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PARENT-OFFSPRING RECOGNITION IN CAVE SWALLOWS

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ABSTRACT

Natural selection favors investment in genetic relatives over giving such aid indiscriminately. Parental care is often easily directed only towards genetic relatives, but under some circumstances, this task can be problematic. For example, in systems where unrelated young are commonly mixed with genetically related offspring (through such mechanisms as brood parasitism and extrapair fertilization), or when young are mobile, there is increased risk of misdirecting parental care. In such cases, parents may evolve mechanisms for recognizing their own young. The colonial cave swallow (*Petrochelidon fulva*) presents an outstanding opportunity for testing predictions about the evolution of parent-offspring recognition in response to the risk of misdirected parental investment. In breeding colonies, offspring are known to mix through brood parasitism; extrapair young are also likely. After juveniles leave the nest, they form mixed-family flocks, crèches, and remain dependent on their parents for food. Provocatively, the nestlings are known to develop idiosyncratic white facial plumage patterns, a feature that has been suggested to provide a visual signature system for parent-young recognition in cliff swallows. The open structure of many cave swallow nests, unlike those of cliff swallows, facilitates observation (and filming) of parental feeds to chicks. In the following chapters, I examine how likely cave swallow parents are to encounter young unrelated to them and whether they have evolved strategies to reduce the risk of caring for unrelated young. Chapter 1 details a series of behavioral studies designed to determine whether parents recognize their own offspring and, if so, when and how such abilities develop. Temporary nestling transfer experiments showed that parents bias feedings toward a chick from their own nest vs. one from another nest

when chicks are older (18 days) but not when they are about halfway through the nesting phase (10 days). To examine the mechanism by which parents learn to identify their young, I also cross-fostered half-broods of young chicks between nests and tested parental recognition of older chicks. The results of the behavioral experiments in the cross-fostering nests suggested that parents learn characteristics of the young present in their nest, perhaps in preparation for finding offspring in the crèche after fledging. However, such a mechanism would not allow parents to identify and reject young that arrive in their nest via extrapair matings or conspecific brood parasitism. Chapter 2 examines whether adults are able to reduce the costs associated with hosting parasitic eggs and chicks by recognizing and rejecting foreign eggs. I analyzed egg shape, size, and spot pattern from photographs of cave swallow clutches and found evidence that egg characteristics differed among clutches, and so contain information that could be used to reject parasitic eggs. However, experimental egg transfers showed that cave swallows rarely ejected eggs from their clutches, and hatching failure was no more likely for eggs transferred into clutches than for the hosts’ own eggs. Thus, cave swallows do not appear to have a mechanism to avoid caring for eggs deposited in their nests by brood parasites. Chapter 3 addresses the possibility that the striking variation in facial plumage present in young cave swallows is used by parents in their efforts to recognize their own offspring. Despite the provocative result in an earlier study, which showed that human subjects can reliably identify individual nestlings from photographs in a closely related species, the cliff swallow, this is the first study to pair molecular methods of identifying nestlings unrelated to their nestmates with quantitative analysis of their facial markings. A cross-fostering experiment revealed that genetic effects far outweighed environmental effects on nestling facial patterns, although neither of these effects was significant. Together, these studies show that cave swallows have evolved to reduce the risk of misdirecting parental care at some stages, (post-fledging crèches), but not others (brood parasitism or extrapair young) and suggest that visual traits, perhaps in tandem with vocal cues, may facilitate parental recognition of young.

CHAPTER 1

RECOGNITION OF YOUNG IN A COLONIALLY NESTING BIRD

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Parents ought to restrict costly parental care to their genetic offspring and, particularly when the risk of misdirecting care is high, parent-offspring recognition may evolve. I tested whether adult cave swallows, which nest in dense colonies and feed fledglings in mixed-family groups, discriminate against unrelated young, using temporary chick transfers at two nestling ages and a cross-fostering experiment. Temporary chick transfers indicated that parents bias feedings toward their own offspring near fledging (18 d) but not at about halfway through the nesting period (10 d). I also examined how parents learn to identify their offspring by cross-fostering young 3 d after hatching and testing parental response 2 weeks later. Adults did not favor their own offspring over unrelated nestlings when both were unfamiliar to the focal parents. However, when parents encountered two of their own offspring, one of which was reared by foster parents, they preferentially fed the familiar nestling. By recognizing young, cave swallow parents reduce some risks of misdirected parental investment (mobile fledglings) but not others (extra-pair young and intraspecific brood parasitism).

INTRODUCTION

Natural selection favors individuals that direct costly assistance to genetic relatives over individuals that give such aid indiscriminately (Hamilton 1964). Thus, breeding adults should restrict parental investment (*sensu* Trivers 1972) to their own offspring. This may be accomplished with simple rules like “feed all young in my nest,” if nest occupancy is a reliable indicator of relatedness. However, such rules may not suffice when unrelated young intermingle, creating a potential selection pressure for parental ability to recognize their own offspring. Empirical studies confirm that parent-offspring recognition tends to evolve in species in which unrelated young can move into the wrong nest or otherwise commingle while dependent on parental care (e.g., Davies & Carrick 1962; Beer 1969; Miller & Emlen 1975; Beecher et al. 1981a; Stoddard and Beecher 1983; Insley et al. 2003b). For instance, colonial species more successfully reject alien young and use more complex individually-specific ‘signatures’ for recognition than their non-colonial counterparts (Beecher 1988). Similarly, penguins that combine nest location and vocal recognition for chick identification have less complex vocal signatures than species that use vocal cues alone (Jouventin & Aubin 2002). Recognition ability also varies within species as a function of the risk of misdirected care. In many species, recognition is delayed until just before the onset of offspring mixing (e.g., Beecher 1991; LeFevre et al. 1998; Insley et al. 2003b). When only one sex encounters unrelated young, sex differences in parental recognition of young arise (e.g., razorbills *Alca torda*: Insley et al. 2003a). Mutual recognition between parents and offspring is common (e.g., Beer 1969; Beecher et al. 1985;

LeFevre et al. 1998; Balcombe & McCracken 1992), although not necessarily symmetrical (Insley 2001).

Recognition is an internal process that cannot be observed directly and therefore must be inferred from differential treatment of individuals (discrimination: Waldman et al. 1988). Many animals use template-based learning to recognize kin. Generally, a mental template is produced during contact with conspecifics (or oneself) during an early stage of development; later, individuals are treated as kin if their phenotypes match the template (e.g., Holmes & Sherman 1982; Mateo & Holmes 2004). Some discrimination mechanisms require direct contact with putative kin during template formation; that is, only previously encountered individuals may be recognized (direct familiarization: Tang-Martinez 2001; prior association: Mateo & Holmes 2004). Others allow recognition of unfamiliar individuals, provided their templates are sufficiently similar to the template (indirect familiarization: Tang-Martinez 2001; phenotype matching: Holmes & Sherman 1982). Indirect familiarization mechanisms allow unfamiliar individuals to be recognized as kin; because recognition by direct familiarization is limited to familiar individuals, this mechanism is most useful when unfamiliar individuals are unlikely to be relatives. These mechanisms can be used by the same species in different contexts. For example, female Belding’s ground squirrels (*Spermophilus beldingi*) use direct familiarization to recognize pups because unfamiliar pups are unlikely to be their offspring (e.g., Holmes & Sherman 1982). However, more distant relatives (e.g., aunts, uncles, cousins) may not be familiar, and so indirect familiarization is used for recognition among adults and juveniles (e.g., Holmes 1986; Mateo 2002).

Cave swallows (*Petrochelidon fulva*) present an opportunity to test for avian parent-offspring recognition. Because of the densely-packed nature of their nesting colonies and additional risks for misdirected parental care, parents would likely benefit from the ability to discriminate against unrelated young. Colony size can reach a few thousand active nests (Strickler & West 2011), and roughly 6% of nests in the study population are subject to conspecific brood parasitism (Weaver & Brown 2004). Cave swallows generally lay 3-5 eggs per clutch, and most birds lay 2 or 3 clutches per year (Weaver 2002). Nestlings remain in the nest for 24-29 d; fledglings from several nests form crèches, and parents continue to provision young for several days after they have fledged (Strickler & West 2011). Nestling cave swallows develop highly variable white-and-dark facial patterns, similar to those of cliff swallows (*P. pyrrhonota*), by 17-18 d (Martin et al. 1986). Cave swallows may also use vocal signatures for parent-offspring recognition, like the congeneric cliff swallow (Stoddard & Beecher 1983; Beecher et al. 1985). Despite the presence of ecological factors that should favor parent-offspring recognition, such as conspecific brood parasitism, densely packed nesting colonies, and crèches, as well as the presence of auditory and visual traits that likely facilitate parent-offspring recognition, cave swallows have not been tested for parent-offspring recognition.

Here, I present a field study examining whether cave swallows are capable of parent-offspring recognition at the nest. To test whether parents discriminate against unrelated young, I observed parental feeding decisions after nestlings were experimentally transferred between nests at two ages, before and after the development of putative recognition cues. In a second experiment, I reciprocally cross-fostered chicks between nests to examine whether parents bias feeding toward familiar or related nestlings.

METHODS

General Methods

The study site included colonies in bridges and culverts in Refugio County, TX (28º09’N, 97º23’W); I selected colonies with nests < 4 m high for accessibility. The study colony in 2009 had 320 active nests (as defined by Brown & Brown 1996); colonies used in 2010 had 270 and 136 active nests. Beginning in late April of 2009 and 2010, I checked nests every 2—3 d to estimate hatch dates (13 d after laying was completed), switching to daily visits when hatching was expected. Eggs within a clutch tend to hatch within 24 h; on the date at which hatching was observed, a nest was considered 0 d old. As part of another study, nestlings from all nests used in these experiments were banded and photographed, and two small (70 µl) blood samples were taken from the brachial vein for DNA analysis. Colonies were visited only after 08h00 to avoid disturbing egg-laying, and visits were limited to 1.5 h (Brown & Brown 1996).

