Age, growth, and sexual development in the self - fertilizing hermaphroditic fish *Rivulus marmoratus*

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Synopsis

We studied age, growth, and sexual development in the early life intervals of the self-fertilizing mangrove killifish, *Rivulus marmoratus*. Newly hatched (day 0) individuals had sagittal otoliths of 60 µm radius, with about 30 increments. Sequential sampling until about day 60 after hatching yielded otoliths with the number of increments outside the 60 µm radius equal to the daily age of the fish. Alizarin complexone marking of otoliths also confirmed the increments were daily, and demonstrated the applicability of this technique to field studies for mark-recapture, or age and growth estimates. Individuals fed a restricted amount of food formed fewer daily otolith growth increments than fish fed to satiation each day. Using histological analysis for identifying gonad morphogenesis, we found no correlation between gonadal development and external appearance (caudal ocellus, orange fin colouration) in young fish of known ages. The caudal ocellus was not present until 9 mm total length, and developed thereafter. Of 136 individuals examined, fish less than 17.2 mm total length (TL, n = 124) were females. Testicular tissue first appeared among individuals 17 - 18 mm TL (n = 3), while some individuals greater than 18 mm TL (n = 8) were functional hermaphrodites. The single male in our study was relatively in small body size (9.6 mm, day 37) with a distinct caudal ocellus, indicating that it is presumably a primary male.

Introduction

Rivulus marmoratus, the mangrove killifish, is the only known self - fertilizing hermaphroditic vertebrate (Harrington 1961, Warner 1978). The fish inhabits brackish mangrove habitats from Brazil to Florida (Harrington & Rivas 1958, Turner[1]). This species is of interest not only for its unique reproductive biology (Harrington 1967, 1968, 1971, 1975) but also because the genetically identical individuals within each self-fertilizing lineage (sometimes referred to as clones) are remarkably suited to studies of developmental biology (Swain & Lindsey 1968a, 1968b), physiology (Ali et al. 1988, King et al. 1988, 1989), toxicology (Abel et al. 1987, Davis 1988, Lin & Dunson 1993, Park et al. 1994) and cancer research (Park & Kim 1984, Koenig & Chasar 1994, Couch 1995).

In general, individual mangrove killifish are reported to be either hermaphrodites which produce both sperm and ova simultaneously, or secondary males which develop from hermaphrodites by loss of ovarian tissue, or primary males which develop directly to produce sperm throughout the rest of their lives (Harrington 1971, Soto et al. 1992). Both primary and secondary males are quite rare in the field and the laboratory. The proportion of males is reported as very much less than 1% in localities in Florida (Davis et al. 1990), to at most 24% at one site in Belize (Turner et al. 1992a). Males develop distinguishing orange colouration on their median fins and posterior parts of their bodies and generally lack caudal ocelli. Hermaphrodites have a distinct caudal ocellus but no orange colouration (Soto & Noakes 1994). It was assumed for many years, and earlier genetic techniques had confirmed, that all reproduction in this species is by internal self-fertilization in hermaphrodites (Harrington & Kallman 1968). However, recent molecular genetic studies have shown that while most individuals appear to be the result of such self-reproduction, there is genetic variation in some wild individuals that must result from cross-fertilization between individuals (Turner et al. 1991, 1992b, Lubinski et al. 1995). Green & Noakes (1996) predicted that the mangrove killifish is likely to have alternating crossing and self-reproduction, based on theoretical considerations. The behaviour

[1] Turner B.J. 1998. The RivMar Webpage (http://www.bsi.vt.edu/rivmar/). Virginia Polytechnic Institute and State University, Blacksburg.

involved in such crossings is not yet known.

Recently, detailed histological observations of young individuals revealed that most contain only ovarian tissue. There is an increasing development of testicular tissue in gonads of these individuals with age, so that they become hermaphrodites (Cole & Noakes 1997). Individuals with only ovarian tissue were found between 0 and 100 days after hatching. Mature ovarian gonads were found only between 60 and 100 days after hatching. This finding suggests the possibility of crossing between hermaphrodites or males and these young individuals with only ovarian tissue.

