EFFECTS OF BEHAVIORAL INTERACTION ON SEX DETERMINATION IN THE MIDAS CICHLID

by

Ronald G. Oldfield

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Doctoral Committee:

Professor Emeritus Gerald R. Smith, Co-chair
Professor William L. Fink, Co-chair
Assistant Professor Thomas Duda Jr.
Associate Research Scientist Edward S. Rutherford
Douglas Y. Shapiro, Pfizer Global Research and Development

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PREFACE

As a child keeping pet fish I developed a fondness for the Midas cichlid, *Amphilophus citrinellus* (Günther, 1864). I became interested in sex determination in this species when I read a popular article by Ron Coleman (Coleman 1997) that described the research of Francis and Barlow (1993) that found that Midas cichlids had sex determined at the juvenile stage by social interactions. I found fascinating the concept that social control of sex determination was a heterchronic variant of the social control of sex change at the adult stage, which is commonly observed in marine fishes. When choosing a project for my Ph.D. research I felt compelled to further investigate social control of sex determination in order to learn more about how it occurred. With a general interest in fish behavior I wanted to know if aggressive interactions directed an individual to differentiate as a female or a male in the same way that they controlled timing of sex change in marine fishes. I also wanted to know whether the cichlids differentiated directly as females or males or if they underwent prematurational sex changes in a manner similar to the post-maturational sex changes observed in marine fishes.

I felt that I could not properly attempt such an investigation without thorough knowledge of sexual lability in fishes. This necessity motivated me to conduct a literature review on the topic. However, I found the topic to be much too broad, and my manuscript seemed to barely skim the surface. Around that time I found that Devlin and Nagahama (2002) had just published their nearly 200 page comprehensive review of sex

determination in fishes. I then re-directed my efforts toward understanding sex determination in cichlids. The resulting paper was published in *Fish and Fisheries* (Oldfield 2005) and serves as Chapter 1 of this dissertation.

In the winter of 2001 I took residence in room 4091 of the University of Michigan Museum of Zoology. I acquired a brood of Midas cichlids from the pet trade and in this laboratory, which contained a small lab bench and was attached to two small aquarium rooms, began a behavior experiment that addressed a question that had long been of interest to me: why do fish become aggressive when placed into small aquaria? An understanding of the effects of ecological conditions on social behavior and social structure was necessary for planning future experiments that tested the effects of social conditions on sexual development. In the context of resource defense theory I analyzed the effects of space, group size, and 3-D structure on aggression, both at a small scale in the lab, and a larger scale by incorporating data aquired at the Toledo Zoo and in the field. The paper has broad animal welfare implications that I hope will revolutionize attitudes toward aquarium keeping. It now serves as Chapter 2 of the dissertation.

My first test of socially controlled sex determination in Midas cichlids was performed in the same laboratory and served as the experiment portion of the qualifying exams for the Ph.D. program. It specifically examined the role of behavioral interaction in sex determination. The results of the experiment were inconsistent with the conclusions of Francis and Barlow (1993), in that they showed no relationship between relative body size, or behavioral interactions, and sex. The resulting manuscript was published in the *Journal of Fisheries International* (Oldfield 2007), and now serves as Chapter 3.

The next project was a study of sex determination under natural conditions. It took me on a 12-day trip to Nicaragua where I collected fishes, snorkeled, and SCUBA dived in Lake Xiloá, Lake Nahapa, and Lake Apoyo. To investigate sexual development I collected data on Midas cichlid social behavior in Lake Apoyo and also captured two groups of juveniles for histological examination. The results of this study were also inconsistent with the reports of Francis and Barlow (1993) because there was no association between body size and sex in either group. The resulting manuscript was published in the *Caribbean Journal of Science* with my two Nicaragua contacts as co-authors and serves as Chapter 4 of this dissertation (Oldfield et al. 2006).

There seemed to be three possible reasons for the inconsistencies between my investigations and the conclusions reached by Francis and Barlow (1993). It seemed that there may have been differences in sex determination mechanisms among different Midas cichlid lineages. It also seemed possible that social factors only determined sex when groups of fish took on a specific social structure, which I found in Chapter 2 to be dependent on the number of fish held together in a captive group. The third possibility was that Francis and Barlow (1993) were incorrect and sex was not determined socially in Midas cichilds. In order to test the effects of lineage and group size on social control of sex determination, I renovated the Museum's primary aquarium laboratory, room 4088, and utilized this room, the two smaller aquarium rooms previously mentioned, and the aquatic facilities at the University's E. S. George Reserve to conduct several experiments that employed hundreds of Midas cichlids. In Chapter 5 I describe these experiments and how they further indicated that social factors were not responsible for sex determination in Midas cichlids. I also discuss how Francis and Barlow (1993) erroneously interpreted

their data in a way that suggested socially controlled sex determination.

In a final effort to seek evidence that Francis and Barlow (1993) were correct in their conclusion that sex may be controlled by social interactions in Midas cichlids, I went to the California Academy of Sciences and accessed specimens from Lake Masaya, Nicaragua, the same locality as the specimens used by those authors, and examined their gonads histologically. No evidence to support a mechanism of socially controlled sex determination was found. In addition, the gonadal structures and the ontogenetic pattern observed indicated that young individuals differentiate directly as either females or males. The manuscript describing this project serves as Chapter 6 of the dissertation.

In Chapter 7 I discuss my conclusion, based on all of these studies, that social conditions do not influence sex determination in Midas cichlids. I also discuss the implications that my conclusion has for theories proposed by Francis (1992) regarding the evolution of sequential hermaphroditism in marine fishes.

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ABSTRACT

Some teleost fishes can change sex according to social conditions, a trait that has been proposed to have evolved through a change in developmental timing. I reviewed literature and found that cichlids exhibit different expressions of lability at each of three life stages, supporting this idea. In Midas cichlids, *Amphilophus citrinellus*, relative body size within a group has been reported to determine sex at the juvenile stage, with larger individuals differentiating as males and smaller fish differentiating as females. In contrast to this report, I found that sex was not associated with either behavioral interaction or relative body size in any of 10 small groups of Midas cichlids that were grown to maturity in the laboratory. In Lake Apoyo, Nicaragua, large juveniles at the onset of sex differentiation exhibited no association between body size and sex in either of two natural social groups. In lab experiments that restricted available space and numbers of competitors, juveniles behaved territorially and performed elevated levels of aggression. Therefore, subsequent lab experiments tested the effects of relative body size on sex determination at larger group sizes and in larger tanks. When eight groups from four different lineages were grown for much longer periods, sex was unrelated to body size initially, but as the fish matured males began to grow faster than females. Maturation in isolation did not affect sex determination. Of 25 wild-caught Midas cichlids of various ages from the same locality as those used in the original reports, none had bisexual gonads although these are often present in sexually labile species. In juveniles, there was

no significant difference in body size between females and males, but in adults males were much larger than females. In each investigation, results were not consistent with a hypothesis of socially controlled sex determination. Larger body size in adult males compared to adult females is attained not because the largest juveniles differentiate as males, but because males experience greater post-maturational growth than females. Sex determination in Midas cichlids therefore does not support any hypothesis regarding the evolution of functional sex change in sequentially hermaphroditic fishes.

CHAPTER 1

GENETIC, ABIOTIC, AND SOCIAL INFLUENCES ON SEX DIFFERENTIATION IN CICHLID FISHES AND THE EVOLUTION OF SEQUENTIAL HERMAPHRODITISM[1]

ABSTRACT

Genetic and environmental factors may interact to control sex determination in fishes. A common pattern of initial female differentiation and subsequent male transformation before maturation in non-hermaphroditic fishes and after maturation in sequentially hermaphroditic fishes has suggested that changes in developmental timing may be responsible for the evolution of various expressions of sexual lability. Sequential hermaphroditism is rare in freshwater fishes, but investigators report degrees of sexual lability at four distinct life stages in cichlid fishes. Some cichlids undergo genetic sex determination and are not labile. Lability at the larval stage allows temperature or pH to determine sex. Social interactions apparently determine sex at the juvenile stage in the Midas cichlid (*Amphilophus citrinellus*). Most reports of post-maturational sex change in cichlids are anecdotal or unsubstantiated. The common observation of same-sex spawning suggests that many species are incapable of sex change. Sequential hermaphroditism is concluded not to be typical, except for the checkerboard cichlid (*Crenicara punctulata*), which apparently undergoes functional female-to-male transformation. Different patterns of sexual development at four life stages in one family of fishes corroborate a role for developmental timing in the evolution of sequential

[1]A version of this chapter was originally published as: Oldfield R.G. 2005. Genetic, abiotic and social influences on sex differentiation in cichlid fishes and the evolution of sequential hermaphroditism. *Fish and Fisheries* 6: 93-110.

hermaphroditism as well as environmentally-controlled sex determination. The broad phylogenetic distribution of sexual lability in cichlids indicates that processes capable of producing sex change are generally present. The rarity of sequential hermaphroditism in cichlids and possibly other freshwater fishes is likely due to unpredictability of food and therefore potential mate distributions compared to coral reef habitats.

INTRODUCTION

Genetic factors control sex determination in most fishes. However, extrinsic factors also influence sex in many species. Before maturity, abiotic factors such as temperature and pH may determine sex. After maturity, behavioral interactions may initiate a functional sex change, i.e. the sequential hermaphroditism observed in many coral reef fishes (Fishelson 1970, Robertson 1972, Fricke and Fricke 1977). In most of these species individuals mature as an initial sex and later transform into the terminal sex. In protogynous species, females are the initial sex and males the terminal sex. In protandric species, individuals are function first as males then transform into females.

The size-advantage model has been proposed to explain why sequential hermaphroditism may be advantageous (Ghiselin 1969). According to this model, an individual may change sex if it would result in an increase in its expected future fitness. Males are generally capable of producing similar amounts of gametes regardless of body size. Eggs are much larger than sperm, and fecundity in females increases with body size. In a group-spawning social structure males of all body sizes have similar chances of fertilizing eggs. When young and small an individual could maximize reproduction by functioning as a male. As the male grows it may reach a point when its future

reproductive potential as a female would exceed that expected if it continued to reproduce as a male. It would then maximize reproduction by undergoing protandrous sex change. If ecological conditions allow the largest males to defend harems, male reproductive potential may increase exponentially with body size, and protogynous sex change would maximize fitness (Warner 1975).

A change in developmental timing has been proposed as a mechanism involved in the evolution of sequential hermaphroditism. In some gonochoristic fishes (species in which individuals are only functional as one sex in their lifetimes), individuals differentiate directly as a male or female early in development. However, in many gonochores all individuals initially differentiate as females, but early in development some are diverted, presumably by genetic factors, to differentiate as males (Uchida et al. 2002). Oocytes often remain in the gonads after these rudimentary hermaphrodites mature as males. This led to the idea that sequential hermaphroditism may evolve from a post-displacement of this pre-maturational sex change into adulthood (reviewed by Atz 1964, Shapiro 1987, Francis 1992).

Large scale variation in the timing of sex-change supports the theory that sequential hermaphroditism evolves by change in developmental timing. Some populations of sequential hermaphrodites contain exceptional individuals that mature as the terminal sex and do not undergo adult sex-changes (Robertson and Warner 1978). However, histological evidence in the protogynous bluehead wrasse (*Thalassoma bifasciatum*, Labridae) has shown that even 'primary males' initially differentiate as females but change sex before maturity (Shapiro and Rasotto 1993). In addition, many gonochoristic fishes that are closely related to sequential hermaphrodites possess

intersexual gonads (gonads that contain both male and female structures) as juveniles, suggesting precocious sex change (Smith and Young 1966, Buxton and Garratt 1990, Baca Hovey et al. 2002). Fishes that have sex determined before maturity by environmental factors provide an example of another variant in timing of gonad development. This suggests that a prolonged period of gonad differentiation may result in different expressions of sexual lability depending on the life-stage at which it terminates.

The initial female differentiation observed in rudimentary hermaphrodites led to the idea that female may generally be the default or initial sex in fishes (Shapiro 1992). Even protandric hermaphrodites do not initially differentiate as males. The gonads of juvenile dusky anemonefish (*Amphiprion melanopus*, Pomacentridae) contain primarily female tissue. Testicular tissue becomes more prominent when fish mature as males, but then regresses when they transform into females (Shapiro 1992, Godwin 1994). Shapiro (1992) noted similar findings in three other *Amphiprion* species, and also in *Sparus aurata* (Sparidae) that were serially sampled from captive groups. Juveniles of protandric creediids also have intersex gonads (Langston 2004). The gonads in these species become mostly male only upon maturation. Therefore, maleness in protandric species is a transitional stage rather than the initial sex, and patterns of sex differentiation as seemingly different as protogyny and protandry share a general female-to-male pattern.

Although hermaphroditic species are common on coral reefs, they are rare in freshwaters. There are no freshwater fishes that have been well-studied and generally accepted to be sequential hermaphrodites. No explanations have been proposed to account for this absence. Possible clues may lie in the biology of cichlid fishes.

Cichlidae have often been considered sister to one of two families, Pomacentridae

and Labridae (Stiassny and Jensen 1987, Streelman and Karl 1997), that contain the most well known sex-changing marine fishes. These relationships have recently been questioned; an alternative phylogeny places Cichlidae sister to a group containing many hermaphrodite-containing perciform lineages (Sparks and Smith 2004). Cichlids are extremely speciose and diverse, and have evolved a broad range of reproductive strategies that include substrate-spawning and mouthbrooding (Barlow 2001); uniparental (Fryer and Iles 1972), biparental (Barlow 1976), communal (Ward and Wyman 1977, Taborski 1984), and interspecies (McKaye 1977) brood care; and mating systems that include monogamy, polygamy, and sneaking strategies (tilapia species discussed in Oliveira and Canario 2001).

Although reports of sequential hermaphroditism are uncommon in cichlids, there are more reports of various expressions of sexual lablity in this group than in other groups of freshwater fishes (Table 1-1). Kallman (1984) found reports by four authors that had claimed to observe sex-change in poeciliids to be inconclusive or unsubstantiated despite the fact that they had been cited often. I will evaluate published reports of sexual lability in cichlids. Analysis of these reports may elucidate mechanistic and phylogenetic patterns within the group that may lead to a better understanding of the processes involved in the evolution of sexual lability and the scarcity of hermaphroditism in cichlids and other freshwater fishes.

Because genetic sex determination (GSD) is generally considered to be the ancestral mechanism of sex determination in fishes (Smith 1975), it needs to be understood to effectively conceptualize how extrinsic factors could interact with genes to control phenotypic sex. The next section provides an overview of our current

understanding of how genetic factors influence sex in cichlids. The following three sections will then review in detail reports of sexual lability in cichlids at successive life stages and identify patterns of initial female development. Reports of same-sex mating without subsequent sex change will be reviewed and interpreted to indicate an absence of lability at the adult stage. These reports will then be evaluated.

Evidence of lability at multiple life stages will support the hypothesis that various expressions of sexual lability, including sequential hermaphroditism, may evolve by ontogenetic extension in the critical period of gonad differentiation (Figure 1-1). In a phylogenetic context, a concentration of labile species in one clade within Cichlidae would indicate that an evolutionary emergence or loss of the developmental process capable of producing sequential hermaphroditism could account for its rarity. A broad distribution of labile species in the cichlid cladogram would indicate that a developmental process capable of producing sequential hermaphroditism is generally present in cichlids, but some aspect of coral reef environments that makes sequential hermaphroditism adaptive for labrids and pomacentrids is absent in freshwater environments.

GENETIC SEX DETERMINATION

Most fishes do not have heteromorphic sex chromosomes (Devlin and Nagahama 2002). Thompson (1976) examined karyotypes of 47 cichlids, concentrating on neotropical species, and found no differences in chromosome shape between the sexes. A later review concluded that there are no morphologically distinguishable sex chromosomes published for any cichlids, perhaps with the exception of *Geophagus brasiliensis* (Kornfield 1984). A region of one chromosome non-homologous between males and

females has been identified in the Nile tilapia cichlid (*Oreochromis niloticus*) by TEM analysis of the synaptonemal complex (Carrasco et al. 1999). Therefore, sex chromosomes may exist in cichlids even though they are not morphologically apparent using traditional observation techniques.

Sex-influencing genes have been identified in some fishes. A male-determining gene, DMY, has been recently identified and sequenced in a strain of medaka (*Oryzias latipes*, Adrianichthyidae), although this species has no visually apparent sex chromosomes (Matsuda et al. 2002, Nanda et al. 2002). DMY evolved recently as a duplicate of the autosomal gene, DMRT1 (Zhang 2004), which had been suspected to cause male differentiation in the rainbow trout (*Oncorhynchus mykiss*, Salmonidae; Marchand et al. 2002). A DNA fragment that is a male sex-linked marker has been isolated for the three-spined stickleback (*Gasterosteus aculeatus*, Gasterosteidae) using a Restriction Fragment Length Polymorphism (RFLP) method. However, no markers could be found for nine-spined (*Pungitius pungitius*, Gasterosteidae) or fifteen-spined sticklebacks (*Spinachia spinachia*, Gasterosteidae; Griffiths et al. 2000). A male-specific DNA probe was isolated from Chinook salmon (*Oncorhynchus tshawytscha*, Salmonidae) by the use of a subtractive hybridization technique (Devlin et al. 1991). Molecular evidence for a sex-determining gene has also been found in a cichlid. Offspring of three mating pairs of *Oreochromis niloticus* were analyzed genetically by bulked segregant analysis for evidence of a male sex-determining region (Lee et al. 2003). The method identified a region of DNA that predicted phenotypic sex in more than 95% of progeny from two of the pairs. However, it was ineffective in the third.

By progeny testing artificially sex-reversed fish, some cichlids have been found to

undergo GSD in a manner indicative of a heterogametic system. Genetic sex can be over-ridden in many fish species by early treatment with exogenous hormones (reviewed by Hunter and Donaldson 1983, Pandian and Koteeswaran 1999). For example, evidence for male heterogamety (XY/XX) may be obtained after larva are masculinized with an androgen (typically 17α-methyltestosterone). If an XY/XX sex-determining mechanism is present, half of the resulting males will be normal (XY), and the other half will be sex-reversed females (XX). When these treated males are then bred to normal females (XX), half of the pairs should yield broods in which all progeny differentiate as females. The males of those pairs must have been sex-reversed genetic females (XX), and males must be the heterogametic sex. A similar method can be used to detect female heterogamety (ZZ/ZW).

Aquaculture researchers have extensively studied sex determination in tilapia. Monosex cultures increase production by preventing mating so that the fish allocate higher proportions of energy toward growth. Interestingly, *Oreochromis niloticus* has been demonstrated to have male heterogamety (XY/XX; Jalabert et al. 1974), and a congeneric species, the blue tilapia (*Oreochromis aureus*), female heterogamety (ZZ/ZW; Guererro 1975). The Mossambique tilapia (*Oreochromis mossambicus*) has been found to have heterogametic males (Clemens and Inslee 1968), although a conspecific strain appears to be female heterogametic (Hickling 1960). The non-tilapiine Egyptian mouthbrooder (*Pseudocrenilabrus multicolor*) and the neotropical convict cichlid (*Archocentrus nigrofasciatus*) have both been found to have sex determined by XY/XX genetic mechanisms (Hackman and Reinboth 1974, George and Pandian 1996). Although genetically sex-reversed fish usually yield monosex broods, there are often a few

anomalous offspring that do not differentiate as expected. When George and Pandian (1996) mated genetically sex-reversed XX *A. nigrofasciatus* males with normal XX females they observed broods that contained nearly all females but also a few males.

Inconsistencies in otherwise straightforward sex determination mechanisms may be results of the multi-locus, multi-chromosomal nature of sex determination mechanisms in fishes. Kallman (1984) found W, X, and Y sex chromosomes in most populations of the platyfish, *Xiphophorus maculatus* (Poeciliidae). In this system chromosome combinations of WY, WX, and XX result in females and XY and YY result in males. These chromosomes share some of the same genes and are morphologically indistinguishable. YY males are possible because Y has not accumulated deleterious mutations typical in Y chromosome evolution (Lui et al. 2004). There are additional male-promoting genes on all of these sex chromosomes, but they are not usually expressed. There are also a number of sex-influencing autosomal genes that can interact with the primary sex determining genes. Autosomal genes have been proposed to account for the many reports of exceptional individuals that contradict their genetic sex, and their effects have been shown to be heritable. The absence of absolute dominance by any one sex determining gene or allele may be what allows environmental factors to have profound effects on sex differentiation in some species.

LABILITY AT THE LARVAL STAGE: ENVIRONMENTAL SEX DETERMINATION

Abiotic environmentally controlled sex determination (ESD) occurs when extrinsic factors such as pH and temperature influence the direction of sex differentiation. The

most well understood case of ESD in a fish is that of the Atlantic silverside (*Menidia menidia*, Atherinidae), on the Atlantic coast of North America (Conover and Heins 1987). Populations of this species that occur at higher latitudes experience a short growing season. These populations undergo GSD. However, populations at lower latitudes experience longer growing seasons and offspring produced early in the season usually grow larger in their first year than those produced later. Large body size is more important to females than to males in this species, as fecundity increases with size in females but not in males. Larvae of these lower latitude populations undergo temperature-controlled sex determination. Those produced earlier in the season experience colder initial temperatures than those produced later. Cold temperature predicts large body size and causes female differentiation that maximizes the future fitness of exposed individuals.

Environmental sex determination has been observed in cichlids in the laboratory. Heiligenberg (1965) described skewed sex ratios in *Pelvicachromis pulcher* that were correlated with pH. In broods of 50 to 80 fish, acidic water (pH 4-5) yielded 90% males and neutral water (pH 7.0) 90% females. Rubin (1985) found three species of *Pelvicachromis* and two species of *Apistogramma* to differentiate as males when in acidic water (pH<6.0) and females in neutral water (pH 7.0). It is interesting to note that the green swordtail (*Xiphophorus helleri*, Poeciliidae) yielded similar results. Römer and Beisenherz (1996) investigated the effects of temperature and pH in 37 species of *Apistogramma*, and in *Pseudocrenilabrus multicolor*, and also in another poeciliid, *Limia melanogaster*. The authors inferred sex from secondary sexual characteristics. Temperature significantly affected sex ratio in 33 species of *Apistogramma*. Generally,

low temperature (23° C) produced females, high temperature (29° C) males, and intermediate temperature (26° C) a 1:1 sex ratio. *Limia melanogaster* showed a similar sensitivity. Sex in *A. caetei* was affected by pH, but not temperature. Temperature and pH did not affect sex determination in *P. multicolor*. Although the labile periods are the same for both hormones and temperature in *Oreochromis niloticus* (see below), sex determination in *P. multicolor* is sensitive to extrinsic hormones (Hackman and Reinboth 1974) but not to temperature or pH.

Temperature-controlled sex determination has also been identified in tilapia. Generally, when larva are subjected to higher temperatures during the period of exogenous steroid sensitivity, sex ratios are skewed toward males. Wang and Tsai (2000) investigated the effects of temperature on young *Oreochromis mossambicus*. After 5 days of exposure at the treatment temperatures, groups were brought to 24° C, raised until they were 150 days old, and then sexed. Method of sexing was not described. Exposure to a cool temperature (20° C), either between days 1-5 or 6-10 post hatching, yielded significantly higher ratios of females than did higher temperatures. Fish exposed to warmer temperatures (either 28° or 32° C) between the ages of 11 and 15 days differentiated significantly more frequently as males. The observed sex ratios are not due to differential mortality, as survival rate was between 95% and 98% in all groups. Intermediate temperatures yielded balanced sex ratios. The observed female-before-male sensitivity is consistent with a pattern of initial female development (Shapiro 1992).

A masculinizing affect of high temperature has also been found in *Oreochromis aureus* (Desprez and Melard 1998). Treatment temperature began at 9 days post spawning and fish were sexed at 69 days. Nearly 100% of the fish reared at high

temperature (34° C) differentiated as males. Mean percentage of males in groups raised at an intermediate temperature (27° C) was 63%, not significantly different from a 1:1 sex ratio. Fish raised at low temperature (21° C) experienced very high mortalities (mean survival = 43%). Survival was also low in the control (73%) and high temperature (82%) treatments. Most of the surviving fish from the low temperature treatments were undifferentiated at 69 days, although some had differentiated as females. Fifty individuals from one low temperature group were raised for an additional 60 days and sexed. At that time the ratio had evened out (46% male). This female-before-male differentiation supports a pattern of primacy of female development.

Baras et al. (2000) observed sex differentiation in groups of *Oreochromis aureus* raised under temperatures that fluctuated daily from 27 to 35° C, in order to better approximate natural conditions. As was found previously (Desprez and Melard 1998), they observed even sex ratios (0.75-0.82:1.00) under constant neutral temperature of 27° C, and male-biased sex ratios (7.33-19.00:1.00) at high temperature (35° C). Sex ratios of groups raised under fluctuating conditions were male-biased (2.33 – 11.50:1.00), although not as strongly as in the constant warm treatment. Intersex gonads were observed at 46 days in some individuals in the high temperature and fluctuating treatments. They were mostly differentiated as testes, with a few oogonia. However, all fish sexed at 90 days were completely differentiated as one or the other sex. This suggests that intersex individuals were genetic females whose sex was reversed by the temperature treatments.

Baroiller et al. (1995, 1996) tested the masculinizing effect of high temperatures in *Oreochromis niloticus*. Each of 10 broods from different breeding pairs was divided

into 2-7 groups. Each group contained an average of 144 fish. Fish were subjected to either the control (27-29° C) or treatment (34-36° C) temperatures over the typical androgen sensitive period, beginning 9-13 days after fertilization and lasting 21 days. The first histological signs of ovarian differentiation begin during this period (Nakamura et al. 1998). Larva were removed from brooding mothers 7 to 9 days after fertilization and subjected to control temperature before and after treatment. After 3 months, over 100 fish in each group were sexed by the aceto-carmine squash method (Guererro and Shelton 1974). Fish generally exhibited significantly higher proportions of males at higher temperatures (up to 81% at 36° C). Survival was similar in control (76.9%) and treatment (71.4%) groups. Duration of treatment was tested in other groups by subjecting them to high temperatures for periods of 10-60 days. Ten days was significant to induce masculinization, and longer treatments did not increase male proportions. Age at treatment was also tested in other groups by beginning treatment at 7-21 days post-fertilization. High temperature caused masculinization in treatments begun 13 days post-fertilization or earlier, but not in treatments beginning 15 – 21 days post-fertilization, demonstrating a close association between period of sensitivity to temperature and the period of hormone sensitivity.

In order to verify that some males observed in high temperature treatments were sex reversed XX females, the sex ratios of broods of temperature-treated males were examined. One 10-day-old brood was divided into two groups and exposed to either normal (27° C) or high (36° C) temperatures for 21 days, which yielded 45.8% and 79.2% males, respectively. Resulting males were then raised to maturity and mated with normal females. All broods derived from 15 control males yielded balanced sex ratios,

however, four of 10 broods derived from the high temperature treated males contained all females. This shows that some of the males observed in the high temperature treatments were genetic females whose sex was reversed by high temperature. Twenty-three pairs derived from these genetic females were then formed and their offspring raised at control and high temperature treatments. All of the broods showed higher frequencies of male differentiation at higher temperatures. Six pairs produced all female offspring at control temperatures and high proportions of males at high temperatures. Surprisingly, some of these supposed genetic female pairs yielded balanced sex ratios at control temperatures. Baroiller et al. (1995) concluded that temperature and sex chromosomes govern sex ratios in *Oreochromis niloticus* and that sex determination is irreversible after 15 days post-fertilization, but labile before the onset of histological evidence of gonadal differentiation. The masculinizing effects of high temperature in this species have been confirmed by other investigators (Abucay et al. 1999).

LABILITY AT THE JUVENILE STAGE: SOCIAL CONTROL OF SEX DETERMINATION

Social control of sex determination might occur if the critical period of gonad lability was prolonged into the juvenile stage, when behavioral interactions before maturity could influence the direction of sex differentiation. A version of this hypothesis was originally proposed by Williams (1972) for the convict cichlid. In adults of this species, males are larger than females. Williams divided each of two broods into experimental groups according to relative body size within their original brood. If large body size was a result of maleness, then experimental groups that were composed of initially large fish would

all differentiate as males, and those that were initially small all as females. After fish reached maturity, Williams was unable to observe significant differences in sex ratio among the groups. It is possible that individual fish assessed their body size relative to other members in their group, and differentiated into males if they were relatively large, and females if relatively small. Williams did not provide the size-sex distributions within the experimental lots, so it is unknown if there was an association between size and sex at the time of sampling. In addition, the very high levels of mortality observed could have obscured disproportionate mortality between the sexes.

Francis (1984, 1990) and Francis and Barlow (1993) have since provided evidence for socially controlled sex determination in two teleost fish species. Initially, Francis (1984) described social control of sex determination in the paradise fish (*Macropodus opercularis,* Anabantidae). Ratios of males in captive populations were found to be inversely correlated with stocking density, and individuals usually developed as males when raised in solitude. In addition, lines selected for dominance yielded high ratios of males and those selected for subordinance high ratios of females.

Francis (1990) and Francis and Barlow (1993) then presented data suggesting that relative body size as a juvenile determines sex in the Midas cichlid, *Amphilophus citrinellus*. Captive groups of this species exhibit a hierarchy effect during growth (Brown 1957). Large individuals in a social group suppress the growth of smaller subordinates through behavioral interactions. Behavioral interactions can affect somatic and gonadal development in both gonochoristic and hermaphroditic fishes. An increase in growth co-occurring with sex change has been documented in the protogynous saddleback wrasse (*Thalassoma duperrey,* Labridae; Ross 1987), and is suspected in

Pseudanthias squamipinnis (Serranidae; Shapiro 1979) and *Amphiprion akallopsis* (Pomacentridae; Fricke and Fricke 1977). As in *Archocentrus nigrofasciatus*, adult male *A. citrinellus* are known to be larger than females, and a captive social group had been shown to maintain a stable size hierarchy from the juvenile stage to adulthood (Francis 1990).

A 6-month old brood of *A. citrinellus* was separated into two experimental groups based on size: those above the median and those below. If size and sex were only controlled by genetic factors, the group containing the smaller individuals would be expected to develop into females, and the group of larger fish into males. The two groups were raised for another 6 months and then sacrificed. Free of the aggressive behaviors of the larger individuals, fish in the 'small' group experienced growth compensation (see Ali et al. 2003). Mean body sizes in the two groups were found to be nearly equal. In addition, each group contained about a 1:1 ratio of females to males, with the females being the smaller, and males being the larger individuals of each group. Francis and Barlow (1993) reasoned that, due to the stable size hierarchy observed by Francis (1990), relatively large size as a juvenile predicts large size as an adult, and large fish could maximize fitness by differentiating as males because competition for breeding sites in the natural habitat is intense and large body size helps males effectively guard the brood site while females tend the offspring. The authors speculated that the fish were interpreting their relative size via aggressive interactions, but did not present behavioral data. They posited that all Midas cichlids may initially differentiate along a default female trajectory, and that being subordinate to conspecifics repressed the prematurational transformation from female to male.

Francis and Barlow (1993) were unsure if the sex-controlling process they observed was a prematurational sex change or social control of initial sex determination and they questioned the distinction between the two concepts. Francis (1990) stated that 12-week-old Midas cichlid gonads were undifferentiated and could not be sexed histologically. Although the European sea bass (*Dicentrarchus labrax*, Moronidae) remains sexually undifferentiated until 9 months of age (discussed in Carrillo et al. 1995), it is unlikely that Midas cichlids delay differentiation for 12 weeks. Other cichlids begin gonad differentiation much sooner. In *Oreochromis niloticus*, differences between testes and ovaries can be distinguished as early as 20 to 23 days after hatching (Nakamura et al. 1998). Similarly, female *Cichlasoma dimerus* begin ovarian differentiation when 15 days old (Meijide et al. 2005). Like the closely related *Archocentrus nigrofasciatus*, *A. citrinellus* may also have a genetic component to sex determination (George and Pandian 1996). If so, it is likely that it begins differentiation at a very early age according to its genotype. Later, social factors either support or compete with the genetic influence, as temperature does in *Oreochromis niloticus*, and the fish perform prematurational sex changes.

Rhodes and Francis later examined sex determination in *Astatotilapia burtoni* (Cichlidae). In this species, there was no indication that sex was determined by social conditions (Fernald, personal communication).

LABILITY AT THE ADULT STAGE: SEQUENTIAL HERMAPHRODITISM

Several authors have reported anecdotes or investigations of adult sex-change in cichlids. Barlow (2000) once separated females from males of black chinned tilapia (*Sarotherodon*

melanotheron) and males later appeared in the group of females. He made a similar observation on orange chromides (*Etroplus maculatus*) that were separated by sex. Females would usually pair with each other, spawn, and produce infertile eggs. However, on one occasion two *E. maculatus* that had both previously spawned with males produced a few eggs that hatched (Barlow 2000).

In a short communication, Heiligenberg (1965) mentioned two cases in which female *Pelvicachromis pulcher* changed into males. In one brood, five females changed into males, and in another case one female changed into a male. These notes were added while the paper was in press and no details were given. We do not know whether the females had been previously observed laying eggs or if their gonads were examined histologically.

Histological examination has revealed intersex gonads in many African cichlids. Peters (1975) examined 65 ovaries and 63 testes of 14 species of haplochromine cichlids representing seven genera. All females had normal ovaries but the gonads of males of all species except *Pseudotropheus livingstonii* were ovotestes. Oocytes were distributed throughout the spermatogenic tissue, attached to the walls of the tubuli. They were much smaller than vitellogenic oocytes found in females and in some individuals showed signs of degeneration. There were no additional indications of sexual transformation in any of the fish examined. Behavior was similar among fishes with differing amounts of oocytes. Within each species there was no difference in body size between sexes, which is often seen in sex-changing animals (Allsop and West 2003). A plausible explanation is that these fishes underwent prematurational sex changes and these species are rudimentary hermaphrodites.

Naish and Ribbink (1990) followed up on the possibility of sex change in haplochromines. Previous observations of wild *Pseudotropheus lombardoi* had found individuals that were colored as males but were mouthbrooding, which is normally performed by females. In the lab the authors isolated five groups, each containing one male and four to eight females in an attempt to stimulate sex change in one of the females. They left each group undisturbed for one month and then removed the male. Three tanks that contained four, six, and eight females served as controls. They also set up three groups that each contained five males and five females. After one month they removed the females in order to stimulate protandric sex change. They did not observe any sex changes. After 2 months one territorial female began to exhibit male coloration, but upon dissection her gonads were found to be female. Like the haplochromines examined by Peters (1975), testes of adult males contained undeveloped oocytes. Of three juveniles, gonads of the smallest two had developing oocytes and the largest had both oocytes and spermatocytes. The authors concluded that males go through an intersexual juvenile period and that females are dichromatic. The evidence presented indicates that sex change does not occur in this species.

Like many African cichlids, *Tramitichromis intermedius* is polygamous and fertilizations are limited to a few dominant males, a social system similar to those of some sex-changing coral reef fishes (e.g., Fishelson 1970, Robertson 1972). Harnish (2004) observed four groups of five previously spawned females for 16 weeks. No male colors or behaviors were observed. In addition, the gonads of 29 males and 30 females were examined histologically for evidence of sex change (Sadovy and Shapiro 1987), but none were found.

Neotropical cichlids have also been investigated for sex change. Loir et al. (1989) examined size distributions and gonad sections in *Satanoperca* aff. *leucosticta*. Males were found to have broader weight distributions than females; while some males were larger than females, many fell within the size range of the females. Distributions that did not overlap would have suggested protogyny (Sadovy and Shapiro 1987). Histological examination revealed all testes to be similar to those found by Peters (1975). They contained previtellogenic oocytes as well as male germ cells in all stages of spermatogenesis. This species is most likely not able to undergo functional sex change.

In his detailed studies of reproduction in *Cichlasoma portalegrense*, Polder (1971) witnessed protogynous sex change. He described a reproductively functional mated pair in which the male became afflicted with an ailment described as a "defect of the swimbladder and respiratory system". While the male was in this condition the female became dominant and grew until she was much larger than him. When Polder noticed secondary male characteristics (thick neck region and small genital papilla), he sacrificed the animal and examined its gonads. Macroscopically and histologically they looked like normal functional testes, complete with masses of spermatozoa. The dorso-cranial end of the testes appeared as ovary walls, but contained no oocytes (Polder 1971).

In addition, Polder (1971) found a large group of captive *C. portalegrense* to be heavily biased toward males (60:2 after the removal of several mated pairs). Two more groups together contained 35 males, 2 females, and 3 intersexes. The intersexes' gonads possessed both female and male tissue macroscopically visible. The ovarian tissue was situated in the anterior end of the gonad and the testicular tissue posterior. Histological sections revealed that all of the largest oocytes showed signs of degeneration, as did

many of those of all sizes that were located near testicular tissue. There was no oviduct in the genital papilla, although the musculature was female. Polder then attempted to stimulate sex reversal, but was unsuccessful. Six recently functional females were placed into an aquarium together for 1 year and spawning did not occur. Polder also noted that he removed three male *Archocentrus nigrofasciatus* from an aquarium containing many individuals, and all had ovotestes containing various amounts of small oocytes. No details or micrographs were provided for *A*. *nigrofasciatus*.

The first studies to investigate sex change in the checkerboard cichlid, *Crenicara punctulata*, were conducted by Ohm (1978, 1980). The resulting publications were magazine articles that did not describe experimental protocol or provide evidence of sex change. However, Zupanc (1985) provided a more complete account of Ohm's work. According to Zupanc (1985), Ohm established 30 groups each containing 4-10 young fish. At an age of 7 to 10 months the dominant fish in each group began to grow faster than the others. This fish differentiated as a male and the rest differentiated as females. If the male was removed then the most dominant female changed sex and began to mate normally with the remaining females, even if it had previously reproduced as a female. During the second year some females in each group began to differentiate into "sub-males". These fish were not the strongest males in each group and were not reproductively active, but did have male coloration. If the original alpha male was removed then these sub-males began to reproduce with the females in the group. Fish raised in isolation all went through female phases, but then changed into males at 7 to 10 months of age.

Zupanc (1985) documented some histological gonad sections from Ohm's work.

A section from an alpha fish showed an intersex gonad with spermatocytes and sperm adjacent to two oocytes. Another section showed testes in a 3 year old sub-male that had previously been a functional female. A gonad section from a 7 month old subordinate female showed an ovary filled with oocytes in various stages of development.

Carruth (2000) gathered further evidence for sex change in *C. punctulata*. She initially maintained a group of 15 juveniles then divided it into four groups, three with four fish each and one with three fish. Initially, all had female coloration. In each group the dominant individual developed male coloration between 5 and 16 weeks. In order to establish that coloration is a reliable indicator of sex, one male, one dominant female, and two subordinate females were sacrificed. Each had gonads consistent with external appearance. The remaining fish were placed into isolation. The three dominant females developed male traits, but the five submissive ones did not. After several weeks, one submissive female was housed with another. One individual developed secondary male traits. Histological examination revealed fish colored as females to have ovaries. Some contained small amounts of undifferentiatied tissue. Fish that had secondary male traits had well differentiated testes. Carruth concluded that the fish had changed sex. Unlike Ohm's sections, testes were found not to contain oocytes, even in males that were thought to have changed sex. The transformed males also had no gonadal lumens, which are often present in transformed males of other protogynous hermaphrodites (Sadovy and Shapiro 1987). Mating did not occur.

ABSENCE OF SEX CHANGE IN SAME-SEX PAIRS

In addition to failures to find sex change in studies where it was sought, evidence that

some cichlids are incapable of sex change can be gathered from investigations of same-sex mating. Some true sequential hermaphrodites begin mating with conspecifics of the same sex prior to physically transforming to the opposite sex. Upon removal of the male in a polygynous group of cleaner wrasse (*Labroides dimidiatus*, Labridae) the largest female changes sex (Robertson 1972). Behavioral sex-change precedes morphological sex-change and takes place immediately after removal of the terminal sex individual in both *L. dimidiatus* and *Thalassoma bifasciatum* (Labridae; Robertson 1972, Warner and Swearer 1991). Several weeks are required for this individual to complete the morphological transformation of the gonads, but it begins courting other females nearly immediately after male removal and performs the 'upward rush', typical in regular spawning, with other females. Nakashima et al. (2000) experimentally removed males from groups of *L. dimidiatus* and observed same-sex spawning behavior in all dominant females. In most of these cases both spawning females released eggs. Upon the return of the removed male, male-role females resumed their previous subordinate role and spawned with the male. The authors concluded that behavioral sex is changeable according to social status, independent of gonad condition, and that females will spawn with the largest member of their group, regardless of sex. Godwin et al. (1996) have shown that behavioral sex-change occurs in female *T. bifasciatum* even after their gonads have been removed.

Same-sex pairing in some cichlids without subsequent sex-change suggests that they possess the ability to change sex behaviorally, but lack the physiological or developmental capabilities required to undergo morphological transformation. Male *Oreochromis mossabicus* often court other males (Oliveira and Canario 2001). Territorial

males of this species have a dark nuptial coloration. Juveniles, females, and non-territorial males have a pale color and generally stay out of the territories. Oliveira and Canario (2001) found that 33% of 618 total courtships by territorial males were directed towards other males, and that territorial males courted non-territorial males with a full courtship repertoire including tilting, signaling the nest, circling, and quivering. Courted males never had dark coloration typical of territorial males, and they frequently responded by behaving like females. They performed female behavior including immobility, following male to the nest, and assuming a pivot position in the nest while being circled by the male. In three of 204 observed cases the courted male placed its mouth near the genital papilla of the male and, when it was quivering, performed a chewing motion typical of a female inhaling sperm to fertilize eggs.

Female-female courtship and spawning has been documented in many cichlids. However, whether or not one of the females behaved in a male role has been a matter of contention. Seitz's (1942) interpretation of female-female spawning in 'Cichlasoma' *octofasciatum* was that one layed eggs while the other 'skimmed' as a male would if attempting to fertilize them. However, both females displayed "symbolic inferiorism" (female behavior). Baerends and Baerends-van Roon (1950) reported spawning in three female jewelfish (*Hemichromis bimaculatus*) in a group of four and when one laid eggs another followed, skimming the eggs like a male.

Aronson (1948) observed spawning of isolated females and female-female pairs of *Sarotherodon melanotheron*. In this species courtship and spawning behavior is similar between males and females. All reproductive behavior patterns are exhibited by both sexes. Even oviposition and fertilization are similar. However, there are differences

between the sexes in amount of time spent performing specific behaviors. Females tail slap and build nests more than males. In courtship males pass over the nest more than females, but this is reversed at the onset of spawning. Each member's behavior in a female-female pair is qualitatively similar, but one female performs behaviors in relative frequencies typical of a male (Aronson 1951). Therefore, one fish was considered to be in the male role. As would be expected from a heterosexual pair, the fish in the male role passed over the nest more during the time preceding spawning, and the female-role fish did so more often immediately before spawning. Immediately after spawning, the fish in the male role rubbed her genital papilla over the eggs as a male would do during fertilization (Aronson 1951). Visual cues alone stimulated spawning, and when physically isolated females were allowed to view males or other females they spawned at similar rates, which were much higher than spawning rates in isolated females (Aronson 1951).

Female-female pairing has also been documented in *Etroplus maculatus*. Barlow (1970) found that females evoke more courtship behavior in both sexes than do males. Threesomes of females often form also. Barlow concluded that females normally do not form pairs because they evoke more attack in other females than do males, but pairs can form when motivation to spawn exceeds aggression. When two males approach each other, their aggressive responses are greatly stimulated and their sexual responses are insufficiently aroused. Therefore they do not form pairs.

