MULTIPLE PATERNITY AND THE BREEDING BIOLOGY OF THE RED-EYED TREEFROG, *AGALYCHNIS CALLIDRYAS*

by

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(Abstract)

External fertilization makes male anurans susceptible to direct intrasexual competition for fertilization opportunities at the egg mass. The red-eyed treefrog, *Agalychnis callidryas*, is one species in which pairs of males appear to simultaneously fertilize the clutch of a single female. DNA fingerprinting revealed the presence of multiple paternity in two egg clutches examined from two matings involving a female with two males. The breeding biology of females and the potential costs and benefits of mating with multiple males were examined. Females were found to decrease the number of eggs in matings with multiple males. In addition, amplexed females moving toward oviposition sites avoided secondary males by moving when approached by secondary males. Mortality to the eggs as a result of multiple males attempting to amplex females is suggested as the reason females avoid multiple males. Males

were found to exhibit calling site defense from other males. Males used a combination of auditory and a visual behavior in defending calling sites. The call types are described and the contexts within which calls occur is discussed. Density of frogs was found to be a better indicator of the occurrence of matings involving multiple males than the operational sex ratio (number of males/number of females).

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CHAPTER 1

Introduction

Darwin (1871) recognized male intrasexual competition as a main component in his theory of sexual selection. He pointed out that intrasexual competition would favor males that developed devices or behaviors preventing interference from other males during copulation. For example, he uses the case of parasitic worms to illustrate the importance of sexual selection versus natural selection: "So again, if the chief service rendered to the male by his prehensile organs is to prevent the escape of the female before the arrival of other males, or when assaulted by them, these organs will have been perfected through sexual selection, that is by the advantage acquired by certain individuals over their rivals." Aristotle stated that "if a bird who carries an egg conceived by copulation, if it then has coitus with another male, will hatch all its chicks thereafter of a genus similar to the latter male." (Lind 1963). Yet, until 1970, intrasexual competition as a form of sexual selection was generally regarded to be precopulatory competition such as fighting between males for

direct access to females.

In 1970 Parker added a significant new dimension to the theory of sexual selection. He demonstrated that in insects, intrasexual competition could continue after copulation and insemination through 'sperm competition', the competition between the sperm from two or more males to fertilize the eggs of a single female during one reproductive cycle (Parker 1970) which can result in multiple paternity of offspring. Parker argued that sperm competition, or mating by multiple males, is a component of intrasexual competition. Selection should favor males that are more successful at fertilizing ova, thus sperm competition can be an important evolutionary force.

Sperm competition creates two parallel selective forces. Selection will favor males with mechanisms for preempting other males or their sperm from fertilization opportunities, while counter selection favors males that can circumvent these preemption mechanisms (Parker 1970, 1984). These two selective forces result in an evolutionary "arms race" (Dawkins & Krebs 1979) in which adaptation in one direction is offset by adaptation in the other.

Theoretical implications of multiple paternity for male and female fitness

Males generally have the capacity to mate with many females, and hence fertilize many eggs. The cost of fertilization is considered low for males compared to females, based on differences in sperm versus egg sizes (Lifjeld & Slagsvold 1989; Maynard Smith 1978; Trivers 1972)(but see Dewsbury 1982). In males, success in securing mating opportunities will directly influence male fitness by determining the number of progeny produced. Thus a strategy of extra-pair mating, resulting in multiple paternity of offspring, could increase a male's lifetime reproductive success (Birkhead & Møller 1992). Presently, the literature lacks evidence for an increase in the lifetime reproductive success of males who mate with previously paired females (Birkhead & Møller 1992). A study of red-winged blackbirds indicates the potential for males to increase their reproductive success by extra-pair matings. Individual male red-winged blackbirds achieved more than 20% of their overall mating success from extra-pair matings, as indicated by genetic analysis of offspring (Gibbs *et al.* 1990).

Three main hypotheses are proposed to explain the benefits a female could acquire from mating with more than

one male: 1) "Good genes," 2) genetic diversity of offspring, and 3) insurance against infertility. The good genes hypothesis proposes that a previously paired female can benefit from mating with another male if the second male has higher genotypic qualities with a high degree of heritability than the male with which she initially paired (Buitron 1983; Hamilton 1990; Wittenberger 1979). Most of the support for this hypothesis comes from studies of "monogamous" birds in which the female seeks extra-pair matings (Birkhead & Møller 1992; Graves *et al.* 1993; Kempenaers *et al.* 1992). Female zebra finches were found to actively solicit extra-pair matings from males that were more attractive than their mates. Males having higher song rates were judged more attractive by females in choice tests. These males produced offspring that fledged heavier and had higher song rates (Houtman 1992). In the butterflies *Colias* species, allelic differences in the phosphoglucose isomerase (PGI) enzyme genotype produce ten different genotypes. Certain PGI genotypes have been found to confer an advantage in a number of diverse fitness-related properties: flight capacity, survivorship, and overall mating success (Watt *et al.* 1986). Flight capacity is an important variable in male courtship of females. Males

with greater flight capacity are more successful in mating with females because they can sustain courtship for longer periods of times (Watt *et al.* 1986).

The genetic diversity hypothesis of offspring suggests a benefit to females who produce offspring of multiple paternity in unstable environments (Caldwell & Rankin 1974; Gladstone 1979). Genetic variability among offspring would increase the probability that some offspring will possess favorable genetic traits to succeed in a heterogenous habitat.

The insurance against infertility of mates hypothesis (Ridley 1988) is a female hedge for the likelihood of male infertility. Because the energetic and reproductive investments by a female are high, non or partial fertilization of an egg clutch will significantly decrease her fitness. Females could protect themselves against this cost by mating with different males and thus assure total fertilization of their eggs. Female birds (razorbills) engaged in extra-pair copulations (EPCs) were also more likely to mate more frequently with their own mates (Wagner 1992). This contradicts the good genes and genetic diversity hypotheses, which both predict that females who engage in EPCs will behave to maximize their chances of

being fertilized by extra-pair males. These findings are compatible with the insurance against infertility hypothesis which only requires females to store sperm from extra-pair males, not necessarily use it (Wagner 1992).

Conversely, two hypotheses propose to explain why females should discourage matings by multiple males: 1) direct cost to females by predation, and 2) loss of fertilization efficiency. If females incur mortality costs associated with matings by multiple males, they should avoid mating with multiple males (Daly 1978; van den Berghe *et al.* 1989).

In organisms with external fertilization, multiple paternity can only occur when the eggs are being oviposited and multiple males are present (Halliday & Verrell 1984). Mortality costs could be incurred if multiple male assemblages attract more predators (van den Berghe *et al.* 1989), or if egg-laying times are longer with multiple males, thus exposing females to increased predation risk (Kasuya *et al.* 1987). In the foam nesting frog, *Rhacophorus arboreus*, there was a strong positive correlation between egg-laying duration and the number of males participating (Kasuya *et al.* 1987).

Females may discourage matings involving multiple males

due to the loss of fertilization efficiency. Among fishes, it has been suggested that the increased activity of multiple males during spawning could generate currents that would disperse sperm away from the eggs, thus decreasing fertilization efficiency (van den Berghe *et al.* 1989). However, in foam nesting frogs, multiple male assemblages might be needed to increase fertilization efficiency if there is physical or chemical impedance of sperm due to the foam material.

Mating systems and opportunities for multiple male paternity

Different mating systems, the way in which individuals obtain mates (Krebs & Davies 1991), are thought to arise as a consequence of differences in dispersion of mates (Krebs & Davies 1991; Rubenstein & Wrangham 1986). Dispersion is influenced by the ecological factors responsible for the spacial and temporal distribution of resources necessary for reproductive activity (Emlen & Oring 1977).

If resources necessary for female reproductive activity are clumped in space, females will be aggregated and a few males will be able to monopolize most of the matings. Conversely, if resources are clumped in time, male monopolization of females becomes less likely because of

breeding synchrony. The operational sex ratio (OSR), the ratio of sexually active males to fertilizable females at any given time, provides a measure of the opportunities for males to monopolize females (Emlen & Oring 1977).

Male reproductive success and the opportunities for extra-pair copulations may depend on the operational sex ratio which influences both the probabilities of attaining a mate and the availability of extra-pair partners for copulation (Birkhead & Møller 1992). As the number of males increase relative to the number of females, male intrasexual competition and the probability of matings by multiple males should increase. Local breeding synchrony increases the population density of males and females. Higher population densities also increase the probability of matings by multiple males (Birkhead & Biggins 1987; Møller 1991; Thornhill & Alcock 1983).

Competition for mates may also shape the intrasexual mating strategies within a species. In the coral reef fish, *Thalassoma bifasciatum*, group spawning-males, which are directly affected by mate competition from other males, spawn selectively with larger, more fecund females. Pair-spawning males, who face little sperm competition, spawn with all females who arrive at their territories (van den

Berghe & Warner 1989).

In Belding's ground squirrel, *Spermophilis beldingi*, females occur in aggregations and males fight for dominance of small mating territories within the aggregations. The heaviest territorial males gain the most matings (Sherman & Morton 1984). Females mate with 3-5 males. The offspring are multiply sired, with the first male siring an average of 60%, the second male siring and average of 30%, and subsequent males siring averages of 10% of the litters (Hanken & Sherman 1981). After mating, the first male leaves the female and searches for other females to mate, rather than remaining to guard her. The first male mating advantage, along with the high density of females, has been suggested to be the reason males do not guard females (Sherman 1989). In the 13-lined ground squirrel, *S. tridecemlineatus*, another species with first male mating advantage (Foltz & Schwagmeyer 1988), females occur in low densities. Males do not guard mates after mating, nor fight with other males, but range widely looking for other females. Here, males with the greatest mate-searching areas are the most successful (Foltz & Schwagmeyer 1988).

In contrast, males guard females in the Idaho ground squirrel, *S. brunneus*. Paternity analysis of litters

guarded by a single male revealed that all pups were sired by the resident male (Sherman 1989). In litters with multiple male copulation, the male who guarded either last or longest, sired the most pups (Sherman 1989).

Anuran mating systems

In anurans, the spatial and temporal distribution of females determines the form of male-male competition (Wells, 1977). There are two generalized patterns of breeding, explosive breeding and prolonged breeding.

Explosive breeders are characterized by short (from a few days to a few weeks) breeding periods. Females arrive simultaneously and exhibit spatial clumping around suitable egg-laying sites. Subsequent behavior of males and females varies with male density (Arak 1983a; Wells 1977). At high male densities, females do not approach individually calling males. Males actively search for females, and amplex both other males and females in a trial-and-error process. In some species clasped males give stereotypical release calls or vibrations, after which, the clasping male releases the clasped male (Bogert 1960). Although males call, they do not defend fixed calling sites. Intense intra-sexual competition for females occurs under high male densities.

Several males may attempt to amplex the same female and struggle to dislodge each other (Wells 1977). In the woodfrog, *Rana sylvatica*, larger unamplexed males can displace smaller amplexed males and thus achieve more matings than the smaller males (Howard 1988a). At low or intermediate densities, males display characteristics associated with prolonged breeders; calling from dispersed locations, and remaining stationary. At low densities, females can approach individual calling males without being intercepted by other males. Explosive breeders are generally characterized as having smaller differences in the number of breeding males to females(e.g., 2:1) than prolonged breeders (up to 31:1)(Arak 1983a).

In contrast, prolonged breeders have protracted breeding seasons generally lasting more than a month (Wells 1977). Territorial defense or lek-behavior is the common pattern (Arak 1983a). Females arrive at the breeding site asynchronously. Under these conditions active searching by males is considered energetically unprofitable (Wells 1977; Arak 1988). Thus, males compete to set up calling sites which may reflect either the best egg-laying sites for females (Howard 1978a, 1988a), or are the best sound propagation sites for males (Fellers 1979a).

Female choice is an important factor in determining individual male mating success (Howard 1988b; Ryan 1985; Sullivan 1983; Wells 1977). Males do not compete physically for possession of females, but vocally. In many species this has led to vocal competition, maintenance of inter-male spacing, or defense of individual territories (see review in Wells 1977). For example, in woodhouse's toad, *Bufo woodhousei*, females discriminate between males with high and low call rates, preferring males with high call rates, independent of male size (Sullivan 1983). In *Hyla chrysoscelis*, females preferred to approach larger males with lower frequency calls over smaller males with higher frequency calls (Morris & Yoon 1989). In bullfrogs, females chose males with the highest quality egg deposition sites (Howard 1978a,b, 1988b).

Multiple paternity in animals with internal fertilization

Until recently, evidence for multiple paternity or fertilization by an extra-pair male in nominally monogamous species, has been assumed through observations of extra-pair matings, but not demonstrated empirically (Gladstone 1979, Maynard Smith & Ridpath 1972, Westneat 1990). For example, in the study of "wife sharing in the Tasmanian native hen"

the presence of extra-pair copulatory behavior was used to presume multiple paternity (Maynard Smith & Ridpath 1972).

DNA fingerprinting has revolutionized the study of mating systems through examination of the actual maternity and paternity of offspring. The ability to detect paternity based on genetic evidence rather than behavioral evidence is crucial for determining true paternity. For example, in the Japanese quail only three percent of observed fertilizations resulted in the fertilization of a full clutch of eggs (Adkins-Regan 1995). DNA fingerprinting reveals multiple paternity in monogamous species where extra-pair copulations were thought not to occur (Burke & Bruford 1987; Swatschek *et al.* 1994; Westneat 1990, 1993; Wetton *et al.* 1987).

Multiple paternity, as measured by either matings by multiple males with a female during one reproductive cycle or multiple paternity of progeny during a single reproductive cycle, has been reported in most animal groups with internal fertilization (Smith 1984): insects (Achmann *et al.* 1992; Müller & Eggert 1989; Waage 1979), arachnids (Watson 1991), fish (Constantz 1984; Greene & Brown 1991; Travis *et al.* 1990; van den Berghe *et al.* 1989; van den Berghe & Warner 1989), amphibians (Houck & Schwenk 1984; Houck *et al.* 1985; Tilley & Hausman 1976), reptiles (Barry

et al. 1992; Galbraith *et al.* 1993; Schwartz *et al.* 1989; Stille *et al.* 1986), birds (see review by Birkhead & Møller 1992; Briskie *et al.* 1992; Graves *et al.* 1993; Houtman 1992; Kempenaers *et al.* 1992; Møller 1991; Wagner 1992; Wetton *et al.* 1987), and mammals (Ginsberg & Huck 1989; Gomendio & Roldan 1991; Harcourt 1991; Smith 1984).

Multiple paternity in animals with external fertilization.

Multiple paternity has been reported in fish with external fertilization, where unpaired males (sneakers and streakers) swim over to spawning pairs and release sperm (Dominey 1980; Gross 1991; Keenleyside 1972; Peterson 1990; van den Berghe, *et al.* 1989). Multiple paternity has also been suggested in aquatically ovipositing anurans where unamplexed males gather near spawning pairs and may be releasing sperm analogously to sneakers and streakers in fish (Halliday & Verrell 1984). However, multiple paternity has not been documented in anurans (Halliday & Verrell 1984; Jennions *et al.* 1992), the only group of tetrapod vertebrates with external fertilization.

The potential for multiple paternity in anurans.

In contrast to the internal fertilization found in arthropods, some fishes, and amniotic vertebrates, male anurans fertilize eggs externally by releasing sperm over the eggs during, or shortly after the eggs leave the female's body (Halliday & Verrell 1984). Females are not known to possess sperm storage organs and males generally lack intromittent organs (Halliday and Verrell 1984). Thus, the potential for more than one male to have direct access to the unfertilized eggs should make mating males particularly vulnerable to multiple fertilization.

In addition, there are a number of factors that should encourage male anurans to engage in extra-pair copulations to increase their reproductive success. First, unlike many birds and mammals, most anurans are characterized by a lack of male parental care, enabling males to seek successive mating opportunities (Duellman & Trueb 1986; Wells 1981). Second, many anurans have mating systems characterized by high OSR, the result of males remaining at their breeding sites throughout the breeding period, while receptive females arrive asynchronously (Wells 1977). As the ratio of males to females increases, intrasexual competition for mates is expected to intensify (Ryan 1985; Wells 1977) and the potential for matings by multiple males should increase

(Birkhead & Møller 1992). Male anurans may also lack the ability to effectively guard an ovipositing female from other male's sperm.

Females also should have a role in determining whether to mate with a single or multiple males because of the potential advantages or disadvantages of mating with multiple males (e.g., "good genes", genetic diversity of offspring, insurance against infertility or direct cost to females by predation and loss of fertilization efficiency). One way females may be able to encourage matings by multiple males is to exhibit behaviors that enable multiple males to join during egg-laying. For example, in racophorid frogs, females construct large foam nests into which multiple males are thought to release their sperm (Coe 1967; Kato 1956; Jennions *et al.* 1992).

Characteristics of anurans in which multiple male mating has been reported

The aggregation of multiple males around an ovipositing female has been reported in three species of arboreal ovipositing hylid anurans (Pyburn 1963, 1970; Roberts 1994; Wiewandt 1971), and five species of terrestrial and arboreal ovipositing rhacophorid anurans (Coe 1967; Feng & Narins

1991; Fukuyama 1991; Jennions *et al.* 1992; Kasuya *et al.* 1987; Kato 1956; Kusano *et al.* 1991).

The site of egg development may limit the potential for multiple-male matings. Of the three main categories for sites of egg deposition- 1) aquatic egg-laying, 2) terrestrial egg-laying or arboreal egg-laying, and 3) eggs carried by the adult (Duellman & Trueb 1986)- matings by multiple males have only been reported in the terrestrial or arboreal egglayers. In the family Hylidae, matings by multiple males have been reported for the arboreal leaf-breeders, *Agalychnis callidryas* (Pyburn 1963, 1970), *A. saltator* (Roberts 1994), and *Pachymedusa dacnicolor* (Wiewandt 1971). These three species lay eggs in jelly masses on leaves overhanging ponds. The eggs undergo development to tadpoles which then hatch and fall into the water below.

Matings by multiple males have also been reported in the family Rhacophoridae. *Rhacophorus schlegelii* deposits eggs terrestrially in a foam nest in a burrow (Fukuyama 1991), while *Chiromantis rufescens*, *C. xeropelima*, *Rhacophorus arboreus*, and *Polypedates leucomystax* oviposit in arboreal foam nest constructed by the females (Coe 1967, 1974; Feng & Narins 1991; Kasuya *et al.* 1987; Kusano *et al.*

1991).

In contrast to species that lay their eggs in water, the sperm of species that oviposit terrestrially or arboreally may be able to place their sperm in close proximity to the eggs without it being washed away by water currents. Additionally, the substrate (ground egg-laying), branch or leaf (arboreal egg-laying) enable intruding males to position their cloacae in close proximity to the egg mass without having to directly amplex the female. In aquatic ovipositers, intruding males have nothing to grasp to position themselves close enough to the emerging eggmass to effectively fertilize the eggs.

Study questions

Agalychnis callidryas was chosen to address the questions: (1) Do matings by multiple males result in multiple paternity, (2) how prevalent is multiple paternity in this species, and (3) what constellation of ecological characteristics might facilitate multiple paternity?

Study organism

Matings by multiple males have been reported by Pyburn (1963, 1970) and observed by me. The information on *A. callidryas* breeding biology is from studies in the Tuxta

Mountains of southern Veracruz, Mexico (Pyburn 1963, 1970) and a species summary by Duellman (1970).