In previous histological studies (Soto et al. 1992, Cole & Noakes 1997) the specimens were mostly from 30 to 100 days after hatching. However, body size of their specimens were not mentioned, and chronological age since hatching cannot be taken as an absolute ontogenetic landmark (Noakes & Godin 1988). Moreover, there is still limited information on the details of early life history of this species, such as age determination and sexual development examined both by size and age. There have been very few studies of early development in mangrove killifish. Koenig & Chasar (1984) clearly demonstrated a simple rearing method of this species, the embryonic development, and growth until 225 days after hatching with 2-4 weeks intervals, but sexual development. Park & Lee (1988) described the development of squamation, but gave no information on sexual development. Ali et al. (1988) described ontogenetic changes in the retina, but again without mention of the reproductive system.

We compared the gonadal development and external appearance (body colouration, caudal ocellus) of known age individuals to confirm the age (size) – specific time course of sexual development (particularly hermaphroditism) in this species. Counts of daily growth increments are commonly used for estimating age and growth of many young fishes (Campana & Neilson 1985, Secor et al. 1995). We validated this technique for the mangrove killifish by comparing otolith increment counts to known ages of fish since release of fertilized eggs from hermaphrodites and from hatching. We also marked otoliths on known days with a fluorescent chemical to validate the daily growth increments. Environmental conditions, including food availability, are believed to vary widely for this species in nature (Davis et al. 1990). We tested the prediction that food restriction would limit somatic and otolith growth. Based on previous studies (Soto

et al. 1992, Soto & Noakes 1994, Cole & Noakes 1997), we tested the hypothesis that this species is a protogynous diandric hermaphrodite. We predicted that fish would initially develop gonads with only ovarian tissue or with only testicular tissue. Those with only testicular tissue, primary males, would not develop ovarian tissue at any time. Those individuals who first developed only ovarian tissue in their gonads would subsequently develop testicular tissue as well, and would become hermaphrodites. We predicted that the orange colouration typical of males would develop concurrently with the development of testicular tissue in males, and that the caudal ocellus typical of hermaphrodites would develop in individuals with only ovarian tissue as they added testicular tissue to their gonads.

Materials and methods

Experimental fish

We studied fish from a single lineage, derived originally from Florida (97-52-7; W. P. Davis, U. S. Environmental Protection Agency, Gulf Breeze, Florida) to minimize genetic variance in our experiments. The original fish was collected in Florida in 1994 (named PAN-RS by W. P. Davis, U. S. Environmental Protection Agency, Gulf Breeze, Florida). The fish we used is the 4th generation from the original fish.

Fish were held individually in translucent plastic containers (FisherBrand Collection Containers, Fisher Scientific) in about 60 ml of brackish water. Water in all containers was changed weekly. We constituted the brackish water from distilled water and marine salt (Instant Ocean, Aquarium Systems). All fish were held in a climate – controlled room (12 : 12 hours light : dark photoperiod, 26 °C, pH of water 8.0, 17 ppt salinity) at the Hagen Aqaualab at the University of Guelph. All fish were fed newly hatched brine shrimp, *Artemia salina*, nauplii and daphnia, *Daphnia pulex*, once daily. Adult hermaphrodites released fertilized eggs on a regular basis (about 5 per week per individual). The developmental state of the fertilized eggs released by hermaphrodites varied somewhat, since eggs are released at different times after internal fertilization in this species (Davis 1988). Developing embryos were placed in individual containers and held under the same conditions as adults. The day of hatching was designated as day 0 for convenience of comparisons among experimental treatments (Cole & Noakes 1997).

Age and growth

We tested the prediction that food restriction would limit somatic and otolith growth by holding young fish under one of two feeding conditions: satiation (n = 106) or restricted (n = 33). Fish in the satiation condition were fed more brine shrimp nauplii than they could eat in 5 minutes, once each day. Fish in the restricted group were fed 5 – 20 individual brine shrimp nauplii once each day. Fish were euthanized with 400 ppm of clove oil (Keene et al. 1998) at various ages (day 0 to 60 after hatching). Total length of each fish was measured to the nearest 0.1 mm, and both saggital otoliths were extracted under a dissecting microscope. After extraction of otoliths, we preserved fish in a 5% formalin solution for histological examination of sexual development. We used total length (TL) as our measure of body size since it can be measured more quickly and with less disruption for live fish than would standard length (SL). At the end of our study we measured both total length and standard length for individuals over the size range in our study. The relationship between standard length and total length is given by the expression SL = 0.8 TL -0.4 (n = 70, r = 0.99).

