

Female-solicited extrapair matings in Humboldt penguins fail to produce extrapair fertilizations

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The study reported in this paper demonstrated that Humboldt penguins at Punta San Juan, Peru, despite forming pair-bonds, are not strictly monogamous in their mating behavior: 19.2% of the study males and 30.7% of the study females (21 nests) engaged in extrapair copulations. The total number of completed matings observed during the course of this study was 106, of which 17.9% were extrapair copulations. Using DNA fingerprinting we demonstrated that none of these extrapair copulations resulted in extrapair fertilizations; all 49 offspring were attributed to the putative father. Location of copulations suggested that females solicited these extrapair copulations because 89.2% of Humboldt penguin within-pair copulations occurred at the home burrow, yet extrapair copulations took place at a different location based on the sex of the penguin. Extrapair copulations by males occurred at their nest, whereas females conducted 92% of their extrapair copulations away from the nest. These results are most consistent with mate-appraisal and epiphenomenal hypotheses. *Key words*: epiphenomenon, extrapair fertilization, extrapair mating, female choice, Humboldt penguins, mate appraisal, *Spheniscus humboldti*. [*Behav Ecol* 10:242–250 (1999)]

Until recently monogamy has been considered the predominant mating system among birds (Lack, 1968). Nevertheless, behavioral studies have shown that many avian species previously believed to be monogamous actually engage in extrapair copulations (EPCs; Birkhead et al., 1990; Gowaty and Bridges, 1991a; Lifjeld et al., 1993; Morton et al., 1990; Westneat, 1990). Individuals may engage in EPCs to increase their fitness relative to others of the same sex who only copulate with their bonded partner. To evaluate the possible adaptive significance of this reproductive tactic, the frequency and nature of EPCs need to be known, as does the extent to which EPCs lead to extrapair fertilizations (EPFs). Developments in molecular genetics have provided important tools such as DNA fingerprinting and DNA microsatellite analyses that allow for accurate assignment of genetic parentage (Burke and Bruford, 1987; Jeffreys et al., 1985; Primmer et al., 1995).

Possible explanations for EPC have been postulated in terms of trade-offs between the costs and benefits in reproductive effort for both sexes. For males it is often believed that the production of sperm is relatively cheap (Trivers, 1972); hence the costs of extrapair mating are minimal. Unless offspring require extensive paternal care, which may compete directly with EPC effort, the male should employ a strategy of seeking EPCs (Westneat et al., 1990, but see Birkhead and Møller, 1992). A variety of costs to the male for participating in EPCs have been noted, all of which must be balanced against the potential increased fitness through in-

creased lifetime reproductive success (Gowaty, 1985; Trivers, 1972; Westneat, 1990).

For males the magnitude of the costs and benefits are mediated through ecological factors and social factors that influence the value of paternal care (Dunn et al., 1994; Freeman-Gallant, 1996; Stuchbury and Morton, 1995). For example, a male that has to make long foraging trips to partition resources for his offspring will spend less time seeking EPCs, as will a male whose offspring benefit from nest guarding (Birkhead and Møller, 1996; Westneat, 1990). It has also been shown that increases in coloniality and density (Dunn et al., 1994; Gowaty and Bridges, 1991b; Møller and Birkhead, 1993; Westneat and Sherman, 1997; Westneat et al., 1990) will increase the male's opportunities for EPCs (Westneat et al., 1990).

Although these social and ecological factors often influence a female as well, the overall costs and benefits are different than for the male. Female costs include retaliation by the paired male if he discovers he has been cuckolded, harassment and possible injury by extrapair males, and increased rates of parasitism or disease transmission (Westneat et al., 1990). The numerous hypotheses proposed to explain female participation in EPCs can be divided into two main categories (reviewed by Birkhead and Møller, 1992; Westneat et al., 1990): those associated with direct benefits, which immediately add to a female's reproductive success, and those associated with indirect benefits, which aid offspring by conferring genotypic advantages. Specifically, the direct benefits females might receive are additional courtship feeding and additional parental care by the extrapair male. The hypotheses based on indirect benefits for females include obtaining better genes from higher quality males (Weatherhead, 1994; Weatherhead and Robertson, 1979), production of a mixed brood with a maximum variety of genotypes (Westneat et al., 1990), induction of sperm competition (Lifjeld et al., 1993), the opportunity for mate appraisal and acquisition in future breeding seasons (Colwell and Oring, 1989; Wagner, 1991), and insurance against male infertility (Westneat et al., 1990).

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These hypotheses have mostly been tested with respect to passerine species (e.g., Birkhead et al., 1990; Morton et al., 1990; Westneat, 1987a,b, 1990) because passerines are often abundant, relatively easy to capture and observe, and produce clutches with numerous offspring. The few studies of nonpasserine species have shown EPCs to be infrequent and to lead to few, if any EPFs (e.g., Decker et al., 1993; Warkentin et al., 1994). Seabirds, in particular, have demonstrated a low rate of EPF, possibly due to the extensive biparental care needed to successfully fledge offspring in an environment of unpredictable food resources (Austin and Parkin, 1996; Birkhead and Møller, 1996; Hunter et al., 1992; Mauck et al., 1995).

Penguins (*Spheniscidae*) provide an extreme example of the biparental care needed to provision young. For many species of penguins both adults embark on foraging trips lasting one or more days, with the foraging trip of one species (the emperor penguin, *Aptenodytes forsteri*) extending to as long as 24 days (Williams, 1995). We therefore might expect many penguin species to exhibit strict monogamy or a low level of EPCs and EPFs.

