

The Role of Dyadic Interactions in the Mixed-mating Strategies of the Mangrove
Rivulus, *Kryptolebias marmoratus*.

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ABSTRACT

The mangrove rivulus, *Kryptolebias marmoratus*, is a small cyprinodont fish native to tropical and subtropical waters of Florida, Brazil, and the Caribbean. It is the only known self-fertilizing, hermaphroditic vertebrate and the only vertebrate to display androdioecy, a complex system of reproduction in which hermaphrodites and males are present. This study describes the behavioral repertoires observed during dyadic interactions staged in the laboratory. *Kryptolebias marmoratus* exhibited a total of 23 distinctive acts or behaviors. Acts were divided into four categories: aggressive, submissive, neutral, and reproductive. Leading and following behaviors played important roles in the behavioral repertoires of these fish. In the hermaphrodite-male dyad, males initiated the reproductive process exclusively and actively pursued the hermaphrodite. When two hermaphrodites were paired together, there was no evidence that hermaphrodites behave like other simultaneous hermaphrodites and alternate sexual roles. Hermaphrodites were extremely aggressive towards one another and the aggressor established dominance rapidly. Male-male dyads were divided into two subdivisions based on the presence or absence of the caudal ocellus. It appeared the presence of the caudal ocellus signaled to males the possibility of a potential mating partner. When no ocellus was present in either male, their behavior was similar to hermaphrodite-hermaphrodite dyads, in that both members of the pair were aggressive towards one another. These observations may be indicative of interactions taking place in natural communities or assemblages of fish where both males and hermaphrodites occur, and provide evidence on the role of dyadic interactions in the mixed-mating strategies of the mangrove rivulus.

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DEDICATION

This thesis is dedicated to my mother, whose love, support, patience, and encouragement has guided me through the years. This thesis is also dedicated in memory of my father, who first introduced me to the love of fish.

Chapter I

INTRODUCTION

The purpose of my thesis is to describe the repertoires of reproductive and agonistic behaviors displayed by the mangrove rivulus, *Kryptolebias marmoratus*, and elucidate the roles played by hermaphrodites and males in a complex, little understood, mating system. *Kryptolebias marmoratus* is a small cyprinodont fish native to tropical and subtropical waters of Florida, the Caribbean, and the South American coastline down to Brazil (Davis *et al.* 1990). It is the only known self-fertilizing, hermaphroditic vertebrate (Harrington 1961). *Kryptolebias marmoratus* is also the only vertebrate that displays androdioecy, a complex system of reproduction in which hermaphrodites and males are present (Mackiewicz *et al.* 2006a). Three sexual types have been indentified: hermaphrodites (undergo self-fertilization), primary males (remain males throughout lifespan), and secondary males (hermaphrodites that have lost female function) (Harrington 1971).

Recent work has raised significant questions about possible mixed mating strategies in *K. marmoratus*, especially in Florida populations. Laboratory hybridization studies (Mackiewicz *et al.* 2006a) showed that male-hermaphrodite crosses can occur and there was evidence of such crosses occurring naturally in various parts of the *K. marmoratus* range (Mackiewicz *et al.* 2006b). Males are rare in Florida, but microsatellite data demonstrated high levels of heterozygosity (Mackiewicz *et al.* 2006c), leading to questions about how mixed mating strategies might occur in this area of their range. Suggestions have been made (Mackiewicz *et al.* 2006b) that outcrossing in

Florida might be attributed to hybridization between two hermaphrodites. If this is the case, there are two possibilities:

- Hermaphrodites could actively seek out one another as mating partners and engage in specific reproductive behaviors designed to achieve fertilization.
- “Accidental” matings between hermaphrodites could occur due to release of sperm and unfertilized eggs when two *K. marmoratus* are in close proximity to each other.

To evaluate these possibilities, I conducted in-depth analyses in the laboratory of the behavioral repertoires exhibited by three experimental dyads: (1) hermaphrodite-male, (2) hermaphrodite-hermaphrodite, and (3) male-male. Jarne and Charlesworth (1993) argued that, because males are not needed for reproduction to occur in normally self-fertilizing species, when males do exist in such species they must be more aggressive/dominant in order to obtain matings. Based on this I made the following predictions about the behavior of individuals in my experimental constructs: (1) males should be more dominant and initiate the reproductive process more frequently than hermaphrodites when the two are paired together; (2) hermaphrodites paired together should behave like other known simultaneous hermaphrodites (e.g., as in the family Serranidae (Fischer and Petersen 1987)) and alternate sexual roles during spawning; and (3) males paired together should show intense aggression toward each other because they are in competition for mating partners.

In order to address these issues, I collected data that allowed me to: (1) construct a detailed description of the reproductive and agonistic behaviors of *K. marmoratus*; (2) describe patterns or trends for each behavior, both within and between experimental

dyads; and (3) use a Markov transition matrix to examine important leading and following acts for the different experimental dyads. I discuss these laboratory results with respect to mixed-mating strategies that may be occurring in wild mangrove rivulus populations.

Chapter II

REVIEW OF THE LITERATURE

2.1 *Kryptolebias marmoratus* Biology

The genders of *K. marmoratus* usually have different phenotypes (Figure 2.1). While two different types of male gonochorists (functional males) exist, females are not evident (Harrington 1971). Hermaphrodites appear a mottled grayish-brown and have a distinct caudal ocellus located on the caudal peduncle (Harrington 1961). Fromm (1986) describes the caudal ocellus as a female-specific trait for most members of the *Kryptolebias* genus. Males appear orange in coloration, especially along the fins and posterior part of the body. Some males possess a black margin along the caudal fin (Soto and Noakes 1994; Costa 2004). Turner *et al.* (1992) report that males collected from the Belize cays also may have hermaphrodite characteristics, including the mottled appearance and caudal ocellus.

Taxonomy and Systematics

The fish was first described by Poey in 1880 from areas in Cuba (Harrington and Rivas 1958); however, it was not until 1945, when Harrington described the species as separate from *Rivulus cylindraceus*, that the name *Rivulus marmoratus* was used (Harrington 1971). Costa (2004) placed *R. marmoratus* and other similar species into the genus *Kryptolebias* based on morphology and molecular data. The new name is derived from the Greek words *kryptos*, meaning hidden, and *lebias*, pertaining to a

cyprinodontid genus. The new name generally suggests that the generic name of the fish was improperly buried under another genus name for years (Costa 2004).

Figure 2.1: *Kryptolebias marmoratus* Genders. (A) Adult hermaphrodite. (B) Adult male.



