
Molecular Evidence for Extrapair Paternity and Female-Female Pairs in Antarctic Petrels

SVEIN-HÅKON LORENTSEN,1,4 TROND AMUNDSEN,2 KRISTIN ANTHONISEN,3 AND JAN T. LIFJELD3
1Norwegian Institute for Nature Research, Tungasletta 2, N-7485 Trondheim, Norway; 2Department of Zoology, Norwegian University of Science and Technology, N-7491 Trondheim, Norway; and 3Zoological Museum, University of Oslo, Sars gate 1, N-0562 Oslo, Norway

Considerable interspecific variation exists in the frequency of extrapair fertilizations (EPFs) in birds. In general, EPFs are more common and occur at higher frequencies in passerines than in nonpasserines (Westneat and Sherman 1997). Lower rates of EPFs are typical for territorial nonpasserines as well as those that breed colonially (Westneat and Sherman 1997). This seems to contradict Birkhead and Møller’s (Birkhead and Møller 1992, Møller and Birkhead 1993) hypothesis of intense sperm competition in colonial birds. Their arguments were based on the assumption that the need for nest defense in dense aggregations restricts the ability of males to guard their mates, and that the high number of potential extrapair mates available in colonies selects for a high rate of extrapair copulations (EPCs).

In contrast, Westneat and Sherman (1997) found no correlation across species between the frequency of EPFs and nesting dispersion, local breeding density, or breeding synchrony, although EPFs were related to nesting density within species. This suggests that EPC rates are not informative regarding EPF rates in colonial birds (Westneat and Sherman 1997), or that the pattern reported by Møller and Birkhead (1993) does not hold true when more species are included. The conflicting evidence regarding the relationship between extrapair activities and breeding density calls for more empirical studies, especially among colonial nonpasserines.

Social monogamy is the predominant mating system in the Procellariiformes (Warham 1990). Several aspects of their breeding biology may, however, provide favorable opportunities for extrapair sexual activity. First, colonial breeding provides ample opportunities for EPCs because many potential partners are available at close range (Birkhead and Møller 1992, Møller and Birkhead 1993). Second, when the sexes are spatially and/or temporally separated, as may be the case in procellariiforms where

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4 E-mail: shl@ninatrd.ninaniku.no
adults seek food far from the colony, males have few cues to assess whether their mates have been unfaithful. Hence, few reasons exist to expect a facultative decrease in male parental investment if cuckolded, in contrast to the case for many territorial species where a male may have more reliable cues to his mate's unfaithfulness (e.g. female disappearance, high intrusion rate, etc.). Accordingly, colonial breeding may facilitate EPCs for both sexes, and colonial species may be expected to display high rates of EPC.

Paternity studies require error-free sex determination of adults. This is straightforward for clearly dimorphic species, or if sex determination can be done from genitalia during the fertile period. But if fieldwork can be performed only during the nestling period, sex determination may be problematic for largely monomorphic species, and indirect methods must be used.

In the Antarctic Petrel (Thalassoica antarctica), the two sexes differ slightly in mean body size. Lorentsen and Røv (1994) used this difference to determine the sex of Antarctic Petrels by discriminant function analysis (DFA). The procedure correctly determined the sex of 92% of the birds in a sample from the same year. This does not necessarily imply a similar resolution if the discriminant function is adopted for samples from other years, or if data are collected by other observers. Moreover, although useful for many purposes, 92% resolution in sex determination is insufficient for paternity studies. Therefore, we performed molecular sexing of all breeding adults according to the PCR-based method of Griffiths et al. (1998).

The main aim of our study was to analyze whether extrapair paternity occurs in a colonial procellariiform, the Antarctic Petrel. We also tested the robustness of morphological sex determination (from DFA) across seasons relative to that obtained from molecular techniques.

**Study area and methods.**—The study was conducted at Svarthamaren (71°53'S, 05°10'E), Mühlig-Hofmannfjella, Dronning Maud Land, Antarctica, during the austral summer of 1993–1994. About 250,000 pairs of Antarctic Petrels bred on a northeast-facing mountain slope at 1,650 m elevation, more than 200 km from the nearest open sea (Mehlum et al. 1988, Røv et al. 1994). Antarctic Petrel nests were relatively evenly spaced at a density of 0.76 nests per m² (Mehlum et al. 1988). The topography of the colony was relatively flat and open. Thus, neighboring pairs often interfered with each other, indicating that many opportunities for EPCs were available.

