Gonad development in Midas cichlids and the evolution of sex change in fishes

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SUMMARY Some fishes mature and function as one sex and later transform to the other sex in response to social interactions. Previous evidence suggested that a change in developmental timing may be involved in the evolution of adult sex change in fishes. The most recent support for this idea came from reports that sex in the Midas cichlid, *Amphilophus citrinellus*, was determined by social conditions experienced at the juvenile stage. Differentiation as a male was reported to be dependent on large body size relative to group-mates, and thought to be mediated through aggressive interactions. Here I demonstrate that socially controlled sex determination does not occur as was originally reported. Previously, I found that sex was not associated with body size in juveniles either in nature or in captivity. Similarly, I found no association between aggressive behavior and sex in juveniles. I later demonstrated that socially controlled sex determination does not typically occur in the Midas cichlid and closely related species and supported an alternative mechanism to explain large body size in adult males. Finally, in the current study I analyze gonad histology of fish from the same population used by the original authors and lay to rest the idea of socially controlled sex determination in this species. Recent observations of socially controlled sex determination in juveniles of species that typically change sex at the adult stage are examples of phenotypic plasticity, not genetic variation. Therefore, juvenile socially controlled sex determination does not support a theory that a change in developmental timing is involved in the evolution of adult sex change in fishes.

INTRODUCTION

Environmental factors play an important role in the development of phenotype in organisms (Lewontin 2000; Gilbert 2001). Social environment is particularly important (Stamps 2003). In fishes, unlike in most vertebrates, reproductive development can be influenced by both abiotic and social environmental factors (Baroiller et al. 2009). Social conditions can control rates of somatic growth (Brown 1957) as well as reproductive physiology (Hofmann and Fernald 2000; Fernald 2002; Gerlach 2006) and development (Borowsky 1973; Fraley and Fernald 1982; Bushman and Burns 1994; Kolluru and Reznick 1996). Extreme examples of social control of reproductive development occur in sequentially hermaphroditic fishes, those that mature and function as one sex and later transform to the other sex, typically in response to behavioral interactions (Devlin and Nagahama 2002).

It has been suggested that a change in developmental timing is involved in the evolution of sequential hermaphroditism in fishes (Bullough 1947; Atz 1964). Many gonochoristic fish species (those that do not change sex as adults) undergo sex changes before maturity. In these species, gonads of all in-

dividuals initially produce oocytes, but in genetic males oocytes degenerate early in development and are replaced by spermatogenic tissue (Yamamoto 1969; Takahashi and Shimizu 1983; Uchida et al. 2002; Maack and Segner 2003). The discovery that sex in several species may be determined by abiotic factors such as pH or temperature has been interpreted as additional evidence for the developmental timing hypothesis, culminating in the theory that there may be an early critical period of gonad plasticity generally present in fishes, when the gonad is capable of differentiating in either direction, and if this period were extended to adulthood then the gonad might undergo functional transformation (Shapiro 1987; Francis 1992). This pattern seemed especially robust in cichlid fishes, because there are published examples of different forms of sexual plasticity at three different life stages in different species within this one family (Oldfield 2005).

One of these forms of plasticity, socially controlled sex determination at the juvenile stage, seemed to represent a midpoint on an ontogenetic continuum, linking sexual plasticity very early in development with sex change at the adult stage, and provided even more support for the developmental timing theory (Francis 1992). Nearly all of the evidence

for socially controlled sex determination has been obtained from one species, the Midas cichlid, *Amphilophus citrinellus* (Francis 1990; Francis and Barlow 1993). Large Midas cichlids aggressively dominate (Barlow 1983), and may suppress growth in (Francis 1988), smaller individuals. As adults, males are larger than females (Barlow 1976). When Francis and Barlow (1993) manipulated relative body size in juveniles, it appeared that sex was a result of relative size, rather than size being a result of sex. A brood of 74 juveniles was divided 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 (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. Apparently, the same aggressive interactions that controlled growth also determined sex, in a manner similar to how aggressive behavior controls adult sex change in sequentially hermaphroditic fishes (Francis and Barlow 1993). This finding received significant attention (Baroiller et al. 1999; Baroiller and D'Cotta 2001; Devlin and Nagahama 2002; Godwin et al. 2003; Oliveira 2006; Baroiller et al. 2009), including discussion in a popular textbook (Helfman et al. 1997).

