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## **Prevalence of hematozoan parasites in Red Kite nestlings from France**

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## Abstract

Blood pathogens may be harmful for birds and thus, represent a potential threat for some populations. To date, only few data are available on the prevalence of hematozoans in the Red Kite, a raptor endemic to the Western Palearctic listed as Near Threatened within its global range. Here, we report the first screening of haemosporidian parasites in Red Kite nestlings from French breeding areas. The blood of 47 nestlings was sampled in three areas in Eastern France and the prevalence of three genera of blood pathogens, *Leucocytozoon*, *Haemoproteus* and *Plasmodium*, was measured based on PCR-assays. Thirteen nestlings were positive to *Leucocytozoon* sp. (n=13) and *Plasmodium relictum* (n=1), which corresponds to an overall prevalence of 28%. This prevalence is higher than those previously reported for the Red Kite in other countries. This is the first record of *P. relictum*, a potential pathogen for birds, in the Red Kite. No relationship was found between the body condition of nestlings and the occurrence of haemosporidians. Our findings suggest that hematozoans could be a limited threat for nestlings but prevalence in adults, a sensitive stage for the population dynamic of Red Kite, remains poorly known and should be further investigated at least in its main breeding areas.

Key words: Haemosporida, *Haemoproteus*, *Leucocytozoon*, *Plasmodium*, raptor

## Declarations

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All the authors consent to participate and for publication

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Authors' contributions: Michael Coeurdassier designed the study. Christophe Morin and Michael Coeurdassier managed and carried out fieldwork and sampling. Eve Afonso and Nicolas Bassin conducted the pathogen analysis. Michael Coeurdassier, Eve Afonso, Thibaut Powolny and Nicolas Bassin analyzed and interpreted the data and wrote the manuscript. All co-authors commented on the manuscript and accepted the final version.

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## Introduction

The Red Kite *Milvus milvus* is an Accipitridae endemic to the Western Palearctic. It breeds mainly in Germany, France and Spain, which together hold around 75% of the global population (Knott et al. 2010). In these countries, the populations have been estimated to be declining, which led the International Union for the Conservation of Nature to assess the Red Kite as Near threatened (BirdLife International 2020) within its global range while it is currently listed as vulnerable in France (UICN France et al. 2016). The main threats identified are poisoning by anticholinesterasic pesticides and rodenticides, decrease of livestock, homogenization of landscapes and potentially wind turbines (BirdLife International 2020). In both Europe and France, the decline reported during the period 1990-2010 has led to the implementation of a Conservation Plan in which the health status of the Red Kite was surveyed, notably on nestlings that were caught at nests for ringing and wing-marking. Among the health indicators monitored and considering that ecotoxicological issues were identified, chemical concentrations in blood were measured and we showed that 50% of nestlings were exposed to organic compounds and/or metals (Coeurdassier et al. 2014; Powolny et al. 2020). If infection by pathogens has not been pointed out as a threat for the Red Kite, only few studies have focused on the prevalence of blood parasites and no data are available on the prevalence of hematozoan pathogens in Red Kite nestlings from France. Haemosporidians belonging to three genera of protozoan (*Haemoproteus*, *Leucocytozoon*, and *Plasmodium*) are intracellular blood parasites transmitted to hosts by bloodsucking dipteran vectors, they have been commonly detected in birds including raptors (Remple 2004). *Haemoproteus* and *Leucocytozoon* are often considered to have little or no pathogenic effects on birds but their presence in some raptor nestlings has been related to subclinical effects and even host death. *Plasmodium*, the causative agent of malaria in animals, can be a serious avian

pathogen, *Plasmodium relictum* being the species most commonly associated with virulent disease (Remple 2004). In raptors, haemosporidians may also negatively affect health such as body condition and life traits (Korpimaki et al. 1993; Hatchwell et al. 2001; Sol et al. 2003; Garvin et al. 2006). Here, we aim to measure the prevalence of common blood parasites in birds of the genera *Haemoproteus*, *Leucocytozoon* and *Plasmodium* in nestlings of three areas monitored in Eastern France. The relationship between prevalence and body condition of the nestlings was also checked to document whether such infection could affect individuals.

