered through the diet or other routes of exposure (Satoh et al. 2002, Çokuğraş 2003). Butyrylcholinesterase is related to food assimilation (Kutty 1980) and amount of fat in the diet (Van Lith et al. 1991). Glutathione peroxidase can be used as an indicator of exposure to pollutants, because it is part of the antioxidant defense mechanism of the organism (Sies 1993) against reactive oxygen species—generated by normal metabolism but also by processes of biotransformation of xenobiotics—that may cause oxidative stress (Kappus 1987, Parkinson 1996). Inflammation, phagocytic activity, and fatty-acid metabolism may also cause oxidative stress (Ames et al. 1993).

Hematocrit is the relative amount of red blood cells in total blood volume. Both HCT and Hb reflect the oxygen-carrying capacity of blood, which is related to amount of muscular activity (Saino et al. 1997), metabolic rate (Kostelecka-Myrcha et al. 1993), and capacity to thermoregulate (Ricklefs 1983, Swanson 1990). High values of HCT and Hb are usually associated with health and nutrition (Rattner et al. 1987, Averbeck 1992, Bańbura et al. 2007) because red blood cells and Hb are costly to produce and because

the presence of parasites may decrease both HCT and Hb levels, leading to anemia (Dein 1986). Interpretation of HCT, however, should be done carefully and should take into account natural influencing factors (reviewed by Fair et al. [2007]) and variation of other measures of condition (e.g., Hb). Dawson and Bortolotti (1997a) suggested that HCT may not be a robust indicator of condition in normal populations of birds (i.e., populations that are not under severe stress or injured; Dawson and Bortolotti 1997a, b). White-blood-cell count (WBC) indicates the overall state of immune function and the presence of current infection–inflammation (high WBC accompanied by a high heterophil count; Campbell 1995, Norris and Evans 2000). Heterophil-to-lymphocyte ratio (H:L), often used as a stress indicator in birds (Gross and Siegel 1983, Maxwell 1993), increases as a response to stressors such as fear, social disruption, food restriction, temperature, and noise, among others (Vleck et al. 2000, Ruiz et al. 2002). However, in the case of viral infection, the increase in H:L may not be noticeable, given an accompanying increase in the number of lymphocytes.

Laboratory analysis.—Total plasma protein (protein, mg mL^{-1}) was measured using the Bradford protein assay based on the Bradford dye-binding procedure (Bradford 1976). Total plasma ChE activity (total ChE, μmol min^{-1} mL^{-1}) was assayed according to the method of Ellman et al. (1961) by following the increase of yellow color produced by the reaction of thiocholine with 5,5-dithio(bis-2-nitrobenzoic acid) (DTNB) at 405 nm. To distinguish between BuChE and AChE activity, we selectively inhibited BuChE activity using tetraisopropylpyrophosphoramide (iso-OMPA). For red-blood-cell GSH-Px measurements (μmol min^{-1} g^{-1} Hb), we used the method of Paglia and Valentine (1967) as modified by da Silva and dos Santos (1991), which consists of measuring the rate of GSSG (oxidized glutathione) formation at 340 nm as NADPH is converted to $NADP^+$. Enzymatic activities and total protein were measured in a Sunrise microplate reader (Tecan, Männedorf, Switzerland).

Red-blood-cell Hb index (g L^{-1}) was measured by the cyanmethemoglobin method at a wavelength of 540 nm (van Kampen and Zijlstra 1961) using a commercial kit (Bio-Systems SA, Simon's Town, South Africa) in the 4× diluted hemolysate of red blood cells and is not equivalent to whole-blood Hb concentration. Hematocrit was measured as the percentage of the length of the part of the capillary tube occupied by red blood cells in relation to the total length of the capillary tube occupied by blood, after its centrifugation during 10 min at 3,200 rpm. Blood smears from each bird were air-dried and stained using the May-Gründwalds-Giemsa procedure and scanned under 1,000× magnification. White-blood-cell count was estimated by counting the number of white blood cells per ~10,000 red blood cells. Heterophil-to-lymphocyte ratio was measured on the basis of examination of 50 white blood cells, because the repeatability of measurements on 50 and 100 white blood cells was 0.94 ± 0.01. In 2003, enzymatic activities and red-blood-cell Hb index were not measured, whereas HCT levels were not measured in 2004.