A



B

Reproduction

Most wild populations are composed entirely of self-fertilizing hermaphrodites (Kallman and Harrington 1968; Turner *et al.* 1990). This unique form of reproduction is accomplished using the ovotestis, a gonad that produces both eggs and sperm at the same time (Soto 1988). Internal fertilization takes place inside the oviduct of the fish. The ovotestis is paired and has an appearance similar to a normal functional ovary (Soto and Noakes 1994). The oogenic and spermatogenic tissues are not separated by individual membranes (Soto and Noakes 1992). The main constituent of the ovotestis is ovarian tissue; testicular tissue usually appears around the edges of the gonad (Soto and Noakes 1994). Fertilization occurs at ovulation with sperm being released from the testicular component of the ovotestis (Harrington 1963). Although females are not evident in natural populations of *K. marmoratus*, Cole and Noakes (1997) documented “females” in juvenile, laboratory reared fish. They reported the gonadal tissue of juveniles (<100d) often contained mature ova, but not testicular tissue, and argued these individuals were true females.

Kryptolebias marmoratus populations are found in two genetic states: homozygous and heterozygous. *Kryptolebias marmoratus* is generally thought to be an obligate self-fertilizer, with most populations consisting of only self-fertilizing hermaphrodites. Originally, Harrington and Kallman (1968) used tissue histocompatibility techniques that involved grafting fins from parent to offspring and vice versa to verify that populations of *K. marmoratus* were homozygous. With the discovery of a high percentage of males at Twin Cays, Belize (Davis *et al.* 1990; Turner *et al.* 1992a), researchers began using multilocus DNA fingerprinting techniques to assess

heterozygosity. These data showed surprising levels of outcrossing in largely hermaphroditic populations of *K. marmoratus* from Twin Cays, Belize (Mackiewicz *et al.* 2006a) and Florida (Mackiewicz *et al.* 2006b).

Production of males

Males can be generated in three ways. First, Harrington (1975) was able to induce primary males in the laboratory by incubating embryos from hermaphrodites at temperatures between 18 and 20° C. Second, adult hermaphrodites can sometimes undergo succession to secondary males. This has been attributed to exposure of the hermaphrodite to periods of short-day seasons. Secondary males can only be induced in the laboratory by exposing juvenile hermaphrodites to high temperatures (Harrington 1971). Turner *et al.* (1992b) discovered there is variation among clones in their response to the previous listed effects. Third, juvenile hermaphrodites can transform into secondary males if subjected to high temperatures (Harrington 1975). In contrast, Turner *et al.* (2006) demonstrated that Twin Cays populations produce a higher percentage of males than other populations and “maleness” is not environmentally controlled, but under genetic control. They were also able to verify that distinct genetic differences exist in male production between the Twin Cays population and other populations, suggesting the Twin Cays population is shifting from a predominately self-fertilizing population to an outcrossing population.

Distribution, Ecology, and Behavior

The mangrove rivulus is distributed from southern Florida to southern Brazil, a total of twenty-nine different countries (<http://www.fishbase.org>). The vast geographic range of *K. marmoratus* coincides with the distribution of the red mangrove, *Rhizophora*

mangle (Taylor 2000). The fish are considered benthopelagic, non-migratory and reside in brackish waters with a temperature range between 18-24° C (Taylor 1988). They inhabit red mangrove swamps, a unique tidal, euryhaline environment that is characterized by cycles of frequent flooding and droughts (Davis *et al.* 1990). Because of this, *K. marmoratus* has evolved tolerance to a number of severe environmental conditions. For example, it can tolerate water low in oxygen content, elevated levels of hydrogen sulfide (H₂S), and a wide array of salinities, including a maximum salinity of 80 ppt (Abel *et al.* 1987; Taylor 2000).

In the late 1800s and throughout most of the 1900s, *K. marmoratus* was thought to be limited to the Caribbean. However, in recent years knowledge of its distribution has grown to include the eastern coasts of South and Central America and the southern coasts of Florida. Harrington and Rivas (1958) and Harrington (1971) were the first to discover the mangrove rivulus in Florida. Huehner *et al.* (1985) reported the fish's location in the mangrove swamps of the Florida Keys. The northernmost locality reported is Volusia County, Florida (Taylor *et al.* 1995).

Kryptolebias marmoratus is very difficult to catch in the wild. Until Huehner and colleagues (1985) captured more than fifty specimens in the Florida Keys, *K. marmoratus* was thought to be rare. This may have been because many populations are cryptic, inhabiting burrows of the great land crab, *Cardisoma guanhumi* (Taylor 1988, 1989; Davis *et al.* 1990). This microhabitat may provide a refuge for the small fish. Individuals are known to cohabit and 26 fish were found in a single crab burrow in Florida (Taylor 1990). Fish not only inhabit crab burrows in southern Florida, but also in the Florida Keys and Belize. In Belize the crab species is *Ucides cordatus*. *Ucides cordatus* is a

large herbivorous crab that is part of the supratidal fauna of Twin Cays. The crab constructs extensive burrows that connect and form little “villages” (McKeon and Feller 2004). *K. marmoratus* is often found dwelling inside the burrows. All fish from the Belize cays have been collected from small pools of water or from burrows of *U. cordatus* (Turner *et al.* 1992). Habitats utilized in other locations include mosquito ditches, ephemeral pools, and underneath logs and leaf-litter (Davis *et al.* 1990; Taylor 1993; Taylor *et al.* 2003). Indian River Lagoon (Brevard County, FL) is characterized by extensive salt marshes and over 500 fish have been caught in this area alone (Davis *et al.* 1995).

Recently, *K. marmoratus* has been discovered out of water, inside decaying driftwood in several locations (Taylor *et al.* 2008). More than 50 fish were found inside one rotten piece of mangrove in the Florida Keys. Taylor and colleagues termed this bizarre behavior “log packing.” The discovery is unique for several reasons: (1) it is the first documented case of its kind, i.e., semi-terrestrial behavior of fish leaving water to cram inside logs; (2) *K. marmoratus* is normally an aggressive fish and to find so many fish dwelling so close together suggests aggressive behaviors may be reduced under such circumstances; (3) it provides insight into how *K. marmoratus* can rapidly colonize remote islands and locations via rafting (Taylor *et al.* 2008).

Locomotion of the fish is primarily swimming, but *K. marmoratus* also has an unusual mode of locomotion known as “flipping” (Taylor 1990; Taylor 2000; Taylor *et al.* 2004). Flipping is an amphibian-like movement and allows the fish to move across land from one pool of water to another or from burrow to burrow (Taylor 1990). This process of terrestrial locomotion (emersion) can be defined as movement outside of water

and is linked to avoidance of high H₂S levels and aggression from other fish (Abel *et al.* 1987; Taylor 2000). Adaptations for emersion involve rich capillary beds along the fins and skin that allow the animal to breathe cutaneously. Abel *et al.* (1987) noted that *K. marmoratus* can survive emersed for 30 days in a moist environment and Taylor (1990) reports a fish may survive more than 60 days without water. Ong *et al.* (2007) reported that gill morphology changes during emersion. Frick and Wright (2002) and Littwiller *et al.* (2006) suggest that NH₃ is excreted through the skin.