## Methods

### Study areas and sample collection

Fieldwork was carried out in 2015 from 4<sup>th</sup> to 25<sup>th</sup> June in Franche-Comté region, Eastern France. Three breeding populations of Red Kite, from three areas (called “Area” factor hereafter) were monitored: Sundgau Belfortain (47°38'0170"N, 6°51'0460"E), Dugeon-Remoray basin (46°54'0220"N, 6°21'0170"E) and Besançon plateau (47°14'0350"N, 6°01'0190"E). Forty-seven chicks (23 males and 24 females) corresponding to 24 nests (called “Nest” factor hereafter) were retained for the study. The sampling was implemented 15 days prior to the expected fledging date, when the chicks were approximately 25–40 days old. Nestlings were removed from the nests by an experienced arborist climber and carried to the ground for data collection. First, the body mass (g) and the wing length (i.e., length from the extremity of the longest primary feather to the wrist of the right wing in mm) were measured. Blood (2 mL) was sampled by trained operators from the brachial vein using lithium-heparin coated syringes. As recommended (Fair et al. 2010), the volume of blood collected was <0.5% of the chick's body mass. Blood samples were kept in the dark at 4°C and then stored at -20°C until processed in the laboratory. The sex of the chicks was determined according to a PCR method (Griffiths et al.

1998) as described in Powolny et al. (2020). All the manipulations were performed with governmental authorizations no. 2015093-0007 and 2015093-0023 in the framework of the Red Kite national conservation plan.

### **Body condition estimate**

In the present study, we define body condition as a proxy of energy reserves, mainly fat mass that represents a broad indicator of the health status of nestlings.

According to Labocha and Hayes (2012), body mass of birds is « *as good or nearly as good an indicator of fat content, as any of the other condition indices* ». Because body mass can be easily and reliably measured, we used it as an estimate of body condition. As we studied growing individuals, difference in body mass could be due to slight differences in specimen age rather than in fat reserve. Mougeot and Bretagnolle (2006) showed that the wing length was closely correlated to the age of Red Kite nestlings. Moreover, the relationship they established is almost linear within the range of ages of the kite chicks we studied, thus, we used the wing length as an estimate of chick age. Body condition was calculated as the residuals of the linear model [body mass (in g) ~ wing length (in mm)], which corresponds to an assessment of the body mass following a statistical elimination (sensus Grafen and Hails 2002) of age differences. Positive residuals were related to chicks in relative good condition while negative residuals represented kites in poor condition.

### **DNA extraction, PCR amplification, sequencing, and parasite detection**

DNA was extracted from blood samples using the DNeasy Blood & Tissue kit (Qiagen, France) and according to manufacturer's instructions (protocol "Purification of Total DNA from Animal Blood or Cells"). A nested PCR was conducted to amplify a fragment of cytochrome b gene (542 bp) from species of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* as recommended by Pérez-Rodríguez et al. (2013). A first PCR was performed in total volumes of 12.5µL including 1 × Accustart



II PCR ToughMix (Quanta Biosciences, USA), 0.6 mM of each primer  
 (Plas1F/HaemNR3), and 22 ng of total genomic DNA. The PCR program consisted  
 in an activation step of 3 min at 94°C, followed by 20 cycles of 30 sec at 94°C, 20  
 sec at 52°C, 30 sec at 72°C, and a final elongation of 5 min at 72°C. We then used  
 PCR products from the first PCR to conduct the following reaction in a total volume  
 of 25µL comprising 1 × Accustart II PCR ToughMix, 0.6 mM of each primer  
 (3760F/HaemJR4) and 2.5µL of PCR products from the first PCR. The PCR  
 program consisted in an activation step of 3 minutes at 94°C, followed by 35 cycles  
 of 30 sec at 94°C, 20 sec at 52°C, 30 sec at 70°C, and a final elongation of 5 min at  
 70°C. PCR products were then separated and visualized using a Qiaxcel device  
 (Qiagen, France) and a DNA High Resolution kit (Qiagen). A negative control (PCR  
 grade water instead of DNA extract) was introduced every ten samples. PCR  
 products which yielded an approximately 590 bp fragment (including primers) were  
 confirmed by sequencing. The amplified products were purified using the QIAquick  
 PCR Purification Kit (Qiagen) according to manufacturer's instructions. Direct  
 sequencing of the purified PCR products was performed with an automated  
 sequencer (Applied Biosystems 3130 Genetic Analyzer) using the primers employed  
 for the second PCR reactions. For each PCR-positive sample, we aligned forward  
 and reverse DNA sequences to obtain a consensus of 479 bp (Pérez-Rodríguez et  
 al. 2013). A homology search of the sequences generated in this study was then  
 performed by conducting an online search with the NCBI Basic Local Alignment  
 Search Tool for nucleotides (BLASTN) in the GenBank database  
[\(<http://blast.ncbi.nlm.nih.gov/Blast.cgi>\)](http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Mixed infections were checked by examining electropherograms using the software  
 BioEdit following the recommendations of Pérez-Tris and Bensch (2005). When  
 double peaks were observed, we compared double peak patterns with the DNA  
 sequences of parasite haplotypes already available on Genbank to identify which

