RESEARCH ARTICLE

Genetic and phenotypically flexible components of seasonal variation in immune function

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ABSTRACT

Animals cope with seasonal variation in environmental factors by adjustments of physiology and life history. When seasonal variation is partly predictable, such adjustments can be based on a genetic component or be phenotypically flexible. Animals have to allocate limited resources over different demands, including immune function. Accordingly, immune traits could change seasonally, and such changes could have a genetic component that differs between environments. We tested this hypothesis in genotypically distinct groups of a widespread songbird, the stonechat (*Saxicola torquata*). We compared variation in immunity during 1 year in long-distance migrants, short-distance migrants, tropical residents and hybrids in a common garden environment. Additionally, we investigated phenotypically flexible responses to temperature by applying different temperature regimes to one group. We assessed constitutive immunity by measuring hemagglutination, hemolysis, haptoglobin and bactericidal ability against *Escherichia coli* and *Staphylococcus aureus*. Genotypic groups differed in patterns of variation of all measured immune indices except haptoglobin. Hybrids differed from, but were rarely intermediate to, parental subspecies. Temperature treatment only influenced patterns of hemolysis and bactericidal ability against *E. coli.* We conclude that seasonal variation in constitutive immunity has a genetic component, that heredity does not follow simple Mendelian rules, and that some immune measures are relatively rigid while others are more flexible. Furthermore, our results support the idea that seasonal variability in constitutive immunity is associated with variability in environment and annual-cycle demands. This study stresses the importance of considering seasonal variation in immune function in relation to the ecology and life history of the organism of interest.

KEY WORDS: Common garden, Constitutive immunity, Genetic components, Phenotypic flexibility, Seasonal variation, Stonechats

INTRODUCTION

In seasonal environments, ecological factors such as temperature, food availability and pathogen pressure are temporally variable (Altizer et al., 2006; Lovegrove, 2003; Thiollay, 1988). Accordingly, organisms have adjusted their life histories and associated physiology to the variability of the environments they live in (Foster and Kreitzman, 2010; Klaassen, 1995; Nelson et al., 2002; Versteegh et al., 2012a). An important physiological system that relates to self-maintenance and that connects life history traits with environmental factors is the immune system (Ricklefs and Wikelski, 2002; Sheldon and Verhulst, 1996). Immune traits may be costly (Klasing, 2004; Schmid-Hempel, 2003), and a high immune defense may not be uniformly valuable over the entire annual cycle. Some immune traits have indeed been shown to vary among seasons in wild (Hegemann et al., 2012; Lochmiller et al., 1994; Machado-Filho et al., 2010; Pap et al., 2010a; Pap et al., 2010b) and captive animals (Buehler et al., 2008; Martin et al., 2004). Understanding the degree to which fluctuations among seasons (i.e. seasonal variation) in immunity are determined by genes versus the environmental and ecological conditions is key for integrative perspectives of the factors that shape the dynamics of life history and physiology (Williams, 2012).

Several hypotheses exist about seasonal variation in immunity. It is often hypothesized to be the outcome of internal trade-offs in response to seasonally changing demands or workloads associated with annual-cycle stages [e.g. reproduction, moult (Moreno-Rueda, 2010; Nordling et al., 1998; Sheldon and Verhulst, 1996)]. These demands and workloads may become more pronounced with increasing seasonality of the environment (Newton, 2008). Alternatively, immunity could be adjusted to changing environmental factors such as pathogen pressure, food availability and temperature (Ljifeld et al., 2002; Maizels and Yazdanbakhsh, 2003; Marais et al., 2011). In both of these scenarios, seasonal variation in immunity could primarily stem from phenotypically flexible adjustments in response to the current environment (e.g. food availability, pathogen abundance) and/or physiological state (e.g. energy reserves, sickness), developmental plasticity, or genetically encoded annual programs in anticipation of, rather than in response to, environmental and physiological changes (Foster and Kreitzman, 2010; Gwinner, 1999; MacDougall-Shackleton and Hahn, 2007; Piersma and Drent, 2003). If seasonal variation in immunity is solely based on phenotypic flexibility, we would expect birds from different environments to display the same variation when they are bred, hand-raised and kept in the same environment. However, if there are genetic differences that contribute to variation in immune traits, we would expect to find differences among these birds under such 'common garden' conditions.

The immune system consists of a complex collection of interrelated and overlapping pathways that protect the body from disease. In our study, we focus on constitutive immunity. This part of the immune system provides the first line of defense against general challenges (Buehler et al., 2008; Janeway et al., 2004). We included three measurements to assess constitutive immunity: (1) the ability of whole blood to limit bacterial growth – a repeatable trait that integrates both humoral and cell-mediated components (Millet et al., 2007; Tieleman et al., 2005; Tieleman et al., 2010); (2) the ability of plasma to agglutinate and lyse foreign cells (Matson et

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al., 2005) – this involves both natural antibodies and the complement system (Ochsenbein and Zinkernagel, 2000); and (3) concentrations of haptoglobin in the blood, an acute phase protein that increases in concentration in response to inflammation or infection (Dobryszycka, 1997; van de Crommenacker et al., 2010).