Greenberg (1961) witnessed several female-female spawns in *Hemichromis bimaculatus* but concluded that females did not behave as males. In total, seven female-female pairs were observed spawning one or more times. Female-female pair formation

occurred when one female blocked aggression from another by quivering or assuming the 'estrous stand' – spreading the median fins and keeping the head raised while avoiding the attacks of the courting fish. Groups that contained even one small male yielded heterosexual pairs, suggesting that females prefer to mate with any male over a female. In some cases fertilized eggs of *Cichlasoma portalegrense* were substituted for the unfertilized eggs of the female-female pair. Sometimes the pair ate the foster eggs, but two pairs cared for the offspring. One of these pairs tended larva for 18 days before one female killed the other, after which the remaining female continued to raise them. The other pair brooded free-swimming larva for at least 21 days (final disposition not reported).

In Greenberg's (1961) observations neither female could be assigned a male role. Males seldom eat the eggs of their partners, but in many cases a female ate some of the eggs of her laying partner. In addition, both females of a pair alternated laying and fanning eggs in most cases, but males do not begin fanning until all eggs are laid. Each female appeared ready to spawn regardless of the actions of its partner. This agrees with Aronson (1951), who doubted the specific stimulating effect of a partner in female-female pairs, suspecting that isolated females might increase spawning frequency in the presence of shadows or other species.

I have observed one female-female pairing in *Archocentrus nigrofasciatus*. In an attempt to stimulate sex change I set up three aquaria, each with four adult females and one overturned clay pot to serve as a spawning site. In one group, two of the females formed a pair bond and spawned in the pot. Unfertilized eggs appeared several times over a period of 6 months until I removed the fish. One of the females adopted a male pattern

of parental care. It frequently drove away its tank mates and left the breeding site to feed, but frequently returned to the breeding site to guard the opening. The other remained at the breeding site most of the time fanning and cleaning the eggs, behavior typical of a female (Itzkowitz et al. 2001).

Male *Oreochromis mossambicus* are apparently capable of assuming a female role and engaging in same-sex mating (Oliveira and Canario 2001). However, Greenberg (1961) and Barlow (2000) disagreed with claims like Aronson's (1948, 1951) that female cichlids mated in the male role. In substrate spawners males and females perform the same repertoire of behavior. The sex roles differ only by time budget differences (which are variable). Itzkowitz et al. (2001) have described this quantitatively in the broodcare behavior of *Archocentrus nigrofasciatus*. The absence of qualitatively different behavior makes it difficult to demonstrate a change in sex role. However, the fact that females form pairs and release gametes together suggests that protogynous sex-change would have been observed if individuals possessed physiological pathways that could instruct the gonad to change sex, and if the gonad was capable of such a transformation. It is unlikely that the species discussed in this section are normally able to change sex.

DISCUSSION

Many species of coral reef fishes are sequential hermaphrodites (Devlin and Nagahama 2002). In contrast, no well-studied freshwater fishes have been found to regularly undergo post-maturational sex changes. It is possible that this difference stems from events in the evolutionary histories of various groups of freshwater fishes. Mechanisms capable of facilitating sex change may have been independently gained in coral reef

ancestors or lost in freshwater ancestors. It is also possible that there is some fundamental characteristic of coral reef environments that favors sequential hermaphroditism that is absent in freshwater systems. While cichlids exhibit a paucity of hermaphroditic species compared to their close marine relatives, there are more reports of different expressions of sexual lability in cichlids than there are in other families of freshwater fishes.

Variation in the timing of gonad lability supports the hypothesis that sequential hermaphroditism may evolve by an ontogenetic extension in the critical period of gonad differentiation. Cichlid fishes exhibit four degrees of variation in timing of gonad development. Some cichlids exhibit no sexual lability and have sex determined genetically. Sex-influencing genes probably occur in all cichlids, although they may be highly variable among species and even among individuals of a single species. These genes are likely distributed throughout the genome as in *Xiphophorus maculatus* (Kallman 1984). Sex chromosomes are hard to identify and appear not to be evolving in cichlids. There has been no chromosome degeneration typical in the evolution of sex chromosomes (Lui et al. 2004). Nevertheless, sex differentiation is canalized in many cichlid species.

In some species exogenous influences may interact with genetic influences early in ontogeny. During a short critical period, sex may be determined by extrinsic factors such as temperature or pH. The corroboration of environmental sex determination in diverse cichlid species by independent investigators that employed well-designed experimental methods and thoroughly communicated results indicates that ESD is normal in many cichlids. These species express gonad lability at the larval stage.

Gonad lability at the juvenile stage, after behavioral interactions have developed,

could result in social control of sex determination (Francis and Barlow 1993). Such individuals would not undergo functional sex changes and would be gonochores. This process has been proposed for two cichlids. An alternative explanation for the observations of Williams (1972) and Francis and Barlow (1993) is that the sexual size dimorphism present in adults begins to develop at the onset of sexual maturity. In introduced populations of the Mayan cichlid ('Cichlasoma' *uropthalmus*) there is no difference in body size between males and females at 1 year of age. In subsequent years, males grow faster than females, presumably because less energy is required for male gamete production (Faunce et al. 2002). Francis and Barlow (1993) did not consider this possibility in *Amphilophus citrinellus* because a stable size hierarchy was observed in a longitudinal study of a captive group of 12 fish that were individually tagged at an age of 10 weeks and followed for 18 months (Francis 1990). The statistical test used to demonstrate the significance of the stability of the size hierarchy was Kendall's Concordance, and was computed for both weight ($W = .912$, $p < .0001$) and standard length ($W = .914$, $p < .0001$). These values indicate that only three fish occupying adjacent ranks changed positions, or possibly two fish from close, but not adjacent ranks switched positions. This is a nearly stable hierarchy, but a small sample upon which to assume that other social groups will be stable as well. The evidence provided by Williams (1972) is not sufficient to conclude that social interactions control the direction of sex differentiation in *Archocentrus nigrofasciatus*. Data provided by Francis (1990) and Francis and Barlow (1993) are more convincing. *Amphilophus citrinellus* is here concluded to undergo social control of sex determination, although the limited amount of evidence begs further study.

If the critical period of gonad differentiation reached into the adult stage, then social interactions could control sequential hermaphroditism. Most reports of sex change are from captive cichlids and are isolated cases, unreplicated, and unlikely to be species-typical. In addition, the common occurrence of same-sex pairing in cichlids suggests that the behavioral components necessary for sex change are present, but gonad lability generally does not reach the adult stage.

The most convincing evidence of sequential hermaphroditism in a cichlid has been provided for *Crenicara punctulata*. However, Carruth (2000) concluded that individuals initially colored as females were truly females based on gonad sections of only three fish (Carruth 2000). Heiligenberg (1965) may have made a similar assumption in *Pelvicachromis pulcher*. An alternative explanation is that juveniles, females, and non-reproductive males all lack male display coloration. Male *Pseudotropheus lombardoi* juveniles begin life looking like females and later develop male coloration (Naish and Ribbink 1990). In *Astatotilapia burtoni* and *Oreochromis mossambicus*, only territorial males exhibit display coloration. Juveniles, females, and non-reproductive males all have drab coloration and shoal together above the territories of reproductive males (Fernald and Hirata 1977, Oliveira and Canario 2001). It is possible that Carruth's "sex-changed" males may not have changed sex, but had maturation delayed through social interactions, as in *Astatotilapia burtoni* (Fraley and Fernald 1982, Davis and Fernald 1990) and other fishes (e.g., Bushman and Burns 1994, Kolluru and Reznick 1996), appearing as females until social conditions allowed them to mature as their genetically predetermined sex. Evidence provided by Zupanc (1985), and Carruth (2000) indicates that *C. punctulata* is a protogynous hermaphrodite, although more data would be desirable to draw a strong

conclusion. Thus we may conclude that most cichlids are normally gonochores, with the exception of *C. punctulata*.

Aspects of the experimental methods in some of the studies reviewed may have reduced the possibility of observing sex change. Some authors either established all female groups (Polder 1971, Harnish 2004), or placed females in isolation (Carruth 2000), and waited for them to change sex. It is important to note that male removal, not absence, has been shown to be important in stimulating sex-change in the protogynous *Pseudanthias squamipinnis* (Shapiro 1979) and *Thalassoma duperrey* (Ross et al. 1983). Shapiro (1979) found that all-female groups of *P. squamipinnis* are common in nature and captivity. Ross et al. (1983) failed to observe sex-change in *T. duperrey* when females were placed into isolation. It is possible that male removal from established social groups of *Cichlasoma portalegrense*, *Tramitichromis intermedius*, and *Crenicara punctulata* would yield stronger sex-change results.

Descriptions of sex differentiation in cichlids corroborate a pattern of initial female development (Shapiro 1987, 1992). Males of gonochoristic haplochromines and *Satanoperca* aff. *leucosticta* appear to undergo prematurational protogynous sex changes as do many other gonochoristic fishes (Francis 1992). Studies on tilapia have indicated initial female differentiation, either by serial sampling or by sensitivity to temperature that is earlier for female than for male differentiation. While high temperatures induced male differentiation in all three species of tilapia examined, low temperatures induced female differentiation in only one of these. Apparently, it is easier for temperature to induce male differentiation in a genetic female than it is to induce female differentiation in a genetic male. Female may generally be the default sex in cichlids, with male

differentiation occurring when male-inducing factors, either genetic or environmental, are superimposed.

Cichlids exhibit great variability in timing of sex determination, with species expressing either no lability at all or lability at one of three distinct life stages (Figure 1-1). This finding supports the hypothesis that variability in developmental timing is involved in the evolution of sequential hermaphroditism, as well as other forms of sexual lability. Changes in relative time of appearance and rates of development of characters in a species relative to its ancestors have traditionally been observed in morphological features and have been termed heterochrony (Gould 1977). Heterochrony can occur in two general patterns. The first, paedomorphosis, is generally characterized by retention of juvenile traits to the adult life stage. The opposite, peramorphosis, is characterized by exaggerated development of some feature. In typical analyses, timing of development in homologous structures is described among a monophyletic group of species. A comparison can then be made between a focal species and its hypothesized ancestral state and any heterochronic patterns identified (Fink 1982). In fishes, gonochorism and genetic sex determination are generally considered to be ancestral (Smith 1975). Some of the gonochores discussed here express brief periods of lability at the larval stage. Sexual lability expressed later in ontogeny would exemplify retention of a larval trait (lability) into a subsequent life stage. Thus, the evolution of sequential hermaphroditism in fishes in general is an example of paedomorphosis. Because *Crenicara punctulata* is the only sequentially hermaphroditic cichlid, and its closest relatives are known to be gonochoristic, it could be described as paedomorphic.

Instead of being a structure, sexual lability can be interpreted as physiological and

developmental potential. As such, it poses exceptional difficulties when considering the specific processes potentially responsible for its late expression. Neoteny, progenesis, and post-displacement are all candidate processes (Alberch et al. 1979).

Some unsubstantiated reports of sequential hermaphroditism in cichlids are not species-typical. If accurate, these may be examples of intra-specific paedomorphosis (Reilly et al. 1997). This interpretation is consistent with the co-occurrence of gonochorism and sequential hermaphroditism within single populations of many marine species (Smith and Young 1966, Robertson and Warner 1978, Warner and Robertson 1978, Baca-Hovey et al. 2001), although more recent data indicate that early terminal sex differentiation may be due to phenotypic plasticity rather than genetic variation (Liu and Sadovy 2004, Munday et al. 2006)

Phylogenetic hypotheses are available for cichlid relationships and a composite phylogeny has been used to map the evolution of parental care behavior (Goodwin et al. 1998). Phylogenetic analysis has recently been successful at mapping emergence and loss of hermaphroditism in Creediidae (Langston 2004). The reports of sexual lability in cichlids reviewed here have been placed into a phylogenetic framework (Figure 1-2). There are insufficient data to conclusively identify the evolutionary emergence of each case of sexual lability, but the cases are dispersed throughout the phylogeny and apparently do not represent one evolutionary transformation. Although the two cichlids that apparently have sex differentiation influenced by social factors (*Amphilophus citrinellus* and *Crenicara punctulata*) are both neotropical, the species concluded to undergo ESD represent various clades of both South American and African taxa.

If the basic developmental mechanism capable of producing sequential

hermaphroditism is present throughout Cichlidae, but sequential hermaphroditism is very rare, some aspect of marine environments that favors sequential hermaphroditism must be absent from freshwater environments. Barlow (1993) contrasted the abundance of fishes that regularly maintain feeding territories in marine environments to their paucity in freshwaters and attributed the difference to permanence of food sources. Quantity and predictability of food influence the costs and benefits of defending feeding territories (Brown 1964). Grant (1997) found feeding territories in 67% of coral reef fishes, but only 6% of marine fishes of Eastern Canada, and 9% of freshwater fishes of Canada. Food also influences social structure by influencing abundance and dispersion of potential mates (Emlen and Oring 1977). Differences in mate defense were not as pronounced as those in food defense. 84% of coral reef fishes defend mates, offspring, or breeding sites, compared to 73% of marine fishes of Eastern Canada, and 68% of freshwater fishes of Canada (Grant 1997). However, mating on coral reefs may be continual (Thresher 1984), while it is seasonal in freshwater habitats, even most tropical freshwaters (Lowe-McConnell 1975). Whereas short term mate defense might be common in freshwater, scarcity of feeding territories may indicate longer term unpredictability of mate distribution and abundance. The size-advantage model requires a reliable estimate of future reproductive potential. As the cost of making a mistake in gonad allocation increases, sex differentiation processes may become increasingly canalized (Francis and Barlow 1993). Ecological stability in coral reef environments may allow more accurate predictions of future mating opportunities necessary for the evolution of sequential hermaphroditism. The absence of sequential hermaphroditism in freshwater habitats is likely due to their unpredictable nature.

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Table 1-1. Cichlids reported to undergo genetic sex determination (GSD), environmental sex determination (temp. or pH), socially controlled sex determination (SSD, before maturation), and sex change (SSC, post-maturation), and same-sex mating (S-S) without subsequent sex change that indicates an absence of sex change ability. '+' indicates presence of lability, '0' absence. Superscripts indicate references.

Range	Species	GSD	Temp.	pH	SSD	SSC	S-S
India	*Etroplus maculatus*					+[1]	0[2]
Africa	*Oreochromis niloticus*	+[3]	+[4]				
	Oreochromis aureus	+[5]	+[6]				
	Oreochromis mossambicus	+[7]	+[8]				0[9]
	Sarotherodon melanotheron					+[1]	0[10]
	Astatotilapia burtoni				0[11]		
	Hemichromis bimaculatus						0[12]
	Pelvicachromis spp. (n=3)			+[13, 14]		+[13]	
	Pseudocrenilabrus multicolor	+[15]	0[16]	0[16]			
	Pseudotropheus lombardoi					0[17]	
	Tramitichromis intermedius					0[18]	
South	'Cichlasoma' *octofasciatum*						0[19]
America	*Apistogramma* spp. (n=37)		+, 0[14, 16]	+, 0[14, 16]			
	Cichlasoma portalegrense					+[20]	
	Crenicara punctulata					+[21]	
Central	*Archocentrus nigrofasciatus*	+[22]			+[23]		0[24]
America	*Amphilophus citrinellus*				+[25]		

References: **1** Barlow 2000. **2** Barlow 1970. **3** Jalabert et al. 1977, Carrasco et al. 1999, Lee et al. 2003. **4** Baroiller et al. 1995, 1996, Abucay et al. 1999. **5** Guererro 1975. **6** Desprez and Melard 1998. **7** Clemens and Inslee 1968. **8** Wang and Tsai 2000. **9** Oliveira and Canario 2001. **10** Aronson 1948, 1951. **11** Fernald, personnal communication. **12** Greenberg 1961. **13** Heiligenberg 1965. **14** Rubin 1985. **15** Hackman and Reinboth 1974. **16** Romer and Beisenherz 1996. **17** Naish and Ribbink 1990. **18** Harnish 2004. **19** Seitz 1942. **20** Polder 1971. **21** Ohm 1978, 1980, Zupanc 1985, Carruth 2000. **22** George and Pandian 1996. **23** Williams 1972. **24** Oldfield unpublished. **25** Francis and Barlow 1993

Figure 1-1. Continuum of various expressions of sexual lability. A different expression may result depending on the life stage during which the critical period of gonad differentiation ends.

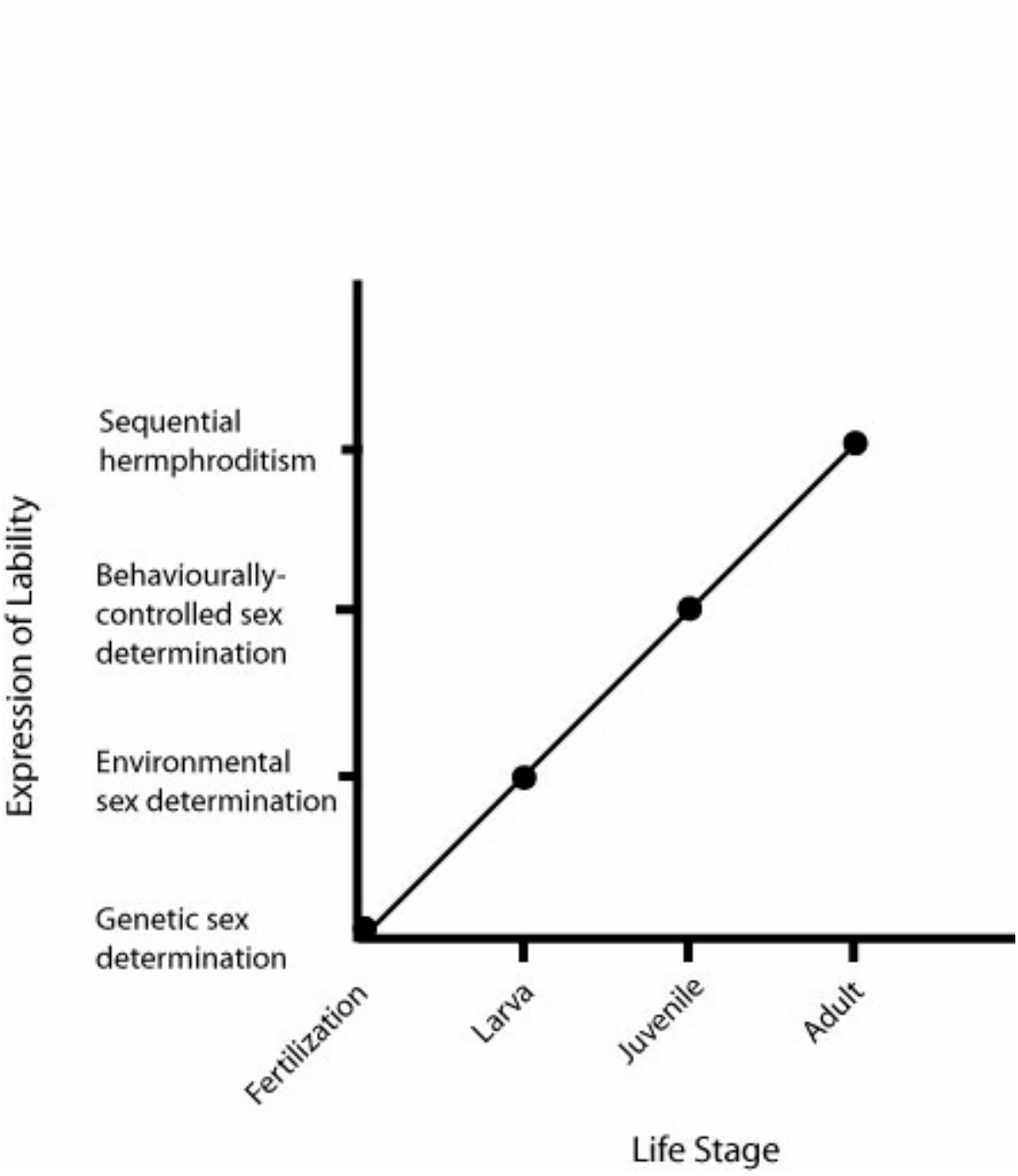
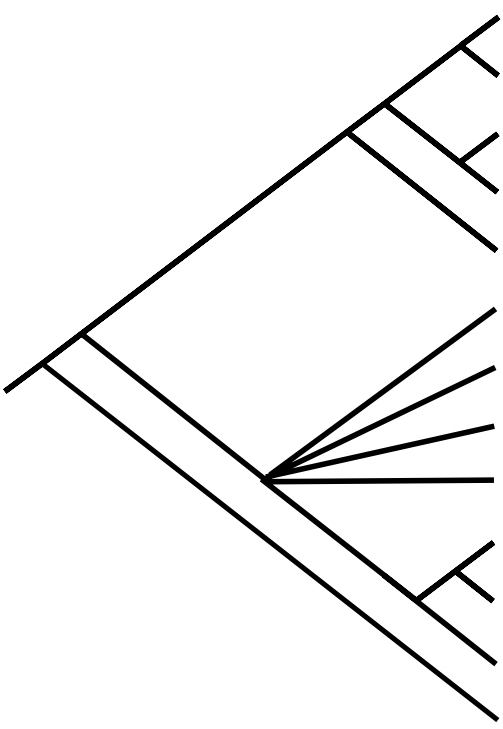


Figure 1-2. Phylogenetic relationships of cichlid taxa discussed in the text as hypothesized by Goodwin et al. (1998). Conclusions reached in this review regarding presence or absence of sex differentiation at successive life stages are indicated. '+' indicates presence of lability, '0' absence.



	fertil	larva	juven	adult
Apistogramma spp.		+		
Cichlasoma portalegrense				0
Amphilophus citrinellus			+	
Archocentrus nigrofasciatus	+			0
Crenicara punctulata				+
Pelvicachromis spp.		+		
Hemichromis bimaculatus				0
Astatotilapia burtoni			0	
Pseudocrenilabrus multicolor	+	0		
Oreochromis spp.	+	+		0
Sarotherodon melanotheron				0
Pseudotropheus lombardoi				0
Etroplus maculatus				0

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CHAPTER 2

EFFECTS OF GROUP SIZE, SPACE, AND 3-D STRUCTURE ON BEHAVIOR IN CAPTIVE MIDAS CICHLIDS

ABSTRACT

Animals may perform elevated levels of aggression in captivity, which may be a response to the modified costs and benefits of resource defense imposed by their artificial environments. The Midas cichlid (*Amphilophus citrinellus*) is a species whose patterns of aggression appear to fit predictions of resource defensibility. Two experiments were performed to test the effects of small-scale changes in group size, available space, and habitat complexity on aggression to determine if Midas cichlids modify behavior under different conditions of defensibility. Proportions of time that dominant fish spent in aggression were not associated with group size or available space, but submissive behavior performed by subordinates and the amounts of body damage received by subordinates decreased as group size and available space increased. Aggression was lower in the presence of 3-D structure. Behavior in the experiments was then compared to that observed in a large zoo exhibit (large group size) and in nature (large available space) to investigate the effects of large-scale differences in defensibility. Aggression was highest under the more defensible, experimental conditions. Aggressive behavior in the absence of food or mating motivation suggests that space was defended as a resource, but it may result as a default due to restrictive artificial conditions that do not provide

opportunities for alternative activities. Elevated aggression has serious animal welfare implications. Some alternative housing tactics that do not promote aggression may nevertheless be suboptimal as they restrict behavioral diversity.

INTRODUCTION

Animals may exhibit modified behavior patterns when maintained in captivity. Many fishes perform elevated levels of aggression (e.g., Okuno 1963, Buchanan 1971), which may be due to artificial ecological conditions that facilitate defense of a particular resource. Guarding a resource is advantageous as long as the benefits received outweigh the costs of defending it against competitors (Brown 1964, reviewed by Grant 1997). To determine if fish optimize behavior, variability in aspects of resources or competitors can be compared to corresponding changes in benefits or costs of defense. Resources that are commonly guarded include food, mates, and shelter. Benefits have been measured in fish in terms of food acquisition (Grand and Grant 1994) and mating success (Warner and Hoffman 1980). Costs of aggressive defense might include injury, expended energy (Haller 1992, 1994), vulnerability to predators, and time not spent exploiting the resource. Changes in costs of defense can be measured in any of these factors or in aggression performed.

A captive environment may be more defensible than a natural environment due to decreased number of competitors and decreased amount of available space. As the number of competitors increases, a defender will be required to increase aggressive behavior in order to maintain monopoly over a resource. When a number is reached at which the costs required to defend the resource exceed its benefits, aggression should

decline (reviewed by Grant 1993). Fish abandon contest competition and resort to scrambling for resources (Kawanabe 1969, Magurran and Seghers 1991, Chapman and Kramer 1996, Syarifuddin and Kramer 1996), and may begin exploiting an alternative resource (Jones 1983). When facing low numbers of competitors, a reduced amount of available space will encourage resource defense because it will result in a shorter distance required to deliver an attack (Schoener 1983). Another factor that may influence social interactions is the presence of 3-D structure, which can increase the amount of usable surface area (Hazlett 1979), provide additional food resources (Semmens et al. 2005), serve as territory boundaries (Breau and Grant 2002), or block visual contact, which will result in fewer opportunities for aggression (Kalleberg 1958, Bronstein 1983).

Midas cichlids, *Amphilophus citrinellus* (Günther, 1864), are extremely aggressive and difficult to house in small groups in aquaria. They become territorial in aquaria and can only successfully be kept either individually or at very high densities, or in large pools (Barlow 1976). This suggests that Midas cichlids modify aggression according to changes in defensibility of some resource that occur with changes in either the number of fish present or amount of space available.

In order to determine if Midas cichlids respond to small-scale changes in defensibility, aggression was observed while manipulating group sizes (experiment I) and aquarium sizes (experiment II). In experiment I aggression was expected to possibly increase as the number of competitors increased, to a point at which net benefits of defense decreased, after which aggression would decrease. In experiment II, aggression was expected to decrease, possibly preceded by an increase, with increase in available space. The effects of 3-D structure were tested in both experiments by repeating some

treatments in complex environments. Midas cichlids are demersal, preferring to remain near benthic structure in their natural habitat (Oldfield et al. 2006), and Barlow and McKaye (1982) suggested that aggression in Midas cichlids was lower in the presence of 3-D shelters. Therefore, aggression was expected to be lower in complex environments.

In order to determine if Midas cichlids behave differently with larger-scale differences in defensibility, behavior observed in experiments I and II was compared to behavior observed in a large zoo exhibit (large group size) and to behavior observed under natural conditions (large available space). For captive conditions that did not promote elevated levels of aggression, behavior diversity and frequencies were compared with diversity and frequencies observed under natural conditions. Such research in a zoo setting has typically been limited to mammals (de Waal 1989, Cassinello and Pieters 2000, Sannen et al. 2004), but captive aggression may pose serious concerns for the welfare of fishes as well.

EXPERIMENTAL SUBJECTS AND HUSBANDRY

One hundred fourteen juvenile Midas cichlids from one brood were obtained from a retail supplier and placed into two large aquaria. The fish were variants of an oligomelanic genotype that undergoes a socially influenced transformation from the usual gray and black barred coloration to orange or white as they mature. On 25 March 2002, 8 days before the trials began, a random sample of 36 individuals had a mean SL (standard length) of 38.0±6.5 (SD) mm, and a mean mass of 2.3±1.8 (SD) g. Sexual immaturity was indicated by small body size, barred coloration, absence of reproductive behavior, and undeveloped state of the genital papilla (Barlow 1976). The use of juvenile fish

prevented aggression that might arise from mating motivation. Experimentation occurred over the course of 2 months. The fish retained barred coloration and showed no signs of maturity throughout this period.

All aquaria were illuminated with natural sunlight through translucent skylights supplemented with fluorescent lighting in a 12 L : 12 D photoperiod. Water (26° C, pH 7.8) was filtered through air powered sponge filters, which were covered by natural gravel (5 cm deep). In addition, complex environments included one stone (10 x 8 cm) placed in the center of the tank, one 15 cm^2 clay tile leaned against each end wall to create two caves, and one bunch of Java moss, *Vesicularia dubyana*, (10 x 8 cm). The ends of each test tank were covered in brown posterboard to prevent inter-group visual communication (Heiligenberg 1965, Oliveira et al. 2001). Data were also collected from replicates in a 380 l 'super-complex' environment in which extensive 3-D structure was erected in order to reduce visual contact and provide excess refuges (Figure 2-1). Fish in holding tanks were fed twice daily on commercial pellet and flake foods, supplemented weekly with frozen brine shrimp (*Artemia* sp.). Experimental groups were fed pellets and flakes once per day.

For each trial, subjects were randomly selected from the two holding tanks and placed into a test tank. The following day data were collected for a 5 min. period, at least 2 hours after feeding. Performers and receivers of all aggressive bouts were recorded. A bout was defined as a string of aggressive acts separated from other bouts by a cessation of at least 1 second, and could include attacks (nip, chase, charge) and displays (lateral display, operculum flare) (Baerends and Baerends van Roon 1950). In addition, time budgets were established by recording behavior at 30-second intervals according to an

ethogram established during preliminary trials (Table 2-1). Data recording was repeated the following 2 days, after which fish were returned to the holding tanks. Due to the limited supply of fish, reuse of fish was considered to be an undesirable (Kuhar 2006) but acceptable option, with the justification that each group was likely composed of a different combination of individuals. The water in the test tanks was changed between trials. Each individual was observed in one preliminary trial within a group of three fish in a 38 l aquarium (except one individual that was used twice). Because all fish experienced preliminary trials, prior residency advantage was not given to any one individual (Frey and Miller 1972).

A disproportionately high rate of aggressive behavior immediately identified one dominant individual in each group, and it always appeared to be the largest fish. Within each experiment these alpha individuals were compared among treatments for the total number of times they exhibited aggression in the three 5 minute periods, and the proportion of time (number out of 30 total observations) that they were seen performing aggressive acts. Aggression scores were not divided by the number of competitors present, as fish were nearly always within visual contact of one another and opportunity for aggression was continuous. For subordinate fish the amount of time spent cowering was compared among treatments, with each group providing one data point due to non-independence of individuals within a group. Proportion of time subordinates spent escaping was also analyzed in experiment II. Data from experiment II were analyzed a second time including data from the 380 l super-complex treatment. Kolmogorov-Smirnov tests found that most data sets did not significantly depart from normality so parametric tests were applied. For each behavior, data were analyzed as a 2-way factorial

ANOVA through a Univariate General Linear Model in SPSS, with behavior as the dependent variable, complexity as a fixed factor, and either group size (experiment I) or tank size (experiment II) as a covariate. Type IV sum of squares was used to account for empty cells in the experimental design.

Each fish was examined at the end of each trial for body damage and assigned to a class between 0 and 3 according to the following criteria. Class 0 – no damage, class 1 – one tear in fin, class 2 – two or more fin tears, class 3 – fin damage and scale loss. No fish were damaged seriously. All healed uneventfully within days after the trial and all fish entered trials at Class 0. No illness or death occurred. Each group provided one data point. Data were transformed by dividing the number of subordinates in each damage class in each treatment by the number of subordinates per group for that particular treatment. Due to the small sample sizes, damage classes 1-3 were grouped, and the number of 'damaged' fish compared to the number 'not damaged' with Cramér's V contingency tables.

The first run consisted of 3-fish trials conducted in 38 l simple environments, the data from which were utilized in both experiments I and II. Subsequent trials were chosen randomly and many treatments ran simultaneously. Eight replications of the original 3-fish, 38 l simple treatment were conducted at the conclusion of the study to test for order effects. Two-tailed *t*-tests revealed that number of aggressive bouts (t_{14} = -1.217, p = 0.244) and proportion of time spent performing aggressive acts (t_{14} = -0.268, p = 0.793) performed by alpha fish did not change by the conclusion of the study. Behavior performed by subordinate fish also did not change between the beginning and end of the experiment (cowering: t_{30} = 1.998, p = 0.061, escaping: t_{30} = 0.437, p = 0.665). All

analyses were performed on Microsoft Excel and SPSS software.

EXPERIMENT I – GROUP SIZE

In experiment I density was varied by changing the number of individuals in each treatment while keeping aquarium size constant. All tests were performed in 38 l aquaria (LxWxH = 51 x 26 x 31 cm), and included 3-fish (n = 8), 5-fish (n = 8), 7-fish (n = 8), and 9-fish (n = 8) treatments. Additional treatments included complex environments at 3-fish (n = 8) and 5-fish (n = 8) group sizes.

Dominant fish increased number of aggressive bouts with increase in number of competitors ($F_{1,45} = 4.961$, $p = 0.031$), but this behavior did not change with complexity ($F_{1,45} = 1.865$, $p = 0.179$) (Figure 2-2a). There was an interaction between group size and complexity ($F_{2,45} = 5.309$, $p = 0.009$). The proportion of time that alpha fish spent performing aggressive behavior did not change with group size ($F_{1,44} = 0.072$, $p = 0.789$) or complexity ($F_{1,44} = 0.405$, $p = 0.528$) (Figure 2-2b), and there was no interaction ($F_{2,44} = 0.221$, $p = 0.803$).

Cowering in subordinate fish showed a strong negative relationship with increasing group size ($F_{1,44} = 42.752$, $p < 0.001$) (Figure 2-3a), but not with complexity ($F_{1,44} = 0.063$, $p = 0.803$). There was an interaction between the two factors ($F_{1,44} = 27.395$, $p < 0.001$). Thirty-one out of 32 alpha fish finished simple trials with 0 body damage and one sustained class 1 damage. In subordinate fish, there were significantly fewer damaged fish at larger group sizes in simple environments (n = 32, V = 0.513, p = 0.038), but not complex environments (n = 16, V = 0.125, p = 0.617) (Figure 2-4a). Subordinate body damage was compared between simple and complex environments and

there was no difference in either 3-fish (n = 16, V = 0.348, p = 0.164) or 5-fish groups (n = 16, V = 0.220, p = 0.379).

In all treatments, alpha fish directed aggression toward, and were able to dominate all group-mates. As number of competitors increased, the costs of maintaining dominance were expected to increase until they exceeded the benefits of defense, at which point alpha fish were expected to decrease aggression. Rate of aggressive bouts increased with group size, but proportion of time spent behaving aggressively did not. This indicates that the dominant fish were stimulated to attack consistently in all of the treatments, but when faced with greater numbers of competitors they may have switched targets more frequently. Aggression of each alpha fish was distributed among more subordinates at the highest densities, with each subordinate receiving a smaller portion. Blackenhorn (1992) observed a similar pattern in groups of pumpkinseed sunfish, *Lepomis gibbosus*, ranging from two to eight fish. Although complexity did not cause a significant reduction in aggression, complex environments consistently resulted in mean values that were lower than in corresponding simple environments.

Subordinate fish in the large group-size treatments often remained within close proximity of one another. It is not apparent whether this was due to shared motivation to reduce inter-individual distance, or if it was the indirect result of independent tendencies to maximize distance toward the alpha fish. The group resembled the 'dominated school' described by Baerends and Baerends van Roon (1950) and Keenleyside and Yamamoto (1962), and may have been similar to a typical shoaling social structure. Increasing numbers of non-territorial fish can eventually overwhelm territory holders (Barlow 1974, Robertson et al. 1976). In the ayu, *Plecoglossus altivelis* (Kawanabe 1969), and in groups

of male guppies, *Poecilia reticulata* (Magurran and Seghers 1991), defenders abandon feeding patches and join schools at high densities. In order to explore the hypothesis that Midas cichlids perform less aggression when faced with a much larger number of competitors, behavior under experimental conditions was compared to behavior observed in a large zoo exhibit (see below).

EXPERIMENT II – AVAILABLE SPACE

In experiment II density was varied by manipulating aquarium size while keeping group size constant at three fish. Aquaria had volumes of 38 l (LxWxH = 51 x 26 x 31 cm) (n = 8), 76 l (61 x 31 x 42 cm) (n = 6), 110 l (76 x 31 x 47 cm) (n = 5), 151 l (122 x 32 x 41 cm) (n = 7), and 380 l (152 x 47 x 51 cm) (n = 2). Behavior in complex environments was tested in 38 l (n = 8) and 110 l (n = 5) tanks.

Number of aggressive bouts performed by dominant fish was not associated with available space ($F_{1,38} = 3.017$, $p = 0.091$) although the number was lower in complex environments than simple environments ($F_{1,38} = 10.611$, $p = 0.002$), and there was a significant interaction between the two factors ($F_{2,38} = 8.596$, $p = 0.001$) (Figure 2-2c). There were no differences in the proportions of time alpha fish spent performing aggressive behavior among different tank sizes ($F_{1,38} = 0.459$, $p = 0.502$) or between simple and complex environments ($F_{1,38} = 4.020$, $p = 0.052$) (Figure 2-2d), and there was no interaction ($F_{2,38} = 3.020$, $p = 0.061$).

Amount of time spent cowering by subordinate fish decreased with increase in aquarium size ($F_{1,38} = 7.886$, $p = 0.008$) (Figure 2-3b) but not with complexity ($F_{1,38} = 1.931$, $p = 0.173$), although there was a strong interaction between the factors ($F_{2,38} =$

7.143, p = 0.002). Proportions of time spent escaping by subordinate fish were not associated with available space ($F_{1,38} = 0.823$, p = 0.370), although they were lower in complex environments than they were in simple environments ($F_{1,38} = 7.236$, p = 0.011), and there was a significant interaction ($F_{2,38} = 4.601$, p = 0.016). All 28 alpha fish in experiment II finished simple trials with 0 body damage. Of subordinates in the simple treatments, there was no significant difference in distribution between damage classes at different aquarium sizes (n = 28, V = 0.289, p = 0.674), although the pattern suggested a decrease in body damage in larger tanks (Figure 2-4b). A similar result was obtained for complex environments (n = 13, V = 0.037, p = 0.894). Subordinate fish did not receive less body damage in complex environments than in simple environments in 38 l (n = 16, V = 0.348, p = 0.164) or 110 l (n = 10, V = 0.105, p = 0.740) aquaria.

When the 380 l super-complex treatment (n = 6) was added to the experiment II data set as a third level of complexity, tank size still had no effect on number of aggressive bouts performed by alpha fish ($F_{1,43} = 3.333$, p = 0.075). Complexity retained an effect on number of aggressive bouts ($F_{2,43} = 15.393$, $p < 0.001$) and there was still an interaction ($F_{3,43} = 10.126$, $p < 0.001$). Proportion of time spent in aggression was still unaffected by tank size ($F_{1,43} = 0.511$, p = 0.478), although it became highly significantly affected by complexity ($F_{1,43} = 5.793$, p = 0.006) and there was an interaction ($F_{3,43} = 4.544$, p = 0.007). Only minor changes in significance occurred regarding behavior of subordinates after the addition of the 380 l super-complex treatment, none of which crossed or neared the p = 0.05 threshold. Body damage in the 380 l super-complex treatment was not different than it was in the 380 l simple treatment (n = 8, V = 0.234, p = 0.509).

When offered more space, energetic costs of aggression increased because more swimming was required to reach a competitor, so aggression was expected to decrease (possibly after an initial increase). Surprisingly, aggression of dominant fish failed to change with increases in aquarium size. Large aquarium size resulted in decreases in cowering but not escaping in subordinates. While escape behavior resulted directly from aggressive behavior performed by alpha fish, which also was not related to tank size, cowering seems indicative of subordinates' interpretations of the general level of danger presented by the dominant fish. Although there were no significant differences in body damage in subordinates, the patterns suggest less damage in larger tanks.

Perhaps the scale of variability in space available in experiment II was not sufficient to elicit a reduction in aggression. Increased inter-individual distance reduced aggression in physically isolated male *Betta splendens* (Bronstein 1981). In group-held *B. splendens*, physical space was inversely related to frequency of attacks, but not displays, indicating that subjects could sense intruders, but were not willing to expend energy to travel to attack them (Cain et al. 1980). Perhaps differences in aggression were not observed in experiment II because Midas cichlids are better swimmers that *B. splendens*. When juvenile Midas cichlids were kept in exceptionally large aquaria, Heijns (2004) observed that fish only discontinued chases after 1.8 – 2.4 m. In mammals, enclosure size should allow sufficient flight distance or pacing may arise (Koontz and Roush 1996).

When the data from experiment II were re-analyzed including the data from the 380 l super-complex treatment, not only was rate of aggression again associated with complexity, but proportion of time spent behaving aggressively also became highly affected by complexity. Aggression in other fishes has been found to be lower in complex

than in simple habitats (Basquill and Grant 1998, Sundbaum and Näslund 1998). Addition of 3-D structure may have provided visual barriers that reduced the rate of reception of sign stimuli (Bronstein 1983), caused smaller territories (Eason and Stamps 1992, Imre et al. 2002), or made territories more difficult to defend (Gray et al. 2000). However, in some replicates of the super-complex treatment fish swam all around the tank often in very close proximity to one another with almost no aggression. Fish sometimes even shared a cave. In the other treatments individuals seemed to maintain a maximum distance between themselves and aggressive conspecifics. The close inter-individual proximity sometimes tolerated suggests that in this environment alpha fish may not have been behaving territorially. Interestingly, vervet monkeys avoided visual contact and dispersed at greater distances under crowded conditions (McGuire et al. 1983). If high levels of aggression in captivity are a form of resource defense, then fish apparently cease defense when enough space or complexity is offered. In order to explore the hypothesis that aggression would eventually decline given enough space, behavior under experimental conditions was compared with behavior observed in nature, where much more space is available and density is much lower (see below).

ZOO EXHIBIT

Behavior under experimental conditions was compared to behavior observed in young individuals in the semi-natural environment of an artificial stream in a large walk-through rainforest exhibit at Toledo Zoological Gardens (Toledo, Ohio, U.S.A.). The stream was hard-bottomed, and did not contain substrate except a limited amount of fallen leaves, logs, and detritus. It was divided into four sections, the lowest of which (a pool 5.08 x

2.74 m x 0.33 m = 4500 l) housed the focal population of cichlids, as well as many *Gambusia affinis*, three *Astyanax* sp., three loricariid catfishes, two turtles (*Podocnemis unifilis*), and three ducks (*Anas versicolor* and *Callonetta leucophrys*). All the Midas cichlids in the exhibit were derived from 10 founders obtained from a commercial dealer (Beldt's Aquarium) in 2000, and therefore were probably genetically similar to the fish used in experiments I and II (Loiselle 1980). On 26 Dec 2002 the population in the pool contained one large breeding pair (male: 150 mm SL, female: 110 mm SL) guarding a brood of offspring (2 cm SL) in a 2 m^2 area, and three additional smaller pairs that each guarded much smaller areas (approximately 0.3 m^2 each) without offspring. This left over half of the total area of the pool for the remaining cichlids: a congregation of approximately 87 fish consisting mostly of juveniles, but ranging in size from approximately 40-120 mm SL due to the presence of seven larger sub-adults. The group moved about as a shoal but generally remained under an overhanging plant in an area of approximately 1 m^2. On two afternoons (28 December 2002 and 20 January 2003) focal individuals were chosen from the shoal and their behavior recorded for 5 minutes. The numbers of aggressive bouts they performed and received were recorded as was their behavior at 30 second intervals. On the first day four fish were observed and the second day 10 fish were observed.

Out of the 14 fish observed three were omitted from analysis due to large size and possible reproductive motivation. Of the remaining 11 fish, some were sub-adults, but although larger than the juveniles used in experiments I and II their behavior seemed to be consistent with that observed in their juvenile co-inhabitants. Approximate total length (TL) of the 11 fish ranged from 45 to 100 mm. Aggressive bouts were performed at a

mean rate of 0.22 per minute and received at 0.29 per minute. The higher rate of aggression received appeared to be due to aggression received from larger, mating individuals in the exhibit. Fish were usually either hovering in the water (69.1 % of the time) or swimming (26.4 % of the time). Other activities were each observed at only one or two time points; the percentage of total time devoted as follows: foraging 1.8 %, attacking 0.9 %, hiding 0.9 %, escaping 0.9 %.

The social structure of the non-breeding fish resembled the groups of subordinates in the 9-fish treatments in experiment I, and the 'dominated school' reported by Baerends and Baerends van Roon (1950). No dominant fish could be identified in the shoal. The levels of aggression were much lower than those observed in the experimental treatments. This observation seemed to indicate that there was a threshold number of competitors above which individuals would form shoals rather than defend territories (Pitcher 1986, Hoare et al. 2004).

