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Factors affecting haematological variables and body mass of reed warblers (*Acrocephalus scirpaceus*) and sedge warblers (*A. schoenobaenus*)

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Energetically demanding life-history stages in birds (reproduction, migration) might be associated with sex- and age-specific physiological changes expressed in suppressed immune function or declined body mass. Here, we examined the numbers of red and white blood cells (RBC and WBC), the ratio of heterophils and lymphocytes (H/L)], and body mass of reed warblers (*Acrocephalus sirpaceus*) and sedge warblers (*A. schoenobaenus*) during the breeding and migration periods. Higher WBC counts and H/L ratio in adults (both warblers) and lower RBC counts (only the reed warbler) during the prelaying/laying period as compared with those during the incubation/chick-rearing period suggest greater energetic demands during the latter period. In adults, higher H/L ratio and WBC counts in males of both species as compared with those in females during the prelaying/laying period indicate higher energy expenditures of males, probably associated with mate guarding and chasing intruders. Lower H/L ratio and WBC counts in adults of both species as compared with those in immatures during the migration period suggest lower stress level of adults, possibly due to their greater experience and/or higher social status.

Introduction

Birds undergo pronounced seasonal changes in physiology (Murton & Westwood 1977). Such energetically demanding activities as reproduction, molt, growth or migration may drain energy and resources from individuals, and are often associated with declined body mass and suppressed immune function (Ricklefs 1974, Drent & Daan 1980, Norris & Evans 2000). Males and females, due to their constraints imposed by physiological (different endocrine profiles, different cost of gametes production), behavioral (usually one sex-biased contribution into brood rearing) and environmental factors (lower susceptibility of bigger sex to deteriorating conditions) may differ in body condition and blood profiles (e.g. Hõrak *et al*. 1998, Ots *et al*. 1998, Klein 2000, Sanz *et al*. 2000, Visser & Lessells 2001, Kilgas *et al*. 2006). Similarly, birds of different age may respond to a given activity in a different way. For instance, migrating adults are often in better body condition (larger fat reserves, higher body mass) than immatures, which is usually explained by the lack of experience and lower social status of the latter (Woodrey & Moore 1997).

Physiological response of birds to particular life-history stages might be expressed by changes in various variables associated with body condition and stress level. In the present study, the following variables were investigated: the numbers of red blood cells (hereafter RBC) and white blood cells (hereafter WBC), the ratio of heterophils and lymphocytes (H/L ratio), and body mass. The RBC number, being an index of the oxygen transfer capacity of the blood, is used as an indicator of health in birds (e.g. Kalmbach *et al*. 2004, Sergent *et al*. 2004). Low RBC numbers can indicate illness (Campbell 1995, Artacho *et al*. 2007), might also be a sign of anaemia, since erythropoesis (RBC production) depends on the nutritional status. Decrease in numbers of RBC is also associated with reduction in body mass (Artacho *et al*. 2007). Thus, individuals in good body condition have greater values of RBC counts than those in poor body state (Sergent *et al*. 2004). Since WBC form the basis of the immune system of an organism, their elevated numbers (leukocytosis) are symptomatic of stress syndrome and inflammatory processes (Fudge 1989, Campbell 1995, Norris & Evans 2000). Leukocytosis is mostly caused by a rising number of heterophils in the peripheral blood (e.g. Dein 1986). The ratio of the most numerous leukocytes in birds, i.e. the heterophils/lymphocytes ratio (hereafter H/L) is often used as a stress indicator in birds (Gross & Siegel 1983, Maxwell 1993). It increases in response to various stressors, including infectious diseases, parasite infestation, food or water deprivation, temperature extremes and psychological disturbance (Ots & Hõrak 1996, Totzke *et al*. 1999, Vleck *et al*. 2000, Ruiz *et al*. 2002, Lobato *et al*. 2005, Davis *et al*. 2008). This increase is caused by redistribution of lymphocytes and heterophils between the blood and other tissues and it is primarily induced by the secretion of corticosterone (Dhabhar 2002, Davis *et al*. 2008). A change in the H/L ratio is detectable up to 1 hour following exposure to a stressor (Davis 2005, Cīrule *et al*. 2012). Thus, unlike the corticosterone level in blood, it is not so rapidly affected by handling or blood sampling stress (Maxwell 1993, Davis *et al*. 2008). Body mass is the simplest, widely used index of body condition. However, it might be affected by body size. Calculation of the body mass corrected for body size is the simplest way of separating the effects of condition from the effects of body size (Reist 1985, Krebs & Singleton 1993, Jakob *et al*. 1996, Ots *et al*. 1998).

The reed warbler (*Acrocephalus scirpaceus*) and the sedge warbler (*A. schoenobaenus*) are two closely related passerines with a similar morphology. Both species are sexually monomorphic in plumage with slight body size dimorphism (males are larger than females, with longer wings; Ellrich *et al*. 2010, Jakubas & Wojczulanis-Jakubas 2010, Wojczulanis-Jakubas & Jakubas 2011). The two species have similar breeding biology, although with some notable exceptions. Basically, the two species are socially monogamous, however, the sedge warbler is facultatively socially polygynous (Hasselquist & Langefors 1998). In both species, a male sings before pair formation but in the reed warbler also during the nesting period (Cramp 1998, Langefors *et al*. 1998). Mate guarding prior to egg-laying is performed by males in both species. A female of the reed warbler usually lays 3–5 eggs whereas the sedge warbler female 5–6. Relative contribution of a male to the whole parental care is larger in the reed warbler (classified as full parental care) than in the sedge warbler (reduced parental care) (Hoi *et al*. 1995, Kleindorfer *et al*. 1995, Leisler *et al*. 2002). Both species perform long-distant, nocturnal migrations to wintering areas in central Africa, south of the Sahara. Adults of both species undergo a complete moult in their wintering areas. However, immature reed warblers undergo a partial post-fledging molt in the natal area whereas sedge warblers do not (Cramp 1998, Hall 1996, Redfern & Alker 1996).

