

MOLECULAR EVIDENCE FOR TWINNING IN WEDDELL SEALS (*LEPTONYCHOTES WEDDELLII*)

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We used microsatellite genotyping to determine the genetic relatedness of 7 apparent twin dyads of Weddell seals (*Leptonychotes weddellii*) born in McMurdo Sound, Antarctica. Genetic evidence for twinning in wild pinnipeds has not been reported previously. A review of 14 years of demographic data combined with pathological exams, behavioral observations, and molecular genetic evidence suggests that twinning is extremely rare in Weddell seals and that females do not wean both pups in the wild. The incidence of live twin births was about 0.1% (2/1,439 births), recorded over 3 seasons in Erebus Bay, McMurdo Sound. Additionally, a single case of true twinning was documented from 23 known pregnancies observed in an isolated population of Weddell seals located within McMurdo Sound. The 3 twin sets were dizygotic full siblings, 1 nontwin dyad represented a case of adoption, and the 3 remaining putative twin sets were identified as instances of foster nursing. These results indicated that observation of mother–offspring behavior was not a reliable method for identifying a twin birth in this species. Use of genetic techniques to verify presence of twins in species with low or unknown twinning rates offers the opportunity for a refinement of estimates in studies of reproductive success, fostering behavior, and adoption.

Key words: adoption, fostering, *Leptonychotes weddellii*, McMurdo Sound, microsatellite, twin, Weddell seal

Twin phocid seals have been reported occasionally in free-ranging populations from observation of in utero twins during field necropsies or have been speculated from observations of “twin” pups nursing (Arnbohm et al. 1997; Spotte 1982). Live twin births have been reported in captive phocids (Spotte 1982; Spotte and Stake 1982), but we could not find any published record of a live birth of twins in the wild corroborated by either genetic data or witness of parturition.

Weddell seals (*Leptonychotes weddellii*)

breed farther south than any other mammal and inhabit pack-ice and fast-ice regions around the Antarctic continent. Females give birth each year on the surface of the ice and then nurse their dependent pup for about 6–7 weeks (Fenwick 1973; Hill 1987; Lindsey 1937; Mansfield 1954; Reijnders et al. 1990) with a minimum reported weaning age of about 33 days (Kaufman et al. 1975). Postparturient females weigh an average of 405 kg but can lose >45% of their body weight during lactation (Hill 1987; Tedman and Green 1987; Testa et al. 1989), indicating a high energetic cost of lactation.

Investigations of the natural history of

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Weddell seals in and around Erebus Bay, Antarctica, were first described in the early 1900s (Wilson 1907). Since that time, accounts of twinning in Weddell seals have been limited to anecdotal reports of twin pups recorded in field notes and publications. Most were of stillborn or fetal twins dissected in utero (Bertram 1940; Lindsey 1937; Smith 1966; Stirling 1969). The only observations of live-born twins were based on circumstantial evidence. Stirling (1972: 110) noted 2 live pups of "identical size, sex and pelage" with the same female on 2 separate occasions during 1 season at White Island, McMurdo Sound, Antarctica. Cournet and Jouventin (1980) reported that an average of 1 set of twins were born each year in a population of about 340 adult Weddell seals near the French Antarctic station, Dumont D'urville, but they did not provide further substantive evidence or indicate if they actually witnessed births.

The population ecology of Weddell seals in Erebus Bay, McMurdo Sound, Antarctica, has been the focus of detailed study since the late 1960s (Siniff et al. 1977; Stirling 1969; Testa and Siniff 1987; Fig. 1). Since 1971, virtually all newborn pups in a designated study area (Fig. 1) have been tagged each Austral spring. Weekly censuses between 1 November and 10 December of each year have provided an extensive database identifying mother-pup relationships at birth and throughout the weaning period. On occasion, females have been observed nursing or attending 2 pups, suggesting that they may be twins. Since 1982, a field for observer comments on each seal sighting in the database has recorded incidental notes on the possible occurrence of twin births. These comments usually refer to 2 newborn pups observed nursing or associated with the same female on ≥ 1 occasions.