Despite a considerable number of studies on penguin copulation behavior, few have focused on extrapair activity (Williams, 1996). Two recent studies provide evidence for relatively low levels of EPCs. Hunter et al. (1995) found that 9.8% of female Adélie penguins (*Pygoscelis adeliae*) engage in EPCs, but genetic analysis was not presented. St. Clair et al. (1995) demonstrated, by the use of DNA fingerprinting, that extrapair males fertilized 7.7% of royal penguins' (*Eudyptes schlegeli*) first eggs. None of the penguins from the genus *Spheniscus* have been examined for EPCs.

In this study, we first addressed the basic questions of whether Humboldt penguins participate in EPCs, and if so, how frequently. We then used DNA fingerprinting to determine the extent to which EPC leads to EPF. To identify which factors might influence the occurrence of extrapair mating activity, we examined the nature and the spatial distribution of this behavior in relation to the sex of each focal animal.

METHODS

Natural history and study site

Based on osteological and molecular data (Olson, 1985; Sibley et al., 1988; Simpson, 1975), the six modern genera of penguins appear to be related to Procellariiformes (e.g., albatrosses) and Gaviiformes (e.g., loons). Thus, penguins are often classified in the superfamily Procellarioidea with Gaviidae, Procellariidae, and Fregatidae (Fordey and Jones, 1990). The Humboldt penguin, *Spheniscus humboldti*, is classified in the genus *Spheniscus* with the Magellanic, Galapagos, and Cape penguins. All members of this genus breed along the South American or African coast line, with the Humboldt penguins breeding in colonies between the islets off Algarobo, Chile (33°20' S) and Isla Foca, Peru (5°12' S; Hays, 1984).

The Humboldt penguin is the least studied of the 17 species of penguins (Hays, 1986), with little data published on its breeding cycle in the wild (Williams, 1995). They breed 10 months of the year, commencing in March with pair formation and nest selection occurring simultaneously (Zavalaga and Paredes, 1993). After the bond is established and the nest is nearly finished, mating begins, usually at a nest site, which is either an excavated burrow, natural crevice, cave, or depression in the surface layer of guano (dried seabird excrement). Many nests are lined with bird feathers and seaweed brought to the nest by either the male or the female.

Several days after successful breeding, two eggs are laid 3–4 days apart. Both parents alternately incubate the eggs for 42 days (Zavalaga and Paredes, 1993). Two peaks of egg laying

occur, one in April and another in August (Murphy, 1936). Some breeding pairs have successive broods, one in each peak, with a maximum of two offspring per brood (Murphy, 1936; Zavalaga and Paredes, 1993). Because such a high level of biparental care is needed to successfully rear offspring, it is generally believed that Humboldt penguins, like most seabirds, are monogamous and maintain a pair-bond throughout the breeding season, similar to other penguin species (Williams, 1995).

This study was conducted at Punta San Juan, Peru (15°22' S, 75°12' W), a guano reserve located on a peninsula in southern Peru, containing the world's largest Humboldt penguin breeding area. The reserve is protected by a 3.0-m high, 1.2-km long concrete wall which isolates the peninsula from landward entry, protecting the breeding colonies from disturbances by humans and other mammalian predators such as foxes (*Dusicyon griseus*) and domestic dogs (*Canis familiaris*). The habitat is a dry desert, with strata of sedimentary material covered by layers of recent guano (Murphy, 1936), a substrate in which the penguins dig burrows. Offshore, the marine environment is best characterized by unpredictable fluctuations, with high variance in food supply for top-chain marine predators, especially during El Niño years (Arntz et al., 1991; Idyll, 1973).

Within the reserve there are several distinct Humboldt penguin colonies. The largest one consists of approximately 1100 breeding pairs of penguins (Zavalaga and Paredes, 1995); the remainder of the colonies mostly comprise 10–50 pairs of penguins. PT colony, the location of our study, is perched on a 20- to 25-m high cliff and consists of approximately 95 penguin burrows, although only 35 breeding pairs used these burrows.

Behavioral observations

During the first breeding peak in 1995 (from the first week in March until the end of July), behavioral observations of penguins at PT were conducted from a blind directly opposite the penguin colony approximately 20 m away. This unique viewing situation provided an excellent vantage point for watching all study nests without visual aides. However, we used a 15–60× spotting scope (Swift) to confirm all penguin identifications.

Individual penguins were recognizable by either flipper bands attached during previous studies or by a unique spotting pattern on the penguin's breast and stomach. The spotting pattern has been shown to remain constant from the first molt onward (Sholten, 1989; C. B. Zavalaga, personal communication).

Between 7 and 24 March 1995, we successfully identified more than 125 individual penguins. This included 26 pairs of birds that were observed throughout the breeding peak to determine the level of pair-bonding behavior and within-pair and extrapair copulations. As copulations are relatively rare events, we observed the penguin colony from dawn to dusk to maximize the number of copulations observed. Furthermore, one or two observers scanned the colony continuously throughout the observation period, specifically searching for copulations or events that were usually precursors to copulation (all-occurrence sampling; Martin and Bateson, 1986). The fact that most observed copulations exceeded 2 min suggests it is unlikely many copulations would have been missed using these observation procedures. We were not able to observe penguins at night. Consequently, our reported rates of within-pair and extrapair copulations will be underestimated insofar as copulatory activity occurs at night. However, there is no reason to expect a bias in estimating either type of copulation.

