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ORIGINAL ARTICLE

No evidence of extra-pair paternity or intraspecific brood parasitism in the Imperial Shag *Phalacrocorax atriceps*

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Abstract In long-lived birds with significant paternal care contribution, as the case of seabirds, extra-pair paternity (EPP) is an infrequent phenomenon. Intriguingly, and in contrast to the general pattern exhibited by seabirds, EPP rates appear relatively high in the two species of cormorants and shags (Phalacrocoracidae family) analyzed so far. We test for EPP in the Imperial Shag *Phalacrocorax atriceps*, a medium-sized colonial seabird, using four DNA microsatellites originally developed for Great Cormorants *P. carbo*, and successfully cross-amplified in our focal species. We assessed the parentage of 110 chicks from 37 broods sampled at Punta León, Argentina, during the 2004 and 2005 breeding seasons. We found no evidence of EPP or intraspecific brood parasitism (IBP). Given our sample sizes, the upper 95% confidence limits for both EPP and IBP were estimated at 3.3% for the chicks and 8.4% for the broods. Our results did not agree with the previous reports of EPP within the family, probably as consequence of different copulation and courtship behaviours, mostly related to male's solicitation display and females active search for extra pair copulations.

Keywords Cormorants · Genetic parents · Microsatellites · Phalacrocoracidae · Seabirds

Zusammenfassung

Keine Belege für Fremdvaterschaft oder intraspezifischen Brutparasitismus bei der Blauaugenscharbe *Phalacrocorax atriceps*

Bei langlebigen Arten, bei denen die Männchen signifikant zur Brutpflege beitragen, wie es bei Seevögeln der Fall ist, ist Fremdvaterschaft (EPP) ein seltenes Phänomen. Interessanterweise sind die EPP-Raten bei den zwei bislang analysierten Kormoran- und Scharbenarten (Familie Phalacrocoracidae) anscheinend relativ hoch, im Gegensatz zum allgemein von Seevögeln gezeigten Muster. Wir haben EPP bei der Blauaugenscharbe *Phalacrocorax atriceps*, einem mittelgroßen koloniebrütenden Seevogel, mit Hilfe von vier DNA-Mikrosatelliten untersucht, die ursprünglich für den Kormoran (*P. carbo*) entwickelt und in unserer Untersuchungsart erfolgreich kreuzamplifiziert wurden. Wir haben die Elternschaft von 110 Küken aus 37 Bruten, die in Punta León, Argentinien, während der Brutsaisons 2004 und 2005 beprobt wurden, ermittelt. Wir haben keine Belege für EPP oder intraspeczifischen Brutparasitismus (IBP) gefunden. Für unsere Stichprobengrößen lagen die oberen 95%-Konfidenzintervalle für sowohl EPP als auch IBP bei 3.3% für die Küken und 8.4% für die Bruten. Unsere Ergebnisse stimmen nicht mit früheren Berichten über EPP in dieser Vogelfamilie überein, wahrscheinlich infolge

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unterschiedlichen Paarungs- und Balzverhaltens, hauptsächlich in Bezug auf das Werbungsverhalten der Männchen und das aktive Suchen von Kopulationen außerhalb des Paarbundes durch die Weibchen.

Introduction

Over the last 20 years, with the advent of high-resolution molecular markers, there has been a revolution in our understanding of avian mating systems and alternative reproductive strategies. Most dramatic has been the distinction between social monogamy (i.e. the association between a male and female for the purpose of reproduction) and genetic monogamy (i.e. an exclusive mating relationship between a male and a female). While social monogamy is common among birds (Lack 1968), genetic monogamy is much less frequent (Griffith et al. 2002). Indeed, true genetic monogamy has been found in less than 25% of socially monogamous birds that have been studied (Griffith et al. 2002).

Extra-pair paternity (hereafter, EPP) and intraspecific brood parasitism (IBP) are two of the best-studied alternative reproductive strategies. EPP refers to situations where chicks are sired by a male other than a female's social mate (Ligon 1999), while IBP occurs when a female lays her eggs in the nest of another individual of the same species, without investing in incubation and/or caring for the chicks (Yom-Tov 1980). Until recently, contemporary ecological factors such as breeding density and breeding synchrony have been considered as the main causes of variation in levels of EPP and IBP among species (Møller and Birkhead 1993; Stutchbury and Morton 1995; Westneat and Sherman 1997; Weatherhead and Yezerinac 1998). However, Arnold and Owens (2002) showed that, even though ecological factors may explain differences between closely related species or even among different populations of the same species, over half the interspecific variation in EPP and IBP rates is attributable to variation among taxonomic levels above family, probably because of fundamental contrasts in life history traits and modes of parental care that exist at these deeper nodes of the avian phylogeny (Owens and Bennett 1995).