The main aim of this study was to investigate how extrinsic (season) and intrinsic (sex and age) factors affect haematological variables and body mass of reed and sedge warblers. To fully comprehend how the factors studied affect the birds physiological response, we chose the two closely related species that have in general similar life-strategies but differ in some traits (*see* above). Moreover, to our knowledge, studies of haematological variables have never been conducted in these two species before. We expected that the body condition variables should differ between spring and summer, reflecting different physiological states and energy demands of the breeding and post-breeding periods. In the light of sex differences in parental duties, we expected differences in the investigated variables between males and females. Considering inter-specific differences in male contribution to the whole parental care (full care in reed warblers *vs*. reduced care in sedge warblers), we expected lower sex differences in haematological variables and body mass in the reed warbler as compared those in the sedge warbler. Finally, we expected that because of different life-experience, effectiveness in foraging, and moulting strategy, the haematological variables and body mass in both studied species should differ between adults and immatures.

Material and methods

Study site and field methods

We conducted the study in the southern part of the "Lake Druzno" reserve (54°05´N, 19°27´E) in northern Poland. Lake Druzno is a large, shallow lake, overgrown for much of its area with reedbeds, which makes it an attractive place for breeding and migrating reed and sedge warblers. We trapped birds in 5–20 mist nets situated in reedbeds controlled every 1–1.5 hours during: early spring (11 days of 2–3 day trapping sessions between 25 April and 23 May), corresponding to the pre-laying and laying periods; mid-spring (5 days of 2–3 day trapping sessions between 4 and 28 June 2008), corresponding to the incubation and chick-rearing periods; and summer (26 days of continuous trapping between 27 July and 22 August 2008), corresponding to the post-breeding dispersal and the start of autumn migration. The division into the seasons was based on avialable phenological data [for breeding from Halupka (1996), Halupka *et al*. (2008); for migration from Cramp (1998)].

We trapped, ringed and aged [immature (caught only in summer) or adult] the birds by external characters (Svensson 1992). We measured wing length (flattened) with a ruler (accuracy ±1 mm), and body mass using an Ohaus CL 201 electronic scale (accuracy ±0.1 g). To examine haematological variables and for molecular sexing, from each bird we took a small blood sample of 10–20 μl by brachial vein puncture (following the standard procedure used in passerines, e.g. Ots *et al*. 1998, Owen 2011).

Laboratory analyses

We divided the collected blood samples into 3 subsamples for the following analyses: RBC counts (5 μl), differential leukocytes counts to establish the WBC number and the H/L ratio (blood smear of 2–5 μl), and molecular sexing (a drop). Air dried blood smears were stained using the May–Grünewald–Giemsa method with cytocentrifuge Wescor "Aerospray Hematology".

To determine the total RBC count, we counted the number of cells using a disposable, plastic counting chamber (Pentasquare Slides, Medlab-Products, Raszyn, Poland), analogous to the widely-used Neubauer chamber. Before the count, the blood was diluted 200 times and mixed with a saline solution. The average time between sampling and the RBC count was 5 h. To obtain the final RBC number (× 10^6) in 1 μl of blood, we multiplied the sum of cells counted in the five squares by 90 (as the chamber volume, 0.011 μl, multiplied by 90 gives 1 μl) and the dilution (200×), then the product was divided by the number of squares in which cells were counted.

We assessed the proportion of different types of leukocytes (lymphocytes, heterophils, monocytes, eosinophils and basophils) based on the examination of 100 leukocytes at 1000× magnification under oil immersion. The WBC count was expressed as the number of all types of leukocytes per 10 000 erythrocytes and was calculated by counting RBC per field and multiplying the outcome by the number of fields within which leukocytes had to be counted to reach 100 (Ots *et al*. 1998). Because of very low numbers or absence of monocytes, basophils and eosinophils in most of the blood smears, we presented and considered in the statistical analyses only ratios of heterophils to lymphocytes (H/L). All leukocyte counts were performed by the same person (IK). The repeatability of the leukocyte counts obtained from repeated scannings of the same blood smear (Krams *et al.* 2010) were high (heterophils, $r = 0.96$; lymphocytes, $r = 0.95$; H/L ratio, $r = 0.95$; basophiles $r = 0.77$; eosinophils, $r = 0.65$; monocytes, $r = 0.63$; all $p < 0.0001$).

We extracted DNA from the blood following evaporation of the alcohol using the Blood Mini kit (A&A Biotechnology, Gdynia, Poland). We performed molecular sexing with the primer pair P2 and P8 according to the protocol described by Griffiths *et al.* (1998), using an annealing temperature of 50 °C for the PCR reaction. These primers amplify a 390-bp fragment on the W chromosome (in females only), and a 370-bp fragment on the Z chromosome (in both sexes) (Griffiths *et al.* 1998). This size difference was clearly visible when separating the fragments on 3% agarose gel.

We screened the adult and immature birds captured in summer for haemoparasites. Since the infected individuals were found to have a higher H/L ratio than the parasite-free birds (Wojczulanis-Jakubas *et al.* 2012), in the present study the infected birds were excluded from all anlyses. In total, we examined 48 adult and 77 immature reed warblers and 96 adult and 75 immature sedge warblers.

Statistical analyses

We performed two separate analyses to examine the effects of: (1) sex and season (early spring, mid-spring and summer, i.e. the pre-laying period, the incubation and chick-rearing period, and post-breeding dispersion and autumn migration, respectively) on the studied adult variables; and (2) sex and age on the studied variables in individuals caught in summer. All analyses were performed separately for reed and sedge warblers.

To analyze the effects of categorical factors on the RBC, WBC counts and H/L ratio, we performed GLM analyses with sex, age and season as independent factors. Interaction terms were also included in the models. In adults, we used an analysis of covariance with sex, age and season as independent factors and date of trapping as a covariate to control for advancement of particular stages of seasonal activities. We tested the data for normality and applied arcsin-square-root or ln-transformation if necessary. The H/L ratio and WBC counts in adult birds were ln-transformed, and the H/L ratio and WBC counts in birds caught in summer were arcsin-square-root-transformed. We analyzed the effects of sex, age and season on body mass (i.e. calculated body mass corrected for body size) using an analysis of covariance with wing length as a covariate, to control for structural size differences (García-Berthou 2001). We chose wing length to represent adult individual's body size due to a significant correlation between this variable and body mass (both ages and sexes combined; Pearson's correlation coefficient, reed warbler: $r_{129} = 0.20$, $p = 0.02$, sedge warbler: $r_{167} = 0.46$, $p < 0.0001$). To control for advancement of particular stages of seasonal activities in adults, we used an additional covariate, date of trapping.

