ABSTRACT

Title of Document: Social vocalizations and their implications for group dynamics of pallid bats (*Antrozous pallidus*)

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Pallid bats (*Antrozous pallidus*) are unusual among vespertilionids (the most common and diverse family of bats in temperate regions of the world) in that they often emit a loud, partially audible social call several times in rapid succession while in flight. This social call appears to function as a contact call in that it is frequently given when bats return from foraging and perform circular flights before entering a crevice roost. In this dissertation, I examine the functional and social significance of this calling behavior by free-flying pallid bats in central Oregon using a combination of observations, audio recordings, audio playbacks, acoustic analysis, and genetic marker analysis. In chapter 1, I found that bats respond to the calls of conspecifics and that call structure is unique to individuals and stable through time, which makes these calls well-suited for roostmate recognition. In chapter 2, I found significant genetic structure among colonies based on sequence variation at the mitochondrial DNA control region but very little structure among colonies for nuclear microsatellites. These data are indicative of female philopatry with male-mediated gene flow and highlight the potential that calls may function in the maintenance of multigenerational social groups. Finally, in chapter 3, I utilized genetic markers to investigate relatedness among individuals sharing a roost and

the extent to which call variation encodes information about relatedness to examine whether calling behavior may assist in maintaining social bonds as individuals switch roosts. I found that while average colony relatedness was low, bats roost with a greater proportion of relatives than expected by chance. In addition, I found that contact call structure encodes information about matrilineal relationship and relatedness as well as individual identity. Overall, these results suggest that calling behavior in pallid bats is important in maintaining social structure at maternity roosts. Given the high roost lability and nocturnal environment of pallid bats, this study offers important insight into how animals in fluid societies mediate interactions with groupmates using acoustic signals.

SOCIAL VOCALIZATIONS AND THEIR IMPLICATIONS FOR GROUP DYNAMICS OF PALLID BATS (*ANTROZOUS PALLIDUS*)

By

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Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2011

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Dedication

To J. Marie, whose unwavering love and support throughout this process kept me from going batty, and to my friends “Lucky”, “Pig-Pen”, “Stellaluna”, and “Darth Nihilus” without whom this would not have been possible.

Acknowledgements

All field protocols were approved by the University of Maryland Institutional Animal Care and Use Committee (Protocol R-08-39). I would like to thank Emily Muns and Stephanie Rihl for assistance with fieldwork, Tom Rodhouse for help with locating study sites, Kevin Misner for assisting with the video tape analysis, and Jason Douglas for help measuring calls. I am also grateful to the Confederated Tribes of Warm Springs, the Cole family, and the National Park Service for allowing access to the field sites as well as the staff at Hancock Field Station in Clarno, Oregon for providing support during the field season. I owe thanks to Lois Reid and the staff in the BEES graduate office for their help and support. Finally, I would especially like to thank my advisor, Jerry Wilkinson, and all members of the Wilkinson lab past and present who provided invaluable support and feedback throughout my doctoral career. This work was supported by research grants from Sigma Xi, the Cosmos Club Foundation of Washington DC, the American Society of Mammalogists, the Animal Behaviour Society, the American Philosophical Society as well as summer support and a Darwin Fellowship provided by the BEES graduate program at the University of Maryland.

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Introduction

Bats have achieved a near worldwide distribution and are the second most speciose order of mammals (Kunz and Pierson 1994). Despite tremendous variability in habitat, diet, and mating systems, the great majority of the over 1,100 species are social (Kerth 2008). In temperate species, the most prevalent and frequently studied social aggregation of bats is that of females comprising a maternity colony. These groups form in the late spring and early summer to give birth and rear their young (McCracken and Wilkinson 2000). Bats in maternity colonies occupy a variety of roosts ranging from man-made structures such as buildings or bridges to natural structures found in the shedding bark of dead trees and rock crevices of large cliff complexes (Lewis 1995; Kunz and Lumsden 2003).

While the roosting ecology of many bat species is well known, studies investigating the social aspects of group living in bats are relatively rare. For example, because echolocating bats are dependent on acoustic signals for both orientation and prey capture, acoustic communication with conspecifics has frequently been proposed as a key mechanism underlying behavioral interactions within social groups (Fenton 2003). However, for the majority of bat species, the extent to which social communication may be used within and among social groups is largely unknown. This is surprising because bats exhibit many characteristics that make them a particularly interesting group for examining the role of acoustic communication in the evolution of sociality. For example, bats exhibit tremendous longevity compared to similar sized mammals (Wilkinson and South 2002), and females of many species display natal philopatry (Burland and Wilmer 2001), both of which result in increased opportunity for social interactions among

individuals in a colony. Finally, there is evidence that several species of bats maintain non-random roosting associations despite frequent roost site movements; however, the mechanisms bats use to locate roostmates are in need of further research (Kerth and König 1999; Willis and Brigham 2004; Garroway and Broders 2007; Popa-Lisseanu et al. 2008).

Like other temperate vespertilionid bats that roost in natural structures, pallid bats (*Antrozous pallidus*) exhibit a dynamic social structure where non-randomly associating individuals move frequently among multiple crevice roosts within a large rocky outcrop (Lewis 1996). However, pallid bats are unusual among vespertilionid bats in that they emit partially audible social calls that appear to function in roostmate recruitment as they fly outside the roosting area (Vaughan and O'Shea 1976). Since these calls have yet to be studied extensively in the field, in the first chapter of my dissertation, I investigate the calling behavior of pallid bats by documenting the context in which calls occur as well as performing playbacks to examine the response of bats to conspecific social calls. As a second related objective, I examine the potential that the calls could have for individual recognition by analyzing calls recorded from free-flying bats to investigate both individual specificity and temporal stability in call structure.

Studying the genetic structure of social groups in conjunction with the mechanisms that those in the group utilize to maintain contact with groupmates is crucial to understanding the evolution and maintenance of sociality in group living species. For example, philopatry can lead to higher relatedness and a greater opportunity for social interactions among group members of the sex that remains at the natal territory (Lukas et al. 2005). However, in species that exhibit high mobility and nocturnal behavior, such as

bats, examining social structure and sex-biased dispersal using the traditional methods of marking and observational data collection is difficult. Thus, the advancement of molecular genetic techniques has been particularly valuable in shedding light on the social structure of bat roosting groups (Burland and Wilmer 2001). In Chapter two, I explore the genetic structure of pallid bat colonies using bi-parentally inherited nuclear microsatellites and maternally inherited mitochondrial DNA (mtDNA) sequence data to determine if females are philopatric to their natal roosting area.

In addition to elucidating the effect of dispersal and gene flow on the structure of current populations, molecular sequence data can also be used to infer historical patterns of gene flow and range expansion. Previous studies on bats have found very limited genetic structuring over broad geographic areas likely owing to the high vagility and enhanced dispersal capability of bats (Petit and Mayer 1999; Russell et al. 2005). Weyandt and Van Den Bussche (2007), however, surveyed multiple locations throughout the range of pallid bats in western North America and identified three geographically distinct clades with over 9% mtDNA control region divergence between them. However, their study contained few samples from the northern part of the range of pallid bats. Thus, in Chapter two, I use samples collected in central Oregon to make inferences about how pallid bats sampled in the study area fit into a broader phylogeographic context.

Finally, in Chapter three, I assess the potential that social calling by pallid bats could aid in maintaining non-random associations among relatives. To examine this hypothesis, I compare calling activity and utilize microsatellite data and mtDNA haplotypes to estimate relatedness of roosting groups at two different types of roosts: a traditional night roost found in a building and several rock crevices used by bats as

temporary day roosts. I also utilize playbacks to test whether bats preferentially respond to the calls of relatives. Lastly, I investigate whether calls might contain information about kinship by examining the relationship between call similarity and both relatedness and matrilineal relationship.

My research provides important insight into both the genetic structure of social groups and the functional and social significance of contact calls in pallid bats. Pallid bats are a difficult species to study because they roost in tight crevices in large cliff complexes and switch roosts frequently. However, their unusual calling behavior provides a rare opportunity to study social communication in the field. In addition, since non-random associations have been found to be stable in multiple seasons in other bat species that exhibit fission-fusion behavior (Patriquin et al. 2010), this study offers important insight into a potential acoustic mechanism to maintain these social bonds given the constraints of a nocturnal environment.

Chapter 1: Individual specific contact calls of pallid bats (*Antrozous pallidus*) attract conspecifics at roosting sites

Abstract

Contact calls are utilized by several bird and mammal species to maintain group cohesion and coordinate group movement. From a signal design perspective, contact calls typically exhibit acoustic features that make them easily localizable and encode information about individual or group identity. Pallid bats (*Antrozous pallidus*) are unusual among vespertilionids in that they often emit a loud, partially audible, frequency modulated social call several times in rapid succession while in flight. This call appears to function as a contact call in that it is frequently given when bats return from foraging and perform circular flights before entering a crevice roost. However, the degree to which pallid bats respond to the calls of conspecifics and what information is provided in the call is unknown. Thus, the goal of this study was to investigate pallid bat calling behavior to determine if calls attract roostmates or elicit responses from them and provide sufficient information for individual recognition. In playback studies, I found that contact calls elicit calls and approaches and that free-flying bats respond more to familiar than unfamiliar calls. In addition, analysis of frequency and temporal measurements of calls collected from multiple sites and spectral cross correlation analysis of calls recorded from the same radio-tagged bats on multiple evenings revealed that the frequency pattern of contact calls is highly repeatable over time within individuals but exhibits significant differences among individuals. Thus, contact call structure appears to be unique to individuals and stable through time, which makes these calls well-suited for roostmate recognition.

Introduction

Benefits of group living in animals include predator detection, information transfer about foraging sites, and social thermoregulation (Krebs and Davies 1993). To obtain these benefits, a mechanism to maintain group cohesion is necessary, especially when individuals in the group are highly mobile. One such mechanism utilized by several bird and mammal species is the use of a specialized vocal signal, typically designated as a contact call. From a signal design perspective, contact calls are often high in amplitude and easily localizable, with the broadcast range of the call often closely tied to the dispersion of the group. In addition, variation in frequency modulation, temporal pattern, and harmonic structure can encode information about the individual or the social composition of its group (Bradbury and Vehrencamp 1998). For example, northern resident killer whales (*Orcinus orca*) live in stable family groups and produce contact calls that are specific to groups, and to a lesser extent, individuals (Nousek et al. 2006).

Alternatively, in more fluid societies there is little benefit to maintaining group signatures due to the frequent immigration and emigration of individuals. Thus, contact calls often encode individual signatures, which may aid in maintaining non-random associations among group members (Cortopassi and Bradbury 2006). For example, fission-fusion social structure is characteristic of many avian and mammalian societies where subgroups are part of a larger group that frequently splits or merges together (Couzin 2006), and many species with this type of social system produce individually specific contact calls [e.g. spider monkeys, *Ateles geoffroyi* (Ramos-Fernandez 2005); orange-fronted parakeets, *Aratinga canicularis* (Cortopassi and Bradbury 2006); and

brown-throated conures, *Aratinga pertinax* (Buhrman-Deever et al. 2008)]. One well-studied species that utilizes contact calls and exhibits fission-fusion social structure is the bottlenose dolphin, *Tursiops truncatus* (Tyack 2003). In this species, individually distinct whistles are produced in both captive and free-living contexts (Sayigh et al. 2007) and are thought to mediate interactions and maintain social bonds within the group (Tyack 2003; Watwood et al. 2005).

Given their relatively dark aquatic habitat where light is quickly attenuated, acoustic signals enable dolphins to orient in their environment and interact with group members. Echolocating bats face similar challenges when flying at night necessitating the use of acoustic signals for both orientation and communication. Calls utilized for social communication in bats have been shown to function as contact calls to locate group members prior to foraging (Wilkinson and Boughman 1998) and recruit roostmates in species that exhibit high roost lability (Chaverri et al. 2010; Schoner et al. 2010). In addition, the information encoded in bat contact calls ranges from group specific via call convergence in stable *Phyllostomus hastatus* social groups (Boughman 1998) to individually specific calls of white-winged vampire bats, *Diaemus youngi* (Carter et al. 2008).

However, the extent to which social calls are used for mediating interactions within and among social groups is largely unknown for most bat species. Here, I report on social calls and associated behaviors of free-ranging pallid bats (*Antrozous pallidus*) a species where individuals in the colony switch roosting sites frequently and thus may benefit from a mechanism to locate roostmates as they return to the maternity colony from foraging at night. My study includes observational data on the behavioral context

of social calls, playback data, and an analysis of the variability and repeatability of social calls to determine if calls contain sufficient information to allow for individual discrimination at the roost site.

Pallid bats range throughout western North America from British Columbia south to central Mexico (Barbour and Davis 1969). In the summer, female pallid bats form maternity colonies in large rock outcroppings near a source of water to give birth and nurse their pups while males typically roost away from the maternity colony in smaller groups (Hermanson and O'Shea 1983). In addition, telemetry data show that pallid bats within a maternity colony exhibit high roost lability, switching among multiple rock crevice roosts within the larger cliff complex every 1-2 days, on average, with inconsistent group association (Lewis 1996). Thus, pallid bats appear to conform to a fission-fusion social structure model similar to that found in other vespertilionid bat species (Kerth 2008).

Although the roost switching behavior of pallid bats is a relatively common phenomenon among bats (Lewis 1995), pallid bats are unusual among vespertilionids in that they often produce a loud, audible call several times in rapid succession while in flight. This directive call (as described by Orr 1954) differs from echolocation calls in both structure and context by having lower frequencies and by occurring in rapid bursts rather than being continuously emitted (Fig. 1). Interestingly, pallid bats exhibit greater auditory sensitivity between 5 and 15 kHz than many other bat species (Brown et al. 1978), which is notable because low frequency hearing in bats is associated with the use of low frequency calls for social communication (Bohn et al. 2006). Pallid bat directive calls are frequently given both when bats leave the roost in the evening and when they

return from foraging and perform "rallying flights" in which individuals give repeated calls as they fly back and forth along the roosting area while being joined by other calling bats (Vaughan and O'Shea 1976). While pallid bat directive calls are also given in other contexts, such as parent-offspring recognition (Brown 1976), several lines of evidence indicate that calling outside the roost does not function solely for this purpose. For example, rallying behavior occurs during all phases of the maternity season (including prior to the birth of pups) with calls typically produced away from the roosting crevice (personal observation). In addition, adult males produce calls that are similar in structure to calls given by females outside the maternity roost (personal observation). Thus, I will hereafter refer to the audible calls given by pallid bats in flight as "contact calls" since the term "directive call" is typically associated with maternal social calls directed at offspring (Bohn et al. 2008).

Alternatively, low frequency calls produced by bats outside roosting crevices may have a limited social function in that calls could potentially be utilized as a specialized autocommunication signal to enhance crevice detection. Playback studies are thus critical for determining if there is a causal relationship between the call and the response of the receiver (Falls 1992). While playback studies on bats in the field have been conducted infrequently, they have been used to examine social call function in *Pipistrellus pipistrellus* (Barlow and Jones 1997), *Phyllostomus hastatus* (Wilkinson and Boughman 1998), *Thyroptera tricolor* (Chaverri et al. 2010), and *Myotis bechsteinii* (Schoner et al. 2010). Thus, my first objective was to assess the extent to which calling behavior assists in forming roosting groups by observing the context in which pallid bats give contact calls to determine whether calling is more frequently associated with

approaching and entering rather than while exiting a crevice roost. In addition, I utilized playbacks to address the following three predictions. First, if calls have a social function, I expect bats to respond more to playbacks of contact calls than to white noise. Second, if calling behavior in pallid bats is associated with roost advertisement, I predict that bats would be attracted or respond more to the calls of multiple than single bats outside the roost since larger roosting groups offer a greater thermoregulatory benefit to cavity dwelling bats (Willis and Brigham 2007). Third, I test if bats can recognize and respond preferentially to familiar calls by broadcasting calls recorded from bats at the same or a different colony.

My second objective was to analyze recordings from free-flying individuals to determine if the acoustic structure of contact calls contains information about individual identity. While the stability of pallid bat social groups is not well known, telemetry data collected by Lewis (1996) showed a greater roosting association among lactating bats than pregnant bats. Thus, given the potential benefits of group roosting for lactating pallid bats [e.g. social thermoregulation (Trune and Slobodchikoff 1976)], Lewis (1996) suggested that contact calls function in roostmate recognition, which would require individually specific call structure with high inter-individual and low intra-individual variability (Beecher 1989).

To examine whether contact calls contain sufficient information for roostmate recognition, I used calls recorded from unmarked free-flying bats at different colonies to determine the proportion of call variation explained at different levels of social affiliation (e.g. differences among colonies and differences among bats within a colony) and the information capacity present in the call (Beecher 1989). In addition, I used calls recorded

on multiple days from free-flying bats carrying radiotransmitters to test whether individual differences in contact calls are present and stable through time, as expected if contact calls are utilized for social communication and roostmate recognition.

Methods

Site Locations

I conducted fieldwork at four different colonies in the Clarno basin of central Oregon, U.S.A (44.94°N lat., 120.38°W long.). Two colonies [designated as Cove Creek North (CCN) and Cove Creek South (CCS)] are located in the Pine Creek Conservation Area, which is managed by the Confederated Tribes of Warm Springs, and the remaining two colonies are located in the Clarno Unit (CU) and Painted Hills Unit (PHU) of the John Day Fossil Beds National Monument (Fig. 2). The habitat in this area is typical of shrub-steppe desert in central Oregon with common vegetation consisting of sagebrush (*Artemisia tridentata*), juniper trees (*Juniperus virginianus*), and cheat grass (*Bromus tectorum*) (Verts and Carraway 1998) and moderate to steep topography with numerous large cliff formations each of which typically contains multiple roosting crevices suitable for pallid bats.

