

# Distress calls reflect poxvirus infection in lesser short-toed lark *Calandrella rufescens*

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Several studies have highlighted the association between bird song and parasite load, but there is no evidence regarding the relationships among pathogens and alarm or distress calls, which are used in antipredator strategies. We analyzed the association between virus infection and the distress calls of lesser short-toed lark (*Calandrella rufescens*), addressing the relationships between call acoustic properties, presence of poxvirus lesions, and other measurements of bird health (body condition, T-cell-mediated immune response, heterophils-to-lymphocytes ratio [H/L ratio], and blood parasites). The study was carried out in Fuerteventura (Canary Islands), where 55% of lesser short-toed larks were infected. Pox infection was associated with changes in the spectrotemporal structure of lark distress calls and affected the condition-dependent nature of these signals. Virus bearers uttered significantly shorter and lower pitched calls than virus-free birds, giving even shorter calls when subject to greater physiological stress (higher H/L ratio), whereas virus-free individuals did the opposite. Call harshness was positively correlated with bird immune and body condition, independently of virus infection, whereas call pulse rate decreased in stressed birds. We hypothesize that healthy birds might reveal their ability to bear the costs of antipredator defense by means of long, harsh, and fast modulated distress calls. Infection not only affects individual state and morphology by means of gross lesions but also may alter a signal used in an antipredator context, thus potentially reducing bird fitness both directly (through disease) and indirectly (through increased predation). *Key words*: acoustic communication, H/L ratio, immune response, island, parasite, poxvirus, prey. [*Behav Ecol*]

The dynamic relationship between host and parasite has broad evolutionary implications and has been the focus of many studies over the past few decades (Hamilton and Zuk 1982; Ebert and Hamilton 1996; Prugnolle et al. 2005). Parasites can profoundly affect animal morphology, behavior, and physiology and can increase variability in sexually selected secondary traits, pain threshold, dominance status, dispersal strategies, and immune system (Hart 1997; Zuk et al. 1998; Lindström et al. 2004; Lion et al. 2006).

Parasites can reduce host fitness both directly, by means of the costs of disease, and indirectly, through decreased reproductive success driven by parasite-mediated sexual selection. In this context, females can use male condition and resistance to parasites as cues to avoid being directly infected (Borgia and Collis 1989) or to obtain resistance genes for their offspring (Hamilton and Zuk 1982). The song is one of the ways male birds communicate their health status to females and rivals (Garamszegi et al. 2005, 2006). Predation is another indirect cause of parasite-induced fitness reduction. Predators might benefit from hunting parasitized individuals, as pathogens can reduce ability of prey to escape predation and make them more vulnerable (Lindström et al. 2003). Temple (1987) showed that prey killed by the predator consistently suffered more from parasitism by trematodes, nematodes, and ectoparasites than randomly collected individuals. In this scenario, natural selection should favor prey that encode information on individual condition and pathogen load in signals used in prey-predator communication, in the same way that sexual selection can drive the evolution of honest sexual secondary traits advertising individual quality (Catchpole 1986; Gil and

Gahr 2002). Although several studies have highlighted the relationship between bird song and parasite load (for a review, see Garamszegi 2005), there is little evidence of the effects of pathogens on signals used in a nonsexual context. In some instances, vocalizations may be used to advertise individual quality to predators (Cresswell 1994; Bergstrom and Lachmann 2001; Laiolo et al. 2004), and we might expect an effect of parasite infection on such "honest" signals.

In this paper, we test for the relationship between pox infection and the distress calls of the lesser short-toed lark (*Calandrella rufescens*). We studied a population severely affected by an epizootic of poxvirus, located in Fuerteventura (Canary Islands), at the western fringes of the species distribution (Smits et al. 2005). Pathogenicity of avian poxviruses can be moderate to severe (Kim et al. 2003); in its cutaneous form, virulence causes hyperplastic lesions in the unfeathered part of the body, whereas the diphtheritic form provokes necrotic lesions in the mucous membranes of the respiratory tract (Smits et al. 2005). Poxvirus disease has been described in diverse avian groups, from fowls through pigeons to passerines, but its prevalence tends to be very low in nature (Tripathy et al. 2000). However, in Fuerteventura, half of the lesser short-toed lark population was poxvirus infected, with a high percentage of birds with manifest gross lesions, missing nails and toes (Smits et al. 2005).