Pair-bonding in penguins involves several behaviors. We recorded the following pair-bonding behaviors, all of which often preceded mating attempts: mutual vocalizations, mutual preening, flipper patting, and bill dueling (Boersma, 1977; see Results). Mating attempts were classified as "incomplete" if the attempted copulation stopped after mounting but before cloacal contact (cloacal contact was presumed when the female lifted her tail feathers and the male arched his tail feathers toward the female's cloaca, apparently touching cloacas). On the other hand, they were considered "successful" if cloacal contact was achieved during the mating ritual. However, a significant proportion of the successful copulations may not have successful sperm transfer (Hunter et al., 1996).

Copulations were also classified as either within-pair or extrapair. Within-pair copulations occurred between pair-bonded individuals, defined as any two birds seen together and engaging in pair-bonding behavior on more than two occasions at the same location. EPCs were defined as copulations with an individual other than the pair-bonded mate (Westneat et al., 1990).

Based on 265 h of behavioral observations during the first breeding peak in March, no extrapair matings or mating attempts were witnessed after 0900 h nor before 1600 h. Therefore, between 7 and 26 August 1995, behavioral observations were only collected between 0530 h and 1000 h and between 1500 h and 1830 h.

Calculation of oviposition

Because Humboldt penguins are burrow nesters and tend to desert their nests if disturbed during the courtship and mating period, we did not repeatedly check nests to determine the exact day of oviposition. Instead, we used weekly culmen-length data collected on nonstudy birds in the same population (R. Paredes and C.B. Zavalaga, unpublished data) to calculate a first-order regression curve for culmen length against the chick's age ($y = 21.36x - 40.0$; $r^2 = .95$; SE of chick age determined from culmen length = 2.29). Using this regression equation, we calculated the chick's age, from which we subtracted 42 days, the mean incubation period at Punta San Juan (R. Paredes and C.B. Zavalaga, unpublished data). Although we do not have variance estimates on the mean incubation duration, there is evidence that penguins in general have an incubation period with a low variance (e.g., the gentoo penguin's incubation period is reported to be 35.3 ± 0.1 days, $n = 48$; Williams, 1990). Thus, we believe we provide a reasonable estimate of hatch date.

Paternity estimations

Blood collection

We collected blood from 21 penguin families (42 adults and 49 chicks). Due to either the extreme depth or narrowness of the burrows or to the failure of the clutch before hatching, only 15 pairs of birds included in the behavioral analysis were accessible for blood collection. Consequently, blood was also collected from six additional families of penguins for which no behavioral data were recorded. We captured birds by hand in their burrows or with the use of a pole with a small noose on the end (or a sigmoidal hook for chicks) and moved them out of the colony for sampling.

Birds were bled from the intradigital vein on the top of the foot, between the second and third digits (Cheney, 1993; G.D. Miller, personal communication) using a 23- or 25-gauge needle with a 3-cc syringe. We collected between 1 ml and 2 ml of blood from adult birds and between 0.1 ml and 0.2 ml from chicks. Occasionally, when no blood was obtained using the intradigital veins of the chicks, the metatarsal vein was used.

Blood was mixed with an equal volume of lysis buffer (Fleischer et al., 1994). As all adult birds that were bled were sighted at the burrow after handling, we presumed there were no substantial negative effects associated with handling birds.

Laboratory analyses

DNA was extracted from the blood samples, quantified, and digested using standard techniques detailed in Fleischer et al. (1994). Four micrograms of digested DNA were electrophoresed through a 1.0% gel in TBE buffer for 36 h at 47 volts and transferred to nylon filters (MSI-NT) by vacuum blotting (Pharmacia, LKB-VacuGene XL). Samples were probed with radiolabeled Jeffreys 33.15 and 33.6 probes (Jeffreys et al., 1985, 1991) following protocols given in Loew and Fleischer (1996). The membranes were washed up to three times under stringent conditions (20-min washes at 60°C with 250 ml of wash solution) and then exposed with intensifying screens at -80°C for 1, 4, 33, and 66 h sequentially (see Fleischer et al., 1994, for details).

Data analyses

We scored all bands between approximately 2 kb and 18 kb for all individuals. Gels were run with the chick DNA nested between that of putative parents and with any potential extrapair mates, as indicated by the behavioral data, run in adjacent lanes to putative family groups. A size marker was run in the outer lanes, and a line was drawn between fragments of the same size. No comparisons were made between gels due to the high level of variation in band mobility curves (Fleischer et al., 1995). We compared band sharing by marking all bands on a transparency film overlay with four permanent, colored ink pens (denoting paternal bands, maternal bands, shared bands, and nonattributable bands).

It is assumed that DNA fingerprinting bands are inherited according to Mendelian laws (Jeffreys et al., 1985). Therefore, nesting bands not found in profiles of either putative parent indicate a mutation in the offspring or a mistake in parent identification. To establish a criterion for excluding a putative male as father, we created a frequency plot of the number of offspring versus the number of nonattributable bands. This distribution did not differ significantly from a Poisson distribution ($p > .5$). Based on our expected distribution, we would have expected to see 1.15 offspring with 2 nonattributable bands and 0.094 offspring with 3 nonattributable bands (using our calculated mutation rate of 0.0145 mutations per band per generation). Thus, we established our criterion at a level similar to Westneat's (1990) and Lifjeld et al.'s (1993) criteria: at least one of the presumed parents was excluded as a parent if an offspring's DNA fingerprint profile had three or more nonattributable bands. In addition, we calculated the probability of assigning an unrelated male as father to be 1.18×10^{-4} with Jeffreys' 33.15 probe (Table 1).