Subsequent studies were surprisingly inconsistent with the original conclusions drawn by Francis and Barlow (1993). The next study to investigate sex determination in Midas cichlids (Oldfield 2007) was an attempt to elucidate the specific behavioral mechanism responsible for socially controlled sex determination. The experiment was terminated earlier than the original experiment done Francis and Barlow (1993), and it was found that males were not larger than females. A field study was then performed in Lake Apoyo, Nicaragua, and again there was no difference in body size between juvenile females and juvenile males (Oldfield et al. 2006). Next, long-term growth experiments were performed using large groups of individually marked fish of different lineages to test the possibilities that sex determination mechanism varied with size of social group and/or with genetic background (Oldfield 2009). These experiments demonstrated an alternative mechanism by showing that large size in adult males is typically due to greater postmaturational growth than in females, and not due to relatively large fish differentiating as males. Finally, the current study used gonad histology, the most powerful tool for interpreting sexual pattern in fishes (Sadovy and Shapiro 1987), and specimens from the same population as the fish used in the original experiments performed by Francis and Barlow (1993), in order to uncover some indication of sexual plasticity in Midas cichlids, to either give credence to the original study or to lay to rest the notion of socially controlled sex determination in this species.

Size distributions of females and males were compared to identify a pattern either consistent with socially controlled sex determination, with juvenile males being larger than juvenile females (Francis 1990), or consistent with more recent studies (Oldfield et al. 2006; Oldfield 2007, 2009) that found no difference in body size between the sexes at the juvenile stage but faster growth in males after the onset of maturity. If this analysis were to find no difference in body size between juvenile females and juvenile males, then the only way that all relatively large juveniles could become males is if those large juveniles that were females were to undergo a gonadal sex change and transform into males (and small males into females). If this were occurring then some bisexual gonads would be observed. The presence of both female and male structures in a fish gonad is commonly interpreted to indicate previous sexual plasticity. For example, remnant oocytes in testicular tissue, sperm sinuses in the gonad wall, and remnants of an ovarian lumen and lamellae, together 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).

MATERIALS AND METHODS

Forty *A. citrinellus* originally collected by J. R. Baylis, C. R. Bleick, and G. W. Barlow from Rotarians Beach on Lake Masaya, Nicaragua, on April 14, 1970, using the ichthyocide rotenone (Barlow 1976) were examined from California Academy of Sciences lot #76050. The smallest and largest fish were intentionally sampled from the lot along with intermediate-sized specimens. Nine additional small fish were collected from Lake Masaya by Jeffrey McCrary in September 2010 to fill gaps in the body size distributions that remained after examining the California Academy of Sciences specimens. These fish were deposited at the Cleveland Museum of Natural History (catalog numbers pending). The fish that were originally concluded to be sexually labile were descended from individuals caught in Lake Masaya (G. W. Barlow, personal communication). Seasonal breeding cycles of Midas cichlids in Lake Masaya are not well understood, but at least some amount of spawning occurs year-round (Barlow 1976). In nearby Lake Xiloa, a crater lake similar to Lake Masaya, Midas cichlids were observed to spawn during the months of July and August, but no spawning was observed in February, March, April, May, or June (McKaye 1977).

Each fish was measured for standard length (SL) and then dissected. The right gonad was removed from each specimen unless it was damaged, in which case the left gonad was removed. An attempt was made to remove the gonoduct along with each gonad. For some particularly small fish, instead of trying to remove the gonad, a transverse section of the entire body was removed. Tissues (either gonads or transverse sections of the entire body) were embedded in paraffin, sectioned longitudinally and/or transversely at 4 or 6 μm, stained with hematoxylin and eosin, and examined with a light microscope. A mean of 19 sections were taken from each individual, and came from the anterior, central, and posterior regions of the gonad. Body sizes (SL) completely overlapped the size

Table 1. Standard lengths of juvenile and adult female and male Midas cichlids

	Juvenile		Adult	
	Female	Male	Female	Male
	49	51	97	97
	49	53	98	97
	57	61	99	116
	60	69	102	117
	64	79	102	121
	68	85	103	124
	73	86	105	127
	75		107	127
	76		109	132
	83		110	133
	84		116	149
	95		117	160
	97		148	161
	101			169
	104			
Mean	75.63	69.07	108.62	130.71
SE	4.68	5.54	3.73	5.94
df		14		21
t-statistic		0.90		− 3.15
Two-tailed P		0.381		0.005

There was no significant difference in body size between the 15 female and the seven male juveniles, but the 14 adult males were much larger than the 13 adult females.