We performed all statistical calculations using STATISTICA 8.0 (StatSoft, Inc., Tulsa, Oklahoma).

Results

RBC counts

In adult reed warblers, the RBC number was significantly, though marginally affected by season and sex (Table 1). However, post-hoc analyses of the season effect did not reveal any significant differences; the difference between early spring [mean ± SD (n): $7.3 \times 10^6 \pm 2.10 \times 10^6$ per 1 μl (7)] and summer [$8.5 \times 10^6 \pm 1.59 \times 10^6$ per 1 μl (21)] were only close to being significant (LSD test: $p = 0.06$). Considering the effect of sex, the RBC number was higher in males [$8.3 \times 10^6 \pm 1.99 \times 10^6$ per 1 μl (26)] than in females [$7.0 \times 10^6 \pm 2.14 \times 10^6$ per 1 μl (13)]. Neither the season × sex interaction nor trapping date affected the RBC number (Table 1). Season, sex, season × sex, and trapping date had no effect on the RBC number in adult sedge warblers (Table 2).

In both species captured in summer, the RBC number was not affected by sex, age or the sex × age interaction (Tables 3 and 4).

Table 1. The effects of season (early spring, mid-spring and summer), sex (male, female), the season × sex interaction, and trapping date (covariate) on the RBC and WBC counts and H/L ratio in adult reed warblers (ANCOVA).

Variable	RBC count			WBC count*			H/L ratio*		
	df	*F*	*p*	df	*F*	*p*	df	*F*	*p*
Intercept	1	2.0	0.16	1	3.9	0.054	1	2.9	0.09
Trapping date	1	2.0	0.17	1	3.8	0.06	1	2.9	0.09
Season	2	3.6	0.04	2	46.7	< 0.001	2	25.4	< 0.001
Sex	1	4.3	0.047	1	40.5	< 0.001	1	11.5	0.002
Season × sex	2	1.4	0.27	2	21.3	< 0.001	2	18.2	< 0.001
Error	32			41			41		

* data ln-transformed.

Table 2. The effects of season (early spring, mid-spring and summer), sex (male, female), the season × sex interaction, and trapping date (covariate) on the RBC and WBC counts and H/L ratio in adult sedge warblers (ANCOVA).

Variable	RBC count			WBC count*			H/L ratio*		
	df	*F*	*p*	df	*F*	*p*	df	*F*	*p*
Intercept	1	0.1	0.78	1	9.8	0.002	1	1.0	0.31
Trapping date	1	0.1	0.79	1	4.8	0.03	1	1.0	0.33
Season	2	0.1	0.90	2	686.1	< 0.001	2	196.2	< 0.001
Sex	1	0.9	0.34	1	65.2	< 0.001	1	35.6	< 0.001
Season × sex	2	1.6	0.21	2	15.4	< 0.001	2	17.1	< 0.001
Error	69			89			89		

* data ln-transformed.

Table 3. The effects of age (immatures, adults), sex (male, female) and the age × sex interaction on the RBC and WBC counts and H/L ratio in reed warblers caught in summer (GLM).

Variable	RBC count			WBC count*			H/L ratio*		
	df	*F*	*p*	df	*F*	*p*	df	*F*	*p*
Intercept	1	1213.8	< 0.001	1	18706.6	< 0.001	1	34884.6	< 0.001
Age	1	0.1	0.75	1	184.3	< 0.001	1	699.8	< 0.001
Sex	1	1.4	0.25	1	1.5	0.23	1	8.3	0.005
Age × sex	1	0.5	0.45	1	2.5	0.12	1	15.4	< 0.001
Error	70			97			97		

* data arcsin-square-root-transformed.

Table 4. The effects of age (immatures, adults), sex (male, female) and the age × sex interaction on the RBC and WBC counts and H/L ratio in sedge warblers caught in summer (GLM).

Variable	RBC count			WBC count*			H/L ratio*		
	df	*F*	*p*	df	*F*	*p*	df	*F*	*p*
Intercept	1	1020.1	< 0.001	1	21347.6	< 0.001	1	29239.4	< 0.001
Age	1	0.4	0.51	1	216.0	< 0.001	1	553.4	< 0.001
Sex	1	0.0	0.84	1	0.8	0.39	1	1.2	0.28
Age × sex	1	0.1	0.73	1	0.8	0.36	1	4.5	0.04
Error	74			90			90		

* data arcsin-square-root-transformed.

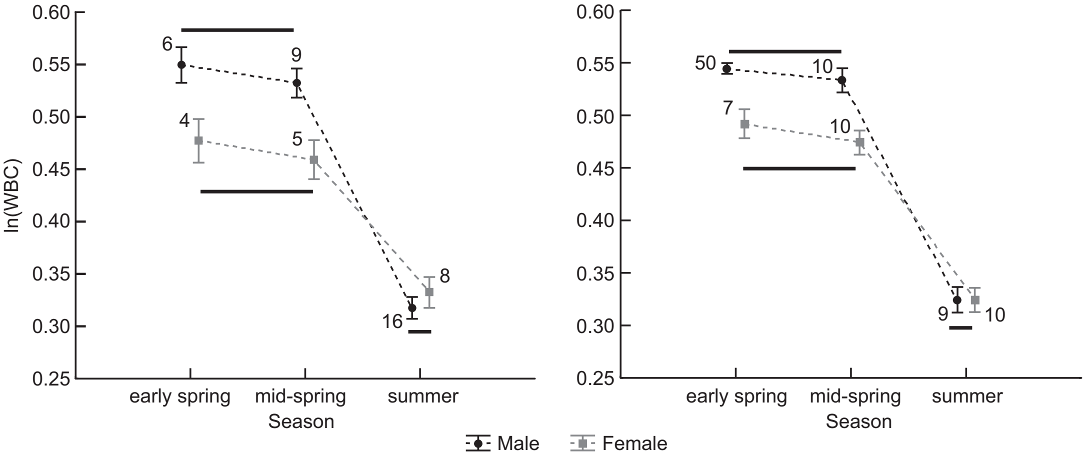

Fig. 1. Mean ± 95% confidence intervals of WBC counts (per 10 000 RBC; ln-transformed data) in adult males and females of reed warblers (left) and sedge warblers (right; presented WBC values are corrected for trapping date) captured in early and mid-spring, and summer. Numbers next to the bars denote sample sizes. Lines indicate lack of significant differences (post-hoc HSD test, $p > 0.05$).