Statistical analysis.—Pearson's correlation coefficients among health-state variables in nestlings were assessed for the populations at Choupal and Wytham, and differences between correlation coefficients for each pair of variables in each population were compared. The relationships between each health-state variable and (1) time of sampling and (2) hatching date were assessed with linear regressions for each population. For hatching date, we used the residuals of health-state variables on time of sampling. Mean brood values were used in these correlation and regression analyses to control for non-independent siblings in the same nest box.

The effects of brood size and year (and their interaction; in Choupal) and the effect of brood size (in Wytham) on each health-state variable were tested using a general liner model (GLM) fitted with restricted maximum likelihood, with "band number" nested in "nest box" as a random effect, to control for siblings sharing the same box. We controlled for potential effects of time of sampling, when significant, on the response variables by including it as a covariable in the models. However, because it was not significant in any of the models, it was dropped from the final models.

The relationships of the health-state variables, computed as their residuals on time of sampling (and their squared terms), with survival to first winter and recruitment into the breeding population were studied by fitting a GLM controlling for overdispersion. The dependent variable was defined as survived–recruited (1) or not survived–not recruited (0) and followed a binomial distribution. First, each health-state variable was tested separately and was included in the multivariate model if $P < 0.25$ (Hosmer and Lemeshow 2000). The final model includes only the variables that were significant. We also fitted the same models including nest box as a random effect, to control for non-independent survival–recruitment among nestlings from the same nest box, but, because the results were very similar, we present the results of the final models with no random effects.

Only first broods were used in all analyses. Differences in health-state variables of nestlings between first and second broods were assessed with a paired *t*-test using the residuals of mean brood values on mean time of sampling. Variables were square-root (sqrtWBC) and logarithmic (H:L, protein, and AChE) transformed for normality. Total ChE (in Choupal) and body condition, Hb, and ln H:L (in Wytham) deviated moderately from normality.

Results

Correlations between health-state variables.—We sampled 238 nestlings from 54 first broods in Choupal and 273 nestlings from 36 first broods in Wytham. Mean ± SD for each health-state variable is presented in Table 1. Leukocyte-related variables (WBC and H:L) had the largest coefficients of variation (Table 1). Pearson's correlation coefficients between health-state variables for both Choupal and Wytham and the *P* value of the difference between correlation coefficients for each pair of variables of the two populations are shown in Table 2. The highest correlation coefficients were found between total ChE and both AChE and BuChE and ranged between 0.50 and 0.80. Therefore, these variables should not be treated independently. The strongest correlation coefficients, consistent in both Choupal and Wytham, were between protein and both total ChE and AChE ($0.40 < r < 0.61$) and between body-condition index and H:L ($r = -0.35$ and $r = -0.28$, respectively). Relationships between AChE and both BuChE and WBC, and between Hb and GSH-Px, differed between populations. This indicates that those variables may have different meanings when evaluating the overall health state of nestlings in different populations.

TABLE 1. Means ± SD and coefficients of variation (CV) for health-state variables of nestling Great Tits in Choupal in 2003 (5 broods), 2005 (22 broods), and 2006 (27 broods) and in Wytham in 2004 (36 broods). For definitions of variables, see text.

	Choupal						Wytham	
	2003		2005		2006		2004	
	CV (%)	Mean ± SD	CV (%)	Mean ± SD	CV (%)	Mean ± SD	CV (%)	Mean ± SD
Body-condition index	—	–0.11 ± 1.68	—	–0.30 ± 1.64	27	0.27 ± 0.97	—	0.03 ± 0.86
Protein (mg mL^{-1})	12	64.87 ± 7.94	18	57.08 ± 10.36	12	66.30 ± 8.19	20	46.16 ± 9.35
Total ChE (μmol min^{-1} mL^{-1})	—	—	12	0.60 ± 0.07	14	0.65 ± 0.09	7	0.58 ± 0.04
BuChE (μmol min^{-1} mL^{-1})	—	—	8	0.30 ± 0.04	18	0.27 ± 0.05	10	0.29 ± 0.03
AChE (μmol min^{-1} mL^{-1})	—	—	18	0.30 ± 0.05	15	0.39 ± 0.06	10	0.29 ± 0.03
GSH-Px (μmol min^{-1} g^{-1} Hb)	—	—	13	8.23 ± 1.05	12	7.10 ± 0.87	18	7.49 ± 1.36
Hb index (g L^{-1})[a]	—	—	29	17.03 ± 4.96	17	23.28 ± 3.92	25	17.82 ± 4.55
HCT (%)	18	44.93 ± 7.92	8	50.01 ± 4.30	7	47.52 ± 3.30	—	—
WBC	37	21.62 ± 7.95	19	16.79 ± 3.28	38	16.29 ± 6.13	37	11.76 ± 4.34
H:L	38	0.61 ± 0.23	74	0.74 ± 0.55	76	0.81 ± 0.62	42	0.33 ± 0.14