Structures were recognized according to previously published descriptions of fish gonad development (Hunter and Macewicz 1985; Wenner et al. 1986; Selman and Wallace 1989; Takashima and Hibiya 1995; Grier and Taylor 1998; Grier 2000; Meijide et al. 2005; Grier and Uribe Aranzábal 2009; Grier et al. 2009). Terminology followed Grier and Uribe Aranzábal (2009) and Grier et al. (2009). Based on the structures observed, each individual was classified according to reproductive phases recently agreed upon by the international fish gonad histology community and presented at the Third Workshop on Gonadal Histology of Fishes (Brown-Peterson et al. 2007). These phases were then interpreted in a context that would allow classification into two categories: those individuals that had not yet reached spawning condition and might possibly be sexually plastic according to a theory of socially controlled sex determination (juvenile), and those that were in spawning condition or had spawned previously and would not be expected to be sexually plastic (adult). SLs were compared between juvenile females and juvenile males, and between adult females and adult males with two-tailed t-tests.

RESULTS

General gonad anatomy

The gonads in both males and females were lobed structures, located beneath the gas bladder, attached by mesentery tissue to the dorsal surface of the body cavity. 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 gonad development

All females possessed gonads that contained exclusively ovarian structures. No structures that could possibly indicate sexual plasticity were observed. Oogenic tissue was organized into lamellae, which projected into an ovarian lumen.

Females classified in the reproductive phases "immature" (Fig. 1A, Fig. S1) and "developing" (Fig. 1B, Fig. S2) were

range reported previously for individuals thought to be undergoing socially controlled sex determination (Francis and Barlow 1993). There were five individuals that were processed but did not yield observable slides (not included in the total numbers given above). Small gonads may have been more likely than large gonads to be destroyed during processing. This may be responsible for the observation of such a small number of juvenile males (Table 1), which typically had noticeably smaller gonads than adult males and juvenile and adult females.

Sex was identified in each individual, and structures known to indicate sexual plasticity (Sadovy and Shapiro 1987) were sought.

Fig. 1. Structure observed in Midas cichlid gonads of different sexes and phases of development. (A) Transverse section of the ovary of a 49 mm standard length (SL) "immature" female, classified as a "juvenile." OG, oogonium; PGmn, multiple nucleoli primary growth oocyte; BV, blood vessel. Scale bar = 200 μm. (B) Longitudinal section of the ovary of a 104 mm SL "developing" female, also classified as a "juvenile," showing densely packed chromatin nucleolus stage oocytes (CN), perinucleolar primary growth stage oocytes (PGpn), and cortical alveolar (PGca) primary growth stage oocytes. Note the thin ovary wall (OW) and the absence of atretic oocytes. Scale bar = 1.0 mm. (C) Longitudinal section of the ovary of a 116 mm SL "spawning capable" female that was classified as an "adult." Oocytes at all stages up to the late vitellogenic/late secondary growth (SGl) step are present as well as alpha- and later-stage atretic follicles (AF). Note the thick ovarian wall compared with (B). Scale bar = 1.0 mm. (D) Longitudinal section of the ovary of a 103 mm SL "regenerating" phase female classified as an "adult." Despite the fact that there are no oocytes present beyond the cortical alveolar step, the ovary is easily distinguished from a "juvenile" ovary by the large number of melano-macrophage centers (MMC), extensive vascularization, and thick ovarian wall. OL, Ovarian Lumen. Scale bar = 1.0 mm. (E) Transverse section of the testis of a 61 mm SL "developing" male, classified as a "juvenile." Spermatogenic activity is sparse, but spermatogonia (Sg) and a spermatocyst containing spermatocytes (Sc) is visible. Scale bar = 100.0 μm (F) Transverse section of the testis of a 169 mm SL "spawning capable" male classified as an "adult." The gonad is large, and contains lobules (LO) with spermatocysts at various stages of development, as well as a large number of sperm ducts (SD) filled with spermatozoa. Scale bar = 1.0 mm.

