EFFECTS OF GONADAL STEROIDS ON SEXUALLY DIMORPHIC CHARACTERS IN *BRIENOMYRUS NIGER* (GÜNTHER, 1866) (MORMYRIDAE, TELEOSTEI): SOLVING A PARADOX

by

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Abstract

EFFECTS OF GONADAL STEROIDS ON SEXUALLY DIMORPHIC CHARACTERS IN *BRIENOMYRUS NIGER* (GÜNTHER, 1866) (MORMYRIDAE, TELEOSTEI): SOLVING A PARADOX

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Sonja K. Stell

Advisor: Professor Peter Moller

This study established the maturational time line for *Brienomyrus niger* and identified discrete developmental stages, characterized by the appearance of prominent osteological sexual dimorphisms (support structures of the anal-fin). The study proceeded with an investigation of androgen-induced transformations in juvenile and adult fish. 17α-methyltestosterone (17MT) exposure induced male-typical transformations of reproductive structural and behavioral traits in juveniles of both sexes and in subadult and adult females. These transformations were partial and masculinized only secondary sexual traits: (1) large expansions of the anal fin ray bases and thickening of fin ray shafts, (2) inferred increases in attached muscle leading to an indentation of the dorsal margin of the anal fin, (3) structural modifications of the electric organ, i.e. a thickening of the anterior face

of the individual electrocytes, and (4) temporal changes in the electric discharge it generates. Following hormone withdrawal, osteological modifications remained permanent, whereas the male-typical elongation of the electric organ discharge and body wall indentation returned to pre-treatment conditions. 17MT treatment did *not* masculinize female gonads, but, on the contrary, resulted in a dramatic hypertrophy of gonadal tissue in females and induction of normal oocytic development. There was no evidence of intersex gonads.

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The support and encouragement of mentors, peers, friends, and family were vital to my completion of this long and sometimes difficult path. I would not be the person I am today without the experiences and opportunities this work provided me with.

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My times spent with Dr. Barbara Brown, Radford Arrundell, Damaris Rodriguez, Erica Detwiler, and Xenia Freilich studying fish at the American Museum of Natural History deepened my knowledge and made my work even more enjoyable.

What would a dissertation be without the friendships it creates?
Now Ph.D., Anika McPhie, who showed me the light, and Judy Choi, who still shares the passion for Sushi with me - thank you both for making me stay sane through all these years.

I would like to thank my parents from the bottom of my heart for giving me the opportunity to come to this country and support me in fulfilling my dreams. My husband Frederic for all his love, support and understanding when I needed it most, my best friend Angela, who always reminds me of the important things in life, and my dog Zoe who started the whole passion for the furry kind.

Only dead fish swim with the stream.

Abbreviations

17MT	17 α-Methyl Testosterone
AFL	anal fin length
AMNH	American Museum of Natural History
ANOVA	analysis of variance
b/a	tangent α
CF	Control female
CM	Control male
DHT	dihydrotestosterone
E_2	17β-estradiol
ED	endocrine disruptors
EOD	electric organ discharge
GSI	gonado somatic index
HPG	Hypothalamo- pituitary-gonadal axis
HSD	Honest Significant Difference test
IACUC	Institutional animal care and use committee
L:D	light/dark cycle
PPSF	peak power spectrum frequency
Q_{10}	temperature coefficient
SL	standard length
T	Testosterone
TF	17MT treated female
TM	17MT treated male

Glossary

anterior	towards the front (head) end of the fish
basal	at or towards the base
caudal	towards the posterior end or caudal fin
dorsal	towards the back or upper part of the body
meristics	is an area of ichthyology, which relates to counting quantitative features of fish, such as the number of fins or scales.
morphometrics	from the Greek: "morph," meaning shape or form, and "metron", meaning measurement comprises methods of extracting measurements from shapes.
posterior	situated at the rear or behind something
rostral	headwards, relating to snout
ventral	underside of fish / lower part of body

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General Introduction

Electric Fish

Electric fishes have caught the interest of naturalists, primitive physicians, philosophers, and scientists since ancient times. These fish played a major role in understanding the nature of electricity and since the discovery of their electric sense in the mid 20th century, have fascinated ethologists, neurobiologists, and molecular taxonomists alike (Moller, 1995, 2006; Sullivan, Lavoué, and Hopkins, 2000). Many species have become 'champion species' (Heiligenberg, 1991; Zupanc and Lamprecht, 2000), and research on electric fishes has contributed to basic understanding of evolutionary principles underlying social communication, molecular taxonomy, neural processing, and stimulus integration, and molecular control of motor output based on characteristics of membrane ion channels (Bullock, Hopkins, Potter, and Fay, 2005). Sharing a considerable amount of developmental plasticity with other teleost fishes (Taborsky, 1994; Foran and Bass, 1999), several groups of electric fish, and the African Mormyridae in particular, have spurred interest in exploring the behavioral endocrinology underlying this plasticity (Bass and Hopkins, 1983, 1985; Landsman, Harding, Moller, and Thomas, 1990; Carlson, Hopkins, and Thomas, 2000).

The African weakly discharging electric fish of the family Mormyridae belong to a small group of extant fishes (about 1.6%) that possess the ability to

generate and detect electric organ discharges (EODs) using specialized, myogenic (or in some cases, also neurogenic) organs, and equally specialized, lateral-line derived electroreceptors (Bennett, 1971 a,b; Bass, 1986a; Zakon, 1986; Bell, 1986; Bullock et al., 2005). EOD-related sexual dimorphisms result from direct gonadal steroid action on the basic element of the electric organ, the electrocytes, typically causing longer lasting EODs in males than in females (Bass, 1986 a, b; Bass and Hopkins, 1983, 1984; Freedman, Olyarchuk, Marchaterre and Bass, 1989; Landsman, 1993 a, 1995). For South American gymnotiforms, using immunocytochemistry, it has been shown that electrocytes immunolabel positive with antibodies to either androgen (*Eigenmannia virescens* Valenciennes, 1842, Dunlap and Zakon, 1998) or estrogen receptors (*Sternopygus* sp.: Dunlap, McAnelly and Zakon, 1997).

EODs facilitate spatial orientation and social communication, and because of their nocturnal habitat, mormyrids rely on these signals for species recognition and mate attraction (Hopkins, 1981, 1986; Kramer, 1990; Moller, 1995). During courtship, the male envelops the female's anal fin with its own, forming a common spawning pouch in which the eggs are fertilized (Kirschbaum, 1987; Moller, Schugardt, and Kirschbaum, 2004). This behavior lasts only a few seconds requiring fast flexing of the anal fin. The expansion of the anal-fin ray surface should provide the increased substrate for muscle attachment and thus facilitate the anal-fin reflex (Brown, Benveniste, and Moller, 1996).

Two sexually dimorphic structures seem to facilitate this behavior as adult males are distinguished from juveniles and adult females by a dorsally directed

indentation of the posterior ventral body wall (Iles, 1960; Nawar, 1959; Okedi, 1969; Kirschbaum, 1987, 1995; Kirschbaum and Schugardt, 1995; Schugardt, 1997; Pezzanite and Moller, 1998; Greisman and Moller, 2005) and massive expansion of the bases of several anal-fin rays (Brown et al., 1996; Pezzanite and Moller, 1998).

As in most vertebrates (e.g. Nottebohm, 1980, 1989; Arnold and Breedlove, 1985; Harding, 1986; Kelley, 1988; McEwen, 1991; Nelson, 1995), gonadal steroids control the normal expression of sexual differentiation and can effect structural, physiological, and behavioral changes (e.g. Bass, 1992; Brantley, Marchaterre, and Bass, 1993 a; Brantley, Wingfield and Bass, 1993 b; Landsman, 1995; Rosa-Molinar, Fritzsch and Hendricks, 1996). The androgen sensitivity of the EOD-generating structures, the electrocytes, led to several studies that investigated the effect of exogenous-hormone manipulation on these structures (Bass and Hopkins, 1983, 1985; Landsman et al., 1990; Herfeld and Moller, 1998; Voustianiouk, 2003). Because of the aforementioned developmental plasticity, it was not surprising that in species with a natural EOD sex difference, gonadal steroids can induce females and juveniles to produce EODs that are typical of sexually mature males (Bass, 1986c; Bass and Volman, 1987).

The goal of the current work was to apply an integrative approach to relate the known normally expressed sexual dimorphisms in mormyrid fish, i.e. behavior (EOD, anal fin reflex) and structure (expanded anal-fin ray bases, and indented ventral body wall in males) with the reported gonadal steroid sensitivity of the electric organ and its motor output, the EOD.

It was hypothesized that the developmental plasticity evidenced by hormone-manipulated adult mormyrids can clarify the organizational-activational effects in these fish. Reproductive structures and behaviors may not only be independent of early organizational effects, but also be bipotential. Thus it should be possible to induce these traits at all times and in both sexes.

Since the female electric organ and the EOD it generates can be masculinized through testosterone administration, it was hypothesized that androgens should also drive other structural and behavioral traits that are intimately involved with the fish's reproductive behavior. Further, as secondary sexual dimorphisms are plastic during adulthood, could primary characters such as gonadal tissue be sex reversed in adulthood, as is demonstrably the case in several reproductively-active teleosts (Shapiro, 1992; Cole, 1990)?

Brienomyrus niger

Several reasons contributed to the choice of the subject, the mormyrid *Brienomyrus niger*. A considerable library of background information on this species covers aspects of both its electric (EOD) and social behavior (Moller, 1970; Squire and Moller, 1982; Moller and Serrier, 1986; Serrier and Moller, 1989; Moller, Serrier, and Bowling, 1989). Seminal work on hormone effects on the electric behavior of Mormyridae involved species belonging to the genus *Brienomyrus* (see reviews: Bass, 1986 a, b). Bass (1986 b) reported that 17MT caused changes in the EOD waveform in *B. niger*. A new structural sexual dimorphism, i.e. the naturally occurring basal anal-fin ray expansion was discovered in specimens (adult

males) belonging to the genus *Brienomyrus* (Brown et al., 1996). This genus with about 10 described species was erected by Taverne (1971). These fish are characterized by a moderately elongated body, dorsal and anal fins approximately equal in length, 5-7 teeth in the upper jaw and 6-8 in the lower jaw, the absence of the lateral ethmoid bone, the presence of 6 circumorbitals, and 4 hypurals. Electric organs are composed of electrocytes with non-penetrating stalks with posterior innervation or electrocytes with penetrating stalks with anterior innervation (Sullivan et al., 2000). *B. niger* is indigenous to West African riverine and lacustrine habitats (Gambia, Niger, Volta, White Nile, Lake Chad, the Senegal basin, and the Sudan region (Lowe-McConnell, 1987)).

Specific aims, questions, and hypotheses

Chapter 1. To determine the natural maturational time course for *B. niger* and identify discrete developmental stages. A large number of preserved specimens, with a wide range of sizes, yielded an ideal 'developmental series'. What was the correlation of anal-fin ray expansion and anal fin indentation with gonadal maturity? At what size (age) will ray expansion begin and end? How is this expansion related to sexual maturity? Does the frequency of fish with and without expanded rays match the sex ratio based on gonadal evidence?

Chapter 2. In a parametric study, to establish the mathematical procedures (Q_{10} values) to standardize EOD measures that could be affected by temperature and water conductivity.

Chapter 3. To systematically follow the effects of externally administered

17α-methyltestosterone (17MT) to juveniles, and adult males and females, on their EOD characteristics (EOD waveform and related Fast Fourier peak power spectra) and structural traits (anal fin support structures, including but not restricted to the ray bases). What is the time course of these induced transformations, and what is their plasticity or permanence?

Chapter 4. To verify for sexually mature female *B. niger* that the structure of the electric organ (electrocytes) can be masculinized through exogenous androgen treatment, and to compare these effects with treated males. What is the time course of change? Are there sex differences in response to the hormone?

Chapter 5. To test two hypotheses that (1) late lifetime exposure to androgens can masculinize primary sexual characters (i.e. induce the development of testicular tissue in treated female ovaries; and (2) that overexposure to 17MT favors ovarian development due to excessive aromatization to estradiol.

Chapter 1

Timing of sexual maturity: An osteological survey of the anal-fin complex in the mormyrid fish *Brienomyrus niger*

Summary

Adult mormyrids can be sexed externally by the presence (in males) or absence (in females) of an indentation of the ventral margin of the anal fin. This indentation is linked to an osteological sexual dimorphism, an expansion of the bases of anal-fin rays in males. A radiographic survey of 249 specimens of *Brienomyrus niger* confirmed the existence of this sexual dimorphism in this species. The expression of basal anal-fin ray expansion was first noticed in specimens of 60 mm SL (standard length). When fish reached 90 mm in length, the ratio of fish with and without expansion was 1:1, reflecting the same sex ratio as confirmed by gonadal inspection. Based on these data, three developmental stages were identified: juveniles measuring up to 60 mm SL, subadults up to 90 mm SL, and adults measuring more than 90 mm SL.

Introduction

The sex of adult mormyrids can be determined externally by inspection of the posterior ventral body wall. In males the body wall is dorsally indented giving the impression of a sigmoid curvature affecting the dorsal margin of the anal fin (Figure 1.1). In females the body wall appears almost straight (Nawar, 1959; Iles, 1960; Okedi, 1969; Kirschbaum, 1987; Pezzanite and Moller, 1998; Moller et al., 2004; Greisman and Moller, 2005). The anal fin itself is much more “feathery” and rounded in males and appears triangular-shaped in females (Schugardt, 1997).

The indentation is linked to a male-typical sexually dimorphic massive bone expansion, which affects the basal portion of anal-fin rays. This was discovered in two species of the genus *Brienomyrus*, *B. brachyistius* (Gill, 1863) *and B. kingsleyae* (Günther, 1896) by Brown, et al. (1996). The expansion is absent in females.

The basal fin ray expansion is apparently a characteristic trait of the family Mormyridae as has now been verified for several other species: (Herfeld, Pezzanite, Voustianiouk, Choi, Gannon, Brown, and Moller, 1997), in *Gnathonemus petersii* (Pezzanite and Moller, 1998; Greisman and Moller, 2005), *Mormyrus rume proboscirostris* Boulenger, 1898 (Moller et al., 2004), and *Pollimyrus adspersus* (Günther, 1866) (Moller and Tong, in prep.).

This sexual dimorphism was also established in three other species in a series of unpublished laboratory rotation studies conducted by students in the CUNY Biopsychology program under the supervision of Dr. P. Moller:

Marcusenius senegalensis Steindachner, 1870, *M. ussheri* Günther, 1867 (Kabitzke, 2000), *Hippopotamyrus batesii* Boulenger 1906, *Paramormyrops gabonensis* Taverne, van den Audenerde, and Heymer 1977 *(*Casriel, 2001; Bogdan, 2002).

