

## VOCAL DEVELOPMENT OF BIG BROWN BATS

DEVELOPMENT OF FREQUENCY MODULATED VOCALIZATIONS  
IN BIG BROWN BAT PUPS

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## **ABSTRACT**

Developing bat pups produce distinct vocalizations called isolation calls (I-calls) that serve to attract the bat's mother. Mothers use spatial memory, auditory and olfactory cues to reunite with their offspring. Because I-calls are unique to individual pups, vocalizations are crucial for the reunion process. How individual pups shift their vocalizations from I-calls to downward frequency modulated (FM) sweeps during development remains unclear. By recording individual bat pups from the day of birth to twenty-five days postnatal age I observed behavioural and bioacoustic (temporal and spectral) changes in pup calls. Temporal characteristics examined were call duration and call rate; spectral characteristics were minimum frequency, maximum frequency, peak spectral frequency, total signal bandwidth, maximum frequency of the fundamental acoustic element and bandwidth of the fundamental. I-calls were produced only until a certain point in development (ca. 8 or 9 days old), after which pups change from emitting long-duration, tonal I-calls to downward FM signals and eventually short-duration biosonar vocalizations used for echolocation. I discovered additional spectral changes in the harmonic structure of pup calls, with the number of harmonic elements decreasing with age. Increased physiological stress and reduced body temperature resulting from isolation may cause pups to alter their I-call content, indicating an increased need for maternal assistance. Alternatively, pups may reduce calling to conserve energy during extended isolation. Therefore, I also recorded pup vocalizations

during prolonged separation from their mothers to determine if extended isolation alters the type, number or acoustic structure of emitted vocalizations. The rate of calling was influenced by prolonged separation. I also compared the temporal and spectral characteristics of spontaneous and provoked calls. By documenting the vocal behaviour and acoustic structure of pups calling in different situations, my research provides the groundwork for further studies on the ontogeny and development of FM vocalizations in bats and other mammals.

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## LIST OF ABBREVIATIONS AND SYMBOLS

AM	Amplitude Modulation
CF	Constant Frequency
FM	Frequency Modulation
I-calls	Isolation Calls
PND x	Postnatal Day x

## **DECLARATION OF ACADEMIC ACHIEVEMENT**

I assisted in the collection, training and care of all bats (including mothers and pups) that were used in this research. I collected and analyzed all data presented in this thesis. Data collection was assisted by members of my laboratory and statistical analyses were assisted by members of my supervisory committee.

Complex societies have evolved in many organisms. These societies consist of co-existing members engaging in co-operative behaviours and interacting with one another based on some form of pre-established social organization (Chesser et al, 1993). Organisms such as termites and bees, for example, have evolved highly intricate societies in which the interactions between members are crucial to the survival of the group (Gordon, 1996). Many social insects, as well as most species of mammals, structure their societies using sophisticated methods of organization and communication within the community, such as dominance hierarchies (Hamilton Kennedy, 1947). Members are able to transmit and receive information pertaining to each individual's role within the group (Gordon, 1996; Van Gelder, 1969). All organisms that engage in social organization require a way to exchange information between society members. For social organizations to remain stable and effective, communication between members must be possible (Van Gelder, 1969).

Communication, or the transfer of information, can exist in many forms (Bradbury and Vehrencamp, 1998). Communication requires a sender to produce a signal and a receiver to receive it. Senders and receivers can be different from one another when communication occurs between individuals or groups, or they can be the same individual. This latter form of communication is referred to as self-communication and can be used to learn about the surrounding environment (Gunderson, 1976). Communication can promote reproductive success and as a result, many forms of

communication have become adaptive, heritable traits. In mammals, examples of adaptive communication include the warning of other society members of approaching predators, the use of visual, auditory and/or chemical cues to attract and maintain mating partners, and for informing individuals of the location of food, water or shelter (Gunderson, 1976; Van Gelder, 1969). These are simply a few examples of how mammalian social organization and specifically communication have evolved to support reproductive success and survival.

Many species of bats (Order Chiroptera) are nocturnal, insectivorous mammals that roost in large colonies. Due to their highly social and gregarious nature, bats require communication between conspecifics as well as between and within individuals in order to survive and reproduce. Bats are also capable of flight, hence communication (both self communication and between conspecifics) often occurs over some distance. Because of these long distances as well as the nocturnal foraging of bats, bat acoustic communication has undergone sophisticated evolution as visual communication at night is more difficult. Bats use vocal signals for many purposes, such as indicating distress, sharing information between conspecifics, and for orientation. Biosonar calls, also referred to as echolocation calls, are used for navigation, object avoidance and prey detection and are therefore particularly important to the survival of bats. Echolocation is an example of self-communication and is the process of sending out signal energy and sensing the time it takes for the sound to return (Gunderson, 1976). During

echolocation, the sender and the receiver of the signals are, ideally, the same bat. Some organisms, such as insects, may become accidental receivers of echolocation signals and can use these signals to avoid predation (e.g. Roeder, 1967; Faure and Hoy, 2000). Bats echolocate with ultrasonic frequencies which are rapidly attenuated in air (Lawrence and Simmons, 1982). As a result, self-communication via echolocation occurs only over relatively short distances. Bats are able to control many parameters of their echolocation calls, such as signal duration, the rate and direction of frequency modulation (FM), the rate and magnitude of amplitude modulation (AM), signal bandwidth, harmonic structure and the number of calls emitted (Gunderson, 1976; Fenton et al, 1987; Fenton et al, 2011).

The vocalizations of bats have been studied with respect to individuality and group distinctiveness. For example, some bats are known to produce calls that reflect a specific social group. Vocalizations of greater spear-nosed bats (*Phyllostomus hastatus*) from the same social group are more similar to one another than vocalizations from bats in different social groups (Boughman, 1997). These bats are also able to discriminate calls from the same versus different groups. Moreover, many bats produce vocalizations that are unique to an individual (Scherrer & Wilkinson, 1993; Bohn et al, 2007). Distinct individualized calls are present at birth (Gelfand & McCracken, 1985). There is also evidence that adult bats emit identifying vocalizations. For example, white-winged vampire bats (*Diaemus youngi*) can identify individuals based solely on contact call social

vocalizations (Carter, Fenton & Faure, 2009). Individual vocal signatures may lead to further sophistication in the evolution of communication in bats.

Another important form of communication is that which occurs between a parent and its offspring. Female big brown bats (*Eptesicus fuscus*) give birth to one or two young per year, often in early June (Hood et al, 2002). The pups are altricial and require the near constant attention and assistance of their mothers during the initial stages of development. Altricial infants need extended assistance from their mothers to survive the early postnatal period, as opposed to precocial young which are born relatively mature and mobile (Leon, 1992; Starck & Ricklefs, 1998). During the early postnatal period, bat pups are completely dependent on their mothers for food, protection, and mobility. Pups require milk from their mothers for nourishment before they begin to forage at the age of approximately four weeks (Kunz, 1974; Burnett & Kunz, 1982). Pup growth and survival is, therefore, critically dependent on maternal care. Furthermore, pups depend on their mothers for social and behavioural learning, such as the development of echolocation and communication skills (Janik & Slater, 2000).

During their development, bat pups produce distinct Isolation calls, also known as I-calls, to facilitate retrieval by the pup's mother (Moss, 1988). Bat pups require retrieval when they are cold, hungry, or have become separated from their mothers. I-calls are simple long-duration tonal calls that are rich in harmonics. There is some

evidence that I-calls may have a familial and/or social group distinctiveness. For example, in some species like the noctule bat, *Nyctalus noctula*, the I-calls of sibling pups from the same social group are more similar to each other than from genetically unrelated pups or pups from different social groups (Knörnschild et al, 2006, Bohn et al, 2007). However, other researchers have failed to replicate this finding as there were no genetic or social group similarities within the I-calls of big brown bat pups (Rasmuson and Barclay, 1991).

Many bats, including the big brown bat, roost in large maternity colonies consisting of tens to up to hundreds of females and their offspring. When mothers leave at night to forage the relatively immobile pups are left behind in the roost. When the mothers return to the roost, they must locate and identify their own offspring. Bats are selective with respect to nursing their own offspring and are not known to engage in cooperative nursing of non-related individuals. Therefore, returning mothers are faced with the problem of identifying and nursing only their young. Previous research suggests that the recognition between mothers and offspring is based on both olfactory and auditory cues, as well as spatial memory for the general area where pups were left. Mother bats discriminate the odours of individual pups in order to identify offspring and use olfactory cues to locate their offspring in crowded colonies (Gustin & McCracken, 1987). It is unclear if pups are also able to discriminate between the olfactory cues of their mother and those of other adult bats (Mayberry, 2009). Mothers and pups,

however, are both capable of identifying vocalizations produced by each another. The use of acoustic cues for mother-infant recognition reinforces the importance of the development of vocal communication in bat pups. Mother bats can identify the retrieval or I-calls of their own pups, and pups are able to recognize the calls emitted by their mothers (Balcombe, 1990, Balcombe and McCracken, 1992; Thomson et al, 1984). The vocalizations emitted by mother bats during mother-infant communication are tonal-like FM calls referred to as directive calls (Balcombe and McCracken, 1992). Acoustic signalling back and forth between mother and pup continues as the mother bat makes her way towards the pup, while also using olfactory cues, and eventually both olfactory and acoustic communication ends with the reunion of mothers with their offspring.

Bat pups emit I-calls only until a certain point in development, after which their calls change from being predominantly simple tonal calls long in duration and rich in harmonics to broadband FM vocalizations and eventually short-duration downward FM sweeps called biosonar vocalizations that are used for echolocation. Precursor vocalizations to FM biosonar calls are also observed during infancy while I-calls are being produced (Moss, 1988). Precursor vocalizations are calls with similar characteristics to both I-calls and adult echolocation calls, but have less sophisticated downward FM sweep structure than typical biosonar calls. Currently, it is unclear how pups shift their vocalizations from I-calls to FM vocalizations during development. Much of the previous work on bat pup vocal development has used pups of unknown ages or

estimated age, or used a very small sample size of pups with known ages, and the findings were typically displayed as a between-subject comparison rather than as a within-subject developmental comparison (Matsumura, 1979; Monroy et al, 2011; Sterbing, 2002). By recording the vocalizations of bat pups born in captivity from the day of birth to 25 days of age, I was able to observe detailed temporal, spectral and behavioural changes in pup vocalizations over the course of postnatal development. Working with bats born in captivity allowed me to record pups of known ages and to standardize the recording conditions. This also allowed me to record each pup on any day and to obtain detailed and precise records about other aspects of pup development (e.g. developmental milestones, morphological changes, etc.).

The switch from using I-calls early in life to FM biosonar sweeps closer to adulthood and the correlations between vocal development and physiological growth of the pups have not been extensively studied. It is unknown whether the change in call structure correlates with the pup's new motor ability of flight and aerial foraging on its own, thus ending the dependence on the mother for food and rendering the need to attract the mother useless. Furthermore, when pups begin to fly they are introduced to a new source of food (i.e. nocturnal flying insects) which must be detected with echolocation. The onset of flight and the subsequent need to catch moving prey may drive the switch from I-calls to echolocation calls. If so, then I would expect pups to begin emitting echolocation calls shortly after the onset of flight. Because pups begin to

emit echolocation calls as early as postnatal day four or five, and flight attempts do not occur until approximately three weeks of age, the switch to echolocation calls as a result of autonomous foraging is unlikely. Alternative explanations could include morphological development of tissues and structures involved in sound production (e.g. trachea, larynx or vocal cords), or extensive neuronal growth and circuit organization in the brain regions used for the control and production of vocalizations. My research will explore physiological correlations with behavioural observations to further refine hypotheses on the evolutionary and functional significance of temporal and spectral shifts in pup vocalizations during development.

## METHODS

All procedures were conducted in accordance with the Canadian Council on Animal Care (CCAC) and approved by the McMaster University Animal Research Ethics Board (AREB).

### *Animal Collection and Housing*

The big brown bats (*Eptesicus fuscus*) used in this study were captured between May 2006 and May 2011 from two separate maternity colonies located in houses in southern Ontario, Canada. Captive bats were housed in a bat husbandry facility at McMaster University and were given *ad libitum* access to food (mealworms [*Tenebrio molitor*]) and water. Pregnant females were housed separately, either alone or with another pregnant female, and were monitored daily to record the date of pup births. In this paper, the day of birth for a pup is referred to as postnatal day zero (PND 0).

### *Pup Identification*

Most pregnant female big brown bats gave birth to two pups (twins) per litter. This finding is in accordance with the observation that big brown bats give birth to two pups in eastern regions and one pup in western regions (Kunz, 1974). Often the twins consisted of one female and one male pup, which made visual identification of each pup easy. When mothers gave birth to two female or two male pups, same sex pups were given identifying marks drawn onto the pup's skin with a permanent marker.

*Morphological Measurements/Observations*

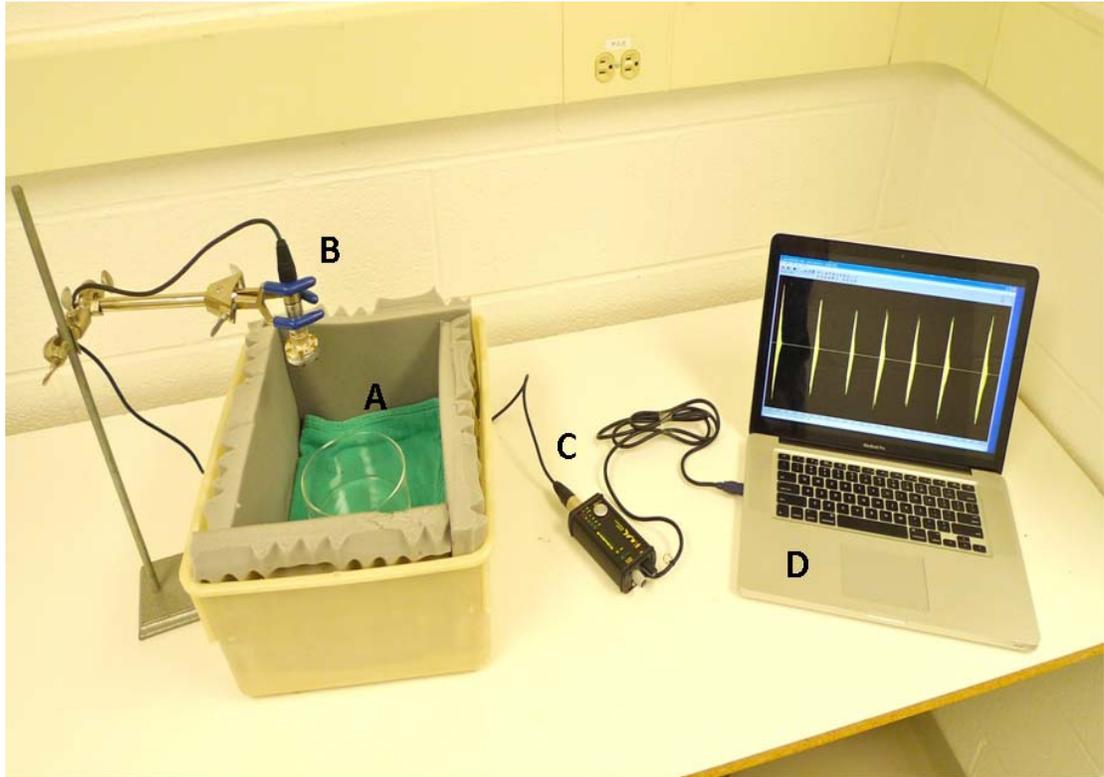
Pups were recorded and measured daily between PND 0 and PND 25 (vocalizations) and PND 35 and when necessary every fifth day thereafter. Pups found attached to their mother were removed by placing a blunt probe into the pup's mouth and easing open the mouth until it was no longer in contact with the mother's nipple. For each pup I measured its mass, left and right forearm length, and scored several developmental milestones, such as whether the pup was attached to the mother, if the pup's eyes were open, the presence of fur on the pup, and its ability to fly. Mass was measured with a Mettler PE3000 balance and forearm length was measured with Manostat 15-100-100 vernier callipers. Flight ability was scored by placing the pup on a flat surface and manually prodding it or by gently holding the pup upside down by its feet and encouraging it to fly. I scored five separate stages of flight ability; no attempt at flight (pups remained motionless when prodded or crawled without extending the wings), wing flapping (pups extended and flapped their wings without forward and/or upward motion), wing hopping (pups achieved some upward and/or forward motion without prolonged airtime), controlled falling (pups unable to remain airborne and without active control over direction of descent), and finally true flight (pups able to remain airborne and in control of their flight).

### *Growth Estimation Equations*

The mass and forearm length of each pup was measured prior to recording its vocalizations. These data were used to develop growth equations for researchers to estimate the age of unknown pups. Two separate equations were developed, one for age as a function of forearm length and one for age as a function of mass. The general validity of each equation was tested with a random sub-sample of pup mass and forearm length data as input to the appropriate equation, and the sub-sample of data points was not used in the development of the growth equations. I then calculated a predicted age for each pup using the average age estimated from each equation and compared this to the observed age in the sub-sample of test pups. I used the average age predicted by the mass and forearm length equations because I had no reason to suspect that one equation was more accurate than the other.