parasite haplotypes were involved in mixed infections (Pérez-Rodríguez et al., 2013).

## Data analysis

Difference of infection status (i.e., presence/absence of hematozoans) between sex was checked with a binomial generalized linear mixed effect model (GLMER) with 'Sex' as fixed factors and 'Nest' nested into 'Area' (Area/Nest) as random effects [corresponding full model:  $\text{Status} \sim \text{Sex} + (1|\text{Area/Nest})$ ]. The relationship between the presence of at least one positive chick in the brood ('Positive brood', binomial variable) and the brood size was tested with a binomial generalized linear mixed effect model (GLMER) with 'Brood size' as fixed factors and 'Area' as random effects [corresponding full model:  $\text{Positive brood} \sim \text{Brood size} + (1|\text{Area})$ ]. Relationship between body condition and infection status of the individual was checked with a linear mixed effect model (LMER) with 'Wing length', 'Sex' (in interaction or not with 'Wing length') and 'Status' as fixed factors and 'Nest' nested into 'Area' (Area/Nest) as random effects [corresponding full model:  $\text{Body mass} \sim \text{Wing length} + \text{Sex} + \text{Status} + (1|\text{Area/Nest})$ ] (Zuur et al., 2009). The null model was also tested and the most parsimonious model with the lowest Akaike information criterion (AICc) was selected, a difference of  $\text{AICc} > 2$  was retained to evidence difference between 2 models (Burnham and Anderson, 2010). Normality of model residuals and variance homoscedasticity were verified. All statistical analyses were performed using R 3.4.2. with the packages *lme4*, *pgirmess* and *lmerTest*.

## Results

Among the 47 chicks studied, 30 came from the Dugeon-Remoray basin (15 females and 15 males), 14 from the Besançon plateau (eight females and six males) and three from the Sundgau belfortain (one female and two males). Haemosporidian

parasites were found in 13 chicks (i.e., 28%), *Leucocytozoon* was detected in eight males and five females from the three studied areas and *Plasmodium* in a single male also infected by *Leucocytozoon* of the Sundgau belfortain population (Fig. 1). No difference of *Leucocytozoon* prevalence was evidenced between sex (binomial GLMER,  $p = 0.33$ ) or between brood size (binomial GLMER,  $p = 0.66$ ). We found two *Leucocytozoon* haplotypes, which perfectly matched (i.e. with a 100% identity on 479bp) with L-MILVUS1 (Genbank accession number JN164716) and L-MILANS4 (JN164713) haplotypes described in Pérez-Rodríguez et al. (2013). The DNA sequence corresponding to L-MILVUS1 also closely matched with *Leucocytozoon toddi* (GenBank accession number DQ177272) with a 99% identity. The DNA sequence amplified in the only sample positive for *Plasmodium* matched with *Plasmodium relictum* (GenBank accession number LN835311) with a 100% identity over 462bp. Although *Leucocytozoon toddi* is a typical parasite of birds (Valkiūnas et al. 2010), we cannot exclude that we detected an unreferenced species genetically close, we thus retained *Leucocytozoon* ssp. hereafter, while we considered that DNA from *Plasmodium relictum* was detected in the corresponding positive nestling. The only PCR-positive sample for *Plasmodium relictum* was also positive for *Leucocytozoon* sp. (L-MILVUS1 haplotype) and we recorded three other individuals with a co-occurrence of both *Leucocytozoon* haplotypes L-MILVUS1 and L-MILANS4, among which two nestlings from the same brood. This suggests that co-infections can occur relatively frequently, both for interspecific (1/47 individual) and inter-haplotypes (3/47) co-occurrences.

