USING PEDIGREE RECONSTRUCTION TO TEST HEAD-STARTING EFFICIENCY FOR ENDANGERED AMPHIBIANS: FIELD TESTED IN THE HOUSTON TOAD (*BUFO HOUSTONENSIS*)

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USING PEDIGREE RECONSTRUCTION TO TEST HEAD-STARTING EFFICIENCY

FOR ENDANGERED AMPHIBIANS:

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ABSTRACT

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FIELD TESTED IN THE HOUSTON TOAD (*BUFO HOUSTONENSIS*)

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The Houston Toad (*Bufo houstonensis*) was first described in 1953 and due to habitat loss and severe droughts the species was placed on the endangered species list in 1970. In 2007, a head-starting project was implemented using tadpoles and juveniles that cannot be marked by traditional means. I developed a method to use molecular genotyping arrays and tested four full sibling reconstruction algorithms to estimate the frequency of head-starts on the landscape. The overall allelic retention of the head-starts was 67% of the wild population. The percent retention ranged from 26% to 90% among subpopulations. COLONY was the most efficient family assignment software placing

97% of samples into their appropriate sib groups. Several of these groups (20%) showed evidence of multiple paternity but it is unclear if this is the result of multiple amplexus events, indirect fertilization, or physical cross contamination of egg strands either in captivity or upon collection from the wild. I estimated the over winter survivorship of post-metamorphosed juveniles to be 0.001. Given the low survivorship and low frequency of head-starts on the landscape, this age group is not efficient for head-starting and adult head-starts should be tested for survivorship in the coming years.

CHAPTER I

INTRODUCTION

More than 30% of amphibians are listed as threatened, endangered or critically endangered and more than 43% of species are in decline (IUCN et al., 2008). The causes most often blamed for this decrease are climate change (including increased UV light and chemical exposure), poor habitat management, invasive species and the damaging effects of chytrid fungus (*Batrachochytrium dendrobatidis*) (Collins and Storfer, 2003). A number of conservation practices like habitat restoration and population supplementation have been applied to address these declines in a number of different groups (Griffiths and Pavajeau, 2008).

Manipulative practices such as population supplementation have been an attractive strategy for conservation biologists and some species have benefited from supplementation through relocation, repatriation, translocation and head-starting (Griffith et al., 1989). Head-starting is a management practice in which wild individuals of early lifestages (eggs, tadpoles, etc.) are protected in the field or raised to a larger size in captivity (Haskell et al., 1996). Many anurans are explosive breeders in which a single

egg strand may consist of thousands of eggs. The mortality rate is highest in the early life stages (eggs, tadpoles and metamorphosed juveniles) (Breden, 1987; Greuter, 2004). By avoiding this mortality through head-starting, it is believed more individuals will be capable of reaching maturity and reproducing (Dodd and Seigel, 1991). Head-starting or population propagation as a conservation practice has yielded success in mammals (Dobson and Lyles, 2000), birds (Cannon, 1996) and reptiles (Shaver and Wibbels, 2007), yet few reintroduction, relocation or repatriation programs have yielded success in amphibians (Dodd and Seigel, 1991; Seigel and Dodd, 2002). Regardless of this lack of success, several groups maintain efforts to supplement wild populations with captive bred or head-started anurans. Population supplementation has been incorporated in the Natterjack toad (*Bufo calamita*) of Great Britain (Denton et al., 1997), The Crested toad (*Peltophryne lemur*) of Puerto Rico (Miller, 1985), the Boreal toad (*B. boreas*) in Rocky Mountain National Forest (Muths et al., 2001) and the Houston toad (*B. houstonensis*) in southeast Texas (Quinn and Ferguson, 1987). Only *B. calamita* has shown positive results from population supplementation (Denton et al., 1997).

Bufo houstonensis was first described in Houston, Texas in 1953 (Peterson et al., 2004). The species belongs to the *B. americanus* species group which also includes *B. americanus*, *B. baxteri*, *B. fowleri* and *B. woodhousii* (Pauly et al., 2004; Goebel et al., 2009). The species is strictly constrained to areas with sandy soils and often associated with loblolly pine (*Pinus tadea*) or mixed hardwood forests (Brown, 1971). In 1970, the Houston Toad was the first animal in Texas and the first amphibian federally listed as an endangered species (Peterson et al., 2004). Critical habitat was designated in Bastrop,

Burleson and Harris counties by the U.S. Fish and Wildlife Service (USFWS) in 1978, although the designation in Harris Co. was later revoked after significant lobbying by real estate interests (USFWS, 1978). *Bufo houstonensis* has historically been detected within 12 counties (Austin, Bastrop, Burleson, Colorado, Ft. Bend, Harris, Lavaca, Lee, Leon, Liberty, Milam and Robertson) of south east Texas, yet recent surveys recorded *B. houstonensis* only in Austin, Bastrop, Colorado, Lee, Leon and Milam counties with low numbers in Austin, Colorado and Leon counties (McHenry, 2010). Population estimates suggest there are fewer than 1,000 breeding adults today (Michael R.J. Forstner, unpublished data).

Population restoration for *B. houstonensis* using releases from captive propagation was first attempted by the Houston Zoo in Colorado Co. at Attwater Prairie Chicken National Wildlife Refuge (APCNWR) in the 1980s. The refuge was selected as what was then considered suitable habitat within an extirpated area of the historical range of *B. houstonensis*. The restoration program included the introduction of wild caught juveniles, adults and captive reared egg strands from Bastrop Co. to ten sites within the APCNWR (Quinn and Ferguson, 1983; Quinn et al., 1984). The program involved the release of approximately 500,000 captive raised and wild caught individuals over the span of several years. Reproduction was recorded in 1985 and males were heard calling in 1984 and 1986 (Quinn et al., 1984; Quinn and Ferguson, 1987). Unfortunately, from 1987 onward, few returns were made to survey the APCNWR for Houston toads. Consequently, the long-term success of the program was not effectively measured and a self-sustaining population was never documented as a result of the propagation program.

Recently, several calling males were collected in north Colorado Co. that had a genetic signature most closely related to individuals from the original source population of the Houston Zoo toads (McHenry, 2010). McHenry (2010) suggested that a self-sustaining population of captive reared *B. houstonensis* migrated from the initial release point and settled north of the NPCNWR. This provides some support that head-starting could be a successful conservation method for the Houston toad.

In 2007 a new program of head-starting was started in cooperation with Texas State University, USFWS, Texas Parks and Wild Department (TPWD) and the Houston Zoo. Several factors differ from the propagation program of the 1980s. First, in conjunction with head-starting, funded work for Houston toad population rehabilitation also includes efforts to improve habitat quality as well as monitor populations through annual chorusing surveys. Second, only extant populations are supplemented in Austin, Bastrop and most recently Leon counties. Third, as opposed to translocation of wild caught juveniles and adults, only egg strands are being collected and reared to different life stages (tadpoles, metamorphs and adults) and released to their natal pond. Finally, adult head-starts are toe clipped or PIT tagged for subsequent identification. The twelve month recapture frequency of adult head-starts has been approximately 10% (Michael R.J. Forstner, unpublished data). Unfortunately, the majority of head-starts are tadpoles or juveniles which cannot be physically marked for future identification making an assessment of head-starting difficult. Molecular tools may provide insight into the success of this conservation strategy.

It is possible to estimate the relatedness among pairs of individuals with genetic markers. Relationship categories such as full sibling (sib) or half sib can be estimated from the probabilities derived from a dyad (a pair of individuals) sharing zero, one or two alleles that are identical by descent (Thompson, 1991). Hamilton (1964) introduced an early attempt to estimate relatedness within a population. The *r* expressed in Hamilton's rule is essentially a genetic correlation or regression coefficient. The relatedness coefficient has been defined as the genetic similarity between two individuals relative to that between random individuals from some reference population (Pamilo, 1990). Since then, many methods have been derived to determine the degree of relatedness among pairs of individuals (Hamilton, 1972; Thompson, 1975; Queller and Goodnight, 1989; Thompson, 1991; Lynch and Ritland, 1999; Wang, 2002). The relative accuracy, precision and robustness of a relatedness estimator are dependent on the number of loci, allele frequency and the true relationships among samples. Each estimator performs differently given the data and a single "best" estimator has yet to be derived (Van De Casteele et al., 2001; Csilléry et al., 2006; Oliehoek et al., 2006).

A powerful tool derived from relatedness estimations yet to be fully exploited is pedigree reconstruction algorithms. These divide a dataset into sibgroups based on codominant genetic marker data (Blouin, 2003). There are two main groups of partitioning algorithms, group methods that involve single generation pedigree reconstruction for all members within a sample, and pairwise approaches that infer relationships of pairs of individuals based on relatedness (Ritland, 1996; Van De Casteele et al., 2001) or based on the likelihood that pairs belong to a specific relationship class

(Thompson, 1975; Herbinger et al., 1997). Large data sets are often plagued by both real mutations and genotyping errors (Taberlet et al., 1996; Pompanon et al., 2005). These errors negatively affect the accuracy of sibship partitions as these prohibit individuals from joining their appropriate sibling family. Some software developers have allowed users to correct for mutations and genotyping errors often associated with real biological datasets.

These partitioning algorithms may be able to determine whether a wild caught toad is directly related to the head-started population providing a unique genetic mark-recapture method to measure the abundance and distribution of head-started Houston toads on the landscape. This is one of the five conditions Dodd and Seigel (1991) suggested conservation biologists should address when designing a manipulative practice including know the cause of decline, understand the biological constraints, evaluate the social structure and population genetics of the species in question, and prevent disease transmission. In the case of the Houston toad, the cause of decline is most likely due to habitat fragmentation and dispersal restriction (Brown, 1971). A long term study has been in place since 2001 to monitor and learn the ecology of the Houston toad (Greuter, 2004; Swannack and Forstner, 2007; Swannack et al., 2009; Gaston et al., 2010). A population genetics study has been conducted (McHenry, 2010) and prevention of disease (*B. dendrobatidis*) is ongoing through monitoring chytrid in the wild and preventing exposure in captive populations (Gaertner et al., 2010). Herein, I describe a method capable of monitoring the supplemented populations through time using molecular markers and sibship algorithms.

I report a new method for monitoring head-start success in juveniles. Because a captive assurance colony has been developed as a direct result of head-starting, I compared the genetic diversity of the captive population to the wild population to measure the degree of retained genetic diversity in the captive population. I determine if the present genetic diversity in both the captive and wild populations was sufficient enough to use pedigree reconstruction as an appropriate mark and recapture tool. Many adults were captured after thousands of head-started juveniles were released onto the landscape. I used pedigree reconstruction to determine how many collected adults were in fact recaptured head-starts thus measuring the impact head-starting has had since 2007.

CHAPTER II

METHODS

Egg strand collection and juvenile tissue acquisition- Between 2007 and 2010, areas within the range of *B. houstonensis* were surveyed for amplextant adults, including Bastrop State Park (BSP) (Bastrop Co.), Bluebonnet Electric (BBE) (Bastrop Co.), Griffith League Ranch (GLR) (Bastrop Co.), Hilltop lakes (HTL) (Leon Co.), Jim Small Family property (JMS) (Bastrop Co.), Musgrave Family pond (MSV) (Bastrop Co.) and Nava Family pond (NAP) (Austin Co.). If amplextant *B. houstonensis* were observed during surveys, the location was marked and the area surveyed for egg strands the following day. Up to 75% of discovered egg strings were removed from wild habitats and brought back to the Houston Zoo to be reared to different life stages. A small percentage (1-10%) of the tadpoles or early metamorphosed juveniles from the generated captive population were sacrificed and accessioned into the Michael R. J. Forstner Frozen Tissue Catalog. Head-started juveniles were released at different life stages post hatch to the same site they were collected.

Adult tissue collections- Tissue was taken from adults in the years after head-starting began to determine how many collected adults were recaptured head-starts.

Adults were sampled in two different ways, audio surveys and pit fall traps. During the springs of 2008-2010, between 16 and 26 auditory surveys were conducted between January and May. Listening posts were located to allow chorus monitoring of potential Houston toad breeding sites (~300), most of these occurring in Bastrop Co. (Jackson et al., 2006). Observers listened for 5 minutes for chorusing. Calling males and observed females were collected and a toe was clipped from specimens using sterile scissors and stored in 95% ethanol. Blood samples were also taken from collected toads using a sterile syringe and stored in a blood storage buffer (Longmire et al., 1997).

Pit fall traps have been employed on GLR to maximize the number of toads captured and evaluate how the Houston toad has used the landscape (Swannack et al., 2009). Sampling efforts were slightly different in 2008 vs. 2009-2010. In 2008, Houston toads were collected from 72, 9 linear pit fall traps associated with 18 Y-shaped arrays. Traps were checked daily from March 1st to May 1st. In 2009 and 2010, 18 Y-shaped and eight linear (seven-short and 1 long-linear) arrays connected with 89, 9 linear pit fall traps were checked daily from February 1st to May 1st. Tissue samples were taken and stored as stated above.

DNA extraction- DNA was extracted from toe clips or tail clips 1-2 mm^{3}or 10-50 µl blood in storage buffer using a DNeasy® DNA Tissue kit (QIAGEN Inc.) or Wizard® SV 96 Genomic DNA Purification System (Promega) on a Biomek® 3000 Laboratory Automation Workstation (Beckman Coulter) following both manufacturer's protocols. Extractions were evaluated by electrophoresis on a 1.5% agarose gel and visualized under UV light after ethidium bromide staining.

DNA haplotyping- To assess haplotype frequency and provide maternal haplotype information among egg strands a 533 base pair (bp) fragment of the control region of the mitochondrial genome (mtDNA) was amplified and sequenced using the primers BHDL1 and BUFOR1 (McHenry, 2010). Refer to McHenry (2010) for complete polymerase chain reaction (PCR) and PCR clean up conditions. Cycle sequence reactions were performed with BigDye ® Terminator v3.1 sequencing kit (Applied Biosystems, Inc.) following manufacturer's instructions. Cycle sequencing products were cleaned using ethanol, EDTA and sodium acetate precipitation recommended by Applied Biosystems Inc. and analyzed on an ABI 3500xL Genetic Analyzer (Applied Biosystems, Inc.). Resultant chromatogram were edited and trimmed in Geneious Pro 5.1.6 (www.geneious.com) and aligned using ClustalW (Thompson et al., 1994). Finally, a statistical parsimony haplotype network (Templeton et al., 1992) was constructed in TCS 1.21 (Clement et al., 2000).

