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HETEROSPECIFIC PAIRING AND HYBRIDIZATION BETWEEN WILD HUMBOLDT AND MAGELLANIC PENGUINS IN SOUTHERN CHILE

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Abstract. The Humboldt (*Spheniscus humboldti*) and Magellanic (*S. magellanicus*) Penguins overlap over 1100 km along the coast of the southeastern Pacific Ocean, and much has been hypothesized about hybridization between them. We visited Puñihuil and Metalqui islands, southern Chile (41–42° S), where both species form mixed colonies; these are also the Humboldt Penguin's southernmost colonies. We observed one mixed pair attending chicks and two adults of intermediate color pattern, one of which tended a chick at a nest. Additionally, on the basis of analysis of 30 blood samples of Humboldt Penguins from the Puñihuil colony, we report the first documented Humboldt × Magellanic Penguin hybrid. Judged from the pattern of restriction fragments, this bird had a Magellanic dam and a Humboldt sire. We sequenced mitochondrial and nuclear copies independently to confirm these results. We suggest that hybridization at

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Metalqui and Puñihuil is encouraged by the low abundance of the Humboldt Penguin rather than by failed mate recognition.

Key words: interbreeding, hybridization, Spheniscus, *mixed colony, DNA*, *heterospecific pairing, penguin*.

Cruzamientos Heteroespecíficos e Hibridación entre los Pingüinos Silvestres *Spheniscus humboldti* y *S. magellanicus* en el Sur de Chile

Resumen. El pingüino de Humboldt (*Spheniscus humboldti*) y de Magallanes (*S. magellanicus*) se sobreponen sobre 1100 km a largo del Pacífico suroccidental y se ha hipotetizado mucho acerca de entrecruzamiento e hibridación entre ambas especies. Visitamos las islas de Puñihuil y Metalqui, en el sur de Chile (41–42° S), donde ambas especies forman colonias mixtas. Aquí el pingüino de Humboldt tiene su límite meridional de distribución. Observamos una pareja mixta en un nido con pollos y dos individuos adultos presentaron caracteres intermedios entre ambas especies nominales; uno de estos individuos estaba en un nido con un pollo. Adicionalmente, sobre la base del análisis de 30 muestras de sangre de pingüinos de Humboldt de Puñihuil, documentamos por primera vez un híbrido Humboldt–Magallanes. Los patrones de los fragmentos de restricción producidos indican que la madre de este individuo era de la especie *S. magellanicus* y el padre, *S. humboldti*. Las copias mitocondriales y nucleares fueron secuenciadas independientemente para confirmar estos resultados. Sugerimos que la hibridación en Metalqui y Puñihuil es fomentada por la baja abundancia de una de las especies (*S. humboldti*) más que por problemas de reconocimiento de pareja.

In birds, natural heterospecific pairing occurs rather frequently, and outcomes may include successful hybrid offspring, breeding attempts, copulation, and behavioral displays (Randler 2002 and references therein). The incidence of hybridization varies geographically and ecologically, and it appears to be more common in temperate regions than in the tropics and more frequent in terrestrial birds, especially passerines, than in seabirds (Grant and Grant 1992). Hybridization is more widespread in areas where two closely related species overlap in distribution and one of them is less common. The combination of these two factors encourages hybridization through restricted mate choice (Randler 2002). Such hybrid zones, defined as "regions in which two genetically distinct populations meet, mate and produce hybrids," can be several hundred kilometers long and provide interesting ways of understanding the process of speciation (Barton and Hewitt 1985, Randler 2008).

Among penguins, hybridization in the wild has been reported in the genus *Eudyptes* at the Falkland Islands. Here, White and Clausen (2002) observed Rockhopper (*E. chrysocome*) × Macaroni (*E. chrysolophus*) hybrids pairing and breeding successfully with Rockhopper Penguins. These authors summarized seven other cases of hybrids of *Eudyptes* reported elsewhere at different Atlantic, Indian, and Pacific Ocean islands.