For both experiments in this study, I observed adults feeding pairs of nestlings placed in the nest. Parental response was assessed with small, portable cameras attached to the adjacent wall and recorded onto portable digital video recorders (Pierce &

Pobprasert 2007); cameras were installed 1 d prior to trials. On the day of the test, chicks were randomly marked with small dots of paint on either the right or left wrist to facilitate identification of chicks on videos. Markings were assigned independently of chick status (e.g., familiar vs. unfamiliar). All nestlings were weighed prior to trials, and then test subjects were placed in the focal nest and video-recorded for 3 h trials. Thus, all nestlings were handled similarly prior to experimental trials. Videos were scored using the paint marks to distinguish chicks, whose status was ascertained only after scoring. Chicks not used in the experimental trials at their nest were held at ambient temperature and fed ~1 ml baby bird food (Kaytee Products, Inc., Chilton, WI, U.S.A.). After trials, all chicks were returned to the nest in which they were reared.

Protocol for this study was approved by the University of Oklahoma Institutional Animal Care and Use Committee and conducted with the required state and federal permits.

Experiment 1: Temporary Nestling Transfers

To test whether parents discriminate against unrelated nestlings, I presented parents with two size- and age-matched chicks: one from their own nest and one from another nest in the colony. Only one chick was used from each donor nest. This experiment was performed twice, first at nests (n = 17) with pairs of 10-day-old chicks, and then, at different nests (n = 24), with pairs of 18-day-old chicks; thus recognition was tested both before and after the visual and vocal cues hypothesized to mediate recognition were expected to have developed (Strickler & West 2011). Trials began at 08h30. Experimental nestling pairs did not differ in mass at either age (10 d: paired t_{16} = -0.09, p = 0.928, familiar 20.56 ± 1.46 g, unfamiliar 20.57 ± 1.53 g; 18 d: Wilcoxon signed ranks T = -0.40, p = 0.069, n = 24, familiar 22.28 ± 1.81 g, unfamiliar 22.79 ± 1.94 g). Descriptive statistics throughout are given as mean ± standard deviation; further details of statistical analysis and video scoring are given in a separate section below.

Experiment 2: Cross-fostering

To test whether parents discriminate against unfamiliar young, I reciprocally cross-fostered half-broods between 24 pairs of nests 3 d after hatching, and then tested parental response to secondary (temporary) chick transfers at 18 d post-hatching. For initial cross-fostering, 1—2 chicks were transferred between broods of 3—5 chicks. Pairs of nests were matched for brood size ± 1 chick; all nestlings were weighed and cross-fostered chicks were matched for mass (Dugas 2012). The number of nestlings cross-fostered depended on the brood size. When the smaller brood of the pair contained 3 chicks, one nestling was transferred; when the smaller brood size was 4 or 5, 2 nestlings were transferred (Dugas 2012). Thus, the cross-fostered broods never contained more non-resident (transferred) chicks than residents. After cross-fostering, all nestlings were temporarily marked on the tarsus with non-toxic ink to denote their resident or non-resident (cross-fostered) status; markings were refreshed every 2—3 d until chicks were banded at 9 d old. To test whether cross-fostering affected chick mass near fledging, all chicks were weighed at 18 d-old, immediately prior to experimental trials.

On day 18, two separate 3 h behavioral trials were conducted with pairs of chicks at each of the focal nests. The chicks used in each trial came from the focal nest and/or the cross-fostering partner nest, where some of the focal parents' offspring had been transferred and reared. In Unfamiliar Unrelated—Unfamiliar Related trials (n = 15), parents encountered two unfamiliar chicks, one that was both unfamiliar and unrelated to the focal parents and another that was the focal parents' offspring that had been cross-fostered to another nest at 3 d old. In the Unfamiliar Related—Familiar Related trials (n = 19), parents encountered a pair of their own genetic offspring: one they reared plus one that had been cross-fostered to another nest and was thus unfamiliar. Nestling pairs were selected to minimize mass differences (Unfamiliar—Unfamiliar pairs: paired $t_{14} = 0.32$, $p = 0.751$, Unfamiliar Related 23.63 ± 2.23 g, Unfamiliar Unrelated 23.53 ± 2.81 g; Unfamiliar—Familiar pairs: paired $t_{18} = 0.28$, $p = 0.785$, Unfamiliar Related 22.92 ± 1.91 g, Familiar Related 23.26 ± 2.04 g). The two behavioral trials were conducted sequentially and in random order; trials began at 08h30 and 12h00. All chicks were removed from focal nests between the trials and, when possible, a different Unfamiliar Related nestling was used for each trial. For 9 nests at which only one Unfamiliar Related chick remained by 18 d old, these chicks were used in both trials; the order of the 2 experimental trials was split approximately evenly among these nests.

Video Scoring and Data Analysis

For each adult arrival, I recorded which chicks gaped (fully opened bill before food was delivered) and which received the food. The subset of deliveries at which both chicks gaped before one was fed was considered separately for some analyses. Adults pack multiple small insects into a bolus held in the beak or throat pouch and provision a single nesting per feeding trip. Food deliveries could be unambiguously assigned to a single recipient for 96% of 694 feeding events in 2009 and 95% of 617 feeding events in 2010; the remaining feeding events were excluded from analysis. Food deliveries from males and females were combined because the sexually monomorphic adults were not marked to allow identification of parental sex. All videos were scored using Zoom Player Standard software (Inmatarix Media Solutions, Haifa, Israel).

Throughout the study, I used two-tailed paired tests with chick status (e.g., familiar vs. unfamiliar) as the independent variable; the number of feedings received and the number of feedings at which each begged were dependent variables. When difference scores were normally distributed, I used paired t tests; otherwise I used Wilcoxon signed-ranks tests. Descriptive statistics are given as mean ± standard deviation.

To test whether cross-fostering affected nestling mass at 18 d, I used linear mixed models with nestling status (resident vs. non-resident) included as a fixed effect, brood size included as a covariate, and colony, dyad (pair of nests), nest-of-origin within dyad, nest-of-rearing within dyad and nest-of-origin x nest-of-rearing within dyad as random effects. Degrees of freedom for fixed effects were calculated with the Kenward Rogers method (Spilke et al 2005; Biard et al 2006). I used SAS v. 9.2 (SAS Institute, Inc., Cary, NC) for the mixed model and SPSS v. 18 (SPSS, Inc, Chicago, IL) for all other analyses.

RESULTS

Experiment 1: Temporary Nestling Transfers

Overall, familiar nestlings were fed significantly more often than unfamiliar ones at 18 d-old (paired $t_{23} = 3.60$, $p = 0.002$, familiar 8.46 ± 5.73 feeds, unfamiliar 3.13 ± 3.87 feeds), but not at 10 d-old (paired $t_{16} = 1.84$, $p = 0.083$, familiar 12.94 ± 4.50 feeds, unfamiliar 10.11 ± 4.59 feeds). However, familiar chicks also gaped at more food deliveries than unfamiliar nestlings at both ages (10 d: paired $t_{16} = 2.29$, $p = 0.040$, familiar 18.56 ± 5.74 gapes, unfamiliar 15.94 ± 6.53 gapes; 18 d: paired $t_{23} = 3.33$, $p = 0.003$, familiar 8.96 ± 6.04 gapes, unfamiliar 4.29 ± 4.61 gapes). When analysis was restricted to parental deliveries where both chicks gaped, familiar chicks were fed significantly more often than unfamiliar nestlings at 18 d-old (Wilcoxon signed ranks $T = 1.99$, $n = 24$, $p = 0.046$, Fig. 1), but not at 10 d (paired $t_{16} = -0.47$, $p = 0.647$, Fig. 1).

Experiment 2: Cross-fostering

There was no evidence that resident chicks were more likely to survive than their non-resident foster-siblings: of 47 nests that were followed to 18 days old, 29 had no mortality, 8 had one resident chick die, 4 had one non-resident chick die, and in 6 nests all chicks died prior to 18 days old. Day 18 mass did not differ for residents or non-residents ($F_{1, 17} < 0.01$, $p = 0.970$), and no other effects in the model significantly predicted nestling mass (all $p > 0.16$).

In Unfamiliar—Unfamiliar trials, related and unrelated nestlings received equal numbers of feeds when both chicks begged (Wilcoxon signed ranks $T = -0.05$, $n = 15$, $p = 0.959$, Fig. 2). By contrast, in Unfamiliar—Familiar trials, familiar nestlings were fed more than unfamiliar ones when both chicks begged, even though both chicks were related to the focal parents (Wilcoxon signed ranks $T = -2.16$, $n = 19$, $p = 0.031$, Fig. 2).

DISCUSSION

These results support the hypothesis that cave swallows direct parental investment selectively toward their nestling young. In simultaneous-choice experiments, parents made significantly more food deliveries to 18 d chicks from their own broods than to those temporarily transferred from other nests. This effect held when analysis was restricted to food deliveries at which both chicks gaped, indicating that parental favoritism was not due solely to differences in begging per se. Parents of younger chicks (10 d) also showed some preference for familiar nestlings, but the weak statistical effect vanished when restricted to feeds at which both chicks begged. Parents' behavior after cross-fostering of nestlings was also consistent with recognition based on familiarity. Basically, parents fed familiar offspring more than unfamiliar young, even when both subjects were their own genetic offspring. However, parents did not differentiate between an unrelated chick and their own offspring when both were unfamiliar; they did not discriminate in favor of their own, unfamiliar offspring. Together, these findings suggest that parents rely, at least in part, on familiarity when discriminating among chicks.