The treefrog, *A. callidryas,* is an arboreal leaf-breeder. Its range extends from southern Mexico through Panama, with breeding activity coinciding with the start of the rainy season (April-June, depending on location), and lasting for the duration of the wet season (August-December, depending on location). Females move from the forest to ponds where males call from the elevated pondside vegetation. Male advertisement calls are described as a single or double note "tlock" (Dunn 1931), "quirt" (Breder 1946), "chock" (Duellman 1970), or "chack" (Pyburn 1970). Females approach single males or groups of males calling from the vegetation. After a female is amplexed by a male, she carries him to the pond where she absorbs water through her cloaca for hydration of the egg clutch. Following hydration she carries the male to a leaf overhanging the water and oviposits on the leaf surface (Pyburn 1963, 1970) and then returns to the water to rehydrate her eggs between clutches.

Unamplexed "secondary" males can be attracted to movement of paired frogs consisting of a female amplexed by a "primary" male. Secondary males may attempt to join the

pair or pull the primary male off of the female (Pyburn 1970). The attempt by secondary males to join pairs does not result in injury to either male, and was not observed between unpaired males (Pyburn 1970). An amplectant female remains stationary while males attempt to dislodge each other. If the female has reached an egg-laying site, she may influence the outcome of a struggle by beginning egg-laying. Once she has started to oviposit, both males stop moving and hold onto the female, but at the cessation of egg-laying, the males may renew the struggle (Pyburn 1970).

CHAPTER 2

Ecology and breeding biology

Introduction

Matings that involve multiple males are relatively uncommon in anurans. Such matings occur in 10 species from two of 21 extant families (Hylidae, Rhacophoridae) that oviposit in terrestrial habitats; *Agalychnis callidryas*, Pyburn 1963, 1970; *A. saltator*, Roberts 1994; *Pachymedusa dacnicolor*, Wiewandt 1971; *Phyllomedusa burmeisteri*, Carduso 1991, personal communication; *Rhacophorus schlegelii*, Kato 1956; *R. arboreus*, Kasuya *et al.* 1987; *Chiromantis rufescens*, Coe 1967; *C. petersii*, Coe 1974; *C. xerampelina*, Jennions *et al.* 1992; and *Polypedates leucomystax*, Feng & Narins 1991. The basic breeding biology and ecology of species with matings that involve multiple males is poorly understood. Thus, the factors that may encourage or limit mating by multiple males are unknown. A preliminary step in understanding this breeding behavior is to gather information on the basic breeding biology and ecology of a

species where matings that involve multiple males occur. This study investigates the breeding biology and ecology of the Red-eyed treefrog, *Agalychnis callidryas*, a species in which matings that involve multiple males occur (Pyburn 1963, 1970), in order to gather data necessary for determining the factors that may influence the presence of matings that involve multiple males.

Information on the basic breeding biology and ecology of *A. callidryas* is limited (Duellman 1970; Pyburn 1963, 1970). For example, basic reproductive parameters such as the number of clutches per female and clutch size are unknown as are the nightly activity patterns of frogs and whether frogs are explosive or long-term breeders. Moreover, while mating that involve multiple males have been observed (Pyburn 1963,1970), the frequency of this unusual reproductive strategy for anurans is unknown. There are also no data on operational sex ratios of breeding aggregations, which could indicate competitive pressures on males for mates (Emlen & Oring 1977).

My objectives were to: (1) determine the numbers and activity patterns of males and females; (2) determine the operational sex ratios of males to females; (3) determine the frequency of matings involving multiple males; (4)

determine the mean number of clutches and eggs produced by females during an egg-laying session; (5) determine the snout-vent lengths (SVL) and masses of females, amplexed males, and unamplexed males; (6) determine site fidelity by males and females.

Materials and Methods

All observations were made at a seasonal pond, "Ocelot pond", located approximately 2 km south of Gamboa, Republic of Panama. The pond increased in size from approximately 1 x 2 m to 25 x 90 m from mid April through November. Second growth rainforest surrounded the pond. The leaves of overhanging shrubbery surrounding the pond served as egg-laying substrates. Censuses took place at two different trees, and along a 50 m transect parallel to the shoreline. All observations were made using a headlamp covered with two layers of red acetate from a distance of 1-5 m. At distances greater than 1.5m, 8x20 binoculars were used. Individuals were identified by individual variation in dorsal striping and/or the presence or absence of a unique pattern of dorsal spots. Male mass as determined using either an electronic balance or Pesola scale to the nearest 0.1 g, and SVL as recorded to the nearest mm. All measurements and data are presented as mean (± SE).

Censuses

To determine the nightly activity patterns of breeding frogs, and the pattern of breeding activity over an extended

period, two types of censuses were conducted. The "nightly activity census" combined the results from a six night census and a eight night census, conducted on two different years, to assess the nightly activity patterns of males, females, pairs, OSRs, and the frequency of matings involving multiple males. The "long-term breeding census" was used to assess the patterns of breeding activity over a period of 80 nights during one breeding season.

Nightly activity patterns

The results from two censuses were combined to determine the nightly activity patterns of males and females. The first census was conducted on eight nights from 8-21 June, 1991 from 2100-0500 h. The census site was a tree (*Alibertia edulis*) the foliage of which encompassed an area of approximately 30 m^3 (4 m high x 2.5 m width x 3 m depth). The tree was situated on the edge of the pond with approximately 70% of the foliage overhanging the water.

The second census was on six nights from 17-22 May, 1994 from 1900-0600 h. The census site was a treefall encompassing an area of approximately 11 m^3 (5 m long x 1.5 m width x 1.5 m depth). Approximately 60% of the tree was over the water.

Variables recorded during censuses were: (1) date; (2) hour; (3) sex; (4) whether an individual was amplexed or not; (5) number of clutches layed by female amplexed by one male; and (6) number of clutches layed by females with multiple males. A nightly OSR of the number of males divided by the number of females was calculated for the 2300 h census. No OSR was calculated when females were not present.

Long-term breeding patterns

To determine long-term breeding patterns, censuses were conducted on 80 nights between 2200-2300 h from May 16 to August 11, 1993. Censusing took place on a 50 m transect that bisected vegetation parallel to the east side of the pond shore. Surveyor's flagging delimited the census site at 10 m intervals. I walked the census route and recorded any frogs seen or heard within approximately 2 m laterally and 5 m vertically on either side of the census trail. Two rain gauges were situated at different locations at the site. Variables recorded each evening were: (1) date; (2) temperature read to the nearest ^{0}C at the time of census; (3) precipitation in mm for previous 24 h; (4) number of females; (5) number of males; (6) number of amplexed pairs;

and (7) number of females with multiple males.

Focal female observations

Continuous focal animal observations (Altmann 1974) were conducted to determine the sequence of activities involved from initial amplexus to the end of egg-laying.

Observations were started when I found an unamplexed female. I following her movements from a distance of 2-5 m using 8x20 binoculars. Actions were recorded in a notebook and timed to the nearest minute and continued through the completion of egg-laying.

A total of six females were observed on four different nights (8 June, 1991; 9 June, 1991; 16 July 1993; 8 August 1993). Variables recorded were: (1) number of clutches oviposited; (2) number of eggs oviposited in each clutch; and (3) total duration of egg-laying activities; and (4) the distance males moved to amplex the females.

Egg number and clutch location

The number of eggs per clutch and the location of clutches were recorded from 105 clutches chosen at random from around the periphery of the pond on various dates from May-July 1993. Variables recorded were: (1) height above

water in cm, (2) position of clutches on the leaf (e.g., topside or underside), (3) egg color (e.g., green or yellow), and the number of eggs per clutch. The height measurements taken underestimate actual mean clutch height because the height of clutches above 3 m could not be measured. Clutches were seen as high as 15 m above the pond. I judge that I was able to reach approximately 70% of all clutches I saw.

Secondary male behavior

Matings that included multiple males were observed to determine: (1) when secondary males joined amplexed pairs; (2) how secondary males joined pairs; (3) how many males were involved and (4) the position of primary males and secondary males in relation to the emerging eggs.

Phenotypic differences between males in matings with multiple males

Snout-vent lengths and masses of 27 pairs of primary and secondary males were compared with a paired t-test. Primary males are males that are first to amplex a female, while secondary males are males that join an already amplexed pair.

Site fidelity re-sighting census

Fifty-five frogs were marked using toe clipping of 1-3 toes (Hero 1989) and released at their site of capture between August 23 and September 23, 1990. The individuals coding number was attached to the perch using surveyor's flagging at the site of capture. All frogs encountered on subsequent nights were identified as previously captured or new captures. Distance in cm from the initial capture site to the recapture site was recorded. Unmarked individuals were marked and their sites of capture flagged.

Results

Nightly activity pattern

Nightly activity patterns varied with sex. Male frogs appeared at the census sites starting at 1900 h, with the highest number of unamplexed males arriving by 2200 h (Fig. 2.1). In contrast, females paired with males arrived gradually starting at 2000 h and reaching maximum numbers at 0200 h (Fig 2.1). Eighty-seven percent of the females (27/31) censused were amplexed before reaching the census sites. At the cessation of egg-laying pairs separated, and both males and females moved into the higher tree branches or disappeared, presumably moving into the canopy.

Frequency of matings that involve multiple males

Matings by pairs and multiple males were observed during the censuses. Of a total of forty-nine matings, 26.5% (13/49) involved matings that involve multiple males.

Matings that involve multiple males were quite variable in occurrence. Egg-laying by females with multiple males was observed on two of the seven nights during the June 1991 census that females oviposited. During the 1994 census, eight of the nine matings that involved multiple males

occurred on a single night of the six nights that egg-laying was observed. During this night, 36% (8/22) of all matings recorded involved multiple males. During the May 1994 census, one female was recorded laying two clutches with multiple males.

Nightly OSR averaged 3.8 (± 0.4) and ranged from 1.7-5.0, males to females. The mean OSR of the four nights when matings involving multiple males were recorded was 2.5 (± 0.4) and ranged from 1.7 to 3.2 males to females.

Long-term breeding patterns

A total of 902 unamplexed males, 33 unamplexed females, and 117 amplexed pairs were observed during the 80 night census. The mean number of frogs observed per night was 14.5 (± 1.5) and the range was between 0-76. A mean of 11.3 (± 1.1) males were recorded per night. Males were present on 78 of the 80 nights. Unamplexed females accounted for 0.4 (± 0.1) frogs per night, while amplexed females represented 1.4 (± 0.3) frogs per night. One hundred and seventeen amplexed pairs were censused on 47 of the 80 nights. Fifteen females with multiple males were recorded on seven of the 80 nights. The nightly amount of rainfall was positively correlated with the total number of frogs

censused per night (Table 2.1).

The number of active frogs, rather than operational sex ratio, was a better predictor of nights when matings that involve multiple males would occur. The mean OSR on nights when matings that included multiple males occurred was 5.2 (± 1.0) males to females, which did not significantly differ from the average nightly mean of 7.4 (± 0.5), (Mann-Whitney U test, z=1.81, P=0.07). In contrast, on nights when matings that included multiple males occurred the mean of 43.0 (± 7.4) frogs differed significantly from the mean of 15.5 (± 1.4) frogs per night when matings that included multiple males did not occur (Mann-Whitney U test; z=-3.72, P<0.001). Nightly operational sex ratios were not correlated with density (Fig 2.3, r^2=0.0001, P=0.94; Least Squares regression).

Site fidelity and recapture

Males exhibited site fidelity. Of the 39 males marked, 12 males were re-sighted a mean of 2 ± 0.4 times (range 1-4 times). The mean recapture distance was 80 ± 22 cm. Males were seen for an average of 20.5 (± 5.0) days after capture (range 1-55), with the exception of one male who was found 1 year later within 2 m of his original site of capture. None

of the eight females marked were recaptured.

Focal female observations

To determine the behavioral sequences involved in amplexus and egg-laying, females were observed continuously from the time that they approached a male until they had completed egg-laying. Females (N=10) were observed moving toward calling males. The males initiated amplexus by moving (jumping or crawling) toward females and clasping the female. After females were amplexed (N=5), they spent a mean of 135 (± 37.8) min moving toward egg-laying sites before egg-laying started. The time between clutches (N=10) ranged from 63-143 min with a mean of 95 (± 11.1) min. During this period females either moved to the margin on the pond to hydrate their next clutch, or to adjacent branches where moisture on the surface of leaves or branches was used to hydrate the clutch. Successive clutches were oviposited on different leaves. Clutches averaged 142.6 (± 6.1) cm above the surface of the water. Most eggs (60%) were oviposited on the underside of leaves, while 40% were on the topside. After all clutches were oviposited, males crawled off females and both males and females moved up branches towards the forest canopy. The total duration, starting

with amplexus and ending after egg-laying of all clutches, was 410 (± 23.3) min, with a range of 325-495 min.

Six females were observed from the beginning to the end of their egg-laying activities to determine the number of clutches laid and the total number of eggs oviposited in a night. Females laid between 2-4 clutches with a mean of 3.0 (± 0.26) clutches. The combined total of eggs layed in all clutches per female varied from 129-182 eggs with a mean of 153 (± 9.0) eggs. The mean number of eggs produced per clutch did not differ significantly between females ($F_{5,17}$=0.70, P=0.64, block design ANOVA) or clutches ($F_{3,17}$=0.89, P=0.48, block design ANOVA).

Variation in egg color has not been previously reported for *Agalychnis callidryas*. Of 105 egg clutches surveyed, approximately 76% were a previously described green color (Duellman 1970), while 24% were yellow. Individual clutches consisted of one color only. The relative proportion of clutches placed on the topside or underside of leaves were similar for both green and yellow eggs.

Primary and secondary males in multiple male matings

Primary males initiated amplexus by crawling or jumping on females from variable distances, with a mean distance of

21.6 (± 5.2) cm (range 3-50 cm). Males remained amplexed until the female had finished laying all clutches.

Ninety-four percent (32/34) of the secondary males that were successful in joining a pair for egg-laying, joined the pair at the egg-laying site. In only two of 34 matings involving multiple males, did secondary males successfully join an already amplexed pair before the female had reached an egg-laying site. In these matings, the females carried both males to the egg-laying site. Secondary males joined pairs by jumping on the pair or leaf where the female was egg-laying. Secondary males then attempted to insert their cloacae into the eggmass.

Secondary males were generally thwarted in their attempts to join pairs prior to egg-laying by either female movement, or by the primary male pushing or kicking the secondary male. However, once the female began to oviposit, primary males generally remained motionless and secondary males pushed their cloacae into various positions near the emerging eggmass.

Positional data was collected from 29 of the 34 matings involving secondary males. Secondary males achieved three general positions in relation to the primary male and female. Fifty-two percent (15/29) of the secondary males

were positioned laterally to the pair and not directly amplexed to either female or primary male. Thirty-one percent (9/29) of the secondary males positioned their cloacae underneath the primary male's cloaca and next to the female's cloaca. Approximately 17% (5/29) of the secondary males were amplexed to the primary male's back and positioned their cloacae posterior to the primary male's cloaca.

The mean SVL and mass of primary and secondary males from the same mating were compared to determine if differences existed between males that could potentially be used by females to assess male quality. Neither mean SVL nor mass of primary males (44.8 ± 0.4 mm, 3.3 ± 0.1 g) differed significantly from the mean SVL and mass of secondary males (45.3 ± 0.3 mm, 3.4 ± 0.1 g) (two-tailed, paired *t*-test, N=27, t=-0.995, P=0.329; t=-0.721, P=0.477, respectively). During the May 1994 census, one male was a primary male on one night, and a secondary male three nights later.

Discussion

Breeding biology

Previous studies report prolonged breeding patterns for *A. callidryas* (Donnelly & Guyer 1994; Pyburn 1970; Wells 1977). This study reveals that *A. callidryas* has breeding patterns with characteristics of both prolonged and explosive breeders. *Agalychnis callidryas* bred throughout the 80 night census but on nights following heavy rains, after periods of little precipitation, there was an increase of 4-9 fold in the number of frogs breeding. The combined patterns of prolonged and explosive breeding are reflected in the mate searching behavior of males (pers. obs.). Males resort to a mixed strategy of calling from a stationary position when under prolonged breeding conditions of low density. When densities increase during explosive breeding conditions, males resort to scramble competition in an attempt to secure mates. This combination of mate acquisition strategies may be one of the reasons why matings that involve multiple males occur in this species.

Nightly activity pattern and the opportunity for matings that involve multiple males

Primary males remained amplexed to females until the females had finished laying all their clutches. Thus, primary males remained amplexed to the female between clutches while females hydrated their next clutch. Because of the duration of egg-laying activities (approximately 7 h.), primary males presumably mated with only one female in a night.

This behavior contrasts with the behavior of primary males of the rhacophorid frog *Chiromantis xerampelina*, where secondary males insert their cloacae into arboreal foam nest produced by the female. Between egg-laying bouts, when the female descends to the water to hydrate, about half of the primary males dismounted from the female and remained at the nest. When the female returned to the nest, only 11% of the primary males were successful in re-amplexing the female (Jennions *et al.* 1992). As a result most primary males became secondary males in the next egg-laying bout. It is unclear why the primary males do not remain amplexed to the female when their chance of being displaced in the next egg-laying bout are so high. *Agalychnis callidryas* males, in contrast, remain amplexed for all egg clutches, a

behavior that insures the highest number of fertilized eggs.

Primary males may also remain with one females because the male biased OSR makes it unlikely that an amplexed primary male will encounter another unamplexed female. Unamplexed females were seen only during the beginning of nightly activity and the majority were amplexed before they reached the egg-laying sites. Thus a primary male would either have to move away from the egg-laying site to increase the probability of encountering an unamplexed female, or a primary male would have to switch to the role of a secondary male at the egg-laying site.

Occurrence of matings that involve multiple males

Matings that included multiple males occurred on nights when the total number of frogs at the breeding site was higher than the average nightly number. In contrast, the OSR on nights when matings that included multiple males took place was less male biased than the average OSR of nights when no matings that included multiple males were recorded. This suggests that the conditions associated with high densities: decreased distances between frogs, and increased movement, may facilitate matings that involve multiple males. For example, in other species, an increase in male

densities results in a shift from stationary calling to searching behavior (Arak 1983a; Wells 1977). In *A. callidryas*, increased searching results in more amplexed pairs being intercepted and thus increases the opportunity for mating by multiple males. Matings that involve multiple males also take place at higher than average density for *A. saltator*, the only other member of the genus for which matings that involve multiple males have been reported (Roberts 1994).

An unexpected result of this study, was that operational sex ratio was not correlated with the occurrence of matings that involve multiple males. Emlen & Oring (1977) suggest that male-male competition for females increases when females arrive asynchronously and the operational sex ratio is male biased. As the OSR becomes more male biased the potential for matings that involve multiple males should increase (Birkhead & Møller 1992). Similarly unexpected results were found in a study of how the occurrence of fighting for females is influenced by OSR in the toad *Bufo calamita* (Tejedo 1988). Male fighting for access to females was associated with less male-biased OSR than with more male-biased OSR. One hypothesis for the decrease in male aggression under male-biased OSR is that

the scarcity of pairs in the breeding area may dilute the aggression against males because males are more likely to encounter other males than pairs under these conditions (Tejedo 1988). Another explanation for the lack of correlation between OSR and matings that involve multiple males is that even when there are a large number of males per female, there can still be low densities of frogs. Under lower densities unamplexed males may not move as frequently, resulting in a lower probability of encountering pairs. Lower densities may also result in increased spacing between individuals resulting in pairs reaching egg-laying sites without being detected by secondary males.