Within the genus *Spheniscus*, only the Humboldt (*S. humboldti*) and Magellanic (*S. magellanicus*) Penguins overlap geographically, along the west coast of South America between Algarrobo (33° S) and Metalqui Island (42° S) in Chile (Murphy 1936, Williams 1995, Simeone and Hucke-Gaete 1997). This extensive continuous overlap (>1000 km) of breeding range raises the potential for interbreeding, particularly in southern Chile (41–42° S), where both species coexist in significant numbers (Wilson et al. 1995, Simeone and Schlatter 1998). Although some authors (e.g., Williams 1995, Davis and Renner 2003) asserted that the species interbreed in this region, they provided no direct evidence in support. In captivity, however, interbreeding between the Humboldt and Magellanic Penguins has been well documented at zoos and aquaria. Outcomes in captive birds have included mixed pairs and fertile offspring (Anonymous 1984/85, 1986, 1987, Thumser and Karron 1994).

In this paper we present evidence that the Humboldt and Magellanic Penguins interbreed in southern Chile, including (a) observation of a heterospecific breeding pair attending a nest with chicks, (b) a bird with characteristics intermediate between the species on a nest, tending a chick, (c) a bird with characteristics intermediate between the species on a nest with an adult Magellanic Penguin, and (d) DNA evidence for hybridization in a phenotypic Humboldt Penguin. Additionally, we describe a method for testing for hybrid status in *Spheniscus* penguins.

METHODS

We have been studying the foraging ecology of the Humboldt and Magellanic Penguins at two colonies in southern Chile, Puñihuil (41° 55′ S, 74° 02′ W) and Metalqui (42° 12′ S, 74° 10′ W) islands. Both colonies are located off the exposed Pacific coast of Chiloe Island (Fig. 1). Puñihuil consists of two islands, which we visited daily between 11 November and 15 December 2008. The smaller ("Island 1") is about 400 m offshore and has a total surface area of 1.54 ha; the larger ("Island 2") is located about 700 m from the coast and has a total surface area of 2.65 ha. The islands, described in detail by Simeone and Schlatter (1998), are approximately 200 m apart. Combined, they support a population of 76 pairs of Humboldt and 458 pairs of Magellanic Penguins (Simeone 2004). We visited Metalqui Island (Chiloe National Park) on 8 December 2008. It is approximately 0.9 km from the coast, has a total surface area of 17 ha, and supports a minimum of 28 pairs of Humboldt and 203 pairs of Magellanic Penguins (unpubl. data).

From 6 to 8 December 1997 we collected blood samples from 30 adult Humboldt Penguins at Puñihuil. We placed a wooden marker at the burrows of all sampled birds to ensure that no individual was sampled more than once. Sampled birds tended either eggs or small chicks (i.e., <2–3 weeks old). Blood (5 ml) was collected from the jugular vein with a 22-gauge needle and a 5-ml syringe.

An aliquot of each blood sample was stored in long-term storage buffer (100 µM Trizma, 100 mM EDTA, and 2% SDS, pH 8.0) for genetic analysis at the Brookfield Zoo lab. DNA was extracted from blood or tissue samples according to the protocol outlined in Sambrook et al. (1989) by overnight digestion with proteinase K and successive washes with phenol, phenol/chloroform/isoamyl alcohol, and chloroform/isoamyl alcohol, followed by precipitation with 3-M sodium acetate and 100% ethanol. Genomic DNA was resuspended in 100 mM Tris-HCl and 10mM EDTA, pH 8.0, and used as a template for PCR amplification of the region coding for mitochondrial NADH subunit 2 (ND2) in *S. humboldti.* The following primers (L-Met 5′-TATCGGGCCCATACCCCGAATAT-3′; H-Trp 5′-CCTTTATTTAAGGCTTTGAAGGC-3′) and PCR conditions were used: 94 °C for 5 min; 94 °C for 40 sec, 57 °C for 45 sec, 72 °C for 45 sec for 35 cycles, followed by a 10-min extension at 72 °C. PCR products were cleaned for sequencing with QIAquick Spin Columns (Qiagen) and sequenced on a Beckman/Coulter CEQ2000XL with the manufacturer's reagents and protocol.