Familiarity is a key component of social interactions that use learned recognition templates, including parental recognition of young (e.g., Holmes & Sherman 1982; Beecher 1988), sibling recognition (e.g., Beecher & Beecher 1983; Porter 1988), and helping behavior in cooperative breeders (e.g., Komdeur 1994; Sharp et al. 2005). When discrimination between familiar and unfamiliar individuals can be used as a proxy for kin and nonkin, direct familiarization is common (Porter 1988; Tang-Martinez 2001). For example, direct familiarization is often the mechanism of parent-offspring and sibling recognition among mammals in which family or individual-specific cues can be learned before young from different litters intermix (e.g., Porter et al. 1978; Holmes & Sherman 1982; Hepper 1983, Porter et al. 1986, Porter 1988; Yamazaki et al. 2000). The results of this study are consistent with direct familiarization as the mechanism of parent-offspring recognition in cave swallows because there was no evidence that parents recognize their own offspring when it is unfamiliar, as would be expected with indirect familiarization. Although direct vs. indirect familiarity mechanisms have not been tested in other colonial swallows, familiarity is likely important to parent-offspring recognition in these species; parents learn chicks' vocal and/or visual signatures prior to discriminating among chicks (Beecher et al. 1981b; Stoddard & Beecher 1983).

In most colonial species, parent-offspring recognition appears to develop just prior to offspring mobility, after which parents' risk of misdirecting care increases sharply (e.g., Tinbergen & Perdeck 1950; Davies & Carrick 1962). Parental recognition of young cave swallows appears to develop just prior to fledging, after which the young form mixed-family crèches and remain dependent on their parents for several days (Strickler & West 2011). At natural sites, cave swallows crèche in cave recesses, where they are fed by adults (Strickler & West 2011). Similarly, recognition in bank swallows (*Riparia riparia*) and cliff swallows, which also provision young in post-fledging crèchesnnnn, coincides with the increased probability of misdirecting care. In cliff and bank swallows, nestlings have non-individualized begging calls when young, but these gradually change until each gives an individualized signature call. Signatures crystallize several days before fledging, at which point parents discriminate against foreign chicks in the nest (Beecher et al. 1981b; Stoddard & Beecher 1983). Both cliff swallow (Stoddard & Beecher 1983) and cave swallow (Martin et al. 1986) nestlings develop highly variable patterns of facial feathers, which may provide an additional visual cue of identity.

Many systems rely on mutual recognition between parents and offspring, rather than solely on parental recognition of young. For example, a vocal exchange between parent and offspring facilitates recognition in some birds and mammals (e.g., Thompson & Emlen 1968; Beer 1979; Trillmich 1981; Balcombe & McCracken 1992). Recognition of parents by offspring may develop earlier or be stronger than parental recognition of offspring (e.g., Beer 1969; Knudson & Evans 1986; Insley 2001). I found that cave swallow nestlings were significantly less likely to beg to an unknown adult than to their own parents. Both nestlings were handled similarly before experimental trials (e.g., removed from nests and weighed), so this difference is probably not an artifact of experimental design. This result is, however, consistent with chicks discriminating between their own parents and unfamiliar adults, although it cannot be separated from the possibility that non-resident chicks were responding to various aspects of the strange nest and/or novel nestmate rather than the parents themselves. In species in which parents recognize offspring, including other swallows, offspring are often capable of recognizing siblings and parents (Beer 1969; Beecher & Beecher 1983; Beecher et al. 1985; Sieber 1985; LeFevre et al 1998). Other swallows recognize their siblings (Sieber 1985) and parents (Beecher & Beecher 1983; Beecher et al. 1985) in addition to parents recognizing their offspring (Beecher & Beecher 1981a; Stoddard & Beecher 1983).

In this study, cave swallows did not eject foreign chicks from the nest, and few aggressive acts (pecking at a chick or tugging at its wings) were observed. The strength of rejection behavior will depend on the likelihood and costliness of identification errors (i.e., rejection own offspring) (Lotem 1993; Shizuka & Lyon 2010). Mistakenly ejecting one's own chick would mean wasting the 3 weeks of investment already provided; perhaps cave swallows have evolved a milder defense that takes the form of simply allocating food preferentially to familiar chicks. Alternatively, adults may be physically unable to eject 18 d-old chicks, which are at least the same mass as adults. A final possibility is that the nest is a sufficiently reliable predictor of relatedness in cave swallows such that evolutionary pressure to reject chicks within the nest has been low. Cave swallow chicks occasionally crawl into neighboring nests, but after fledging they generally do not return to the colony. Bank swallows, on the other hand, return to the nest for several days after they begin flying. These fledglings often fly into the wrong nest, where they are forcibly ejected by the parents tending that nest (Beecher et al.1981a).

When the risk of misdirecting parental care is high, as with species in which families intermingle, parent-offspring recognition is expected to evolve. Cave swallows encounter unrelated young both early and late in the nesting cycle, and their ability to discriminate against unrelated young supports the expectation that parents should preferentially allocate care toward their own offspring. Although it appears that the discriminative abilities of cave swallow parents protect them from caring for unrelated chicks they encounter near fledging, they still risk caring for parasitic or extra-pair young, which arrive in the nest as eggs. This suggests that the selective pressures on parents and young vary with time through the nesting cycle, or that the cues necessary for identifying offspring are unavailable early in the nesting cycle. Further investigation of the cues used in parent-offspring recognition and the frequency with which parents encounter unrelated young in the nest would facilitate a better understanding of the selective forces that limit early development of parent-offspring recognition.

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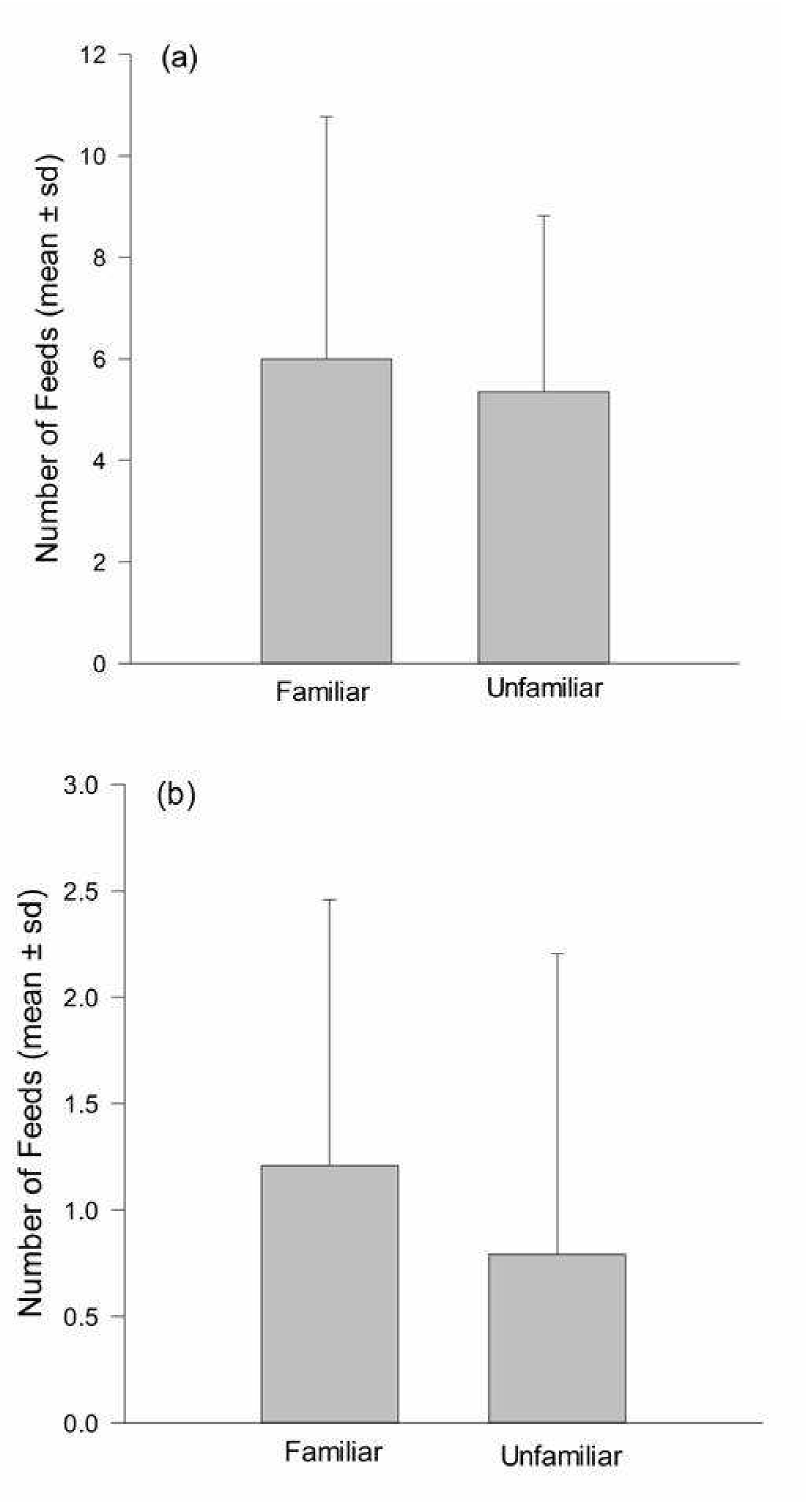

Fig. 1: Number of feeds received by familiar and unfamiliar young (a) at 10 d old and (b) at 18 d old.