Foraging can be aquatic and terrestrial (Taylor *et al.* 2004). *Deinocerites cancer*, commonly referred to as the crab-hole mosquito, is frequently preyed upon by *K. marmoratus* (Taylor 1992). Besides mosquito larvae, *K. marmoratus* also feeds on other insects, polychaetes, copepods, and detritus (Taylor 1992). Many believe *K. marmoratus* is also a piscivore; unidentifiable fish scales and bones have been found in the intestines (Taylor 1992). Many researchers believe that the fish feeds opportunistically, whenever food becomes available inside the burrow (e.g., when *D. cancer* larvae are present), but extensively when the habitat is flooded (Taylor 2000). Because the invertebrate fauna inside crab burrows is not well documented, it is not clear what prey species are available inside a burrow (Taylor 1988). The species is also cannibalistic, eating its own eggs, young, and those of other clones (personal observation).

Although there are a tremendous number of publications focusing on the biology, ecology, and life history of the mangrove rivulus, little information exists concerning most forms of behavior, probably because the cryptic nature of the fish makes it extremely difficult to make observations in the field. Most of what we know comes from the following laboratory-based studies. Kristensen (1970) first described emersion and

also compared courtship in *Kryptolebias* to other rivuline species. Abel *et al.* (1987) also reported on the emersion behavior of *K. marmoratus* and Taylor (1990) documented that *K. marmoratus* flips between burrows and can survive drought within a salt marsh.

Huehner *et al.* (1985) reported that *K. marmoratus* is very aggressive and often bites other less aggressive fish. In fact, attacked fish sometimes leapt out of the aquarium to escape the aggressor, adopting an S-shaped posture before flipping out of the water. Huehner *et al.* also noted that aggressors tended to have the brightest colors of all fish in the tank. *Kryptolebias marmoratus*'s aggressive nature and the fact that fish set up territories in less than one hour when observed in the lab makes them perfect for conducting fighting behavior studies (Hsu and Wolf 1999). Experiments have shown that aggressive behavior in the mangrove rivulus is influenced by previous encounters (Hsu and Wolf 1999) and an individual's history of wins or losses (Hsu and Wolf 2001). Earley *et al.* (2000) used several standard aggression assays including the mirror test, model test, and standard opponent test to determine if one could forecast the winner and loser of a paired encounter. They discovered that aggression varied among clonal lineages and biting frequency was a reliable predictor of the outcome of a contest (Earley *et al.* 2000). Recently, Earley and Hsu (2007) were the first to demonstrate a connection between hormone levels and contest behavior in the mangrove rivulus, indicating their endocrine system is much more fine-tuned to initiation, progression, and the duration of the attack than to previous wins or losses.

Besides Kristensen (1970) no one has provided an in-depth description of reproductive behaviors in mangrove rivulus. Martin (2007) was able to show that hermaphrodites spent more time with males in an artificial crab burrow than with other

hermaphrodites, suggesting crab burrows play an important role in the mixed-mating strategies of *K. marmoratus*.

2.2 Modes of Reproduction in Fish

Fish show the greatest diversity in reproduction of any vertebrates (Helfman *et al.* 1997). Fish can reproduce by separate sexes, by unisexual reproduction, or hermaphroditically (Vrijenhoek 1994). Most bony fish follow the first mode, in which both sexes are present and fixed upon maturation (Helfman *et al.* 1997).

Gynogenesis and hybridogenesis are distinct modes of unisexual reproduction (Dawley and Bogart 1989). In gynogenetic species there is no male contribution, only egg activation. The best example of this is in *Poecilia formosa* (Avise *et al.* 1991). *Poecilia formosa* instantaneously became a new species from the hybridization of *P. latipinna* and *P. mexicana*. *Poecilia formosa* is diploid and gynogenetic. Sperm from *P. mexicana* or *P. latipinna* activate the egg but provide no genetic input. Embryogenesis is triggered by RNA left inside of the egg by the female. Because no male contribution is needed, *P. formosa* is essentially clonal within lineages.

Hybridogenesis occurs when a male and female each contribute a haploid set of chromosomes during a fertilization event; but when the resulting female embryo matures and reproduces, only the original female's contribution is passed on to the egg. In the Mexican livebearer, *Poeciliopsis monacha-lucida*, hybridogenesis occurs as females produce haploid eggs that contain only the mother's genome. Sperm from *P. lucida* fertilize the haploid egg, and syngamy occurs to produce a diploid daughter. However, the male contribution is eliminated from the genome during subsequent gamete

production. Each newly produced egg will have only *P. monacha* genes. This system is unique in that only hemiclonal daughters are produced (Dawley and Bogart 1989).

Hermaphroditism is divided into two categories: simultaneous and sequential. Sequential hermaphroditism occurs when an organism starts its life as one sex and then changes to the opposite sex. This is further subdivided into two categories: protandry and protogyny. Protandrous species begin life as a male and then undergo a sex change to a female. Examples of protandrous fish include moray eels, snook, porgies, threadfins, damselfish, and clown or anemonefish (Helfman *et al.* 1997). The genus *Amphiprion* from the family of clownfish, *Pomacentridae*, live in groups of fish among sea anemones. A hierarchy exists and only the two largest fish are sexually active, with the largest fish being the dominant female and the next being the male. If the dominant female dies, the male will switch sex to become the dominant female. One of the smaller fish living in the anemone becomes the male. Social cues and behavior from the dominant pair keep smaller fish from maturing and reproducing until one mate dies (Dawley and Bogart 1989). Protogyny occurs when a female fish changes to a male fish. Protogyny is the most common type of hermaphroditism among fish. Examples include the wrasses, parrotfish, gobies, and humbug damselfish (Helfman *et al.* 1997). Robertson (1972) was the first to report that social dominance controlled the sex reversal of *Labroides dimidiatus*, a wrasse from the Indo-Pacific Ocean. *Labroides dimidiatus* males set up harem systems, guarding as many as ten females. A dominance hierarchy exists among the females and only the top female gets to breed with the male. Kuwamura *et al.* (2002) reported that the alpha female begins courting females within an hour after the alpha male dies and develops testes in as little as two weeks.