The double-stranded sequence from repeated amplifications consistently produced doublet peaks in several complementary positions on both strands. We suspected that two copies of this gene, the mitochondrial and nuclear pseudogene, were being

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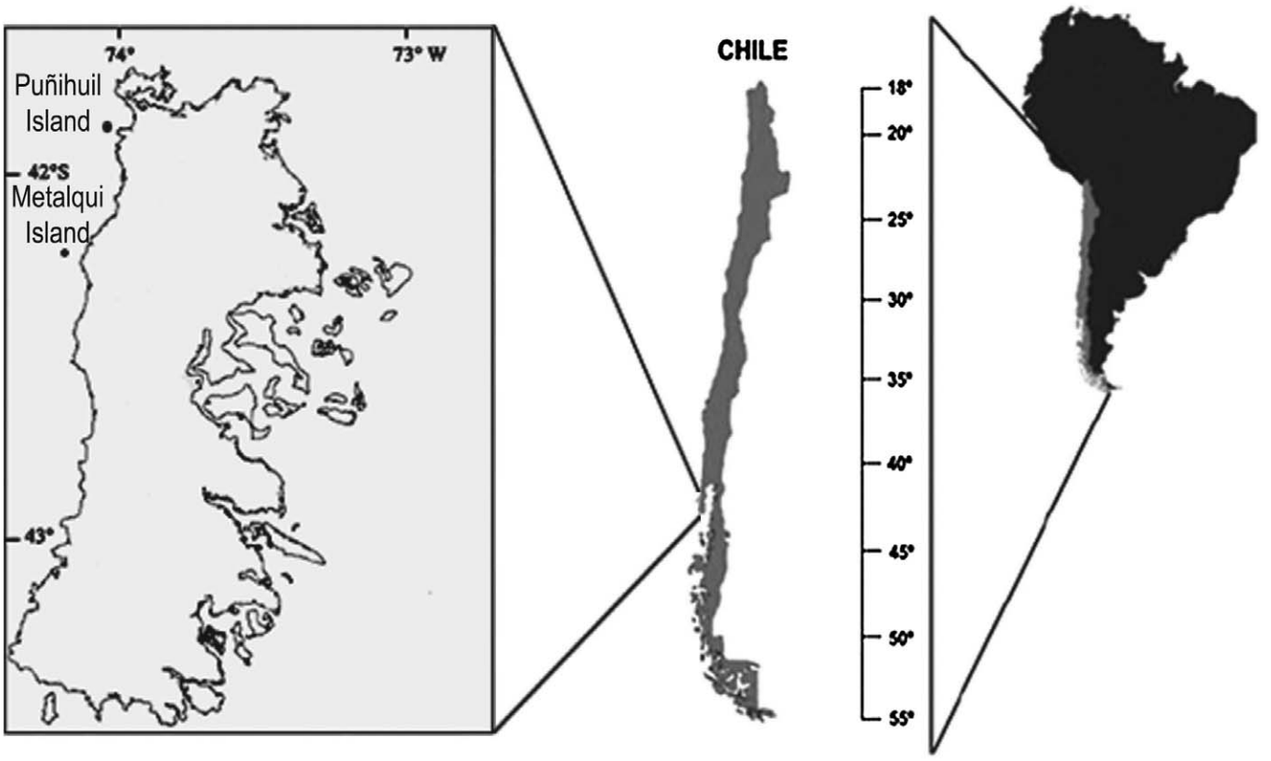


FIGURE 1. Location of Puñihuil and Metalqui islands in Chiloé, southern Chile.