Call Terminology

Pallid bat contact calls typically consist of a series of 2-4 vocalizations with frequency modulated (FM) sweep structure separated by short periods of silence (avg. 40 ms). Following Kanwal et al. (1994) and Bohn et al. (2008), I refer to the elements of the call as "syllables," a "call" as a group of syllables each separated by less than 80 ms, and a call "bout" as two or more calls separated by at least 500 ms of silence (see Fig. 1).

Calling Behavior

I videotaped bats entering and exiting seven different roosting crevices for one hour on each of nine nights at the CCN and CCS colonies using a Sony DCR-TRV320 night shot digital video camera along with an LED infrared spotlight (model # 15-IL07, Cop Security System Corp. Taiwan) to illuminate the roosting area. Video recording times varied but were typically between midnight and 0500 when bats returned from foraging. Vocalizations were recorded into the video camera with a shotgun microphone (model AT4071A, Audio Technica, Japan) that was oriented toward the roost approximately 6 m above ground on extension poles. For each 1 h video recording, I scored call occurrence, approaches of a bat to the roosting crevice, and entries into the roosting crevice. I scored calls as being associated with approaching or entering the roost if the call occurred less than five seconds before either event on tape. Videotapes were scored using JWatcher v. 1.0 (JWatcher.ucla.org).

To determine if calling is associated with bats exiting a roost, on 25 nights I counted the number of bats that did or did not call as they exited 12 different roosts using a night scope (Noctron V, Varo, Inc. Garland, TX, U.S.A.) and the LED spotlight. Counts were carried out until no bats exited for more than 5 min or until bats began to return to the roost after foraging. I determined if calling behavior is associated with approaching, entering, and exiting the roosting crevice using a two-way contingency table analysis conducted using JMP v.5.0 (SAS Institutes Cary, NC, U.S.A.).

Response to Call Playback

I conducted playbacks on 21 different evenings and pre-dawns in 2006 and 2008 at the CCN and CCS colonies. On each night or pre-dawn I conducted two to five trials

in a 1 h period (80 total trials). Each trial consisted of a series of calls from either a single bat or multiple bats that were recorded from the same or a different colony as the playback site. For a control stimulus, I broadcast pulses of white noise equivalent to the duration of a call. All playback files were 30 s long and consisted of calls recorded from free flying bats at the CCN, CCS, and PHU colonies. All calls utilized for playback consisted of 2-5 syllables per call and only calls with sufficient signal to noise ratio were selected. To determine whether bats respond more to recordings from multiple bats than an equal number of repeated recordings of a single bat, I created playback sound files using the program Raven Version 1.3 (Cornell University Lab of Ornithology, Ithaca, NY, U.S.A.). Single bat recording files consisted of the same call recorded from a single bat repeated 13 times in the following pattern: two bouts consisting of calls repeated 5 times with each call spaced approximately 1.2 s apart followed by 5 s of silence and finishing with one bout with the call repeated three times. Multiple bat playback files consisted of single calls recorded from three different bats with calls from two bats repeated five times and calls from one bat repeated three times with the same silent intervals as the single bat treatment. For the multiple bat treatment call order was randomly determined.

Calls were broadcast using a Marantz PMD671 flash recorder (sampling frequency 96 kHz, Marantz Inc., Mahwah, NJ, U.S.A.) connected to a Radio Shack 40 W stereo amplifier and a Realistic portable loudspeaker (flat frequency response to 45 kHz) mounted on a 6 m extension pole above ground or lowered from the top of the cliff using a pulley. All playback stimuli were equalized to have amplitudes similar to calls made by free flying bats (approximately 50-54 dB at 6-8 m).

All playback trials were videotaped using a Sony nightshot video camera (Sony Inc.) focused on the speaker, which was illuminated with infrared light. The number of social calls and passes of bats by the speaker were counted 1 min prior to the initiation of each trial to assess background activity. Responses were scored as the difference between the 1 min playback and the 1 min background level. If there was no bat activity either prior to the playback or after the playback the observation was removed from the analysis. Both passes by the speaker and calls in response were analyzed using a mixed effects model Analysis of Variance (ANOVA) with day and trial included as random effects and colony (CCN or CCS), time of day (evening or pre-dawn), call treatment (contrast between all bat call playback files together and the white noise control), bat number (single or multiple), and colony origin (same or different colony) included as fixed effects.

Assessment of Call Variability

Recording Methods

I conducted 39 recording sessions outside pallid bat crevice roosts between June and August 2005-2008. Although recording sessions often lasted throughout the night, the majority of calls were recorded between 2100 and 2300 h as bats exited the day roost and 0300 and 0500 h as bats returned to the day roost. I recorded calls using the high frequency output of an Ultrasound Advice S-25 bat detector (Ultrasound Advice Inc., London, U.K) and a custom built filter/amplifier (bandpass 4 kHz-100 kHz) connected in 2005 to a Gateway laptop (Gateway, Inc., Irvine, CA, U.S.A.) with a DAQ i508 sound card sampling at 250 kHz (INEES Inc., New South Wales, Australia) running Batsound Pro (Pettersson Elektronik AB, Sweden) or in 2006-2008 to the Marantz PMD671 flash

recorder sampling at 96 kHz. The microphone was positioned 6 m in the air using extension poles to get it as close as possible to free flying bats, which were typically at or near the top of the rock formation (approx. 10-20 m above ground depending on the site).

Pallid bat contact calls are partially audible which allowed the observer to easily determine that a call was given and often permitted observing the bat that called. However, because not all bats were individually marked, I limited recordings to a single calling bout. To minimize the chance of analyzing multiple calling bouts from the same bat, I included no more than three calling bouts separated by at least 5 minutes per recording session (range 3-8 hours of recording). A total of 189 calls (58 calls from CCN, 57 calls from CCS, 55 calls from CU, and 19 calls from PHU) from 74 calling bouts with at least two calls per bout were analyzed (range 2-5 calls per bout). Calls used for the analysis had sufficient signal relative to noise to measure frequency and time variables. In situations where a recording file contained calls from multiple bats, I used amplitude differences and syllable intervals to assign calls to bat.

Call Measurements

I performed spectrographic analyses using Raven Version 1.3 (Cornell University Lab of Ornithology, Ithaca, NY, U.S.A.) with a 128-point Hanning window and 512-point Fast Fourier Transform (FFT). Pallid bat contact calls contain multiple harmonics (Fig. 1b). However, because contact calls were recorded from flying bats at different heights and trajectories above the recording set-up, I could only reliably measure the fundamental frequency of each syllable from each call. Three variables were measured from the waveform of the call (call duration, syllable duration, and inter-syllable interval) and twenty-three frequency, time, and amplitude variables were measured from the

spectrogram of the first and last syllable of each call (see Table 1 for the description of variables measured). Variables were log transformed to meet the assumption of normality if necessary.

Statistical Analyses

Prior to analysis, I examined the correlation matrix for all call variables and found that the first and last syllable 1st quartile frequency and time, as well as the first and last syllable 3rd quartile frequency and time, were highly correlated ($r > 0.8$) with the center frequency and time measurements. Thus, I removed the 1st and 3rd quartile measurements from the subsequent analyses since any attempts to include these variables resulted in a singular covariance matrix. To reduce the dimensionality of the dataset, I performed a factor analysis with varimax rotation in SAS v 9.1 (SAS Institutes, Cary, NC, U.S.A.) to extract orthogonal factors. After applying the selection criteria outlined in Tabachnick and Fidell (2001), I retained six factors, which explained 78% of the variation in the data. These six factors were then included in a multivariate analysis of variance (MANOVA) to test if call characteristics differed among bats and colonies, both of which were designated as random effects. Finally, I used Proc Varcomp in SAS v. 9.1 to estimate the variance explained by colony, bats within colony, and calls within bat for each retained factor using restricted maximum likelihood. The variance estimates for between bat differences (S_B^2) and within bat differences (S_W^2) were then used to calculate the total variance (S_T^2) and the information capacity for each factor [$H_i = \log_2(S_T/S_W)$] as well as the total information capacity present in the call [$H_s = \sum H_i$] (Beecher 1989). Finally, these estimates were also used to calculate the repeatability of each factor as $S_B^2/(S_B^2+S_W^2)$.

Temporal Stability of Calls

Recording Methods

Ten pregnant or lactating female pallid bats were captured using mist nets placed at a spring fed water trough located between the CCN and CCS colonies (Fig. 2) during the 2007 and 2008 field seasons. Each bat was weighed and marked with a numbered band (National Band and Tag, Newport, KY, U.S.A.) so that individuals could be identified if recaptured during future mist net sessions. Radiotransmitters were built (Wilkinson and Bradbury 1988), marked with colored reflective tape (3m Inc., St. Paul, MN, U.S.A.), and placed in the interscapular region of each bat using Skinbond adhesive (Torbot Group, Inc., Cranston, RI, U.S.A.). To minimize disruption to normal flight behavior, transmitters weighed less than 5-8% of total body weight (Aldridge and Brigham 1988). I tracked each bat to their roosting crevice during the day and set up recording equipment (Marantz PMD671 and high frequency microphone, see above) to attempt to record a contact call from the bat as it either exited or returned to the day roost during the night. I was able to verify that the bat wearing the radio called by monitoring the pulse from the radio with a telemetry receiver (Custom Electronics, Inc., Urbana, IL, U.S.A.) and using a spotlight to highlight the colored reflective tape on the radio to identify the location of the bat with respect to the microphone after the call occurred. Since radio-tagged bats often returned to the roost several times in a night, I was able to reliably assign calls to the radio-tagged bats by using calls recorded when the bat either returned or exited by itself or in small groups (less than 3 bats).

Statistical Analyses

For six bats, I recorded at least two contact calls during the same recording session and one contact call on an additional day. Thus, I had at least three calls recorded for six bats to examine differences in call structure within bats over time. Each call spectrogram was first partitioned into separate spectrograms for each syllable and then band-pass filtered between 5 kHz and 45 kHz to remove excess noise present in the recordings. To compare the similarity of syllable structure among and within bats, I used SPCCA, spectrographic cross correlation analysis (Clark et al. 1987) in Raven. In this procedure, two spectrograms are overlapped in time and cross-correlated frame by frame. The peak of the resulting correlation function represents the time frame where the two sounds are most similar.

I performed SPCCA on all possible combinations of each syllable from each radio-tagged bat's call. To test whether calls recorded from the same bat have a higher peak cross correlation value than calls recorded from different bats, I conducted a permutation test (Manley 1997) using R (V. 2.7.2, http://www.R-project.org). Here the observed test statistic was computed as: $\overline{X}_{\text{(peak r same bat)}} - \overline{X}_{\text{(peak r between bats)}}$ and tested for significance against the permuted distribution (10,000 permutations). Since calls recorded on the same day and at the same site could artificially inflate within bat similarity, I only included the two calls recorded on different days and different sites in this analysis. If calls from the same bat are structurally similar across time, I predicted that there should be no difference in correlation values between calls recorded from the same bat on different days and calls recorded from the same bat on the same day. To test this prediction I used a second permutation test in R. Here I computed the observed test

statistic as: $\overline{X}_{\text{(peak r same day)}} - \overline{X}_{\text{(peak r different day)}}$ and tested for significance against the permuted distribution (1,000 permutations).

Results

Calling Behavior

Calling was not independent of the position of a bat relative to the crevice in that calls occurred more frequently when bats were approaching (χ^2=312.82, P<0.0001) and entering a roost (χ^2=132.88, P<0.0001) than while exiting the roost (Table 2). The number of calls emitted when approaching versus when entering the roost did not differ (χ^2=1.02, P=0.314).

Response to Call Playback

Bats both called in response ($F_{(1,366)}$=27.67, P<0.0001) and flew past the speaker ($F_{(1,366)}$=8.09, P=0.0047) significantly more often during playbacks of contact calls than of white noise (Fig. 3). There was no significant effect of either the location where the playbacks were conducted [calls in response ($F_{(1,366)}$=0.567, P=0.452), passes by speaker ($F_{(1,366)}$=0.676, P=0.412)] or time of day [calls in response ($F_{(1,366)}$=0.499, P=0.48, passes by the speaker ($F_{(1,366)}$=0.001, P=0.92)].

There was a significant effect of colony origin in that bats called in response more frequently to the playbacks of calls recorded from their own roosting area ($F_{(1,286)}$=6.036, P=0.0146) while colony origin had no significant effect on of the number of bats flying by the speaker ($F_{(1,286)}$=1.38, P=0.241). The number of bats included in the playback file had no significant effect (Fig. 3) on either calls in response ($F_{(1,286)}$=3.196, P=0.075) or passes by the speaker ($F_{(1,286)}$=0.167, P=0.683).

Variability of Calls Among Bats and Colonies

In general, the intensity of pallid bat contact calls recorded in the field was high, allowing me to make recordings even when bats were flying 20 m above the recording apparatus. The majority of the recorded contact calls consisted of two to four syllables, although several recordings contained up to six syllables. In addition, the frequency modulation pattern of the syllables within each call was relatively consistent with correlations of the first and last syllable measurements ranging from 0.69 to 0.81. Thus, pallid bat contact calls appear to consist of a single FM syllable type repeated 2 to 6 times. The mean, standard error, and range of the temporal and frequency variables measured from the contact calls are summarized in Table 3.

Factor analysis revealed that temporal and frequency variables tend to load independently on each factor with mid-time, peak, and center frequency loading predominantly on factor one, syllable duration and center time loading on factor two and end frequency loading heavily on factor three (Table 4). Together, the first three factors explained 55%, and the six extracted factors explained 78% of the variation in the calls. MANOVA using the six extracted factors as variables in the analysis showed that there were significant differences among bats but not among colonies (Colony – Wilks' Lambda= 0.80, $F_{(18, 184.33)}$ =0.84, P=0.66; Bat– Wilks' Lambda=0.000027, $F_{(420, 667.22)}$ =7.63, $P < 0.0001$). This result is consistent with nested univariate ANOVAs, which revealed that the majority of the variance in call structure as measured by the six factors is explained by differences among bats with little to no variance explained by differences among colonies (Table 5). The variance estimates for differences among bats for all six factors equates to a total information capacity of H_S = 7.83 bits (Table 5).

Temporal Stability of Calls Within Bats

The spectrograms of contact calls recorded from the same bat were visually similar with consistent frequency modulation patterns and syllable durations, while differences in these call features are evident when comparing calls recorded from different bats (cf. examples of syllable spectrograms from radio-tagged bats BB#49 and BB#76 shown in Fig. 4). The permutation test confirmed that SPCCA values of calls recorded from the same radio-tagged bat on different days and recording sites were significantly greater than SPCCA values calculated from recordings between bats [$\overline{X}_{\text{(peak r same bat)}} = 0.714$, $\overline{X}_{\text{(peak r different bat)}} = 0.586$, P=0.0063]. In addition, a second permutation test to examine temporal stability of contact calls showed that the peak correlation for syllables from calls recorded from the same bat on different days did not differ from the peak correlation of calls recorded from the same bat on the same day [$\overline{X}_{\text{(peak r different day)}} = 0.714$, $\overline{X}_{\text{(peak r same day)}} = 0.802$, P=0.11)].

Discussion

Social Function of Pallid Bat Contact Calls

Pallid bat contact calls have been suggested to function in facilitating roosting group formation (Vaughan and O'Shea 1976) either by advertising the location of a suitable crevice for roosting or by recruiting individuals to maintain social bonds. However, low frequency calls could potentially function as a specialized echolocation signal to detect roosting crevices as bats approach the roost. Since pallid bat calling behavior has yet to be thoroughly investigated in the field, one of the objectives of this

study was to determine the context in which contact calls occur using observations and playbacks and then infer the extent to which contact calls perform a social function.

First, if contact calls are used to facilitate roosting group formation, I expected that calling behavior would be associated with bats approaching or entering a crevice more than when bats exit a roosting crevice. In addition, if calls are being used to advertise roost location, one might expect that calling would occur almost ubiquitously with approaching while if bats are using calls to maintain contact with other bats, calling may occur opportunistically depending on the bat's motivation to locate roostmates. While bats were more likely to call when entering or approaching a crevice than when exiting, there were 247 instances of bats calling while exiting the roost, and the frequency of bats approaching silently was almost equal to that of bats calling while approaching (Table 2). These results suggest that calls have a social function to maintain contact with roostmates in that if calls were utilized as a method of autocommunication to locate roosting crevices or roost advertisement I would expect few bats to approach silently or call while exiting a crevice roost.

The playback data also support the hypothesis that contact calls serve a social function. For example, bats responded both by calling in response and passing by the speaker more during playback of call stimuli than during white noise as expected if they are utilizing the calls for social communication. Interestingly, playbacks of calls recorded from the same colony as the location of the playback elicited significantly more calls than playbacks of calls from a different colony, which suggests that bats can differentiate familiar from unfamiliar calls as has been shown in playbacks of orange-fronted parakeets (*Aratinga canicularis*) (Vehrencamp et al. 2003) and lesser bulldog bats

(*Noctilio albiventris*) (Voigt-Heucke et al. 2010). However, there was no significant difference between passes by the speaker for playbacks of colony versus non-colony member calls, which could be due to a tendency for bats to approach the source of an unknown call to obtain more information about the calling bat. Finally, neither calling nor passes by the speaker differed when playback stimuli contained calls from one or more than one bat. This result also suggests that calls are used more for roostmate identification than for advertisement of a roosting area. If the latter was correct, then bats would be expected to be more attracted to the calls of multiple bats if they were searching for an occupied roost.