LAKE APOYO, NICARAGUA

Behavior in experiments I and II and the zoo stream was also compared to behavior observed in *Amphilophus* cf. *citrinellus* in a natural environment, Lake Apoyo, Nicaragua, which has been reported previously (Oldfield et al. 2006, Chapter 4). In this lake, large juveniles and adults were found in deep water. Small juveniles (2.5 to 5 cm TL) were absent from deep water and bare sandy areas in shallow water, but were common in rocky habitats in shallow (1 m) water. In one rocky area they were observed and found to be solitary, but frequently came into contact with one another to form brief groups of 2-6 fish, when aggressive behavior was usually exchanged. Fish (n = 32)

performed 0.76 mean aggressive bouts per minute. Submission was observed 0.37 times per minute. The difference between aggression and submission was attributed to aggressive bouts that did not result in obvious submissive reactions. A mean time budget was calculated as in experiments I and II and the zoo exhibits. Fish (n = 29) spent most of their time swimming (33.7%), hovering (21.9%), foraging (20.0%), and hiding under rocks (17.6%). They engaged in attacks or displays 4.6% of the time and submissively responded to the aggression of other individuals 2.2% of the time.

When aggression rates were compared among the 38 l 3-fish simple treatment, the 380 l 3-fish super-complex environment, the artificial stream, and the Lake Apoyo habitat (Table 2-2), there was a significant difference (Kruskal-Wallis test: $\chi^2 = 19.501$, $df = 3$, $p < 0.001$). Proportion of time spent in aggression was different among the different environments (Kruskal-Wallis: $\chi^2 = 8.608$, $df = 3$, $p = 0.035$). Foraging was also significantly different (Kruskal-Wallis: $\chi^2 = 23.175$, $df = 3$, $p < 0.001$) as was proportion of time spent swimming (Kruskal-Wallis: $\chi^2 = 12.924$, $df = 3$, $p < 0.005$).

DISCUSSION

Elevated aggression in fishes when they are placed into small aquaria has been noted by previous authors. Okuno (1963) studied the behavior of 52 species of marine fishes in the ocean and at the Suma Aquarium of Kobe City, Japan in a large aquarium (359,100 l: 19 m x 7 m x 2.7 m deep) and in small aquaria similar in size to those employed in experiments I and II. Of the 52 species, 33 schooled in nature. Of these 33, 27 were found to be highly aggressive to conspecifics when housed in small tanks. Buchanan (1971) described Texas cichlids, *Herichthys cyanoguttatus*, in the San Marcos

River as shoaling and performing little aggression outside of the breeding season, but if two or more individuals were caught and placed into an aquarium they always attacked each other. Excessive aggression in captivity similarly occurs in isolated mating pairs (Rasa 1969). Differences in aggression have been observed between captive fish and wild fish as a result of either artificial selection or differences in previous experience (Keenleyside and Yamamoto 1962, Ward 1967, Fenderson and Carpenter 1971, Swain and Riddell 1990, Mesa 1991, Siikavuopio et al. 1996). However, the large differences observed here seem to be due to group size, available space, and habitat complexity.

With food and mates eliminated as possible resources in the current experiments, the fish apparently recognized space as a limited resource and defended it. Through his experiments at Vancouver Public Aquarium, Magnuson (1962) questioned the interpretation that aggression occurs over competition for space "...where space...does not include any specified limiting resources..." However, defense of space (territories) may allow greater avoidance of predators and provide differential access to future resources (Huntingford and Turner, 1987). Although freshwater fishes rarely possess feeding territories in nature (Barlow 1993, Grant 1997), absence of food may cause an increase in aggression in captivity (Barlow et al. 1975, Barlow and McKaye 1982, Blackenhorn 1992). In the current experiments, control over space may have resulted in preferential access to future food. There is an advantage for dominant fish in captivity; they grow faster than subordinates (e.g., Koebele 1985, Blackenhorn 1992, Fernandes and Volpato 1993, Ryer and Olla 1995, Fox et al. 1997, Hofmann and Fernald 2000).

Elevated aggression in small artificial environments could be a result of conditions that restrict natural behavior (see Kolter 1995). In Lake Apoyo, Midas cichlids

spent much of their time foraging, swimming, and avoiding detection by predators. These activities take time to perform and therefore leave little time for aggression. Order of behavior priority in Midas cichlids is likely: (1) avoiding predation, (2) foraging, (3) avoiding aggression, (4) distributing aggression. For a captive alpha fish, the first three of these are not necessary most of the time. The predominant stimuli are other fish, and their presence elicits aggression. In typical experimental treatments visual contact was continuous and may have provided a constant stimulus for aggression (Bronstein 1983).

ANIMAL WELFARE IMPLICATIONS

Koontz and Thomas (1989) suggested that behavior in zoo animals should be studied in order to improve their quality of life. It has been acknowledged that an understanding of natural behavior in fishes may aid in exhibit design (Spotte 1992), but no ethological research has yet been conducted on a fish in order to improve living conditions in zoos and public aquaria. The current research demonstrates that both aggression and activity budgets in captive fishes deserve more attention than they have historically received.

Housing fish in isolation is a common practice and may eliminate concerns regarding aggression, but it can cause problems. This practice may be appropriate for animals that are solitary in nature (Seidensticker and Doherty 1996), but some may exhibit stereotypic behavior and high glucocorticoid levels in isolation (Sachser and Beer 1995). Social housing may result in more species-typical behavior patterns (Schapiro et al. 1996), and an under-stimulating environment may affect mammals by increasing lethargy or causing self-stimulation in the form of appetitive or social behavior in unnatural contexts (Carlstead 1996). Pumpkinseed sunfish did not eat and lost weight

while isolated, but ate more and gained weight when put back into a group (Blackenhorn 1992). Isolated mammals may be hyper-aggressive when later placed into social groups (Brain 1972, Benus 1995, Carlstead 1996). Elevated aggression after isolation has been also been observed in fishes (Rasa 1971, Hinkel and Maier 1974, Coss and Globus 1979), as has decreased aggression (Gallagher et al. 1972, Fernö 1977). Private aquarists have reported that housing fish singly but near other aquaria so that they can communicate visually with other individuals alleviates problems associated with isolation, without the danger presented by cohabitation (e.g., Perrson 2003). In mammals, visual contact between separate cages may be beneficial (increased activity) or detrimental (lowered reproduction) depending on the species (Koontz and Roush 1996).

Although fish in the super-complex treatment and the zoo stream were not subjected to excessive aggression, their environments may not have been sufficiently rich to provide for healthy development. Mellen and MacPhee (2001) argued that a captive animal's environment should maximize variety and range of species-appropriate opportunities and that captive time budgets should match wild time budgets (although they acknowledged that not all natural elements are beneficial (see Dawkins 1998)). Environments that allow animals to behave naturally may also make them more visible and engaging to visitors (Myers 1978, Seidensticker and Doherty 1996). In both environments the cichlids spent less time foraging than did fish in Lake Apoyo. Fish in the zoo stream spent much more time hovering motionless than did wild fish. Although they swam nearly as often, they covered very little distance. Artificially high densities that restrict natural behavior are known to arrest neurological development (Burgess and Coss 1981, 1982).

Improving the conditions under which captive animals are held is often attempted through environmental enrichment. Mellen and MacPhee (2001) considered a critical element of effective environmental enrichment to be species-appropriate behavioral opportunities (choices in the environment). Environmental enrichment increases behavioral diversity and activity and reduces stereotypic behavior and feeding anticipation in mammals (Carlstead et al. 2004). Providing foraging opportunity is one of the most effective forms of enrichment (Carlstead 1996, Maple and Perkins 1996, Seidensticker and Doherty 1996, Mellen and MacPhee 2001), as is adding objects to an enclosure (Carlstead 1996). The purpose of typical enrichment objects such as those Schapiro et al. (1996) used with primates (balls, dog toys, perches, fleeces, food puzzles, mats, etc.), and furniture generally offered to mammals (nest boxes, loose browse, berms, ropes, manipulable objects, toys (Maple and Perkins 1996)), is to increase habitat complexity. Therefore the interpretations of the importance of habitat complexity and behavioral diversity made above for Midas cichlids are congruent with current thinking regarding environmental enrichment in mammals.

Midas cichlids are extremely common in the pet trade, and the data presented here raise ethical questions about their widespread availability. In the aquarium hobby this species is usually maintained in aquaria similar in size to those used in experiment II. Such aquaria have been shown here not to be suitable to house juveniles, save a 380 l aquarium filled with 3-D structure. Even if a private aquarist established such an aquarium to house juveniles, this species commonly grows to 20-30 cm SL, at which size the only remaining options are housing in isolation or an aquarium so large as to be impractical for the typical hobbyist. It is suspected that in the pet trade each year

thousands of cichlids are killed after being purchased by aquarists that lack an understanding of the excessive levels of aggression these fish can perform in captivity and how inappropriate small aquaria are for them. Perhaps zoos and public aquaria should play a role in educating the public in this matter (Marliave et al. 1995).

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Table 2-1. Midas cichlid behavior recorded at periodic intervals.

Behavior	Description
aggression	Attack (nip, chase, charge) or display (lateral display, operculum flare).
territoriality	Stationed near a structure or pit. Not randomly positioned, but usually facing outward near the entrance so that darting out or in is immediately possible.
foraging	Sifting particles from the gravel or engulfing and chewing floating particles.
cowering	Maximum distance possible between subject and dominant fish. Subject usually near water surface (Baerends and Baerends-van Roon, 1950), in a corner, or in plants (if present). Displaying any two of the following: accelerated beating of pectoral fins, accelerated respiration, in contact with wall of aquarium, near water surface, body held obliquely.
escaping	Moving away from another individual in response to its movement, display, or attack.
resting	Resting on substrate. Coloration often dark and fins not moving.
hovering	Motionless in water column.
hiding	Body positioned behind object or against gravel slope, inconspicuous to observer. Little or no fin movement, and may have body pressed against object.
swimming	Locomotion through water.
digging	Moving gravel with mouth, resulting in a pit in which the fish resides.

Table 2-2. Water volume, density, and selected behavior patterns (mean±SE) in Midas cichlids in different habitats.

Habitat	Volume available (l)	Density (# fish/m^3)	Rate of aggression (#/min.)	% Time spent in aggression	% Time spent hovering	% Time spent foraging	% Time spent swimming
Lake Apoyo	∞	1.5±0.1	0.8±0.2	4.6±1.7	21.9±3.6	20.0±3.2	33.7±3.6
super-complex	380	7.9	0.9±0.2	3.3±1.2	not recorded	0	17.8±6.9
38 l 3-fish simple	38	79.0	2.6 ±0.5	9.2±3.4	37.9±5.1	6.3±2.3	10.0±2.1
Zoo stream	2250	42.2	0.2±0.1	0.9±0.9	69.1±5.8	1.8±1.2	26.4±5.8

Figure 2-1. Super-complex environment erected in 380 l aquarium (top view).

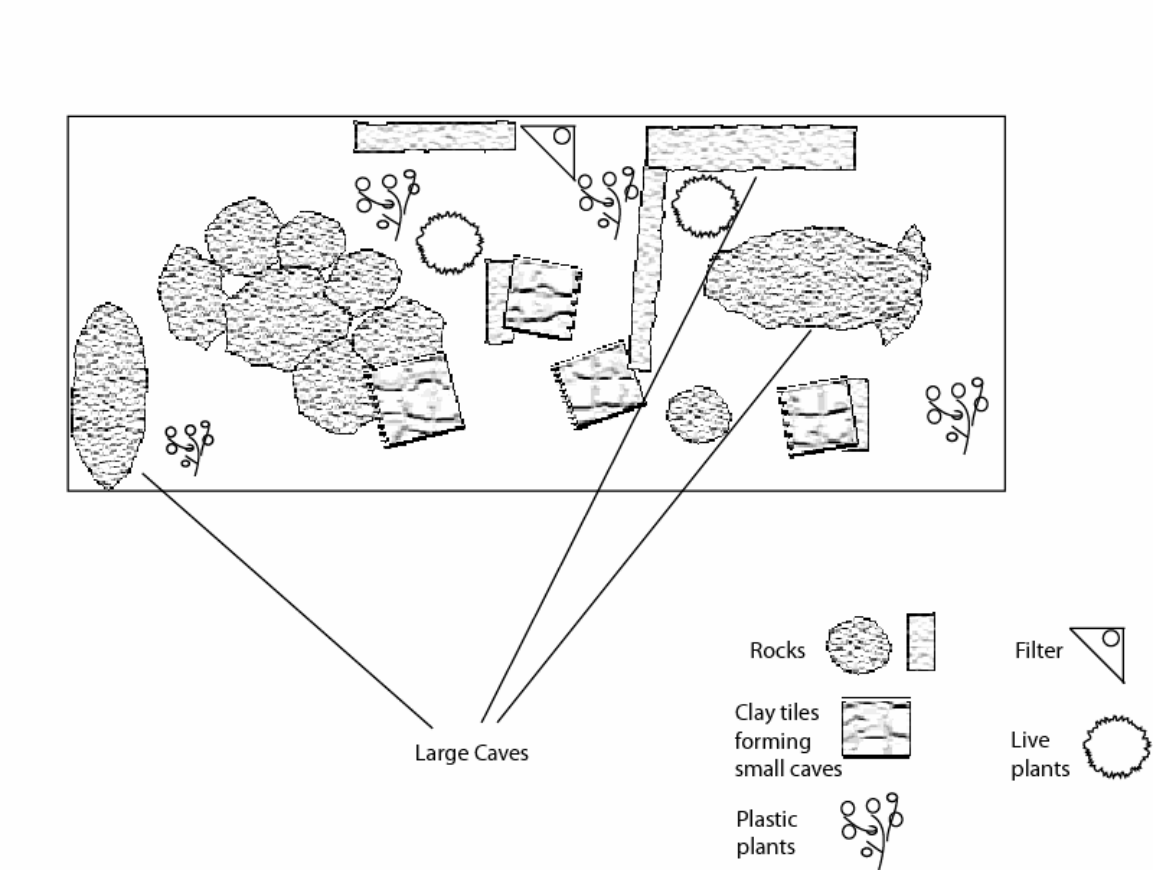
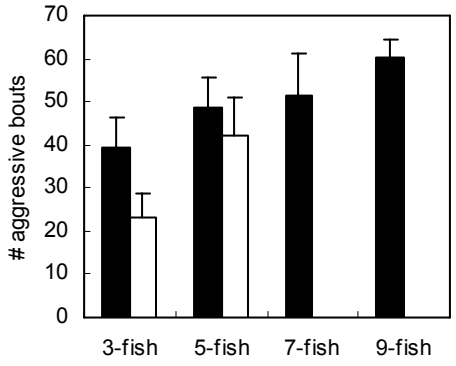
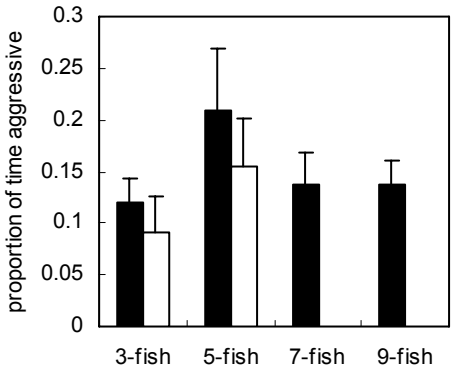


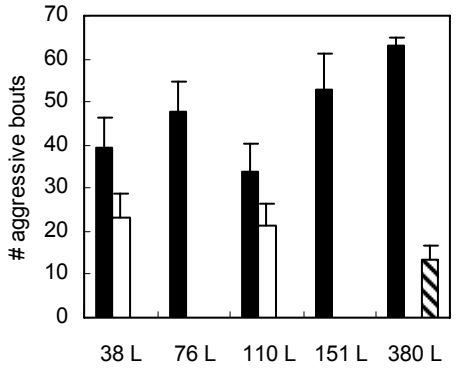
Figure 2-2. Mean numbers of aggressive bouts performed and proportion of time spent behaving aggressively in three 5-minute observation periods by alpha fish at different group sizes in a 38 l tank (a and b), and at different tank sizes when held in a 3-fish group (c and d) in simple (black bars), complex (white bars), and super-complex (hashed bars) environments. Bars indicate SE. Statistical significance discussed in text.



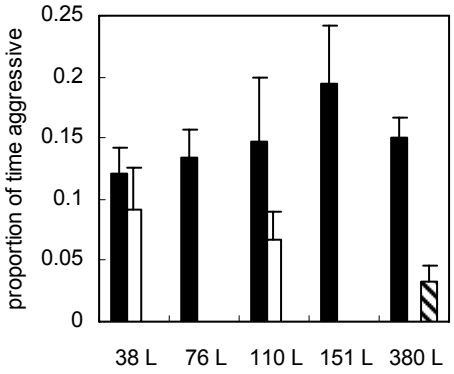
a.



b.

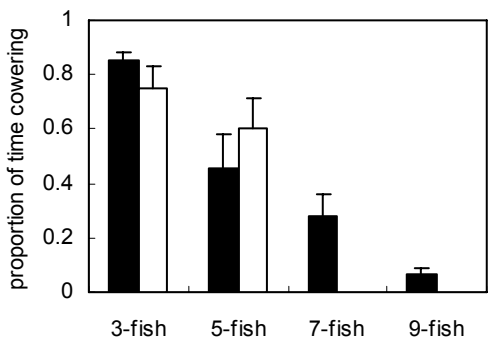


c.

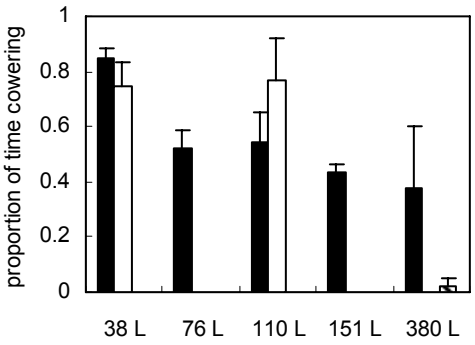


d.

Figure 2-3. Proportion of time subordinate fish spent cowering at different (a) group and (b) tank sizes in simple and complex environments (bars as in Figure 2-2).

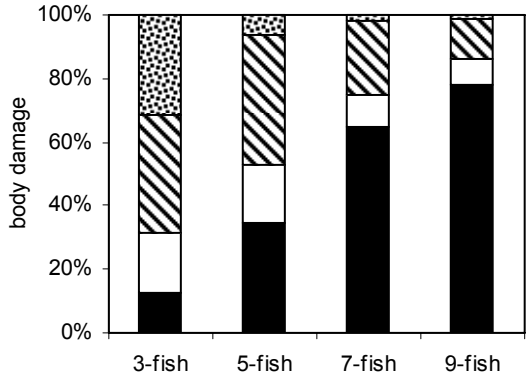


a.

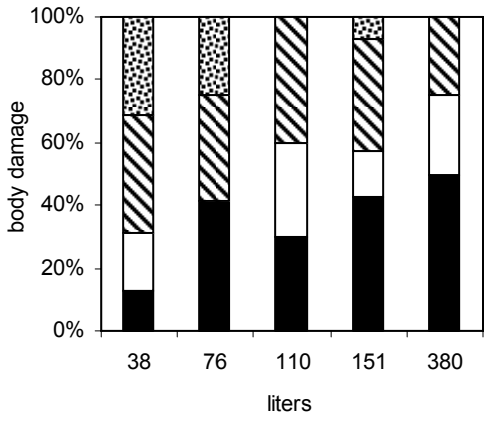


b.

Figure 2-4. Distribution of subordinate fish among categories according to amount of body damage received in simple environments at different (a) group and (b) tank sizes. Bar patterns represent categories of increasing damage: black – 0, white – 1, hatched – 2, stippled – 3 (see text for category explanations).



a.



b.

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CHAPTER 3

THE EFFECT OF BEHAVIORAL INTERACTION ON SEX DETERMINATION IN THE MIDAS CICHLID[2]

ABSTRACT

Social control of sex determination has been reported in juvenile Midas cichlids, *Amphilophus citrinellus*, and was thought to be a heterochronic variant of functional sex change at the adult stage, as observed in some marine fishes. Large body size relative to group-mates was interpreted to cause male differentiation. To test the hypothesis that relative body size and behavioral interactions affect sex determination, one brood of juvenile Midas cichlids was divided into 13 experimental groups. Fish were predicted to differentiate as males if they were larger than most of their group-mates, and if they performed more aggressive behavior than they received. Several individuals were isolated and predicted to differentiate as females due to absence of conspecific stimulus hypothesized to be necessary to induce male differentiation. Neither relative body size nor behavioral interactions were found to affect sex determination. These results raise doubts concerning the reports that originally claimed that sex is socially determined in Midas cichlids. Possible explanations for the inconsistency with these reports are discussed.

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INTRODUCTION

In most fishes sex is determined by genetic factors, but environmental factors can influence sex determination in a large number of species (Devlin and Nagahama 2002). Most sexually labile species are marine, but there are reports of cichlid species that exhibit different expressions of lability at each of four life stages (reviewed by Oldfield 2005). There are species that differentiate directly as one sex or the other and are apparently not labile (e.g., Meijide et al. 2005). At the larval stage some cichlids are labile and have sex determined by abiotic factors like temperature or pH (e.g., Rubin 1985, Römer and Beisenherz 1996). Others apparently undergo a transient female stage as larva, indicated by the presence of oocytes in the testes of adult males (e.g., Peters 1975, Loir et al. 1989). The Midas cichlid, *Amphilophus citrinellus* (Günther, 1864), has been reported to have sex determined at the juvenile stage by social conditions (Francis and Barlow 1993), and one cichlid, *Crenicara punctulata*, is thought to be a sequential hermaphrodite that changes sex at the adult stage (Carruth 2000). This pattern suggests a continuum in the timing of gonad lability, and that adult sex change may evolve through a change in developmental timing (Atz 1964, Shapiro 1987, Francis 1992).

Socially controlled sex determination at the juvenile stage has been proposed for only a small number of fishes. It was first suspected in the convict cichlid, *Archocentrus nigrofasciatus*, but was not conclusively demonstrated (Williams 1972). Francis (1984) reported an association between stocking density and sex ratio in the paradise fish, *Macropodus opercularis*, and interpreted it as social control of sex determination. Social control of sex determination can also occur in some typically sequentially hermaphroditic marine fishes (Bruslé-Sicard et al. 1994, Liu and Sadovy 2004, Munday et al. 2006).

A series of investigations led Francis and Barlow (1993) to conclude that sex is determined by social factors in juvenile Midas cichlids. Adult Midas cichlid males are larger than females (Barlow 1976), and larger individuals aggressively dominate smaller ones (Barlow 1983). Francis (1988) demonstrated that there are no inherent differences in aggression between relatively small and large fish within a group and that individuals obtain their size ranks by chance early in ontogeny. Next he followed a group of 12 marked juveniles to adulthood. During this period individuals maintained their size ranks and at the end the fish were sexed and the smallest male was larger than the largest female (Francis 1990), suggesting that whichever fish were initially larger developed as males and the smaller fish as females. Francis and Barlow (1993) then divided a brood of 74 juveniles into two groups based on size, one containing the smallest 37 fish and the other the largest 37. Once free from the aggressive dominance of the larger fish, individuals in the group of smaller fish experienced growth compensation (reviewed by Ali et al. 2003), and after 6 months they were as large as the individuals in the group of initially larger fish. The relatively larger fish in each of these new social groups were males and the smaller fish were females. Francis and Barlow (1993) concluded that the same aggressive interactions that controlled growth also controlled sex determination, in a manner similar to how aggressive behavior controls sex change in sequentially hermaphroditic marine fishes.

Francis and Barlow (1993) have been cited 25 times (e.g., Helfman et al. 1997, Baroiller et al. 1999, Baroiller and D'Cotta 2001, Devlin and Nagahama 2002, Godwin et al. 2003, Liu and Sadovy 2004, Oliveira 2006) although their experiments remain unreplicated. Oldfield et al. (2006) found no association between sex and body size in

wild juvenile Midas cichlids – a pattern inconsistent with Francis and Barlow (1993).

The current experiment was performed in order to identify aspects of aggressive behavior that affect sex in this species. The first expectation was that the results would corroborate those of Francis (1990) and Francis and Barlow (1993) in that males would be larger than females within each experimental group. Because threshold levels of encounters result in sex change in some sequentially hermaphroditic marine fishes (Shapiro 1979, Ross 1990, Lutnesky 1994), it was hypothesized that Midas cichlids that initiated relatively large numbers of aggressive bouts would differentiate as males more often than would those that behaved aggressively less often. A separate hypothesis predicted that fish that received large numbers of aggressive bouts would differentiate as females, and those that received fewer would differentiate as males. To test the hypothesis that Midas cichlids interpret numbers of encounters in which they are dominant relative to numbers in which they are submissive, these values were compared for each individual. Fish that won more encounters than they lost were predicted to differentiate as males, and fish that lost more often were predicted to differentiate as females. Female is thought to be the default sex (Shapiro 1992, Francis and Barlow 1993), so it was also hypothesized that the presence of smaller conspecifics would be required for male differentiation and that fish raised in isolation would differentiate as females.

MATERIALS AND METHODS

One group of 95 juvenile Midas cichlids spawned from a single pair of adults was purchased from a tropical fish dealer and placed into two large glass holding aquaria (147

l: 46 x 90 x 36 cm). The brood remained in the holding aquaria for approximately 4 months, until they reached a size similar to that used by Francis and Barlow (1993). On 4 June 2002 the median SL (standard length) of the brood was 51.5 mm (compared to 52.2 mm in Francis and Barlow 1993). The following day (day 1) the largest individual fish and the smallest three fish were set aside, raised to maturity, and allowed to produce offspring for later use (see below). The size distribution was then used to divide the remaining fish into 13 groups each containing seven fish of similar size (groups 1-13, with higher group numbers representing groups that contained larger individuals). For individual identification these fish were anesthetized with tricaine methanesulfonate (MS-222) and freeze branded with silver wire super-cooled with dry ice (frozen carbon dioxide). One fish from each group was then randomly selected and placed in isolation. Group-held fish were branded again on day 49, and pelvic fin tissue was clipped on day 61.

Each group of six fish was placed into a 76 l (61 x 31 x 42 cm) aquarium. Each of these tanks was equipped with an air-powered sponge filter, a clay pot, one stone (8 cm x 10 cm), one bunch of Java moss, *Vesicularia dubyana* (8 cm x 10 cm), two clay tiles (15 cm^2) leaned obliquely against the end glass panels, and 5 cm of natural gravel substrate. Later addition of PVC pipe curtailed aggression-induced mortalities. Fish were fed ad libitum once per day on commercial dry food supplemented with commercial frozen brine shrimp, *Artemia* sp., once per week. Natural sunlight penetrated opaque skylights and was supplemented with fluorescent lighting set on a 12:12 hour light:dark schedule. Water temperature was maintained at approximately 27º C and pH was 7.6 +/- 0.2, conditions similar to the fish's native habitat (Meral 1973, Cole 1976). Water quality was

maintained by vacuuming the gravel and regularly replacing part of the water with aged tap water.

Each group was observed using continuous recording (Martin and Bateson 1993) for five 5-minute observation periods randomly distributed between the time of fin clipping and the end of the experiment, beginning on day 65 and ending on day 126. Observations were conducted between 1500 h and 1910 h, after waiting a minimum of 95 minutes after feeding. Behavior sampling (Martin and Bateson 1993) was used to record the performer and receiver of all bouts of aggressive behavior, defined as any deviation from an individual's previous action or trajectory with redirection toward another individual in an apparent effort to displace it, including both displays and attacks. Displays were relatively infrequent, but if a fish received either form of aggression it usually resulted in submission. Submission was defined as a change in position or posture on the part of the receiving fish apparently to avoid physical contact with the aggressive fish. In addition, time budgets were interpreted by recording the behavior of each individual at 30-second intervals. Time budget behavior analyzed included aggression, as described above, territoriality, which was characterized by digging a pit in the gravel or guarding the entrance to the pot, and subordinate behavior, characterized by cowering near the surface or hiding in the moss or a PVC tube (Baerends and Baerends-van Roon 1950). Fish held in groups were removed from their tanks and killed with MS-222 on day 126. All fish were sexed externally by examination of the genital papillae (Barlow 1976). Sex was later confirmed by gross examination of the gonads.

The 13 isolated specimens were each housed in a 38 l (51 x 26 x 31 cm) aquarium equipped in a manner similar to the other tanks. Cardboard dividers prevented visual

communication among isolated fish. Isolated fish were killed on day 138. To increase the sample size of isolated fish this portion of the experiment was repeated with additional fish produced in the lab. The four individuals mentioned above produced offspring that hatched on 19 May 2003. Fry were removed and maintained in a 147 l aquarium (46 x 90 x 36 cm) until 182 days old, when 18 were removed, weighed (mean, 5.7 ± SD 3.1 g), measured (mean, 52.3 ± SD 10.2 mm SL), and placed individually into aquaria set up similarly to those used for the initial 13 isolates. The 18 fish were killed when 423 days old. All 31 isolated fish were sexed in the manner described above for group-held fish.

Data analysis

Kolmogorov-Smirnov analysis was used to compare the number of males at each size rank in order to test the hypothesis that sex was associated with relative size in a social group. Kolmogorov-Smirnov analysis was used a second time to compare the number of males across groups to determine if relative size within the original brood was related to sex. Body mass was compared between all females and all males across all groups with a two-tailed Mann-Whitney U test in order to determine if males were generally larger than females.

To determine if aggression was associated with body size, numbers of aggressive bouts performed and received were compared across size ranks with Kruskal-Wallis tests. Aggressive behavior, submissive avoidance, and territoriality were similarly compared across ranks as proportions of time budgets. Dominance relationships were analyzed and hierarchies evaluated for linearity. Dominance was considered to be a relationship between two individuals. This relationship was assessed for each dyad within every group by comparing the number of times each fish displaced the other. Hierarchies were

constructed from these dyadic relationships. Similarities between size hierarchies and dominance hierarchies were tested with Spearman rank correlation analyses.

Two-tailed Mann-Whitney tests were used to detect differences between fish that differentiated as males and those that differentiated as females in number of aggressive bouts performed, number received, number received from the alpha fish, number received from the alpha fish when the alpha fish was female, and number received from the alpha fish when it was male. In addition, a dominance index (D) was calculated for each fish:

$$D = (\#\text{ attacks and displays performed}) - (\#\text{ received})$$

The numbers of winners ($D > 0$) that differentiated as males and as females were compared with the numbers of losers ($D < 0$) that differentiated as males and as females with a chi-square test.

The number of the total 31 isolated fish that differentiated as females was compared to the sex ratio observed in the group-held fish and to the number expected given the typical 1:1 sex ratio observed in nature (Barlow 1976).

All data analyses were performed either by hand or with Microsoft Excel or SPSS software according to Zar (1999). Some fish died during the experiment, so most analyses included fewer than 13 groups.

RESULTS

After separation from larger fish in the original brood, dominant individuals in each experimental group began to exhibit growth compensation, although they never reached the size of the largest fish in group 13 (Figure 3-1). Neither grouped fish nor isolates had external sexual characteristics by day 49 or day 55, but by the time they were killed

almost all had genital papillae that were identifiable as either female or male. Gonads were well developed in all individuals except one. Near the end of the experiment, two fish in group 13 had begun to exhibit reproductive behavior, which was absent in all other groups.

The null hypothesis that sex was independent of size rank within each group could not be rejected (n = 30 males, k = 6, $d_{max} = 2$, $p > 0.50$, Figure 3-2), indicating that relative size within each group did not affect sex determination. Sex was not differentially distributed among the experimental groups (n = 30 males, k = 10, $d_{max} = 3$, $p > 0.50$, Figure 3-3), indicating that size rank in the original brood did not affect sex determination. However, males (n = 30, 44.85 ± 24.14 g) were generally larger than females (n = 30, 31.78 ± 13.27 g, U = 302.0, p = 0.029).

Larger fish performed more aggressive bouts ($\chi^2 = 27.5$, df = 5, $p < 0.001$) and received fewer ($\chi^2 = 25.0$, df = 5, $p < 0.001$) than smaller fish (Figure 3-4). Larger individuals spent more time behaving aggressively ($\chi^2 = 29.1$, df = 5, $p < 0.001$) and territorially ($\chi^2 = 25.4$, df = 5, $p < 0.001$) and less time behaving in a subordinate manner ($\chi^2 = 22.2$, df = 5, $p < 0.001$) than smaller fish (Figure 3-5). (N = 9 for each rank except rank 6, where n = 8.)

Behavior data in many of the groups indicated linear dominance hierarchies. Of the 10 groups with complete data sets, groups 2, 3, 7, 11, 12, and 13 exhibited differential numbers of aggressive bouts between members of each pair of individuals, implying dominance relationships and hierarchies, although in one dyad the difference was small (four vs. five aggressive bouts). Interaction data were lacking between some individuals in groups 1, 8, and 10, resulting in tied ranks. In group 6, there were no interactions

recorded between two specific individuals but differential relationships with other group members implied difference in rank. Dominance hierarchies were significantly correlated with size hierarchies in five of the 10 groups and the two measures were identical in two of the 10 (Table 3-1).

No significant difference in number of aggressive bouts performed was found between fish that differentiated as males (n = 24, mean = 19.46) and those that differentiated as females (n = 29, mean = 14.28, U = 285.5, p = 0.262). Similarly, there was no significant difference in number of aggressive bouts received between males (n = 24, mean = 18.13) and females (n = 29, mean = 15.17, U = 310.5, p = 0.501). When considering only the number of aggressive bouts that subordinates received from alpha fish, females (n = 26, mean = 7.04) received fewer than males (n = 18, mean = 13.06, U = 131.0, p = 0.014). When the alpha was female, other females received fewer aggressive bouts (n = 7, mean = 4.29) from the alpha fish than did males (n = 8, mean = 13.63, U = 7.5, p = 0.017). There was no difference between males (n = 10, mean =12.30) and females (n = 19, mean = 8.21) in number of aggressive bouts received by the alpha fish when it was male (U = 57.5, p = 0.085). Fish that won most of their encounters (D > 0) did not differentiate as males (9 of 22) more often than those that lost (D < 0, 15 of 31) most of their encounters ($\chi^2 = 0.289$, df = 1, p > 0.50).

The 13 fish initially isolated were identified as nine females and four males. One of the 18 fish in the second group of isolates had gonads that were not distinguishable as either male or female so a total of 30 isolated fish were included in the analysis. Fifteen out of 30 differentiated as females. Sizes of isolated fish were not consistently similar to the sizes of fish at any one particular rank from the experimental groups from which they

were taken (Table 3-1).

DISCUSSION

Francis and Barlow (1993) suspected that social interactions affected sex determination in the same way they affected growth. In the current study social factors affected growth but not sex determination. Dominant individuals in each of the 12 smallest experimental groups underwent growth compensation after separation from the larger fish in their initial brood, although they never reached the size of the largest fish in Group 13. Fish that remained subordinate to other members of their experimental groups failed to experience compensatory growth (Koebele 1985, Fernandes and Volpato 1993, Fox et al. 1997, Hofmann and Fernald 2000).

According to Francis and Barlow (1993), *A. citrinellus* differentiate as males if they are larger, and females if they are smaller, than most conspecifics they encounter at the juvenile stage. It was therefore expected that the largest three fish in each of the 13 groups would differentiate as males and the smallest three as females. However, sex was independent of size rank within groups. This absence of association could have arisen if the critical period of sexual lability terminated before the fish were divided into experimental groups. However, if sex determination was influenced by relative size within the original brood then sex would be biased toward males in higher numbered groups and toward females in lower numbered groups, which was not the case.

It was further expected that aggressiveness would be associated with sex. Although larger members of a social group were more aggressive than smaller members in terms of both numbers of bouts and time spent, dominance hierarchies were not always

congruent with size hierarchies so the possibility remained that some aspect of aggressive behavior might be associated with sex. Aggressive behavior was not associated with sex when considered in terms of absolute values or as a dominance index that also considered aggression received from other fish. Only when restricting consideration to aggression received from an alpha fish was it received differently between males and females. Because this difference occurred only when the alpha fish was female it is unlikely that aggression could function in sex determination.

To control for the effects of social conditions, sex determination was analyzed in isolated fish. Female is thought to be the default sex in most fish species (Shapiro 1992), so isolated fish were expected to differentiate as females in the absence of social stimulus that was hypothesized to be necessary to induce male differentiation. Fifteen of the 30 isolated fish that were examined developed as females. It is remarkable that an exact 1:1 sex ratio was observed in both the 30 isolated fish and the 60 group-held fish (those from groups in which data were obtained for all members). A 1:1 sex ratio is seen under natural conditions (Barlow 1976). Isolation did not affect sex determination. The sizes of the isolated fish were not consistently similar to any particular size rank in group-held fish (Table 3-1), suggesting that there was no association between sex determination and growth in isolates.

There are three possible explanations for the absence of social control of sex determination in this experiment. There may be variation in sexual lability among Midas cichlid lineages. Recent research has found that the Midas cichlid may actually represent a species complex consisting of as many as 15 to 30 species in eight crater lakes in Nicaragua (McKaye et al. 2002). Unlike the fish used by Francis and Barlow (1993), the

animals used in the current experiment may not have had the genetic components that allow sex to be influenced by behavior. Baroiller et al. (1995) found a high level of variability in susceptibility to temperature controlled sex determination among broods from different parents in the cichlid *Oreochromis niloticus*.

A second possibility involves the formation of small groups, which may have elicited a social structure different from that elicited by the large (37 fish) groups created by Francis and Barlow (1993). In small captive groups fish often behave territorially, but in large groups they may form shoals (Grant 1997). This change in behavior with density also occurs in the Midas cichlid (Chapter 2). Territorial behavior may have somehow 'short-circuited' social control of sex determination.

The third possible explanation is that sex is determined not by social factors, but by genes, as it is in the closely related convict cichlid, *Archocentrus nigrofasciatus* (George and Pandian 1996). Under genetic sex determination, larger body size in adult males could be attained by faster growth than in females. In fact, this is the pattern observed in the closely related Mayan cichlid, 'Cichlasoma' *urophthalmus*. In this species there is no difference in size between the sexes at one year of age, but with each subsequent year males become increasingly larger than females (Faunce et al. 2002). This pattern is also consistent with the lack of association between sex and body size observed in social groups of juvenile Midas cichlids sampled from nature (Oldfield et. al. 2006). Francis and Barlow (1993) did not consider this possibility because Francis (1990) had observed a stable size hierarchy in a single captive group of 12 fish. This was a small sample upon which to generalize that size ranks in other groups would be stable as well. In the current experiment fish were killed earlier than were those used by Francis and

Barlow (1993). They were at the onset of maturity and showed no association between maleness and large size within or across groups. However, males were generally larger than females. Perhaps a differentially faster growth rate in males was just beginning to be expressed. This may account for the lack of correlation between size hierarchies and dominance hierarchies in five of the 10 groups analyzed. The current results suggest that sex in the Midas cichlid is not determined by social factors as claimed by Francis and Barlow (1993).

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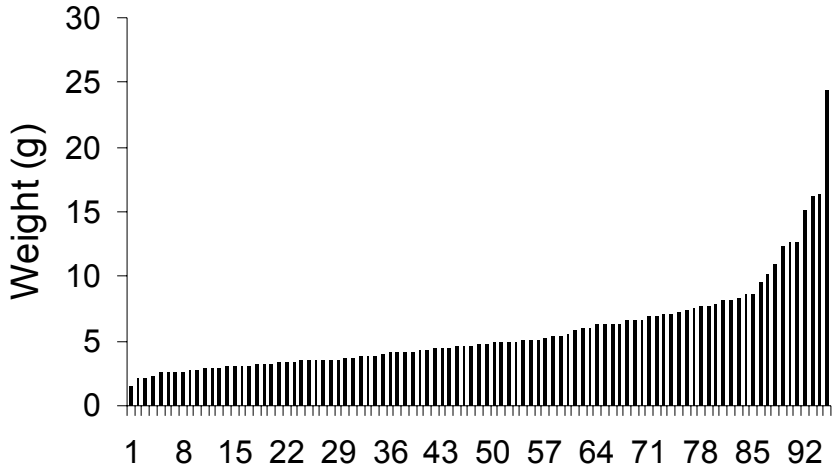
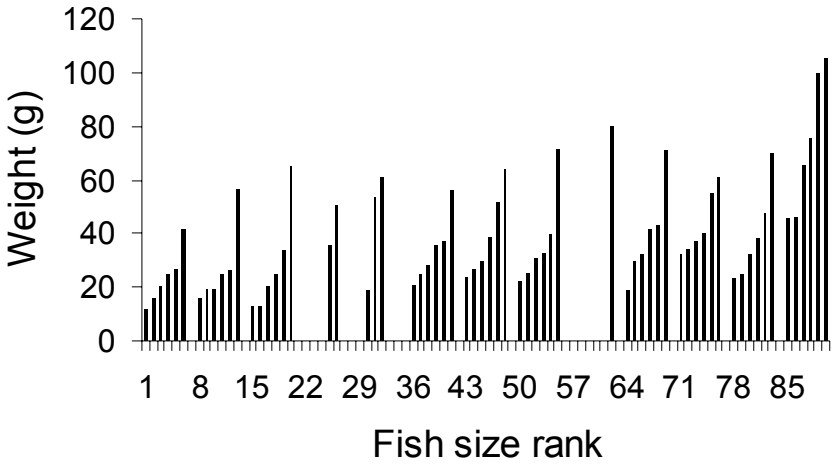
Table 3-1. The size (mass) and dominance hierarchies within 10 groups and their correlations as determined by Spearman rank analyses. Bold font indicates males. Large/dominant fish are assigned lower ranks. Final size of fish placed in isolation (I) relative to the ranks of fish from which group they came is indicated in the bottom row.

Size	Dominance Rank									
Rank	Grp. 1	Grp. 2	Grp. 3	Grp. 6	Grp. 7	Grp. 8	Grp.10	Grp.11	Grp.12	Grp.13
1	1	**1**	**1**	1	**1**	**1**	**2.5**	1	**1**	**1**
2	2.5	2	2	**4**	**2**	2	2.5	**3**	2	**2**
3	2.5	3	3	**2**	**3**	5.5	1	5	**3**	**3**
4	5	4	6	**5**	**4**	3	**4**	2	**4**	4
5	**5**	**5**	**5**	**3**	**5**	4	**5.5**	**6**	6	**6**
6	5	6	**4**	**6**	6	5.5	5.5	4	5	**5**
r =	0.93	1.00	0.77	0.71	1.00	0.75	0.79	0.60	0.94	0.94
p	<0.05	<0.005	>0.10	>0.10	<0.025	>0.10	>0.10	>0.20	=0.02	=0.02
Isolate:	1<I<2	I = 2	3<**I**<4	4<I<5	3<I<4	1<I<2	1<I<2	2<I<3	1<I<2	4<I<5

Figure 3-1. Size distribution of one brood of Midas cichlids (a) at the beginning and (b) at the conclusion of the experiment, after being divided into 13 groups of like-sized individuals (isolated and deceased individuals omitted from b).



a.



b.

Figure 3-2. Sex ratio for each size rank across the 10 groups (n = 10 for each rank) in which data were obtained from all six fish (rank 1 = largest fish in group, rank 6 = smallest). Sex was not associated with rank ($p > 0.50$). If sex were determined by relative size then ranks 1-3 would be all male and 4-6 all female.