WBC counts

In adult reed warblers, the WBC number was significantly affected by season, sex and the sex × season interaction, though trapping date did not affect the WBC count (Table 1). The WBC number in summer [mean ± SD (*n*): 0.32 ± 0.02 per 10 000 RBC (24)] was significantly lower than in early [0.52 ± 0.04 per 10 000 RBC (10)] and mid-spring [0.50 ± 0.05 per 10 000 RBC (14)] (HSD tests, $p = 0.0001$). Regarding the sex effect, the WBC counts were lower in females [0.40 ± 0.07 per 10 000 RBC (17)] than in males [0.42 ± 0.11 per 10 000 RBC (31)]. Considering the sex × season interaction, the WBC counts generally differed among all compared groups (HSD tests, $p < 0.007$). However, the values recorded in males in early and mid-spring were similar ($p = 0.70$). Also in females, there was no significant differences between those two seasons ($p = 0.82$). Similar WBC counts were found in both sexes in summer ($p = 0.71$) (Fig. 1).

In sedge warblers, the WBC number was significantly affected by trapping date, season, sex and the sex × season interaction (Table 2). The WBC count corrected for trapping date was significantly lower in summer [mean ± SD (*n*): 0.32 ± 0.01 (19)] than in early [0.52 ± 0.01 (57)] and mid-spring [0.50 ± 0.04 (20)] (HSD tests, $p = 0.0001$). Regarding the sex effect, the values found in females [0.42 ± 0.08 (27)] were lower than those found in males [0.51 ± 0.08 (69)]. Considering the sex × season interaction, the WBC number generally differed among all compared groups (HSD tests, $p = 0.0001$). However, similarly to reed warblers, the values recorded in males in early and mid-spring were similar ($p = 0.76$). Also in females, there was no significant differences between those two seasons ($p = 0.38$). Similar WBC counts were found in both sexes in summer ($p = 1.00$) (Fig. 1).

In reed warblers caught in summer, the WBC number was significantly affected by age (Table 3). The values in immatures [mean ± SD (*n*): 0.40 ± 0.02 per 10 000 RBC (77)] were higher than in adults [0.32 ± 0.02 per 10 000 RBC (24)]. Neither sex nor the age × sex interaction affected the WBC number (Table 3).

Similarly, in sedge warblers caught in summer, the WBC number was significantly affected by age (Table 4). The WBC number in immatures [mean ± SD (*n*): 0.40 ± 0.02 per 10 000 RBC (75)] was higher than in adults [0.32 ± 0.01 per 10 000 RBC (19)]. Neither sex nor the age × sex interaction affected the WBC number (Table 4).

H/L ratio

In adult reed warblers, the H/L ratio was significantly affected by season, sex and the sex ×

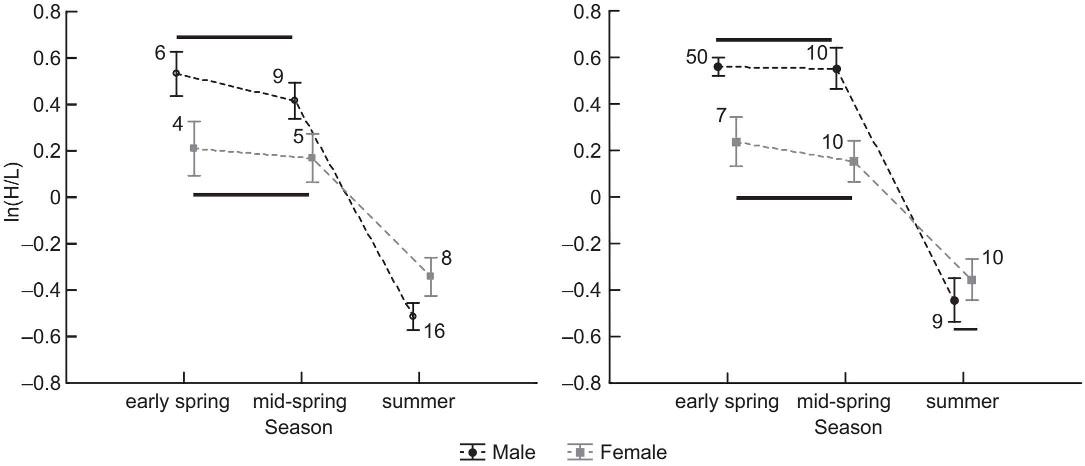

Fig. 2. Mean ± 95% confidence intervals of H/L ratios (ln-transformed data) in adult males and females of reed warblers (left) and sedge warblers (right) captured in early and mid-spring, and summer. Numbers next to the bars denote sample sizes. Lines indicate lack of significant differences (post-hoc HSD test, $p > 0.05$).

season interaction. Trapping date did not affect H/L ratio (Table 1). Considering the season effect, the H/L ratio in summer [mean ± SD (n): –0.45 ± 0.13 (24)] was significantly lower than in early [0.40 ± 0.20 (10)] and mid-spring [0.32 ± 0.17 (14)] (HSD tests: $p = 0.0001$). The values recorded in females [–0.06 ± 0.28 (17)] were lower than those found in males [–0.04 ± 0.51 (31)]. Considering the sex × season interaction, the H/L ratios generally differed among all compared groups (post-hoc HSD tests, $p < 0.05$). However, the values recorded in males in early and mid-spring were similar ($p = 0.42$). Also in females, there was no significant differences in the H/L ratios between those two seasons ($p = 0.99$) (Fig. 2).

In adult sedge warblers, the H/L ratio was significantly affected by season, sex and the sex × season interaction. Trapping date did not affect the H/L ratio (Table 2). The H/L ratios differed among all compared seasons (HSD tests: $p = 0.0002$). Regarding the sex effect, the values in females were significantly lower [mean ± SD (n): –0.01 ± 0.29 (27)] than in males [0.43 ± 0.37 (69)]. Considering the sex × season interaction, the H/L ratios generally differed among all compared groups (HSD tests: $p < 0.002$). However, the values recorded in males in early and mid-spring were similar ($p = 1.00$). Also in females, there was no significant differences in the H/L ratios between those two seasons ($p = 0.87$). Similar H/L ratios were found in both sexes in summer ($p = 0.77$) (Fig. 2).