Evidence for Signature Calls in Adult Pallid Bats

Both enhanced variability among individuals and reduced variability within individuals are key characteristics of signature calls in acoustically mediated recognition systems (Beecher 1989). If contact calls are used for individual recognition by pallid bats, I would expect calls to exhibit significant differences among individuals and stereotypy within individuals. I characterized the variability in adult pallid bat contact calls by analyzing recordings of 189 calls from 74 bats from four colonies to determine the level of information provided in the call. In addition, I used radio-telemetry to find and record the same bat repeatedly over a period of at least two days to examine temporal stability of call structure, which has yet to be reported for any bat species in the field.

Random effects nested ANOVAs using factors extracted from acoustic characteristics of calls recorded from bats at multiple colonies showed that most of the variability in call structure is explained by differences among bats and little to no variability is explained by differences among colonies. The second and third factors

showed the highest variance explained by differences among bats (80% and 87%), and therefore the highest repeatabilities (Table 5). These factors are loaded heavily by both temporal variables (Factor two – syllable duration and syllable center time) and frequency characteristics (Factor three – mid-time frequency and end frequency) indicating that both temporal and frequency characteristics of the call carry individual information and may be important for distinguishing among bats. Since these calls are given predominantly while bats are flying outside the roost (Vaughan and O'Shea 1976), features of the call that will be less susceptible to distortion over distance will be most beneficial for carrying information about the sender. Thus, syllable duration, mid-time frequency, and end frequency may be especially useful for individual discrimination of different bats because, unlike high frequency portions of the call, they will be less affected by attenuation as the distance from the receiver to the sender increases (Bradbury and Vehrencamp 1998).

Encoding of individuality by differences in the frequency and temporal structure of pallid bat contact calls is similar to how individual distinctiveness arises among pup isolation calls in both evening bats (*Nycticeius humeralis*) (Scherrer and Wilkinson 1993) and greater spear-nosed bats (*Phyllostomus hastatus*) (Bohn et al. 2007) where the majority of information is carried by the spectral features of the call while temporal characteristics also carried significant, but lesser amounts of information. One hypothesis for the development of adult contact calls in pallid bats is that they are derived from infant isolation calls. Evidence in support of this hypothesis comes from Brown (1976) who reported that pallid bat pup isolation calls decrease in frequency and resemble contact calls after eight weeks of age and Esser and Schmidt (1989) who

reported that maternal directive calls resemble the isolation calls of pups in the lesser spear-nosed bat, *Phyllostomus discolor*. However, isolation calls of both evening bats (Scherrer and Wilkinson 1993) and greater spear-nosed bats (Bohn et al. 2007) increase in frequency as pups age. Furthermore, isolation calls are given only by pups at rest while contact calls are primarily given by bats in flight. Thus, more longitudinal studies are needed to determine if contact calls are ontogenetically related to or distinct from isolation calls in pallid bats.

The total information capacity of pallid bat contact calls is 7.83 bits, which would allow for the identification of approximately 228 unique call signatures (Beecher 1989). Colony size in pallid bats typically varies depending on maternal period, and groups of up to 200 bats and their young have been reported (Hermanson and O'Shea 1983). Based on roost exit counts at both CCN and CCS colonies at the study area using an infrared spotlight and night vision spotting scope, roosting group sizes ranged from 7-100 (average 48) bats in the same crevice with bats occupying up to four different crevices within the same colony at a given time (B. Arnold, unpublished data). Thus, the information content provided by the signal is consistent with the potential discrimination required by the roosting habits of pallid bats in central Oregon. When compared to other acoustically mediated individual recognition systems, the information content encoded in pallid bat contact calls also falls well within the range expected for individual recognition in a gregarious species. For example, similar analyses conducted on mew calls in cooperatively breeding bell miners (*Manorina melanophrys*) (McDonald et al. 2007) and pup isolation calls in Mexican free-tailed bats (*Tadarida brasiliensis*) (Wilkinson 2003) found a total information capacity of 9 bits. In addition, the information capacity of

alarm calls in yellow-bellied marmots (*Marmota flaviventris*) was calculated as 3.37 bits (Blumstein and Munos 2005), the latter of which has been shown to discriminate between calls of different individuals (Blumstein and Daniel 2004) even though the estimated information content is relatively low.

Finally, SPCCA of calls recorded from bats wearing radiotransmitters also supports the contention that adult pallid bat contact calls encode individual signatures in that calls recorded from the same individual are significantly more correlated than calls recorded from different bats. One advantage of SPCCA over the MANOVA approach is that it considers all features of the spectrogram rather than an arbitrary set of acoustic variables. Thus, it may provide a better representation of the information available to a bat as it extracts information from another individual's contact call.

By recording the same bat on multiple days, I also demonstrated that contact call structure is maintained across time, i.e. calls recorded on different days and at different sites are correlated no less than calls recorded on the same day. Although the calls used for this analysis were recorded over the course of up to only three days, I also obtained evidence that call structure is maintained over longer periods of time. During the study, a bat designated as BB#9 was captured and recorded as it called immediately after being released and then recaptured and recorded again over two weeks later, at which time it gave a structurally similar call (Fig. 4). Thus, evidence indicates that pallid bat contact calls contain enough information and are sufficiently repeatable to function as signature calls.

Social Communication in Bats

While I describe contact calls as "social calls" throughout this paper, distinguishing between vocalizations used for social communication or for echolocation can be difficult, since any call can potentially be used by conspecifics to gain information about the sender (Fenton 1985). Furthermore, echolocation calls have been shown to encode individual identity both in the lab [e.g. *E. fuscus* (Kazial et al. 2001)] and in the field [e.g. African large-eared free-tailed bats, *Otomops martiensseni* (Fenton et al. 2004)]. However, Siemers and Kerth (2006) failed to find evidence of individual signatures in *M. bechsteinii*, a bat species where females live in closed groups that are stable across years (Kerth et al. 2000). Arguably, echolocation calls may be poorly designed for encoding individual identification given their relatively simple acoustic structure. In addition, some bats vary call structure depending on the context in which the call is given to maximize returning information (Fenton 2003). In contrast, several studies that have analyzed variation in acoustic structure of social calls in adult bats have found evidence for individual signatures (Balcombe and McCracken 1992; Pfalzer and Kusch 2003; Carter et al. 2008). This study adds a new dimension to this body of work by using calls recorded from radio-tagged individuals in the field to assess call stability over time and by demonstrating with playbacks that calls preferentially attract familiar individuals.

Potential Benefits of Calling in Pallid Bats

Pallid bats are often regarded as highly social in that females have been reported to preferentially place juveniles in the center of a roosting group (Trune and Slobodchikoff 1978), guard juveniles (Beck and Rudd 1960), and guide mothers to

distressed offspring (Brown 1976). While most of the evidence for cooperative behavior is limited to bats in captivity, the potential for contact calls to function in maintaining roosting associations among bats and to facilitate kin-selected (Hamilton 1964) or reciprocity-based (Trivers 1971) cooperative behavior warrants further study. Although relatively little is known about the stability of pallid bat social groups, mist-netting efforts in the study area have resulted in re-capture of twenty banded female bats in more than one field season and telemetry data has confirmed that bats roost in the same colony in multiple years (B. Arnold, unpublished data). Thus, the available evidence to date indicates that female pallid bats are philopatric to their maternity colony. In addition, pallid bats have been reported to live over nine years in the wild (Tuttle and Stevenson 1982), which along with female philopatry increases the possibility that calling behavior may facilitate the maintenance of multigenerational social groups. Future studies incorporating playbacks of calls in pallid bats to test whether bats can discriminate between individually specific calls of other bats will be necessary to determine the role they may play in maintaining social structure.

Table 1. Description of variables measured from pallid bat contact calls

Variable Measured	**Description**
Call Duration	Duration of call measured from waveform
First Inter-Syllable Interval	Time between first and second syllable
First and Last Syllable Duration	Duration of syllable measured from waveform
First and Last Syllable Mid-Time Frequency	Frequency measured at the middle time of the syllable
First and Last Syllable End Frequency	End frequency of the syllable
First and Last Syllable Peak Frequency	Peak frequency of the syllable
First and Last Syllable Center Frequency	Frequency that divides the syllable into two intervals of equal energy
First and Last Syllable Center Time	Time of the center frequency
First and Last Syllable 1st Quartile Frequency	Frequency that divides the syllable into 25% and 75% of the total energy in the syllable
First and Last Syllable 1st Quartile Time	Time of the 1st quartile frequency
First and Last Syllable 3rd Quartile Frequency	Frequency that divides the syllable into 75% and 25% of the total energy in the syllable
First and Last Syllable 3rd Quartile Time	Time of the 3rd quartile frequency
First and Last Syllable Inter-Quartile Range (IQR) Bandwidth	Difference between 1st and 3rd quartile frequencies
First and Last Syllable Inter-Quartile Range (IQR) Duration	Difference between 1st and 3rd quartile times

Table 2. Occurrence of contact calls outside a roost categorized by context

Bat Behavior	**Call Occurrence**	
	Yes	**No**
Approaching Roost	897	790
Entering Roost	202	199
Exiting Roost	247	956

Table 3. Descriptive statistics for variables measured from contact calls

Call Variable Measured	**Mean ± SE**	**Range**
Call Duration (ms)	138.83±3.04	68–281
First Inter-Syllable Interval (ms)	38.67±0.64	23–79
First Syllable Duration (ms)	22.89±0.40	13–47
Last Syllable Duration (ms)	24.30±0.39	15–42
First Syllable Mid-Time Frequency (kHz)	15.46±0.27	9.36–27.70
Last Syllable Mid-Time Frequency (kHz)	17.33±0.30	9.03–32.92
First Syllable End Frequency (kHz)	7.55±0.06	5.72–13.34
Last Syllable End Frequency (kHz)	7.76±0.06	5.92–11.82
First Syllable Peak Frequency (kHz)	21.54±0.33	9–33
Last Syllable Peak Frequency (kHz)	21.94±0.28	13.5–35.25
First Syllable Center Frequency (kHz)	22.00±0.26	11.25–30.75
Last Syllable Center Frequency (kHz)	22.55±0.24	15.75–31.25
First Syllable Center Time (ms)	9.05±0.22	3.3–20
Last Syllable Center Time (ms)	10.07±0.26	3.7–23
First Syllable IQR Bandwidth (kHz)	6.98±0.23	1.56–17.25
Last Syllable IQR Bandwidth (kHz)	6.64±0.21	0.78–16.41
First Syllable IQR Duration (s)	7.07±0.21	2–18.6
Last Syllable IQR Duration (ms)	6.83±0.17	2–14.1

Table 4. Varimax-rotated loadings for the first six factors extracted from contact call measurements

Variable Measured	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6
Call Duration	-0.08	-0.07	-0.29	0.03	-0.34	**0.54**
First Inter-Syllable Interval	0.01	0.02	0.02	-0.10	**0.73**	-0.11
First Syllable Duration	-0.15	**0.79**	-0.14	-0.21	0.12	-0.06
Last Syllable Duration	-0.04	**0.86**	-0.17	-0.11	0.15	0.01
First Syllable Mid-Time	**0.78**	0.14	0.44	-0.03	-0.07	-0.12
Last Syllable Mid-Time	**0.73**	0.38	0.35	-0.09	-0.24	-0.02
First Syllable End Frequency	0.12	-0.05	**0.91**	0.05	0.10	-0.07
Last Syllable End Frequency	0.14	0.01	**0.90**	0.06	-0.00	-0.01
First Syllable Peak Frequency	**0.69**	-0.20	0.01	0.06	0.35	0.14
Last Syllable Peak Frequency	0.49	-0.12	0.05	0.09	0.08	**0.69**
First Syllable Center Frequency	**0.88**	-0.19	0.02	0.15	0.08	0.11
Last Syllable Center Frequency	**0.75**	-0.16	0.16	0.13	-0.11	0.45
First Syllable Center Time	-0.25	**0.77**	0.26	0.00	-0.22	-0.04
Last Syllable Center Time	0.17	**0.88**	0.07	0.07	-0.08	-0.20
First Syllable IQR Bandwidth	-0.09	-0.04	0.04	**0.81**	-0.27	0.18
Last Syllable IQR Bandwidth	0.24	-0.11	0.05	**0.86**	0.13	-0.07
First Syllable IQR Duration	**-0.59**	0.46	0.19	0.07	0.17	0.26
Last Syllable IQR Duration	-0.33	0.47	0.20	0.29	0.44	0.21

Call variables with loadings greater than 0.5 are shown in bold

Table 5. Variance component estimates, repeatabilities, and information content in bits, H_i, for factors extracted by factor analysis of contact calls

	Colony (N=4)		Bat (N=74)		Call (N=189)		
	F†	VCE‡	F†	VCE‡	VCE‡	Repeatability	H_i
Factor 1	1.6	0.03	9.35*	0.76	0.23	0.77	1.49
Factor 2	0.96	0	11.81*	0.80	0.20	0.80	1.58
Factor 3	0.76	0	16.64*	0.87	0.13	0.87	1.83
Factor 4	1.25	0	3.12*	0.47	0.55	0.46	0.95
Factor 5	0.33	0	5.34*	0.62	0.38	0.62	1.18
Factor 6	0.5	0	2.48*	0.35	0.65	0.35	0.80

† F values for random effects univariate ANOVA conducted on each factor

‡ Variance component estimates (VCE) obtained by restricted maximum likelihood (REML) indicate the proportion of variance explained by differences among colonies (Colony), among bats within colony (Bat) and calls within bats (Call)

* $P < 0.0001$

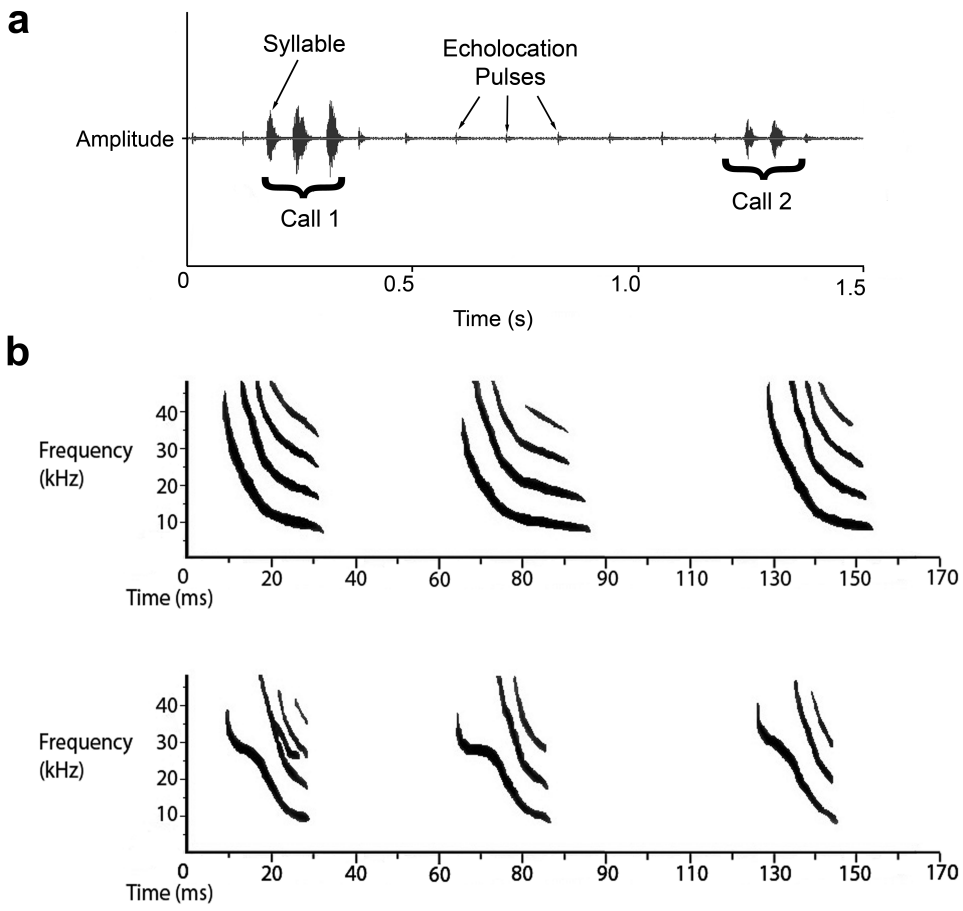
Fig. 1 – Examples of contact calls recorded from free flying adult pallid bats. **a**-Waveform showing a "call bout" consisting of two contact calls flanking a series of echolocation pulses recorded from a single bat flying away from the microphone; **b**-Spectrograms of contact calls recorded from two different bats.

Fig. 2 – Map showing the relative locations in central Oregon, USA of the colonies where bats were recorded for the analysis of contact calls.

Fig. 3 – Histograms showing the response of pallid bats to the playback treatments (**a**-contrast between call treatments and white noise control; **b**-Contrast between same and different colony playbacks; **c**-Contrast between single and multiple call playbacks).

Fig. 4 – Spectrograms showing the similarity of contact calls recorded from the same pallid bat on multiple days. Numbers designate bat identity. For presentation purposes, only one syllable from each contact call is shown.