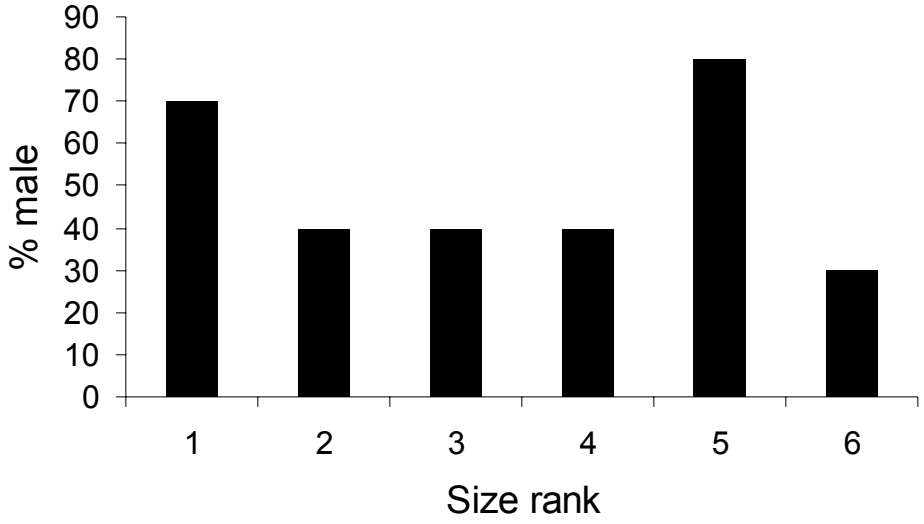


Figure 3-3. Sex ratio within each of the 10 groups (group 1 = smallest fish in original brood, group 13 = largest) in which data were obtained from all six fish. N = 6 for each group. If social control of sex determination occurred before the formation of the experimental groups, groups containing the larger fish (groups 8-13) would be expected to be all males, and groups of smaller fish (groups 1-6), all females. Instead, sex was not associated with group ($p > 0.50$).

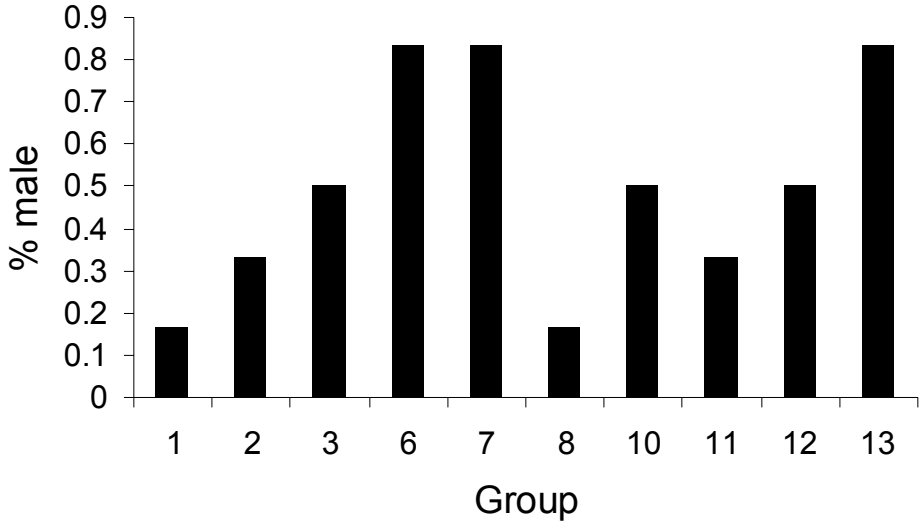


Figure 3-4. Mean number of aggressive bouts performed (white bars, $p < 0.001$) and received (black bars, $p < 0.001$) by size rank. Lower rank numbers represent larger fish. N = 9 for each rank except rank 6, where n = 8. Bars represent standard error.

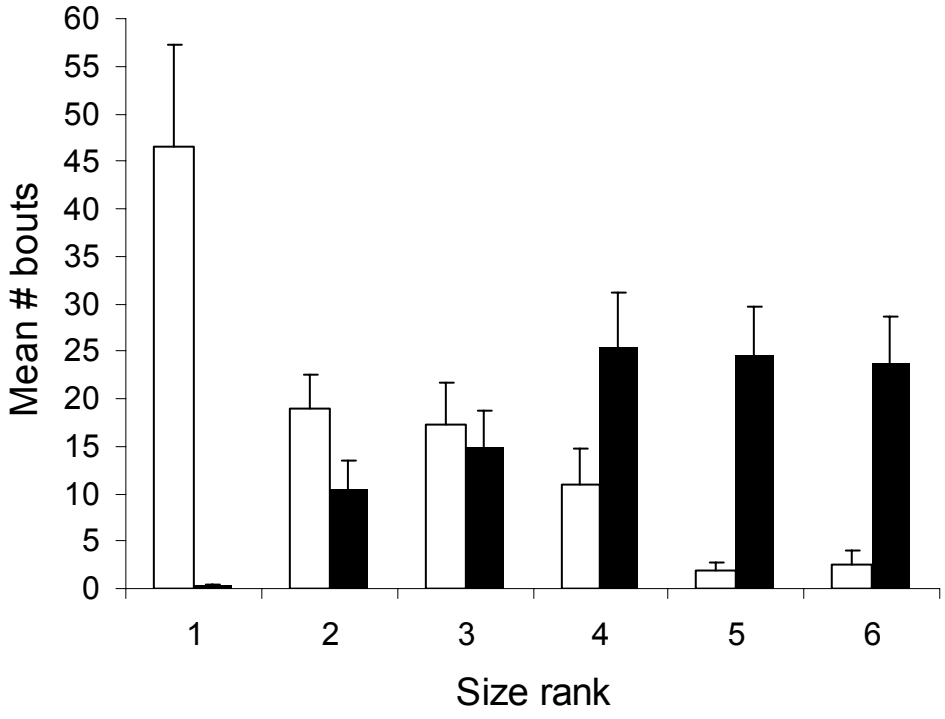
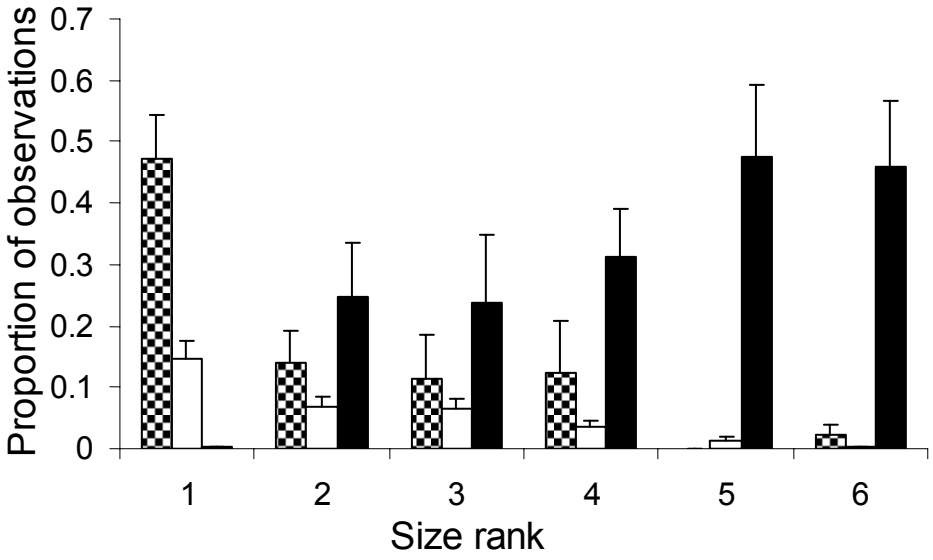


Figure 3-5. Proportions of 50 total observations spent performing various types of behavior for fish of different size ranks across nine groups (n-values as in Figure 3-4). Checkered bars represent territorial behavior, including guarding a pot or digging a pit in the gravel ($p < 0.001$). White bars represent aggressive behavior, including attacking or displaying ($p < 0.001$). Black bars represent behavior typical of subordinate fish including cowering near the surface, hiding in plants, or hiding in a PVC tube ($p < 0.001$). Bars represent standard error.



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CHAPTER 4

HABITAT USE, SOCIAL BEHAVIOR, AND FEMALE AND MALE SIZE DISTRIBUTIONS OF JUVENILE MIDAS CICHLIDS, *AMPHILOPHUS* CF. *CITRINELLUS*, IN LAKE APOYO, NICARAGUA[3]

ABSTRACT

The Midas cichlid complex contains many closely related species throughout Nicaragua. One of the species from Lake Masaya, *Amphilophus* cf. *citrinellus*, has been previously described to undergo socially controlled sex determination. A field study of the Midas cichlid was conducted in Lake Apoyo, a clear crater lake near Lake Masaya. I used visual observations to quantify habitat use of Midas cichlids and other fishes at three shallow water sites. Behavior was recorded and aggression rates and time budgets were calculated for juvenile Midas cichlids. Three predictions were tested that would be consistent with socially controlled sex determination: (1) Aggressive interactions potentially capable of influencing direction of sex differentiation would be common, (2) juveniles would be expected to be socially assorted by age/body size, and (3) males would be larger than females in individual social groups. Small juvenile Midas cichlids were present at one of the shallow water sites, which was characterized by large boulders and complex three-dimensional structure. These juveniles were solitary, but temporarily formed small groups in which aggressive interactions occurred. Midas cichlids were socially assorted by body size. Two shoals of larger juveniles were captured in deeper water and their gonads analyzed histologically. In one group, some of the fish were sexually

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undifferentiated. Sex was not associated with body size in either group. The results were not consistent with a hypothesis of socially controlled sex determination. Greater post-maturational growth in males is an alternative process that could result in the sexual size dimorphism found in adults of this species.

INTRODUCTION

Cichlid nomenclature in Central America is in a state of change. Stauffer and McKaye (2002) discussed the nomenclatural problems surrounding the Midas cichlid, *Amphilophus citrinellus*, complex in Nicaragua. Stauffer et al. (1995) have been cautious in assigning specific status to the many newly discovered forms and instead have referred to various taxa as evolutionarily significant units. Nevertheless, the Midas cichlid taxa in the various crater lakes are most likely different species (McKaye et al. 2002). There is a rich literature by George Barlow and his students concerning the behavior of the Midas cichlid (Barlow 2000). The majority of Barlow's work focused on Midas cichlids from Lake Masaya, Nicaragua.

Francis and Barlow (1993) reported evidence for social control of sex determination at the juvenile stage in the Midas cichlid, and their study posed interesting questions concerning the evolution of sequential hermaphroditism in fishes. They proposed that the social control of sex determination observed in this species is a heterochronic variant of the behaviorally controlled sex change observed in sequentially hermaphroditic marine fishes. Variation in timing of the sexually labile period has suggested that changes in developmental timing may be involved in the evolution of sequential hermaphroditism (Shapiro 1987, Francis 1992). Cichlids are the only group of

fishes known to exhibit sexual lability at three distinct life stages (Oldfield 2005, Chapter 1).

Growth can be controlled socially in fishes (Brown 1957), especially in cichlids (Koebele 1985, Fernandes and Volpato 1993, Hofmann and Fernald 2000). In the Midas cichlid, differences in body size occur randomly soon after hatching. Larger fish are not intrinsically more aggressive than smaller ones, but once they have a size advantage their aggressive dominance may suppress growth in smaller members of their social group (Francis 1988). Francis (1990) found a stable size hierarchy in a captive group of 12 individuals from the early juvenile (10 weeks) to adult stage (18 months). He proposed that the social interactions that control growth also control direction of sex differentiation.

Because Midas cichlid males are generally larger than females in adult groups, it was hypothesized that juveniles that are small relative to other group members differentiate as females, and those that are relatively large as males. Under laboratory conditions, Francis and Barlow (1993) divided a brood of juveniles into two groups based on body size. If sex were determined only by genetic factors, then all of the fish in the 'large' group would mature as males, and all of the fish in the 'small' group as females. Those in the 'small' group experienced growth compensation (see Ali et al. 2003), and became as large as the fish in the 'large' group at maturity, and both groups had similar male/female proportions and gendered size rankings, with males larger than females. The authors concluded that the fish assessed their size via aggressive interactions and differentiated as the sex that would grant them the best reproductive success, because in this species large body size is more important to males than to females. Although Francis

and Barlow (1993) have been cited 25 times (e.g., Helfman et al. 1997, Baroiller et al. 1999, Baroiller and D'Cotta 2001, Devlin and Nagahama 2002, Godwin et al. 2003, Oliveira, 2006, Liu and Sadovy 2004), their findings remain unreplicated.

Although *in situ* behavior of adult Midas cichlids from several crater lakes has been well described (Barlow 1976, McKaye and Barlow 1976, McKaye and van den Berghe 1996, Murry et al. 2001, Vivas and McKaye 2001), juveniles have received little attention. Francis and Barlow (1993) predicted that juveniles would be socially segregated from fish of other ages/sizes during the period of sex differentiation. I therefore hypothesized that (1) there would be aggressive intraspecific interactions in social groups of juvenile Midas cichlids, (2) fish would assort into social groups according to body size, and (3) the males in given social groups would be larger than the females.

I studied habitat use and social behavior in juvenile Midas cichlids under natural conditions in order to better understand how communicatory interactions might function in controlling sex differentiation. In order to obtain a more complete understanding of behavior in Midas cichlids, co-occurring fish species were also noted and interactions with these species recorded. Gonad development was compared among Midas cichlids within social groups and compared to the patterns observed by Francis and Barlow (1993).

MATERIALS AND METHODS

Lake Apoyo

Field work took place in February 2004, and was based at Proyecto Ecológico, a field

station situated near the western shore of Lake Apoyo, Nicaragua (Figure 4-1). The Lake formed from a volcanic explosion approximately 20,000 years ago and measures 6 km across and 200 m deep (Waid et al. 1999). The terrestrial ecosystem in the crater has been classified as a tropical dry forest with a wet season occurring between May and November (Incer 1995). At least five fish species are native: *Atherinella sardina*, *Parachromis managuensis*, *Poecilia sphenops*, and *Amphilophus* cf. *citrinellus*, which may be a complex of four Midas cichlid species (McKaye et al. 2002), including *Amphilophus zaliosus* (Barlow and Munsey 1976). Introductions to the lake include the blue tilapia, *Oreochromis aureus*, introduced in 1983, the eleotrid goby *Gobiomorus dormitor* in 1990 (Berdarf et al. 2002), and the Nile tilapia, *Oreochromis niloticus*, in 1992 (McCrary et al. 2001).

Lake Apoyo was an ideal setting in which to observe social behavior in juvenile Midas cichlids. Its low species richness allowed observation with few interspecific interactions, which might complicate interpretation in a more species-rich environment such as Lake Xiloa. Excellent visibility was afforded by the clear water of Lake Apoyo, unlike the muddy or green waters found in some of the other Nicaraguan lakes. Lake Masaya, the source of the fish used by Francis and Barlow (1993), had become too polluted in which to work safely (Heijns 2002).

Habitat use

Three sites with differing substrate characteristics and anthropogenic influences were selected in different areas of Lake Apoyo. At each site, one transect of approximately 25 continuous 1 m^2 quadrats was laid-out parallel to the shore so that the deepest edge was 1 m deep (Figure 4-2). Each transect was traversed between two and four times, never more

than once in the same day. No effort was made to traverse the quadrats at the same time each day. At each observation session, percent cover of different types of substrate was estimated in each quadrat. Substrate was classified as one of three types: 'boulder', which was a large (> 0.5 m) flat rock surface, 'rubble', which represented smaller rocks, and grain-sized 'sand'.

The number of fish of each species observed was recorded according to body size (fry, juvenile, adult). The mobile nature of the fish indicated that each observation could be considered independent in data analysis. When multiple Midas cichlids were observed, I noted whether at least two of them were in close proximity (< 20 cm) to one another.

Site 1

Site 1 was located on the northern shore of the lake, at the first rocky outcropping east of the row of houses that lined the lake shore (Figure 4-1). Part of the site included a rock wall that rose 5 m vertically out of the water, and continued down below its surface. Rocks ranging from large boulders to small stones were scattered in the vicinity. Access to the site would have been difficult from land. There were cattle paths directly above the site along the crater wall, but the site was reached by kayak. The shallow (< 1 m deep) area of the site was between 0 and 3 m from the shore. After this distance the bottom sharply dropped off.

The transect at this site consisted of 27 quadrats, and was traversed four times. Due to the rocky, uneven nature of this site, water depth on the shallow edge of the transect was variable, but was generally around 0.75 m. After the final observation at Site 1, rocks were overturned in order to determine how many fish were hiding and therefore overlooked during the data collecting periods. Fish were absent except for one small

Midas cichlid, which indicated that the previous observations represented nearly all of the fish in the transect.

Site 2

Located on the southern shore of Lake Apoyo, Site 2 was reached by following the low road south from Proyecto Ecológico. Past the old walkways and many sets of stone stairs that descended to the shore, the water line turned to the right. At this point the stairs no longer reached the water. The transect was located about halfway between this turn and an isolated hut (Figure 4-1). The substrate here was clay, intermittently covered by a thin layer of sand. The water was shallow (< 1 m) until it dropped off at about 20 m off shore. From the shore, about ¾ of this distance was covered in rubble (20-30 cm diameter). The lakeside border of the transect followed the lakeside border of this stone field and was approximately 1 m deep. The transect consisted of 26 quadrats and was traversed twice.

Site 3

Site 3 was located just off the heavily populated western shore of Lake Apoyo, directly in front of Proyecto Ecológico. It was used as a boat launch by the field station and was commonly used by swimmers, people washing clothes, etc. Trash was scattered on the shore. Approximately 95% of all the houses on the lake were located on the western shore. One couple was in the water at the time of sampling on Day 1, and two groups of people were in the water on Day 2. The substrate was thick sand. The slope descended slowly until about 20 m from shore, to a depth of about 1.5 m, and then dropped off to about 10 m deep. Rubble (20 cm diameter) overlaid the sand for about the first 3 m off shore. The transect was about 10 m off shore, over sand mixed with a few small pebbles. It consisted of 22 quadrats and was traversed twice.

Amphilophus cf. *citrinellus*, *P. managuensis*, and *G. dormitor* were tested for associations between density and substrate characteristics within each site with linear regressions. Also, substrate characteristics were compared between observations in which a species was present and those in which it was absent within a site with Mann-Whitney tests. Fish densities among sites were compared using Kruskal-Wallis tests. Statistical analyses were performed using SPSS 11.0 computer software with alpha at 0.05.

Social behavior

Each of 33 juvenile Midas cichlids were individually chosen and followed for the duration of a preset period, either 2.5 or 5 minutes, or until it was lost, whichever occurred first. Numbers of aggressive and submissive interactions were recorded for each focal fish. A bout of aggressive behavior was defined as any deviation from an individual's previous action or trajectory with redirection toward another individual in an apparent effort to displace it, followed by cessation of aggression for more than 1 s. Displays and attacks (Baerends and Baerends van Roon 1950) were not differentiated, as an approach seemed to be the most important component of an aggressive action. Submission was defined as a change in position or posture apparently to avoid physical contact with an antagonistic fish. Behavior was recorded at predetermined time points (either every 15 s or every 30 s) and used to calculate time budgets. Behavioral categories were based on previous laboratory observations (Table 4-1, Chapter 2, Oldfield 2007, Chapter 3). In the current *in situ* observations, a fish 'hiding' may also have been performing other acts that went unrecorded. It was usually under a rock and temporarily out of view. All data were taken from Site 1, although observations were not restricted to the transect.

Sex distributions

Gonad differentiation was investigated in two social groups of Midas cichlids. Two professional divers employed an underwater seining technique with a nylon gillnet to capture all of the members of each of two shoals of juveniles offshore near Site 3 at depths between 12 to 15 m. The first shoal, Group 1, contained 14 specimens. The second, Group 2, contained 15 specimens. All specimens from both groups were fixed in modified Davidson's fluid and were later transferred to 70% ethanol. They were measured for Standard Length (SL) and dissected. Their gonads were removed, embedded in paraffin, sectioned at 4 μm, stained with hematoxylin and eosin, and examined under a light microscope. Specimens were deposited at the University of Michigan Museum of Zoology (Group 1: UMMZ #246209, Group 2: UMMZ #246213).

To determine if males and females were assorted by size within each group, a chi-square test was used to compare number of males and number of females above and below the median SL. Standard Length was compared between males and females with a Mann-Whitney U-test.

RESULTS

Habitat use

During data collecting observations, no Midas cichlids were seen in the transects at Sites 2 and 3, and only juveniles were observed at Site 1. This indicated an affinity for rocks. Association of juvenile Midas cichlids with both rubble and boulders at Site 1 was tested by performing a regression to determine if the number of Midas cichlids decreased with increasing proportions of sand. No association was found ($R^2 = 0.057$). However,

quadrats that contained Midas cichlids had significantly greater percent cover (combined 'rubble' and 'boulder') than those that did not (Mann-Whitney test, one tailed: Z = -2.303, p = 0.011, Figure 4-3). Similar results were found for *P. managuensis* ($R^2 = 0.048$ and Mann-Whitney test, one tailed: Z = -2.418, p = 0.008, Figure 4-3). Whereas no adult Midas cichlids were observed, adult *P. managuensis* were occasionally observed and included in the analysis. The occurrences of these two species were also tested for associations with 'rubble' alone and found to be insignificant ($R^2 = 0.039$, $R^2 = 7.47 \times 10^{-5}$ respectively).

Out of 104 total quadrat observations at Site 1, Midas cichlids were observed in 68. Of these 68 observations, two or more individuals were in close proximity (< 20 cm) to each other in 22 cases. *Parachromis managuensis* were observed in 10 of 104 observations and they were always solitary.

Gobiomorus dormitor was common at all three sites. As in the cichlids, there was no linear association between the occurrence of *G. dormitor* and percent sand at Site 1 ($R^2 = 0.001$). A similar lack of association was found at Sites 2 ($R^2 = 0.029$) and 3 ($R^2 = 0.018$). In contrast to the cichlids, there was no difference in percent cover between the quadrats that contained *G. dormitor* and those that did not at Site 1 (Mann-Whitney test, two tailed: Z = -1.414, p = 0.157, Figure 4-3), or at Site 2 (Mann-Whitney test, two tailed: Z = 0.954, p = 0.34) or Site 3 (Mann-Whitney test, two tailed: Z = 0.183, p = 0.855). In addition, differences in *G. dormitor* density among sites were tested by the Kruskal-Wallis test and found to be insignificant ($\chi^2 = 0.349$, p = 0.840). Individuals of *G. dormitor* in the shallow waters were mostly juveniles (approximately 5 cm Total Length (TL) or less), but adults were recorded occasionally. No adults were observed at

Sites 2 or 3, but 10 of the total 140 *G. dormitor* observed at Site 1 were adults.

Occurrence of *A. sardina* and *P. sphenops* would not be expected to be associated with substrate type, as these species are not demersal, but they were only observed at Site 1. Out of the 104 quadrat observations at Site 1, *A. sardina* was observed 27 times, with 15 of these being only 1 or 2 fish. However, groups of up to approximately 40 fish were observed. The mean of approximated group sizes was 8.3 fish per group. The large groups caused *A. sardina* to be the most abundant fish at Site 1 (Figure 4-4). *Poecilia sphenops* was observed on two occasions. The first was an observation of a school of seven fish, each approximately 5 cm TL, moving very quickly through the area. The second was a sighting of one large (15 cm TL) individual which was seen hiding in a cave deep in the rock face. It was positioned vertically in the cave and had two wounds on its body, and appeared to be stressed (rapid breathing and pectoral fin movement). No *Oreochromis* species were observed.

Social behavior

One Midas cichlid was omitted when calculating mean rate of aggression because it was observed for less than 30 s, leaving data from 32 fish in the analysis. Fish performed a mean of 0.76 aggressive bouts per minute. Submission was observed 0.37 times per minute. The difference between aggression and submission was attributed mostly to aggressive displays that did not result in obvious submissive reactions. Aggressive interactions also occurred between Midas cichlids and *P. managuensis* and *G. dormitor*, but much less frequently (only six with *P. managuensis*, and one with *G. dormitor*).

Four fish were excluded from the time budget analysis because only three behavior samples were recorded for each. This was not considered a sample sufficient to

be representative of their time budgets. The remaining 29 fish were used to calculate a mean time budget (Figure 4-5). *Amphilophus* cf. *citrinellus* spent most of their time swimming (33.7%) or hovering in the water (21.9%). They also spent considerable proportions of time foraging (20.0%) and hiding under rocks (17.6%). Aggressive interactions made up considerably smaller proportions of time. Fish engaged in attacks or displays 4.6% of the time and submissively responded to the aggression of other individuals 2.2% of the time.

Sex distributions

Gonad structure was consistent with the descriptions provided for *Cichlasoma dimerus* by Meijide et al. (2005). Females were identified by the presence of oocytes at various stages of development and an early ovarian lumen (Figure 4-6a). Males were identified by testicular lobules consisting of spermatocysts and sperm ducts that contained sperm in various stages of development (Figure 4-6b). No bisexual gonads were observed: all contained exclusively either female or male tissue.

A two-tailed t-test revealed that the Midas cichlids in Group 1 were significantly larger than those in Group 2 ($t = 2.428$, d.f. = 27, $p = 0.022$), and they were more developed (Table 4-2). Sex was identified in all of the fish in Group 1, whereas three fish in Group 2 were undifferentiated and not identifiable to sex even through histological analysis (Figure 4-6c). Within the smallest seven individuals of Group 1, four were females and three were males. There were also four females and three males among the largest seven fish. Therefore, sexes were not assorted by size ($\chi^2 = 0.000$, $p = 1.000$). Males were not generally larger than females in Group 1 (Mann Whitney U = 23.500, Z = -0.065, p (two tailed) = 0.948). In Group 2, three of the four fish identifiable as females

were above the median body size, and there were no identifiable females below the median.

DISCUSSION

Habitat use

Juvenile cichlids only occupied a portion of the shallow water habitats of Lake Apoyo. They were absent from habitats without rock structure. The presence of three-dimensional structure seemed to provide juvenile cichlids with shelter from predation by larger cichlids, *G. dormitor*, and presumably predatory birds.

Midas cichlids were more common than *P. managuensis* at Site 1. While no adult Midas cichlids were present during data collecting periods, adult *P. managuensis* were. *Parachromis managuensis* had been previously observed frequently in Lake Apoyo (Barlow 1976), and they were fairly common at Site 1. Neither adults nor juveniles were observed feeding, and they were always solitary. Large *P. managuensis* were more common than large Midas cichlids at Site 1. Perhaps their more predatory nature brought them into the shallow water to prey upon young cichlids. Juvenile *G. dormitor* did not require extensive structure, and were found in more diverse habitats than the cichlids. Their cryptic coloration and demersal behavior probably allowed them to avoid predation in the more barren habitats of the lake. Both large and small *G. dormitor* at Site 1 were observed preying on *A. sardina*. Although *A. sardina* was observed considerably less often than most other species, its large schools compensated to make it the most abundant fish at Site 1. Schools of many hundreds were observed outside of the data collecting periods. *Poecilia sphenops* was very rare. The exotic fishes may have detrimentally

affected the *P. sphenops* population as exotic fishes have affected native fishes in other Nicaraguan Lakes (McKaye et al. 1995). This species seemed to be under predation pressure from the introduced *Gobiomorus* and from habitat (*Chara* sp.) loss induced by introduced tilapia (McCrary et al. 2001). No *Oreochromis* species were observed at any time, even outside of the data collecting periods. However, only limited conclusions can be reached from a preliminary survey limited to shallow water; tilapia have been caught recently in deep water.

Outside of data collecting periods, larger and smaller fish were present at the sites. Large Midas cichlids and *P. managuensis* were present at low densities at Site 1, but were easily scared off and were generally gone by the beginning of each sampling period. Many large Midas cichlids were seen by free diving. On Day 1, a free-diving harpoon fisherman had two adult *P. managuensis* that he reported to have captured at about 10 m depth. On Day 1 one school (approximately 30 fish) of Midas cichlid fry and one school (approximately 20 fish) of *G. dormitor* fry were observed at Site 1. Near Site 2, two anglers were observed with one large Midas cichlid and one large *P. managuensis* on Day 2. Near Site 3, a shoal of nine adult Midas cichlids was observed in shallow (1 m) water on Day 1.

Most of the Midas cichlids observed in shallow water were very small (2.5 cm TL). Unquantified SCUBA observations and free dives found larger fish and fewer small fish at depths greater than 1 m. Individuals smaller than 2.5 cm TL occurred in tight schools that appeared to be natal broods, and occupied a variety of depths. After the fish leave these broods they apparently move into shallow water and are solitary until they reach between 2.5 and 5 cm TL, when they move into deeper water and form small shoals

that contain similarly sized individuals. Considering the seasonality of cichlid reproduction in Lake Xiloa (McKaye 1977), this size segregation may represent age segregation. If so, then intra-cohort social interactions may be much more common than inter-cohort interactions, and social interactions at the late juvenile stage would be particularly informative of an individual's size relative to other members of its cohort.

Social behavior

Juvenile Midas cichlids in the shallow water were very active, feeding on periphytic algae and associated fauna as well as free vegetable material that appeared to be allochthonous – probably fruit and pollen from shoreline trees. They were generally solitary, although they did engage in frequent social interactions that could possibly influence sex differentiation. Some interactions occurred with only an approach followed by a submissive acknowledgement. An aggressive approach elicited a response 50% of the time. Analyses by numbers of aggressive and submissive bouts and by proportions of time spent performing aggressive and submissive behavior both indicated that submissive behavior occurred at levels of about half of those of aggressive behavior.

Sex distributions

All of the individuals from Group 1 were differentiated sexually, but three of the fish in Group 2 had undifferentiated gonads. This finding is consistent with the predictions of Francis and Barlow (1993). If juveniles were sexually undifferentiated at the time they formed small shoals in deeper waters, then behavior within each shoal could provide a good indicator of relative size and possibly influence direction of gonad differentiation.

Distribution of males and females among the size ranks within each shoal did not support the Francis and Barlow (1993) hypothesis. According to their hypothesis, size

hierarchies within social groups are stable over time. Because adult males are larger than adult females, at any point past the onset of sex differentiation individuals larger than the median size should be predominantly males and those smaller should be females. Instead, there was no association between large size and maleness in either group. Sex is determined genetically in the closely related *Archocentrus nigrofasciatus* (George and Pandian 1996). Genetic factors cannot be ruled out in sex determination in Midas cichlids, with size differences between adult males and females resulting from intrinsically greater post-maturational growth rates in males. This type of growth pattern seems to be responsible for sexual size dimorphism in the closely related Mayan cichlid, 'Cichlasoma' *urophthalmus* (Faunce et al. 2002).

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Table 4-1. Behavior observed in juvenile *Amphilophus* cf. *citrinellus* used to describe the fish's time budget.

Behavior	Description
Hovering	Hanging motionless in the water column.
Swimming	Locomotion through water.
Hiding	Avoiding a line of site between itself and other animals, usually under a rock or in a crevice.
Foraging	Looking at substrate, sifting the particles in the gravel, or approaching and chewing a particle in water column or at the surface.
Aggression	Any kind of chase, bite, or display.
Submission	Evading an attack or display.

Table 4-2. Size distributions of two shoals of *Amphilophus* cf. *citrinellus*. Three individuals in Group 2 were undifferentiated. The 72.5 mm specimen in Group 2 was not sexed due to problems in preservation. Bold font indicates median sizes.

Group 1		Group 2	
SL (mm)	Sex	SL (mm)	Sex
63	F	60	M
64	F	61	? (undifferentiated)
65	F	61	M
65.5	M	61	M
67	M	64	? (undifferentiated)
67	M	65	? (undifferentiated)
70	**F**	**66**	**F**
70	**M**	**66**	**M**
71	F	**66**	**M**
72	F	66.5	M
72.5	M	69	F
73	M	69	M
77	F	70	F
77	F	70	F
		72.5	? (poorly preserved)
Mean SL:	69.6	Mean SL:	65.8
Std. Dev.:	4.5	Std. Dev.:	3.9
Std. Error:	1.2	Std. Error:	1.0

Figure 4-1. Map of Lake Apoyo, Nicaragua (11° 56' N, 83° 3' W). The field station Proyecto Ecológico (PE) and the three field sites are indicated.

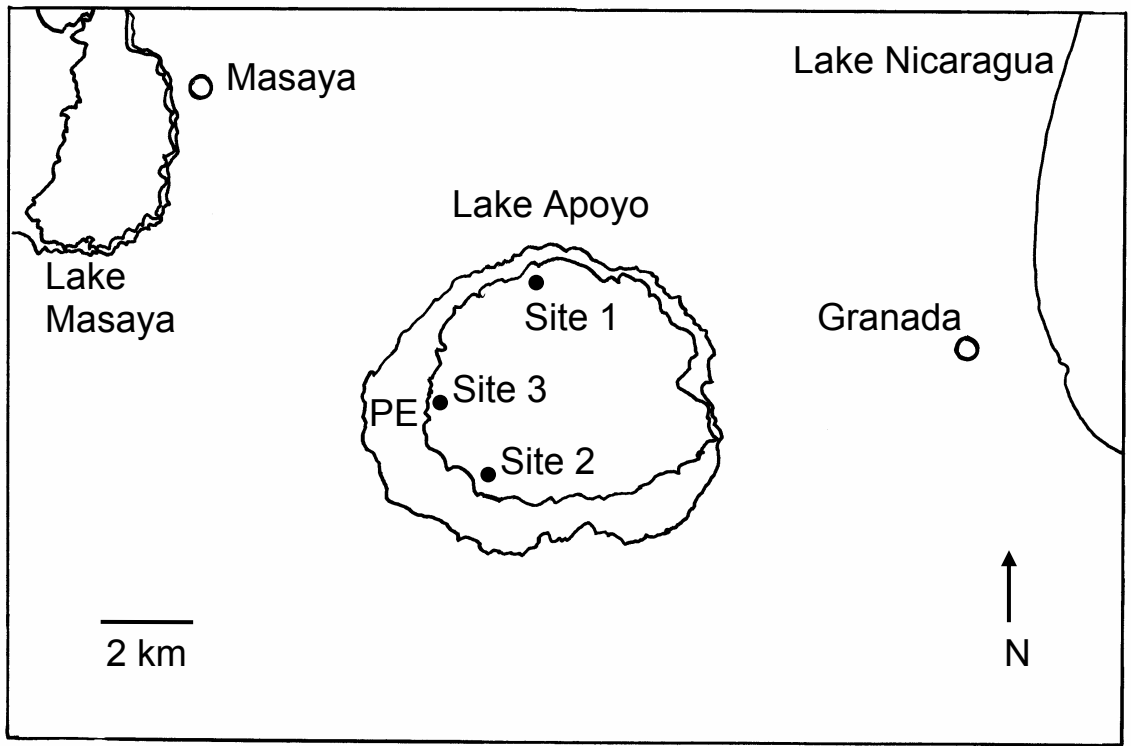


Figure 4-2. At each site a transect of approximately 25 m was laid parallel to the shore in 1 m of water. The transect was composed of adjacent 1 m^2 quadrats.

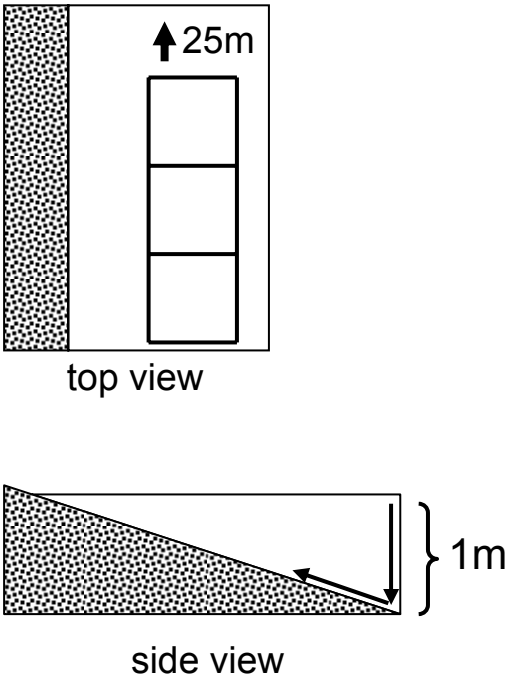


Figure 4-3. Mean percent cover (combined 'rubble' and 'boulder') for quadrats at Site 1 in which fishes were present (white bars) or absent (black bars). Significantly (*) lower percent cover observed in quadrats where individuals were absent indicates a preference for rubble and boulders (see text for substrate descriptions) in *Amphilophus* cf. *citrinellus* and *Parachromis managuensis*, but not in *Gobiomorus dormitor*. Bars represent SE.

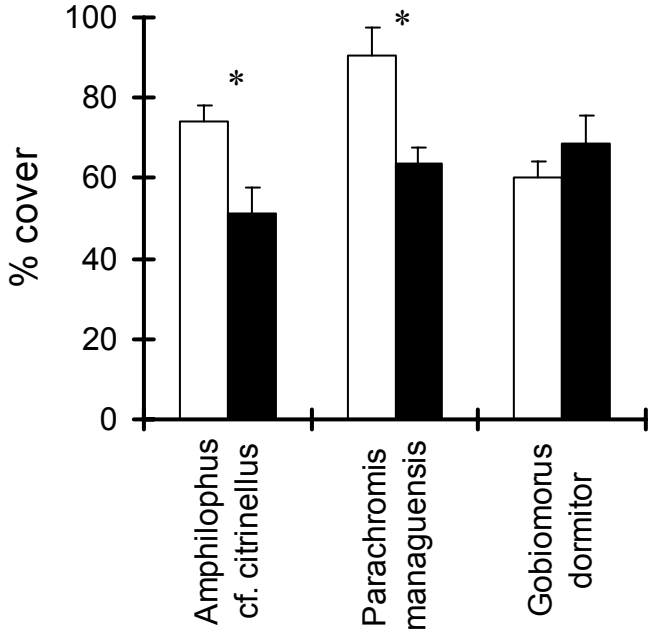


Figure 4-4. Density (# fish per m^2) of fishes regularly observed at Site 1 in Lake Apoyo. Bars represent Std. Dev.

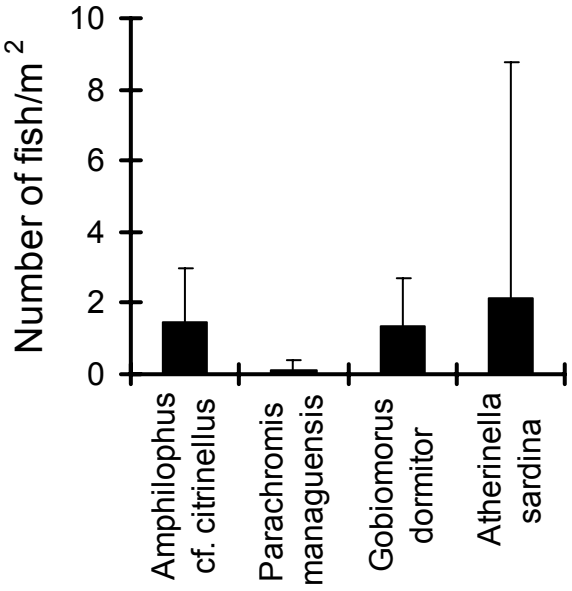


Figure 4-5. Time budget of solitary young juvenile *Amphilophus* cf. *citrinellus*.

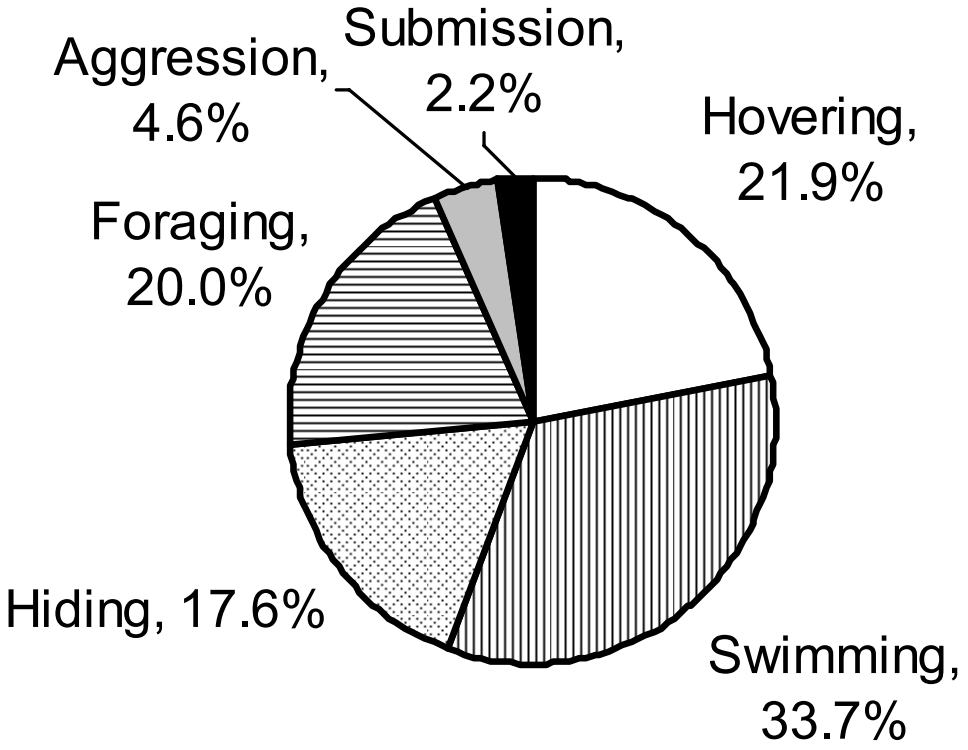
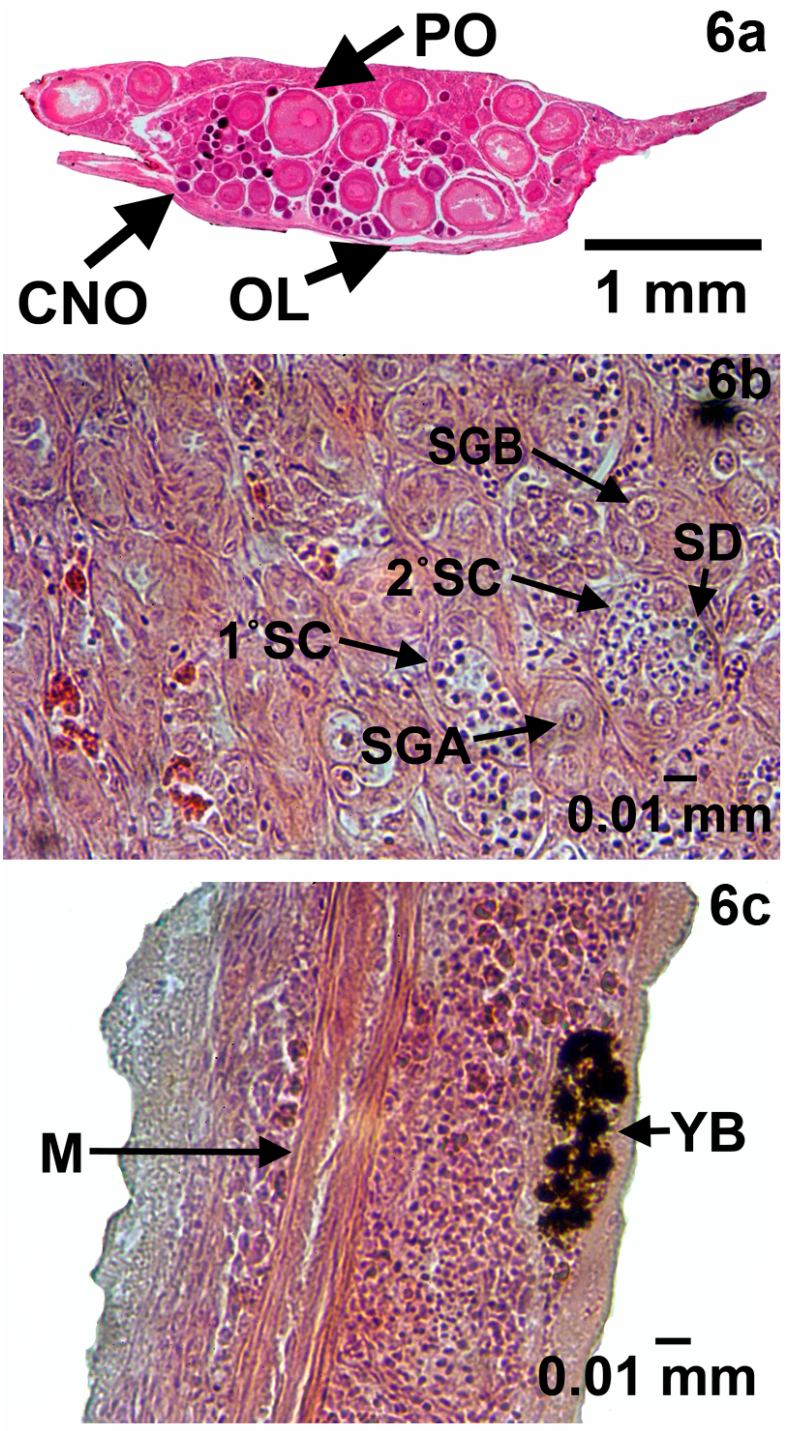


Figure 4-6. Histological sections of typical (6a) female, (6b) male, and (6c) undifferentiated gonads observed in two shoals of late juvenile *Ampilophus* cf. *citrinellus* from deep water. 6a and 6b are transverse sections, 6c is a longitudinal section. CNO = chromatin nucleolus phase oocyte, M = mesentery, OL = ovarian lumen, PO = perinucleolar phase oocyte, 1ºSC = primary spermatocyte, 2ºSC = secondary spermatocyte, SD = sperm duct containing spermatids and spermatozoa, SGA = spermatogonia A, SGB = spermatogonia B, YB = yellow body.



