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Abstract: Experimental assessment of mating opportunities in three shorebird species

Mating opportunities may differ between closely related species, although the evidence for such variation is scant. Here we compare remating opportunities and courtship behaviour between three shorebird species: the Kentish plover (Charadrius alexandrinus), the Kittlitz's plover (C. pecuarius) and the white-fronted plover (C. marginatus), using data and an experimental approach previously developed for the Kentish plover. By experimentally creating unmated males and females, we show that remating opportunities are different between closely related plover species (Charadrius spp): remating times were shorter for females than males in a Kentish plover population that exhibits a male-biased adult sex ratio, and where the majority of brood care after hatching is carried out by males. In contrast, remating times were male-biased in the uniparental Kittlitz's plover and unbiased in the biparental white-fronted plover. We also show that male Kentish plovers spend significantly more time on courtship than females, whereas courtship behaviour is not sex biased in the other two plover species. The mate-removal experiments also provided insights into pair bond stability. In the Kittlitz's plover, all 16 newly formed pairs remained together after the release of their former mates from captivity, whereas newly established pairs were replaced by their former mates upon release in 12 out of 12 white fronted plover pairs. Taken together, these results are important in highlighting interspecific variation in mating activities, and suggest that both operational sex ratio (OSR) and pair bond stability may differ between closely related species. These variations in turn, may influence mating systems and parental care.

Experimental assessment of mating opportunities in three shorebird species

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***Detailed Response to Reviewers**

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Dr. Per Smiseth
Editor
University of Edinburgh

Dear Dr. Smiseth

Parra et al: Experimental assessment of mating opportunities in three shorebird species

Please find attached our revised manuscript for consideration as a research article in Animal Behaviour.

We much appreciate the constructive and detailed comments of the Editor before the paper will be publishable. We have carefully considered all comments and have revised the manuscript accordingly. Please find our detailed responses below. All changes have been highlighted in the uploaded revision.

Best regards,

Jorge E. Parra

PhD student

Editorial letter (original text italicised, and our responses are in plain font)

Formatting changes:

1. Put keywords in alphabetical order.
This has been modified in the revised manuscript, (lines 22 – 23)

2. Use APA style for citations and references.
This has been modified in the revised manuscript.

3. For 'unpublished data' references include authors' initials and also a date if available.
Done, thank you (see lines 97 - 98, 101 and 129)

4. Tables should have a short one-sentence title above the table. Put other information below the table.
Done, (see lines 545, 554 – 555 and 561)

5. Tables 2, 3. Remove the internal horizontal lines.
Done, (see tables 2 and 3)

6. Make the supplementary table into an appendix table and include it with the other tables in the manuscript.
Thanks, this has been changed accordingly (see lines 566 – 571)

Editor's additional comments:
(1) Please delete the reference to Liker et al. unpublished data. This reference is not useful to the reader as the data are unpublished and it does not appear to be required given that you cite published work by the same author. If the reference is required, I would suggest that you cite it as personal communication.
Thanks, this has been deleted accordingly.

(2) Line 90: Delete the word 'the' to read 'White-fronted plovers and Kittlitz's plovers .'.
Done, the word was deleted accordingly (see line 94)

(3) Line 134: Change 'arthropods' to 'invertebrates' as earthworms are annelids (and not arthropods).
Done, thank you (see line 138)

(4) Lines 149, 150 and elsewhere: Change 'mins' into 'min'.
Done, thank you (see lines 153 and 155)

(5) Line 158: Change semicolon into period.
Done, thank you (see line 162)

(6) Line 207: Change 'minutes' into 'min'.
Done, thank you (see line 214)

(7) Line 237: Consider changing the subheading 'Between Species Prediction' to 'Between Species Comparison'.

Thanks, this has been changed accordingly; (see lines 78 and 248)

(8) Line 247: Please name the test statistic of the Gehan-Wilcoxon test and report the degrees of freedom.

Done, thank you (see line 258)

(9) Line 249: Consider changing the subheading 'Between Sexes Prediction' to 'Between Sexes Comparison'

Thanks, this has been changed accordingly; (see lines 82 and 260)

(10) Lines 257 and 273-275: Please name the test statistic and report the degrees of freedom for the LSD tests.

Thanks, the test statistic and degrees of freedom have now been added (see lines 265, 268, 284 – 287)

In addition, Fisher's least significant difference test (LSD test) has now been explained in the manuscript as follows (see lines 202 – 204):

“Pairwise multiple comparisons were performed to compare mean differences of mating time between males and females of the three species using Fisher's least significant difference tests (LSD test)”

See also lines 222 – 223:

“Pairwise multiple comparisons were performed to compare mean differences of courtship behaviour between males and females in the three species of plovers using LSD tests”

(11) Line 266: Consider changing the subheading 'Courtship Prediction' to 'Courtship Behaviour'.

Thanks, this has been changed accordingly; (see lines 84 and 277)

(12) Lines 284-285: Please name the test statistic and report the degrees of freedom of the Fisher's exact test

Fisher's exact test does not have degrees of freedom. This test has been explained in the manuscript (see lines 224 – 225):

“Finally, pair bond stability was analysed comparing the frequencies of mate replacement between white-fronted plover and Kittlitz’s plover with Fisher's exact test”

In addition (see lines 296 – 297):

“Fisher’s exact test: mate replacement in white-fronted plover =12, N = 12; mate replacement in Kittlitz´s plover = 0, N = 16; P < 0.001”

(13) Lines 291-292: Change '. ecological explanations are unlikely to explain .' to something like '. ecological factors are unlikely to explain .'.

Thanks, this has been changed accordingly; (see line 304)

(14) Lines 334-335: Please reword this sentence as it is unclear whether you mean to say that male and female plovers behave similarly despite having conventional sex roles or whether you mean to

say that the finding that male and female plovers behave similarly suggests that do not have conventional sex roles

Thanks, we have changed the manuscript as follows (lines 347 – 349):

"The latter result indicates that males and females may compete similarly for available mates, suggesting that they do not have conventional sex roles: male-male competition and female choice for mates (Vincent, Ahnesjö, & Berglund, 1994)"

(15) Line 357: Change 'albatross' to 'albatrosses'.

Done, thank you (see line 370)

Reviewer #1:

I am pleased to see that the authors have carefully reviewed this manuscript according to previous suggestions. In my view these changes improved the paper and are sufficient for making the paper a highly interesting contribution within the field of animal mating behaviour.

Thank you

Reviewer #2:

I am satisfied with the changes/improvements done by the authors.

Thank you

HIGHLIGHTS

- Biparental species mated more quickly than uniparental species
- Uniparental plover species exhibited sex differences in mating opportunities
- Courtship behaviour by males differed significantly between plover species
- Newly established pair bonds were weaker in biparental than in uniparental plovers
- Mating opportunities and pair bond stability may influence breeding systems

Experimental assessment of mating opportunities in three shorebird species

Mating opportunities may differ between closely related species, although the evidence for such variation is scant. Here we compare remating opportunities and courtship behaviour between three shorebird species: the Kentish plover (*Charadrius alexandrinus*), the Kittlitz's plover (*C. pecuarius*) and the white-fronted plover (*C. marginatus*), using data and an experimental approach previously developed for the Kentish plover. By experimentally creating unmated males and females, we show that remating opportunities are different between closely related plover species (*Charadrius spp*): remating times were shorter for females than males in a Kentish plover population that exhibits a male-biased adult sex ratio, and where the majority of brood care after hatching is carried out by males. In contrast, remating times were male-biased in the uniparental Kittlitz's plover and unbiased in the biparental white-fronted plover. We also show that male Kentish plovers spend significantly more time on courtship than females, whereas courtship behaviour is not sex biased in the other two plover species. The mate-removal experiments also provided insights into pair bond stability. In the Kittlitz's plover, all 16 newly formed pairs remained together after the release of their former mates from captivity, whereas newly established pairs were replaced by their former mates upon release in 12 out of 12 white fronted plover pairs. Taken together, these results are important in highlighting interspecific variation in mating activities, and suggest that both operational sex ratio (OSR) and pair bond stability may differ between closely related species. These variations in turn, may influence mating systems and parental care.

Keywords: adult sex ratio, mating systems, operational sex ratio, pair bond, parental care, remating opportunity.

The different evolutionary interests of males and females over reproduction (termed sexual conflict; Parker, 1979) are a pervasive evolutionary force influencing the behaviour, ecology and life histories of many organisms (Arnqvist & Rowe, 2005; Chapman, Arnqvist, Bangham, & Rowe, 2003). A common issue when the interests of males and females are antagonistic concerns offspring care (Trivers, 1972; Maynard Smith, 1977; Houston, Székely, & McNamara, 2005; Lessells, 2012). By caring for the offspring, parents often improve the growth and survival of the young (Clutton-Brock, 1991; Klug, Alonzo, & Bonsall, 2012); for example, by gestating, nursing, protecting and feeding the young, the offspring have improved chances of survival (Tyler, Shearman, Franco, O'Brien, Seamark, & Kelly, 1983; Balshine-Earn & Earn 1998; Baeza & Fernández, 2002; Klug, Alonzo, & Bonsall, 2012). However, care is costly in terms of time and energy, and the caring parent can be killed by predators or may lose additional mating opportunities (Veasey, Houston, & Metcalfe, 2001; Li & Jackson, 2003; Klug et al., 2012). Therefore, whilst both biological parents benefit from providing care for the offspring, each parent is expected to withhold his (or her) parental contribution in order to raise further offspring in future (Houston et al., 2005; Lessells, 2012; McGraw, Székely, & Young, 2010).

Theory suggests that a key component of conflict resolution between male and female parents is mating opportunity (Székely, Webb, & Cuthill, 2000; McNamara, Székely, Webb, & Houston, 2000; Kokko & Jennions, 2008; Klug et al. 2012). If one sex has more favourable mating opportunities than the other, the former parent is expected to reduce (or completely terminate) care more often that its mate, and seek out a new partner (Balshine-Earn & Earn, 1998; Owens, 2002; Pilastro, Biddau, Marin, & Mingozzi, 2001). One approach used by researchers to assess mating opportunities is to estimate the ratio of sexually active males to females (operational sex ratio, OSR; Kvarnemo & Ahnesjö, 1996; Forsgren, Amundsen, & Bjelvenmark, 2004). An alternative approach to estimating mating opportunities is to experimentally create unmated individuals, and to quantify their remating behaviour, e.g. time to remate, remating success and reproductive success with the new mate (Lessells, 1983; Székely, Cuthill, & Kis, 1999). This experimental approach is powerful, since it directly assesses the mating potential of unmated individuals at a given time in a population.