Vector-borne virus disease can become extremely deleterious for isolated bird populations and island endemics, affecting both the health status and behavior of individuals (Yorinks and Atkinson 2000). In the light of this, we predicted that infection influences lesser short-toed lark distress calls, given that call spectral features can vary in relation to individual body condition (size-corrected body mass) and T-cell-mediated immune response (Laiolo et al. 2004). We focused on the same data set of distress calls used by Laiolo et al. (2004), to analyze the relationship between call spectrotemporal features and

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poxvirus infection, and address to the interplay between pox infection and other measures of health status, such as body condition, T-cell-mediated immune response, leukocyte profile (heterophils-to-lymphocytes ratio [H/L ratio]), and occurrence of blood parasites. The effects of poxvirus on wildlife are poorly studied (Tripathy et al. 2000), and nothing is known about the consequences for the host communication system. This study can therefore offer insight into how naturally isolated populations respond behaviorally to the invasions of pathogens, as most of previous studies on host-parasite interaction on islands have addressed host ecophysiology or immunogenetics (Lindström et al. 2004; Wikelski et al. 2004; Woodworth et al. 2005; Whiteman et al. 2006; Wikelski and Cooke 2006).

## METHODS

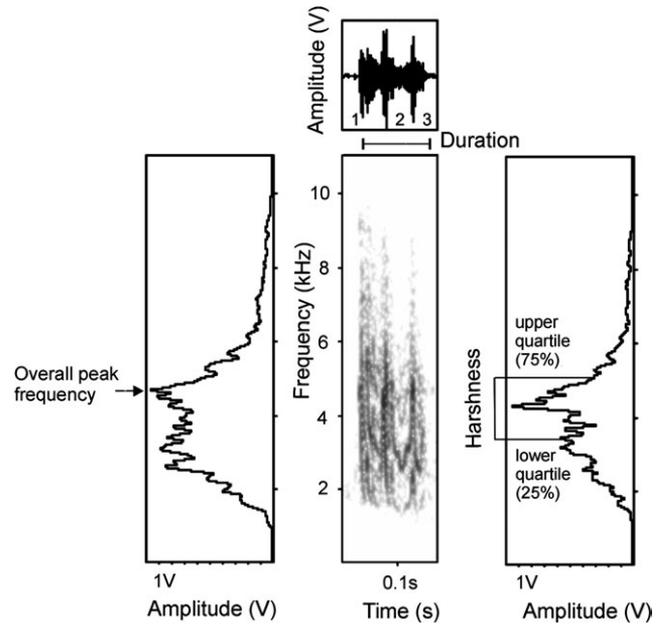
### Measurements of bird health

In November 2003, we captured lesser short-toed larks in Fuerteventura (Canary Islands) by means of spring-released nets baited with ground corn. These nets were located close to farmyards, where birds used to gather and feed on animal fodder. We examined each captured bird for anomalies related to pox infection, such as hyperplastic lesions on legs, feet, face and eyelids, or missing nails and toes (for details of histopathological and molecular virus identification, pathology, and inspection methodology, see Smits et al. 2005); infection was scored using presence or absence of lesions.

As indices of bird health, we used body condition (size-corrected body mass), T-cell-mediated immune response, H/L ratio, and blood parasite occurrence. Bird body condition was expressed as the residuals of the linear regression of log body mass on log wing length ( $r = 0.45$ ,  $P < 0.01$ ) (Brown 1996). Wing length is the best estimator of body size in the species (Laiolo P, Serrano D, Tella JL, Carrete M, Lopez G, Navarro C, unpublished data).

Immune function is a key physiological trait that influences fitness in animals, and its cell-mediated component is most frequently quantified (Smits et al. 1999; Tella et al. 2002). T-cell-mediated immune response was measured overnight, using the phytohemagglutinin (PHA) test (following Smits et al. 1999). The injection of PHA as a mitogen results in local activation and proliferation of T cells, followed by increased expressions of major histocompatibility complex molecules and, in turn, swelling of the skin at the point of injection. We injected 10  $\mu$ l of 5 mg/ml of PHA in wing patagium and estimated cellular immunocompetence by measuring swelling using a pressure-sensitive micrometer (details in Laiolo et al. 2004).