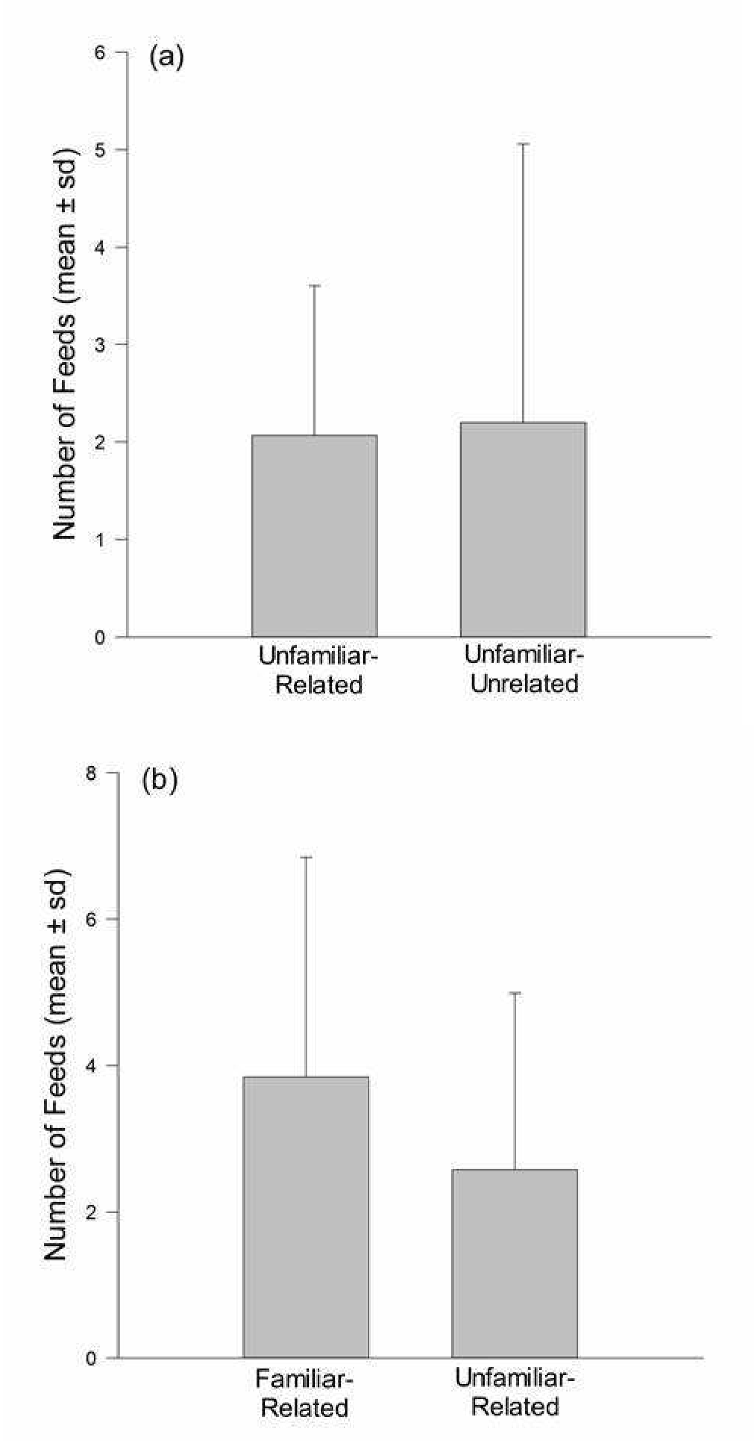

Fig. 2: Comparison of the number of feeds received by experimental pairs of 18 d old nestlings. (a) shows Unfamiliar Related vs. Unfamiliar Unrelated chicks; (b) shows Familiar Related vs. Unfamiliar Related nestlings.

CHAPTER 2

FAILURE TO REJECT CONSPECIFIC BROOD PARASITIC EGGS DESPITE HIGH VARIATION IN EGG SHAPE AND PATTERN AMONG CLUTCHES

(Formatted for *Behavioral Ecology and Sociobiology*)

To reduce the costs of brood parasitism, hosts often evolve resistance measures, including egg recognition based on egg coloration, pattern or, less frequently, shape. This study examines the potential for rejection of conspecific parasitic eggs by a colonial passerine, the cave swallow (*Petrochelidon fulva*). Analysis of photographs revealed that egg pattern and shape were more similar within than between clutches; thus, eggshell characteristics could inform adults about the presence of parasitic eggs. However, experimental transfers of half-clutches between nests rarely resulted in egg losses (inferred rejection), and transferred eggs were equally likely to hatch as non-transferred ones. Cave swallows may not reject parasitic eggs due to constraints on the evolution of recognition. Alternatively, the net costs or the probability of being parasitized may be sufficiently low to counter the potential benefits of egg recognition.

INTRODUCTION

Many species in which parents frequently encounter offspring other than their own have evolved strategies to reduce the risk of misdirecting parental care. Defenses against avian brood parasitism commonly include guarding the nest from parasites, removing eggs in the nest prior to laying a clutch, and/or rejecting parasitic clutches or eggs (Payne 1977). The evolution of egg recognition may be more difficult for hosts of conspecific brood parasites than for hosts of interspecific brood parasitism because the initial differences between host and parasite eggshells are likely to be lower when host and parasites are different species (Rothstein and Robinson 1998). Accordingly, many reports of egg recognition by hosts of conspecific brood parasites come from species with remarkably high variation in eggshell appearance; in several species successful rejection can be predicted by the magnitude of visual contrast between host and parasite eggs (Victoria 1972; Moksnes 1992; Lyon 2003). Recent work in cuckoo hosts, however, suggests that high within-clutch variation in appearance may facilitate the rejection of mimetic parasitic eggs, perhaps because a single parasitic egg is unlikely to match an entire clutch if host eggs are highly variable (Avilés et al. 2004; Cherry et al. 2007).

Birds may use one or a combination of egg characteristics to reject parasitic eggs (e.g., Lyon 2003; Victoria 1972; Braa et al. 1992). Background color (Lyon 2003; Jackson 1998) and spot pattern (Lopez-del-Hierro and Moreno-Rueda 2010), alone or in tandem (Victoria 1972), are frequently used in egg recognition. The blunt egg pole, often the most densely spotted egg region, is essential for egg recognition in some species (Lahti and Lahti 2002; Polačiková et al. 2007, Polačiková et al. 2010; Polačiková and Grim 2010). These studies highlight the importance of considering the relative contribution of each egg region to recognition, rather than averaging pattern characteristics over the entire egg (Polačiková et al. 2007, Honza and Polačiková 2008). Egg size can also inform hosts about parasitic eggs (Davies and Brooke 1988; Rothstein 1982; Marchetti 2000). In parallel, Bán et al. (2011) suggest that egg shape may perform a similar function in species that base discrimination on egg size, but this needs further study.

Cave swallows (*Petrochelidon fulva*) are colonially-nesting passerines whose risk of misdirecting parental investment spans the entire nesting cycle. Conspecific brood parasitism, including the transfer of eggs from one nest to another, has been reported in up to 6% of nests (Weaver and Brown 2004). Additionally, fledglings form mixed-family crèches while still dependent on parental care (Strickler and West 2011). Although experimental evidence shows that cave swallow parents discriminate against unfamiliar nestlings, this method of recognition leaves parents vulnerable to brood parasitism (Strickler 2013). I estimated the incidence of conspecific brood parasitism in cave swallows, and I examined whether cave swallows reduce the costs of conspecific brood parasitism via egg rejection by experimentally transferring eggs between pairs of nests and observing hatching success of resident (non-transferred) and transferred eggs. I also photographed clutches and analyzed within- and among-nest variation in egg shape and spot pattern.

METHODS

From April-July 2008-2010, I visited seven cave swallow colonies in highway culverts in Refugio Co., TX, (28°09'N, 97°23'W) and checked nests every 2 d during egg laying and incubation, switching to daily visits when hatching was expected (13 d after laying ended). Colonies were selected for nest accessibility (nests <4m high) and contained 14-130 active nests. I avoided checking nests before 0800 hours or disrupting colonies for longer than 1.5 h (Weaver and Brown 2004). I monitored 244 nests during egg laying to assess the frequency of brood parasitism and followed 213 of those nests until hatching to evaluate the rate of clutch failure. Nests were considered parasitized if more than one egg was laid per day (Brown 1984), and a clutch was considered to have failed if the nest was found empty before the estimated hatch date.

At a subset of 52 unparasitized nests, I experimentally manipulated clutches to mimic parasitism. The day after laying was completed (when clutch size stopped increasing), I removed all eggs from the nest, photographed the clutch, and individually marked eggs with a small dot of non-toxic ink on the blunt pole. The eggs were placed near a ruler, 38 cm from the camera (Camedia 4000, Olympus Co., Tokyo) and photographed in a portable dark box against a standard gray card (Kodak). To avoid pseudoreplication, each nest was used only once and all experimental manipulations were performed within a 2-week period in 2010, so these clutches were laid by different females.

After photography, I replaced half of the eggs from each clutch in their own nest; the remaining eggs were reciprocally transferred with another clutch at which laying had begun on the same day. I used clutches of 4 or 5 and transferred 2 eggs. This produced experimental clutches with 2-3 own and 2 transferred eggs; after transfers, no clutch had more transferred eggs than own eggs. I recorded how many transferred and non-transferred eggs remained in the nest 1 and 3 d after egg transfers, on the estimated hatching date and 2 d after chicks began hatching. Cave swallow clutches usually hatch within 24 h (unpub. data), so eggs remaining 2 d after hatching began were considered to have failed.