Here we estimate remating opportunities for two small plover species, the white-fronted plover (*Charadrius marginatus*) and the Kittlitz´s plover (*C. pecuarius*), and compare these data with the results of a previous study on the Kentish plover (*C. alexandrinus*, Székely et al., 1999). Small plovers (*Charadrius spp.*) exhibit substantial variation in their breeding systems, since some of these species are monogamous and both parents rear the young, whereas others exhibit polygyny and/ or polyandry whereby a single parent (the male or the female) raises the young to independence (Székely, Thomas, & Cuthill, 2006; Thomas, Székely, & Reynolds, 2007). In addition, plovers typically breed in open areas, and their nests and broods are therefore accessible for experimental manipulations (Székely & Cuthill, 2000).

A previous experiment established that remating opportunities were female-biased in the Kentish plover (Székely et al., 1999), and this result was consistent with demographic analyses that estimated about 6 times more adult males than females in the population (Kosztolányi, Barta, Küpper, & Székely, 2011). Skewed adult sex ratios (ASRs) are common in wild populations (Donald, 2007), and recent works suggest that biased ASRs predict sex roles, mating systems and pair-bonds (Liker, Freckleton, & Székely 2013). Here we use an identical experimental protocol in two close relatives of the Kentish plover, the white-fronted plover and Kittlitz’s plover, to compare remating opportunities between these three plover species. All three species are insectivorous ground-nesting birds that exhibit similar life-histories and ecology (adult body masses, Kentish plover: 41.8 g; white-fronted plover: 37.1 g; Kittlitz’s plover: 35.3 g, Urban, Fry, & Keith, 1986; Hockey, Dean, & Ryan, 2005). The latter two species are common breeding birds in Africa, and their parental care systems differ from the Kentish plover which exhibits male-biased parental care after hatching (Lessells, 1984; Székely & Lessells, 1993; Amat, Fraga, & Arroyo, 1999). White-fronted plovers exhibit biparental brood care, whereas Kittlitz's plovers are reported to exhibit uniparental brood care, carried out by either the male or the female parent (Hockey et al., 2005; Tree, 1974; Urban et al., 1986).

Based on theoretical models (Klug et al., 2012; Kokko & Jennions, 2008) and available information on patterns of parental care (Hockey et al., 2005; Urban et al., 1986), we derived three predictions. First, we predicted higher remating opportunities in uniparental species (Kittlitz´s plover) than in biparental species (white-fronted plover), since in biparental species both parents are engaged with care until the offspring are fully independent (henceforth, between species comparison). Second, we predicted no difference in remating opportunities between males and females in biparental white-fronted plover given that both sexes are fully engaged in parental care. Similarly, no difference in remating opportunities between males and females was predicted for the uniparental Kittlitz´s plover in which either parent is free to seek a new mate (henceforth, between sexes comparison). Third, we predicted intense courtship behaviour by males and females both in biparental white-fronted plover and uniparental Kittlitz´s plover where care is provided by either parent (henceforth, courtship behaviour). In addition to the experimental assessment of remating opportunities, we also monitored pair bond stability among newly established pairs. We include the Kentish plover in our analyses (using the data from Székely et al., 1999), since the same experimental methodology was used in all three species. Nevertheless, our main conclusions remain consistent when restricting the analyses to the white-fronted and Kittlitz´s plovers.

METHODS

Study Species and Study Sites

White-fronted plovers and the Kittlitz’s plovers were investigated in SW Madagascar (for Kentish plover, see details in Székely et al., 1999). Kittlitz’s plovers were studied between 6 February 2010 and 13 May 2010 in Andavadoaka (22° 02’ S, 43° 39’ E) where they breed around alkaline lakes.

Approximately 300 Kittlitz's plovers breed in Andavadoaka (J.E. Parra, S. Zefania, & T. Székely, unpubl. data). Fieldwork with the white-fronted plover was carried out between 1 April 2011 and 23 June 2011 at Lake Tsimanampetsotsa National Park (24° 3' S, 43°44' E), a large alkaline lake (approx 15 km x 0.5 km), surrounded by sandy shores, short grass and saltpans. Approximately 150 white-fronted plovers breed around the lake (J.E. Parra et al., unpubl. data).

In the field, we searched for nests on foot, identified incubating parents and watched the parent(s) returning to nests in potential breeding sites. In total, we captured 18 Kittlitz's plover pairs (36 individuals) and 14 white-fronted plover pairs (28 individuals) with funnel traps placed on their nests. The traps were continuously monitored until a parent entered the trap and sat on the eggs. Parents were immediately removed from the traps to reduce stress and the risk of injury. Morphological traits (body mass, tarsus length, wing length and bill length) were measured using a spring balance, a sliding calliper and wing ruler (see details in Kentish plover field guide, www.bath.ac.uk/bio-sci/biodiversity-lab/pdfs/KP_Field_Guide_v3.pdf). All adults were ringed with an individual combination of colour rings and a numbered SAFRING metal ring from the University of Cape Town, South Africa.

Experimental Manipulation

We used the methodology developed by Székely et al. (1999) to estimate remating times in the Kentish plover. Briefly, both parents were trapped, ringed, measured and a blood sample was taken for sex determination (see below). One parent was then selected at random (the male or the female) and was released at the capture location immediately. The other parent was taken into captivity (see below). In both Kittlitz´s and white-fronted plovers, both the male and female incubate the eggs (Hockey et al., 2005; Urban et al., 1986). Only pairs incubating two eggs (modal clutch size in both species) were manipulated. Egg length and breadth were measured with a sliding calliper, and the number of days the eggs had been incubated for was estimated based on the floatation stage of the egg in a transparent jar with clean water (mean ± SD no. of days incubated: Kittlitz´s plover: 9.0 ± 4.32 days, *N* = 36; white-fronted plover: 11.5 ± 3.16 days, *N* = 20). Eggs were distributed to other non-experimental plover clutches at approximately the same stage of incubation in the local populations. Monitoring the augmented clutches was beyond the scope of the experiment, although casual nest checks suggest that at least 33.3% and 19.4% of augmented nests survived until hatching in the Kittlitz´s plover (*N* = 36 nests) and the white-fronted plover (*N* = 20 nests), respectively. Survival in these nests appeared to be higher than for unmanipulated nests (13.4% and 8.9%, based on *N* = 101 Kittlitz's plover nests and *N* = 56 white-fronted plover nests, respectively; J.E. Parra et al., unpubl. data).

Removed plovers were transported in an air-conditioned vehicle to a purpose-built aviary near the field camp at both study sites. Lightweight bird bags were used to keep the plovers undisturbed and ventilated during the transport. Distance from capture areas to the aviaries varied between 1 and 10 km in both study sites. The aviaries had four units for Kittlitz's plovers and six units for white-fronted plovers. Each unit consisted of a 1 m x 1.5 m x 1.5 m (height x length x width) wood frame fitted with chicken mesh (13 mm x 13 mm). To provide shade for the captive birds, we covered the outside of the aviary with papyrus, *Cyperius sp.*, and fitted 50 cm of cloth at the base of the mesh inside the units. Captive plovers were provided with appropriate food and drink three times every day to maintain their good health using high protein meals: dried invertebrates for wild birds (dried mealworms, dried earthworms, shrimps and dried waterfly; shop.naturesgrub.co.uk/), bird supplement vitamins (Vitacombex V; www.petland.co.uk) and pinhead oatmeal (Prosecto InsectivorousTM; www.haiths.com). Captive plovers were also supplied with fresh insects twice a day using pit fall traps set-up in the salt-marsh. In addition, two water recipients were set for drinking and bathing in each unit. Captive plovers were released after their former mate found a new mate or their former mate was not seen in the study sites for at least 12 days. Time in captivity was comparable between white-fronted plovers (mean ± SD no. of days in captivity: 8.0 ± 1.71 days, *N* = 14) and Kittlitz's plovers (7.12 ± 2.57 days, *N* = 18). Captive plovers were measured before release. Although captive plovers appeared to lose a small amount of body mass during their time in captivity (2.77 ± 0.51 g in Kittlitz’s plover, and 0.73 ± 0.22 g in white-fronted plover), they were in good condition as indicated by the fact that many remated shortly after release from captivity (see Results).

Behavioural Observations

The released plovers were searched for every day in the field using a car and mobile hide. When a focal plover was found, we recorded its behaviour for 30 min at 30 seconds intervals. Attempts were made to record the behaviour of focal plovers on at least two occasions before they found a new mate. 30 min is sufficient to establish whether a plover is mated or not (Székely et al., 1999). We used behavioural categories of courting and self-maintenance behaviours that were previously developed for the Kentish plover (Székely et al., 1999). Courtship behaviours included: (1) Courting: male plovers perform upright posture and high-stepping movements and female plovers perform a lower head position. (2) Copulations: courting pairs frequently copulated. (3) Scraping: male and female plovers dig several scrapes in a territory. (4) Fighting: focal individuals chase away intruders with buffed-out plumage and buzzing calls. Self-maintenance behaviours included: (1) Feeding: individuals pick up food items followed by a short run, (2) Preening: an individual groomed its own feathers. Mated individuals were identified based on courtship behaviours including courting, scrape ceremony and copulation (Urban et al., 1986; Hockey et al., 2005, see video of Kittlitz´s plover courting behaviour in the Supplementary Material, Video S1). New pairs were checked for clutches every day, and eggs were measured as described above. Two observers recorded all behavioural observations (M. Beltrán and J.E. Parra).

Molecular Sexing

Both plover species have sexually monomorphic plumage (Hockey et al., 2005; Urban et al., 1986), therefore we used molecular sex-typing to determine the sex of individuals (dos Remedios, Lee, Székely, Dawson, & Küpper, 2010). A small blood sample was taken from each adult's brachial wing vein, by puncturing, collecting drops of blood (~25 ul) in capillary tubes, and storing this in Eppendorf tubes of Queen's Lysis Buffer. DNA was extracted from blood samples using the Ammonium Acetate extraction method (Miller, Dykes, & Polesky, 1988; Richardson, Jury, Blaakmeer, Komdeur, & Burke, 2001). For molecular sex-typing, Z- and W-chromosome specific genes were amplified via polymerase chain reaction (PCR) using the Z-002B/Z-002D primers (Dawson, 2007). For additional certainty in sex assignment, the W-chromosome specific Calex-31 primers, developed in the genus *Charadrius* were utilized (Küpper, Horsburgh, Dawson, Ffrench-Constant, Székely, & Burke, 2006). PCR amplification was conducted on a DNA Engine Tetrad 2 Peltier Thermal Cycler under the following conditions: 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 56°C for 90 s, 72°C for 60 s with a final extension of 60°C for 30 min. Samples were visualized on an ABI 3730 automated sequencer. IR Dye-labelled tailed primers separated the products of Z-002B/Z-002D primers into either one (ZZ) or two bands (ZW), indicating male or female respectively. The W-specific Calex-31 product appeared as one band indicating female only. Images were scored using GeneMapper software version 4.1 (Applied Biosystems). To maximize reliability, all samples were sexed using two markers. Additionally, for 8% of samples (11 Kittlitz's plover and 10 white-fronted plover individuals) molecular sexing was repeated; in all cases, repetitions concurred with the original results.