DNA Genotyping and allelic diversity- PCR was performed at five microsatellite loci: BBR36 (Simandle et al., 2006), BC52.10, bco15 (Chan, 2007), BM224 (Tikel et al., 2000) and IHHH (Gonzalez et al., 2004) that were previously shown to be highly polymorphic within *B. houstonensis* populations (McHenry, 2010). It was revealed in McHenry (2010) that BM224 contained two unique motifs separated by a conserved region where electromorph size homoplasy was present. An additional reverse primer (BM224DJM) was designed to anneal within the conserved region to amplify the first half of the locus. The BM224DJM locus was subtracted from the total length to determine the length of the second more polymorphic locus, BM224other. Through the remainder of this manuscript only BM224other was used in analyses as BM224DJM was

primarily monomorphic and linked to BM224other. PCR conditions and fragment analysis were the same as in McHenry (2010). Single locus statistics such as observed number of alleles, allelic richness (El Mousadik and Retit, 1996), allele frequency per egg strand, global allele frequency, observed heterozygosity (H_O), expected heterozygosities (H_E) and tests for deviations from Hardy-Weinberg Equilibrium before and after sequential Bonferroni correction (Rice, 1989), were estimated in FSTAT (Goudet, 2001). The informativeness of inferring sibling relations for the microsatellite marker set was assessed using KinInfor version 1.0 (Wang, 2006). I used the estimated population allele frequencies to test the power of relationship inference for three genealogical relationship comparisons: 1) full sibs vs. unrelated 2) half-sibs vs. unrelated and 3) full-sibs vs. half sibs. In each test a total of 100,000 dyads were simulated, a prior Dirichlet distribution of (1,1,1) was assumed and the significance and precision level were set to 0.05 and 0.01 respectively.

Assessing genetic diversity of captive and wild populations- McHenry (2010) provided evidence of nine different subpopulations in *B. houstonensis*. I compared the allelic richness (number of alleles per locus) and heterozygosity of wild adults collected in McHenry (2010) and this study to the captive population generated since head-starting began to estimate the amount of genetic diversity retained in the captive population. Allelic richness can be a more effective measurement of genetic diversity compared to H_O because allelic richness is initially more vulnerable to a decrease in population size (Nei et al., 1975). The units of allelic richness are the mean number of alleles per locus averaged over loci after rarefaction was used to correct for sample sizes (El Mousadik and Retit, 1996). I corrected for sample sizes among pairwise comparisons of captive and

wild populations within individual subpopulations. Mean allelic richness differences between captive and wild populations were measured using a paired t-test. Allelic retention was calculated for each captive subpopulation by dividing the allelic richness of the captive subpopulation by the allelic richness of the wild subpopulation from which the captive subpopulation was derived (Wilson et al., 2009). I measured genetic differentiation by calculating pairwise F_{ST} values of all wild and derived captive subpopulations using Arlequin 3.1(Excoffier et al., 2005).

Sibship reconstruction- Sibship partitions were constructed from a dataset of all sampled individuals within egg strands. Different sibship algorithms vary in their approach and behave differently given the parameters of a dataset. Therefore, four different algorithms were compared to assess the most efficient algorithm for this dataset. These algorithms were assessed using the minimum partition distance method (Grusfield, 2002). The efficiency was measured by the percentage of individuals correctly assigned to their appropriate egg strand. If several individuals within an egg strand partitioned separately from all other egg strands including other members of the egg strand from which it came (as expected under half sibling constraints), and shared a reconstructed maternal genotype with members of the same egg strand, it was considered an accurately assigned full sibling group within a half sibling family. All sibship algorithms assume loci are in Hardy-Weinberg and linkage equilibrium, and follow Mendelian rules of inheritance.

COLONY (Wang, 2004; Jones and Wang, 2009) uses an algorithm for calculating and maximizing the likelihood function to reconstruct full sibling families. COLONY accounts and corrects for genotyping errors such as allelic drop out, real and apparent

mutations (Wang, 2004). This method uses a simulated annealing algorithm to search parameter space for a relationship configuration of the entire sample that maximizes the likelihood based on population allele frequencies that were estimated from the sample. COLONY was run using an full-likelihood approach assuming polygamous females and monogamous males. These assumptions may not be biologically accurate for the Houston toad, but this does produce the most accurate reconstructed families (data not shown). An allelic dropout rate was set to 0.01 and other mutation rates were set to 0.015.

The second algorithm implemented in PEDIGREE 2.2 constructs possible partitions based on DNA marker data of individuals into either full-sib partitions or kin groups (usually a mixture of full and half sibs) depending on the user's specifications. The genotype information is used to construct pairwise likelihood ratios of being full sibs (or half sibs) vs. being unrelated for every pair of individuals in the data set. These ratios are then used to build an overall sibship partition score that is maximized by a Markov chain Monte Carlo (MCMC) algorithm (Herbinger et al., 2006). Thirty separate iterations were ran using a MCMC chain length of 1,000,000 iterations with an annealing temperature of 10 (Herbinger, 2005), a weight of 5 as a greater weight did not improve group coalescence (data not shown). The partition with the high likelihood was chosen as the "best" partition.

KINALYZER (Ashley et al., 2009) uses a combinatorial approach that searches for an optimum partition as opposed to a maximal collection of sibling sets (Berger-Wolf et al., 2007). Contrary to a statistical likelihood approach, the combinatorial approach constructs sibling groups only from Mendelian properties to search for the most parsimonious (smallest number of sibgroups) solution (Berger-Wolf et al., 2007).

Finally, the last full sibling reconstruction implements a maximum likelihood approach for a given partition that is calculated from pairwise likelihood ratios of being related vs. unrelated. A 'Descending Ratio' search algorithm groups pairs of individuals with the clearest relationships (highest likelihood of being related) first, which provides more information for subsequent and less clear assignments (Konovalov et al., 2004). A Simpson-assisted Descending Ratio heuristic algorithm was shown to be more accurate than the Descending Ratio algorithm in a simulation study (Konovalov, 2006). This method uses a Simpson index as a scoring function that optimizes the best partition of full sibling groups.

Detecting head-starts in the wild- The algorithm in COLONY was the most successful at correctly assigning individuals to families (See Results). COLONY was run using a full-likelihood analysis method assuming polygamous females, monogamous males and no inbreeding (see above). All collected adults from Bastrop and Austin counties between 2008 and 2010 were combined with samples from strands 1 and 3 (2007), 4-8 and 10 (2009) to determine the frequency of recaptured head-starts in these counties. Included in the analysis were strands 25-28 and eight adults collected from Leon Co. (see results) the same year to determine if wild individuals could be differentiated from the captive population given similar allele frequencies in the captive and wild populations from Leon Co. (see results). Allelic dropout rates and mutation rates were set to 1% and 1.5%, respectively as recommended by Wang (2004). Adults collected between 2008 and 2010 that partitioned with samples taken from captive egg strands were considered potential head-starts given congruent temporal and spatial data. To account and correct for inaccurately scored genotypes from wild caught adults, the

allelic dropout rate and mutation rate was increased to 3% and 2%, respectively. If a wild adult partitioned under these relaxed constraints, they were subjected to a second round of genotyping to confirm or falsify inconsistent alleles.

CHAPTER III

RESULTS

Head-starting captures and releases- A total of 31 egg strands were collected for head-starting from localities within extant populations of the Houston Toad (Figure 1, Table 1). Zero data were collected from strands 2 and 9 as these were never released due to species misidentification or hybridization. Data were collected from strand 14 but juveniles were never released due to high mortality at the zoo and all strands collected from Hilltop Lakes have yet to be released (Paul Crump, personal communication). Head-starting releases took place between March and September of each year. A mean of 698 individuals were released from each egg strand, the majority of which were tadpoles or newly metamorphosed juveniles (Table 1). Only a fraction of the egg strands were raised past metamorphosis. On average, 77% of an egg strand survived to metamorphosis in captivity (Table 1) and when releases were accounted for, 24.6% of metamorphs survived to one year (Table 2). A mean of 26 samples were collected from each strand for genotyping.

Adult collection- Two hundred and fifty-five toads were collected between the audio and pit fall trap surveys (Table 3, Appendix 1). Most toads were collected from

GLR as this is a highly surveyed area in the species range. Due to drought conditions relatively few toads were heard calling or collected in years 2008 and 2009, but a wetter 2009-2010 winter yielded many more in the spring of 2010. The wet winter also yielded more toads collected from Leon and Austin counties than any year prior to this study. Almost all were males given a significant male bias within the species (Swannack and Forstner, 2007).

Haplotype analysis- A 506 bp fragment from one individual in each egg strand was used for the haplotype analysis to determine the haplotype frequency among egg strands. Eight haplotypes were found in the 29 egg strands (Figure 2). The most common haplotype, houB, was recorded from eight strands. HouD was the least common haplotype recorded from one strand. Two egg strands (strands 19 and 21) had a wooA haplotype originating from *B. woodhousii*. Two *B. woodhousii* haplotypes (wooA and wooC) are relatively common in *B. houstonensis* (See McHenry, 2010), hybridization has been a common occurrence in *B. houstonensis*, but these strands do not appear to be the result of F_1 hybridization. The strands were completely void of species specific microsatellite alleles from *B. woodhousii*. More likely, these haplotypes are the remnants of hybridization events from several generations ago or possibly the retention of ancient haplotype diversity. A previously unnamed haplotype was recovered from strands 25-28 (now houI). This haplotype was previously recorded by McHenry (2010) from the only individual previously sampled from Leon Co., but a haplotype name was never designated.

Egg strand genotyping- Seven hundred and sixty eight individuals were genotyped at four or five loci. One missing genotype per individual was allowed, which resulted in 687 completely genotyped samples and 81 samples were missing information at one locus. This sums to only 1.8% missing data. Loci from a previous study were shown to be in linkage and Hardy-Weinberg equilibrium (McHenry, 2010). The number of alleles per locus varied from 1 to 6 with a mean of 2.05 alleles per locus per egg strand (Table 4). Average observed heterozygosities within egg strands ranged between 0.32 and 0.9 with an overall mean of 0.73. Based on population allele frequencies, KinInfor was able to differentiate 99.8% of simulated full sibs from unrelated individuals. The simulated power in differentiating half sibs from unrelated toads was 71% and the simulated power in differentiating full sibs from half sibs was 78%.

Genetic diversity of captive and wild populations- Samples were collected between 2001-2007 prior to head-starting. Fewer alleles were found across five loci in the captive population than in the wild population both pre and post head-starting. On average, 20 alleles per locus were present prior to head-starting while 15 alleles per locus were recovered from 2008-2010. The majority of private alleles were observed from the samples collected prior to head-starting with an average of 5.8 per locus (Table 5). The mean allelic richness of the captive population is 69% of that of the wild population (t = -5.572, df = 5, p = 0.002). This is not surprising given a minimum of 58 individuals contributed genetic information to the founding population.

Egg strands were collected among six previously identified subpopulations, Austin Co. (U), Bastrop Co. north (N), Bastrop Co. south 1 (S_1), Bastrop Co. south 2 (S_2),

Leon (LEOp), and GLR Pond 12 (BAPp). See Table 6 for a list of sampling localities within subpopulations. Both captive and wild subpopulations had similar heterozygosities (Table 7), but the allelic richness within the captive populations is significantly reduced in all subpopulations except LEOp and U (Table 8). The values of pairwise F_{ST} are significantly different among all wild and captive populations except within LEOp and U (Table 9).

Efficiency of partitioning algorithms- All partitioning algorithms were ran with the four putative egg strands from Leon Co. and without provided it is possible more than four egg strands were unintentionally collected and putative egg strand assignments may not be 100% accurate in this group. The number of sib groups reconstructed ranged between 22 and 44. Individuals were correctly assigned to their appropriate sib group between 67 and 97% of the time (Table 10). The algorithm implemented in COLONY was above all the most efficient, reconstructing 40 groups and placing individuals within an appropriate sib group 97% of the time. In a few instances, especially in strands collected from Austin Co., strand assignments were inefficient most likely because of the reduced allelic diversity in that specific subpopulation. Many of the several smaller groups consisting of one or two individuals were the result of genotyping errors (Figure 3). Finally, strand 20 appeared to be made up of more than one egg strand as several maternal and paternal genotypes can explain the multiple groups. The Simpson assisted Descending Ratio algorithm was computationally the least demanding but the least efficient. KINGROUP assigned individuals to 25 (with strands 25-28) and 20 (without strands from 25-28) sib groups. KINGROUP was more likely to group many members of different egg strands together, especially where egg strands were collected from the same

pond in the same year (e.g MSV, NAP and HTL). The algorithm implemented in PEDIGREE was efficient but because of an inability to correct for genotyping errors it was not quite as efficient as COLONY.

Frequency of recaptured head-starts- Two hundred and fifty five (60% were collected from GLR and 81% were collected within Bastrop Co.) adults were collected between the audio and pit-fall trap surveys. Four adults had data missing at more than one locus and were not included. Only one individual had a genotype 100% consistent with a head-started egg strand in Bastrop Co. (Figure 4). This adult was a non-PIT tagged male collected from GLR Pond 2, in March of 2008 that was assigned to a kin group within strand 1. The probability an individual having this exact genotype by chance was $1.31e^{-8}$ and the probability a random individual has a genotype consistent with egg strand 1 was $1.48e^{-5}$ based on global allele frequencies. Four separate releases occurred for this strand: July 9th, May 27th and September 11th of 2007 and April 30th in 2008. No individuals were PIT tagged on any release prior to September 11, 2007 (Paul Crump, personal communication). This male could have been released on July 9th or May 27th of 2007. The other strand released at GLR was strand 10 on June 4th 2009. No adults captured in 2009 or 2010 had genotypes 100% consistent with strand 10. Two individuals had similar genotypes (80-90% consistent) but these were confirmed to not belong to this group after a second round of genotyping. Seventy seven percent of all adults captured from Austin Co. partitioned with strands 5-10 including two adults collected in 2008 and eight adults collected in 2009, prior to any releases (Figure 5). This is most likely an artifact given that there is not a significant difference in the number or frequency of alleles in the captive and wild population in Austin Co. A similar result was recorded among the

captive Leon Co. population and the eight individuals caught in Leon Co. the same year. Six of the eight adults collected in 2010 partitioned with the captive population as full siblings (Figure 6).

CHAPTER IV

DISCUSSION

Here I present information evaluating the efficacy of head-starting the Houston toad. The population genetics of the Houston toad was well studied in McHenry (2010). The study gave insight into the genetic diversity of all extant populations and provided a baseline for future comparisons. I have gathered data that provided more insight into this species including mating ecology, juvenile survivorship and the retention of genetic diversity in the captive population. Generally speaking, the recently generated captive population has reduced allelic diversity compared to the wild population. Pedigree reconstruction resulted to an efficient method of assigning individuals to appropriate sibgroups and juveniles head-starting has not made a significant impact on the abundance of Houston toads as only one head-start was recollected the year following release.