Detailed observations of known mother-pup pairs throughout the nursing period indicate that pups sometimes wander through a colony and attempt to nurse multiple females. Nursing females will occasionally allow these pups to suckle (Kaufman et al.

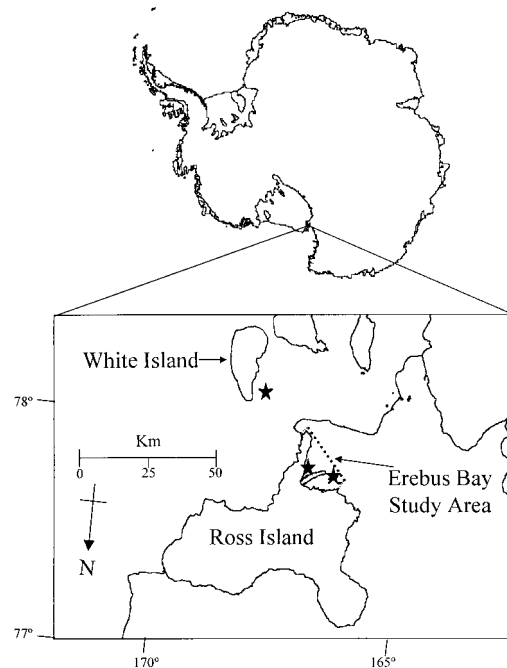


FIG. 1.—Locations (★) of sampling sites of 3 mother-twin sets of Weddell seals (*Leptonychotes weddellii*) at White Island and Erebus Bay, McMurdo Sound, Antarctica, 1995–1998.

1975; Smith and Burton 1970; Stirling 1975; Tedman and Bryden 1979). These reports suggest that behavioral observations alone may not be an accurate method of identifying a twin birth. Here, we refer to occurrence of females nursing foreign pups as foster nursing, and females foster nursing a foreign pup until weaning is considered adoption.

We used microsatellite genotyping to examine all apparent cases of twins born in the study area and at the isolated population of White Island (Stirling 1972; Testa and Scotton 1999) in 1996–1998. Our objectives were to use behavioral observations to identify potential mother-twin groups and use molecular genetic analysis to affirm or deny live twin births of Weddell seals. We also assessed reliability of using the discarded placenta as an indicator of twin birth by sampling 2 placentas found frozen in the ice near a female with 2 nursing pups. Finally, we used the comments in the long-

term database to estimate historical occurrence of twinning within the McMurdo population.

MATERIALS AND METHODS

We collected skin samples (1–3 mm²) for DNA extraction from the rear flippers of each putative mother–twin group using a hog ear notcher. Additionally, 224 samples collected from Erebus Bay adults in 1996–1997 and 1997–1998 and 28 samples from all adults at the White Island population were used to create population allele frequency distributions for parent–offspring calculations. All samples were stored in 70% ethanol and kept at –20°C until DNA extraction. Possible twins were identified by the presence of 2 newborn pups attended by a single mother with no other single adult female in the immediate vicinity at the time of tagging. In each case, we observed the adult female nursing or defending both pups similarly.