Data Processing and Statistical Analyses

Date of mating was defined as the mean date between the date when a plover was last seen single and the first date it was seen with a new mate. Remating time was the difference between date of release (either on the day of manipulation or from captivity) and date of mating. The response variable, remating time, was analyzed using generalized linear models (GLM) with Tweedie (1.5) error structure and a log link function (Smyth & Verbyla, 1999). The models investigated the effects of two main variables:

species (Kentish, white-fronted and Kittlitz's plovers) and sex; and three additional fixed variables: type of manipulation (released in the field or released from captivity), release date, and number of days in captivity (see Table 1). Dates were expressed as Julian dates, i.e. number of days since 1 January. Results of backward elimination based on Akaike's information criterion for small sample sizes (AICc) are presented for variable selection of the GLM models where lowest AICc score is the best supported model (Symonds & Moussalli, 2010; Table 1). Pairwise multiple comparisons were performed to compare mean differences of mating time between males and females of the three species using Fisher's least significant difference tests (LSD test).

Remating time was also analyzed using survival analyses and these estimates are referred to as expected remating times (see rationale in Székely et al., 1999). In these analyses, the terminal event (outcome) was the occurrence of mating, defined as the first observation when a plover was seen with a mate. Several individuals did not find a new mate when we saw them for the last time, and these were treated as censored observations. First, we used a Gehan-Wilcoxon test to compare expected remating times curves (survival curves) for three species by sex. Survival curves were generated by the Kaplan-Meier method. Second, for each plover species a separate Cox regression model was constructed to investigate the probability of remaining single from the day of release (season), sex and their interaction as covariates (Table 2).

Courting behaviour was estimated as percentage obtained from each 30 min sample. For individuals with several behavioural observations, we calculated the mean percentage of courting. Courting behaviour was analyzed using GLMs with Tweedie (1.5) error structure and a logarithmic link function per individual plover. The model included two main factors: species of plover and sex; and three additional fixed variables: type of manipulation (released in the field or from captivity), released date, and number of days in captivity. Model selection and statistical parameters estimated for each independent variable in the models are provided in the Appendix, Table A1. In addition, for each sex a separate GLM model was constructed to investigate the effect of three species of plovers on courting behaviour (Table

3). Pairwise multiple comparisons were performed to compare mean differences of courtship behaviour between males and females in the three species of plovers using LSD tests.

Finally, pair bond stability was analysed comparing the frequencies of mate replacement between white-fronted plover and Kittlitz's plover with Fisher's exact test. Data were analyzed by using SPSS statistics for Windows version 19 and figures were made in R (R Development Core Team, 2008) using the package ggplot2 (Wickham, 2009).

Ethical Note

The experiments in Madagascar were approved by the Ministry of Environment, Forests and Tourism of the Republic of Madagascar (Research permit No: 053/11/MEF/SG/DGF/DCB.SAP/SCB of 11 March 2011 and 132/10/MEF/SG/DGF/DCB.SAP/SSE of 06 May 2010) and Madagascar National Parks (No: 398-10/MEF/SG/DGF/DVRN/SGFF of 18 May 2011). Blood sampling was also covered by these research permits. The blood transport permit was approved by Service de la Gestion de la Faune et de la Flore, Direction de la Valorisation des Resources Naturelles, Ministère de l'Environnement et des Forêts Madagascar (authorization number 080N-EA06/MG11). The Kentish plover experiment was approved by the Turkish Ministry of Environment (see Székely et al., 1999). The Kittlitz´s and the white-fronted plovers are common breeding birds in much of Africa and Madagascar and they are not considered threatened by the IUCN (BirdLife International, 2012). Captive plovers were monitored daily and kept under standard conditions (see Experimental Manipulation) to reduce their stress levels. In addition, translocated eggs coped with the natural breeding conditions of local clutches in the two plover populations (see above). The experiment was designed to reduce adverse effects on plover welfare and their local populations.

RESULTS

Between Species Comparison

Remating opportunities differed significantly between the three plover species (Fig. 1): white-fronted plovers mated significantly more quickly (median = 2.0 days, range 0.5 – 4.5 days, *N* = 12) than both Kittlitz's plovers (median = 5.1 days, range 1.0 – 11.5 days, *N* = 16) and Kentish plovers (median = 6.3 days, range 0.5 – 47.5 days, *N* = 34; Table 1).

These results remained consistent using survival analyses that also included the individuals that were not successful in finding a new mate (Fig. 2, Table 2; see Methods). The proportion of plovers remaining single was significantly lower for the white-fronted plover (median = 4 days, *N* = 14) than both the Kittlitz's plover (median = 14.6 days, *N* = 33) and Kentish plover (median = 13.4 days, *N* = 59; testing the three species, Wilcoxon–Gehan test: χ^2_2= 16.316, $P < 0.001$).

Between Sexes Comparison

A significant species by sex interaction suggested a sex-biased difference in remating opportunities (GLM: $\chi^2_2 = 47.62$, $P < 0.001$, Table 1). Female Kittlitz's plovers took significantly longer to mate (median = 6.5 days, range 3.5 – 11.5 days, *N* = 6) than males (median = 3.3, range 1.0 – 7.5 days, *N* = 10; LSD test: pairwise mean difference = -0.66, *df* = 1, $P = 0.047$) whereas the opposite was found in the Kentish plover (Székely et al. 1999). However, male and female remating times were not significantly different in white-fronted plovers (male: median: 2.0 days, range 0.5 – 3.5 days, *N* = 6; female: median: 2.0 days, 1.0 – 4.5 days, *N* = 6, LSD test: pairwise mean difference = -0.11, *df* = 1, $P = 0.823$).

These results remained consistent using survival analyses (Table 2): the proportion of female Kittlitz's plovers remaining single was higher than that of males (male median: 11.0 days, *N* = 17, female median: 21.0 days, $N = 16$, Fig. 2), whereas the proportion of single males and females were not significantly different in the white-fronted plover (male median: 3.0 days, $N = 7$, female median: 4.0 days, $N = 7$, Fig. 2). Remating time increased over the season only for female Kentish plovers (Cox regression: $\chi^2_1 = 7.66$, $P = 0.014$), suggesting an influence of time of breeding season on mating opportunities in the Kentish plover, although this was not the case in the other two species (Table 2).

Courtship Behaviour

Courtship behaviour had a significant species by sex interaction (GLM: $\chi^2_2 = 6.329$, $P = 0.042$, Supplementary Material: Table S2, Fig. 3). Courtship behaviour by males differed significantly between species (GLM: $\chi^2_2 = 10.689$, $P = 0.005$, Table 3), male Kentish plovers spent significantly more time on courtship than males of the other plover species; whereas courtship behaviour by females did not differ between species (GLM: $\chi^2_2 = 1.437$, $P = 0.487$, Table 3). In contrast to the Kentish plover, which exhibited male-biased courtship behaviour (LSD test: pairwise mean difference = -3.29, $df = 1$, $P = 0.005$), males and females of the other two species spent comparable times on courtship (LSD test white-fronted plover: pairwise mean difference = 1.06, $df = 1$, $P = 0.252$; LSD test Kittlitz’s plover: pairwise mean difference = -0.36, $df = 1$, $P = 0.679$, Fig. 3).

Pair Bonds

The new pair bonds in experimentally-induced white-fronted plovers were significantly weaker than in Kittlitz’s plover: in 12 white-fronted plovers that remated after their former partner was removed (6 males, 6 females), all experimentally-induced pair bonds were replaced by the original mates after they were released from captivity. In contrast, in 16 Kittlitz’s plovers that remated after their former partner was removed (10 male and 6 female), none were replaced by their former mates once their former mates were released from captivity (Fisher's exact test: mate replacement in white-fronted plover =12, $N = 12$; mate replacement in Kittlitz´s plover = 0, $N = 16$; $P < 0.001$)

DISCUSSION

These experiments provided four key results. First, they show that mating opportunities are significantly different between closely related species. This result is striking because two of these plover species (white-fronted and Kittlitz's) breed in the same habitat in Madagascar, and therefore, ecological factors are unlikely to explain the differences in remating opportunities. The rapid remating of white-fronted plovers – a pattern we did not expect – suggests that there is a large pool of floating individuals that can rapidly move in to pair up with unmated individuals. Consistent with the latter argument, both male and female white-fronted plovers stayed in the same territory, and new individuals moved in to replace the removed mates. Mating opportunities seem to be an important factor in the evolution of breeding systems across a range of species (Balshine-Earn & Earn, 1998; Magrath & Komdeur, 2003). For example, as in the white-fronted plover, male dunlin (*Calidris alpina*) were rapidly replaced by other males after experimental removal from their breeding territories (Holmes, 1970; Pitelka, Holmes, & Maclean, 1974). In the Eurpoean starling (*Sturnus vulgaris*), after mating opportunities were increased by the provision of additional nest-boxes, males increased their mating effort to attract additional mates and also reduced parental care effort (Smith, 1995), and in St. Peter´s fish (*Sarotherodon galilaeus*), males and females were more likely to desert the offspring when remating opportunities were increased experimentally (Balshine-Earn & Earn, 1998).