Presence of multiple male fertilization- Before 2000, only four reports of polyandry existed for amphibians (Jennions and Passmore, 1993; D'Orgeix and Turner, 1995; Laurila and Seppä, 1998; Roberts et al., 1999). Within the last decade codominant molecular markers like microsatellites have become almost ubiquitously used to describe polyandry in vertebrates (Byrne and Roberts, 2000; Foerster et al., 2003; Herbinger et al.,

2006; McVay et al., 2008; Knopp and Juha, 2009). As many amphibians fertilize their eggs externally, it is not surprising that egg clutches can be fertilized by multiple males (Byrne and Roberts, 2000; Sztatecsny et al., 2006; Doody et al., 2009). Modeling and empirical evidence has shown polyandry is a strategy which can increase the heterozygosity and effective population size (Sugg and Chesser, 1994; Foerster et al., 2003). I present evidence that suggests like many other anurans, polyandry is a part of the mating ecology in *B. houstonensis*. As there is a higher degree of allelic diversity and heterozygosity than one would expect given the low population numbers, this may account for the present allelic diversity.

Data from five (strands 1, 7, 14, 15 and 23) out of the 25 available strands for accurate analysis suggest two or more fathers could have contributed to egg clutches (Figure 3). At least 24% of each egg strand was sired by at least one peripheral male. This frequency of multiple paternity is similar to other anurans (Lodé and Lesbarrères, 2004; Knopp and Juha, 2009). All of these egg strands have more alleles/locus than expected under the assumption of single parental pairs and more than one full sibling group was constructed in all of these strands. It is unclear whether egg strands are directly fertilized by multiple amplectant fathers or if this is the result of indirect fertilization from a nearby amplectant pair. Oppositely, this result could be an artifact of egg strand mishandling during and after collection as two or more egg strand fragments being unintentionally collected (this most likely happened in 20 and 25-28), or inaccurate handling and donation could yield conflicting sibship assignments which did occur in the first year of head-starting, but was later resolved. Regardless of whether this is polyandry as a natural phenomenon or an artifact, this issue must be resolved and its consequences

incorporated into the head-starting efforts and should start with more precise handling of egg strands.

While human error could account for these results, a shared parental genotype was reconstructed in egg strands with multiple full sibling groups. Two separate egg strands collected as one typically do not meet this requirement needed to support polyandry. In strands 1, 7, 14, 15 and 23 this requirement is met suggesting polyandry is a probable explanation of these observations.

A detailed study of amplexus and polyandry has yet to be undertaken for the Houston toad. Five males in Bastrop State Park have been observed in amplexus with a single female (J.R. Dixon, personal communication). Here, multiple paternity was recorded throughout a majority of the extant range of *B. houstonenesis* (GLR, BSP, MSV and NAP). Interestingly, all the strands that exhibit multiple male fertilizations except strand 1 were collected after a large chorus when multiple pairs were seen in amplexus and several clutches were collected. It is unclear whether these strands were fertilized by multiple amplectant males or indirectly fertilized by free floating sperm from a neighboring amplectant pair. Interestingly, Sztatecsny et al. (2006) found multiple male amplexus was more likely to occur in high male density in *Bufo bufo*. The data collected from this study should be corroborated by both field and lab experiments to understand exactly how multiple males are contributing genetic information as secondary males were never witnessed in amplexus with the founding females. As an example of unique mating strategies, multiple paternity was detected in *Rana temporaria* and the proposed explanation was free floating sperm in the water column (Laurila and Seppä, 1998). After

several years of study, it was revealed that “pirate” males directly amplexing with an egg clutch explained how peripheral males contributed to the progeny (Vietes et al., 2004). This is not as likely to explain what is happening in *B. houstonensis*, but emphasizes the importance of determining the exact mechanism of multiple paternity in *B. houstonensis* as a detailed record of mating strategies do not exist for this species. This is an important observation in regards to head-starting. If it is a sampling and an organization artifact, it needs to be addressed and corrected in the future. If it is a natural phenomenon and a breeding colony is established, multiple males from the same subpopulation should be allowed to mate with a female in efforts to increase the effective population size in the breeding colony and preserve genetic diversity.

Genetic diversity of captive and wild populations- There are subpopulations throughout the range of *B. houstonensis* with both high and low allelic richness (McHenry, 2010). More specifically, subpopulations within Bastrop Co. have the highest levels of genetic variation. Also to note, both captive and wild populations have similar heterozygosities but the allelic richness of the captive population was 67% of the wild. The current captive population contains the product of at least 58 founding pairs. Nei et al. (1975) showed that for two loci, 100 individuals sampled from a founded population had 99.5% of the average herterozygosities of the parental population but only 42-53% of its allelic richness. A single egg strand, even if sired by multiple males can only represent a very small fraction of the wild genetic diversity. The number of strands collected within subpopulations ranged between 1 and 9 (See Table 6). Thus a general pattern arises; the more egg strands collected from a subpopulation, the more alleles are represented in the captive population and increasing allelic richness within the captive population may

simply be a function of increasing the number of egg strands collected within subpopulations. The captive populations derived from Austin and Leon counties are the most similar to the wild. Unfortunately, this is not necessarily a function of increased diversity in the captive population but decreased diversity in the wild. Compared to the subpopulations throughout Bastrop Co., Austin and Leon counties are genetically impoverished.

The captive populations are genetically dissimilar from each other and wild populations, except in Leon and Austin counties (Table 9). The captive and wild populations of Austin and Leon counties are so similar, it is nearly impossible to detect differences among wild and captive reared individuals (Figure 5 and 6). This raises a concern about monitoring these supplemented populations through time. The system I have developed here is not powerful enough to recognize population changes associated with the current system of population propagation. Head-starting can only be assessed in these areas through traditional mark-recapture techniques.

It is common for captive populations to experience a reduction in genetic diversity (Winsely et al., 2003; Forstmeier et al., 2007). Captive breeding colonies are subject to strong founder affects, high inbreeding and relaxed selection that allows deleterious alleles that are normally selected against to accumulate and potentially fix in the captive population and persist in the wild (Lynch and O'Hely, 2001; Woodworth et al., 2002). It is too soon to tell explicitly, but head-starting may be a preferential way of buffering a captive population from these genetic problems if the goal is to maximize genetic variation in the captive population. By collecting offspring from wild pairs and releasing

them at relatively early life stages (metamorphosed juveniles and intermediates) selection can still act on deleterious alleles before head-starts are capable of reproducing. As of now, *B. houstonensis* do not breed in captivity naturally, but only through hormonal injection (Quinn and Mengden, 1984). It may turn out this is an unappreciated advantage as the captive population is continuously being repopulated with wild bred individuals and the recurrent stocking and releasing may help protect the wild population from the adverse genetic effects of a captive bred population. However, it is still possible that head-starting a wild strand can potentially over-represent the recruitment for those strands in the wild. Thus, an assessment of the genetic outcomes in the wild over multi-generational time will likely need to be modeled to ensure that this is not simply another mechanism for negatively impacting the variation in the wild populations.

Assessment of head-starting detection method- This power of differentiating full siblings vs. unrelated toads was 0.998. Using COLONY, sibship was correctly assigned 97% of the time. Given there were 25 accurately collected families, the efficiency of COLONY provides confidence when a wild individual is assigned to an apparent sibgroup. It is the most computationally expensive (which some authors criticize), but given all other choices, the program is currently the best available although it would be wise to test new partitioning software as it is being developed.

This program is efficient for several reasons. First, among these five loci, there is sufficient diversity within the population (and among egg strands) to efficiently identify sibling relationships. When data are insufficient, family sizes are overestimated and relationship inferences are inaccurate. Because COLONY uses a full likelihood approach

that takes all sample information into consideration when building a partition, the larger the family sizes, the more information is contributed to relationship inferences (Jones and Wang, 2009). Interestingly, all individuals of strand 24 were accurately grouped regardless of the small sample size (n = 8). For future studies, sample sizes that range around 25-30 individuals/egg strand provide more than adequate information for reconstructing family relationships and also increase the probability of detecting and accounting for multiple paternity. Finally, the efficiency of COLONY is improved because the algorithm recognizes and corrects for genotyping errors that are frustratingly common in large genotypic datasets like this (Pompanon et al., 2005).

This method of detecting recaptured head-starts is appropriate where genetic diversity is present and the probability of an individual randomly partitioning with a sibgroup by chance is low. Unfortunately, the probability of unrelated toads being assigned to the captive population is high (~77%) in both Austin and Leon counties. Thus, the genetic mark recapture method is not appropriate in these areas. It could be possible if more loci were sampled, but allelic diversity at additional loci would be expected to be similarly reduced and the effectiveness would remain the same. For this method to truly be effective, some aspects need to be taken into consideration. First, collected strands need to be handled carefully. I sampled less than 1% of a collected egg strand for genotyping. If multiple egg strands are collected as one, there is a realistic probability that unintentionally collected egg strands could be grown and released undetected, thus under representing the number of head-started toads present in the wild. Second, this method should be corroborated with both spatial and temporal data. As adult and egg strand sample sizes increase it is more likely wild caught adults will partition

with a sibgroup by chance but when temporal and spatial data are accounted for, these individuals can be excluded if data are inconsistent.

Juvenile survivorship- Through this study I had the availability to estimate the over winter survivorship of post metamorphic juveniles. This is the first empirical estimate of survivorship in this age group. Greuter (2004) estimated the frequency of survivors from fertilization to metamorphosis and also from metamorphosis to 13 weeks. She estimated a 4.6% probability for any fertilized egg to survive to metamorphosis. She also recognized the survivorship from metamorphosis to 13 weeks was about 84%. Estimated male survivorship was shown to be between 0.15 and 0.27 through mark-recapture survey data from 2001-2004 (Swannack, 2007) and the yearly female survivorship has been calculated to be 0.2 (Hatfield et al., 2004). Through modeling and algebra, Swannack (2009) estimated the probability for a metamorphosed juvenile to survive to maturity was 0.15. Given that the majority of head-starts were immature juveniles, I can indirectly measure survivorship of this age group from this genetic mark-recapture study.

GLR is the most intensely examined area at the current time and the consequence of this is increased data depth and more intense population supplementation efforts. At GLR two egg strands were head-started between 2007 and 2009. Three hundred and nineteen juveniles were released from strand 1 in 2007 and 660 from strand 10 were released at pond 13 in 2009. It is very likely that one individual that was released in the spring of 2007, had matured by spring 2008 and was found courting at its natal pond. No other head-starts released prior to 2010 were recaptured. Strand 10 was not generated

until April 19, 2009 (Table 1) and maturation time for male *B. houstonensis* is 12 months. It is possible that a majority of releases had not fully matured by the breeding season in 2010 and are not represented. This study provides a new estimation of over winter juvenile survivorship that is significantly lower than previous estimations, 0.001 or 0.003 if the released toads from the 2009 egg strand were not present during the breeding season. There is uncertainty surrounding these estimates, given that this is a direct frequency and juvenile dispersal or variation in maturation rates and an exact probability of recapture was not accounted for. Also, during 2008 and 2009, drought conditions were at their worst ever in southeast Texas (Nielsen-Gammon and McRoberts, 2009) adversely affecting Houston toad reproduction and potentially juvenile survivorship, consequently skewing this survivorship estimate. Ongoing work should support or refute this preliminary estimate.

Typically, if genotyping errors are present, they happen at only one locus, very rarely would two genotyping errors exist in the same individual. If an allele was miscalled in a wild adult and incorrectly classified as a non-captive, when it was in fact a recaptured head-start, increasing the mutation or allelic dropout rate should have forced an individual to partition with its appropriate family. This did occur, in two individuals (see Results), but after subsequent genotyping they were confirmed to be derived from the wild population.

There are obviously significant differences between the prior estimations of juvenile survivorship and mine. At the Houston zoo, the average percent survivorship from fertilized egg to metamorphosed juvenile was 77% and the average survivorship of

metamorphosed juveniles to mature adults was 24.6%. These estimates can be considered background survivorship estimates. Captive juveniles are not subjected to predation, competition is explicitly minimized and disease is controlled. The only issue the captive juveniles face is significant crowding compared with the wild individuals of similar sizes. Under optimum conditions, eggs were 3.1 times more likely to reach metamorphosis than metamophs were to reach one year. Given the wild survivorship from egg to metamorph was 5% (0.061 of the optimum) the previous juvenile 15% over winter survivorship frequency may be significantly overestimated.

This genetic mark recapture method was a unique attempt to gain an over winter survivorship for metamorphic juveniles. My estimations were similar to a previous study in *Bufo woodhousii fowleri* where only 0.4% of marked metamorphs were recaptured the following year as adults (Breden, 1987). In many instances, juveniles have been shown to be the most vulnerable age group in the Houston toad and reduced survivorship of this age group can likely lead to extinction (Greuter, 2004; Hatfield et al., 2004; Swannack, 2007). My data for this estimation were collected during the worst drought on record in Bastrop Co. which may be an indicator of how drought can adversely affect recruitment in the Houston toad. Hatfield (2004) calculated that if recruitment is less than 1% of the total reproduction, populations have a high probability of extinction. The results from this study may reveal one of the factors by which drought is a significant threat to the Houston toad.

Recommendations for future head-starting- The majority of head-start releases were either tadpoles or juveniles (Table 1). Given the low survivorship of both tadpoles

(Greuter, 2004) and juveniles, it may be inefficient or unpractical to release these age groups. In 2010, more than 5,000 tadpoles and juveniles were released at GLR and MSV, five times the number of individuals released between 2007 and 2009. The data collected from the breeding season on 2011 would be helpful to estimate the success. Unfortunately significant chorusing or breeding has not occurred. This makes it impossible to assess the overwinter survivorship and efficiency of the 2010 juvenile head-starts.

Modeling has suggested the most advantageous method of head-starting for the Houston toad is adult female releases (Dunham et al., unpublished data). Although this may help increase population sizes, this strategy may be genetically the unhealthiest assuming head-starts have a higher survivorship than their wild counterparts. Given the low overall allelic richness of the captive population to the wild population, introducing a few individuals per year with the maximum survivorship may result in an unintended genetic bottleneck leaving the species more vulnerable to a changing environment and disease in an already declining species.