The H/L ratio is often used as an indicator of chronic stress, as blood-circulating lymphocytes (L) decrease whereas circulating heterophils (H) increase in stressing conditions, resulting in an alteration of the overall H/L ratio (Davison et al. 1983). This parameter is known to increase in response to infectious diseases, parasitemia, food or water deprivation, psychological stress, and injury (Horak et al. 1998; Work et al. 1999; Groombridge et al. 2004; López et al. 2005; Alvarez et al. 2006). As H/L ratio can be related to plasma corticosterone levels, it can also indicate immediate stress (Morici et al. 1997; Post et al. 2003). To quantify individual H/L ratio, a drop of blood was extracted from captured lesser short-toed larks to prepare a smear on a microscope slide, which was then air-dried and fixed with ethanol (Bennett 1970). For each smear, the cellular type (heterophils, lymphocytes, eosinophils, monocytes, and basophils) of 100 leukocytes was identified according to Campbell (1995), and the H/L ratio was calculated as the ratio of the numbers of heterophils and lymphocytes.



**Figure 1**  
Distress call of a lesser short-toed lark from Canary Island, Spain. Waveform (top), sonogram (center), mean power spectrum (left), and power spectrum of pulse 2 (right). Numbers in the waveform indicate the pulsed units of calls.

Based on examination of blood smears, we also recorded presence or absence of blood parasites (following Garamszegi et al. 2006). *Haemoproteus* was the only parasite found.

### Recordings and sound analysis

Distress calls are a particular category of alarm vocalizations, uttered when the predator is very close. In birds, they can be studied quite easily, as several species utter them when trapped for banding purposes (Jurisevic and Sanderson 1998). We recorded captured lesser short-toed larks during release, when birds uttered bouts of calls while flying away. Recordings were carried out in open habitat in conditions of low background noise, when individuals were  $\leq 10$  m of the recorder. We used a Sony TC-D8 DAT recorder and a Sennheiser ME67 microphone. Sound analyses were carried out with Avisoft SASLab Pro by R. Specht (Berlin), performing a Fast Fourier Transform (FFT) (sampling frequency 22050 Hz, FFT length 512, time resolution 8.9 ms, frequency resolution 43 Hz, window function: Bartlett, high-pass filter: cutoff frequency 900 Hz). Following Laiolo *et al.* (2004), we measured the following acoustic parameters (Figure 1): 1) number of calls uttered in the first 40 s after release, 2) call duration, 3) overall peak frequency (the frequency with the greatest energy, measured on the average spectrum), 4) pulse rate (number of pulses/call duration), and 5) call harshness (measured as the frequency range in which the signaler concentrate 50% of the call energy). The latter parameter is evaluated as the distance between the upper (75%) and lower (25%) quartiles in the pulse carrying the greatest call energy. Analyses were carried out using individual mean values of call duration, harshness, pulse rate, and overall peak frequency, while only one value was available for the number of calls given.

### Data analysis

Correlations among acoustic variables were very low ( $r = -0.22$  to 0.22,  $P = 0.10-0.77$ ), thus they were treated separately in

all the analyses. The use of the original and uncorrelated acoustic variables rather than, for example, derived factors of principal component analysis, also make results easier to interpret from a biological point of view. We performed generalized linear mixed model analyses with the macro GLIMMIX of SAS (version 8.2, Cary, NC) using call duration, harshness, pulse rate, and overall peak frequency as dependent variables; body condition, immune response, H/L ratio, virus, and blood parasite occurrence were used as independent variables, and the individual was entered as a random factor. Independent variables were added using a forward procedure, and the fit of the model was calculated as the proportion of explained deviance. The use of generalized linear mixed models permitted us to control for individual identity (and thus avoid pseudoreplication) and account for the natural variability within individuals. The relationship between call number and health indices was analyzed with a generalized linear model (GENMOD procedure in SAS), as only one value per individual was available. Generalized linear models were also used to examine the relationships between health indices and the coefficients of variation (standard deviation/mean) of call duration, harshness, overall peak frequency, and pulse rate. Quadratic terms were also incorporated into the models for continuous descriptors (immune response and H/L ratio) to account for potential nonlinear relationships. A normal distribution with an identity link function was used, and H/L ratio values were log transformed to attain normality. We did not control for body size and sex, as Laiolo et al. (2004) showed that these had no effect on this sample of distress calls.

Information on virus infection and distress calls was obtained from 45 individuals (giving 443 calls, on average  $9.8 \pm 0.9$  standard error [SE] per individuals), and immune response and body condition were measured in 41 birds,

whereas H/L ratio and parasite occurrence were estimated for 28 individuals. Because of this unequal sample size, we first obtained a model for virus infection, and then tested the effects of immune response, parasite infection, and H/L ratio incorporating these variables into the resulting models (following Serrano et al. 2003).