Table 2.1 Pearson correlation coefficients with *P* values, for the variables: precipitation, number of males, number of females, number of amplexed frogs, and total number of frogs. An asterisk denotes statistical significance.

	Males	Females	Amplexed	Total
Rain/mm	0.59	0.41	0.56	0.64
	0.001*	0.001*	0.001*	0.001*

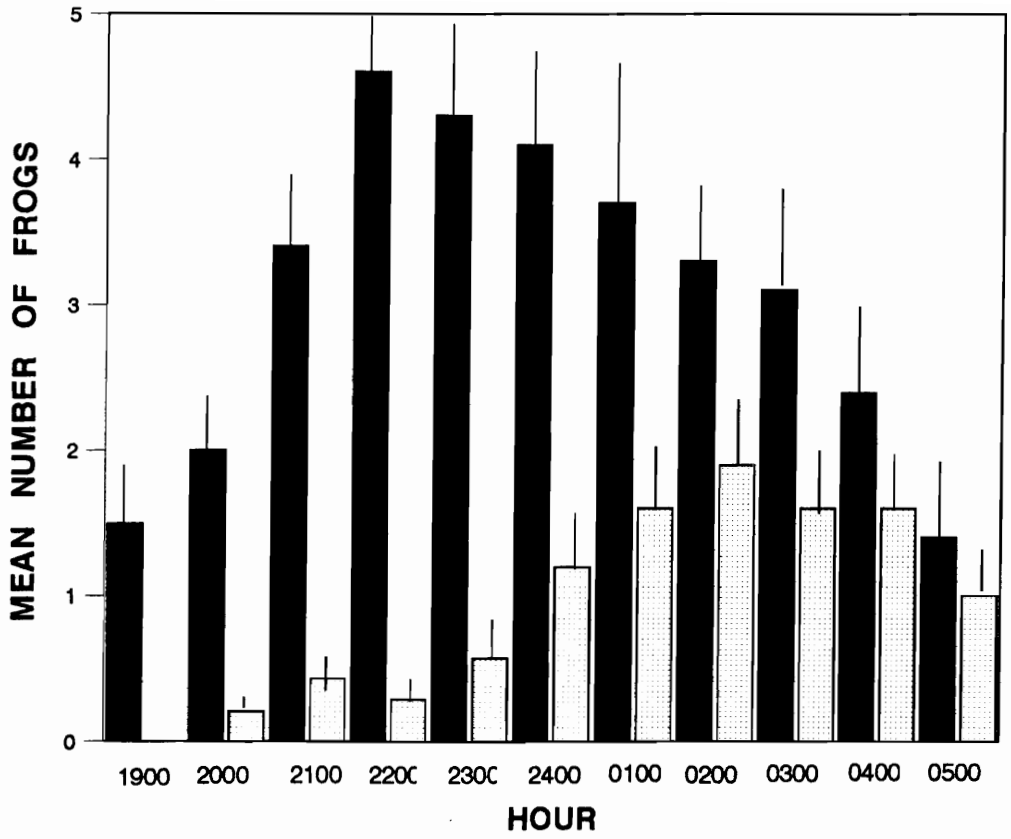

Figure 2.1 The combined mean (+1 SE) hourly number of active males and pairs of *Agalychnis callidryas* censused near Gamboa, Panama, from 1900-0500 h on eight nights in June 1991 and six nights from May 17-22, 1994. Solid bars indicate mean number of males. Stippled bars indicate mean number of pairs.

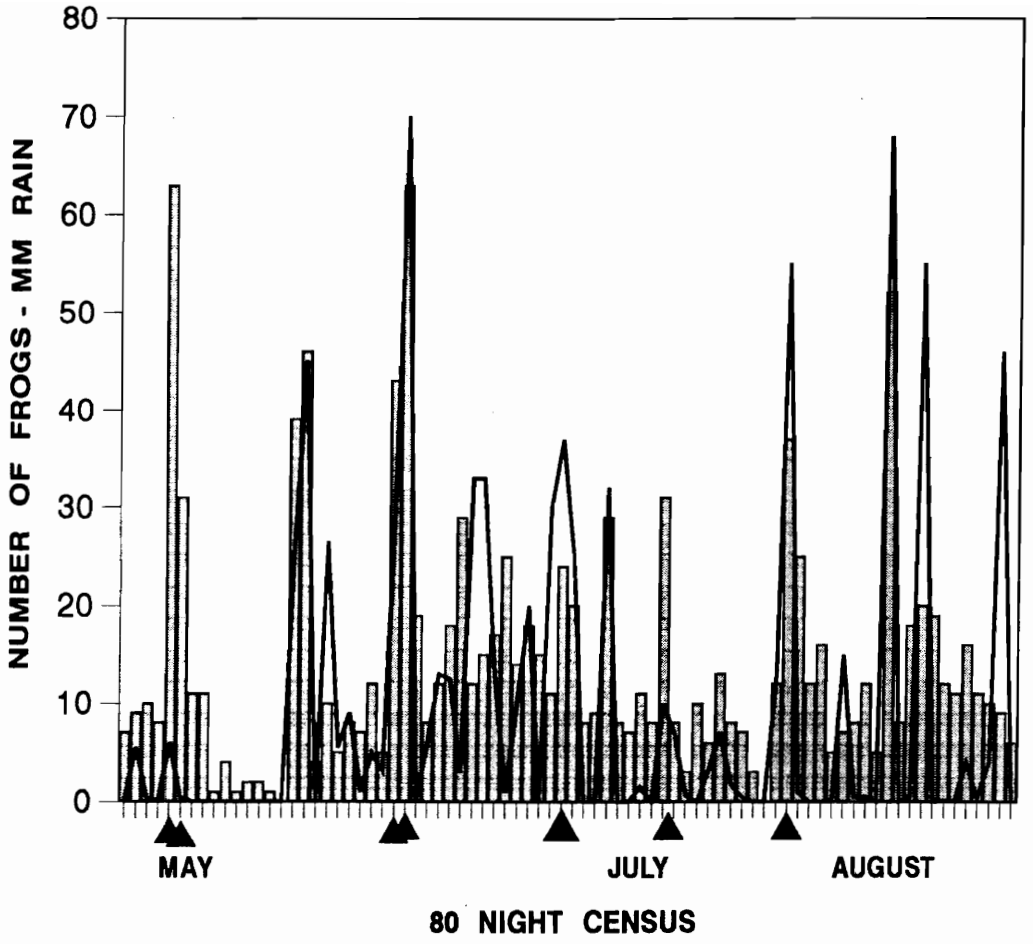

Figure 2.2 The number of *Agalychnis callidryas* censused nightly and the amount of precipitation in mm between May 16-August 11, 1993. Stippled bars indicate the number of frogs. Lines indicate the amount of precipitation in mm. Triangles denote nights on which matings that involve multiple males were recorded.

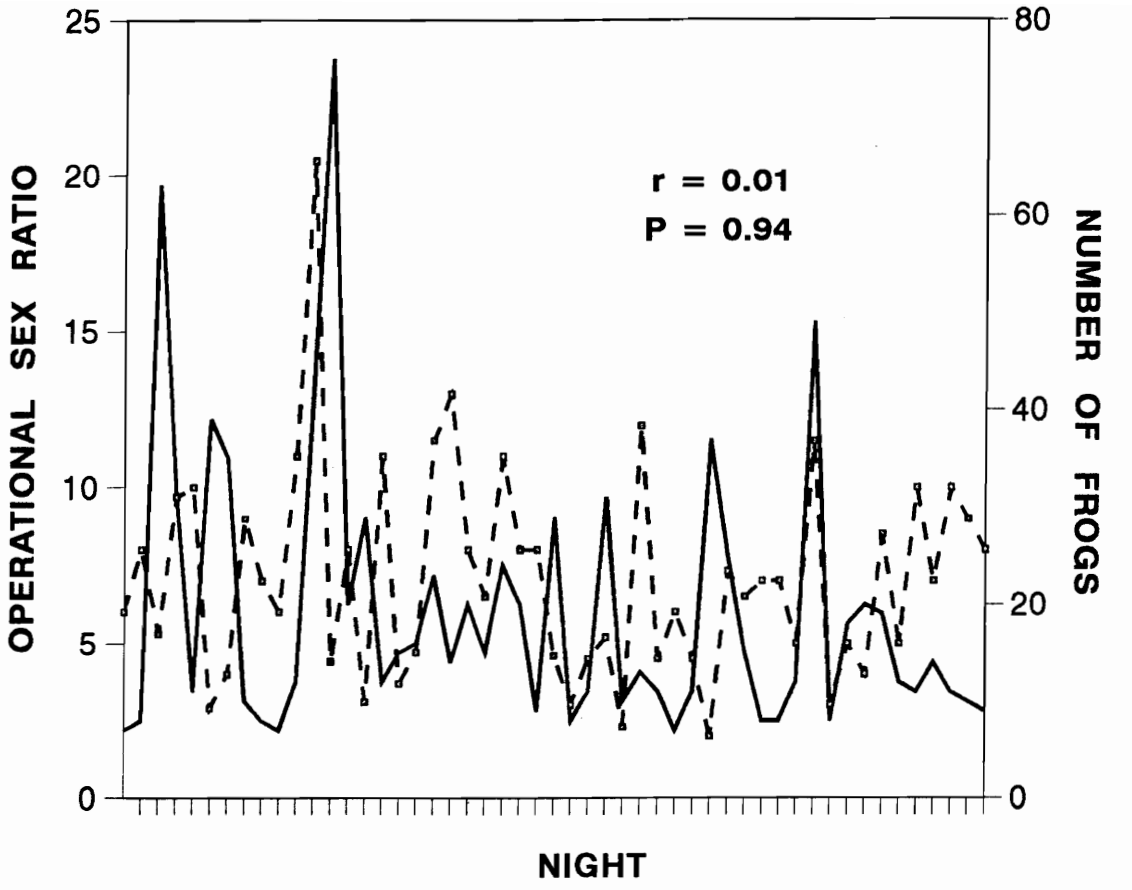

Figure 2.3 The number of *Agalychnis callidryas* frogs present and their operational sex ratio (number of males/number of females) on nights when both males and females were present between May 16-August 11, 1993. The number of frogs are indicated by the solid line. The operational sex ratio is indicated by the dotted line. No correlation was found between number of frogs and operational sex ratio.

CHAPTER 3

DNA evidence for multiple paternity

Introduction

External fertilization creates the potential for direct access to the eggs by unpaired males. In contrast to insects and most vertebrates, male anurans (frogs and toads) fertilize eggs externally as they are released by the female (Halliday & Verrell 1984). Thus, primary males (first male to pair with female) should be particularly vulnerable to competition from secondary males (males who join pair) that release sperm on the eggs during egg-laying. For example in the African frog, *Chiromantis xerampelina*, secondary males fertilized eggs when the sperm from the primary male was prevented from reaching the eggs (Jennions & Passmore 1993).

Although multiple paternity has been reported in widely disparate groups of animal: insects (Achmann *et al.* 1992; Parker 1970, 1984), fishes (Travis *et al.* 1990), amphibians (; Houck *et al.* 1985; Tilley & Hausman 1976), reptiles (Barry *et al.* 1992; Galbraith 1993; Stille *et al.* 1986), birds (Birkhead & Møller 1992; Burke & Buford 1987; Wetton

et al. 1987), there is no direct evidence for multiple paternity in anurans.

Aggregations of secondary males around a spawning pair have been reported in 10 species from two families (Hylidae, Rhacophoridae) that oviposit in terrestrial habitats *Agalychnis callidryas*, Pyburn 1963, 1970; *A. saltator*, Roberts 1994; *Pachymedusa dacnicolor*, Wiewandt 1971; *Phyllomedusa burmeisteri*, Carduso 1991, personal communication; *Rhacophorus schlegelii*, Kato 1956; *R. arboreus*, Kasuya *et al.* 1987; *Chiromantis rufescens*, Coe 1967; *C. petersii*, Coe 1974; *C. xerampelina*, Jennions *et al.* 1992; and *Polypedates leucomystax*, Feng & Narins 1991. Multiple paternity may occur in these species and aquatic ovipositors in which secondary males gather near spawning pairs (Halliday & Verrell 1984).

Despite the potential for secondary males to fertilize eggs, aggregations of multiple males around a spawning pairs have not been reported for the majority of anuran species. One potential explanation for the lack of multi-male matings is absolute sperm priority by primary males (Jennions & Passmore 1993). Absolute sperm priority could result from the position of the primary male's cloaca directly over the emerging eggs, thus his sperm should reach the eggs first

and preclude fertilization by other males. Here we report that in two multi-male matings of the red-eyed treefrog, *Agalychnis callidryas*, both primary and secondary males fathered progeny. The results do not support a hypothesis of first male sperm priority in anurans.

Materials and methods

The red-eyed treefrog is distributed from southern Mexico through the Republic of Panama. The study site was located at a seasonal pond, approximately 2 km south of Gamboa, Republic of Panama, where nine multi-male matings occurred out of a total of 29 matings during a six night census. The nightly frequency of multi-male matings was highly variable, ranging from 0% (0/2) to 36% (8/22) during the census.

Females moved from the surrounding forest canopy to pondside vegetation from which males called. After a female was amplexed (male's nuptial clasp prior to egg-laying) by a primary male, she carried that male to an egg-laying site on a leaf overhanging the water. Females laid from 2-4 separate clutches ($\bar{x}$=47 eggs/per clutch). Between successive clutches pairs usually moved to the pond margin, where the female absorbed water to hydrate the next clutch. After hydration, pairs moved to a different egg-laying site to lay another clutch. Secondary males frequently attempted to intercept an amplectant pair on their way to an egg-laying site or during egg-laying. Successful secondary males gained positions on the primary male's back, female's

back, or aligned themselves laterally to the pair during egg-laying (Fig. 3.1). As egg-laying began, males scuffled to position their cloacae against the eggs. Thus, the assignment of paternity can be unequivocally limited to the males observed during egg-laying.

Two observed multi-male matings, each consisting of two males and a female, were collected along with released eggs.

Tissue collection

DNA fingerprinting analysis of parents used three toes per frog, in accordance with guidelines for the treatment of animals in behavioral research (Animal Behaviour 1995). DNA analysis of offspring used a portion of tail from randomly chosen tadpoles reared to stages 43-44 (Goner 1960). Tissues were stored in liquid nitrogen or methanol and transported to Blacksburg, Virginia.

DNA fingerprinting

DNA isolation

Methods of DNA isolation and fingerprinting were modified from those of Laughlin *et al.* (1994). DNA was obtained from frog toes or tadpole tails that were finely minced with a razor and placed in individually labeled test tubes containing 1 ml of extraction buffer. The buffer

contained 10 mM Tris-Cl, 0.1 M ethylenedinitrilo-tetraacetic acid disodium salt (EDTA) and 0.5% Sarkosyl at pH 8.0. One hundred *ug* proteinase K was added to the extraction buffer and the homogenate was incubated overnight at 50^0 C in a water bath.

The homogenates were then subjected to two extractions using equal volumes of phenol:chloroform equilibrated with 1 X TE (10 mM Tris, 1 mM EDTA). After the phenol:chlorophorm was added to each homogenate, the test tube was covered with parafilm and gently inverted 20 times to mix homogenate with phenol:chloroform. The mixture was then centrifuged at 5,000 x G for 30 min and the aqueous phase drawn off with a wide bore transfer pipette, and used in the next extraction. Following the phenol:chloroform extractions, two extractions using equal volumes of 24:1 chloroform:isoamyl alcohol were preformed.

To precipitate DNA, two volumes of -20^0 C 95% ethanol in the presence of 2 - 3 M ammonium acetate. Samples were then covered with parafilm and gently inverted 5-10 times until DNA became visible, then centrifuged at -20^0 C for 20 minutes at 5,000 x G. DNA pellets were washed with 70% ethanol and allowed to air dry after removing ethanol. The pellet was redissolved in 1 ml of 1 X TE and incubated

overnight at 37° C. Samples were placed in dialysis tubing and dialyzed in 1X TE for 24-72 h.

Measurement of DNA concentration

DNA concentration was calibrated using a fluorometer with *A. callidryas* DNA (100-200/*u*g/ml) as a standard and Hoechst dye 33258. Four *u*l of frog DNA were placed in a cuvette containing 2 ml of 0.1 *u*g/ml Hoechst dye and 1 X TNE (10mM Tris, 1 mM EDTA, 0.1 M NaCl, pH 7.4). The cuvette was rinsed in distilled water and vacuumed dry between samples.

DNA digestion and electrophoresis

Fifteen *u*g of genomic DNA per sample were digested overnight at 37°C with Pal I enzyme or Alu I enzyme following manufacturers specifications. DNA was fractionated on 1.0% agarose gels in the presence of 0.5 *ug*/ml ethidium bromide at 25 V. One lane was used as a molecular weight marker by loading 10 *u*g of BstE II-digested lambda DNA, five *u*l ethidium bromide and 10 *u*l distilled water. Gels were run at 25 V from 72-150 h in a recirculating solution of 1 X TAE buffer. Depending on the gel, fragments smaller than 1.5-6.0 kilobase pairs of DNA were run off the gel. The gel was photographed with UV

transillumination with a mm ruler placed next to the lane containing the molecular weight marker.

Gels were placed on two sheets of Whatman 3M paper and covered with plastic wrap, then placed on a vacuum gel drier. Gels were dried for 2.5 h at 55^0 C. After drying, gels were rinsed in distilled water and denatured. Denaturation of DNA in the gel was accomplished with two 30 m washes using 0.5M NaOH in a 1L solution. Between washes the gel was rinsed in distilled water. Neutralization followed, using two 30 minute washes in a 1L solution of 0.5 M Tris containing 0.15 M NaCl at pH 7.4.

Hybridization and autoradiography

The oligonucleotide $(GGCAGG)_4$ (Mitani *et al.* 1990) or $(CAC)_5$ (Schafer *et al.* 1988) were used as probes. Fifty *u*Ci [gamma-^{32}P]ATP at 6000 Ci/mmol with 20 units T4 polynucleotide kinase, at 37 ^{0}C for one hour, was used to label probes. In-gel hybridization took place overnight in a rotisserie type hybridization unit. The gel was placed into a hybridization cylinder with 25 ml of hybridization buffer containing 5 X SSPE (0.15 M NaCl, 10 mM sodium phosphate monobasic, 1 mM EDTA), 0.1% sodium dodecyl sulfate (SDS), and 1 *u*g sonicated transfer RNA. Two hundred ng of labeled probe were then added to the hybridization cylinder.

Temperature of hybridization was approximately 55 ^{0}C for $(GGCAGG)_4$, and 48 ^{0}C for $(CAC)_5$.

After hybridization the gel was first placed in a stringency wash of 1L of 2 X SSPE (0.15 M NaCl, 10 mM sodium phosphate monobasic, 1 mM EDTA) with 0.05% SDS at room temperature, and agitated for an hour. The gel was then rinsed in distilled water and placed in 1L 5 X SSPE and 0.1% SDS for 30 minutes at hybridization temperature.

Gels were blotted dry on Whatman 3MM paper, wrapped in plastic, and placed between two intensity screens in a film cassette containing a sheet of Kodak XAR-5 diagnostic film. The cassette was placed at -80 ^{0}C for 2-10 days, depending on activity level.

Paternity analysis

Paternity analysis was based on the presence of characteristic paternal bands found in the offspring but not shared by the mother or other putative father (Jeffreys *et al.* 1985; Burke & Bruford 1987). To facilitate scoring of paternal bands, the three putative parents were run in adjacent lanes on both sides and in the middle of the gel (Fig. 3.2).