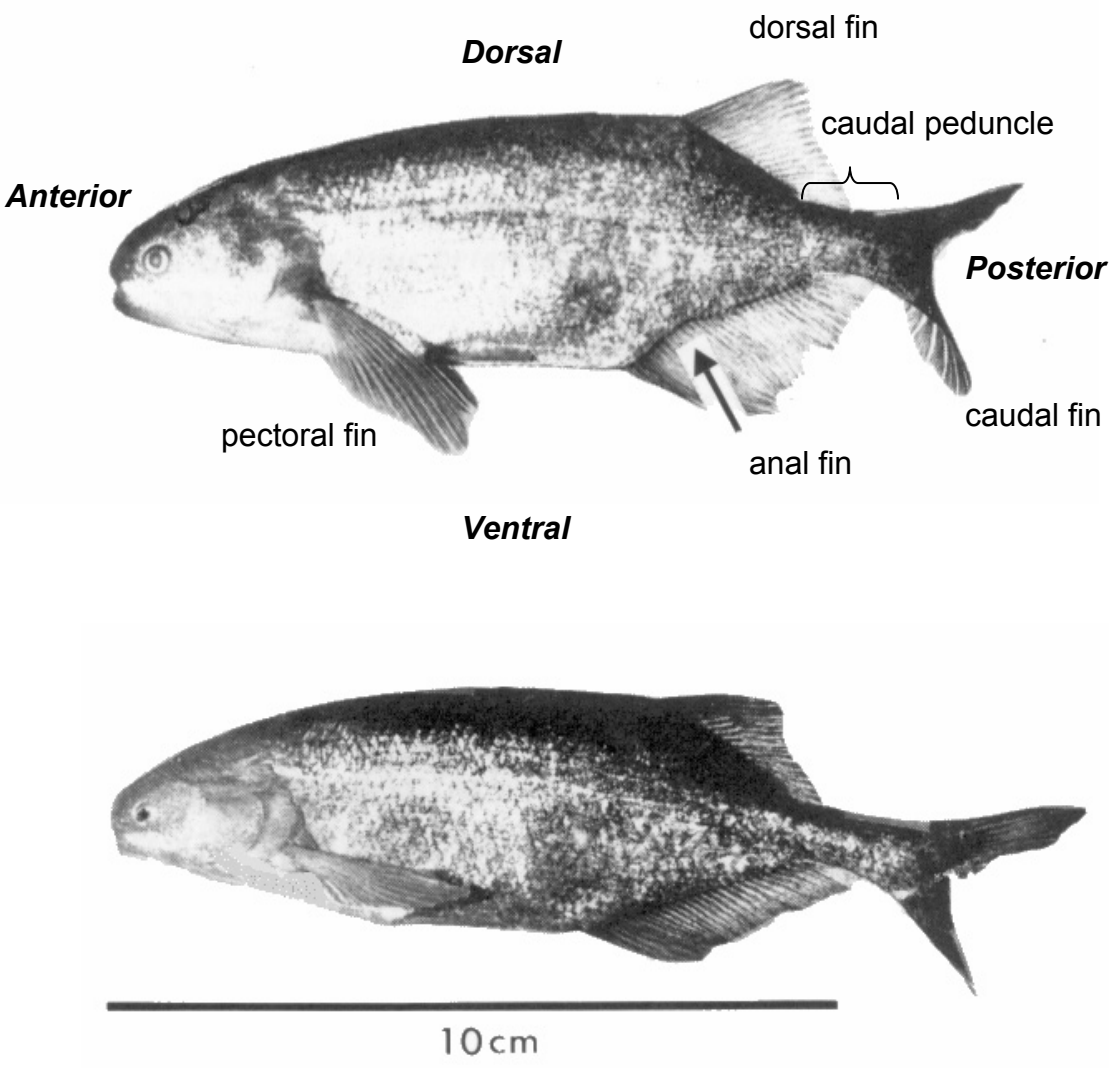

Figure 1.1. Brienomyrus niger. Upper panel: male; arrow points to male-typical indentation of the posterior ventral body wall. Lower panel: female; the dorsal margin of the anal fin is straight (adapted from Serrier and Moller, 1989).

The functional significance of this sexual dimorphism has been attributed to the fish's courtship behavior during which the male typically envelops the female's anal fin with its own in a series of rapid flexes forming a common spawning pouch (anal-fin reflex) (Kirschbaum, 1987; Kirschbaum and Schugardt, 1995). The anal-fin complex comprising of the rays and their osteological support structures facilitate the anal-fin reflex. During the first year, presumably driven by circulating androgens (see Chapter 3, this thesis; Herfeld and Moller, 1998), the characteristic dimorphic basal fin-ray transformation begins to develop in males. Moller et al. (2004) established that in fact, in a related species (*Mormyrus rume proboscirostris*), the structures defining the anal fin undergo a permanent sexually dimorphic transformation at a time when ripe spermatozoa first appear in the testis of young males. However, the expression of the dorsally directed indentation of the ventral body wall is plastic and correlated with the fish's gonadal status. It is assumed that the sex ratio in mormyrid fish is 1:1 (Moller, pers. communication). Thus, the presence of expanded and non-expanded fin ray bases should attain this ratio at that developmental stage (as reflected by size) when fish become sexually mature for the first time.

This study was designed to determine (1) at what size range and, by implication, at what developmental stage this transformation is initiated, (2) what is the time course for fish to reach sexual maturity, and (3) what are the specific sexually dimorphic structural characters that distinguish males from females. Access to a large collection of preserved *B. niger* allowed screening of

specimens widely differing in size. This sample was considered a representative 'developmental series' including juveniles, subadults, and adults.

Materials and Methods

A total of 249 *B. niger*, ranging in standard length (SL) from 44 to 137 mm, were analyzed from the collection at the American Museum of Natural History, New York. Fish were radiographed (Hewlett-Packard, model Faxitron 43807 N) under low-intensity radiation (30-40 KVP). All radiographs were scanned into a computer and analyzed with professional imaging software (ScanPro™) (Figure 1.2). Specimens surveyed: AMNH 36924, 36985, 36986, 36987, 36988, 57114, 57144, 71881, 219145 and P. Moller's collection, Hunter College, CUNY.

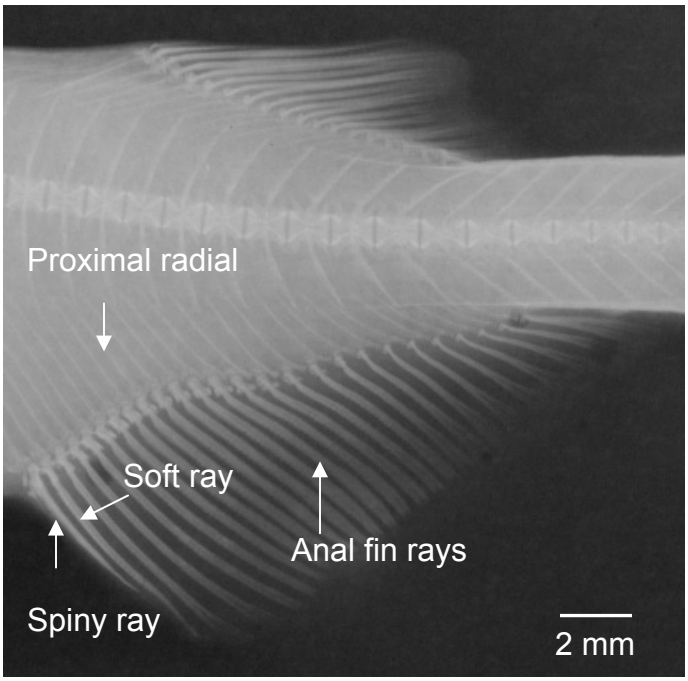

Figure 1.2. Radiographic image of the anal fin complex in a male *B. niger*

Morphometrics collected included standard body length (SL) as defined by the distance from the tip of the snout to the end of the hypural bones, i.e. the bones at the end of the vertebral column to which the caudal rays attach, body depth, body width, and the distance between the base of the first and the last anal-fin rays (anal fin length, AFL). To quantify the extent of the indentation (i) the maximum, dorsally-directed indentation using the base of the associated ray as a point of reference was determined, (ii) from this point straight lines were drawn rostrad (headwards) and caudad (tailwards), respectively, using the bases of the adjoining next four rays as a reference, (iii) from the respective first and last fin-ray bases, the perpendicular projections on these lines were obtained, (iv) the endpoints of these projections were connected, and (v) the resulting triangle allowed the computation of 'b/a' (tangent β) as indentation measure (Figure 1.3).

Meristics collected included the number of anal-fin rays, the number of fin rays with expansion, the ray count of the most dorsally directed indentation, and the ray count of the first and last rays with expansion. Bone expansion was judged from radiographs and assigned to two groups: fish with bone expansion (males) and fish without expansion (females or juveniles). The decision as to whether or not fin ray bases were expanded was based on comparing the juvenile conditions (44-60mm) with later stages (61-137mm) (Figure 1.7). Sex of adult fish was verified by gonadal inspection following Blake (1977). Data were analyzed using Excel for WIN 95 and STATISTICA™ (regression analyses, Mann-Whitney U-test).

Results

Sexual maturity

Specimens were assigned to 10 mm size classes from 40-49 mm to 130-139 mm SL, and sorted by size and presence or absence of bone expansion (Table 1.1). Bone expansion appears for the first time in fish of approximately 60 mm SL. By the time fish measured 90 mm SL, the ratio of specimens with and without basal fin-ray expansion and a clearly discernible body wall indentation had reached 1:1, reflecting the same sex ratio as confirmed by gonadal inspection.

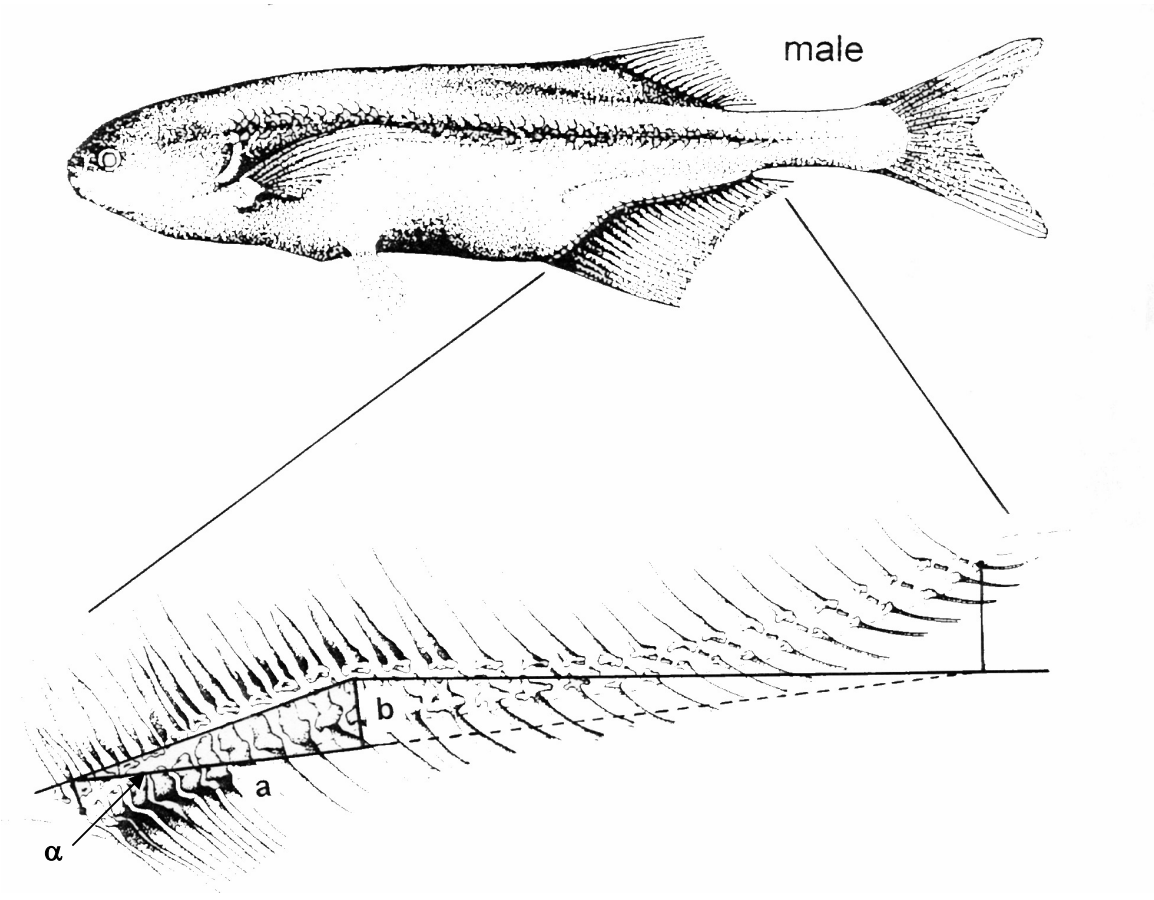

Figure 1.3. Illustration of morphometrics and meristics applied to the anal-fin of *B. niger.* Indentation angle α is identified by measures a and b (tangent α = b/a).

B. niger measuring up to 60 mm SL were considered juveniles of both sexes with no signs of the two noted sexual dimorphisms present. Fish measuring 60-90 mm SL showed a beginning indentation and bone expansion, and were considered subadults as the sex ratio was still skewed in favor of fish with no expansion and indentation (68-78% versus 22-32%). Fish exceeding 90 mm SL were adult males and females, with both sexes equally represented in this sample, as unambiguously confirmed by gonadal inspection, and the presence or absence of basal fin-ray expansion and associated body wall indentation.

In addition, meristics and morphometrics were analyzed to assess differences in secondary sexual characters in *B.niger* (Table 1.2). The length of the anal-fin ranged from 10.5 to 36 mm. As was to be expected, standard length and anal-fin length were positively correlated in both groups, i.e. in fish with and without expanded fin-ray bases (Figure 1.4, Table 1.2).

Distribution of fish with and without anal-fin ray expansion

Developmental stage	*Size mm (SL)*	*n*	*n of No-Expansion group*	*%*	*n of Expansion group*	*%*
Juveniles	40-49	3	3	100	0	0
	50-59	4	4	100	0	0
Subadults	60-69	18	14	77.8	4	22.2
	70-79	31	21	67.7	10	32.3
	80-89	32	25	78.1	7	21.9
Adults	90-99	31	17	54.8	14	45.2
	100-109	62	35	56.5	27	43.5
	110-119	47	22	46.8	25	53.2
	120-129	19	9	47.4	10	52.6
	130-139	2	0	0	2	100
Total # of fish		249				

Table 1.1. Stages of development. Fish were grouped in 10-mm size classes and, depending on the expression of anal-fin ray expansion, to 'expansion' or 'no-expansion' groups. Expansion begins when males reach approximately 60 mm SL. Specimens with and without expansion attain a 1:1 ratio at about 90 mm SL. In fish exceeding 90 mm SL, bone expansion is either fully developed, resulting in male-typical anal-fin indentation, or remains absent, resulting in the female-typical straight ventral body wall of the anal-fin.

Expansion Group

Measurement	Slope	r^2	F	df	p
Body depth	y = 0.24x+1.53	0.66	192.8	1,98	<.0001
Body width	y = 0.09x-0.49	0.39	62.7	1,98	<.0001
Anal fin length	y = 0.26x+1.32	0.89	755.4	1,98	<.0001
First Bone	y = -5x+120.2	0.51	102.5	1,98	<.0001
Last Bone	y = 4.52x+22	0.43	72.5	1,98	<.0001
Rays expanded	y = 0.2x-4.8	0.53	112.6	1,98	<.0001
Indentation	y = 0.002x+0.09	0.13	15.0	1,98	.0002

No Expansion Group

Measurement	Slope	r^2	F	df	p
Body depth	y = 0.28x+ 2.27	0.91	1465.7	1,146	<.0001
Body width	y = 0.14x-3.86	0.82	666	1,146	<.0001
Anal fin length	y = 0.26x+0.81	0.93	1915	1,146	<.0001
Indentation	y = 0.0004x+0.15	0.04	6.5	1,146	.0116

Table 1.2. Results of regression analyses. Correlation between standard length and different measurement for the expansion and no expansion group.