considered to be juveniles possibly capable of socially controlled sex determination. Ovaries of "immature" females were very small and contained only oogonia, chromatin–nucleolus-stage oocytes, and primary growth oocytes up to the perinucleolar-step (Fig. 1A). "Developing" females possessed larger gonads tightly packed with oocytes up to the cortical alveolar step of the primary growth stage, with very little interstitial tissue. Vascularization was minimal. No
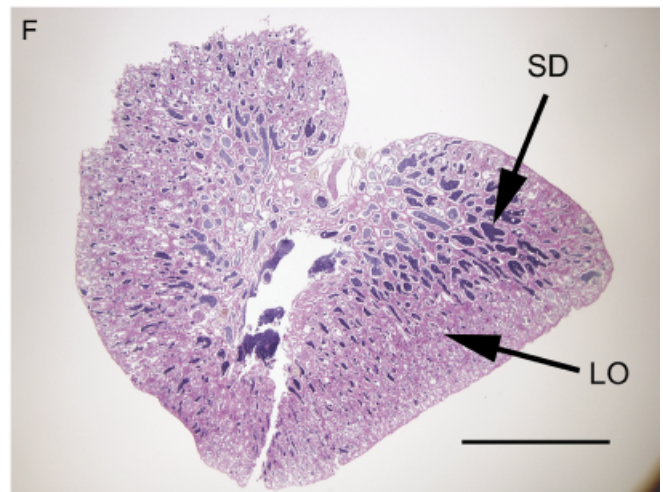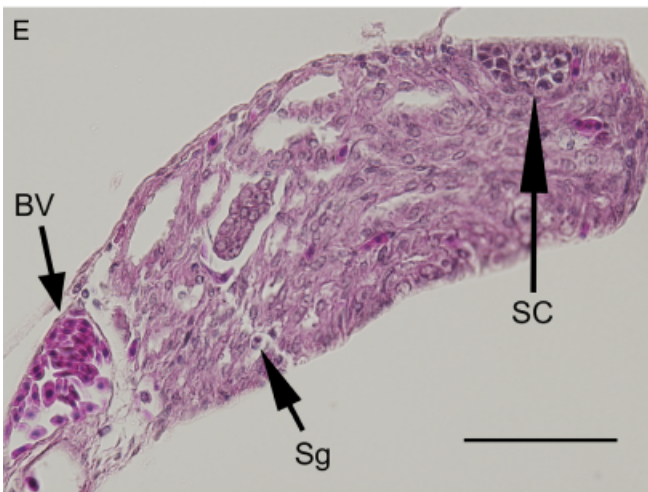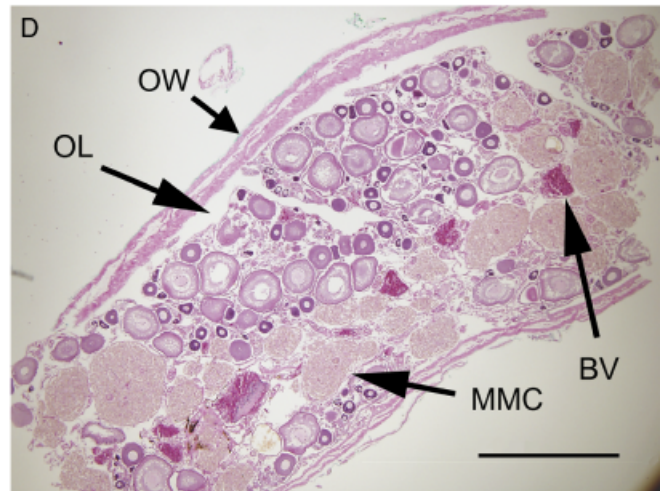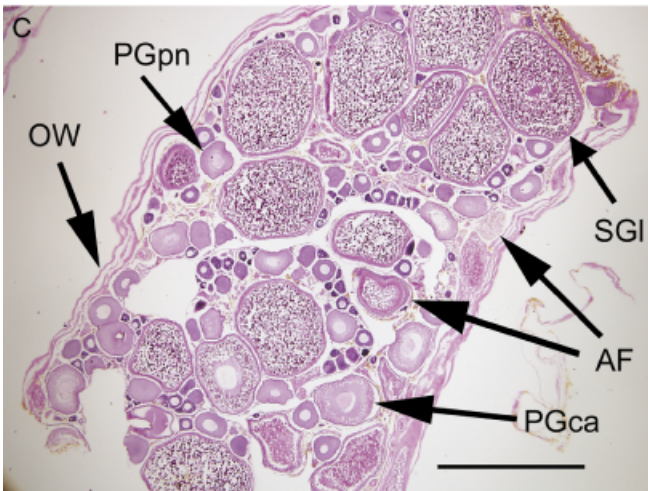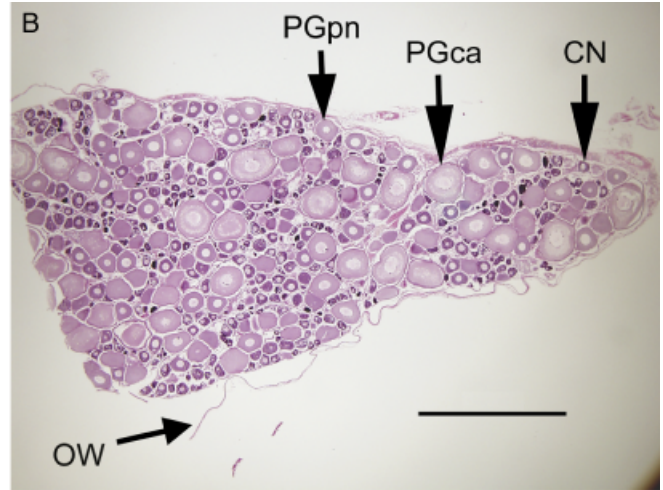
postovulatory follicles were present and there was little to no follicular atresia (Fig. 1B, Fig. S2B). Follicle (granulosa) cells were small and amorphous and thecal cells were not easily discernable (Fig. S2C).