Molecular analysis and calculations.—We genotyped each mother–twin set and the 2 placentas at 18 microsatellite loci. Seventeen markers were isolated from Antarctic seals (Davis et al. 2000; GenBank Accession numbers LC6, AF140580; LC13, AF140581; LC18, AF140582; LC26, AF140583; LC28, AF140584; HL18, AF140585; HL14, AF140586; HL15, AF140587; HL16, AF140588; HL20, AF140589; LW4, AF140590; LW7, AF140591; LW10, AF140592; LW11, AF140593; LW16, AF140594; LW20, AF140595; LW18, AF140596) and 1 marker (G1A, UAU22095) isolated from North American black bears (*Ursus americanus*—Paetkau and Strobeck 1994). Total genomic DNA was extracted from tissue samples using QIAmp[®] spin columns (QIAGEN, Inc., Chatsworth, California). About 100 ng of DNA template were used in 15- μ l PCR reactions also containing 0.16 μ M (4 pmols) of each primer, 120 μ M dNTP's, 2 nM MgCl₂, 0.3 units of *Taq* DNA polymerase (Engelke et al. 1990), and 1 \times PCR buffer (10 mM Tris buffer, pH 8.8, 0.1% Triton X100, 50 mM KCl, and 0.16 mg/ml BSA). The PCR reactions were performed in a Perkin Elmer 9600 thermal cycler under the following conditions: 1 min at 94°C, 3 cycles of 30 s at 94°C, 20 s at 49°C, and 5 s at 72°C, followed by 33 cycles of 15 s at 94°C, 20 s at 50°C, and 1 s at 72°C. Final extension at 72°C for 30 min preceded ramping to 4°C. The PCR products were diluted and resolved on an Applied Biosystems 373A DNA

Sequencer and analyzed using Genescan software (Applied Biosystems, Foster City, California). Genotyper software (Applied Biosystems) was used to determine allele sizes in base pairs (Davis and Strobeck 1998).

We used the Markov chain method of the exact test (Guo and Thompson 1992) as calculated in the program GENEPOP version 3.1d (Raymond and Rousset 1995) to test allele frequencies in each population for deviation from Hardy–Weinberg equilibrium at each locus. We used the same program to test for genotypic linkage disequilibrium at each pair of loci and a Bonferroni correction to account for the 18 loci tested.

We compared each pup genotype with the genotype of the putative mother. If the adult female shared ≥ 1 allele at every locus with its assumed offspring, we calculated the probability of parent–offspring exclusion ($P[POE]$) for each mother–pup dyad to determine if the 2 offspring seen with a single female were twins. $P[POE]$ was the probability that an individual, sampled at random, would be rejected as the parent of each pup. That was calculated for 18 loci as the product of $(1 - p)^2$ for each locus where the pup was homozygous and p was the frequency of the homozygous allele in the population and $(1 - q - r)^2$ for each heterozygous locus where q and r were frequencies of heterozygous alleles. We then searched for any male genotype that could have contributed the pup's paternally inherited alleles and calculated the probability of paternal exclusion ($P[PE]$). We determined the $P[PE]$ as the product over 18 loci of $(1 - p)^2$ for each locus where the paternally inherited allele in the pup could be unambiguously determined and $(1 - q - r)^2$ for each locus where either of the pup's alleles could have been inherited from the father. The frequency of the unambiguously scored paternal allele was p , while q and r were frequencies of alleles that could have been inherited from either parent. We used allelic frequencies specific to the population where samples were collected to calculate exclusion probabilities. Individual probabilities for seals at White Island were calculated separately because there is no immigration–emigration between populations (Stirling 1972; Testa and Scotton 1999) and the allelic frequencies differed from those in Erebus Bay.

We used the log-likelihood-ratio tests in the program Kinship 1.3.1 (Goodnight and Queller

1999; <http://www.gsoft.smu.edu/Gsoft.html>) to test the relatedness of twin pups with ambiguous paternity. The program used simulations generated from the allelic distribution of genotyped adults in the local population ($n = 298$) to determine significance values. We compared the likelihood that a pup dyad included maternal half siblings only versus the likelihood that they were full siblings to test for the possibility of multiple paternity. We queried the long-term database for any note or comment referring to twin pups seen or tagged during 14 previous field seasons in 1982–1995 and used that as a reference for a prior incidence of twin observations.