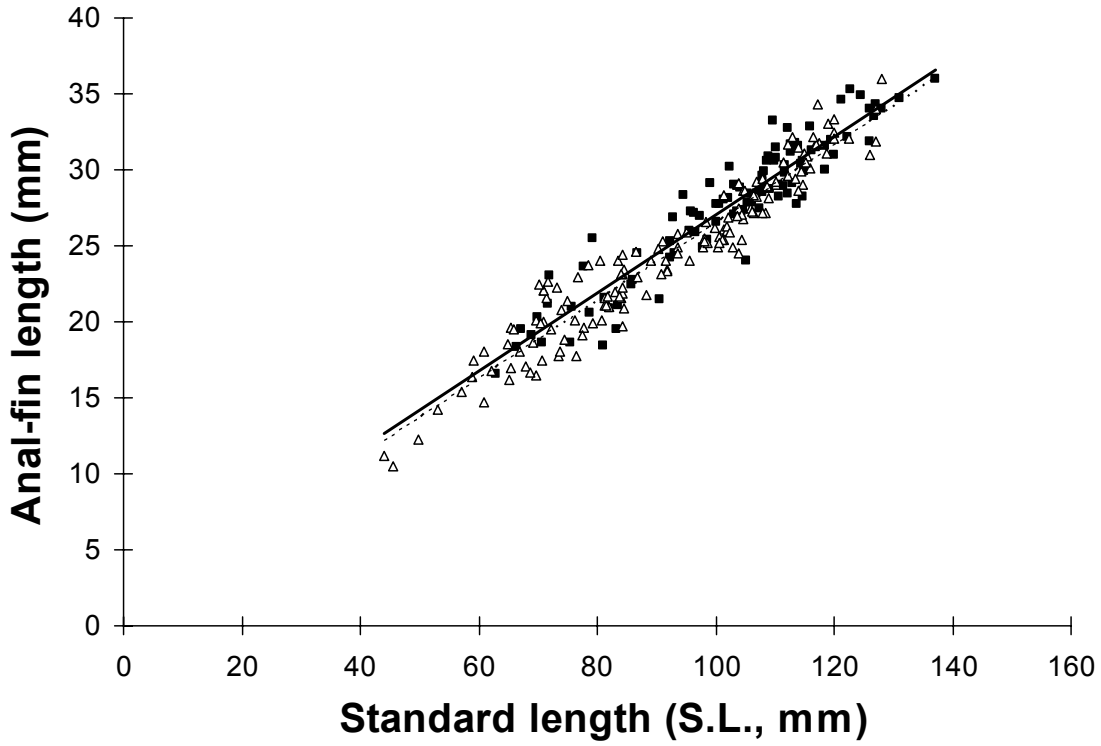

Figure 1.4. Anal-fin length as a function of standard length; ■ 'expansion' group (solid line), Δ 'no expansion' group (dotted line).

Figure 1.4 illustrates the relationship between anal fin length and standard length of the fish. Fish in both groups showed the same allometric growth. There was, however, a significant, difference in the ratio of anal-fin length to standard length between groups, thus signaling a sexual dimorphism not described before (Mann Whitney U-test; U = 5938.5, Z = 2.6373, p = .0084).

Body depth ranged from 10.7 to 38 mm in both groups (Figure 1.5). Again, there was a positive correlation between standard length and body depth in both groups (Table 1.3). There was no difference between groups (Mann-Whitney U-test; U = 6349.5, Z = -1.89564, p = .058).

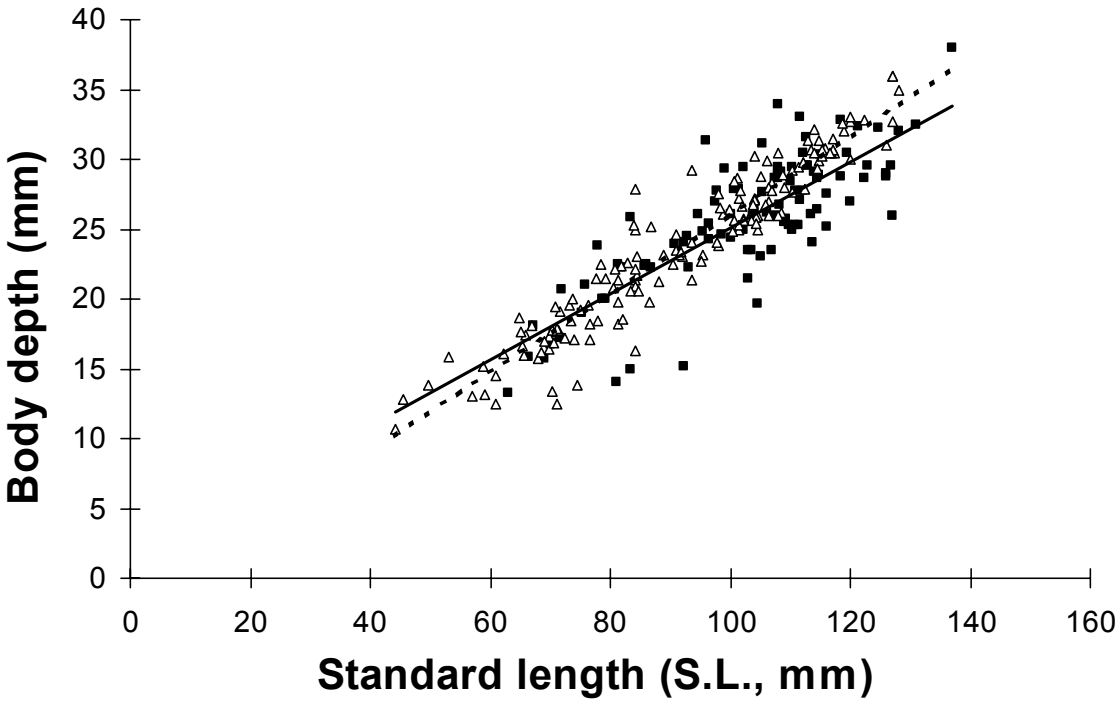

Figure 1.5. Body depth as a function of standard length. ■ 'expansion' group (solid line), Δ 'no expansion' group (dotted line).

Body width ranged from 3.2 to 16.3 mm. In both groups, standard length and body depth were positively correlated (Figure 1.6; Table 1.2). Fish with expanded bone differed significantly from those without expanded bone (t_{249} = 4.193, $p < .001$) with females exhibiting thicker body width.

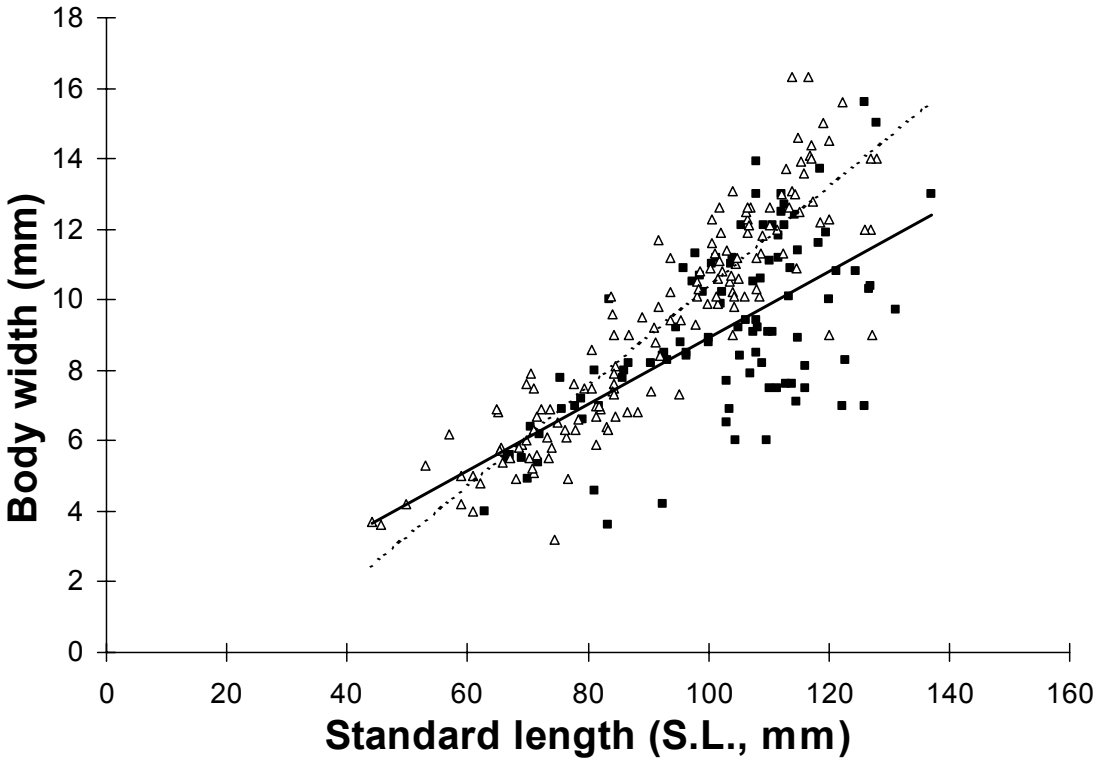

Figure 1.6. Body width as a function of standard length. ■ 'expansion' group (solid line), Δ 'no expansion' group (dotted line). Significant difference between groups ($p < .05$).

The morphometrics on anal-fin length, body depth and width, reveal novel findings in that, at the time of phenotypic sexual differentiation, i.e. the appearance of expanded rays (see Table 1.1: 60-80 mm), female body depth and width overtake the corresponding measures in males (body width: 70 mm, body depth: 80 mm). This sexual dimorphism was significant for body width only. These findings were supported by the comparison of the slopes of the associated regression functions (body depth: $t = 173.8$, $df = 244$, $p = .001$, body width: $t = 5.8$, $df = 244$, $p = .001$). Over the entire size range sampled, the female anal fin length, however, was always, but not significantly, shorter, while the derived measure AFL/SL ratio was.

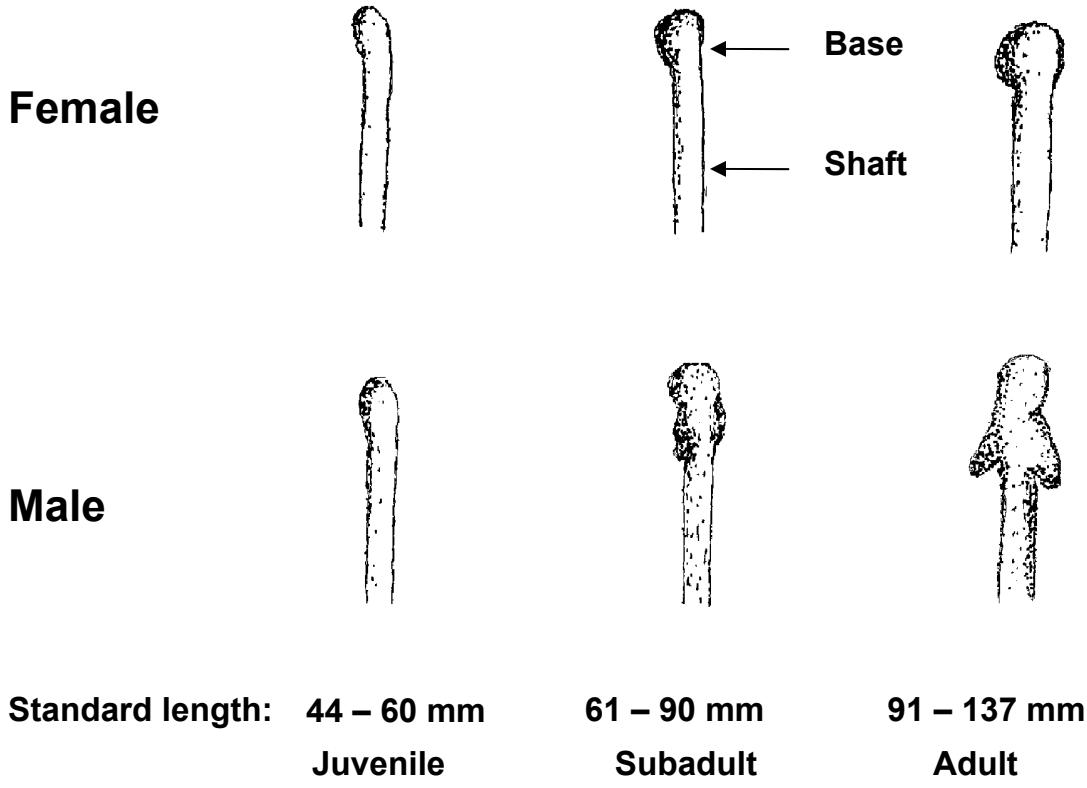

Figure 1.7. Illustration of basal anal-fin ray expansion in three developmental stages (juvenile, subadult and adult fish) and their size ranges. The two parts of a ray are the base and the shaft.

Figure 1.7 illustrates the change in bone structure during development. In juveniles the structure of the fin-ray bases is similar in both sexes. Bone expansion becomes apparent at 60 mm SL, and distinguishes males from females. The transformation of the fin-ray base begins with the development of a small knob on the posterior edge of the ray and continues to grow into the shape

seen in mature males. The bases of the fin rays in adult males (90 mm SL and up) are larger and by inference also thicker (see also chapter 4). There was a difference between adult male and female bone types. The bases of the fin rays in females are rounded dorsally with a slight thickening anteriorly and a larger expansion posteriorly (Figure 1.7).

Permanent structures of the anal-fin complex

The total anal-fin ray count ranged from 25 to 30 rays. As fish grow, additional anal-fin ray bases expand suggesting a linear relation within the range sampled (Figure 1.8; Table 1.2). The most dorsally directed (and expanded) ray was always associated with ray counts 10, 11, or 12 (central ray). Only rays 2 through 21 were affected by this structural transformation. Rays 1 and 22-30 never expanded. We assessed whether the increase in number of expanded rays with growth followed a particular growth pattern (Figure 1.8). Indeed, the expansion appeared to originate around the central ray and spread in a similar, linear fashion both rostrally and caudally, affecting an increasing number of fin-ray bases (Figure 1.7; Table 1.2). Thus, as fish grow, more fin-ray bases expand (Figure 1.8; Table 1.2).