Second, we found sex-bias in remating opportunities: the male-biased remating opportunities in Kittlitz's plover were the opposite of those found in the Kentish plover (Székely et al., 1999), whereas in white-fronted plovers remating opportunities did not differ between males and females. As far as we are aware, our study is the first to experimentally demonstrate differences in sex-biased remating opportunities between closely related species in wild populations. Sex-biased mating opportunities may emerge in two ways. One explanation is that the ratio of sexually active males to females (operational sex ratio, OSR) may not be at parity. OSR may be biased due to differences in the reproductive schedules of males and females (“time in”: time spent in the mating pool, sexually active; “time out”: time spent out of the mating pool, not sexually active), or biased adult sex ratios (ASR; Donald, 2007). Evidence suggests that OSR can vary due to mating and parental care activities (Forsgren et al., 2004, Symons, Svensson, & Wong, 2011; LaBarbera, Lovette, & Llambías, 2011; Canal, Jovani, & Potti, 2012). In addition, recent studies found substantial difference in ASR between closely related shorebird species (Liker et al., 2013). Further works are needed to separate whether biased remating opportunities emerge via different reproductive scheduling or biased ASR in plovers. For one of these species, the Kentish plover, a demographic study confirmed male-biased ASR (Kosztolányi et al., 2011), although ASR has not been estimated for the white-fronted and the Kittlitz’s plover. Alternatively, sex-biased mating opportunities may arise due to differences in the willingness of males and females to remate. For instance, the post-breeding refractory periods, the recovery phase spent preparing for another breeding attempt, may differ between males and females (Balshine-Earn & Earn, 1998; Cantoni & Brown, 1997): females typically need more time to recover than do males. However, the latter explanation is unlikely, since the adult plovers used in our experiments had breeding efforts interrupted and sought new mates shortly after removal of their mate (or on release from captivity). Furthermore, several female Kentish plovers remated within less than a day – a pattern that is inconsistent with the explanation that females need more time to recover than do males.

Third, male courtship behaviour was different between the three species, since male Kentish plovers spent more time on courtship than male white-fronted and Kittlitz’s plovers. This pattern is consistent with the explanation that ASR is male-biased in the Kentish plover. The significance of this result is that courtship behaviour is variable between closely related species and suggests that ASR, and in turn the OSR, is probably related to the intensity of mating competition in males. Consequently, comparable intensity of courtship behaviour was observed between sexes in the Kittlitz´s and white-fronted plovers. The latter result indicates that males and females may compete similarly for available mates, suggesting that they do not have conventional sex roles: male-male competition and female choice for mates (Vincent, Ahnesjö, & Berglund, 1994). Variation in resources for breeding has also been suggested to influence OSR, and in turn, the intensity of mating competition (Forsgren, Kvarnemo, & Lindstrom, 1996). Availability of breeding territories, for example, may affect the OSR, since the sex that holds the territories will be limited by scarcity of nest sites. In a sand goby population (*Pomatoschistus minutes*), for instance, nest-site abundance can influence the intensity of male mating competition (Forsgren et al., 1996). Hence, the dynamic of OSR, and in turn mating competition, is probably modulated by both ASR and resource availability (Kvarnemo & Ahnesjö, 1996, Forsgren et al., 2004).

Finally, the new pair bonds were significantly weaker in white-fronted plovers than in Kittlitz's plover suggesting that the former species exhibits long-term pair bonds whereas the latter has short-term pair bonds. Mate fidelity may emerge in two ways. On the one hand, former mates may actively seek out each other *per se*, and prefer to mate with each other. On the other hand, mate fidelity may emerge via site fidelity: white-fronted plovers are highly territorial (Lloyd, 2008), and therefore upon release from captivity, individuals return to their former territories and chase out their former partner's new mates. Established pairs may prefer to reunite because of the fitness benefits in terms of synchronisation of behavioural and physiological characteristics such as defence of breeding territories, courtship behaviours, laying date, incubation, chick-raising between others which have been shown to improve with time and experience of the pair (Bried, Pontier, & Jouventin, 2003; Rowley, 1983). An experiment carried out in bearded reedling *Panurus biarmicus* found that long-term pair bond formation improved coordination of breeding activities and reproductive success (Griggio & Hoi, 2011). In another example, newly formed pairs (either due to divorce or loss of a mate) had lower reproductive success than established pairs in black turnstone *Arenaria melanocephala* (Handel & Gill, 2000). Improved breeding with increased experience is also well known in geese, albatrosses and other long-term pair bonding animals (Angelier, Weimerskirch, Dano, & Chastel, 2006; Black, 2001). Overall, the consequences of pair bond and site fidelity on mating opportunities could be significant since the ability of an individual to mate may be limited by their access to mates and breeding sites.

In conclusion, using an experimental approach we found significant differences in remating opportunities between closely related plover species. As mating opportunity is linked to OSR and ASR, our work suggests that substantial variation in OSR (and possibly ASR) is exhibited among closely related species. Such variation may influence the direction and intensity of competition in males and females for mates and breeding territories. These differences in OSR, in turn, may facilitate different intensities of sexual selection and induce different mating systems and patterns of parental care.

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Figure Legends

Figure 1. Remating times in three plover species. The lower and upper borders of the box are lower and upper quartiles, respectively, the horizontal bar is the median and whiskers represent the lowest and highest observations.

Figure 2. Proportion of males and females remaining single in three plover species: Kentish plover (top), white-fronted plover (middle) and Kittlitz's plover (bottom). Dotted lines show the expected mating time of males and females after release. Number of individuals: 32 male and 27 female Kentish plovers; 7 male and 7 female white-fronted plovers; and 17 male and 16 female Kittlitz´s plovers.

Figure 3. Courtship behaviour in three plover species. The lower and upper borders of the box are lower and upper quartiles, respectively, the horizontal bar is the median and whiskers represent the lowest and highest observations. Circles denote outliers that are between the first and third interquartile from the nearer edge of the box.

Table 1.

Remating times (response variable) of males and females in three species of plover.

Independent variable	Full model (AICc = 346.004)			Best model (AICc = 341.088)		
	Wald χ^2	*Df*	*P*	Wald χ^2	*Df*	*P*
(Intercept)	38.596	1	<0.001	49.365	1	<0.001
Between species comparison						
Species	11.248	2	0.004	11.595	2	0.003
Between sexes comparison						
Sex	4.072	1	0.044	3.974	1	0.046
Species * sex	39.65	2	<0.001	47.620	2	<0.001
Manipulation	0.290	1	0.59	-	-	-
Release date	4.818	1	0.028	5.007	1	0.025
Captive days	0.646	1	0.422	-	-	-

GLMs were used to analyze mating time using Tweedie (1.5) error structure and a log link function. Model selection was carried out using Akaike information criterion for small sample sizes (AICc). Manipulation refers to whether a plover was kept in captivity or not. Release date refers to the date when a plover was released to find a new mate (Julian dates). Captive days are the number of days that a plover was kept in captivity.

Table 2.

Remating time in plovers based on Cox proportional hazard models, to examine the relationship of the survival distribution which includes censored observations.

Species	Variable	B	Wald χ^2	*df*	p
Kentish plover	Sex	1.541	12.07	1	0.001
	Release date	-0.024	6.073	1	0.014
White-fronted plover	Sex	-0.18	0.083	1	0.77
	Release date	0.002	0.004	1	0.95
Kittlitz's plover	Sex	-1.342	4.864	1	0.027
	Release date	-0.01	0.088	1	0.767

For each species a separate model was constructed. Number of individual Kentish plovers, mated = 34, censored = 19; white-fronted plover, 12, 2; Kittlitz’s plover, 16, 17, respectively.

Table 3.

Courtship behaviour (response variable: % of time courting) in three plover species.

Sex	Variables	Wald χ^2	*Df*	*P*
Male	(Intercept)	13.176	1	< 0.001
	Species	10.689	2	0.005
Female	(Intercept)	0.155	1	0.694
	Species	1.437	2	0.487

GLMs were used to analyse percentage of time courting using Tweedie (1.5) error structure and a log link function. Model selection was carried out using Akaike information criterion for small sample sizes (AICc).

APPENDIX

Table A1.

Courtship behaviour in plovers (response variable: proportion of time courting).

Independent variable	**Full model (AICc = 269.035)**			**Best model (AICc = 261.316)**		
	Wald χ^2	**d.f.**	**p**	**Wald χ^2**	**d.f.**	**P**
(Intercept)	2.215	1	0.137	2.914	1	0.088
Species	0.621	2	0.733	0.791	2	0.673
Sex	5.713	1	0.017	5.381	1	0.020
Species * sex	6.084	2	0.048	6.329	2	0.042
Manipulation	0.057	1	0.811	-	-	-
Release date	0.056	1	0.813	-	-	-
Captive days	0.477	1	0.490	-	-	-

GLMs were used to analyse courtship behaviour using Tweedie (1.5) error structure and a log link function. Model selection was carried out using Akaike information criterion for small sample sizes (AICc).

Experimental assessment of mating opportunities in three shorebird species

Mating opportunities may differ between closely related species, although the evidence for such variation is scant. Here we compare remating opportunities and courtship behaviour between three shorebird species: the Kentish plover (*Charadrius alexandrinus*), the Kittlitz's plover (*C. pecuarius*) and the white-fronted plover (*C. marginatus*), using data and an experimental approach previously developed for the Kentish plover. By experimentally creating unmated males and females, we show that remating opportunities are different between closely related plover species (*Charadrius spp*): remating times were shorter for females than males in a Kentish plover population that exhibits a male-biased adult sex ratio, and where the majority of brood care after hatching is carried out by males. In contrast, remating times were male-biased in the uniparental Kittlitz's plover and unbiased in the biparental white-fronted plover. We also show that male Kentish plovers spend significantly more time on courtship than females, whereas courtship behaviour is not sex biased in the other two plover species. The mate-removal experiments also provided insights into pair bond stability. In the Kittlitz's plover, all 16 newly formed pairs remained together after the release of their former mates from captivity, whereas newly established pairs were replaced by their former mates upon release in 12 out of 12 white fronted plover pairs. Taken together, these results are important in highlighting interspecific variation in mating activities, and suggest that both operational sex ratio (OSR) and pair bond stability may differ between closely related species. These variations in turn, may influence mating systems and parental care.

Keywords: adult sex ratio, mating systems, operational sex ratio, pair bond, parental care, remating opportunity.

