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Behavioral Interaction, Body Size and Sex Determination in the Midas Cichlid, *Amphilophus citrinellus*

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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. In order to test the hypothesis that relative body size and behavioral interactions affect sex determination, thirteen experimental groups of juvenile Midas cichlids were raised to maturity. 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. Greater body size typically observed in adult males appears to be due to faster post-maturational growth rather than relatively large juveniles differentiating as males.

Key words: Aggression, development, growth, hermaphroditism, body size, determination

INTRODUCTION

Most fishes have sex 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 (Oldfield, 2005). There are species that differentiate directly as one sex or the other and are apparently not labile (Meijide *et al*., 2005). At the larval stage some cichlids are labile and have sex determined by abiotic factors like temperature or pH (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 (Peters, 1975; Loir *et al*., 1989). The Midas cichlid, *Amphilophus citrinellus* 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 *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). However, 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 that were initially larger developed as males and the smaller fish as females. Francis and Barlow (1993) then divided a brood of 74 juveniles into 2 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 (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 (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. In fact, 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× 90× 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. 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 6 fish was placed into a 76 l (61×31×42 cm) aquarium. Each of these tanks was equipped with an air-powered sponge filter, a clay pot, one stone (8×10 cm), one bunch of Java moss, *Vesicularia dubyana* (8×10 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 translucent skylights and was supplemented with fluorescent lighting set on a 12:12 h light: Dark schedule. Water temperature was maintained at approximately 27°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 min 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 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 sec 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, 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.

Total 13 isolated specimens were each housed in a 38 l (51×26×31 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 (46×90×36 cm) until 182 days old, when 18 were removed, weighed (5.7±3.1 g, mean±SD) measured (52.3±10.2 mm SL, mean±SD) 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.

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.

In order 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 U 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 = (# attacks and displays performed)-(# 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 (Fig. 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, $k = 6$, $d_{max} = 2$, $p>0.50$, Fig. 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, $k = 10$, $d_{max} = 3$, $p >0.50$, Fig. 3), indicating that size rank in the original brood did not affect sex determination. However, males

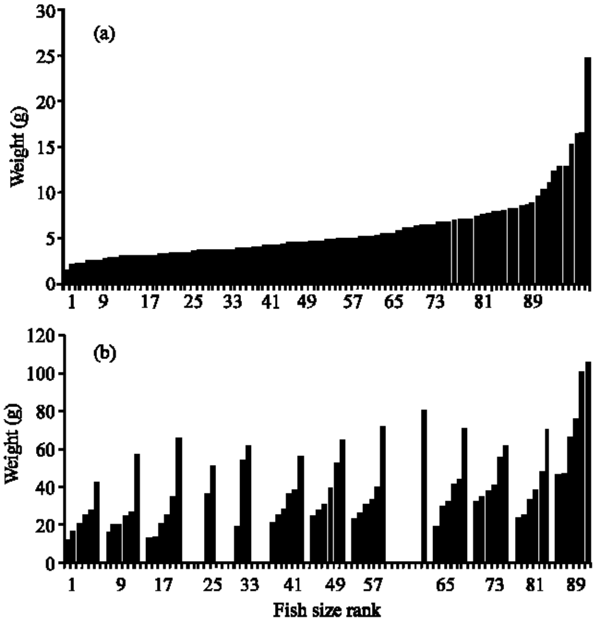

Fig. 1: Size distribution of one brood of Midas cichlids (a) at the beginning and (b) at the conclusion of the experiment, after being divided into 13 groups of like-sized individuals. (Isolated and deceased individuals omitted from b)

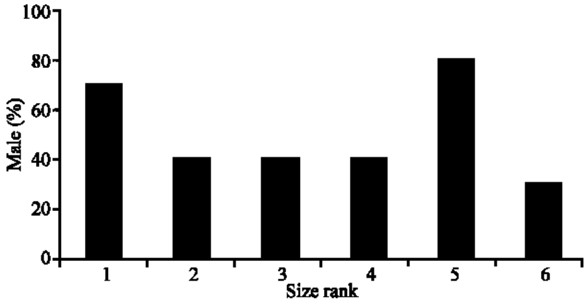

Fig. 2: Sex ratio for each size rank across the 10 groups (n = 10 for each rank) in which data were obtained from all six fish (rank 1 = largest fish in group, rank 6 = smallest). Sex was not associated with size rank (p>0.50)

(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 (Fig. 4). Larger individuals spent more time behaving aggressively (χ^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 (Fig. 5). (N = 9 for each rank except rank 6, where n = 8).