On day 13 we anaesthetized 22 of the 106 fish in the satiation group with 100 ppm clove oil and measured their total lengths by caliper to the nearest 0.1 mm. Following this measurement we returned all fish to their original containers and held them until they recovered from the anaesthesia. We then labelled 15 of the 22 fish in their individual containers with 100 ppm of ALC (alizarin complexone, Sigma Co.) for 12 hours, following the methodology of Tsukamoto (1988). The other 7 fish were transferred to containers with brackish water, as controls. All fish were euthanized with 400 ppm clove oil 7 days after marking with ALC (day 20). We measured each fish again for total length, and extracted otoliths under a dissecting microscope. Fish were then preserved in 5% formalin solution for histological observations for sexual development.

Validation of otoliths daily increments

Each otolith was mounted in epoxy resin (Bond E-set, Konishi Bond, Japan) with its lateral side on a glass microscope slide. Otoliths were ground to a plane with the

nucleus using fine abrasive paper (Sakakura & Tsukamoto 1997). We used a camera lucida to measure the shortest radius of the otolith (radius, μm) and to count increments between the nucleus and otolith edge. A bipartite structure of a narrow opaque band (discontinuous zone) and an adjacent translucent (incremental zone) was counted as one growth increment. Widths of growth increments were also measured for 20 specimens each for the satiation and restricted feeding groups. To minimize counting errors, the off-focusing technique was used (Tsukamoto & Kajihara 1987). The ALC label in otoliths was examined with an ultraviolet illumination microscope (Tsukamoto et al. 1989a). Growth increments were counted outside the ALC label. All counts of growth increments were repeated three times by the same observer for each specimen. The mean value of increment counts was calculated for each individual.

Sexual development

We observed the external appearance of all specimens under a dissecting microscope prior to otolith extraction to judge the development of the caudal ocellus and orange colouration. The caudal ocellus was recorded as present when well defined black or slightly faded (Soto & Noakes 1994).

All preserved specimens were processed for histological study by dehydrating and embedding in paraffin following the procedure of Cole & Noakes (1997). They were serially sectioned at 7μm in their entirety, mounted on glass slides, stained with hematoxylin and eosin, and examined with a light microscope. Following Cole & Noakes (1997), we classified sexual development of gonads as: (1) immature female, with only immature oocytes; (2) mature female, with vitellogenic oocytes; (3) immature hermaphrodite, with vitellogenic oocytes and spermatocytes; (4) mature hermaphrodite, with vitellogenic oocytes and spermatozoa; (5) male, with only spermatocytes and/or spermatozoa.

Statistical analyses

For the relationship between age and TL, between TL and otolith radius, and between age and counts of otoliths increments, linear fitting was applied (Sokal & Rohlf 1995). Between-group comparisons of widths of otolith increments for satiation and restricted

groups, and comparison of TL for the ALC – labelled and control fish were undertaken using Student's t-test (Sokal & Rohlf 1995).

Results

Age and growth

The interval between release of the fertilized egg by the hermaphrodite to hatching ranged from 20 to 90 days (mean ± standard deviation = 36.3 ± 13.8, n = 39) and average TL at hatching was 6.1 ± 0.2 mm (n = 7). Young fish started feeding immediately after hatching (day 0) even though yolk could be detected histologically until day 3 (data not shown). The age – TL relationship was fitted closely by a linear regression, and the growth of the satiation group was about 3 times higher than that of the restricted group (Figure 1).

The otolith of the mangrove killifish was a cardioid shape at all sampling ages. We examined 131 of 139 from satiation, restricted and ALC experiments (8 specimens were lost during otolith extraction). Mean otolith radius was 59.9 ± 5.6 µm (n = 7) on day 0. The relationship between TL (x mm) and otolith radius (y µm) was described by the following equation: $y = 10.0 x + 7.3$ ($r = 0.97$, $n = 131$). Otoliths did not have clear check marks around the 60 µm radius, and increments were faint from the nucleus to the outer edge. The relationship between day from release of the fertilized egg (day x) and number of otolith increments (y) was $y = 0.8 x – 2.0$ ($r = 0.9$, $n = 76$) in the satiation group, and $y = 0.4 + 29.8$ ($r = 0.53$, $n = 33$) in the restricted group, respectively.