## RESULTS

Overall, 55% of all recorded lesser short-toed larks were poxvirus infected (25 out of 45 individuals). Infected and virus-free individuals did not differ in body condition ( $t_{39} = 0.93$ ,  $P = 0.18$ ), H/L ratio ( $t_{27} = 1.44$ ,  $P = 0.08$ ), immune response ( $t_{39} = 1.45$ ,  $P = 0.08$ ), or blood parasite occurrence ( $\chi^2 = 0.05$ ,  $P = 0.82$ ). There was no significant association among the 4 health measures as well (all  $P > 0.11$ ).

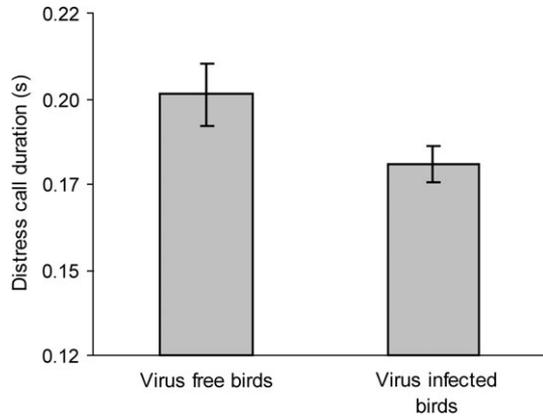
Call duration was significantly affected by pox infection, H/L ratio in its quadratic term, and the interaction of infection with H/L ratio (Table 1). Overall, call duration decreased in virus-infected birds ( $P < 0.001$ ; Figure 2). It also depended on infection in its relationship with H/L ratio, that is, virus-infected birds uttered long calls when possessing high H/L ratio, whereas the opposite occurred in the case of virus-free birds ( $P < 0.001$ ; Figure 3). The significant, quadratic relationship between call duration and H/L ratio thus resulted from the latter pattern ( $P = 0.021$ ; Table 1).

Call harshness was significantly affected by immune response and body condition ( $P = 0.003$  and  $0.044$  respectively; Table 1), but no significant effect of infection was found ( $P = 0.50$ ; Figure 4). Birds in better immunological condition uttered consecutive calls that were less variable in harshness than those of birds in poor condition ( $P = 0.019$ ; Table 1).

**Table 1**  
**Results of generalized linear mixed models and generalized linear models for acoustic parameters and health indices**

Acoustic variable	Parameter	Parameter estimate	SE	F	P
Generalized linear mixed models					
Duration	Intercept	0.24	0.02		
	Infection	-0.06	0.02	11.84	0.0007
	H/L ratio	-0.02	0.09	0.26	0.6098
	(H/L ratio) <sup>2</sup>	0.32	0.14	5.37	0.0212
	Infection $\times$ H/L ratio	-0.32	0.12	23.16	0.0000
	Explained deviance (%)	25.15			
	Residual deviance	1.04			
Harshness	Intercept	1.42	0.06		
	Immune response	1.34	0.45	8.89	0.0031
	Body condition	0.10	0.05	4.08	0.0440
	Explained deviance (%)	3.10			
	Residual deviance	94.18			
Overall peak frequency	Intercept	3.51	0.06		
	Infection	0.19	0.09	4.07	0.0440
	Explained deviance (%)	0.02			
	Residual deviance	135.2			
Pulse rate	Intercept	34.27	1.60		
	H/L ratio	-9.76	4.60	4.50	0.0350
	Explained deviance (%)	73.72			
	Residual deviance	16829.3			
Generalized linear models					
Coefficient of variation of harshness	Intercept	1.46	0.07		
	Immune response	-0.98	0.41	5.54	0.0190
	Explained deviance (%)	14.9			
	Residual deviance	2.88			

Only results from variables significantly affected by health indices are shown.



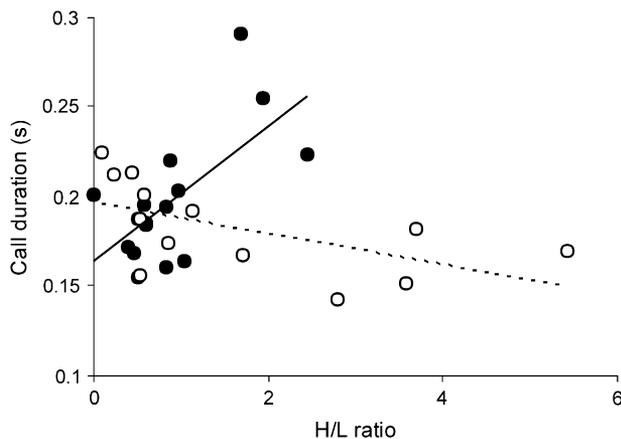
**Figure 2**  
Mean ( $\pm$ SE) distress call duration from virus-infected and virus-free lesser short-toed larks.