We studied these immune traits in three subspecies and two hybrids of stonechats [*Saxicola torquata* (Linnaeus 1766)], kept and, for the majority, bred and hand-raised in a common environment during a full annual cycle. Stonechats are ideal for investigating seasonal variation as they have an extensive distribution (Urquhart, 2002), spanning southern Africa to northeastern Asia, that includes considerable variation in seasonal patterns of environmental conditions (Gwinner et al., 1995a; Helm, 2009; Helm et al., 2009; König and Gwinner, 1995). We studied long-distance migrant Kazakh stonechats (*S. t. maura*), originating from a continental climate; short-distance migrant European stonechats (*S. t. rubicola*), originating from a temperate climate; and resident Kenyan stonechats (*S. t. axillaris*), originating from a tropical climate. Additionally, we studied hybrids between Kazakh and European stonechats, and between European and Kenyan stonechats. The phylogenetic relationships among these genotypic groups have been described in detail previously (Illera et al., 2008; Zink et al., 2009). Differences in life history traits (e.g. clutch size) and physiology (e.g. metabolic rate, measures of immunity) among these five genotypic groups are well described and remain present in captivity (Gwinner et al., 1995a; Helm, 2009; Helm et al., 2009; Klaassen, 1995; König and Gwinner, 1995; Tieleman, 2007; Tieleman et al., 2009). For a number of traits, seasonal variation has been shown to persist in captivity, to differ between geographical populations, and to be based on underlying, genetically encoded circannual programs (Gwinner, 1999; Helm, 2009; Helm et al., 2009; Versteegh et al., 2012a). Inheritance of seasonal variation and seasonal traits (e.g. clutch size) has further been documented by crossbreeding and subsequent study of hybrids (Gwinner et al., 1995a; Helm, 2009; Tieleman et al., 2009; Versteegh et al., 2012a). If seasonal variation of constitutive immunity is driven more heavily by genetic components than environmental components, we would expect it to persist in captivity and to differ between subspecies. Depending on the pattern of inheritance, hybrid groups could show values intermediate to their parental subspecies. The environments of origin of these subspecies differ in seasonality and associated variation in annual-cycle demands. Therefore, we expected that seasonal variation in immune function would be largest in Kazakh stonechats, which experience high seasonal variation, smallest in Kenyan stonechats, which experience little seasonal variation, and intermediate in European stonechats, which experience intermediate seasonality. Birds in captivity were not able to migrate or to breed. Therefore, arising differences in seasonal variation would be based on underlying programs that anticipate seasonally changing demands, rather than on direct trade-offs with different workloads. To further investigate phenotypic flexibility of seasonal variation in immunity in response to temperature, we compared groups of European stonechats kept at year-round constant temperature and seasonally variable temperature. If phenotypic flexibility in response to temperature is an important (additional) mechanism, we would expect greater variation in immune indices under variable temperature than under constant temperature.

RESULTS
Differences in seasonal variation among genotypic groups
Exploring whether annual-cycle stage had a different effect in the different genotypic groups, we found that the interaction annual-

cycle stage × genotypic group had a significant effect on hemagglutination (χ^2_{16}=30.48, P=0.02; Fig. 1A), hemolysis (χ^2_{16}=36.89, P=0.002; Fig. 1B) and bactericidal ability against *Escherichia coli* (χ^2_{16}=59.96, P<0.001; Fig. 1C) and *Staphylococcus aureus* (χ^2_{16}=26.83, P=0.04; Fig. 1D). This indicates that seasonal variation in these traits differed among genotypic groups. The interaction annual-cycle stage × genotypic group did not have a significant effect on haptoglobin (χ^2_{16}=14.29, P=0.58; Fig. 1E, note the large error bars). We explored seasonal variation in hemagglutination, hemolysis and bactericidal ability against *E. coli* and *S. aureus* further by first comparing patterns of seasonal variation among Kazakh, European and Kenyan stonechats, and then comparing patterns among hybrid and parent groups.
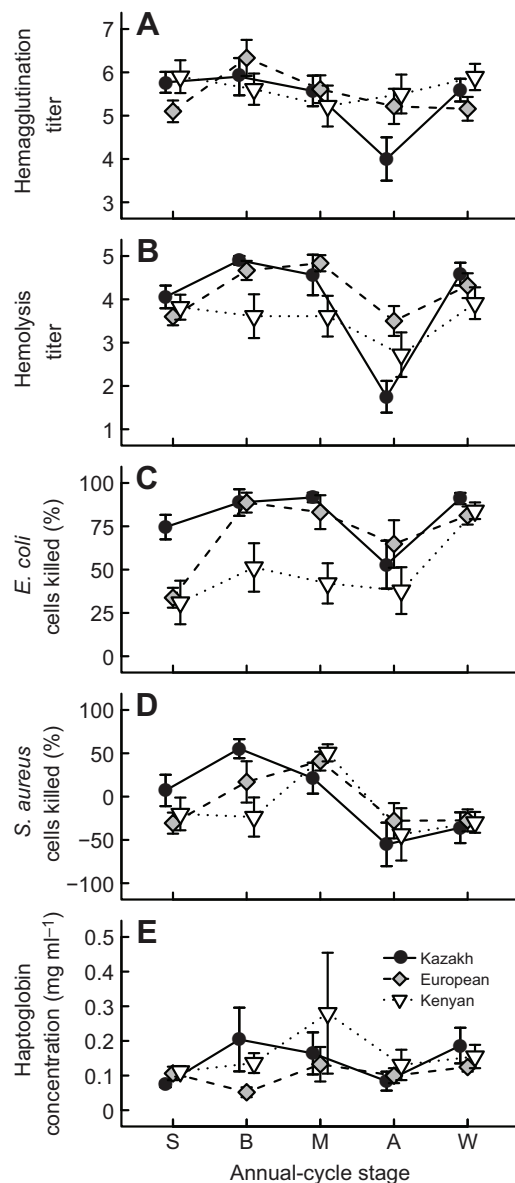

Fig. 1. Seasonal variation in constitutive immunity of subspecies of stonechats. Graphs show (A) hemagglutination, (B) hemolysis, (C) bactericidal ability against *Escherichia coli,* (D) bactericidal ability against *Staphylococcus aureus* and (E) haptoglobin concentration. Kazakh stonechats are shown in black circles, European stonechats in gray diamonds and Kenyan stonechats in white triangles. Data are means ± s.e.m. Annual-cycle stages: S, spring migration; B, breeding season; M, moult; A, autumn migration; W, winter.