At a same wing length, females are heavier than males (best LMER selected with 'Sex' as factor,  $p < 0.0001$ , Table 1. Estimate for males = -84.8,  $df = 31.1$ ,  $t = -5.45$ ). Thus, the body condition of the nestlings is presented for both sex separately in Fig. 1. No difference of body condition was detected between infected and non-infected kites whatever the sex ( $p = 0.15$ , Table 1).

## Discussion

Our findings showed that nestlings of Red Kite are infected by haemosporidians, notably *Leucocytozoon* spp. and *P. relictum*, in three French breeding areas. The prevalence of 28% we measured was higher than those commonly reported for *Leucocytozoon* in Red Kite chicks from German, Spanish, UK or Danish populations while *Plasmodium* was less frequently found (Table 2). Overall, if we aggregate all the studies reported in Table 2 except our and without distinction of age, the prevalences measured in the Red Kite (n=366) were 3.8%, 0.3% and 0.0% for *Leucocytozoon*, *Haemoproteus* and *Plasmodium*, respectively. Co-infection by two haplotypes of *Leucocytozoon* corresponding to some of those previously described in Spanish populations of Red Kite (Pérez-Rodríguez et al. 2013) were found in three nestlings of the French populations studied. We also point out that the detection of *P. relictum* in one single nestling of a French population is the first record for the Red Kite while several haplotypes of *Plasmodium* were detected in the Black Kite *Milvus migrans* (Pérez-Rodríguez et al. 2013; Nourani et al. 2020). More generally, *P. relictum* has already been reported in species belonging to Accipitridae and Falconidae families (Bennett et al. 1993). It is considered as a serious pathogen for birds and has been associated to virulent diseases and even death for translocated Gyrfalcon *Falco rusticolus* (Remple 2004). However, to our knowledge, no information is available on its harmfulness for Accipitridae and the individual infected by *P. relictum* we found did not show obvious health troubles.

Prevalence of blood parasites in diurnal raptors is highly variable and may reach 50% or more notably in adults (e.g., Forrester et al. 1994; Castellucci et al. 1998), the genera *Haemoproteus* and *Leucocytozoon* being the most frequently detected while *Plasmodium* commonly has lower prevalence (Remple 2004). Overall,

nestlings have been reported to exhibit relatively low prevalence of Haemosporida in numerous studies (e.g., Gutiérrez-López et al. 2015; Hanel et al. 2016) even if notable exceptions exist (Toyne and Ashford, 1997, Jeffries et al. 2015). Such differences of prevalences between juveniles and adults have been explained by the higher probability of adults contracting an infection in successive year (Podmokla et al. 2014). For the Red Kite, available data does not allow to identify a difference of prevalence between adults and nestlings mainly because of the low number of positive individuals and unbalanced samples (Table 2).

The comparison of Haemosporida prevalence of Red Kite in Spain to those determined in other studies (Table 2) suggests that it could be higher in more northern areas such as France and potentially in Denmark, UK and Western Europe despite low sample sizes. The prevalence of haemosporidians in diurnal raptors has been explained by different factors. Some are related to populations such as the abundance of Diptera vectors within the environment (Sol et al. 2003, Sehgal 2015) while others rely more on species features, such as the world geographic range, breeding habitat and embryonic development (Tella et al. 1999). In the case of the Red Kite, abundance of vectors has never been investigated directly in the previous studies published but it was assumed that the low prevalence measured in Spain could be due to the dryness of the environment, making it unsuitable for the main vectors of haemosporidian parasites (Tella et al. 1999). In Franche-Comté, the climate is more wet (annual rainfall from 1000 to 1500 mm against 600 mm in Spain) and numerous ponds, lakes and streams composed the landscape of the studied areas, which could at least partly explain the relatively high prevalence of *Leucocytozoon*. Moreover, the world range of the Red Kite limited to Western Europe, the absence of inter-continental migration notably through Africa and the fact that it breeds in open-woodland habitat are all together factors that may explain

relatively low Haemosporida prevalence in raptors and more generally animals (Tella et al. 1999; Altizer et al. 2011).