To further confirm that these fingerprinting data could resolve paternities, the putative father of the family group was substituted with a presumably unrelated "sham" male, and the number of nonattributable bands was recounted (Fleischer et al., 1994). When a sham male is substituted into a monogamous family group, the number of nonattributable bands in the offspring's DNA profile should increase above the threshold used to resolve paternities (see above). Thus, we used this analysis to demonstrate our ability to detect extrapair copulations.

We calculated band-sharing coefficients (S; Lynch, 1988, 1991) between parent-offspring dyads, offspring-offspring dyads, and among presumably unrelated birds. The variance around the theoretical mean for first-degree relatives was calculated to determine 95% confidence intervals (CI) around the theoretical mean predicted for first-degree relatives.

Table 1
Results of analysis of penguin DNA fingerprints

Analysis	Result
Number of bands (f)	17.100
Mean proportion of bands shared by nonrelatives (x)	0.340
Mean allele frequency (q) ^a	0.171
Heterozygosity (h) ^b	0.991
Sibling band sharing (s) ^c	0.515
Expected number of maternal bands (m) ^d	8.718
Expected number of exclusively paternal bands (p) ^e	8.382
Probability of assigning an unrelated male as father (p_u) ^f	1.18×10^{-4}
Probability of assigning an uncle as father (p_r) ^g	3.84×10^{-3}

^a $x = 2q - q^2$ (Jeffreys et al., 1985).

^b $h = 2(1 - q)/(2 - q)$ (Georges et al., 1988).

^c $s = (4 + 5q - 6q^2 + q^3)/4(2 - q)$ (Georges et al., 1988).

^d $m = f(1 + q - q^2)/(2 - q)$ (Georges et al., 1988).

^e $p = f - m$ (Burley et al., 1996).

^f $p_u = x_p$ (Burley et al., 1996).

^g $p_r = s_p$ (Burley et al., 1996).

When comparing between S (i.e., first-degree relatives versus unrelated individuals), t tests were used, and each individual's DNA profile was used only once to maintain independence.

RESULTS

Pair-bonding behavior

The majority of pair-bonding behaviors occurred at the nest between 0 and 10 days before the first within-pair mating (mean = 1.77 ± 2.9 days, median = 1.0 day), with the four most common behaviors being mutual preening, mutual vocalization, bill dueling, and flipper patting. Mutual preening usually occurred after a bout of self-preening and consisted of one penguin using its bill to preen the other penguin's head or neck. Often the preened bird would reciprocate preening or continue to self-preen. Mutual vocalization and bill dueling often occurred when one member of the pair-bond returned to the nest. During mutual vocalization, the two birds faced each other, threw their heads back simultaneously, and commenced braying (similar to the well-documented "ecstatic call" that occurs in the *Pygoscelis* genus; Williams, 1995). Similarly, bill dueling displays also occurred when the pair reunited and the two animals, facing each other, moved their heads back and forth, banging their bills. Bill dueling was always followed by flipper patting.

Flipper patting was common and was solely a male behavior directed toward females. In all cases the male approached the female from behind and rapidly moved his flippers along her body. Twenty-one percent ($n = 33$) of the recorded flipper pattings led to the male rapidly rubbing his bill along the neck of the female, forcing her horizontal to the ground in the process, and ultimately mounting her.

Mating behavior

Despite pair-bonding behavior, not all within-pair copulation attempts succeeded; often the female rejected the male's initial attempts at mating or the mating was incomplete. Rejected mating attempts occurred when the female refused to lie on her ventrum, preventing the male from mounting her. As the study was not designed to collect detailed behavioral data, we were unable to accurately assess the proportion of male mating attempts that were refused by the female. However, incom-

Table 2
Frequency of within-pair copulations and extrapair copulations observed during the March and August study periods

	March	August	Total
Within-pair copulations	73 (2.81)	14 (1.75)	87
Extrapair copulations	15 (0.58)	4 (0.5)	19
Total	88	18	

The numbers in parentheses are the numbers of observed copulations divided by the total number of nests observed in March (26) and in August (8).

plete copulations, defined as a mating in which the male mounted the female but cloacas never touched, accounted for 26.3% of the within-pair copulation attempts which the female initially accepted.

In addition to copulations between bonded partners, 19 EPCs were witnessed. Therefore, the total number of completed matings observed during the course of this study was 106, of which 17.9% were EPCs (Table 2). Six of the EPCs involved males from one of the study nests; 13 involved focal females. The relative frequency of EPCs varied slightly between study nests (from one to three for females, and from one to two for males). Overall, 42.3% (11/26) of the bonded pairs observed had at least one mate participating in at least one EPC, either at the nest or away from it.

Timing of mating behavior relative to oviposition

Copulations that occur during a female's fertile period but before eggs are laid are more likely to result in fertilizations than those occurring either too early or too late. We therefore examined when copulations occurred relative to oviposition. Using the regression equation for nestling growth in culmen length (described in methods) and subtracting 42 days from each estimated hatch date, we found all observed extrapair and within-pair copulations occurred before the estimated oviposition date calculated for individual nests.