In addition, band-sharing between offspring and

putative parents was used to calculate similarity coefficients which were compared with the initial paternity assignments based on the presence of characteristic bands. The proportion of shared bands was calculated for the statistic $D = 2N_{AB}/(N_A + N_B)$, where N_{AB} is the number of fragments shared between two individuals, and N_A and N_B are the number of fragments in individuals A and B (Wetton *et al.* 1987). Band-sharing coefficients are expressed as means ± 1 SD.

Adult band-sharing

Band-sharing coefficients were calculated for 32 adults involved in 12 multi-male matings. Band-sharing coefficients in each mating were calculated between the female and both males; and between the primary and secondary male. DNA fingerprints for each mating were resolved on separate gels, therefore band-sharing coefficients were only calculated between individuals from the same gels (Table 3.2).

Results

Paternity of offspring

Mating #1.

Two males were positioned side-by-side on the female's back during egg-laying. Their relatively equal positioning prevented us from assigning primary or secondary status to either male. The probe $(GGCAGG)_4$ resolved 12 scorable bands for the female, 15 bands for each male, and 7-13 bands for each of the nine offspring examined ($\bar{x}$ = 9.1 ± 2.5) using fragments of approximately 4.8 kb and greater. Band-sharing coefficients between Female - Male 1 were (0.30), Female - Male 2 (0.22), and Male 1 - Male 2 (0.20).

Five offspring were assigned to Male 1 based on the presence of from 2-3 shared characteristic bands (Table 3.1; Fig. 3.2). The mean band-sharing coefficient between these five offspring and Male 1 was 0.58 ± 0.11. Four offspring not assigned to Male 1 had a mean band-sharing coefficient with Male 1 of 0.14 ± 0.10.

Four offspring were assigned to Male 2 based on the presence of 2-4 shared characteristic bands (Table 3.1, Fig. 3.2). These offspring had a mean band-sharing coefficient with Male 2 of 0.46 ± 0.03. Five offspring not assigned to Male 2 had a mean band-sharing coefficient with Male 2 of

0.26 ± 0.08. The mean band-sharing coefficient between mother and offspring was 0.49 ± 0.07. Paternity could not be determined for three offspring due to poor resolution of individual lanes (insufficient DNA or incomplete digestion) and for two offspring because of lack of diagnostic bands shared with a putative father. No offspring had bands that could not be attributed to either the mother or one of the putative fathers.

Mating #2.

The female and primary male (Male 1) were joined by a secondary male (Male 2) who was situated posteriorly to the amplexed pair. The probe $(CAC)_5$ resolved 12 scorable maternal bands, 12 and 13 scorable bands for Male 1 and Male 2 respectively, scoring fragments larger than 6.3 kb (Fig. 3.3). Twelve offspring exhibited from 8-14 scorable bands ($\bar{x}$ = 10 ± 2.0). Band-sharing coefficients between Female-Male 1 were (0.33), Female-Male 2 (0.32), and Male 1-Male 2 (0.24).

Seven offspring were assigned to Male 1 based on the presence of 2-4 shared characteristic bands (Table 3.1; Fig.3.3). The mean band-sharing coefficient between assigned offspring - Male 1 was 0.58 ± 0.10. The mean band-

sharing coefficient between offspring not sharing characteristic bands with Male 1 was 0.20 ± 0.02.

Four offspring were assigned to Male 2 based on the presence of 2-4 shared characteristic bands (Table 3.1; Fig. 3.3). The mean band-sharing coefficient between these offspring and Male 2 was 0.64 ± 0.10. Six offspring not sharing characteristic bands with Male 2 had a mean band-sharing coefficient of 0.30 ± 0.14. The mean band-sharing coefficient between mother and offspring was 0.56 ± 0.08. Because one offspring (#9) showed no characteristic bands with either male, its paternity was not assigned. This offspring's band-sharing coefficient was 0.40 with Male 1, and 0.29 with Male 2.

Adult band-sharing

Nine female-primary male pairs had a mean band-sharing coefficient of 0.43 ± 0.17 bands-shared. Seven female-secondary male pairs had a mean band-sharing coefficient of 0.27 ± 0.12 bands shared. Ten primary-secondary male pairs had a mean of 0.39 ± 0.19 bands shared.

Discussion

This study demonstrates that matings that involve multiple males in the red-eyed treefrog, can result in multiple paternity. No evidence was found for complete sperm priority by the primary male. The lowest paternity value attributed to a secondary male was 36% (Mating #2). In Mating #1 where it was not possible to determine the relative status of individual males, the "secondary" male was responsible for either 44% or 56% of the fertilizations. These matings represent high levels of fertilization success by secondary males and indicate a potentially important way by which reproductive success can be increased in male-biased mating systems.

In addition, the majority (79-87.5%) of females are already amplexed when they enter the area of highest male densities to lay eggs. Therefore, the best option these males may have to increase their fitness is to join an already amplexed female and attempt to fertilize at least some of her clutch.

Secondary males may fertilize eggs either by partial physical displacement of the original male, or simply achieving proximity to the eggs. Thus, multiple paternity

should be common in species of anurans where secondary males join already paired females.

Table 3.1 Shared bands x 2 over total bands (s/b), band-sharing coefficients (c), and characteristic bands (u) shared between offspring and putative parents in 2 matings. Male 1 is symbolized as ♂▷, Male 2 is symbolized as ♂▶.

Offspring #		♀			♂▷			♂▶		
		s/b	c	u	s/b	c	u	s/b	c	u
M	1	12/23	0.55	2	6/26	0.23	0	12/26	0.46	3
a	2	10/21	0.48	1	4/24	0.17	0	12/24	0.50	4
t	3	10/20	0.50	0	4/23	0.17	0	10/23	0.43	2
i	4	10/19	0.53	0	10/22	0.45	2	6/22	0.27	0
n	5	8/20	0.40	2	0/22	0.00	0	10/22	0.45	3
g	6	10/24	0.42	1	18/27	0.67	3	8/27	0.30	0
	7	8/18	0.44	1	10/21	0.48	2	4/21	0.19	0
#	8	16/25	0.64	1	20/21	0.71	3	10/28	0.36	0
1	9	10/21	0.48	1	14/24	0.58	2	4/24	0.17	0
	1	10/21	0.48	1	12/21	0.57	2	10/22	0.46	0
M	2	14/25	0.56	1	18/25	0.72	3	12/26	0.46	0
a	3	12/22	0.55	3	4/22	0.18	0	12/23	0.52	4
t	4	10/20	0.50	1	10/20	0.50	2	6/21	0.29	0
i	5	18/26	0.69	4	6/26	0.23	0	16/27	0.60	4
n	6	10/21	0.48	1	4/21	0.19	0	16/22	0.73	3
g	7	16/23	0.70	4	10/23	0.44	2	8/24	0.33	0
	8	14/23	0.60	3	12/23	0.52	4	4/24	0.17	0
#	9	12/20	0.60	3	8/20	0.40	0	6/21	0.29	0
2	10	12/22	0.55	3	14/22	0.64	3	2/23	0.09	0
	11	14/25	0.56	3	16/25	0.64	4	6/26	0.23	0
	12	10/21	0.48	1	4/21	0.19	0	16/22	0.73	2

Table 3.2 The total number of bands for females and each of two males the female was mated with. The shared bands x 2 and band-sharing coefficients (coef) between female and each male and between males are given.

Mating	Total bands			2X Shared bands			Band-sharing coef		
	♀	♂1	♂2	♀♂1	♀♂2	♂1♂2	♀♂1	♀♂2	♂1♂2
1	0	20	16	-	-	20	-	-	.56
2	20	13	16	12	10	8	.37	.28	.28
3	8	4	4	4	2	6	.33	.17	.75
4	11	8	6	10	4	2	.53	.12	.14
5	0	16	15	-	-	10	-	-	.32
6	6	10	0	4	-	-	.25	-	-
7	13	12	12	18	8	6	.72	.32	.21
8	12	8	13	6	8	8	.30	.32	.38
9	0	9	9	-	-	8	-	-	.44
10	14	13	0	12	-	-	-	-	.44
11	22	18	18	13	10	10	.65	.50	.56
12	11	16	13	5	3	4	.31	.25	.27

Fig. 3.1 A mating of *Agalychnis callidryas,* in Gamboa, Panama, involving a light colored primary male amplexed to a larger female, and a dark colored secondary male positioned behind the primary male, inserting his cloaca into the eggs.

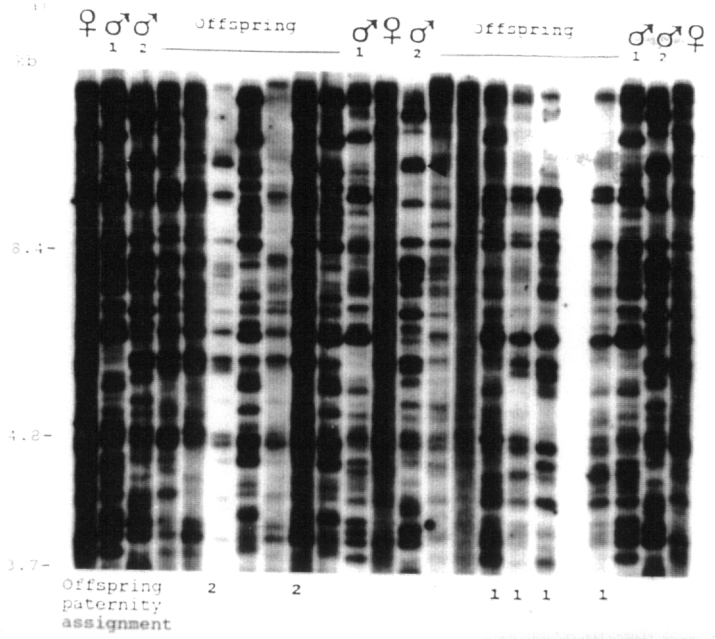

Fig. 3.2 DNA fingerprint of *Agalychnis callidryas* collected near Gamboa Panama, putative parents and offspring assignment. The probe $(GGCAGG)_5$ was used to assign paternity of 4 offspring to Male 1 and 5 offspring to Male 2. Critical bands used in paternity assignments are marked with open triangles for Male 1 and closed triangles for Male 2. *Bst II* digest lambda DNA size markers are shown on left.

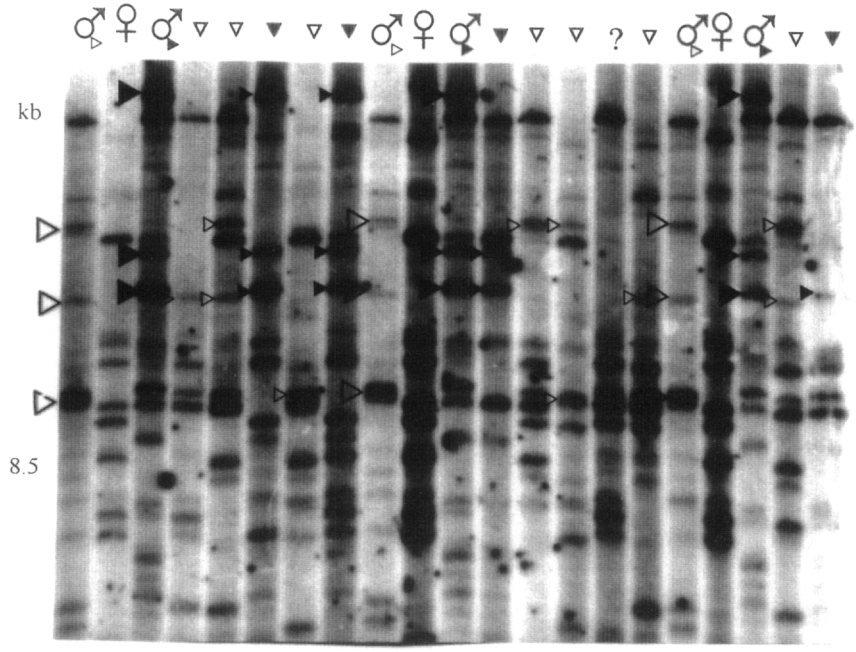

Fig. 3.3 DNA fingerprint of Mating #2. Putative parents and offspring assignment. Bands characteristic to each male are indicated by: open triangles for Male 1, filled triangles for Male 2. The triangle above a lane indicates the paternity assignment for that offspring. The question mark indicates an offspring of unresolved paternity (see text). *Bst II* digested lambda DNA 8.5 kb size marker is shown on left.

CHAPTER 4

The breeding biology of females: costs and benefits of matings involving multiple males

Introduction

Females in species with external fertilization are unable to manipulate sperm after mating. In contrast, females in species with internal fertilization may be able to influence the paternity of offspring through differentiated release of sperm stored from multiple males (Achmann *et al.* 1992; Birkhead & Møller 1992; Parker 1970, 1984). However, females of species with external fertilization can influence the paternity of offspring through varying the number of eggs produced when mating with single or multiple males. In some externally fertilized fishes, for example, females increase the number of spawn five- to eight-fold within minutes of the removal of peripheral males from the nest of a dominant male (van den Berghe *et al.* 1989).

Explanations given for why females should vary clutch size in the presence of single or multiple males can be

categorized into two groups: those in which females encourage multiple males to mate, and those in which females discourage multiple males from mating.

Females may mate with multiple males to increase the genetic diversity of offspring or to mate with males of higher genetic quality than their original mates (Birkhead & Møller 1992, Kempenaers *et al.* 1992). A number of studies of sexual selection in anurans suggest that either male-male competition (Davies & Halliday 1977; Davies & Halliday 1978; Howard & Kluge 1985; Wilbur *et al.* 1978) or female choice (Arak 1983b; Ryan 1985; Morris 1989) result in females pairing with larger males. Size and mass in both fishes and anurans may reflect either ability to effectively gain resources needed for growth, or be an index of longevity (because of continuous growth after reaching sexual maturity). Another explanation is that females mate with multiple males to ensure sufficient sperm to fertilize all eggs (Birkhead & Møller 1992). In the frog *Rana sylvatica*, Smith-Gill and Berven (1980) reported that fertilization potential decreased with subsequent matings by males.

Females might discourage matings that involve multiple males because of the potential for reduction in fertilization efficiency. In fish, the increased activity

of multiple males during spawning generates currents that might disperse sperm away from the eggs, thus decreasing the fertilization efficiency (van den Berghe *et al.* 1989). This situation is applicable to anurans (frogs & toads) that oviposit aquatically, but it should not apply to terrestrial or arboreal breeders. The potential for injury to the female may discourage females from mating with multiple males. In the European common toad, *Bufo bufo*, for example, the struggles of competing males to gain access to a female can result in the female's death by drowning (Davies & Halliday 1979).

There are a number of reasons why female anurans that produce jelly-like eggmasses on leaves should avoid mating with multiple males: (1) the weight or struggles of multiple males may prevent a female from reaching an egg-laying site; (2) struggles of multiple males may result in damage to eggs; (3) the weight of additional males may be too great for a single leaf to support; (4) fertilization efficiency may be reduced in matings involving multiple males; and (5) there may be intersexual selection for traits such as primary male size and secondary males may be significantly smaller. In contrast to the hylids, rhacophorid males are positioned laterally to the female and

hang onto a branch while releasing sperm in a foam nest (Jennions & Passmore 1993). Males also do not remain amplexed to the female while she moves to the water between egg-laying bouts, thus the female does not have to bear the weight of secondary males while moving to egg-laying sites. In rhacophorids, matings involving multiple males are the predominant mating type.

Anurans have several characteristics that make them particularly suitable subjects for addressing questions of female control over the number of eggs oviposited, and whether females encourage or discourage matings that involve multiple males. External fertilization enables observers to unequivocally determine the composition of mating groups and quantify the number of eggs oviposited.

Reports of assemblages of males attempting to join ovipositing pairs are relatively rare, occurring in four of 21 families of anurans. Two of these families (Hylidae & Rhacophoridae) contain species of arboreally or terrestrially ovipositing anurans. Aggregations of multiple males are reported for 9 species of arboreal and terrestrial breeders (Coe 1967, 1974; Feng & Narins 1991; Jennions *et al.* 1992; Kasuya *et al.* 1987; Kato 1956; Pyburn 1963, 1970; Roberts 1994; Wiewandt 1971). Multiple male assemblages

have also been reported for two families of aquatic breeders (Ranidae & Bufonidae; see Halliday & Verrell 1984). In the red-eyed treefrog, *Agalychnis callidryas*, matings that involve multiple males (primary & secondary) can result in multiple paternity (chapter 3).

Females in many species of arboreal breeders are amplexed in one location and then carry the male to another location to oviposit. During this time unamplexed males may attempt to join the pair, thus giving a female a post-amplexus, pre-egg-laying period to encourage or discourage additional males from joining her.

The primary questions addressed in this section are: (1) Do females vary egg number based on mating context (e.g., matings with single or multiple males)?; (2) Can females influence male success in achieving amplexus prior to egg-laying; (3) What is the sequence of behaviors from amplexus through egg-laying? (4) How do primary and secondary males vary in amplexus behavior? (5) Do phenotypic differences exist between primary and secondary males? and (6) What is the fertilization efficiency of primary and secondary males?

Materials and methods

Study Site and Observations

The study site was a seasonal pond located approximately 2 km south of Gamboa, Republic of Panama. The pond was surrounded on all sides by overhanging vegetation that was used by frogs for egg-laying sites. Frogs were watched from approximately 1-2 m distance using a headlamp filtered with two sheets of red acetate. My presence did not appear to disturb their behavior (but see Buchanan 1993). Focal animal observations (Altmann 1974) were made comparing the responses of unamplexed and amplexed females towards males attempting to amplex. The durations of behaviors were recorded with a stopwatch. Times were rounded to the nearest minute or second, depending on the type of observation. Data were collected on egg-laying patterns, primary and secondary male behavior, phenotypic characters of males, fertilization efficiency, and the frequency of matings involving single and multiple males. Six independent data sets were used to address the questions in this study.

Data sets

Set 1. Continuous focal observation of females and

primary males (N=6) were conducted on June 8 & 9, 1991 and June 21, July 16, and August 8, 1993. Observations of females started before amplexus and continued until the termination of egg-laying. Variables recorded were: (1) the number of times females stopped before reaching egg-laying site after amplexus; (2) number of eggs oviposited in each clutch; (3) time in min between clutches; (4) whether females moved from the trees to the water to hydrate between clutches; (5) total duration of egg-laying activities and (6) female response to male attempts to amplex: movement or stationary. A response was scored as stationary if the female remained at the location of her interception by the male for > 10 s. Movements were defined as directional motion greater than 5cm, when a male touched or attempted to amplex the female (or pair). A stopwatch was used to determine the 10 s period. Times for other behaviors were recorded to the nearest minute.

Set 2. Female-primary male pairs (N=69) were observed during egg-laying of from 1-3 clutches (N=92). Variables recorded were: (1) number of eggs laid; (2) number of clutches observed; (3) female response to males attempting to amplex: movement or stationary. Data were recorded on various dates in August 1990, May-July 1991, May-June 1993.

Set 3. Females with multiple males (N=38) were observed during May and June 1991 (N=14), May - July 1993 (N=15), and May 1994 (N=9). Variables recorded were: (1) snout-vent lengths measured to nearest mm; (2) masses rounded to 0.1g; (3) number of eggs oviposited; (4) number of clutches; and (5) the number of eggs fertilized. Frogs usually voided water from their cloacae when handled, thus, they were not assumed to be fully hydrated during mass measurements.