Lower body condition has been related to infection status (i.e., infected or not) by haemosporidians in both passerines (Garvin et al. 2006, Hatchwell et al. 2000) and raptors (Dawson and Bortolotti 2000). We did not show any effect of infection of Red Kite by *Leucocytozoon* on body condition, which suggests that these blood parasites might have no or weak pathogenic consequences on nestlings and/or that the infection intensity is too low to impair condition. In the American kestrel *Falco sparverius*, Dawson and Bortolotti (2000) found that a reduced condition of males was associated with higher intensity of infections by *Haemoproteus* using blood smear as a diagnostic tool. Moreover, Schoenle et al. (2017) reported that measuring intensity infection rather than only presence/absence could be important when evaluating the role of infection in influencing hosts' behaviour, physiology or fitness. If the molecular methods we used are considered as relevant to diagnose infection status of Red Kite by blood parasites (Martinsen et al. 2006, Krone et al. 2008), blood smears remain useful to measure the infection intensity in individuals and should be implemented complementarily to PCR screening in further studies.

Overall, both the low to medium prevalences reported in the different European populations studied and the absence of negative correlation with body condition we show suggest that hematozoans in Red Kite nestlings could be a limited threat for this life stage even if the detection of *P. relictum* in one chick questions about its occurrence at a larger scale and possible health effects on some individuals. In addition, recent findings showed that low-level chronic malaria infection reduced life span as well as the lifetime number and quality of offspring in a wild population of great reed warblers *Acrocephalus arundinaceus*, these delayed fitness effects of malaria being mediated by telomere degradation (Asghar et al. 2015). This stresses the need for further research on “subtle” hematozoan effect on individual in different

avian groups including raptors. Moreover, the prevalence in adults has been only assessed in Spain, a country where it is particularly low, while breeder's survival is a critical parameter in the dynamic of raptor populations (Saether and Bakke 2000). Further investigation associating both qualitative and quantitative methods on a significant number of Red Kites and including adults, from other European areas, notably in Germany and France, would be needed to confirm whether blood pathogens can be considered as negligible threat for the Red Kite population.

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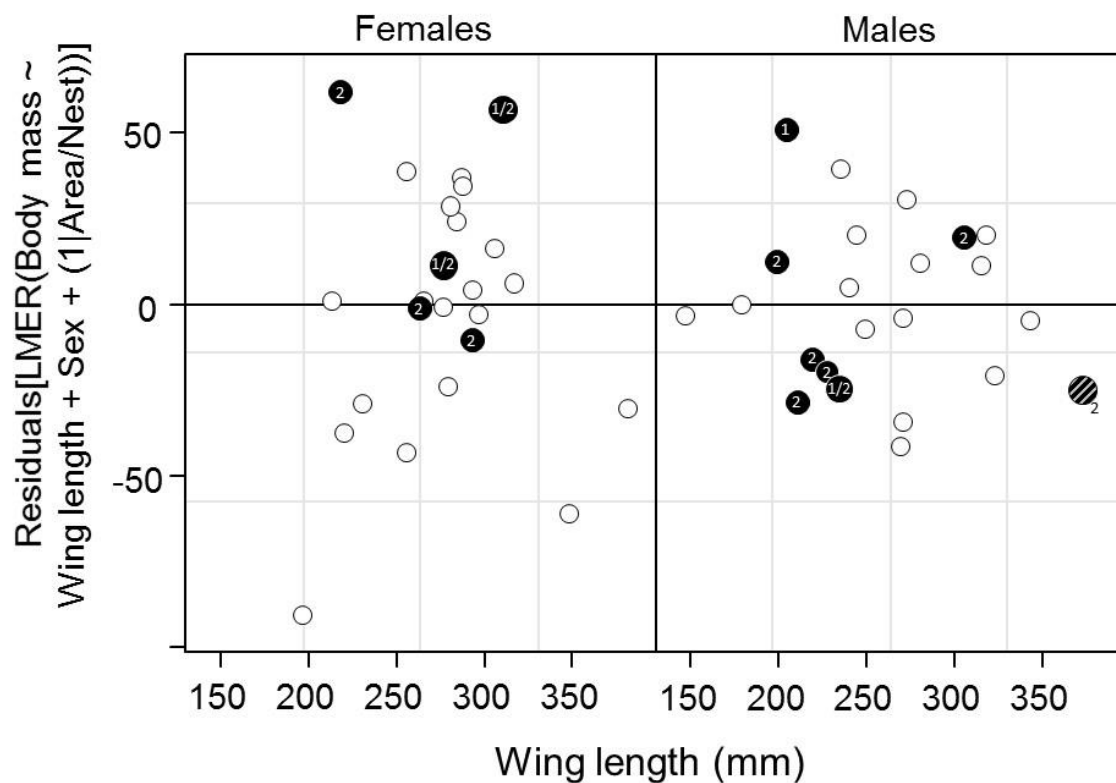
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Figure caption