amplified, and we investigated this further in two ways. First, from the blood of a dead penguin we prepared genomic DNA and enriched mtDNA from the heart tissue by using the Wizard Minipreps Purification System (Promega, Inc.) and protocol (Beckman et al. 1993). Second, from sequence data we identified two restriction enzymes (Nci I and Ssp I) that each cut only one strand of a doublet, leaving one or the other DNA strand intact for reamplification and sequencing under the same conditions described above. The genomic and mtDNA preparations and 10 µl of each ND2 PCR product from the same individual as well as previous PCR templates were incubated with each restriction enzyme at the recommended incubation temperature (Promega, Inc.) and each was used as a template for PCR reamplification. PCR products were sequenced on both strands from the following templates: genomic DNA from blood, mitochondrial DNA from heart, PCR products from genomic DNA or mitochondrial DNA from heart, and PCR products from genomic DNA from other penguin species. All templates were amplified "as is" and after separate digestion with Nci I or Ssp I restriction enzymes. We designed internal primers specific to the mitochondrial (mt) or nuclear (nc) copy (mtL-510 5′-CTTCTCATCTATCTCCCATC-3′; mtL-630 5′-TATACTCCCTAATAACCATCAC-3′; mtH-510 5′-GATGGGAGATAGATGAGAAG-3′; ncF-510 5′-CTTCTCATCTATCTCCCATT-3′; ncR650 5′-GTTTTAGGGTTTTAGTTGTG-3′; ncR-950 5′-AGGGGTAGGAGTAGGGTTGT-3′). Sequences were aligned by the sequence editor of MacDNASIS v3.2 (Hitachi Software Engineering America, Ltd.).

To identify interspecific differences and screen captive penguins for possible hybrids, including those with the African Penguin (*S. demersus*), we included two captive Magellanic Penguins and two captive African Penguins in the study. Genomic DNA from each individual was used to amplify ND2; 10 µl of each PCR product was restricted as described above and reamplified for sequencing. The sequences of each species were aligned and compared for species-specific substitutions. We identified three restriction enzymes that selectively cut the mitochondrial (EcoR V, Bgl I) or nuclear pseudogene (Taq I) copy and produced fragments that can be used to identify maternal (mitochondrial) or paternal species when separated on a 2% agarose gel. Wild and captive Humboldt Penguins were screened with the three restriction enzymes to determine if any Humboldt × Magellanic or Humboldt × African hybrids were present in either population.

Gene sequences were deposited in GenBank (accession numbers GQ354789 to GQ354794).

RESULTS

At Puñihuil Island 2 we observed a breeding pair, comprising a Humboldt and a Magellanic Penguin, tending two chicks, both estimated to be 4 to 5 weeks old. We marked the nest (a dirt burrow covered by vegetation) with a wooden marker and a numeric code and checked it daily from 25 November to 10 December 2008. During this period, each bird spent 8 days in the nest divided among three shifts of brooding.

At Metalqui Island we observed two birds showing characteristics intermediate between the Humboldt and Magellanic Penguins (Fig. 2A, B). The first was on a nest with a typical adult