### *Pup Vocal Recordings*

Pup vocalizations were recorded and analyzed with Avisoft Bioacoustics hardware and software (Berlin, Germany) as depicted in Figure 1. Individual pups were placed in the middle of a recording arena lined with sound attenuating foam to reduce echoes. Spontaneous calls emitted by pups were recorded with a CM 16 ultrasonic condenser microphone suspended 17 cm above the centre of the arena floor. Calls were digitized at 250 kHz using an UltraSoundGate 116 and stored as .wav files on a laptop computer. Pups were typically recorded for 1 minute. Early in development when pups



**Figure 1:** Vocal recording apparatus. Calls were recorded and analyzed with Avisoft Bioacoustics hardware and software. Individual pups were placed in the middle of the recording arena (A). The walls and floor of the arena were surrounded by foam to attenuate echoes. A glass restraint was used to position the pup below the microphone and to keep it from crawling out of the arena. Calls were recorded with a CM ultrasonic condenser microphone (B) placed 17 cm above the arena and were digitized at 250 kHz using an UltraSoundGate 116 (C) and stored on a laptop computer (D).

were small and relatively immobile, they were placed directly in the centre of the foam-lined recording arena. Older and more mobile pups were increasingly likely to crawl around and leave the centre of the arena and hide in the foam lining, and this resulted in muffled and/or distorted recordings. When this occurred, pups were handheld 17 cm from the microphone and their vocalizations recorded. Not every pup emitted spontaneous vocalizations, and in some instances it was necessary to provoke calling by gently pressing on the back or tail of the pups. The location of the pup (recorded in the arena or handheld) and the type of vocalization (spontaneous or provoked) was documented for each recording.

After vocal recordings and morphological measurements were obtained, pups were returned to their mother. Pups found attached to their mother were reattached by holding the mother and allowing the pup to reattach to her nipple. If the pup had previously been found unattached to the mother, the pup was placed back in the cage. Upon reaching PND 45, pups were banded with a numbered, coloured plastic split-ring bird band (L&M Bird Leg Bands Inc., California), reunited with their mother, and returned to the colony.

#### *Adult Morphology and Vocal Recordings*

Each parameter was compared to the corresponding values in adult big brown bats. Adult vocalizations from nine adult big brown bats were recorded and analyzed

using the same UltraSoundGate recording hardware and Avisoft analysis software. Adult recordings were all spontaneously emitted by animals flying towards a microphone.

#### *Prolonged Isolation Recordings*

I recorded pup vocalizations while isolated from their mother to determine the types of spontaneous signals emitted by pups during prolonged separation and without human interaction. I obtained prolonged isolation recordings from 15 pups on PND 2, 4 and 8. After recording initial spontaneous vocalizations, pups remained in the recording arena for up to two hours (except for a single PND 2 pup that, after 90 minutes of separation, began showing signs of physical distress and was reunited with its mother) and their calls were spot recorded at 15, 30, 45, 60, 90 and 120 minutes post-separation. For each recording I measured call duration, proportion of call types, call rate, the number of harmonic elements, maximum fundamental frequency and peak spectral frequency of the call, and compared these parameters over the 2 hours of separation and between the three days of development (PND 2, 4 and 8).

#### *Spontaneous vs. Provoked Recordings*

I also examined differences between spontaneous and provoked vocalizations on PND 13 to determine if human interaction with pups caused changes in call parameters. Spontaneous vocalizations were recorded immediately after separation from their mothers and compared to a second, separate set of recordings collected immediately after the same pups were provoked into calling via manual stimulation. Spontaneous

and provoked vocalizations were recorded and analyzed using the same equipment and I compared call duration, proportion of call types, total signal bandwidth, number of harmonic elements, bandwidth of the fundamental acoustic element and peak spectral frequency. Call rate was not compared between spontaneous and provoked recordings because this parameter would be directly related to the rate of manual stimulation.

### *Sound Analysis*

Vocalizations were analyzed using SASLab Pro software (Avisoft Bioacoustics). Calls in each .wav file were automatically detected and given a unique label. The following parameters were measured from each call: duration, call rate, maximum frequency, minimum frequency, peak spectral frequency, bandwidth, maximum frequency of fundamental acoustic element and the bandwidth of fundamental. Duration was measured from the start to the end of call. Call rate was determined by counting the number of calls in each file and converting this value in Hz. The maximum frequency is the highest frequency in the call. The minimum frequency of the fundamental acoustic element is also the minimum frequency of the entire call (Fenton et al, 2011). The peak spectral frequency is the frequency of maximum energy in the call. Signal bandwidth is the difference between the maximum and minimum frequencies of the entire call, whereas the bandwidth of the fundamental is the difference between the maximum and minimum frequency within the fundamental acoustic element. The fundamental acoustic element is the lowest spectral element in a

call (Fenton et al, 2011). The number of harmonic elements in a call, a measure of spectral complexity, was determined by counting the fundamental acoustic element plus any harmonics.

Calls were classified into one of five types using both quantitative and qualitative acoustic parameters (Table 1). Two levels of classification were required. The first level of classification was based on call duration and the second level of classification was based on the presence and direction of FM, unless the call contained obvious upward and downward FMs (call Type 4) or no FM (Type 5). Type 1 calls had durations greater than 20 ms, Type 2 calls had durations between 5 and 20 ms and Type 3 calls had call durations below 5 ms. Type 4 calls had varying durations, although they were measured to be between 5 and 65 ms, and Type 5 calls had durations less than 20 ms. Although the duration of Type 4 and Type 5 calls were temporally similar to Type 2 and Type 3 calls, spectrally these call types were quite distinct. Type 4 calls contained both upward and downward FM and shared no spectral similarities to any other call type, whereas Type 5 calls were tonal and contained little or no FM. To differentiate Type 4 and Type 5 calls from other call types, a second level of classification was required: the presence and direction of changes in frequency throughout the call (i.e. FM). Type 1 calls either had no FM or downward FM. Occasionally, Type 1 calls had some upward and downward FM, but such spectral fluctuations were small and occurred only during the long duration, tonal component of the call. Type 2 and Type 3 calls were always

**Table 1:** Criteria for classification of call types. The first level of classification was based on call duration (exception: Type 4 calls). Type 1 calls had the longest durations ( $\geq 20$  ms) and Type 3 and Type 5 calls had the shortest durations ( $< 5$  ms and  $< 20$  ms, respectively). Type 2 and 4 calls had intermediate call durations. The direction of frequency modulation (FM) was a second level of classification used to differentiate calls that fit into more than one duration category. Type 4 calls had an unique up and down frequency modulation structure, while Type 2 and 3 calls always had downward frequency modulation and Type 5 calls did not have any frequency modulation. Type 1 calls had downward or no frequency modulation.

Call Type	Duration (ms)	FM Direction
1	$\geq 20$	Down or None
2	$\geq 5$ and $< 20$	Down
3	$< 5$	Down
4	$> 5$ and $< 65$	Up and Down
5	$< 20$	None

downward FM. Type 4 calls always had distinct upward FM followed by downward FM and were easily differentiated from the small upward or downward FMs occasionally present in Type 1 calls.

### *Statistical Analysis*

All data are reported as the mean  $\pm$  standard error (SE), unless noted otherwise. Because not every pup called on each day, it was not possible to perform a daily repeated measures analysis on each acoustic parameter throughout development. Therefore, changes in spectral and temporal parameters are described with respect to their direction and magnitude. Comparisons between the non-defining acoustic parameters of Type 1, Type 2 and Type 3 calls were conducted using a Student's t-test using sequential Bonferroni corrections for statistical significance (Rice 1989). For the prolonged isolation study, repeated measures ANOVAs were conducted on call parameters measured after 0 minutes of separation from their mothers between PND 2, 4 and 8 for animals that vocalized on all three days. Additional paired t-tests were conducted on PND 2 and PND 4 data to compare differences between the increments of separation which gave the highest and lowest values for each measured parameter. For calling rate, paired t-tests were performed to compare the time separation at which the call rate fell to 0 Hz on PND 2, 4 and 8. A Chi-square test was used to compare the distribution of the proportion of call types on PND 2, 4 and 8. In this test, comparisons were made using Type 1, 2 and 3 calls and with Type 4 and Type 5 calls collapsed into a

single fourth group. The analysis of spontaneous vs. provoked recordings was restricted only to calls recorded on PND 13 because I wanted to look at the differences between spontaneous and provoked vocalizations and was no longer interested in how these parameters change over development. I used paired t-tests to compare vocal parameters measured from spontaneous and provoked vocalizations and Chi-square tests to compare the proportion of emitted call types.

## RESULTS

A total of 52 pregnant female *Eptesicus fuscus* gave birth in captivity from 2006 to 2011. The sex ratios of the pups born each summer are listed in Table 2. Of the 97 births, 47 consisted of two pups and 7 were single pups. Of 47 sets of twins, 23 were same-sex pairs and 24 had one male and one female pup.

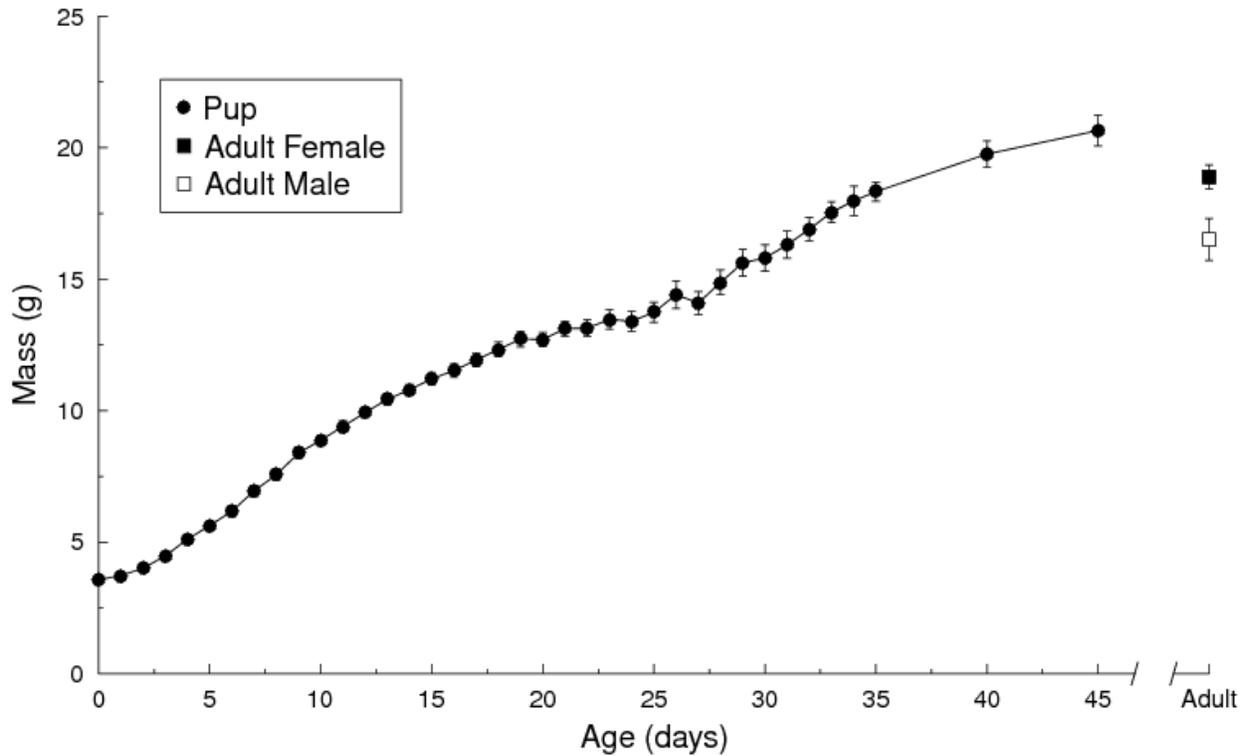
### ***Morphology***

Pup mass increased in a linear manner throughout development (Fig. 2). There was no significant difference between female and male pup masses at birth ( $t = 1.13$ ,  $d.f. = 21$ ,  $p = 0.269$ ). Male and female pups had approximately equivalent mass development rates and therefore only a single curve is illustrated in Figure 2. By PND 45, female pups weighed 21.1 grams, which is slightly higher than the average wild-caught adult female mass of 18.9 grams, and male pups weighed 20.1 grams, which was also slightly higher than the average wild-caught adult male mass of 16.5 g. There was no significant difference in male and female pup mass at PND 45 ( $t = 0.65$ ,  $d.f. = 5$ ,  $p = 0.546$ ). The variance in pup mass became greater as pups aged. There was a difference in mass between wild-caught adult male and female bats; however, most adult females were captured in May and were thus likely pregnant.

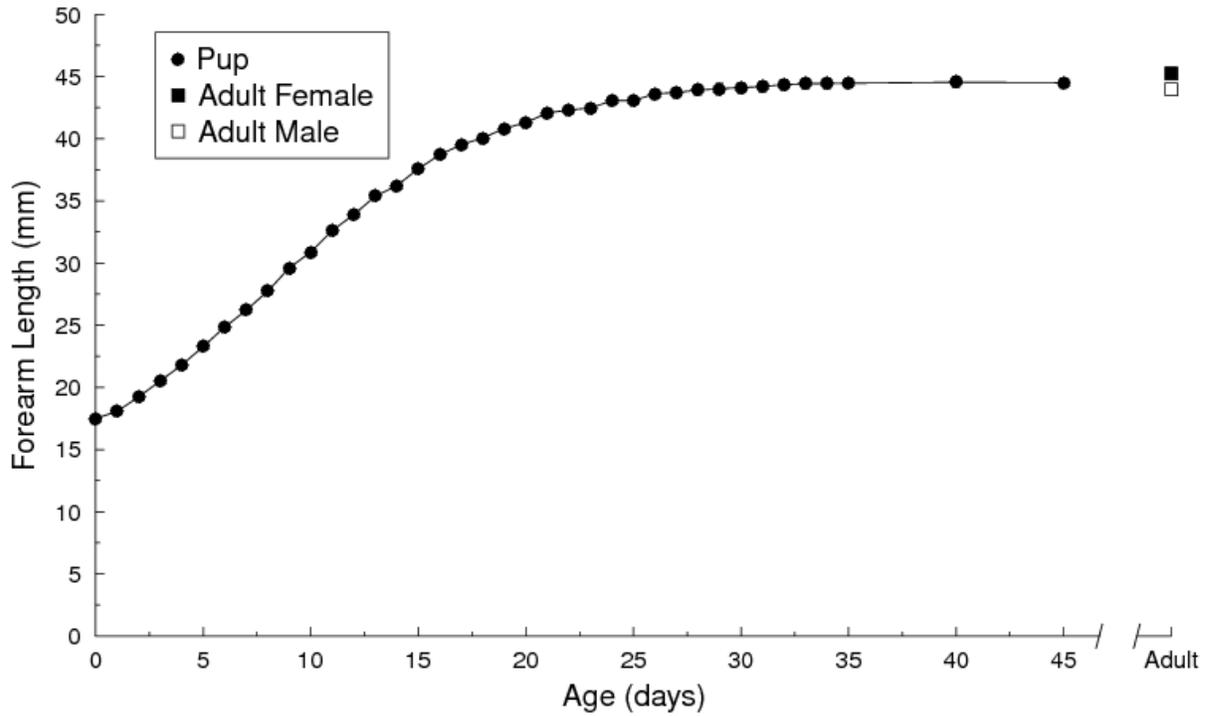
Forearm length increased linearly early in development and eventually plateau (Fig. 3). The variance in pup forearm length was smaller than the variance in pup mass

**Table 2:** Sex patterns of births in *E. fuscus* recorded for six years. Most births were twins of different sexes (1 female pup and 1 male pup). Twin births of the same sex were less common, but when they occurred they were slightly biased toward two female pups. Single births were rare (13%). Other than the total number of births per year, the ratios of single vs. twin and male vs. female births were similar across all summers.

Year	Total # of Births	Total # of Pups	Single Births		Twin Births		
			Female	Male	Female-Female	Male-Male	Female-Male
2006	3	4	0	2	1	0	0
2007	1	2	0	0	0	0	1
2008	8	14	1	1	1	2	3
2009	6	12	0	0	3	2	1
2010	20	38	0	2	2	5	11
2011	14	27	1	0	5	2	8
<b>TOTAL:</b>	52	97	2	5	12	11	24



**Figure 2:** Trajectory of growth in pup mass throughout development. Each point represents data from a range of 15 to 28 pups and depicts the mean  $\pm$  SE mass on each day. Adult values are means from 128 female and 40 males. Adult female masses were significantly greater than adult male masses ( $t = 2.57$ ,  $d.f. = 68$ ,  $p = 0.012$ ). Only a single pup growth curve is shown because there were no significant difference between male and female pup mass. The average increase in mass for the first 25 days was 0.41 g/day and the average increase in mass for the entire 45 day period was 0.38 g/day.



**Figure 3:** Trajectory of growth in pup forearm length throughout development. Each point represents data from a range of 15 to 75 pups and depicts the mean  $\pm$  SE forearm length on each day. Adult values are means from 128 females and 40 males. Adult female forearm lengths were significantly greater than adult male forearm lengths ( $t = 3.48$ ,  $d.f. = 63$ ,  $p = 0.001$ ). Only a single pup growth curve is shown because there was no significant difference between male and female pup forearm length. The average increase in forearm length for the first 25 days was 1.05 mm/day and the average increase in forearm length for the entire 45 day period was 0.60 mm/day. Pup forearm length reached adult values by approximately PND 25.

throughout development, although there was also a slight increase in the variance of forearm length over time. There was no significant difference in forearm length at birth between female and male pups ( $t = 0.43$ ,  $d.f. = 64$ ,  $p = 0.670$ ). Male and female pups also had approximately equivalent forearm length growth rates. By PND 45, the average forearm length in female pups was 44.43 mm, approximately 98% of an average adult female bat (45.25 mm), and the average male pup forearm length was 45.01 mm, which is slightly larger than an average adult male bat's forearm length (43.98 mm). Average forearm lengths at PND 45 were not significantly different between the sexes ( $t = -0.76$ ,  $d.f. = 9$ ,  $p = 0.464$ ).