Among socially monogamous birds, there is a wide range of interspecific variation in the level of EPP (Arnold and Owens 2002; Griffith et al. 2002). While EPP has shown to be common in short-lived species (e.g. 86% of species studied in the Passeriformes order contained extra-pair young; Griffith et al. 2002), it has been proved to be rather infrequent in long-lived birds as in the case of seabirds. In general, lineages that show low rates of EPP are those with significant parental care from males, low adult annual mortality, and long life span (Arnold and Owens 2002). Given the particular life-history characteristics of seabirds (e.g. extended parental care, low adult annual mortality), it is not strange that most of the studies carried out in this group reported low or no EPP (Mauck et al. 1995; Dearborn et al. 2001; Baumgarten et al. 2001; Quillfeldt et al. 2001; Lijfeld et al. 2005; Anderson and Boag 2006; Anker-Nilssen et al. 2008, 2010; but see Huyvaert et al. 2000; Ležalová-Piálková 2011). On the other hand, IBP has been reported for 234 bird species (Yom-Tov 2001). However, the study of the underlying causes of interspecific variation in IBP had received less attention than EPP (Yom-Tov 2001; Arnold and Owens 2002). IBP does appear to be more frequent in precocial species than in altricial ones, implying that in the former group the cost of caring for unrelated young may be lower than in the latter (Yom-Tov 2001).

The Phalacrocoracidae family (order Pelecaniformes) is comprised of 39 species of cormorants and shags characterized by a relatively long life span (average 10–15 years), low rate of annual adult mortality (10–15%) and social monogamy with a strong role of males in brood care (Orta 1992; Nelson 2005). Intriguingly, and contrary to expectation based on these life-history traits, phalacrocoracid species studied thus far show moderate to high levels of EPP within broods (~13–16%; Graves et al. 1992, 1993; Piertney et al. 2003). Based on those studies, Piertney et al. (2003) suggested that EPP may be relatively common in cormorants and shags, and certainly more frequent than in the majority of seabird species. The records for IBP are rare for the group. Graves et al. (1992) reported that one European Shag *Phalacrocorax aristotelis* chick sampled in their study was not the offspring of either member of the social pair, although the authors could not unequivocally assign its origin. Aside from that, we know of no other study that has investigated IBP in cormorants and shags.

Here, we report our findings of a parentage analysis on Imperial Shags *Phalacrocorax atriceps*. Imperial Shags are medium-sized colonial seabirds that inhabit southern South America (Orta 1992; Nelson 2005). Although there are no obvious differences between the sexes in appearance and ornamentation, males are larger and heavier (~18%) than females (males: 2,317 ± 153 g, $n = 139$; females: 1,965 ± 119 g, $n = 152$; Svagelj and Quintana 2007). They are also socially monogamous and raise a single brood per year (Svagelj and Quintana 2011a); clutches are usually comprised of three eggs, with both parents sharing incubation of the eggs for approximately 29 days (Malacalza and Navas 1996; Svagelj and Quintana 2011a, b). Once chicks hatch, both parents play an active role in brood care and chick feeding duties for more than 2 months (Svagelj 2009; Svagelj and Quintana 2011a, b, unpublished data). Given these characteristics, we would predict low or negligible EPP and IBP rates; however, previous work on related species suggests that detecting EPP would not be unexpected.