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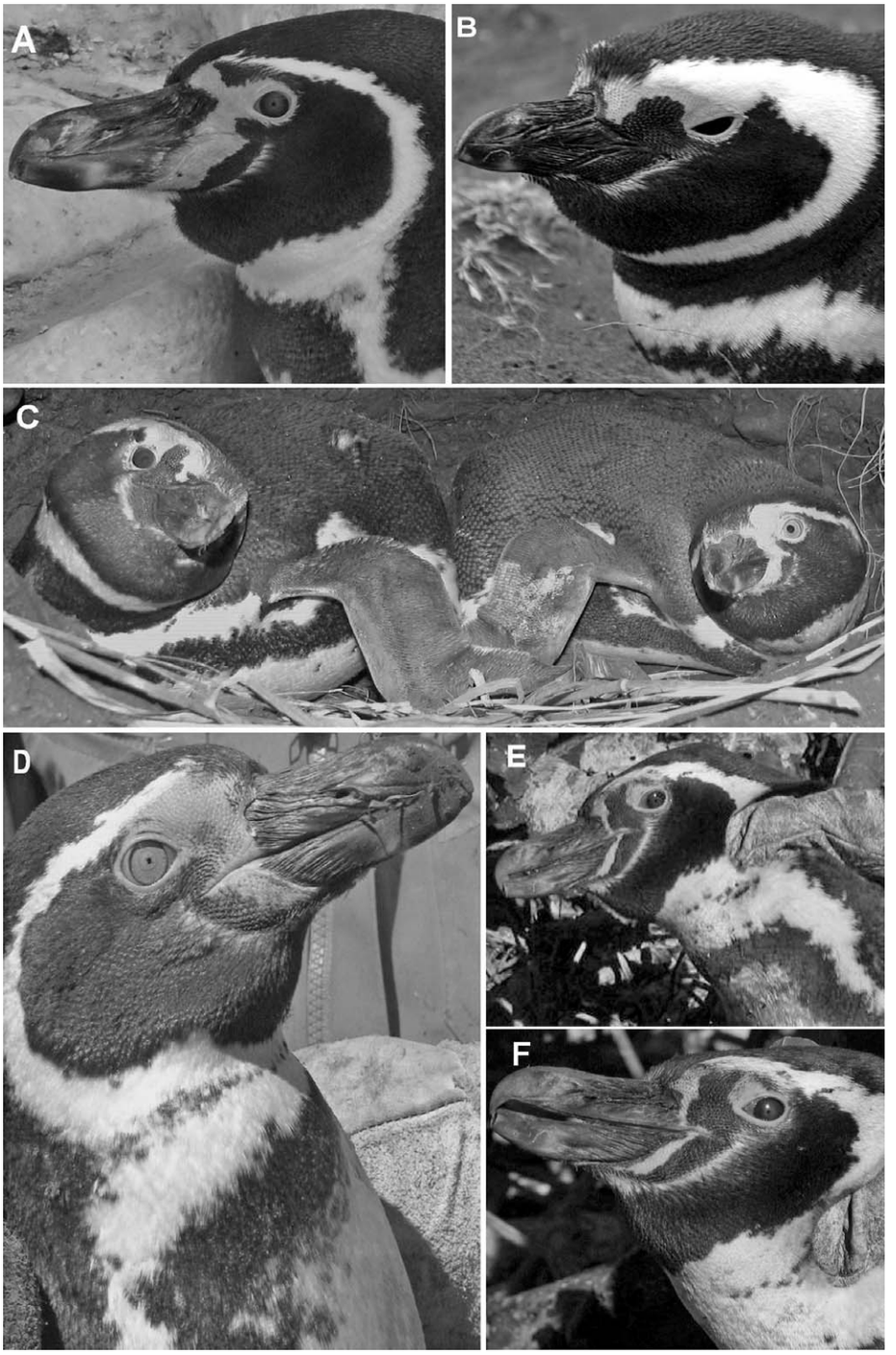


FIGURE 2. (A) Typical head and breast-band pattern of the Humboldt Penguin. (B) Typical head and breast-band pattern of the Magellanic Penguin. (C, D) Penguin (on the right in C) intermediate a typical Humboldt (face coloration) and Magellanic (breast and thin neck band) penguin. This bird was on a nest with a Magellanic Penguin (on the left in C). (E, F) Penguin showing an intermediate facial pattern (pink with an atypically large black band) and a thin neck band as in the Magellanic Penguin. This bird was attending a nest with a single 3-week-old chick.