To determine if secondary males remained with pairs, I recorded the movements of secondary males after a clutch had been laid. The variable recorded was whether secondary males remained with the female after she had laid a clutch.

Fertilization efficiency (percentage of eggs fertilized) of the primary male was measured for both the first clutch, in which two or more males were present, and subsequent clutches in which the primary male was the only male. After the first clutch, the amplexed pair was placed in a zip-lock bag and brought to the laboratory in Gamboa, where they were placed in 20 l aquaria. The aquaria had plants for egg-laying sites and approximately 10 cm of water. In the aquaria, pairs produced 1-3 additional clutches. Eggs were scored as fertilized (color and

textural change indicative of gastrulation) or unfertilized (discolored) after a period of 24-48 h.

Set 4. The number of eggs per clutch for matings in which egg-laying was not observed were counted on various dates from May-July 1993 (N=105 clutches). Clutches were randomly chosen from vegetation around the pond.

Set 5. Reproductive activities of females grasped by three or more males were recorded (N=13) on various dates in May-June 1991, and May-July 1993. Variables were: (1) number of males involved; (2) success of amplexus attempts by secondary males; (3) if females oviposited prematurely; (4) the number of eggs prematurely oviposited; and (5) if female movement was restrained.

Questions addressed and data sets used

Egg-laying sequence (Set 1)

These data were used to determine the sequence of reproductive events including behaviors displayed by females during a complete breeding session starting from the time of amplexus and ending when the male released the female at the end of egg-laying. The number of eggs per clutch in relation to the order of clutch and the individual female laying clutches, are compared using a block design ANOVA.

Data are presented as means (± SE).

Primary male amplexus behavior (Sets 1 & 2)

The amplexus behavior of primary males during egg-laying was recorded to determine if primary males remain with females after egg-laying of a clutch, or if primary males release females after fertilization of a clutch (see Backwell *et al.* 1990; Jennions *et al.* 1992). Data were taken from Set 1 of continuous focal observation of males observed throughout the egg-laying of all clutches and from Set 2 where primary males were observed during the egg-laying of 1-3 clutches. Data are presented as the percentage of males remaining amplexed to females.

Secondary male amplexus behavior (Set 3)

Secondary males could increase their fitness by fertilizing as many clutches as possible. One way to fertilize more eggs would be to remain with a pair (female and primary male) until the female has finished laying all her clutches. Alternatively, secondary males could release the pair and search for another pair getting ready to lay their eggs. Data are presented as the percentage of secondary males that remain amplexed to females after the

female has laid a clutch.

Phenotypic differences between males (Set 3)

The hypothesis that females pair with larger males, was examined using measurements from individuals from matings involving multiple males. Snout-vent lengths and masses of 27 pairs of primary and secondary males were compared using a two-tailed paired *t*-test.

Fertilization efficiency of males (Set 3)

Data were collected to test the hypotheses of male sterility (Knowlton & Greenwell 1984), or that males may decrease in fertilization efficiency in subsequent matings (Halliday 1976, Smith-Gill & Berven 1980). Either of these conditions would result in decreased fertilization efficiency, and would favor females mating with multiple males. Data were analyzed by taking the percentage of eggs fertilized in each clutch and transforming the data (arcsine $\sqrt{\%}$) for analysis using a one-way ANOVA.

Pre-egg-laying behavior of females (Sets 1 & 2)

To test the hypothesis that females display pre-egg-laying behaviors that could either facilitate or hinder the

mating attempts of secondary males, the responses (movement or stationary) of 15 unamplexed females were compared to the responses of 45 amplexed females when males attempted to amplex them. A chi-square test was used to compare the responses of amplex and unamplexed females.

Female response to 3 or more males (Set 5)

These data enabled me to determine if egg were oviposited before the female had reached a proper egg-laying site (over water) when she was grasped by more than 2 males. In these situations egg mortality was assumed to be total. These data are presented as percentages of females extruding eggs, and the number of eggs extruded.

Female egg-laying behavior (Set 1, 2, 3 & 4)

To determine how secondary males affect the number of eggs oviposited, the number of eggs laid per clutch in the field were counted for: 1) paired females (N=34), 2) females with more than one male (N=34), and 3) the number of eggs in a survey of egg clutches (N=105) where egg-laying was not observed. The latter data set was used as a control to test for the effect of an observer on the number of eggs laid.

I examined the hypothesis that females compensate for laying fewer eggs in a clutch with multiple males by laying more eggs in the next clutch. Pairwise comparisons (N=21) were made of clutches from the same female. Twenty one females were first observed in a mating with multiple males in the field and then the female oviposited again with the primary male in the laboratory. Both the first clutch and pair were collected after egg-laying in the field and moved to the laboratory in Gamboa, where they were placed in 20 l aquaria. The aquaria had plants for egg-laying sites and approximately 10 cm of water. In order to factor out the potential that first clutches are always smaller than subsequent ones, these data are compared with egg-laying data from females (N=6) laying their total clutch complement (2-4 clutches) with a single male in the field.

A one-way ANOVA was initially used to determine if significant differences existed between the number of eggs produced by paired females, females with multiple males, and egg clutches of unknown mate composition. Significant differences were then examined using Fisher's LSD test to determine which groups differed. A paired t-test was used to determine if differences existed between the number of eggs in a female's first and second clutch when the first

clutch was laid in the presence of multiple males and the second clutch with a single male present. A block design ANOVA was used to examine the difference between first, second and third eggclutches laid with a single male.

Results

Amplexus and egg-laying

Females (N=6) laid 2-4 clutches (3.0 ± 0.3). Overall females laid a mean of 153 (± 9.0) eggs, with a range of 129-182 eggs. Females took a mean time of 410 (± 23.3) min from the time of amplexus to the time she finished the last clutch. The mean number of eggs produced per clutch did not differ significantly between females ($F_{5,17}$=0.70, P=0.64, block design ANOVA) or clutches ($F_{3,17}$=0.89, P=0.48, block design ANOVA). After being amplexed females initially remained motionless for 18 (± 10.5) min and then moved toward the egg-laying site. Females moved in spurts, stopping occasionally ($\bar{x}$ = 4.2 ± 0.9 times) before reaching the egg-laying site. Females paused from 63-143 min (N=10, 95 ± 11.1 min) between clutches. During this period females either moved to the margin of the pond to hydrate their next clutch, or to adjacent branches where moisture on the surface of leaves or branches was used to hydrate the clutch.

Primary males (N=6) observed from amplexus until termination of egg-laying, and primary males (N=69) observed during egg-laying of 1-3 clutches, remained amplexed to the

female until she had completed egg-laying of all clutches. In contrast, 95% (36/38) of the secondary males separated from the pair after egg-laying of a single clutch. In two exceptions, the secondary male remained with the pair for more than one egg-laying sequence. One exception involved two males that were amplexed in a side-by-side fashion, and the other mating involved a large secondary male that had sandwiched a small primary male when he amplexed to the female.

Phenotypic differences between males

Neither SVL nor mass of primary males (44.8 ± 0.4 mm, 3.3 ± 0.1 g) differed significantly from the SVL and mass of secondary males (45.3 ± 0.3 mm, 3.4 ± 0.1 g) (N=27, two-tailed paired t-test, t=-0.995, P=0.329; t=-0.721, P=0.477, respectively).

Fertilization efficiency of males

Fertilization efficiencies were determined for a sequence of clutches produced by females. The first clutches all involved a primary male with one or more secondary males (Table 4.1, Fig. 4.1). Only a primary male was present during egg-laying of the second and third

clutches. A one-way ANOVA resulted in no significant differences in fertilization efficiency between 1st, 2nd, and 3rd clutches ($F_{2,49}$=0.41, P=0.67).

Pre-egg-laying behaviors of females

The behavioral responses of unamplexed (N=15) and amplexed (N=45) females to males attempting to amplex with them were compared to determine if females displayed different behaviors towards males before (primary) and after (secondary) they were amplexed (Fig. 4.2) Significant differences in female responses to males were found between the groups (χ^2=32.4, df=1, P<0.001). Ninety-three percent of the unamplexed females remained stationary when amplexed by a male. In contrast, after amplexus, 87% of the females moved when touched by a secondary male. Secondary males were not successful in joining these moving pairs. However, at the egg-laying site, where the female assumed a stationary egg-laying stance on a leaf, three secondary males (8%) successfully trailed and joined the pair during egg-laying. Of the 13% of amplexed females that remained stationary when touched by secondary males, one of six (17%) was amplexed by secondary males. This females carried both males to an egg-laying site.

Another group of amplexed females (N=13) were grasped by two to six secondary males while moving towards egg-laying sites. Secondary males immobilized the amplexed female by either anchoring themselves to branches with their hindlegs while grasping the pair (Fig. 4.3), and/or through the sheer mass of the male assemblage. Movement by the amplexed female was curtailed until either the secondary males pulled each other off, released the pair, or lost their grip when either the primary male kicked them off and/or the female moved or jumped. No attempts by secondary males to join pairs were successful if two or more secondary males were involved before the pair reached an egg-laying site.

Twenty-three percent (3/13) of the females grasped by two to six secondary males extruded eggs before reaching an egg-laying site (Fig. 4.4). Grasped females extruded a mean of 5.7 (± 1.8) eggs. Extruded eggs were not encased within the normal protective jelly matrix (Fig. 4.4), and were scattered among the leaves, branches, or fell to the ground.

Egg-laying behavior of females

The mean number of eggs produced per clutch by females with single males (N=34, 48 ± 2.3), multiple males (N=34,

40.1 ± 1.7) and the number of eggs per clutch seen in the field (N=105, 47.2 ± 1.7), were compared. The number of eggs produced by the three groups differed significantly ($F_{2,170}$=3.19, P<0.05, one-way ANOVA). Fisher's LSD test indicated a significant difference between matings with multiple males when compared to single male and matings of unknown male composition. No significant differences were found between single male and matings of unknown male composition.

Females that first produced eggs with multiple males and then laid a second clutch with a single male, significantly increased the mean number of eggs from 40.1 (± 2.2) to 52.8 (± 3.9) respectively (N=27, paired t-test, t=-2.67, P<0.05). Females that produced clutches with single males only did not significantly alter clutch size ($F_{3,1}$=0.89, P=0.48, block design ANOVA).

Discussion

Eighty-seven percent of the amplexed females displayed pre-egg-laying avoidance behaviors by moving when encountering secondary males. When amplexed females moved, only 5% of the secondary males continued to pursue and successfully join the pair during egg-laying. When amplexed females did not display avoidance behaviors toward secondary males, approximately 17% of the secondary males were successful in achieving amplexus. In contrast, 93% of the unamplexed females remained stationary when amplexed by a male. The behaviors of unamplexed and amplexed females toward primary and secondary males indicate that females are actively avoiding secondary males, not all males. The low rate of success (5%) by secondary males suggests that female avoidance of secondary males before reaching the egg-laying site is a relatively successful strategy.

When secondary males joined amplexed females at egg-laying sites, females laid significantly fewer eggs than with single males. When females laid the following clutch with a single male, they increased the number of eggs. Thus, because females reduce the number of eggs available in clutches involving multiple males, the potential number of

eggs available for secondary males is reduced by 15%. In contrast, the overall loss for primary males in eggs to fertilize is approximately 13% (assuming both males fertilize 50% of one clutch) through shared paternity of one clutch, rather than through a reduction in the total number of eggs available to fertilize. This is because primary males remain with females until females finish laying all their clutches. In contrast, in the fishes *Symphodus tinca* and *S. ocellatus*, females have multiple bouts of egg-laying, spawning in one or several nests guarded by different males. When secondary males are present the female decreases by 5-8 fold the number of eggs spawned which results in an overall reduction in the number of eggs for the primary male to fertilize. The main cost of secondary males to primary males is through the reduction of total eggs available for fertilization (van den Berghe *et al.* 1989), not shared fertilizations, as in *A. callidryas*.

No support was found for the hypothesis that females mate with multiple males because primary males lack sufficient sperm to fertilize all clutches. The percent of eggs fertilized by individual primary males (99-100%) was similar between clutches, providing no evidence for decreased levels of fertilization over successive clutches.

A similar lack of evidence for decreased fertilization efficiency over successive clutches was found in the American toad, *Bufo americanus* (Kruse & Mounce 1982). The high rates of fertilization (99%) for clutches of *A. callidryas* are comparable to those of the gladiator frog, *Hyla rosenbergi* (Kluge 1981), and the tungara frog, *Physalaemus pustulosus* (Ryan 1985).

The hypothesis that females might discourage matings involving multiple males due to a reduction in fertilization efficiency was not supported. Clutches fertilized with secondary males present showed similar rates of fertilization (99%) providing no evidence that the activity of secondary males decreased fertilization efficiency. Because I lack data on fertilization efficiency of a series of clutches all fertilized by a single male, I cannot rule out the possibility that primary males restrict the release of sperm during matings involving multiple males, thus reserving sperm to fertilize subsequent clutches. However, several lines of evidence suggest this is not the case. Primary males fertilized approximately 50% of the eggs in matings involving multiple males. Primary males have no guarantee that only one clutch will involve multiple males, thus primary males who withhold sperm during matings where

secondary males are in attendance, could lose all their fertilization opportunities.

The hypothesis that females pair with larger males due to female choice or male-male competition was not supported. Neither size nor mass of primary males differed significantly from secondary males in matings involving multiple males. In another closely related species *Agalychnis saltator*, where matings involving multiple males occur during explosive breeding, amplecting males were also of similar size to unpaired males (Roberts 1994). Explosive breeding occurred in *A. callidryas* on nights when matings involving multiple males occurred, with approximately 36% more active frogs than nights where matings involving multiple males did not occur (see chapter 2). The high density of males suggests that if intersexual selection through female choice for male size exists in this species, it could be masked through scramble competition of males. It is also possible that intersexual selection exists for traits not measured in this study such as male call rate or intensity. Female selection for call rate or call intensity has been documented in a number of hylid species (see review by Andersson 1994).

The sequence of egg-laying behaviors, male biased OSR,

and secondary male behavior should reflect a potentially higher rate of matings involving multiple males. The combination of laying three clutches over a protracted period of 7 h, and moving between clutch sites and the water to hydrate should increase the probability of pairs being intercepted by unpaired males. Thus the most reasonable explanation for the relatively low matings involving multiple males (chapter 2) is that females are actively discouraging secondary males from amplexing. Egg loss through the struggles of multiple males to grasp females, and difficulty in reaching an egg-laying site, appear to be the most likely reasons for females to avoid secondary males. Twenty-three percent of the amplexed females intercepted by two or more secondary males before reaching the egg-laying site released approximately 13% of the normal clutch prematurely. The premature release of eggs appeared to be the result of males exerting pressure on the female's abdomen as they struggled to amplex her. Survivorship of the eggs was unlikely because the eggs were not encased in a protective jelly matrix, and even those stuck to branches were positioned over land rather than the pond.

Intersexual selection should favor females who avoid secondary males. Assuming secondary males attempted to

intercept all females, total egg loss could increase as females were intercepted between each clutch. Presently, females that move away from secondary males risk a small increase in energetic demand compared to the energetic cost of being immobilized by multiple males prior to reaching the egg-laying site and/or suffering egg mortality. Both the male biased OSR and the attempts by secondary males to join pairs provide females with the option of soliciting matings involving multiple males, but females are choosing to avoid matings involving multiple males.

Table 4.1 The percentage of eggs fertilized by clutch order and the number of males mating. Total clutch size is followed by the percent fertilized. Clutch 1 always involves multiple males, a primary male and 1 or more secondary males, while clutches 2 & 3 involve only the primary male.

Males	Clutch 1		Clutch 2		Clutch 3	
N	N	%	N	%	N	%
2	30	98	63	--	--	
2	34	88	65	100	35	100
2	31	100	49	98	42	98
2	36	100	55	97	46	100
2	47	100	75	98	--	
2	45	100	19	100	39	100
2	49	91	39	99	--	
2	39	100	--	--	--	
2	35	100	84	100	--	
3	48	100		--	--	
2	14	100	45	100	44	100
2	47	98	48	100	52	100
4	33	97	--	--	--	
2	44	97	--	--	--	
2	40	100	44	100	50	100
4	52	100	39	100	--	
2	36	100	37	100	--	
2	39	100	51	100	--	
2	37	97	26	96	--	
2	37	100	79	100	--	
2	37	100	72	100	--	
2	40	100	47	100	--	
2	51	100	--	--	--	
2	58	100	--	--	--	
2	39	100	--	--	--	
2	43	100	--	--	--	
2	22	100	--	--	--	
MEAN 2.2	39.4	98.7	52.1	99.3	44.0	99.7
SE 0.11	1.78	0.56	4.23	0.31	2.26	0.29

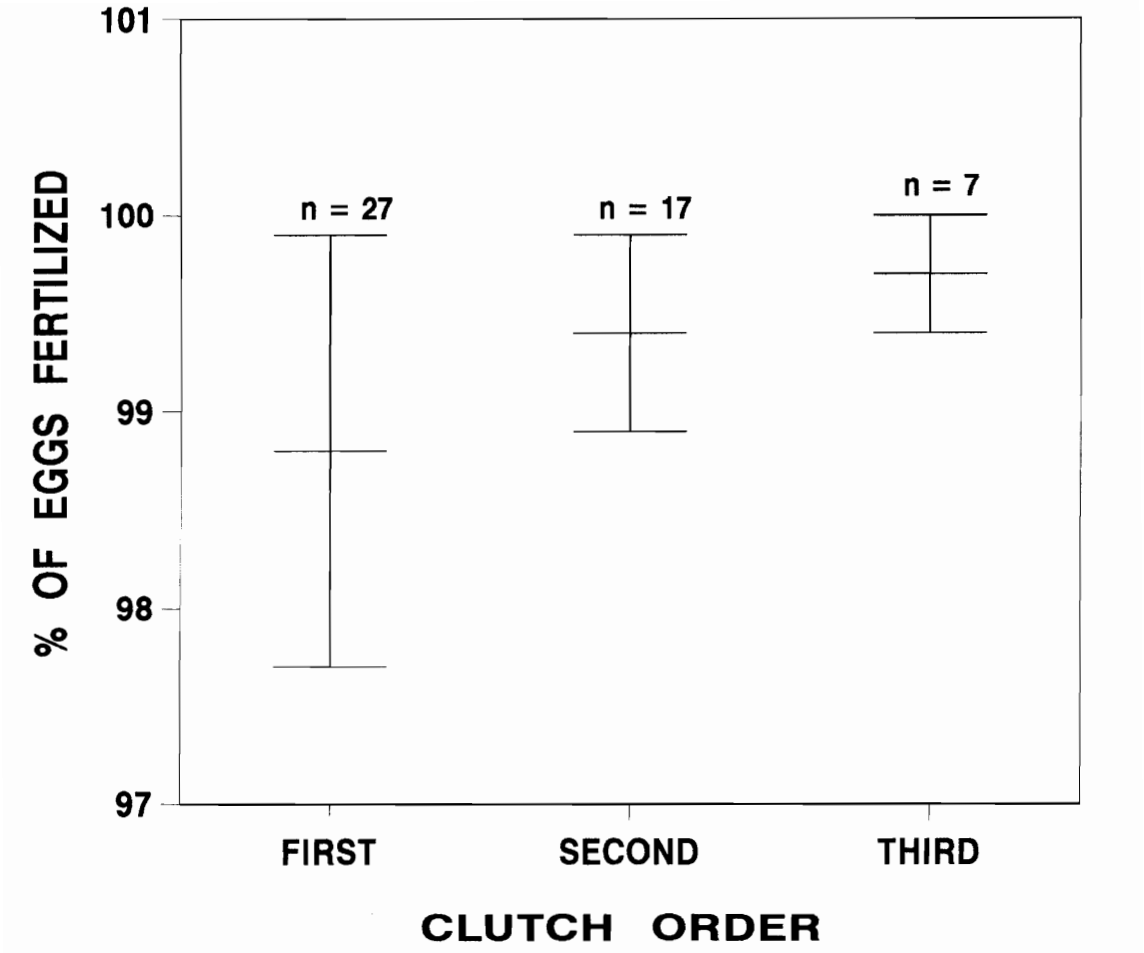

Figure 4.1 The fertilization efficiency (% of eggs fertilized) of male *Agalychnis callidryas*, from near Gamboa Panama, over successive clutches. Means ± 2SE are shown. In the first clutch (n=27) a primary male is joined by a secondary male. Only the primary male is involved in the fertilization of the second (n=17) and third (n=7) clutch.