RESULTS

We observed and genotyped 7 putative twin groups (adult female and both pups; 1 in 1996, 3 in 1997, 3 in 1998; Table 1). In 1997, we sampled 1 group that included 1 living and 1 dead pup. We included that group because the attending female vigorously defended both pups (Table 1). All other pups were alive when sampled. The goodness-of-fit test in GENEPOP indicated that all but 1 locus in the Erebus Bay population and all loci in the White Island population did not deviate from Hardy–Weinberg equilibrium and that all loci in both populations were in linkage equilibrium ($P < 0.05$ for each).

At least 1 pup in every group shared ≥ 1 maternal alleles at all 18 loci and had a $P(POE) > 95\%$, demonstrating that, at minimum, 1 of each pup dyad was the biological pup of the attending female (Table 1). We found that 3 of the 7 putative twin pairs were genetic twins (Table 1). This is the 1st genetic evidence of twinning in wild pinnipeds. The 2 cases of twinning in Erebus Bay had $P(POE) > 99.9\%$. We determined paternity of the White Island twins by examining genotypes of all adult males in the population (Table 1). Male 30036 was the only individual that could have contributed the paternally inherited alleles, and the $P(PE)$ for each pup was $>99.95\%$ (Table 1). Each of the 2 placentas found frozen in the ice near the female twin group at White Island was genetically identical to 1 of the

twin pair. No paternal matches were found for the twin pairs in Erebus Bay. However, the log-likelihood-ratio tests confirmed that both pairs had a greater likelihood of being full siblings than half siblings ($P < 0.001$), indicating that there was no evidence of multiple paternity.

All 3 twin sets were dizygotic, as evidenced by the presence of different maternally inherited alleles in both pups. Pup survival beyond maternal dependence is unknown because all but 1 of the twin pups were still alive when last seen, but only pup 13182 was old enough to have weaned.

Six of the 1,439 births (0.4%) recorded in Erebus Bay in 1996, 1997, and 1998 were noted as putative twin births, and 0.1% (2 of 1,439) were confirmed to be twins. The White Island twins were the only putative twin births observed in 23 births within that population during the 3-year period.

Between 1982 and 1995, there were 23 of 5,628 births (0.4%) recorded in the study area in which 2 pups were tagged as newborns attended by or nursing the same female. However, in 9 of those cases, the 2 pups were tagged as singletons on separate days with the same female or seen nursing or attended by a different female at a later time. We interpreted those reports as cases of orphaned pups and foster nursing, not as instances of twinning. The other 14 reports (0.2%) were of 2 newborn pups tagged at the same time with the same mother and may be regarded similar to the observations in Table 1. Likewise, we believe that all previously observed cases probably were not legitimate twins. Frequency of those reports was less than we found during the 3 seasons of analyses and supports our observation that twinning is extremely rare in Weddell seals.

DISCUSSION

Twinning in other marine mammals (excluding polar bears, *U. maritimus*) has been reported on the basis of behavioral observations of sea otters (*Enhydra lutris*—Ja-

meson and Bodkin 1986; Snow 1910) and from necropsies in sea otters (Barabash-Nikiforov et al. 1947; Kenyon 1969; K. B. Schneider, in litt.; Snow 1910; Williams et al. 1980), various phocids and otariids (Spotte 1982), walrus (*Odobenus rosmarus*—Fay et al. 1991), and ceteceans (Harrison 1969). However, we found no reported cases of known twin offspring surviving to weaning in the wild. Those marine mammals without a terrestrial phase during weaning must constantly attend to their offspring to prevent separation in a 3-dimensional environment. This would be intensified with multiple offspring as they swim or drift in opposite directions and may simply be near impossible for mammals. Similarly, Weddell seals leave their pups alone on the ice for increasingly longer periods as pups approach weaning age. The ability to relocate pups on surfacing becomes more difficult as pups gain independence and move about the colony during the mother's absence (Thomas and DeMaster 1983). Relocating 2 dependent pups in separate locations when returning from a dive could increase the likelihood of premature pup abandonment. In general, mortality of 1 or both twin offspring in marine mammals is most likely due to an inability of the mother to attend and effectively care for both neonates until weaning.