Methods

Study area

The study was carried out at the Imperial Shag colony of Punta León (43°05′S, 64°30′W), Chubut, Argentina, during 2004 and 2005 seasons. This colony is located in a flat and elliptical area, approximately 130 m length by 15 m width, with nests uniformly distributed with a density of ca. 2 nests m^{-2} (Yorio et al. 1994; Svagelj and Quintana 2011a). During the 2004 and 2005 seasons, the breeding population of the Imperial Shags at this colony comprised a total of 3,200–3,300 breeding pairs (Svagelj 2009; Svagelj and Quintana, unpublished data). Neither mean clutch size (2004: 2.79 ± 0.04 eggs, $n = 143$; 2005: 2.82 ± 0.03 eggs, $n = 195$) nor breeding success (2004: 1.14 ± 0.07 chicks, $n = 143$; 2005: 1.25 ± 0.05 chicks, $n = 195$) differed between the two seasons (Svagelj and Quintana 2011a).

Sampling and genotyping

A total of 37 complete families were sampled, 27 in 2004 and 10 in 2005. Families with three hatchlings were chosen randomly from different parts of the colony, including both early and late breeding attempts at inner and peripheral locations. Each sampled family had a social father, a social mother and three chicks for a total of 185 individuals. All sampled families belonged to the *albiventer* morph (Orta 1992). Adult Imperial Shags were caught at the nest using a pole with a crook on the end, sampled, measured and subsequently ringed with plastic and aluminum rings, allowing us to follow the behaviour of individuals through the breeding seasons. Using heparinised syringes, blood samples were taken from jugular or brachial veins in adults and from tarsal or brachial veins in chicks. We sexed all adults on the basis of morphological measurements (i.e. bill depth and tarsus length), and also by their vocalisations displayed during the courtship and nest defence behaviour (for details, see Svagelj and Quintana 2007). Hence, we had no doubt about the identities of the social father and the social mother for each nest.

Genotyping was performed on blood samples (approximately 50 μl) directly taken from individuals. Samples from 2004 ($n = 135$) were preserved in lysis buffer (Tris 0.1 M, NaCl 0.1 M, EDTA 50 mM, SDS 1%, pH 8), and those from 2005 ($n = 50$) were stored on common filter paper (see Quintana et al. 2008). Whichever preservation method was used, all the DNA extractions were made following the inexpensive silica-based method proposed by Ivanova et al. (2006) adapted for individual spin columns (Epoch Life Sciences, Missouri City, TX, USA). One of the 37 families was incomplete because we discarded one chick sample with an aberrant genotype that resulted from a degraded DNA sample; thus, our results were based on 110 chick samples.

We genotyped all individuals using four polymorphic microsatellites (PcD2, PcT3, PcT4 and PcD5) originally developed for Great Cormorants *P. carbo* (Piertney et al. 1998). Two other microsatellites described in Piertney et al. (1998) were discarded for this study because they showed low variability (only two alleles; PcD6) or high rates of allele size inconsistency (i.e. each individual presented a different allele; PcT1). All forward primers were modified, adding the universal M13 sequence to the 5′ end of each of them, to allow the correct binding of the fluorescent-labeled M13 forward primer (Neilan et al. 1997). PCR reactions were conducted in 10-μl final volumes with PCR cocktail composition and cycling conditions identical for all markers: 10X PCR buffer (200 mM Tris-HCl, 500 mM KCL), ~10 ng genomic DNA, 2.5 mM $MgCl_2$, 0.15 μM of each primer, 0.2 units of Taq polymerase (Fermentas), 0.1 mM dNTPs and 0.06 μM of the FAM blue fluorescent-labelled M13 primer. PCRs were performed in a GenAmp 9700 (Applied Biosystems), and thermal cycling conditions included an initial denaturing step at 95°C for 3 min, then 34 cycles of 95°C for 30 s, 55°C for 45 s, and 72°C for 45 s, with a final extension at 72°C for 20 min. We genotyped the samples on a Beckman-Coulter CEQ^{TM} 8000 (Genetic Analysis System) and microsatellites sizes were scored using CEQ 8000 Genetic Analysis System with proprietary size standards.

Parentage analysis

Summary statistics of the polymorphic markers and allele frequencies were estimated using CERVUS v3.0.3 (Kalinowski et al. 2007). In all cases, the social parents were confirmed as the genetic parents if the multilocus genotype of an offspring was consistent with Mendelian parental inheritance at the four loci considered here. In those cases where a mismatching locus was observed, we calculated the cumulative resemblance probability (P_{RCum}). This method allows discrimination between the possibilities of true parentage or sharing alleles by chance, based on the population frequencies of the alleles shared by two individuals (Ibarguchi et al. 2004). We calculated EPP and IBP 95% confidence intervals based on the binomial distribution according to Sokal and Rohlf (2009).