Increments of the otolith outside the 60 µm radius showed almost the same number as its known age in days (Figure 2). The linear regression between age (day x) and number of otolith increments (y) was $y = 0.9 x – 0.1$ ($r = 0.99$, $n = 76$) in the satiation group, and $y = 0.7 x – 0.4$ ($r = 0.92$, $n = 33$) in the restricted group, respectively. The mean number of increments between the core region and 60 µm radius was 24.5 ± 10.0 (n = 109). The widths of growth increments inside the 60 µm radius were not significantly different between the satiation group (1.2 ± 0.6 µm, n = 20) and the restricted food group (1.0 ± 0.4 µm, n = 20; t test, $p = 0.24$). The widths of increments outside the 60um radius of the satiation group (3.2 + 1.2 µm, n = 20) were significantly larger than those of the restricted group (1.5 + 0.6 µm, n = 20; t – test, $p < 0.01$).

The initial TL of the fish before ALC marking was 10.6 ± 0.6 mm (n = 22, day 13). By 7 days after ALC marking the TL of marked fish was 12.6 ± 0.7 mm (n = 15), and of the control fish was 13.0 ± 0.7 mm (n = 7), respectively. There was no significant difference in TL between ALC marked and control fish (t – test, p = 0.2). A clear fluorescent mark was observed in the otoliths of the ALC marked fish under UV illumination. The number of otolith increments outside the ALC mark was 6.4 ± 1.8 (n = 15, range 4 – 10 increments).

Sexual development

Orange colouration on the body or fins was not seen in our sample of 139 fish (ages 0 to 60 days). The minimum TL at which the caudal ocellus was present was 9.1 mm (satiation group, day 10), and the maximum TL at which the caudal ocellus was not yet present was 10.7 mm (satiation group, day 14, Figure 3), respectively.

Sections of gonads were present for 136 of 139 fish (some sections for the other 3 fish were lost during processing). Since all 22 fish in the ALC labelled group had only immature ovarian tissue in their gonads (day 20), they were combined with the other groups (Figure 3). From hatching until 17 mm TL, all fish had only immature ovarian tissue in their gonads. Thereafter they rapidly started to develop mature ovarian and testicular tissue in their gonads (Figure 3). The body size of fish with only mature ovarian tissue was 17.2 ± 0.5 mm (n= 5). Immature hermaphrodites were 17.2 ± 0.1 mm (n = 3), and mature hermaphrodites were 18.3 ± 1.1 mm (n = 8), respectively. The largest individual with ovarian tissue only was 17.2 mm TL (satiation group, day 36). The smallest individual with a mature hermaphroditic gonad was 16.3 mm TL (satiation group, day 34, Figure 3). We found only one male, and it was a primary male, in our study. It had a distinct caudal ocellus and no orange colouration (9.6 mm TL, restricted group, day 37, Figure 3) when it was examined histologically.

Discussion

Age and growth

We have confirmed that *R. marmoratus* deposits daily otolith growth increments after hatching. Otolith increments of the food restricted treatment were fewer than

increment counts of the satiation treatment. There are several possible explanations for this difference. Campana & Neilson (1985) suggested that the resolving power of light microscopy might cause underestimation of true age by missing increments narrower than the theoretical resolution limit (0.2 µm). In *R. marmoratus*, the width of otolith increments is 1.8 $\pm$ 1.1 µm (n = 80) and the minimum increment width is 0.3 µm. Both these are larger than the resolution limit of the light microscope, so this is not likely to account for the differences we observed between the diet treatment groups.

Food restriction is known to affect not only somatic growth but also the formation of otolith increments in fishes (Campana 1983, Molony 1996, Payan et al. 1998). For example, juvenile Japanese eel, *Anguilla japonica*, and milkfish larvae, *Chanos chanos*, held without feeding showed very little somatic growth and did not deposit daily otolith increments (Umezawa & Tsukamoto 1991, Tzeng & Yu 1992). In our study, the somatic growth as well as the width of otolith growth increments outside the 60 um radius (after hatching) of the food restricted treatment group were significantly lower than those of the satiation group. Growth increments within the 60 um radius (before hatching) were the same width in both treatment groups. We conclude from this evidence that the lower counts than age in the food restricted group in our study resulted from the same mechanism as the food restricted studies cited above.