At weaning, mass of Weddell seal pups may exceed one-half their mother's mass (Tedman and Green 1987). Successfully nursing 2 pups to weaning in the wild seems physiologically impossible based on this constraint. However, death or abandonment of 1 pup early in lactation could allow its mother to maintain sufficient resources to nurse its sibling to weaning. Female 13180 successfully raised pup 13182 to weaning after its twin died within the first 3 days. The greater frequency of twin fetuses reported from dissections of the reproductive tract or at birth compared with those seen nursing may be a consequence of the relatively greater energetic demands of lactation relative to gestation. The dif-

ference between nourishing single and twin fetuses is probably less than the difference between nursing single and twin pups to weaning. This effect would be amplified if the nursing female is feeding little or not at all during lactation (Clutton-Brock et al. 1989). Diving profiles of Weddell seals suggest that some females forage during lactation, possibly on an opportunistic basis (Kooyman 1968; Siniff et al. 1977; Testa et al. 1989). Given ample forage and a habitat with no possibility of mother-pup separation, a female might be able to wean both pups as has been seen in captive phocids (Spotte 1982).

The low incidence of twinning observed in the population is most likely due to a reduced survivorship for twin pups and should be selected against. However, if dizygotic twinning in Weddell seals is hereditary, as has been shown for humans (Haukioja et al. 1989), then the trait could persist in the surviving twin or any of its siblings. Adult females 5002 and 30019 successfully weaned single offspring in the season following the birth of their twins, suggesting that any heritable tendency for twinning could remain in the population (Haukioja et al. 1989).

The identical matches between the 2 placentas and twins at White Island indicated that presence of placentas alone without the pups would have been sufficient to reveal a twin birth. Again, use of log-likelihood tests in Kinship would permit confirmation of full siblings based on relatedness values (Goodnight and Queller 1999). However, of greater use is the implication that a single placenta found adjacent to or guarded by an adult female could provide genetic verification of a pup birth even if the pup is no longer present.

Fostering and adoption.—Four of the 7 cases we sampled had only 1 true offspring and 1 foster-nursed pup (Table 1). In 3 cases where the sex of each pup was known, pairs were of mixed sex, and the female was always the true offspring. This is a very small sample but may indicate that

TABLE 1.—Genotypes of all putative twin sets of Weddell seals (*Leptomychotes weddellii*) sampled in McMurdo Sound, Antarctica, 1996–1998. Each individual was typed at 18 microsatellite loci.