The Antarctic Petrel is an open-nesting, medium-sized petrel (500 to 675 g) and lays a single egg after a prelaying exodus of 20 to 23 days (Pryor 1968, Luders 1977). Both parents incubate (Lorentsen and Røv 1995), and hatching occurs synchronously within the colony in the middle of January (Haftorn et al. 1991, Amundsen et al. 1996). After the egg hatches, parents brood their chick constantly for 9 to 13 days (Bech et al. 1988, Røv et al. 1994) before it is left unattended. Chicks at Svarthamaren are fed by both parents, which on average return from feeding areas in the open sea every second day with 80 to 250 g of food (Haftorn et al. 1991, Lorentsen 1996).

We marked 75 nests containing an egg on 5 January 1994. For 42 (56%) of these nests, we obtained blood samples for DNA fingerprinting from both parents and the chick. We also obtained blood from seven additional pairs that were used for molecular (and morphological) sex determination. The adult on duty was banded, measured (wing length, ± 1 mm; head plus bill length, ± 0.1 mm; bill depth, ± 0.1 mm), and dyed on the back with highly diluted picric acid. Measurements were taken by the same person as in 1992 (N. Røv) to control for effects of the measurer (Lorentsen and Røv 1994) and hence, to specifically test for temporal reliability of morphological sex determination.

We collected about 50 µL of blood in capillary tubes and immediately suspended the sample in 1 mL of Queen's lysis buffer (Seutin et al. 1991). When the mate returned to the nest, the same procedure except dyeing was repeated. All adult birds were caught during incubation or early in the brood-rearing period. Blood samples were collected from chicks a few days after hatching, using the same methods as for adults. The samples were deep frozen until analysis. The sex of adults was determined using the discriminant function of Lorentsen and Røv (1994), and by molecular techniques (see below). When using the discriminant function, the pair member with the lowest discriminant score was considered to be the female.

DNA isolation, gel electrophoresis, and Southern blotting followed the protocol of Krokene et al. (1996), with some minor adjustments (see Bjørnstad and Lifjeld 1997). Briefly, DNA was isolated through a standard procedure of proteinase K digestion, phenol/chloroform washes, and isopropanol precipitation. DNA was cut with the restriction enzyme HaeIII, electrophoresed in 0.8% agarose in 0.8% agarose with TBE-buffer, and blotted onto Hybond N+ nylon membranes according to the manufacturer's protocol. The minisatellite probe per (Shin et al. 1985) was radioactively labeled with Redivue (α-32P)dCTP (Amersham) using the Prime-a-Gene labeling kit (Promega). The hybridization procedure followed the manufacturer's protocol for the Hybond N+ membrane. Filters were autoradiographed with or without an intensifying screen at −80°C for one to seven days using Kodak BioMax MS film.

Scoring of fingerprint bands was done by marking each nestling band on an acetate overlay with a specific color according to whether a similar band was in the fingerprint of the father, the mother, both, or neither of them. Bands not present in either parent,
i.e. novel bands, may arise through scoring error, mutation, or mismatched parentage. Scoring was carried out by KA and JTL. Only a few disagreements occurred between scorers (ca. 20 bands), and in these cases the scorings of JTL were used. Because mutations of minisatellite DNA typically occur randomly at a frequency of 1 in 100 to 300 bands (e.g. Burke and Bruford 1987, Westneat 1990, Lifjeld et al. 1993), chick fingerprints normally will contain none or only a few mutated bands, depending on the number of bands. In the present study, nestling fingerprints contained either 0 to 1 or 8 or more novel bands. We consider cases of a single novel band to be the result of mutation, and those with eight or more novel bands to result from mismatched parentage.

Band sharing was calculated by the formula of Wetton et al. (1987). Parentage was excluded when band sharing was lower than the lowest value recorded for chicks related to both parents and within the limits for band sharing between mates, which we assume are genetically unrelated.

The sex of adults was determined by PCR amplification of the CHD-Z and CDH-W genes on the sex chromosomes (Z and W) using the primer pair P2 and P8 described by Griffiths et al. (1998). These primers typically amplify one band in males and two bands in females, because the female is the heterogametic sex in birds. Analyses were out carried according to the protocol of Griffiths et al. (1998), except that we radioactively labeled the P2 primer with (gamma 32P) ATP (Amersham) and electrophoresed the products on 6% polyacrylamide gels. Products were visualized by autoradiography.