Females classified in the phase "spawning capable" were considered to be adults, past any possible period of sexual plasticity. The ovaries of these females were characterized by having oocytes at the secondary growth/vitellogenic stage, as well as smaller oocytes at earlier stages (Fig. 1C, Fig. S3). Many oocytes appeared to be at the onset of the maturation stage because the germinal vesicles had an eccentric position, as if beginning migration to the animal pole (Fig. S3B). Vitellogenic oocytes were filled with dark-stained yolk globules. Transparent lipid droplets were visible within the ooplasm and cortical alveoli could be discerned at the oocyte periphery, adjacent to the zona pellucida. Follicle cells surrounding late vitellogenic oocytes were large and arranged as a simple columnar layer outside the zona pellucida, which was thick and striated in appearance. Thecal cells were sometimes discernable lying outside of the follicle cells (Fig. S3C). Compared with the juvenile fish, vascularization was extensive and there was more stromal tissue between follicles. Various stages of follicular atresia were common (Fig 1C, Fig. S3D). Postovulatory follicles were not present. The absence of postovulatory follicles and late vitellogenic oocytes indicated that no individuals were in the "actively spawning" or "regressing" phases, which follow the "spawning capable" phase.

Ovaries in the "regenerating" phase (Fig. 1D, Fig. S4) were easily distinguished from "immature" and "developing" ovaries, and were considered to indicate an adult fish. "Regenerating" ovaries possessed oocytes up to the primary growth stage, as well as atretic oocytes and melano-macrophage centers. The ovaries were extensively vascularized, and considerable stromal tissue lay between follicles. Ovarian walls were much thicker than in juvenile females.

Male gonad development

All males possessed gonads that contained exclusively testicular structures. No female structures were observed. Testes were organized into lobules surrounded by interstitial tissue. The lobules contained spermatogenic cysts, which enclosed male germ cells at various stages of development and eventually released spermatozoa into the lumena of lobules and the sperm ducts.