In areas like Leon and Austin counties where population numbers and allelic richness is low, it appears there is not a significant difference in richness between the wild and captive populations so it might be an acceptable plan to continually supplement these wild populations with adults to keep *B. houstonensis* on the landscape in these areas. Regardless, to create healthy, self-sustaining populations in these areas, habitat restoration is required. Continuously supplementing a population where resources are

unavailable for population growth will not improve abundance (Diemer, 1986; Dodd and Seigel, 1991).

This is the first attempt to monitor and track captive reared juvenile amphibians. Previous head-starting programs have not implemented a way to monitor supplemented populations beyond presence/absence. The next step in this head-starting program is to test the efficiency of releasing captive reared adults. Head-starting and captive breeding has had variable degrees of success in amphibians (Dodd and Seigel, 1991; Griffiths and Pavajeau, 2008). Here I have addressed the problem of monitoring life stages that previously couldn't be tracked. This genetic mark recapture method could be implemented in other amphibian head-starting or captive breeding programs in an effort to accurately assess the impact supplementation has had on wild populations. This technique provided insight into the juvenile ecology previously unattainable and provided evidence of multiple males contributing genetic information to egg strands. Most importantly, this study illustrates the importance of assessing these populations to determine the impact of population propagation, as head-starting juveniles has not been efficient and a different approach should be evaluated.

Table 1. Locality and release description for captive egg strands. The total number released is the total number of individuals released up to this study. The %tadpole, %juvenile and %adult describe the proportion of the release consisting of each age group and the sample size (*n*) describes how many were sacrificed for genotyping in this study.

Egg Strand	County	Specific Locality	Date Collected	Latitude	Longitude	Estimated collected	Number released	%tadpole	%juvenile	%adult	*n*
1	Bastrop	GLR P-2	3/14/2007	30.216471	-97.241783	1000	384	-	83	17	80
3	Bastrop	BBE P-3	3/14/2007	30.216261	-97.241722	700	151	-	93	7	20
4	Bastrop	BSP P-19	3/20/2009	30.090160	-97.238510	1300	936	-	100	-	14
5	Austin	NAP P-1	4/18/2009	29.883560	-96.361229	1700	968	-	100	-	23
6	Austin	NAP P-1	4/18/2009	29.883560	-96.361229	1250	336	-	100	-	22
7	Austin	NAP P-1	4/18/2009	29.883560	-96.361229	1500	622	-	100	-	17
8	Austin	NAP P-1	4/18/2009	29.883560	-96.361229	1300	672	-	100	-	18
10	Bastrop	GRL P-13	4/19/2009	30.188950	-97.232536	3300	660	-	100	-	66
11	Bastrop	BSP P-11	2/21/2010	30.114355	-97.276980	1000	651	70	30	-	15
12	Bastrop	BSP P-8	2/21/2010	30.095699	-97.238586	1400	865	80	20	-	19
13	Bastrop	BSP P-8	2/21/2010	30.095699	-97.238586	1400	805	96	4	-	20
14	Bastrop	BSP P-8	2/22/2010	30.095699	-97.238586	1200	-	-	-	-	23
15	Bastrop	BSP P-8	2/22/2010	30.095699	-97.238586	2100	1528	91	9	-	18
16	Bastrop	BSP P-14	2/22/2010	30.107121	-97.241810	900	350	100	-	-	22
17	Bastrop	BSP P-14	2/22/2010	30.107121	-97.241810	1400	520	100	-	-	21
18	Bastrop	GLR P-9	2/22/2010	30.199181	-97.221970	1600	1275	100	-	-	30
19	Bastrop	MSV P-1	3/4/2010	30.245670	-97.221352	700	585	100	-	-	26
20	Bastrop	MSV P-1	3/4/2010	30.245670	-97.221352	800	675	100	-	-	20
21	Bastrop	MSV P-1	3/4/2010	30.245670	-97.221352	1300	120	100	-	-	23
22	Bastrop	JMS P-4	3/6/2010	30.126381	-97.239342	500	329	-	100	-	27
23	Bastrop	GLR P-12	3/6/2010	30.194889	-97.243584	900	331	-	100	-	32
24	Bastrop	GLR P-5	3/7/2010	30.209320	-97.242912	1200	1000	100	-	-	8

Table 1-Continued

Egg Strand	County	Specific Locality	Date Collected	Latitude	Longitude	Estimated collected	Number released	%tadpole	%juvenile	%adult	*n*
25	Leon	HTL P-1	3/9/2010	31.055962	-96.161090	3100	-	-	-	-	42
26	Leon	HTL P-1	3/9/2010	31.055962	-96.161090	2500	-	-	-	-	37
27	Leon	HTL P-1	3/9/2010	31.055962	-96.161090	2500	-	-	-	-	36
28	Leon	HTL P-1	3/9/2010	31.055962	-96.161090	2500	-	-	-	-	24
29	Austin	NAP P-1	3/26/2010	29.883560	-96.361229	1400	399	-	100	-	20
30	Austin	NAP P-1	3/27/2010	29.883560	-96.361229	2100	-	-	-	-	16
31	Bastrop	GRL P-13	4/18/2010	30.188950	-97.232536	2100	1908	-	100	-	29

Table 2. Zoo survivorship. Metamorphosis and 1st year survivorship data for 15 egg stands reared at the Houston Zoo. The 1st year survivorship was estimated from the number of retained juveniles.

Strand ID	Eggs	Completed metamorphosis (%)	Released	Retained	Survived 1st year (%)
1	1000	84.6	384	462	21.6
3	700	61.1	151	277	16.6
4	1300	90.3	936	238	25.2
5	1700	90.8	968	575	10.4
6	1250	92.2	336	816	19.0
7	1500	86.1	622	670	9.6
8	1300	92.6	672	532	19.0
10	3300	91.3	660	2354	37.3
22	500	94.4	329	143	32.2
23	900	73.3	331	329	14.9
25*	3100	95.2	-	NA	29.4
26*	2500	86.6	-	NA	32.8
27*	2500	91.3	-	NA	45.2
28*	2500	46.6	-	NA	30.2
29	1400	45.4	399	237	25.3

* Strands have not been released thus 1st year survivorship was estimated from the number total number of juveniles that completed metamorphosis

Table 3. Adult collection. The number of samples collected for this study by specific locality and year.

County	Site	Latitude	Longitude	2008	2009	2010
Austin						
	FM 1084	29.8725	-96.3639	1		
	Hall Rd	29.8839	-96.3616	1		
	McMurray Pond	29.8799	-96.3596		2	3
	Nava Pond	29.8836	-96.3612		9	17
	TWC Pond	29.8779	-96.3529	4		
	Waldrop Pond	29.8900	-96.3624			1
Bastrop						
	Bob Long	30.1423	-97.1958			1
	Clay Pond B	30.0000	-97.0000			4
	Dube Ln	30.2375	-97.2115			4
	GLR L2	30.2166	-97.2417			2
	GLR L2-E	30.2166	-97.2417			1
	GLR L7	30.2125	-97.2301		1	
	GLR L9	30.1989	-97.2219			1
	GLR L10	30.1981	-97.2133			1
	GLR L12	30.1947	-97.2442		1	14
	GLR L12-N	30.1955	-97.2436			2
	GLR L16	30.1693	-97.2324			1
	GLR-P2	30.2163	-97.2417	20	1	2
	GLR P4	30.0000	-97.0000			3
	GLR-P5	30.2093	-97.2429		2	8
	GLR-P6 A1	30.2145	-97.2328	2		
	GLR-P7	30.2124	-97.2300		1	4
	GLR P8	30.2056	-97.2342			1
	GLR-P9	30.1992	-97.2220		1	13
	GLR P10	30.1978	-97.2133			1
	GLR P10-A2	30.1995	-97.2104			1
	GLR P11	30.2020	-97.2090			5
	GLR-P12	30.1949	-97.2436		6	37
	GLR P12-A1-B1	30.1961	-97.2437	1		
	GLR-P13	30.1889	-97.2325		4	3
	GLR P-14	30.1787	-97.2325		1	
	GLR P14-A3	30.1772	-97.2342			1
	GLR P15	30.1779	-97.2338			2
	GLR T7	30.2162	-97.2306			2
	GLR T10-1	30.2001	-97.2227			1

Table 3- Continued

County	Site	Latitude	Longitude	2008	2009	2010
	GLR T10-5	30.2002	-97.2214			1
	GLR T15	30.1961	-97.2437			1
	GLR TB	30.2105	-97.2383			1
	GLR TC	30.2099	-97.2400			1
	JMS P2	30.1377	-97.2434			7
	JMS P3	30.1400	-97.2425			3
	JMS P4	30.1264	-97.3934			12
	JMS P5	30.1263	-97.2337			5
	MSV	30.2457	-97.2216			19
Leon						
	HTL	31.0670	-96.1712			10

Table 4. Egg strand genetic diversity. The number of alleles per locus (*A*) and observed heterozygosities (H_O) are recorded for each egg strand.

Strand	BBR36		BC52.10		bco15		BM224other		IHHH		Mean	
	A	H_O	*A*	H_O	*A*	H_O	*A*	H_O	*A*	H_O	*A*	H_O
1	4	0.70	6	0.57	5	0.97	3	0.81	4	0.57	3.43	0.60
3	3	0.90	2	0.56	4	1.00	4	1.00	4	1.00	3.00	0.74
4	2	0.00	3	0.71	2	0.50	3	0.71	4	0.93	2.71	0.48
5	3	0.61	3	0.70	3	0.95	3	0.70	3	0.70	3.00	0.61
6	3	0.24	3	0.71	4	0.95	3	0.91	2	0.59	3.14	0.57
7	5	0.82	3	0.56	5	0.88	3	0.71	5	1.00	4.14	0.66
8	3	0.83	2	0.35	3	0.41	3	0.94	3	1.00	3.29	0.59
10	2	0.44	4	0.65	2	1.00	4	0.98	3	0.76	3.71	0.64
11	3	1.00	4	1.00	3	0.93	2	0.60	4	1.00	4.00	0.76
12	3	0.68	3	0.61	4	0.84	3	0.68	4	1.00	4.29	0.64
13	3	0.80	2	0.53	3	1.00	3	1.00	4	1.00	4.14	0.72
14	4	0.91	5	0.95	4	0.95	3	0.78	3	0.95	3.33	0.76
15	4	0.47	5	0.65	5	0.89	3	0.83	3	0.76	3.50	0.60
16	3	0.95	3	0.73	4	1.00	3	0.73	2	1.00	2.67	0.73
17	3	1.00	2	0.24	3	1.00	3	1.00	3	0.52	2.50	0.63
18	4	1.00	4	1.00	4	1.00	2	0.30	4	1.00	3.17	0.72
19	4	1.00	3	1.00	3	1.00	3	0.85	2	0.31	2.67	0.69
20	6	1.00	5	0.65	3	0.65	2	0.45	2	0.40	3.17	0.53
21	2	0.57	3	0.55	4	1.00	3	0.78	3	0.78	2.67	0.61
22	3	0.63	3	1.00	2	0.48	1	0.00	2	0.48	2.00	0.43
23	2	0.53	2	0.53	2	0.34	2	0.50	3	0.81	2.17	0.47
24	4	0.88	4	0.88	4	1.00	2	0.50	4	1.00	3.00	0.73
25	4	0.97	4	0.97	5	0.80	2	0.51	3	0.26	3.33	0.48
26	4	0.27	4	0.27	3	0.40	2	0.60	2	0.06	2.50	0.31
27	4	0.88	3	0.83	3	0.77	2	0.39	4	0.97	2.83	0.64
28	6	0.75	5	0.25	5	0.41	2	0.39	4	0.58	3.83	0.40
29	3	0.79	2	0.47	1	0.00	3	0.80	3	0.95	2.17	0.50
30	4	0.94	3	1.00	4	1.00	2	0.50	3	1.00	2.83	0.74
31	4	1.00	3	0.79	3	0.79	2	0.96	4	1.00	2.83	0.76
Total	19	NA	11	NA	9	NA	6	NA	18	NA	10.67	NA
Mean	2.20	0.74	2.09	0.67	2.18	0.79	1.70	0.69	2.06	0.77	1.79	0.61

Table 5. Single locus statistics for wild and captive *B. houstonensis*. Sample size (*n*), number of alleles (*A*), number of private alleles (A_P), allelic richness (A_R), expected (H_E) and observed (H_O) heterozyosities and F_{IS} are reported. Allelic richness is based off of 245 individuals. Asterisks represent significant deviations from HWE before Bonferroni correction.

Locus	all individuals	2001-2007	HS	2008-2010
BBR36				
n	1418	417	751	250
A	27	25	19	21
A_P	0	5	0	1
A_R	22.51	23.90	18.94	21.00
H_E	0.91	0.91	0.91	0.90
H_O	0.67	0.62	0.72	0.61
F_{IS}	0.26*	0.32*	0.21*	0.32*
BC52.10				
n	1416	434	737	245
A	17	17	11	12
A_P	0	5	0	0
A_R	13.37	15.49	10.91	12
H_E	0.89	0.89	0.89	0.87
H_O	0.61	0.55	0.65	0.58
F_{IS}	0.32*	0.38*	0.26*	0.34*
bco15				
n	1441	433	753	255
A	15	15	9	9
A_P	0	6	0	0
A_R	11.14	13.90	9	9
H_E	0.86	0.87	0.86	0.86
H_O	0.77	0.71	0.80	0.77
F_{IS}	0.11*	0.18*	0.07*	0.11*
BM224other				
n	1446	435	761	250
A	14	12	6	9
A_P	0	5	0	2
A_R	8.52	10.31	6.00	8.94
H_E	0.72	0.76	0.69	0.72
H_O	0.66	0.60	0.69	0.68
F_{IS}	0.08*	0.21	-0.01	0.05
IHHH				

Table 5-Continued

Locus	all individuals	2001-2007	HS	2008-2010
n	1439	434	754	251
A	33	31	19	24
A_P	0	8	1	1
A_R	27.23	28.10	18.28	23.88
H_E	0.87	0.85	0.87	0.84
H_O	0.71	0.67	0.72	0.75
F_{IS}	0.18*	0.21*	0.17*	0.11*
Average				
A	21.20	20	12.80	15
A_P	0	5.80	0.20	0.8
A_R	16.55	18.34	12.63	14.96
H_E	0.85	0.85	0.84	0.84
H_O	0.68	0.63	0.72	0.68
F_{IS}	0.19*	0.26*	0.14*	0.18*

Table 6. Samples collected from subpopulations. Sites within subpopulations and concurrent egg stands collected from each subpopulation. The number of samples from the captive population used is listed under the column Captive *n.* The column Wild *n* lists all of the wild samples collected from each subpopulation from 2001-2010.