Fig. 1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Time (ms)
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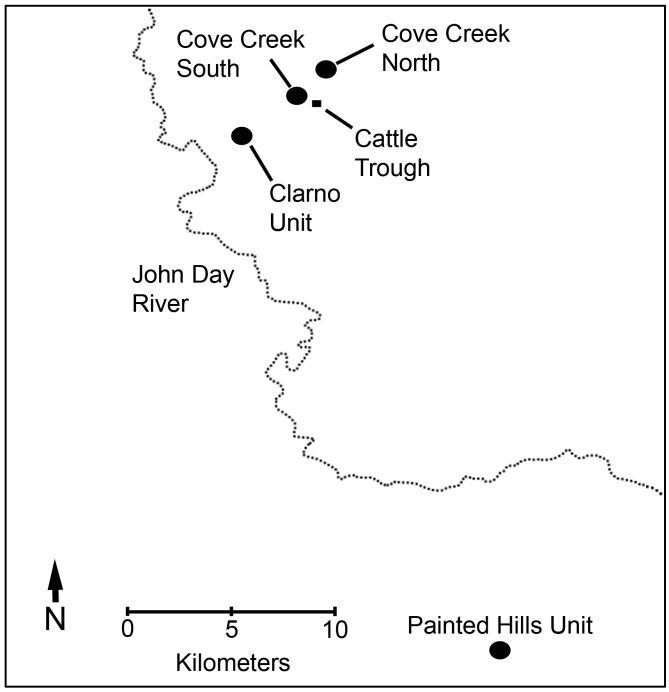
Fig. 2


Cove Creek
South
Cove Creek
North
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John Day
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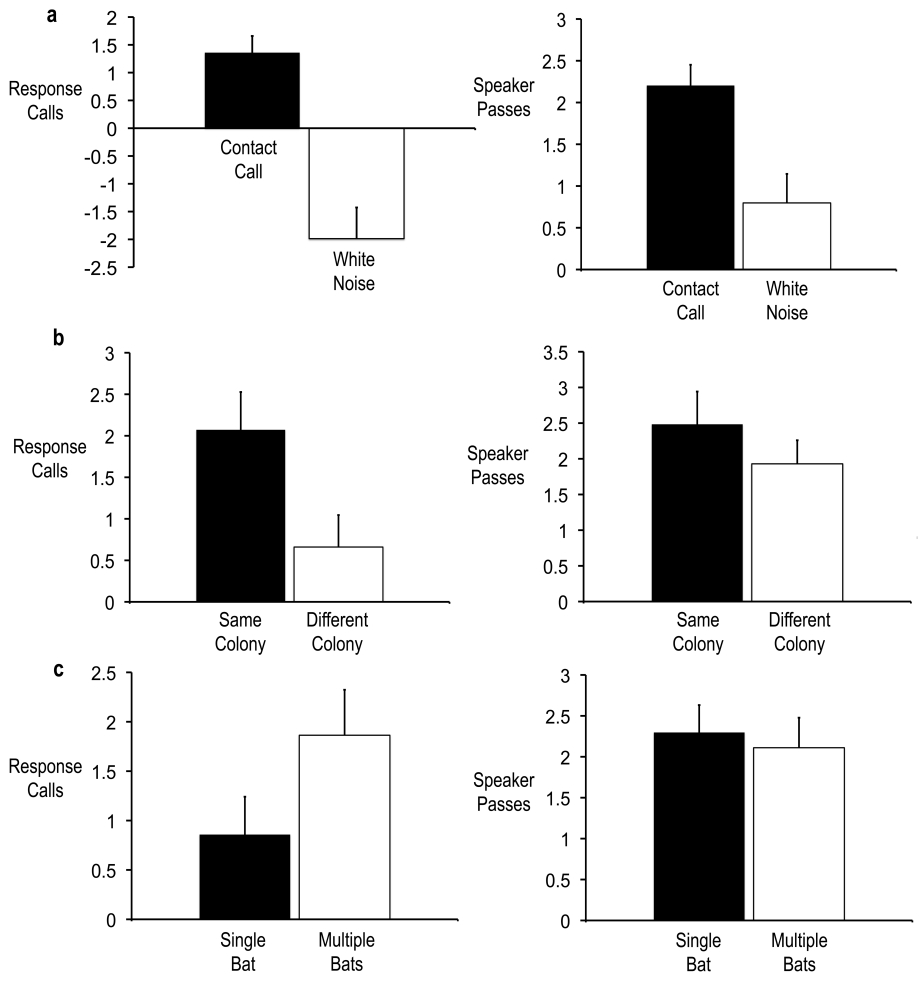
Painted Hills Unit

Fig. 3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Response Calls
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Contact Call
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Speaker Passes
3
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2
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Contact Call
White Noise
b
Response Calls
3
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2
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Same Colony
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Speaker Passes
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Same Colony
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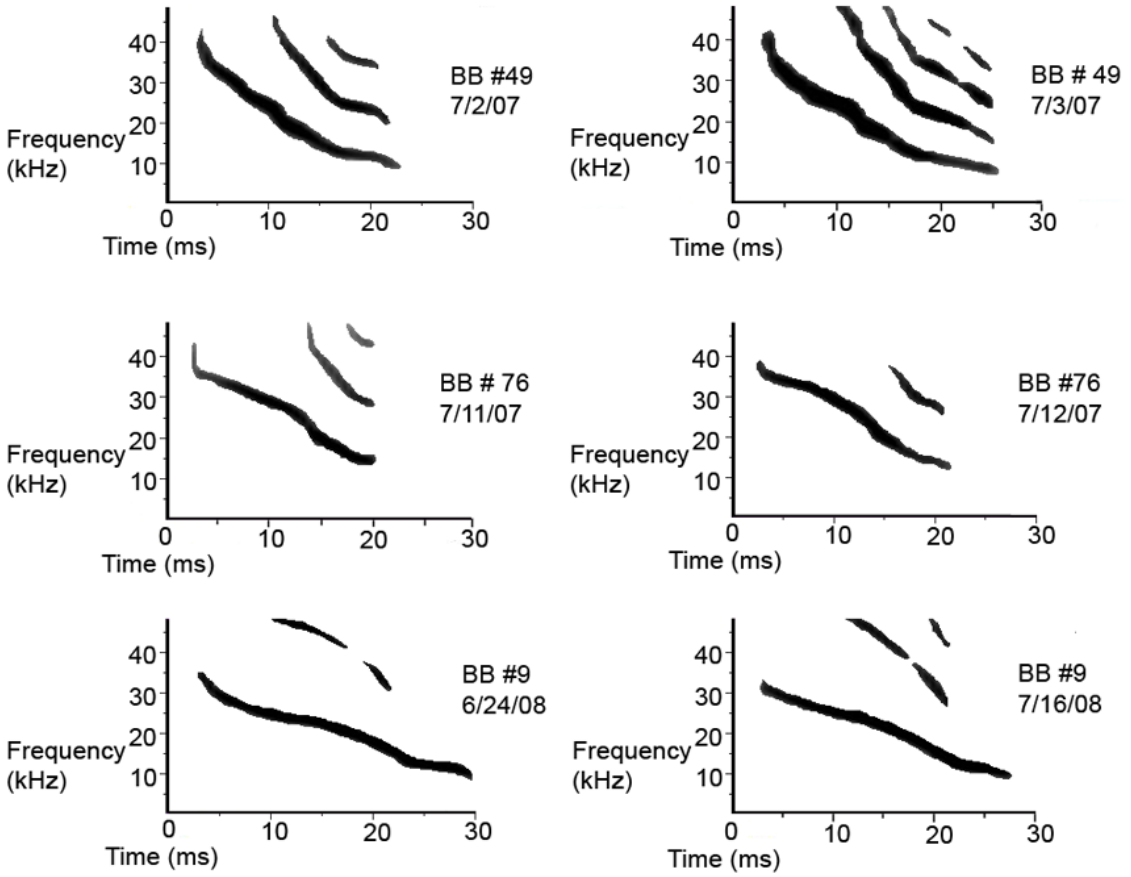
Fig. 4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(kHz)
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Chapter 2: Female natal philopatry and gene flow between divergent clades of pallid bats (*Antrozous pallidus*)

Abstract

In long-lived temperate bats, female philopatry can influence the genetic structure of roosting groups and the potential for individuals to interact across generations. Direct observation of dispersal between social groups is difficult given the vagility and nocturnal activity of most bats; however, molecular markers can be used to infer dispersal and mating patterns. Here I report on female philopatry among pallid bats, *Antrozous pallidus*, a species that exhibits dynamic fission-fusion roosting behavior in which philopatry and familiarity with a roosting area may help individuals choose roost sites and gain benefits associated with social roosting. I also use genetic data to draw inferences about how dispersal and mating in this region relates to the presence of divergent mitochondrial clades, which have been previously reported for pallid bats in western North America. I found significant genetic structure among colonies based on sequence variation at the mitochondrial (mt) DNA control region (pairwise Φ_{ST} - 0.08 – 0.678) but very little structure among colonies for nuclear microsatellites (pairwise F_{ST} - 0.004 – 0.01) indicative of female philopatry and male-mediated gene flow. Some bats captured in the same colony had mtDNA haplotypes that differed by more than 12% yet failed to exhibit differences at nuclear markers. Thus, even though such divergence values are sometimes associated with species differences, evidence indicates that bats from these clades freely interbreed.

Introduction

Patterns of genetic variation detectable among natural populations are a result of both historical patterns of vicariance and colonization, as well as contemporary behavioral factors such as mating behavior and dispersal that can cause gene flow among populations. For example, natal dispersal, defined as the permanent movement of juveniles from their birth location in order to breed, can have significant effects on the genetic structure of animal social groups (Handley and Perrin 2007). In most vertebrates, dispersal is sex-biased, with mammals typically displaying male-biased dispersal and female philopatry to their place of birth (Greenwood 1980). One proposed explanation for this general pattern is that males are more likely to be the dispersing sex in polygynous mating systems, the system most often identified in mammals, because they have greater expected variation in reproductive potential and stand to benefit by dispersing to reduce local competition for mates (Greenwood 1980). Females, being the sex that typically provides the majority of care for the young, benefit by remaining on their natal territory through greater familiarity with foraging and roosting areas, as well as benefits from kin such as increased acquisition of resources (Handley and Perrin 2007). Such cooperative behaviors can be enhanced either actively through the establishment of a kin recognition system, or passively through indiscriminate cooperation maintained by population viscosity of the sex that benefits by being philopatric (Gardner 2010).

Because the degree to which females are philopatric and males disperse critically impact the social structure of mammalian groups, estimating the relative strength of these two parameters can help shed light on the evolution of sociality in a given species. However, direct observation of dispersal using traditional methods of mark-recapture or

radio-telemetry is difficult and can often underestimate the degree to which dispersal is occurring, especially if the spatial scale over which dispersal occurs is large. Thus, indirect estimation of these factors using molecular markers has been crucial to making inferences about how their relative strength and direction contribute to the social structure of mammalian groups (Handley and Perrin 2007). This is especially true in species, such as bats, which are highly mobile and often difficult to observe (Burland and Wilmer 2001).

In most temperate bat species, females form maternity colonies in the summer to give birth and nurse their pups while males roost away from the maternity colony in smaller bachelor groups (McCracken and Wilkinson 2000). Recent work has shown that the majority of bats fit the mammalian pattern of female philopatry and male-biased dispersal (Castella et al. 2001; Kerth et al. 2002; Arnold 2007; Vonhof et al. 2008; Piaggio et al. 2009c) although there are exceptions, such as the brown long-eared bat (*Plecotus auritus*) (Burland et al. 2006) and Schreibers' long-fingered bat (*Miniopterus schreibersii natalensis*) (Miller-Butterworth et al. 2003), where both sexes are philopatric, and the white-lined bat (*Saccopteryx bilineata*) in which females disperse (McCracken 1984; Nagy et al. 2007).

There are several lines of evidence that suggest that female philopatry may be an important factor influencing bat sociality. For example, bats exhibit tremendous longevity compared to similar sized mammals (Wilkinson and South 2002), which along with female natal philopatry results in increased opportunity for social interactions among individuals in a maternity colony across multiple generations. In addition, female philopatry may be especially important in species that exhibit high roost lability since

experience with potential roosting areas may be important in selecting optimal roosts for rearing young (Kerth and Reckardt 2003; Kerth et al. 2006).

Pallid bats (*Antrozous pallidus*) exhibit labile roosting behavior frequently switching among multiple rock crevice roosts within larger rocky outcrops comprising a maternity colony (Lewis 1996). Like most temperate vespertilionid bats, male and female pallid bats are spatially segregated during the maternity season, with males roosting in small groups (Hermanson and O'Shea 1983), although mixed sex maternity colonies have been observed (Orr 1954). Mating is thought to occur in the fall when bats travel short distances from their summer roosting areas prior to hibernation with females storing sperm over the winter during hibernation (Barbour and Davis 1969). However, the effect that these seasonal movement patterns have on the genetic structure of maternity colonies, including the degree to which females return to their natal roosting area after hibernation, are unknown. Female philopatry in pallid bat maternity colonies is of special interest since pallid bats have been reported to emit individually specific low frequency vocalizations to assist in the formation of roosting groups (Chapter 1; Vaughan and O'Shea 1976). Thus, examining the genetic structure of pallid bat maternity colonies may provide insight into the role these vocalizations play in the maintenance of social bonds across generations.

In addition, while examining population structure on a microgeographic scale is informative for investigating the potential effects that biased dispersal and gene flow have on the genetic structure of social groups, analyzing molecular variation on a macrogeographic scale allows for greater inference about how these behavioral patterns shape genetic differences among populations over the range of a species. For example,

sequence variation in the rapidly evolving mitochondrial DNA (mtDNA) control region has been used to infer long distance seasonal migration patterns (Wilkinson and Fleming 1996; Russell et al. 2005) as well as historical patterns of vicariance and gene flow in bats (Ruedi and Castella 2003). Weyandt and Van Den Bussche (2007) utilized mtDNA control region variation to examine the phylogeography of pallid bats across most of their range in North America. They identified three distinct clades corresponding to major desert regions (Fig. 5). Sequences from these clades differ by more than 9%, which corresponds to an estimated divergence time of approximately 3.8 MYA (Weyandt and Van Den Bussche 2007). However, the study contained few samples from the northern part of the range and only used mtDNA, which as a maternally inherited marker cannot be used to determine if male mediated gene flow occurs between clades.

Therefore, I had two goals for this study. First, I examined the genetic structure of pallid bats in central Oregon within and among roosting areas using both bi-parentally inherited microsatellite markers and maternally inherited mtDNA sequences to infer whether females are philopatric to their natal maternity colony. Following Castella et al. (2001), if female philopatry and male-biased dispersal are strong factors affecting genetic variation at pallid bat maternity colonies, I expected to observe differentiation among colonies at the mitochondrial level and limited genetic structure at the nuclear level. Second, I examined mtDNA variation in a broader phylogeographic context by combining sequence data collected from bats captured in Oregon to the previously published homologous sequences from pallid bats sampled throughout western North America (Weyandt and Van Den Bussche 2007).

Methods

Sampling and DNA Extraction

I conducted fieldwork in the Clarno basin of central Oregon, U.S.A (44.94°N lat., 120.38°W long.). The habitat and vegetation of the study area was described in Chapter 1. Bats were captured at six sites (Fig. 6) using either mist nets extended over a water source (Site 4, CCT), mist nets placed outside the entrance to night roosts (CSH, WCB), or a 0.75 m triangular shaped net attached to a 3 m extension pole to capture bats as they exited day roosting crevices (CCS, CCN). Each bat was weighed and marked with a numbered band (National Band and Tag, Newport, KY, U.S.A.) so that individuals could be identified if recaptured. Tissue samples were obtained from wing membranes using 3 mm biopsy punches (Worthington-Wilmer and Barratt 1996) and stored in 95% ethanol until DNA was extracted from each wing-punch using a Qiagen DNeasy Tissue Kit (Qiagen Inc., Valencia, CA, U.S.A.) following the manufacturer's instructions.

Mitochondrial Control Region Sequencing

I sequenced approximately 450 bp of mtDNA control region for 113 captured bats using primers P and E (Wilkinson and Chapman 1991). In addition, DNA extractions of wing punches taken from 18 recaptured bats were independently sequenced and compared to sequences obtained from their previous extractions to verify the repeatability of the sequence data. Polymerase Chain Reaction (PCR) amplifications were carried out in 25 μl reaction volumes consisting of 10-20 ng of template DNA, 10x PCR Buffer, 2.5mM MgCl2, 0.48μM of each primer, 200 μM dNTPs and approximately 1.4 units of Taq polymerase. PCR thermocycling conditions consisted of an initial incubation at 95°C for 5 min followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1

min with a final extension at 72°C for 30 min. Each sample then went through an initial purification process by treatment with ExoSap (USB Corp.) to digest unincorporated primers and dNTPs. Each sample was then sequenced in both the forward and reverse directions using a BigDye Terminator V.3.1 cycle sequencing kit (Applied Biosystems). For cycle sequencing, I used 9.5 μl PCR reaction volumes consisting of 1 μl of cleaned product, 1 μl of Big Dye, 1.5 μl of Big Dye buffer, and 0.35 μM forward or reverse primer in a PCR cycle consisting of 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Sequenced products were then precipitated with isopropanol and re-suspended in 10 μl of Hi-Di Formamide (Amresco) prior to fragment sizing in a 3730 capillary sequencer (Applied Biosystems).

Control region sequences obtained from bats in the family Vespertilionidae often contain variable numbers of an 81 bp repeat resulting in sequences of differing length within species (Wilkinson et al. 1997). However, all pallid bats sequenced in this study contained three repeats and yielded sequences that were approximately equal in size. Forward and reverse sequences for each bat were aligned using Sequencher 4.0 (Genescan) and checked for ambiguities. All sequences were trimmed to a common length of 425 bp and a representative of each haplotype was entered into Genbank (Genbank accession numbers XXXXX – XXXXXX).