Virus-infected birds called with slightly lower peak frequency than virus-free birds ( $3.51 \pm 0.38$  vs.  $3.69 \pm 0.40$  kHz,  $P = 0.044$ ), although the deviance explained by this model was quite low (Table 1).

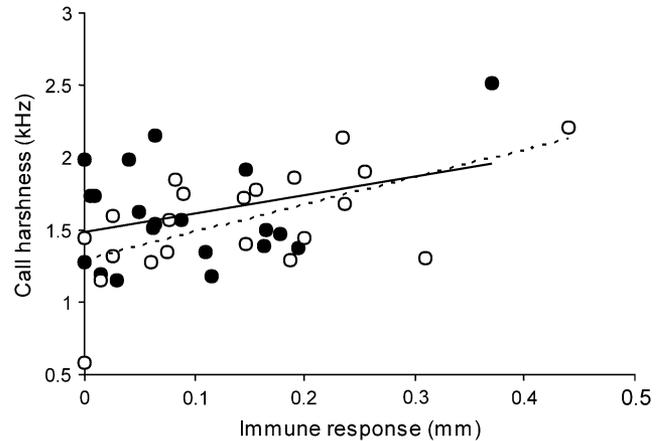
Pulse rate was lower in birds with greater H/L ratio ( $P = 0.035$ ); the interaction between H/L ratio and pox infection had no significant effect ( $P = 0.67$ ), although the relationship was slightly stronger in virus-infected birds (Figure 5). The number of calls given was not affected by any health parameter ( $P = 0.17$ – $0.93$ ), as well as the coefficient of variation of call duration ( $P = 0.10$ – $0.69$ ), pulse rate ( $P = 0.30$ – $0.80$ ), and peak frequency ( $P = 0.051$ – $0.85$ ).

## DISCUSSION

Infection in lesser short-toed larks was associated with changes in the spatio-temporal structure of distress calls and affected the condition-dependent nature of these signals. Virus-infected individuals uttered significantly shorter and lower pitched calls than virus-free birds, and infection was associated with the relationship of health indices to spatio-temporal variation in calls. It is worth noting that we found no significant relationship between infection and the other health status indices commonly used in avian research. This may be related to low sample sizes that condition the strength of the tests (several  $P$  values ranged from 0.05 to 0.1). Alter-



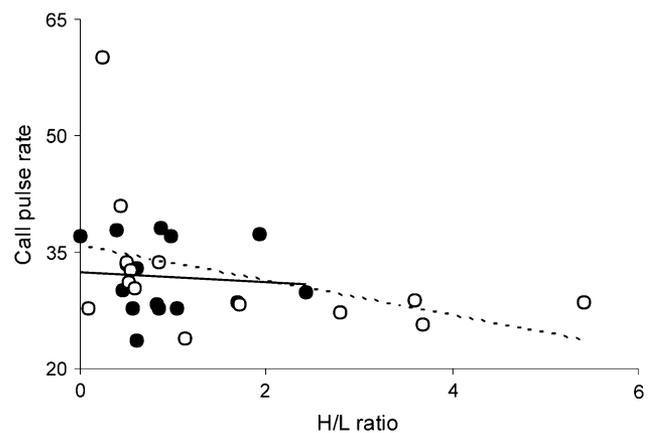
**Figure 3**  
Relationship between mean distress call duration and H/L ratio in virus-infected (open dots and dashed line) and virus-free birds (filled dots and line).



**Figure 4**  
Relationship between mean distress call harshness and immune response in virus-infected (open dots and dashed line) and virus-free birds (filled dots and line).

natively, it may result from the great availability of predictable food supplies in farmyards, which permit birds to maintain a good state in spite of the infection (Carrete M, Serrano D, Tella JL, Lopez G, Illera JC, Vögeli M, Delgado A, unpublished manuscript).