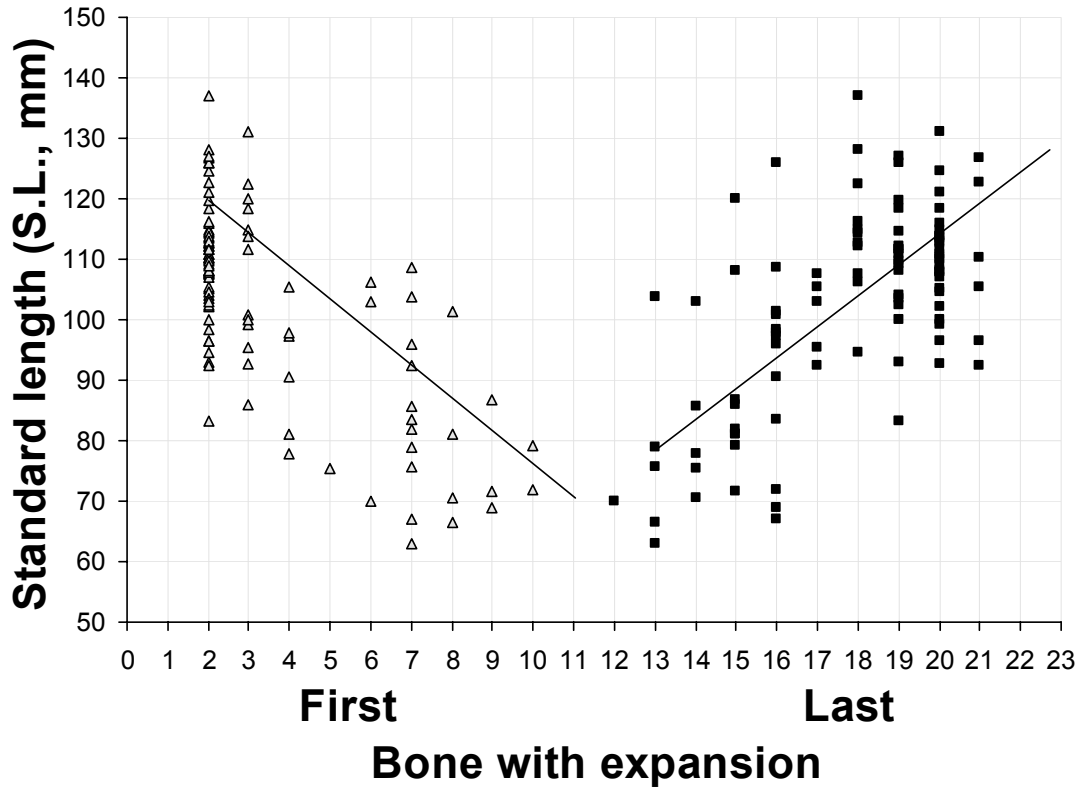

Figure 1.8. Standard length as a function of first (Λ) and last bone (■) with expansion. Lines are regression lines.

Body wall indentation

Fish that exhibited expanded fin-ray bases also showed varying degrees of an externally visible body-wall indentation. In fish with expanded rays, the indentation angle appeared to increase with size (Figure 1.9) ($r = 0.268$; $p < .001$; $n = 249$), but only 13% of this increase was attributable to growth (Table 1.2). The apparent indentation in fish with non-expanded fin-ray bases (subadults and females) determined at the 'typical male ray site' was not affected by size (Figure 1.10; Table 1.2).

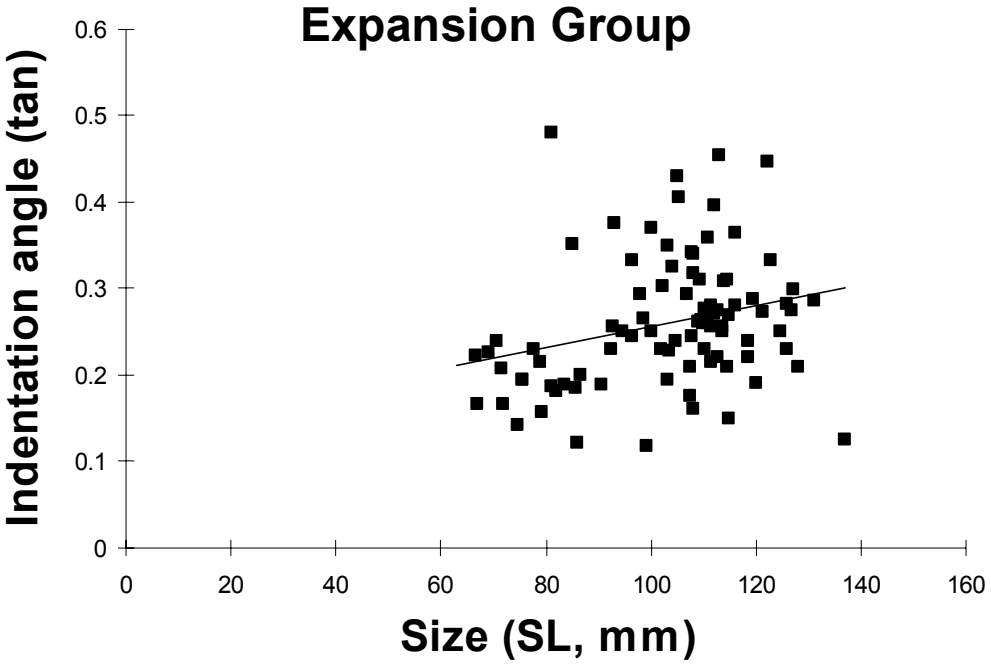

Figure 1.9. Body wall indentation as a function of standard length in fish with expanded anal fin ray bases.

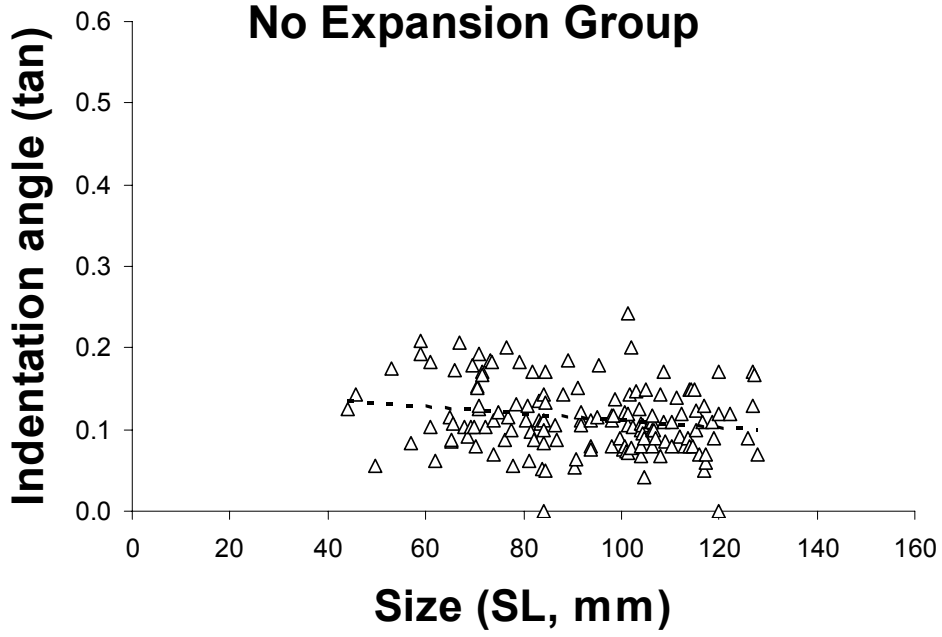

Figure 1.10. Body wall indentation as a function of standard length in fish without expanded anal fin ray bases.

Discussion

This osteological survey of *B. niger* confirmed the existence of a sexual dimorphism affecting the anal-fin ray complex in this mormyrid fish and further corroborated its occurrence in several other species of mormyrids (as referenced in introduction of this chapter). As fish grow (spanning a range in this population of subadult and adult fish from 60 to 137 mm SL), body depth, width and length of the anal-fin follow proportionally. The larger the fish, the deeper and thicker were their bodies and the longer their anal fins. There was a significant difference between the two groups, with females having thicker bodies and a slightly shorter anal fin. There was no difference in depth of the body between males and females.

It appeared that the indentation was significantly affected by growth. The results in *B. niger* are in line with findings by Brown et al. (1996) who reported that in young male *B. brachyistius* the anal fin rays were expanded to a lesser degree than in larger, older males. Pezzanite and Moller (1998) made a similar observation in male *Gnathonemus petersii*.

While the significant correlation between indentation and size of the fish is supported by these data, growth alone, however, cannot be the only determinant for the basal anal fin transformation for the following reason. Unlike the other morphometrics (anal-fin length, fish body depth and height), the angular measure is a ratio measure (tangent) and thus independent of size. A low coefficient of determination (13%) signaled a caveat. The question arose as to what caused the transformation of the fish's body wall?

We know today that while the basal expansion of anal-fin rays in mormyrids is permanently expressed by the time males attain sexual maturity, the extent of the indentation can undergo cyclic variations that are driven by the fish's gonadal status during and out of breeding season (Kirschbaum, 1987). This is certainly the case for two large species, *M. r. proboscirostris* (Moller et al., 2004) and *G. petersii* (Greisman and Moller, 2005), and doubtful for a small species, *P. adspersus* (Moller and Tong, in prep.).

For *G. petersii* and *M. r. proboscirostris* there was a significant correlation between gonadal status and body wall indentation such that high gonadosomatic indices (GSI) correlated with large indentation angles and low indices with small angles (Moller et al., 2004; Greisman and Moller, 2005). The indentation angle was fully expressed at the time fish attained first sexual maturity and did not increase further irrespective of further growth. It was hypothesized that (a) the expanded fin ray bases provide substrate of the anal-fin musculature, and (b) that anabolic effects of increased circulating androgen levels on these muscles during the breeding season affect the apparent indentation (pushing the dorsal margin of the anal fin "upward", i.e. dorsally).

When gonads regress, muscle regresses, and the indentation appears less visible. From data on androgen-manipulated mormyrids (*G. petersii*), there is first direct evidence that androgens do in fact affect an increase in anal-fin muscle volume (Voustianiouk, 2003). The time course of androgen effects on those structures that are intimately involved in reproduction has been

investigated by Herfeld and Moller (1998). More detailed analyses are provided in chapters 3 and 4 of this thesis.

What explains the observed positive correlation between indentation and size in *B. niger*? At the time the data were collected and analyzed, the relationship between gonadal status (androgen action) and musculature driving the anal-fin reflex had not been worked out. In this study, the fish were pooled and sorted by the presence or absence of expanded ray bases alone, thus regardless of (a) whether they had been caught during or out of their breeding seasons, and (b) whether a sex ratio of 1:1 had been attained. The values of the reported indentation angles are thus seriously confounded by the uncertainty of the season the fish were captured and by pooling subadult and adult fish. Unless we have detailed knowledge about the gonadal status of the fish, the question concerning the cause of the transformation of the indentation angle remains open.

The prediction would be that the degree of indentation in fish sampled/caught during their breeding season would be significantly higher than in fish monitored out of breeding season. Recently, Greisman and Moller (2005) have reexamined the material originally used by Pezzanite and Moller (1998) (who had been unaware of the androgen action on anal-fin musculature) and made a plausible case for a causal relation between GSI and body wall indentation in *G. petersii*. Recrudesced testes were indeed highly correlated with an indentation of the dorsal margin of the anal fin. This analysis has provided a means to assess sex ratio in *B. niger* based on the anal-fin ray expansion.

Chapter 2

Environmental conditions modulate the electric organ discharge (EOD) in *Brienomyrus niger*

Summary

The electric organ discharge (EOD) waveform can change long term due to seasonal changes in water temperature and conductivity, but can also change short-term as a result of abrupt changes in temperature, conductivity, and water current. This experiment documented the influence of conductivity and temperature on the duration of the EOD in *Brienomyrus niger*. While differences in conductivity (under current experimental conditions) had no effect on the EOD waveform, temperature significantly affected EOD duration. All four phases constituting the EOD decreased with increased temperature. The results provided Q_{10} values that will be used to normalize all data to 25 ˚C (phase 1: 0.519, phase two: 0.539, phase three: 0.421, and phase four: 0.513; peak power spectrum frequency: 1.595).

Introduction

Weakly electric fish can modulate components of their EOD such as individual waveform and/or rate of emission depending on a number of environmental, biological, and social factors. To assess the exclusive effects of exogenous hormone treatment on the fish's EOD and anal-fin morphology (see chapter 3), it was necessary to understand and correct for the impact of confounding, EOD-modulating variables such as temperature and conductivity.

Cyclic changes in the aquatic environment such as temperature, ionic concentration, and photoperiods have a powerful influence on growth, development, and reproductive behavior of many aquatic organisms. It follows that depending on the species' adaptation to tropical or temperate climates, only one or a combination of these factors is effective. Light-dark cycles (photoperiod) in the tropics remain fairly unchanged, but are crucial in eliciting gonadal maturation and reproductive behavior in fish indigenous to temperate zones (Kirschbaum, 1979).

Effects of External Factors on the Electric Organ Discharge

In their natural aquatic habitats, weakly electric fish encounter temperature and conductivity fluctuations with little change in photoperiod (Moller, Serrier, Belbenoit, and Push, 1979; Quintana, Silva, Berois, and Macadar, 2004; Crampton and Albert, 2006). The tropical habitats preferred by most electric fish species are characterized by seasonal rainy and dry seasons (Kirschbaum, 1995). During the dry season the ion concentration of the water is very high, and

the aquatic conductivity can increase to 400 µS/cm and higher. During the rainy season, i.e. during the fish's breeding season, conductivity can decrease to 5 µS/cm due to the high water influx (Moller et al., 1979; Bénech and Quensière, 1985; Kirschbaum, 1987, 1995). Temperature changes (Harder, Schief and Uhleman, 1964; Enger and Szabo, 1968; Boudinot, 1970; Toerring and Serrier, 1978; Lewis and Kay, 1991) as well as water conductivity (Bell, Bradbury and Russell, 1976; Squire and Moller, 1982; Bratton and Kramer, 1988) affect the EOD discharge rate. Several papers quantitatively illustrate the effects of ambient aquatic temperature on fish discharge rates (Gymnotiformes: Grundfest, 1957; Lissmann, 1958; Watanabe and Takeda, 1963; Enger and Szabo, 1968; Boudinot, 1970; Bullock, Hamstra and Scheich, 1972; Schwassmann, 1978; Mormyriformes: Toerring and Serrier, 1978; Serrier and Graff, 1985; Lewis and Kay, 1991). Higher temperatures increased the discharge rates whereas lower temperatures reduced the discharge rates. The reported median Q_{10} is 1.5 (Enger and Szabo, 1968).

The EOD rate in mormyrids is highly variable. In addition to temperature affecting the metabolism of the electric organ that generates EODs, a wide range of other environmental factors can affect the EOD rate. This can easily be demonstrated by subjecting the fish to brief stimulation with visual, mechanical, olfactory, or chemical stimuli. The fish will respond through its electromotor output in the form of brief bursts of discharges, the EOD startle, prolonged cessations, or prolonged rate increases with invariable inter-discharge intervals

(Ciali, Gordon and Moller, 1997; Post and von der Emde, 1999; reviews: Moller, 1995; Crampton and Albert, 2006).

In electrocommunication, the fish's EODs serve as a social medium. In response to the presence of another fish, pulse-type fish cease discharging briefly (Hopkins, 1986; Kramer, 1990; Moller, 1995, 2006), decrease the variability of interpulse-intervals, show brief accelerations in EOD rate and synchronize their discharges with those of the other fish (echo response; preferred latency response; Kramer, 1974; Russell, Myers and Bell, 1974).