[a] Hb index is not equivalent to whole-blood Hb concentration.

Effect of time of sampling.—Time of sampling explained 24.8% (Choupal) and 32.7% (Wytham) of the variation in nestlings' plasma protein, which increased during the day (Table 3). In Choupal, total ChE, AChE, and Hb also increased significantly during the day, but in the case of Hb, the variance explained was smaller (8.4%). In Wytham, AChE also had a tendency to increase during the day, but the effect of time of sampling was only nearly significant (Table 3).

Effect of hatching date.—Hatching date ranged between 20 April and 22 June in Choupal and between 29 April and 22 May in Wytham. Total ChE decreased significantly during the season in Choupal, whereas in Wytham it decreased at the start of the season but increased again at the end of the season (Table 4). BuChE increased significantly with hatching date in Wytham, but in Choupal there was a tendency for a decreasing trend. Late nestlings in Choupal and nestlings hatching in the middle of the season in Wytham had significantly lower levels of Hb. Late nestlings in Choupal also had significantly lower HCT (Table 4).

Effects of year and brood size.—No significant effects of brood size on health-state variables were detected in Wytham. In Choupal, there were significant effects of year ($F = 10.49$, df = 2 and 177, $P < 0.0001$), brood size ($F = 40.11$, df = 1 and 177, $P < 0.0001$), and an interaction between year and brood size ($F = 14.07$, df = 2 and 177, $P < 0.0001$) on HCT. HCT increased with brood size in all years, but the interaction should be interpreted cautiously because of the small sample size for 2003. In Choupal, year significantly affected protein ($F = 13.99$, df = 2 and 178.1, $P < 0.0001$), Hb ($F = 41.50$, df = 1 and 166, $P < 0.0001$), and GSH-Px ($F = 22.86$, df = 1 and 53.8, $P < 0.0001$). In 2005, Choupal nestlings had significantly lower protein and Hb and higher GSH-Px than in 2006 (Table 1).

Health-state variables and survival.—Of 273 nestlings, only 40 were recaptured during their first winter in Wytham. Only hatching date and plasma BuChE activity were related to probability of survival to first winter. Nestlings hatching later in the season and with intermediate values of plasma BuChE activity had a larger probability of not surviving (Table 5).

Only 19 nestlings were recaptured breeding in Wytham. We found evidence that GSH-Px activity was related to the probability of recruitment into the breeding population, because nestlings with very low or very high levels of GSH-Px activity had higher probability of not recruiting (Table 5).

Health-state variables in nestlings from first and second broods.—Thirteen pairs of Great Tits had second broods in Choupal in 2003, 2005, and 2006. Nestlings from second broods had significantly lower HCT (first broods: 48.95 ± 4.94; second broods: 43.68 ± 4.15; $t = 3.14$, df = 1 and 12, $P = 0.009$) and nearly significantly lower Hb (first broods: 23.24 ± 4.46; second broods: 20.22 ± 4.54; $t = 2.08$, df = 1 and 12, $P = 0.06$). No significant differences were found in body-condition index, protein, total ChE, AChE, BuChE, GSH-Px, WBC, or H:L between first and second broods ($0.90 > P > 0.13$).

DISCUSSION

We have reported how time of sampling, hatching date, year, brood size, and brood type affected morphological, biochemical, and hematological variables of nestlings in two populations of Great Tits. Apart from hatching date, our results suggest that the activities of BuChE and GSH-Px were related to the probability of a nestling surviving or recruiting into the breeding population.