The different evolutionary interests of males and females over reproduction (termed sexual conflict; Parker, 1979) are a pervasive evolutionary force influencing the behaviour, ecology and life histories of many organisms (Arnqvist & Rowe, 2005; Chapman, Arnqvist, Bangham, & Rowe, 2003). A common issue when the interests of males and females are antagonistic concerns offspring care (Trivers, 1972; Maynard Smith, 1977; Houston, Székely, & McNamara, 2005; Lessells, 2012). By caring for the offspring, parents often improve the growth and survival of the young (Clutton-Brock, 1991; Klug, Alonzo, & Bonsall, 2012); for example, by gestating, nursing, protecting and feeding the young, the offspring have improved chances of survival (Tyler, Shearman, Franco, O'Brien, Seamark, & Kelly, 1983; Balshine-Earn & Earn 1998; Baeza & Fernández, 2002; Klug, Alonzo, & Bonsall, 2012). However, care is costly in terms of time and energy, and the caring parent can be killed by predators or may lose additional mating opportunities (Veasey, Houston, & Metcalfe, 2001; Li & Jackson, 2003; Klug et al., 2012). Therefore, whilst both biological parents benefit from providing care for the offspring, each parent is expected to withhold his (or her) parental contribution in order to raise further offspring in future (Houston et al., 2005; Lessells, 2012; McGraw, Székely, & Young, 2010).

Theory suggests that a key component of conflict resolution between male and female parents is mating opportunity (Székely, Webb, & Cuthill, 2000; McNamara, Székely, Webb, & Houston, 2000; Kokko & Jennions, 2008; Klug et al. 2012). If one sex has more favourable mating opportunities than the other, the former parent is expected to reduce (or completely terminate) care more often that its mate, and seek out a new partner (Balshine-Earn & Earn, 1998; Owens, 2002; Pilastro, Biddau, Marin, & Mingozzi, 2001). One approach used by researchers to assess mating opportunities is to estimate the ratio of sexually active males to females (operational sex ratio, OSR; Kvarnemo & Ahnesjö, 1996; Forsgren, Amundsen, & Bjelvenmark, 2004). An alternative approach to estimating mating opportunities is to experimentally create unmated individuals, and to quantify their remating behaviour, e.g. time to remate, remating success and reproductive success with the new mate (Lessells, 1983; Székely, Cuthill, & Kis, 1999). This experimental approach is powerful, since it directly assesses the mating potential of unmated individuals at a given time in a population.

Here we estimate remating opportunities for two small plover species, the white-fronted plover (*Charadrius marginatus*) and the Kittlitz´s plover (*C. pecuarius*), and compare these data with the results of a previous study on the Kentish plover (*C. alexandrinus*, Székely et al., 1999). Small plovers (*Charadrius spp.*) exhibit substantial variation in their breeding systems, since some of these species are monogamous and both parents rear the young, whereas others exhibit polygyny and/ or polyandry whereby a single parent (the male or the female) raises the young to independence (Székely, Thomas, & Cuthill, 2006; Thomas, Székely, & Reynolds, 2007). In addition, plovers typically breed in open areas, and their nests and broods are therefore accessible for experimental manipulations (Székely & Cuthill, 2000).

A previous experiment established that remating opportunities were female-biased in the Kentish plover (Székely et al., 1999), and this result was consistent with demographic analyses that estimated about 6 times more adult males than females in the population (Kosztolányi, Barta, Küpper, & Székely, 2011). Skewed adult sex ratios (ASRs) are common in wild populations (Donald, 2007), and recent works suggest that biased ASRs predict sex roles, mating systems and pair-bonds (Liker, Freckleton, & Székely 2013). Here we use an identical experimental protocol in two close relatives of the Kentish plover, the white-fronted plover and Kittlitz’s plover, to compare remating opportunities between these three plover species. All three species are insectivorous ground-nesting birds that exhibit similar life-histories and ecology (adult body masses, Kentish plover: 41.8 g; white-fronted plover: 37.1 g; Kittlitz’s plover: 35.3 g, Urban, Fry, & Keith, 1986; Hockey, Dean, & Ryan, 2005). The latter two species are common breeding birds in Africa, and their parental care systems differ from the Kentish plover which exhibits male-biased parental care after hatching (Lessells, 1984; Székely & Lessells, 1993; Amat, Fraga, & Arroyo, 1999). White-fronted plovers exhibit biparental brood care, whereas Kittlitz's plovers are reported to exhibit uniparental brood care, carried out by either the male or the female parent (Hockey et al., 2005; Tree, 1974; Urban et al., 1986).

Based on theoretical models (Klug et al., 2012; Kokko & Jennions, 2008) and available information on patterns of parental care (Hockey et al., 2005; Urban et al., 1986), we derived three predictions. First, we predicted higher remating opportunities in uniparental species (Kittlitz´s plover) than in biparental species (white-fronted plover), since in biparental species both parents are engaged with care until the offspring are fully independent (henceforth, between species comparison). Second, we predicted no difference in remating opportunities between males and females in biparental white-fronted plover given that both sexes are fully engaged in parental care. Similarly, no difference in remating opportunities between males and females was predicted for the uniparental Kittlitz´s plover in which either parent is free to seek a new mate (henceforth, between sexes comparison). Third, we predicted intense courtship behaviour by males and females both in biparental white-fronted plover and uniparental Kittlitz´s plover where care is provided by either parent (henceforth, courtship behaviour). In addition to the experimental assessment of remating opportunities, we also monitored pair bond stability among newly established pairs. We include the Kentish plover in our analyses (using the data from Székely et al., 1999), since the same experimental methodology was used in all three species. Nevertheless, our main conclusions remain consistent when restricting the analyses to the white-fronted and Kittlitz´s plovers.

METHODS

Study Species and Study Sites

White-fronted plovers and the Kittlitz's plovers were investigated in SW Madagascar (for Kentish plover, see details in Székely et al., 1999). Kittlitz's plovers were studied between 6 February 2010 and 13 May 2010 in Andavadoaka (22° 02' S, 43° 39' E) where they breed around alkaline lakes.

Approximately 300 Kittlitz’s plovers breed in Andavadoaka (J.E. Parra, S. Zefania, & T. Székely, unpubl. data). Fieldwork with the white-fronted plover was carried out between 1 April 2011 and 23 June 2011 at Lake Tsimanampetsotsa National Park (24° 3’ S, 43°44’ E), a large alkaline lake (approx 15 km x 0.5 km), surrounded by sandy shores, short grass and saltpans. Approximately 150 white-fronted plovers breed around the lake (J.E. Parra et al., unpubl. data).

In the field, we searched for nests on foot, identified incubating parents and watched the parent(s) returning to nests in potential breeding sites. In total, we captured 18 Kittlitz’s plover pairs (36 individuals) and 14 white-fronted plover pairs (28 individuals) with funnel traps placed on their nests. The traps were continuously monitored until a parent entered the trap and sat on the eggs. Parents were immediately removed from the traps to reduce stress and the risk of injury. Morphological traits (body mass, tarsus length, wing length and bill length) were measured using a spring balance, a sliding calliper and wing ruler (see details in Kentish plover field guide, www.bath.ac.uk/bio-sci/biodiversity-lab/pdfs/KP_Field_Guide_v3.pdf). All adults were ringed with an individual combination of colour rings and a numbered SAFRING metal ring from the University of Cape Town, South Africa.

Experimental Manipulation

We used the methodology developed by Székely et al. (1999) to estimate remating times in the Kentish plover. Briefly, both parents were trapped, ringed, measured and a blood sample was taken for sex determination (see below). One parent was then selected at random (the male or the female) and was released at the capture location immediately. The other parent was taken into captivity (see below). In both Kittlitz´s and white-fronted plovers, both the male and female incubate the eggs (Hockey et al., 2005; Urban et al., 1986). Only pairs incubating two eggs (modal clutch size in both species) were manipulated. Egg length and breadth were measured with a sliding calliper, and the number of days the eggs had been incubated for was estimated based on the floatation stage of the egg in a transparent jar with clean water (mean ± SD no. of days incubated: Kittlitz´s plover: 9.0 ± 4.32 days, *N* = 36; white-fronted plover: 11.5 ± 3.16 days, *N* = 20). Eggs were distributed to other non-experimental plover clutches at approximately the same stage of incubation in the local populations. Monitoring the augmented clutches was beyond the scope of the experiment, although casual nest checks suggest that at least 33.3% and 19.4% of augmented nests survived until hatching in the Kittlitz´s plover (*N* = 36 nests) and the white-fronted plover (*N* = 20 nests), respectively. Survival in these nests appeared to be higher than for unmanipulated nests (13.4% and 8.9%, based on *N* = 101 Kittlitz's plover nests and *N* = 56 white-fronted plover nests, respectively; J.E. Parra et al., unpubl. data).

Removed plovers were transported in an air-conditioned vehicle to a purpose-built aviary near the field camp at both study sites. Lightweight bird bags were used to keep the plovers undisturbed and ventilated during the transport. Distance from capture areas to the aviaries varied between 1 and 10 km in both study sites. The aviaries had four units for Kittlitz's plovers and six units for white-fronted plovers. Each unit consisted of a 1 m x 1.5 m x 1.5 m (height x length x width) wood frame fitted with chicken mesh (13 mm x 13 mm). To provide shade for the captive birds, we covered the outside of the aviary with papyrus, *Cyperius sp.*, and fitted 50 cm of cloth at the base of the mesh inside the units. Captive plovers were provided with appropriate food and drink three times every day to maintain their good health using high protein meals: dried invertebrates for wild birds (dried mealworms, dried earthworms, shrimps and dried waterfly; shop.naturesgrub.co.uk/), bird supplement vitamins (Vitacombex V; www.petland.co.uk) and pinhead oatmeal (Prosecto InsectivorousTM; www.haiths.com). Captive plovers were also supplied with fresh insects twice a day using pit fall traps set-up in the salt-marsh. In addition, two water recipients were set for drinking and bathing in each unit. Captive plovers were released after their former mate found a new mate or their former mate was not seen in the study sites for at least 12 days. Time in captivity was comparable between white-fronted plovers (mean ± SD no. of days in captivity: 8.0 ± 1.71 days, *N* = 14) and Kittlitz's plovers (7.12 ± 2.57 days, *N* = 18). Captive plovers were measured before release. Although captive plovers appeared to lose a small amount of body mass during their time in captivity (2.77 ± 0.51 g in Kittlitz’s plover, and 0.73 ± 0.22 g in white-fronted plover), they were in good condition as indicated by the fact that many remated shortly after release from captivity (see Results).