#### *Developmental Milestones*

Big brown bat pups follow relatively stable morphological and behavioural trajectories (Fig. 4). Very young pups are born naked, blind and incapable of flight. They are dependent on their mothers for nutrition and are usually found attached to and nursing from their mother's nipple. Pups are consistently found attached to their mothers until around PND 13/14. As pups mature, their eyes open, they grow fur and eventually develop the motor skills necessary for flight. Pups usually open their eyes on PND 1/2. Pups are born completely hairless and remain naked until PND 3/4. After PND 4, pups begin to grow sparse hairs on the lower back and stomach and by PND 7/8 sparse hairs cover the entire body. The fur is fine and light until PND 8/9 and changes to dark, coarse, adult-like fur by PND 10/11. Pups do not make flight attempts until PND



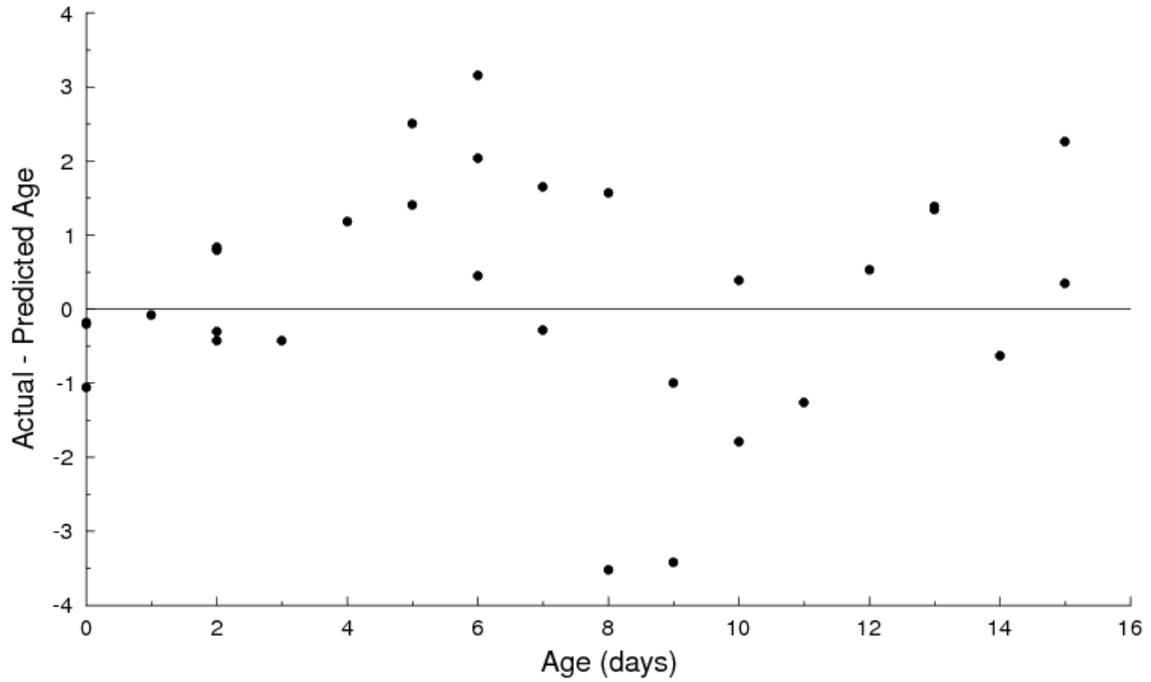
**Figure 4:** Milestones of pup development. Horizontal bars represent each of four aspects of morphological development: attachment to the mother, opening of the eyes, presence and type of fur and the ability to fly. Vertical lines in each bar represent the average time in days at which the various developmental milestones were achieved.

7/8, after which they begin to flap their wings when encouraged to fly. By PND 13, pups begin wing hopping but are unable to perform controlled falls/early flight attempts until PND 18-20. Most pups were capable of true flight by PND 27/28.

### *Growth Equations*

I first developed a set of growth equations for mass and forearm length using data from healthy female and male pups for ages up to PND 15 to directly compare my results to growth equations previously developed by Kunz and Burnett (1974). In total, 1 male pup and 13 females were not used in developing the final equations because these pups had unhealthy growth trajectories. Two estimated ages for each pup were obtained, one from the mass equation and one from the forearm length equation. The two estimated ages were averaged to give a final estimated age for each pup.

The validity of the growth equations was tested with 30 randomly selected data points that were not used in the development of the equations. The estimated ages of these 30 points are plotted as the difference between the actual and estimated ages as a function of the age of the pup being estimated (Fig. 5). If the equations were accurately predicting the age of unknown pups, then I would expect the difference between the observed and estimated values to be zero. The average absolute value difference between the estimated age and the actual age for the growth equations using data from PND 0 to PND 15 was  $1.22 \pm 3$  days. When I measured the accuracy of



**Figure 5:** Average difference between predicted and actual pup age estimated from growth equation data up to PND 15. Predicted ages were calculated using the average age predicted by the mass equation ( $\text{age} = -1.78667 * (3.0916 - \text{mass})$ ) and the forearm length equation ( $\text{age} = -0.705517 * (16.614 - \text{forearm length})$ ).

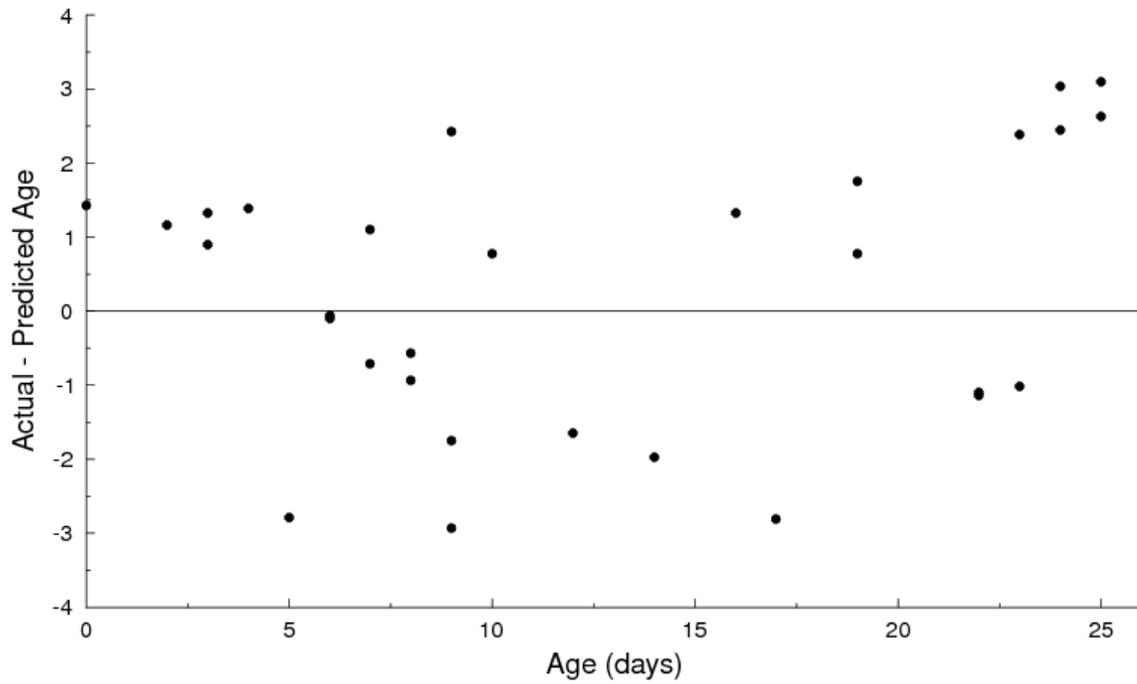
aging bats using either the mass or forearm equation, the mass equation had an average absolute value difference of 1.33 days and the forearm length equation had an average difference of 1.18 days.

I also developed a set of mass and forearm length growth estimation equations using 30 different data points for pup ages from PND 0 to PND 25. This second set of equations was developed to see if I could extend the previous growth estimation work and accurately predict ages of older pups. The PND 25 equations were slightly less accurate than the PND 15 equations, but were still relatively good at predicting the age of pups (Fig. 6). The averaged estimated ages from these equations had an average absolute value difference of  $1.58 \pm 3$  days. When data up to PND 25 were used to derive growth equations, the forearm length equation was still more accurate than the mass equation and the averaged equations (average absolute value difference = 1.46 days). The average absolute value difference between the predicted and actual age using only the mass equation was 1.97 days.

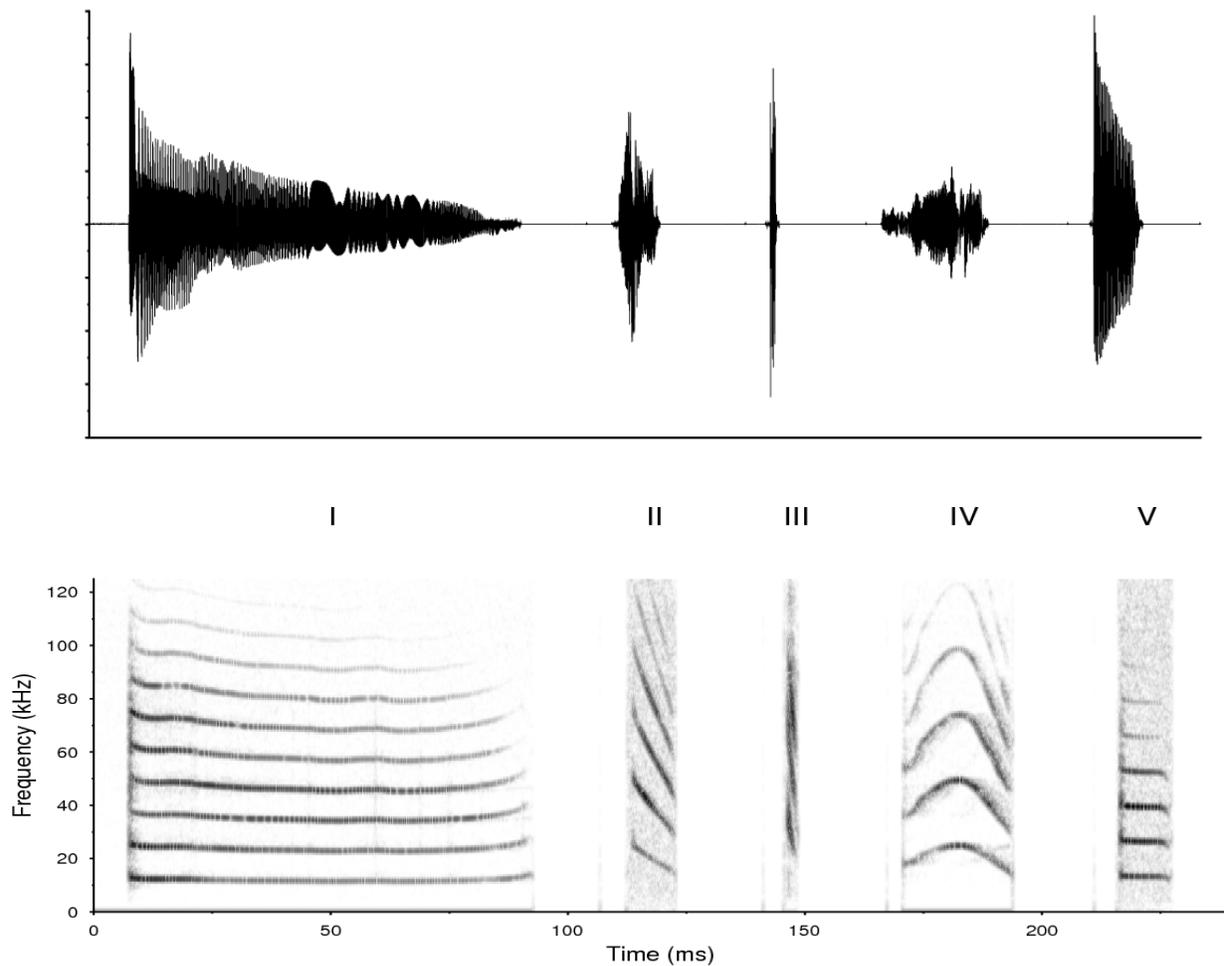
### ***Call Parameters***

#### *Call Types*

Figure 7 shows the five different call types emitted by developing big brown bat pups. Type 1 calls are typical Isolation calls (I-calls). The production of Type 1 calls early in development was expected, based on previously published studies (e.g. Moss, 1988;



**Figure 6:** The average difference between the estimated and the actual pup age from equations up to PND 25. The estimated ages were determined using the average of the mass equation ( $\text{age} = -2.23914 * (3.8333 - \text{mass})$ ) and the forearm length equation ( $\text{age} = -0.861623 * (18.192 - \text{forearm length})$ ).

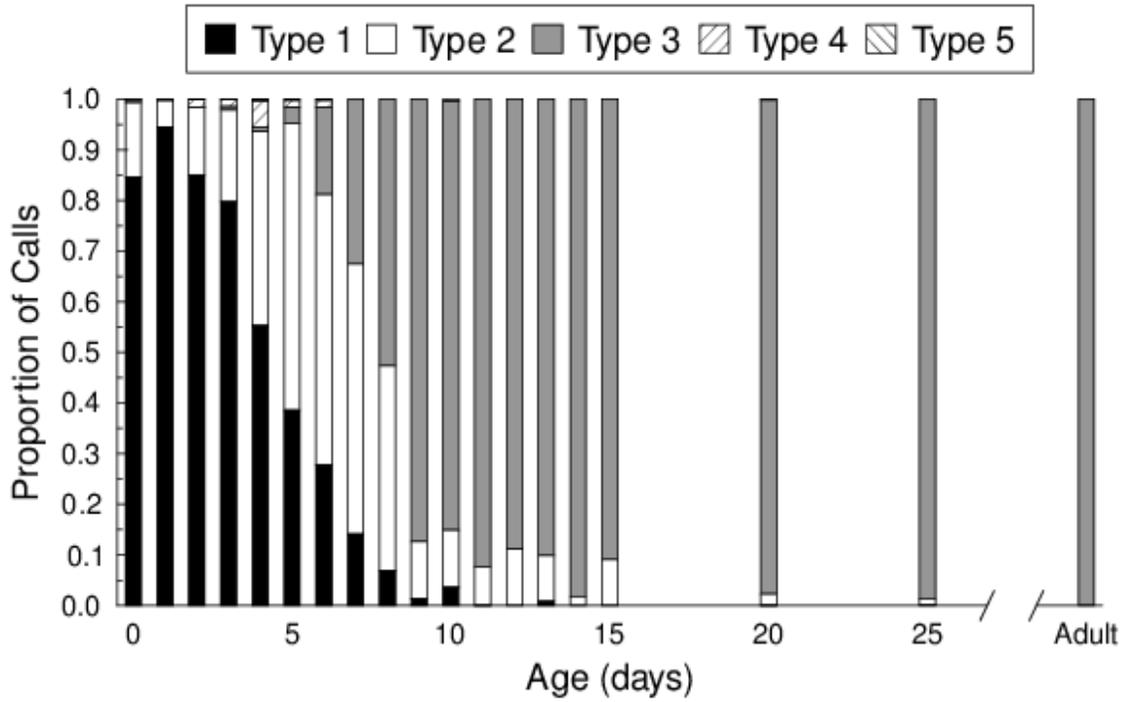


**Figure 7:** Oscillogram (top) and spectrogram (bottom) views of the five call types emitted by big brown bat pups during development. From left to right: Type 1 (I-call), Type 2 (intermediate transition call), Type 3 (biosonar/echolocation call), Type 4 (social-like call) and Type 5 (short duration, tonal call lacking FM).

Koehler and Barclay, 1987; Thomson et al, 1985) because this is when pups require the most attention and assistance from their mothers (Kunz and Hood, 2000). Here I describe Type 2 calls as intermediate vocalizations between I-calls and echolocation calls. Type 3 calls look like typical echolocation calls produced for the purpose of foraging and navigation, and were similar in spectral and temporal parameters to those emitted by adult bats. Type 4 calls were similar to adult social calls and may reflect a different sort of intermediate call type, those between I-calls and social calls. Type 4 calls have a unique spectral structure, in having an upward FM sweep followed by a downward FM sweep. Type 5 calls were similar in duration to Type 2 and 3 calls, but were spectrally more similar to Type 1 calls.

Type 1 calls were produced earliest in development. The average age of pups emitting I-calls was 2.2 days. Type 2 calls were produced in intermediate stages between when Type 1 and Type 3 calls were most common. The average age of pups producing Type 2 calls was 6.1 days. Type 3 calls were produced later in development when pups averaged 14.4 days. Type 4 and 5 calls were emitted early in development; the average ages of pups producing Type 4 and 5 calls were 4.0 and 4.1 days, respectively.