Microsatellite Genotyping

I obtained genotypes from captured bats at 12 microsatellite loci (Table 6). I used the M-13 tagging method developed by Schuelke (2000) to label the forward primer for loci EF4, EF5, EF21, G07, E07, H10, and G02 so that fragments could be sized by a DNA fragment analyzer. Briefly, in this method an 18 bp fragment is added to the 5' end

of each forward primer sequence. Three primers for each locus are then added to the PCR mixture: the forward primer with the added sequence, the reverse primer, and a fluorescently labeled 18 bp fragment corresponding to the sequence added to the forward primer. The forward primer is added at half the concentration of both the reverse and labeled primers so that the forward primer will be exhausted and the fluorescently labeled primer will be incorporated into the microsatellite amplicons. For this procedure, the PCR thermocycler conditions for loci EF4, EF5, and EF21 were 3 minutes at 95°C to denature and a touchdown protocol consisting of 20 cycles of 30 seconds at 95°C, 30 seconds at 10°C above the primer specific annealing temperature indicated in Table 6 dropping ½°C per cycle, and 30 seconds at 72°C, followed by 30 cycles of 30 seconds at 95°C, 30 seconds at the primer specific annealing temperature, and 30 seconds at 72°C with a final extension of 20 minutes at 72°C. For loci G07, E10, H10, and G02 I used the M-13 tagging method along with the thermocycling protocol outlined below.

For the remaining microsatellite loci (EF6, EF15, EF20, B02, and G25) I used fluorescently labeled forward primers and a PCR thermocycling profile consisting of an initial denature period of 3 minutes at 95°C followed by 35 cycles of 30 seconds at 95°C, 30 seconds at the primer specific annealing temperature (Table 6), and 30 seconds at 72°C, followed by an extension period of 20 minutes at 72°C. Amplifications were carried out in 12 μl reaction volumes consisting of 10-20 ng of template DNA, 0.5μM of each primer, and 2X PCR Master Mix (Lucigen Corp) containing 0.6 units of Taq DNA polymerase, 200 μM each dNTP, and 1.5 mM MgCl2 along with PCR reaction buffer (ph 9.0). For microsatellite loci EF15 and EF20, 0.5μg/μl of Bovine Serum Albumin (BSA)

and 5% Dimethyl Sulfoxide (DMSO) were added to enhance amplification of the PCR products.

Following amplification, the PCR products were run on a 3730 DNA analyzer (Applied Biosystems) and fragment sizes scored using Genemapper v.3.7 (Applied Biosystems). I used the program GENEPOP (Raymond and Rousset 1995) to test loci for conformity to Hardy-Weinberg Equilibrium using exact tests and to examine all possible pairs of loci for linkage disequilibrium using a log likelihood ratio test. A Bonferroni correction was applied to account for multiple comparisons when considering significance for these tests (Rice 1989).

Genetic Diversity Within and Among Day Roosting Areas

Bats were assigned to one of two day roosting areas (CCN or CCS) if they were either captured exiting a day roost in that area or tracked to the roosting area using radio-telemetry. Radiotransmitters were built (Wilkinson and Bradbury 1988) and placed in the interscapular region of each bat using Skinbond adhesive (Torbot Group, Inc., Cranston, RI, U.S.A.). To minimize disruption to normal flight behavior, transmitters weighed less than 5-8% of total body weight (Aldridge and Brigham 1988). In addition, I also included bats captured at two night roosts (CSH and WCB) to examine the genetic structure of pallid bat colonies. While CSH and WCB appear to be used strictly as night roosts in that I never observed bats present in the buildings during the day, Lewis (1994) captured females at these sites and tracked them to day roosts within 2 km. Thus, I assumed bats captured at these night roosts used day roosts nearby. In addition, because only one bat was captured at the WCB site, I included this bat with those captured at the CSH night roost.

Mitochondrial sequence variability was characterized for each roosting area (CCN, CCS, and CSH) by the number of haplotypes (N_h), haplotype diversity (h) defined as the probability that two randomly chosen haplotypes from one area are different, and nucleotide diversity (π) calculated as the probability that two randomly chosen homologous sites are different. For the microsatellite data, I calculated the average number of alleles per locus (N_a) and the average observed (H_O) and expected (H_E) heterozygosities. All calculations were conducted using Arlequin v. 3.5 (Excofier and Lischer 2010).

Genetic subdivision among the roosting areas was calculated using pairwise estimates of Weir and Cockerham's (1984) Φ_{ST} (mitochondrial data) and F_{ST} (microsatellites) in Arlequin v. 3.5. In addition, I tested for any relationship between geographic distance and genetic differentiation of the mitochondrial data set by grouping sites into a western "population" (CSH and WCB) and an eastern "population" (CCS, CCN, CCT, and Site 4) to calculate the proportion of the genetic variance explained by differences among populations and differences among sites within populations using an Analysis of Molecular Variance (AMOVA) implemented in Arlequin. I used a maximum likelihood approach to identify the best fitting model for nucleotide substitutions using estimates of the Akaike information criterion (AIC) calculated in the program JModeltest v. 1.0 (Posada 2008). The value of the gamma shape parameter from the best fitting model was utilized in the calculations of pairwise differences among populations for the AMOVA.

Phylogeographic Patterns

To visualize haplotype relationships among pallid bats in the study area, I constructed haplotype networks with the program TCS (Clement et al. 2000) incorporating all of the sequences obtained from all capture sites (Fig. 6). In this procedure, haplotype networks are generated under statistical parsimony where haplotypes are split into separate groups if the number of mutational steps required to join them exceeds the number of steps allowed using a 95% parsimony criterion. In addition, to place the mitochondrial sequences of pallid bats in Oregon into a broader phylogeographic context, I downloaded 80 homologous control region sequences from pallid bat specimens collected throughout western North America (British Columbia Canada, N=5; California, N=6; Utah, N=6; Arizona, N=2; Nevada, N=1; Texas, N=28; Oklahoma, N=2; New Mexico, N=17 and Mexico, N=13) from Genbank (accession numbers AY706769–AY706887). I aligned these sequences with the Oregon sequence data and trimmed them to a common length using Sequencher 4.0 (Genescan). I then used the program JModeltest (Posada 2008) to determine the appropriate model of evolution and nucleotide substitution for the data and used these parameters to create a maximum likelihood tree in the program Phyml (Guindon and Gascuel 2003) with the initial tree created using the BioNJ algorithm and the nearest neighbor interchange (NNI) algorithm used for the tree topology search. Node support was determined using 1000 bootstrap replicates.

Results

Genetic Diversity and Roost Composition

I obtained mtDNA control region sequence data from 108 females and 5 males. These 113 sequences contained eight unique haplotypes with 57 variable sites (5 insertions/deletions, 44 transitions, and 8 transversions) (Table 7). Within roosting areas, mitochondrial sequence diversity was relatively low with 3-5 haplotypes identified per roosting area (Table 8). In addition, while there was some overlap in the distribution of haplotypes among roosting areas, I identified several haplotypes that were unique to one area (e.g. haplotypes 5 and 7 from the CSH and WCB night roosts and haplotypes 2 and 3 found only at the CCS and CCN roosting areas, Fig. 6).

Females captured exiting the same day roost crevice at the CCN and CCS sites comprised only two matrilines (haplotypes 2 and 8) and all females shared a haplotype with at least one additional captured bat. In contrast, males captured in the same day roosts shared a haplotype with the females they were roosting with on one occasion (haplotype 2) while two males exiting a day roost at CCS belonged to different matrilines than the females they were captured with (males - haplotypes 1 and 3, females - haplotypes 2 and 8).

All 113 bats and the 18 recaptures referenced above were genotyped at 12 microsatellite loci (Table 6). Two loci were excluded from subsequent analyses due either to lack of variation (E10) or to failure to meet Hardy Weinberg expectations (H10). The remaining 10 loci did not deviate from Hardy Weinberg expectations ($P = 0.032 – 0.81$) or exhibit significant linkage disequilibrium ($P = 0.02 – 1.0$) after sequential Bonferroni correction (Rice 1989) and had 2-24 alleles with an average heterozygosity of

0.72 ± 0.07 for all genotyped bats. Within roosting areas, the 10 microsatellite loci showed considerable variability averaging 7.6 – 9.4 alleles per locus with high observed heterozygosities (Table 8).

Population Structure Among Roosting Areas

As shown in Table 9, for the microsatellite data pairwise F_{ST} values among the roosting areas were low with none significantly greater than zero after Bonferroni correction. In contrast, the mitochondrial sequence data showed evidence of significant population structure between CSH and both the CCN and CCS roosting areas while there was no evidence of population subdivision between the CCN and CCS roosting areas. Excluding the three males captured in the CCS roosting area resulted in evidence for marginally significant population structure between CCS and CCN using the microsatellite data, but otherwise did not alter the results (Table 9). Finally, AMOVA revealed significant genetic structuring (Φ_{ST} of 0.496, $P < 0.0001$, 10,000 randomizations) between the eastern and western sites. This result is due to 46.0% of the mtDNA sequence variation attributable to differences between the east vs. west sites and 3.6% of the mtDNA variation due to differences among sites within groups.

Phylogeographic Patterns

Statistical parsimony grouped the eight haplotypes into two distinct clades with over 12% sequence divergence (Fig. 7). However, evidence of this mitochondrial structure is not reflected in the microsatellite markers (F_{ST} between the two clades = 0.004). Haplotype 8 in clade A was found in 65 (57%) of the sequenced bats while in clade B, most haplotypes were evenly distributed with the exception of haplotype 6, which was found in only one bat from the CSH night roost. Finally, there appears to be a

relationship between geographical location and haplotype frequency in that the clade A haplotypes were found almost exclusively in the eastern sites while clade B haplotypes were more common in the western sites (Fig. 6).

When the control region sequences obtained for this study were aligned with those collected by Weyandt and Van Den Bussche (2007), I found that the two clades identified in this study correspond to two of the clades they located either in northern California (clade A) or in British Columbia extending south to Mexico (clade B) (Figs. 5 and 8). The bats sampled in Texas, Oklahoma, New Mexico, and three samples from Mexico designated as clade C by Weyandt and Van Den Bussche (2007) did not align with the sequences sampled in Oregon and were excluded from the tree for clarity.

Discussion

Population Structure and Female Philopatry

In this study, I estimated population structure from maternally inherited mtDNA haplotypes and bi-parentally inherited nuclear microsatellites to detect evidence of female philopatry and sex-biased dispersal and mating in cliff roosting pallid bats in central Oregon. Overall, similar to the findings from other temperate bats, I found evidence of strong genetic structure from maternally inherited markers and little structure from bi-parentally inherited markers. These differences indicate that female dispersal is restricted to neighboring roosting sites while male dispersal enables inter-colony mating. While in most temperate bats it is thought that mating occurs either in a swarm outside or en route to a hibernaculum (e.g. (Kerth and Morf 2004), the extent to which pallid bats engage in similar behavior is unknown, although pallid bats have been reported to vacate summer

diurnal roosting areas in the early fall presumably to hibernate (O'Shea and Vaughan 1977; Hermanson and O'Shea 1983). Regardless, given the difference in population structure revealed by the two types of genetic markers, gene flow among nuclear markers must result from males mating with females from different colonies while females remain philopatric to their natal roost sites. This conclusion is also supported by re-capture of twenty banded female bats in more than one field season at the same site and telemetry data which has shown that bats roost in the same roosting area in multiple years (B Arnold, unpublished data).

Relative to some studies conducted on temperate vespertilionid bats, the haplotype diversity exhibited by pallid bats in central Oregon may appear to be low. For example, in a study on *Eptesicus fuscus*, Vonhof et al. (2008) found haplotype diversity ranging from 0.615 to 0.948 within colonies and 5-15 haplotypes per colony, which is more than I found (Table 8). However, the *E. fuscus* maternity colonies sampled for the Vonhof et al. (2008) study were in buildings that have maintained stable colonies for at least 17 years. Tree roosting bats, such as *E. fuscus* in Canada and *M. bechsteinii* in Germany, that exhibit fission-fusion behavior and high roost lability may offer a better comparison to this study. In these species, the number of unique haplotypes per roosting area range from two (*M. bechsteinii*, (Kerth et al. 2000) to six (*E. fuscus*, (Metheny et al. 2008b), which is comparable to my results. Finally, the low haplotype diversity found in this study could be explained by the relatively small sample size of bats captured or tracked to roosting areas. However, multiple bats captured at the cattle trough located adjacent to the two roosting areas also showed limited haplotype diversity comparable to

the two maternity sites (Fig. 6, Table 8) and did not add any additional haplotypes beyond those found at either the CCS or CCN roosting areas.

The low Φ_{ST} estimate of 0.077 between the CCS and CCN roosting areas suggests that female movement between these two cliff complexes is relatively high, which is not surprising given that they are only 1 km apart. In fact, two bats with a pairwise relatedness estimate of R=0.42 (see Chapter 3) were captured exiting a crevice in the CCN roosting area in 2007 and re-captured exiting a crevice at the CCS roosting area in 2008 indicating that closely related females visit and utilize roost sites in both areas over time. Thus, as in other bat species that exhibit high roost lability such as *M. bechsteinii* (Kerth and König 1999), *E. fuscus* (Willis and Brigham 2004), and the northern myotis (*Myotis septentrionalis*) (Garroway and Broders 2007), a colony may be defined not by a given structure that bats return to in multiple years, but by a geographic area with multiple potential roosts to which females are faithful.

The high Φ_{ST} between the night roosts and both of the maternity roosting areas along with the AMOVA results indicating significant genetic differences between the eastern and western sites, suggests the presence of a geographic or behavioral barrier to female dispersal between these two areas even though they are separated by only a few kilometers. Behavioral barriers to mitochondrial gene flow have been found in *M. bechsteinii* as a result of forest fragmentation even though both males and females can regularly cross clearings between forest patches when moving to swarming sites to mate (Kerth and Petit 2005). Kerth and Petit (2005) argue that dispersal by both males and females to swarming sites resulting in the spread of autosomal genes and females crossing boundaries to colonize new roosting areas can be viewed as behaviorally

distinct. Thus, colonization of new roosting areas followed by female philopatry can lead to genetic divergence in maternally inherited markers over short distances, as was found in tree roosting *E. fuscus* where females moving to new roosting areas consist of closely related maternal kin (Metheny et al. 2008a). As with most temperate bats, we know little about the behavioral mechanisms behind maternity colony formation and where and when mating takes place in pallid bats. Additional research on these topics is needed to understand how such deep mtDNA divergence between groups can persist over such a small geographic area.

Phylogeography

The finding that pallid bats in the study area are representative of two divergent mitochondrial lineages is unexpected in that the two clades differ by over 12%, which is greater than the level of divergence reported for many mammalian subspecies and some species (Baker and Bradley 2006; Mayer et al. 2007). However, both weight ($\overline{X}_{\text{(Clade A)}}$ = 22.2 g, $\overline{X}_{\text{(Clade B)}}$ = 22.3 g, P=0.85) and forearm length ($\overline{X}_{\text{(Clade A)}}$ = 53.0 mm, $\overline{X}_{\text{(Clade B)}}$ = 53.6 mm, P=0.23) measured from bats captured at the study area are not significantly different indicating that there are no obvious morphological differences between the two groups. More importantly, the lack of structure at microsatellite loci used in this study indicates that the two groups freely interbreed. There also appears to be a geographic pattern in that clade A is represented by two haplotypes, one of which is found only in the eastern sites, and clade B is represented by six haplotypes which are found predominantly in the two westernmost sites. Finally, the pattern identified by the haplotype network (Fig. 7) suggests the expansion of bats from clade A into central Oregon is relatively recent given the predominance of haplotype 8 with only two individuals represented by

haplotype 7 and few presumed haplotypes not found in this study. In contrast, the pattern exhibited by clade B haplotypes is indicative of a large population maintained over a long period of time with a greater abundance of haplotypes with evenly distributed frequencies (Avise 2009).

When the sequences obtained for this study were aligned with those collected by Weyandt and Van Den Bussche (2007), I found that the two clades identified in this study correspond to two distinct geographic clades (Figs. 5 and 8) located either in northern California (clade A) or from British Columbia extending south to Mexico (clade B). Recent phylogeographic studies on mammals in the Pacific Northwest have identified similar divergence zones resulting in clades corresponding to the geographic locations of northern California into central Oregon and British Columbia (Chavez and Kenagy 2010; Galbreath et al. 2010). These distributions are indicative of post-glacial range expansions from southern refugia northwards on either side of the Sierra Nevada Mountains with secondary contact occurring recently in central Oregon. Further sampling both west and east of the study area is needed to determine if this interpretation of range expansion of pallid bats in northwestern North America is correct.

Female Philopatry and Social Behavior

The finding of strong female natal philopatry in pallid bats is especially interesting given that pallid bats are often regarded as being highly social when compared to other vespertilionid bats (Hermanson and O'Shea 1983). For example, pallid bats have been reported to engage in cooperative behaviors in the roost such as preferentially placing juveniles in the center of a roosting group (Trune and Slobodchikoff 1978), guarding juveniles (Beck and Rudd 1960), and guiding mothers to distressed offspring

(Brown 1976). Pallid bats are also relatively long-lived with reports of individuals surviving over nine years in the wild (Tuttle and Stevenson 1982), which along with female natal philopatry, creates opportunities for the maintenance of cooperative behaviors through kin selection if roosting groups consist of relatives and bats have a mechanism to identify relatives. Interestingly, pallid bats emit a loud, partially audible contact call several times in rapid succession while in flight outside roosting areas, primarily as bats return to the roosting area after foraging (Vaughan and O'Shea 1976). These calls are individually specific and stable through time and playbacks show that bats respond to calls by approaching the source of the sound and calling in response (Chapter 1). Thus, calling appears to facilitate roosting group formation. To determine the degree to which calling may assist in the maintenance of multigenerational social groups during roost switching, acoustic analysis of call structure to determine whether calls encode any relatedness information in conjunction with more detailed analysis of the genetic relatedness of bats sharing a crevice roost will be an important avenue for future research.