Results.—On average, we counted 30.9 ± SE of 0.9 (range 17 to 43, n = 42) scorable bands per nestling; 38 nestlings (90%) had a fingerprint profile that matched (0 to 1 novel bands) both putative parents. Band-sharing coefficients averaged 0.57 ± 0.01 (range 0.37 to 0.72) with the putative mother and 0.58 ± 0.01 (range 0.39 to 0.73) with the putative father, and we considered 0.37 to be the lower limit for band sharing with a genetic parent.

The remaining four nestlings had between 8 and 14 novel bands (Fig. 1). Each chick had high band sharing with one parent (range 0.40 to 0.77) and low with the other (0.15 to 0.19). The latter values fall within the range of values observed for paired birds (0.00 to 0.28; \( \bar{x} = 0.13 \pm 0.01, n = 42 \)). The putative father was excluded from being the genetic father in three cases, and the excluded parent was one of two female parents in the fourth case. In summary, 3 of 41 nestlings (7%) resulted from EPFs (excluding the young of the female-female pair).

We did not actively seek among fingerprinted males to find possible extrapair fathers. However, we happened to find the genetic father of one of the extrapair chicks among the males fingerprinted on the same gel: he was the male that bred at the closest of the neighboring nests. This male also was the father of the chick in his own nest.

Mean discriminant scores based on morphology were 0.97 ± SD of 1.06 (range -2.06 to 2.93, n = 49) for individuals assessed as males and -1.05 ± 1.11 (range -3.11 to 1.18, n = 49) for individuals assessed as females. Discriminant scores from 1992 (Lorenzen and Rov 1994) did not differ significantly from those of 1994 (males, \( t = 1.47, df = 116, P = 0.14 \); females, \( t = 0.97, df = 103, P = 0.33 \)).

Using molecular sexing, the P2/P8 primers gave one band (ca. 350 base pairs) present in all adults (n = 98) and a slightly larger band present in 51 adults. Hence, this pattern agrees with the sex-specific pattern described by Griffiths et al. (1998) that individuals with one band are males, and those with two bands are females. The female-specific band was the larger one. Among the 49 pairs analyzed, two (4%) consisted of two females.

The DFA correctly determined the sex of 88% of the males and 92% of the females (\( \bar{x} = 90\% \)). Discriminant scores for one of the two female-female
pairs assessed by the molecular method clearly indicated that both were females. For the other pair, discriminant scores indicated that the pair members were of opposite sex. The mean discriminant score of males whose sex was determined incorrectly (-0.09 ± 1.15) was significantly lower than that of males whose sex was determined correctly (1.09 ± 0.96; t = 2.32, df = 45, P < 0.05). For females, mean discriminant scores for the two groups did not differ significantly (-0.43 ± 1.59 and -1.10 ± 1.08 for incorrect and correct, respectively; t = 1.48, df = 49, P = 0.15).

Discussion.—The main findings of our study were: (1) three nestlings (7%) were sired by extrapair males; (2) two pairs (4%) consisted exclusively of females; and (3) DFA based on morphological measurements correctly determined the sex of 88% of the males and 92% of the females, as confirmed by molecular sexing.

Our study is the second to demonstrate extrapair paternity in a species of procellariiform. In the Short-tailed Shearwater (Puffinus tenuirostris), Austin and Parkin (1996) found that 11% of the chicks were sired by extrapair males. This value is close to that found in our study of Antarctic Petrels (7%). Studies of three other procellariiforms (Cory's Shearwater [Calonectris diomedea], Swartschek et al. 1994; Leach's Storm-Petrel [Oceanodroma leucorhoa], Mauck et al. 1995; Northern Fulmar [Fulmarus glacialis], Hunter et al. 1992) failed to find evidence for EPFs. This does not necessarily imply an absence of extrapair sexual relationships. In Northern Fulmars, 2.4% of all copulations were extrapair, but these apparently were unsuccessful in fertilizing the eggs. Taken together, it seems clear that extrapair paternity is relatively infrequent among procellariiforms, including the Antarctic Petrel. However, the apparent absence of EPFs in certain species should be interpreted cautiously because of the limited power in detecting rare EPFs with small sample sizes.