ALC marking of otoliths further confirms that *R. marmoratus* deposits daily otolith growth increments and validates the bipartite structure as the daily increment. However, there is some variance in days compared to otolith increment counts. This is partly because the growth increments tend to be faint. Otolith growth increments of wild-caught fishes are invariably more distinct than those of laboratory-reared individuals (Campana 1983, Campana & Neilson 1985, Umezawa & Tsukamoto 1991, Sakakura & Tsukamoto 1997), so we believe that aging from otolith increments can easily be applied to wild-caught specimens of *R. marmoratus*. Moreover, this aging technique can add more useful and reliable ecological aspects to field studies, since there is at present a relatively limited knowledge of the distribution and ages of young fish in the wild (Davis et al. 1990, Taylor 1992, Taylor et al. 1995). ALC marking of otoliths does not affect the growth, behaviour or survival of fishes (Tsukamoto 1985), including *R. marmoratus*. Both ALC and control fish developed mature ovarian tissue and grew at comparable

rates. ALC marking of otoliths would be useful for marking small *R. marmoratus* for behavioural or other investigations of young individuals, as it has been in comparable studies of other species (Tsukamoto 1985, Tsukamoto et al. 1989, Sakakura & Tsukamoto 1999). In particular, ALC marking of otoliths could be used for mark-recapture, movement, and growth studies in the field.

There was a very wide range in days from release of the fertilized egg by the hermaphrodite to hatching (20 to 90 days), even though all fish were from the same lineage, kept under the same environmental conditions and virtually all developed as hermaphrodites. This wide variation in time to hatching has been reported by others for this species (e.g. Ritchie & Davis 1986). On the other hand, the slope of the equation between days to hatching and the number of otolith increments inside the 60 µm radius which were formed before hatching was almost flat (0.3). This indicates that while some otolith increments do form before hatching, there is no correlation between age at hatching and the formation of otolith increments up to that time. This means that the rate of embryonic development before hatching is different for each individual. It also means that the formation of otolith increments before hatching is not a function of daily rhythms but rather a consequence of some other biological (epigenetic) events.

The mummichog, *Fundulus heteroclitus*, is in the same Order as the mangrove killifish. However, in the mummichog otolith daily increments are formed about 3 days before hatching (14 days after activation), and they are based on daily rhythms (Radtke & Dean 1982.). Similar daily otolith growth increments before hatching have been reported in the rainbow trout, *Oncorhynchus mykiss* (Mugiya 1987). In contrast, otolith increments before hatching in the ayu, *Plecoglossus altivelis*, do not reflect an exact daily basis, but are also affected by parental genotype and the incubation temperature of the embryos (Tsukamoto 1987), similar to what we have found for the mangrove killifish. Therefore, if juveniles of any population of the mangrove killifish were to be aged from otoliths, the size of otolith and of the entire body would have to be determined using *in situ* incubation experiments as we have done, e.g. 60 µm radius and 6.1 mm TL in our study.

Ritchie & Davis (1986) reported that embryonic diapuse is possible in this species, because they observed emergence of 10 mm TL juveniles in a Florida site following a

monsoon flood after several months of dry season when no adults could have been present. The differences we found among hatching dates for different individuals, despite the same genetic constitution and the same controlled environment, could be the result of embryonic diapuse. Further incubation studies would be required to resolve this issue, and to investigate the mechanism(s) required to trigger hatching in this species.

Sexual development

In adult mangrove killifish there is a strong correlation between sex and external appearance (orange colouration for males and caudal ocellus for hermaphrodites) (Soto & Noakes 1994). However, the caudal ocellus developed around 10 mm TL (about day 10 – 20), earlier than sexual maturation (about 17 mm TL, and day 30 – 40, Figure 3). On the other hand, the one male in our study had a distinct caudal ocellus (Figure 3) and no orange colouration (9.6 mm TL, day 37). Therefore, there may be no correlation between sexual development and colouration in the early life of this species. The 10 mm TL size corresponds to the time of onset of agonistic interactions in this species (Sakakura, unpublished observations). This suggests that the development of the caudal ocellus reflects the onset of social interaction, rather than sexual development. The critical involvement of brain development in addition to morphological development for social interactions such as schooling behaviour, have been reported in some marine species (Masuda & Tsukamoto 1998, Masuda et al. 1998). Further studies focused on morphological and physiological development, including skeletal and neurological features, are required for the mangrove killifish, in addition to the study of development of sex hormones.