Simultaneous hermaphroditism is a unique system in which an organism can produce both sperm and eggs at the same time. According to Sadovy and Shapiro (1987), the sex ratio for a simultaneous hermaphrodite is 1:1 and only a size frequency distribution exists. However, in all but one vertebrate, synchronous hermaphrodites still cross fertilize (Helfman *et al.* 1997). For example, black hamlets, *Hypoplectrus nigricans*, have adopted an egg-trading mechanism in which an individual fish lays a small number of eggs and then fertilizes a batch of eggs laid by the partner. Egg fertilizing is much less costly than egg laying (Dawley and Bogart 1989). There is one exception to the rule that simultaneous hermaphrodites still cross-fertilize. *Kryptolebias marmoratus* is the only known simultaneously hermaphroditic vertebrate that can self-fertilize.

Androdioecy

Androdioecy is the complex mating system of *K. marmoratus* and is defined as a reproductive system in which both hermaphrodites and males are present. Several plants are androdioecious, including some species of maple (*Acer sp.*), a Spanish fern (*Culcita macrocarpa*), a perennial herb (*Datisca glomerata*), and a wind-pollinated shrub (*Phillyrea augustifolia*) (Gleiser and Verdu 2005; Hollenbeck *et al.* 2002). Far fewer animals are androdioecious; all are invertebrates with the exception of the mangrove rivulus. Barnacles (*Balanus gealeatus*), nematodes (*Caenorhabditis elegans*), and many branchiopod crustaceans like the clam shrimp (*Eulimnadia texana*) are androdioecious (Hollenbeck *et al.* 2002). Weeks *et al.* (2006) noted two similarities shared by all androdioecious animals. First, androdioecy usually only exists in one species, or a small number of related species, within a larger taxonomic group. Second, all males in

androdioecious systems arose in one of two ways: (1) androdioecy evolved from separate sex ancestors, resulting in males and hermaphrodites of similar size; or (2) androdioecy evolved from hermaphroditic ancestors, with males complementary to hermaphrodites.

The freshwater clam shrimp, *E. texana*, has been extensively studied by Weeks, Zucker, and colleagues to understand how androdioecy is maintained in populations. In populations of *E. texana*, males occur along with two genetically distinct types of hermaphrodites: amphigenic and monogenic. Maleness is controlled by a recessive allele (s) and hermaphroditism is produced through a dominant allele (S). Therefore, homozygous dominants are monogenic hermaphrodites (SS), homozygous recessives are males (ss) and heterozygotes are amphigenic hermaphrodites (Ss) (Sassaman and Weeks 1993). Unlike many androdioecious plants that can hybridize with other hermaphroditic plants, in both *E. texana* and *C. elegans* cross-fertilization between hermaphrodites is not possible because hermaphrodites do not possess specialized mating appendages as males do (Sassaman and Weeks 1993). Therefore, all outcrossing occurs by hermaphrodites breeding with males, with outcrossing decreasing inbreeding depression (Sassaman and Weeks 1993). Inbreeding depression can best be defined as a decrease in fitness of a population over time due to reproduction between related individuals.

Weeks *et al.* (2000) used two separate populations of *E. texana* to calculate an estimate of inbreeding depression. Their results were phenomenal: the population with greater genetic diversity showed reductions in fecundity and size and higher mortality of inbred shrimp, while the population with less diversity showed no fitness loss among shrimp produced by selfing. This suggests inbreeding depression occurs to a greater extent in more genetically diverse populations. The researchers also concluded that

because males have a greater mortality rate than hermaphrodites, androdioecy is either maintained in *E. texana* by hermaphrodites incapable of fertilizing their eggs or by successful male matings. Later Zucker *et al.* (2001) reported an increase in male longevity when there were also increased opportunities to mate. Hollenbeck *et al.* (2002) concluded that outcrossing between males and hermaphrodites is influenced by environmental factors such as age and distribution of males. For example, the best scenario for high encounter rates and multiple matings per male is when male clam shrimp are young and males are rare; overall, the density of the population, male frequency, and age work together to influence the success of males.

Chapter III

MATERIALS AND METHODS

3.1 Study Species and Husbandry

Behavioral observations on *K. marmoratus* were based on fish reared in the aquatic laboratory at Valdosta State University (VSU) and males provided by the laboratories of Bruce Turner (Virginia Polytechnical Institute and State University) and Edward Orlando (Florida Atlantic University). Fish were housed in plastic 11.3 or 14 l Sterilite storage containers with lids. The storage containers were aerated by an air hose running through the lid, with an air stone attached. Unlike many other facilities, this laboratory is unique in that it also housed the fish in community tanks; as many as ten fish of similar size from the same clone were kept in one container. Rocks, shells, and cut pieces of PVC pipe provided both habitat and refuges for fish.

Fish were fed newly hatched brine shrimp (*Artemia* sp.) nauplii daily and Tetramin tropical flake food on alternate days. Water salinity in the tanks was approximately 10-13 ppt and room temperature was maintained at approximately 27° C. The room containing fish operated on a 14:10 h light: dark cycle. For more information on additional protocols, visit the website:

<http://chiron.valdosta.edu/dbechler/vsu%20k%20mar%20clones.htm>.

Laboratory stocks consisted of highly inbred individuals of clonal lineages propagated from generation to generation from single, selfing hermaphrodite progenitors. Each line of fish was identified and named using various designations devised by original collectors to reflect the location and other conditions involved in the collection of the

original specimens. For example 50.91 was the 50th fish caught in 1991 from Belize. See Table 3.1 for a list of clonal lines that were used in the behavioral analyses.