Males classified in the phase "developing" (Fig. 1E, Fig. S5) possessed very small testes and were considered to be juveniles possibly capable of socially controlled sex determination. Spermatocysts with germ cells at early stages of spermatogenesis were observed in these males. Primary and secondary spermatogonia, primary and secondary spermatocytes, and spermatids were present. Small patches of spermatozoa could be seen occasionally in isolated sper-

matocysts, but had not yet been released into lumena of lobules or sperm ducts. Males observed with spermatozoa in the lumena of lobules and/or sperm ducts and therefore classified in the phase "spawning capable" were considered to be past the point of possible sexual plasticity, and were designated as adults (Fig. 1F, Fig. S6). "Immature" males possessing only spermatogonia and no other germ cell stages were not observed. "Regressing" males, with only residual sperm present in their sperm ducts, were also not observed. "Regenerating" males, in which both residual spermatozoa and spermatogonia would be expected to present, were not observed either.

Body size distributions

Juvenile females ranged in size from 49 to 104 mm and adult females from 97 to 148 mm SL (Table 1). Juvenile males ranged from 51 to 86 mm and adult males from 97 to 169 mm SL. None of the four data sets significantly differed from a normal distribution (Kolmogorov–Smirnov: 0.124, 0.228, 0.179, 0.173; $P > 0.150$, $P = 0.064$, $P > 0.150$, $P > 0.150$, respectively). There was no significant difference in SL between juvenile females and juvenile males ($t = 0.90$, two-tailed $P = 0.381$). In adult fish, males were much larger than females ($t = -3.15$, two-tailed $P = 0.005$).

DISCUSSION

No difference in body size was observed between juvenile females and males. Therefore, if socially controlled sex determination was occurring, then a gonadal sex change would be necessary if those relatively large juveniles that were females were to become males (and if those relatively small juveniles that were males were to become females), and some bisexual gonads would have been observed. Instead, no bisexual gonad was observed in any individual at any stage of development. Gonad structure in Midas cichlids was similar to that reported for other cichlids (Hyder 1969; Polder 1971; Cichocki 1976; Msiska 2002; Meijide et al. 2005), and provided no indication of sexual plasticity. This is consistent with the histological patterns in 28 Midas cichlids analyzed from Lake Apoyo (Oldfield et al. 2006), and 30 specimens analyzed in long-term growth experiments (Oldfield 2009).

Female structures in testes are commonly interpreted to indicate previous protogynous sex change (Sadovy and Shapiro 1987), and bisexual gonads have been observed in adult cichlids that were thought to have changed sex. An unusual *Cichlasoma portalegrense* that purportedly underwent protogynous sex change was found to have ovarian tissue in the cranial region of the gonad and testicular tissue in the caudal region (Polder 1971). *Crenicara punctulata* has been reported to be a protogynous hermaphrodite, and oocytes have been observed in the testes of a male (Zupanc 1985).

Small, nonviable oocytes have also been found in the testes of cichlids not thought to have undergone functional sex change: *Amatitlania nigrofasciata* (Polder 1971), 13 species of haplochromine cichlids (Peters 1975), *Tilapia zilli* (Yoshikawa and Oguri 1978), *Satanoperca* aff. *leucosticta* (Loir et al. 1989), *Pseudotropheus lombardoi* (Naish and Ribbink 1990). These oocytes may be remnants (Francis 1992) of the pre-maturational sex change that is typical of gonochoristic species that undergo a "hermaphroditic" process of development (Yamamoto 1969; Uchida et al. 2002; Maack and Segner 2003). It is not possible to be certain without histological analysis of recently differentiated individuals, but the exclusively female or male gonads observed in Midas cichlids suggest a "differentiated" developmental process (Yamamoto 1969), which has been observed in cichlid species such as *Cichlasoma dimerus* (Meijide et al. 2005), and *Oreochromis mossambicus* and *Oreochromis niloticus* (Nakamura et al. 1998), in which the gonads differentiate directly as testes or ovaries. Adult *Oreochromis karongae* and *Tramitichromis intermedius* do not exhibit bisexual gonads and may develop in a similar fashion (Msiska 2002; Harnish 2004).