Behavioural Observations

The released plovers were searched for every day in the field using a car and mobile hide. When a focal plover was found, we recorded its behaviour for 30 min at 30 seconds intervals. Attempts were made to record the behaviour of focal plovers on at least two occasions before they found a new mate. 30 min is sufficient to establish whether a plover is mated or not (Székely et al., 1999). We used behavioural categories of courting and self-maintenance behaviours that were previously developed for the Kentish plover (Székely et al., 1999). Courtship behaviours included: (1) Courting: male plovers perform upright posture and high-stepping movements and female plovers perform a lower head position. (2) Copulations: courting pairs frequently copulated. (3) Scraping: male and female plovers dig several scrapes in a territory. (4) Fighting: focal individuals chase away intruders with buffed-out plumage and buzzing calls. Self-maintenance behaviours included: (1) Feeding: individuals pick up food items followed by a short run, (2) Preening: an individual groomed its own feathers. Mated individuals were identified based on courtship behaviours including courting, scrape ceremony and copulation (Urban et al., 1986; Hockey et al., 2005, see video of Kittlitz´s plover courting behaviour in the Supplementary Material, Video S1). New pairs were checked for clutches every day, and eggs were measured as described above. Two observers recorded all behavioural observations (M. Beltrán and J.E. Parra).

Molecular Sexing

Both plover species have sexually monomorphic plumage (Hockey et al., 2005; Urban et al., 1986), therefore we used molecular sex-typing to determine the sex of individuals (dos Remedios, Lee, Székely, Dawson, & Küpper, 2010). A small blood sample was taken from each adult's brachial wing vein, by puncturing, collecting drops of blood (~25 ul) in capillary tubes, and storing this in Eppendorf tubes of Queen's Lysis Buffer. DNA was extracted from blood samples using the Ammonium Acetate extraction method (Miller, Dykes, & Polesky, 1988; Richardson, Jury, Blaakmeer, Komdeur, & Burke, 2001). For molecular sex-typing, Z- and W-chromosome specific genes were amplified via polymerase chain reaction (PCR) using the Z-002B/Z-002D primers (Dawson, 2007). For additional certainty in sex assignment, the W-chromosome specific Calex-31 primers, developed in the genus *Charadrius* were utilized (Küpper, Horsburgh, Dawson, Ffrench-Constant, Székely, & Burke, 2006). PCR amplification was conducted on a DNA Engine Tetrad 2 Peltier Thermal Cycler under the following conditions: 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 56°C for 90 s, 72°C for 60 s with a final extension of 60°C for 30 min. Samples were visualized on an ABI 3730 automated sequencer. IR Dye-labelled tailed primers separated the products of Z-002B/Z-002D primers into either one (ZZ) or two bands (ZW), indicating male or female respectively. The W-specific Calex-31 product appeared as one band indicating female only. Images were scored using GeneMapper software version 4.1 (Applied Biosystems). To maximize reliability, all samples were sexed using two markers. Additionally, for 8% of samples (11 Kittlitz's plover and 10 white-fronted plover individuals) molecular sexing was repeated; in all cases, repetitions concurred with the original results.

Data Processing and Statistical Analyses

Date of mating was defined as the mean date between the date when a plover was last seen single and the first date it was seen with a new mate. Remating time was the difference between date of release (either on the day of manipulation or from captivity) and date of mating. The response variable, remating time, was analyzed using generalized linear models (GLM) with Tweedie (1.5) error structure and a log link function (Smyth & Verbyla, 1999). The models investigated the effects of two main variables:

species (Kentish, white-fronted and Kittlitz's plovers) and sex; and three additional fixed variables: type of manipulation (released in the field or released from captivity), release date, and number of days in captivity (see Table 1). Dates were expressed as Julian dates, i.e. number of days since 1 January. Results of backward elimination based on Akaike's information criterion for small sample sizes (AICc) are presented for variable selection of the GLM models where lowest AICc score is the best supported model (Symonds & Moussalli, 2010; Table 1). Pairwise multiple comparisons were performed to compare mean differences of mating time between males and females of the three species using Fisher's least significant difference tests (LSD test).

Remating time was also analyzed using survival analyses and these estimates are referred to as expected remating times (see rationale in Székely et al., 1999). In these analyses, the terminal event (outcome) was the occurrence of mating, defined as the first observation when a plover was seen with a mate. Several individuals did not find a new mate when we saw them for the last time, and these were treated as censored observations. First, we used a Gehan-Wilcoxon test to compare expected remating times curves (survival curves) for three species by sex. Survival curves were generated by the Kaplan-Meier method. Second, for each plover species a separate Cox regression model was constructed to investigate the probability of remaining single from the day of release (season), sex and their interaction as covariates (Table 2).

Courting behaviour was estimated as percentage obtained from each 30 min sample. For individuals with several behavioural observations, we calculated the mean percentage of courting. Courting behaviour was analyzed using GLMs with Tweedie (1.5) error structure and a logarithmic link function per individual plover. The model included two main factors: species of plover and sex; and three additional fixed variables: type of manipulation (released in the field or from captivity), released date, and number of days in captivity. Model selection and statistical parameters estimated for each independent variable in the models are provided in the Appendix, Table A1. In addition, for each sex a separate GLM model was constructed to investigate the effect of three species of plovers on courting behaviour (Table

3). Pairwise multiple comparisons were performed to compare mean differences of courtship behaviour between males and females in the three species of plovers using LSD tests.

Finally, pair bond stability was analysed comparing the frequencies of mate replacement between white-fronted plover and Kittlitz’s plover with Fisher's exact test. Data were analyzed by using SPSS statistics for Windows version 19 and figures were made in R (R Development Core Team, 2008) using the package ggplot2 (Wickham, 2009).

Ethical Note

The experiments in Madagascar were approved by the Ministry of Environment, Forests and Tourism of the Republic of Madagascar (Research permit No: 053/11/MEF/SG/DGF/DCB.SAP/SCB of 11 March 2011 and 132/10/MEF/SG/DGF/DCB.SAP/SSE of 06 May 2010) and Madagascar National Parks (No: 398-10/MEF/SG/DGF/DVRN/SGFF of 18 May 2011). Blood sampling was also covered by these research permits. The blood transport permit was approved by Service de la Gestion de la Faune et de la Flore, Direction de la Valorisation des Resources Naturelles, Ministère de l'Environnement et des Forêts Madagascar (authorization number 080N-EA06/MG11). The Kentish plover experiment was approved by the Turkish Ministry of Environment (see Székely et al., 1999). The Kittlitz´s and the white-fronted plovers are common breeding birds in much of Africa and Madagascar and they are not considered threatened by the IUCN (BirdLife International, 2012). Captive plovers were monitored daily and kept under standard conditions (see Experimental Manipulation) to reduce their stress levels. In addition, translocated eggs coped with the natural breeding conditions of local clutches in the two plover populations (see above). The experiment was designed to reduce adverse effects on plover welfare and their local populations.

RESULTS

Between Species Comparison

Remating opportunities differed significantly between the three plover species (Fig. 1): white-fronted plovers mated significantly more quickly (median = 2.0 days, range 0.5 – 4.5 days, *N* = 12) than both Kittlitz's plovers (median = 5.1 days, range 1.0 – 11.5 days, *N* = 16) and Kentish plovers (median = 6.3 days, range 0.5 – 47.5 days, *N* = 34; Table 1).

These results remained consistent using survival analyses that also included the individuals that were not successful in finding a new mate (Fig. 2, Table 2; see Methods). The proportion of plovers remaining single was significantly lower for the white-fronted plover (median = 4 days, *N* = 14) than both the Kittlitz's plover (median = 14.6 days, *N* = 33) and Kentish plover (median = 13.4 days, *N* = 59; testing the three species, Wilcoxon–Gehan test: χ^2_2= 16.316, $P < 0.001$).

Between Sexes Comparison

A significant species by sex interaction suggested a sex-biased difference in remating opportunities (GLM: $\chi^2_2 = 47.62$, $P < 0.001$, Table 1). Female Kittlitz's plovers took significantly longer to mate (median = 6.5 days, range 3.5 – 11.5 days, *N* = 6) than males (median = 3.3, range 1.0 – 7.5 days, *N* = 10; LSD test: pairwise mean difference = -0.66, *df* = 1, $P = 0.047$) whereas the opposite was found in the Kentish plover (Székely et al. 1999). However, male and female remating times were not significantly different in white-fronted plovers (male: median: 2.0 days, range 0.5 – 3.5 days, *N* = 6; female: median: 2.0 days, 1.0 – 4.5 days, *N* = 6, LSD test: pairwise mean difference = -0.11, *df* = 1, $P = 0.823$).

These results remained consistent using survival analyses (Table 2): the proportion of female Kittlitz's plovers remaining single was higher than that of males (male median: 11.0 days, *N* = 17, female median: 21.0 days, $N = 16$, Fig. 2), whereas the proportion of single males and females were not significantly different in the white-fronted plover (male median: 3.0 days, $N = 7$, female median: 4.0 days, $N = 7$, Fig. 2). Remating time increased over the season only for female Kentish plovers (Cox regression: $\chi^2_1 = 7.66$, $P = 0.014$), suggesting an influence of time of breeding season on mating opportunities in the Kentish plover, although this was not the case in the other two species (Table 2).

Courtship Behaviour

Courtship behaviour had a significant species by sex interaction (GLM: $\chi^2_2 = 6.329$, $P = 0.042$, Supplementary Material: Table S2, Fig. 3). Courtship behaviour by males differed significantly between species (GLM: $\chi^2_2 = 10.689$, $P = 0.005$, Table 3), male Kentish plovers spent significantly more time on courtship than males of the other plover species; whereas courtship behaviour by females did not differ between species (GLM: $\chi^2_2 = 1.437$, $P = 0.487$, Table 3). In contrast to the Kentish plover, which exhibited male-biased courtship behaviour (LSD test: pairwise mean difference = -3.29, $df = 1$, $P = 0.005$), males and females of the other two species spent comparable times on courtship (LSD test white-fronted plover: pairwise mean difference = 1.06, $df = 1$, $P = 0.252$; LSD test Kittlitz's plover: pairwise mean difference = -0.36, $df = 1$, $P = 0.679$, Fig. 3).