Cole & Noakes (1997) found individuals with gonads containing only mature ovarian tissue between 60 and 100 days of age, and thus we hypothesized that the mangrove killifish is a possible protogynous hermaphrodite. In the present study we confirmed the existence of individuals with only mature ovarian tissue (Figure 3). However, the interval for individuals with only mature ovarian tissue in their gonads is somewhat limited (from about 16 to 18 mm TL, corresponding to about day 34 to 46). The mating of these individuals with either males or hermaphrodites, was suggested by

Cole & Noakes (1997) as a possible mechanism to produce the outcrossed individuals found in some localities. If it does occur, it would be limited to a relatively short interval early in the life of individuals who soon become hermaphrodites. However, without further data we cannot reject either that possibility or the alternate possibility of outcrossings between mature hermaphrodites and males, or between mature hermaphrodites.

There is only one published report of an outcross mating in the mangrove killifish. Harrington & Kallman (1968) observed a pairing between an adult male and a hermaphrodite. The latter fish was notable because it had oviposited only unfertilized eggs before this mating. The mating was reported to have produced two fertilized eggs which both developed to become hermaphrodites. For any hermaphrodite to be involved in an outcross mating would require that the hermaphrodite would have to be able to constrain its own internal fertilization so as to release unfertilized eggs for external fertilization by another individual. Whether this is possible is at present an open question. Our results, as those of Cole & Noakes (1997), suggest that the most likely scenario for outcross matings would involve a male (primary or secondary) and a young fish with only ovarian tissue in its gonads. Clearly a study of the mating behaviour and other social interactions of the mangrove killifish is required to resolve these important questions.

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Figure legends

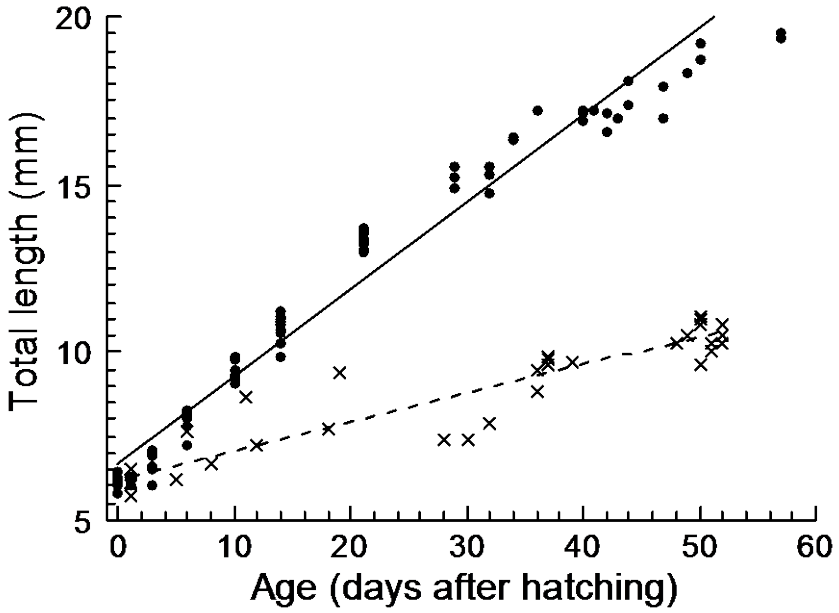

Figure 1. Size of *Rivulus marmoratus*. (total length, mm) fed to satiation (dots solid line, y = 0.25x + 6.7; r = 0.98; n = 84) and on a restricted diet (crosses, dotted line, y = 0.08x + 6.2; r = 0.93; n = 33) at different ages (days after hatching).