The observation that there was no significant difference in SL between juvenile females and juvenile males is inconsistent with relatively large juveniles differentiating as males and relatively small juveniles differentiating as females, but it is consistent with juvenile size distributions observed in each of 21 social groups from a series of previous studies (Oldfield et al. 2006; Oldfield 2007, 2009). The larger body size observed in adult males compared with adult females must be due to faster growth after the onset of maturity, as demonstrated previously in nine groups grown out over long periods (Oldfield 2009). A similar pattern has been found in the closely related cichlid "Cichlasoma" *urophthalmus* (Faunce et al. 2002). A similar but opposite process—greater female growth after the onset of maturity—is responsible for sexual size dimorphism in a fish species in which adult females are larger than adult males (Magnuson 1962). The overlap in size ranges between juvenile and adult females in the current study provides further support for this pattern, indicating that growth in females slows upon reaching maturity. Faster male growth is most likely responsible for the size differences that led to the original conclusion of socially controlled sex determination in this species (Francis and Barlow 1993). In that experiment faster male growth was not considered as a possible explanation because stable size ranks had previously been observed in a captive group (Francis 1990), and were assumed to be typical. However, it has recently been shown (Oldfield 2009) that the observation of stable size ranks was an exception rather than the rule.

Because the current subjects came from the same population as the fish used by the original investigators to conclude that this species did undergo socially controlled sex determination (Francis and Barlow 1993), we can now confidently conclude that socially controlled sex determination does not occur in this species, and that those authors came to their conclusion in error. Sex in Midas cichlids is most likely determined genetically, as it is in the closely related convict cichlid, *Amatitlania nigrofasciata* (George and Pandian 1996).

The conclusion that socially controlled sex determination does not occur in Midas cichlids has implications for the theory that sequential hermaphroditism in fishes evolves through changes in developmental timing (Bullough 1947; Atz 1964; Shapiro 1987; Francis 1992; Oldfield 2005). Social control of sex determination has not been conclusively demonstrated in any gonochoristic fish species. Williams (1972) considered the possibility that sex is determined socially in the convict cichlid but his methods were insufficient to make such a conclusion (Oldfield 2005). After working with the Midas cichlid, Richard Francis attempted to demonstrate socially controlled sex determination in another cichlid, *Astatotilapia burtoni*, and social conditions were found not to determine sex (R. D. Fernald, personal communication). Francis (1984) had originally suggested that sex was socially determined in the Paradise fish, *Macropodus opercularis* (Osphronemidae), but this seems unlikely considering what is now known about sex determination in Midas cichlids and in *A. burtoni*. Therefore, sex is not known to be socially determined in any gonochoristic fish, and there is no example of sexual lability at the juvenile stage to support a continuum in developmental timing.

Social control of sex determination may 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 trio 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 (Liu and Sadovy 2004). When juveniles of the typically protandrous (male-to-female changing) clownfishes *Amphiprion bicinctus* or *Amphiprion frenatus* (Pomacentridae) are housed in groups, the largest member differentiates as a female, the second largest a male, and smaller members remain undifferentiated. Juveniles differentiate as females when they mature in isolation (Bruslé-Sicard et al. 1994). In the typically protogynous bluehead wrasse, *Thalassoma bifasciatum*, juveniles differentiate as the initial sex (female) when raised in isolation. In groups of three, one individual develops as a male (Munday et al. 2006). Social control of sex determination in these typically sequentially hermaphroditic fishes are examples of phenotypic plasticity in the timing of sex change, and therefore do not provide any evidence to support a theory that change in developmental timing is involved in the evolution of sequential hermaphroditism.

The idea that heterochrony may play a role in the evolution of sequential hermaphroditism remains plausible and may yet be supported by social control of sex determination.

Genetic factors, rather than phenotypic plasticity, might 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 fair amount of evidence remains that suggests that sequential hermaphroditism might evolve through a change in developmental timing (Bullough 1947; Atz 1964; Shapiro 1987; Francis 1992; Oldfield 2005). This paradigm may yet be beneficial for future investigations attempting to elucidate the proximate mechanisms and evolutionary processes responsible for reproductive development and plasticity in animals.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. The external appearance of a 49 mm SL female *Amphilophus citrinellus* classified in the reproductive phase "immature", and the macroscopic appearance of its gonad. The microscopic appearance of an ovary at this phase was shown completely in Fig. 1A.