Subpopulation	Sites within	Strands collected within sites	Captive *n*	Wild *n*
BAPp	GLR P12	23	32	85
LEOp	Hilltop Lakes	25-28	139	8
N	GLR except P12	1, 10, 18, 24, 31	282	337
	MSV	19-21		
S1	BSP P8	12-15	179	81
	BSP P11	11		
	BSP P19	4		
	BSP P14	16, 17		
	JMS P2			
	JMP P3			
	JMP P4	22		
S2	BBE	3	20	74
	JMS P1			
	JMS P5			
	BSP P1			
	BSP P10			
U	All Austin Co.	5-8, 29, 30	116	39

Table 7. Comparison of genetic diversity from wild and captive subpopulations. The sample size (*n*), number of alleles (*A*) and private alleles (A_P), allelic richness (A_R), expected (H_E) and observed (H_O) heterozygosities and F_{IS} are listed. F_{IS} in bold indicate significance departures from HWE before sequential Bonferroni correction for multiple tests. Allelic richness is corrected for the smallest sample size in each paired comparison of captive and wild populations. Refer to Table 6 for sampling localities.

Locus	U		N		S_1		S_2		LEOp		BAPp	
	Captive	Wild	Captive	Wild	Captive	Wild	Captive	Wild	Captive	Wild	Captive	Wild
BBR36												
n	87	39	280	320	177	81	20	74	123	8	32	82
A	7	5	12	22	10	19	3	15	5	5	2	15
A_P	2	1	0	10	0	9	0	12	0	0	0	13
A_R	5.825	5	11.978	21.812	9.975	18.950	3	11.989	4.229	5	2	10.425
H_E	0.711	0.710	0.859	0.912	0.781	0.878	0.678	0.898	0.756	0.817	0.396	0.663
H_O	0.609	0.744	0.743	0.616	0.740	0.778	0.900	0.689	0.691	0.875	0.531	0.301
F_{IS}	**0.144**	**-0.048**	**0.135**	**0.325**	**0.053**	**0.115**	**-0.339**	**0.234**	**0.086**	**-0.077**	**-0.348**	**0.547**
BC52.10												
n	85	36	274	330	172	81	18	74	127	8	31	84
A	3	4	8	14	10	14	2	11	5	4	3	10
A_P	0	1	0	6	1	5	0	9	1	0	0	7
A_R	3	4	8	13.802	9.941	13.963	2	8.976	3.720	4	3	8.868
H_E	0.560	0.447	0.846	0.875	0.826	0.894	0.413	0.829	0.642	0.642	0.675	0.840
H_O	0.588	0.444	0.712	0.512	0.715	0.802	0.556	0.662	0.488	0.875	0.613	0.536
F_{IS}	**-0.050**	**0.006**	**0.159**	**0.414**	**0.135**	**0.103**	**-0.360**	**0.203**	**0.240**	**-0.400**	**0.094**	**0.364**
bco15												
n	86	39	277	336	177	81	20	74	131	8	32	85
A	5	6	8	11	9	10	4	12	6	4	2	11
A_P	0	1	0	3	0	1	0	8	2	0	0	9
A_R	5	5.923	8	10.782	8.992	9.975	4	8.931	3.485	4	2	9.861

Table 7-Continued

Locus	U		N		S_1		S_2		LEOp		BAPp	
	Captive	Wild	Captive	Wild	Captive	Wild	Captive	Wild	Captive	Wild	Captive	Wild
H_E	0.747	0.756	0.857	0.842	0.831	0.848	0.768	0.858	0.688	0.600	0.289	0.864
H_O	0.733	0.615	0.946	0.708	0.842	0.827	1	0.811	0.617	0.625	0.344	0.753
F_{IS}	**0.020**	**0.186**	**-0.104**	**0.158**	**-0.013**	**0.025**	**-0.313**	**0.055**	**0.103**	**-0.045**	**-0.192**	**0.129**
BM224other												
n	88	39	279	233	179	81	20	74	130	8	32	85
A	3	3	4	13	5	8	4	5	2	3	2	6
A_P	0	0	0	9	0	3	0	1	0	1	0	4
A_R	3	3	4	12.375	5	7.988	4	4.982	1.997	3	2	5.346
H_E	0.622	0.633	0.704	0.725	0.647	0.673	0.736	0.759	0.415	0.575	0.508	0.683
H_O	0.818	0.632	0.778	0.673	0.682	0.519	1	0.689	0.477	0.625	0.500	0.553
F_{IS}	-0.316	0.003	0.085	0.154	-0.053	0.231	-0.372	0.093	-0.149	-0.296	0.016	0.191
IHHH												
n	87	39	276	333	176	81	19	74	131	8	32	85
A	6	9	9	27	11	18	4	15	5	4	3	15
A_P	0	3	0	18	0	7	0	11	1	0	0	11
A_R	5.895	8.764	9	26.246	10.876	18	4	11.260	4.379	4	3	11.781
H_E	0.758	0.810	0.760	0.817	0.808	0.885	0.767	0.867	0.724	0.758	0.649	0.831
H_O	0.816	0.718	0.696	0.691	0.830	0.688	1	0.824	0.435	1	0.813	0.753
F_{IS}	**-0.077**	**0.114**	**-0.104**	**0.072**	**-0.026**	**0.224**	**-0.315**	**0.050**	**0.400**	**-0.349**	**-0.256**	**0.094**
All												
n	88	39	282	336	179	81	20	74	134	8	32	85
A	24	27	41	87	45	69	17	58	23	20	12	57
A_P	2	6	0	51	1	26	0	41	4	1	0	46
Mean H_E	0.566	0.559	0.671	0.705	0.649	0.698	0.560	0.702	0.537	0.565	0.420	0.653
Mean H_O	0.594	0.525	0.646	0.543	0.635	0.604	0.743	0.613	0.451	0.667	0.467	0.489
F_{IS}	**-0.090**	**0.054**	**0.017**	**0.208**	**0.011**	**0.151**	**-0.343**	**0.123**	**0.125**	**-0.207**	**-0.092**	**0.242**

Table 8. Allelic retention. The allelic retention among pairs of captive and wild subpopulations. *P* values were derived from a paired t-test. Percent retention is the mean captive allelic richness divided by the wild mean richness.

Population		mean allelic richness	p value	% retention
BAPp				
	captive	2.40	0.0060	25.92
	wild	9.26		
LEOp				
	captive	3.56	0.1385	89.00
	wild	4.00		
N				
	captive	8.10	0.0122	48.20
	wild	17.00		
S1				
	captive	8.96	0.0260	65.02
	wild	13.78		
S2				
	captive	3.40	0.0220	36.85
	wild	9.22		
U				
	captive	3.95	0.2602	85.71
	wild	4.61		
Total				
	captive	12.63	0.0020	68.84
	wild	18.34		

Table 9. Pairwise F_{ST} for all captive and wild populations. Wild populations were previously assigned in McHenry (2010). All pairwise values are significant ($p < 0.05$) except those listed with an asterisk.

Group	Uhs (n = 88)	Uwild (n = 39)	LEOphs (n = 134)	LEOpwild (n = 8)	Nhs (n = 282)	Nwild (n = 337)	S_1hs (n = 179)	S_1wild (n = 81)	S_2hs (n =20)	S_2wild (n = 74)	BAPphs (n =32)	BAPpwild (n = 85)
Uhs	-											
Uwild	0.0074*	-										
LEOphs	0.22942	0.24977	-									
LEOpwild	0.21743	0.22645	0.03705*	-								
NHS	0.13843	0.13113	0.15506	0.10522	-							
Nwild	0.11377	0.10784	0.11387	0.0722	0.01539	-						
S_1hs	0.16588	0.16663	0.1594	0.12596	0.08578	0.06575	-					
S_1wild	0.1355	0.1324	0.1315	0.09477	0.05227	0.03633	0.01866	-				
S_2hs	0.25478	0.26326	0.2661	0.22198	0.14405	0.1269	0.12918	0.11864	-			
S_2wild	0.15631	0.15417	0.15039	0.09614	0.0382	0.03154	0.05178	0.02148	0.09026	-		
BAPphs	0.33126	0.33696	0.38669	0.37013	0.17373	0.18554	0.25364	0.23753	0.30709	0.21108	-	
BAPpwild	0.1743	0.15954	0.19559	0.13712	0.04256	0.0453	0.11896	0.09154	0.16395	0.06532	0.1174	-

Table 10. Sibship efficiency. The number of groups and efficiency of each partitioning algorithm each ran with and without the Leon Co. egg strands.

Partitioning Program	Number of Groups	Individuals correctly assigned (%)
Without HTL		
COLONY	40	96.82
KINGROUP	22	67.75
KINALYZER	40	86.50
PEDIGREE	45	94.00
With HTL		
COLONY	50	93.75
KINGROUP	25	66.14
KINALYZER	52	83.85
PEDIGREE	58	89.32

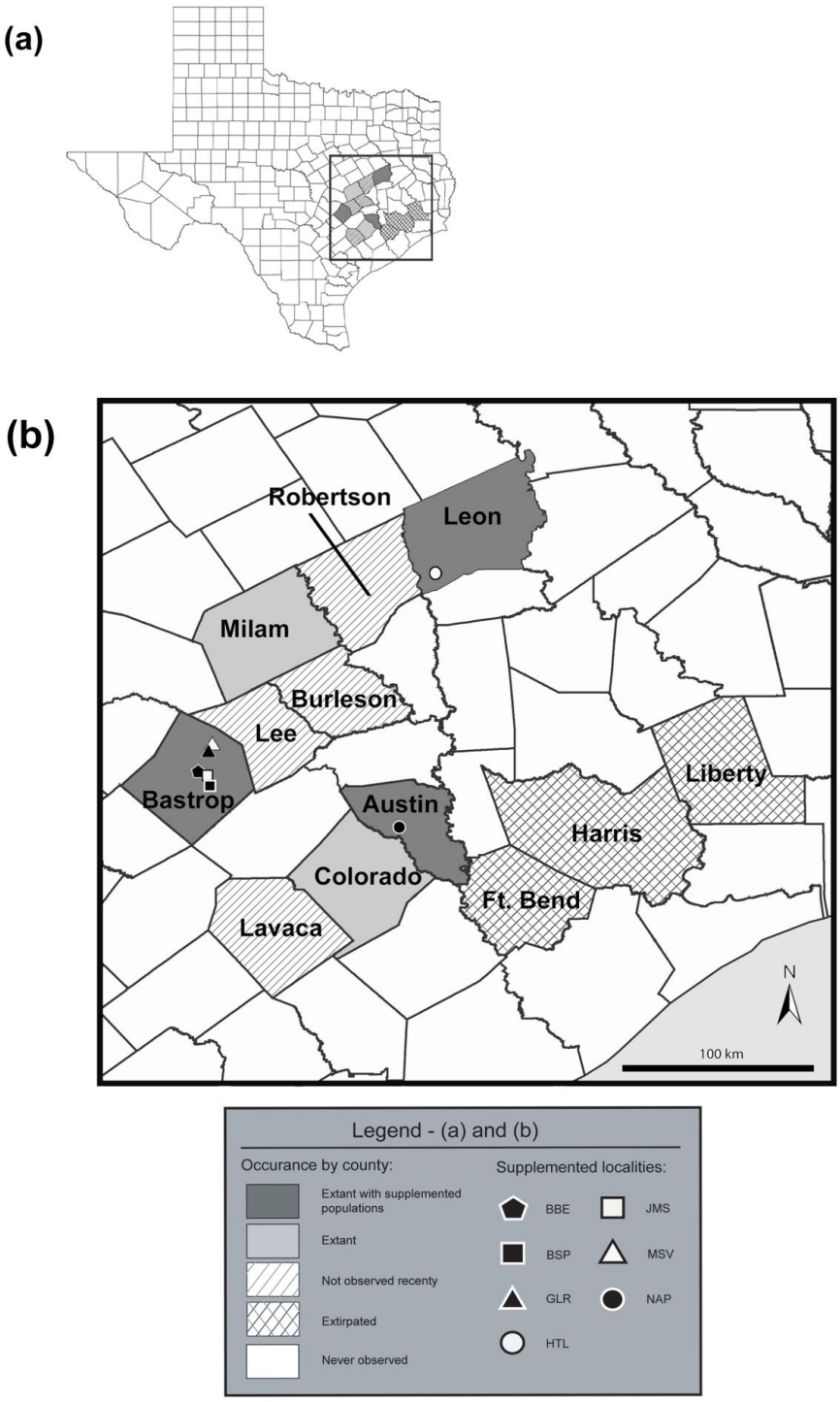
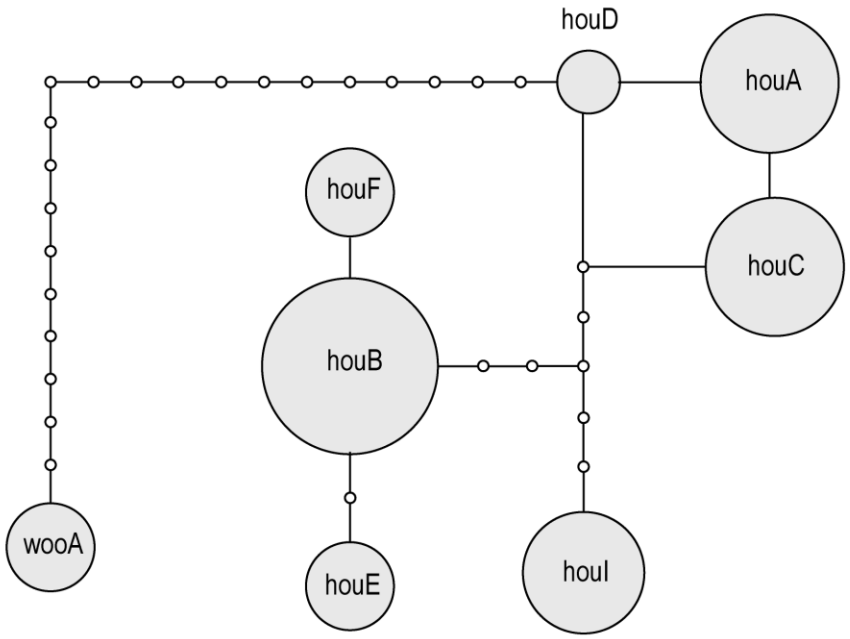


Figure 1. Range map of *B. houstonensis*. (a) Occurrence of *B. houstonensis* in Texas by county. (b) Counties surveyed and distribution of supplemented sites.