Table 1. Statistics and P-values for effects of annual-cycle stage, age and sex on immune indices in Saxicola torquata

	d.f.	Hemagglutination[a]			Hemolysis[a]			Microbicidal ability against E. coli[a,b]			Microbicidal ability against S. aureus[a,b]		
		χ^2	P	Age/Sex	χ^2	P	Age/Sex	χ^2	P	Age/Sex	χ^2	P	Age/Sex
Constant temperature treatment													
Kazakh													
Annual-cycle stage	4	14.29	0.01		41.90	<0.001		21.70	<0.001		28.14	<0.001	
Age	1	0.90	0.34		0.95	0.33		0.25	0.62		2.12	0.15	
Sex	1	2.71	0.10		6.32	0.01	F	1.75	0.19		0.72	0.40	
Kazakh × European													
Annual-cycle stage	4	17.67	0.001		14.98	0.005		23.44	<0.001		7.75	0.10	
Age	1	0.13	0.72		2.25	0.13		0.27	0.61		0.42	0.52	
Sex	1	0.90	0.34		4.31	0.038	M	3.98	0.046	M	4.09	0.04	M
European													
Annual-cycle stage	4	6.18	0.19		24.12	<0.001		35.57	<0.001		11.89	0.02	
Age	1	0.65	0.42		10.22	0.001	+	5.72	0.02	+	0.06	0.81	
Sex	1	2.90	0.09		0.45	0.50		0.74	0.39		0.63	0.43	
European × Kenyan													
Annual-cycle stage	4	13.22	0.01		8.08	0.09		31.26	<0.001		19.41	0.001	
Age	1	2.94	0.09		3.65	0.06		7.22	0.01	+	1.09	0.30	
Sex	1	0.01	0.94		0.06	0.81		2.71	0.10		0.002	0.97	
Kenyan													
Annual-cycle stage	4	2.25	0.69		4.39	0.36		23.16	<0.001		11.46	0.02	
Age	1	5.62	0.02	+	5.56	0.02		1.52	0.22		0.37	0.54	
Sex	1	<0.001	0.98		0.52	0.47		0.95	0.33		3.98	0.046	F
Variable temperature treatment													
European													
Annual-cycle stage	4				7.27	0.12		24.71	0.000				
Age	1				3.77	0.052		3.48	0.06				
Sex	1				0.62	0.43		0.06	0.81				

[a]Included as covariate in the model is the time between entering the room and completion of sampling.
[b]Included as covariate in the model is age of the microbial stock solution.

Shown are values for hemagglutination titers, hemolysis titers and bactericidal ability (% cells killed) against Escherichia coli and Staphylococcus aureus for the five genotypic groups of stonechats kept at constant temperature, and for European stonechats kept at variable temperature. Results are from mixed-effects models after backward elimination of non-significant terms (P>0.05). Whether age has a positive or negative effect on immune traits is indicated with + or −, and the sex that has the higher value is indicated by M (male) or F (female).

Comparing seasonal variation among Kazakh, European and Kenyan stonechats

Hemagglutination

Annual-cycle stages differed from each other in Kazakh stonechats, but not in European and Kenyan stonechats (Table 1, Fig. 1A). Kazakh stonechats showed a substantial decrease in hemagglutination during autumn migration compared with moult (supplementary material Table S1). In European and Kenyan stonechats, there were no significant differences among annual-cycle stages (Table 1), although hemagglutination in European stonechats tended to be higher during breeding than during the other seasons. Also, during spring migration, European stonechats had a lower hemagglutination than the other two subspecies.

Hemolysis

Annual-cycle stages differed from each other in Kazakh and European stonechats, but not in Kenyan stonechats (Table 1, Fig. 1B). Patterns of seasonal variation were comparable in Kazakh and European stonechats (supplementary material Table S1). Both subspecies had low lysis during spring and autumn migration, although this decrease was more pronounced in Kazakh than European stonechats. Kenyan stonechats showed less seasonal variation, also with a dip during autumn, but none of the annual-cycle stages differed significantly from each other.

Bactericidal ability against *E. coli*

Annual-cycle stages differed from each other in all three subspecies with respect to bactericidal ability against *E. coli* (Table 1, Fig. 1C). Also, patterns of seasonal variation differed among stonechat subspecies (supplementary material Table S1). In Kazakh stonechats, bactericidal ability was lowest during the autumn migration, but was high during the rest of the year (supplementary material Table S1). European stonechats had low microbicidal ability during spring migration, but the ability notably increased during breeding and was similar to that of Kazakh stonechats for the rest of the year (supplementary material Table S1). The resident Kenyan birds had low microbicidal ability year-round, but increased it to the level of the other two subspecies during winter (supplementary material Table S1). All three subspecies showed a lower bactericidal ability in the migration periods than in the other annual-cycle stages, although in Kenyan stonechats this pattern was non-significant and less pronounced than in the other two subspecies.

Bactericidal ability against *S. aureus*

Bactericidal ability against *S. aureus* showed large variation among annual-cycle stages in all subspecies (Table 1, Fig. 1D), and patterns of seasonal variation differed among Kazakh, European and Kenyan stonechats (supplementary material Table S1). Kazakh stonechats had a high bactericidal ability during the breeding period and a low ability during autumn migration (supplementary material Table S1). The seasonal patterns of European and Kenyan stonechats were similar to each other, with peaks during moult and the lowest values during spring and autumn migration (supplementary material Table S1). Only during breeding did European stonechats have a slightly higher ability than Kenyan stonechats.

Comparing seasonal variation of hybrid and parent groups

Patterns of seasonal variation of hybrid and parent groups differed in hemagglutination, hemolysis and bactericidal ability against *E. coli* and *S. aureus*, but not in haptoglobin (Table 1, Fig. 2, supplementary material Table S1). There was no consistent pattern in how the hybrid groups differed from their parent groups, neither within nor among immune measures. In some cases the immune indices of the hybrid groups showed intermediate values to parental subspecies (e.g. *E. coli* in European × Kenyan hybrids during breeding and moult, but not during spring migration, autumn migration or winter; Fig. 2H), and in others resembled one of the parent groups (e.g. *E. coli* in Kazakh × European stonechats; Fig. 2C) or were dissimilar to both parent groups (e.g. hemagglutination in European × Kenyan stonechats; Fig. 2F).