| Year | Female ID | Offspring ID | Age ^a | Relationship | Number of shared loci ^b | <i>P</i> (<i>POE</i>) ^c | <i>P</i> (<i>PE</i>) ^d | Condition at last observation ^e |
|------|-----------|--------------|------------------|----------------------|------------------------------------|--------------------------------------|-------------------------------------|--|
| 1996 | 5002 | | ≥17 | Mother | | | | With pup 11945 at pup age 28 days. Observed with single pup in 1997, 1998, and 1999. |
| | | 11944 | | Female twin | 18 | 100.00 | | Alone on the surface at age 16 days while mother and twin sister were together in the water. |
| 1997 | 12180 | | | Female twin | 18 | 100.00 | | With mother at age 28 days. |
| | | | | Mother | | | | With pup 12812 at pup age 41 days; observed with single pup in 1998 and 1999. |
| | | 12181 | | Male foster | 11 | | | Alone at age 22 days and with a different female (not its mother) at age 36 days; possibly weaned. |
| | 6584 | | | Female pup | 18 | 100.00 | | With mother on 7 occasions; the last at age 41 days; probably weaned. |
| | | 12182 | 13 | Mother | | | | With adopted pup 12554 at pup age 29 days; observed with single pup in 1998 and 1999. |
| | | 12554 | | Female foster | 9 | | | Observed on 6 occasions with female 6584. Last time at age 29 days. |
| | 30019 | | 6 | Pup sex ^f | 18 | 100.00 | | Dead at time of tagging and sex unknown. |
| | | | | Mother | | | | Alone at pup age 42 days; observed with single pup in 1998 and alone in 1999. |
| | | 30065 | | Female twin | 18 | 95.09 | 99.98 | With mother and twin brother at age 30 days; pup not seen again. |
| | | 30066 | | Male twin | 18 | 98.93 | 99.96 | With mother and twin sister at age 30 days; pup not seen again. |
| | | 30065 | | Placenta | | | | |
| | | 30066 | | Placenta | | | | |
| | 30036 | | | Father | | | | Alone in November 1998. |
| 1998 | 9810 | | 7 | Mother | | | | With adopted pup 13292 at pup age 31 days; observed with single pup in 1999. |
| | | 13291 | | Female pup | 18 | 99.98 | | Alone at age 2 days and age 7 days; dead at age 13 days. |
| | | 13292 | | Male foster | 12 | | | With female 9810 on 6 separate occasions between ages 7 and 31 days; alone and molted at age 39 days; probably weaned. |
| | 7483 | | 12 | Mother | | | | With pup 13279 at pup age 25 days; observed alone in 1999. |
| | | 13278 | | Male foster | 11 | | | With female 7483 at age 11 days and alone at age 25 days. |
| | | 13279 | | Female pup | 18 | 99.98 | | With mother at age 25 days and alone at age 40 days (mother not seen in area); probably weaned. |

TABLE 1.—Continued.

| Female ID | Offspring ID | Age ^a | Relationship | Number of shared loci ^b | $P(POE)^c$ | $P(PE)^d$ | Condition at last observation ^e |
|-----------|--------------|------------------|--------------|------------------------------------|------------|-----------|--|
| 13180 | | | Mother | | | | With pup 13182 at pup age 35 days; observed alone in 1999. |
| | 13181 | | Female twin | 18 | 100.00 | | Dead at age 3 days. |
| | 13182 | | Female twin | 18 | 100.00 | | With mother on 5 separate occasions between ages 4 and 35 days; alone at age 43 days; probably weaned. |

^a All known age adults were tagged as pups except 5002 tagged as an adult, age ≥ 2 in 1981; pups were all tagged within 3 days of birth, and date of tagging was designated as birth day for aging purposes.

^b Number of loci where pup shares ≥ 1 allele with the putative mother, and in pups 30065 and 30066, to both parents.

^c Probability of parent-offspring exclusion refers to the probability that a randomly sampled individual from the population will be excluded as a parent or offspring of the known individual at ≥ 1 of the 18 tested loci.

^d Probability of paternal exclusion refers to the probability that a randomly sampled male from the population will be excluded as the father of the known individual at ≥ 1 of the 18 tested loci, given the mother's genotype.

^e Pups ≥ 33 days of age at the time of last observation were assumed to have survived to independence; observations of adult females in subsequent years indicate reproductive status.

^f Pup 12555 born to mother 6584 was dead when found and could not be reliably sexed.

male pups are more likely to stray or be abandoned than females, or they simply have a greater likelihood of eliciting adoption or foster nursing. One adopted male pup survived to weaning, while the biological offspring, a female pup (13291), died in the 2nd week following birth. Similarly, female 6584 lost her pup (sex unknown) soon after birth and nursed her adopted female pup for ≥ 29 days (Table 1). These observations indicate that a twin dyad might be able to survive if, early in development, 1 pup separated from its mother and its sibling was adopted by a single lactating female. Fostering behavior in phocids is relatively common (Stirling 1975), and Boness (1990) reported an incidence of 87% in Hawaiian monk seals (*Monachus schauinslandi*). We do not know what percentage of Weddell seals foster nurse in the McMurdo population, but this combination of behavioral and molecular genetic methods ultimately could be applied to quantitative analyses of fostering and adoption in Weddell seals.

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