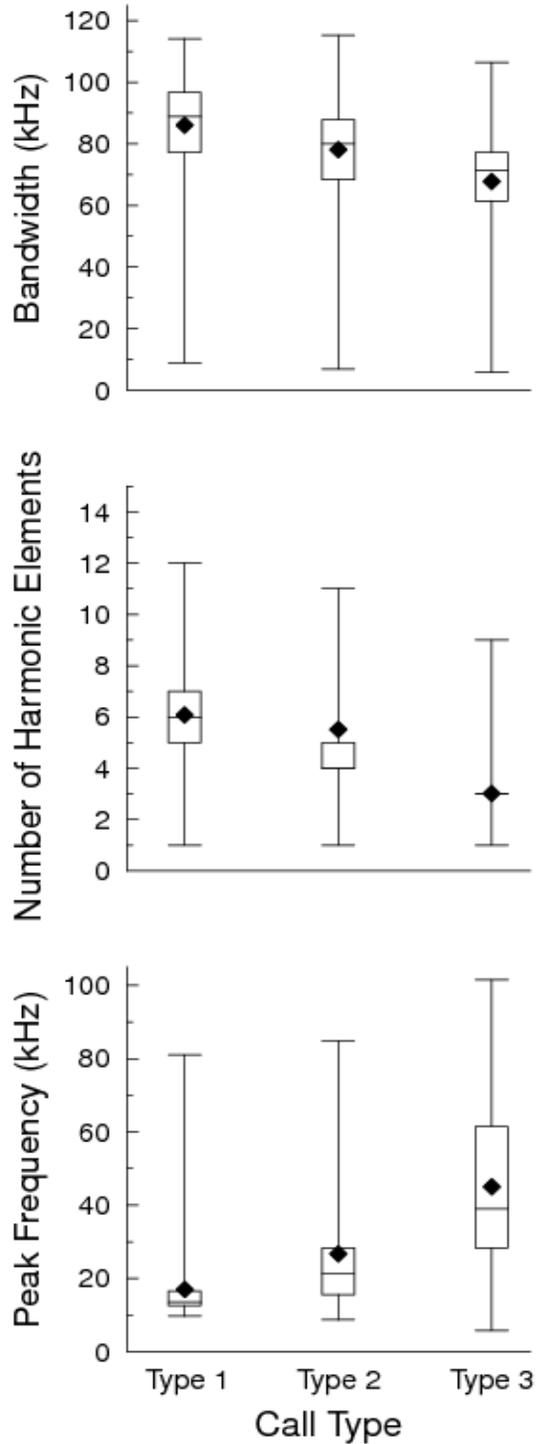
The proportion of emitted call types changed dramatically throughout development (Fig. 8). During the first post-natal days of life approximately 80 to 90% of all vocalizations emitted were Type 1 calls. The proportion of Type 1 calls decreased



**Figure 8:** Change in call type proportions throughout development. Each bar represents data from 16 to 22 individuals. The final bar on the right is the average call type proportions measured from 9 adult bats.

with age, such that they were practically nonexistent by PND 15. Pups emitted relatively few Type 2 calls during the first days of life. Peak production of Type 2 calls occurred between PND 5 and 8. At this time, approximately 50 to 60% of all vocalizations were Type 2 calls. The proportion of Type 2 calls rapidly decreased after PND 9/10. Type 3 calls did not become part of the pup's vocal repertoire until PND 5, after which the proportion of Type 3 calls increased dramatically and remained high throughout development so that almost all vocalizations emitted by pups after PND 10 were echolocation calls. The proportion of Type 3 calls increased from about 15% on PND 6 to 30% by PND 7, to 50% by PND 8, to 90% by PND 9, to 96-97% by PND 20 and finally to 98-99% by PND 25.

All of the vocalizations of adult bats recorded and analyzed in my study were Type 3 biosonar calls. For the description of call development, all five call types were included to illustrate how call parameters change with age. When comparing call types with respect to the call parameters measured, I chose to compare only call Types 1, 2 and 3. Call Types 4 and 5 were excluded from this analysis because they occurred relatively infrequently. The average spectral bandwidth, number of harmonic elements and peak spectral frequency for call Types 1, 2 and 3 are shown in Figure 9. Type 1 calls were significantly larger in bandwidth than Type 2 and Type 3 calls ( $t = 25.81$ ,  $d.f. = 5384$ ,  $p = 1.3E-138$ ;  $t = 66.8$ ,  $d.f. = 5701$ ,  $p = 0$ ), and Type 2 calls were significantly larger in bandwidth than Type 3 calls ( $t = 31.73$ ,  $d.f. = 6363$ ,  $p = 2.9E-205$ ). Type 1 calls



**Figure 9:** Spectral differences between Type 1, 2 and 3 calls with respect to bandwidth, spectral complexity (number of harmonic elements) and peak spectral frequency. Each box and whisker plot shows the median value for each call type, 25<sup>th</sup> and 75<sup>th</sup> percentile, and the maximum and minimum values. The mean values are represented by filled diamonds.

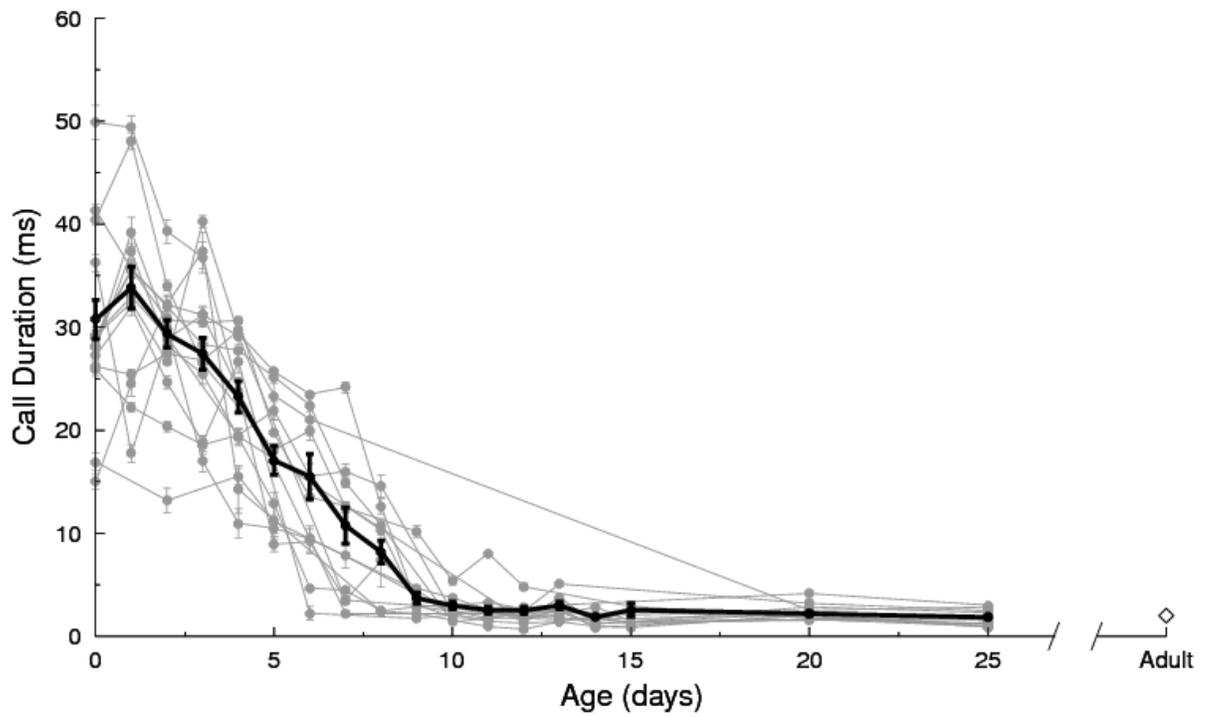
contained significantly more harmonic elements than Type 2 and Type 3 calls ( $t = 62.2$ ,  $d.f. = 6179$ ,  $p = 0$ ;  $t = 183.19$ ,  $d.f. = 10006$ ,  $p = 0$ ), and Type 2 calls also had significantly more harmonic elements than Type 3 calls ( $t = 73.27$ ,  $d.f. = 4933$ ,  $p = 0$ ). Type 1 calls had significantly lower peak spectral frequencies than Type 2 and Type 3 calls ( $t = -32.63$ ,  $d.f. = 4143$ ,  $p = 4.5E-208$ ;  $t = -83.92$ ,  $d.f. = 10746$ ,  $p = 0$ ), and Type 2 calls were also significantly lower in peak spectral frequency than Type 3 calls ( $t = -2.22$ ,  $d.f. = 3532$ ,  $p = 0.027$ ).

#### *Call Duration*

Average call duration decreased with pup age (Fig. 10). Pups used relatively long call durations immediately after birth (e.g. 30 ms) and call duration decreased sharply to 2 and 3 ms by PND 10. Calls remained at this duration for the rest of the recordings. Adult bats produced calls with an average duration of 2 ms, which is similar to the call durations emitted by the eldest pups in this study.

#### *Call Rate*

The spontaneous rate of calling, measured in hertz (calls per second), was extremely variable through development. On average, there was a slight decrease in calling rate from PND 0 to PND 10, after which the rate of calling increased steadily until PND 25. The youngest pups emitted calls at approximately 4 Hz, which decreased to 1.5-2 Hz by PND 10. By PND 25, pups were producing calls at a rate of 5 Hz. As shown in



**Figure 10:** Change in call duration throughout development. The grey traces represent call duration data from 15 individual pups, and the thick black line is the mean  $\pm$  SE call rate. The open diamond is the mean  $\pm$  SE call duration measured from 9 adult bats.

Figure 11, there was significant individual variation in the rate of calling. The spontaneous rate of calling in adult bats was approximately 10.8 kHz.

#### *Bandwidth*

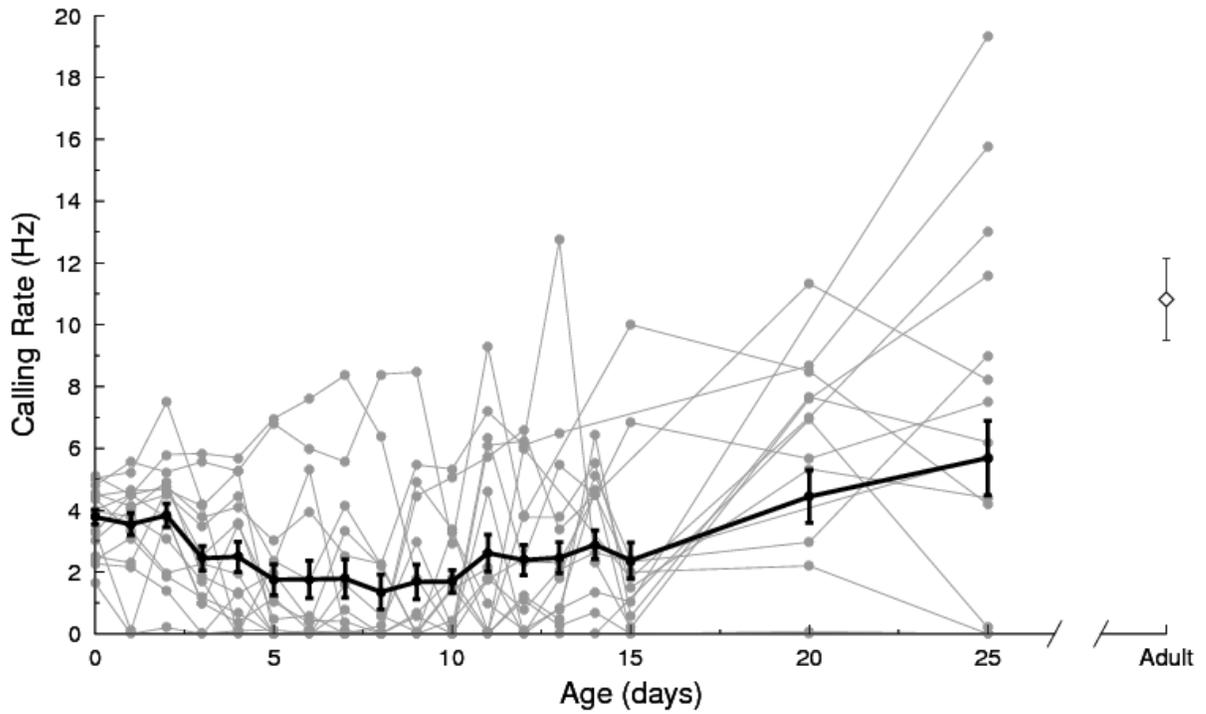
There was a slight decrease in average signal bandwidth throughout development. Call bandwidths emitted by the youngest pups were approximately 80 or 85 kHz, but decreased to 70 or 75 kHz by PND 25 (Fig. 12). This trend appears to continue into adulthood as adult bats produced calls with typical bandwidths of approximately 65 kHz.

#### *Fundamental Bandwidth*

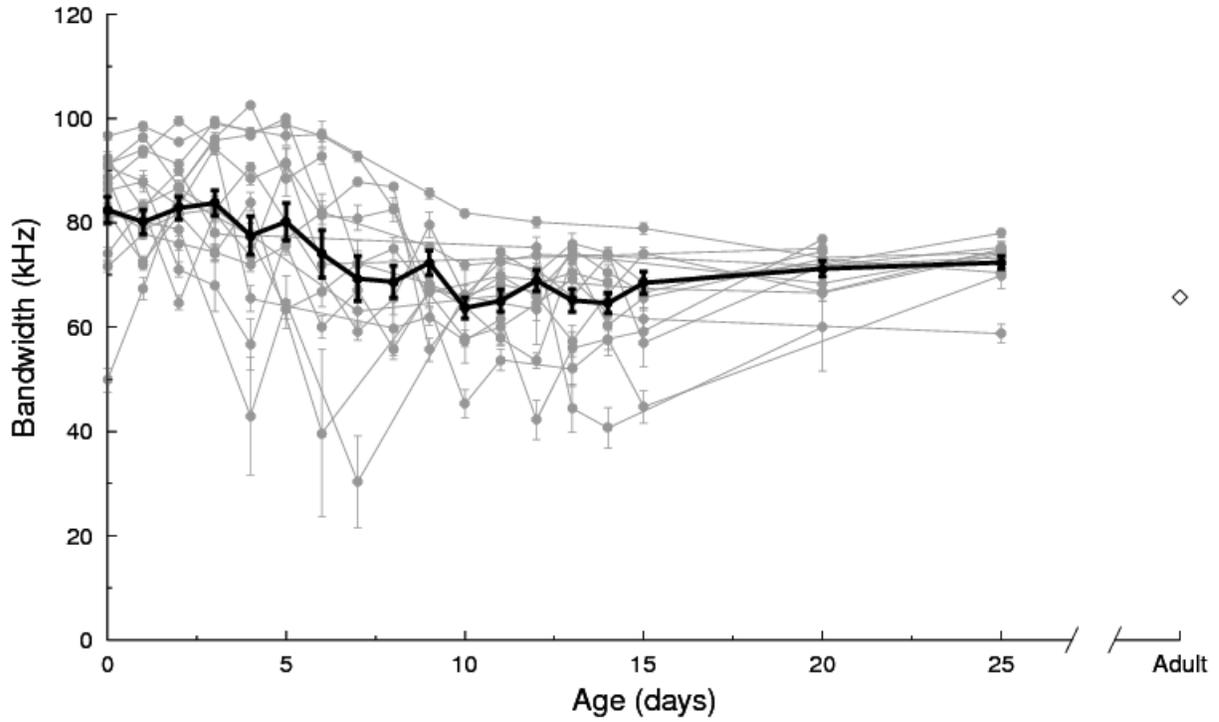
In contrast to total signal bandwidth, the bandwidth of the fundamental acoustic element increased from 10 kHz immediately after birth to approximately 20 kHz by PND 25 (Fig. 13). Adult bats emitted calls with an average fundamental bandwidth of approximately 25 kHz.

#### *Maximum Frequency*

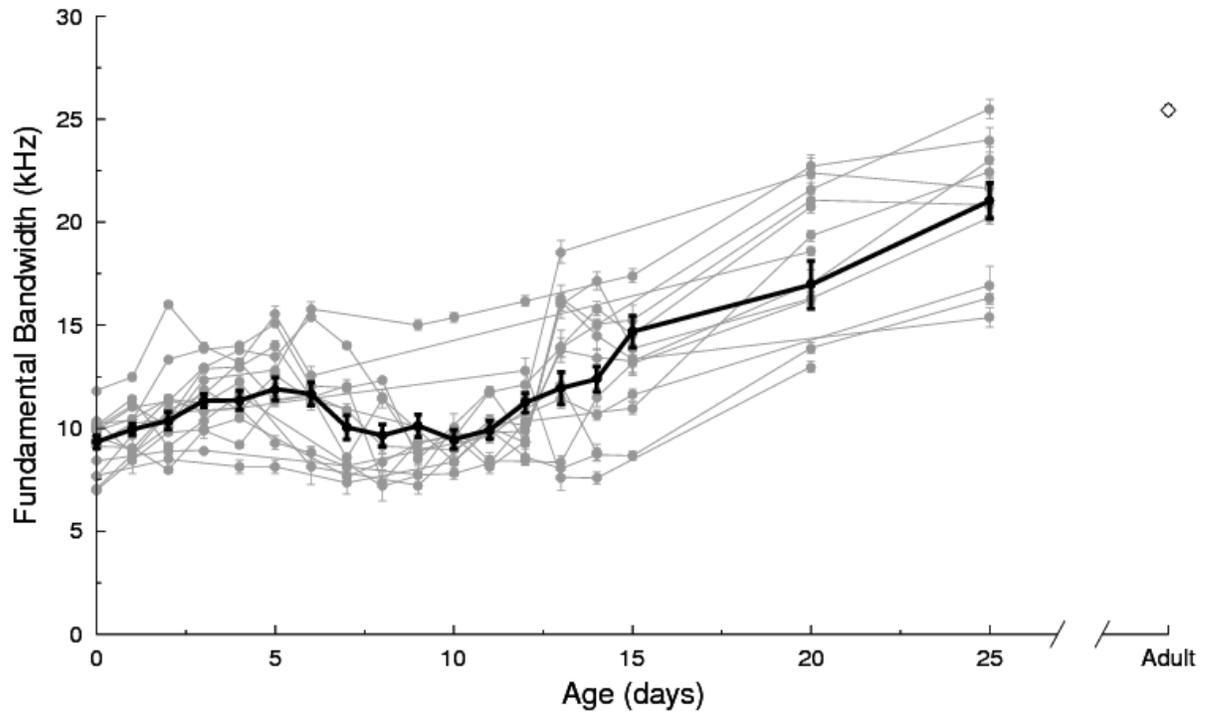
The maximum frequency of pup vocalizations remained relatively constant throughout development. Very young pups produced calls with maximum frequencies between 90 and 95 kHz, whereas both older pups and adults emitted calls with equivalent maximum frequencies (Fig. 14).