Phenotypic flexibility of seasonal variation under different temperature regimes

Exploring the interaction term annual-cycle stage × treatment, we found that housing European stonechats under different temperature regimes affected two of the five immune indices. Patterns of seasonal variation of hemagglutination (χ^2_4=3.80, P=0.43; Fig. 3B), bactericidal ability against *S. aureus* (χ^2_4=4.42, P=0.35; Fig. 3E) and haptoglobin (χ^2_4=4.51, P=0.34; Fig. 3F) did not differ between stonechats kept under variable and constant temperature regimes. However, the patterns of seasonal variation in hemolysis (χ^2_4=12.20, P=0.02; Fig. 3C) and bactericidal ability against *E. coli* (χ^2_4=9.60, P=0.048; Fig. 3D) were significantly different between treatment groups. For hemolysis, annual-cycle stages differed from each other in stonechats kept at constant temperature, with a decrease during spring and autumn migration, but no such differences were found at variable temperature (Table 1). Annual-cycle stages differed from each other in both treatment groups in bactericidal ability against *E. coli* (supplementary material Table S2), showing an increase from spring migration to breeding in stonechats kept at variable and constant temperature. However, stonechats kept at variable temperature maintained this high level during the other annual-cycle stages, while stonechats kept at constant temperature reduced bactericidal ability against *E. coli* during autumn migration. As a result, during spring migration, bactericidal ability did not significantly differ from that during autumn migration in stonechats kept at constant temperature (supplementary material Table S2).

DISCUSSION

We showed that closely related birds with different life histories, and originating from environments that differ in seasonality, maintained different patterns of seasonal variation in constitutive immunity. Stonechat subspecies reared in a common garden environment showed differences in seasonal patterns of hemagglutination, hemolysis and bactericidal ability against *E. coli* and *S. aureus*, throughout the year. No such differences were found for measures of haptoglobin. However, large standard errors in some subspecies and annual-cycle stages may indicate that some individuals were combating an infection (Dobryszycka, 1997; van de Crommenacker et al., 2010). These results indicate that seasonal variation in four of the five measures of immunity had a genetic component. Studying seasonal variation in hybrids confirms this finding, but the results were inconsistent. Studying the phenotypically flexible response to temperature in European stonechats showed that keeping them at two temperature regimes did not influence the patterns of seasonal variation of haptoglobin, hemagglutination and bactericidal ability against *S. aureus*. However, patterns of variation of hemolysis and bactericidal ability against *E. coli* differed between the two treatment groups. These findings show that there is a genetic component underlying seasonal variation in immune indices of different genotypic groups, and that, at least in one subspecies, phenotypic flexibility in response to temperature accounts only for a small amount of variation.
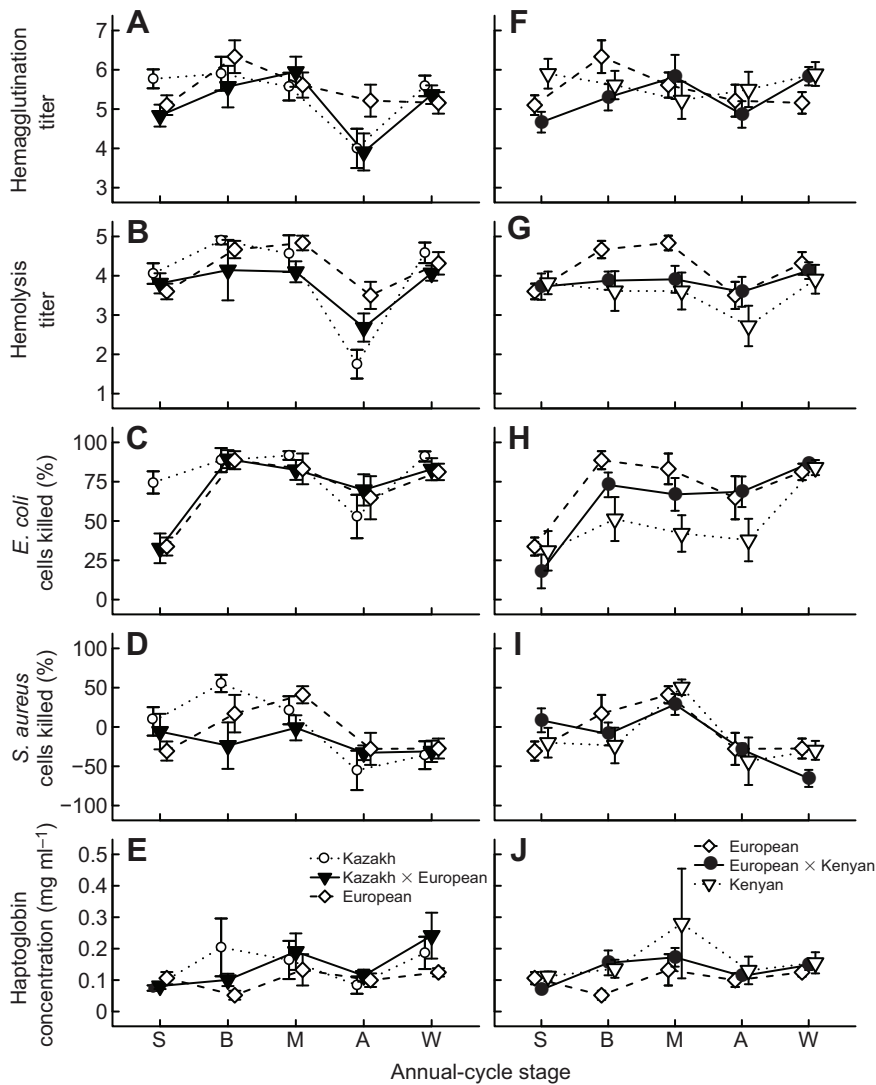
Fig. 2. Seasonal variation in constitutive immunity of hybrid stonechats and their parental subspecies. Graphs show seasonal variation in (A,F) hemagglutination, (B,G) hemolysis, (C,H) bactericidal ability against *E. coli*, (D,I) bactericidal ability against *S.aureus* and (A,J) haptoglobin concentration. (A–E) Values for Kazakh × European hybrids (black triangles) and Kazakh (white circles) and European (white diamonds) stonechats. (F–J) Values for European × Kenyan hybrids (black circles) and European (white diamonds) and Kenyan (white triangles) stonechats. Data are means ± s.e.m. Annual-cycle stages: S, spring migration; B, breeding season; M, moult; A, autumn migration; W, winter.