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CHAPTER 5

GROWTH PATTERNS IN MIDAS CICHLIDS ARE INCONSISTENT WITH A HYPOTHESIS OF SOCIALLY CONTROLLED SEX DETERMINATION

ABSTRACT

Social control of sex determination has been reported in juvenile Midas cichlids, *Amphilophus citrinellus*, held in large captive groups. Large body size relative to group-mates was proposed to cause male differentiation. More recent evidence has failed to corroborate this phenomenon in individuals held in small social groups. In a series of experiments, juvenile Midas cichlids from four lineages were individually marked and divided into large experimental groups to test the hypothesis that sex determination is dependent on relative body size. Fish were predicted to differentiate as males if they were larger than most of their group-mates, and females if they were smaller. An alternative hypothesis predicted that social conditions would not affect sex, and that the greater body size typically observed in males at the adult stage is due to faster growth in individuals predetermined to differentiate as males. Hunger was measured in isolated juveniles to test the hypothesis that intrinsic differences were responsible for differences in growth between females and males. Relative body size did not affect sex determination. The observed growth patterns indicate that dimorphism in body size between the sexes in adults is due to faster growth in males than in females. There were no differences in hunger between isolated females and males, and differences in growth between females

and males were much smaller in isolates than in group-held fish. Social conditions were not responsible for sex determination, but they appear to be responsible for the size differences typically observed between the sexes.

INTRODUCTION

Many fishes have sex determined by environmental factors. Some species are developmentally labile as larvae, and some as adults (Devlin and Nagahama 2002). This variation, as well as the observation that some gonochoristic fishes (those that only function as one sex as adults) undergo a period of gonadal bisexuality (bearing both female and male tissue) very early in ontogeny, have suggested that the different expressions of gonad lability in fishes have evolved through ontogenetic extensions of an initial brief critical period of gonad lability (Atz 1964, Shapiro 1987). Social control of sex determination at the juvenile stage is thought to represent a midpoint on an ontogenetic continuum between these gonochores and functional sequential hermaphrodites (Francis 1992). In cichlids, there are reports of species that have no lability at all and other species labile at one of three distinct life stages (reviewed by Oldfield 2005, Chapter 1), further supporting this hypothesis.

The Midas cichlid, *Amphilophus citrinellus*, has been reported to undergo socially controlled sex determination (Francis 1990, Francis and Barlow 1993). Growth is socially controlled in this species, as in many other fishes (reviewed by Johnsson et al. 2006). The same behavioral interactions that allowed large, dominant juveniles to suppress growth in smaller subordinates were proposed to cause large fish to differentiate as males and smaller fish to differentiate as females. Despite having been extensively cited (e.g.,

Helfman et al. 1997, Baroiller et al. 1999, Baroiller and D'Cotta 2001, Godwin et al. 2003, Oliveira 2006), social control of sex determination has not been subsequently tested in Midas cichlids until recently (Oldfield 2007, Chapter 3, Oldfield et al. 2006, Chapter 4), and in both studies the results were inconsistent with those of Francis and Barlow (1993). Oldfield (2007, Chapter 3) and Oldfield et al. (2006, Chapter 4) sampled fish at the onset of maturity in both nature and the laboratory and found no differences in body size between males and females. Oldfield (2007, Chapter 3) proposed three possible explanations for the inconsistency between these results and those of Francis and Barlow (1993). One possible explanation is that processes of sex determination are different among different lineages of Midas cichlids. McKaye et al. (2002) have recently estimated that the Midas cichlid species complex may contain 15 to 30 different species.

A second possibility is that behavior only determines sex under particular social conditions. In many fishes, social structure changes with group size or density (reviewed by Grant 1997). In small captive groups dominant juvenile Midas cichlids become territorial and behave despotically, but as group size increases a typical shoaling social structure forms (Chapter 2). Francis and Barlow (1993) divided a brood of 74 fish into two groups, one containing the smallest 37 fish, and the other the largest 37. Oldfield (2007, Chapter 3) tested sex determination in groups that each contained six fish. Territorial behavior may somehow have 'short-circuited' social control of sex determination.

The third possible explanation is that the large size typically observed in adult males (Barlow 1976) is due to greater post-maturational growth relative to females, rather than due to social control of sex determination. Francis and Barlow (1993) assumed that

this was not the case because Francis (1990) had observed stable size ranks in a group of 12 fish raised in captivity. However, differential growth between the sexes is typical in some other cichlids (see Chapter 3). In Midas cichlids, Barlow (1976) had earlier acknowledged that males grow faster than females, but under the Francis and Barlow (1993) hypothesis, faster male growth must occur after relatively larger juveniles have differentiated as males.

In order to choose between the three possible explanations for the inconsistency between Oldfield (2007, Chapter 3) and Oldfield et al. (2006, Chapter 4), and Francis (1990) and Francis and Barlow (1993), I performed additional variations of the Francis and Barlow (1993) experiment. Several broods from different Midas cichlid lineages were tested. Within each brood, fish were individually marked and assigned to one of two sub-groups based on relative body size: those greater than the median size in one sub-group and those smaller than the median in the other. Sub-groups contained numbers of individuals similar to the sub-groups used by Francis and Barlow (1993). Additional data on the isolated fish reported by Oldfield (2007, Chapter 3) regarding hunger and growth were also analyzed in an attempt to identify intrinsic mechanisms that may be responsible for differences in growth rates between the sexes. The gonads of several of the group-held and isolated fish were examined histologically to confirm sex inferred from macroscopic features and to identify any bisexual structures that could be interpreted as an indicator of gonad lability.

The ability to track individuals over time made it possible to compare an individual's relative size within its sub-group at the beginning of the experiment with its relative size at the end. If sex was determined socially within a sub-group, individuals

identified as males at the end of the experiment would be larger than those identified as females, both at the beginning of the experiment and at the end. An alternative hypothesis predicted that there would be no difference in size between females and males initially, but that greater post-maturational growth in males would eventually lead to sub-groups in which males were larger than females.

MATERIALS AND METHODS

Over a period of nearly 3 years, four broods of separate origins, all belonging to the Midas cichlid species complex, were divided in the manner of Francis and Barlow (1993) and raised to maturity. Taxonomic identity was determined from original species descriptions and comparisons with type specimens (Günther 1864, Barlow and Munsey 1976). From one of the broods, some individuals were removed and placed into isolation before the remaining members of the brood were divided into sub-groups of relatively small and large fish. The numbers of individuals in each sub-group of each lineage that survived the experimental period are summarized in Table 5-1. Subjects, husbandry procedures, marking techniques, and experimental manipulations were as follows:

Pet-trade lineage

Some individuals from the brood used by Oldfield (2007, Chapter 3) were raised to maturity and allowed to reproduce. The parents were purchased from a pet dealer and their original geographic origin was unknown. A resulting brood hatched on 19 May 2003 and was free-swimming on 25 May 2003 (day 1). The fry were maintained in a 147 l aquarium (46 x 90 x 36 cm) until day 123, when 60 fish were removed and placed into their own 147 l aquarium. On day 182 another 18 were removed, weighed (5.7 ± 3.1 g,

mean ± SD), measured (52.3 ± 10.2 mm SL), and placed individually into 18 tanks. Another four were maintained and the remaining 55 were killed with an overdose of tricaine methanesulfonate (MS-222), preserved in Bouin's fluid, and deposited in the collection at the University of Michigan Museum of Zoology (UMMZ #247657 and #247658).

On day 185, the 60 fish were weighed (4.2 ± 3.5 g), measured (47.1 ± 10.4 mm) and divided into two sub-groups, one containing the largest 30 fish and the other containing the smallest 30. Each fish was anesthetized with MS-222 and marked by sewing a short strand of nylon monofilament line though the dorsal musculature and tying one colored bead to each end. Similar methods have been successful with cichlids in the field (Oliver 1984, Swanson et al. 2003). Each sub-group was then transferred to its own 147 l aquarium. Approximately once per month all individuals were removed, weighed, measured for SL, and checked for tag retention. Beads were replaced on individuals from which they were lost. This marking technique proved less than ideal. After marking, the fish appeared frightened and remained on the gravel near the rear of their aquaria. The tags also caused infection in many individuals, especially the smallest ones. Despite the addition of salt (NaCl) to the water (Aronson 1951), and the application of Mercurochrome to their wounds, the tags resulted in the deaths of five individuals in the sub-group of small fish.

Aquaria contained natural gravel substrate (5 - 8 cm deep) and air-powered sponge filters (electric external filters were later added). Water temperature varied (21º - 30º C) but was generally around 25º C, pH was 7.6 ± 0.2. Natural sunlight penetrated skylights and was supplemented with standard florescent lights turned on during regular

working hours. Fish were fed ad libitum once per day on a variety of commercial pellet and flake foods, supplemented once per week with frozen brine shrimp (*Artemia* sp.), frozen blood worms (*Chironomus* sp.), live wax worms, or live earthworms. Water quality was maintained through frequent partial water changes and detritus removal with a gravel vacuum.

In the summer, after the weather was deemed warm enough, fish from each sub-group were transferred outdoors to a 1300 l black plastic tub (1.90 m diameter x 0.45 m deep). Several tubs were set up outdoors at the E. S. George Reserve (Hell, Michigan) and filled with well water on 12 June 2004 (day 384) and the sub-group of large fish and the sub-group of small fish were each added to a tub on day 387. The tubs contained only water, thermometers, and six short lengths of PVC pipe. Fish were fed ad libitum three times per week and also ate arthropods that fell into the vats and large amounts of algae. The practice of periodically removing the fish continued as before, and each time they were removed their water was completely replaced with aged well water. The summer of 2004 was unusually cool (water temperature mean: 20.6º C, range: 10.6º - 27.8º C), pH ranged from 8.0 to 8.8. On day 469 (5 September 2004) all individuals were removed, killed with an overdose of MS-222, identified, weighed, measured, dissected, and fixed in 10% formalin. They were later transferred to 70% ethanol and sexed by macroscopic and microscopic examination of their genital papilla and gonads (UMMZ #247659 and #247660).

Isolated individuals

The 18 fish removed from the brood of pet-trade fish on day 182 were each isolated in a 38 l (50 x 26 x 30 cm) aquarium, as described by Oldfield (2007, Chapter 3). The effects

of isolation on sex determination were assessed by comparing the number of fish that differentiated as females with the number that differentiated as males. The results of this analysis were reported previously (Oldfield 2007, Chapter 3). In addition, fish were periodically removed and weighed and measured to determine if intrinsic growth rate differences were responsible for the differences in body size observed between adult females and males. Hunger was quantified by recording the time elapsed until first feeding and the amount of food consumed. Each day, each fish was fed the same amount of food as every other fish. The amount of food and food type varied by day, but the amount was generally close to enough to satiate the hungriest fish. For the first measure of hunger, individuals were observed for 30 s after the introduction of food. If they consumed food within this period they were classified as hungry. If they did not, they were considered not to be hungry. For the second measure, the aquarium of each fish was examined for remaining food between 15 and 56 minutes after feeding. If a fish had eaten all of its food it was considered to be hungry. If it had not, it was classified as not hungry. On day 423 the fish were killed with an overdose of MS-222, weighed, measured, dissected, fixed in 10% formalin, and later examined to determine sex (UMMZ #247663).

'Hybrid' lineage

The second brood was also produced in the lab and was manipulated in a manner similar to that described for the pet-trade lineage. Offspring came from a second generation captive bred female *A. citrinellus* descended from individuals wild-caught in Lake Nicaragua (the type locality for the species, Günther, 1864) and a male Midas cichlid obtained from a pet dealer in a city different than the source of the pet-trade lineage fish.

The brood hatched on 6 June 2003, and the larvae were free-swimming on 11 June 2003 (day 1). Shortly after becoming free-swimming the larvae were removed to a separate tank. Sixty fish were removed on day 110 and the rest of the brood was killed (UMMZ #247664). These 60 were weighed (8.1 ± 4.1 g) and measured (59.1 ± 9.3 mm) on day 228 and divided into 'small' and 'large' sub-groups. Four fish from each group jumped out of their tanks and died the first night after marking, leaving 26 fish per group. Few fish died beyond these initial losses. They were moved to outdoor tubs on day 371 (16 June 2004) and killed on day 452 (5 September 2004) (UMMZ #247661 and #247662).

Lake Nicaragua lineage

A brood of *A. citrinellus* were produced from second generation captive parents descended from individuals wild-caught in Lake Nicaragua. Eggs were laid on 11 June 2004. The brood was free-swimming on 19 June 2004 (day 1) and was removed from their parents to a 147 l aquarium on day 3. On day 120 all of the fish were removed and counted and it was found that there were 51 individuals. They were weighed (5.4 ± 1.6 g), measured (50.7 ± 5.2 mm), anesthetized with MS-222, and marked on one side of the body with either a red or a green visible implant elastomer (VIE) produced and marketed by Northwest Marine Technologies. Fish were marked at two of four possible sites: above the anal fin, above the pectoral fins, below the dorsal fin, and in front of the dorsal fin. Each fish was marked several times at each site to ensure tag retention. They were removed from the aquarium again on day 142 and examined and found to have retained the marks. On day 158 the fish were removed again, anesthetized, weighed (7.9 ± 2.5 g), and measured (57.4 ± 6.3 mm SL). Blue VIE was applied to two sites on the unmarked side of each individual to make it individually identifiable. The 26 smallest individuals

were separated from the 25 largest and each sub-group placed into its own 147 l aquarium. The fish from each sub-group were removed and weighed and measured again on day 207 and on day 252, when they were transferred to two 416 l acrylic aquaria (168 x 88 x 27 cm + 47 x 46 x 11 cm) that were set up similarly to the other aquaria except that they lacked gravel. They were removed and weighed and measured again on day 313 and moved to two 596 l steel vats (229 x 60 x 43 cm) that each contained air powered sponge filters, 5 cm natural gravel substrate, and two cinder blocks. Fish that were not clearly marked were remarked as needed, although no fish needed remarking after day 313. They were removed and measured again on day 366 and day 419 and finally on day 434 (27 August 2005) they were killed with an overdose of MS-222, identified, weighed, measured, dissected, sexed, and fixed in 10% formalin (UMMZ #247655 and #247656). Water temperature varied (range: 22º - 29º C, mean for the sub-group of small fish: 25.4º C, mean for large fish: 24.6º C), and pH was always 7.6.

Lake Apoyo lineage

Seventy-five fish from a brood of second generation *Amphilophus zaliosus* descended from fish wild caught in Lake Apoyo, Nicaragua were purchased from Tangled up in Cichlids, a commercial cichlid importer, breeder, and distributor, on 18 Aug 2004 and placed into a 147 l aquarium. Fish were manipulated in the same manner as the Lake Nicaragua lineage. Before the experiment began, an unidentified illness killed 36 individuals. On 12 January 2005 (day 1) the remaining 39 fish were removed, weighed (8.4 ± 5.9 g), and measured (63.5 ± 14.4 mm). Nine of these were killed and preserved (UMMZ #247666). The remaining 30 were marked with VIE and the larger 15 fish were separated from the smaller 15. Temperature varied (range: 20º - 29º C, mean for the sub-

group of small fish: 25.6º, mean for the large fish: 25.3º C), and pH varied but was generally 7.6. On day 269 (7 October 2005) they were killed with an overdose of MS-222, identified, weighed, measured, dissected, sexed, and fixed in 10% formalin (UMMZ #247653 and #247654).

Histological analysis

Gonads from several individuals were analysed histologically. In most cases gonads were excised and then processed. For some smaller individuals, transverse sections of the visceral area approximately 2 mm thick were removed and processed. Tissue was embedded in paraffin, sectioned at 8 μm, placed on glass slides, and stained with hematoxylin and eosin. Five individuals from the pet-trade lineage and eight individuals from the Lake Apoyo lineage preserved at the time of sub-group formation were analyzed to determine if sex differentiation had begun before the initiation of experimentation. At the conclusion of the experiments, some specimens from the sub-groups were analyzed to confirm sex inferred from underdeveloped macroscopic characters and to seek bisexual gonads that might serve as an indication of some degree of sexual lability. One individual from the small sub-group and one isolated fish from the pet-trade lineage, eight individuals from the small sub-group and one individual from the large sub-group of the 'hybrid' lineage, and four individuals from the small sub-group and two from the large sub-group of the Lake Apoyo lineage were analyzed. Sex was verified by the exclusive presence of either ovarian or testicular structures.

Data analysis

Body sizes and growth of females were compared with males over the durations of the experiments. In each sub-group the significance of correlation between the size (body

weight) ranks of individuals at the beginning of the experiment with their ranks at the end of the experiment was tested with Kendall's Coefficient of Concordance. Mann-Whitney U-tests were applied to body weight data from each sampling date in order to determine if there were significant differences between fish that would eventually be identified as females and those as males. Mean daily growth rates were calculated for each weighing date throughout each experiment. The proportion weight change was calculated at each weighing by dividing the difference between the current weight and the previous weight by the previous weight. The proportion weight change was then divided by the number of days passed between readings. Data were analyzed with SPSS 13.0 software.

In isolated fish, hunger was compared between females and males with logistic regressions in SAS to determine the effects of sex on hunger, while also considering differences that may have been due to subject, day, and weight of the subject, as well as an interaction between sex and day. The model included 'day' as a variable equal to 'time', because data were repeated for each subject on different days. The factors 'sex', 'subject', and 'day' were classed as categorical variables, with 'time' remaining by default as a continuous variable. The dependent variable 'feedlatency' was binomial. The test was repeated for 'foodremaining'.

RESULTS

At the conclusion of each experiment, males were generally larger than females within each sub-group. However, at the beginning of each experiment males were not larger than females within each sub-group, indicating that the juveniles that differentiated as males were not the relatively larger fish within each sub-group. In no sub-group were

individual size ranks at the beginning of the experiment correlated with the ranks held at the end (Table 5-1), meaning that some individuals grew faster than others. Males generally grew faster than females, but the strength of this pattern varied according to sub-group.

Pet-trade lineage

In the sub-group of small fish, 22 individuals survived, of which there were 11 females and 11 males. At the beginning of the experiment, the size distribution of females was very similar to that of the males (Figure 5-1a). By the end several males had grown larger than the largest female, but a number of them remained within the size distribution of the females (Figure 5-1b). At no point during the experiment were males significantly larger than females (Appendix A, Figure 5-2).

In the sub-group of large fish, three died late in the experiment, one of which was identified as a male, leaving 13 females and 15 males. As in the sub-group of small fish, the size distribution of females was very similar to the distribution of males at the beginning of the experiment (Figure 5-3a). At the end of the experiment there was still no significant difference in weight between females and males, although additional males had grown larger than the largest female (Figure 5-3b), and the difference approached significance (Appendix A, Figure 5-4).

Isolated individuals

The 18 isolates included five females, 12 males, and one individual of unidentified sex. Unlike the group-held fish, several males were larger than females at the beginning of the experiment (Figure 5-5a). Although growth rates were very similar between the sexes, by the end of the experiment all the males had grown larger than the largest female (Figure

5-5b). Males in general were significantly larger than females consistently throughout the experimental period (Appendix A, Figure 5-6).

For latency to feeding there were 342 total responses as 'hungry', and only 117 as 'not hungry'. The model was a good fit to the data as the GENMOD value divided by the degrees of freedom was generally around 0.84 (a quotient over 1.0 would have indicated a failure of the data to fit the model). "Analysis of Initial Parameter Estimates" indicates that, for 'time', as time went on in the experiment there was a slight increase (Estimate = 0.0486) in the chance of getting a 'not hungry' response, and that this trend was highly significant ($p < 0.0001$). Also, as indicated by 'weight', as an animal got larger there was less of a chance (Estimate = -0.1061) that it would exhibit a 'not hungry' response (and therefore a greater chance that it would be hungry), and this was also highly significant ($p < 0.0001$). For females, there seemed to be less of a chance that an individual would be not hungry compared to males (Estimate = -0.1778), but this was not significant ($p = 0.6561$). When the program was modified to test for interaction between 'time' and 'sex', the interaction was found to be insignificant ($p = 0.8419$).

Similar results were obtained when comparing the amount of uneaten food among subjects. Of 425 total observations, 270 found a fish to be hungry, and 155 not hungry. The model was a good fit for these data also, as indicated by goodness of fit value of 0.7653. For food remaining there was also an increasing chance (Estimate = 0.1115) of fish not being hungry as time progressed ($p < 0.0001$). Again, the more a fish weighed, the less of a chance there was of it being not hungry (Estimate = -0.1621, $p < 0.0001$). Females seemed to be less likely not to be hungry (-0.3902), but again this was not significant ($p = 0.4038$). When the program was run again to test for interaction between

'time' and 'sex', the interaction was insignificant ($p = 0.1042$).

'Hybrid' lineage

In the sub-group of small fish, three died late in the experiment. One of these was identified as female, for a total of 11 females and 13 males. At the beginning of the experiment there was only one male larger than the largest female (Figure 5-7a). By the end of the experiment there were six males with weights similar to females and seven larger than the largest female (Figure 5-7b), but at no time were males significantly larger than females (Appendix A, Figure 5-8).

In the sub-group of large fish, two died during the experiment, leaving eight females and 15 males. At the beginning of the experiment, there were only two males larger than the largest female (Figure 5-9a). By the end of the experiment there were only four males smaller than the largest female, and 11 males larger than the largest female (Figure 5-9b). There were no significant differences in body size between males and females from the beginning of the experiment to day 367, but weights recorded on days 401, 432, and 452 revealed significant differences between males and females (Appendix A). Whereas females and males began the experiment at similar sizes, males generally expressed faster growth rates than females, which resulted in an increasing disparity in size between the two sexes (Figure 5-10).

Lake Nicaragua lineage

Four individuals from the sub-group of small fish jumped out of their aquarium during a single night, possibly due to work going on in the aquarium room that caused the lights to turn on and off several times. The 22 surviving fish included 18 females and four males. At the beginning of the experiment, the size distribution of females overlapped the

distribution of males (Figure 5-11a). At the end of the experiment males were significantly larger than females (Appendix A), and all were larger than the largest the female (Figure 5-11b). Males and females began the experiment at similar sizes but males grew faster than females (Figure 5-12).

All 25 fish in the sub-group of large Lake Nicaragua fish survived. These fish developed as 10 females and 15 males. At the beginning of the experiment on day 158, only five of the 15 males were larger than the largest female (Figure 5-13a), and there was no significant difference in weight between females and males. At the second weighing on day 207, and each one thereafter, males were significantly larger than females (Appendix A). By the end of the experiment on day 434 the smallest male was much larger than the largest female (Figure 5-13b, 5-14).

Lake Apoyo lineage

In the sub-group of small fish, 14 survived, and were identified to be five females and nine males. At the beginning of the experiment, the size distribution of females overlapped the distribution of males (Figure 5-15a). By the end of the experiment three males had grown to be larger than the largest female (Figure 5-15b), but males wre still not significantly larger than females (Appendix A, Figure 5-16).

All 15 fish in the sub-group of large fish survived, of which there were seven females and eight males. At the beginning of the experiment, there were three males larger than the largest female (Figure 5-17a). At the end of the experiment one additional male grew to be larger than the largest female (Figure 5-17b), but there was still no significant difference in weight between females and males (Appendix A, Figure 5-18).

Gonad histology

Females possessed ovaries that contained open lumina and tissue was divided into lamellae that contained oocytes at various stages of development (Figure 5-19a). Testes of males lacked lumina. Spermatogenic tissue was divided into lobules, which consisted of sperm ducts and spermatocysts, which contained sperm at various stages of development (Figure 5-19b). All gonads contained exclusively either female or male tissue, although two males in the small sub-group and one in the large sub-group of the 'hybrid' lineage possessed genital papilla that appeared to have lateral slots typical of females (see Chapter 6). In no individual was macroscopic gonad appearance inconsistent with histological evidence. These observations are consistent with those made by Oldfield et al. (2006, Chapter 4).

Four of the five fish from the pet-trade lineage that were killed at the time of sub-group formation were found to have begun gonad differentiation. Among these were three females, characterized by the presence of oocytes in the perinucleolar phase, and one male. Of the eight Lake Apoyo fish killed at the time of sub-group formation, six were not identifiable as either female or male, although two had begun differentiation as females, as indicated by the presence of perinucleolar oocytes.

DISCUSSION

The results of the current experiments are not consistent with the pattern of development interpreted by Francis (1990) and Francis and Barlow (1993). Those authors concluded that juveniles that were larger than most of their group-mates differentiated as males and those that were smaller as females. In the current experiments, males were not

significantly larger than females in several sub-groups. Because males are typically larger than females at the adult stage (Barlow 1976), the males in these sub-groups must had not yet attained their characteristically large size, indicating that they would have eventually grown larger than the females if the experiments had been allowed to continue.

The alternative hypothesis that greater adult body size in males results from greater post-maturational growth rates was demonstrated in some sub-groups. In the sub-group of large fish formed from the 'hybrid' lineage, females and males were similar in size as juveniles, but as they aged males began to grow faster than females until a significant weight difference was observed between the two sexes. Differential post-maturational growth was most pronounced in fish descended from Lake Nicaragua *A. citrinellus*. In both sub-groups formed from this lineage, significant differences in weight between females and males appeared early in the experiments, and eventually developed into mutually exclusive dimorphisms between the sexes in which the smallest males were larger than the largest females. Faster growth in males may explain why size ranks were not correlated over time in any sub-group. Social control of sex determination did not occur in any sub-group.

The histological analyses revealed no bisexual gonads, although common in sexually labile fish species. Histological observations from individuals killed at the time of sub-group formation revealed that some fish had begun sex differentiation before the division of the groups into sub-groups. However, this is irrelevant – the absence of a difference in body size between females and males as juveniles, and subsequent faster growth in males, refutes the interpretation that sex determination is dependent on relative body size.

The argument could be made that sex was determined by social interactions that occurred before an initial brood was divided into experimental sub-groups. If this had occurred, then those sub-groups containing fish that were relatively large in their initial brood would have contained all males, and those with small fish would have been all females. As in Chapter 3 (Oldfield 2007), this pattern was not observed.

Whereas the fish used by Francis and Barlow (1993) exhibited sexual size dimorphism at 1 year of age after being held in cages in an outdoor heated pool in California, fish in most sub-groups in the current experiments did not, possibly due to the restricted space available in their aquaria, or to low water temperatures in the outdoor tubs. In general, fish in sub-groups that showed the most disparity in body size between females and males by the end of the experiment also attained larger overall sizes than those in sub-groups with little or no disparity. For example, mean weight of females from the pet-trade and 'hybrid' lineages was around 30 g and males around 50 g by the end of the experiments, and these groups showed little disparity in size between females and males. In Lake Nicaragua fish, however, females weighed around 60 g and males around 140 g by the end of the experiment. It seems that the first two groups would have exhibited greater size disparity had they been provided conditions that would have allowed greater growth. The sub-group of large *A. zaliosus* does not fit this pattern as well, as the mean weight of females was 76.6 g and males was 132.5 g, yet at no point were males significantly larger than females. The *A. zaliosus* were maintained at the smallest sub-group sizes and were held in the smaller aquaria for a longer period than the Lake Nicaragua *A. citrinellus*. These conditions may have been more defensible by large males and facilitated aggression-induced growth depensation in smaller males. In fact, in

sub-groups that failed to show differences in weight between females and males, variance in body weight among males was very high – much higher than the variance observed among females in the same sub-groups and higher than both sexes in sub-groups in which males were larger than females. These large variances in body weight suggest that large males suppressed growth in smaller males.

Isolated fish were expected to differentiate as females, but as reported previously (Oldfield 2007, Chapter 4) isolation did not affect sex determination. After being placed in isolation both females and males experienced exponential compensatory growth (Ali et al. 2003) compared to group-held fish. Rapid growth was also observed in the largest individual in each experimental group of six fish in Chapter 4 (Oldfield 2007). When an individual is free to grow uninhibited by the presence of larger, dominant individuals, growth is rapid. Interestingly, isolated females had growth rates similar to isolated males. Also, there were no differences in hunger between females and males. It seems that intrinsically, growth rates are similar between females and males, but under group conditions the presence of males suppresses female growth after the onset of sexual development.

The patterns of growth observed in the nine sub-groups described here, created by dividing broods from four different lineages, strongly indicate that sex is not affected by social conditions in the Midas cichlid species complex. Larger adult body size in males than in females is the result of faster growth after the onset of maturity. This is consistent with the findings that young juvenile Midas cichlids do not exhibit an association between sex and body size either in Lake Apoyo, Nicaragua (Oldfield et al. 2006, Chapter 4) or in small groups in the laboratory (Oldfield 2007, Chapter 3). It is also

consistent with growth patterns observed in a population of the closely related Mayan cichlid ('Cichlasoma' *urophthalmus*) introduced into Florida. In this species there is no difference in body size between females and males at one year of age. In subsequent years, males become increasingly larger than females (Faunce et al. 2002). In medaka, Magnusson (1962) observed that females and males were similar in size until the onset of maturity, when females began to grow faster than males, which resulted in the sexual size dimorphism typical of that species. Sex in Midas cichlids is most likely determined genetically, as it is in the closely related convict cichlid, *Archocentrus nigrofasciatus* (George and Pandian 1996).

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Appendix A. Significance of differences between the weight distributions of males and females at different times throughout the experiments. Day numbering began on the first day free-swimming, except for *A. zaliosus* from Lake Apoyo, in which numbering began at the time of sub-group formation.

Pet-trade lineage – 'small' sub-group

Day	185	221	252	286	315	346	380	416	444	469
Mann-Whit. U	48.0	46.5	47.5	51.5	59.0	50.0	50.0	52.0	48.0	47.0
Z	-0.826	-0.921	-0.855	-0.591	-0.098	-0.689	-0.689	-0.558	-0.821	-0.886
p (two-tailed)	0.409	0.357	0.393	0.554	0.922	0.491	0.491	0.577	0.412	0.375

Pet-trade lineage – 'large' sub-group

Day	185	221	252	286	315	346	380	416	444	469
Mann-Whit. U	94.0	96.0	96.0	95.0	97.5	97.0	82.0	73.0	63.0	54.0
Z	-0.161	-0.069	-0.069	-0.115	-0.000	-0.023	-0.714	-1.129	-1.589	-1.795
p (two-tailed)	0.872	0.945	0.945	0.908	1.000	0.982	0.475	0.259	0.112	0.073

Isolates

Day	182	314	365	403	423
Mann-Whit. U	11.0	3.0	0.0	0.0	0.0
Z	-2.004	-2.848	-3.162	-3.162	-3.162
p (two-tailed)	0.045	0.004	0.002	0.002	0.002

'Hybrid' lineage – 'small' sub-group

Day	228	270	299	331	364	401	432	452
Mann-Whit. U	52.0	61.5	65.5	70.0	62.5	45.0	48.0	42.0
Z	-1.134	-0.581	-0.348	-0.087	-0.522	-1.536	-1.362	-1.426
p (two-tailed)	0.257	0.561	0.733	0.931	0.602	0.125	0.173	0.154

'Hybrid' lineage – 'large' sub-group

Day	228	270	298	331	367	401	432	452
Mann-Whit. U	54.0	56.0	44.0	43.0	37.0	19.5	17.0	15.0
Z	-0.388	-0.258	-1.033	-1.097	-1.485	-2.615	-2.776	-2.905
p (two-tailed)	0.698	0.796	0.301	0.272	0.138	0.009	0.006	0.004

Lake Nicaragua lineage – 'small' sub-group

Day	155	207	252	313	366	419	434
Mann-Whit. U	17.0	14.0	13.0	4.0	10.0	0.0	0.0
Z	-1.710	-1.947	-2.028	-2.758	-2.271	-3.082	-3.083
p (two-tailed)	0.087	0.052	0.043	0.006	0.023	0.002	0.002

Lake Nicaragua lineage – 'large' sub-group

Day	155	207	252	313	366	419	434
Mann-Whit. U	59.5	38.5	12.5	2.0	0.0	0.0	0.0
Z	-1.195	-2.284	-3.634	-4.178	-4.282	-4.282	-4.283
p (two-tailed)	0.232	0.022	<0.001	<0.001	<0.001	<0.001	<0.001

Lake Apoyo lineage – 'small' sub-group

Day	1	53	107	169	228	269
Mann-Whit. U	17.5	22.0	21.0	22.0	21.0	20.0
Z	-0.667	-0.067	-0.200	-0.067	-.200	-0.333
p (two-tailed)	0.505	0.947	0.841	0.947	0.841	0.739

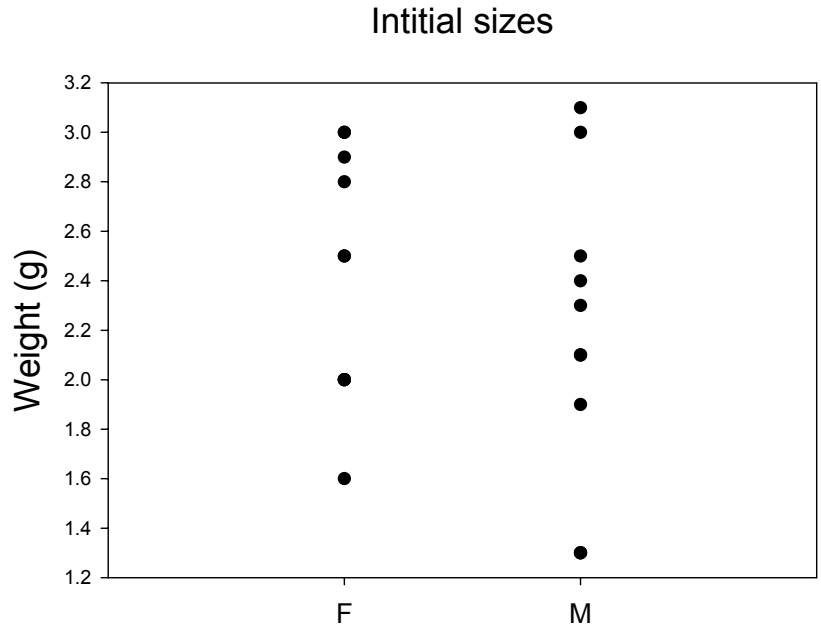
Lake Apoyo lineage – 'large' sub-group

Day	1	53	107	169	228	269
Mann-Whit. U	26.0	25.0	26.0	24.0	20.0	18.5
Z	-0.232	-0.347	-0.231	-0.463	-.926	-1.100
p (two-tailed)	0.817	0.728	0.817	0.643	0.355	0.271

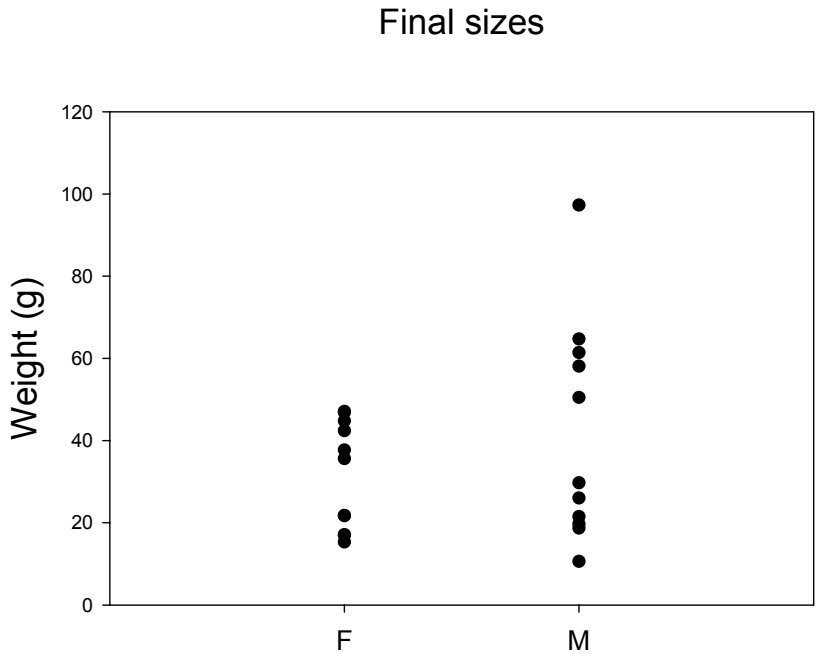
Table 5-1. Correlations of size (body weight) ranks of individuals at the time of sub-group formation with their ranks on the date of termination as determined by Kendall's Coefficient of Concordance (W).

Lineage	Sub-group	n	W	p
Pet trade	small	22	0.000	1.000
	large	27	0.001	0.841
	isolate	17	0.067	0.285
'Hybrid'	small	23	0.019	0.513
	large	23	0.002	0.827
Lake Nicaragua	small	22	0.002	0.827
	large	25	0.007	0.683
Lake Apoyo	small	14	0.024	0.564
	large	15	0.005	0.782

Figure 5-1. Weight distributions of females (F) and males (M) in the sub-group of pet-trade fish that contained small individuals, at the (a) beginning and (b) end of the experiment.

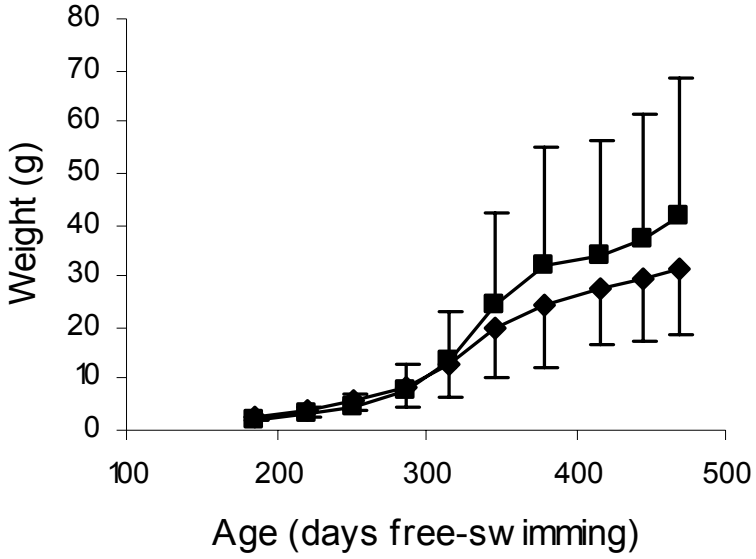


a.

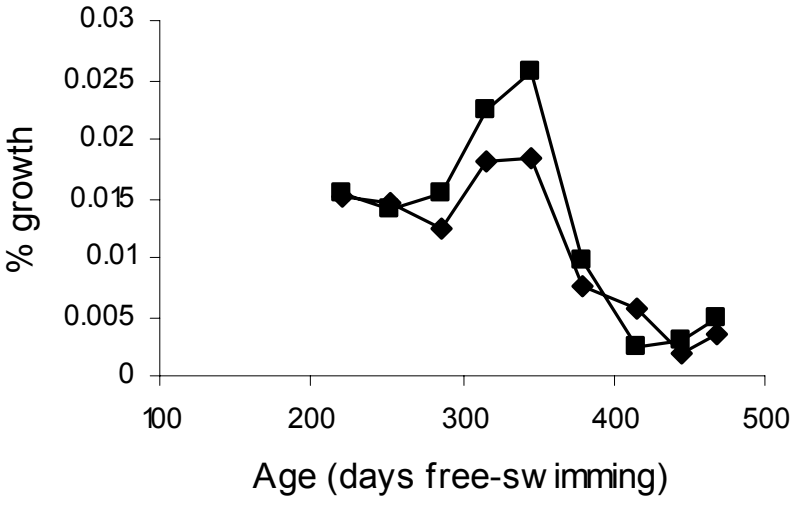


b.

Figure 5-2. Mean (a) weights and (b) growth rates of females (diamonds) and males (rectangles) in the sub-group of the pet-trade lineage that contained small fish. Males were never significantly larger than females (see Appendix A). Bars indicate that standard deviation was greater among males than among females.

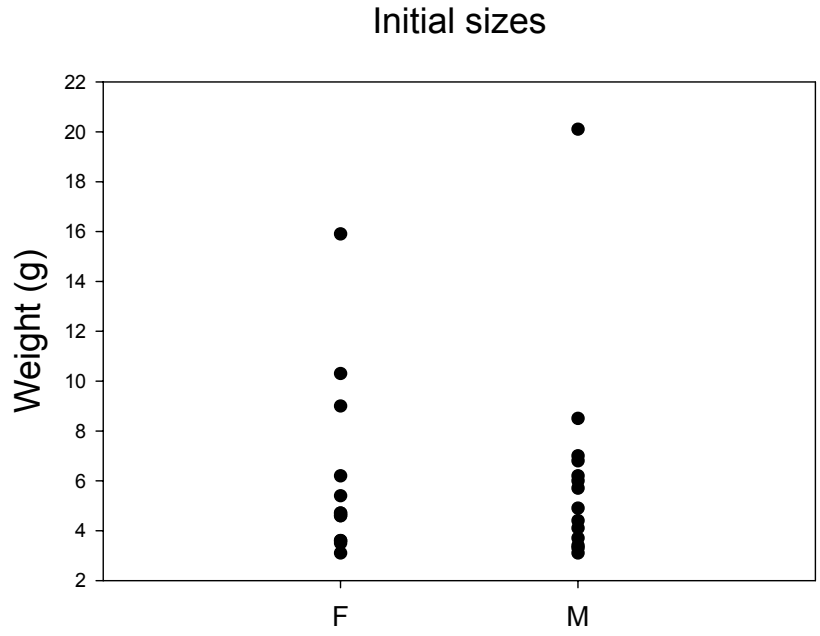


a.

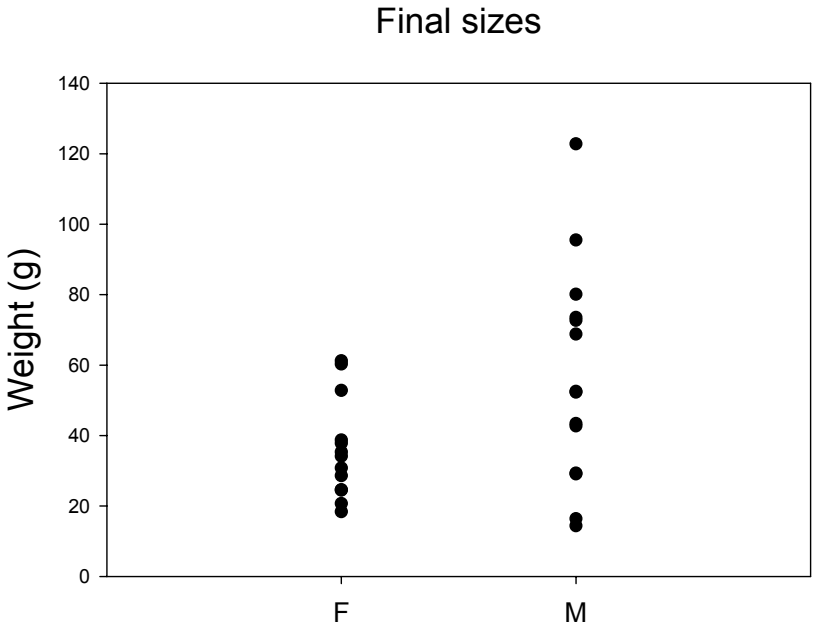


b.