In reed warblers captured in summer, the H/L ratio was significantly affected by age, sex and the age × sex interaction (Table 3). Considerng the age effect, the H/L ratio was significantly higher in immatures [mean ± SD (n): 0.11 ± 0.004 (77)] than in adults [0.08 ± 0.005 (24)]. The values recorded in females [0.103 ± 0.01 (47)] were higher than those found in males [0.099 ± 0.01 (54)]. Considering the sex × season interaction, the H/L ratios differed among all compared groups (HSD tests, $p < 0.007$), except for immatures of both sexes (HSD test: $p = 0.69$) (Fig. 3).

In sedge warblers caught in summer, the H/L ratio was significantly affected by age, and the age × sex interaction. Sex did not affect the H/L ratio (Table 4). Regarding the age effect, the H/L ratio was significantly higher in immatures [mean ± SD (n): 0.11 ± 0.004 (75)] than in adults [0.08 ± 0.006 (19)]. Considering the sex × season interaction, the H/L ratios differed among all compared groups (HSD tests: $p = 0.001$), except for immatures ($p = 0.66$) and adults ($p = 0.31$) of both sexes (Fig. 3).

Body mass

In adult reed warblers, none of the analyzed

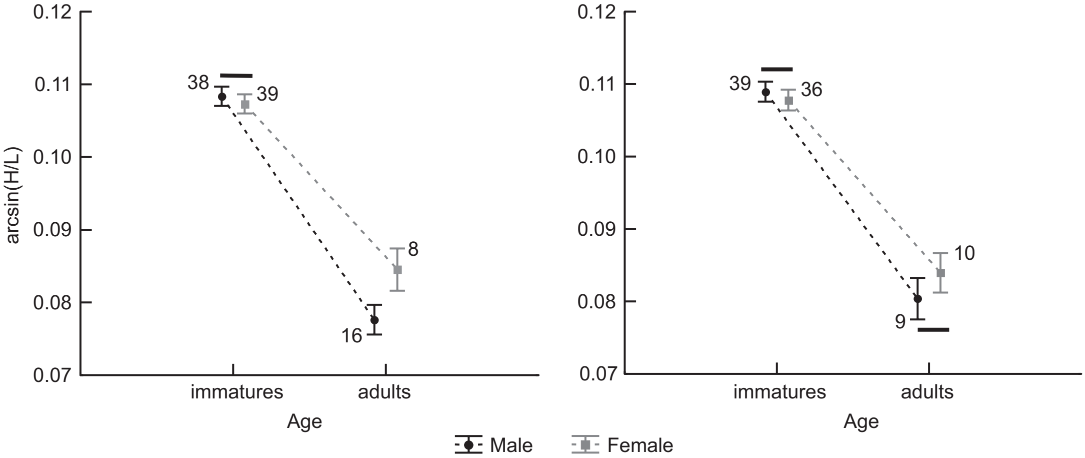

Fig. 3. Mean ± 95% confidence intervals of H/L ratios (arcsin-square-root-transformed data) in males and females of immature and adult reed warblers (left) and sedge warblers (right) captured in summer. Numbers next to the bars denote sample sizes. Lines indicate lack of significant differences (post-hoc HSD test, $p > 0.05$).

variables (season, sex, interaction season × sex, trapping date, wing length) affected body mass significantly (Table 5). In adult sedge warblers, body mass was affected by wing length and interaction season × sex (Table 5). Body mass corrected for body size in summer was significantly higher in males [mean ± SD (n): 12.4 ± 1.38 g (8)] compared to females [11.9 ± 1.61 g (10)] (LSD test, $p = 0.02$). Other factors, i.e. season, sex, trapping date did not affect body mass significantly (Table 5).

In reed warblers captured in summer, body mass was affected only by age (Table 6) with adults [mean ± SD (n): 12.4 ± 0.88 g (24)] being heavier than immatures [11.5 ± 0.64 g (77)]. Wing length, sex and interaction age × sex did not affect body mass significantly (Table 6). In sedge warblers, body mass in summer was affected only by age and sex (Table 6). Adults [mean ± SD (n): 12.2 ± 0.89 g (18)] were heavier than immatures [11.1 ± 0.68 g (75)]. Considering sex effect, males [11.6 ± 0.77 g (47)] were heavier than females [10.9 ± 0.77 g (46)]. Wing length and interaction age × sex did not affect body mass significantly (Table 6).

Discussion

Adult reed and sedge warblers captured in early and mid-spring (breeding) had higher WBC counts and H/L ratios than in summer (post-

Table 5. The effects of season (early spring, mid-spring and summer), sex (male, female), and the season × sex interaction, as well as trapping date and wing length (covariates) on body mass in adult reed warblers and sedge warblers (ANCOVA).

Variable	Reed warbler			Sedge warbler		
	df	*F*	*p*	df	*F*	*p*
Intercept	1	2.6	0.11	1	0.03	0.87
Trapping date	1	2.7	0.11	1	0.03	0.87
Wing length	1	0.1	0.73	1	13.41	0.0004
Season	2	1.9	0.16	2	0.04	0.96
Sex	1	4.0	0.051	1	0.47	0.49
Season × sex	2	0.0	0.95	2	4.37	0.02
Error	44			84		

breeding). Elevated H/L ratios during the breeding period were also reported for other birds (e.g. Mallory *et al*. 2005, Norte *et al*. 2009, Pap *et al*. 2010, Jakubas *et al*. 2011). It probably reflects the cost of reproduction in terms of physiological stress and immune suppression (Pap *et al*. 2010). Alternatively, an elevated H/L ratio and glucocorticoid levels (Romero 2002) found in breeding birds may maintain their internal systems within a heightened operating range to support expected reproduction demands (allostasis concept; McEwen 2002, Schulkin 2003). Thus, this elevation represents rather pre-breeding adjustment than a reproduction stress response (Landys *et al*. 2006).

In contrast to adult sedge warblers, in the reed warbler adults, the RBC numbers differed between the seasons, with marginally (possibly due to relatively low sample size) higher values during the post-breeding period. Since a low RBC number is usually recorded in birds in poor body condition (Kalmbach *et al*. 2004, Sergent *et al*. 2004), the lower values of RBC counts in spring (together with elevated H/L ratio and WBC) may indicate physiological constraints and trade-offs of breeding. The inter-species differences in the RBC counts may be a consequence of the reed warbler breeding in a relatively poor-quality habitat (in terms of food availability and size) as compared with the sedge warbler (Leisler *et al*. 2002).