Affected by changes in aquatic ionic concentration due to changes in temperature and/or influx of minerals, the electric load on the electric organ output (EOD) changes and results in EOD amplitude changes (Harder et al., 1964; Bell et al., 1976; Squire and Moller, 1982).

The fish's individual EOD waveform was considered a fairly fixed trait as it communicates species membership, developmental stage, and sex. But the EOD waveform, too, is much more plastic than was assumed, and is affected by biological and non-biological factors. The EOD waveform changes due to seasonal changes in conductivity. Conductivity changes trigger gonadal recrudescence (Kirschbaum, 1987), but can also change abruptly and short term as a result of changes in abiotic environmental factors such as temperature, conductivity, and water current. Recently, Quintana et al. (2004) discovered that a change from low to high environmental temperature is sufficient to elicit gonadal maturation in the gymnotiform knifefish, *Brachyhypopomus pinnicaudatus*, a species abundant in the temperate regions of South America.

An extensive review of the effects of abiotic conditions on the EOD waveform in gymnotiforms was provided by Crampton and Albert (2006). Short-term changes in EOD waveform have also been attributed to circadian control, social contexts and/or environmental stressors (Hagedorn and Heiligenberg, 1985; Franchina and Stoddard, 1998; Carlson et al., 2000; Terleph and Moller, 2003). There is also empirical evidence that low-frequency electromagnetic radiation modulates the EOD waveform in mormyrids (Berry and Moller, in prep.).

The following experiments will establish the quantitative relationship between EOD waveform duration and aquatic temperature in *Brienomyrus niger (B. niger)*. If this relationship is significant, the results will be used to normalize all electric organ discharge data to a temperature of 25 ºC.

Material and Methods

Subjects

For this experiment, 17 African weakly electric freshwater fish, *B. niger* (see chapter 1: Fig. 1.1) were imported through a local tropical fish vendor (Quality Tropicals, Inc. Wallington, NJ). Fish were originally imported from Nigeria at the end of their breeding season (November 1995) and were maintained at our laboratory at the *American Museum of Natural History* (AMNH). Two weeks prior to the start of the experiment, subadult and mature males and females were weighed, measured (length and body depth), and transferred to the laboratory at Hunter College. Sexually mature males were identified by their dimorphic external indentation along the ventral body wall (see

chapter 1: Fig 1.1). Females do not have such an indentation (Iles, 1960; Kirschbaum, 1987). There were 8 males and 9 females. Standard length of the fish was 89.2 ± 6.9 mm, ranging from 76-104 mm. Weight was 8.95 ± 1.46 g ranging from 7-13 g. Body depth was 23.5 ± 2.4 mm ranging from 19-27 mm.

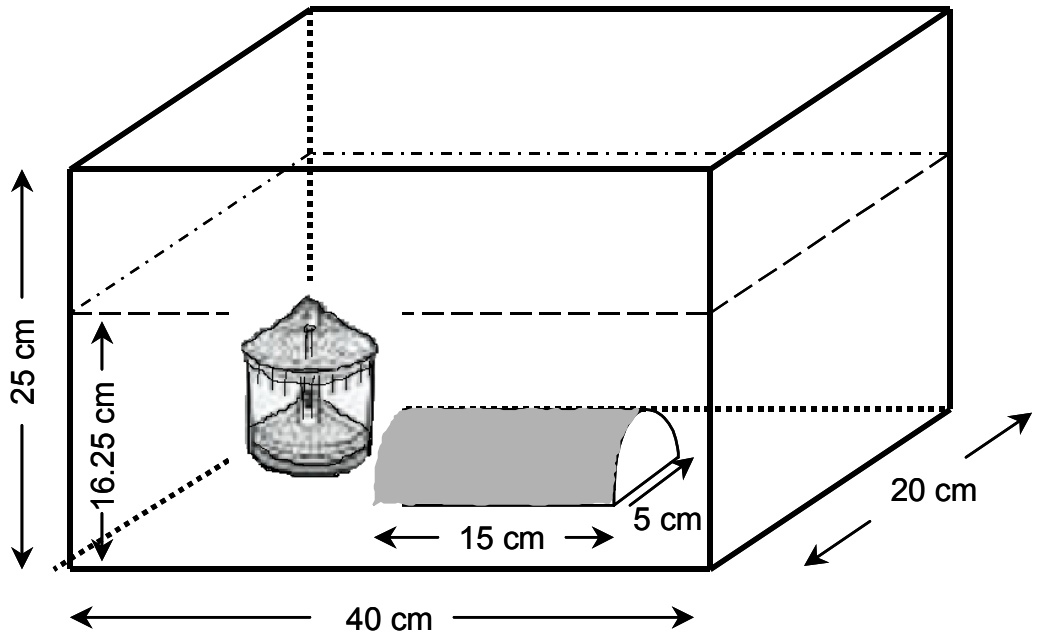

Figure 2.1. The 20-l test aquarium measured 40 cm L x 20 cm D x 25 cm H. Fish remained in a porous clay shelter (15 x 5 cm) while EODs were monitored.

Fish were kept in individual 20-liter aquaria with a water volume of 13 liters (Fig. 2.1). Each aquarium was equipped with a porous ceramic shelter (150 mm long and 50 mm in bottom width) and a standard corner filtration device. The aquarium was covered with a glass plate to prevent dirt and dust particles from polluting the water and to prevent the fish from jumping out of the tank during their active phase at night. Aquaria were placed on a three-level rack in the test room. Fish were acclimated to their new environment for 15 days. The

photoperiod was adjusted to L12 : D12 hours with lights on at 8:30 a.m. Water temperature ranged from 20.0-25.5º C. The temperature fluctuations during the experiment mimiced the natural fluctuation of the temperature in their environment (Moller et al., 1979). Water conductivity ranged between 130-180 µS/cm. One third of the water volume was changed weekly to keep the conductivity within this range. Fish were fed three times a week with tubifex worms (freshwater bristle worms), frozen mosquito larvae (Chironomidae) and small saltwater brine shrimp (*Artemia*).

Recording and measurement of the electric organ discharge and conductivity

EODs were recorded from singly housed fish when the fish was calm and resting in its shelter. The discharge was monitored with a pair of silver/silverchloride electrodes (Ag/AgCl) that were affixed at the narrow sides of the aquarium. EOD recordings were only taken when fish were resting in their shelter (Figure 2.1), thus the fish-to-electrode distance remained constant. The EOD of *B. niger* consists of four phases (Figure 2.2); the duration of these phases were read from the screen of a Hitachi Oscilloscope (Model VC-6023). The EOD-associated fast Fourier spectra as well as the peak power spectrum frequencies (PPSF) were obtained using an Hewlett Packard real-time analyzer (model 3582 A, range 0-25 kHz, resolution 100 Hz). Water conductivity measurements were taken daily with a conductivity meter (YSI Model 33S-C-T meter) from all tanks.

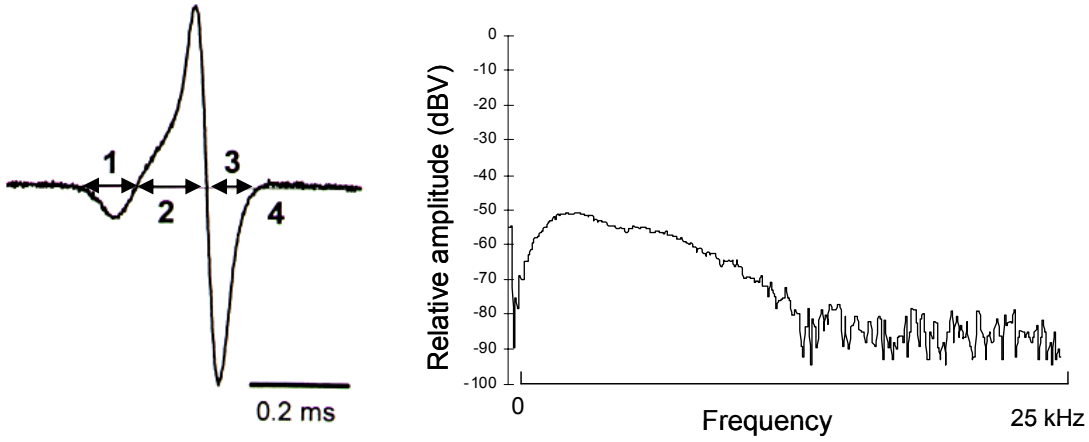

Figure 2.2. A representative EOD illustrates the typical four phases (1-4) and the associated PPSF. Arrows indicate phase duration measures.

Temperature coefficient, Q_{10}

This coefficient allows an estimate of changes in the dynamics of temperature-dependent physiological processes, i.e. a change in activity or performance caused by a 10° Celsius increase. EOD generation is such a process and is as such affected by temperature. EOD measurements were obtained under different temperature conditions. In order to compare these measures, all EOD data were adjusted to 25 ºC conditions as follows:

D (at 25º) = [DxQ_{10} / 10] x (25-t), with D being the duration of the measured phase and/or peak power spectrum frequency, and t being the recorded temperature of the water in ºC.

Statistical Analysis

Data were subjected to parametric statistical procedures: regression analysis, analyses of variance with post-hoc tests (Neuman-Keuls; $\alpha = 0.05$) using commercially available programs (EXCEL, Windows, STATISTICA 6.0, and Statsoft Inc.). The relation between conductivity and water temperature, excretion, food residues, and water changes, as well as the effect of temperature on phase duration and peak power spectrum frequency were examined by regression analysis.

All experiments were in compliance with federal, state, and local laws and regulations. The work was approved by the Hunter College IACUC.

Results

Temperature

Figure 2.3 illustrates a positive correlation between temperature and conductivity, which according to laws of physics was to be expected. The regression analysis yielded COND = 3.21T+ 83.3 ($r^2 = 0.049$) with T denoting temperature in degrees Celsius. Over the sampled temperature range under the current conditions, however, the increase in conductivity was not significant.

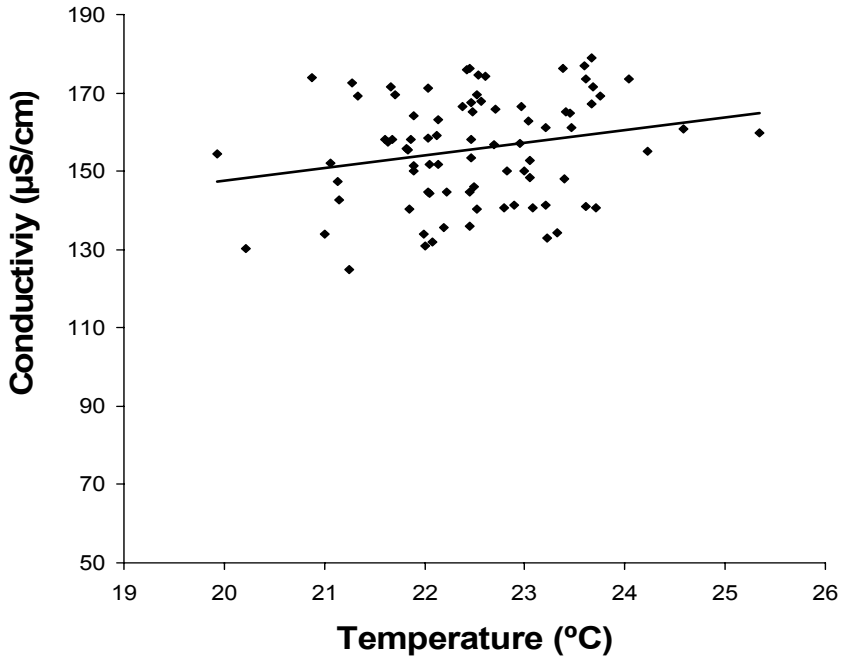

Figure 2.3. Mean daily water conductivity measures recorded from all tanks as a function of temperature. Regression line: y = 3.21x+83.31; r^2 =0.0489.

Food residue and Excretion (Temperature range: 20.0 –25.5 ºC)

Under the given experimental conditions, accumulation of food residue and excretion over time, as well as routine water changes influenced water conductivity (Figure 2.4). Due to food residue and excretion, conductivity increased over 42 days (day 16-57) in a linear fashion by about 40-50 µS/ cm. Water change on four days (between days 57-64) resulted in a drop of conductivity from 186 µS/cm to 140 µS/cm. In the following days the conductivity increased by 26 µS/cm. A regression analysis over the two sampling periods, i.e. days 16-52 and 57-100 days indicated significant increases in conductivity (Table 2.1).

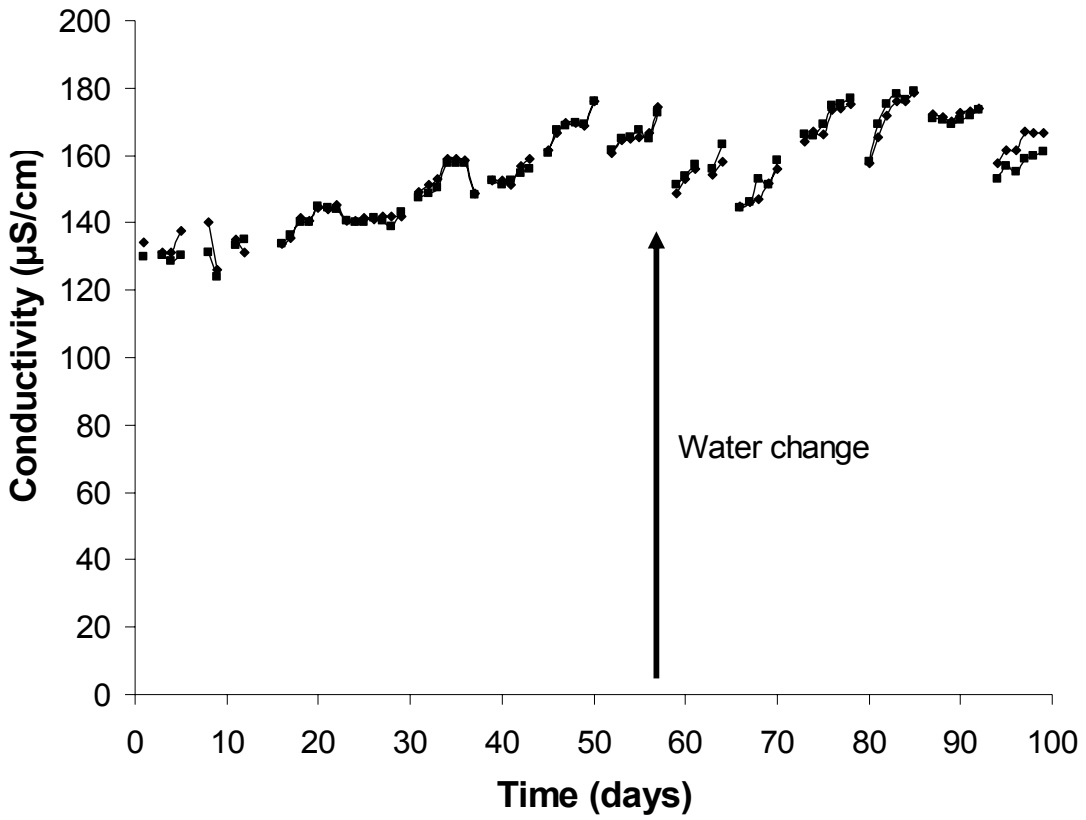

Figure 2.4. Mean daily water conductivity measures over the 100-day sampling period. Arrow marks the start of 4 daily water changes (1/3 of water volume was replaced each time).