I analyzed egg shape from the photographs by setting 9 points along one edge of each egg, with one point at each egg pole and the others evenly spaced between (Fig. 1), and then fitting a 3^{rd} degree polynomial function using ImageJ software (Todd and Smart 1984; Abramoff et al. 2004; Mónus and Barta 2005). This fits the points to the function $f(x) \rightarrow Y = \pm\sqrt{(a^2 - X^2)(c_0 + c_1x + c_2x^2 + c_3x^3)}$, where 2a is the length of the egg, X and Y represent the coordinates of the outline points, x = X/a, and $2ac_0$ = the width of the egg. The function parameters define the shape of the egg; higher values of c_1 correspond to more pointed eggs, c_2 determines the bicone character of the egg (Preston 1968), and c_1 and c_3 together determine the egg's asymmetry (Mónus and Barta 2005).

To analyze egg pattern, I quantified the average size of spots, spot density, and pattern coverage (proportion of each egg region covered by spots). Because egg pattern often varies along the longitudinal axis of eggs, I divided the longitudinal axis into thirds and sampled egg pattern in each third (Stoddard and Stevens 2010). Spots were analyzed within a rectangle covering 90% of the length of the egg and the maximum width possible (Avilés et al. 2010); this rectangle was divided into equal thirds representing the blunt, middle, and sharp portions of the egg (Fig. 1; Antonov et al. 2010). I converted each image to binary format using the Threshold function in ImageJ, so that each pixel had a value of 1, corresponding to a spot, or 0, corresponding to the background color. Differences in egg curvature made it difficult to set accurate thresholds automatically, so I followed Stoddard and Stevens (2010) and manually adjusted the threshold to capture egg patterns. The number of egg spots (adjacent pixels with a value of 1) and average area of each spot (in cm^2) were calculated using the Analyze Particles function of ImageJ. Spot density was calculated by dividing the number of egg spots by the total sample area. Pattern coverage was calculated as the number of pixels with a value of 1 (corresponding to an egg spot) divided by the total number of pixels in that egg region (Stoddard and Stevens 2010; Spottiswoode and Stevens 2010).

I examined whether there was significant among-clutch variation in egg shape and pattern using a MANOVA with Nest ID as the independent variable and egg shape parameters c_0-c_3 and pattern coverage for each third of the egg as dependent variables. I did not use spot density or average spot size as dependent variables because they were highly correlated with pattern coverage in each of the egg regions (all $r > 0.7$, $p < 0.001$). The MANOVA was performed using PASW Statistics 18 (SPSS Inc., 2009, Chicago).

To determine whether egg status (own vs. transferred egg) affected hatch failure, a proxy for rejection, I used a generalized linear mixed model (Proc GLIMMIX in SAS 9.2; Littell et al. 2006) with egg status as a fixed effect and dyad, nest(dyad), and nest*status as random effects, where dyad represents the identity of the pair of nests whose eggs were swapped. The dependent variable was the ratio of the number of eggs present after hatching (hatch failure) to the initial number of eggs. This was modeled using the events/trials syntax with a binomial distribution and a logit link function; degrees of freedom were calculated using the Kenward-Rogers method (Spilke et al 2005; Biard et al 2006). I tested whether the full model differed from a model with no random effects using tests of covariance parameters based on the residual pseudo-likelihood (Littell et al. 2006) and removed the random effects, which did not affect the model ($X^2_3 > 0.01$, $P > 0.99$). Nests that were empty prior to hatching (n = 10) were excluded from this analysis. The presence of eggs 1 and 3 days after egg transfers was not modeled, because no eggs disappeared from nests after egg transfers.

RESULTS

Brood parasitism was detected in 8.2% of unmanipulated nests (n = 244). Few (4.5%) clutches failed prior to hatching (n = 213). Out of 19 parasitized nests followed to hatching, two failed before hatching.

Analysis of photographs showed that egg shape and pattern coverage differed significantly among clutches (MANOVA Wilks' Lambda > 0.001, $F_{816, 2373.9} = 3.297$, $P < 0.001$, partial $eta^2 = 0.504$). One shape parameter c_0 (= width/length) did not differ among clutches, but all other shape and pattern variables did (Table 1).

After egg transfers, the proportion of eggs that failed to hatch was similar for transferred eggs (0.11 ± 0.03) than for own eggs (0.08 ± 0.03; Table 2).The probability of hatch failure was unrelated to egg status ($F_{1, 82} = 0.41$, $P = 0.521$).

DISCUSSION

This study found no support for the hypothesis that cave swallows use among-clutch differences in shape or pattern to reject parasitic eggs. Egg pattern and shape differed significantly among clutches. However, egg loss was surprisingly rare in experimental nests, and there was no evidence that birds targeted alien eggs for rejection. Furthermore, no eggs in experimental nests were found buried in the nest lining or punctured, though such have occasionally been observed during routine nest checks (pers. obs.).

The lack of egg rejection observed in this study might be due to parental inability to recognize parasitic eggs. Cave swallows may be unable to recognize eggs if the variations in eggshell pattern and shape are too small to be reliably perceived by the birds. In this study, the variation in eggshell appearance was significantly higher among clutches than within them, however, this accounted for only about 50% of the total variation in appearance. This moderate effect size suggests that, while females tend to lay distinctive eggs, these differences may be too small to allow birds to discriminate reliably against the eggs of conspecifics (Brown and Sherman 1989). Another possibility is that egg pattern is obscured by dim light levels at the nest, which might impede egg recognition (Endler 1993). Two recent studies suggest that ambient light conditions can influence the likelihood of egg rejection by cuckoo hosts. Egg rejection was affected by an interaction between photosynthetically active radiation and chromatic contrast in great reed warblers (*Acrocephalus arundinaceus*) (Honza et al.

2011). Nightingales (*Luscinia megarhynchos*), which build open nests in deeply shadowed places, use achromatic contrast rather than eggshell patterns to reject parasitic eggs (Antonov et al. 2011). The dim light within cliff swallow (*P. pyrrhonota*) nests, which are fully enclosed gourds with a narrow entrance tunnel, has been speculated to increase difficulty with egg recognition (Brown and Brown 1988). Cave swallow nests comprise a continuum of shapes ranging from barn swallow-like (*Hirundo rustica*) open cups to flared cups and enclosed gourds with or without entrance tunnels (Strickler and West 2011), so many are more open than nests of cliff swallows, and may admit more light. However, cave swallows typically choose dimmer nesting sites (e.g., by avoiding the sunnier areas) than cliff swallows in mixed-species colonies and in colonies with only cave swallows (pers. obs.). Even if nesting in sunnier areas could help adults recognize parasitic eggs, such brighter nesting spots became available only recently, when cave swallows began nesting on man-made structures in the 1970's (Martin 1974; Palmer 1988).

Alternatively, cave swallows may perceive differences among eggs and recognize their own, but fail to reject foreign eggs. For instance, yellow warblers (*Setophaga petechia*) spend more time looking at and probing or shuffling their clutch once it is experimentally parasitized (Guigueno and Sealy 2012) and olivaceous warblers (*Hippolais pallida*) peck at parasitic eggs (Antonov et al. 2009). However, both these hosts frequently accept parasitism because their bills are too small to puncture-eject parasite eggs effectively (Antonov et al. 2009; Guigueno and Sealy 2012). In cave swallows, the lack of egg ejection is unlikely due to physical inability to remove parasitic eggs (or the risk of damaging own eggs in that process), as physical transfer of eggs between nests has been reported in this study area (Weaver and Brown 2004): eggs can be lifted and carried, so it seems clear that they could be dropped anywhere.

Although egg recognition is a very common response to brood parasitism, that ability may not evolve if the costs of parasitism are sufficiently low (Rothstein and Robinson 1998; Davies 2000; Broom and Ruxton 2002). An earlier study of cave swallows could not detect differences between parasitized vs. control nests in gross success rate of young reaching the nestling period midpoint, but survival was defined only as one or more young remaining by day 10 (Weaver and Brown 2004). These results do not point toward heavy costs associated with conspecific brood parasitism, as found in some species (e.g., Lyon 2003), but more sensitive measurements of host fitness costs are needed to determine whether cave swallow hosts would benefit from anti-parasite defenses. For example, it is not known whether parasitized cave swallow nests produce fewer or lower-quality fledglings than unparasitized nests, nor whether parental effort is harmfully inflated by alien young.

Alternatively, the rarity of parasitism in cave swallows, rather than the absence of unit costs, may reduce selective pressure imposed by conspecific brood parasitism. Over 3 years of study, I found ~8% of nests were parasitized, comparable to the 5% reported previously for this species (Weaver and Brown 2004). The lower rate of conspecific brood parasitism in cave swallows than in cliff swallows (11.9% in Texas: Weaver and Brown 2004; 22-43% in Nebraska: Brown and Brown 1989), has been attributed to reduced exposure to blood-feeding nest parasites at this study site plus cave swallow nesting colonies generally being less densely-packed and smaller than those of cliff swallows, which may complicate depositing eggs in a neighboring nest (Weaver and Brown 2004).

Cave swallows do not appear to have well-developed defenses against eggs inserted by conspecific brood parasites, despite the presence of noticeable variation in eggshell appearance among clutches. In cave swallows (this study), as in cliff swallows (Brown and Brown 1989), brood parasitism appears to be balanced by other factors, such as constraints on egg rejection and the likelihood or magnitude of costs incurred by hosts. Behavioral observations of parasites and hosts will be essential for understanding the ecology of conspecific brood parasitism in this species.