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TABLE 1. Main identification features used to distinguish the Humboldt and Magellanic penguins. Comparisons are referred to the other species.[a]

	Humboldt	Magellanic
Black breast bands	One, inverted horseshoe shape	Two, lower inverted horseshoe shape, upper wider
Bill	Sturdy, thick	Thinner, thus appearing longer
Head pattern	Narrow white supercilium that curls around ear coverts and meets white of underparts	Broad white supercilium that curls around ear coverts and meets below throat, bordering upper breast band
Face pattern	Extensive pink fleshy area at base of bill (upper and lower mandible), from lores to chin	Pink on face restricted to orbital ring and supraloral area; indistinct pink line at base of lower mandible

[a] Sources: Williams (1995), Davis and Renner (2003), and Jaramillo (2003).

Magellanic Penguin on the northwest side of the island (Fig. 2C); no chicks were observed at the nest. This bird (on the right in Fig. 2C) had the face coloration and crown stripe typical of the Humboldt Penguin (Table 1, Fig. 2A), but after we removed the bird from the nest, we noticed a thin and irregular band crossing its upper breast (Fig. 2D). This type of breast band is characteristic of the Magellanic Penguin (Table 1, Fig. 2B), although the one on this bird was considerably thinner than typical for the species.

The second bird, tending a single chick ~3 weeks old, was on a vegetation-covered nest on the northwest side of the island (Fig. 2E, F); no other adult was at the nest. The facial pattern was intermediate between that of the two species, with pink at the base of the beak (typical for the Humboldt Penguin) but a large fleshy black area crossing it (Fig. 2E, F). Again, a thin and irregular band crossed the upper breast.

We obtained a total of 1050 bp of mitochondrial ND2 sequence and 1041 bp of nuclear ND2 sequence from six penguins of these three species of *Spheniscus*. The Humboldt Penguin's mitochondrial gene has a 9-bp duplication starting at position 1035 not present in the Magellanic or African Penguin sequences. This duplication, resulting in a repetition of three additional amino acids, does not interfere with translation. The enriched mitochondrial preparation from the Humboldt Penguin produced a sequence with no doublet peaks and had one of the nucleotides from each doublet observed in the original sequence data. The sequence from the DNA and PCR products digested with Nci I matched the mitochondrial sequence, and the DNA and PCR products digested with Ssp I contained all alternative bases from each doublet. Therefore, Nci I restricts the nuclear pseudogene, leaving the mitochondrial product intact, while Ssp I cuts the mitochondrial product, leaving the nuclear copy intact. These results were consistent whether genomic DNA was restricted and amplified or genomic DNA was amplified and the PCR product restricted and reamplified. In all three species, these PCR products were separated in the same manner (Table 2).

We screened 30 wild Humboldt Penguins from Puñihuil for hybridization, discovering one Humboldt × Magellanic hybrid. On the basis of the pattern of restriction fragments produced, this individual had a Magellanic dam and a Humboldt sire. As expected, there was no evidence of African Penguins in the Puñihuil population. Captive penguins are occasionally maintained in mixed-species groups; therefore, we screened 150 individuals from this population, identifying two Humboldt × African hybrids. Both the mitochondrial and nuclear copies were independently sequenced to confirm these results.

DISCUSSION

Considering the divergence of color patterns of the birds we observed from the patterns described in the literature (Table 1), we propose that the cases we report resulted from heterospecific crossing of Humboldt and Magellanic Penguins. These birds could have been aberrant Humboldt or Magellanic Penguins, although this alternative seems unlikely. In captivity, putative Humboldt Penguins with double thin pectoral bands (like the ones we describe) have been regarded as Humboldt × Magellanic hybrids (McCarthy 2006). Williams (1995) stated that in the wild Magellanic Penguins infrequently lack neck bands, and Davis and Renner (2003) attributed variations in both banding pattern and facial coloration to individual variation and hybridization with the Humboldt Penguin. Our phenotypic and DNA data for the bird at Puñihuil are, to our knowledge, the first documented evidence of hybridization between the Humboldt and Magellanic Penguins in the wild.