Fish	*Regression*	*F*	*df*	*p*
CF (16- 57 days)	y = 0.8619x+122.09	208.14	1,39	< .001
CF (58-100 days)	y = 0.4516x+127.27	13.29	1,40	< .0008
CM (16- 57 days)	y = 0.8823x+120.73	200.91	1,39	< .001
CM (58-100 days)	y = 0.277x+140.82	4.193	1,40	.047

Table 2.1. Increase in conductivity due to food residue and excretion. Results of regression analyses indicate that during two consecutive sampling periods (days 16-57 and 58-100) water conductivity increased significantly in tanks housing males (CM) and females (CF).

Food residue and Excretion (constant temperatures: 22 and 23 °C)

Although temperature had no significant effect on conductivity over the current working range, conductivity data were sampled for two selected temperatures, 22 and 23 °C, and analyzed (Fig. 2.5). Clearly, under constant temperature conditions, food residue and excretion had a significant effect on conductivity (females: $F(1,86) = 133.33$, $p < .001$; males: $F(1,83) = 119.95$, $p < .001$). The effect of conductivity on the EOD-associated PPSFs for these two temperatures is illustrated in Fig. 2.5. Regression analyses showed that under constant temperatures (22 or 23 °C) there was no significant impact of water conductivity on the PPSF (Table 2.2).

	Regression	r^2	*F*	*df*	*p*
A: CM (22 °C)	y= 0.11x+4565.6	4E-0.5	0.00	1,47	.96
B: CF (22 °C)	y= 2.2x+3904.7	0.014	0.85	1,60	.36
C: CM (23 °C)	y= -3.4x+5400.5	0.032	1.718	1,52	.196
D: CF (23 °C)	y= -1.4x+4588.1	0.002	0.09	1,557	.77

Table 2.2. Regression analyses on the effect of conductivity on the EOD-associated peak power spectrum frequency. CM – males, CF – females. At constant temperatures there was no conductivity effect.

These data indicate (1) that temperature, food residue, and excretion influence conductivity, and (2) that as expected food and excretion affect conductivity with water temperature kept constant. These conductivity fluctuations (40 μS/cm at constant temperature), however, did not significantly affect PPSF and thus EOD duration.

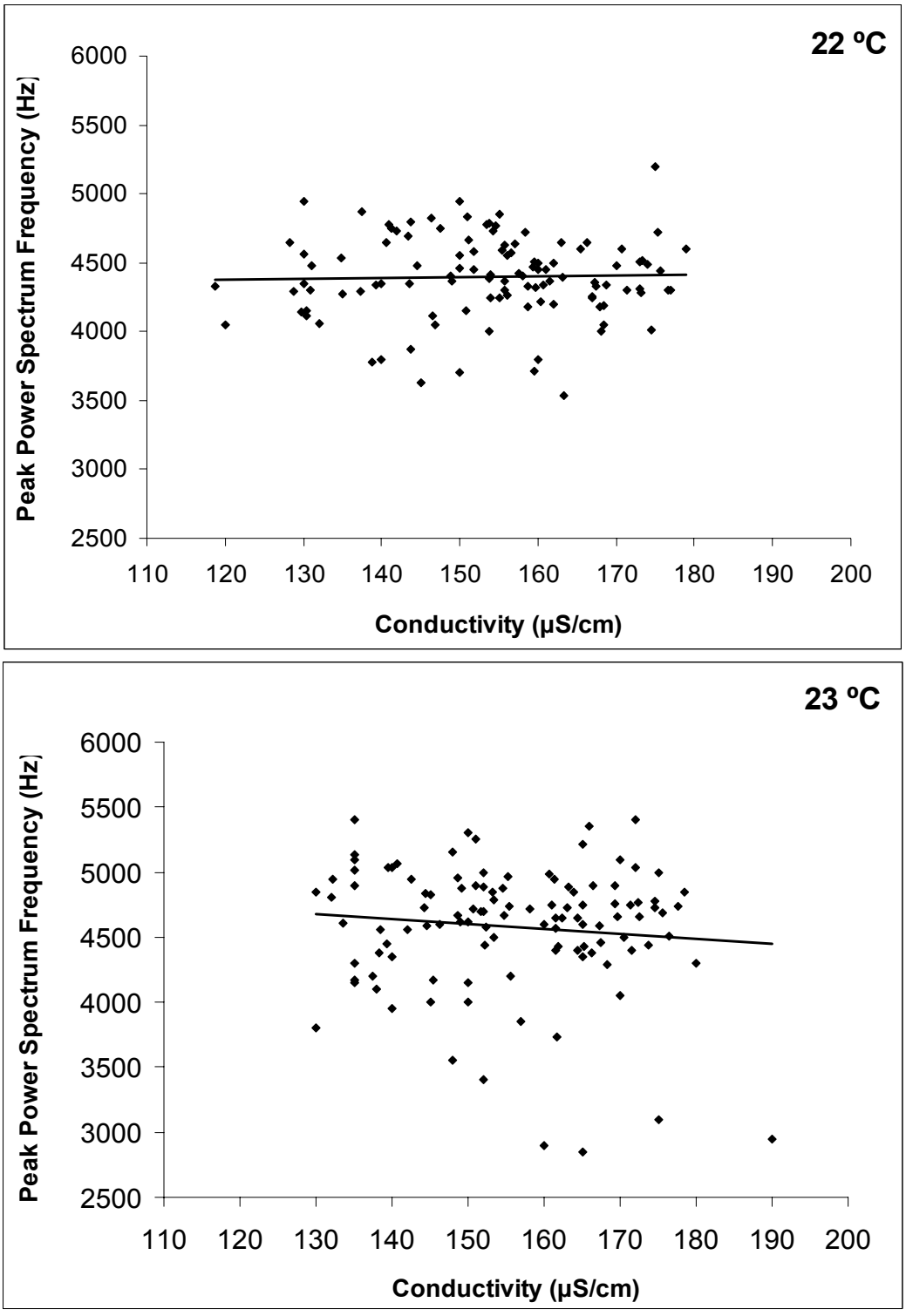

Figure 2.5. Peak Power Spectrum Frequency measured at 22 °C as a function of water conductivity. Regression function: $y = 0.6x + 4304.8$; $r^2 = 0.0008$.
Peak Power Spectrum Frequency measured at 23 °C as a function of water conductivity. Regression function: $y = -3.69x + 5153.7$; $r^2 = 0.0107$

Influence of temperature on the electric organ discharge

Seasonal and periodic daily changes in the fish's natural habitat affect water conductivity and temperature (Kirschbaum, 1979). Moller et al. (1979) reported daily temperature variations ranging from 17 to 32 °C. Under the current experimental conditions, water temperature fluctuated between 19.5 and 25.5 °C.

The effect of water temperature on the duration of the four phases of the fish's EOD waveform (P1-P4) and the EOD-associated PPSFs were analyzed separately for males and females (Figure 2.6 A-J). The duration of all four phases decreased significantly with increased water temperature (Fig 2.6 A-H; Table 2.3). This decrease was best described by a linear best-fit analysis; the rate of decrease did not differ significantly among phases and between sexes. The corresponding PPSFs increased significantly in a linear manner with an increase in temperature (Fig. 2.6 I, J; Table 2.3). There was no difference between males and females.

Temperature Coefficient: normalizing data to 25° C

To allow comparisons, phase duration and PPSF data were normalized to reflect metabolic conditions at 25° C. The temperature coefficient (Q_{10}) was calculated using the individual regression analysis (Fig. 2.6 A-J). The individual measurements were converted using a Q_{10} - Value [M x Q_{10} / 10] x (25- T) with T being the measured temperature, and M being the duration of the individual EOD phase. In Table 2.4 an example calculation of a Q_{10} for P1 (normalized to 25º C) is illustrated. Table 2.5 lists all Q_{10} values.

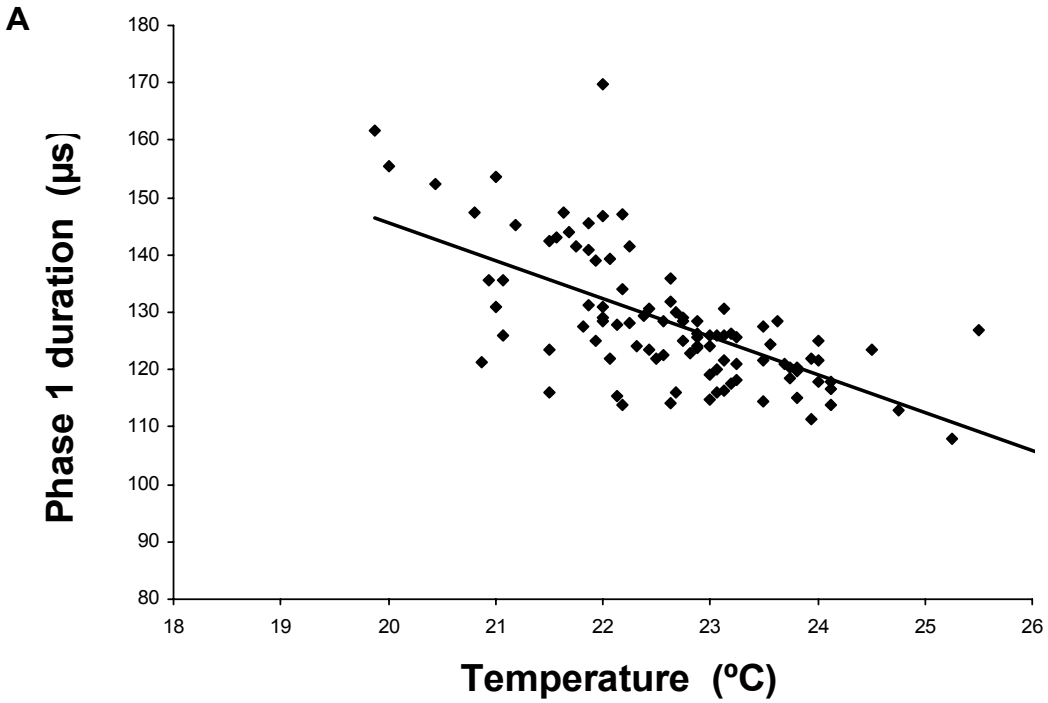

Figure 2.6. A. Mean daily phase duration of Phase 1 in males as a function of water temperature. The best-fit regression function is: y = -6.6x+279.05; r^2 = 0.43.

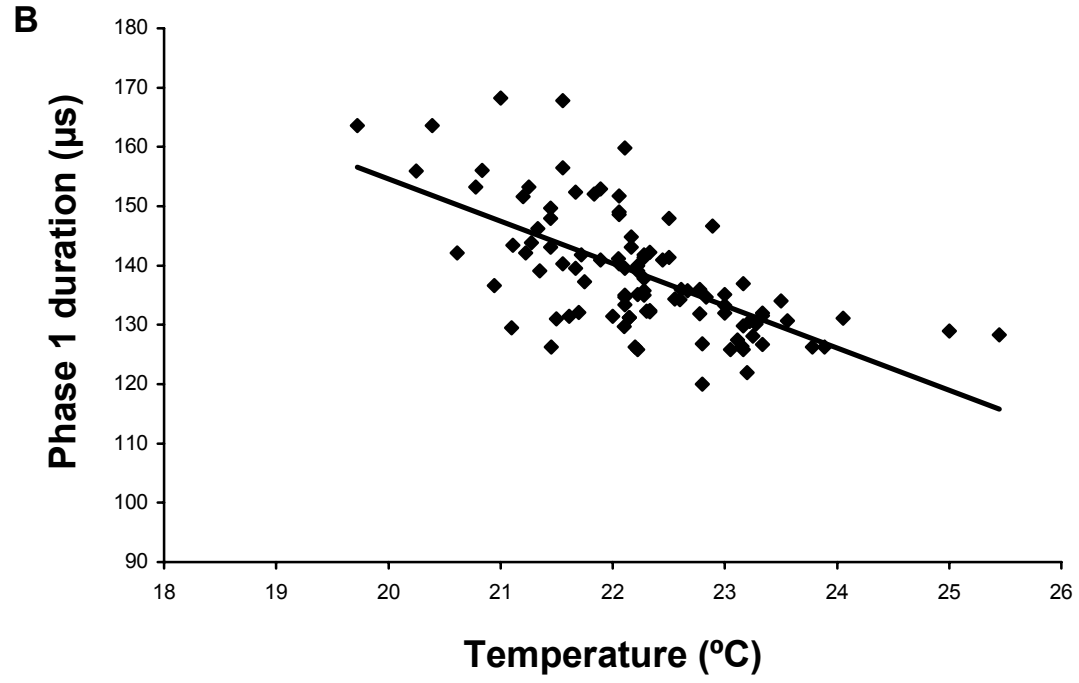

Figure 2.6. B. Mean daily phase duration of Phase 1 in females as a function of water temperature. The best-fit regression function is: y = -7.13x+297.18. r^2 = 0.41.

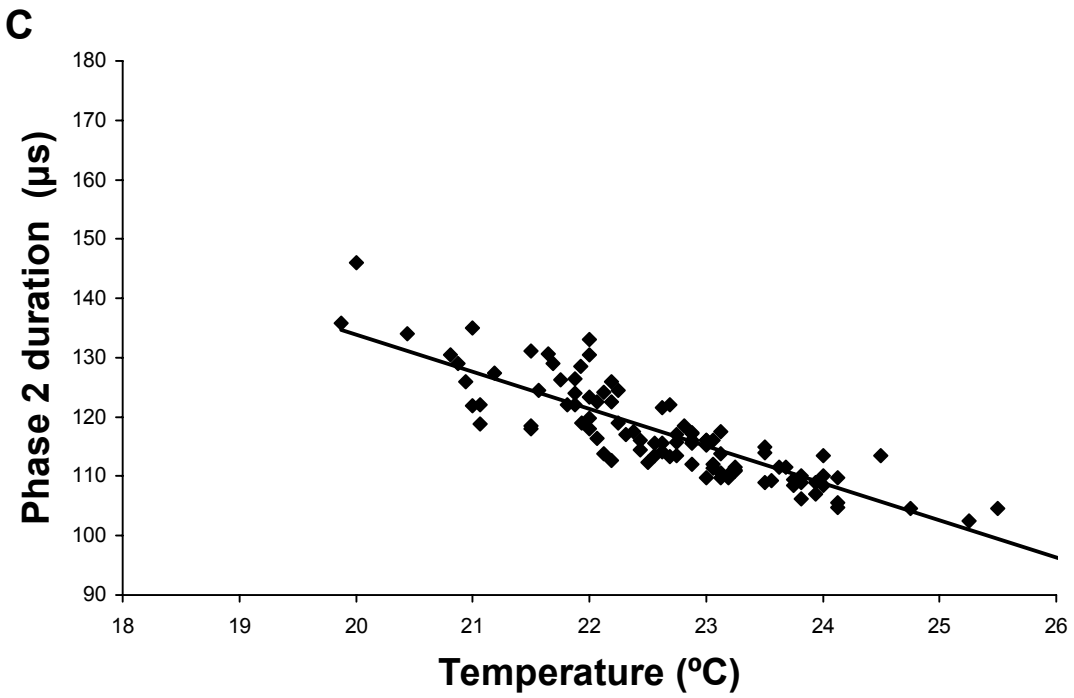

Figure 2.6. C. Mean daily phase duration of Phase 2 in males as a function of water temperature. The best-fit regression function is: y = -6.26x+259.14. r^2 = 0.75.