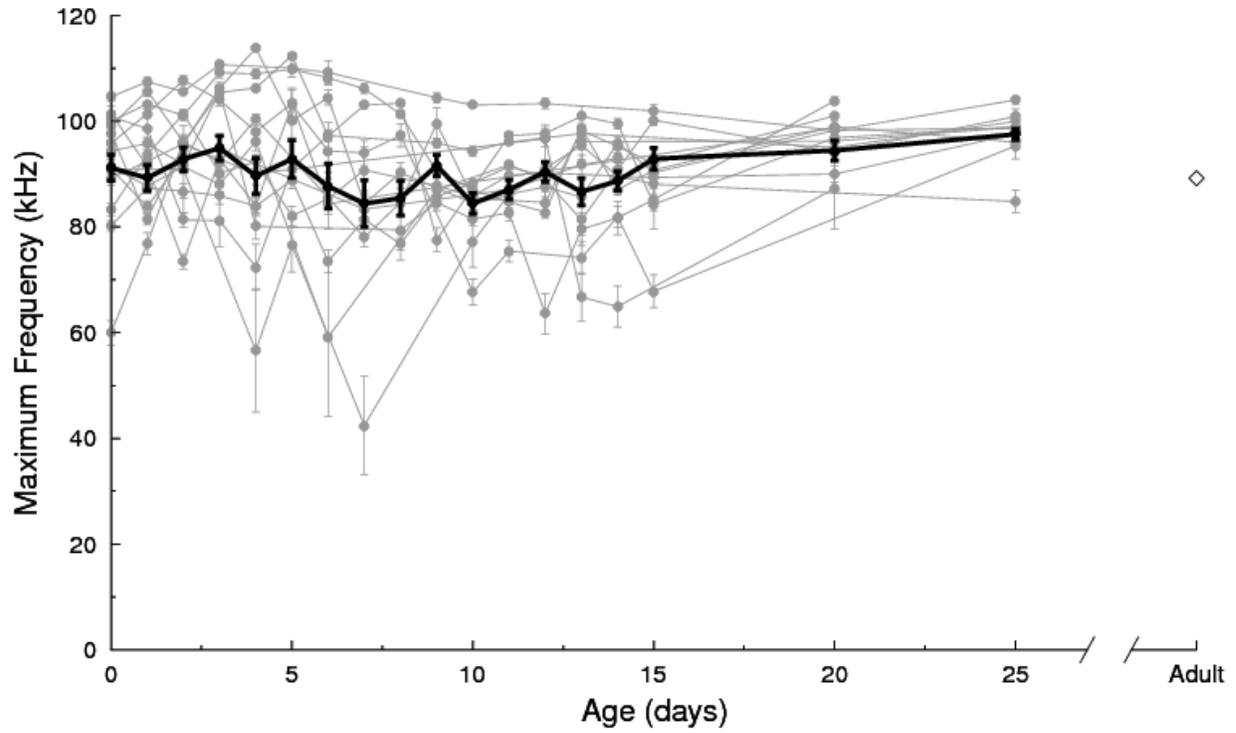
**Figure 11:** Change in call rate throughout development. The grey traces represent call rate data from 15 individual pups, and the thick black line is the mean  $\pm$  SE call rate. The open diamond is the mean  $\pm$  SE call rate measured from 9 adult bats.



**Figure 12:** Change in signal bandwidth throughout development. The grey traces represent bandwidth data from 15 individual pups, and the thick black line is the mean  $\pm$  SE signal bandwidth. The open diamond is the mean  $\pm$  SE signal bandwidth measured from 9 adult bats.



**Figure 13:** Change in fundamental bandwidth throughout development. The grey traces represent fundamental bandwidth data from 15 individual pups, and the thick black line is the mean  $\pm$  SE fundamental bandwidth. The open diamond is the mean  $\pm$  SE fundamental acoustic element bandwidth measured from 9 adult bats.



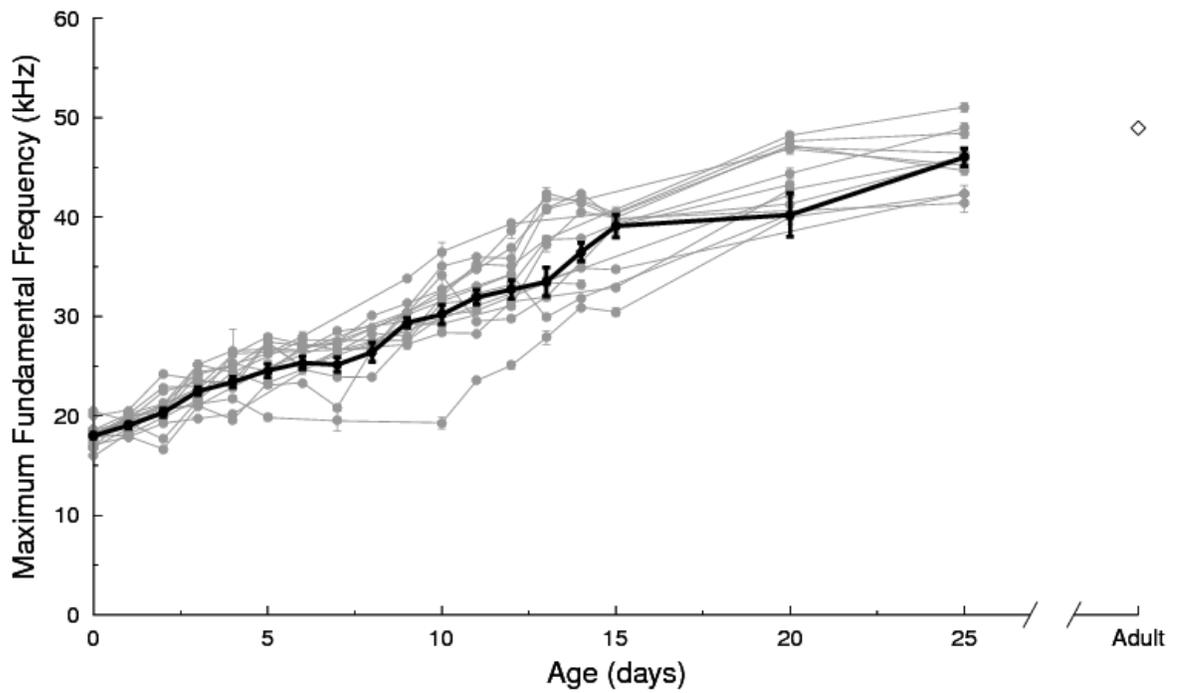
**Figure 14:** Change in maximum frequency throughout development. The grey traces represent maximum frequency data from 15 individual pups, and the thick black line is the mean  $\pm$  SE maximum frequency. The open diamond is the mean  $\pm$  SE maximum frequency measured from 9 adult bats.

### *Maximum Fundamental Frequency*

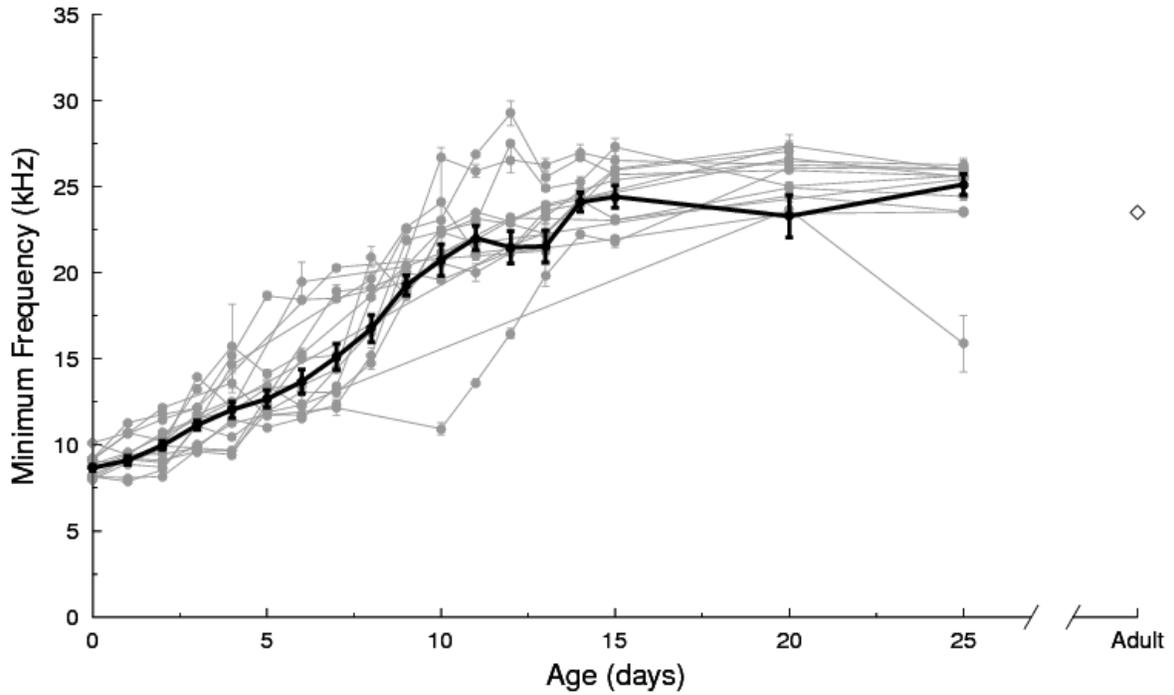
The maximum frequency of the fundamental acoustic element increased linearly throughout development (Fig. 15). At birth, initial maximum fundamental frequencies began between 15 and 20 kHz, and by PND 25 pups produced calls with maximum fundamental frequencies of approximately 45 kHz. The majority of changes taking place with respect to maximum fundamental frequency appear to occur during early development, as adult bats emitted calls with an average maximum fundamental frequency of approximately 49 kHz, a value not much greater than that of the eldest pups I measured.

### *Minimum Frequency*

The minimum frequency of the fundamental is also equal to the minimum frequency of the entire call. The minimum frequency of pup vocalizations increased throughout development. Newborn pups emitted calls with minimum frequencies of 8 or 9 kHz, and by PND 15 this increased to approximately 23 or 25 kHz, after which the minimum frequency remained relatively constant (Fig. 16). As with the maximum fundamental frequency, most changes in minimum frequency occurred during early development. Adult bats emitted calls with average minimum frequencies equivalent to those of pups on PND 25 (23.5 kHz).



**Figure 15:** Change in maximum fundamental frequency throughout development. The grey traces represent maximum fundamental frequency data from 15 individual pups, and the thick black line is the mean  $\pm$  SE maximum fundamental frequency. The open diamond is the mean  $\pm$  SE maximum frequency of the fundamental acoustic element measured from 9 adult bats.



**Figure 16:** Change in minimum frequency throughout development. The grey traces represent minimum frequency data from 15 individual pups, and the thick black line is the mean  $\pm$  SE minimum frequency. The open diamond is the mean  $\pm$  SE fundamental minimum frequency measured from 9 adult bats.

### *Peak Frequency*

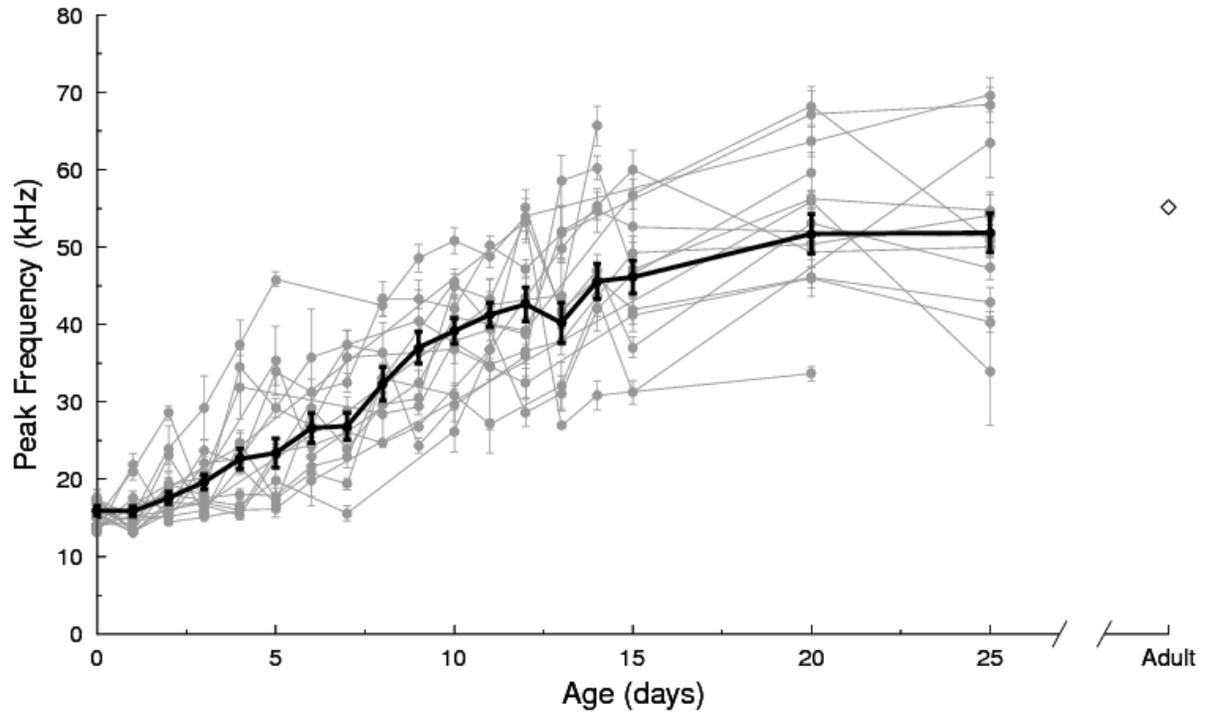
The peak spectral frequency of calls produced by developing bat pups increased nearly linearly over development (Fig. 17). Peak frequencies at birth were approximately 15 kHz, whereas PND 25 pups produced calls with peak frequencies around 50 kHz. Adult bats emitted calls with average peak frequencies of approximately 55 kHz, suggesting that pup peak frequencies would have continued to increase past the final day of recording.

### *Harmonic Elements*

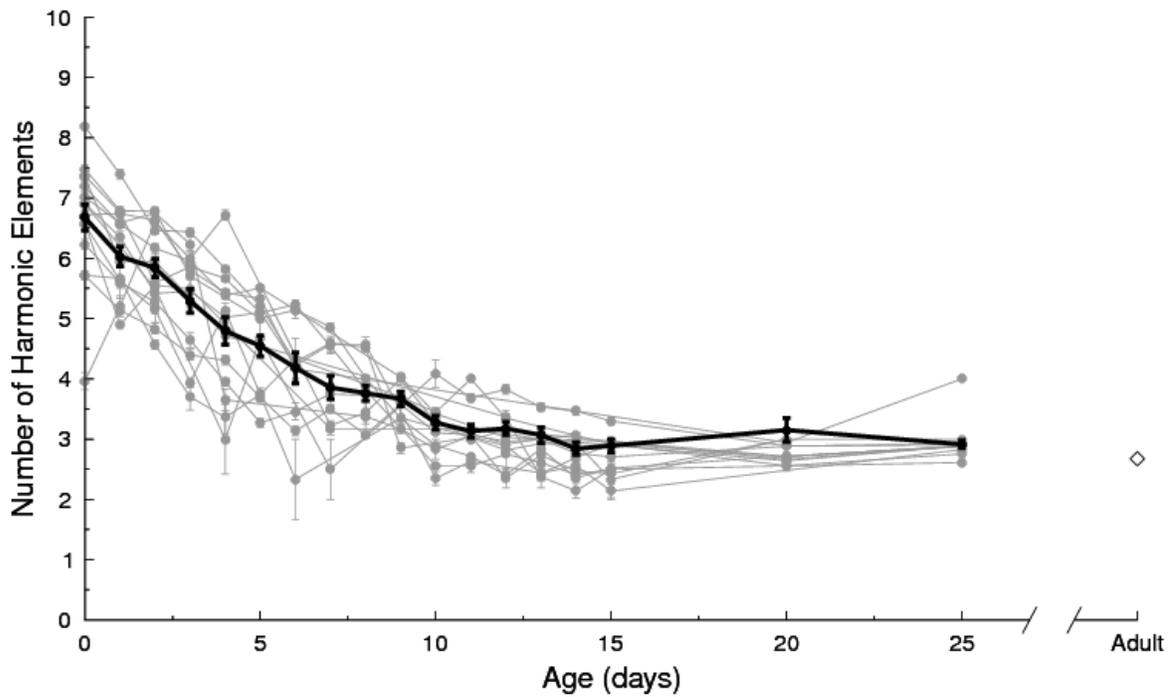
Call spectral complexity, or number of harmonic elements, decreased throughout development (Fig. 18). Very young pups produced calls with an average of 7 elements (fundamental element plus 6 harmonics). By the time pups reached PND 13 or 14, their calls typically contained only 3 harmonic elements (i.e. the fundamental plus 2 harmonics). The number of harmonic elements then remained consistently low at an average of 3 elements through adulthood, as adult bats emit biosonar calls with an average of 2 or 3 harmonic elements.

### ***Prolonged Separation***

For the prolonged separation experiments, pups were isolated from their mother for two hours on postnatal days 2, 4 and 8. I hypothesized that younger pups would be more likely to vocalize during prolonged separation due to a greater need for maternal



**Figure 17:** Change in peak spectral frequency throughout development. The grey traces represent peak frequency data from 15 individual pups, and the thick black line is the mean  $\pm$  SE peak frequency. The open diamond is the mean  $\pm$  SE fundamental peak spectral frequency measured from 9 adult bats.