Haplotype	houA	houB	houC	houD	houE	houF	houI	wooA
Strands	1, 4, 10, 23, 24	3, 6, 7, 12, 13, 16, 29, 30	11, 14, 15, 17, 22	20	18, 31	5, 8	25, 26, 27, 28	19, 21

Figure 2. Egg strand haplotype network. The network is constructed from 506 bp for the control region of 29 *B. houstonensis*. Circle size is proportional to frequency of the haplotype. Each line represents a base substitution and small circles represent extinct or unsampled haplotypes.

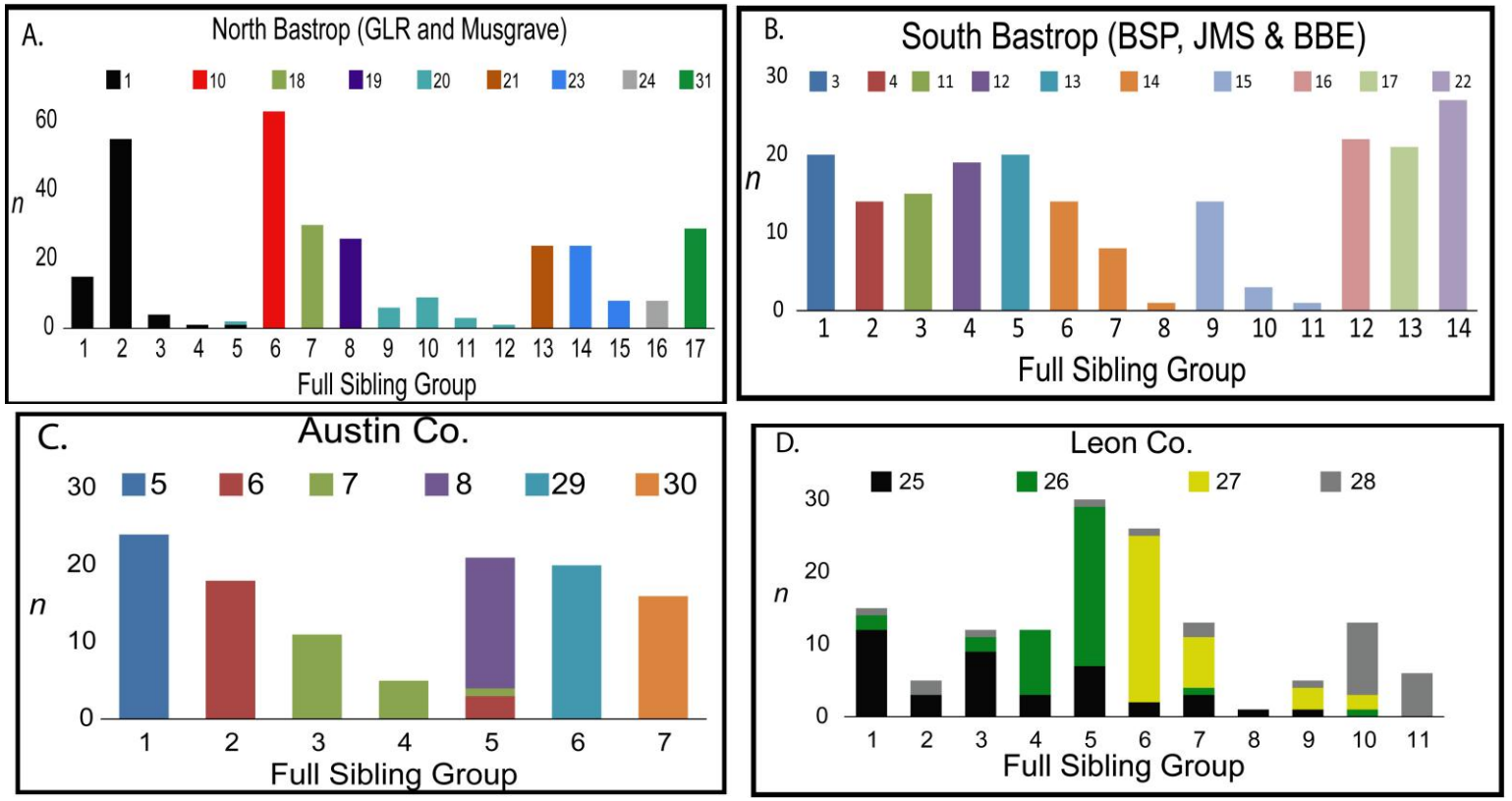


Figure 3. Reconstructed egg strands partitioned by geography. A. are groups constructed from egg strands collected in North Bastrop Co., B. groups constructed for South Bastrop Co., C. groups constructed from egg strands collected from Austin Co. and D. groups constructed from egg strands collected from Leon Co. On the x axis are the number or reconstructed sibling groups from each locality and each bar represents a different sibling group. The number of individuals are represented on the y axis and each color is a representation of a putative egg strand. On occasion (especially in all groups reconstructed from Leon Co.), members of different egg strands have grouped together. This is the result of a sampling artifact or a reconstruction artifact caused by a similarity of allele frequencies within egg strands.

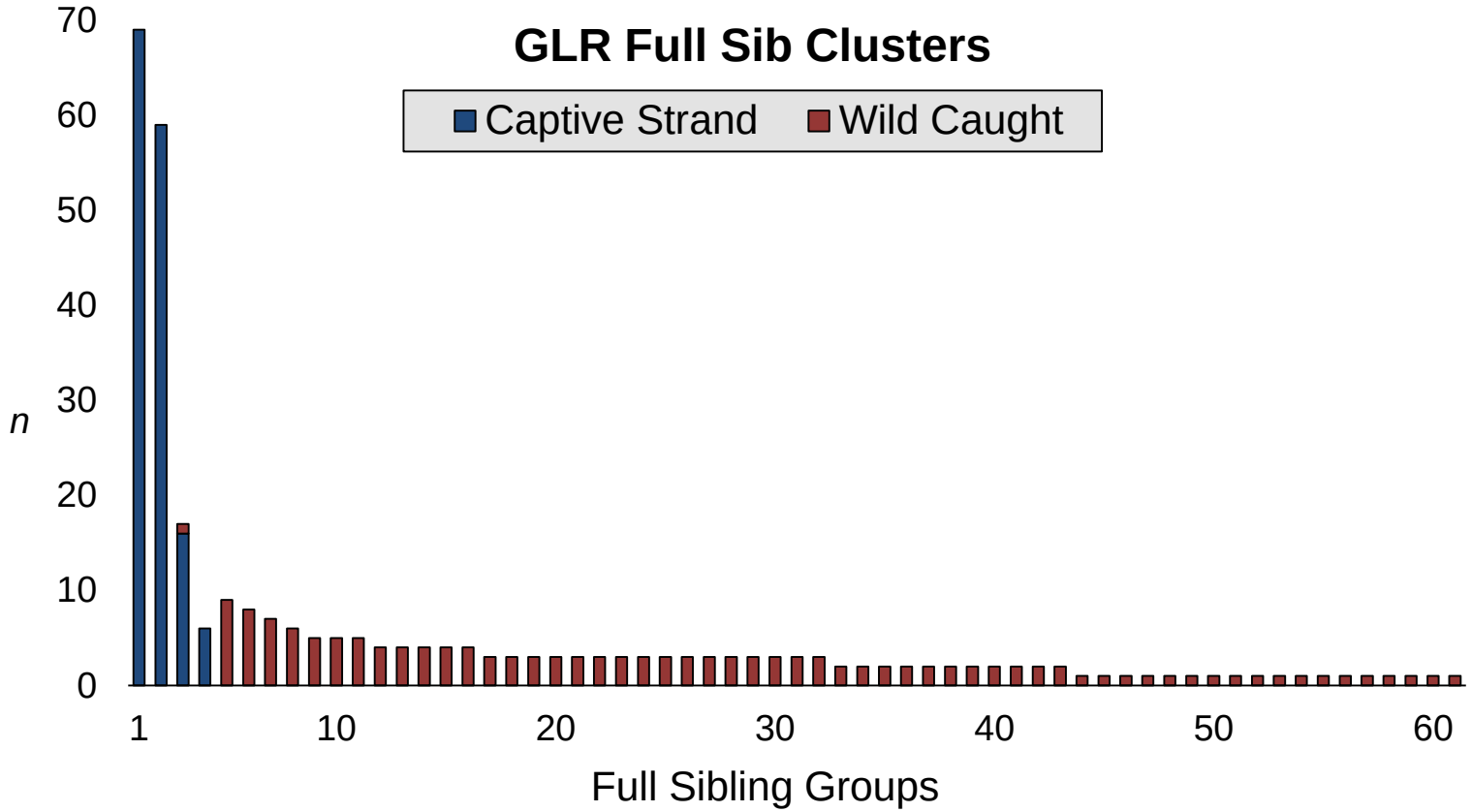


Figure 4. Full sibling reconstruction from GLR. Full sibling partition was constructed from both captive egg strands (1 and 10) and wild caught individuals from GLR collected between 2008 and 2010. Each bar is a different reconstructed sibling group. The y axis represents the number of individuals that partitioned within a single sibling group. Blue represents members from the captive population and red represents individuals collected from the wild. Notice only one individuals partitioned with a captive egg strand. This individual portioned with a kin group in egg strand 1 and was collected in 2008.

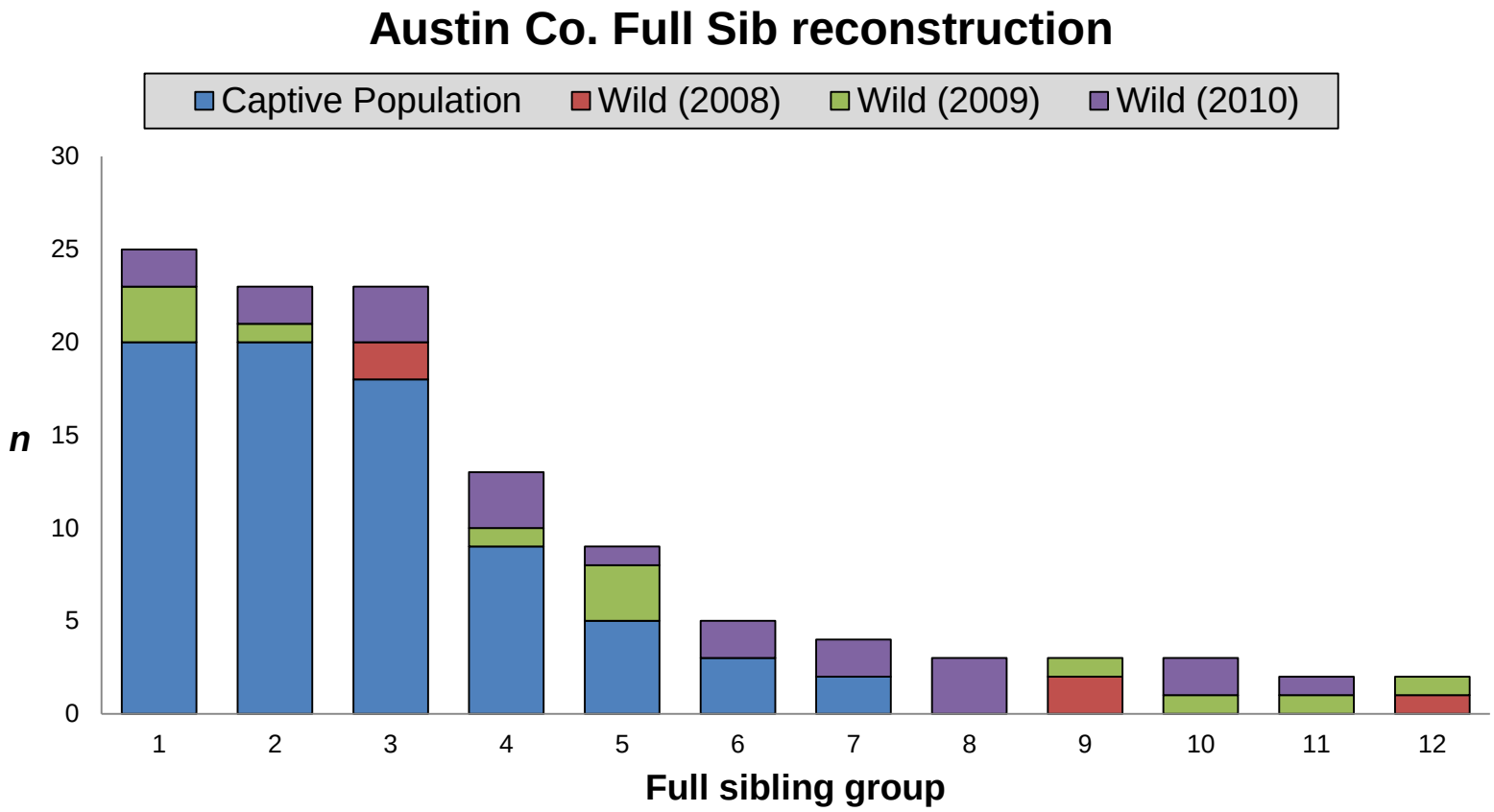


Figure 5. Full sibling reconstructions from Austin Co. Partition was made from captive egg strands (5, 6, 7 and 8) and wild caught individuals from Austin Co. from 2008-2010. Notice wild caught individuals partitioned with the captive egg strands, even when toads collected in prior to juvenile releases in 2009. This is an artifact of reduced genetic diversity in Austin Co.