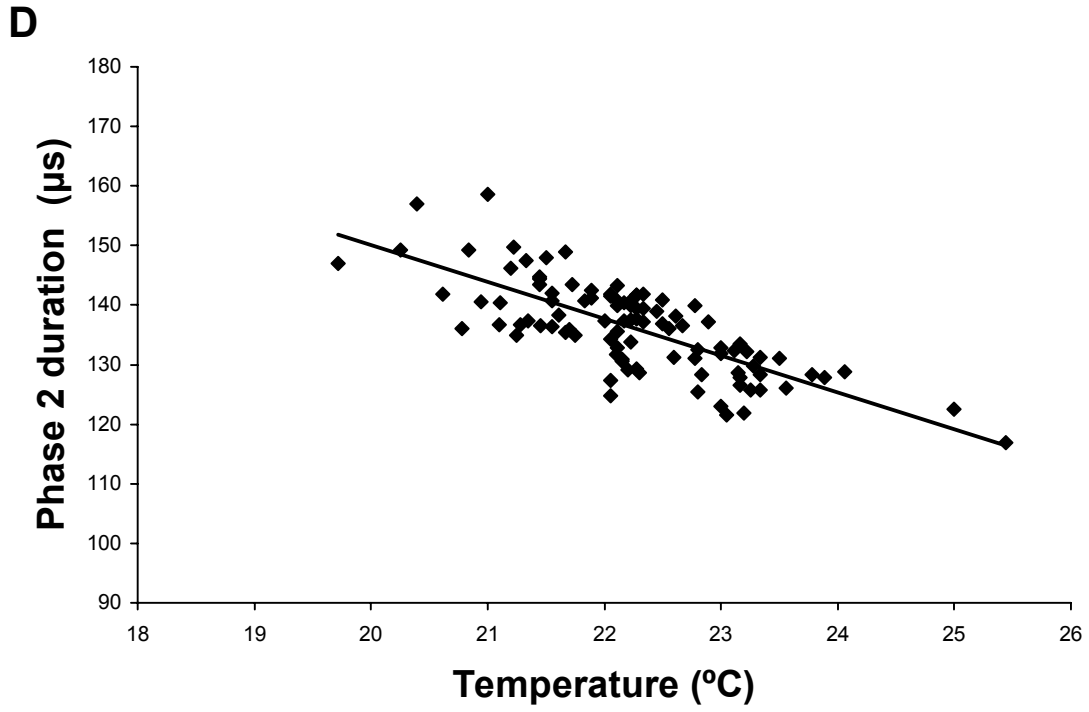

Figure 2.6. D. Mean daily phase duration of Phase 2 in females as a function of water temperature. The best-fit regression function is: y = -6.20x+274.11. r^2 = 0.57.

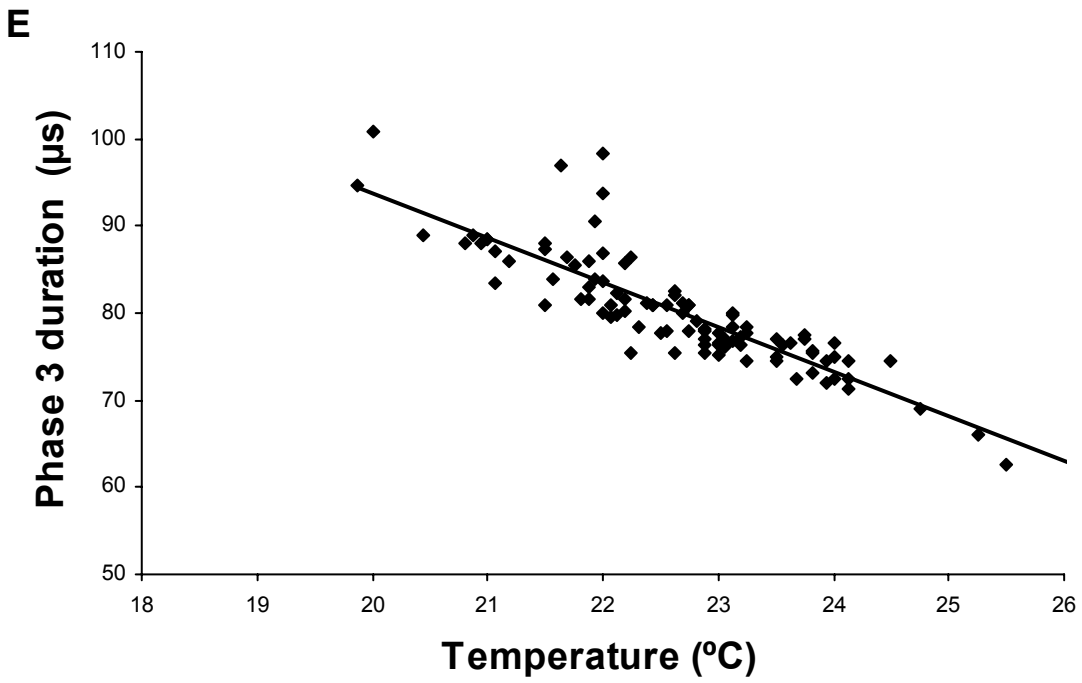

Figure 2.6. E. Mean daily phase duration of Phase 3 in males as a function of water temperature. The best-fit regression function is: y = -5.11x+196.03. r^2 = 0.77.

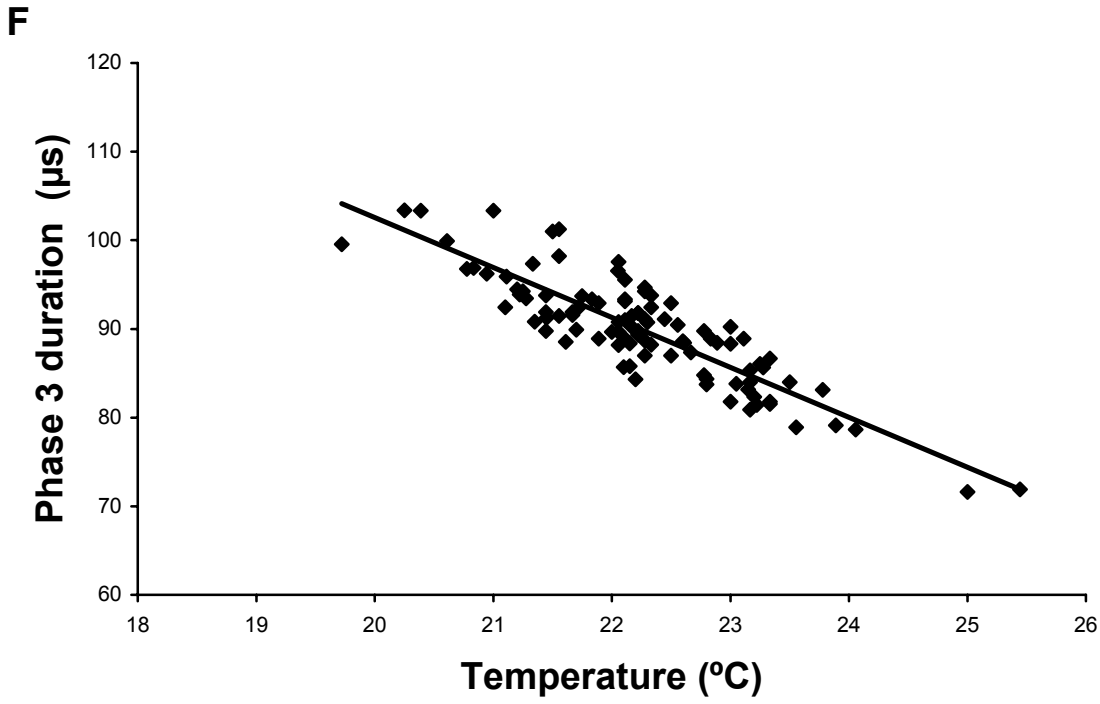

Figure 2.6. F. Mean daily phase duration of Phase 3 in females as a function of water temperature. The line is the regression line with y = -5.634x+215.24. r^2 = 0.76.

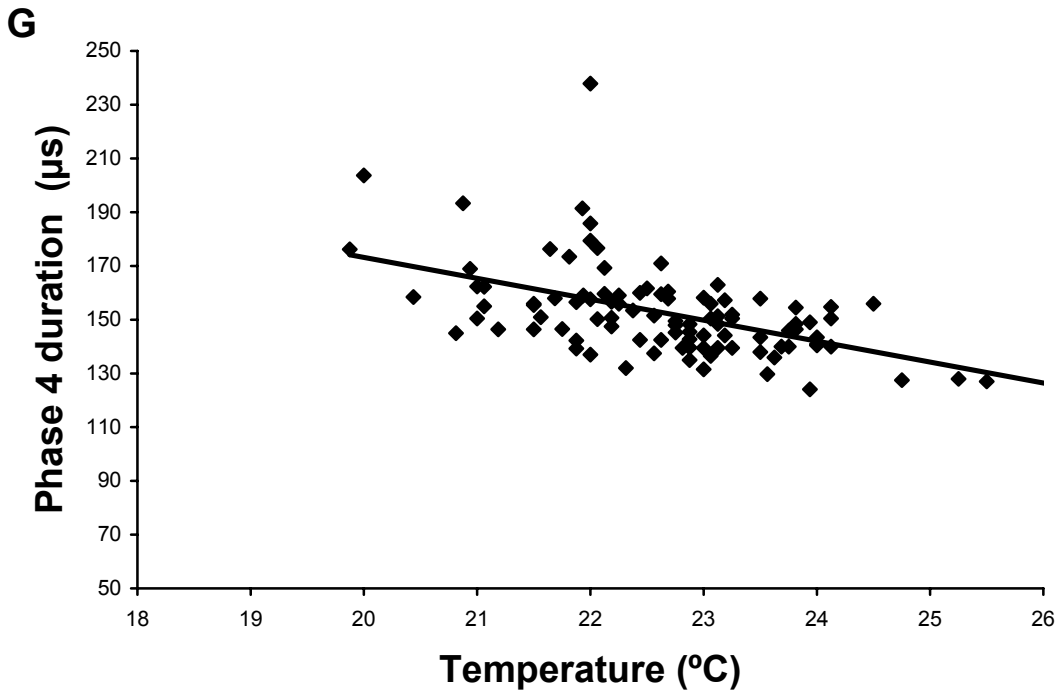

Figure 2.6. G. Mean daily phase duration of Phase 4 in males as a function of water temperature. The line is the regression line with y = -7.79x+328.95. r^2 = 0.28.

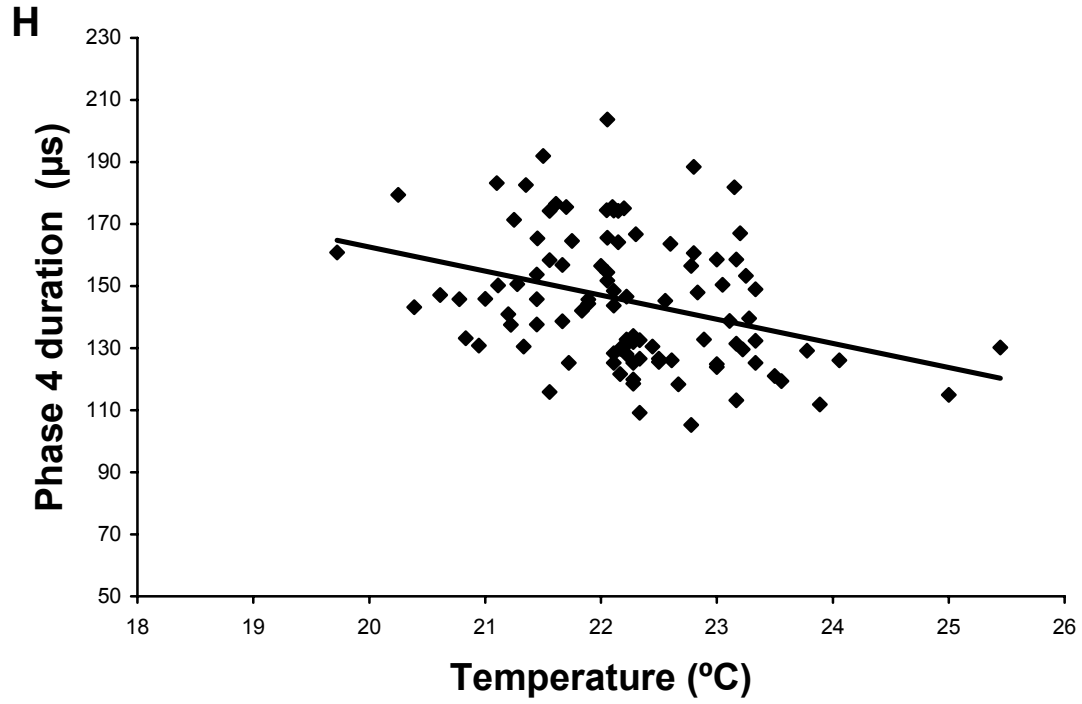

Figure 2.6. H. Mean daily phase duration of Phase 4 in females as a function of water temperature. The line is the regression line with y = -7.78x+318.19. r^2 = 0.12.

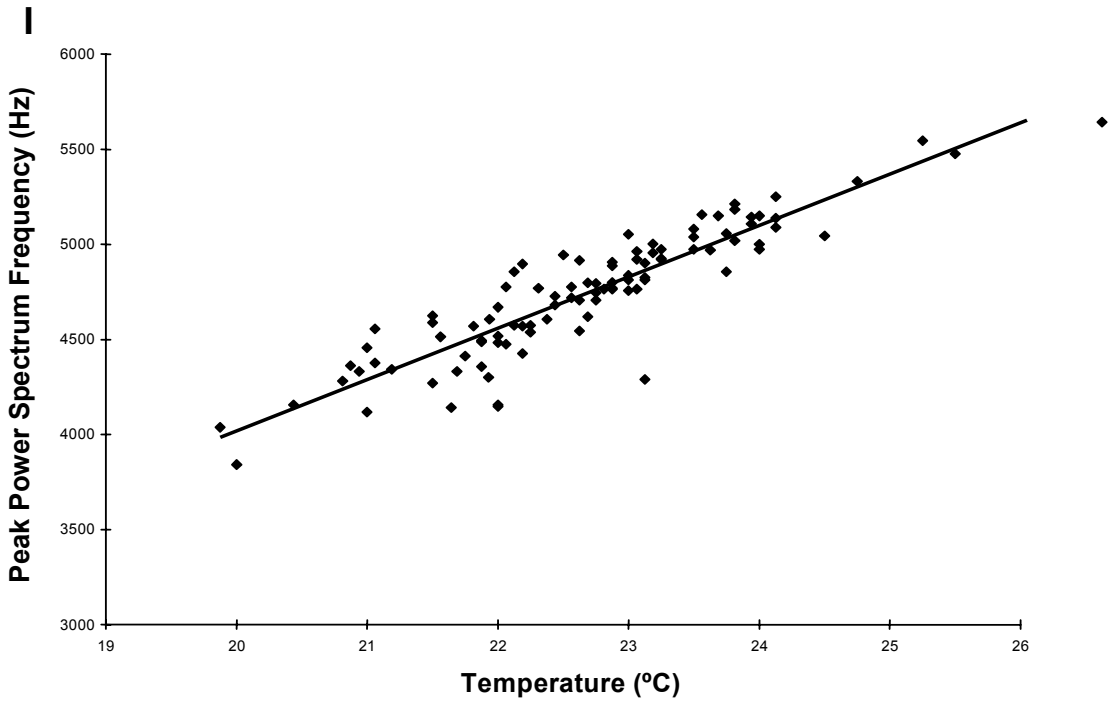

Figure 2.6. I. Mean daily duration of the PPSF in males as a function of water temperature. The line is the regression line with y = 270.13x-1383.9. r^2 = 0.82.