Table 6. Annealing temperatures, observed size ranges, and genetic diversity (heterozygosity expected (H_E), heterozygosity observed (H_O) for the 12 microsatellite loci used in the genetic analyses of 113 *Antrozous pallidus* samples collected in central Oregon, U.S.A.

Locus	Observed Size Range (bp)	T_A (°C)	No. of Alleles	H_E	H_O	Source Species	Primer Source
EF4	232-260	50	11	0.86	0.84	*Eptesicus fuscus*	(Vonhof et al. 2002)
EF5	157,159	50	2	0.49	0.39	*Eptesicus fuscus*	(Vonhof et al. 2002)
EF6	174-205	47	9	0.74	0.72	*Eptesicus fuscus*	(Vonhof et al. 2002)
EF21	217-242	50	10	0.77	0.81	*Eptesicus fuscus*	(Vonhof et al. 2002)
EF15	77-108	55	12	0.87	0.86	*Eptesicus fuscus*	(Vonhof et al. 2002)
EF20	90-228	47	24	0.92	0.93	*Eptesicus fuscus*	(Vonhof et al. 2002)
G25	129-151	53	6	0.59	0.54	*Myotis myotis*	(Castella and Ruedi 2000)
B02	160-192	53	16	0.91	0.88	*Corynorhinus townsendii*	(Piaggio et al. 2009b)
G07	338,340	53	2	0.26	0.27	*Corynorhinus townsendii*	(Piaggio et al. 2009b)
G02	185-210	53	12	0.85	0.87	*Corynorhinus townsendii*	(Piaggio et al. 2009b)
H10	255-317	53	20	0.93	0.82	*Corynorhinus townsendii*	(Piaggio et al. 2009b)
E10	307	53	1	NA	0	*Corynorhinus rafinesquii*	(Piaggio et al. 2009a)

Table 7. Haplotypes obtained from the 425 bp mtDNA control region sequences of 113 *Antrozous pallidus* captured at six sites in central Oregon (Sample sizes for each haplotype are: H1–7, H2–15, H3–3, H4–9, H5–11, H6–1, H7–2, H8–65)

	Nucleotide Position																			
	28	**30**	**31**	**34**	**36**	**41**	**48**	**51**	**52**	**70**	**72**	**73**	**74**	**78**	**79**	**87**	**96**	**99**	**101**	**105**
H1	A	A	A	A	C	A	T	G	C	G	C	T	G	C	T	A	T	G	A	A
H2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.
H4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H5	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.
H6	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.
H7	–	G	C	G	T	G	C	A	T	A	T	C	A	T	A	.	C	A	G	–
H8	–	G	C	G	T	G	C	A	T	A	T	C	A	T	A	.	C	A	.	–

	Nucleotide Position																			
	111	**114**	**115**	**117**	**118**	**127**	**138**	**141**	**159**	**160**	**188**	**190**	**195**	**197**	**201**	**219**	**224**	**225**	**228**	**235**
H1	C	–	C	G	G	C	T	A	C	C	A	T	–	T	A	T	C	T	G	C
H2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H7	T	T	T	A	T	T	C	G	T	T	C	A	A	C	.	C	T	C	A	A
H8	T	T	T	A	T	T	C	G	T	T	C	A	A	C	G	C	T	C	A	A

	Nucleotide Position																
	236	**237**	**238**	**248**	**252**	**255**	**272**	**281**	**298**	**306**	**334**	**344**	**350**	**356**	**360**	**371**	**414**
H1	C	T	C	T	T	–	C	T	C	A	C	T	C	T	C	T	A
H2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
H3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
H4	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
H5	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
H6	.	.	.	.	.	.	T	.	.	.	.	.	.	C	.	.	.
H7	T	C	T	C	C	A	A	.	T	G	A	C	T	.	T	C	.
H8	T	C	T	C	C	A	A	.	T	G	A	.	T	.	T	C	.

Table 8. Measures of genetic variation for *A. pallidus* at six sites in central Oregon. Sample size (N), number of haplotypes (N_h), haplotype diversity (h), nucleotide diversity (Π), mean number of alleles per microsatellite locus (A), observed (H_O) and expected (H_E) heterozygosities

		Mitochondrial Variability			**Microsatellite Variability**		
Site Location	N (n_f/n_m)	N_h	h	Π	A	H_O	H_E
CCS	25 (22/3)	5	0.597	0.061	7.7	0.68	0.702
CCN	18 (18/0)	3	0.307	0.036	7.6	0.72	0.734
Site 4	2 (2/0)	2	1	0.122	3	0.83	0.778
CCT	47 (45/2)	5	0.456	0.050	9.4	0.706	0.732
CSH	20 (20/0)	5	0.663	0.02	8.2	0.735	0.712
WCB	1 (1/0)	1	–	–	–	–	–

Table 9. Weir and Cockerham's estimates of F_{ST} (microsatellite data, lower diagonal) and Φ_{ST} (mitochondrial data, upper diagonal) between *A. pallidus* roosting sites. Significance was determined using a permutation procedure implemented in the program Arlequin with alpha set to 0.017 to correct for multiple testing. Values in parentheses were calculated excluding the three males captured at the CCS roosting area.

	CCS	**CCN**	**CSH**
CCS	–	0.077 (0.045)	0.391*** (0.438) ***
CCN	0.01 (0.017)*	–	0.678***
CSH	0.0083 (0.0086)	0.0035	–

* P=0.013

*** P<0.001

Fig. 5 – Map showing the location of the study area relative to the known occurrence of mtDNA clades of pallid bats in North America. The figure was modified from Weyandt and Van Den Bussche (2007) with dashed lines encompassing the sampling locations utilized for their study.

Fig. 6 – Map of the study area showing the capture sites for *Antrozous pallidus*. Pie charts indicate mtDNA control region haplotype frequencies at each site. Capture site abbreviations are: Cove Creek North (CCN), Cove Creek South (CCS), Cove Creek Cattle Trough (CCT), Clarno School House (CSH), and Will Cole Barn (WCB).

Fig. 7 – Parsimony network of mtDNA control region haplotypes showing clades identified using 95% confidence in the program TCS. A bar between haplotypes represents a single mutation and solid squares designate presumed haplotypes not identified in this study. Percent sequence divergence is shown within clades (in box) and between clades (outside box).

Fig. 8 – Unrooted maximum likelihood tree obtained for mtDNA control region sequences of bats sampled in Oregon (shown in bold italics) or obtained from Genbank and representative of clades A and B (Weyandt and Van Den Bussche 2007). The tree was constructed based on the TrN + G (gamma=0.15) model of nucleotide substitution obtained from JModeltest (see methods). Bootstrap support (percentage of 1000 replicates) is shown for the nodes leading to major groups.

Fig. 5

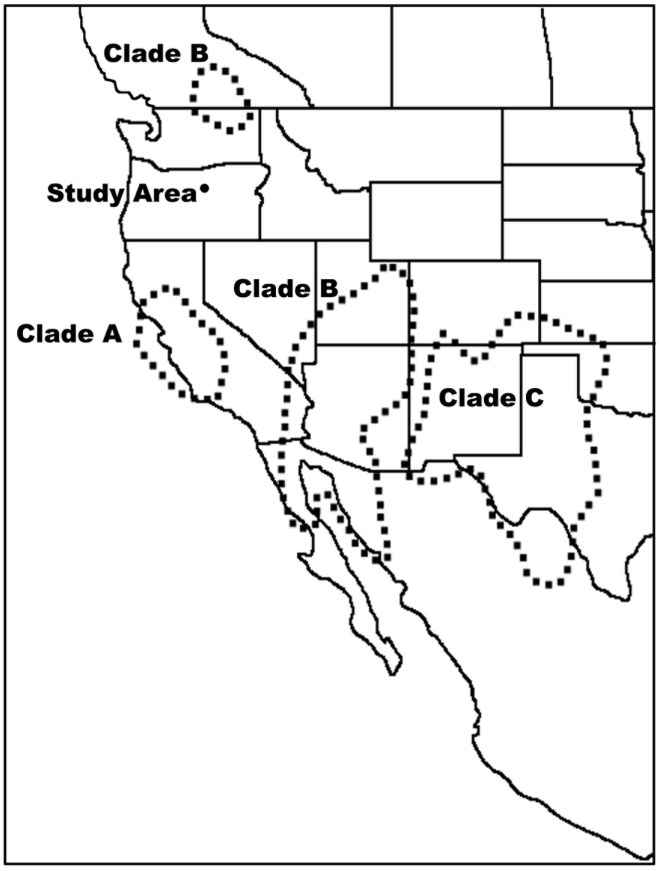


Fig. 6

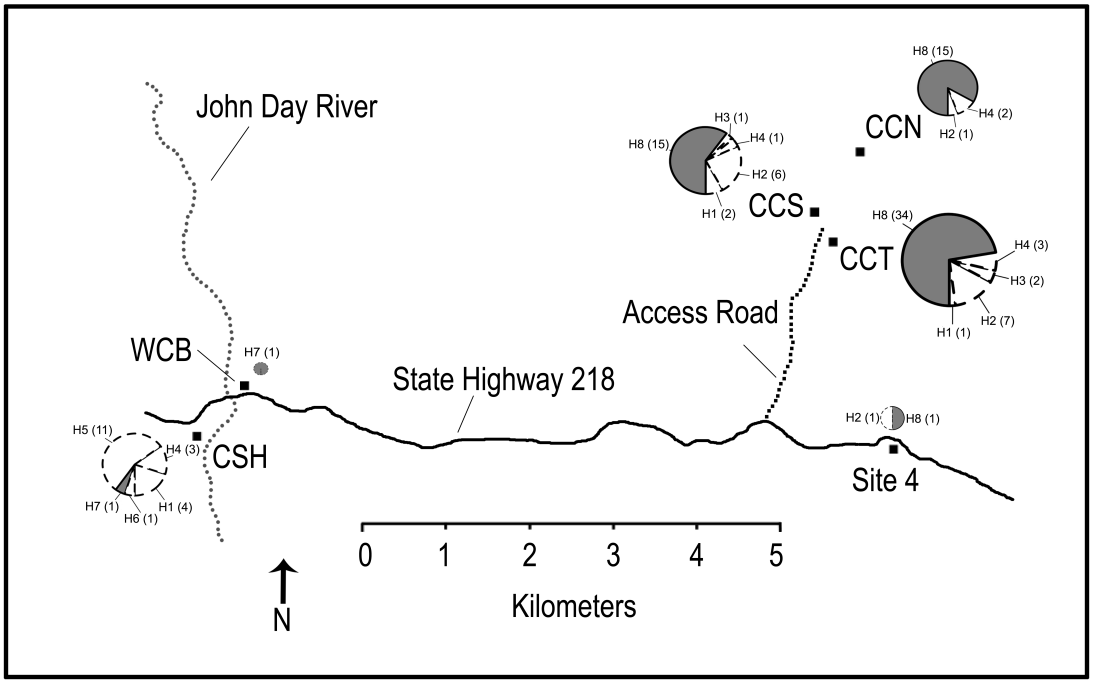

John Day River
H8 (15)
H4 (2)
H2 (1)
CCN
H3 (1)
H8 (15)
H4 (1)
H2 (6)
H1 (2)
CCS
H8 (34)
H4 (3)
CCT
H3 (2)
H2 (7)
H1 (1)
Access Road
WCB
H7 (1)
State Highway 218
H2 (1)
H8 (1)
H5 (11)
H4 (3)
CSH
Site 4
H7 (1)
H1 (4)
H6 (1)
0 1 2 3 4 5
Kilometers
N

Fig. 7

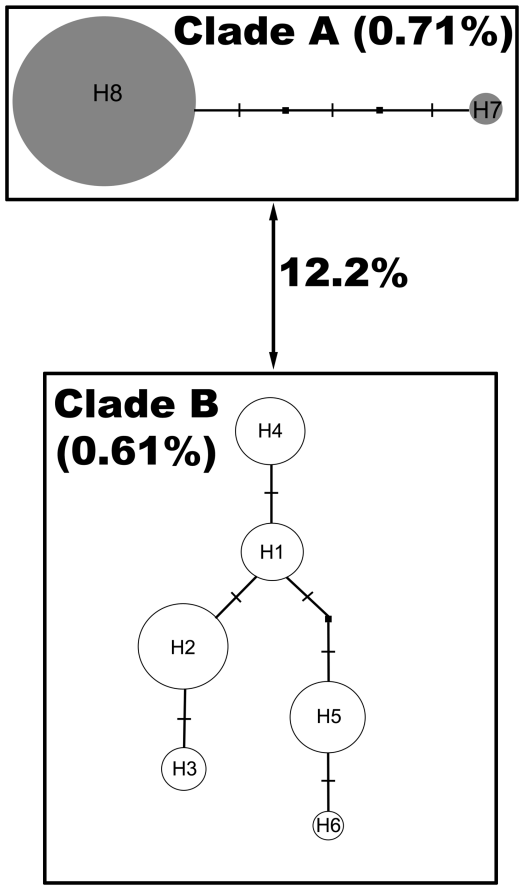
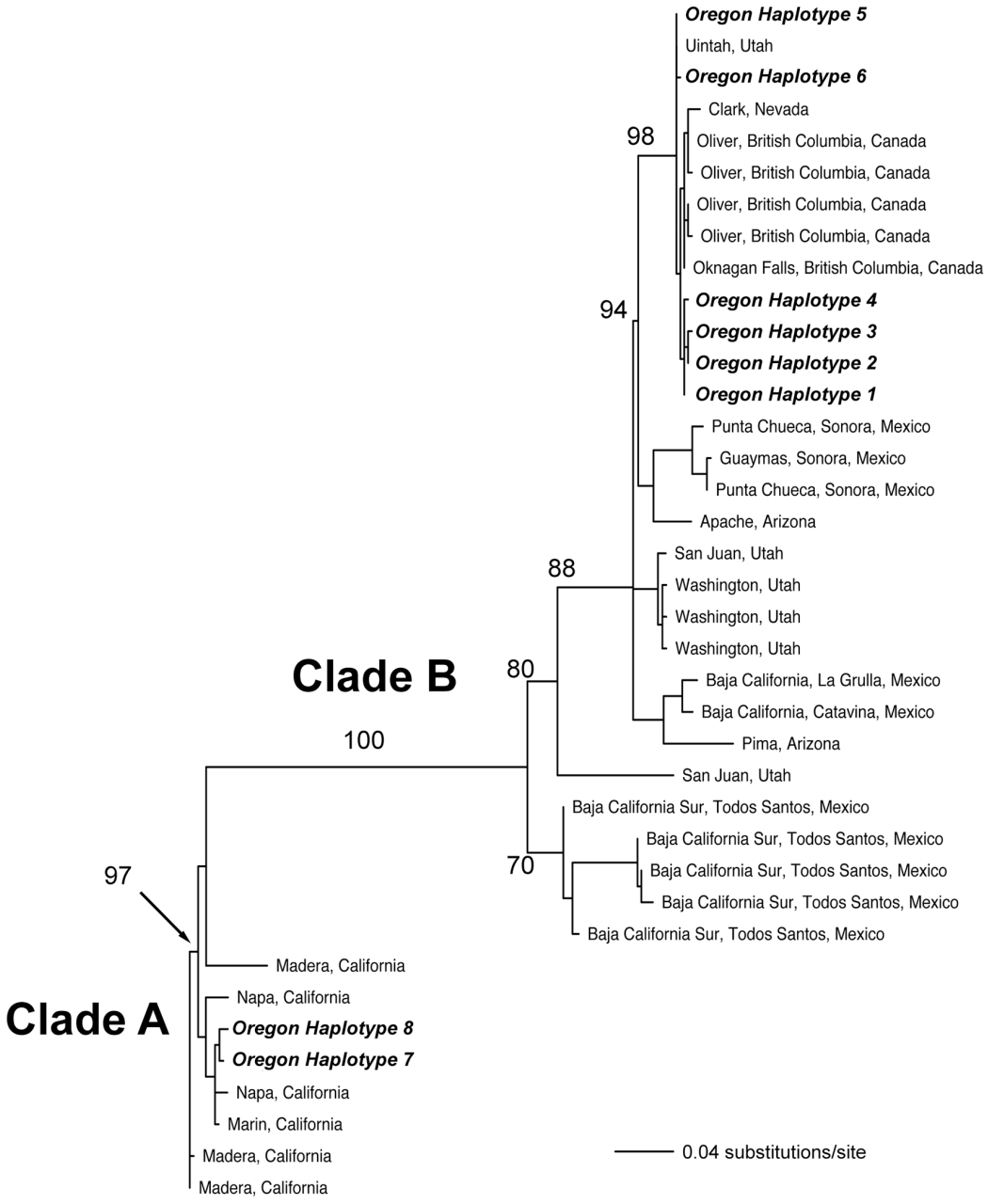


Fig. 8


Oregon Haplotype 5
Uintah, Utah
Oregon Haplotype 6
Clark, Nevada
98
Oliver, British Columbia, Canada
Oliver, British Columbia, Canada
Oliver, British Columbia, Canada
Oliver, British Columbia, Canada
Oknagan Falls, British Columbia, Canada
94
Oregon Haplotype 4
Oregon Haplotype 3
Oregon Haplotype 2
Oregon Haplotype 1
Punta Chueca, Sonora, Mexico
Guaymas, Sonora, Mexico
Punta Chueca, Sonora, Mexico
Apache, Arizona
88
San Juan, Utah
Washington, Utah
Washington, Utah
Washington, Utah
Clade B
80
Baja California, La Grulla, Mexico
Baja California, Catavina, Mexico
100
Pima, Arizona
San Juan, Utah
Baja California Sur, Todos Santos, Mexico
Baja California Sur, Todos Santos, Mexico
70
Baja California Sur, Todos Santos, Mexico
Baja California Sur, Todos Santos, Mexico
Baja California Sur, Todos Santos, Mexico
97
Madera, California
Clade A
Napa, California
Oregon Haplotype 8
Oregon Haplotype 7
Napa, California
Marin, California
Madera, California
Madera, California
0.04 substitutions/site

Chapter 3: Contact calls provide kinship information in a fission-fusion society of pallid bats (*Antrozous pallidus*)

Abstract

Bats exhibit a diverse array of social systems including dynamic fission-fusion associations in species that exhibit high roost lability. However, the mechanisms that bats utilize to locate and choose roostmates have received little study and the relatedness among individuals within a colony are known for only a few species. Previous work has shown that contact calls emitted by pallid bats (*Antrozous pallidus*) flying outside a roosting area aid in the formation of roosting groups and could, therefore, provide a mechanism for recognizing kin. Here I investigate the relatedness of bats in roosting groups, whether calls encode information about kinship, and whether bats preferentially respond to the calls of relatives. While I did not detect any preference for the calls of relatives, I did find that roosting groups contain a greater proportion of related individuals than expected by chance. Moreover, by comparing calls from individuals with known microsatellite genotypes and mtDNA haplotypes I found that the acoustic structure of contact calls encodes information about kinship and matrilineal relationship. These results provide evidence that acoustic cues can convey kinship information and thereby provide a mechanism to influence social group formation in animals that rely on vocal communication.