3.2 Experimental Set-up

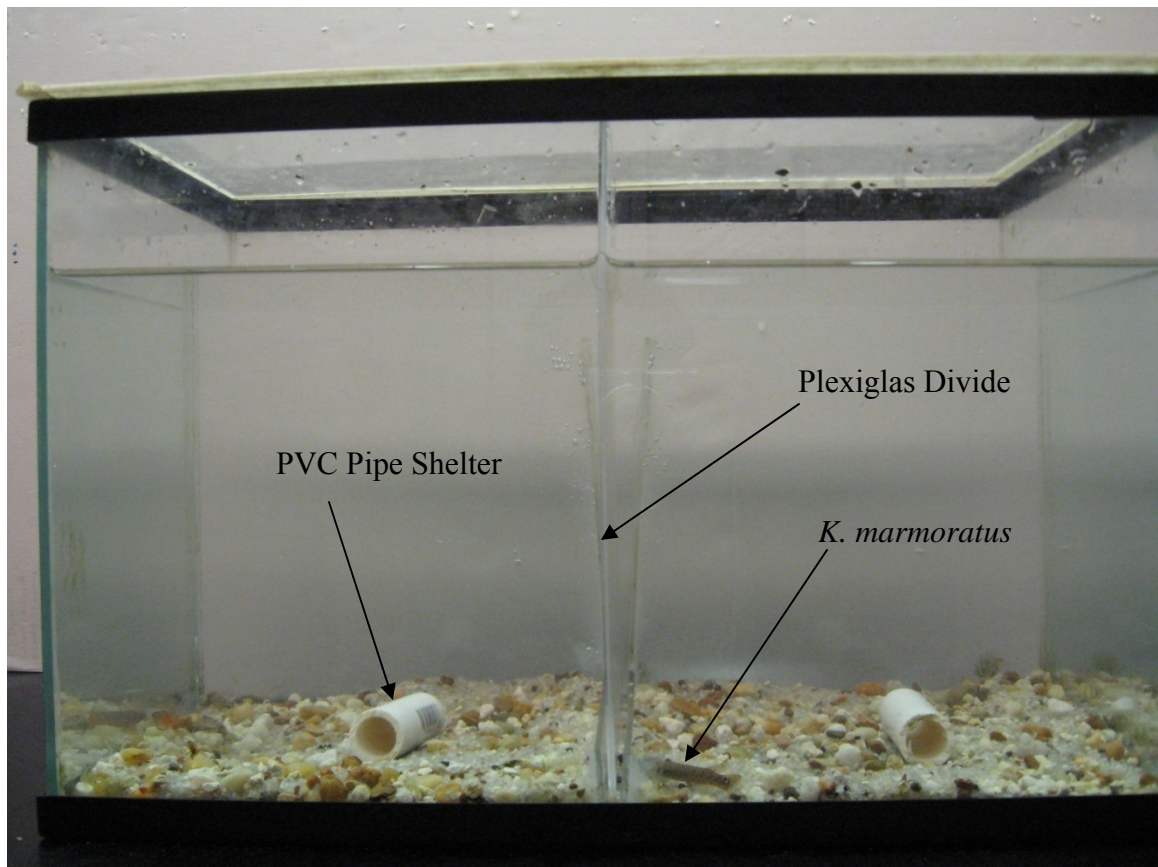
Experimental pairs of fish were observed in a 19 l glass tank filled to a capacity of 93% with brackish water at a salinity of 13 ppt (Figure 3.1). To ensure that the same amount of water was used repeatedly, a fill line was marked on the outside of the tank. Gravel was placed to a depth of 1.5 cm along the bottom of the tank and two plastic PVC pipes measuring 2 cm x 10 cm served as artificial burrows or refuges. The tank was separated into two regions by a removable, transparent, permeable Plexiglas partition held in place by Plexiglas ribs cemented on the sides and bottom of the tank. A glass plate served as lid for the tank. A fluorescent light was placed above the tank to light it from above and all other lighting was turned off during data collection. Cardboard partitions were set up around the perimeter of the observation tank to prevent any external disturbances from affecting the fish, and a small window was cut to allow filming by a camera. Pairs of fish of similar size from different clonal stocks were placed in the partitioned tank for an acclimation period of 22 hours. Choosing fish of similar size was intentional so as to eliminate any bias that may have resulted from differences in body size. In addition, using fish of similar size was most likely to elicit the greatest number and diversity of responses (Bechler 1983), therefore adding to our understanding of the behaviors engaged in by the mangrove rivulus. After 22 hours, the Plexiglas partition was pulled and data collection began.

Table 3.1: List of Fish Used in Behavioral Observations. Unknown clones were fish whose identity could not be confirmed. VSU WF-1 is the first documented laboratory hybrid and the result of a cross between a Hon 2 male x ENP-12 hermaphrodite (Mackiewicz *et al.* 2006c). An asterisk indicates that two of the male VSU clones were used once in the herm-male observations and then later in the male-male observations. All other fish were used only once.

Stock designation	Geographic origin	Number of fish used from stock
Dan 2K	Dangriga, Belize	6 males
50.91	Belize	7 hermaphrodites
DAN	Dangriga, Belize	1 hermaphrodite
ENP 02-2	Everglades National Park, Florida	2 hermaphrodites
ENP-12	Everglades National Park, Florida	5 hermaphrodites
EP 18	Everglades National Park, Florida	1 male
Hon 2	Utila Island, Honduras	3 hermaphrodites; 4 males
Hon 7	Utila Island, Honduras	2 hermaphrodites
Hon 11	Honduras	2 hermaphrodites
Hon 9	Honduras	2 hermaphrodites
R2	Roatan Island, Honduras	2 hermaphrodites
RHL	San Salvador	1 hermaphrodite
SSH	Brevard County, Florida	2 males
SS-LL	San Salvador	4 hermaphrodites; 1 male
SS-RHL	San Salvador	1 hermaphrodite
Vol	Volusia County, Florida	6 males
TC2K	Twin Cays, Belize	4 males
Bel 01	Belize	1 male
VSU WF-1	VSU Hybrid	3 hermaphrodites; 9 males*
Unknown	n/a	1 hermaphrodite

Figure 3.1. The Testing Aquarium with a Transparent, Permeable Plexiglas Partition.

The partition was removed at the beginning of each observation session.



3.3 Data acquisition for the Different Constructs and Subconstructs

The term construct is used to refer to data obtained from specific pairings of fish. For example, data was combined from 15 hermaphrodite-male pairings into a single herm-male construct. Similarly, data was combined from 11 hermaphrodite-hermaphrodite pairings into a single herm-herm construct and from 10 male-male pairings into a single male-male construct.

Behavioral observations of pairs of fish in each construct were recorded for 90 minutes with a digital video recorder (Sony HDD Handycam DCR-SR85) mounted on a tripod. Specific behaviors from the pair interactions were quantified and analyzed using *JWatcher Video*, a component of *Jwatcher*TM 1.0 developed by Blumstein *et al.* (2006). *JWatcher Video* is an event recorder software package that works with QuickTime 7 (Apple Software) to allow video files to be analyzed while deriving time sequences from the digital movie file. Specific alphabetic keys on the keyboard were assigned to specific behaviors. Behaviors were treated as mutually exclusive events, allowing the press of one keystroke to signal the end of the behavior event. Numbered keys represented modifiers. A modifier was defined as indicating which member of a pair performed the behavior. For example, if the behavior was performed by the hermaphrodite of a hermaphrodite-male pairing, a one (1) was pressed directly after the key representing the specific behavior by the hermaphrodite and a two (2) was pressed when the male performed the behavior.

Following the 90 minute observation period was a 30 minute break-down and re-set period. Fish were removed from the tank and the tank was checked for deposited eggs. It was standard procedure to siphon off the water and rinse the tank and gravel with

distilled water between observations in case pheromones were released. Fresh saltwater was added to the fill line and a new pair was introduced into the tank.

Sample sizes for each construct are provided above. It was intended that a minimum sample size of 12 pairs/construct would be filmed. However, due to equipment malfunctions, three observations were lost and not used in the analyses. Most fish were used just once. However, because of the rareness of males of equal size, two males were used once in a hermaphrodite-male dyad and then later in a male-male dyad. A total of 68 different fish were used in the experiment (Table 3.1). All observations were recorded between December 2006 and July 2007.