Adult males of both species captured in both early and mid-spring had significantly higher WBC counts and H/L ratios than females. This result was unexpected, since in other passerines with biparental care [great tit (*Parus major*) and Siberian tit (*Poecile cinctus*)], the H/L ratio in females during breeding was higher than in males, possibly due to incubating alone and nestling brooding, i.e. greater contribution to offspring feeding and/or high initial breeding investments by females (Cramp 1998, Hõrak *et al*. 1998, Ots *et al*. 1998, Kilgas *et al*. 2006, Krams *et al*. 2010). This different pattern of the sex differences in the physiological profiles in the two studied species may have resulted from greater contribution of reed and sedge warbler males to brood rearing (participation in incubation and nestlings brooding; Cramp 1998) as compared with the tits.

There was no sex difference in the H/L ratio in adult sedge warblers captured in summer. However, in reed warblers, the H/L ratio in males was significantly lower than in females. In contrast, body mass corrected to body size (wing length) of adult sedge warblers captured in summer was significantly higher in males than in females, whereas in the reed warbler, body mass was similar in both sexes. Considering similar H/L ratios and body mass in females of both warblers in summer, the significantly lower H/L ratio in the reed warbler males possibly reflects faster post-breeding relaxation of physiological stress related to reproduction as compared with the sedge warblers. Despite greater relative contribution of reed warbler males to parental care (Leisler *et al*. 2002), sedge warbler males during breeding perform some species-specific behaviours such as singing in flight (Cramp 1998, Buchanan & Catchpole 2000) or mate guarding and chasing intruders at consecutive nests in polygynous individuals, which may be stressful and result in slower post-breeding relaxation as compared with reed warblers. On the other hand,

Table 6. The effects of age (immatures, adults), sex (male, female) and the age × sex interaction, as well as wing length (covariate) on body mass in reed warblers and sedge warblers caught in summer (ANCOVA).

Variable	Reed warbler			Sedge warbler		
	df	*F*	*p*	df	*F*	*p*
Intercept	1	10.2	0.002	1	6.2	0.01
Wing length	1	0.3	0.58	1	1.0	0.33
Age	1	24.4	< 0.001	1	43.2	< 0.001
Sex	1	1.1	0.29	1	12.4	< 0.001
Age × sex	1	0.7	0.41	1	0.4	0.55
Error	96			88		

as sedge warbler males (especially polygynous ones) participate in chick feedings to a smaller extent than reed warbler males (Hoi *et al*. 1995, Leisler *et al*. 2002), it gives them a chance to conserve their own body reserves (body mass was higher than in the reed warbler males). In summing, at the end of the breeding season, the sedge warbler males (less involved in parental duties, more in mating activities) had similar stress levels but were heavier than females. The reed warbler males (more involved in parental care) were less stressed than females but did not increase their body reserves.

The opposite pattern of the sex differences in hematological variables and body mass in both species clearly indicates that those two variables respond independently to physiological stress related to reproduction. That was also found to be the case in other bird species [e.g. the zebra finch (*Taeniopygia guttata*) (Ewenson *et al*. 2001), little auk (*Alle alle*) (Jakubas *et al*. 2008)]. Thus, reliable assessment of physiological responses to different stressors should be complex and based on different variables.

Age-related differences were found in both species in the WBC counts, the H/L ratio and body mass. Immatures of both reed and sedge warblers had higher values of the WBC counts an H/L ratios, and lower body mass than adults. This pattern is likely to result from a higher stress level and/or less efficient foraging by inexperienced or having lower social status immatures at stopover sites (Woodrey & Moore 1997). Similar results were reported for the studied warbler species (body mass, Gyurácz & Bank 1996, Yosef & Chernetsov 2004, Zakala *et al*. 2004) and the reed bunting *Emberiza schoeniclus* (H/L ratio, Jakubas *et al*. 2011). However, for some species of birds an opposite pattern of age differences in the H/L ratio (i.e. elevated values in adults) was reported (e.g. Fairbrother & O'Loughlin 1990, Alonso *et al*. 1991, Quillfeldt *et al*. 2008, Norte *et al*. 2009). This discrepancy between the studies may be due to the variability of environmental (food abundance) and social (intensity of competition) conditions affecting stress levels.

Since we captured the birds in nets controlled every 1.0–1.5 h, we were not able to measure the exact time between the capture and blood sampling. Davis (2005) showed that routine handling times up to 1 h affect WBC counts but do not affect H/L ratios. Cīrule *et al*. (2012) reported even more rapid changes (within 30–60 min) in the leukocyte profile. This time constraints might raise a concern about the results obtained in the present study. However, 1.0–1.5-h period was a maximum time which birds had to wait for blood sampling, Had that maximum sampling time occurred frequently, we should have obtained a considerable variance in the data but that was not the case (*see* range of confidence intervals, Figs. 2 and 3). Also, even if the time had affected the leukocyte profiles, it should have worked in the similar manner in the birds of different ages and sexes. Thus, any comparisons performed in the present study should be robust for the potential sampling-time violation.

In conclusion, we found that physiological processes associated with important life-history stages, as breeding and migration, affected haematological variables and body mass of reed and sedge warblers. Some responses were sex- and/or age-specific. Inter-species differences in physiological and body mass responses probably reflect different breeding strategies of both warbler species.

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References

Alonso, J. C., Huecas, V., Alonso, J. A., Abelenda, M., Muñoz-Pulido, E. & Fuerta, M. L. 1991: Hematology and blood chemistry of adult white storks (*Ciconia ciconia*). — *Comparative Biochemistry and Physiology A* 98: 395–397.

Artacho, P., Soto-Gamboa, M., Verdugo, C. & Nespolo, R. F. 2007: Using haematological parameters to infer the health and nutritional status of an endangered blacknecked swan population. — *Comparative Biochemistry and Physiology A* 147: 1060–1066.

Buchanan, K. L. & Catchpole, C. K. 2000: Song as an indicator of male parental effort in the sedge warbler. — *The Proceedings of the Royal Society B* 267: 321–326.

Campbell, T. W. 1995: *Avian hematology and cytology*. — Iowa State University Press, Ames, Iowa.