Results

All markers exhibited inheritance patterns that were consistent with Mendelian expectations, and we found no evidence for linkage disequilibrium. Despite using only

Table 1 Summary statistics for the four microsatellite loci screened for the Imperial Shag *Phalacrocorax atriceps*

Locus	k	Allele size (bp)	He	Ho	F_{null}	NE-1P	NE-2P
PcD2	10	204–230	0.844	0.792	0.031	0.487	0.319
PcT3	14	212–264	0.858	0.863	−0.006	0.459	0.295
PcT4	15	218–282	0.915	0.932	−0.014	0.314	0.186
PcD5	7	209–223	0.499	0.459	0.034	0.863	0.700

Microsatellite names are as in Piertney et al. (1998). Values are based on the genotypes of 74 parents and calculated employing Cervus v3.0.3 (Kalinowski et al. 2007)

k number of alleles, *He* expected heterozygosity, *Ho* observed heterozygosity, F_{null} estimated frequency of null alleles, *NE-1P* non-exclusion probability of the first parent, *NE-2P* non-exclusion probability of the second parent

four markers, our ability to assign parentage was high, with a combined non-exclusion probability for the first or known parent (i.e. mother) of 0.060, and for the second or unknown parent (i.e. father) of 0.012. Although these markers were developed for a congener, they proved to be highly variable within our focal species with mean number of alleles of 11.5 and an average observed heterozygosity of 0.784. PcD5 showed fewer alleles than the other loci (Table 1). Despite that, PcD5 was used for the analysis since non-exclusion probabilities for the first and second parents were lower when we included it. Estimated null allele frequencies for all loci were below the maximum recommended value of 0.05 (Marshall et al. 1998), and thus we retained all microsatellites for our analysis (Table 1).

In all cases, social parents were confirmed as the genetic parents. In each locus, and for the four loci considered here, the offspring showed one allele inherited from the mother and one allele inherited from the father. This was true for all but three parent–offspring pairs, from different families, showing one mismatching locus each. In those cases, the cumulative probability of resemblance (P_{RCum}) was very low: 3.3×10^{-3} for a mother–offspring pair, and 6.6×10^{-5} and 1.6×10^{-6} for two father–offspring pairs. Because the probability of sharing the remaining three alleles by chance was very low, we considered them as true relatives, contemplating mutation or null alleles as possible alternative explanations (see “Discussion”). Therefore, we concluded that there is no evidence for EPP or IBP for any of the 110 Imperial Shag chicks analysed here. Given our sample sizes, and disregarding any interdependency in the dataset, we estimate the maximum level of EPP in our study population to be 3.3% per young and 8.4% per brood, calculated as the upper 95% confidence limit for 0/110 chicks and 0/37 broods, respectively.

Discussion

We assessed the parentage of Imperial Shag chicks and found neither EPP nor IBP. This certainly does not mean that these phenomena do not occur in this species, as there is a strong correlation between sample size, error estimates and confidence intervals (Griffith et al. 2002). However, assuming that our sample comes from a homogenous population, the true rate of both EPP and IBP per young for the Imperial Shag population of Punta León is very low and would not exceed 3.3%.

In the three instances of parent–offspring mismatch, the parentage relationship was confirmed based on the low P_{RCum}. For the mother–offspring pair there is evidence of mutation at locus PcT4. Both parents and the chick were heterozygous, but the latter had a novel allele that was not found in any prospective parent, and was one repeat shorter than that observed in the mother, consistent with mutation via polymerase slippage (Ellegren 2000). For the two father–offspring pairs, the mismatch occurred at loci PcD2 and PcT4. These could have been caused by the presence of a null allele, as both suspected fathers and the chicks were homozygous for the mismatched locus.

Our results contrast with the two previous studies conducted in cormorant and shag species. Using hypervariable minisatellite DNA fingerprinting to assess EPP, Graves et al. (1993) found that 9.3% (15/161) of European Shag chicks were the product of EPP, while 12.6% (11/87) of broods included at least one chick with EPP. Employing DNA microsatellites, Piertney et al. (2003) analyzed EPP frequency in Great Cormorants, finding that 16.1% (10/62) of broods contained extra-pair young. Clearly, Imperial Shags from the Punta León colony contrast with these two species, not supporting the prediction made by Piertney et al. (2003) that EPP would be a relatively common occurrence in cormorants and shags.