The association between call and virulence was striking when considering the relationship between H/L ratio and call duration, given that virus bearers gave shorter calls when undergoing greater stress (higher H/L ratio), whereas virus-free individuals did the opposite. The quadratic up and down relationship between call duration and H/L ratio might actually reflect these contrasting trends. Although virus infection did not significantly affect H/L ratio, some infected birds showed the highest H/L ratios among the whole sample of individuals (Figure 3). Virulence reached its maximum severity in these birds, by means of multiple and coalescent lesions (the individual with the highest H/L ratio had 5 large swelling areas). We hypothesize that stressed virus-free birds may invest in long calls, whereas infected birds may not because of their extreme stress conditions. H/L ratio may also reveal psychological stress (Morici et al. 1997) and, as a consequence, may affect animal alarm responses (Blumstein et al. 2005). Hence, scarcely stressed birds might not be alerted enough to respond to capture stress with long signals, whereas birds might



**Figure 5**  
Relationships between mean distress call pulse rate and H/L ratio in virus-infected (open dots and dashed line) and virus-free birds (filled dots and line).

not afford the cost of a vigorous reaction at the opposite stress extreme.

Laiolo et al. (2004) showed that birds in good immune or body condition produced the harshest calls. This study suggests that birds in prime immune condition also keep more constant harshness values over consecutive calls. Call pulse rate was neither affected by bird immune response or infection, but appeared to decrease linearly with bird stress (H/L ratio; Figure 5). Fast trill rate and wide bandwidth (a parallel of "harshness" for pure tones) are difficult to produce because of mechanical constraints in the beak region (Westneat et al. 1993; Podos et al. 2004) and can signal male condition in a sexual selection context (Ballentine et al. 2004). This study shows that these acoustic features might signal bird quality to predators as well.

By using a larger set of health indicators, here we confirm the hypothesis of Laiolo et al. (2004) that distress calls could be condition dependent. Healthy birds might reveal their ability to bear the costs of antipredator defense by means of long, harsh, and fast modulated distress calls and, on the other hand, predators might mutually benefit from this signaling by focusing on the most vulnerable prey, in order to avoid wasting energy with birds with good escape abilities (Møller and Erritzoe 2000; Cresswell and Quinn 2004). Based on our results, it seems intuitive that poor-condition birds should avoid signaling in order to prevent being identified as poor-quality birds. In contrast, all captured larks alarmed irrespective of their state. This strategy may derive from the gregarious behavior of lesser short-toed larks, in which an individual may have no knowledge of its relative condition with respect to that of flock mates. Further analysis of the potential costs and benefits of prey calling could help in fully understanding the nature of this behavior. Cresswell (1994) showed that the skylark (*Alauda arvensis*) used the song as pursuit-deterrent signal. Distress-like sounds are also uttered when free-ranging lesser short-toed larks are threatened by an approaching predator and sometimes are included in song performances. In the latter context, they might function as pursuit-deterrent signal for potential predators spotted while singing.

The structural similarities found in the distress calls of a wide range of bird species suggest that predators (either generalists or specialists) are driving the evolution of avian distress calls (Hope 1980; Jurisevic and Sanderson 1998). However, given the lack of experimental data on receivers (i.e., predators), we cannot rule out the alternative explanation that birds in poor condition modify their behavioral activities (including calling) in general (Yorinks and Atkinson 2000). Controlled experiments on predator hunting strategies according to prey call quality need to be performed to better support the hypothesis that selective pressure by predators affects the evolution of distress calls.

Selective processes apart, this study shows that distress signals can reflect virus infection. Lindström et al. (2001) and (2003) found out that predator escape performance of the common greenfinch *Carduelis chloris* decreased in birds infected by Sindbis virus, an *Alphavirus* that has no apparent pathological and morphological effect (Lindström et al. 2003; Lindström 2004). In the case of lesser short-toed larks, pox infection did affect bird morphology, through lesions, swelling, and tumors (Smits et al. 2005). Thus, the potential direct fitness decrease caused by disease might be coupled with the indirect negative effect of reduced antipredator defense.

Virus-induced changes in alarm calls might not be noticeable in continental zones with low virus prevalence, but clearly become manifest in Fuerteventura, where 55% of lesser short-toed larks are infected. Virus spread in the island might be the consequence of low disease resistance caused by inbreeding,

which can become prominent in isolated systems (Frankham 1998; Whiteman et al. 2006), or by the larks' tendency to forage close to human settlements and farms, where birds come in contact with a series of possible disease reservoir hosts (poultry and other domestic animals; Gottdenker et al. 2005). In the light of this, we stress that more work should be addressed to the behavioral consequences of pathogen infection and epizootics in isolated ecosystems, in order to fully understand, and properly manage, pathogen effects on island endemic or endangered species.

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