Effect of time of sampling.—Time of sampling explained a large proportion of the variation in plasma protein levels in both populations. This is because plasma protein reflects what the bird ate recently and what has been absorbed and transported to the tissues (Brown 1996) and, therefore, increases during the day through continuous feeding of nestlings (Perrins 1979). Total ChE and AChE, which are themselves proteins and are correlated with plasma protein levels, also increased through the day in Choupal. Circadian rhythms of plasma ChE and carboxilesterase activities were reported by García-Rodríguez et al. (1987) and Thompson et al. (1988); presence of sunlight (Bieth et al. 1970) and both activity and feeding patterns may trigger variation in the activities of these enzymes. Also, a small percentage of the variation in the red-blood-cell Hb index was attributable to time of sampling. Variations in the oxygen transport capacity during the day, which have been described in birds and mammals (Fox and Laird 1970, Rehder et al. 1982, Durotoye et al. 2000), could reflect variations in their activity and may arise through contraction of the spleen (Kleiner and Orten 1966).

Table 2. Pearson's correlation coefficients (*r*) between health-state variables (mean brood values) of populations of nestling Great Tits in Choupal (C) and Wytham (W) (upper table), and *P* values for the comparison between correlation coefficients of the two populations for each pair of variables (lower table). For definition of variables, see text; for variable units, see Table 1.

	Body-condition index		Protein		Total ChE		AChE		BuChE		GSH-Px		Hb		HCT		sqrtWBC		ln H:L	
	C	W	C	W	C	W	C	W	C	W	C	W	C	W	C	W	C	W	C	W
Body-condition index			0.33	0.06	−0.02	−0.14	−0.10	−0.48	0.10	0.36	−0.06	−0.45	0.23	−0.02	0.10	—	0.18	−0.08	−0.35	−0.28
Protein	0.22				0.51	0.49	0.40	0.61	0.31	−0.09	−0.26	−0.03	0.25	0.29	0.33	—	−0.09	0.32	0.01	−0.05
Total ChE	0.60		0.91				0.80	0.59	0.60	0.50	−0.24	0.32	0.42	0.20	0.32	—	−0.07	0.23	0.26	−0.04
AChE	0.07		0.22		0.07				0.00	−0.40	−0.34	0.09	0.59	0.24	0.04	—	−0.17	0.40	0.18	0.22
BuChE	0.24		0.08		0.54		0.07				0.06	−0.44	−0.10	−0.03	0.47	—	−0.06	−0.16	0.18	−0.29
GSH-Px	0.07		0.32		0.02		0.06		0.03				−0.46	−0.01	0.14	—	−0.09	−0.06	−0.23	0.06
Hb index	0.28		0.85		0.30		0.07		0.76		0.04				−0.10	—	0.08	0.26	0.12	−0.04
HCT	—		—		—		—		—		—		—				−0.07	—	0.14	—
sqrtWBC	0.25		0.07		0.20		0.01		0.67		0.90		0.43		—				−0.04	−0.21
ln H:L	0.73		0.80		0.20		0.86		0.04		0.22		0.50		—		0.45			

Effect of hatching date.—A seasonal decline of nestling condition was found in several studies of insectivorous bird species (Barba et al. 1995; Dubiec and Cichoñ 2001, 2005) and is generally explained by a seasonal decline in food availability or differences in parental quality between early and late breeders. In both the Choupal and the Wytham populations, effects of hatching date could be detected in total ChE, BuChE, and Hb. In Choupal, HCT was also affected by hatching date. Late nestlings in Choupal had lower levels of total ChE, BuChE, HCT, and Hb. Lower levels of HCT and Hb later in the season could indicate poor health and anemia, caused either by poorer nutrition (e.g., lack of certain nutrients such as iron [Kubena et al. 1972] or low food availability–quality [Merino and Potti 1998, Bańbura et al. 2007]) or by higher levels of parasite transmission later in the season (blood loss by ectoparasites or red-blood-cell destruction by endoparasites; Campbell 1995, Słomczyński et al. 2006). However, the fact that late nestlings had neither lower body-condition index nor increased WBC in response to infection gives no support to these hypotheses. It is also possible that, because of warmer ambient temperatures later in the season, the need for increased metabolic rate for thermoregulation was lower (Swanson 1990). The fact that late nestlings in Choupal and early nestlings in Wytham had lower levels of BuChE is difficult to explain. It could reflect variations in diet fat content (Van Lith et al. 1991), but we have no data to support this hypothesis. Also, we cannot explain the finding that nestlings hatching in the middle of the season in Wytham had lower levels of total ChE and Hb.