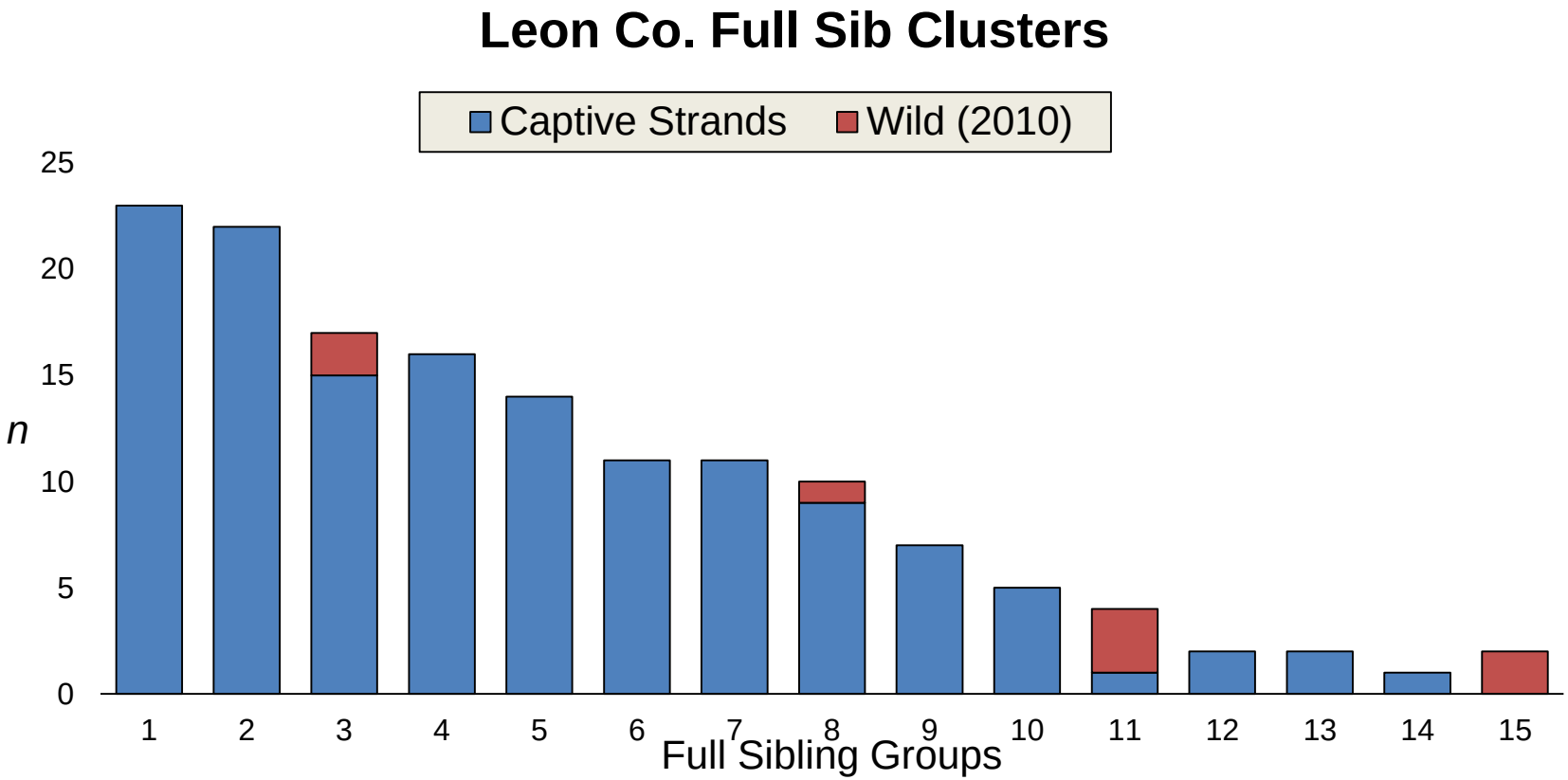


Figure 6. Full sibling reconstruction from Leon Co. Partition was made from both captive egg strands (25, 26, 27 and 28) and wild caught individuals from Leon Co. from 2010. Both egg strands and adults were collected in the same breeding season, yet wild individuals are assigned with the captive population. As in Austin Co., this is an artifact of reduced genetic diversity preventing noticeable differentiation between the captive and wild population.

APPENDIX 1

MF no. (Michael R.J. Forstner Frozen Tissue Catalog identification number), sex, date sampled, coordinates (WGS84), county, state, county and locality description for all captured adults and juveniles. GLR = Griffith League Ranch.

MF no.	Sex	Date Sampled	Latitude	Longitude	Country	State	County	Locality Description
25880	Male	3/14/08	30.21450043	-97.23284912	USA	Texas	Bastrop	GLR P6-A1
25886	Female	3/14/08	30.21626091	-97.24172211	USA	Texas	Bastrop	GLR pond 2
25888	Male	3/14/08	30.21626091	-97.24172211	USA	Texas	Bastrop	GLR pond 2
25889	Male	3/14/08	30.21626091	-97.24172211	USA	Texas	Bastrop	GLR pond 2
25890	Male	3/14/08	30.21626091	-97.24172211	USA	Texas	Bastrop	GLR pond 2
25891	Male	3/14/08	30.21626091	-97.24172211	USA	Texas	Bastrop	GLR pond 2
25892	Male	3/14/08	30.21626091	-97.24172211	USA	Texas	Bastrop	GLR pond 2
25893	Male	3/14/08	30.21626091	-97.24172211	USA	Texas	Bastrop	GLR pond 2
25894	Male	3/14/08	30.21626091	-97.24172211	USA	Texas	Bastrop	GLR pond 2
25895	Male	3/14/08	30.21626091	-97.24172211	USA	Texas	Bastrop	GLR pond 2
25896	Male	3/14/08	30.21626091	-97.24172211	USA	Texas	Bastrop	GLR pond 2
25897	Male	3/14/08	30.21626091	-97.24172211	USA	Texas	Bastrop	GLR pond 2
25898	Male	3/14/08	30.21626091	-97.24172211	USA	Texas	Bastrop	GLR pond 2
25899	Male	3/14/08	30.21626091	-97.24172211	USA	Texas	Bastrop	GLR pond 2
25900	Male	3/14/08	30.21626091	-97.24172211	USA	Texas	Bastrop	GLR pond 2
26041	Male	3/27/08	30.21626091	-97.24172211	USA	Texas	Bastrop	GLR pond 2
26045	Female	4/1/08	30.21450043	-97.23284912	USA	Texas	Bastrop	GLR P6-A1
26100	Male	3/28/08	30.21626091	-97.24172211	USA	Texas	Bastrop	GLR pond 2
26115	Male	3/26/08	30.21626091	-97.24172211	USA	Texas	Bastrop	GLR pond 2
26116	Male	3/26/08	30.21626091	-97.24172211	USA	Texas	Bastrop	GLR pond 2
26117	Male	4/18/08	30.21615982	-97.24143982	USA	Texas	Bastrop	GLR pond 2

Appendix 1-Continued

MF no.	Sex	Date Sampled	Latitude	Longitude	Country	State	County	Locality Description
26267	Male	4/2/08	29.87788963	-96.35294342	USA	Texas	Austin	TCW pond
26268	Male	4/2/08	29.87788963	-96.35294342	USA	Texas	Austin	TCW pond
26269	Male	4/2/08	29.87788963	-96.35294342	USA	Texas	Austin	TCW pond
26270	Male	4/2/08	29.87788963	-96.35294342	USA	Texas	Austin	TCW pond
26271	Male	4/10/08	29.87245941	-96.36386108	USA	Texas	Austin	Pond 500 m E of jct FM-1094 and Hinkel Rd
26383	Male	4/18/08	30.19611931	-97.24373627	USA	Texas	Bastrop	GLR P12-A1-B1
26405	Male	4/26/08	30.21635628	-97.24165344	USA	Texas	Bastrop	GLR pond 2
26416	Male	4/26/08	29.88394928	-96.36161041	USA	Texas	Austin	Hall Rd at jct of Hall Rd and Hinkel Rd
27583	Male	4/18/09	29.88334084	-96.36154938	USA	Texas	Austin	Road next to Nava pond
27584	Male	4/18/09	29.88373947	-96.36165619	USA	Texas	Austin	Road next to Nava pond
27585	Male	4/19/09	29.88373947	-96.36165619	USA	Texas	Austin	Road next to Nava pond
27586	Female	4/19/09	29.8837204	-96.36160278	USA	Texas	Austin	Road next to Nava pond
27587	Male	4/18/09	29.87990952	-96.35961914	USA	Texas	Austin	McMurray Pond
27588	Male	4/18/09	29.87990952	-96.35961914	USA	Texas	Austin	McMurray Pond
27589	Male	4/19/09	29.88356018	-96.36122894	USA	Texas	Austin	Nava Pond
27590	Male	4/19/09	29.88356018	-96.36122894	USA	Texas	Austin	Nava Pond
27593	Male	4/20/09	29.88356018	-96.36122894	USA	Texas	Austin	Nava Pond
27594	Male	4/20/09	29.88356018	-96.36122894	USA	Texas	Austin	Nava Pond
27595	Male	4/20/09	29.88356018	-96.36122894	USA	Texas	Austin	Nava Pond
27689	Male	4/18/09	30.18894958	-97.23253632	USA	Texas	Bastrop	GLR pond 13
27690	Male	4/18/09	30.18894958	-97.23253632	USA	Texas	Bastrop	GLR pond 13
27691	Male	4/18/09	30.18894958	-97.23253632	USA	Texas	Bastrop	GLR pond 13
27692	Male	4/18/09	30.18894958	-97.23253632	USA	Texas	Bastrop	GLR pond 13
27728	Male	3/19/09	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
27749	Male	4/17/09	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
27750	Male	4/17/09	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
27751	Male	4/17/09	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12

Appendix 1-Continued

MF no.	Sex	Date Sampled	Latitude	Longitude	Country	State	County	Locality Description
27759	Male	4/17/09	30.21235085	-97.2299881	USA	Texas	Bastrop	GLR pond 7
27760	Male	4/17/09	30.1991806	-97.2219696	USA	Texas	Bastrop	GLR pond 9
27788	Female	4/18/09	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
27789	Female	4/18/09	30.17873001	-97.23246765	USA	Texas	Bastrop	GLR pond 14
27790	Female	4/18/09	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
27812	Male	4/18/09	30.20932007	-97.24291229	USA	Texas	Bastrop	GLR pond 5
27813	Male	4/19/09	30.21627045	-97.24172211	USA	Texas	Bastrop	GLR pond 2
27816	Female	4/20/09	30.19893074	-97.22187805	USA	Texas	Bastrop	GLR L9 B-W
27852	Female	4/28/09	30.19611931	-97.24373627	USA	Texas	Bastrop	GLR 15 B-S
27870	Male	2/10/09	30.19474983	-97.24420166	USA	Texas	Bastrop	GLR L12 B-N
27873	Male	2/11/09	30.21253967	-97.23008728	USA	Texas	Bastrop	GLR L7 B-N
28626	Male	2/20/10	30.24567032	-97.22164917	USA	Texas	Bastrop	Musgrave pond
28627	Male	2/20/10	30.24567032	-97.22164917	USA	Texas	Bastrop	Musgrave pond
28628	Male	2/20/10	30.24567032	-97.22164917	USA	Texas	Bastrop	Musgrave pond
28629	Male	2/20/10	30.24567032	-97.22164917	USA	Texas	Bastrop	Musgrave pond
28630	Male	2/20/10	30.24567032	-97.22164917	USA	Texas	Bastrop	Musgrave pond
28631	Male	3/6/10	30.24567032	-97.22135162	USA	Texas	Bastrop	Musgrave pond
28632	Male	3/9/10	30.24567032	-97.22135162	USA	Texas	Bastrop	Musgrave pond
28634	Male	3/9/10	30.24567032	-97.22135162	USA	Texas	Bastrop	Musgrave pond
28635	Male	3/9/10	30.24567032	-97.22135162	USA	Texas	Bastrop	Musgrave pond
28636	Male	3/9/10	30.24567032	-97.22135162	USA	Texas	Bastrop	Musgrave pond
28637	Male	3/9/10	30.24567032	-97.22135162	USA	Texas	Bastrop	Musgrave pond
28638	Male	3/6/10	30.24567032	-97.22135162	USA	Texas	Bastrop	Musgrave pond
28639	Male	3/6/10	30.24567032	-97.22135162	USA	Texas	Bastrop	Musgrave pond
28640	Male	3/6/10	30.24567032	-97.22135162	USA	Texas	Bastrop	Musgrave pond
28642	Male	3/6/10	30.24567032	-97.22135162	USA	Texas	Bastrop	Musgrave pond
28643	Male	3/6/10	30.24567032	-97.22135162	USA	Texas	Bastrop	Musgrave pond
28644	Male	3/6/10	30.24567032	-97.22135162	USA	Texas	Bastrop	Musgrave pond
28645	Male	3/6/10	30.24567032	-97.22135162	USA	Texas	Bastrop	Musgrave pond
28646	Male	3/6/10	30.24567032	-97.22135162	USA	Texas	Bastrop	Musgrave pond

Appendix 1-Continued

MF no.	Sex	Date Sampled	Latitude	Longitude	Country	State	County	Locality Description
28647	Male	3/6/10	30.23752022	-97.21151733	USA	Texas	Bastrop	Dube Ln
28648	Male	3/6/10	30.23752022	-97.21151733	USA	Texas	Bastrop	Dube Ln
28649	Male	3/6/10	30.23752022	-97.21151733	USA	Texas	Bastrop	Dube Ln
28650	Male	3/6/10	30.23752022	-97.21151733	USA	Texas	Bastrop	Dube Ln
28651	Male	2/19/10	30.20932007	-97.24291229	USA	Texas	Bastrop	GLR pond 5
28652	Male	2/19/10	30.20932007	-97.24291229	USA	Texas	Bastrop	GLR pond 5
28653	Male	2/19/10	30.20932007	-97.24291229	USA	Texas	Bastrop	GLR pond 5
28654	Male	2/19/10	30.20932007	-97.24291229	USA	Texas	Bastrop	GLR pond 5
28981	Male	2/20/10	30.1991806	-97.2219696	USA	Texas	Bastrop	GLR pond 9
28982	Male	2/20/10	30.1991806	-97.2219696	USA	Texas	Bastrop	GLR pond 9
28983	Male	2/20/10	30.1991806	-97.2219696	USA	Texas	Bastrop	GLR pond 9
28984	Male	2/20/10	30.1991806	-97.2219696	USA	Texas	Bastrop	GLR pond 9
28985	Male	2/20/10	30.1991806	-97.2219696	USA	Texas	Bastrop	GLR pond 9
28986	Male	2/20/10	30.1991806	-97.2219696	USA	Texas	Bastrop	GLR pond 9
28987	Male	2/20/10	30.1991806	-97.2219696	USA	Texas	Bastrop	GLR pond 9
28988	Male	2/20/10	30.1991806	-97.2219696	USA	Texas	Bastrop	GLR pond 9
28989	Male	2/20/10	30.1991806	-97.2219696	USA	Texas	Bastrop	GLR pond 9
28990	Male	2/20/10	30.1991806	-97.2219696	USA	Texas	Bastrop	GLR pond 9
28991	Male	2/20/10	30.20560074	-97.23423004	USA	Texas	Bastrop	GLR pond 8
28992	Female	2/21/10	30.19474983	-97.24420166	USA	Texas	Bastrop	GLR L12
28993	Female	2/21/10	30.19474983	-97.24420166	USA	Texas	Bastrop	GLR L12
28994	Male	2/21/10	30.19474983	-97.24420166	USA	Texas	Bastrop	GLR L12
28995	Male	2/21/10	30.19474983	-97.24420166	USA	Texas	Bastrop	GLR L12
28996	Male	2/21/10	30.19474983	-97.24420166	USA	Texas	Bastrop	GLR L12
28997	Male	2/21/10	30.1991806	-97.2219696	USA	Texas	Bastrop	GLR pond 9
28998	Male	2/22/10	30.19474983	-97.24420166	USA	Texas	Bastrop	GLR L12
28999	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29000	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29001	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29002	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12