Figure 5-3. Weight distributions of females (F) and males (M) of the sub-group of the pet-trade lineage that contained large fish at the (a) beginning and (b) end of the experiment.

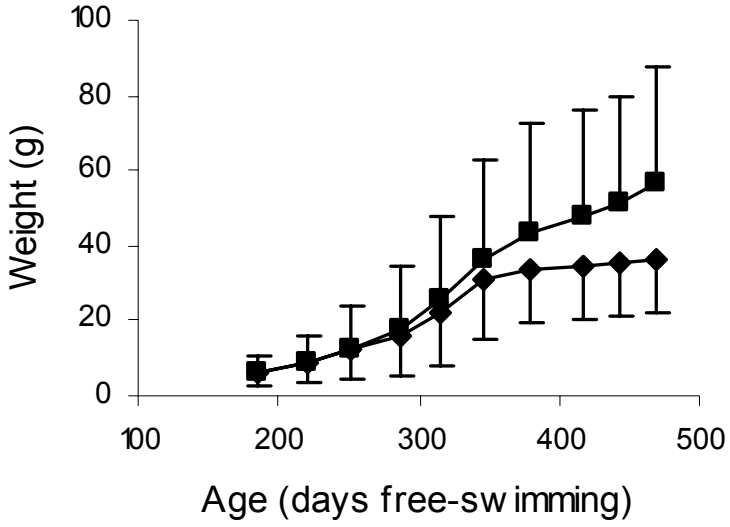


a.

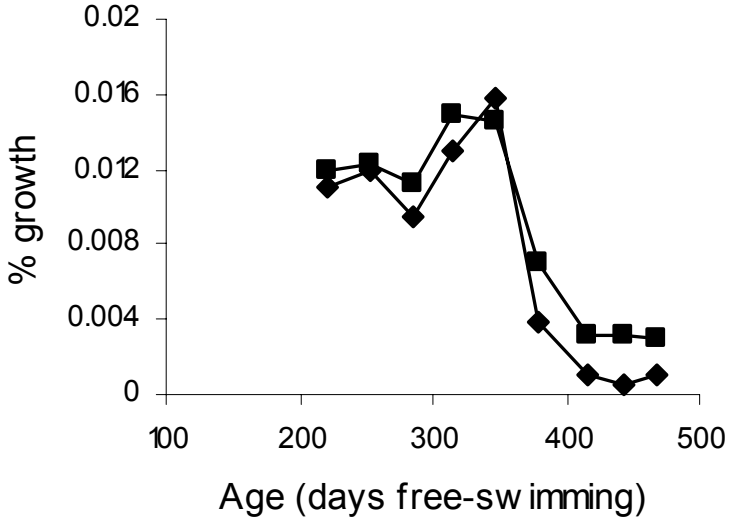


b.

Figure 5-4. Mean (a) weights and (b) growth rates of females (diamonds) and males (rectangles) in the sub-group of the pet-trade lineage that contained large fish. Males were never significantly larger than females, although the difference in weight approached significance toward the end of the experiment (see Appendix A). Bars indicate that standard deviation was greater among males than among females.

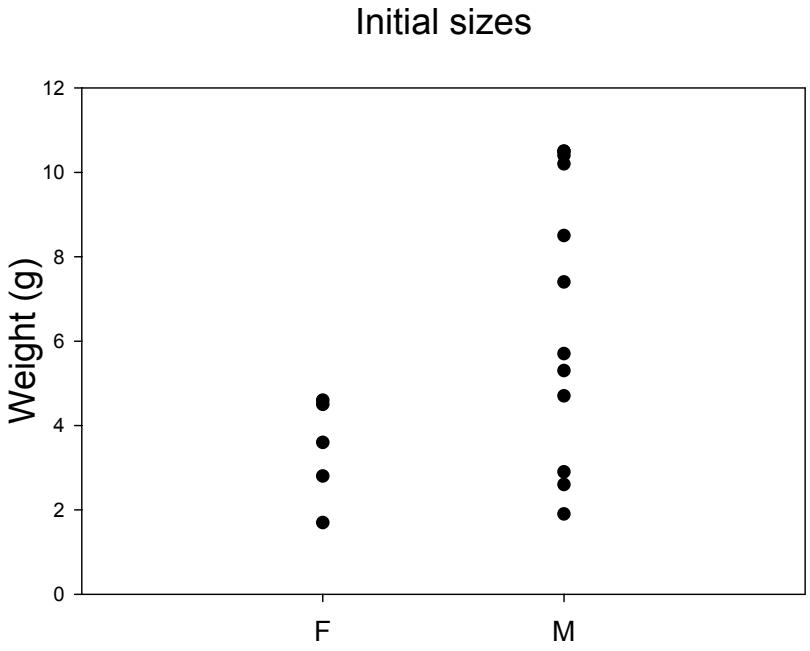


a.

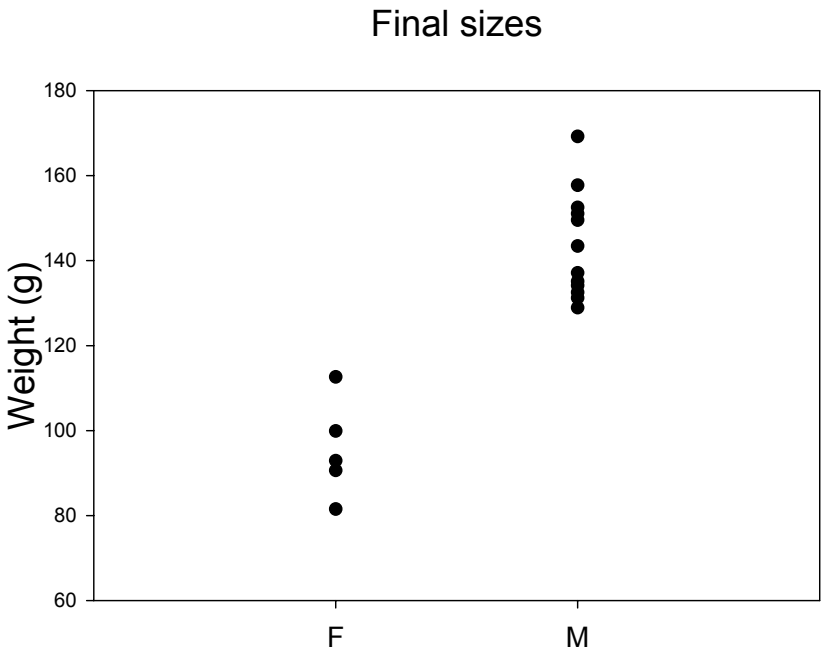


b.

Figure 5-5. Weight distributions of females (F) and males (M) of isolated fish from the pet-trade lineage at the (a) beginning and (b) end of the experiment.

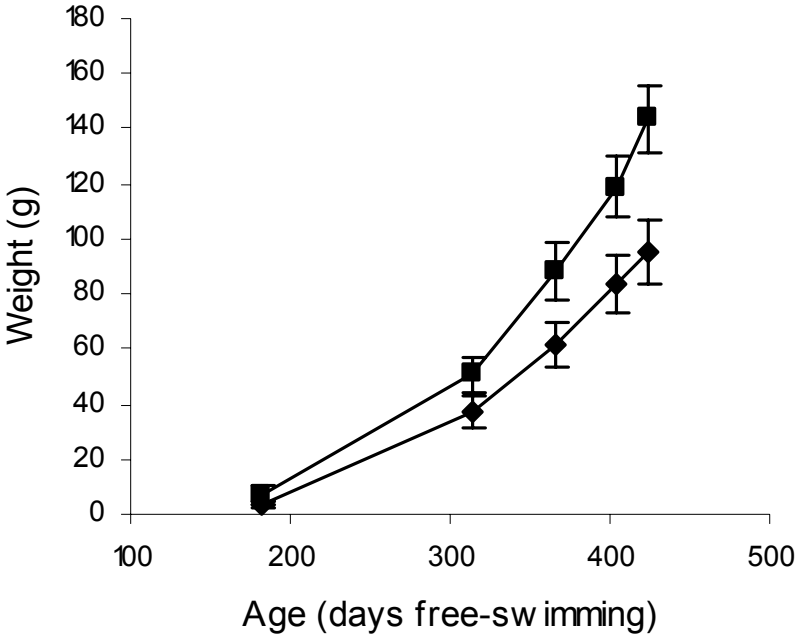


a.

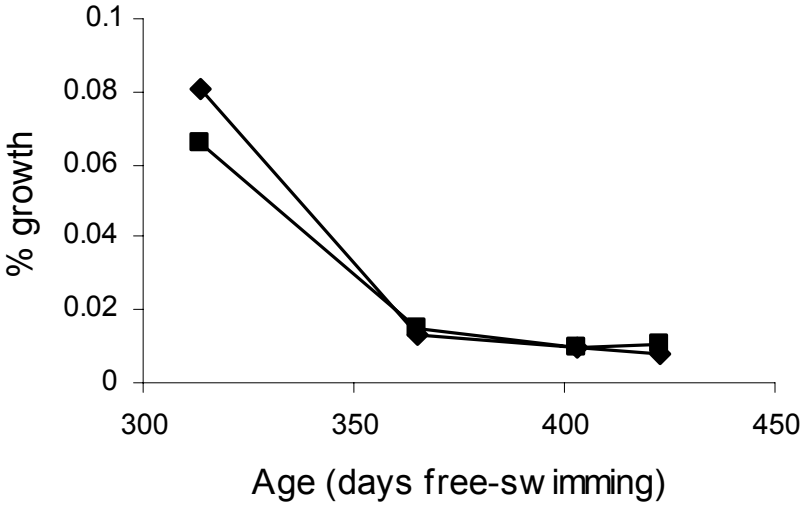


b.

Figure 5-6. Mean (a) weights and (b) growth rates of isolated females (diamonds) and males (rectangles) from the pet-trade lineage. Males were significantly larger than females throughout the duration of the experiment (see Appendix A). Bars indicate standard deviation.

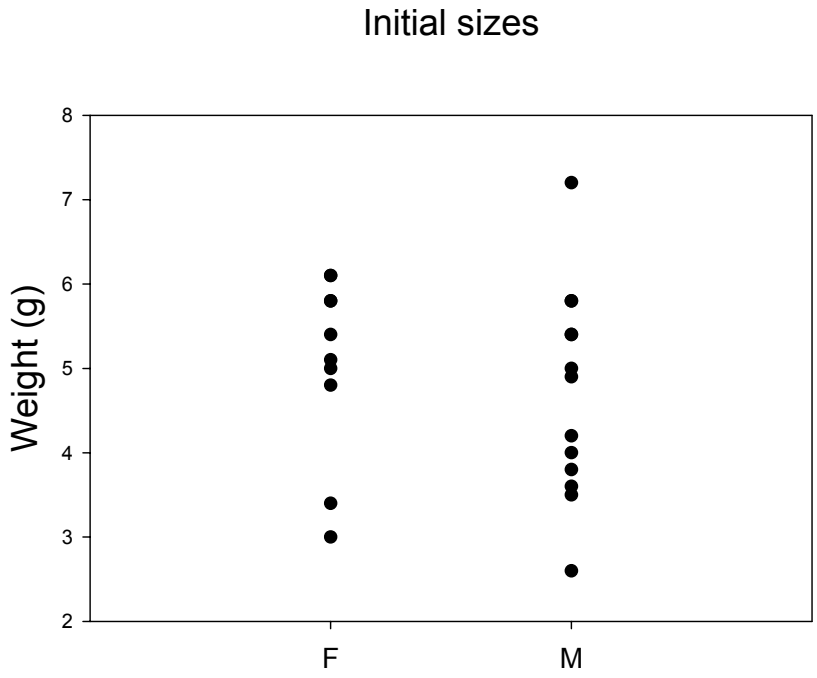


a.

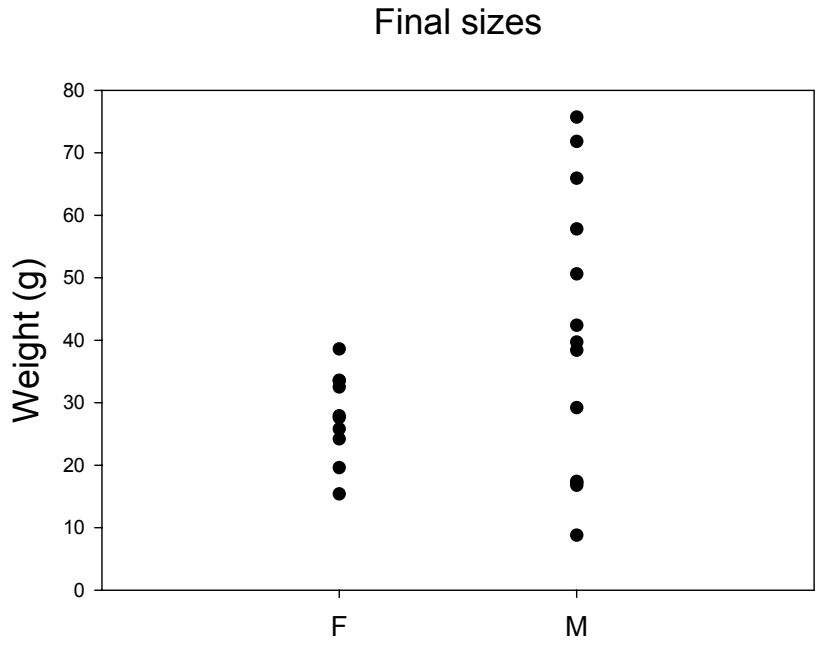


b.

Figure 5-7. Weight distributions of females (F) and males (M) in the sub-group of the 'hybrid' lineage that contained small fish at the (a) beginning and (b) end of the experiment.

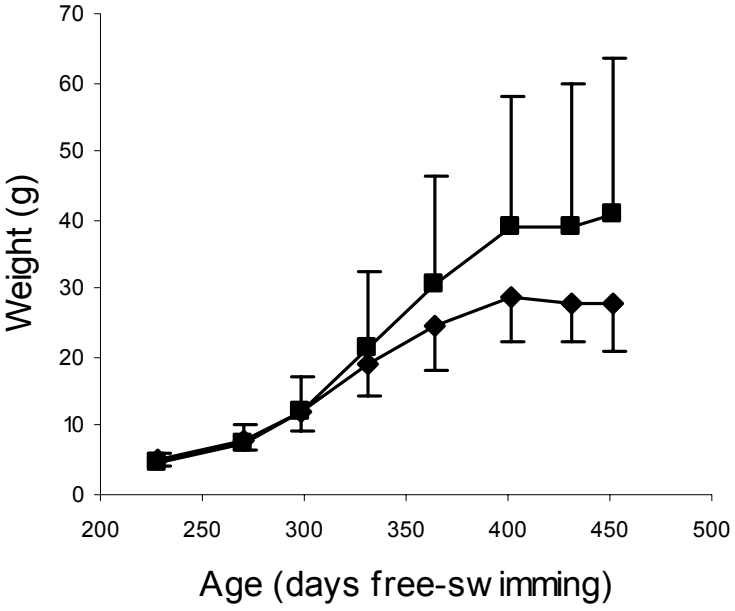


a.

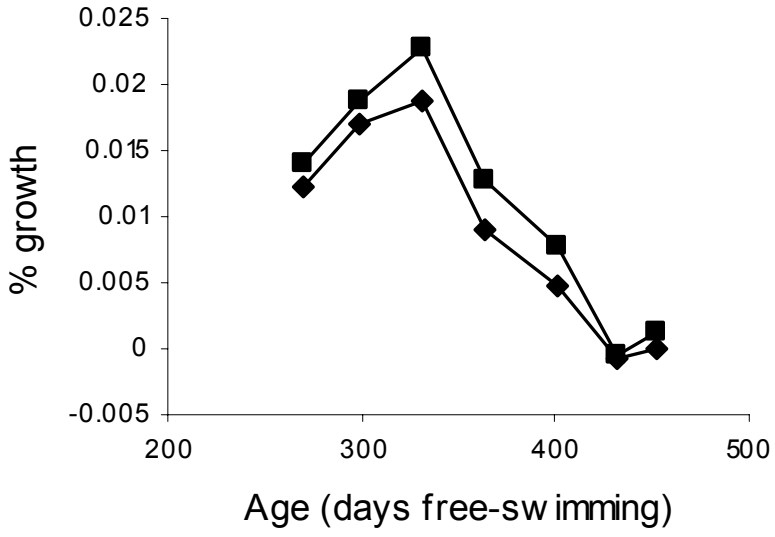


b.

Figure 5-8. Mean (a) weights and (b) growth rates of females (diamonds) and males (rectangles) in the sub-group of the 'hybrid' lineage that contained small fish over the course of the experiment. Males were never significantly larger than females (see Appendix A). Bars indicate that standard deviation was greater among males than among females.

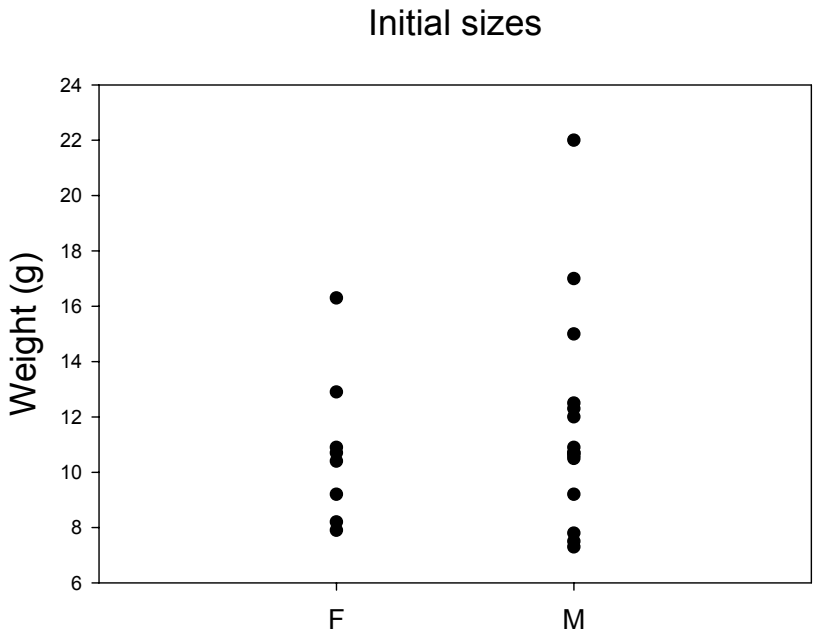


a.

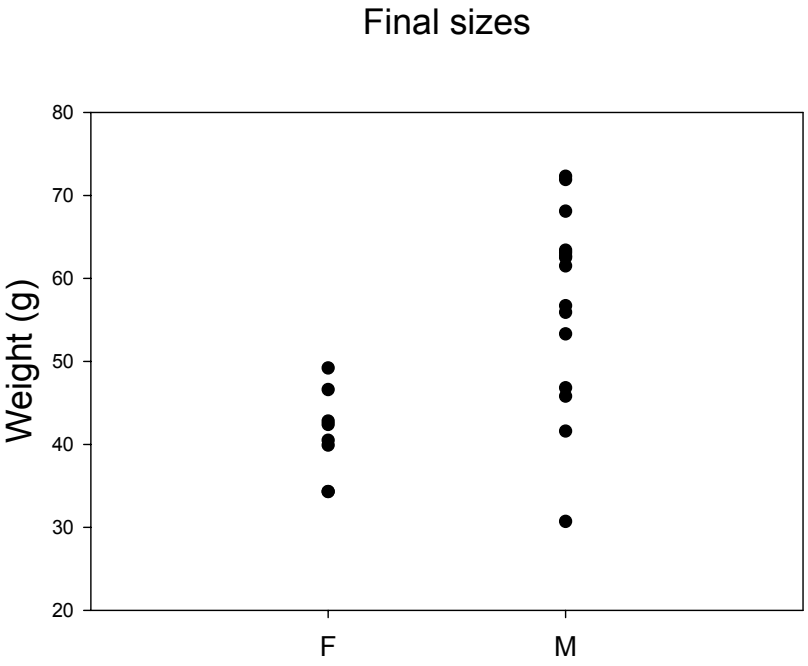


b.

Figure 5-9. Weight distributions of females (F) and males (M) in the sub-group of the 'hybrid' lineage that contained large fish at the (a) beginning and (b) end of the experiment.

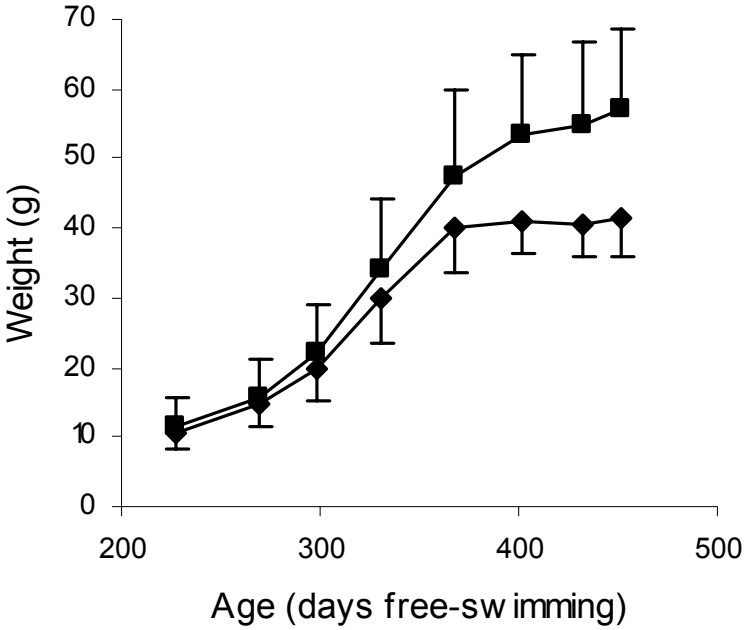


a.

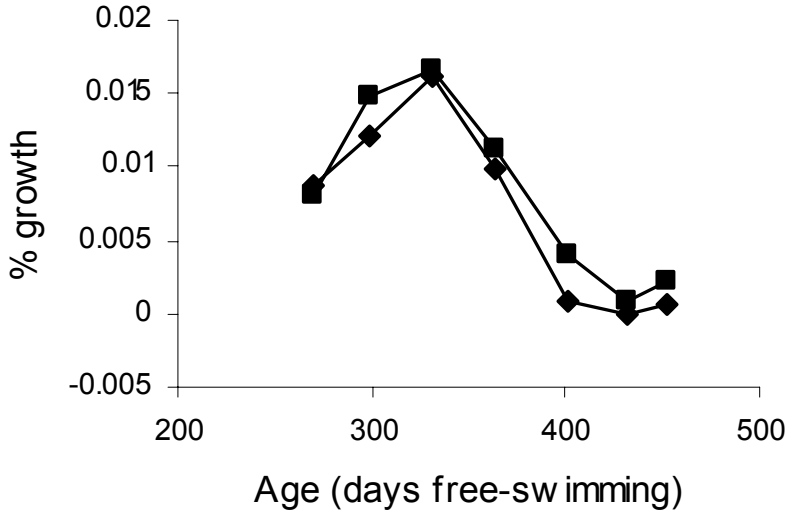


b.

Figure 5-10. Mean (a) weights and (b) growth rates over the course of the experiment of females (diamonds) and males (rectangles) in the sub-group of the 'hybrid' lineage that contained large fish. Males became increasingly more significantly larger than females towards the end of the experiment (see Appendix A). Bars indicate that standard deviation was greater among males than among females.

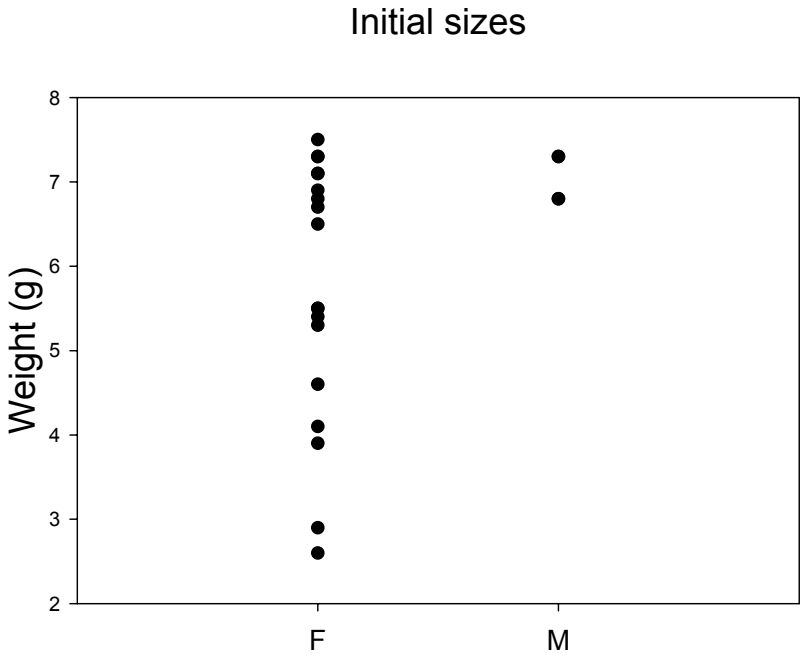


a.

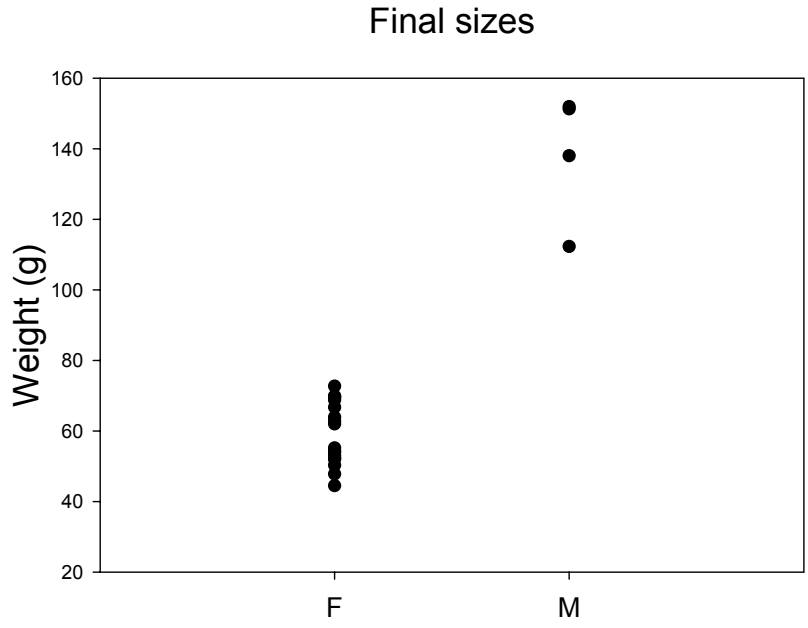


b.

Figure 5-11. Weight distributions of females (F) and males (M) in the sub-group of Lake Nicaragua fish that contained small fish at the (a) beginning and (b) end of the experiment.

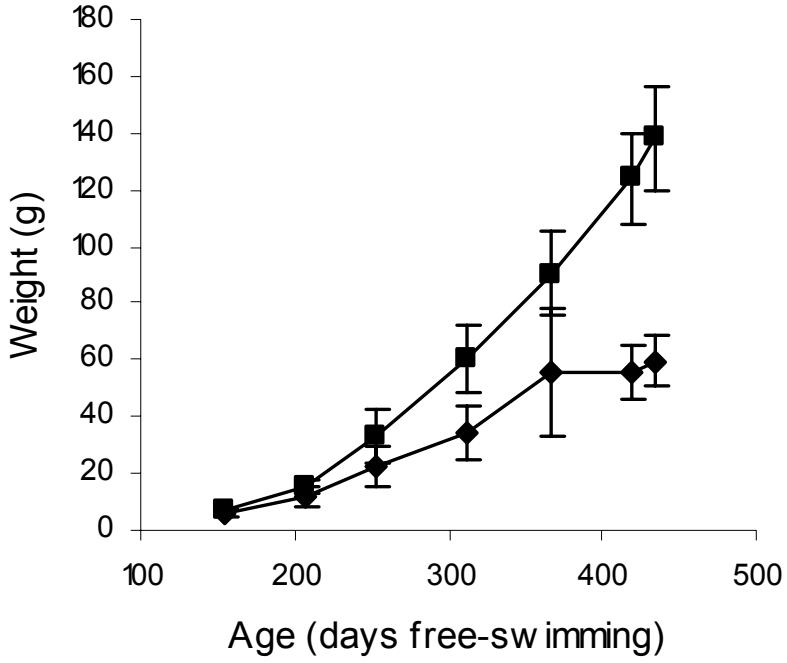


a.



b.

Figure 5-12. Mean (a) weights and (b) growth rates of females (diamonds) and males (rectangles) in the sub-group of the Lake Nicaragua lineage that contained small fish over the course of the experiment. Males became increasingly more significantly larger than females throughout the experiment (see Appendix A). Bars indicate standard deviation.



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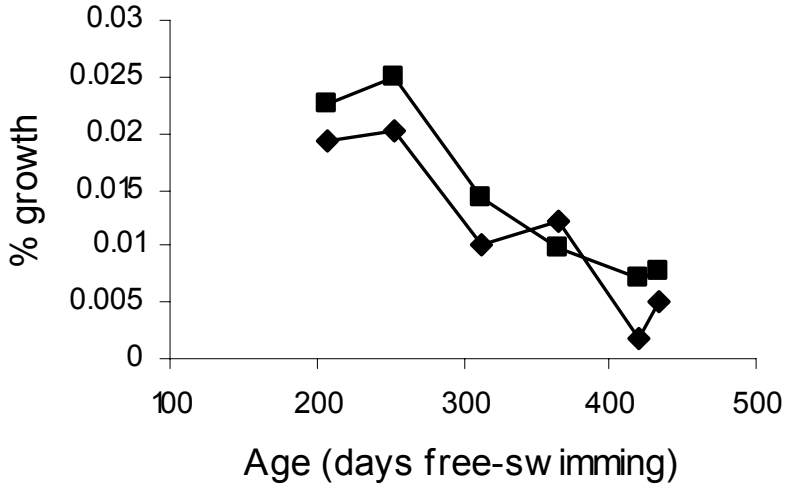
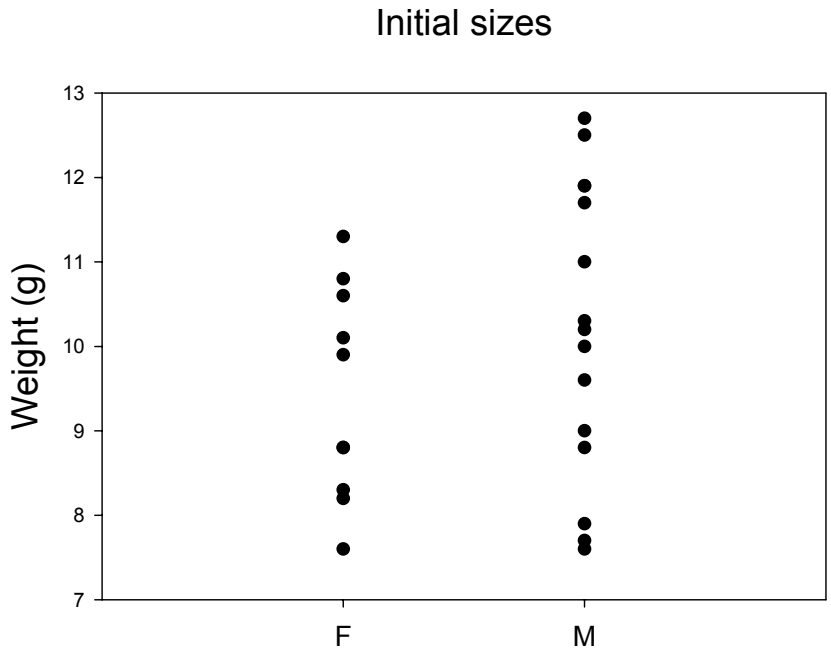
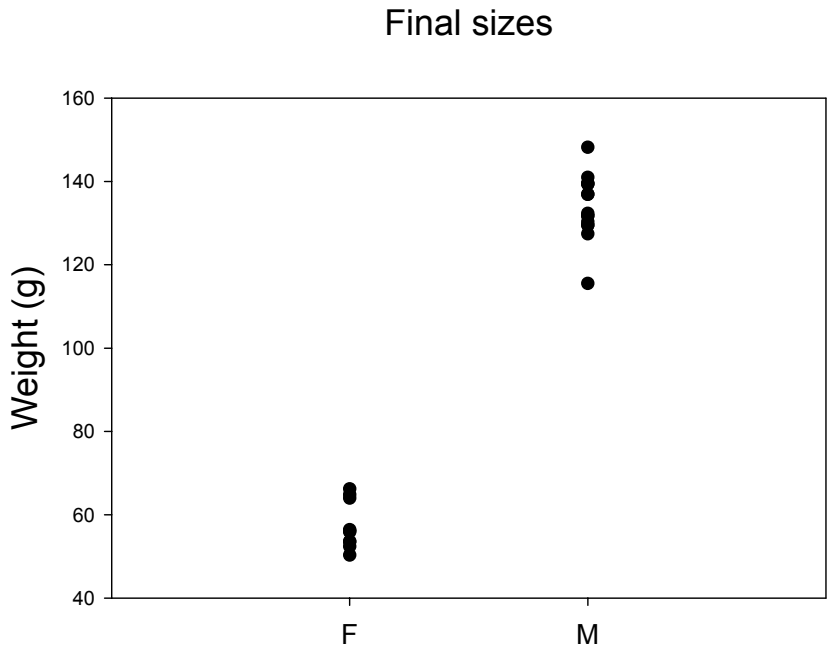


b.

Figure 5-13. Size distributions of females (F) and males (M) in the sub-group of the Lake Nicaragua lineage that contained large fish at the (a) beginning and (b) end of the experiment.

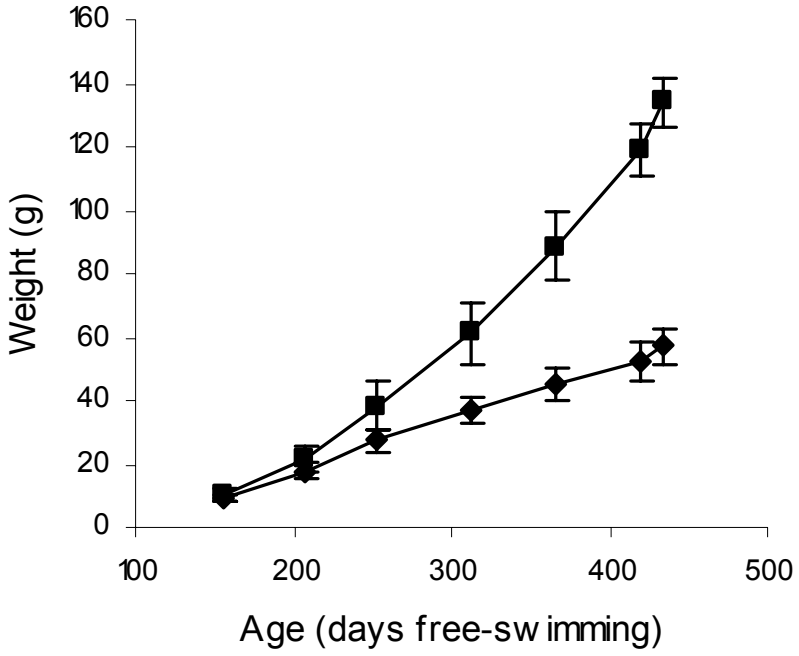


a.



b.

Figure 5-14. Mean (a) weights and (b) growth rates of Lake Nicaragua females (diamonds) and males (rectangles) in the sub-group large fish over the course of the experiment. Males became increasingly more significantly larger than females throughout the experiment (see Appendix A). Bars indicate standard deviation.



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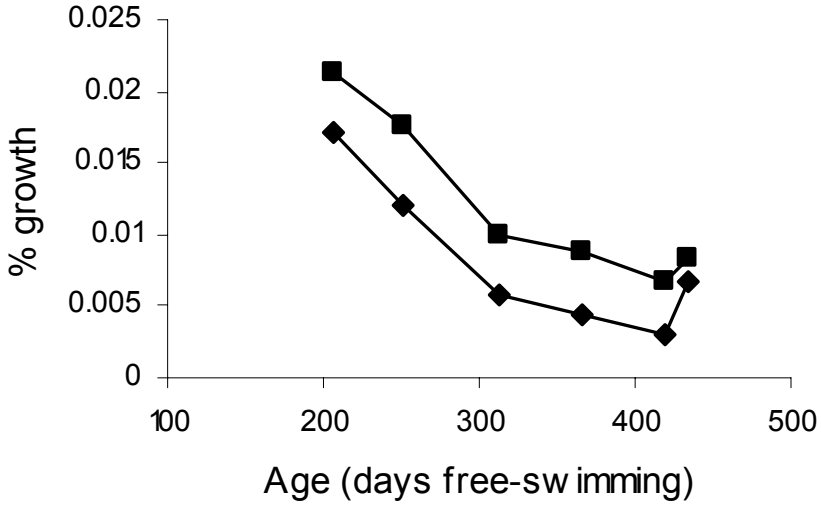
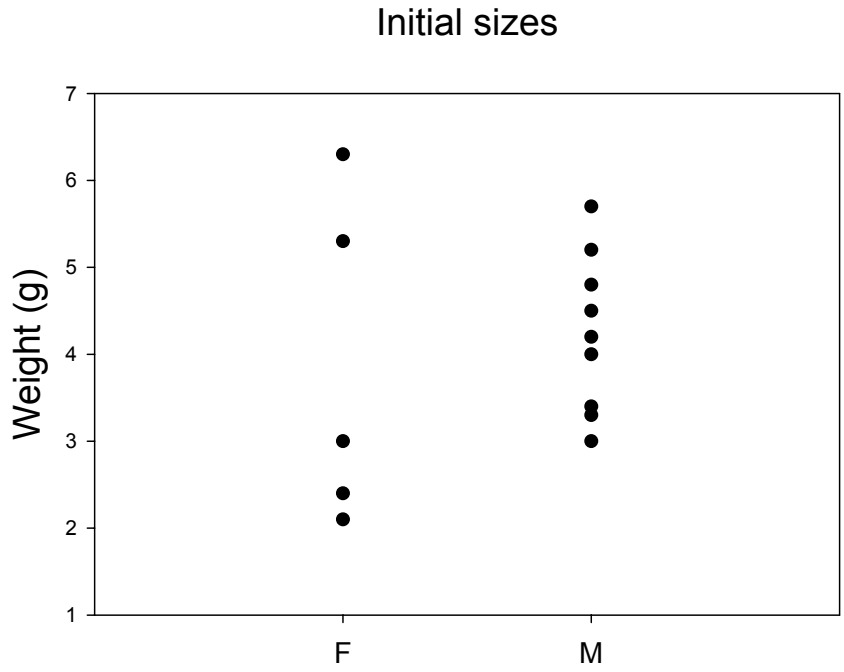
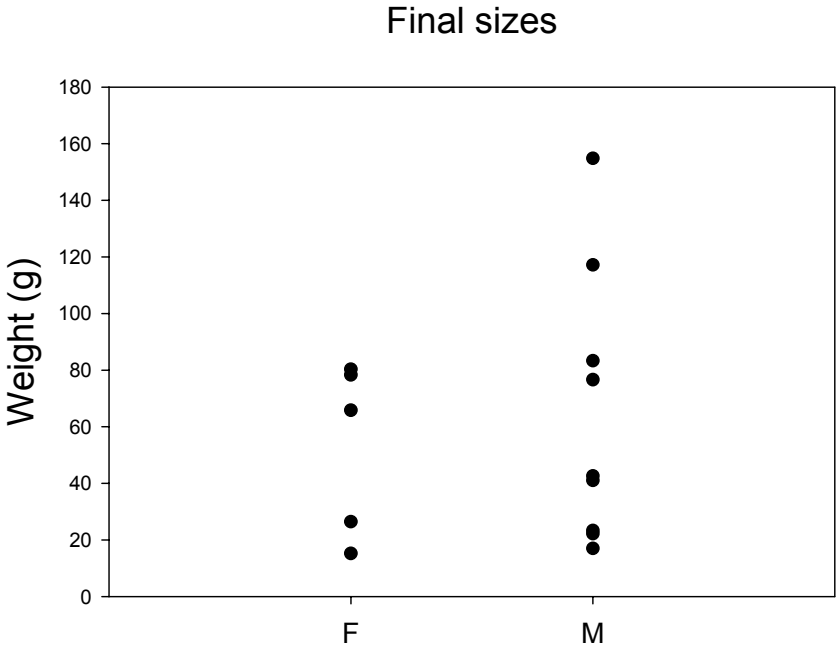


b.

Figure 5-15. Weight distributions of females (F) and males (M) in the sub-group of the Lake Apoyo lineage that contained small fish at the (a) beginning and (b) end of the experiment.

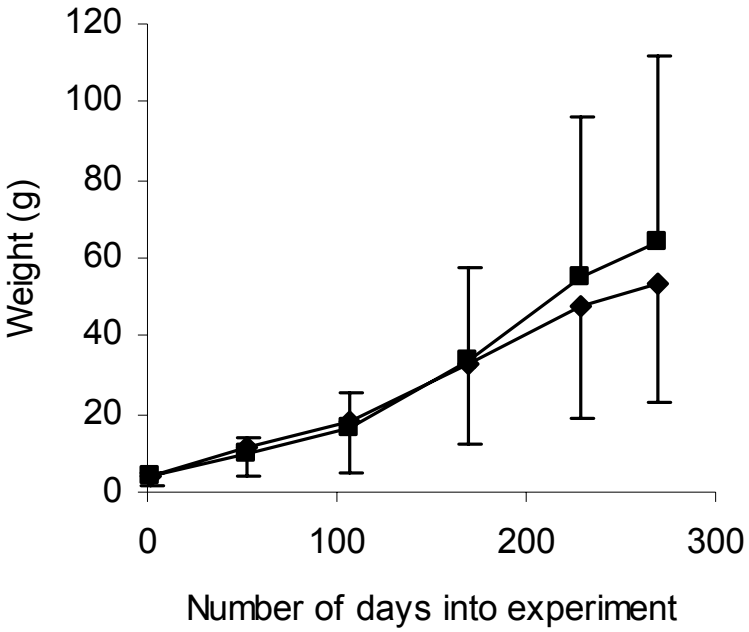


a.



b.

Figure 5-16. Mean (a) weights and (b) growth rates of females (diamonds) and males (rectangles) in the sub-group of the Lake Apoyo lineage that contained small fish. There was never a significant size difference between females and males (see Appendix A). Bars indicate that standard deviation among males was greater than among females towards the end of the experiment.