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	Variable	**$F_{51, 165}$**	**P**
Egg Shape	C_0	0.789	0.836
	C_1	12.313	<0.001
	C_2	4.654	<0.001
	C_3	2.613	<0.001
Blunt	Number of Spots	3.112	<0.001
	Mean spot size	3.430	<0.001
	Pattern coverage	4.745	<0.001
Middle	Number of Spots	2.069	<0.001
	Mean spot size	3.402	<0.001
	Pattern coverage	4.790	<0.001
Sharp	Number of Spots	3.683	<0.001
	Mean spot size	3.313	<0.001
	Pattern coverage	4.292	<0.001

Table 1 Post-hoc ANOVAS of each variable included in the MANOVA, including egg shape parameters c_0-c_3 as well as the number of spots, average spot size, and pattern coverage for the blunt, middle, and sharp egg regions. Variables are defined in text.

Egg status	Accepted (hatched)	Rejected (hatch failure)
Transferred	35	7
Non-transferred	33	9

Table 2 Response to egg transfers at 42 nests. Number of nests that accepted all transferred (or non-transferred) eggs vs. rejected at least one transferred (or non-transferred) egg.

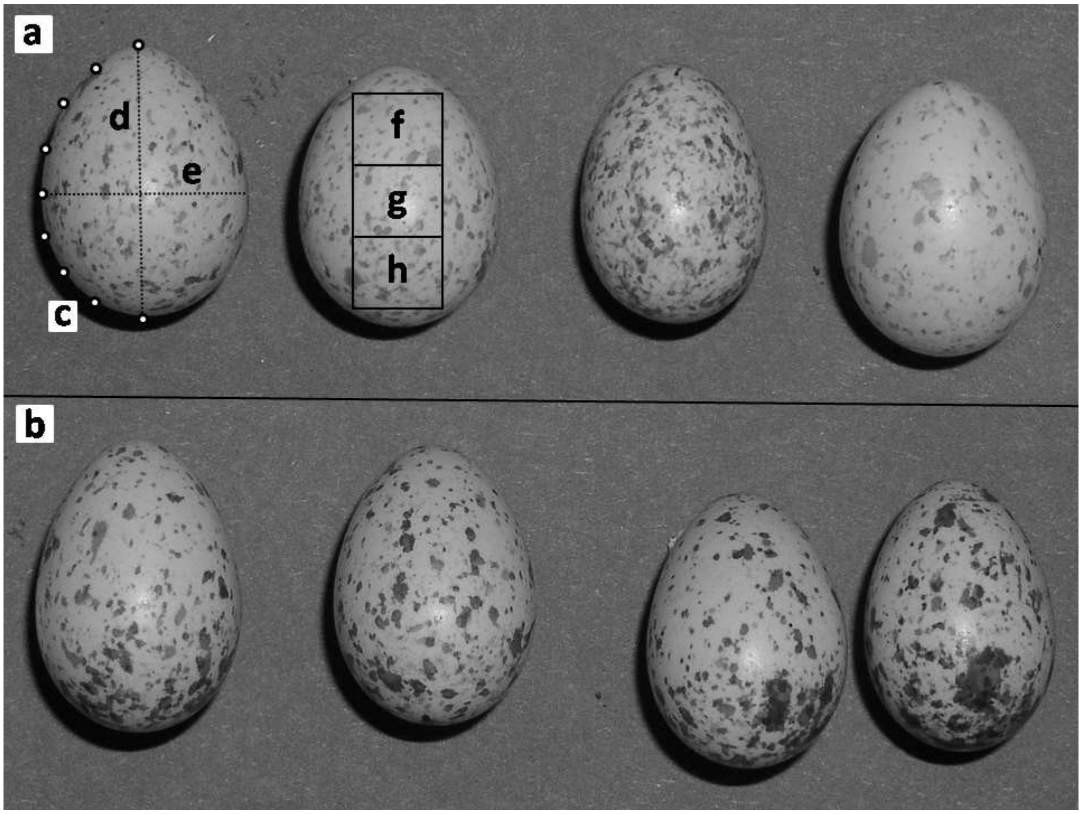

Fig. 1 (a) and (b) Two clutches of cave swallow eggs showing intraclutch variation in egg shape and pattern. Letters indicate landmarks used for analysis: (c) points set along one edge used to analyze egg shape; (d) egg length; (e) egg width; sections used to analyze pattern coverage of the (f) sharp, (g) middle, and (h) blunt egg regions.

CHAPTER 3

POTENTIAL FOR VISUAL RECOGNITION OF YOUNG IN A COLONIAL BIRD

(Formatted for *Behavioral Ecology and Sociobiology*)

This study examines the hypothesis that the highly variable facial plumage patterns present in nestling cave swallows encode information about relatedness among nestlings, which may allow parents to reduce their risk of misdirected parental care. Previous work suggests that the onset of parental discrimination against unfamiliar young coincides with the development of facial feathers. Seventy-five nests chosen subjectively for high or low within-clutch similarity in facial markings showed significantly higher variation in the number of white spots and percent white in the facial markings. A cross-fostering experiment using 14 different pairs of nests did not reveal strong genetic or environmental effects on percent white or number of spots, and heritability was relatively low ($h^2 = 0.23$). In 23 nests selected quantitatively for high or low within-clutch similarity in facial markings, similarity in facial markings was not associated with relatedness within broods. Two of those 23 nests (8.7%) contained one extrapair chick, and none contained brood parasitic chicks. The results of this study are consistent with the hypothesis that nestling facial patterns could provide parents with some information about nestling identity, which could be used to mediate recognition of young after fledging, when juveniles in mixed-family crèches remain dependent on their parents.

INTRODUCTION

When animals engage in social interactions, the recognition and discrimination of individuals can have fitness consequences. Such communications necessarily involve a sender of information, expressed as variable phenotypic traits that convey information about its status, mate quality, identity, etc., and a receiver that must perceive, interpret, and act upon that information. These social exchanges occur in various contexts, including antagonism, cooperation, courtship, parental care, etc., that routinely affect their meanings. During recognition, the receiver forms a mental template based on characteristics of the signal sender. Later, individuals' traits are compared with that template and classified accordingly (Holmes and Sherman 1982; Beecher 1988; Mateo and Holmes 2004; Tang-Martinez 2001). Because recognition is an internal process, it cannot be observed directly and must be inferred from the differential treatment of individuals (discrimination: Waldman et al. 1988). Discrimination may be based on traits that represent individual identity, such as vocal ‘signatures’, or group membership, as in colony-specific chemical profiles in some social invertebrates (e.g., Beecher 1982; Greenberg 1988; Jouventin et al. 1999; Wagner et al. 2000; Tibbetts and Dale 2007).

Visual recognition based on highly variable color patterns may be common. For instance, many birds prone to brood parasitism use egg color and pattern as a basis for rejecting parasitic eggs or clutches (Davies 2000, Rothstein and Robinson 1998). Caspian terns (*Hydroprogne caspia*) learn the color patterns of their hatchling’s down, and reject young that appear dissimilar to their own (Shugart 1990). Visual recognition is also prevalent invertebrates: Lobsters (*Homarus americanus*), crayfish (*Cherax destructor*), and fiddler crabs (*Uca capricornis*) identify familiar opponents visually (Detto et al. 2006; Van der Velden et al. 2008; Gherardi et al. 2010). One well-established visual signature is the facial patterning of the brown paper wasp (*Polistes fuscatus*), which signals individual identity and dominance rank (Tibbetts 2002). Nestling cliff swallows (*Petrochelidon pyrrhonota*) possess striking variation in facial plumage, wherein chicks differ in background coloration, the amount of white feathers present, and the number and placement of white spots (Stoddard and Beecher 1983). Human subjects are able to match photographs of single nestlings with another photograph of the same chick when it is offered in an array of eight photographs (Stoddard and Beecher 1983). This provocative result suggests that nestling facial pattern may differ noticeably among individuals and may be useful to parents for recognizing their offspring.

Juvenile cave swallows (*P. fulva*) possess similar facial patterns and adults risk misdirecting parental care throughout the nesting cycle, providing an opportunity to examine the potential for visually mediated parent-offspring recognition. Conspecific brood parasitism affects at least 6-8% of nests (Weaver and Brown 2004; Strickler *unpubl. data*). After fledging, juveniles form mixed-family crèches, where they are fed by parents before becoming fully independent (Strickler and West 2011). Parents learn to recognize familiar nestlings by ca. 1-1.5 weeks prior to fledging (18 days old) and, during experimental trials, allocate more food to familiar chicks (Strickler 2013). The distinctive patterns of facial feathers vary markedly in the amount of white present, the number and arrangement of white feathers and, to a lesser extent, background color

(Fig. 1); these markings are lost during the first prebasic molt, after which all individuals look much more similar (Martin et al. 1986; Strickler and West 2011). I explored whether the facial patterns of young cave swallows inform parents about chick identity and relatedness, testing two interconnected hypotheses. First, if facial patterns reflect genetic relatedness, they should be strongly influenced by pedigree. Accordingly, facial markings should be more similar within than among broods, and a cross-fostering experimental protocol should reveal both high heritability and greater facial similarity among chicks from the same natal nest than among chicks reared in the same nest. Second, similarity in facial pattern should coincide with relatedness, as revealed by molecular assay. To test this, I predicted that broods containing high variation in nestling facial markings should have lower average relatedness than broods in which chicks showed greater phenotypic similarity.

METHODS

Censusing

Cave Swallow colonies were located in culverts and bridges under highways in Bee, Live Oak, and Refugio Counties, TX (ca. 28°09’N, 97°23’W). Colonies contained 14-130 active nests and were selected to maximize nest accessibility (nests < 4 m high). From April-July of 2007-2011, I censused nests every 2 days during the laying and incubating periods and on a daily basis when hatching was anticipated (13 days after laying was completed). Nests were not checked earlier than 0800 hours and colonies were not disturbed for more than 1.5 h (Weaver and Brown 2004). Nestlings were fitted with individually numbered aluminum bands when they were at least 9 days old.