Appendix 1-Continued

MF no.	Sex	Date Sampled	Latitude	Longitude	Country	State	County	Locality Description
29003	Female	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29004	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29005	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29006	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29007	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29008	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29009	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29010	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29011	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29012	Female	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29013	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29014	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29015	Female	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29016	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29017	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29018	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29019	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29020	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29021	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29022	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29023	Female	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29024	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29025	Female	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29026	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29027	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29028	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29029	Female	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29030	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29031	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29032	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12

Appendix 1-Continued

MF no.	Sex	Date Sampled	Latitude	Longitude	Country	State	County	Locality Description
29033	Male	3/6/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29034	Male	3/7/10	30.19474983	-97.24420166	USA	Texas	Bastrop	GLR L12
29035	Male	3/7/10	30.19474983	-97.24420166	USA	Texas	Bastrop	GLR L12
29036	Male	3/7/10	30.19474983	-97.24420166	USA	Texas	Bastrop	GLR L12
29037	Male	3/7/10	30.19474983	-97.24420166	USA	Texas	Bastrop	GLR L12
29038	Male	3/7/10	30.19474983	-97.24420166	USA	Texas	Bastrop	GLR L12
29176	Male	3/6/10	30.1991806	-97.2219696	USA	Texas	Bastrop	GLR pond 9
29177	Male	3/7/10	30.20932007	-97.24291229	USA	Texas	Bastrop	GLR pond 5
29178	Male	3/7/10	30.20932007	-97.24291229	USA	Texas	Bastrop	GLR pond 5
29179	Male	3/7/10	30.20932007	-97.24291229	USA	Texas	Bastrop	GLR pond 5
29180	Male	3/7/10	30.20198059	-97.208992	USA	Texas	Bastrop	GLR pond 11
29181	Male	3/7/10	30.20198059	-97.208992	USA	Texas	Bastrop	GLR pond 11
29182	Female	3/9/10	30.19474983	-97.24420166	USA	Texas	Bastrop	GLR L12
29183	Female	3/9/10	30.19611931	-97.24373627	USA	Texas	Bastrop	GLR T15
29184	Female	3/9/10	30.19893074	-97.22187805	USA	Texas	Bastrop	GLR L9
29185	Male	3/9/10	30.20198059	-97.208992	USA	Texas	Bastrop	GLR pond 11
29186	Male	3/9/10	30.20198059	-97.208992	USA	Texas	Bastrop	GLR pond 11
29187	Male	3/9/10	30.20198059	-97.208992	USA	Texas	Bastrop	GLR pond 11
29188	Male	3/9/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29189	Male	3/9/10	30.19488907	-97.24358368	USA	Texas	Bastrop	GLR pond 12
29190	Male	3/16/10	30.16929054	-97.23236084	USA	Texas	Bastrop	GLR L16
29191	Female	3/16/10	30.20985985	-97.24002838	USA	Texas	Bastrop	GLR TC
29192	Male	3/19/10	30.21235085	-97.2299881	USA	Texas	Bastrop	GLR pond 7
29193	Male	3/19/10	30.21235085	-97.2299881	USA	Texas	Bastrop	GLR pond 7
29194	Male	3/19/10	30.21235085	-97.2299881	USA	Texas	Bastrop	GLR pond 7
29195	Female	3/25/10	30.19474983	-97.24420166	USA	Texas	Bastrop	GLR L12
29196	Male	3/26/10	30.21627045	-97.24172211	USA	Texas	Bastrop	GLR pond 2
29197	Male	3/26/10	30.21235085	-97.2299881	USA	Texas	Bastrop	GLR pond 7
29199	Female	4/1/10	30.19474983	-97.24420166	USA	Texas	Bastrop	GLR L12
29200	Male	4/1/10	30.21660042	-97.24172211	USA	Texas	Bastrop	GLR L2

Appendix 1-Continued

MF no.	Sex	Date Sampled	Latitude	Longitude	Country	State	County	Locality Description
29201	Male	4/2/10	30.2064991	-97.24990082	USA	Texas	Bastrop	GLR pond 4
29202	Male	4/2/10	30.21627045	-97.24172211	USA	Texas	Bastrop	GLR pond 2
29203	Female	4/3/10	30.21660042	-97.24172211	USA	Texas	Bastrop	GLR L2
29204	Male	4/2/10	30.2064991	-97.24990082	USA	Texas	Bastrop	GLR pond 4
29205	Male	4/2/10	30.2064991	-97.24990082	USA	Texas	Bastrop	GLR pond 4
29206	Male	4/15/10	30.19951057	-97.21044922	USA	Texas	Bastrop	GLR P10-A2
29207	Male	4/15/10	30.18880081	-97.23177338	USA	Texas	Bastrop	GLR pond 13
29208	Male	4/15/10	30.18880081	-97.23177338	USA	Texas	Bastrop	GLR pond 13
29209	Male	4/15/10	30.1991806	-97.2219696	USA	Texas	Bastrop	GLR pond 9
29210	Male	4/16/10	30.19779968	-97.21327209	USA	Texas	Bastrop	GLR pond 10
29211	Female	4/16/10	30.18880081	-97.23177338	USA	Texas	Bastrop	GLR pond 13
29212	Male	4/17/10	30.20932007	-97.24291229	USA	Texas	Bastrop	GLR pond 5
29215	Female	4/23/10	30.1980896	-97.21334076	USA	Texas	Bastrop	GLR L10
29217	Male	3/7/10	30	-97	USA	Texas	Bastrop	Clay Pond B
29218	Male	3/7/10	30	-97	USA	Texas	Bastrop	Clay Pond B
29219	Male	3/7/10	30	-97	USA	Texas	Bastrop	Clay Pond B
29220	Male	3/19/10	30	-97	USA	Texas	Bastrop	GLR pond 4
29221	Male	3/19/10	30	-97	USA	Texas	Bastrop	GLR pond 4
29222	Male	2/21/10	30.19545937	-97.24360657	USA	Texas	Bastrop	GLR L12-N
29223	Male	3/5/10	30.19545937	-97.24360657	USA	Texas	Bastrop	GLR L12-N
29224	Female	3/8/10	30.20008087	-97.22265625	USA	Texas	Bastrop	GLR T10-1
29225	Female	3/8/10	30.20015907	-97.22141266	USA	Texas	Bastrop	GLR T10-5
29227	Male	3/9/10	30.17794991	-97.2338028	USA	Texas	Bastrop	GLR pond 15
29228	Male	3/9/10	30.17794991	-97.2338028	USA	Texas	Bastrop	GLR pond 15
29229	Male	3/24/10	30.21660042	-97.24172211	USA	Texas	Bastrop	GLR L2-E
29230	Male	3/8/10	29.88356018	-96.36122894	USA	Texas	Austin	Nava Pond
29231	Male	3/8/10	29.88356018	-96.36122894	USA	Texas	Austin	Nava Pond
29232	Male	3/8/10	29.88356018	-96.36122894	USA	Texas	Austin	Nava Pond
29233	Male	3/8/10	29.88356018	-96.36122894	USA	Texas	Austin	Nava Pond
29234	Male	3/8/10	29.88356018	-96.36122894	USA	Texas	Austin	Nava Pond

Appendix 1-Continued

MF no.	Sex	Date Sampled	Latitude	Longitude	Country	State	County	Locality Description
29235	Male	3/8/10	29.88356018	-96.36122894	USA	Texas	Austin	Nava Pond
29236	Male	3/8/10	29.88356018	-96.36122894	USA	Texas	Austin	Nava Pond
29237	Male	3/8/10	29.88356018	-96.36122894	USA	Texas	Austin	Nava Pond
29238	Male	3/8/10	29.88356018	-96.36122894	USA	Texas	Austin	Nava Pond
29239	Male	3/9/10	31.06698036	-96.1712265	USA	Texas	Leon	Hilltop Lakes subdivision, 0.5 mi N of 2010 headstart collection point
29240	Male	3/9/10	31.06698036	-96.1712265	USA	Texas	Leon	Hilltop Lakes subdivision, 0.5 mi N of 2010 headstart collection point
29241	Male	3/9/10	31.06698036	-96.1712265	USA	Texas	Leon	Hilltop Lakes subdivision, 0.5 mi N of 2010 headstart collection point
29242	Male	3/9/10	30.17794991	-97.2338028	USA	Texas	Leon	Hilltop Lakes subdivision, 0.5 mi N of 2010 headstart collection point
29243	Male	3/9/10	31.06698036	-96.1712265	USA	Texas	Leon	Hilltop Lakes subdivision, 0.5 mi N of 2010 headstart collection point
29245	Male	3/9/10	31.06698036	-96.1712265	USA	Texas	Leon	Hilltop Lakes subdivision, 0.5 mi N of 2010 headstart collection point
29246	Female	3/9/10	31.06698036	-96.1712265	USA	Texas	Leon	Hilltop Lakes subdivision, 0.5 mi N of 2010 headstart collection point
29247	Male	3/9/10	31.06698036	-96.1712265	USA	Texas	Leon	Hilltop Lakes subdivision, 0.5 mi N of 2010 headstart collection point
29248	Male	3/8/10	29.88356018	-96.36122894	USA	Texas	Austin	Nava Pond
29249	Male	3/23/10	29.88995934	-96.3624115	USA	Texas	Austin	Waldrop Pond
29250	Male	3/24/10	29.87990952	-96.35961914	USA	Texas	Austin	McMurray Pond
29251	Male	3/24/10	29.87990952	-96.35961914	USA	Texas	Austin	McMurray Pond
29252	Male	3/24/10	29.87990952	-96.35961914	USA	Texas	Austin	McMurray Pond
29253	Male	3/24/10	29.88356018	-96.36122894	USA	Texas	Austin	Nava Pond

Appendix 1-Continued

MF no.	Sex	Date Sampled	Latitude	Longitude	Country	State	County	Locality Description
29254	Male	3/24/10	29.88356018	-96.36122894	USA	Texas	Austin	Nava Pond
29255	Male	3/24/10	29.88356018	-96.36122894	USA	Texas	Austin	Nava Pond
29256	Male	3/24/10	29.88356018	-96.36122894	USA	Texas	Austin	Nava Pond
29257	Male	3/24/10	29.88356018	-96.36122894	USA	Texas	Austin	Nava Pond
29258	Female	3/25/10	29.88356018	-96.36122894	USA	Texas	Austin	Nava Pond
29259	Male	3/26/10	29.88356018	-96.36122894	USA	Texas	Austin	Nava Pond
29261	Male	3/6/10	30.14230728	-97.19580078	USA	Texas	Bastrop	Bob Long marsh
29262	Male	3/6/10	30.13772202	-97.2433548	USA	Texas	Bastrop	Jim Small Pond 2
29263	Male	3/6/10	30.13772202	-97.2433548	USA	Texas	Bastrop	Jim Small Pond 2
29264	Male	3/6/10	30.13772202	-97.2433548	USA	Texas	Bastrop	Jim Small Pond 2
29265	Male	3/6/10	30.13772202	-97.2433548	USA	Texas	Bastrop	Jim Small Pond 2
29266	Male	3/6/10	30.13772202	-97.2433548	USA	Texas	Bastrop	Jim Small Pond 2
29267	Male	3/6/10	30.12632942	-97.23370361	USA	Texas	Bastrop	Jim Small Pond 5
29268	Male	3/6/10	30.12632942	-97.23370361	USA	Texas	Bastrop	Jim Small Pond 5
29269	Male	3/6/10	30.12632942	-97.23370361	USA	Texas	Bastrop	Jim Small Pond 5
29270	Male	3/7/10	30.12638092	-97.23934174	USA	Texas	Bastrop	Jim Small Pond 4
29271	Male	3/7/10	30.12638092	-97.23934174	USA	Texas	Bastrop	Jim Small Pond 4
29272	Male	3/7/10	30.12638092	-97.23934174	USA	Texas	Bastrop	Jim Small Pond 4
29273	Male	3/7/10	30.12638092	-97.23934174	USA	Texas	Bastrop	Jim Small Pond 4
29274	Male	3/7/10	30.12638092	-97.23934174	USA	Texas	Bastrop	Jim Small Pond 4
29275	Male	3/10/10	30.13721275	-97.2433548	USA	Texas	Bastrop	Jim Small Pond 2
29276	Male	3/10/10	30.13721275	-97.2433548	USA	Texas	Bastrop	Jim Small Pond 2
29277	Male	3/10/10	30.140028	-97.24250031	USA	Texas	Bastrop	Jim Small Pond 3 outflow
29278	Male	3/10/10	30.140028	-97.24250031	USA	Texas	Bastrop	Jim Small Pond 3 outflow
29279	Male	3/10/10	30.140028	-97.24250031	USA	Texas	Bastrop	Jim Small Pond 3 outflow
29280	Male	3/10/10	30.12632942	-97.23370361	USA	Texas	Bastrop	Jm Small Pond 5
29281	Male	3/10/10	30.12632942	-97.23370361	USA	Texas	Bastrop	Jm Small Pond 5
29282	Male	3/5/10	30.12638092	-97.39341736	USA	Texas	Bastrop	Jim Small Pond 4
29283	Male	3/5/10	30.12638092	-97.39341736	USA	Texas	Bastrop	Jim Small Pond 4

Appendix 1-Continued

MF no.	Sex	Date Sampled	Latitude	Longitude	Country	State	County	Locality Description
29284	Male	3/5/10	30.12638092	-97.39341736	USA	Texas	Bastrop	Jim Small Pond 4
29285	Male	3/5/10	30.12638092	-97.39341736	USA	Texas	Bastrop	Jim Small Pond 4
29286	Male	3/5/10	30.12638092	-97.39341736	USA	Texas	Bastrop	Jim Small Pond 4
29287	Male	3/5/10	30.12638092	-97.39341736	USA	Texas	Bastrop	Jim Small Pond 4
29288	Male	3/5/10	30.12638092	-97.39341736	USA	Texas	Bastrop	Jim Small Pond 4
29683	Juvenile	7/19/10	30.2161808	-97.23059082	USA	Texas	Bastrop	GLR T7
29684	Juvenile	7/19/10	30.21051979	-97.23825836	USA	Texas	Bastrop	GLR TB
29688	Juvenile	7/22/10	30.17715073	-97.23423004	USA	Texas	Bastrop	GLR P14-A3
29689	Juvenile	7/23/10	30.2161808	-97.23059082	USA	Texas	Bastrop	GLR T7

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