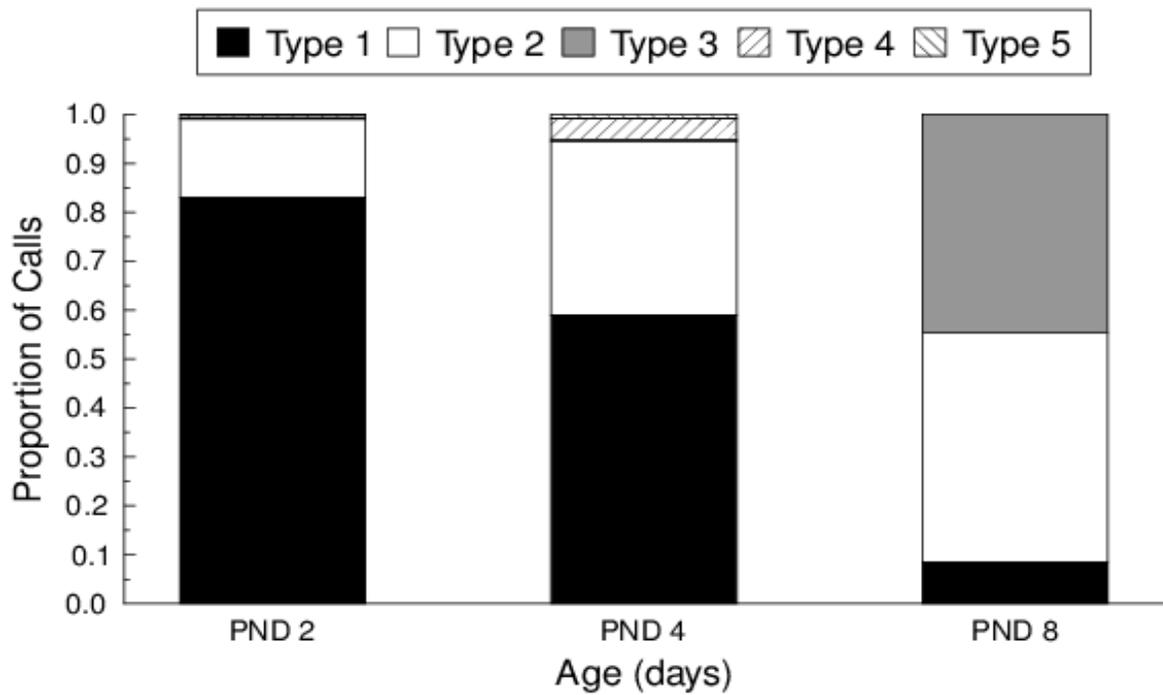
**Figure 18:** Change in number of harmonic elements throughout development. The grey traces represent harmonic element data from 15 individual pups, and the thick black line is the mean  $\pm$  SE number of harmonic elements. The open diamond is the mean  $\pm$  SE number of harmonic elements measured from 9 adult bats.

assistance because older pups would be less likely to attempt to vocalize and signal a need for retrieval by their mothers.

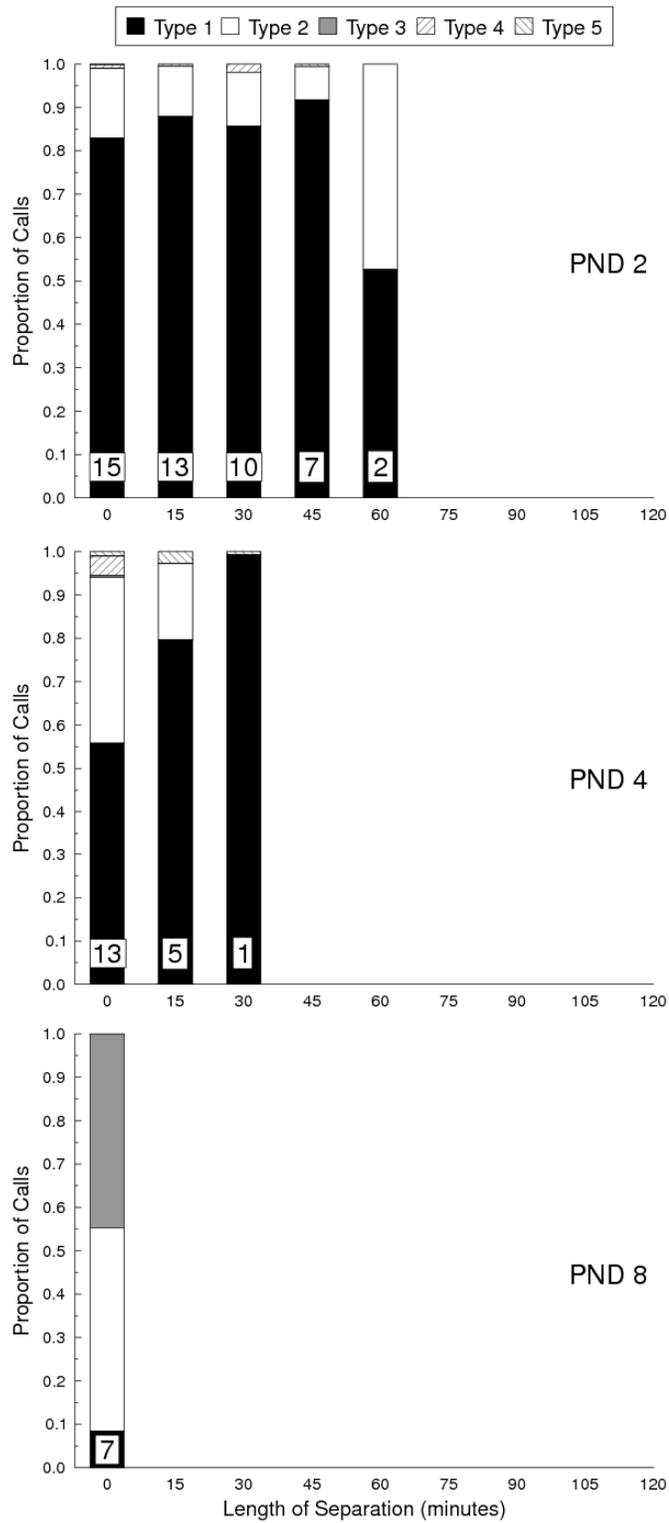
### *Call Types*

The distribution of call types emitted by isolated PND 2, 4 and 8 pups after 0 minutes of separation was significantly different ( $\chi^2 = 1252.58$ , d.f. = 6, critical  $\chi^2 = 22.46$ ,  $p < 0.001$ ; Fig. 19). The proportions of call types emitted by PND 2, 4 and 8 pups immediately after separation supports my previous result that younger pups were more likely to emit Type 1 calls while older pups were more likely to emit Type 2 and Type 3 calls (see Fig. 8). The proportions of call types emitted by PND 2 and PND 4 pups changed as the length of separation increased (Fig. 20). As separation time increased during the first 45 minutes of isolation, the proportion of Type 1 calls emitted by PND 2 pups remained relatively high. On PND 4 the proportion of Type 1 calls was also high throughout prolonged separation. No pups on PND 8 emitted calls after 0 minutes of separation.

The proportion of Type 1 calls emitted by the 4 pups on PND 4 who produced calls at both time 0 and time 15 were not significantly different from one another ( $t = 0.17$ ,  $df = 3$ ,  $p = 0.876$ ). Because these proportions did not differ, I assume that the apparent change in proportion of Type 1 calls between 0 and 15 minutes of separation was the result of a lower sample size after 15 minutes. The animals that called both



**Figure 19:** Distribution of call types emitted by isolated pups on PND 2, 4 and 8 after 0 minutes of separation from their mother. Distributions from 15, 14 and 7 pups were averaged to produce the proportions on PND 2, 4 and 8, respectively.

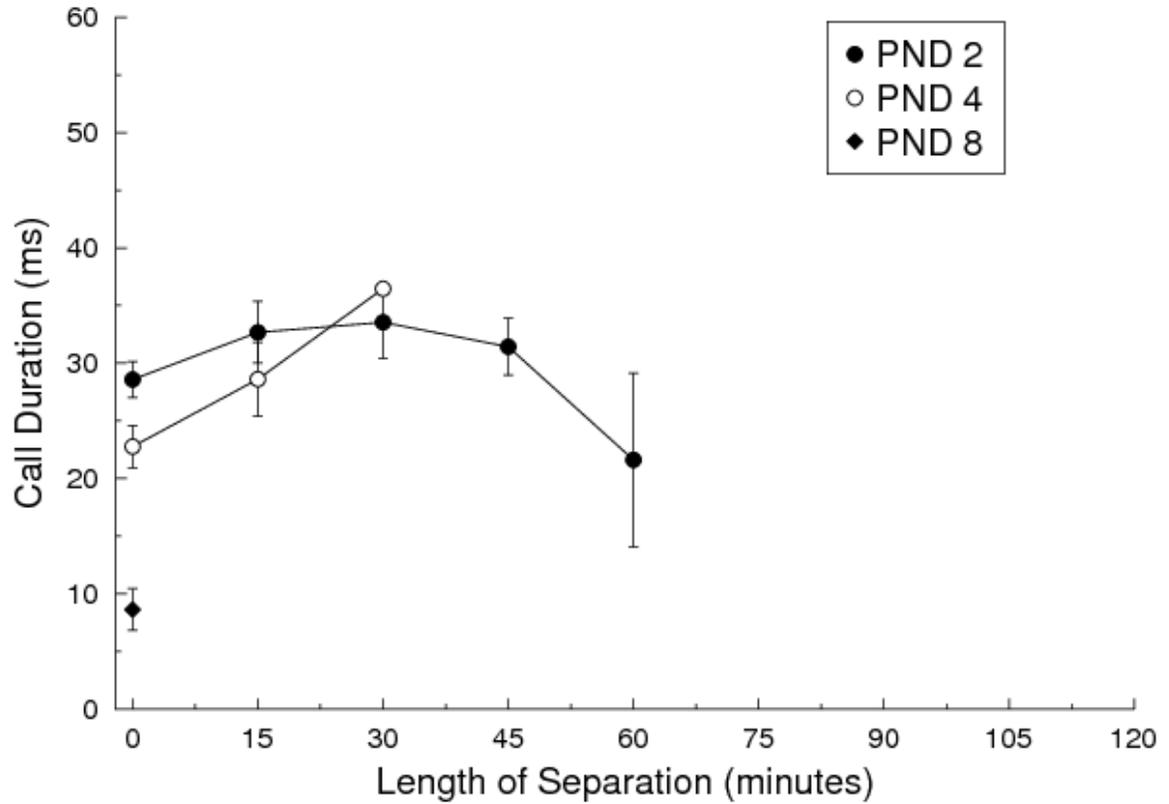


**Figure 20:** Distributions of call types on PND 2, 4 and 8 throughout prolonged separation from the mother. The number of pups is shown in each bar.

after 0 and 15 minutes of separation had no change in Type 1 call production.

### *Call Duration*

When comparing call duration between the three postnatal days over which the prolonged separation experiments were conducted, PND 2 calls were significantly longer in duration than calls emitted by PND 4 and PND 8 pups. Calls emitted by PND 4 pups were also longer in duration than calls emitted by PND 8 pups immediately after separation ( $F = 32.62$ ,  $d.f. = 12$ ,  $p = 1.407E-5$ ; Fig. 21). These results are consistent with my previous developmental data describing call duration as a function of age (Fig. 10). As the length of separation from the mother increased, average call durations emitted by PND 2 pups increased slightly and then decreased. Call durations for individual pups either increased or remained constant, as the decrease in call duration after 45 minutes of separation was observed in only 2 of 15 pups. Call durations emitted after 0 minutes and 30 minutes of separation on PND 2 were not significantly different ( $t = -1.96$ ,  $d.f. = 9$ ,  $p = 0.082$ ). Pups on PND 2 never emitted calls past 60 minutes of separation. Average call durations emitted by PND 4 pups after 0 and 15 minutes of separation were also not significantly different ( $t = -1.28$ ,  $d.f. = 3$ ,  $p = 0.289$ ). No pup on PND 4 emitted calls past 30 minutes of separation, and no pup on PND 8 emitted calls after 0 minutes of separation.



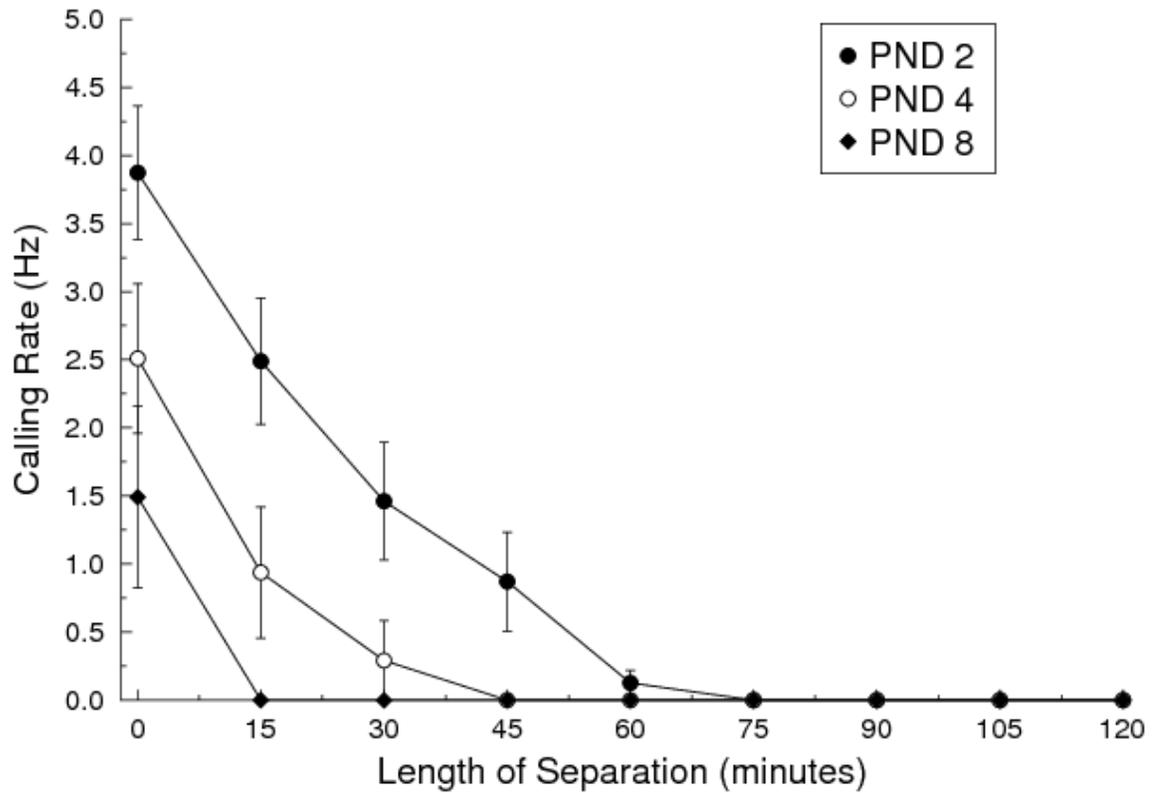
**Figure 21:** Change in call duration throughout prolonged separation on PND 2, 4 and 8. Each point represents the mean  $\pm$  SE duration at each time point throughout separation on each of the three test days.

### *Call Rate*

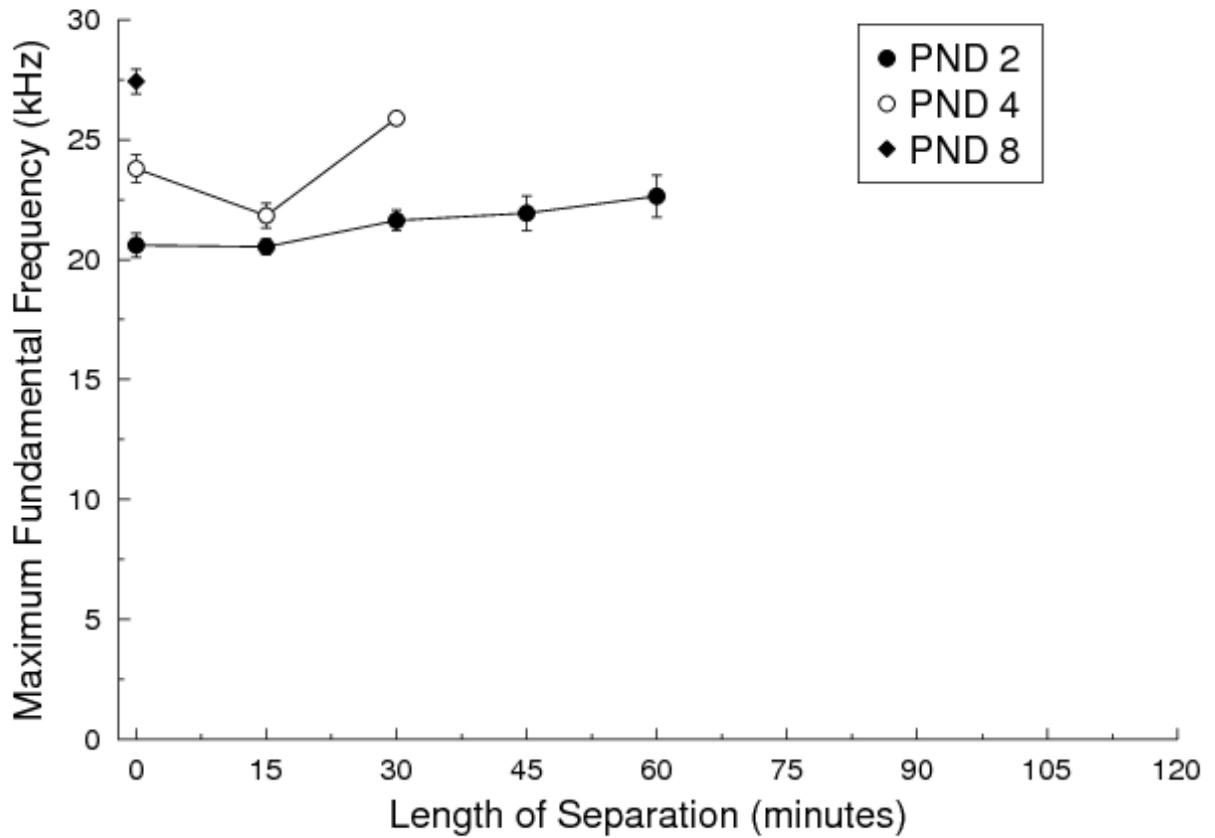
PND 2 pups had higher average calling rates immediately after separation than PND 4 and PND 8 pups. PND 4 pups also had higher calling rates than PND 8 pups ( $F = 8.86$ , d.f. = 28,  $p = 0.001$ ; Fig. 22). There was a significant difference in the time after separation at which the calling rates went to zero between PND 2 and PND 4 pups, such that pups on PND 2 reached a calling rate of 0 Hz significantly later than pups on PND 4 ( $t = 10.68$ , d.f. = 6,  $p = 0.00004$ ). There was also a significant difference in the time at which the calling rates dropped to zero for PND 2 and PND 8 pups, with PND 2 pups continuing to call for much longer times after separation than PND 8 pups ( $t = 4.73$ , d.f. = 6,  $p = 0.003$ ). Although there was no significant difference between the time at which the calling rates go to zero for PND 4 and PND 8, it was very close to significant ( $t = 2.33$ , d.f. = 6,  $p = 0.059$ ). Because all pups on PND 8 reached a calling rate of 0 Hz at the same time (recall that no pup on PND 8 emitted vocalizations after 0 minutes of separation), there was no variation and therefore a repeated measures ANOVA was not performed on these data. Instead, t-tests using corrected p-values were employed.