Photography and DNA sampling

After facial feathers developed fully (17-18 days), 440 nestlings from 130 broods were digitally photographed (Camedia 4000, Olympus Co., Tokyo) against a standard gray background (Kodak) from a standardized distance of 38 cm. For genetic analysis, two small (70 μl) blood samples were collected from the brachial vein of each nestling using pre-heparinized capillary tubes and transferred immediately into lysis buffer (Longmire et al. 1988), inverted several times to mix, and subsequently stored at -20°C.

Scoring facial markings

I analyzed facial markings from the photographs by quantifying two features, number of white spots in each forehead patch and percentage of the forehead patch covered by those white spots. Using Image J software (Abramoff et al. 2004), I selected a trapezoid shape to define the outer bounds of the forehead patch (Fig. 1). I converted each photograph to binary format using the Threshold function in ImageJ, so that each pixel had a value of either 1, (a white spot) or 0 (the background color). Slight differences among nestlings made it difficult to capture facial patterns accurately with automatically set thresholds, so I manually adjusted the threshold for each image (Stoddard and Stevens 2010). The number of white spots in that zone was counted for each nestling. The percentage of each forehead patch covered by spots was calculated in ImageJ as the number of pixels with a value of 1 divided by the total number of pixels in the specified forehead patch; this is analogous to the pattern coverage variable calculated for eggshell spots (Spottiswoode and Stevens 2010, Stoddard and Stevens 2010).

Similarity of facial markings

Because previous reports indicated that brood parasitism was infrequent (4.6-6%; Weaver and Brown 2004), nests were chosen for analysis of facial markings in order to increase the odds of including nests with extrapair or parasitic young. I looked at photographs from 130 broods and scored percent white and number of spots for 75 broods in which the facial markings appeared subjectively either very similar across all broodmates or where one chick appeared very different from its nestmates (Table 1). To assess whether facial patterns were more similar within broods than among broods, I performed ANOVAs with brood identity as the independent variable and percentage of the forehead patch that was white or the number of white spots as dependent variables. Average percent white correlated highly with average number of spots per nest ($r = 0.79$, $n = 75$, $p < 0.001$), so only percent white was used to select broods with high and low variation in facial markings. I used standardized residuals from a regression of the mean and standard deviation percent white for each brood as a quantitative measure of within-brood similarity in facial pattern and then selected 13 broods with the highest and 10 broods with the lowest similarity in facial markings for genotyping. These analyses were performed in PASW Statistics v.18 (SPSS Inc., Chicago, IL).

Cross-fostering experiment

At 14 pairs of nests (Table 1), half-broods were reciprocally cross-fostered 3 days after hatching for a separate behavioral experiment (Strickler 2013). These nest pairs were matched for brood size (±1 chick) and the number of chicks transferred depended on initial brood size. When the smaller brood contained 3 chicks, 1 nestling was transferred; when the smaller brood contained 4 or 5 chicks, 2 nestlings were transferred. All nestlings were weighed and cross-fostered chicks were matched for mass ± 1 g. Each nestling's tarsus was temporarily marked with non-toxic ink to denote resident or non-resident (transferred) status; chicks were re-marked as necessary until banding at 9 days old. In these broods, resident chicks did not differ in mass at 18 days old from their non-resident (transferred) nestmates and chick status (resident vs. non-resident) did not influence survival to day 18 (Strickler 2013). Nestlings were weighed on day 18 and then photographed and bled as detailed above.

The cross-fostered nests were analyzed to assess the influence of genetic and environmental factors on nestling facial pattern. I used a random effects model with the percent white as the dependent variable and four random factors: (i) dyad (pair of nests), which accounts for environmental variability faced by parents and nestlings; (ii) natal nest, which represents genetic effects as well as variation in parental efforts during incubation and the first 3 days after hatching; (iii) rearing nest, which includes variability in parental care after cross-fostering; and (iv) natal nest * rearing nest interaction, which compares chicks from the same natal rest reared in their own vs. a foster nest. Natal nest, rearing nest, and the interaction were nested within dyad (Fitze et al. 2003, Biard et al. 2006, Isaksson et al. 2006, Dugas 2012). To improve approximation of normality, percent white was $\log_{10}$-transformed. A -2 residual log likelihood ratio test was used to test the significance of parameter estimates of the random effects by comparing the full model to a reduced model omitting one random effect (Quinn and Keough 2002, Agresti 2007, Dickey 2008). The *p*-value for this test statistic (G^2: Quinn and Keough 2002) was estimated from a chi-square distribution with 1 degree of freedom (Sokal and Rohlf 1995, Quinn and Keough 2002, Agresti 2007, Dickey 2008) and divided by 2 to produce a *p*-value for a one-tailed test (Littell et al. 2006). This analysis was performed in SAS 9.2 (Cary, NC). Heritability of percent white (h^2) was estimated as twice the variance attributable to genetic effects (i.e., natal nest) divided by the total variance from genetic effects, environmental effects (i.e., rearing nest), interaction between genetic and environmental effects, and the residual error (Gebhardt-Henrich and van Noordwijk 1991, Smith and Wettermark 1995, Littell et al. 2006).

Laboratory protocol

For chicks in the 23 nests with high or low within-brood variation in facial pattern (Table 1), genomic DNA was extracted from the blood samples using DNeasy kits and the accompanying protocol (Qiagen, Valencia, CA). Twelve microsatellite markers originally developed for barn swallows (*Hirundo rustica*) were screened for use in this study: HrU2, HrU3, HrU6, HrU7, HrU8 (Primmer et al. 1995); HrU9, HrU10 (Primmer et al. 1996); Hir4, Hir5, Hir6, Hir7, and Hir8 (Tsukyo et al. 2007). Five of these primers (HrU2, HrU3, HrU6, HrU7, and HrU8) have been used previously on cave swallows (Kirchman et al. 2000). Amplification of each primer was tested in separate 25 µl PCR reactions containing 1x Go Taq Green Master Mix (Promega, Madison, WI), 0.12 µM forward and reverse primers, and approximately 10 ng template DNA. A touchdown PCR protocol was used with 94°C for 3 min followed by 25 cycles of 94°C for 30 s, X°C for 30 s, and 72°C for 40 s, and then a final extension step at 72°C for 10 min; the annealing temperature (X) stepped down by 2°C every 4 cycles, from 60-52°C. PCR products were visualized on 1.5% agarose gels stained with ethidium bromide. The 6 primers that amplified reliably were chosen for the relatedness analysis (Table 2). For microsatellite genotyping, primers were multiplexed in two groups and the 5' ends of forward primers were labeled with a fluorescent dye (Table 2). Each 12.5 µl PCR reaction contained 1x Type-it Multiplex PCR Master Mix (Qiagen), 0.12 µM each primer, and approximately 10 ng template DNA. The PCR protocol was 5 min at 95°C to activate the DNA polymerase followed by 33 cycles of 95°C for 30 s, 57°C for 90 s, and 72°C for 30 s; reactions ended with 30 min at 60°C to permit extension of longer fragments. PCR products were prepared for microsatellite analysis by adding 0.5- 2 µl PCR product to 10.5 µl HiDi Formamide (Applied Biosystems, Foster City, CA) and 0.5 µl ROX size standard (Applied Biosystems). Reactions were then heated at 95°C for 2 min, put on ice for 2 min, briefly centrifuged, and electrophoresed and scored on a 3130XL Genetic Analyzer (Applied Biosystems) using a 36 cm capillary array with POP7 Polymer for 20 min at 15 kV and an injection time of 23 s at 1.2 kV. Fragments were sized with the program Peak Scanner 1.0 (Applied Biosystems,), and alleles were binned using Flexibin v.2 (Amos et al. 2007).

Exact tests for deviations from Hardy-Weinberg equilibrium were performed in GENEPOP 4.2 using the Markov Chain algorithm to estimate exact *p* values (Rousset

2008). The default settings were used: 1000 dememorization steps, 100 batches, and 1000 iterations per batch. Observed (H_O) and expected (H_E) heterozygosity, allele frequency, number of alleles, frequency of null alleles, and the exclusion probability for all loci combined were estimated for each locus using CERVUS 3.0.3 (Marshall et al. 1998). One locus, HrU3, was omitted from estimates of relatedness because it differed significantly from Hardy-Weinberg equilibrium and likely contained null alleles (Table 2). After omitting HrU3, combined non-exclusion probability for sibling identity was 0.1035.

Relatedness estimates

Estimated relatedness (r) between pairs of nestmates was calculated using Queller and Goodnight's (1989) unbiased formula, implemented in KINGROUP 2 software (Konovalov et al. 2004). I also identified nestlings that likely resulted from brood parasitism or extrapair mating. The probability that a pair of nestmates were siblings or half siblings was estimated by comparing hypotheses about the coefficient of relatedness (r) between pairs of chicks using maximum likelihood methods in KINGROUP 2 (Konovalov et al. 2004). To estimate the probability that two chicks are full siblings, the program compares a primary hypothesis ($r = 0$) to the null hypothesis that the chicks are full siblings ($r = 0.5$) and provides the probability that the null hypothesis is true. A low *p*-value ($p < 0.05$) supports the primary hypothesis that the chicks are not full siblings. To test whether two chicks are likely half siblings, the primary hypothesis that $r = 0$ is compared to the null hypothesis that $r = 0.25$. A low *p*-value ($p < 0.05$) supports the primary hypothesis that the chicks are unrelated. A nestling was considered a product of brood parasitism if the *p*-value of both tests (full and half siblings) was lower than 0.05 for all pairs of that nestling and its broodmates; this indicated that the nestling was unlikely to be related to its broodmates. A nestling was considered a product of extrapair mating if the *p*-values associated with full sibling status were less than 0.05 but the *p*-values for half sib status were greater than 0.05, indicating that it was a half-sibling to its nestmates.