**Fig. 1** Body mass of the 47 Red Kite chicks studied standardised against wing length taken as a proxy of age (Mougeot and Bretagnolle 2006) for females and males separately. The individuals for which DNA from *Leucocytozoon* ssp. was detected in blood were shown as a black circle in which the corresponding haplotype 1 (L-MILANS4, Genbank accession number JN164713) and/or 2 (L-MILVUS1, JN164716) is reported. The grey and black hatched dot corresponds to the male infected by both *Leucocytozoon* (haplotype 2) and *Plasmodium relictum*.



442 **Table 1** Linear mixed-effect models tested to explain body mass as response. The best  
 443 model selected based on corrected Akaike information criteria is reported in bold (LL:  
 444 maximized log-likelihood; K: number of estimated parameters; AICc: Corrected Akaike's  
 445 information criterion; w\_ic: Akaike weight)

Model tested (Body mass ~ )	LL	K	AICc	$\Delta$ AICc	w_ic
Wing length + Sex + (1 Area/Nest)	<b>-258.9</b>	<b>6</b>	<b>532.0</b>	<b>0.00</b>	<b>0.39</b>
Wing length + Sex * Status + (1 Area/Nest)	-256.5	8	532.9	0.89	0.25
Wing length + Sex + Status + (1 Area/Nest)	-258.1	7	533.0	1.03	0.23
Wing length * Sex + (1 Area/Nest)	-258.7	7	534.2	2.20	0.13
Wing length + (1 Area/Nest)	-266.1	5	543.6	11.57	0.00

446

**Table 2** Summary of the data available on Red Kite infection by the haemosporidian parasites *Leucocytozoon* spp. (*Leuco.*), *Haemoproteus* spp. (*Haemop.*) and *Plasmodium* spp. (*Plasmo.*). (n = total number of screened individuals; *nr* = not reported; *ns* = not screened)

Area/country	Age	n	Prevalence in % [ <i>n</i> of infected kites]			Reference
			<i>Leuco.</i>	<i>Haemop.</i>	<i>Plasmod.</i>	
Western Europe	<i>nr</i>	4	25 [1]	25 [1]	0 [0]	1
Germany	Nestling	13	15 [2]	0 [0]	0 [0]	2
	Juvenile	2	0 [0]	0 [0]	0 [0]	
	Adult	9	0 [0]	0 [0]	0 [0]	
	<i>nr</i>	2	0 [0]	0 [0]	0 [0]	
UK	<i>nr</i>	1	0 [0]	0 [0]	0 [0]	4
Spain	<i>nr</i>	46	0 [0]	0 [0]	0 [0]	5
	Juvenile	164	4 [6]	0 [0]	0 [0]	6
	Adult	104	2 [2]	0 [0]	0 [0]	
Denmark	Nestling	17	18 [3]	0 [0]	0 [0]	7
	Juvenile	1	0 [0]	0 [0]	0 [0]	
	Adult	1	0 [0]	0 [0]	0 [0]	
France	Nestling	47	28 [13]	0 [0]	2 [1]	This study

1: Peirce (1981), 2: Krone *et al.* (2001), 3: Krone *et al.* (2008), 4: Peirce *et al.* (1983), 5: Tella *et al.* (1999), 6: Pérez-Rodríguez *et al.* (2013), 7: Laursen (2008).