Effect of brood size and year.—In Choupal, nestlings from larger broods had significantly higher HCT, though the magnitude of this difference varied among years. This could be explained by higher competition among nestlings in larger broods, which increases their metabolic activity and, consequently, tissue oxygen demand (Costantini et al. 2006). Potti et al. (1999) studied nestlings' HCT in another insectivorous passerine species, the Pied Flycatcher (*Ficedula hypoleuca*), but found no effect of brood size. This difference between studies and among years may be related to the capacity of parents to increase the feeding rate to larger broods. If the amount of food delivered per capita decreases in larger broods, it is most likely that nestlings will be unable to increase their HCT in response to competition and, in more severe cases, their body-condition index will probably decrease. Two of the study years in Choupal differed in relation to weather conditions and, consequently, probably in terms of food availability. The year 2005 was rather dry, and fledging success was lower than in 2006 (A. C. Norte unpubl. data). This difference was reflected in nestlings' physiology, namely in protein, GSH-Px, and Hb. In 2005, nestlings had lower levels of protein and Hb than in 2006, which may reflect poorer nutrition in that year (Rattner et al. 1987, Bańbura et al. 2007). In 2005, nestlings also had higher levels of GSH-Px, which indicates a higher level of oxidative stress. Oxidative stress arises when there is an imbalance between antioxidant defenses and reactive oxygen species (ROS) production, which originates from normal metabolism or inflammation processes and phagocytic activity toward bacteria and parasites, as well as from fatty-acid metabolism (Ames et al. 1993). If the increase in oxidative stress in 2005 was caused by higher levels of infection by blood parasites, this could explain the lower levels of Hb in that year, because of red-blood-cell destruction (Campbell 1995).

Table 3. Linear regressions of health-state variables of nestling Great Tits on time of sampling (hour) in Choupal and Wytham. Mean brood values were used as response variables. *F* and *P* values are from analyses of variance for linear fit.

	Choupal					Wytham				
	Coefficient time	Whole model				Coefficient time	Whole model			
		F	df	*P*	Adj. R^2 (%)		*F*	df	*P*	Adj. R^2 (%)
Body-condition index	0.0016	1.63	1 and 50	0.21	1.2	0.00048	0.58	1 and 34	0.45	–1.2
Protein (mg mL^{-1})	0.032	16.80	1 and 47	0.0002	24.8	0.023	17.52	1 and 33	0.0002	32.7
Total ChE (μmol min^{-1} mL^{-1})	0.00033	16.12	1 and 43	0.0002	25.6	0.000012	0.20	1 and 33	0.66	–2.4
AChE (μmol min^{-1} mL^{-1})	0.00026	15.54	1 and 43	0.0003	24.8	0.000045	3.5	1 and 33	0.07	6.9
BuChE (μmol min^{-1} mL^{-1})	0.000068	1.54	1 and 43	0.22	1.2	–0.000032	1.94	1 and 33	0.17	2.7
GSH-Px (μmol min^{-1} g^{-1} Hb)	–0.0023	4.13	1 and 43	0.05	6.8	0.0008	0.68	1 and 33	0.41	–0.9
Hb index (g L^{-1}) [a]	0.012	5.03	1 and 43	0.03	8.4	0.0044	1.76	1 and 33	0.19	2.2
HCT (%)	0.005	1.43	1 and 47	0.24	0.9	—	—	—	—	—
sqrtWBC	0.00009	0.02	1 and 50	0.88	–2.0	–0.0003	0.46	1 and 33	0.50	–1.6
ln H:L	0.0002	0.05	1 and 49	0.82	–1.9	0.00015	0.21	1 and 33	0.65	–2.4

[a] Hb index is not equivalent to whole-blood Hb concentration.