Five study females laid their eggs at the end of our observation period. For these birds we failed to detect a difference in the timing of within-pair copulations (11.7 days prior to egg laying ± 5.51) compared to the timing of EPCs (13.7 days prior to egg laying ± 4.46 ; Figure 1), but had low power to detect a difference ($\beta < 0.5$). In all cases except one (nest

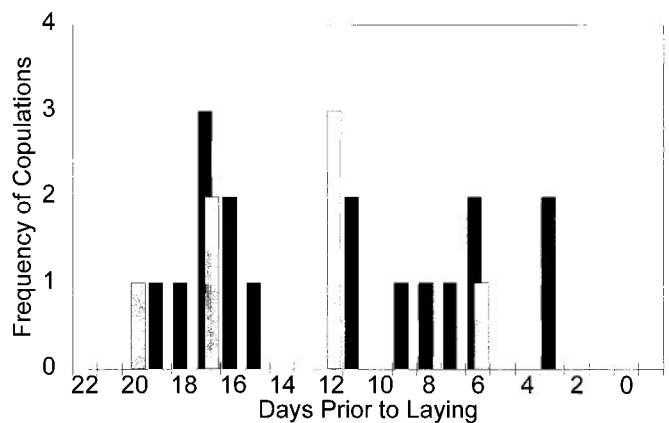


Figure 1
Temporal distribution of extrapair (light bars) and within-pair (dark bars) copulations for all nests where the estimated oviposition date is within the observation period.

104), the pair male was the last one to copulate with his female. The last observed within-pair copulation occurred on average 9.8 days (± 5.17) before the estimated oviposition of the first egg.

Other patterns of EPCs were examined to determine if any trends in the timing of within-pair versus extrapair copulation could be established. Two of the 15 (13.3%) females participated in EPCs before any recorded pair behavior with their ultimate mate. In one other case (nest 133, second breeding peak), after 5 days of pair-bonding behaviors (without observed copulation) with her mate from the previous breeding peak, the female participated in an EPC. A within-pair mating did occur subsequently.

Seasonal differences

Because approximately half of the Humboldt penguins at Punta San Juan breed twice within one year (C. B. Zavalaga, personal communication), we had the opportunity to examine differences that may exist between the two breeding peaks. Of the 26 pairs that were observed in March, 30.7% ($n = 8$) laid a second clutch in August. Seventy-three of 87 (83.9%) within-pair copulations recorded during the two breeding peaks were recorded in March. However, because observations in August were made only on those animals that laid a second clutch and observation periods were shorter, we standardized these data for the number of nests observed and by observer effort. Thus, we compared only afternoon and evening sessions during those days that the nest was active in March and August. This reduced the proportion of copulations in March (relative to August) to 65.9%.

There was no significant difference in the number of within-pair copulations observed in March (0.29 ± 0.41 within-pair copulations per day per nest) compared to August (0.15 ± 0.12 within-pair copulations per day per nest; Wilcoxon, $p = .44$, $n = 8$). None of the birds engaged in EPCs in both March and August, nor was the overall frequency of EPCs different for the two time periods (Wilcoxon, $p = .57$, $n = 8$). However, due to the extremely small sample size and hence the ability to detect a significant difference, the power of the Wilcoxon test was calculated and determined to be low.

Distribution of matings

All observed copulations except one occurred at a burrow. The one exception took place on the beach where animals stage before going to sea to preen and feed. Not all within-pair copulations occurred at the pair's burrow, although most (89.2%) of the 83 within-pair copulations from the 26 study pairs occurred at the home burrow. Because pairs often engage in multiple copulations, to test whether within-pair copulations occurred significantly more often at the home burrow, a Wilcoxon paired-sample test was performed ($p < .01$). Interestingly, 66.7% (6/9) of within-pair copulations away from the home burrow occurred at an adjacent burrow, while others occurred more than one nest away (Table 3). In contrast to within-pair copulations, 68.4% (13/19) of EPCs tended to occur away from the focal animal's burrow (Table 3). Furthermore, only 1 of these 19 matings occurred at an adjacent burrow, the remainder being at least two burrows away.

EPCs might be expected to occur in some parts of the colony more than others (e.g., opportunities for EPCs might be greater for birds in those areas near the ocean, where all birds need to travel for food). To further examine the distribution of EPCs within the colony, we divided the colony into north, central, and south sections, in accordance with natural breaks in the topography. Penguins did not preferentially engage in

Table 3

The distribution of within-pair and extrapair copulations relative to the home burrow

	At home burrow	One burrow away	More than one burrow away
Within-pair	74	6	3
Extrapair			
Females	1	0	12
Males	5	1	0
Total	6	1	12

Four within-pair copulations were not used for this analysis because information on their location was not collected.

EPCs in one particular area of the colony versus another (G test, $p = .25$).

Similarly, EPCs did not tend to occur more frequently with individuals from nests along the path leading from burrows to the ocean than those from nests off the path (G test, $p > .5$). Female EPCs had the same probability of occurring at burrows in their path to the ocean (50%, $n = 12$) as in the other direction, at burrows located away from this path.

To determine if there was a sex difference in location of EPCs, we used a Fisher's Exact test to examine the association between the sex of the animal engaging in an EPC and the location of the copulation. Most individuals engaged in only one EPC, which could be easily classified, as either at burrow or away from burrow. One female had both an at-burrow and an away-from-burrow EPC. Regardless of whether that female's copulations were classified as at burrow or away from burrow, female EPCs occurred away from their burrows (92.3% of EPCs), whereas focal male EPCs occurred at their burrows (83.3% of EPCs; $p = .032$).