### *Maximum Fundamental Frequency*

Calls emitted by PND 8 pups had higher average maximum fundamental frequencies than calls emitted by PND 4 and PND 2 pups, and calls recorded on PND 4 were higher in average maximum fundamental frequency immediately after separation than calls recorded on PND 2 ( $F = 48.64$ , d.f. = 12,  $p = 1.752E-6$ ; Fig. 23). Again, these



**Figure 22:** Change in call rate throughout prolonged separation by PND 2, 4 and 8 pups. Each point represents the mean  $\pm$  SE call rate at each time point throughout separation on each of the three test days.



**Figure 23:** Change in maximum fundamental frequency throughout prolonged separation on PND 2, 4 and 8. Each point represents the mean  $\pm$  SE maximum fundamental frequency for each time point throughout separation on each of the three postnatal test days.

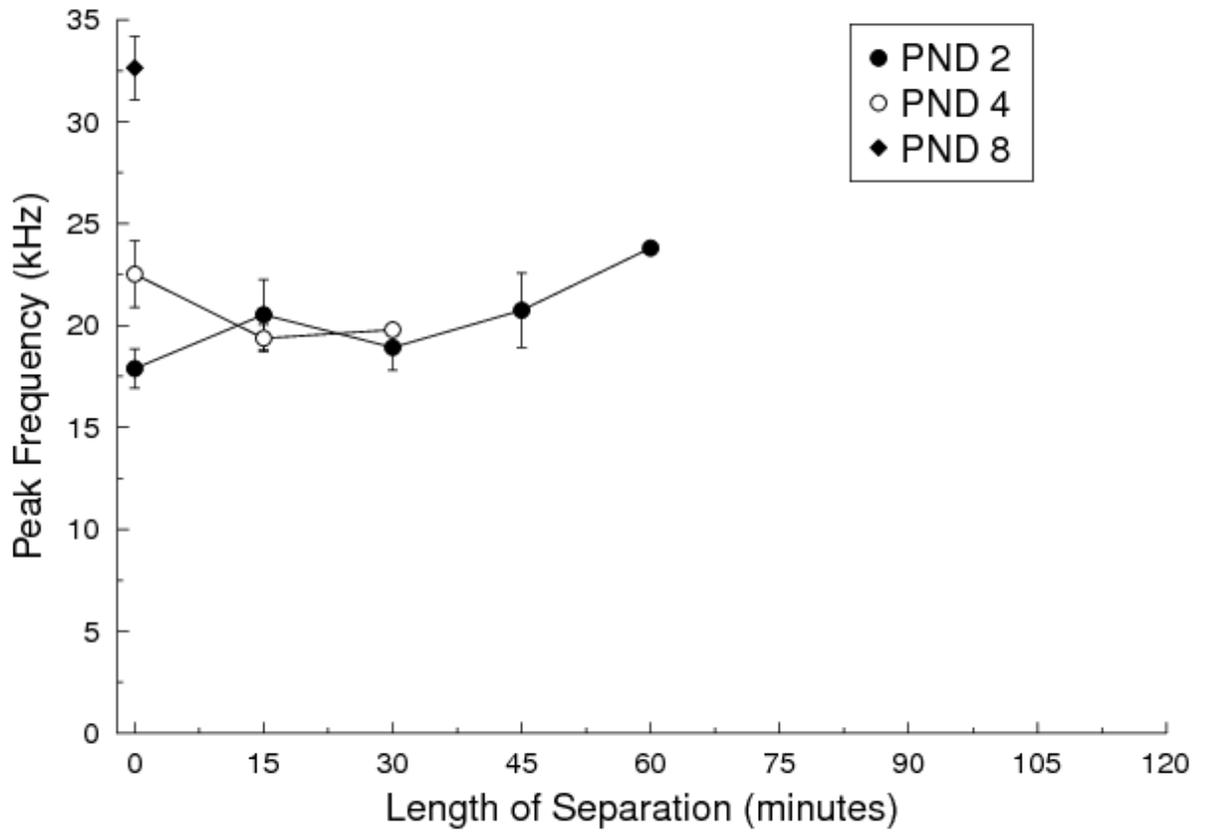
results reflect my developmental descriptions of older pups having higher maximum frequencies than younger pups.

The maximum fundamental frequency increased by 2 or 3 kHz on PND 2 as length of separation increased, but there was no significant difference between the values measured at 0 minutes and after 45 minutes of separation ( $t = -0.24$ ,  $d.f. = 6$ ,  $p = 0.815$ ). On PND 4, the maximum fundamental frequency decreased slightly from immediate separation to 15 minutes of separation by approximately 2 kHz. After 30 minutes of separation, only one pup produced calls with a slight increase in maximum fundamental frequency. The maximum fundamental frequency of calls recorded after 0 and 15 minutes of prolonged separation on PND 4 were not significantly different ( $t = 2.69$ ,  $d.f. = 3$ ,  $p = 0.075$ ). No pup on PND 8 produced calls after immediate separation.

#### *Peak Frequency*

During prolonged separation, PND 8 pups emitted calls with higher peak frequencies than PND 4 and PND 2 pups. Moreover, PND 4 pup calls had higher peak frequencies than PND 2 pup calls immediately after separation ( $F = 19.23$ ,  $d.f. = 12$ ,  $p = 0.0002$ ; Fig. 24). Again, these results match those of general changes in peak frequency throughout development, as older pups emitted calls with higher peak frequencies than younger pups (Fig. 17).

On PND 2, the peak frequencies of calls emitted after 0 and 45 minutes of separation were not significantly different ( $t = -1.41$ ,  $d.f. = 6$ ,  $p = 0.208$ ). The peak



**Figure 24:** Change in peak frequency throughout prolonged separation on PND 2, 4 and 8. Each point represents the mean  $\pm$  SE peak frequency for each time point throughout separation on each of the three postnatal test days.

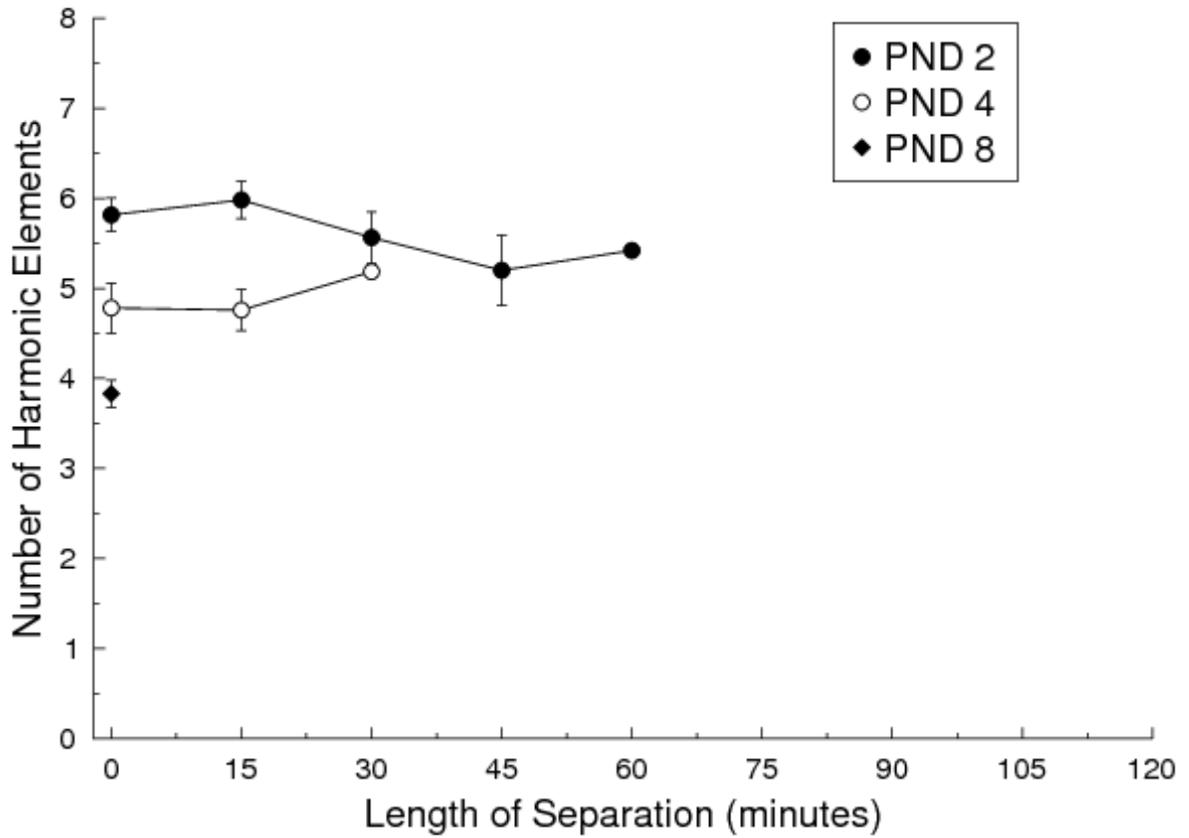
frequency of the calls produced on PND 4 remained fairly constant throughout the period of separation and there were no significant difference between peak frequencies of calls emitted after 0 and 15 minutes of separation ( $t = -2.07$ ,  $d.f. = 3$ ,  $p = 0.130$ ). No pup emitted calls after immediate separation on PND 8.

#### *Harmonic Elements*

During prolonged separation, calls emitted by PND 2 pups contained significantly more harmonic elements than calls emitted by PND 4 and PND 8 pups, and calls emitted by PND 4 pups had a higher number of harmonic elements than calls of PND 8 pups after 0 minutes of separation from the mother ( $F = 16.92$ ,  $d.f. = 12$ ,  $p = 0.0003$ ; Fig. 25). Again, these results are in accordance with my descriptive data showing that younger pups produce calls with a higher number of harmonic elements than older pups (Fig. 18). On PND 2, the number of harmonic elements remained constant after 15 and 45 minutes of separation ( $t = 1.62$ ,  $d.f. = 5$ ,  $p = 0.166$ ). On PND 4, the number of harmonic elements in calls emitted after 0 and 15 minutes of separation did not differ significantly ( $t = 1.03$ ,  $d.f. = 3$ ,  $p = 0.379$ ). No pup on PND 8 produced calls after 0 minutes of separation.

#### ***Spontaneous vs. Provoked Vocalizations***

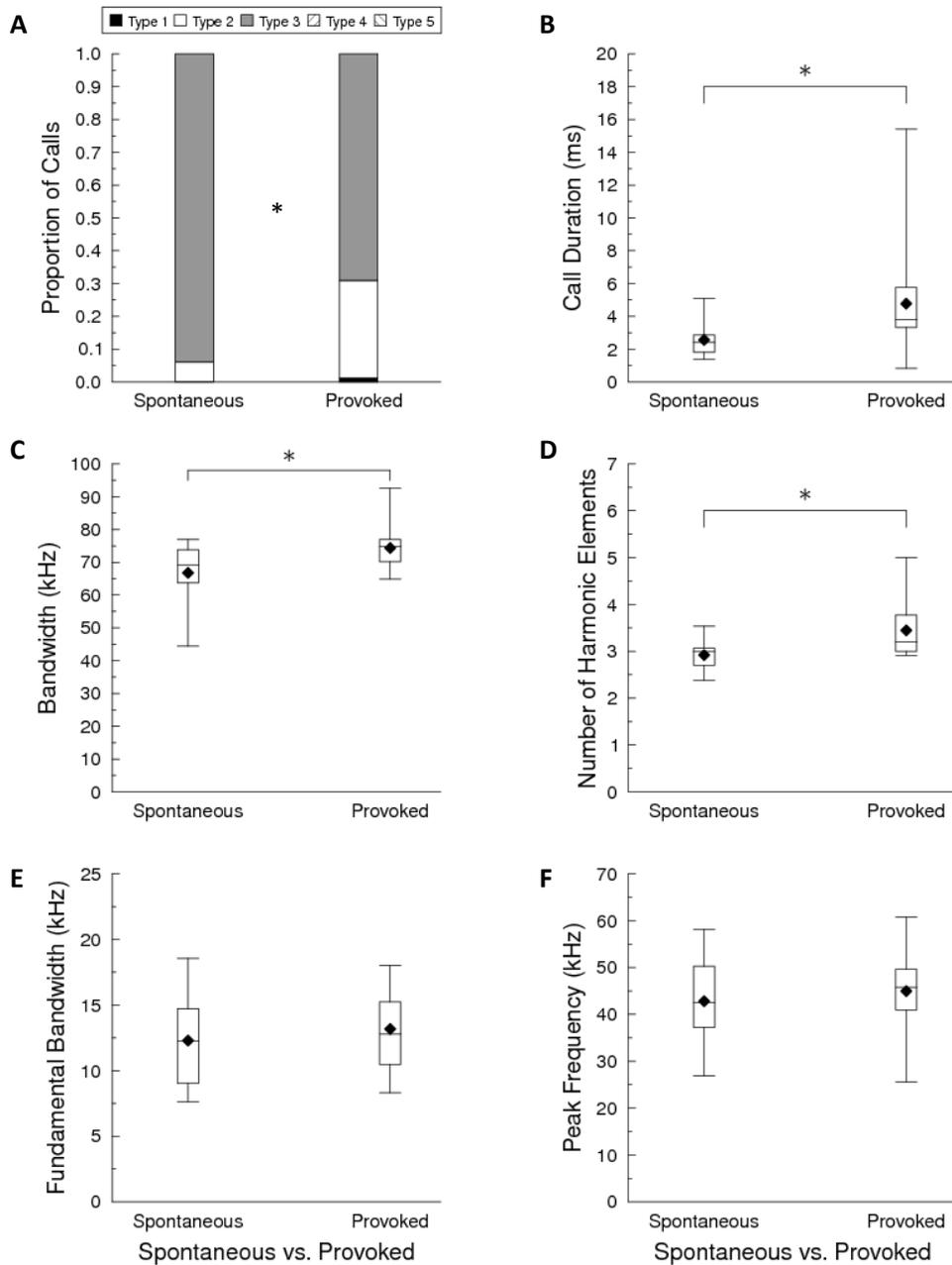
I hypothesized that manual stimulation of pups during recording would result in increased distress calling, and therefore provoked calls would appear to be more similar



**Figure 25:** Change in number of harmonic elements throughout prolonged separation on PND 2, 4 and 8. Each point represents the mean  $\pm$  SE number of harmonic elements for each time point throughout separation on each of the three postnatal test days.

to the vocalizations of younger pups because such calls are more likely to promote maternal retrieval.

The distribution of call types for provoked and spontaneous calls were different ( $\chi^2 = 230.65$ , d.f. = 2, critical p value = 13.82,  $p < 0.001$ ; Fig. 26A). Provoked calls had a greater proportion of Type 1 and Type 2 calls and a lower proportion of Type 3 calls. On PND 13, no Type 4 or 5 calls were emitted by the pups in this experiment. Provoked calls were significantly longer in duration ( $t = -2.46$ , d.f. = 15,  $p = 0.027$ ; Fig. 26B) and were significantly larger in bandwidth than spontaneous calls ( $t = -3.08$ , d.f. = 15,  $p = 0.008$ ; Fig. 26C). Provoked calls also contained significantly more harmonic elements than spontaneous calls ( $t = -2.95$ , d.f. = 15,  $p = 0.010$ ; Fig. 26D), which was expected given their larger spectral bandwidths. There were no significant difference in the bandwidth of the fundamental ( $t = -1.41$ , d.f. = 15,  $p = 0.180$ ; Fig. 26E) or the peak spectral frequency ( $t = -0.89$ , d.f. = 15,  $p = 0.387$ ; Fig. 26F) between spontaneous and provoked vocalizations.



**Figure 26:** Spontaneous and provoked calls of PND 13 big brown bat pups (n = 16). Comparisons between the distribution of emitted call types (A), call duration (B), signal bandwidth (C), number of harmonic elements (D), fundamental bandwidth (E) and peak spectral frequency (F). Box and whisker plot represent the median value for each parameter, the 25<sup>th</sup> and 75<sup>th</sup> percentiles, and the minimum and maximum values. The mean value for each parameter is represented by filled diamonds. A single asterisk represents statistically significant differences (p < 0.05).