Introduction

Fission-fusion social systems are widespread among mammals and include species of elephants (Wittemyer et al. 2005), cetaceans (Lusseau et al. 2006), bats (Kerth and König 1999; Willis and Brigham 2004), hyenas (Smith et al. 2010), and primates (Amici et al. 2008; Ramos-Fernandez et al. 2009). Within these societies, subgroups often associate non-randomly and the social and ecological circumstances that influence these non-random associations are crucial to understanding the benefits of group living in these species. For example, social structure in African elephants (*Loxodonta africana*) is multi-tiered with most primary core groups consisting of related individuals. Thus, the social group can potentially provide indirect benefits via kin selection (Wittemyer et al. 2009). In contrast, when core groups merge to form higher order social groups during times when resources are plentiful (Wittemyer et al. 2005), average group relatedness decreases, which increases the importance of direct benefits, such as predator defense, for maintenance of these larger aggregations (Wittemyer et al. 2009).

Given the fluid nature of fission-fusion societies, a mechanism for identifying and locating groupmates is needed to maintain non-random associations of individuals over time. One such mechanism is the use of specialized vocal signals, termed contact calls, that encode information about group identity via call convergence (e.g. chimpanzees (*Pan troglodytes*) (Crockford et al. 2004) and killer whales (*Orcinus orca*) (Miller et al. 2004) or about individual identity (e.g. African elephants, (McComb et al. 2003) and dolphins, (*Tursiops truncatus*) (Janik et al. 2006). In addition, recent work by Deecke et al. (2010) has shown that killer whales, another species with a fission-fusion social system, produce calls that carry information about kinship as revealed by a correlation

between relatedness of group matriarchs and call similarity. Thus, killer whales may use calls to identify related individuals during fusion with other social groups. Similarly, Sharp et al. (2005) used playbacks to show that long-tailed tits (*Aegithalos caudatus*) can discriminate kin from non-kin using individually specific contact calls. Both of these results support the contention that kin recognition cues can be extracted from social calls in species that depend on vocal communication. Therefore, the possibility that other species that exhibit fission-fusion social structure may also utilize acoustic kin recognition deserves additional study.

Bats (Order Chiroptera) are one of the most socially diverse groups of mammals with some species exhibiting permanent groups that utilize the same roost across years to others with fission-fusion societies in which roost location and group composition change frequently (Kerth 2008). Yet, the benefits of group living in bats and whether acoustic signals are involved in maintaining social groups are largely understudied due to the high mobility and nocturnal lifestyle of bats (Kerth 2006).

However, one species, Bechstein's bat (*Myotis bechsteini*), has been valuable in shedding light on the sociobiology of bats that exhibit dynamic fission-fusion social structure. In *M. bechsteini*, non-random associations among individuals in the group are influenced more by reproductive condition than relatedness with lactating females preferentially roosting together over time (Kerth and König 1999). In addition, colonies of Bechstein's bats typically consist of both related and unrelated individuals (Kerth et al. 2002). Thus, cooperative behaviors at the roost, such as information transfer about roost location, could be maintained by kin selection if bats have a mechanism to identify relatives (Kerth 2006). However, Siemers and Kerth (2006) failed to find differences

among individuals in any acoustic features of echolocation calls of *M. bechsteini*. While bats emit low frequency social calls that attract conspecifics while flying outside the roost (Schoner et al. 2010), the variability in social call structure in this species is unknown.

In arid parts of their range, pallid bats (*Antrozous pallidus*) form maternity colonies in which individuals move among rock crevice roosts every few days and exhibit variable degrees of association (Lewis 1996). In addition, prior to entering a roost pallid bats emit low frequency calls that attract conspecifics and contain sufficient information for individual identification (Chapter 1; Vaughan and O'Shea 1976). Bats that circle and call outside a day roost seem likely to have increased energetic expenditure, reduced potential time spent foraging, and increased exposure to predators, such as owls (O'Shea and Vaughan 1977). These potential costs could, however, be offset if these behaviors facilitate the formation of roosting associations in which individuals benefit indirectly by preferentially associating with kin. Genetic information provided by nuclear microsatellites and maternally inherited mitochondrial DNA (mtDNA) indicate that female pallid bats are philopatric to their natal roosting area (Chapter 2), which along with their longevity (estimated at 9 yrs, (Tuttle and Stevenson 1982), enhances the opportunity for social groups to contain relatives from multiple generations. Pallid bats have also been reported to utilize traditional night roosts for consumption of prey (Lewis 1994) which in other temperate bat species, such as the greater horseshoe bat (*Rhinolophus ferrumequinum*), often contain roosting groups consisting largely of matrilineal kin and are thought to be important in maintaining mother-daughter foraging associations (Rossiter et al. 2006). However, the genetic relationships among pallid bats in day or night roosts or the extent to which calls encode

relatedness remains to be studied.

Thus, the primary objective for this study was to determine if contact calls enable pallid bats to form roosting groups with kin. To test this hypothesis, I collected data from two roosting situations, i.e. either groups of bats that were captured while roosting together during the day or together at night. If calls enable the formation of kin groups, I predicted that calling behavior would be more prevalent outside temporary diurnal roosts than traditional nocturnal roosts, diurnal roosts would have more related individuals than expected by chance, and calls would contain information about kinship. I also utilized playbacks to test if bats responding to playbacks of contact calls exhibit a higher level of relatedness to the caller than a random sample from the population.

Methods

Study Area

I conducted fieldwork at two different diurnal roosting areas located in the Pine Creek Conservation Area in the Clarno basin of central Oregon, U.S.A (44.94°N lat., 120.38°W long.). These sites are located approximately 0.75 km apart and are designated as Cove Creek North (CCN) and Cove Creek South (CCS). The habitat and vegetation of the study area have been described previously (Chapter 1). In addition, I collected observational data and captured bats entering and exiting an abandoned dwelling designated as Clarno Schoolhouse (CSH) which is used as a traditional night roost by pallid bats as well as other bat species (e.g. *Corynorhinus townsendii* and *Myotis yumanensis*). The CSH night roost is located approximately 8 km west of the Pine Creek

Conservation Area colonies (Fig. 9). This building appears to be used exclusively as a night roost in that I never observed bats present in the building during the day.

Call Observation

I used observations of calling behavior at day roosts previously reported in Chapter 1 to compare to pallid bat calling behavior at the night roost. However, since bats call while flying outside roosts more frequently while lactating (personal observation), I only included observational data collected after captured bats were found to be lactating. Thus, I used data collected from videotapes of bats entering and exiting three different roosting crevices for one hour on each of four nights at the CCN roosting area. Video recording times varied but were typically between midnight and 0500 when bats returned from foraging. Vocalizations were recorded into the video camera with a shotgun microphone (model AT4071A, Audio Technica, Japan) that was oriented toward the roost approximately 6 m above ground on extension poles. From each 1 h video recording session, I scored call occurrence, entries into the roosting crevice, and exits from the roosting crevice. I scored calls as being associated with entering or exiting the roost if the call occurred less than five seconds before either event on tape. Videotapes were scored using JWatcher v. 1.0 (JWatcher.ucla.org).

At the CSH night roost, I observed calling behavior between 2100 and 0300 on three separate sampling days during the period when bats were lactating. I observed bats entering and exiting the night roost using a night vision scope (Noctron V, Varo, Inc. Garland, TX, U.S.A.) and an LED infrared spotlight (model # 15-IL07, Cop Security System Corp. Taiwan) and scored calls as described above. I determined if calling behavior is more likely to be associated with entering and exiting the day roosting crevice

than the night roost using a two-way contingency table analysis conducted using JMP v.5.0 (SAS Institutes Cary, NC, U.S.A.).

Capture Techniques

In 2007 and 2008 at the CCN and CCS roosting areas, bats were captured on three occasions as they exited day roosting crevices using a 0.75 m triangular net attached to a 3 m extension pole. Because I did not capture every bat in each crevice roost, I will refer to bats captured in the same roost hereafter as part of a "roosting subgroup". In addition, on one night in 2005 and three nights in 2008 at the CSH site, a 6 m mist net was placed outside the main entrance and bats were captured as they attempted to enter or exit the building between 2100 and 0130 h. Each bat was weighed and marked with a numbered metal band (National Band and Tag, Newport, KY, U.S.A.) so that individuals could be identified if recaptured. Tissue samples were obtained from wing membranes using 3 mm biopsy punches (Worthington-Wilmer and Barratt 1996) and stored in 95% ethanol until extraction. See Table 10 for the number of bats captured at each site.

Relatedness Estimation

DNA was extracted from each wing-punch using a Qiagen DNeasy Tissue Kit (Qiagen Inc., Valencia, CA, U.S.A.) following the manufacturer's instructions. To estimate relatedness, bats were genotyped at 12 microsatellite loci. Complete details including the identity of the primers and specifics of the PCR amplification methods are included in Chapter 2.

I tested all loci for conformity to Hardy-Weinberg equilibrium expectations using exact tests conducted in GENEPOP (Raymond and Rousset 1995). Two loci were excluded from subsequent analyses due either to lack of variation (E10) or to failure to

meet Hardy Weinberg expectations (H10). The remaining 10 loci did not deviate from Hardy Weinberg expectations after sequential Bonferroni correction (Rice 1989) and had 2-24 alleles with an average heterozygosity of 0.72 ± 0.07.

To determine whether the number of genotyped microsatellite loci was sufficient to provide accurate estimates of relatedness, I performed a rarefaction simulation to calculate the change in the standard deviation for relatedness estimates as additional loci are included using the program RE-RAT (http://people.musc.edu/~schwaclh/). This analysis showed that the standard deviation of pairwise relatedness estimates diminished after addition of the eighth locus (data not shown) indicating that the loci sampled for this study provide reliable estimates of relatedness.

Pairwise relatedness estimates were calculated using the program Relatedness V. 5.0.8 (Queller and Goodnight 1989). Calculated relatedness values vary from -1 to 1 with negative values interpreted as a biological relatedness of 0. To estimate background allele frequencies for relatedness calculations of roostmates, I included 67 individuals captured in mist nets at a cattle trough located near the CCS and CCN sites, two bats captured exiting a roosting crevice in CCS, two bats captured over a stream designated as Site 4, and one bat entering a night roost designated as WCB (Fig. 9). To test whether roosting subgroups were more closely related than expected by chance, I calculated the average pairwise relatedness of bats captured exiting a day roost or entering the night roost and compared this average to 1,000 random samples of equal size from the population of genotyped bats with a permutation test (Manley 1997) written in R (V. 2.7.2, http://www.R-project.org). In addition, I tested if bats roosting together in a day roost were more closely related than bats at the night roost using a two sample

permutation test with the test statistic calculated as $\overline{X}_{\text{(pairwise r day roosts)}} - \overline{X}_{\text{(pairwise r night roost)}}$ and evaluated against a distribution created by 1,000 permutations.

Finally, all captured bats were sequenced at a 450 bp region of the mitochondrial DNA control region to identify eight unique haplotypes (Chapter 2) based on 57 variable sites (5 insertions/deletions, 44 transitions, and 8 transversions). I then tested if captured bats were more likely to share a haplotype, and thus matrilineal relationship, with a roostmate at the day roost than at the night roost using a contingency table analysis conducted using JMP.

Call Recording and Similarity Analysis

I used two methods to obtain contact call recordings from 15 bats for which wing biopsies had been taken. First, I recorded contact calls from eight radio-tagged bats while they were flying near the roost area (Chapter 1). Second, I recorded contact calls given by bats immediately after they were released from capture. To record each bat, one researcher held the bat in hand while the other prepared to record it using the high frequency output of an Ultrasound Advice S-25 bat detector (Ultrasound Advice Inc., London, U.K) and a custom built filter/amplifier (bandpass 4 kHz-100 kHz) connected to a PMD671 flash recorder sampling at 96 kHz (Marantz Inc., Mahwah, NJ, U.S.A.). Calls were included only if the signal to noise ratio was comparable to calls recorded from radio-tagged bats. Although the majority of bats do not call when released, I was able to obtain calls from seven individuals (six females and one male) after capture.

Call similarity was assessed using SPCCA, spectrographic cross correlation analysis (Clark et al. 1987) with a 128-point Hanning window and 512-point Fast Fourier Transform (FFT) in the sound analysis program Raven V. 1.4 (Cornell University Lab of

Ornithology, Ithaca, NY, U.S.A.). In this procedure, two spectrograms are overlapped in time and cross-correlated frame by frame. The peak of the resulting correlation function represents the time frame where the two sounds are most similar. Thus, I obtained peak SPCC values for all possible pairwise combinations of the 15 bats.

To determine if contact calls contain information about matrilineal relationships, I utilized mtDNA haplotypes (Chapter 2) in a permutation test (Manley 1997) conducted in R to test if calls recorded from bats that shared the same haplotype were more similar than calls from bats that had a different haplotype. The test statistic was computed as $\overline{X}_{\text{(peak R same haplotype)}} - \overline{X}_{\text{(peak R different haplotype)}}$ and evaluated for significance with a distribution created by 1,000 permutations.

To determine if contact calls also contain information about relatedness, I calculated pairwise relatedness using the method of Queller and Goodnight (1989) as stated above. Correlations between call similarity and relatedness for the full data set of 15 bats and data sets consisting of bats that did or did not share the same haplotype were then evaluated for significance using permutation tests written in R (10,000 permutations).

Playbacks

To assess whether social calls attract related individuals, I conducted playback trials where the playback stimuli consisted of calls recorded from free-flying bats wearing radiotransmitters (Chapter 1). At least three call exemplars from each of three bats were presented as playback stimuli. Recorded calls were broadcast using a Marantz PMD671 flash recorder (sampling frequency 96 kHz) connected to an amplifier and a Realistic portable loudspeaker (Tandy Corp., Ft. Worth, Tx) (flat frequency response to 45 kHz).

The speaker was mounted on a 3 m extension pole attached to a tripod and placed behind one of two mist nets (6 m and 9 m) arranged adjacent to a water trough (Fig. 9). Tissue samples were taken from all bats captured both during the presentation of the stimulus (30 s playback file with calls repeated in 5 s bouts spaced 5 s apart) and a 1 min time period after the stimulus ended. Pairwise relatedness between the bats recorded for the playback stimuli and the bats captured was calculated using the methods described above. Significance was determined using a permutation test where the average pairwise relatedness of the responding bats and the stimulus bat was calculated and compared to an equally sized random sample of bats captured at the water trough (see Table 11 for sample sizes). I also tested whether bats responding to the playbacks shared the same haplotype as the stimulus bat more often than expected by chance using a contingency test conducted in JMP.

Results

Calling at Roosting Sites

Even though bat activity was comparable between the two roost types, I found that calling occurred much more frequently (57%) while bats were entering or exiting day roosts than while entering or exiting the night roost (7.5%, Table 12, $\chi^2 = 136.62$, $P<0.0001$).

Relatedness of Roosting Groups

The average pairwise relatedness for all bats captured in the Cove Creek area (including CCN, CCS, and bats captured at the cattle trough) was -0.003. While bats roosted both with closely related and unrelated individuals, average pairwise relatedness

among bats roosting together in a day roost was significantly greater than random expectation (Table 13). Of the two roosting subgroups captured in the CCS roosting area, 13 of 91 pairwise combinations of bats were closely related (i.e., $r \geq 0.25$). In addition, the three males captured in the CCS roosting area exhibited relatively low relatedness to the females (CCS1 $\overline{X}_{\text{(pairwise r)}} = 0.070$, CCS2 $\overline{X}_{\text{(pairwise r)}} = -0.0717$) and when males were excluded, average pairwise relatedness within groups increased (Table 13). In contrast, there were fewer pairs of closely related bats in the CSH night roost (7 of 100 pairs) although the average pairwise relatedness was greater than expected for all but one roosting group capture (Table 13). Overall, the average pairwise relatedness of bats captured at the day roost ($\overline{X}_{\text{(pairwise r Day Roost)}} = 0.084$) was greater than the average pairwise relatedness of bats captured at the CSH night roost after excluding males ($\overline{X}_{\text{(pairwise r Night Roost)}} = 0.046$), although a permutation test showed that this difference did not reach significance (P=0.07 1,000 permutations). While I recaptured bats roosting together both in different years and within the same year at the night roost, there was limited evidence that recaptured bats exhibited high relatedness ($\overline{X}_{\text{(pairwise r)}} = 0.031$, range -0.090 – 0.224). Finally, I found that pairs of bats captured at day roosts were more likely to share the same haplotype than bats captured at night roosts ($\chi^2_{\text{(day roost males included)}} = 9.303$, P=0.003, $\chi^2_{\text{(day roost males excluded)}} = 17.819$, P<0.0001).