DNA fingerprinting to assess parentage

DNA fingerprinting was completed on a total of 21 family groups, with a mean of 2.4 ± 1.3 offspring per year (1.7 ± 0.47 offspring per clutch), to determine whether putative parents were the actual parents. Putative parents were identified for 15 of these families using the extensive behavioral data collected at colony PT. Six additional families from a smaller colony for which behavioral data were not available were included. For these families, adults were sampled sequentially while at the nest and presumed to be the parents.

The DNA fingerprints produced from Jeffreys 33.15 probes had a mean of 17.8 ± 4.23 bands scored for adults (males, 18.8 ± 4.1 ; females, 16.9 ± 4.1 ; t test, $p = .14$, $n = 21$) and 16.8 ± 2.8 bands scored for offspring. For the second probe, Jeffrey's 33.6, a mean of 19.6 ± 1.23 scorable bands were produced between 2 kb and 18 kb, with no significant differences in the number scored for adult males and females or adults and offspring (t test, $p > .5$, both cases).

Jeffreys 33.15 produced an S of 0.34 ± 0.15 among presumed unrelated individuals ($n = 126$, 95% CI, 0.17–0.52) and was not significantly different using pairings of presumed unrelated individuals from different age–sex classes (Table 4). The S between parents and offspring and between full-siblings did not differ from the theoretical S for related animals (Lynch, 1991; Table 5).

In most cases the DNA fingerprints of the putative parents accounted for all of a nestling's bands. However, to ensure that this result only occurred with the parent–offspring combinations, the analysis for nonattributable bands was redone

Table 4
Mean band-sharing coefficients between presumed unrelated individuals calculated from using Jeffreys probe 33.15 with standard deviations and sample sizes

Unrelated individuals	Mean	SD	<i>n</i>
Male–male	0.34	0.24	10
Female–female	0.34	0.13	11
Male–female	0.34	0.16	18
Mates	0.37	0.12	21
“Sham” male–offspring	0.38	0.08	49
Total unrelated individuals	0.34	0.15	

with the putative male being substituted with a presumed unrelated “sham” male. The number of nonattributable bands produced for the putative parents–offspring analysis was significantly lower than the number of nonattributable bands produced for the sham male (plus parental female)–offspring analysis (*t* test, $p < .001$, $\{x\}_{\text{real}} = 0.26 \pm 0.49$, $\{x\}_{\text{sham}} = 4.23 \pm 2.05$; Figure 2A versus 2B). Hence resolution between putative males and extrapair males was possible.

There were five cases where *S* of chicks and sham males were outside the upper limit of the 95% CI surrounding the mean for unrelated individuals (Figure 2b). In four of these cases, the offspring were from the same nest. Therefore, two additional sham males were substituted sequentially for the original sham male, both times significantly reducing *S* to well within the 95% CI. The *S* between the original sham male and the putative father was found to be high (0.88), strongly suggesting a first-order relationship. Parsimony suggests that the putative father is the biological father. The four offspring–putative father comparisons produced only one nonattributable band and displayed band-sharing coefficients between 0.82 and 0.83, whereas the apparently related sham male produced 14 nonattributable bands (in total), despite its high degree of similarity to the offspring.

When examining putative fathers and offspring, two points were above the upper limit of the 95% CI for the theoretical *S* of first-degree relatives. In both cases, we considered the offspring to be products of within-pair fertilizations because *S* with the putative male is higher than with the sham male, and the number of nonattributable bands with the putative family is lower. However, we used a second and third sham male to test these dyads. Again, there were more nonattributable bands and a lower *S* with the sham males than with the putative male, failing to provide evidence for exclusion of the presumed father. There was only one family group that had two nonattributable bands in one chick (Figure 2A). We used Jeffreys 33.6 to reexamine family groups in which chicks displayed two nonattributable bands with Jeffreys 33.15. The mean *S* between unrelated individuals was 0.94 ± 0.03 ($n = 25$) and between first-degree relatives was 0.98 ± 0.02 ($n = 28$). Although these means are significantly different (*t* test, $p < .005$), this probe is not useful to exclude presumed parents. Given the high *S* between unrelated individuals once maternal bands are accounted for in an offspring’s DNA fingerprinting profile, on average less than one band remained for use in paternal identification (well below the exclusion criteria of three bands). Therefore, Jeffreys 33.6 does not provide necessary data needed to identify putative males.

Comparison of EPC and EPF rates

The failure of EPCs to yield EPFs might be explained by the proportional representation of sperm favoring the pair-bonded male, due to multiple copulations by the pair-bonded male.

Table 5
Mean band-sharing coefficients between presumed first-degree relatives calculated from using Jeffreys probe 33.15 with standard deviations and sample sizes

Related individuals	Mean	SD	<i>n</i>
Female–offspring	0.68	0.13	21
Male–offspring	0.68	0.11	21
PT colony, parent–offspring	0.69	0.09	30
S3 cave colony, parent–offspring	0.67	0.13	12
Theoretical ^a	0.67 ^b		

No significant differences were detected between any of these pairings.

^a The theoretical band-sharing coefficient is based on Lynch (1991).

^b The 95% confidence interval for this value is 0.51–0.83.

Therefore, we tested to see if the rate of within-pair copulation is significantly different from the rate of EPC for each pair of penguins. Within-pair copulations occurred a greater number of times than EPCs (Wilcoxon, $p = .002$), yet the proportion of extrapair to within-pair copulations was different from the proportion of EPFs to within-pair fertilizations (proportions test, $p < .05$).