The relatively low incidence of EPFs found in procellariiforms is inconsistent with the prediction of high rates of EPC and EPF for colonial birds as suggested by Birkhead and Møller (1987, Birkhead and Møller 1992, Møller and Birkhead 1993). Several reasons may account for this apparent contradiction. First, increasing density (i.e. colonial breeding) increases the encounter rate with other males and at the same time often prevents males from guarding their mates. Hence, colonial species generally have high levels of within-pair copulations (Birkhead et al. 1987; Møller and Birkhead 1991, 1993), which may serve as an effective paternity guard (Møller and Birkhead 1991, Hunter et al. 1992). Second, a large potential for sperm competition may not imply that sperm competition actually occurs. For instance, Wagner (1991, 1992) found evidence for female control of EPCs in Razorbills (Alca torda). This was achieved by females resisting EPCs during the fertile period, allowing EPCs when the probability of fertilizing the egg was low during the prelaying period, and possibly by expelling extrapair sperm. Thus, empirical studies on colonial seabirds suggest that EPC rates are low and that EPFs can be ineffective in fertilizing eggs (cf. Scwhartz et al. 1999).

We found the genetic father of one of the extrapair chicks to be the male at the closest neighboring nest. This male also was the genetic father of the chick in his own nest, so we know that his social status at the neighboring nest was determined correctly. Neighborhood males often are the genetic fathers of extrapair offspring (Björklund and Westman 1983, Alatalo et al. 1984, Westneat 1990, Yezerinac et al. 1995, Johnsen et al. 1998).

If a surplus of females exists in the population, "pairing" with another female may be a favorable strategy for some females because it allows them to get established in the colony. The experience with a site gained through forming a female-female pair may greatly improve chances for future successful breeding for the non-genetic parent. Female-female pairs are frequently observed in gull and tern populations with female-biased sex ratios (Nisbet and Hatch 1999). Despite the short-term costs, joining another female and taking care of her young may lead to increased fitness.

Austin and Parkin (1995) found that the majority (75%) of unrelated adults caught in nest burrows of Short-tailed Shearwater were females. They attributed this finding to breeding birds entering the wrong burrow, or prebreeding or failed breeders entering burrows at random. Similarly, Swartschek et al. (1994) found that about 30% of all Cory's Shearwaters captured entering burrows at night were nonbreeders or unrelated breeders entering the wrong burrows. Approaching alien nests apparently is not uncommon in procellariiforms, especially those that enter their burrows at night. Such behavior can be a means of prospecting for future breeding sites. However, a considerable difference exists between burrow-nesting procellariiforms where prospectors might have to enter the burrow to check whether it is occupied, and open-nesting species (like the Antarctic Petrel) where prospectors can observe nest sites from a distance. In our study, we could not completely rule out that the two female-female pairs included a female that only visited the nest for a short time period because we only handled the adults when blood samples were taken. On the other hand, blood samples were taken during late incubation or early in the brood-rearing period, at which time it is relatively unlikely that alien individuals should take over. Moreover, the unrelated females provided care (incubation and/or brooding) for the chick, a type of behavior that would not be expected from prospectors.

Agreement between the results from DFA and the
molecular sexing procedure confirms that DFA provides a relatively reliable means of assessing the sex of individuals (Lorentsen and Røv 1994). The relatively low discriminant scores of incorrectly classified males, and the relatively high scores of incorrectly classified females, show that sex determination by DFA of morphological data may fail for small males and large females. Also, molecular sexing revealed two female-female pairs. Although DFA may detect such cases, a general assumption when using such tools is that pair members are of opposite sexes. Consequently, if female-female pairs are a regular phenomenon, as observed in several gull and tern species, then results from DFA must be interpreted with caution.

Our findings are in line with the statement of Westneat and Sherman (1997) that EPF rates generally are low for colonial birds. Infrequent extrapair fertilizations may be due to little or inefficient extrapair sexual activity. EPCs may be infrequent among many colonial seabirds because of difficulties in assessing the quality of potential extrapair partners. Moreover, the few cases of EPC may have limited influence on realized paternity because of efficient paternity guards, or because females selectively reject sperm from such copulations. The finding that both adults attending two nests were females suggests that prospecting females use “adoption” of eggs or chicks laid by other females as a means of establishing themselves in the colony. Further studies are needed to answer whether such adoptions are only short term, or whether they include taking on full parental duties, including the feeding of nestlings until independence.

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