Call Similarity, Matrilineal Relationship, and Relatedness

Call similarity among the 15 bats from which I was able to record calls and obtain genetic data differed depending on whether bats shared mtDNA haplotypes. When all possible pairwise combinations of calls were compared, pairwise SPCC values were larger for bats that shared the same haplotype than for bats that had different haplotypes (

$\overline{X}_{\text{(Peak R same haplotype)}} = 0.60$, $\overline{X}_{\text{(Peak R different haplotype)}} = 0.46$, $P < 0.0001$, 1,000 permutations, Fig. 10).

I also found that call similarity was positively correlated with relatedness ($r = 0.22$, $P = 0.013$, Fig. 11). Furthermore, this relationship depends on whether or not bats share the same haplotype. For those pairs of bats that do not share the same haplotype the correlation is much higher ($r = 0.32$, $P = 0.004$) than it is for those pairs of bats that share the same haplotype ($r = 0.06$, $P = 0.35$).

Playbacks

The relatedness between bats captured responding to playbacks and the bats used for the playback stimuli ranged from -0.288 to 0.367 (Table 11), which, on average, did not differ from pairwise values generated by randomly selecting all bats captured at the cattle trough ($\overline{X}_{\text{(Pairwise r playback)}} = -0.006$, $P=0.47$). Similarly, while the majority of responding bats shared the same haplotype as the stimulus bat (12 out of 16 responding bats), this frequency did not differ from what would be expected by chance ($\chi^2 = 0.025$, $P = 0.57$).

Discussion

Pallid bats exhibit a fission-fusion social structure with individuals in the colony moving regularly among multiple crevice roosts located in a large cliff complex. My main objective for this study was to assess whether low frequency contact calls emitted as bats fly outside the roosting area could be used to identify relatives as bats search for roosting sites. I collected data on calling behavior and genetic relationships from bats at temporary day roosts and a traditional night roost to allow comparison between two

behaviorally distinct social aggregations of pallid bats. If calls are used to locate roostmates and maintain social bonds, I expected increased calling behavior outside temporary day roosting crevices as compared to the permanent night roost location. Confirming this prediction, observational data collected outside the CSH night roost indicates that bats rarely call while entering or exiting this isolated night roost. In contrast, pallid bats occupying night roosts in multiple rock crevices in Arizona give contact calls frequently while approaching the roost indicating that calling may be more important when night roost location varies over time (O'Shea and Vaughan 1977).

When I examined pairwise relatedness of pallid bat roosting subgroups using microsatellite markers, I found that relatedness, both among bats roosting together during the day and bats entering a night roost, differed significantly from random expectations. However, mean pairwise relatedness of bats captured in day roosts showed a trend towards being greater than bats captured at night roosts and there were more closely related ($r \geq 0.25$) pairs of bats in day roosts than in night roosts. In addition, bats were more likely to share the same haplotype at day roosts than at the night roost. Together, these data indicate that day roosting subgroups typically contain a relatively high proportion of closely related individuals.

While roosting subgroups exhibited higher relatedness than expected by chance, average relatedness among subgroups of bats in this study was still fairly low, although not zero, indicating that roosting groups often contain a mixture of related and unrelated individuals. This result is similar to the findings for other mammalian species that display fluid social structure (i.e. *Desmodus rotundus* (Wilkinson 1985), *Myotis bechsteinii* (Kerth et al. 2002), *Crocuta crocuta* (Van Horn et al. 2004), *Pan troglodytes*

(Lukas et al. 2005), *Eptesicus fuscus*, (Metheny et al. 2008b), *Loxodonta africana* (Wittemyer et al. 2009), and *Tursiops aduncus* (Wiszniewski et al. 2010) likely owing to factors such as high juvenile mortality, low fecundity, low reproductive skew and male-mediated gene flow. Low average relatedness among individuals in many mammal social groups is also supported by simulation data which indicates that even in species with strong natal philopatry, as has been found in pallid bats (Chapter 2), average relatedness of the philopatric sex will likely remain low unless social groups are very small (Lukas et al. 2005). Thus, as shown by Aviles et al. (2004), optimal group size in social animals represents a trade off between group size and kin composition, with groups often containing a mixture of kin and non-kin, particularly in cases where ecological constraints confer advantages to larger groups and individuals benefit directly through group living. For example, roosting group size can be important for bats occupying roosts in cold climates, especially during lactation, in that larger roosting groups increase the temperature within a roosting crevice through social thermoregulation, thereby saving energy for bats in the roost (Willis and Brigham 2007). Roosting group size in pallid bats may, therefore, be as important as group composition which predicts groups consisting of a mixture of related and unrelated bats.

In groups containing individuals of mixed relatedness, cooperative benefits can still be maintained by kin selection if a mechanism exists to identify relatives within the group. Echolocating bats are dependent on acoustic signals for both orientation and prey capture. However, some bats can obtain social information from the vocalizations of conspecifics in that both echolocation signals (Kazial et al. 2001; Fenton et al. 2004; Voigt-Heucke et al. 2010) and social vocalizations (Carter et al. 2008) encode

information about signaler identity. In this study, I found that calls contain information about matrilineal relationships in that calls were more similar among bats that shared the same mtDNA haplotype than those that had different haplotypes. I also found a significant correlation between relatedness and contact call similarity among bats that do not share haplotypes. Thus, call structure appears to encode information about matrilineal relationship and relatedness, as well as individual identity (Chapter 1). To my knowledge, this is the first study to report that social calls contain information about relatedness in adult bats. In addition, the finding that diurnal roosting groups share haplotypes more often than expected by chance (this study), and previous work showing that colony genetic structure is strongly influenced by haplotype differentiation (Chapter 2), suggests that the use of calls to obtain information about the sender in regards to identity, relatedness or matrilineal relationship may influence the formation of roosting groups over time. Anecdotally, two related bats ($r = 0.42$) that shared the same haplotype were captured exiting a roost at CCN in 2007 then recaptured exiting a roost in CCS in 2008. Thus, there is some evidence that pairs of bats roost together across years, although further data on the presence of long-term associations will be an important direction for future research.

The prediction that bats preferentially respond to the calls of relatives was not supported in this study. One potential explanation for this result is that the location used for the playbacks was not suitable to test this prediction. For example, the ideal playback location would be at the roosting site of the stimulus bat. This would increase the likelihood that bats in the area are familiar with the calling bat since I found that radio-tagged bats are faithful to a roosting area for the duration of the time they are tracked

(typically 4-7 days) and bats have been captured in the same roosting area in different seasons (B. Arnold unpublished data). However, playbacks behind a mist net at the roost site were not feasible given the steep terrain. Thus, the cattle trough was chosen for playbacks because it was an area known to attract bats from both the CCN and CCS roosting sites. A more controlled playback experiment presenting call stimuli from both related and unrelated bats in a paired choice design will be necessary to determine whether bats use the encoded information in calls to distinguish relatives (Rendall et al. 1996; Sharp et al. 2005).

Another possibility is that other cues not investigated in this study, such as olfactory signals, may be used by bats for roostmate identification (Dechmann and Safi 2005). For example, olfactory communication has been shown to play a role in colony member recognition in pipistrelle bats (*Pipistrellus pipistrellus*) (De Fanis and Jones 1995). Similarly, Safi and Kerth (2003) found that secretions from the interaural gland of Bechstein's bats could enable individuals to roost together across seasons since they provide information on individual identity and matriline although they do not carry information on kinship. Pallid bats have pararhinal glands distributed across their muzzle (Orr 1954; Hermanson and O'Shea 1983), which may allow for a more complex identification system utilizing a combination of individually specific acoustic signals that convey information about kinship and olfactory information to identify roostmates. Future studies incorporating both acoustic and olfactory signals presented to pallid bats that differ in relatedness will be key to determining the signals that pallid bats utilize for roostmate identification.

Table 10. Sample sizes for the analysis of roostmate relatedness in pallid bats. Sites are abbreviated as follows: CSH – Clarno School House, CCS- Cove Creek South, CCN- Cove Creek North

Roosting Subgroup	**Date**	**Number of Bats Captured (Sex)**
CSH1	6/24/05	10(F)
CCS1	6/18/07	10(F), 1(M)
CCN	7/27/07	3(F)
CCS2	6/13/08	7(F), 2(M) ‡
CSH2	6/24/08	9(F)*
CSH3	6/29/08	7(F)**
CSH4	7/16/08	4(F)***

* - Includes recaptures of BB#6, #9, #1, and #4 previously captured at CSH on 6/24/05

** - Includes recaptures of BB#4 (6/24/05 and 6/24/08) and BB#103 (6/24/08)

*** - Includes recaptures of BB#1 (6/24/05 and 6/24/08), BB#9 (6/24/05 and 6/24/08), BB#109 (6/29/08) and BB#104 (6/24/08)

‡ - Includes recapture of BB#88 and BB#84 previously captured at CCN on 7/27/07

Table 11. Sample sizes, haplotype frequencies, and relatedness estimates obtained for the playback analysis

Stimulus Bat ID (Haplotype)	**Responding Bats (Haplotype Frequency)**	**Average Relatedness to Stimulus Bat**
BB #40 (Hap. 8)	5 (Hap. 8 – 4 bats, Hap. 2 – 1 bat)	0.008
BB #49 (Hap. 8)	5 (Hap. 8 – 3 bats, Hap. 4 – 1 bat, Hap. 2 – 1 bat)	-0.067
BB #15 (Hap. 8)	6 (Hap. 8 – 5 bats, Hap. 4 – 1 bat)	0.032

Table 12. Table showing the occurrence of *A. pallidus* contact calls observed outside day roosts in the CCN roosting area and outside the CSH night roost

Roost Type	**Enter/Exit with Call**	**Enter/Exit No Call**
Day Roost	300	226
Night Roost	14	172

Table 13. Pairwise relatedness estimates for pallid bat roosting subgroups

Roosting Subgroup	**Average Pairwise Relatedness**	**Pairwise Relatedness Range**	**P***
Night Roost Captures	**0.046**		
CSH1	0.040	-0.2653 - 0.5877	0.04
CSH2	0.058	-0.1815 - 0.5315	0.02
CSH3	0.0645	-0.2009 - 0.4688	0.04
CSH4	-0.048	-0.4178 - 0.1744	0.739
Day Roost Captures (Males Included/Males Excluded)	**0.058/0.084**		
CCS1	0.050/0.046	-0.3086 - 0.4241/-0.3086 - 0.4241	0.01/0.02
CCS2	0.0684/0.162	-0.3227 - 0.4691/-0.2943 - 0.4691	0.008/<0.0001
CCN	0.116	-0.0743 - 0.416	0.12

*P based on 1,000 randomizations

Fig. 9 – Map showing the sites in central Oregon where bats were captured to estimate relatedness within *A. pallidus* roosting subgroups.

Fig. 10 – Boxplots showing the range, lower quartile, median (dark line), mean (open box) and upper quartile for the distribution of pairwise spectral cross correlation (SPCC) values for contact calls given by pairs of bats that either shared the same haplotype or had different haplotypes. The two means are significantly different ($P < 0.0001$, permutation test with 1,000 randomizations). Sample sizes for each haplotype are: H1-2, H2-1, H4-2, H7-1, H8-9.

Fig. 11 – Plot showing the relationship between call similarity measured as the peak spectral cross correlation for contact calls and pairwise relatedness estimated from nuclear microsatellite data. Open squares indicate pairs of bats that share the same mtDNA haplotype while black diamonds indicate pairs of bats with different mtDNA haplotypes.

Fig. 9

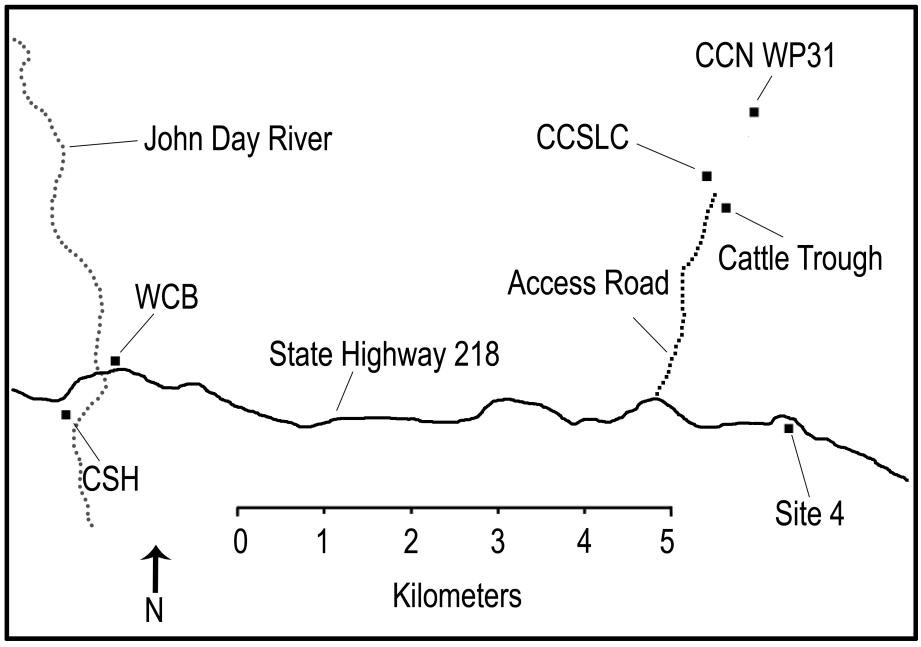

CCN WP31
John Day River
CCSLC
Cattle Trough
Access Road
WCB
State Highway 218
CSH
Site 4
0 1 2 3 4 5
Kilometers
N

Fig. 10

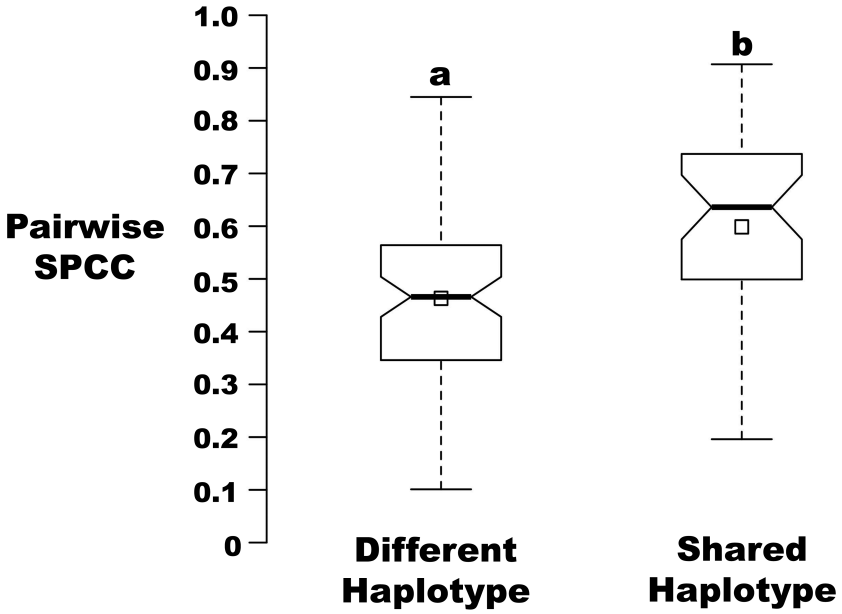
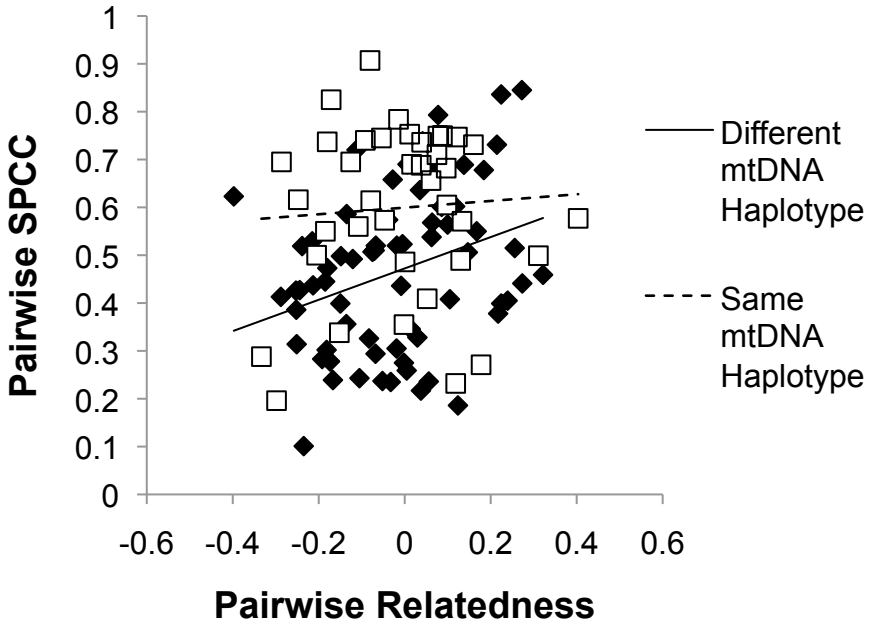

Pairwise SPCC
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
b
Different Haplotype
Shared Haplotype

Fig. 11


Pairwise SPCC
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
Pairwise Relatedness
Different mtDNA Haplotype
Same mtDNA Haplotype

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