DISCUSSION

Frequency of extrapair copulations and fertilizations

This study has shown that Humboldt penguins, despite forming pair-bonds, are not strictly monogamous in their mating behavior, as 19.2% of the study males and 30.7% of the study females engaged in EPCs (affecting a total of 42.3% of the nests). None of these EPCs apparently resulted in EPFs. We were able to assess paternity for 21 families and 49 offspring. The *S* among unrelated Humboldt penguins was 0.34 and 0.94 for Jeffreys 33.15 and Jeffreys 33.6, respectively. Therefore, only the Jeffreys 33.15 data were useful for paternity analysis. Assigning paternity based on both *S* and the presence of nonattributable bands was straightforward for most of the offspring.

Since molecular genetic techniques became more accessible to behavioral ecologists, numerous studies have shown that behavioral observations alone may not be a good indicator of an individual’s reproductive success (Yamagishi et al., 1992). In most cases the observed level of EPCs (Liffeld et al., 1993; Møller, 1987; Westneat, 1987a,b) has drastically underestimated the actual level of EPFs. Our finding of a high rate of EPCs, but no evidence of these yielding fertilizations is highly unusual. Only one other study has reported such results. Hunter et al. (1992) showed that in another seabird, the Northern fulmar (*Fulmarus glacialis*), 2.4% of all copulations involved an extrapair male (16% of the breeding females participated in EPCs), with EPCs occurring throughout the breeding cycle. However, none of these copulations led to EPFs.

Prelying copulation hiatus

The large temporal gap we found between the last breeding attempt by a pair and the oviposition of the female is consistent with the idea that long-term sperm storage occurs in some seabirds (Hatch, 1983; Birkhead and Møller, 1992). Although there is a possibility that this long interval is an artifact of the back-calculation method we used to estimate the date of egg laying, or an artifact of a small sample size, these results are consistent with several other seabird studies. Imber (1976) reported that the grey-faced petrel (*Pterodroma macroptera*)

laid fertilized eggs 2 months after copulation, a presumed adaptation allowing for extremely long prelaying absences. Hatch (1987) reported similar results for northern fulmars, and A. Chiaradia (personal communication) reported similar results for little blue penguins (*Eudyptes minor*).

If sperm is stored and released sequentially, then the last male to copulate with the female has an advantage over previous males. However, if sperm is released simultaneously, mixing can occur, and the likelihood of fertilization will be based on the relative proportion of each male's sperm in the female's reproductive tract (Hunter et al., 1992). The fact that we found no EPFs, but saw EPCs, and in all but one case the pair-bonded male was the last to copulate with a female, suggests a recency effect in sperm storage in Humboldt penguins.

Female control of mating

Westneat et al. (1990) suggested that females act in one of three ways with respect to EPCs. They either actively solicit, apparently resist, or passively accept EPC attempts from the male. Although penguins are capable of severe fights leading to major injury or occasionally death, in this study females rejected copulations from both extrapair males and their mates without any physical punishment, suggesting that female cooperation is essential for mating to occur.

The majority (89.2%) of Humboldt penguin within-pair copulations occur at the home burrow, yet this trend does not hold true for EPCs. When separated by sex, it becomes apparent that EPCs by males tend to occur at their nest. Females, however, conduct 92% of their EPCs away from the nest. They show no pattern to where they mate away from the nest. For example, they do not tend to mate with males along the path to the ocean, as one might expect if females were simply being coerced or attracted by males occupying nests along travel routes.

Female solicitation of EPCs has been reported in only a few species of birds (Birkhead et al., 1990; Fujioka and Yamagishi, 1981; Hatch, 1987; Møller, 1990; Smith, 1988; Venier et al., 1993). Venier et al. (1993) suggested that tree swallow females solicit EPCs because those copulations that occurred when females visited neighboring nests resulted in more fertilizations than copulations initiated by males. Hatch (1987) found female northern fulmars not only soliciting extrapair matings, but also visiting numerous nests and engaging in pair-bonding behaviors.

Westneat et al. (1990) argued that soliciting EPCs costs little for some species, such as female northern fulmars. Northern fulmars are a synchronous breeding species, producing a single egg per year, and require biparental care to successfully fledge their offspring. If a male discovers that his female has engaged in an extrapair mating, it is unlikely that he will re-nest in that year because all females will have already initiated breeding. His choice then is either to abandon the clutch for the year, guaranteeing that the offspring at the nest will perish, or to provide parental care to offspring of uncertain paternity. If he has copulated multiple times with his pair-bonded female, the probability of the offspring being his may be greater than the probability of it being the extrapair male's, and hence he may still provide parental care.

Humboldt penguins differ from northern fulmars in that the breeding cycle is not as synchronous, and males may be more likely to abort the clutch and remate if they discover their pair-bonded female has participated in an EPC. Despite this, female Humboldt penguins solicited extrapair matings, suggesting some net benefit to these matings that outweigh the added risks of being abandoned by her mate.