Pair Bonds

The new pair bonds in experimentally-induced white-fronted plovers were significantly weaker than in Kittlitz's plover: in 12 white-fronted plovers that remated after their former partner was removed (6 males, 6 females), all experimentally-induced pair bonds were replaced by the original mates after they were released from captivity. In contrast, in 16 Kittlitz's plovers that remated after their former partner was removed (10 male and 6 female), none were replaced by their former mates once their former mates were released from captivity (Fisher’s exact test: mate replacement in white-fronted plover =12, $N = 12$; mate replacement in Kittlitz´s plover = 0, $N = 16$; $P < 0.001$)

DISCUSSION

These experiments provided four key results. First, they show that mating opportunities are significantly different between closely related species. This result is striking because two of these plover species (white-fronted and Kittlitz’s) breed in the same habitat in Madagascar, and therefore, ecological factors are unlikely to explain the differences in remating opportunities. The rapid remating of white-fronted plovers – a pattern we did not expect – suggests that there is a large pool of floating individuals that can rapidly move in to pair up with unmated individuals. Consistent with the latter argument, both male and female white-fronted plovers stayed in the same territory, and new individuals moved in to replace the removed mates. Mating opportunities seem to be an important factor in the evolution of breeding systems across a range of species (Balshine-Earn & Earn, 1998; Magrath & Komdeur, 2003). For example, as in the white-fronted plover, male dunlin (*Calidris alpina*) were rapidly replaced by other males after experimental removal from their breeding territories (Holmes, 1970; Pitelka, Holmes, & Maclean, 1974). In the Eurpoean starling (*Sturnus vulgaris*), after mating opportunities were increased by the provision of additional nest-boxes, males increased their mating effort to attract additional mates and also reduced parental care effort (Smith, 1995), and in St. Peter´s fish (*Sarotherodon galilaeus*), males and females were more likely to desert the offspring when remating opportunities were increased experimentally (Balshine-Earn & Earn, 1998).

Second, we found sex-bias in remating opportunities: the male-biased remating opportunities in Kittlitz’s plover were the opposite of those found in the Kentish plover (Székely et al., 1999), whereas in white-fronted plovers remating opportunities did not differ between males and females. As far as we are aware, our study is the first to experimentally demonstrate differences in sex-biased remating opportunities between closely related species in wild populations. Sex-biased mating opportunities may emerge in two ways. One explanation is that the ratio of sexually active males to females (operational sex ratio, OSR) may not be at parity. OSR may be biased due to differences in the reproductive schedules of males and females ("time in": time spent in the mating pool, sexually active; "time out": time spent out of the mating pool, not sexually active), or biased adult sex ratios (ASR; Donald, 2007). Evidence suggests that OSR can vary due to mating and parental care activities (Forsgren et al., 2004, Symons, Svensson, & Wong, 2011; LaBarbera, Lovette, & Llambías, 2011; Canal, Jovani, & Potti, 2012). In addition, recent studies found substantial difference in ASR between closely related shorebird species (Liker et al., 2013). Further works are needed to separate whether biased remating opportunities emerge via different reproductive scheduling or biased ASR in plovers. For one of these species, the Kentish plover, a demographic study confirmed male-biased ASR (Kosztolányi et al., 2011), although ASR has not been estimated for the white-fronted and the Kittlitz's plover. Alternatively, sex-biased mating opportunities may arise due to differences in the willingness of males and females to remate. For instance, the post-breeding refractory periods, the recovery phase spent preparing for another breeding attempt, may differ between males and females (Balshine-Earn & Earn, 1998; Cantoni & Brown, 1997): females typically need more time to recover than do males. However, the latter explanation is unlikely, since the adult plovers used in our experiments had breeding efforts interrupted and sought new mates shortly after removal of their mate (or on release from captivity). Furthermore, several female Kentish plovers remated within less than a day – a pattern that is inconsistent with the explanation that females need more time to recover than do males.

Third, male courtship behaviour was different between the three species, since male Kentish plovers spent more time on courtship than male white-fronted and Kittlitz's plovers. This pattern is consistent with the explanation that ASR is male-biased in the Kentish plover. The significance of this result is that courtship behaviour is variable between closely related species and suggests that ASR, and in turn the OSR, is probably related to the intensity of mating competition in males. Consequently, comparable intensity of courtship behaviour was observed between sexes in the Kittlitz´s and white-fronted plovers. The latter result indicates that males and females may compete similarly for available mates, suggesting that they do not have conventional sex roles: male-male competition and female choice for mates (Vincent, Ahnesjö, & Berglund, 1994). Variation in resources for breeding has also been suggested to influence OSR, and in turn, the intensity of mating competition (Forsgren, Kvarnemo, & Lindstrom, 1996). Availability of breeding territories, for example, may affect the OSR, since the sex that holds the territories will be limited by scarcity of nest sites. In a sand goby population (*Pomatoschistus minutes*), for instance, nest-site abundance can influence the intensity of male mating competition (Forsgren et al., 1996). Hence, the dynamic of OSR, and in turn mating competition, is probably modulated by both ASR and resource availability (Kvarnemo & Ahnesjö, 1996, Forsgren et al., 2004).

Finally, the new pair bonds were significantly weaker in white-fronted plovers than in Kittlitz's plover suggesting that the former species exhibits long-term pair bonds whereas the latter has short-term pair bonds. Mate fidelity may emerge in two ways. On the one hand, former mates may actively seek out each other *per se*, and prefer to mate with each other. On the other hand, mate fidelity may emerge via site fidelity: white-fronted plovers are highly territorial (Lloyd, 2008), and therefore upon release from captivity, individuals return to their former territories and chase out their former partner's new mates. Established pairs may prefer to reunite because of the fitness benefits in terms of synchronisation of behavioural and physiological characteristics such as defence of breeding territories, courtship behaviours, laying date, incubation, chick-raising between others which have been shown to improve with time and experience of the pair (Bried, Pontier, & Jouventin, 2003; Rowley, 1983). An experiment carried out in bearded reedling *Panurus biarmicus* found that long-term pair bond formation improved coordination of breeding activities and reproductive success (Griggio & Hoi, 2011). In another example, newly formed pairs (either due to divorce or loss of a mate) had lower reproductive success than established pairs in black turnstone *Arenaria melanocephala* (Handel & Gill, 2000). Improved breeding with increased experience is also well known in geese, albatrosses and other long-term pair bonding animals (Angelier, Weimerskirch, Dano, & Chastel, 2006; Black, 2001). Overall, the consequences of pair bond and site fidelity on mating opportunities could be significant since the ability of an individual to mate may be limited by their access to mates and breeding sites.

In conclusion, using an experimental approach we found significant differences in remating opportunities between closely related plover species. As mating opportunity is linked to OSR and ASR, our work suggests that substantial variation in OSR (and possibly ASR) is exhibited among closely related species. Such variation may influence the direction and intensity of competition in males and females for mates and breeding territories. These differences in OSR, in turn, may facilitate different intensities of sexual selection and induce different mating systems and patterns of parental care.

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Figure Legends

Figure 1. Remating times in three plover species. The lower and upper borders of the box are lower and upper quartiles, respectively, the horizontal bar is the median and whiskers represent the lowest and highest observations.

Figure 2. Proportion of males and females remaining single in three plover species: Kentish plover (top), white-fronted plover (middle) and Kittlitz’s plover (bottom). Dotted lines show the expected mating time of males and females after release. Number of individuals: 32 male and 27 female Kentish plovers; 7 male and 7 female white-fronted plovers; and 17 male and 16 female Kittlitz´s plovers.

Figure 3. Courtship behaviour in three plover species. The lower and upper borders of the box are lower and upper quartiles, respectively, the horizontal bar is the median and whiskers represent the lowest and highest observations. Circles denote outliers that are between the first and third interquartile from the nearer edge of the box.

Table 1.

Remating times (response variable) of males and females in three species of plover.

Independent variable	Full model (AICc = 346.004)			Best model (AICc = 341.088)		
	Wald χ^2	*Df*	*P*	Wald χ^2	*Df*	*P*
(Intercept)	38.596	1	<0.001	49.365	1	<0.001
Between species comparison						
Species	11.248	2	0.004	11.595	2	0.003
Between sexes comparison						
Sex	4.072	1	0.044	3.974	1	0.046
Species * sex	39.65	2	<0.001	47.620	2	<0.001
Manipulation	0.290	1	0.59	-	-	-
Release date	4.818	1	0.028	5.007	1	0.025
Captive days	0.646	1	0.422	-	-	-

GLMs were used to analyze mating time using Tweedie (1.5) error structure and a log link function. Model selection was carried out using Akaike information criterion for small sample sizes (AICc). Manipulation refers to whether a plover was kept in captivity or not. Release date refers to the date when a plover was released to find a new mate (Julian dates). Captive days are the number of days that a plover was kept in captivity.

Table 2.

Remating time in plovers based on Cox proportional hazard models, to examine the relationship of the survival distribution which includes censored observations.

Species	Variable	B	Wald χ^2	*df*	p
Kentish plover	Sex	1.541	12.07	1	0.001
	Release date	-0.024	6.073	1	0.014
White-fronted plover	Sex	-0.18	0.083	1	0.77
	Release date	0.002	0.004	1	0.95
Kittlitz's plover	Sex	-1.342	4.864	1	0.027
	Release date	-0.01	0.088	1	0.767

For each species a separate model was constructed. Number of individual Kentish plovers, mated = 34, censored = 19; white-fronted plover, 12, 2; Kittlitz’s plover, 16, 17, respectively.

Table 3.

Courtship behaviour (response variable: % of time courting) in three plover species.

Sex	Variables	Wald χ^2	*Df*	*P*
Male	(Intercept)	13.176	1	< 0.001
	Species	10.689	2	0.005
Female	(Intercept)	0.155	1	0.694
	Species	1.437	2	0.487

GLMs were used to analyse percentage of time courting using Tweedie (1.5) error structure and a log link function. Model selection was carried out using Akaike information criterion for small sample sizes (AICc).

APPENDIX

Table A1.

Courtship behaviour in plovers (response variable: proportion of time courting).

Independent variable	Full model (AICc = 269.035)			Best model (AICc = 261.316)		
	Wald χ^2	**d.f.**	**p**	**Wald χ^2**	**d.f.**	**P**
(Intercept)	2.215	1	0.137	2.914	1	0.088
Species	0.621	2	0.733	0.791	2	0.673
Sex	5.713	1	0.017	5.381	1	0.020
Species * sex	6.084	2	0.048	6.329	2	0.042
Manipulation	0.057	1	0.811	-	-	-
Release date	0.056	1	0.813	-	-	-
Captive days	0.477	1	0.490	-	-	-

GLMs were used to analyse courtship behaviour using Tweedie (1.5) error structure and a log link function. Model selection was carried out using Akaike information criterion for small sample sizes (AICc).