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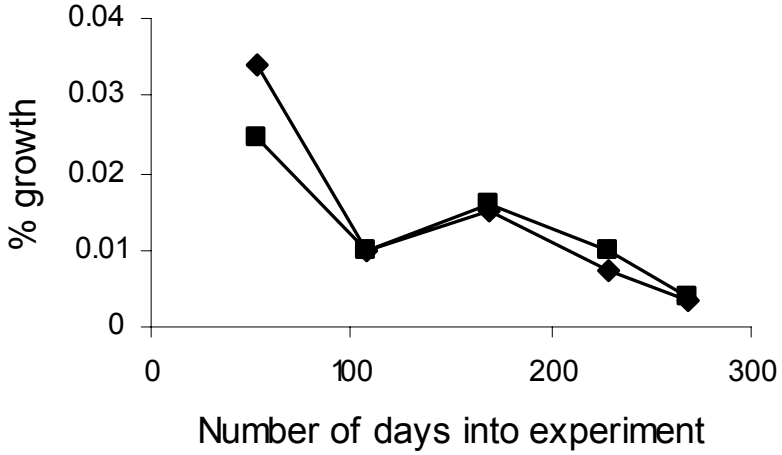
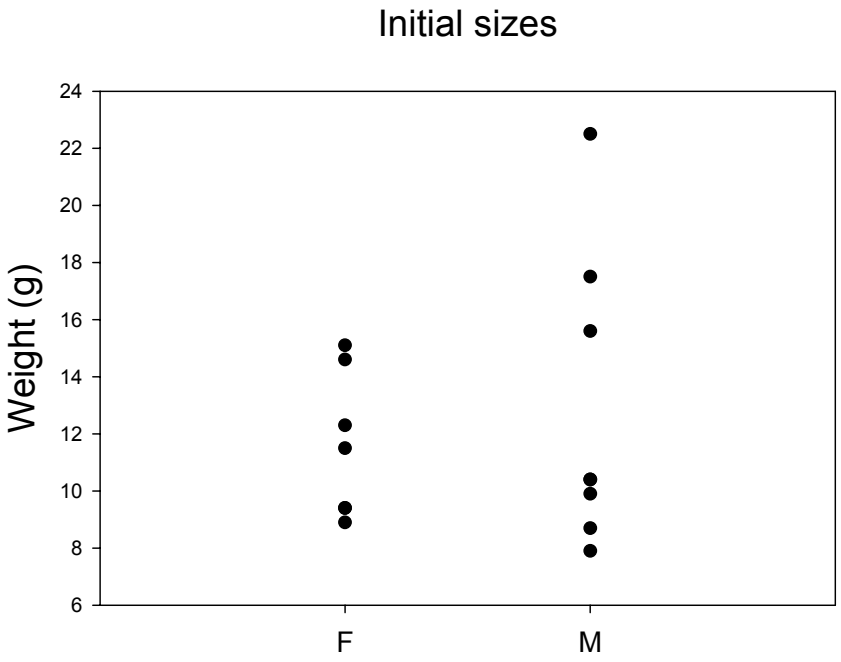
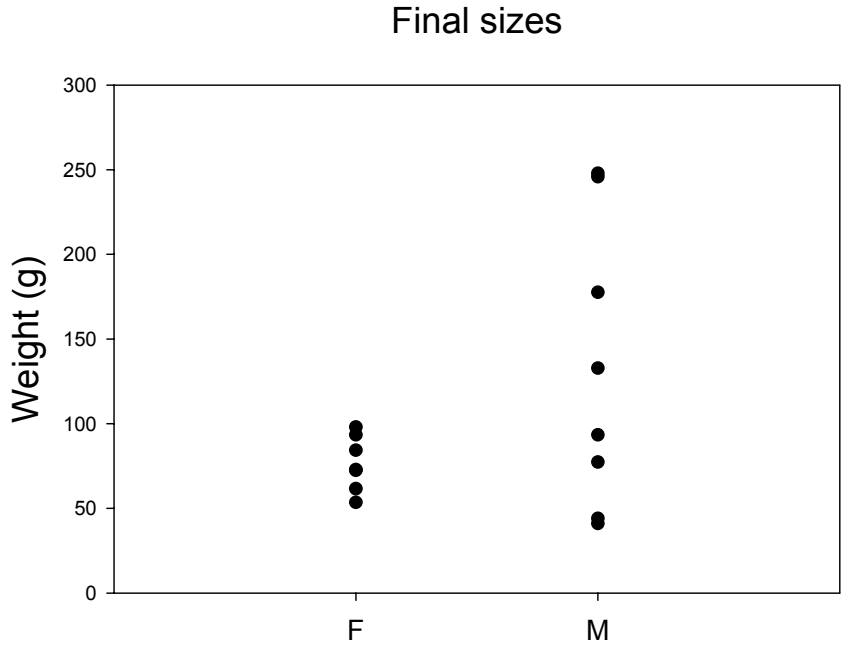


b.

Figure 5-17. Weight distributions of females (F) and males (M) in the sub-group of the Lake Apoyo lineage that contained large fish at the (a) beginning and (b) end of the experiment.

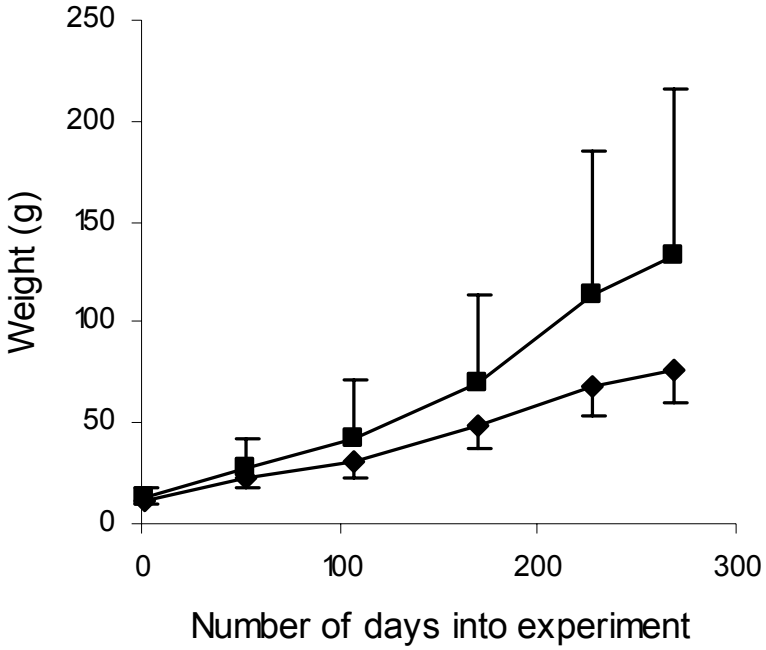


a.



b.

Figure 5-18. Mean (a) weights and (b) growth rates of females (diamonds) and males (rectangles) in the sub-group of the Lake Apoyo lineage that contained large fish. There was never a significant size difference between females and males (see Appendix A). Bars indicate that standard deviation among males was greater than among females.



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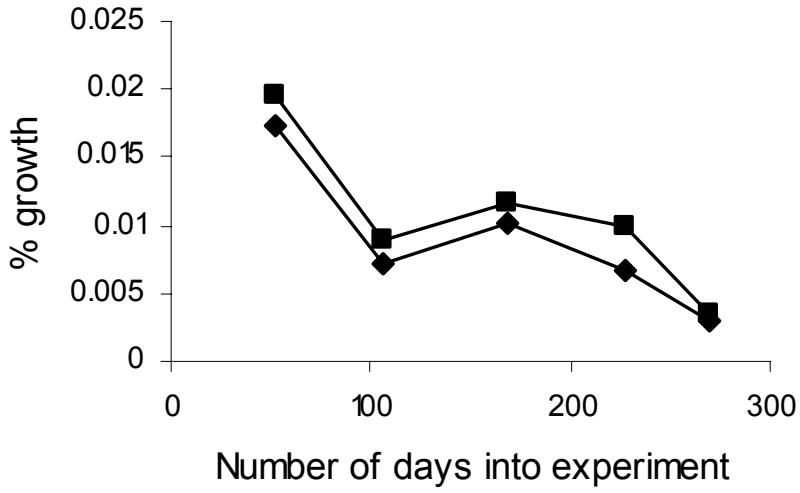
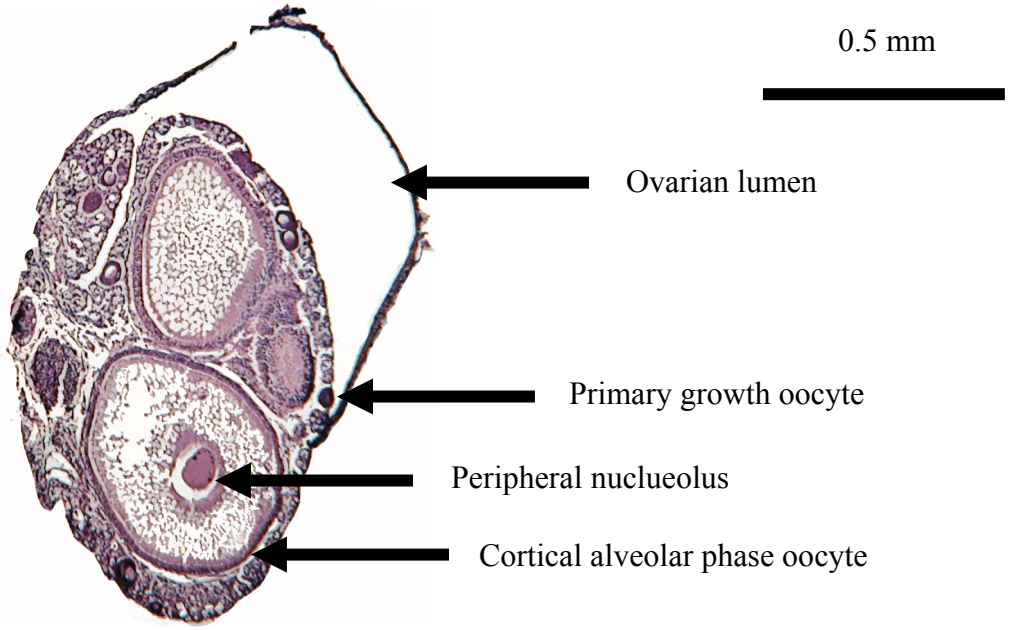
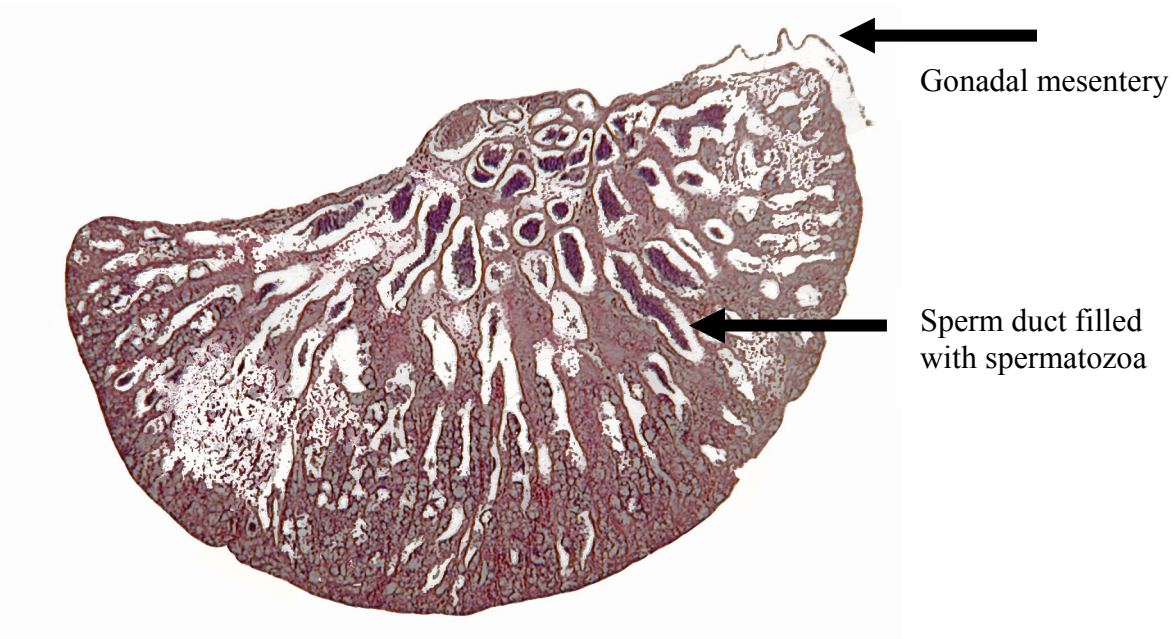


b.

Figure 5-19. Histological structure of an early gonad of a female from the Lake Apoyo sub-group that contained large fish (a), and a gonad of a male from the sub-group of small fish of the 'hybrid' lineage (b).



a.



b.

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CHAPTER 6

GONAD DEVELOPMENT IN MIDAS CICHLIDS AND THE QUESTION OF SEXUAL PLASTICITY

ABSTRACT

Some cichlid fishes differentiate directly as either males or females. In others, males possess testes that contain oocytes, and appear to undergo a prematurational female-to-male sex change. The Midas cichlid, *Amphilophus citrinellus*, has been reported to undergo socially controlled sex determination, which might have evolved from an ontogenetic delay in a prematurational sex change. Gonads of *A. citrinellus* from Lake Masaya, the source of the fish from which the original claims of socially controlled sex determination were based, were analyzed histologically in order to determine the pattern of sex differentiation in this species. Body size distributions were compared between females and males. One individual from the pet-trade that was suspected of post-maturational sex change was also examined. No bisexual gonads were observed. *Amphilophus citrinellus* is a 'differentiated' species, in which ovaries and testes develop directly from undifferentiated gonads. This pattern provides no indication of sexual lability in this species. There was no significant difference in body size between juvenile females and males, but adult males were much larger than adult females. This pattern is not consistent with a hypothesis of socially controlled sex determination, but is consistent with recent studies that have found that large body size in adult males is due to faster

post-maturational growth in males than in females.

INTRODUCTION

Juvenile Midas cichlids, *Amphilophus citrinellus*, have been reported to have sex determined by social interactions (Francis and Barlow 1993), and anecdotal evidence of an exceptional case of protogynous sex change in an adult was described by Barlow (2000). However, a series of investigations involving Midas cichlids from several populations has shown that sex determination is generally not affected by social conditions (Oldfield 2007, Chapter 3, Oldfield et al. 2006, Chapter 4, Chapter 5). In an attempt to find an indication of some form of sexual lability in Midas cichlids, gonads from a series of individuals were analysed histologically.

The presence of both female and male structures in a fish gonad is typically interpreted to indicate sexual lability. For example, remnant oocytes in testicular tissue, sperm sinuses in the testes wall, and remnants of an ovarian lumen have served as the strongest indicators that a fish has undergone protogynous (female to male) sex change, the most common form of hermaphroditism in fishes (Sadovy and Shapiro 1987). Protogynous sex change at the adult stage has been reported in the cichlids *Cichlasoma portalegrense* (Polder 1971), where it is likely unusual, and in *Crenicara punctulata* (Carruth 2000), where it is thought to be typical.

Gonochoristic fishes, those that function as only one sex as adults, can undergo either ‘differentiated’ or ‘hermaphroditic’ processes of gonad differentiation (Yamamoto 1969). In differentiated species, gonads differentiate directly as testes or ovaries. The cichlids *Cichlasoma dimerus* (Meijide et al. 2005) and *Oreochromis mossambicus* and *O*.

niloticus (reviewed by Nakamura et al. 1998) differentiate directly as either males or females. In the hermaphroditic process, gonads undergo a period of bisexuality before maturation. In the zebrafish, *Brachydanio rerio*, gonads of all individuals initially produce oocytes (Maack and Segner 2003), but in genetic males oocyte apoptosis occurs and testicular tissue proliferates before maturity (Uchida et al. 2002). Remant oocytes are often found in the testes of adult males in these rudimentary hermaphrodites (Francis 1992). Non-viable oocytes have been found in the testes of the cichlids *Archocentrus nigrofasciatus* (Polder 1971), 13 species of haplochromine cichlids (Peters 1975), *Satanoperca* aff. *leucosticta* (Loir et al. 1989), and *Pseudotropheus lombardoi* (Naish and Ribbink 1990).

The purpose of the current study was to describe sex differentiation in *Amphilophus citrinellus* and identify either a differentiated or hermaphroditic pattern. Although Francis (1990) reported that *A. citrinellus* remain sexually undifferentiated until at least 12 weeks of age, no histological gonad sections have been published. Social control of sex determination is thought to be an ontogenetic variant of functional sequential hermaphroditism. Therefore, bisexual gonads were expected in *A. citrinellus*. In addition, size distributions of females and males were compared to identify a pattern either consistent with Francis (1990), with juvenile males being larger than females, or consistent with Oldfield (2007, Chapter 3), Oldfield et al. (2006, Chapter 4), and Chapter 5 with no size difference between the sexes at the juvenile stage and faster growth in males after the onset of maturity. I also examined gonads from a captive adult suspected of undergoing protogynous sex change in an attempt to confirm or refute this suspicion.

MATERIALS AND METHODS

Twenty-five *Amphilophus citrinellus* were taken from the 166 specimens that make up lot # 76050 at the California Academy of Sciences (Table 6-1). These fish were originally collected on 14 April 1970 by J. R. Baylis and C. R. Bleick from Rotarians Beach on Lake Masaya, Nicaragua, and were identified by G.W. Barlow. The fish that Francis and Barlow (1993) concluded to be sexually labile were descended from fish caught in Lake Masaya (Barlow, personal communication). The smallest and largest fish were intentionally sampled from the lot along with intermediate-sized specimens. Each specimen was measured for standard length (SL). The fish selected contained both normal and amelanistic and large- and small-lipped morphs. I intended to remove the right gonad from each specimen. However, the fish had previously been dissected on the right side and many of the right gonads were cut. In some cases I removed the left gonad because it was more intact than the right gonad. An attempt was made to remove the gonoduct along with each gonad. Tissues were examined histologically.

One 'gold' morph specimen of unknown origin was purchased as a previously-spawned female from a trusted retailer (Anthony Jones, Toledo, Ohio). This individual apparently changed sex after it courted and mated with another female. The pair produced several batches of infertile eggs over several months until a fertile batch was produced. On 14 April 2003 this suspected hermaphrodite was 188 mm SL and had a mass of 272.3 g. It was killed and its right gonad excised before it was fixed in 10% formalin then transferred to 70% ethanol. Its genital papilla was examined microscopically and compared with those of an adult female and male (UMMZ 188309). Its right gonad was fixed in Bouin's fluid for 22 hours and examined histologically.

For histological analysis gonads were embedded in paraffin. Lake Masaya tissues were sectioned either longitudinally or transversely at 4 μm and the pet trade fish sectioned transversely at 3 μm. Three sections were taken from each of three regions of the gonad: anterior, central, and posterior. stained with hematoxylin and eosin, and examined with a light microscope. After the sex of each fish had been determined, the distribution of female body sizes was compared graphically with the male distribution. Standard lengths were compared between immature females and immature males, and between adult females and males with two-tailed t-tests.

RESULTS

The gonads in both males and females were tubular structures, located beneath the gas bladder, attached by mesentery tissue to the dorsal surface of the body cavity. Progressing caudally, the dorsal wall of the body cavity sloped ventrally until it intersected with the ventral wall of the cavity. The gonads began at the cranial margin of the body cavity. As they neared the ventral surface, mesentery and gametogenic tissue disappeared and the gonads merged to form the gonoduct. The gonoduct merged with the urinary bladder before it traversed the genital papilla and reached the outside of the body.

Female development

All females possessed gonads that contained exclusively ovarian structures. Females generally possessed oocytes at various phases of development, although all but the largest females lacked vitellogenic and mature oocytes (Table 6-2).

The 49 mm SL fish was the second smallest fish sampled, and the smallest for which any sections were recovered. Few oogonia or oocytes were present. An oogonium

possessed a nucleus nearly as dark as its cytoplasm. One nucleolus was present in this oogonium and was darker than the surrounding nucleoplasm. A leptotene stage oocyte appeared similar to an oogonium, although thin threads of chromosomes were visible. No oocytes were observed in the zygotene stage, but one appeared to be leaving the pachytene stage, as its nucleus appeared to contain patches of chromatin. Five nucleoli were near the periphery of the nucleus, typical of the diplotene stage. A thin basement membrane surrounded the oocyte, thecal cells were not apparent.

At 60 mm SL a small lumen was identified in the caudal region of the ovary. The lumen invaded the oogenic tissue, dividing it into lamellae. Zygotene oocytes were identified by a bouquet-like chromosome arrangement. Diplotene oocytes exhibited nucleoli arranged around the periphery of the nucleus (Figure 6-1). Threadlike chromosomes were sometimes visible in diplotene oocytes (Figure 6-2).

Cortical alveoli appeared as spheres in the cytoplasm in oocytes of females 75 mm SL and larger. Distinct granulosa and thecal cell layers were visible in cortical alveoli-phase oocytes (Figure 6-3).

Vitellogenic oocytes were apparent in females as small as 102 mm SL (Figure 6-4), although larger females did not necessarily possess vitellogenic oocytes: The most developed oocytes were at the cortical alveoli phase in females at 103 mm (Figure 6-5) and 104 mm SL, and the perinucleolar phase at 110 mm SL. In the proximal region of the gonad of the 102 mm specimen, most of the oocytes were in the perinucleolar stage or earlier, but some were larger and possessed well developed thecal cells and had reached the mature stage, as germinal vesicles were no longer present (Figure 6-6). Eleven mature oocytes were present in the central region. One was in early maturation and showed the

beginning of germinal vesicle breakdown, thecal cell development, and accumulation of lipids into a single large central droplet. At 116 mm SL, most oocytes were pachytene or diplotene, but one was at the onset of maturation as indicated by a migrating germinal vesicle. The ovary of the 102 mm SL specimen exhibited one empty follicle.

The largest female was 148 mm SL and possessed a gonad that was much larger than that found in the other females. Many mature oocytes were present. Approximately 90% of the cross section in the middle region was filled with mature oocytes, 5% perinucleolar oocytes, and 5% was either interstitial tissue or unidentified oocytes.

Unidentified amoeboid cells were present in the caudal regions of the gonads of the 82.5 and 99 mm SL females. The tissue appeared as a matrix of connective tissue and open chambers. The amoeboid cells appeared to be emerging from and migrating along its surface (Figure 6-7).

Male development

All males possessed gonads that contained exclusively testicular structures. Testes were organized into lobules surrounded by interstitial tissue. The lobules contained spermatogenic cysts and sperm ducts. Meiosis and spermiogenesis occurred within the spermatocysts.

The smallest individual identified as male was the 68.5 mm SL specimen. The gonad was small. The cranial sections consisted mostly of connective tissue and small unidentified cells. A few spermatogonia and spermatocytes were observed, although there were no well-developed spermatocysts. Tissue was organized into chambers that appeared to be rudimentary seminiferous lobules. In the central region of the gonad one small chamber containing about 24 spermatozoa was observed. Spermatozoa appeared as

small, dark-staining, round structures without visible tails. A 79 mm SL male had a small testis at a similar stage of development, containing small patches of spermatozoa and small patches of spermatocytes, but no well-developed spermatocysts. Amoeboid cells such as in the female specimens were also observed in this specimen.

Additional signs of spermatogenesis were observed in males larger than 79 mm SL. At 85 mm SL, one spermatocyst was filled with pachytene spermatocytes, some of which appeared to be leaving the zygotene stage. Zygotene spermatocytes exhibited bouquet chromosomes as described for oocytes (Figure 6-8). Interstitial cells occurred between lobules. At 86 mm, several early spermatocysts appeared in the caudal region of the gonad. Many were filled with small nucleated type B spermatogonia. Type A spermatogonia were isolated cells, embedded within the walls of the lobules. Spermatocytes were found in groups within spermatocysts and were synchronized in development (Figure 6-9). Small sperm ducts were present and filled with spermatozoa. Many chambers in the caudal sections enclosed spermatids along with free spermatozoa, others were empty (Figure 6-10). Spermatids were round cells with round nuclei. Amoeboid cells were also observed in this specimen.

Males 149 mm SL and larger were mature and possessed active spermatocysts and well-developed ducts filled with many spermatozoa (Figure 6-11). In the cranial sections there was a concentration of large, oblong, vertically oriented sperm ducts and another concentration of smaller lobules with fewer ducts and more spermatocysts.

In the largest specimen (169 mm SL), gonad tissue appeared to have expanded around the mesorchium. The proximal region of the gonad consisted of long strands of mesorchium and open space, surrounded by large sperm ducts positioned perpendicularly

to the mesorchium. Further caudally there were many empty chambers near the centers of the sections. Lobules in the caudal region were sparse and appeared inactive.

In gonads of both sexes, the germinal tissue disappeared caudally toward the gonoduct, leaving only connective tissue interspersed with blood vessels, yellow bodies, mitotic figures, and small unidentified cells. In males, small groups of spermatozoa were sometimes present, but tissue was not organized into lobules and there was no evidence of gametogensis.

Size distributions

A bimodal size distribution was observed in males. The four males in the smaller cluster were classified as immature and the four in the larger cluster mature (Figure 6-12). Ten females were classified as mature. These included the smallest female observed to possess mature oocytes (102 mm SL), all females larger than this individual, and three females that were slightly smaller, but very similar in size to this individual. The remaining six females were classified as immature. There was no significant difference in standard length between immature females and immature males ($t = 1.9522$, $p = 0.09$). In adults, males were much larger than females ($t = 6.3601$, $p < 0.0001$).

Sex change

The individual suspected of protogynous sex change possessed a genital papilla with characters typical of both males and females. Male papillae typically possess only one opening, a urogenital pore, located at the distal end. This opening has a ragged appearance and is used to release urine and sperm. Female papillae possess a similar urinary pore, but they also possess a broad transverse ovipore located between the base and the tip of the papilla that is used to lay eggs. An ovipore seemed to be present on the

specimen suspected of sex change (Figure 6-13).

The gonads were extremely large compared to those of the Lake Masaya specimens. A column of mesorchium extended down to the center of the gonad. In the most cranial sections of the gonad the proximal region consisted of large round sperm-filled ducts. There were many spermatocysts exhibiting all stages of spermatogenesis in the peripheral region. Very little interstitial, undifferentiated, or ambiguous tissue was present. More caudally, the ducts elongated and oriented to the center of the gonad (Figure 6-14a). The area around the central column became an invagination. An increasing amount of undifferentiated tissue appeared throughout the gonad. In the absence of the mesorchium in the most caudal sections, the gonad coiled around itself and formed a large, round, empty lumen (Figure 6-14b). The lumen was lined by a membrane indistinguishable from the typical membranes surrounding the lobules, and the sperm ducts did not open into it. A blood vessel was adjacent to the lumen, apparently part of the inside of the fold. The sperm ducts were smaller and many were empty or contained only few spermatozoa. No oocytes were present anywhere in the gonad.

DISCUSSION

All of the specimens for which gonad sections were recovered were identifiable as either female or male through the use of histological procedures. No bisexual gonad was observed in any individual at any stage of development, and the smallest females and males examined were at the early stages of sex differentiation. This is consistent with the histological patterns in the 28 specimens described by Oldfield et al. (2006, Chapter 4) and the 30 specimens analyzed in Chapter 5. *Amphilophus citrinellus* is therefore a

differentiated species, in which ovaries or testes develop directly from undifferentiated gonads without any intermediate bisexual stages. Body size distributions of females and males also provided no indication of sexual lability, and instead were consistent with previous studies (Oldfield 2007, Chapter 3, Oldfield et al. 2006, Chapter 4, Chapter 5) by indicating that adult males are larger than adult females because males grow faster than females after the onset of maturation, and not because relatively large juveniles differentiate as males.

Superficial configuration of gonads was as described for other cichlids (Polder 1971, Carruth 2000). The progressive emergence of gonadal structures observed was consistent with the pattern of differentiation described by Takashima and Hibiya (1995) and Meijide et al. (2005). In females, egg development begins with the differentiation of oogonia stem cells, which enter the first meiotic division to become primary growth oocytes. Growth phases of oocytes progress from oogonia through chromatin-nucleolus, perinucleolar, cortical alveoli, vitellogenic, maturation, and ovulation phases.

In the chromatin-nucleolus phase the oocyte becomes partially surrounded by granulosa cells and a basement membrane, and a series of chromosomal stages occurs. During the leptotene stage the oocyte appears similar to an oogonium, although thin threads of chromosomes might be visible. The pairing of chromosomes occurs during the zygotene stage and a bouquet distribution is apparent. The chromosomes are gathered to one side of the nucleus and a large nucleolus is sometimes visible at the opposite side. The chromosomes then dissociate and small clumps of chromatin may be visible during the pachytene stage.

The oocyte then leaves the chromatin-nucleolus phase and the pachytene stage

and enters the perinucleolar phase, which is characterized by the pause of meiosis in the diplotene stage of prophase. Several nucleoli are visible around the margin of the nucleus. Distinct granulosa and thecal cell layers become visible. At this point the nucleus is called the germinal vesicle. The oocyte becomes enclosed by a continuous layer of follicle cells.

The cortical alveoli phase begins with the appearance of clear vesicles in the cytoplasm that contain a transparent colloidal material. The contents of the vesicles are released into the perivitelline space at fertilization. Oil droplets may also form around the germinal vesicle.

Most oocyte growth occurs during the vitellogenic phase when large clear lipid droplets and smaller dark yolk globules are deposited in the cytoplasm. Maturation begins when the germinal vesicle migrates to one side of the oocyte and lipids accumulate into a single large central droplet. The germinal vesicle then breaks down and thecal cells show extensive development. Ovulation occurs when the mature oocyte is expelled from the follicle.

As in females, male development followed descriptions provided by Takashima and Hibiya (1995) and Meijide et al. (2005). Cytoplasmic extensions of Sertoli cells surround the germ cells and form the spermatocysts. Type A spermatogonia are isolated cells, embedded in the walls of testicular lobules, individually surrounded by cytoplasmic extentions of Sertoli cells. They undergo mitosis to form type B spermatogonia, at which time the surrounding tissue expands and forms a spermatocyst. Type B spermatogonia undergo mitosis to form primary spermatocytes.

The meiotic stages described above for oocytes are also applicable to primary

spermatocytes. In the current study, stages of spermatocytes were not identified, except for zygotene spermatocytes, which exhibited bouquet chromosomes. Primary spermatocytes undergo the first meiotic division to form secondary spermatocytes. Groups of cells undergoing spermatogenesis are usually found at the same phase of development because their divisions are incomplete; after division they remain bound by cytoplasmic bridges that allow synchronous development.

Secondary spermatocytes undergo the second meiotic division to form spermatids. Spermiogenesis occurs when spermatids become spermatozoa by separating from residual bodies, which retain a large portion of cytoplasm and organelles not essential to the sperm.

Amphilophus citrinellus was expected to exhibit bisexual gonads because it had been described to undergo socially controlled sex determination (Francis and Barlow 1993), which was proposed to be an ontogenetic variant of adult-stage sequential hermaphroditism. Bisexual gonads have been observed in adult cichlids that were thought to have changed sex. Polder (1971) found bisexual gonads in an unusual *Cichlasoma portalegrense* that underwent protogynous sex change. The ovarian tissue was situated in the cranial region of the gonad and the testicular tissue in the caudal region. The largest oocytes showed signs of degeneration, as did many of those of all sizes that were located near testicular tissue. There was no oviduct in the genital papilla, although the musculature was female. *Crenicara punctulata* is thought typically to be a protogynous hermaphrodite. Zupanc (1985) provided a histological gonad section from this species that showed a male gonad with spermatocytes and spermatozoa adjacent to two oocytes. Bisexual gonads have also been observed in many other cichlids. Polder (1971) reported

small oocytes in the gonads of three male *Archocentrus nigrofasciatus*. Peters (1975) found that gonads of males of 13 species of haplochromine cichlids contained oocytes as well as spermatogenic tissue. All male *Satanoperca* aff. *leucosticta* had gonads that contained previtellogenic oocytes as well as male germ cells in all stages of spermatogenesis (Loir et al. 1989). When the gonads of three juvenile *Pseudotropheus lombardoi* were examined, those of the smallest two fish contained developing oocytes and those of the largest contained both oocytes and spermatocytes (Naish and Ribbink 1990). Because all gonads examined in the present study, and in previous studies (Oldfield et al. 2006, Chapter 4, Chapter 5), were exclusively female or male, gonad development more closely resembled development in differentiated cichlid species such as *Cichlasoma dimerus* (Meijide et al. 2005), and *Oreochromis mossambicus* and *O. niloticus* (Nakamura et al. 1998). Adult *O. karongae* and *Tramitichromis intermedius* do not exhibit bisexual gonads and may develop in a similar fashion (Msiska 2002, Harnish 2004).

Body size distributions were not consistent with a scenario in which relatively large juveniles differentiate as males and relatively small juveniles differentiate as females, as was proposed by Francis and Barlow (1993). There was no significant difference in SL between females and males as juveniles. However, this pattern is consistent with juvenile size distributions observed in each of 21 social groups from a series of previous studies (Oldfield 2007, Chapter 3, Oldfield et al. 2006, Chapter 4, Chapter 5). On the other hand, males were much larger than females at the adult stage. This indicates that the adult males attained their larger body size sometime after the juvenile stage. This is consistent with the pattern of faster post-maturational growth in

males than in females that was observed when Oldfield (in prep.) followed growth of individuals over long periods in nine of the above 21 groups.

Examination of the adult suspected of protogynous sex change also failed to confirm sexual lability, as its gonadal structure was exclusively male. The presence of oocytes, a lumen, and sperm sinuses in the wall of the testes have been used to identify adult stage protogynous sequential hermaphroditism in fishes (Sadovy and Shapiro 1987). No oocytes were present in the gonad of the 'sex-changed' specimen, and neither were sperm sinuses. Sperm ducts were centrally located and occupied large portions of the transverse sections of the gonad. A lumen was present in the individual suspected of sex change, but it did not resemble lumina typical of ovaries in *A. citrinellus*. A typical ovarian lumen forms at the periphery of the gonad and its space divides the ovarian tissue into distinct lamellae. Lumina have been reported in males of another cichlid, *Cichlasoma portalegrense*, although in that species the sperm ducts emptied into the lumen (Polder 1971). The lumen in the 'sex-changed' specimen had a smooth border with no indication of previous lamellae or connections to efferent ducts. It appeared to be a non-functional result of overgrowth and fusion of testicular tissue, possibly due to the plentiful supply of food and continuous breeding season offered under captive conditions.

The specimen suspected of undergoing protogynous sex change did however appear to have a genital papilla with characters typical of both females and males. The structure of genital papillae in *A. citrinellus* have previously been described (Barlow 1976), and the papillae observed in male and female museum specimens in the current study were consistent with these descriptions. Although the tip of the genital papilla of the 'sex-changed' fish was pointed as in typical males, there appeared to be an ovipore

typical of females. This opening was very difficult to discern, although it also appeared to be present on a few individuals examined previously (Chapter 5). Although the specimen came from a trusted source, the possible presence of a single female character is insufficient to demonstrate that it underwent protogynous sex change. It seems more likely that the retailer was incorrect about the fish's initial sex.

Despite reports of sexual lability in *A. citrinellus* (Francis 1990, Francis and Barlow 1993, Barlow 2000), histological observations failed to provide evidence for any form of lability. Neither rudimentary hermaphroditism in the form of pre-maturational sex change nor adult protogynous sex change seem to occur in this species.

ACKNOWLEDGEMENTS

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Table 6-1. Notes on the specimens examined. 'Tag, new' corresponds to the number on a paper tag placed inside the mouth of the specimen. SL = standard length. 'Tag, pre-existing' refers to numbers on tags that were found already attached to body. 'Notes' describe peculiar features of the specimens. Almost all individuals were of the small-lipped morphology, but a few had large lips. Two specimens examined had their color bleached out on one side of the body, seemingly a result of the preservation process rather than natural amelanism. For body color, N = normal, barred coloration, while G = the oligomelanic 'gold' color. 'Gonad side' refers to the side of the body from which the gonad was taken. As many of the gonads had been previously removed and replaced this was not always certain. 'Sex ext.' refers to sex inferred from external characterists. 'Sex hist.' refers to the sex as identified after histological examination.

Tag, new	SL(mm)	Tag, pre-existing	Notes	Body color	Gonad side	Sex, ext.	Sex hist.
1	160	16		N	R	M	M
2	169	-		N	L	M	M
3	161	11		N	L	M	M
4	149	2	lg. lips	G	L	M	M
5	148	25		N	L	F	F
6	20.5	-		N	?	?	?
7	49	-		N	?	?	F
8	60	-		N	?	?	F
9	68.5	-		N	R	?	M
10	75	-		N	R	?	F
11	68	85	bleach	N	?	?	F
12	82.5	81	bleach	N	L	?	F
13	64	-		N	L	M?	F
14	79	-		N	L	?	M
15	86	-		N	L	M?	M
16	97	-		N	L	F?	F
17	110	-		N	L	F	F
18	103	-		N	L	F	F
19	99	-		N	R	F	F
20	116	-		N	L	F	F
21	109	-		N	L	M	F
22	104	-	lg. lips	G	?	F?	F
23	85	-	lg. lips	N	R	M?	M
24	101	-		N	L	F	F
25	102	-		N	L	F	F

Table 6-2. Structures observed in gonads of *Amphilophus citrinellus* from Lake Masaya, Nicaragua. oo = oogonia, cn = chromatin-nucleolus phase oocyte, pn = perinucleolar (diplotene) oocyte, ca = cortical alveoli oocyte, vo = vitelline oocyte, mo = mature oocyte, l = lumen or lamella, sg = spermatogonium, sc = spermatocyte, st = spermatids, sp = spermatozoa

Female

SL (mm)	oo	cn	pn	ca	vo	mo	l
49	+	+					
60	+	+	+				+
64	+	+	+				+
68	+	+	+				+
75	+	+	+	+			+
82.5	+	+	+	+			+
97	+	+	+	+			+
99	+	+	+	+			+
101	+	+	+	+			+
102	+	+	+			+	+
103	+	+	+	+			+
104	+	+	+	+			+
109	+	+	+	+	+		+
110	+	+	+				+
116	+	+	+	+	+	+	+
148	+	+	+	+		+	+

Male

SL (mm)	sg	sc	st	sp
68.5	+	+		+
79	+	+		+
85	+	+	+	+
86	+	+	+	+
149	+	+	+	+
160	+	+	+	+
161	+	+	+	+
169	+	+	+	+

Figure 6-1. Longitudinal section from Lake Masaya *A. citrinellus* showing immature ovary. Diplotene oocytes in the perinucleolar phase lie adjacent to a large blood vessel. BV = blood vessel, PN = peripheral nucleolus.

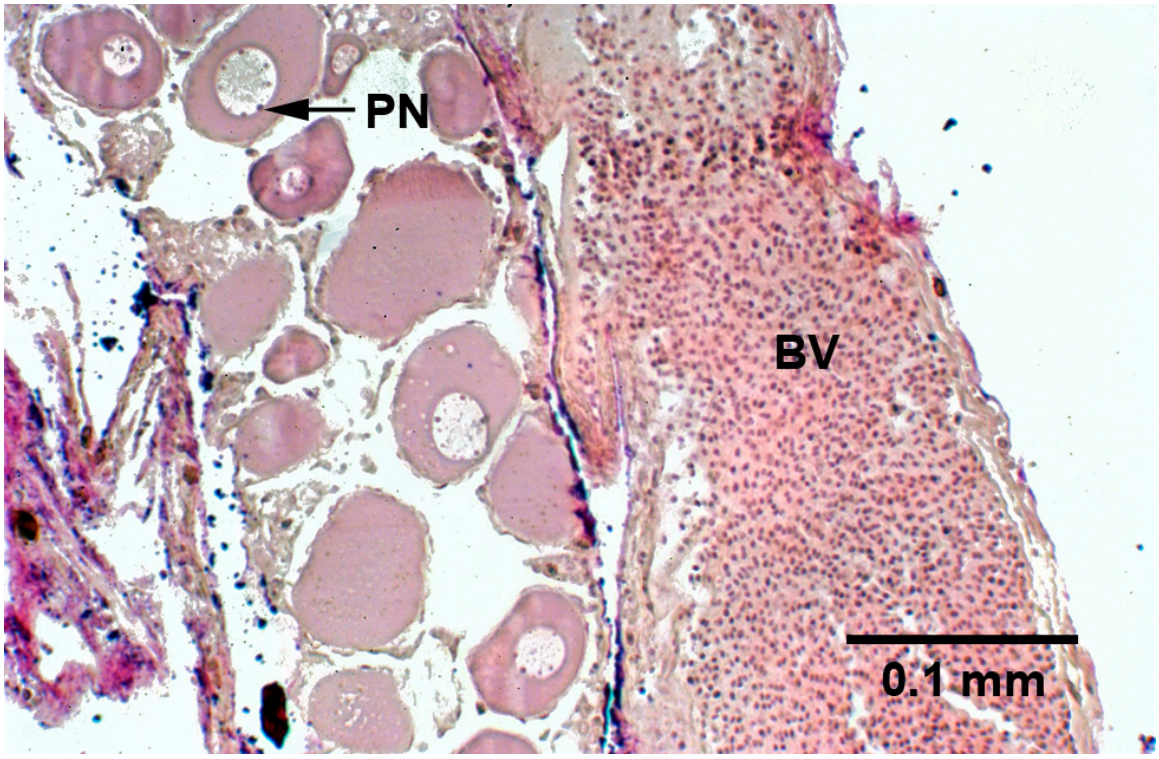


Figure 6-2. Perinucleolar (diplotene) oocytes with chromosomes visible. CH = chromosomes, PN = peripheral nucleolus.

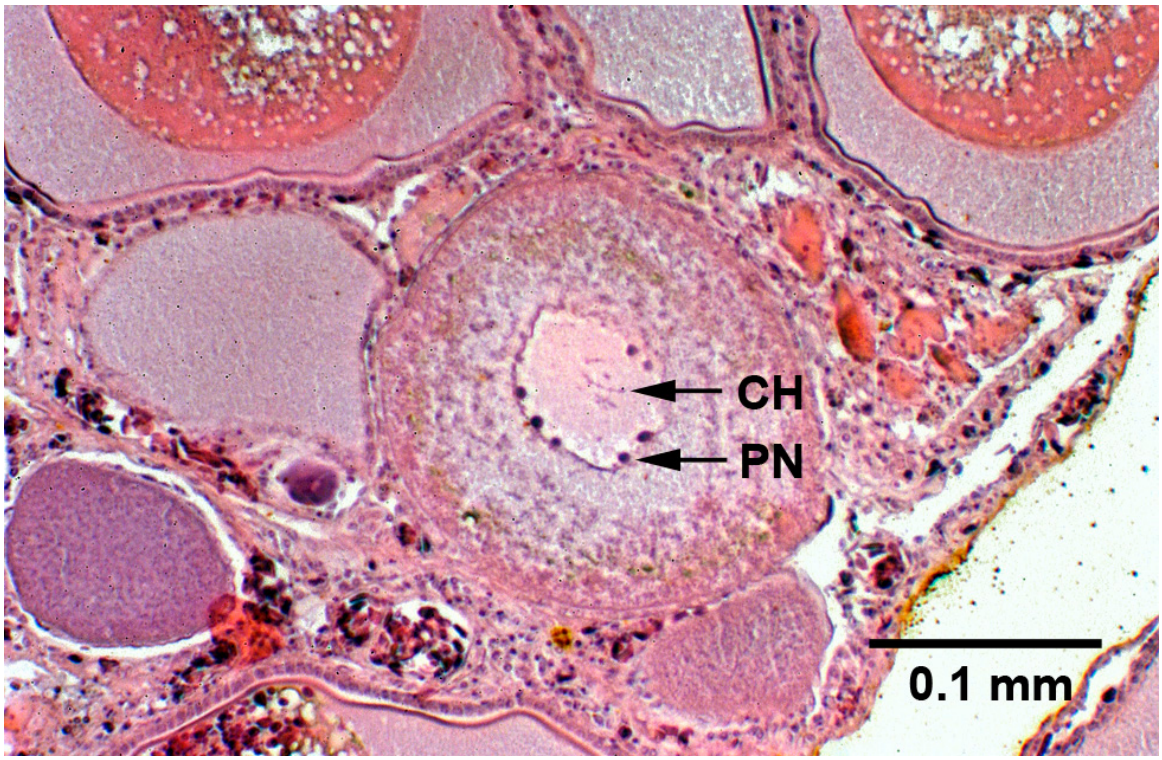


Figure 6-3. Cortical alveolar oocytes with chromosomes visible. CA = cortical alveolus, CH = chromosomes, CM = cell membrane, G = granulosa.