Cīrule, D., Krama, T., Vrublevska, J., Rantala, M. J. & Krams, I. 2012: A rapid effect of handling on counts of white blood cells in a wintering passerine bird: a more practical measure of stress? — *Journal of Ornithology* 153: 161–166.

Cramp, S. 1998: *The complete birds of the Western Palearctic on CD-ROM*. — Oxford University Press, Optimedia, Oxford.

Cuervo, J. J., Møller, A. P. & de Lope, F. 2007: Haematocrit is weakly related to condition in nestling barn swallows *Hirundo rustica*. — *Ibis* 146: 128–134.

Davis, A. K. 2005: Effects of handling time and repeated sampling on avian white blood cell counts. — *Journal of Field Ornithology* 76: 334–338.

Davis, A. K, Maney, D. L. & Maerz, J. C. 2008: The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. — *Functional Ecology* 22: 760–772.

Dein, J. F. 1986: Hematology. — In: Harrison, G. J. & Harrison, L. R. (eds.), *Clinical avian medicine and surgery*: 174–191. Saunders, London.

Dhabhar, F. S. 2002: A hassle a day may keep the doctor away: stress and the augmentation of immune function. — *Integrative and Comparative Biology* 42: 556–564.

Drent, R. H. & Daan, S. 1980: The prudent parent: energetic adjustments in avian breeding. — *Ardea* 68: 225–252.

Ellrich, H., Salewski, V. & Fiedler, W. 2010: Morphological sexing of passerines: not valid over larger geographical scales. — *Journal of Ornithology* 151:449–458.

Ewenson, E. L., Zann, R. A. & Flannery, G. R. 2001: Body condition and immune response in wild zebra finches: effects of capture, confinement and captive-rearing. — *Naturwissenschaften* 88: 391–394.

Fairbrother, A. & O'Loughlin, D. 1990: Differential white blood cell values of the mallard (*Anas platyrhynchos*) across different ages and reproductive states. — *Journal of Wildlife Disease* 26: 78–82.

Fudge, A. M. 1989. Avian hematology: identification and interpretation. — *Proceedings of Association of Avian Veterinary Annual Meeting*: 284–292.

García-Berthou, E. 2001: On the misuse of residuals in ecology: testing regression residuals *vs*. the analysis of covariance. — *Journal of Animal Ecology* 70: 708–711.

Griffiths, R., Double, M. C., Orr, K. & Dawson, R. J. G. 1998: A DNA test to sex most birds. — *Molecular Ecology* 7: 1071–1075.

Gross, W. B. & Siegel, H. S. 1983: Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens. — *Avian Disease* 27: 972–979.

Gyurácz, J. & Bank, L. 1995: Autumn migration dynamics of the sedge warbler (*Acrocephalus schoenobaenus*) in southern Hungary. — *Ornis Hungarica* 5: 23–32.

Hall, S. 1996: The timing of post-juvenile moult and fuel deposition in relation to the onset of of autumn migration of reed warblers *Acrocephalus scirpaceus* and sedge warblers *Acrocephalus schoenobaenus*. — *Ornis Svecica* 6: 89–96.

Halupka, L. 1996: Breeding ecology of the sedge warbler *Acrocephalus schoenobaenus* in the Biebrza Marshes (NE Poland). — *Ornis Hungarica* 6: 9–14.

Halupka, L., Dyrcz, A. & Borowiec, M. 2008: Climate change affects breeding of reed warblers *Acrocephalus scirpaceus*. — *Journal of Avian Biology* 39: 95–100.

Hasselquist, D. & Langefors, A. 1998. Variable social mating system in the sedge warbler, *Acrocrphalus schoenobaenus*. — *Ethology* 104: 759–769.

Hoi, H., Kleindorfer, S., Ille, R. & Dittami, J. 1995: Prey abundance and male parental behaviour in *Acrocephalus* warblers. — *Ibis* 137: 490–496.

Hõrak, P., Jenni-Eiermann, S., Ots, I. & Tegelmann, L. 1998: Health and reproduction: the sex-specific clinical profile of great tits (*Parus major*) in relation to breeding. — *Canadian Journal of Zoology* 76: 2235–2244.

Jakob, E. M., Marshal, S. D. & Uetz, G. W. 1996: Estimating fitness: a comparison of body condition indices. — *Oikos* 77: 61–67.

Jakubas, D., Wojczulanis-Jakubas, K. & Kreft, R. 2008: Sex differences in body condition and hematological parameters in little auk *Alle alle* during the incubation period. — *Ornis Fennica* 85: 90–97.

Jakubas, D. & Wojczulanis-Jakubas, D. 2010: Sex and age related differences in the timing and body condition of migrating reed warblers *Acrocephalus scirpaceus* and sedge warblers *Acrocephalus schoenobaenus*. — *Naturwissenschaften* 97: 505–511.

Jakubas, D., Wojczulanis-Jakubas, K. & Glac, W. 2011: Variation of the reed bunting (*Emberiza schoeniclus*) body condition and haematological parameters in relation to sex, age and season. — *Annales Zoologici Fennici* 48: 243–250.

Kalmbach, E., Griffiths, R., Crane, J. E. & Furness, R. W. 2004: Effects of experimentally increased egg production on female body condition and laying dates in the great skua *Stercorarius skua*. — *Journal of Avian Biology* 35: 501–514.

Kilgas, P., Mand, R., Magi, M. & Tilgar, V. 2006: Hematological parameters in brood-rearing great tits in relation to habitat, multiple breeding and sex. — *Comparative Biochemistry and Physiology A* 144: 224–231.

Klein, S. L. 2000: The effects of hormones on sex differences in infection: from genes to behavior. — *Neuroscience and Biobehavioral Reviews* 24: 627–638.

Kleindorfer, S., Fessl, B. & Hoi, H. 1995: More is not always better: male incubation in two *Acrocephalus* warblers. — *Behaviour* 132: 607–625.

Krams, I., Cīrule, D., Krama, T., Hukkanen, M., Rytkönen, S., Orell, M., Iezhova, T., Rantala, M. J. & Tummeleht, L. 2010: Effects of forest management on haematological parameters, blood parasites, and reproductive success of the Siberian tit (*Poecile cinctus*) in northern Finland. — *Annales Zoologici Fennici* 47: 335–346.

Krebs, C. J. & Singleton, G. R. 1993: Indices of condition for small mammals. — *Australian Journal of Zoology*

41: 317–323.