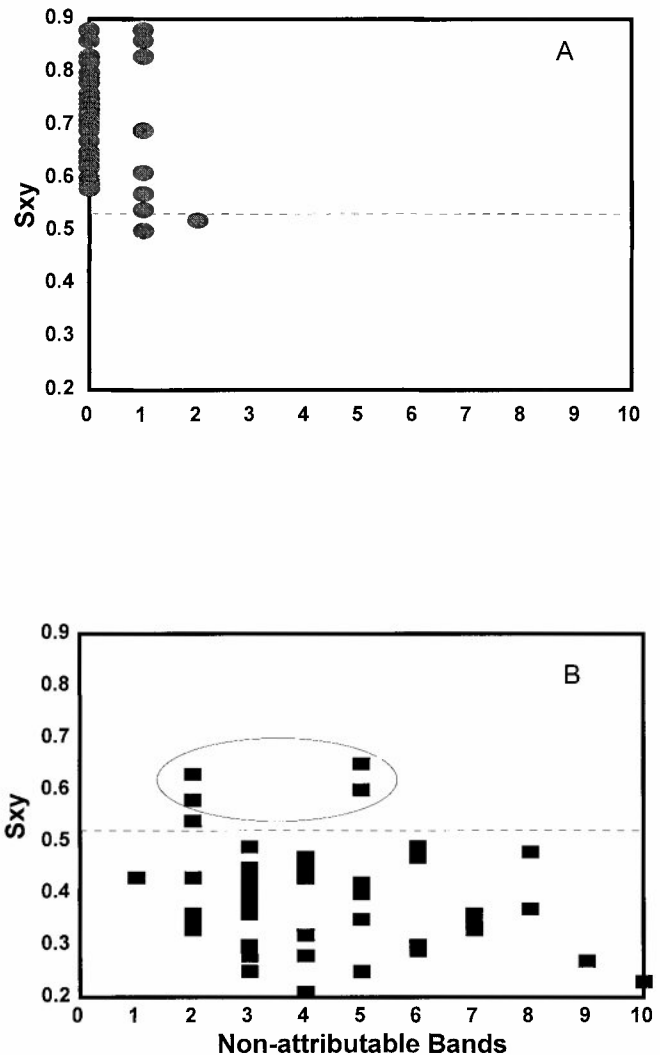


Figure 2
(A) The relationship between band-sharing coefficients from DNA fingerprinting between 49 chicks and their putative fathers (determined by behavioral data) and the number of nonattributable bands in chicks. The dotted line is the lower limit of the 95% confidence interval for unrelated individuals (determined theoretically from unrelated individuals). (B) Distribution of the 49 chicks that were DNA fingerprinted versus "sham" males. The dotted line is the upper limit of the 95% confidence interval for unrelated individuals. The oval encloses a family of four chicks (see text for details).

Hypothesis addressing costs and benefits to female penguins

Several hypotheses about benefits that females might acquire from extrapair mating have been suggested for other species. However, the majority of these hypotheses require the existence of EPF, which we did not find with Humboldt penguins. Therefore, hypotheses that state that females will seek EPCs to (1) increase the genetic diversity of the brood or (2) obtain better genes from an extrapair male (sexy-son hypothesis) or to induce sperm competition, seem implausible as no EPFs were found in this study. Two additional hypotheses, which better explain the results of this study, are presented below in detail.

Colwell and Oring (1989) suggested that EPCs might be used for appraisal and acquisition of future mates. They found that female spotted sandpipers (*Actitis macularia*) engaged in

extrapair mating after their clutch was completed, suggesting the function of this behavior was not for current fertilization. Subsequently, in the next breeding season, the spotted sandpiper females paired with males with whom they had previously engaged in EPCs. Wagner (1991) found that EPCs in razorbill (*Alca torda*) females occurred during infertile periods, again suggesting EPC is used for future mate appraisal and acquisition. Wagner also argued that the use of EPCs for mate appraisal is not uncommon and can be applied to other published studies (e.g., Fujioka and Yamagishi, 1981).

Females using copulation to assess males should participate in many EPCs with multiple males. Furthermore, Wagner (1991) predicted that females using this strategy would position themselves to receive EPCs, but would successfully resist insemination. Our data are somewhat consistent with this; 25% of the females copulated with multiple mates, and these copulations did not lead to fertilizations. However, at least 33% of the female Humboldt penguins that were in a pair-bond for multiple seasons still participated in EPCs and began breeding with the same mate the year after this study (C.B. Zavalaga and R. Paredes, personal communication). If penguins were using EPCs to appraise future mates, we would have expected to see some mate switching between March and August. Yet, no females that sought EPCs in the March breeding peak changed mates in the subsequent August breeding peak.

In both the Colwell and Oring (1989) study and the Wagner (1991) study, the EPCs were done after the breeding cycle. This was not the case for Humboldt penguins. While this alone would not preclude the mate appraisal and mate acquisition hypothesis, the combination of data suggest that mate appraisal alone does not explain EPCs in Humboldt penguins.

It is possible that EPCs are not the product of selection. They may occur simply because they are not selected against. However, if this were true, we would expect that male and female Humboldt penguins would participate in EPCs in a random pattern around the colony and their nests. In this study females appeared to solicit extrapair matings away from their nest, while males stayed at their nests, suggesting it is not an epiphenomenal event. It is possible that without selection, the male's breeding system drifted in one direction (waiting at the nest to solicit extrapair mating), while the female's breeding system drifted in another (roaming the colony to seeking extrapair mating). Hence EPC as an epiphenomenon is still a likely explanation for these data.

In summary, EPCs occur in Humboldt penguins and appear to be under female control. Although no single hypothesis alone explains our results, our data best support the hypotheses that female Humboldt penguin use EPCs either to appraise future mates or that this behavior is epiphenomenal.

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