As noted by Randler (2002), congeneric species of birds frequently hybridize where their ranges overlap. Here, we analyze

TABLE 2. Restriction-enzyme cutting sites within the mitochondrial and nuclear pseudogene regions of ND2 for three species of *Spheniscus* penguins. The enzyme Bgl I cuts the nuclear copy in all species, and these fragments are recovered with the mitochondrial fragments.

		Mitochondrial[a]		Nuclear[a]	
Species	Approximate PCR product size (bp)	Bgl I	EcoR V	Taq I	Bgl I
Humboldt	1150	1150	305 845	208 933	536 605
Magellanic	1141	1141	1141	208 228 705	536 605
African	1141	210 931	1141	208 933	536 605

[a] Restriction fragment sizes that would be observed in a 2% agarose gel.

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two of Randler's (2002) possible scenarios under which the observed hybridization between Humboldt and Magellanic Penguins may have occurred. First, hybridization between congeneric species may be more common when one of the two species is rare in the contact zone. In this situation, hybridization may be encouraged when all conspecific mates are paired and the remaining unpaired individual has a choice between mating heterospecifically or not breeding. Some birds will mate heterospecifically rather than not mating at all, providing that doing so will produce viable hybrids (Randler 2002, 2008). In terms of lifetime reproductive success, hybridization with a related species may be a better alternative than remaining unpaired (Randler 2006). At Metalqui and Puñihuil islands, the Humboldt Penguin is at the edge of its breeding range (Simeone and Hucke-Gaete 1997, Simeone and Schlatter 1998), where the species is expected to be rare (Williams 1995); in this region the ratio of Humboldt to Magellanic Penguins ranges from 1:6 to 1:7 (Simeone 2004). Furthermore, the second phenotypic hybrid we report (Fig. 2) tended a chick, indicating that this adult was fertile and able to raise its offspring.

A second hypothesis that may explain hybridization at Metalqui is failure in mate recognition, which could be the result of mistaking acoustic, visual, or behavioral cues (Randler 2002). Similar calls, for instance, may disrupt mate choice, especially when the heterospecific male's song contains elements that act as a cue for mate recognition. Although plausible, we consider this hypothesis unlikely for the birds we observed. Thumser et al. (1996) found that the bray calls (which are used by *Spheniscus* penguins to establish a territory and advertise availability for pairing) of the Humboldt and Magellanic Penguins are markedly dissimilar; the structure of bray calls of the Magellanic and African Penguins, however, are more similar.

Although the Humboldt and Magellanic Penguins are sympatric over 1100 km of the Chilean coast (Simeone and Hucke-Gaete 1997, Simeone et al. 2003), several studies (e.g., Grant et al. 1994, Thumser and Karron 1994, Thumser et al. 1996, Baker et al. 2006) have shown that these species belong to separate evolutionary lineages, with the Humboldt sharing a common ancestor with the Galapagos Penguin (*S. mendiculus*), the Magellanic with the African Penguin. These two species pairs originated recently and almost contemporaneously in the Pacific and Atlantic Oceans, respectively, in the last 4 million years (Baker et al. 2006). The current overlap in distribution and interbreeding implies secondary contact between the species rather than their sharing an ancestral area. This refutes also character displacement as an explanation for the genetic and vocal differences observed today (see Thumser et al. 1996).

Current evidence supports the hypothesis that hybridization at Metalqui and Puñihuil is boosted by the low abundance of one of the species (Humboldt) rather than by failed mate recognition. Although some authors (e.g., Araya and Todd 1988) have suggested that the overlap in the ranges of Humboldt and Magellanic Penguins resulted from recent phenomena such as strong El Niño weather patterns that have driven the Humboldt south, Schlosser et al. (2009) demonstrated that southern populations of the Humboldt (Puñihuil islands) are genetically well structured and distinct from other known and well-established populations in Chile and Peru. These findings indicate that Humboldt Penguin populations in this region are more ancient than previously thought. Consequently, it is conceivable that hybridization has been occurring for a much longer time than previously thought and was simply overlooked.

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