3.4 Data analyses

The behavioral data were statistically analyzed for: (1) associations among individual behaviors; (2) trends in the frequencies of behaviors over time; (3) differences in behavior between members of a pair construct; (4) differences in behavior between constructs; and (5) important leading and following acts for the different experimental dyads.

Associations among behaviors were examined using factor analysis. I used the totals for each behavior for each fish across all constructs. The factor analysis was performed using StatView, with orthogonal, varimax rotation. Clusters of related behaviors were then identified by listing behaviors by the factor on which each had the highest loading after rotation.

In order to analyze how individual behaviors changed in frequency over time, the 90-minute observation period was divided into six 900 s or 15 minute time bins. The number of times the behavior occurred was calculated for each of the six time bins and

plotted against time. It should be noted that data in the first time bin may be under-represented for some events because this is the bin in which the first approach by one member of a pair was made and in some cases there was a delay in first approach time. Likelihood-ratio chi-square (χ^2) tests were used to compare the frequency of events for the time bins. The G-square statistic indicated the degree of independence between the six time bins, i.e., testing the distribution of each behavior for each fish against the null hypothesis of an equal distribution of behavior across all time bins.

The comparative analyses between the members of the pair consisted of two main parts. First, a nonparametric Friedman's test was used to compare the frequency of each behavior between one fish and the other. Second, Spearman's rank correlations were used to test for association between the frequencies of one fish's behaviors compared to the other fish's frequencies for the same behavior.

Comparative analyses between constructs involved: (1) Contingency chi-square tests (χ^2) to compare the total numbers of each behavior displayed; (2) One-way ANOVAs to compare total frequencies of each pooled behavioral category (i.e., aggression, reproductive, submission, and neutral); and (3) Kendall Tau correlation tests to compare similarities in overall behavioral profiles.

Finally, recorded behaviors were analyzed as paired events. Event pairs were defined as two behaviors that were performed in series, one as a leading act, the other has a following act, regardless of whether or not one member of a pair followed its own behavior with a second behavior or the other member of the pair followed with a behavior. Baylis's (1976) approach of combining each individual dyadic observation as a single unit of data was employed; i.e. the data for all herm-male observations were

lumped together to form one Markovian matrix. *Jwatcher* allowed each behavior to be recorded as a mutually exclusive event; therefore, one fish could follow itself with a second behavior before the other fish responded to a lead behavior. The observed value used was the actual number of events in each category following a given event. An expected value was generated for each cell within each row by using the overall frequency of occurrence for all individual event pairs. As will be demonstrated, *K. marmoratus* has a highly diverse repertoire of behaviors; however, only a few behaviors occurred at high frequencies. As a result, the lead- follow matrices ended up with a large number of cells that contained zeros or no data. Therefore, while it would be desirable to compute Shannon-Weaver diversity indices on entire matrices, it did not prove feasible because of the large number of empty cells. However, because diversity values are valuable and allow comparisons to be made between different data sets or constructs, row and column totals were used in goodness of fit chi-square tests of association, with the Bonferonni correction to adjust for multiple tests. Statistics were based on the chi-square sum for all of the cells in the row. If a row was found to have a significant chi-square value at $\alpha = .010$ (incorporating the Bonferonni correction factor), then the individual cells inside of the row were inspected for values that exceeded the expected value. Statistical tests were conducted using StatsDirect for Windows version 2.6.6 (StatsDirect Limited, 1990-2007).

Chapter IV

RESULTS

4.1 A Catalog of Behaviors for *K. marmoratus*

Kryptolebias marmoratus exhibited a total of 23 distinctive acts or behaviors. Acts were assigned into four categories based on the type of behavior that was performed: aggressive, submissive, neutral, and reproductive. Table 4.1 provides a comprehensive list of the behaviors observed in the experimental pairs. A definition of each behavior follows below. Because mouth gape was used in both agonistic and reproductive contexts, the subscript A and R is used respectively to indicate which context the behavior was observed in.

Aggressive acts were often quick and characterized by swift, bold attacks on an opposing fish or performed in response to a preceding attack. The following is a description of the aggressive behaviors of *K. marmoratus*:

- Charge (C)—Fish swims aggressively towards opposing fish; act begins by fish tilting body in streamline posture. Different from swim away because this is an attack.
- Nudge (NU)—Fish uses body to attack opposing fish.
- Tail flap (TF)—Caudal fin is used to swat at other fish.
- Jaw-lock (LJ) —Fish lock mouths and wrestle. This is similar to mouth wrestle described by Martin (2007).

- Head-butt (HB) — Fish swims straight at opposite fish and uses head to butt other fish's head.
- Nip (N) — One fish bites the other, most of the time ending in rapid displacement. This is similar to the biting behavior described by Hsu and Wolf (1999, 2001); Earley *et al.* (2000); and Martin (2007).
- Mouth gape (MG_A) — Fish opens mouth widely in display.
- Pursuit chase (PC) — Fish swims aggressively after retreating fish. This is similar to chase described by Martin (2007).

Submissive acts are those in which one fish yields to another fish's aggressive behavior. The following is a description of the submissive behaviors of *K. marmoratus*:

- Retreat (R) — Quick movement away from other fish. This is similar to the behavior described by Hsu and Wolf (1999, 2001); Earley *et al.* 2000; and Martin (2007).
- Swim away— (SA) Fish moves more than 3 body lengths away from other fish, occurs at a slower pace than retreat.
- Emerge/escape— (EE) Fish flips out of water adhering to glass lid. This is similar to the behavior described by Hsu and Wolf (1999, 2001); Earley *et al.* 2000; and Martin (2007).

Neutral acts are those that are not associated with antagonistic or reproductive behaviors.

The following is a description of the neutral behaviors of *K. marmoratus*:

- Inspect (I) — Fish swims towards other fish without contact and swims away.

- Swim toward (ST) — Fish approaches or moves within two body lengths of opposing fish and remains in proximity until swim away or retreat occurs. Also includes stationary sitting of pair at bottom of tank. This is similar to the approach behavior described by Hsu and Wolf (1999, 20001); Earley *et al.* 2000; and Martin (2007).
- Idle (Z) — No activity between the two fish, includes sitting motionless at bottom of tank more than two body lengths apart, non-directed swimming, and moving to top of tank to gulp air bubbles. Idle was measured as a duration (s) rather than an event.
- Hover (H) — Pair floats in water column adjacent to one another. This can be subdivided into four positions: side-by side, facing one another with slight space, horizontally on top of one another, and in a criss-cross.
- Inside burrow (IB) — Fish moves inside the artificial burrow.