Some of the patterns of seasonal variation in immunity were qualitatively similar but quantitatively different among subspecies. For example, hemolysis was high during breeding and moult and showed a reduction during the autumn migration period in all three subspecies. However, this reduction during autumn migration was larger in Kazakh than in tropical Kenyan stonechats, while European stonechats showed intermediate levels. Although less clear, a similar pattern can be observed in bactericidal ability against *E. coli*. These findings support the idea that variability of annual cycles in constitutive immunity is associated with variability in environment and annual-cycle demands. Regions with a continental climate are characterized by short breeding seasons and Kazakh stonechats generally produce large clutches and moult at a fast rate before their long southward migration (Benson and Winker, 2001; Helm et al., 2009). The costs of breeding and moult have been proposed to be traded off against immune traits (e.g. Martin, 2005; Moreno-Rueda, 2010). Moreover, migrating Kazakh stonechats will be exposed to multiple environments that may differ in pathogen composition. Tropical stonechats, in contrast, have a long breeding season and do not migrate, so demands may be more constant across the year (Dittami and Gwinner, 1985; Helm, 2009). Furthermore, pathogen pressure may be less variable or diverse for resident than migrating stonechats because they experience only a single environment, although tropical environments have also been hypothesized to be

pathogen-rich (Guernier et al., 2004; Martin, 2005; Møller, 1998). An alternative hypothesis is that tropical environments are less predictable (Helm, 2009; Rubenstein et al., 2008), and that this may have led to greater flexibility of immune measures. As a result, in a relatively stable captive environment, with food available *ad libitum* and constant temperature, immune indices in captive Kenyan stonechats may be relatively constant. Data about pathogen pressure throughout the year and knowledge about immune function of wild populations can test these scenarios in the future.

We found remarkable similarities when comparing seasonal variation of stonechat subspecies within our study, as well as similarities and differences when comparing our stonechat results with the literature concerning other birds. All stonechat subspecies show conspicuous differences among annual-cycle stages in some immune parameters, especially changes in immune indices during migration and moult. During autumn migration, hemagglutination, hemolysis and bactericidal ability against *E. coli* were low compared with during breeding, moult and winter in long-distance migrant Kazakh and short-distance migrant European stonechats. During spring migration, these measures were similarly reduced in European stonechats, but in Kazakh stonechats the changes were less clear. Other studies have also reported changes (increases and/or decreases) in immune indices during migration periods (Buehler et al., 2008; Hegemann et al., 2012; Machado-Filho et al., 2010; Owen
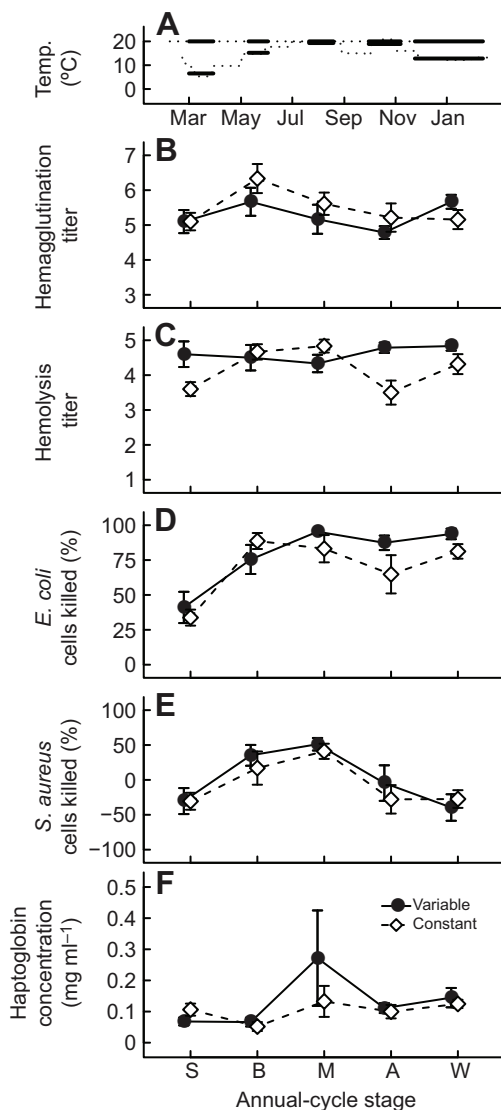
Fig. 3. Seasonal variation in constitutive immunity of stonechats kept under constant and variable temperature. Panels show (A) the temperature birds were exposed to (dotted lines) and the measurement periods (solid lines), and values for (B) hemagglutination, (C) hemolysis, (D) bactericidal ability against *E. coli*, (E) bactericidal ability against *S. aureus* and (F) haptoglobin concentration of European stonechats kept at constant (white diamonds) and variable (black circles) temperature treatments. Data are means ± s.e.m. Annual-cycle stages: S, spring migration; B, breeding season; M, moult; A, autumn migration; W, winter.

considerable variation of patterns among species when we compare our study with others that have used the same immune indices (Buehler et al., 2008; Hegemann et al., 2012; Pap et al., 2010b). This variation may stem from species-specific and index-specific costs and benefits of immune capacity relative to costs and benefits of other annual-cycle stage demands, and from the characteristics of the environments that different species occupy.