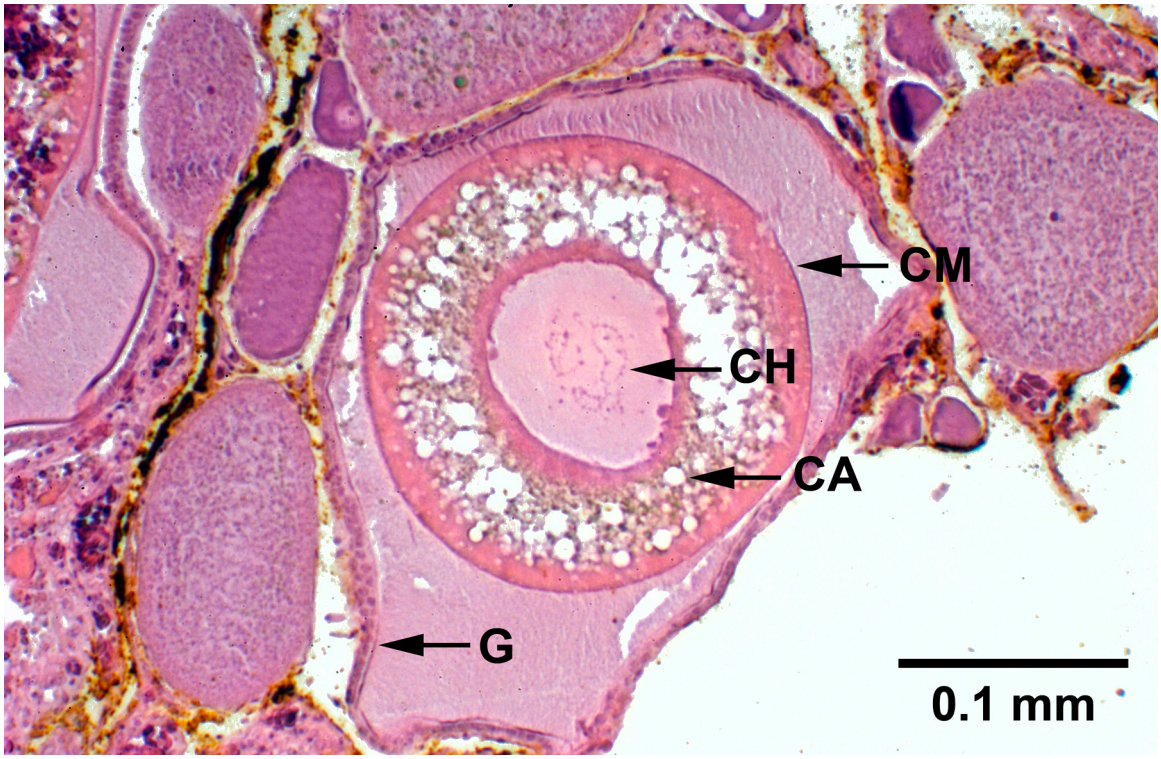


Figure 6-4. Transverse section of ovary with oocytes showing many stages of development up to the vitellogenic phase. Ovarian lumen is present and ovarian tissue is divided into distinct lamellae. LA = lamellae, OL = ovarian lumen, VO = vitellogenic oocyte.

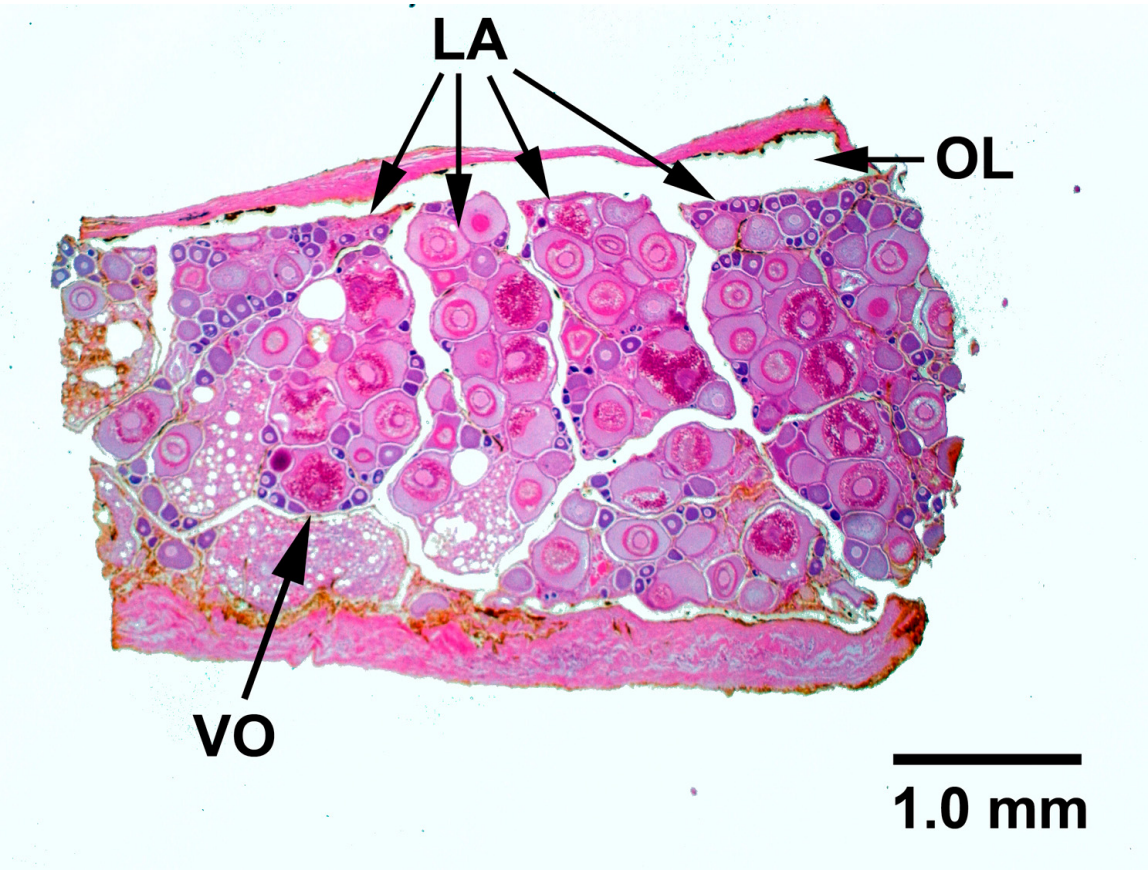


Figure 6-5. Entire ovary showing oocytes up to the cortical alveoli phase and large yellow bodies. OC = oocyte, YB = yellow body.

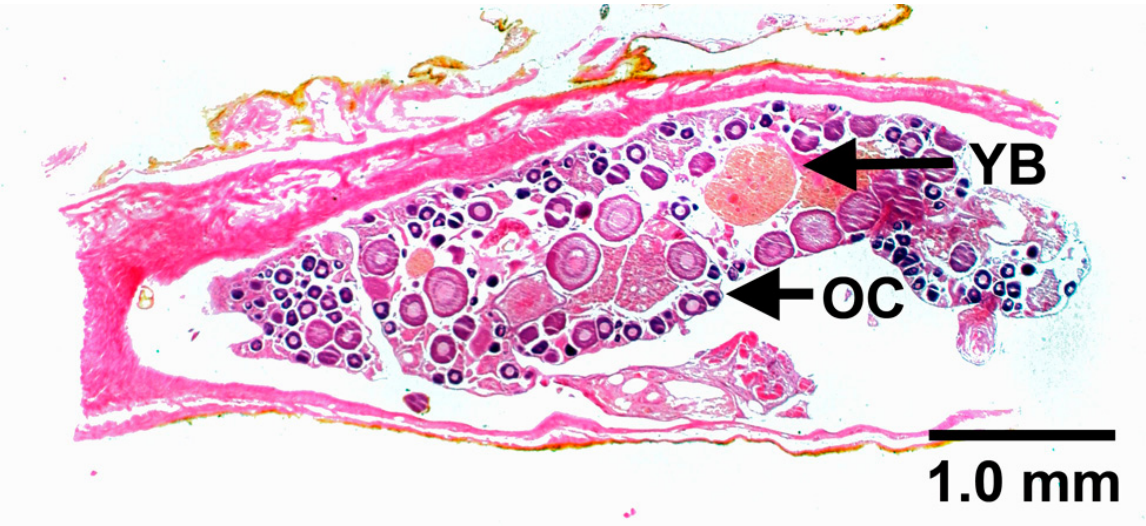


Figure 6-6. Mature oocyte. T = thecal cell layer, YG = yolk globule.

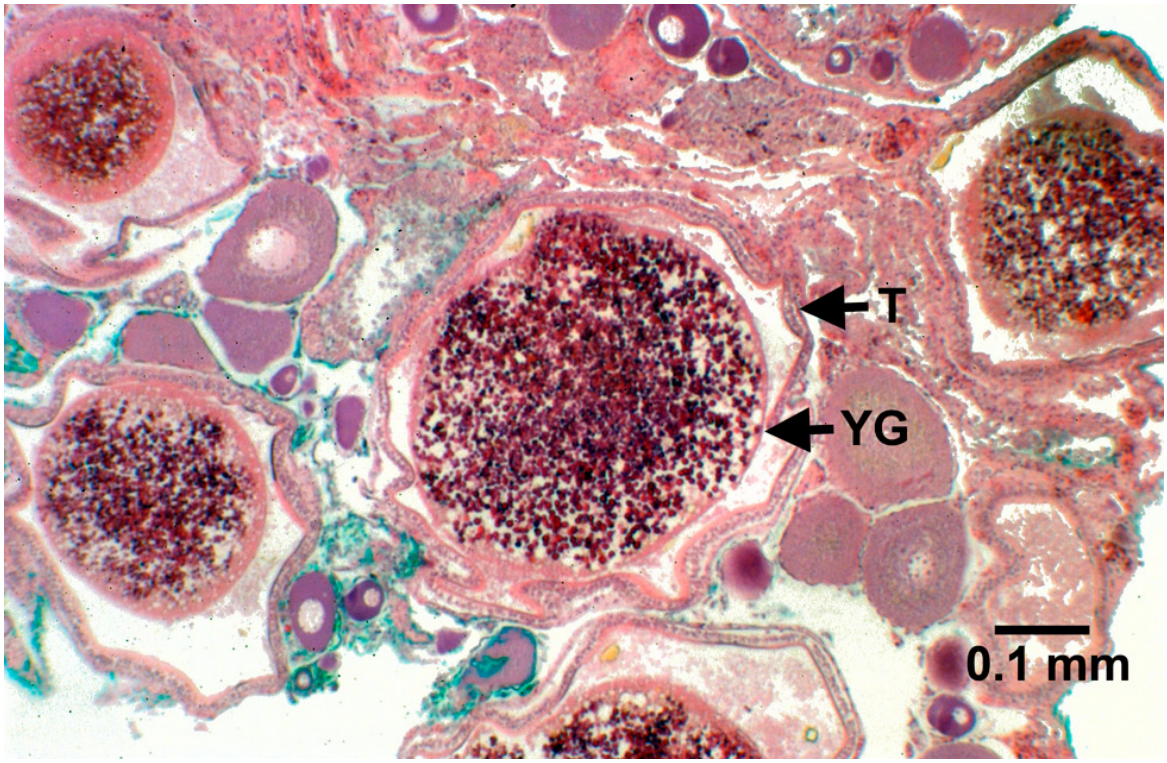


Figure 6-7. Unidentified amoeboid cells. Cells seem to be emerging from and migrating across a matrix of connective tissue. Patches of amoeboid cells were observed in caudal sections of both ovaries and testes. AC = amoeboid cells.

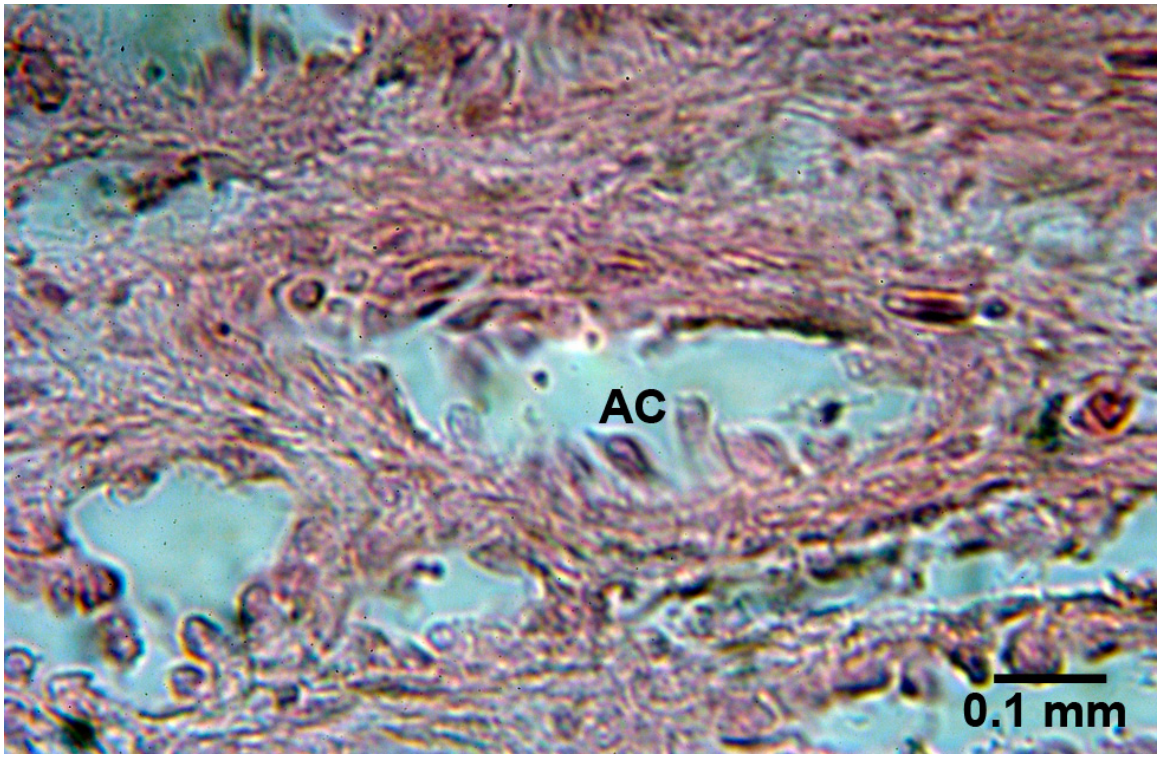


Figure 6-8. Spermatocyst with zygotene spermatocytes exhibiting bouquet chromosome distributions. ZS = zygotene spermatocytes.

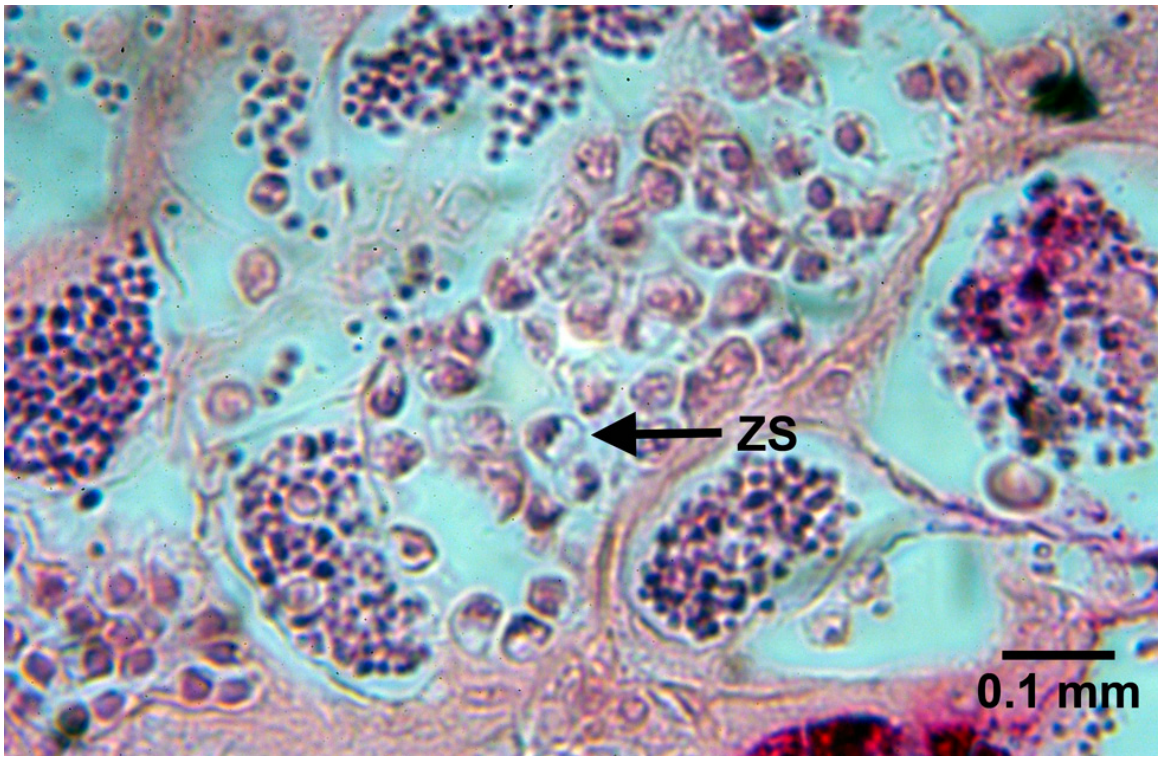


Figure 6-9. Transverse section of testes showing testicular lobules consisting of spermatocysts and sperm ducts. SC = spermatocyte, SZ = spermatozoa.

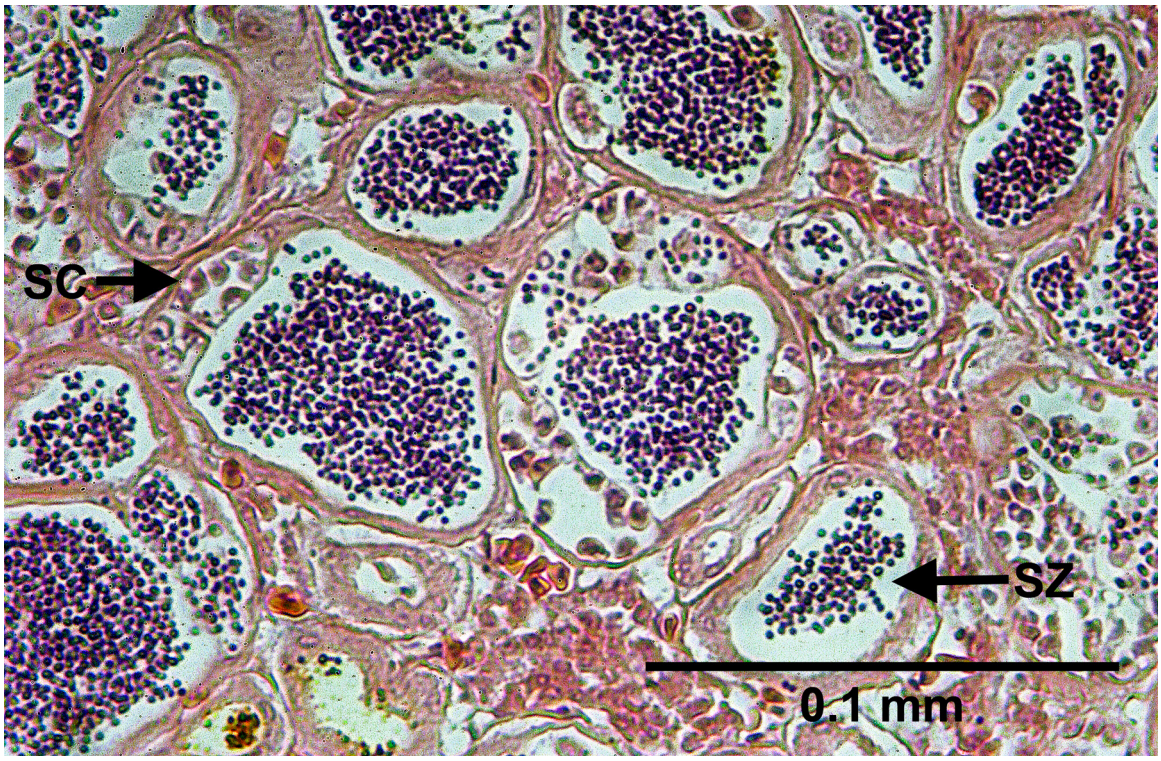


Figure 6-10. Spermiogenesis. Spermatids with cytoplasm and spermatozoa without cytoplasm sharing the same spermatocyst. The spermatocyst will open into the sperm duct and release the spermatozoa. Interstitial tissue and various stages of spermatocytes are also visible. ST = spermatid, SZ = spermatozoa.

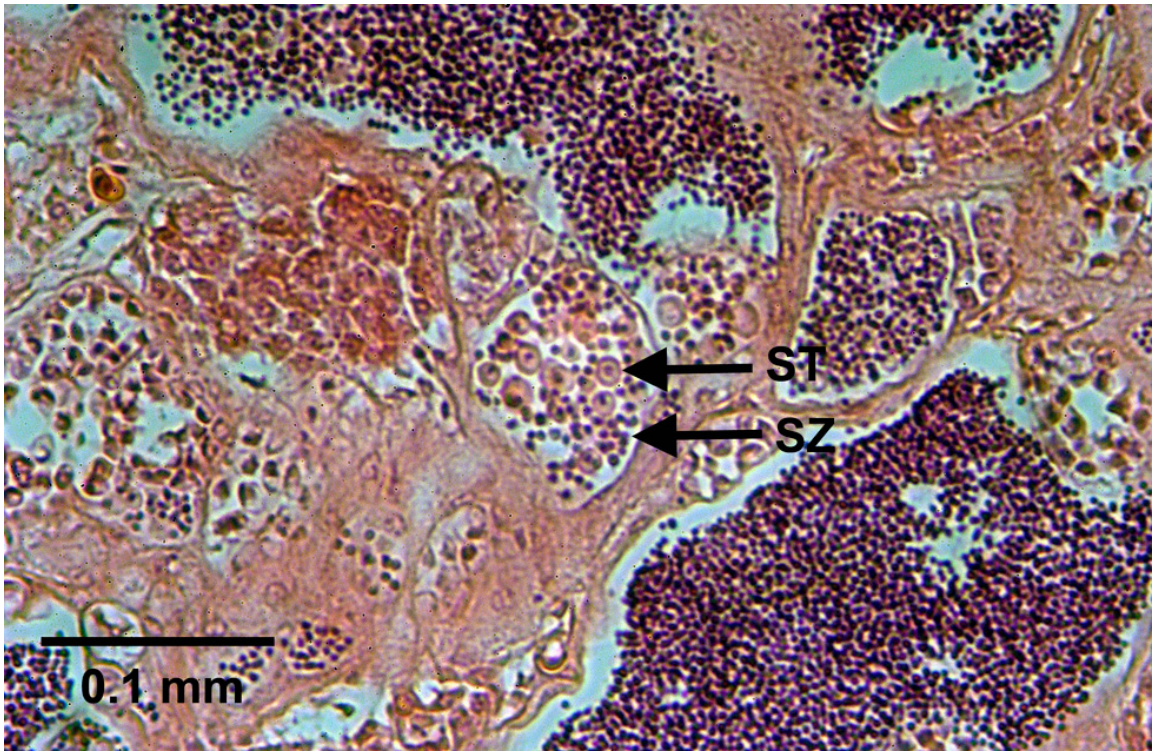


Figure 6-11. Transverse section of testes. LO = lobule, SD = sperm duct.

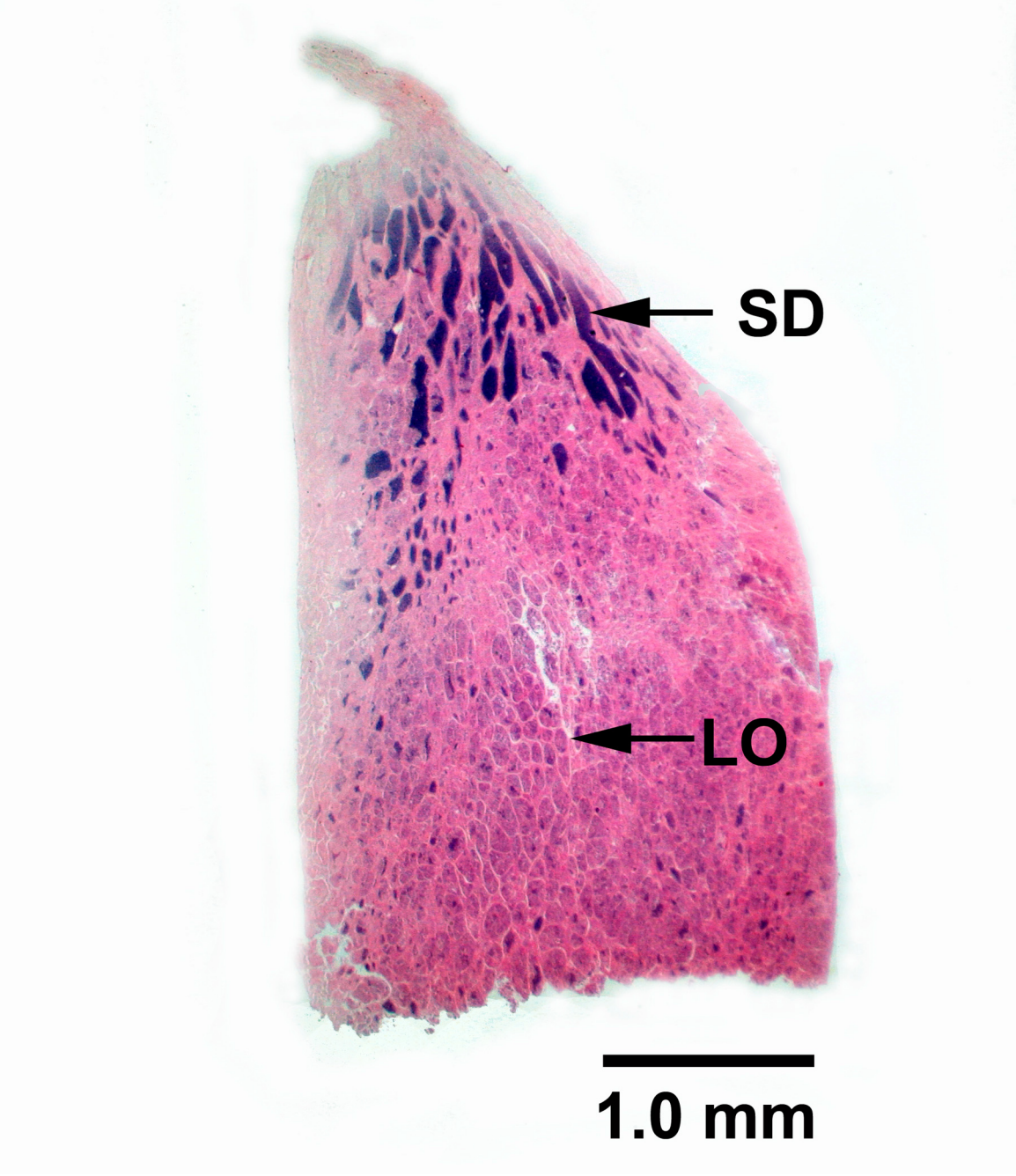


Figure 6-12. Standard length distributions of the females (diamonds) and males (squares) from which gonads were excised for histological examination.

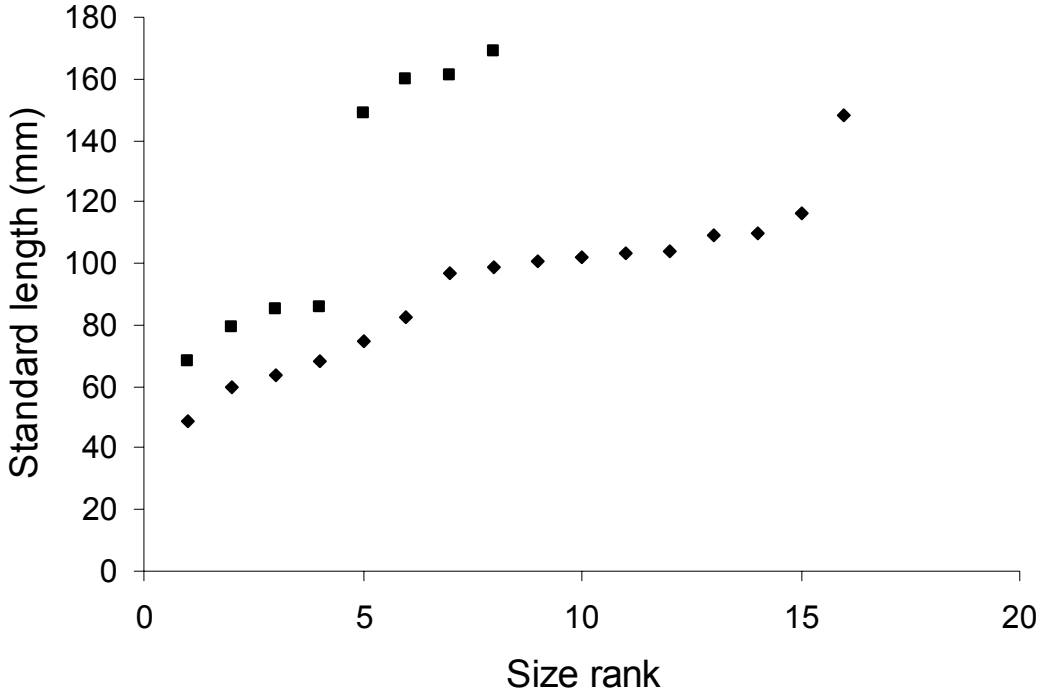
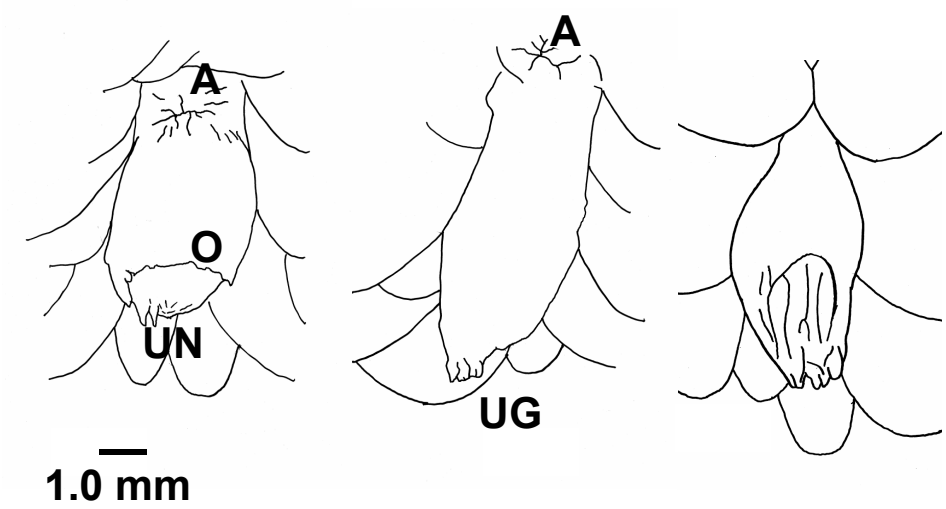


Figure 6-13. Typical genital papillae of a (a) female and a (b) male *A. citrinellus* (UMMZ 188309), and (c) an adult suspected of undergoing protogynous sex change. A = anus, O = ovopore, UG = urogenital pore, UN = urinary pore.



a. b. c.

Figure 6-14a. Gonad structure of an adult Midas cichlid suspected of undergoing protogynous sex change. (a) Cranially, the gonad has expanded up around the mesorchium. BV = blood vessel, M = mesorchium, SD = sperm duct.

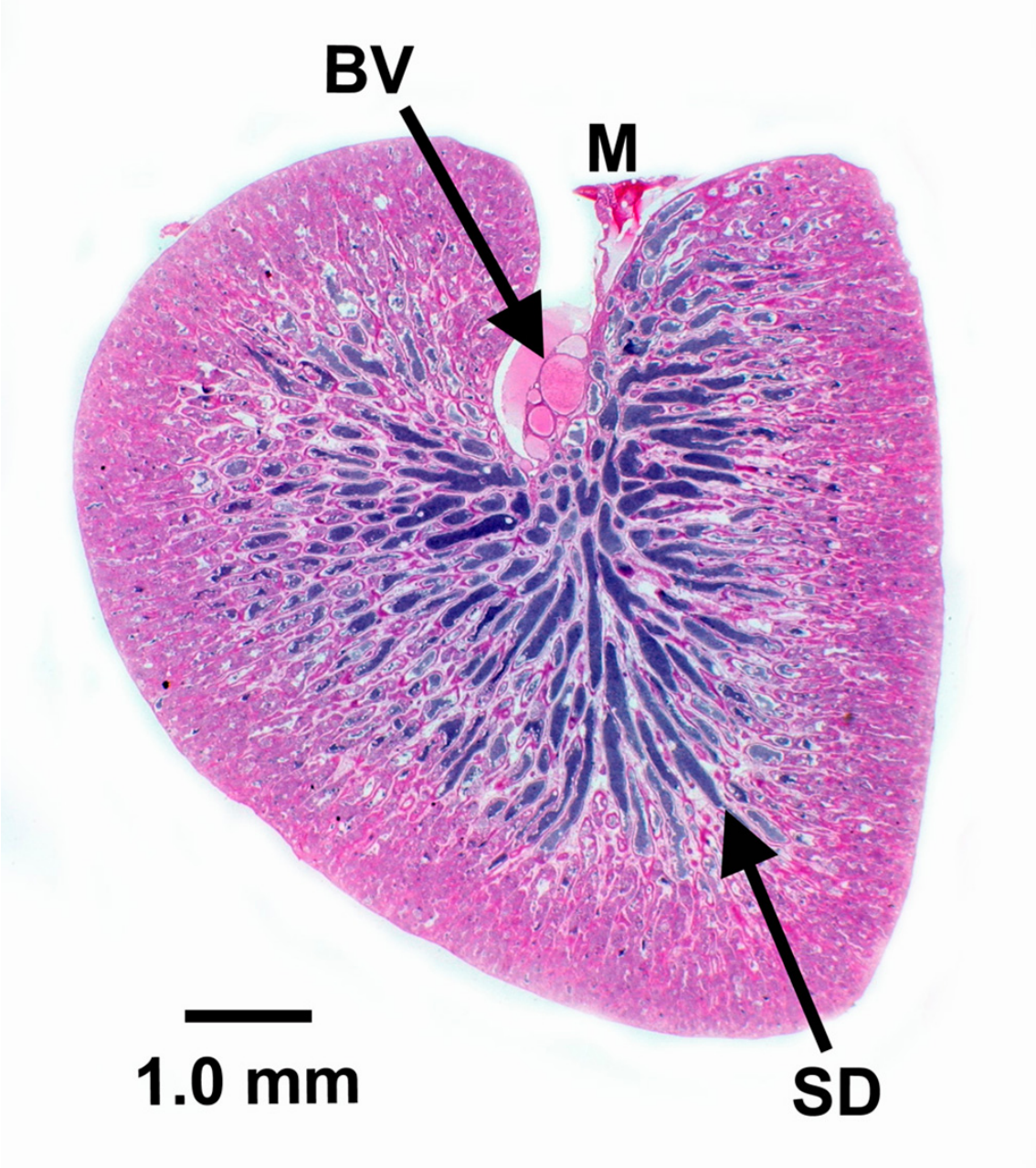
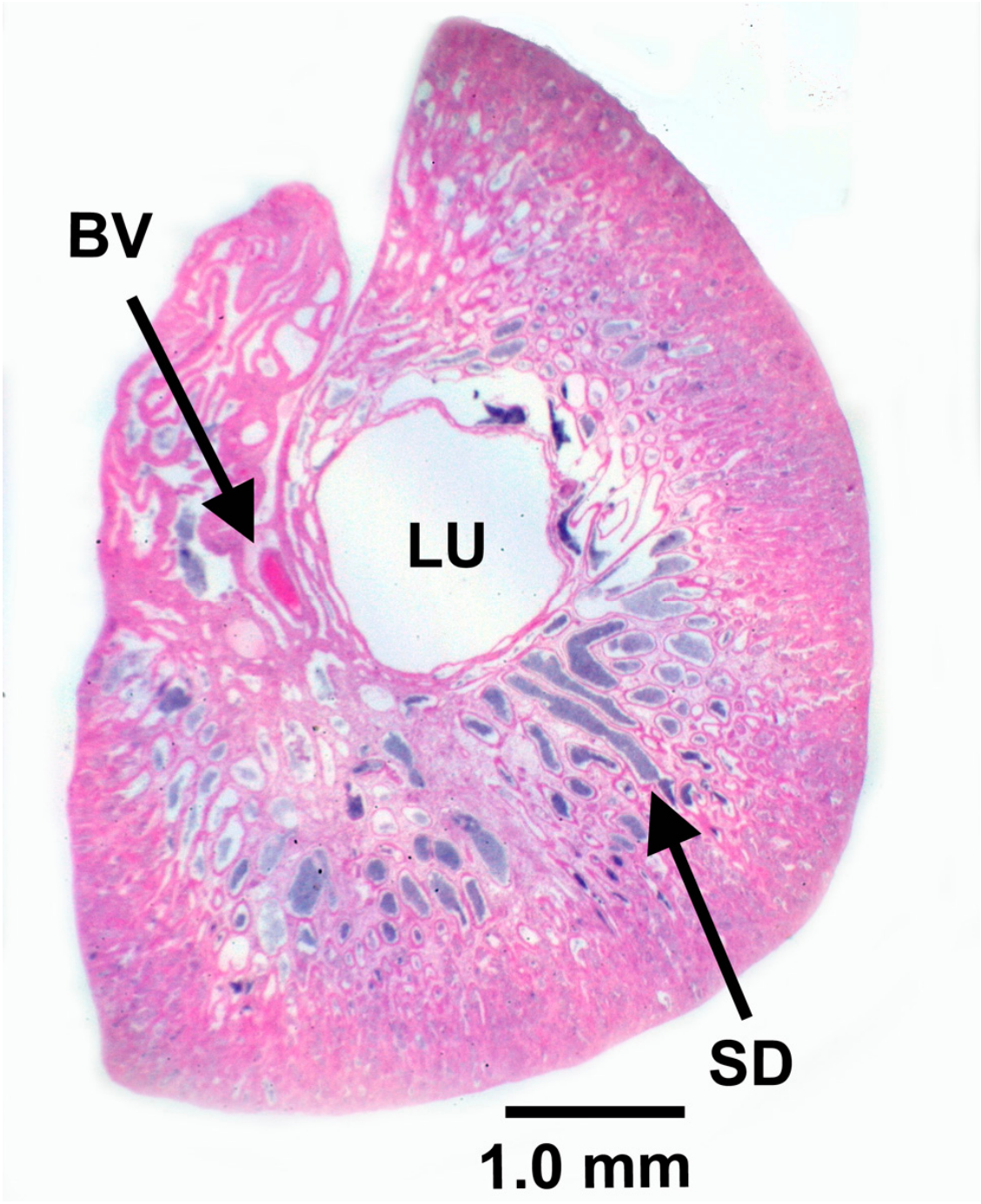


Figure 6-14b. Gonad structure of an adult Midas cichlid suspected of undergoing protogynous sex change. (b) Caudally the gonad separates from the mesorchium and wraps around itself forming an empty lumen. BV = blood vessel, LU = lumen, SD = sperm duct.



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CHAPTER 7

CONCLUSIONS

Francis and Barlow (1993) claimed that sex was determined socially in juvenile Midas cichlids. They came to the conclusion that individuals that were larger than most of their group-mates developed as males and those that were smaller developed as females because (1) as adults, males are larger than females (Barlow 1976), (2) Francis (1990) observed stable size ranks in a captive group of 12 juveniles raised to maturity, and (3) when relatively large juveniles (presumed males) were separated from relatively small juveniles (presumed females) and raised to maturity each group contained large males and smaller females instead of containing all males or all females (Francis and Barlow 1993). These authors considered social control of sex determination to be an example of genetic variation in the timing of sexual lability, supporting a hypothesis that different forms of sexual lability evolve through changes in developmental timing.

In a laboratory experiment, I divided juvenile Midas cichlids into groups that each contained six fish and raised 10 such groups to maturity. If relative body size determined sex, then the largest three fish in each group would have developed as males and the smallest three as females. Instead, there was no association between relative body size and sex (Oldfield 2007, Chapter 3). Findings were also inconsistent with a hypothesis of socially controlled sex determination in Lake Apoyo, Nicaragua, where I captured two groups of Midas cichlids at the onset of sex differentiation, and sex was not associated

with body size in either group (Oldfield et al. 2006, Chapter 4). These findings suggested that size ranks within a group are not typically stable over time, as was concluded by (Francis 1990), and that the large size of males compared to females typically observed in adults is due to greater post-maturational growth in males, rather than due to social control of sex determination. Additional experiments confirmed that differences in growth rates were responsible for the relatively large body size of adult males (Chapter 5). Finally, histological examination of gonads of specimens collected from the same locality as Francis's (1990) and Francis and Barlow's (1993) fish failed to provide any indication of sexual lability (Chapter 6). Thus, social control of sex determination has not been demonstrated in Midas cichlids.

Social control of sex determination has not been conclusively demonstrated in any gonochoristic fishes. Williams (1972) considered the possibility of social control of sex determination in the convict cichlid, *Archocentrus nigrofasciatus*, but his methods were insufficient to make such a conclusion (see Oldfield 2005, Chapter 1). Francis (1984) had originally suggested that sex was socially determined in the Paradise fish, *Macropodus opercularis*, but his conclusion may have been in error, as it was in *Amphilophus citrinellus*. Social conditions most likely do not determine sex in this species either. The last species for which Richard Francis attempted to demonstrate socially controlled sex determination was another cichlid, *Astatotilapia burtoni*, and social conditions were found not to determine sex (Fernald, personal communication).

Social control of sex determination does occur in some typically sequentially hermaphroditic marine fishes. In the typically protogynous (female-to-male sex-changing) species, *Cephalopholis boenak* (Serranidae), the largest juvenile in a captive

pair or group of three differentiates as a male, the second largest as a female, and smaller individuals generally remain undifferentiated or differentiate as females. Isolated individuals differentiate as males (the terminal sex) (Liu and Sadovy 2004). When juveniles of the typically protandrous (male-to-female changing) clownfishes *Amphiprion bicinctus* or *A. frenatus* (Pomacentridae) are housed in groups, the largest member differentiates as a female, the second largest a male, and smaller members remain undifferentiated (von Brandt 1979, Bruslé-Sicard et al. 1994). Juveniles differentiate as females when they mature in isolation (von Brandt 1979, Wood 1986). The typically protogynous bluehead wrasse, *Thalassoma bifasciatum*, also undergoes social control of sex determination. However, isolated individuals were found to differentiate as the initial sex (female) when raised in isolation. In groups of three, one individual developed as a male (Munday et al. 2006). Social control of sex determination in these sequential hermaphrodites is an example of phenotypic plasticity in the timing of sex change that typically occurs at the adult stage, and therefore does not support a theory of change in developmental timing in the evolution of sequential hermaphroditism.

Social control of sex determination at the juvenile stage was proposed by Francis (1992) to represent an ontogenetic midpoint on a continuum of developmental timing. Because this form of sexual lability is not known to exist in any gonochoristic fishes, it does not support Francis' (1992) hypothesis. However, the model proposed by Francis (1992) is reasonable and may yet be supported. Genetic factors could yet account for early terminal sex differentiation in some sequential hermaphrodites (Shapiro 1992), and social control of sex determination may yet be discovered in some gonochores. Such cases would indicate genetic variability in the critical period of gonad lability. A

significant amount of evidence remains that suggests that labile developmental processes evolve via changes in developmental timing (Atz 1964, Shapiro 1987, Francis 1992, Oldfield 2005, Chapter 1), so this hypothesis deserves continued attention.

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