Reproductive acts are those associated with courtship or mating behavior. The following is a description of the reproductive behaviors of *K. marmoratus*:

- Mouth gape— (MG_R) Fish opens mouth widely in display.
- Fin fan (FF) — Male oscillates fins in display.
- Tandem swim (TF) — Fish pair up and move through the water column. Includes side-by-side swimming or one fish following closely behind the other.
- Vertical rub (VR) — Fish positions body vertically and uses entire body to make contact with opposing fish, which is suspended horizontally in water column
- Head rub (HR) — Fish uses head to make contact with opposing fish, often the point of contact is underneath the vent of the opposing fish

- Backroll (B) — Fish swims backwards until the vent is facing up; sometimes a complete backflip is observed

Although not treated as a mutually exclusive event, color change by some fish was documented. Color change for males involved darkening of the orange portions of the caudal, pelvic, and pectoral fins and/or the black margin of the caudal fin, in hermaphrodites the posterior portion of the body begins to darken.

Members of the herm-male construct exhibited a total of 21 behaviors which included all four behavioral categories: aggressive, reproductive, neutral, and submissive. Members of the herm-herm construct displayed the least number of behaviors at 17, with no reproductive behavior being observed, and the male-male construct displayed 21 behaviors with all four categories being represented. When breaking down the Male-Male construct into the two subdivisions based on the presence or absence of an ocellus, the No Male with Ocellus Construct displayed a total of 16 behaviors with no reproductive behavior being noted. The One Male with Ocellus Construct had a total of 19 behaviors, representing all four behavioral categories. There was no statistical difference in the number of behaviors observed among the constructs (Chi-square = 1.53; $df = 3$; $p\text{-value} = 0.821$).

Factor analysis revealed a total of eight factors and the factor score weights for individual behaviors were for the most part evenly distributed, suggesting the described behaviors were distinct (Table 4.2). Factor analysis also confirmed the legitimacy of dividing the behaviors of *K. marmoratus* into four categories. For example, the behaviors with the highest weights for Factor 1 were vertical rub, head rub, and color change, indicating reproductive behaviors. Behaviors loading highest on Factor 4 were also

reproductive: backroll and swim over. Factor 2 represented aggressive behaviors, with charge, nip, and pursuit chase having the highest loadings. Factor 5 also represented aggressive behaviors, with head-butt having the highest factor loading. Factor 3 represented submissive behavior with retreat and emerge/escape having the highest weightings. Factor 6 represented display behaviors with fin fan and mouth gape having their highest loadings on this factor. Factor 8 represented neutral behaviors, with inside burrow having the highest factor loading. For the most part, Factor 7 represented neutral behaviors, with the highest loadings coming from idle and swim toward; however, lock-jaw also had a high loadings on this factor.

Table 4.1: Incidence of Behaviors Exhibited in the Different Constructs. A plus sign indicates the behavior was observed, and a minus sign indicates the behavior was not observed.

Behavior	Herm-Male	Herm-Herm	No ocellus present on either male	Ocellus present on 1 male
Swim away	+	+	+	+
Backroll	+	-	-	+
Charge	-	+	+	+
Nudge	+	+	+	+
Emerse/escape	+	+	+	-
Tail flap	-	+	+	+
Color change	+	+	+	+
Head rub	+	-	-	+
Inside burrow	+	+	+	+
Jaw-lock	-	+	+	-
Nip	+	+	+	+
Pursuit chase	+	+	+	+
Retreat	+	+	+	+
Swim over	+	-	-	-
Swim toward	+	+	+	+
Hover	+	-	-	-
Vertical rub	+	-	-	+
Tandem swim	+	-	-	+
Fin fan	+	-	-	+
Mouth gape	+	+	+	+
Idle	+	+	+	+
Head –butt	-	+	+	-
Inspect	+	+	-	+

Table 4.2 Factor Score Weights for Individual Behaviors. The proportion of total variance explained by each factor is listed parenthetically.

Behavior	Factor 1 (.18)	Factor 2 (.19)	Factor 3 (.14)	Factor 4 (.15)	Factor 5 (.09)	Factor 6 (.09)	Factor 7 (.08)	Factor 8 (.07)
Charge	.04	.33	.01	.03	-.08	-.13	-.06	.06
Nudge	-.05	.09	-.10	-.03	.19	-.11	.24	-.25
Tail flap	.03	-.05	.33	.03	.09	.01	-1.77E-3	-.01
Jaw-lock	.10	.08	.10	.03	.32	.06	-.46	.09
Head-butt	-.02	-.10	-6.15E-7	.07	.51	.07	.01	-.06
Nip	.03	.33	.02	.04	-.09	-.04	-.14	.05
Mouth Gape	-.21	-.03	-.01	.07	.09	.52	-.02	-.12
Pursuit Chase	.01	.31	.02	.04	-.11	.01	-.10	.08
Color Change	.21	.04	.02	-.02	.17	.09	-.07	.04
Retreat	.07	.05	.36	-.01	-.04	4.98E-3	-.08	2.51E-3
Swim Away	-.11	-.07	.09	.27	.08	-.01	.19	-.06
Emerse/Escape	.04	-1.91E-3	.36	-.03	-.08	.04	-.08	-.08
Inspect	.14	.02	-.07	.03	.33	-.20	.22	.50
Swim Toward	.13	-.06	-.07	-.12	.13	-.05	.35	-.20
Idle	-.01	-.03	.05	.07	.04	-3.00E-3	.51	.22
Hover	.15	.01	.01	.16	-.02	.02	-.01	.08
Inside burrow	.06	.03	-.03	.04	-.05	.07	.01	.55
Fin Fan	-.04	-.11	.05	.01	-.05	.59	-.08	.17
Tandem Swim	.13	.03	-.02	.04	-.15	-.15	-.10	-.20
Vertical rub	.44	.01	.06	.01	-3.61E-3	-.19	-.02	.12
Head rub	.33	.01	.04	.03	-.02	-.07	-.04	.11
Backroll	-.09	.06	-.02	.38	.10	.06	-.03	.11
Swim over	-.11	.06	-.03	.38	.03	.03	-.04	.03

4.2 Trends in Behavior and Activity for the Herm-Male Construct

All four of the behavior categories (aggressive, reproductive, neutral, and submissive) were observed in the male-herm construct. In all fifteen pairs the male approached the hermaphrodite first. There was an interval of 59 milliseconds to 7 minutes between removal of the Plexiglas partition and the first approach by the male (mean = 1.59 min). After the initial approach, the male actively pursued the hermaphrodite, exhibiting reproductive behaviors. During the first few minutes of courtship investigative behavior predominated, which involved the male approaching the hermaphrodite and then swimming away. There was a great deal of overlap between aggressive and reproductive behaviors for the herm-male construct. Nipping, nudging, and pursuit chase were all classified as antagonistic behaviors, but were observed in the herm-male construct in association with reproductive behaviors. For example, a common event sequence observed was: male swims toward hermaphrodite, male nips hermaphrodite, hermaphrodite responds with a backroll.