Health-state variables and survival.—The probability of survival to first winter was negatively related to hatching date and to the squared term of BuChE activity. Hatching date is well known to have an important effect on survival of fledgling Great Tits; generally, fledglings that hatch later in the season have less probability of surviving and recruiting (Perrins 1965, Verhulst and Tinbergen 1991, Verboven and Visser 1998). This relationship between hatching date and survival may arise because differences in food availability or in the quality of parental care between early and late broods lead to differences in weight and body condition of nestlings; chicks in good body condition perform better in adverse environmental conditions (Perrins 1965), and in competition among fledged birds (Verhulst and Tinbergen 1991, Both et al. 1999). Therefore, this effect of hatching date is often mediated by an effect of fledging mass–condition on survival, and several studies found a positive correlation between fledgling mass–condition and survival (Perrins 1965, Tinbergen and Boerlijst 1990, Lindén et al. 1992, Verboven and Visser 1998, Perrins and McCleery 2001, Monrós et al. 2002). However, we did not find evidence of a relationship between body-condition index at day 14 and survival. It may be that early nestlings fledge in a more favorable period than late nestlings, which favors their immediate survival after leaving the nest and renders them better competitors than late nestlings. Nestlings with either low or high values of BuChE had a higher probability of surviving, but we have no explanation for this finding.

The fact that nestlings with very high or very low GSH-Px activity levels had disproportionally lower chances of recruitment into the breeding population may be related to the levels of oxidative stress to which they are exposed, of which GSH-Px is an indicator (Sies 1993). The organism compensates for increased production of ROS with activation of defense mechanisms, which include enzymatic activities such as GSH-Px. If ROS production is not completely compensated for, ROS will interact with cell macromolecules, contributing to their damage, to consequent cell senescence and death, and to aging of the organism (Ames et al. 1993, Møller et al. 2000), which can impair nestlings' health and recruitment. Low levels of GSH-Px activity may be associated with lower recruitment probability when the levels of oxidative stress are so elevated that they damage the enzyme by oxidative modification or decrease its activity by negative feedback from excess substrate (Pippenger et al. 1998). Oxidative stress was also pointed out as the mechanism of the tradeoff between breeding effort and future survival and fecundity in adult birds (Wiersma et al. 2004, Alonso-Alvarez et al. 2006). In other studies, hematological variables related to immune function (cellular immune response assayed with PHA test [Moreno et al. 2005], immunoglobulin levels [Christe et al. 2001], and WBC [Lobato et al. 2005, Hylton et al. 2006]) were found to be good predictors of survival of fledglings. However, in the present study, this was not the case, and only BuChE and GSH-Px reflected survival prospects of nestlings.

Health-state variables in nestlings from first and second broods.—In Choupal, nestlings from second broods had significantly lower Hb levels and HCT and, therefore, lower oxygen carrying capacity. This may reflect malnutrition (Merino and Potti 1998, Bańbura et al. 2007), higher parasite loads (de Lope and Møller 1993, Campbell 1995), or less need for thermoregulation in second broods (Swanson 1990). Dubiec and Cichoñ (2001) also reported that nestling Great Tits in second broods had lower HCT and body condition than those in first broods, which they attributed to a diminishing food supply as the season progressed. In the present study, there was also a trend, though not significant, for a decreased body-condition index and an increase in WBC in second-brood nestlings, which suggests that the third hypothesis is less likely and that second-brood nestlings were, indeed, in poorer health. The small sample sizes probably influenced the nonsignificant differences in health-state variables between first and second broods, especially for variables with large coefficients of variation. Measurements of food availability and parasite loads throughout the season would improve our understanding of the variation in health state of nestlings, but these were not evaluated in the present study.

Nestling health-state variables as bioindicators.—Both HCT and red-blood-cell Hb concentration were responsive to environmental variables, reflecting both seasonal (hatching date and type of brood) and yearly effects. We conclude that, if evaluated together, they have the potential to provide valuable information concerning the health state of nestling passerines and the

TABLE 4. Linear regressions of health-state variables of nestling Great Tits in Choupal and Wytham on hatching date (in days; day 1 = 1 January). Response variables were computed as residuals of mean brood values on mean time of sampling. *F* and *P* values are from analyses of variance for linear fit.