## DISCUSSION

### ***Morphology***

The mass and forearm length of big brown bat pups increased at a rate of approximately 0.40 g/day and approximately 0.844 mm/day, respectively. These results are very similar to those estimated previously (mass growth rate of 0.3-0.47 g/day, forearm growth rate of 0.8-1.4 mm/day) by Burnett and Kunz (1982). I was also to confirm their observation that there were no differences between male and female pup growth trajectories. There were, however, significant differences in the average mass and forearm length of adult male and female bats ( $t = 2.57$ , d.f. = 68,  $p = 0.123$  and  $t = 3.48$ , d.f. = 63,  $p = 0.001$ , respectively). The difference in adult masses are likely due to the common occurrence of pregnancy in the adult females I weighed. Furthermore, non-pregnant female mammals are often larger than males in mammals, such as the spotted hyena (*Crocuta crocuta*), spider monkeys (Family Cebidae) as well as many other species of bat, including the red bat (*Lasiurus borealis*) and the pallid bat (*Antrozous pallidus*) (Isaac, 2005; Ralls, 1976). Captive male and female PND 25 pups reached masses that were greater than wild-caught adult male and females. The greater mass of captive pups may be due to the constant availability of food for mothers, the restrictions on flight experienced by the pups as well as constant conditions with respect to temperature.

### *Growth Equations*

The growth equations I developed using data from healthy pups up to PND 15 were more accurate in predicting pup age than the equations developed using data up to PND 25. Because growth trajectories for both mass and forearm length were more linear early in development, this explains why the PND 15 equations were better predictors of pup age than the PND 25 equations. The accuracy of predicting age decreases as pups age because the variance of both mass and forearm length also increases with age (Figs. 2 and 3). Because the forearm length equation was more accurate in estimating age than the age estimated from the equations for forearm length and mass from PND 0 to PND 15, I conclude that age estimations for very young pups should be based solely on forearm length. Furthermore, mass may change over the course of a single day; it varies with nutritional state and health, whereas forearm length is expected to be more stable.

### ***Call Parameters***

#### *Call Types*

Type 1 I-calls were the most frequent vocalization emitted early in development. Type 2 calls were emitted during intermediate stages of development and Type 3 calls (typical echolocation calls) were emitted later. Type 4 and 5 calls were recorded most often early in development, usually at the same time as Type 1 and Type 2 calls. The shift from emitting almost exclusively I-calls early in development to more adult-like

echolocation calls at older ages has also been reported by other researchers studying big brown bats (Gould, 1975a, b; Monroy et al, 2011; Moss, 1988) as well as other species of bat (Gould, 1979; Brown et al, 1983; Habersetzer and Marimuthu, 1986; Rübsamen, 1987; Sterbing, 2002; Lui et al, 2007; Vater et al, 2003).

I-calls and echolocation calls appear to serve two distinct functions related to the needs of the animal at a given age; I-calls are produced by very young pups to facilitate retrieval and maternal assistance whereas echolocation calls are produced by pups that are nearing an age where they begin to navigate, explore and forage independently. Intermediate vocalizations with no obvious retrieval or echolocation function have also been reported by Jones et al, (1991) and Moss (1988). The Type 2, 4 and 5 calls identified in this study are examples of intermediate calls which may reflect vocal learning, a lack of vocal control or possibly a currently unknown form of additional pup communication. I recorded very few social-like vocalizations in my set-up, which was expected because pups were isolated and thus were not provided the pups with clear social situation cues. Precursors to adult social calls may have been observed if I had allowed for such social interactions between mothers and infants (e.g. Monroy et al, 2001; Moss, 1988).

The categorization of calls into types was based initially on quantitative measurements as opposed to other vocal classification schemes based entirely on descriptive or visual categories (e.g. Monroy et al, 2011). A quantitative classification

scheme reduces the likelihood of misclassification based on subjective interpretation of call shape. My categories were specified based on call duration and the presence and direction of FM because I wanted to explore the types of vocalizations produced early in development and how I-calls calls transitioned into biosonar calls in adults. Many other researchers have described I-calls and echolocation calls using quantitative measures such as call duration and frequency modulation (Gould, 1975a, b; Brown, 1976; Habersetzer and Marimuthu, 1986), as well as qualitative measures such as shallow vs. steep FM and the presence/characteristics of the tail portion of FM and calls (Moss, 1988; Monroy et al, 2011). Several researchers have recorded and described pup calls in isolation as well as during the reunion process with mothers (Moss, 1988; Monroy 2011; Gould, 1979).

#### *Call Duration*

I-calls are longer in duration than most other vocalizations produced by bats (i.e. echolocation calls or social calls). Longer call durations may make I-calls more informative and conspicuous, but they also appear to be less sophisticated with respect to the direction and magnitude of FM. For pups simply trying to attract their mothers' attention, longer duration calls would provide more energy for the adult bat to identify and localize their offspring at a distance. Shorter call durations are typical of echolocation calls. Shorter durations allow bats to produce more calls in the same time period and reduce the probability of overlap between outgoing calls and received

echoes. Therefore, shorter calls are better for sophisticated navigation and prey detection, but may be less helpful in providing a cue that other bats may use for information regarding identity or location of conspecifics.

#### *Fundamental Bandwidth*

The echolocation calls of adult big brown bats have larger fundamental bandwidths than those of pup I-calls. This follows the observation that total signal bandwidth remains relatively constant throughout development, even though the number of harmonic elements decreases (Figs. 12 and 18). Therefore, the fewer harmonics present in the echolocation calls of adults must be larger in bandwidth in order to fill the same spectral space. The increase in fundamental bandwidth with age is expected because there is also an increase in the maximum fundamental frequency over time, and the maximum fundamental frequency increases at a faster developmental rate than the minimum call frequency. One reason why echolocation calls may be selected to have higher fundamental bandwidths is because calls of larger bandwidth contain more frequency channels for auditory processing and sound localization (Yost, 2007).

#### *Minimum Frequency, Maximum Frequency and Bandwidth*

Because the minimum call frequency increases with age and the maximum call frequency remains relatively constant, this explains the slight overall decrease in total signal bandwidth observed over development. The increase in minimum frequency with

age may reflect the switch from I-calls to echolocation calls, as echolocation calls in general have more energy at higher frequencies. Very young pups may lack the motor skills necessary to provide proper tension on their vocal cords to produce high frequency vocalizations. Furthermore, higher frequency signals are attenuated in air more quickly than lower frequency signals (Lawrence and Simmons, 1982; Marten and Marler, 1977; Marler, 1955). For young pups trying to attract the attention of their mother, natural selection would favour calls that experience less atmospheric attenuation. I-calls also have more energy at lower frequencies than Type 2 and Type 3 calls.

#### *Peak Frequency*

The average peak frequency of pup vocalizations increased with age and adult echolocation calls had higher peak frequencies than all pup calls except Type 3 calls. Adult echolocation calls had similar peak spectral frequencies to those of average Type 3 pup calls. The difference in peak frequency may be the result of immature pup vocal cord development. The tension and resonant frequency of the vocal cords may be undergoing sophistication and fine-tuning during development, causing younger pups to produce calls with lower peak frequencies. The natural or resonant frequency of the vocal cords will be determined by the stiffness and mass of the vocal cords and their tension. Because young bats are still growing, their vocal cords would be expected to have a lower resonant frequency.

### *Harmonic Elements*

Longer duration sounds have more energy. Additionally, sounds with more harmonic elements will have higher bandwidths because they contain more sound frequencies. Longer duration vocalizations with higher frequency components and larger bandwidths should be easier to localize compared to shorter and more tonal signals. Bat calls can have a variable number of harmonic elements. Big brown bats typically produce echolocation calls with multiple frequencies and acoustic elements (Fenton et al, 2011). A greater number of harmonic elements present in calls emitted earlier in development may provide multiple reference points for interaural time and intensity differences that mothers can use for pup localization (Marler, 1955). Alternatively, younger pups may not have the vocal control necessary to produce sounds with lower number of harmonic elements or with shorter call durations. If so, the higher number of harmonic elements present in I-calls could reflect immature tracheal, laryngeal and/or vocal cord development.

An increased number of harmonic elements may also allow for more precise sound localization, which could explain why adult big brown bats continue to emit echolocation calls with multiple harmonic elements, although fewer elements than the I-calls of pups. Big brown bats produce echolocation calls with downward FM sweeps that typically contain 2 or 3 harmonic elements (including the fundamental). It has been shown that FM signals are better for range discrimination than CF signals (Simmons and

Stein, 1980; Jones and Teeling, 2006). The downward FM sweep of the signal allows bats to differentiate between targets that are very close together. Discrimination is enhanced because the FM sweep allows for better temporal delay precision between outgoing calls and returning echoes (Simmons and Stein 1980; Jones and Teeling 2006). When the number of harmonic elements in a given call increases, the total energy becomes spread out across all spectral components. This spreading reduces the effective energy per Hz in the signal. Spreading also decreases the total distance that ultrasonic frequencies can travel in air and therefore decreases the operating distance that echolocating bats are able to use for prey detection and navigation. For situations that require long distance communication, lower frequency calls with fewer harmonics would be more effective; however, signals with a greater number of harmonics are more useful in cluttered environments that can potentially create background echoes from which the bats need to differentiate their prey (Simmons and Stein, 1980). Therefore, a greater number of harmonic elements will provide for better sound localization of objects in cluttered environments (Jones and Teeling 2006; Fenton 1997). An intermediate number of harmonics emitted by adult big brown bats might therefore be a useful compromise between sophisticated localization and long-range communication.

### ***Prolonged Separation***

#### *Call Types*

Pups on PND 8 were more likely to produce Type 2 and 3 calls, while pups on PND 2 and 4 were more likely to produce Type 1 and 2 calls (Fig. 19). Almost all pups on PND 2 emitted exclusively Type 1 calls. This pattern fits in well with the observation that older pups were more likely to produce Type 3 and less likely to emit Type 1 and 2 calls. With respect to prolonged separation, there was not a substantial change in the proportion of Type 1 and 2 calls emitted on PND 2; however, the proportion of Type 1 calls on PND 4 increased with the period of separation, and the proportion of Type 2 and 3 calls decreased. These changes suggest that younger pups were attempting to produce more I-calls and signal their mothers as the length of separation increased, thereby increasing the likelihood of stimulating mothers to engage in retrieval behaviour. By PND 8, pups emitted mostly Type 2 and Type 3 calls, suggesting that as pups mature they require less retrieval/maternal assistance during separation.

#### *Call Duration*

Call durations of younger pups, such as most individuals on PND 2 and some individuals on PND 4, increased with the length of separation. The lengthening of call duration suggests that pups were attempting to make their calls more similar to I-calls (Type 1), which are known to stimulate retrieval behaviour in mothers. Shorter call durations emitted by PND 4 and PND 8 pups are indicative of general vocal development

and reflect an increased independence of the pups, such that they no longer require retrieval and constant attention from their mothers. As pups age and become more independent, I predict that there should be less selection for making their calls more similar to those of younger bats.

### *Call Rate*

The rate of calling by pups always decreased with the length of separation, regardless of pup age (Fig. 22). Pups on PND 2 emitted more calls and signalled longer throughout the period of separation, with some individuals calling for up to 60 minutes. Pups on PND 4 stopped calling much earlier and no calls were produced after 30 minutes of isolation. However, no pup produced calls on PND 8 on or after 15 minutes of separation. The overall decrease in calling rate at all three ages could represent pups attempting to conserve energy during maternal separation. For example, over time the pup may realize that the reunion process may not happen immediately and therefore continuing to call would waste energy. Alternatively, PND 2 and PND 4 pups may have tired or became too cold to continue calling and expending energy; however, older pups were generally mobile during the experiment, so this explanation seems unlikely for PND 8 pups. The differences in calling rate at each of the three ages may reflect the maturity and independence of the pups, with younger pups (PND 2) more likely to call over longer periods of separation because they require more maternal assistance. Pups on PND 4 are still dependent on their mothers and therefore call more than pups on

PND 8, but stop calling sooner than those on PND 2. Pups on PND 8 did not call past immediate separation because they are less dependent on their mothers for thermoregulation and because they can go for longer intervals without feeding.

#### *Harmonic Elements*

There was no change in the number of harmonic elements emitted by isolated pups at the three developmental ages (Fig. 25). If pups are selected to produce younger-sounding I-calls as the length of separation from their mothers increased, then I would expect PND 2 and PND 4 pups to produce calls with more harmonics after longer periods of isolation. This pattern was not observed, perhaps because very young pups are unable to control the number of harmonics produced during vocalizations. The number of harmonic elements in the calls of pups in the age groups matched the typical developmental pattern observed, such that older pups emitted calls with fewer harmonics.

#### *Maximum Fundamental Frequency*

There was no effect of prolonged separation on the maximum fundamental frequency of the calls emitted by pups on PND 2 and PND 4. If pups were attempting to make their vocalizations appear younger in order to facilitate retrieval, I would have expected a decrease in maximum fundamental frequency during maternal separation; however, this pattern was not observed. The differences in maximum fundamental

frequency between pups of different ages matched the typical developmental pattern of calls, in that older pups produced calls with higher fundamental frequencies (Fig. 15).

#### *Peak Frequency*

As separation time increased, call peak frequencies for PND 2 pups increased significantly between 0 and 15 minutes of separation. On PND 4, there was no increase in peak frequency over time. These patterns were counterintuitive with respect to the trends observed throughout the prolonged separation experiment, because higher peak frequencies were typically emitted by older pups. Most other changes taking place throughout prolonged separation suggest that the pups may be trying to appear younger, more vulnerable and in greater need of maternal assistance. Although the change in peak frequency during prolonged separation was not in accordance with my hypothesis, the differences between the three age groups in terms of initial peak frequency (calls emitted after 0 minutes separation) match the typical developmental pattern of calls initially described (Fig. 17).

#### ***Spontaneous vs. Provoked Vocalizations***

##### *Bandwidth*

Provoked calls had significantly larger bandwidths than spontaneous calls. Pups in distress who are in need of maternal assistance may produce calls of larger bandwidth to mimic pups at a younger age. The bandwidth of spontaneous calls emitted by PND 13 pups were similar to the average bandwidth of calls produced by all pups on

PND 13, whereas the bandwidth of provoked calls on PND 13 were more similar to those produced by pups on PND 6. Therefore, these data are consistent with the hypothesis that larger signal bandwidths are easier to localize and thus are more likely to be produced by younger pups (Marler, 1955).

#### *Call Duration*

Provoked calls were significantly longer in duration than spontaneous calls emitted by the same pup. This result is also consistent with the hypothesis that since longer duration calls contain more energy/information and therefore are easier to localize when bat pups are in distress. Calls with long durations are more likely to be produced by younger pups, as I-calls have longer call durations than echolocation calls (Fig. 10). The duration of spontaneous calls emitted by pups on PND 13 were similar to the average call duration of all pups on PND 13, while provoked call durations on PND 13 were more similar to the duration of pups calls on PND 7 or 8.

#### *Call Types*

Provoked vocalizations on PND 13 contained more Type 1 and Type 2 calls than Type 3 calls. This pattern also matched the predicted hypothesis because younger pups emit more Type 1 and Type 2 calls and less Type 3 calls. Spontaneous vocalizations on PND 13 had a higher proportion of Type 3 calls (Fig. 26). The proportion of Type 1, 2 and 3 spontaneous vocalizations matched the average proportions of call types emitted by

PND 13 pups, whereas the proportions of call types of provoked vocalizations more closely resembled those emitted by PND 8 pups.

#### *Harmonic Elements*

Provoked calls contained significantly more harmonic elements than spontaneous calls, which is also in accordance with the hypothesis that younger pups emit calls with more harmonic elements to increase the likelihood that their mothers locate and retrieve them. The spectral complexity of spontaneous calls emitted on PND 13 was similar to the average number of harmonic elements typically emitted by PND 13 pups during development (Fig. 18); however, the number of harmonic elements in provoked calls on PND 13 more closely resembled that of younger pups (e.g. PND 9 or 10). This also supports the hypothesis that provoked pups were attempting to emit vocalizations that resembled younger pups.

#### *Fundamental Bandwidth and Peak Frequency*

Provoked and spontaneous calls had similar fundamental bandwidths and peak spectral frequencies. If these parameters also followed the expected prediction, then provoked calls should have contained lower peak frequencies and a lower minimum frequency, as younger pups produce calls with these patterns. These results suggest that although pups appear to alter their vocalizations to sound more similar to younger pups while they are being provoked/distressed, they may not be able to adjust all aspects of their calls.

### ***Summary***

Big brown bat pups follow relatively stable developmental trajectories with respect to morphology, behaviour and vocal change. As pups age, their physical appearance changes from being naked, blind and altricial at birth to essentially adult-like size and appearance by PND 45. Pup vocalizations switch from long duration, tonal I-calls with many harmonics to short duration, downward FM biosonar signals with few harmonics used for echolocation. Call rate and maximum frequency do not change throughout development, whereas call duration, total signal bandwidth and the number of harmonic elements decrease. The bandwidth of the fundamental acoustic element, maximum fundamental frequency, minimum frequency and peak spectral frequency also increase throughout development.

By recording vocalizations emitted by pups during prolonged separation from their mothers, I was able to demonstrate that younger pups call for a longer period of time and at a faster rate during isolation, suggesting that younger pups have a greater need for maternal assistance. The prolonged separation data collected after 0 minutes of isolation allowed me to perform repeated measures ANOVAs on the various call parameters across each of the three postnatal testing days, thus providing quantitative support of the call parameters changes described previously. The differences between spontaneous and provoked vocalizations recorded on PND 13 indicated that provoked vocalizations were spectrally and temporally more similar to those of younger bats,

suggesting that pups may attempt to mimic younger bats during distress in order to encourage maternal assistance and retrieval. Additional research into morphological changes in vocal cord and tract anatomy and its influence on pup acoustic signal production is necessary to further understand the development of vocalizations in bats and other mammals.

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