Association between facial markings and relatedness

I examined the association between relatedness and similarity in facial markings. First, I compared broods that had met the quantitative criteria for having high and low similarity in the number of spots (or percent white) on the forehead patch by averaging the estimated relatedness for all nestling pairs within a brood and using an independent samples t-test. For a secondary analysis, I used linear regression with mean relatedness per brood as the predictor and facial pattern similarity (both metrics) as the dependent variable. These statistical analyses were two-tailed and performed in PASW Statistics v.18 (SPSS Inc., Chicago, IL). Except when otherwise stated, means are given ± 1 standard deviation. All methods were approved by the University of Oklahoma IACUC and performed under the necessary state and federal permits.

RESULTS

In the 75 nests chosen based on subjective similarity or dissimilarity in facial markings, the white percentage of the forehead patch differed among nests (ANOVA $F_{74, 156} = 1.39$, $p = 0.045$), as did number of white spots (ANOVA $F_{74, 156} = 2.39$, $p <$

0.001). Facial patterns of broods included in the cross-fostering study had significantly lower mean percent white than the non-transferred nests (t_{102} = -3.11, p = 0.002). In those nests, 21.5% of total variation in percent white was explained by the random effects; all random effects were non-significant (-2 residual log likelihood ratio tests; Table 2). Heritability of percent white was relatively low (h^2 = 0.23) in the cross-fostered nests.

In the sample of 23 broods chosen either for strong quantitative similarity or dissimilarity in percent white, facial marking similarity was not clearly associated with relatedness among broodmates. Relatedness explained virtually no variation in percent white ($F_{1, 21}$ = 0.02, p = 0.878, R^2 = 0.001) or number of white spots ($F_{1, 21}$ = 0.26, p = 0.615, R^2 = 0.01. Nests with high and low similarity in facial markings did not differ in relatedness among broodmates: percent white (t_{18} = 0.72, p = 0.479); number of spots (t_{18} = -0.31, p = 0.760). Of the 23 nests, two (8.7%) were found to contain an extrapair chick and none had been parasitized, as determined by maximum likelihood tests of pairwise relatedness. One nest with an extrapair chick had highly similar facial markings; the other nest had dissimilar faces.

DISCUSSION

This study provides some support for the hypothesis that cave swallow facial markings carry information about genetic relationships among nestlings. As predicted, both the number of white spots and the percentage of the forehead patch containing white feathers varied among nests in the larger sample. Although the cross-fostering experiment did not reveal significant effects related to genetic or environmental factors and heritability of percent white was lower than expected, genetic factors accounted for far more of the variability in percent white than did environmental factors. Microsatellite DNA results established no clear connection between facial markings and relatedness among nestlings, but this small sample included only two instances of extrapair young and none of brood parasitism.

If facial markings are used by parents to infer relatedness among nestlings and perhaps to detect extrapair young, they should be strongly influenced by genetic factors, and thus should be both similar within nests and different among nests. In one sample of nests, this prediction was supported by a strong effect of nest identity on both percent white and number of spots. In the cross-fostering nests, the highest percentage of variation in percent white was explained by genetic factors, whereas variance explained by environmental factors was minute. Although these effects were not significant, they do point toward genetic factors outweighing environmental ones. The non-significant effects in this model may be at least partially attributed to differences between the cross-fostering nests and the regular nests. In the larger sample, the significant effect of nest on facial markings among nests appears to be driven by the presence of nests in which chicks have much more and much less white than average. The subjects used in the cross-fostering experiment, though selected blindly (well before facial feathers developed) turned out to be unusually dark-faced, relative to the larger sample used in the overall analysis-of-variance. Nonetheless, the heritability estimate ($h^2 = 0.23$) is lower than most reported values for melanin-based plumage traits in other species, which cluster around 0.4 – 0.8 (Mundy 2006).

Exploring whether the facial patterns of extrapair or brood parasitic young differed markedly from their nestmates was not possible in this sample. Although the genotyped chicks belonged to a group of nests selected to increase the chances of detecting extrapair and parasitic nests (i.e. by inflating the sample of nests with low similarity in facial markings), no instances of brood parasitism were found. Extrapair young were detected in only two (8.7%) nests; one nest was highly similar in facial markings, the other dissimilar. Previous studies indicated that brood parasitism may affect only 4.6-6% (Weaver and Brown 2004) or 8% (Strickler, *unpub. data*) of nests; these figures were generated solely from checking nests and may be underestimates (Weaver and Brown 2004). An altered sampling technique, coupled with improved relatedness calculations may allow estimation of the frequency of extrapair young and brood parasitism and/or examination of the association between facial pattern and relatedness between chicks. In particular, future studies should focus on using more broods, randomizing the sampling regime, and employing more powerful molecular methods, preferably by genotyping one or both putative parents as well as offspring.

As fledging approaches, the selective pressures associated with providing a reliable basis for recognition are likely to strengthen, and so facial markings may be used by parents to recognize young after they leave the nest. Whereas young nestlings would not benefit from advertising their identity, fledglings can no longer enjoy parental care merely by being present in one place (the nest), so the presence of reliable identity signals at that late stage should benefit both chicks and parents (Beecher 1988). Several features of cave swallow facial markings support the hypothesis that they are used as individual recognition signals for parental recognition of young (Stoddard and Beecher 1983). Traits used for parent-offspring recognition often develop shortly before young reach such a level of inter-nest mobility that they are needed (Tinbergen 1953; Cullen 1957; Davies and Carrick 1962; Beecher 1988), and the presence of fully developed facial feathers coincides with the onset of parental discrimination in cave swallows (Strickler 2013). Parents appear to learn the identities of chicks in their nest and base future discrimination on familiarity rather than categorizing unfamiliar but related chicks as offspring (Strickler 2013). Such a mechanism is consistent with recognition at the level of either individuals or broods, but the weak similarity of facial markings within broods uncovered in this study suggests that individual recognition may be likely. As needed for individual recognition, facial patterns are highly polymorphic and individually variable; the percent white and number of spots differ significantly even between full siblings. Furthermore, facial patterns are stable during the period of recognition and so could be used for individual recognition despite the apparent absence of strong genetic effects on facial pattern (Roulin 2004).

This paper is the first step in examining the significance of young swallows' facial patterns in parent-offspring recognition. Previously, variation in the facial feathers of young cliff swallows was suggested to play a role in parental recognition of young (Stoddard & Beecher 1983), and their facial patterns have been cited as probable examples of visual recognition signals (e.g., Tibbetts and Dale 2007; Bradbury and Vehrencamp 2011). The current study, which paired quantitative analysis of facial markings with molecular methods to estimate relatedness, suggests genetic effects likely influence the percent white in cave swallows' facial markings and that those markings may allow adults to learn to identify their chicks. Cave swallow nestlings also seem likely to develop individually-specific begging calls, as do the other colonial North American swallows (Beecher et al. 1981; Stoddard and Beecher 1983), leaving open the intriguing possibility of multimodal signals containing both visual and vocal components.

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Group of Nests	**Number of Nests**	**Description**
All Nests	130	Brood size ≥ 3; photographs and DNA samples for all chicks
Face Photo Analysis	75	Subset of all nests in which chicks appeared very similar or very dissimilar; chosen subjectively by looking at photographs
Genotyped	23: total 13: high similarity 10: low similarity	Subset of nests for which face photos were analyzed. Standardized residuals of a regression of mean vs. standard deviation percent white were used to select the 13 nests with the highest and 10 nests with the lowest within-brood similarity in facial pattern.
Cross-Fostered	28	Subset of all nests. At these 14 pairs of nests, half-broods were reciprocally cross-fostered at 3 days after hatching. Facial patterns were analyzed to estimate genetic vs. environmental effects and heritability.

Table 1 The nests used for analysis of facial markings were chosen from 130 nests with at least 3 chicks, and for which DNA samples and photographs were available for each chick.

Group	**Locus**	**Alleles**	**N**	**Label**	**H_O**	**H_E**	**HW** (*p*±SE)	**Null**
1	HrU2	3	72	NED	0.15	0.14	1.00±0.00	-0.031
	HrU6	12	72	6-FAM	0.65	0.68	0.70±0.03	0.017
	HrU8	2	71	HEX	0.04	0.07	0.07±0.00	0.214
2	HrU3	11	59	HEX	0.05	0.74	<0.001±0.00	0.871
	HrU7	3	72	6-FAM	0.03	0.03	1.00±0.00	-0.002
	Hir6	18	72	NED	0.99	0.92	0.065±0.01	0.040

Table 2 Description of loci used to genotype indiviudals, including group used for multiplex PCR, number of alleles, fluorescent label, observed and expected heterozygosity, Hardy-Weinberg equilibrium test, and frequency of null alleles

Random effect	**Estimate**	**SE**	**% Variation**	G^2	p
Dyad	0.000	.	0	0	0.500
Natal nest (dyad)	0.100	0.116	11.46	0.8	0.186
Rearing nest (dyad)	$4.09\text{x}10^{-18}$	.	$4.7\text{x}10^{-16}$	0	0.500
Natal nest x rearing nest (dyad)	0.087	0.140	9.99	0.4	0.264
Residual	0.684	0.134	78.55		

Table 3 Results of random effects model assessing the sources of variation in nestling facial patterns

Fig. 1: The faces of young cave swallows vary in color and pattern, especially the amount and distribution of white feathers. Each row shows chicks from one nest. The trapezoid-shaped area in which facial markings were analyzed has been marked on the bottom right chick.