	Choupal						Wytham					
	Coefficient		Whole model				Coefficient		Whole model			
	Date	Date^2	*F*	df	*P*	adj R^2 (%)	Date	Date^2	*F*	df	*P*	Adj. R^2 (%)
Body-condition index	–0.0089	—	0.74	1 and 50	0.39	–0.5	0.027	—	0.88	1 and 34	0.35	–0.3
Protein (mg mL^{-1})	0.0095	—	0.02	1 and 47	0.89	–2.1	0.136	—	0.23	1 and 33	0.63	–2.3
Total ChE (μmol min^{-1} mL^{-1})	–0.0016	—	6.37	1 and 43	0.015	10.9	–0.04	0.00039	4.03	2 and 32	0.028	15.1
AChE (μmol min^{-1} mL^{-1})	–0.00074	—	1.92	1 and 43	0.17	2.0	–0.001	—	0.71	1 and 33	0.40	–0.8
BuChE (μmol min^{-1} mL^{-1})	–0.00083	—	3.74	1 and 43	0.06	5.9	0.0028	—	6.96	1 and 33	0.013	14.9
GSH-Px (μmol min^{-1} g^{-1} Hb)	0.0039	—	0.17	1 and 42	0.68	–2.0	–0.07	—	2.3	1 and 33	0.14	3.7
Hb index (g L^{-1})[a]	–0.098	—	5.34	1 and 43	0.026	9.0	–5.92	0.058	8.32	2 and 32	0.0012	30.0
HCT (%)	–0.96	—	7.51	1 and 47	0.0086	11.9	—	—	—	—	—	—
sqrtWBC	0.0043	—	0.72	1 and 50	0.40	–0.6	0.022	—	0.94	1 and 33	0.34	–0.2
ln H:L	–0.0007	—	0.014	1 and 49	0.90	–2.0	–0.023	—	2.03	1 and 33	0.16	2.9

[a] Hb index is not equivalent to whole-blood Hb concentration.

TABLE 5. Generalized linear models of survival to first winter and recruitment into the breeding population for nestling Great Tits in relation to health variables. Final models retaining only the significant variables are presented. Reference category is no survival–no recruitment. Date = hatching date; BuChE = residuals of butyrylcholinesterase activity on time of sampling; GSH-Px = residuals of glutathione peroxidase activity on time of sampling. For further explanation of variables, see text.

Term	Estimate ± SE	χ^2	*P*
Survival to first winter (χ^2 = 9.06, df = 3, *P* = 0.028)			
Intercept	–3.15 ± 2.41	1.71	0.19
Date	0.096 ± 0.046	4.25	0.04
BuChE	–3.93 ± 3.30	1.42	0.23
BuChE^2	–98.51 ± 44.96	4.80	0.028
Recruitment (χ^2 = 6.61, df = 2, *P* = 0.037)			
Intercept	1.97 ± 0.35	31.86	<0.0001
GSH-Px	–0.33 ± 0.28	1.33	0.25
GSH-Px^2	0.33 ± 0.18	3.19	0.074

environmental conditions they are exposed to. However, if one of these two variables has to be chosen, Hb may be a more robust indicator of environmental quality, because it reflects the development of blood function (Kostelecka-Myrcha et al. 1973), which is based on the food supply to nestlings. Also, the slower regeneration rate of this molecule in the organism, compared with the production of red blood cells to increase HCT, may render it a more sensitive bioindicator (O'Brien et al. 2001). Hemoglobin was also reported to positively affect chick survival in the nest (Bańbura et al. 2007).

Because their function in plasma is not understood, seasonal variation in plasma ChE is difficult to explain (Satoh et al. 2002). Therefore, the use of ChE as a bioindicator may be limited to specific situations of exposure to pesticides (e.g., organophosphates and carbamates; Greig-Smith et al. 1992). Contrary to our expectations, H:L was not correlated with date or brood size and was not affected by year or type of brood. The presence of a viral infection could prevent an increase of H:L ratio in stressed nestlings. The H:L ratio may be a more reliable stress indicator in adults than in nestlings, whose immune systems are still developing (Lowry et al. 1997).

Use of BuChE and GSH-Px activities as bioindicators has the advantage of allowing inferences concerning the effects of environmental quality at the population level, because these two enzymatic activities were found to be related to the probability of survival to first winter and recruitment into the breeding population of nestling Great Tits. On the other hand, we have no evidence that any of the other measured health-state variables are related to survival and recruitment of nestling Great Tits. Our results further suggest that when studying experimental effects on health-state variables or their response to environmental cues (e.g., presence of pollutants), appropriate controls must be used to account for natural variation.

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