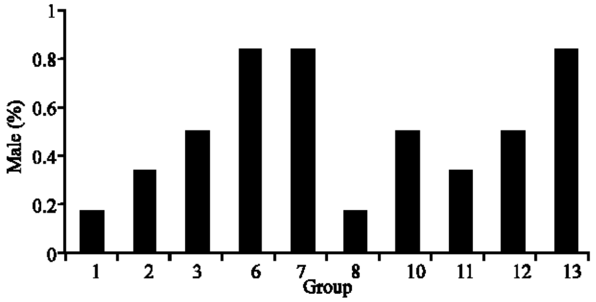

Fig. 3: Sex ratio within each of 10 groups (group 1 = smallest fish in original brood, group 13 = largest). Sex was not associated with group (p >0.50). N = 6 for each group

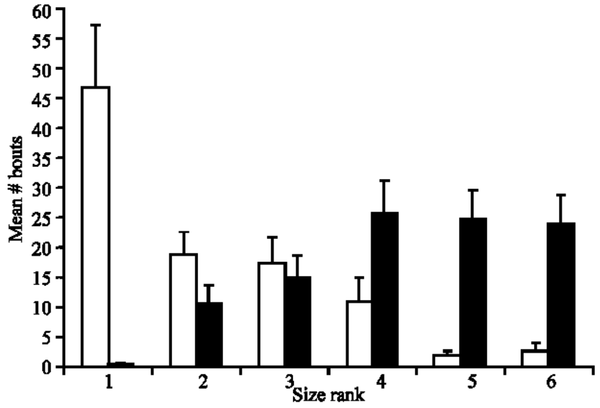

Fig. 4: Mean number of aggressive bouts performed (white bars, p<0.001) and received (black bars, p<0.001) across groups by size rank. Lower rank numbers represent larger fish. N = 9 for each rank except rank 6, where n = 8. Error bars represent standard error

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 (4 vs. 5 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 to size hierarchies in 5 of the 10 groups and the two measures were identical in 2 of the 10 (Table 1).

No significant difference in number of aggressive bouts performed was found between fish that

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

	Dominance rank									
Size	Groups									
rank	1	2	3	6	7	8	10	11	12	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

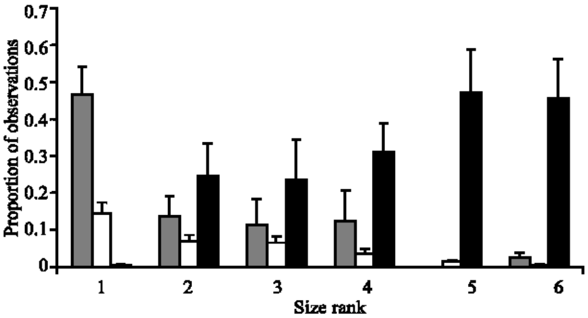

Fig. 5: Proportions of 50 total observations spent performing various types of behavior for fish of different size ranks across nine groups (n-values as in Fig. 4). Lower rank numbers represent larger fish. Checkered bars represent territorial behavior, including guarding a pot or digging a pit in the 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 ($p<0.001$). Error bars represent standard error

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 females (n = 19, mean = 8.21) and males (n = 10, mean =12.30) 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 ($\div^2 = 0.289$, df = 1, p>0.50).

Total 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 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 also 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 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 sex determining mechanisms 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 offspring from different sets of parents in the cichlid *Oreochromis niloticus*.

A second possibility involves the formation of small groups, which may have elicited a social structure different than 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). Territorial behavior may have somehow 'short-circuited' social control of sex determination.

The third possible explanation is that sex in Midas cichlids 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 post-maturational 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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