Patterns of seasonal variation of constitutive immune indices of hybrid groups differed from parental subspecies, but were not always intermediate. This implies that seasonal variation does have a genetic component, but that heredity does not follow simple Mendelian rules. The immune system consists of many components, each having their own costs and benefits (Janeway et al., 2004). Measures of bactericidal ability and complement are characterized by their integration of many cellular and/or humoral components and are not species-specific, which makes them particularly useful for comparative ecological studies (Lee et al., 2008; Millet et al., 2007; Tieleman et al., 2005). The integrated nature of these immune measures may also cause complexity of inheritance and may explain in part the variation in seasonal patterns between hybrids and parental subspecies.

In European stonechats, temperature treatment did have an effect on the seasonal variation of hemolysis and bactericidal ability against *E. coli*. The differences between patterns of seasonal variation of hemolysis and bactericidal ability against *E. coli* in the two treatment groups are somewhat counterintuitive. Stonechats kept at variable temperature showed less variation in these indices than stonechats kept at constant temperature. Moreover, hemolysis was most dissimilar during autumn migration, when ambient temperature was very similar between the two treatment groups. This flexibility in seasonal variation of immunity in response to temperature was not found in red knots, in the only previous study investigating the effect of different temperature regimes on these traits (Buehler et al., 2008). The difference between these results suggests that the extent of flexibility in response to temperature is species-specific. In stonechats, constitutive immune indices may not be immediately influenced by current temperature, as seems to be the case in red knots, but variability of the environment may trigger some immune indices to be kept at high levels throughout the year. Keeping immune indices high year round may be costly in terms of resources. Giving stonechats limited access to food could change this result, although in red knots this had little effect (Buehler et al., 2009a).

In conclusion, we found that seasonal variation of constitutive immunity has a genetic component and seems to coincide with the degree of seasonal variation that a species experiences within its geographic distribution. This study shows the importance of considering seasonal variation in immune function in relation to the ecology and life history of the organism of interest.

MATERIALS AND METHODS
Birds and treatments
Stonechats were kept at the Max Planck Institute for Ornithology, Andechs, Germany (Gwinner et al., 1987), and originated from three different locations: Kazakhstan (*n*=24; 9 females, 15 males), Central Europe (*n*=57, 34 females, 23 males) and Kenya (*n*=15; 9 females, 6 males). Free-living Kazakh stonechats migrate over long distances (i.e. conservatively estimated ~2600 km) and have a short breeding season during which they lay a single clutch with generally five to six eggs; free-living European stonechats are short-distance migrants (i.e. ~1700 km) and lay two to three clutches with generally five eggs; free-living Kenyan stonechats are resident birds and lay generally one clutch with three eggs (Helm, 2009; Baldwin et al., 2010). In addition, we bred and studied

and Moore, 2006). In association with migration, birds go through changes in body composition. During this period, some organs change in size or activity, including those related to immunity (Bauchinger and Biebach, 2006; Bauchinger et al., 2005; Møller and Erritzøe, 1998). The changes during spring and/or autumn migration in different indices reported in our study and in the literature suggest that shifts in immunity during these periods are a general phenomenon. During moult, European and especially Kenyan stonechats showed a relatively high bactericidal ability against *S. aureus*. Two other studies investigated bactericidal ability against *S. aureus* during moult. Pap et al. (Pap et al., 2010b) found that house sparrows (*Passer domesticus*) show high levels during moult while Buehler et al. (Buehler et al., 2008) found that red knots (*Calidris canutus*) do not. Zooming out to the full year, we encounter

hybrids between Kazakh and European subspecies (*n*=20; 5 females, 15 males) and between European and Kenyan subspecies (*n*=21; 7 females, 14 males). Birds were from the first (*n*=33), second (*n*=42), third (*n*=22) or fourth (*n*=5) generation, bred at the Max Planck Institute for Ornithology, or had been taken as nestlings (<9 days old) from the field, moved to the institute and hand-raised (*n*=35). All chicks were hand-raised in a single room. After fledging, birds were kept in separate cages which were randomly assigned to rooms with respect to genotypic group as much as possible. During the overall captive breeding program, new birds were repeatedly added to reduce inbreeding [for details about breeding and keeping of stonechats, see Gwinner et al. (Gwinner et al., 1987)]. All individuals were fully grown, ranging in age from 0 to 8 years, except one Kenyan individual that was 12 years old. The term 'subspecies' is used to refer to birds originating from the three locations (i.e. Kazakh, European and Kenyan stonechats), 'hybrid groups' to collectively refer to the hybrids between Kazakh and European and between European and Kenyan stonechats, and 'genotypic groups' to collectively refer to the combination of the two hybrid groups and the three parental subspecies.

Birds were housed with eight to 12 individuals per room and individuals were kept in separate cages. They were randomly housed in rooms with respect to genotypic group. Food and water were provided *ad libitum*. Stonechats were kept under standard conditions, which consisted of year-round constant ambient temperatures of 20–22°C and day length following the natural day length of European subspecies (Helm et al., 2009). In addition to this standard treatment, a subset of European stonechats (*n*=14) was kept under a variable temperature regime, changing temperature each week to mimic the average natural temperature cycle of free-living European stonechats (see Fig. 3A).

Annual-cycle stages and sampling

Constitutive immunity was quantified during five periods between February 2005 and March 2006, when all subspecies go through the corresponding annual-cycle stages: spring migration (24 February–30 March), breeding (10 May–2 June), moult (1 August–17 August), autumn migration (11 October–8 November) and winter (24 November–18 February). In the migration periods, all genotypic groups of stonechats are active during the night (Helm et al., 2005), which we monitored with a constant infrared light-beam installed in every cage. We defined winter as the quiescent period after autumn migration and before spring migration. In captivity, stonechats go through body and wing moult, and birds were checked for moult twice a week. Stonechats were unpaired during the breeding season, but physiologically they are in breeding condition from late stages of spring migratory restlessness until moult starts (Gwinner et al., 1995b; Helm et al., 2005). Birds were sterilely bled two times per annual-cycle stage by puncturing the brachial vein: once for microbicidal ability assays, and once for haptoglobin, hemagglutination and hemolysis. Collections on the same individual were at least 7 days apart (mean: 18.9 days, range: 7–75 days). We recorded time between entering the room and the end of blood sampling of an individual (sampling time, mean: 4 min; range: 1–22 min). Blood for hemagglutination, hemolysis and haptoglobin was centrifuged within 1 h of sampling to separate plasma from red blood cells, and the plasma was stored at −80°C until further analysis.