Fig. S2. The external appearance and gonad histology observed in juvenile female *Amphilophus citrinellus* classified in the reproductive phase "developing". (A) A 101 mm SL female and its excised left ovary. (B) A higher magnification of a "developing" ovary than is shown in Fig. 1B: a longitudinal section of the ovary of a 68 mm SL female showing perinucleolar (PGpn) and cortical alveolar (PGca) step oocytes close together with little vascularization, thin ovarian wall (OW), and absence of atretic ooctyes. Scale bar = 200 μm. (C) A close up of the cortical alveolar oocytes from a 97 mm SL female showing the cortical alveoli (ca), the zona pellucida (ZP) and surrounding follicle (granulosa) cell (F) and thecal cell (T) layers. Scale bar = 100 μm.

Fig. S3. The external appearance and gonad histology observed in adult female *Amphilophus citrinellus* classified as "spawning capable". (A) A 109 mm SL female and its excised left ovary. Some oocytes in this ovary were in the vitellogenesis/secondary growth stage. (B) Transverse section of the ovary of a 148 mm SL female containing late vitellogenic ooctyes, as well as oocytes in less-developed stages. When visible, germinal vesicles (gv) within late vitellogenic oocytes possessed a jagged margin and consistently occurred off-center as if beginning migration to the oocyte periphery, which would indicate the onset of maturation. Scale bar = 500 μm. (C) A 100 × objective lens oil immersion photomicrograph of the same gonad showing the

simple columnar layer of follicle cells (F) lying outside of the thick, striated zona pellucida (ZP). Thecal cells (T) can be seen lying deep to a single-layered epithelium (E). Large yolk globules (yg) and transparent oil droplets (od) are observed within the ooplasm, as well as small cortical alveoli (ca) that lie immediately adjacent to the ZP. Scale bar = 50 μm. (D) An ovary from a 116 mm SL female displaying alpha (αAF) and beta (βAF) atretic follicles. Scale bar = 200 μm.

Fig. S4. The external appearance and gonad histology observed in an adult female *Amphilophus citrinellus* classified as "regenerating". (A) A 103 mm SL female and its excised left ovary. (B) Longitudinal section of the same ovary. The most advanced oocytes are in the cortical alveolar (PGca) step. The oocytes are dispersed and the ovarian wall (OW) is thick. There are several blood vessels (BV) running through the ovary, adjacent to melano-macrophage centers (MMC), which are characterized by containing yellow flocculent material. Scale bar = 200 μm.

Fig. S5. Juvenile 69 mm SL male *Amphilophus citrinellus*, and its excised right testis, which was classified as "developing". The microscopic appearance of a testis at this phase was shown completely in Fig. 1E.

Fig. S6. The external appearance and gonad histology observed in "spawning capable" adult male *Amphilophus citrinellus*. (A) A 161 mm SL male and its excised left testis. (B) Transverse section of a "spawning capable" testis from a 133 mm SL male, demonstrating lobular structure. The low numbers of spermatozoa and continuous germinal epithelium (GE) of spermatocysts that extends the entire proximal to distal distance of the lobules indicate a state of early GE development/early maturation and preparation for spawning. Sc = spermatocyte, Sg = spermatogonium, St = spermatid, Sz = spermatozoa. (C) Transverse section of a "spawning capable" testis from a 124 mm SL male. The reduced density of spermatocysts and large numbers of spermatozoa within the lumena of lobules and sperm ducts indicates a more advanced state of development than in Fig. S6B. The discontinuity of the germinal epithelium reaches the periphery of the testis in some places, indicating late GE development/late maturation and readiness for spawning. (D) "Spawning capable" testis of a 149 mm SL male showing regions with large amounts of spermatozoa and other, peripheral, regions devoid of spermatogenesis, possessing only spermatogonia, indicating a more advanced state of late GE development/late maturation than is shown in Fig. S6C. Scale bar = 100 μm for B–D.

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