Male fish used several tactile behaviors in courting hermaphrodites including head rubs, vertical rubs, and nips. Hermaphrodites responded to the tactile interactions with backrolls, swim overs, or a head rub. Often, the fish would pair up and swim through the tank together, with the male always directly behind the hermaphrodite. This allowed the male to make contact with the hermaphrodite as they tandem swam through the water. During the 90-minute observation period no spawning events by the pair or any ovoposition of eggs by the hermaphrodite were observed, nor were any eggs deposited in the gravel or inside of the artificial burrows of the observation tank.

Temporal differences

As time progressed, the overall level of activity between hermaphrodites and males generally remained constant, with only individual behavior types varying. Display behaviors by the male such as fin fanning (Figure 4.2C), mouth gaping (Figure 4.2A), and color change (Figure 4.2B) were highest at the beginning of the observation period and then decreased over time. However, fin fan was the only display behavior to show a statistically significant difference in frequency over time ($G^2 = 11.90$, $p = 0.036$). Other notable differences in reproductive behaviors included: backrolls performed by the hermaphrodite leveled off slightly over time (Figure 4.2C, $G^2 = 19.58$, $p = 0.001$), vertical rubs by the male escalated during the fourth time bin (Figure 4.2D, $G^2 = 19.58$, $p = 0.001$), swim over events by the hermaphrodite were the highest during the first two time bins, but then tapered off in the final four time bins (Figure 4.2B, $G^2 = 21.31$, $p = 0.001$), and tandem swimming was constant for most of the time bins except for the 5th, when it increased significantly (Figure 4.2E, $G^2 = 16.36$, $p = 0.002$).

Trends in aggressive behaviors were as follows. Pursuit chase by hermaphrodites remained almost constant over the six time bins ($G^2 = 9.96$, $p = 0.076$); however, males seemed to perform pursuit chases more during the last time bins ($G^2 = 12.32$, $p = 0.040$). Both males and hermaphrodites nipped one another more during the beginning of the observation period than at the end, with males increasing their nipping again towards the end of the observation period ($G^2 = 11.96$, $p = 0.035$). Finally, nudging was infrequently observed in males, but hermaphrodites nudged males more during the beginning and end of the observation period ($G^2 = 12.78$, $p = 0.021$).

Noteworthy trends in submissive behavior included hermaphrodites swam away from males at a constant rate over time; however as time progressed males were less likely to swim away from a hermaphrodite than at the beginning ($G^2 = 11.90$, $p = 0.036$). No significant difference was detected among the time bins for retreat ($G^2 = 3.62$, $p = 0.604$).

Neutral behaviors, such as idle (away from other fish) were almost constant over time ($G^2 = 1.47$, $p = 0.916$). Time spent hovering together in the water column increased over time, with the most hover events recorded during the final time bin ($G^2 = 19.58$, $p = 0.0015$). Males swam toward hermaphrodites more during the first time bin, and then a slight decrease was observed in the number of times a male swam toward a hermaphrodite ($G^2 = 11.96$, $p = 0.0354$). Hermaphrodites swam toward males during the middle of the observation period ($G^2 = 22.04$, $p = 0.001$).

Differences between pair members

The frequency of behaviors between hermaphrodites and males varied quantitatively. Males swam toward hermaphrodites three times more often than the reverse (Figure 4.5 B, $T_2 = \# \text{ INF}$, $p < 0.0001$; note that $T_2 = \# \text{ INF}$ simply means the number exceeds the minimum value to get a p -value ≤ 0.0001). Hermaphrodites swam away at a higher rate than did males (Figure 4.4 A, $T_2 = \# \text{ INF}$, $p < 0.0001$). Nipping (Figure 4.3 C) and head rubbing (Figure 4.1 A, $T_2 = \# \text{ INF}$, $p < 0.0001$) occurred more frequently in males than in hermaphrodites. Vertical rub (Figure 4.1 D) and fin fanning (Figure 4.2 C) only occurred in males and swim over (Figure 4.1 B), nudge (Figure 4.3 b), and back roll (Figure 4.2 C) were only observed in hermaphrodites. Mouth gape ($T_2 = 25$, $p = 0.0041$) and color change ($T_2 = 10$, $p = 0.025$) occurred more frequently in males

than hermaphrodites. Emersion/escape ($T_2 = 0$, $p > 0.9999$), inside burrow ($T_2 = 0$, $p > 0.9999$), pursuit chase ($T_2 = 2.14$, $p = 0.203$), and retreat ($T_2 = 0.294$, $p = 0.611$) did not differ significantly between males and hermaphrodites. Emersion or flipping out of the water was only observed a total of four times and occurred equally in hermaphrodites and males. Finally, retreating behavior was very rare by both hermaphrodites and males

Similarities between pair members

Results of the Spearman rank correlation indicated that there was a strong positive correlation between the number of times a male swam toward a hermaphrodite and the number of times a hermaphrodite swam toward a male (Table 4.2). Nudge showed a perfect negative correlation (Table 4.2).

Table 4.3: A Comparison of the Frequency of Hermaphrodite Behavior versus Male Behavior using Spearman's Rank Correlation (N = 15). The total number of events for each behavior performed by the hermaphrodite was compared to the total number of events of the same behavior performed by the male for each of the 6 time bins.

Behavior	Rho	95 % CI	p-value
Head rub	0.493	-0.531 to 0.932	0.356
Swim away	0.714	-0.231 to 0.966	0.136
Emerse/Escape	-0.500	-0.933 to 0.524	0.242
Mouth Gape	0.664	-0.320 to 0.959	0.175
Nudge	-1.00	Not calculated	< 0.0001
Color change	0.674	-0.303 to 0.960	0.175
Inside burrow	0.583	-0.433 to 0.947	0.297
Nip	0.371	-0.630 to 0.909	0.497
Pursuit chase	-0.372	-0.909 to 0.630	0.419
Retreat	-0.217	-0.874 to 0.722	0.564
Swim toward	0.886	0.264 to 0.987	0.033

Figure 4.1: Frequency of Reproductive Behaviors in the Herm-Male Construct. (A) Head rub. (B) Swim over. (C) Backroll. (D) Vertical rub. (E) Tandem swim. Time bins are for each successive 15 min of the 90 min observation period. Frequency is the total number of events observed per time bin. Male behavior is represented in light gray and hermaphrodite behavior is represented in dark gray. Behaviors performed in tandem are represented by a pattern.