Our finding of no EPP in Imperial Shags, however, is consistent with most other seabird species where low or no EPP is the norm (see Griffith et al. 2002 for a review; but see Huyvaert et al. 2000; Ležalová-Piálková 2011). Moreover, our findings are consonant with other pelecaniform species that are closely related to cormorants and shags (see Hackett et al. 2008): EPP per offspring; Masked Booby *Sula dactylatra*: 0%, Baumgarten et al. (2001); Nazca Booby *S. granti*: 0%, Anderson and Boag (2006); Red-footed Booby *S. sula*: 0%, Baião and Parker (2009);

Great Frigatebirds *Fregata minor*: 1%, Dearborn et al. (2001). Except for the aforementioned single case in European Shags (Graves et al. 1992), IBP has not been reported in any pelecaniform species (Yom-Tov 2001). Given that pelecaniforms are altricial birds (Nelson 2005), our findings of no IBP cases in the Imperial Shag supports the notion that IBP rates are lower in altricial species than in precocial ones (Yom-Tov 1980, 2001).

Variation in the frequency of EPP depends on the relative benefits and costs that accrue to males and females (Westneat et al. 1990; Birkhead and Møller 1992; Petrie and Kempenaers 1998; Griffith et al. 2002; Westneat and Stewart 2003). Females involved in extra-pair copulations could benefit directly (e.g. fertility insurance) or indirectly (e.g. through genetic benefits for their offspring) (reviewed in Westneat et al. 1990; Birkhead and Møller 1992; Petrie and Kempenaers 1998; Petrie et al. 1998). However, if males retaliate with reduced parental care in response to low paternity certainty, and if females cannot compensate for the loss, then those females will be less likely to be involved in EPP (Mulder et al. 1994; Gowaty 1996; Møller 2000; Arnold and Owens 2002). Therefore, a negative relationship between level of male parental care and frequency of EPP has been predicted (Gowaty 1996). In the Imperial Shag, the lack of EPP could be consequence of the potentially high costs to females in relation to the retaliation that they could suffer from their social mate for engaging in extra pair copulations (e.g. reduced parental care, nest abandonment or divorce). Because in the Imperial Shag both sexes have an active role in egg incubation, nest defence and chick feeding for more than 2 months (Svagelj 2009; Svagelj and Quintana, unpublished data), we posit that the cost of nest abandonment or diminished parental care by the male would be high in this species.

A positive correlation between adult mortality rate and EPP frequency among species has also been predicted, because males should be less likely to desert their mates or reduce levels of parental care in response to paternity uncertainty if there is a high probability of mortality and thus low opportunities for future reproduction (Wink and Dyrcz 1999; Mauck et al. 1999). Wink and Dyrcz (1999) and Arnold and Owens (2002) found a significant positive relationship between EPP and adult mortality rate among bird species. Preliminary data from our ringed population of Punta León show that apparent annual survival probability is ~0.9 (Svagelj and Quintana, unpublished data), implying that males with paternity uncertainty would not be constrained in this way.

In summary, both intensive paternal care and high adult annual survival are two very important predictors of EPP when we compare major avian lineages (Arnold and Owens 2002). However, these traits do not explain the contrast between Imperial Shags and the two other phalacrocoracid species analysed so far, since they are closely related and as expected very similar in both attributes (Nelson 2005). A possible explanation for the differences in EPP rates among them could be related to differential courtship and copulation behaviours, probably influenced by the contemporary ecological and genetic benefits of alternative reproductive strategies (Arnold and Owens 2002). In Great Cormorants, males have been observed to copulate with different females in the same nest, and females have been observed moving between males already settled in nests (Kortlandt 1995). Similarly, most extra-pair copulations observed in European Shags took place at the male's nest site, where females responded to the solicitation display of the male (Graves et al. 1993). Although we have no specific data on these behaviours in the Imperial Shag, our preliminary observations suggest that male solicitation displays and females active searches for extra-pair copulations are not as common as in other species of the family (W.S. Svagelj, personal observation). Detailed field studies testing for the existence and circumstances that underpin these behaviours in the Imperial Shag are needed.

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