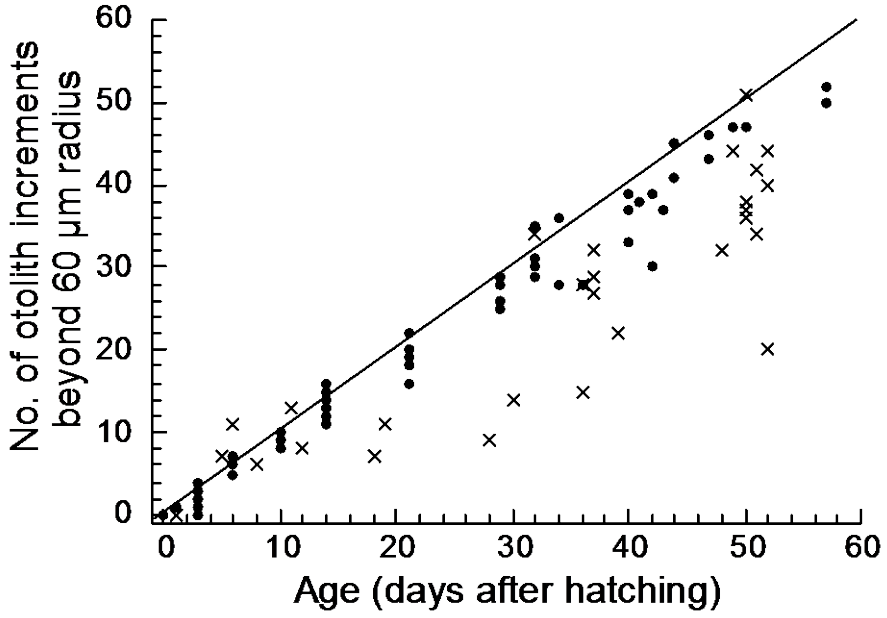

Figure 2. The relationship between age (days after hatching) and the number of otolith increments beyond the 60 µm radius in *Rivulus marmoratus* fed at different food regimes (satiation = dots, restricted = crosses). The diagonal line indicates the relationship predicted if there is one increment per day after hatching (y = x).

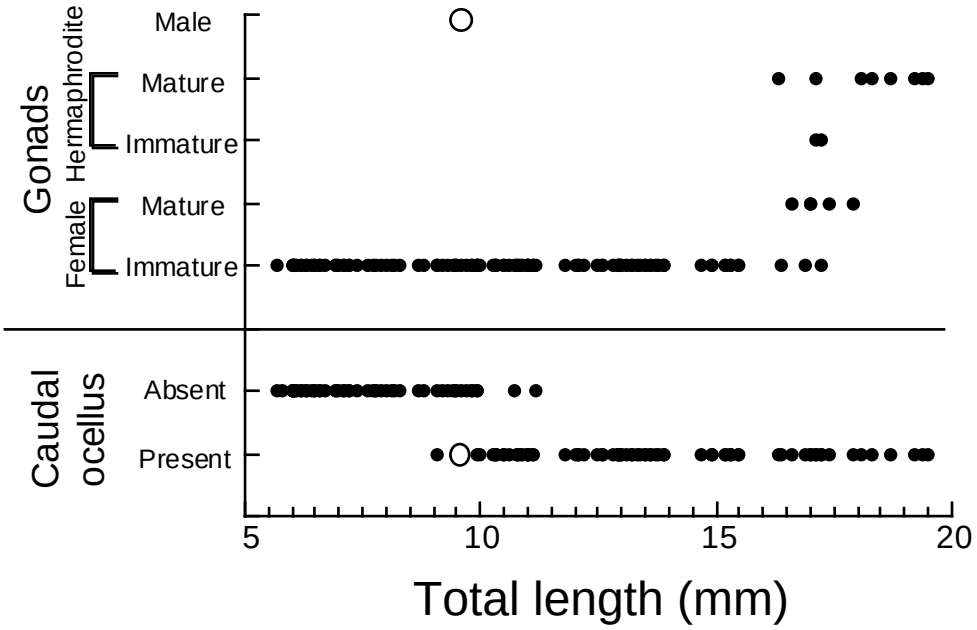

Figure 3. The relationship between sexual allocation and external appearance of *Rivulus marmoratus*. Each dot indicates one individual (n = 136 for gonads; n = 139 for external appearance). The open circle indicates the one male found in our study.