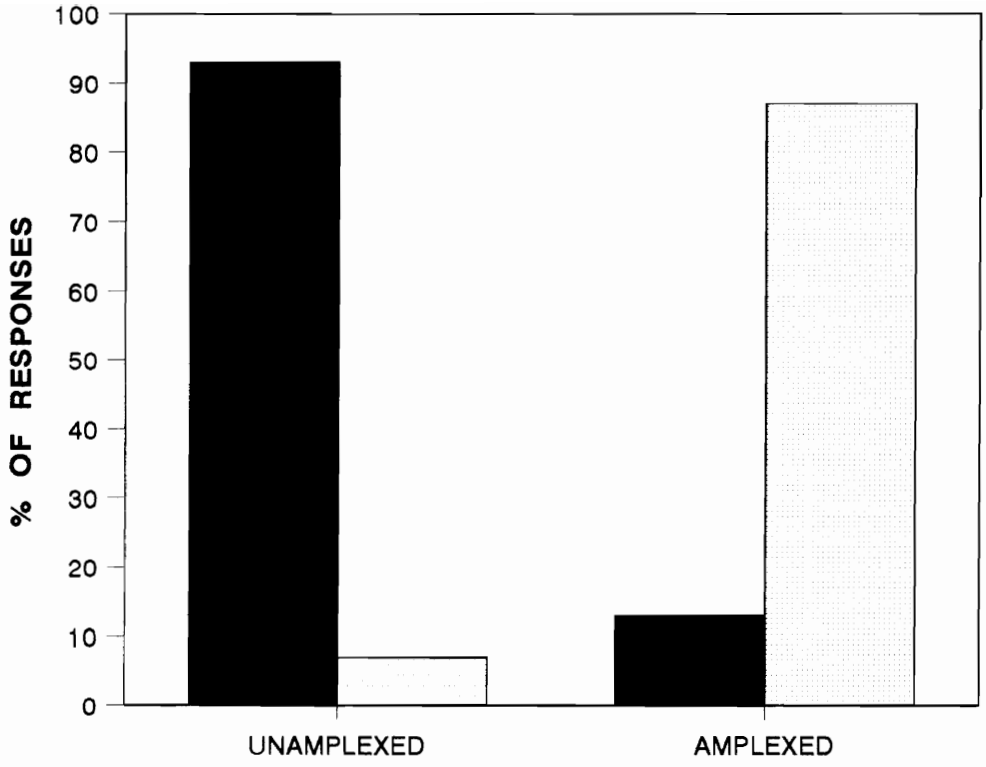

Figure 4.2 The responses of 15 unamplexed and 45 amplexed female *Agalychnis callidryas* from Gamboa, Panama, to males attempting to amplex with them. Black bars indicate that the female remained stationary when touched by a male. Stippled bars indicate that the female moved when touched by a male.

Figure 4.3 Secondary male *Agalychnis callidryas*, from near Gamboa, Panama, hindering female movement to egg-laying site. The secondary male (posterior position) has anchored his hindlimbs on the tree trunk preventing the female from moving forward.

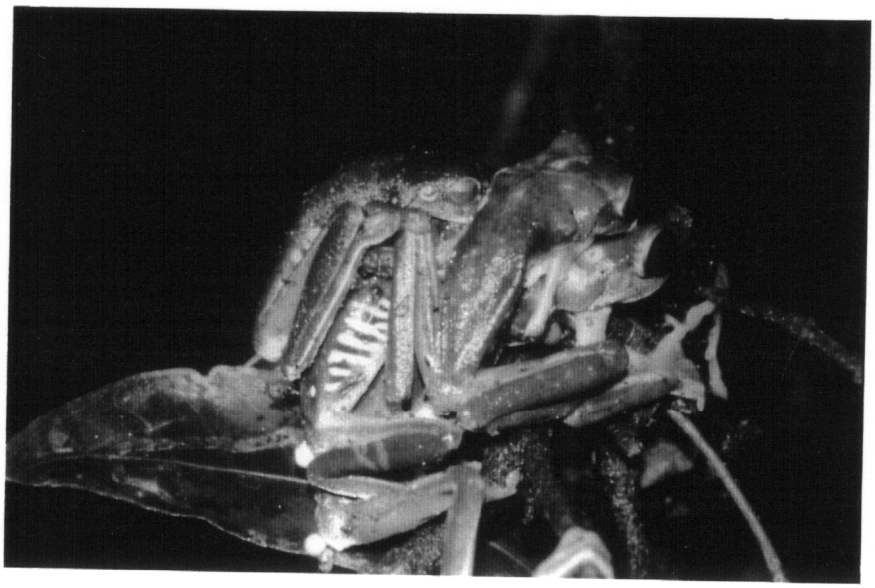

Figure 4.4 Egg loss through secondary males attempts to amplex female in the frog, *Agalychnis callidryas*, from Gamboa, Panama. Two prematurely oviposited eggs (white) can be seen at lower left center.

CHAPTER 5

The behavioral repertoire of males

Darwin (1871) proposed that sexual selection arises from differences in reproductive success caused by competition over mates. In anurans two basic forms of mate acquisition occur: scramble competition and female choice (Wells 1977). In scramble competition, sexual selection will favor male traits for locating females (Andersson 1994). Males in species where scramble competition occurs actively search for females. Fighting or struggling in which males attempt to dislodge each other from a female also occur. Larger males should have an advantage over smaller males in this form of competition. A mating advantage for larger males is documented for a number of anuran species (Arak 1983ab; Berven 1981; Davies & Halliday 1977; Howard & Kluge 1985; Howard 1988a; Wells 1979). For example, in the wood frog *Rana sylvatica*, larger males are more efficient at remaining amplexed to females when rivals attempt to depose them (Howard & Kluge 1985). In wood frogs, sexual selection may influence male arm length, males that have longer arms have a higher level of reproductive

success (Howard & Kluge 1985).

In anurans, mate choice by females primarily occurs in prolonged breeders. Males typically call from stationary positions and females are free to move among males. Females initiate amplexus by making physical contact with a male (Ryan 1985; Wells 1977). Sexual selection in species with prolonged breeding should influence male characteristics associated with female choice. Female choice does not preclude intrasexual competition between males because "the struggle is likewise between the individuals of the same sex, in order to excite or charm those of the opposite sex, generally the females, which no longer remain passive, but select the most agreeable partners" (Darwin 1871).

In anurans, some traits that have been associated with sexual selection through female choice are: call rate (Schwartz 1986; Sullivan 1982, 1983); call complexity (Rand & Ryan 1981; Ryan 1985; Wells & Schwartz 1984); and call loudness (Arak 1983b; Fellers 1979a). Females in territorial species, for example, the American bullfrog *Rana catesbeiana*, may use the quality of male territory which also represents a resource needed by females, an egg-laying site, to choose males (Howard 1978a). Unlike bullfrogs, male red-eyed treefrogs do not defend resources need by

females. Females carry males to an egg-laying site after amplexus. Therefore, if female choice is an important criteria of mating success for males, females should base their choices on characteristics of the males themselves.

A description of the male behavioral repertoire, and the contexts within which specific behaviors occur, are a necessary first step in determining the behaviors that may be used in intrasexual and intersexual competition. Despite previous work on *A. callidryas* (see literature review by Duellman 1970), there is a general lack of information on the both the social behavior of male *A. callidryas* and the call repertoire and contexts within which calls occur. For example, Duellman (1970) suggested the chuckle call is associated with rain and may function as an "awakening" call, while Pyburn (1970) suggested it may have a territorial function. An 'advertisement function', has been variously attributed to the "tlock" (Dunn 1931), "quirt" (Breder 1946), "chack" (Pyburn 1963), and "chock" calls (Duellman 1970). Furthermore, it has been suggested that prolonged breeders should have a greater call repertoire than explosive breeders (Duellman & Trueb 1986; Wells 1977).

The primary objectives of my study of male signal behavior were to: (1) describe the signal repertoire of males in male-alone, and male-male contexts; (2) to indicate how the different signals are used within the given contexts; (3) relate these signals to those described for other anurans; and (4) to determine if males display behaviors associated with territorial defense.

Materials and methods

One laboratory and two field experiments were conducted to determine the auditory and visual signal repertoire of males within controlled social contexts. These experiments used the controlled social contexts to: (1) elucidate and describe male signal repertoire in male-alone and male-male context; (2) compare the signals recorded in male-alone and male-male contexts; (3) compare the signals recorded in these experiments with behaviors expected by frogs with different mating systems.

Two experiments were conducted to determine the signal repertoire of males in male-alone and male-male context. Experiment 1, conducted in the laboratory, videotaped male-alone and staged male-male interactions. The videotapes served as a visual and auditory record of male behaviors that was used to ascertain and describe the different types and frequencies of displays exhibited in male-alone and male-male contexts. Experiment 2, conducted under natural conditions in the field, used a similar protocol to Experiment 1, with the main exception being that the males were not videotaped. A comparison is made between the results of the laboratory and field experiment to determine

if behaviors exhibited in the laboratory were similar to those exhibited in the field.

Experiment 3 was designed to test the hypothesis that male tlock calls function to advertise male presence to other males and resident males will respond to the presumed encroachment of another male on the resident male's calling site. Experiment 3 involved the playback of synthesized tlock calls to 12 free-ranging males.

Experiment 1

Twenty male-male encounters of sexually active (calling) males were videotaped between 24 September and 10 November 1992. Forty individuals were originally collected from four separate localities (as tadpoles *N*=35, or adults *N*=5) in the Canal Zone, Republic of Panama during September-November 1990 and May-August 1991 and were transported to Blacksburg, Virginia.

After metamorphosis animals were housed in a variety of glass fronted enclosures (2.4 x 0.6 x 0.7 m and 1.2 x 0.6 x 0.7 m). Enclosures contained live plants, plastic vegetation, branches, and a substrate composed of peatmoss, bark and soil. Moisture was provided in containers of standing water and by misting daily. A 12L:12D photoperiod

approximating natural photoperiod, was maintained using a combination of coolwhite and vitalite fluorescent lamps suspended above the enclosures. Temperatures varied between 24 to 26 C, within the ambient temperatures for breeding aggregations in Panama (23-27 C) recorded during September-November 1990 and May-August 1991. Frogs were fed every other day on crickets that were dusted in a mineral and vitamin powder.

Experimental setup

Frogs were videotaped in semi-darkness from a permanent blind 1.5 m from the experimental enclosure. The enclosure was illuminated using two 10 W red lightbulbs suspended over the cage. Two layers of plastic hardware cloth were placed between the lights and the cage to further decrease illumination. The cage contained a 36 x 26 x 6 mm container serving as a water source. The container was placed toward the far side of the cage approximately 60 cm from the side entrance door. A potted plant, approximately 40 cm in height, and two branches approximately 2-5 cm in diameter were placed in the cage to serve as calling sites. The branches led from the cage floor, by the entrance door, to the plant top. Entering frogs were placed on a branch by

the entrance door.

Behaviors were recorded by two Panasonic cameras (Model-WV 1550) using 16-160 mm manually operated zoom lenses. A split-screen generator (Vicon Model V270SP) produced side-by-side images on videotape, and a time-date generator (Odetics Model G-77) superimposed elapsed time in 0.01 s increments. Each camera recorded the behavior of one subject. Audio recordings of frog vocalizations were made on the same videotape using an Electro-voice dynamic omni-directional microphone, placed on the screen cagetop.

Behaviors were analyzed on a Panasonic video cassette recorder (Model AG-7300) with a resolution of 30 frames/s. Behaviors could be played back and examined in a frame-by-frame analysis for measurements of duration or amplitude.

Based on preliminary observations, behaviors were divided into vocalizations, visual displays, and physical interactions between the males.

Experimental Design

Two unfamiliar individuals from different caged populations were used in each encounter. I selected males giving tlock calls, which I considered a criterion for sexually active males. Male snout-vent lengths ranged

between 41.5-53.9 mm, $\bar{x}$=45.1, SE=0.35, *N*=40. One male was chosen as a "resident" and placed in the experimental enclosure (1.2 x 0.6 x 0.8 m) 2-3 nights before videotaping interactions, enabling him to establish familiarity with the enclosure. Videotaping was conducted between 1900-0100 h to match the circadian period when the frogs would be most active. The resident male was videotaped in two contexts over a 1 h period.

Based on preliminary videotaping, behaviors were divided into three major display types: (1) auditory (calling) displays; (2) visual displays; and (3) physical displays (wrestling). Calls were subdivided into: (1) single tlock; (2) soft tlock; (3)double tlock; and (5) chuckle calls. Visual displays were classified as posterior vibrations. Physical displays were classified as wrestling. Wrestling consisted of males grasping each other with their forelegs.

Male-alone

"Context 1" was a "male-alone" context during which the resident male was videotaped for 30 min to evaluate resident male behavioral displays in the absence of other male conspecifics. It was expected that males in the male-alone

context would display the flock call which is used by males gathered at breeding sites and has been hypothesized to function in advertising male presence to females (Breder 1946; Duellman 1970; Dunn 1931; Pyburn 1963).

Male-male

"Context 2" was used to determine if "resident" males altered their displays in reaction to the presence of an "intruder". Males in species that breed explosively generally do not alter their calling behavior in response to the presence of other nearby males. In contrast, males in species that have extended breeding seasons defend calling sites from intrusion from other males. Calling site defense is initiated by either altering characteristics of their calls (e.g., frequency or duration), changing call type, or engaging in physical interactions such as wrestling (Fellers 1979; Wells 1977). "Context 2" began when an "intruder" male was introduced into the experimental enclosure with the "resident". The behaviors of both males were videotaped for 30 minutes.

Experiment 2

Because behaviors displayed in laboratory studies may

not accurately reflect the behaviors of animals under natural condition, this field experiment was used to evaluate the data from Experiment 1 and to further elucidate male behavioral displays. Twenty trials of male-male interactions between "residents" and "intruders" were staged in Panama to record the behavioral repertoire and signal of use *A. callidryas* under natural conditions. I used 20 males giving tlock calls from perches as "residents" males. An additional 20 males giving tlock calls from perches >10 m from the residents perches were selected as "intruders" males. Trials were initiated by recording a resident male's behavior using a Marantz PDM 201 cassette recorder with an Electret condenser Telescopic microphone V-6502 with a frequency range of 80-12,000 Hz. The frequency range of the microphone extended both above and below the frequency ranges of approximately 1200-2400 Hz previously recorded for calls of this species (Duellman 1970). The microphone was placed at a distance of between 30-50 cm from the resident male. In addition, oral or written notes of behaviors were taken using a data sheet or microcassette recorder. Resident males were first observed for 15 min to obtain baseline data for comparing the resident male's behavior during the subsequent 15 min in which the intruder male was

introduced. The distance of >10 m was chosen to minimize the possibility of prior association between males. To diminish handling effects on intruding males, the males were captured by removing the substrate they were sitting on (branch or leaf) and carried to the residents site, where the males were placed approximately 50 cm from the resident. The distance of 50 cm was chosen both because it approximated the distance of introduced males in the experimental chamber to the resident, and because of the increased chance that the resident would not see the nonresident through the foliage at greater distances.

Along with time, date, and air temperature, I recorded snout-vent lengths to the nearest mm and mass to nearest 0.1 g for both males using a mm ruler and a Pesola 5 g scale. These measurements were taken after the 30 min period of behavioral observations. The behavioral variables recorded were the same as those described for Experiment 1.

Experiment 3

Experiment 3 used playback of synthesized tlock calls to record the response of residents in the field. The tlock call was used to test the hypothesis that males would display behaviors associated with site defense when hearing

display behaviors associated with site defense when hearing another males call. Two single tlock calls were recorded in the field from two different males in the male-alone context. Calls were recorded with a Marantz PDM 201 cassette recorder with an Electret condenser Telescopic microphone V-6502 with a frequency range of 80-12,000 Hz and digitized at 20khz, 8 bits/sample on a Future Sound digitizer, Amiga 600, Tandy Corp. Output was 1/30 s at 20 kHz, 8 bits per sample, low pass filtered at approximately 5 kHz on an Amiga 600's low-pass filter. After being digitized, the calls were alternately used as stimuli.

Experiment 3 used a 5 W speaker-amplifier Unit SP5 with 4 watts amp power and a frequency response of 80-18,000 Hz. The frequency response encompassed the stimulus tlock call frequency of approximately 2000 Hz used as playback stimulus. The speaker was placed approximately a meter from a calling male. Calls were played every 30 s with sound pressure level (SPL) output measured at 93 dB from the calling male to speaker. Call rate of playback was close to the mean 36.5 (± 5.1) s and within the range (7-89 s) of inter-call durations recorded from 16 calls of four males recorded in the male-alone context in the field. The SPL of 93 dB's approximates the call intensity of a male at 50 cm.

Distance from the frog was measured using a m stick from frog to sound-meter. Sound pressure levels of acoustic stimuli were measured using a GenRad GR 1982 Precision Sound-level meter and analyzer set at 70-120 dB range, with the octave filter at flat weighting mode. The detector was set for peak sound detection. Recordings were made using a Marantz PDM 201 tape recorder with an Electret condenser Telescopic microphone V-6502 with a frequency range of 80-12,000 Hz. The microphone was placed at a distance of approximately 50 cm in front of the frog.

Twelve males were used in the 30 min trials. Trials were initiated after finding a calling male. First the male was recorded for 15 min to serve as a baseline recording. The stimulus tape was then played for 15 min. Simultaneously, vocal behaviors of the male were recorded using a Marantz PDM 201 tape recorder. Visual displays were recorded on a data sheet and/or recorded on microcassette and later transcribed to a data sheet.

Call Analysis

To describe the different call types, I used tapes recorded from individual males in male-alone and male-male interactions in the field (Experiment 2 and Experiment 3).

A Kay Elemetrics Corporation Model 5500 Signal Analysis Workstation was used to determine duration, dominant frequency, and Hz of calls.

To describe the occurrence, duration, and pattern of visual displays, I analyzed videotapes from the Experiment 1 on a Panasonic video cassette recorder (Model AG-7300) with a resolution of 30 frames/s. Behaviors were played back and examined in a frame-by-frame analysis.