Complement and natural antibodies

Natural antibody and complement activity was quantified following the hemagglutination-hemolysis assay described by Matson et al. (Matson et al., 2005) with modifications described by Mauck et al. (Mauck et al., 2005). Hemagglutination and hemolysis titers were scored as the last titer at which the rabbit red blood cells were agglutinated or lysed. Wells that showed partial agglutination or lysis were given half scores.

Haptoglobin

Haptoglobin concentrations in plasma were measured with a commercial kit following the manufacturer's instructions (Tridelta Development Ltd., Maynooth, Ireland). The kit uses a colorimetric method to quantify the concentration of haptoglobin. Plasma and reagents were mixed in a 96-well flat-bottom plate and the absorbance was read at 630 nm using a Molecular Devices Spectra Max 340 plate reader.

Bactericidal ability

The bactericidal ability of blood was tested against two types of microbes that represented the classes of gram-negative bacteria (*Escherichia coli*) and gram-positive bacteria (*Staphylococcus aureus*) (Millet et al., 2007). Lyophilized pellets of *E. coli* and *S. aureus* (Epower Microorganisms, MicroBioLogics, St Cloud, MN, USA; *E. coli*: 0483E7, ATCC 8739, *S. aureus*: 0485E7, ATCC 6538) were reconstituted in phosphate-buffered saline to form a stock solution. Age in days of this stock solution was recorded, and a new stock solution was made at least once every 7 days. Methods described in Tieleman et al. (Tieleman et al., 2010) and Versteegh et al. (Versteegh et al., 2012b) were followed. The blood–*E. coli* mixture was incubated for 7.5 min, and the blood–*S. aureus* mixture was incubated for 180 min. *Staphylococcus aureus* is not always killed by stonechat blood, and also shows growth. However, the amount of growth differs among individuals (ranging from 96.9% killed to 393.4% growth) and we interpret it as a measure of how well stonechat blood inhibits growth.

Statistical analysis

We used R version 2.12.1 for statistical analyses (R Development Core Team, 2010). Variables were normally distributed (Kolmogorov–Smirnov $D<0.07$, $P>0.08$). Mixed-effects models were used to analyze seasonal variation in haptoglobin, hemagglutination, hemolysis and bactericidal ability against *E. coli* and bactericidal ability against *S. aureus* of the genotypic groups or treatment groups (Pinheiro et al., 2013). Because the same individuals were measured during multiple annual-cycle stages, individual was included as random effect. We tested the interaction annual-cycle stage × genotypic group to investigate whether annual-cycle stage had a different effect in the different genotypic groups. Because sex and age can be important factors in determining immune measures (Buehler et al., 2009b; Lee, 2006), we included them as main effects and show, but do not discuss, the results. The covariates age of the bacterial stock solution (in models for bactericidal ability) and sampling time were included in the models. Including or excluding the 12-year-old Kenyan bird did not change the significance of any of the interactions or main effects, so we included it in the analysis. Generations in captivity was not significant ($\chi^2_1<0.91$, $P>0.34$) in any of the immune indices except hemagglutination ($\chi^2_1=4.76$, $P=0.03$). In this case, generations in captivity did not influence the significance of the other main effects or interactions. Therefore, we did not include generations in captivity in the models.

The response to temperature treatment in European stonechats was analyzed in a similar manner. We investigated the effect of treatment on annual-cycle stages by testing the interaction annual-cycle stage × treatment, and included sex and age and the covariates age of the bacterial stock solution (when appropriate) and sampling time in the models.

If the interaction annual-cycle stage × genotypic group or annual-cycle stage × treatment was significant, the significance of annual-cycle stage in each genotypic group or treatment group was tested separately. If annual-cycle stage was significant in these separate analyses, *post hoc* Tukey tests were subsequently used to highlight which and in what manner annual-cycle stages differed from each other. First, seasonal variation of Kazakh, European and Kenyan subspecies was compared. To further investigate genetic components of patterns of seasonal variation, hybrid groups (Kazakh × European or European × Kenyan) were compared with their parental subspecies.

Author contributions

M.A.V. contributed to the execution of the work, the design, the analysis and the writing of the article; B.H. contributed to the execution of the work and the design

Supplementary material
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1 **Table S1 Statistics of pair-wise post hoc tests between an nual-cycle stages by genotypic group.** In the comparison among genotypic groups the

2 interaction term genotypic group*annual-cycle stage was not significant for haptoglobin, and results are not shown.