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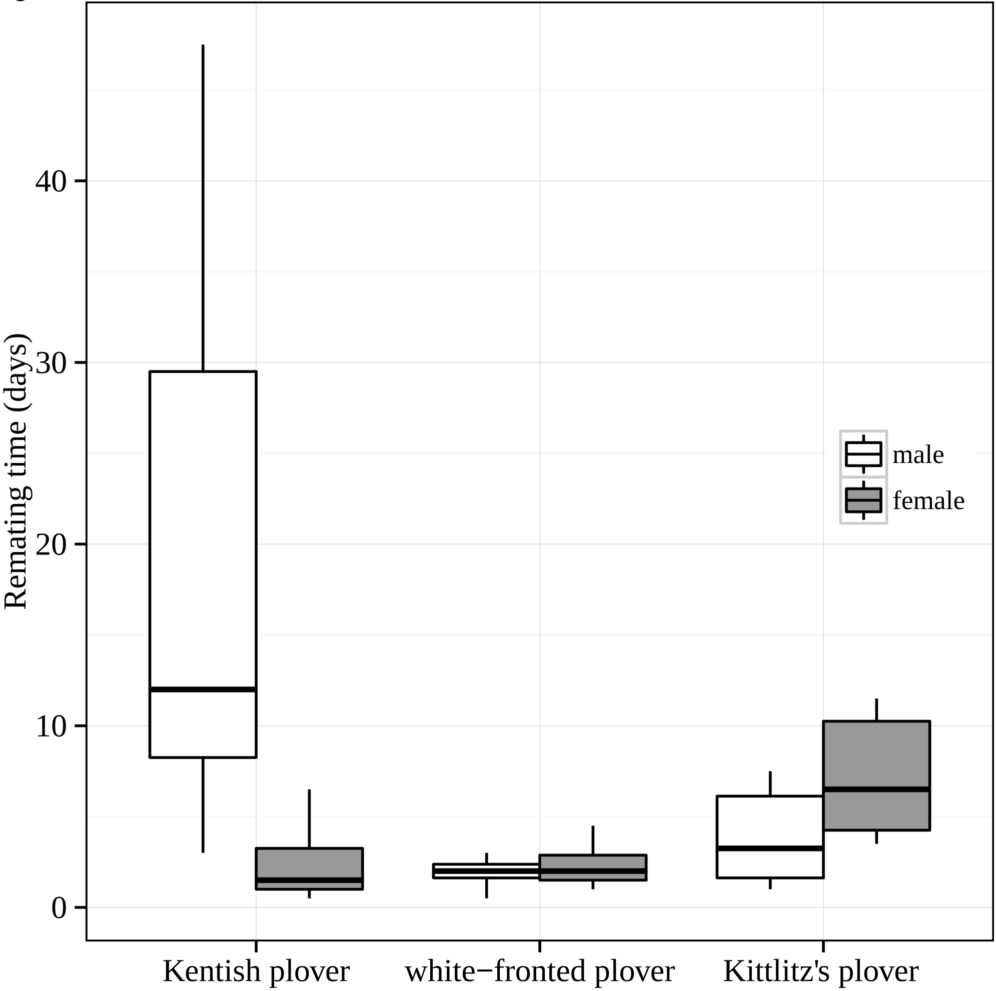
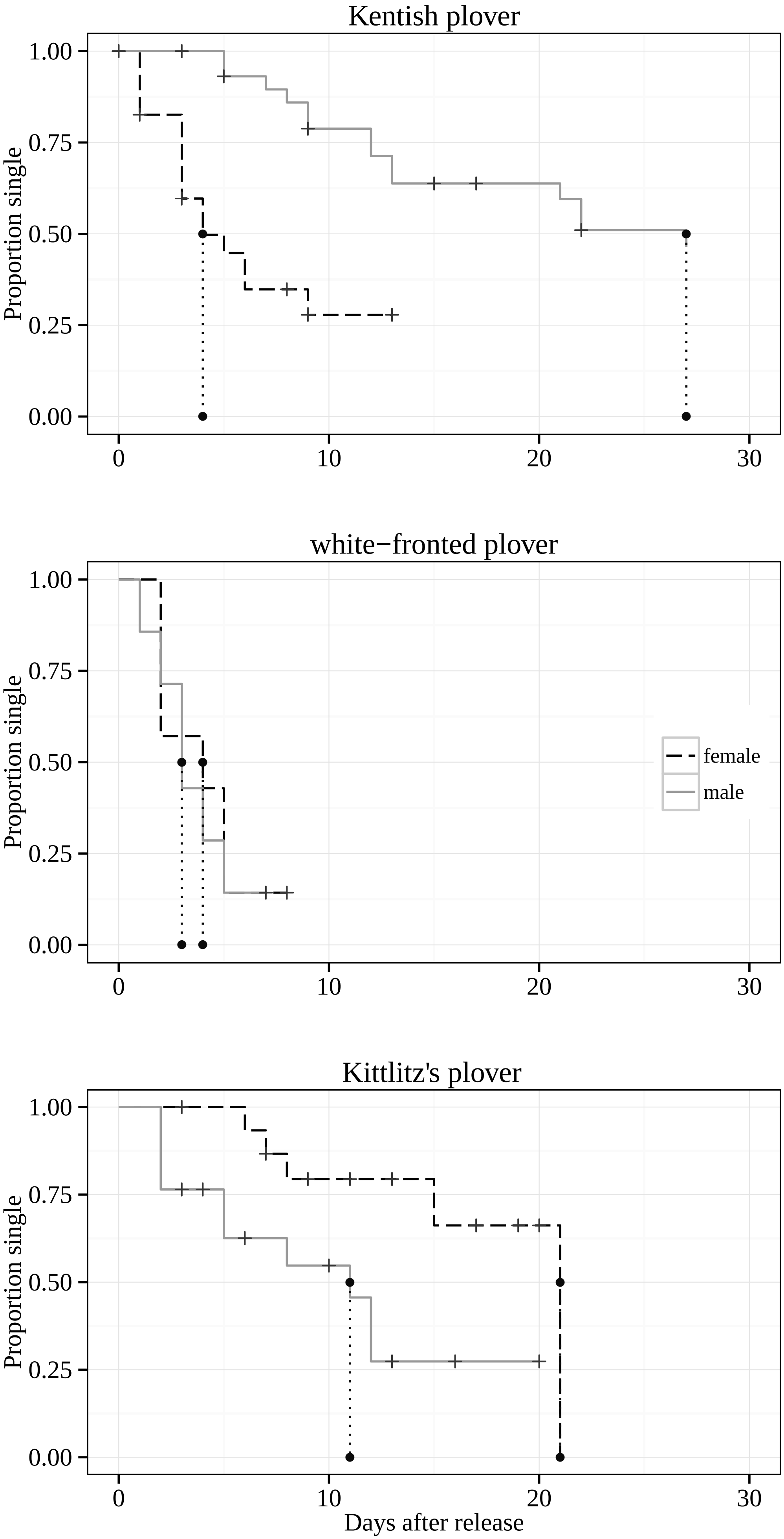
Figure 1



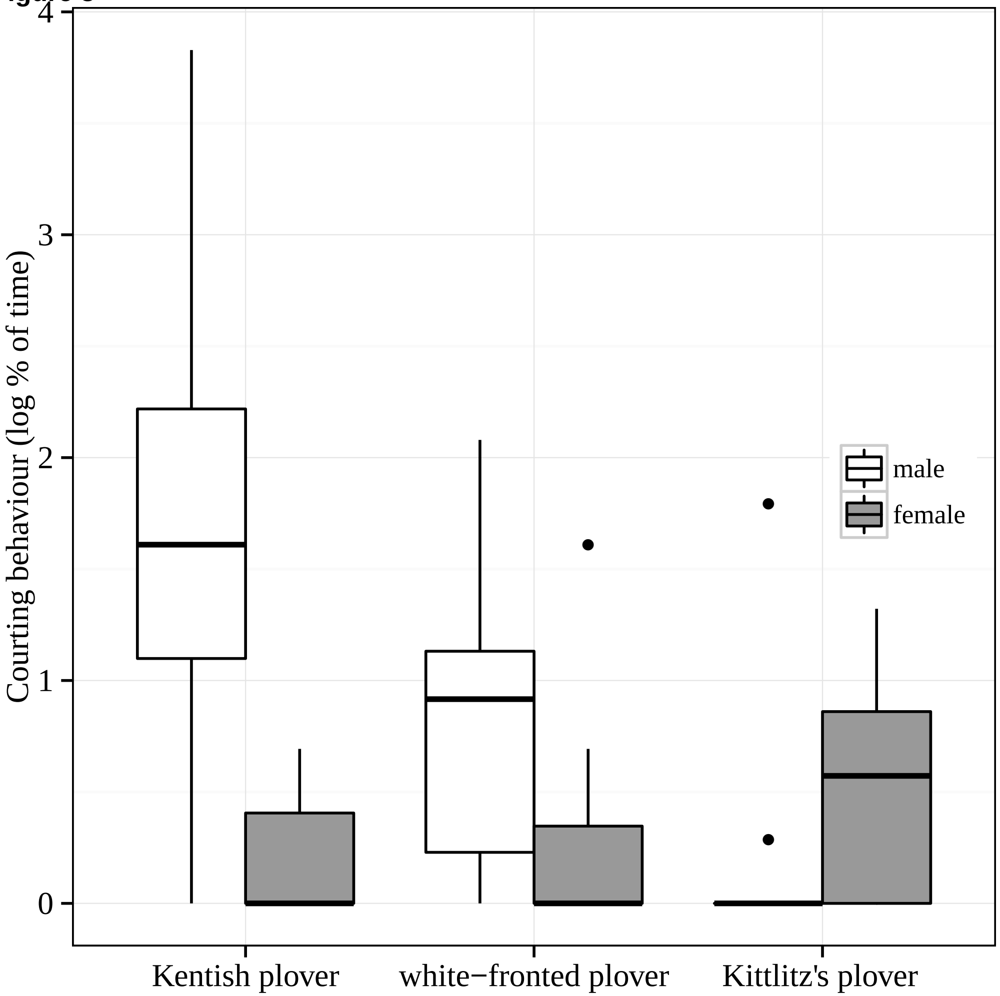
Remating time (days)
0
10
20
30
40
Kentish plover
white−fronted plover
Kittlitz's plover
male
female

Figure 2



Kentish plover
Proportion single
1.00
0.75
0.50
0.25
0.00
0
10
20
30
white−fronted plover
Proportion single
1.00
0.75
0.50
0.25
0.00
0
10
20
30
female
male
Kittlitz's plover
Proportion single
1.00
0.75
0.50
0.25
0.00
0
10
20
30
Days after release

Figure 3


4
3
2
1
0
Courting behaviour (log % of time)
male
female
Kentish plover
white−fronted plover
Kittlitz's plover

Video S1

Caption Video S1

Video Still