Landys, M. M., Ramenofsky, M. & Wigfield, J. C. 2006: Actions of glucocorticoids at a seasonal baseline as compared to stress-related levels in the regulation of periodic life processes. — *General and Comparative Endocrinology* 148: 132–149.

Langefors, Å., Hasselquist, D. & von Schantz, T. 1998: Extra-pair fertilizations in the sedge warbler. — *Journal of Avian Biology* 29: 134–144.

Leisler, B., Winkler, H. & Wink, M. 2002: Evolution of breeding systems in acrocephaline warblers. — *Auk* 119: 379–390.

Lobato, E., Moreno, J., Merino, S., Sanz, J. J. & Arriero, E. 2005: Haematological variables are good predictors of recruitment in nestling pied flycatchers (*Ficedula hypoleuca*). — *Ecoscience* 12: 27–34.

Mallory, M. L., Braune, B. M. & Forbes, M. R. L. 2005: Contaminant concentrations in breeding and non-breeding northern fulmars (*Fulmarus glacialis* L.) from the Canadian high arctic. — *Chemosphere* 64: 1541–1544.

Maxwell, M. H. 1993: Avian blood leucocyte responses to stress. — *Worlds Poultry Science Journal* 49: 34–43.

McEwen, B. S. 2002: *The end of stress as we know it*. — Joseph Henry Press, Washington D.C.

Murton, R. & Westwood, N. 1977: *Avian breeding cycles*. — Clarendon Press, Oxford.

Norris, K. & Evans, M. R. 2000: Ecological immunology: life history trade-offs and immune defense in birds. — *Behavioral Ecology* 11: 19–26.

Norte, A. C., Ramos, J. A., Sousa, J. P. & Sheldon, B. C. 2009: Variation of adult great tit *Parus major* body condition and blood parameters in relation to sex, age, year and season. — *Journal of Ornithology* 150: 651–660.

Ots, I. & Hõrak, P. 1996: Great tits *Parus major* trade health for reproduction. — *The Proceedings of the Royal Society B* 263: 1443–1447.

Ots, I., Murumägi, A. & Hõrak, P. 1998: Haematological health state indices of reproducing great tits: methodology and sources of natural variation. — *Functional Ecology* 12: 700–707.

Owen, J. C. 2011: Collecting, processing, and storing avian blood: a review. — *Journal of Field Ornithology* 82: 339–354.

Pap, P. L., Vágási, C. I., Tökölyi, J., Czirják, G. Á. & Barta, Z. 2010: Variation in haematological indices and immune function during the annual cycle in the great tit *Parus major*. — *Ardea* 98: 105–112.

Quillfeldt, P., Ruiz, G., Rivera, M. A. & Masello, J. F. 2008: Variability in leucocyte profiles in thin-billed prions *Pachyptila belcheri*. — *Comparative Biochemistry and Physiology A* 150: 26–31.

Redfern, C. P. F. & Alker, P. J. 1996: Plumage development and postjuvenile moult in the sedge warbler *Acrocephalus schoenobaenus*. — *Journal of Avian Biology* 27: 157–163.

Reist, J. D. 1985: An empirical evaluation of several univariate methods that adjust for size variation in morphometric data. — *Canadian Journal of Zoology* 63: 1429–1439.

Ricklefs, R. E. 1974: Energetics of reproduction in birds. — In: Paynter, R. A. Jr. (ed.), *Avian energetics*: 152–292. Publication of Nuttall Ornithological Club No. 15.

Romero, L. M. 2002: Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. — *General and Comparative Endocrinology* 128: 1–24.

Ruiz, G., Rosenmann, M., Novoa, F. F. & Sabat, P. 2002: Hematological parameters and stress index in rufous-collared sparrows dwelling in the urban environments. — *Condor* 104: 162–166.

Sanz, J. J., Kranenbarg, S. & Tinbergen, J. M. 2000: Differential response by males and females to manipulation of partner contribution in the great tit (*Parus major*). — *Journal of Animal Ecology* 69: 74–84.

Schulkin, J. 2003: *Rethinking homeostasis*. — The MIT Press, Cambridge, Mass.

Sergent, N., Rogers, T. & Cunningham, M. 2004: Influence of biological and ecological factors on hematological values in wild Little Penguins, *Eudyptula minor*. — *Comparative Biochemistry and Physiology A* 138: 333–339.

Svensson, L. 1992: *Identification guide to European passerines*. — published by the author, Stockholm.

Totzke, U., Fenske, M., Hüppop, O., Raabe, H. & Schach, N. 1999: The influence of fasting on blood and plasma composition of herring gulls (*Larus argentatus*). — *Physiological and Biochemical Zoology* 72: 426–437.

Visser, M. E. & Lessells, C. M. 2001: The costs of egg production and incubation in great tits (*Parus major*). — *The Proceedings of the Royal Society B* 268: 1271–1277.

Vleck, C. M., Vertalino, N., Vleck, D. & Bucher, I. T. 2000: Stress, corticosterone, and heterophil to lymphocyte ratios in free-living Adélie penguins. — *Condor* 102: 392–400.

Woodrey, M. S. & Moore, E. R. 1997: Age-related differences in the stopover of fall landbird migrants on the coast of Alabama. — *The Auk* 114: 695–707.

Wojczulanis-Jakubas, K. & Jakubas, D. 2011: Predicting the sex of the sedge warbler (*Acrocephalus schoenobaenus*) by discriminant analysis. — *Ornis Fennica* 88: 90–97.

Wojczulanis-Jakubas, K., Jakubas, D., Czujkowska, A., Kulaszewicz, I. & Kruszewicz, A. G. 2012: Blood parasite infestation and the leukocyte profiles in adult and immature reed warblers (*Acrocephalus scirpaceus*) and sedge warblers (*Acrocephalus schoenobaenus*) during autumn migration. — *Annales Zoologici Fennici* 49: 341–349.

Yosef, R. & Chernetsov, N. 2004: Stopover ecology of migratory sedge warblers (*Acrocephalus schoenobaenus*) at Eilat, Israel. — *Ostrich* 75: 52–56.

Zakala, O., Shydlovskyy, I. & Busse, P. 2004: Variation in body mass and fat reserves of the sedge warbler *Acrocephalus schoenobaenus* on autumn migration in the L'viv Province (W Ukraine). — *The Ring* 26: 55–69.