In all data sets, means are presented ± 1SE. A one-way analysis of variance procedure (ANOVA) is used to analyze differences in call durations and dominant frequencies from data in Experiment 1 and Experiment 2. A one-way ANOVA is also used to analyze differences in vibration rates of posterior vibrations. Nonparametric procedures were used to compare differences between the same males in Context 1 and Context 2, and between males in the same contexts in Experiments 1 and 2. A Wilcoxon Signed Rank test is used for comparing the same male in different contexts (Experiment 1, context 1 & 2; Experiment 2, context 1 & 2; Experiment 3, context 1 & 2). The Mann-Whitney U test is used for comparing independent groups (e.g., Experiment 1 vs. Experiment 2). Data were analyzed with either the Statistical Analysis system software program (SAS Institute,

1985) or the Number Cruncher Statistical System (Hintze 1992).

Results

Display Descriptions

Visual displays

Experiment 1 documented a visual display labelled "posterior vibrations" that was only performed in the male-male context. This behavior also occurred in the male-male contexts in Experiment 2 and Experiment 3.

Posterior vibrations involve the elevation of the body from the substrate by extending the fore and hindlimbs, and elevating the pelvic region. The posterior end is vibrated though up and down contractions and extension of the hindlimbs. Thirteen bouts of two hundred and two posterior vibrations measured from five males consisted of 10.1 ± 3.3 up and down pulses per bout (range 3-36), produced at a mean rate of 5.5 ± 0.3 vibrations/s (range 0.5-5.1 s) in duration. Individual bouts are defined as a series of vibrations separated by >5 sec. Because of large variability, vibratory rates were not significantly different among males (one-way ANOVA, $F_{3,16} = 0.15$, $P=0.930$).

Auditory displays

Auditory displays were separated into two main call

types, "tlock" calls and "chuckle" calls, based on duration, and dominant frequency in Hz. Tlocks calls were further subdivided into "double-note tlock" calls and "soft tlock" calls based on the intensity (loudness) measured in dB on a peak-sound-level meter and the number of notes, respectively.

Tlock call

Tlock calls were recorded in both male-alone, and male-male contexts. Single note tlock calls occurred in all three experiments. Eighty-six single note tlock calls recorded from 18 males in Experiment 2 were used for analysis of duration, dominant frequency in Hz. Decibel levels were analyzed from 64 calls recorded from 13 males in Experiment 2.

Mean call duration was 0.063 ± 0.03 s (range 0.016-0.125 s). Calls differed significantly in duration among males (one-way ANOVA, $F_{17,68} = 20.02$, $P < 0.0001$). The mean dominant frequency was 1985 ± 57 Hz (range 1160-2840 Hz). Dominant frequencies also varied significantly between males (one-way ANOVA, $F_{17,64} = 4.36$, $P < 0.0001$). The call rate of 114 tlock calls recorded from 10 individuals during a 15 min period ranged from 0.2-1.8 calls per min. Decibel levels of 64 calls recorded from 13 males had a mean of 92.9 ± 0.3

dB's (range 85.7-98.6 dB's).

Double-note tlock call

Double-note tlock calls occurred in both male-alone and male-male contexts in Experiments 1 & 3. In Experiment 3, double-note tlocks only occurred in the stimulus playback context. Thirty-two double note tlock calls recorded from two males in Experiment 1 and two males in Experiment 3, had mean total durations of 0.247 ± 0.008 s (range 0.183-0.366 s). Durations of first and second notes differed significantly (0.105 ± 0.006 s, range 0.047-0.175 s and 0.035 ± 0.005 s, range 0.009-0.142 s respectively; Wilcoxon Signed Ranks test, z=-4.92, P <0.0001). Due to large variances, dominant frequencies of first and second notes did not differ significantly (1882 ± 47, 1175 ± 76 dB); Wilcoxon Signed Ranks test, N=23, z=-1.57, P=0.117). The inter-note interval was 0.107 ± 0.003 s (range 0.073-0.156 s).

Soft Tlock Call

Tlock calls were labelled as "soft tlock" calls if their loudness measured in dB's was less than 85 dB's. This division was based on the observation that in the male-male context or in response to the playback of a tlock call, males produced a tlock call that was significantly less loud

when compared with the normal tlock call (79.5 ± 0.4 dB's, 92.9 ± 0.3, respectively; Mann-Whitney U-test, N =85, z=-7.70 $P < 0.001$). Three soft tlock calls recorded from a single male had a mean call duration of 0.028 ± 0.003 s (range 0.020-0.037 s). The mean dominant frequency was 1565 ± 46 Hz, range 1338-1654 Hz. Decibel levels recorded from 29 calls of seven males had a mean of 79.5 ± 0.4 dB's (range 73.9-82.7 dB's).

Chuckle Call

Chuckle calls were recorded in male-alone and male-male contexts in all three experiments. Three chuckle calls recorded from a single male were used for analysis. Chuckles had a mean total duration of 347.7 ± 61.3 ms. Each call consisted of 3-5 notes of 11.7 ± 0.1 ms, range 6.5-17.3 ms. Notes had a mean frequency of 1285 ± 16 Hz, N=13. Decibel levels measured from 11 males had a mean of 77.3 ± 1.8 dB's (range 70.9-88.8 dB's).

Physical interactions

Wrestling. Males engaged in "wrestling" behavior which involved a male grasping another male with his forelimbs. Wrestling behavior occurred in both Experiment 1 and Experiment 2.

Signal Use

Male-alone

The primary display of solitary males was the tlock call. Tlock calls occurred at a mean display rate of 1.7 (± 0.7) calls per 15 min in Experiment 1 (Table 5.1; Fig. 5.1); 8.2 (± 1.1) calls per 15 min in Experiment 2 (Table 5.1; Fig 5.2); and 9.6 (± 1.2) calls per 15 min in Experiment 3 (Table 5.2). Tlock calls represented 75%, 95%, and 99% of the displays given in the male-alone context in Experiments 1, 2 and 3, respectively. The tlock call has been described as an advertisement call given by reproductively active males to attract females (Breder 1946; Duellman 1970; Dunn, 1931; Pyburn 1963).

Chuckle calls were given in the male alone context in Experiments 1,2 and 3 (Figs. 5.1 and 5.2; Tables 5.1 and 5.2). Although chuckle calls represented approximately 25% of the calls produced in the "male-alone" context in Experiment 1, they were associated with either jumping activity, or in response to the call of another frog. Fifty percent of these chuckle calls were produced within 2 s of jumping from one location to another. The other chuckle calls followed immediately after tlock call from other males in the laboratory. In Experiment 2, chuckle calls

represented only 3% of male-alone calls and followed the calls of other males within approximately 4 s. In Experiment 3, a single chuckle call was recorded for a male during baseline recording. The experimental designs of the male-alone context in Experiments 1 and 2 prevented the subject male from visual observation of other males, but did not prevent the male from hearing calls of other nearby males.

Other calls given in the male-alone context were recorded in very low frequencies, or by single individuals. One male produced a double tlock call in Experiment 1. A single soft tlock call was given in Experiment 1. Two males gave soft tlock calls in Experiment 2 (Fig. 5.2, Table 5.1).

Male-male

Soft tlock calls, chuckle calls, and posterior vibrations occurred in 80% of the male-male interactions (Experiments 1 and 2) and preceded wrestling in 83% of the field trials where wrestling between males occurred. In 15 of 20 trials in Experiment 1 and 17 of 20 trials in Experiment 2, males gave either chuckle calls, soft tlocks or posterior vibrations in the male-male context. All call types recorded in male-alone context were also recorded

(Figs. 5.1 and 5.2; Table 5.1). In addition, posterior vibrations were recorded in male-male contexts (Figs. 5.1 and 5.2; Table 5.1). Wrestling between males was recorded in both Experiment 1 and Experiment 2. Males engaged in wrestling in 3 of 20 trials (Experiment 1) and 5 of 20 trials (Experiment 2). Resident males initiated the wrestling bouts in 88% (7/8) of the trials. In Experiment 1 two different pairs of males remained clasped together for a mean of 2.5 (± 0.5) s. In Experiment 2 five pairs of males remained clasped together for a mean of 197.5 (± 67.8) s. These encounters ended when males broke apart. In the min immediately preceding the grasping of an opponent, 75% (6/8) males displayed one of four different behaviors: soft tlock call (1x), tlock call (1x), chuckle call (2x), or posterior vibrations (2x). Two males did not display calls or vibrations within the min immediately preceding the grasping of another male. There was a high degree of individual male variation in the total combination of displays types used and number of each type of display (e.g., eight chuckle calls and one vibrations; twelve soft tlocks and two vibrations), preceding attacks. Thus, no predictable escalation in the use of call type or posterior vibration discernable.

The number of tlock calls given in the male-male context were not significantly different between the male-male and male-alone contexts (Figs. 5.1 and 5.2; Table 5.1). The number of "soft tlocks" increased in the male-male context in both Experiment 1 and Experiment 2 (Figs. 5.1 and 5.2; Table 5.1) when compared with the male-alone context, but they were not significantly different due to high variance (Table 5.1). The number of chuckle calls increased significantly in the male-male context in both Experiments 1 and 2.

Posterior vibrations only occurred in the male-male context (Figs. 5.1 and 5.2; Table 5.1). Posterior vibrations represented approximately 24% and 21% of the total display types given in male-male interactions in Experiment 1 and Experiment 2 respectively and had mean display rates of 2.6 (± 1.3) per 15 min in Experiment 1 (Fig. 5.1), and 2.2 (± 0.8) per 15 min in Experiment 2.

Male response to synthesized tlock call

In Experiment 3, males decreased the number of tlock calls significantly from the mean pre-stimulus rate (Table 5.2). Males significantly increased the rate of chuckle calls, soft tlock calls and posterior vibrations displayed per 15 min from their mean pre-stimulus rate (Table 5.2).

Comparison of Experiment 1 and Experiment 2:

Tlock calls

Resident males in the field produced significantly more tlock calls than resident males in Experiment 1, both when alone (8.2 ± 4.9, 1.7 ± 3.1, respectively; Mann-Whitney U-test, N =20, z=-4.26 $P < 0.001$) and when with another male (6.05 ± 7.51, 2.10 ± 3.09, respectively; Mann-Whitney U-test, N=20, z=-2.73, $P < 0.006$). Non-resident's display rates were not significantly different between males in Experiments 1 and 2 (2.1± 5.75, 1.92 ± 4.33, Mann-Whitney U-test, respectively; N=20, z=-0.744, P= 0.46).

Soft Tlock Calls

The rate of soft tlock calls were not significantly different between resident males in context 1 (male-alone) in Experiments 1 and 2 (Mann-Whitney U-test; N=20, z=-0.500, P=0.62). The display rates of resident males in context 2 (male-male interactions) were not significantly different (Mann-Whitney U-test; N=20, z=-0.365, P=0.72).

Chuckle Calls

There were no significant differences between lab and field resident males in the number of chuckle calls produced in context 1 (0.4 ± 1.1, 0.3 ± 0.6, Mann-Whitney U-test, N=20, z=-0.041, P=0.97). Resident males in context 2 also

did not display significant differences (1.3 ± 1.6, 1.4 ± 2.1, respectively; N=20, z=0.270, P=0.787). Non-residents did not display significant differences between lab and field behavior (0.6 ± 0.9, 0.7 ± 0.6, respectively; N=20, z=0.514, P=0.607).

Posterior Vibrations

The number of posterior vibrations displayed were not significantly different between resident males in context 2 in Experiment 1 and Experiment 2 (Mann-Whitney U Test, N=20, z=-0.054, P=0.957). Non-resident males were not significantly different in the number of displays between Experiments 1 and 2 (0.4 and 1.1 respectively; N=20, z=-0.271, P=0.787).

Discussion

Signal use

The tlock call was the primary display given by solitary males in all three experiments. The tlock call has been classified as an advertisement call which functions to attract females (Breder 1946; Duellman 1970; Dunn 1931; Pyburn 1963). My study shows that the tlock call also functions in male-male interactions. Resident males respond to tlock calls of intruders <50 cm in distance from the resident (Experiments 1 and 2), and synthesized tlock calls broadcast at the loudness (dB) of a calling male from 50 cm distance (Experiment 3). For example, when males were presented with a synthesized tlock call (Experiment 3), males increased the frequency of soft tlock calls, chuckle calls, and posterior vibrations significantly, while reducing the frequency of tlock calls given significantly.

These data suggest that one function of tlock calls is to advertise calling site occupancy to other males. The significant increases in frequency of behaviors associated with male-male interactions when synthesized tlock calls were broadcast to resident males, indicates that resident males react aggressively when hearing the tlock call of

another male within 50 cm of their calling site.

In male-male interactions resident males gave chuckle calls, displayed posterior vibrations, and increased the frequency of soft tlock calls in comparison to the male-alone context. Soft tlock calls, chuckle calls, and posterior vibrations occurred in response to the playback of tlock calls in Experiment 3. Combinations of soft tlock calls, chuckle calls, and posterior vibrations preceded wrestling in 83% of the field trials where wrestling between males occurred. No predictable escalation in either the rate of displays or type of displays was observed, due to high variability between males in the sequence and type of displays given preceding an attack.

The lack of predictability in type or sequence of male auditory or visual displays may be an artifact of my experimental design which subject males to either other males or synthesized tlock calls at a distance of 50 cm or less. An experiment using a gradation of distances between males, or varying the loudness (dB) of synthesized calls to reflect different distances between males might be able to elucidate the specific function of each of the auditory and visual displays.

Despite the lack of information on specific functional

differences between soft tlock calls, chuckle calls and posterior vibrations, these behaviors are agonistic signals that may reduce the chance of physical interactions between males. Wells (1977) suggested that encounter calls, calls that occur in agonistic interactions between males, may function to warn an intruding male to leave the area. Although 80% of male-male interactions involved soft tlock calls, chuckle calls or posterior vibrations, only 19% of these encounters escalated further into physical interactions.

Wells (1977) predicted that territoriality should occur in anurans with prolonged breeding seasons where females arrive at breeding sites at irregular intervals. In six species of North American *Hyla*, males exhibit territorial defense, switching from advertisement calls to encounter calls when approached by other males. If intruding males continue to approach resident males, wrestling may result. These wrestling matches ended when intruding males freed themselves and left the resident male's territory, or remained in the territory, but did not call (Fellers 1979a, b). Similar types of behaviors were recorded for male *A. callidryas*. Recapture data collected from marked males (chapter 2), supports a hypothesis of male calling site

fidelity. The presence of site fidelity and the agonistic behaviors recorded in male-male interactions suggest that male *A. callidryas* exhibit calling site defense.

Agalychnis callidryas displays: Previous descriptions compared

The genus *Agalychnis* is composed of eight species. With the exception of *A. callidryas*, only a single call type, which functions to attract females, has been described for each of these species (Duellman 1977; Marquis *et al.* 1986; Roberts 1994). In *A. callidryas*, the chuckle call has been described as a "rain-call" occurring at dusk that might serve as an "awakening" call (Duellman 1970). Chuckle calls were also described as occurring at dawn and possibly having a territorial function (Duellman 1970). Pyburn (1970) observed chuckle calls being used by amplectant males when approached by an unattached male, or by two approaching males. Pyburn (1964) also noted the call when there was no direct confrontation between males. In my study, chuckle calls were both associated with the male-alone context, male-male interactions, and in response to playback of a synthesized tlock call. In cases where chuckle calls were produced in male-alone contexts, the chuckle call came right

after a male jumped. Chuckle calls produced after jumping may be emitted to alert other nearby males that a male is moving to a new calling location.

A single observation of posterior vibrations was described by Pyburn (1970). Posterior vibrations occurred between two males, when one male was amplexed to a female and the other male attempted to join the pair. Both males attempted to use posterior vibrations to "bluff each other" before engaging in attempts to displace each other physically (Pyburn 1970). In my study, posterior vibrations occurred in male-male interactions without a female present and when a resident male responded to a synthesized tlock call. Posterior vibrations appear to be visual display which may be enhanced through the displaying of the male's flanks which consist of contrasting lateral striping of white and dark blue. This pattern becomes visible when the frogs elevate their bodies to vibrate. There may also be a tactile component to the display. Vibrations could be transmitted as seismic waves through a branch from the displaying frog to the receiving frog. Seismic displays, and the ability to detect seismic vibrations, have been described for the white-lipped frog, *Leptodactylus albilbris*, which incorporates a seismic "thump" into its

advertisement call (Lewis & Narins 1985).

Comparison with other Anurans

Males in a number of species alter their vocal behavior, switching from advertisement to aggressive calls in response to the advertisement call (actual or synthesized) of another conspecific (Bogert 1960; Brzoska *et al.* 1982; Grafe 1995; Jenssen & Preston 1968; Littlejohn 1977; Ramer *et al.* 1983; Stewart & Bishop 1994; Wells & Schwartz 1984; Whitney 1980). In three species of neotropical hylids *(Hyla ebbraccata, H. microcephala,* and *H. phlebodes)*, males switch from advertisement to aggressive calls by increasing the pulse rate of the introductory note(s) without changing pulse rate of the clicks that follow the introductory notes (Wells 1988). The Puerto Rican frog, *Eleutherodactylus coqui*, has a two note advertisement call. When inter-male distances become closer than 50 cm, males drop the second note and produces a multi-note call by rapidly repeating the first note. This new call then functions as an aggressive call (Stewart & Bishop 1994). Thus, some species have graded signalling systems that allow males to change some temporal aspect of a call to indicate increasing levels of aggressive behavior (Grafe

1995; Schwartz & Wells 1984; Wells 1989).

The advertisement call of *Agalychnis callidryas* is a single note call. Males respond to the presence of other males within 50 cm, or playback calls at sound pressure levels (SPL) equivalent to a male calling from 50 cm (93dB), by reducing the sound level of the advertisement call (tlock to soft tlock), or producing a different type of aggressive calls (e.g., chuckle) or visual displays. Reducing the dB level of tlock calls to soft tlock calls may enable males to continue to broadcast a call to attract females and also signal that the intruding male has been detected by the caller.

A change from advertisement calls to aggressive call types, posterior vibrations, or silence were observed in *A. callidryas* in response to the playback of a synthesized tlock call broadcast at 93 dB. For example, male *E. coqui* (Stewart & Bishop 1994) and *Hyperolius marmoratus* (Grafe 1995), increased the proportion of aggressive calls in response to increasing sound pressure levels of advertisement calls played to them. In the study of *E.coqui*, six SPL levels (75-102 dB) were used in playback experiments. As the intensity of playback increased beyond 89 dB, the intensity of a call broadcast at a distance of 25

cm, males became silent, exhibited attack behavior, or moved away from the speaker (Stewart & Bishop 1994). In *Hyla versicolor* males switched from advertisement calls to aggressive calls and physical interactions after a threshold of 93dB was reached in the broadcast of an advertisement call.

The use of different signals (calls and a visual display) in *A. callidryas* also compares in some respects to the signal use of *H. rosenbergi*, a territorial species of frog in which males build nests that females use for breeding (Kluge 1981). *Hyla rosenbergi* is reported to have eight different kinds of vocalizations (Kluge 1981). Males give five different call types in male-male interactions. These call types are of low intensity (dB) compared to the advertisement call. Both male *A. callidryas* and *H. rosenbergi* give different types of calls in a similar social context (male-male interaction). Both species share a chuckle type call which Kluge (1981) describes as having a low intensity territorial function.