	Kazakh		Kazakh x European		European		European x Kenyan		Kenyan	
	|z|-value	P	|z|-value	P	|z|-value	P	|z|-value	P	|z|-value	P
Hemagglutination										
Spring-Breeding	0.40	0.99	1.09	0.81	2.25	0.15	1.41	0.61	0.31	1.00
Breeding-Moult	0.65	0.97	0.79	0.93	1.48	0.57	1.26	0.71	1.01	0.85
Moult-Autumn	2.78	0.04*	3.97	<0.001**	0.91	0.89	2.03	0.25	0.98	0.86
Autumn-Winter	3.62	0.003**	3.68	0.002**	0.67	0.96	2.80	0.04*	0.25	1.00
Winter-Spring	0.32	1.00	1.48	0.57	0.24	1.00	3.19	0.01*	0.14	1.00
Spring-Moult	0.39	0.99	2.18	0.18	0.59	0.98	2.54	0.08	1.33	0.67
Breeding-Autumn	3.19	0.01*	2.78	0.04*	2.23	0.16	0.81	0.92	0.02	1.00
Moult-Winter	0.17	1.00	1.15	0.77	0.43	0.99	0.29	1.00	1.41	0.62
Spring-Autumn	3.65	0.002**	2.11	0.21	0.49	0.99	0.64	0.97	0.33	1.00
Breeding -Winter	0.62	0.97	0.08	1.00	2.15	0.19	1.81	0.36	0.23	1.00
Hemolysis										
Spring-Breeding	1.38	0.63	0.41	0.99	2.40	0.11	0.71	0.95	0.19	1.00
Breeding-Moult	0.30	1.00	0.10	1.00	0.52	0.99	0.35	1.00	0.17	1.00
Moult-Autumn	5.95	<0.001**	3.12	0.02*	3.36	0.007**	0.96	0.87	1.63	0.47
Autumn-Winter	7.56	<0.001**	3.90	<0.001**	3.80	0.001**	2.38	0.12	1.82	0.36
Winter-Spring	2.15	0.19	0.84	0.91	3.95	< 0.001**	2.24	0.16	0.13	1.00
Spring-Moult	1.27	0.70	0.59	0.98	3.00	0.02*	1.01	0.85	0.02	1.00
Breeding-Autumn	5.51	<0.001**	2.71	0.051	2.82	0.04*	0.64	0.97	1.82	0.36
Moult-Winter	0.40	1.00	0.07	1.00	0.15	1.00	1.18	0.76	0.14	1.00
Spring-Autumn	5.51	<0.001**	2.81	0.04*	0.99	0.85	0.12	1.00	1.67	0.45
Breeding -Winter	0.02	1.00	0.18	1.00	0.45	0.99	1.63	0.47	0.33	1.00

3

Table S1 continued

	Kazakh		Kazakh x European		European		European x Kenyan		Kenyan	
	|z|-value	P	|z|-value	P	|z|-value	P	z|-value	P|	|z|-value	P
Bactericidal ability against *E.coli*										
Spring-Breeding	0.82	0.92	4.71	<0.001**	5.02	<0.001**	4.28	<0.001**	2.12	0.20
Breeding-Moult	0.23	1.00	0.22	1.00	0.11	1.00	1.49	0.56	1.71	0.42
Moult-Autumn	4.48	<0. 001**	1.34	0.66	1.80	0.37	1.85	0.34	0.80	0.93
Autumn-Winter	5.09	<0.001**	1.45	0.59	1.62	0.48	2.49	0.09	3.86	0.001**
Winter-Spring	1.99	0.27	4.84	<0.001**	5.21	<0.001**	5.95	<0.001**	4.01	<0.001**
Spring-Moult	1.17	0.76	4.97	<0.001**	4.73	<0.001**	2.71	0.05	0.49	0.99
Breeding-Autumn	3.32	0.008**	0.96	0.87	1.73	0.41	0.58	0.98	0.95	0.87
Moult-Winter	0.49	0.99	0.09	1.00	0.46	0.99	4.20	<0.001**	4.31	<0.001**
Spring-Autumn	3.12	0.02*	3.38	0.006**	2.36	0.12	4.15	<0.001**	1.17	0.77
Breeding -Winter	0.69	0.96	0.15	1.00	0.36	1.00	3.07	0.0177 *	2.84	0.03*
Bactericidal ability against *S.aureus*										
Spring-Breeding	2.82	0.04*	1.24	0.73	2.22	0.17	0.28	1.00	0.16	1.00
Breeding-Moult	2.88	0.03*	1.43	0.60	0.78	0.94	3.27	0.009**	2.53	0.08
Moult-Autumn	3.47	0.005**	2.56	0.08	2.45	0.10	2.46	0.10	3.23	0.01 *
Autumn-Winter	2.50	0.09	0.54	0.98	0.47	0.99	1.81	0.36	0.32	1.00
Winter-Spring	2.24	0.16	1.75	0.40	0.51	0.99	0.67	0.96	0.66	0.96
Spring-Moult	0.60	0.97	0.29	1.00	2.96	0.03*	2.81	0.04*	1.91	0.31
Breeding-Autumn	5.73	<0.001**	0.83	0.92	1.75	0.40	0.43	0.99	0.90	0.90
Moult-Winter	1.18	0.76	2.18	0.18	2.30	0.14	4.52	<0.001**	3.27	0.009**
Spring-Autumn	4.36	<0.001**	2.04	0.24	0.09	1.00	0.55	0.98	0.85	0.91
Breeding -Winter	4.31	<0.001**	0.45	0.99	1.53	0.53	1.34	0.65	0.67	0.96

8 **Table S2 Statistics of pair-wise post hoc tests between annual-cycle stages by treatment group.** In the comparison between treatment groups the

9 interaction annual-cycle stage*treatment was significant for hemolysis and bactericidal ability against *E.coli*, and we only performed a post-hoc test on

10 these indices.

	Temperature treatment			
	Constant		Variable	
	|z|-value	P	|z|-value	P
Hemolysis				
Spring-Breeding	2.40	0.11	0.64	0.97
Breeding-Moult	0.52	0.99	0.47	0.99
Moult-Autumn	3.36	0.007**	2.13	0.20
Autumn-Winter	3.80	0.001**	0.40	0.99
Winter-Spring	3.95	< 0.001**	0.71	0.95
Spring-Moult	3.00	0.02*	1.13	0.79
Breeding-Autumn	2.82	0.04*	1.54	0.53
Moult-Winter	0.15	1.00	2.42	0.11
Spring-Autumn	0.99	0.85	0.93	0.88
Breeding -Winter	0.45	0.99	1.58	0.50
Bactericidal ability against *E.coli*				
Spring-Breeding	5.02	<0.001**	2.89	0.03*
Breeding-Moult	0.11	1.00	1.91	0.31
Moult-Autumn	1.79	0.37	0.40	0.99
Autumn-Winter	1.62	0.48	0.53	0.98
Winter-Spring	5.21	<0.001**	5.05	<0.001**
Spring-Moult	4.73	<0.001**	4.79	<0.001**
Breeding-Autumn	1.73	0.41	1.43	0.61
Moult-Winter	0.46	0.99	0.13	1.00
Spring-Autumn	2.36	0.12	4.52	<0.001**
Breeding -Winter	0.36	1.00	1.95	0.29

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