Male mating strategies are considered to be adaptations to the length of the breeding season (Wells 1977). Thus the behavioral repertoire of prolonged breeders that call from stationary sites differs from explosive breeders who engage

in scramble competition. My census data (chapter 2) shows that *A. callidryas* has a prolonged breeder with periods of explosive breeding. The behavioral repertoire of males appears to more closely resemble male behaviors described for prolonged breeders (Duellman & Trueb 1986; Fellers 1979a; Wells 1977). Males gave advertisement calls from generalized calling sites and defended these sites from other males who approached within 50 cm of the resident male. In addition to the advertisement call, males had an agonistic behavioral repertoire consisting of two different calls and a visual display. Yet, in contrast to most species of prolonged breeders where mating success depends primarily on attraction of females to the males territory, male *A. callidryas* did not wait for females to make physical contact with them, but crawled or jumped on approaching females. Males also attempted to displace or join already amplexed females, a behavior associated with explosive breeding at high male densities.

The communication system employed by males should reflect the non-static nature of the breeding system. In *A. callidryas* males employ behaviors used by both prolonged breeders and explosive breeders. Males using chuckle calls, soft tlock calls, and posterior vibrations may be more

did not display significant differences (1.3 ± 1.6, 1.4 ± 2.1, respectively; N=20, z=0.270, P=0.787). Non-residents did not display significant differences between lab and field behavior (0.6 ± 0.9, 0.7 ± 0.6, respectively; N=20, z=0.514, P=0.607).

Posterior Vibrations

The number of posterior vibrations displayed were not significantly different between resident males in context 2 in Experiment 1 and Experiment 2 (Mann-Whitney U Test, N=20, z=-0.054, P=0.957). Non-resident males were not significantly different in the number of displays between Experiments 1 and 2 (0.4 and 1.1 respectively; N=20, z=-0.271, P=0.787).

Discussion

Signal use

The tlock call was the primary display given by solitary males in all three experiments. The tlock call has been classified as an advertisement call which functions to attract females (Breder 1946; Duellman 1970; Dunn 1931; Pyburn 1963). My study shows that the tlock call also functions in male-male interactions. Resident males respond to tlock calls of intruders <50 cm in distance from the resident (Experiments 1 and 2), and synthesized tlock calls broadcast at the loudness (dB) of a calling male from 50 cm distance (Experiment 3). For example, when males were presented with a synthesized tlock call (Experiment 3), males increased the frequency of soft tlock calls, chuckle calls, and posterior vibrations significantly, while reducing the frequency of tlock calls given significantly.

These data suggest that one function of tlock calls is to advertise calling site occupancy to other males. The significant increases in frequency of behaviors associated with male-male interactions when synthesized tlock calls were broadcast to resident males, indicates that resident males react aggressively when hearing the tlock call of

another male within 50 cm of their calling site.

In male-male interactions resident males gave chuckle calls, displayed posterior vibrations, and increased the frequency of soft tlock calls in comparison to the male-alone context. Soft tlock calls, chuckle calls, and posterior vibrations occurred in response to the playback of tlock calls in Experiment 3. Combinations of soft tlock calls, chuckle calls, and posterior vibrations preceded wrestling in 83% of the field trials where wrestling between males occurred. No predictable escalation in either the rate of displays or type of displays was observed, due to high variability between males in the sequence and type of displays given preceding an attack.

The lack of predictability in type or sequence of male auditory or visual displays may be an artifact of my experimental design which subject males to either other males or synthesized tlock calls at a distance of 50 cm or less. An experiment using a gradation of distances between males, or varying the loudness (dB) of synthesized calls to reflect different distances between males might be able to elucidate the specific function of each of the auditory and visual displays.

Despite the lack of information on specific functional

differences between soft tlock calls, chuckle calls and posterior vibrations, these behaviors are agonistic signals that may reduce the chance of physical interactions between males. Wells (1977) suggested that encounter calls, calls that occur in agonistic interactions between males, may function to warn an intruding male to leave the area. Although 80% of male-male interactions involved soft tlock calls, chuckle calls or posterior vibrations, only 19% of these encounters escalated further into physical interactions.

Wells (1977) predicted that territoriality should occur in anurans with prolonged breeding seasons where females arrive at breeding sites at irregular intervals. In six species of North American *Hyla*, males exhibit territorial defense, switching from advertisement calls to encounter calls when approached by other males. If intruding males continue to approach resident males, wrestling may result. These wrestling matches ended when intruding males freed themselves and left the resident male's territory, or remained in the territory, but did not call (Fellers 1979a, b). Similar types of behaviors were recorded for male *A. callidryas*. Recapture data collected from marked males (chapter 2), supports a hypothesis of male calling site

fidelity. The presence of site fidelity and the agonistic behaviors recorded in male-male interactions suggest that male *A. callidryas* exhibit calling site defense.

Agalychnis callidryas **displays: Previous descriptions compared**

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The communication system employed by males should reflect the non-static nature of the breeding system. In *A. callidryas* males employ behaviors used by both prolonged breeders and explosive breeders. Males using chuckle calls, soft tlock calls, and posterior vibrations may be more

likely to maintain a calling space and attracting females, than males using only advertisement calls. Under conditions of high density and explosive breeding, moving males that use agonistic behavioral displays may be less likely to be attacked by other males or mistake other similarly displaying males for females. These combination of behaviors would thus not only conserve energy, but increase the probability of encountering females.

Table 5.1 Wilcoxon Signed Ranks test for differences in mean number of displays per 30 min duration by resident male *Agalychnis callidryas* in male-alone context (15 min) and male-male context (15 min) in Experiment 1 and Experiment 2. Means ± 1SE were derived from 20 males each in Experiment 1, Experiment 2. Significant differences in display rates by context are denoted with an asterisk.

	Experiment 1		Experiment 2	
Display	Male-alone	Male-Male	Male-alone	Male-Male
Tlock call	1.7±0.7	2.1±1.3	8.2±1.1	6.1±1.7
	z=-0.51	P= 0.613	z=-1.61	P=0.101
Soft tlock call	0.2±0.1	1.6±0.8	0.3±0.2	1.9±1.1
	z=1.86	P=0.063	z=1.52	P=0.128
Chuckle call	0.4±0.2*	1.3±0.4*	0.3±0.1*	1.4±0.5*
	z=2.00	P<0.046	z=2.13	P<0.033
Posterior vibrations	0.0*	2.5±1.4*	0.0*	2.2±0.8*
	z=-2.52	P<0.012	z=-2.67	P<0.008

Table 5.2 Experiment 3. Wilcoxon Signed Ranks test for differences in mean number of displays per 30 min duration by 12 resident male *Agalychnis callidryas* during 15 min baseline recording and during 15 min playback of a synthesized tlock call. Means ± 1SE were derived from 12 males. Significant differences in display rates by context are denoted with an asterisk.

Display	Base line recording	Response to stimulus
Tlock call	9.6±1.2*	1.9±0.8*
	z=-3.05	*P*< 0.002
Soft tlock call	0.0*	10.2±4.0*
	z=2.52	*P*<0.01
Chuckle call	0.08±0.08*	5.8±2.4*
	z=2.36	*P*<0.01
Posterior vibrations	0.0*	1.6±0.6*
	z=-2.20	*P*<0.02

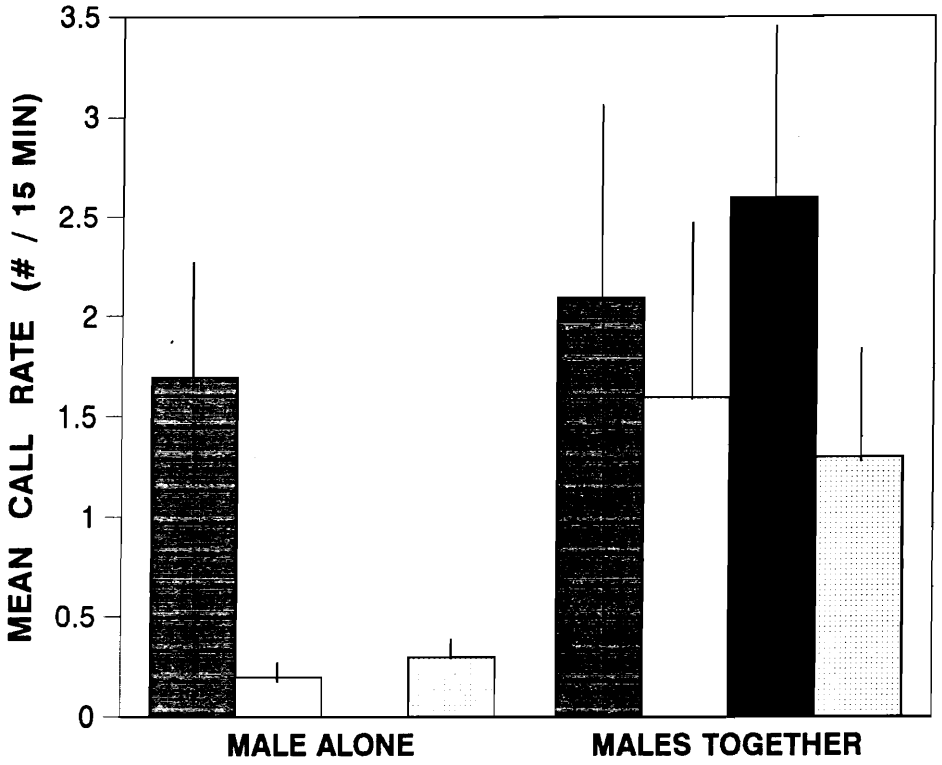

Figure 5.1 Resident male behaviors in "male alone" and "male-male" contexts in Experiment 1 (laboratory trials) (*N*=20) of *Agalychnis callidryas*. Mean display rates + SE are shown. Darkly stippled bars represent tlock calls. White bars represent soft tlock calls. Black bars represent posterior vibrations. Lightly stippled bars represent chuckle calls.

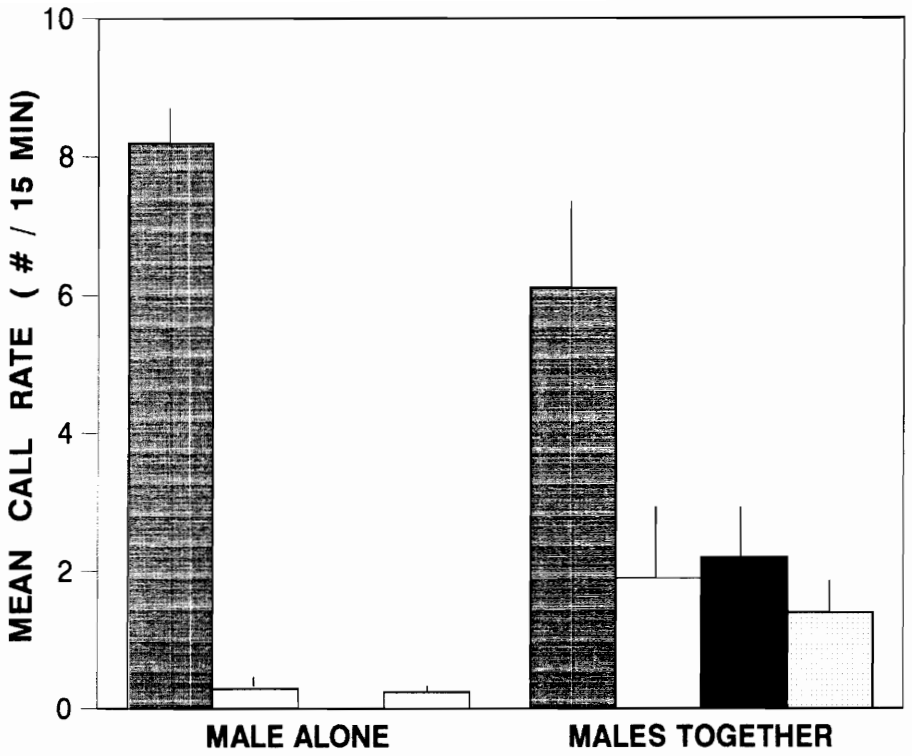

Figure 5.2 Resident male behaviors in "male alone" and "male-male" contexts in Experiment 2 (field trials) (*N*=20) of *Agalychnis callidryas*. Mean display rates + SE are shown. Darkly stippled bars represent tlock calls. White bars represent soft tlock calls. Black bars represent posterior vibrations. Lightly stippled bars represent chuckle calls.

Chapter 6

Synthesis

This final chapter summarizes and addresses the main questions posed by this research, and discusses the potential roles of sexual selection in the population of *Agalychnis callidryas* studied. Also, there is speculation on how sexual selection theory could be tested in future studies. The main questions addressed in this dissertation were: (1) Do matings that involve multiple males result in multiple paternity of offspring? (2) What was the role of multiple paternity in this species? (3) What factors influenced the occurrence of multiple paternity?

Fertilization is generally external in anurans, and occurs when the amplexing male releases sperm in synchrony with female egg-laying. Multiple paternity can only evolve if unpaired males can access to a paired female's eggs. It has been generally proposed (Halliday & Verrell 1984) that the position of the primary male, who is amplexed to the female's back, makes it unlikely for unpaired males to gain access to the eggs. It has also been hypothesized that the sperm from the primary male may have physiological

properties which prevent sperm from secondary males reaching the eggs (Baker & Bellis 1988; Sivinsky 1986). However, in at least 10 species of frogs from two families, matings that involve multiple males have been recorded (chapter 1).

In an attempt to address this inconsistency, this dissertation examined the potential for secondary males to fertilize eggs in *Agalychnis callidryas*, a species in which multiple males may join a pair during egg-laying. The result was the first evidence to document multiple paternity in a tetrapod species with external fertilization (chapter 3). Multiple paternity resulted in matings where two males fertilized almost equal percentages of eggs. Therefore the hypothesis that primary males can exclude secondary males is not valid for this case and the position of a primary male in amplexus is no guarantee of paternity assurance. Fertilization by secondary males suggests that sperm from primary males do not have physiological mechanisms (e.g., "Kamikaze sperm", Baker & Bellis 1988; Baker *et al.* 1990) to inhibit the ability of sperm from secondary males to fertilize eggs. The lack of success by primary males in preventing secondary males from fertilizing eggs in *A. callidryas* suggests that secondary males in other species with matings that involve multiple males should also be

successful. These data infer that external fertilization may enhance rather than limit the opportunity for multiple males to mate simultaneously. This inference is in contrast to previous hypotheses concerning multiple paternity and external fertilization in anurans (Halliday & Verrell 1984; Jennions & Passmore 1993). The adaptive significance of multiple paternity and the potential to influence the frequency of matings that involve multiple males is different for females, primary males, and secondary males. Results presented in chapter 4 showed that females displayed behaviors that minimized mating success of secondary males. There was no evidence to support the hypothesis that females should mate multiply because single males are unable to fertilize the complete complement of female eggs. Additionally, results presented in chapter 2 provided no support for the hypothesis that females might encourage matings by a second, larger male, if females were first paired with a smaller male. Genetic diversity of offspring has been proposed as a reason for females to encourage matings that involve multiple males (Birkhead & Møller 1992, Kempenaers *et al.* 1992). The relatively high degree of bandsharing between adults (chapter 3), however, makes it unlikely that a female would gain a significant increase in

the genetic diversity of her offspring through mating with multiple males.

Females minimized secondary male success by two different behaviors. First, when a secondary male attempted to amplex females, females moved away by jumping or crawling. Second, females oviposited fewer eggs in the presence of secondary males. The only hypothesis my data supports is that female avoidance behavior results from the potential of damage to the eggs which may occur when multiple males attempted to grasp a female (chapter 4).

The loss in fitness to primary males by matings shared with secondary males appears to be minimal. Primary males remain with the female until she has completed egg-laying of all clutches minimizing fertilization opportunities for secondary males (chapter 2). The behavior of females in laying fewer eggs in clutches when secondary males are present and increasing the number of eggs in the following clutch also reduces the potential impact of lost fertilization opportunities for primary males (chapter 4).

Secondary males have the most to gain through multiple paternity. Despite female attempts to avoid secondary males, successful secondary males may be able to increase their fitness without incurring significant costs in joining

paired females. Secondary males potentially gain in fertilization opportunities with little risk of physical injury or missed mating opportunities with unpaired females. As stated previously, the percentage of eggs fertilized was similar for primary and secondary males. Because of the scarcity of unpaired females available for the majority of males were who were unpaired, any eggs fertilized by secondary males should enhance fitness of secondary males.

A number of factors in the breeding biology and behavior of *A. callidryas* influence the occurrence of matings that involve multiple males (chapter 2). Male biased OSR produce a surplus of unpaired males at egg-laying sites. Because a majority of females are already amplexed when they reach the sites, unpaired males are further limited in gaining access to exclusive mating opportunities. Females oviposit multiple clutches over a period of almost seven hours during which females move between the water and egg-laying sites and hence may increase the probability of attracting secondary males.

Matings that involve multiple males were found to be highly variable in frequency, occurring primarily when frogs were at high densities (chapter 2). Density of frogs rather than OSR was a more important factor in the occurrence of

matings that involve multiple males (chapter 2) suggesting that when frogs are in higher densities, unpaired males encounter pairs more often. Conversely, a highly male biased OSR does not insure that males will encounter pairs at lower densities.

The reproductive behavior of *A. callidryas* may be categorized as a combination of prolonged breeding and explosive breeding. The behavioral characteristics of prolonged breeders, males calling from stationary perches, and aggressive behavior toward other males (chapter 5) were recorded in both field and laboratory studies. Yet, frogs bred explosively when periods of little precipitation are followed by periods of heavy rain. When densities increase during explosive breeding conditions, it appeared that males resorted to scramble competition in an attempt to secure mates, which, in turn, results in an increase of matings that involve multiple males (chapter 2). Therefore, I would predict multiple paternity to exist in species of explosive breeders with male biased operational sex ratios. The species for which matings that involve multiple males have been reported offer preliminary support this hypothesis (chapter 1). In addition most of these species lay multiple clutches over extended time periods during a single evening.

The influence of sexual selection on males by females may be tempered by the combination of a prolonged and explosive breeding system. In prolonged breeders, females arrive asynchronously over an extended period of time. Males call from stationary locations and females move toward individual calling males. In contrast, in explosive breeders, males and females arrive at the breeding site synchronously, and scramble competition, in which males grasp any moving individual, results (Wells 1977). Sexual selection may have a stronger influence in prolonged breeders where some males have the ability to obtain more than one mating. In explosive breeders, however, the brief period for breeding activity limits opportunity for males to obtain more than one mating. Because *A. callidryas* has breeding patterns and behaviors characteristic of both prolonged and explosive breeders (chapter 2, 4,& 5), there may be a lesser potential for sexual selection to work than in species with only prolonged breeding. In addition, in the population of *A. callidryas* studied, the effects of matings that involve multiple males, which were both larger and smaller than the primary males with whom they shared fertilizations, should further dilute the effects of sexual

selection.

The information presented in this dissertation provides a basis to test the hypothesis that in prolonged breeders increases in selection for phenotypic characteristics associated with sexual selection would be greater than those in explosive breeders. Populations of *A. callidryas* from geographic areas with daily precipitation patterns during the breeding season could be compared with populations from areas where precipitation was interspersed with period of no precipitation. It would be proposed that prolonged breeding would occur in populations subjected to daily precipitation, while explosive breeding should be characteristic of the populations in areas of intermittent precipitation.

In populations of prolonged breeders the effects of sexual selection, through female choice, should have a greater opportunity to evolve as demonstrated in other species of prolonged breeders (Ryan 1985; Wells 1977). Males with phenotypic characteristics, such as large size or specific call parameters, should gain a greater number of matings than comparable males in explosive breeders. Matings that involve multiple males should also occur at lower frequencies in the population of prolonged breeders thus minimizing secondary male effect on sexual selection.

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VITAE

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