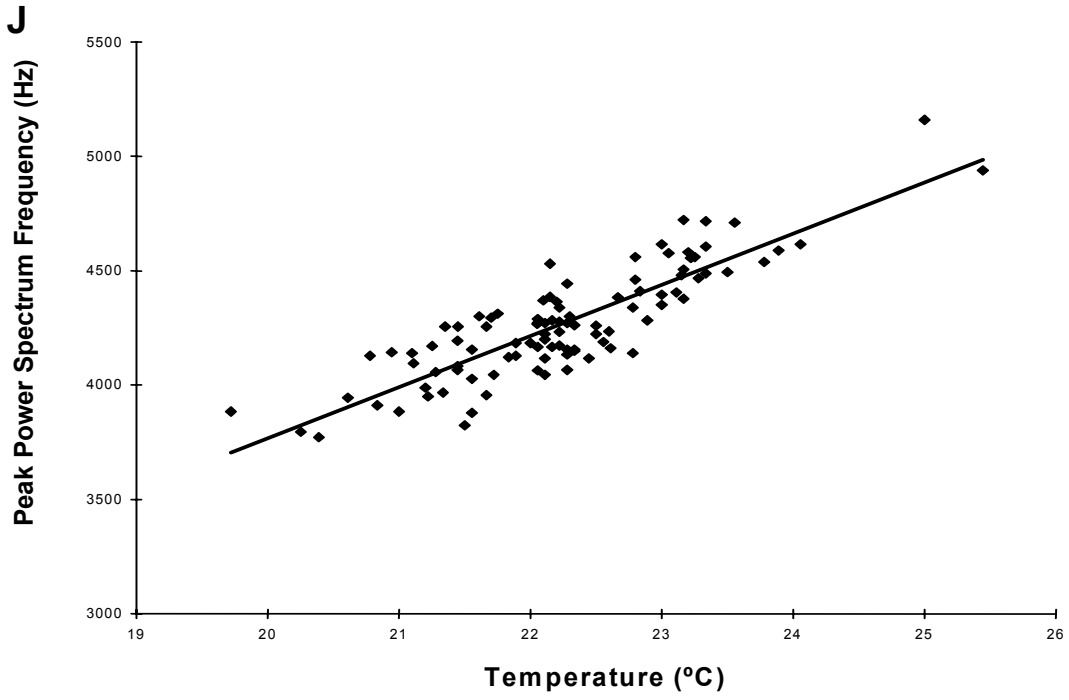

Figure. 2.6. J. Mean daily duration of the PPSF in females as a function of water temperature. The line is the regression line with y = 223.83x-710.79. r^2 = 0.74.

Groups		*F*	*df*	*p*
A Phase 1	♂	73.05	1,97	< .000
B	♀	68.7	1,97	< .000
C Phase 2	♂	285.27	1,97	< .000
D	♀	130.01	1,97	< .000
E Phase 3	♂	319.02	1,97	< .000
F	♀	303.74	1,97	< .000
G Phase 4	♂	37.16	1,97	< .000
H	♀	12.89	1,97	< .000
I PPSF	♂	445.1	1,97	< .000
J	♀	281.0	1,97	< .000

Table 2.3. Regression analyses (n = 99)

Phase 1	Q_{10} = *0.519*
Phase 2	Q_{10} = 0.539
Phase 3	Q_{10} = 0.421
Phase 4	Q_{10} = 0.513
PPSF	Q_{10} = 1.595

Table 2.5. Q_{10} values

Females	y = -7.1304x + 297.180	
	y = 7.130 x 21° C = -149.738 + 297.18 =	147.442
	y = 7.130 x 31° C = -221.042 + 297.18 =	76.138
(a)	76.138 / 147.442 =	0.516
Males	y = -6.664x + 279.050	
	y = -6.664 x 21° C = -139.940 + 279.05 =	139.110
	y = -6.664 x 31° C = -206.578 + 279.05 =	72.472
(b)	72.472 / 139.110 =	0.521
Mean	(a) + (b) / 2 = 0.516 + 0.521 / 2 =	0.519

Table 2.4. Example calculation for Q_{10} Value (underlined) for the first phase.

Discussion

The results of this experiment showed how temperature affects the waveform, i.e. duration and PPSF of the electric organ discharge in the weakly electric fish *B. niger*. Under the given conditions, the influence of conductivity fluctuations was negligible. Using the empirically obtained data from this study, Q_{10} values were calculated from linear regression functions to normalize all phase duration and PPSF measurements. This normalization was based on the assumption that within the prevailing temperature range (19.5-25.5 °C) metabolic processes affected EOD generation in a linear fashion. This assumption seemed to be justified over the noted temperature range. Unpublished data from our laboratory (Higgins, 1997) showed a curvilinear relationship between temperature and EOD duration over a range of 10 °C from 20.0-30.0 °C.

Fluctuations between 16 and 30 °C are not uncommon in the fish's natural habitats (Moller et al., 1979; review Moller, 1995, p.370) and affect both EOD rate and waveform as explained by principles of biophysics. The lateral line, cutaneous nerve endings have been implicated in temperature perception (Murray, 1974). Enger and Szabo (1968) found that temperature has a direct influence on the electric pacemaker system whereas temperature was not perceived through the skin.

Communication

Mormyrids use their EODs to communicate species-specific and sexual differences. Temperature-induced changes of the EOD could therefore impact on

the communication system in these fish. To avoid such temperature-induced distortions of communication signals, *B. niger* as well as other weakly electric fish could employ various strategies to be able to interpret EODs correctly: a) compensate and readjust the EOD to normalcy (Kramer and Kuhn, 1993), b) change electroreceptor tuning in tandem with EOD distortions (Hopkins, 1976), or c) keep the putative communication receptors (knollenorgans) sufficiently broadly tuned (Hopkins, 1976; Zakon, 1986).

EODs as species markers

Form and shape of the mormyrid electric organ discharge have become a powerful taxonomic tool (Sullivan and Hopkins, 2004; Arnegard, Bogdanowicz, and Hopkins, 2005). Socially or environmentally induced variability of the EOD as taxonomic character must be considered by establishing normalized conditions. The current data set could be utilized to determine such normalized data with a caveat that there may be interspecific differences.

Chapter 3

Effects of 17α-methyltestosterone on sexually dimorphic characters in the weakly discharging electric fish, *Brienomyrus niger* (Günther, 1866) (Mormyridae): Electric organ discharge and anal-fin complex*

Summary

Adult males of African weakly discharging electric fish (family: Mormyridae) are distinguished from juveniles and adult females by a dorsally directed indentation of the posterior ventral body wall, and by massive expansion of the bases of several anal-fin rays. These sexually dimorphic structures seem to facilitate the anal-fin reflex that is displayed during courtship. Since the expression of the male sexually dimorphic electric organ discharge (EOD) is under androgenic control, and the adult female EOD can be masculinized through testosterone administration, it was hypothesized that androgens should also drive anal-fin ray expansion in adult fish. Exogenous androgen treatment (17α-methyl-testosterone, 17MT) of adult female *Brienomyrus niger* resulted in male-typical structural transformations (body wall indentation and basal anal-fin ray expansions) and elongated EODs. Some of these changes were immediate and receded following hormone withdrawal (EOD), while others developed more slowly and were apparently permanent (bone formation and indentation). 17MT administration affected only those targets in females that are normally involved in the male's reproductive behavior, i.e. its courtship signal (EOD) and two morphological features (body-wall indentation and expansion). Rays of the dorsal or caudal fins were never affected.

* This is an expanded version of a paper published in *Hormones and Behavior,* 34, 303-319, 1998 (Herfeld, S. and Moller, P.). It includes new data that were collected subsequent to publication of the earlier results. The literature citations have been updated. The bibliography of this article was incorporated with the general bibliography.

Introduction

In most vertebrates, steroids exert profound effects on sexual differentiation and the expression of structural and behavioral dimorphisms. Steroids can cause structural, physiological, and behavioral changes (e.g. Nottebohm, 1980, 1989; Arnold and Breedlove, 1985; Harding, 1986; Kelley, 1988; McEwen, 1991; review: Nelson, 1995). Teleost fishes are no exception in that androgen-directed sexual dimorphisms are manifest in their morphology, physiology, and behavior (e.g. Pickford and Atz, 1957; Fernald, 1976; Hannes and Franck, 1983; Bass and Hopkins, 1983, 1985; Bass, 1992; Brantley et al., 1993 a; Brantley et al., 1993 b; Landsman, 1995; Rosa-Molinar et al., 1996; Moller et al., 2004; Greisman and Moller, 2005).

It is therefore not surprising that administration of synthetic androgens will interfere with the normal expression of primary and/or secondary sexual characters. Androgen-treated adult female poeciliid fishes (*Xiphophorus helleri*: Regnier, 1938; *Gambusia affinis affinis*: Turner, 1941; *Poecilia maculatus*, *P. variatus*: Grobstein, 1940, 1948) developed a masculinized anal fin, but failed to develop normal gonopodia suggesting that administration occurred outside of a critical 'developmental window'. Landsman, David, and Drew (1987) treated adult female *Poecilia reticulata* with 17MT (by way of ingestion) or with silastic time-released testosterone or DHT implants. Treated females developed secondary male-like sexual characteristics and adult male-like reproductive behavior. Within 14 days, treated females developed gonopodia and the associated musculature and fish were able to perform the 'gonopodial swings' necessary to initiate

copulation when paired with normal females. They also developed male-typical coloration and body size (standard length and body weight both decreased) and eventually exhibited the phenotypic appearance of a mature male (Landsman et al., 1987).

Timing of normal androgen exposure (organizational effects) in vertebrates is crucial for normal sexual differentiation (see e.g. Adkins-Regan and Ascenzi, 1990; Kelley, 1992). Timing is critical in the development of a functional male 'genital area' in the Western mosquitofish, *G. affinis affinis* (Rosa-Molinar, Hendricks, Rodriguez-Sierra, and Fritzsch, 1994; Rosa-Molinar *et al*., 1996). The authors established that the critical period for the anterior transposition of the male anal fin and its appendicular support occurs during the late embryonic period and is under androgenic control. The Japanese rice fish, the medaka, *Oryzias latipes*, has become a "poster" species in endocrinological studies on teleost fish. Koger, Teh, and Hinton (2000) exposed early lifetime stages of this species to 17β-estradiol (E_2) or testosterone (T) to determine the effective developmental windows for hormonal effects. Late embryos (stage 10) and larvae (1, 7, and 21 days old) were exposed to E_2 or T for 6 days, and tested at 5 months of age. There was no difference between treated and control groups with regard to mortality, body weight or time to reach sexual maturity; however, the sex ratio became significantly biased toward females in 1 and 7 day old E_2-treated fish. T-treatment did not affect any change in sex ratio regardless of time of treatment. Intersex gonads (ovotestis) developed in all E_2 treated fish, but only in 1 and 7 day old T-treated animals. Mormyrids, in contrast, responded to early

lifetime exposure to dihydrotestosterone (17-DHT) with males developing ovotestis, and females developing secondary male-typical basal anal-fin expansions (Moller, Kirschbaum, Schugardt, and Dowling, in prep.).

African weakly discharging electric fish (family: Mormyridae) can generate and detect electric organ discharges (EODs). Direct gonadal steroid action on the electrocytes, the basic structural elements of the electric organ, results in dimorphic EOD waveforms (Bass, 1986 a, b; Bass and Hopkins, 1983, 1984; Landsman, 1993 a, 1995). This sex difference in the EOD can be permanent in some species or expressed only during the breeding season in others (reviewed in: Landsman, 1995). Typically, the female EOD is shorter than the male's and considerable differences in shape and amplitude are widespread throughout the family Mormyridae (Hopkins, 1981). In the mormyrid, *Gnathonemus petersii* (Günther, 1862), both the male-typical EOD and structural changes in the anal fin can be induced through either testosterone implants (Landsman and Moller, 1988; Voustianiouk, 2003) or administration of 17MT via the aquarium water (Voustianiouk and Perrotti, cit. in Landsman, 1995).

EODs facilitate spatial orientation and social communication, and because of their nocturnal habits mormyrids rely on these signals for species recognition and mate attraction (Hopkins, 1981, 1986; Kramer, 1990; Moller, 1995). During courtship behavior the male in several species envelopes its anal fin around the female's (anal-fin reflex) to form a common spawning pouch (Kirschbaum, 1987).

Two sexually dimorphic structures distinguish adult males from juveniles and adult females, (a) a dorsally directed indentation of the posterior ventral body

wall (Iles, 1960; Nawar, 1959; Okedi, 1969; Kirschbaum, 1987, 1995; Kirschbaum and Schugardt, 1995; Schugardt, 1997; Pezzanite and Moller, 1998; Moller et al., 2004) (see Fig. 1.3 in Chapter 1), and (b) massive fin ray expansion of the bases of a select number of anal-fin rays (Brown et al., 1996; Pezzanite and Moller, 1998).

It can be hypothesized that these sexually dimorphic structures facilitate the anal-fin reflex that is displayed during copulation when the male envelops the female's anal fin with its own forming a common spawning pouch. Expanded bone could provide additional surface for muscle attachment and thus assist in part with the courtship sequence.

Based on the fact that the expression of the males sexually dimorphic electric organ discharge (EOD) is under androgenic control, and that the adult female EOD can be masculinized through testosterone administration, it was hypothesized that androgens should also drive anal-fin ray expansion in male mormyrids and equally effect male-like changes in treated juveniles and adult females.

Material and Methods

Experiment 1

Subjects

Twenty-seven female and eight male *B. niger* (SL 89.2 ± 6.9 mm; range: 76-104 mm; weight 9.0 ± 1.5 g; range: 7-13 g; body depth 23.5 ± 2.4 mm; range: 19-27 mm), imported from Nigeria at the end of the purported breeding season (November 1995) were obtained through a local tropical fish vendor (Quality Tropicals, Inc. Wallington, N.J.). Typically, specimens measuring from 120 mm upward had a fully developed gonad. Thus, fish in this study were just approaching sexual maturity and had not yet spawned which was confirmed by gonadal inspection. Fish were housed separately throughout the study in 20 l aquaria (actual water volume: 13 l) and maintained on an L:D = 12:12 regimen with lights on at 8:30 h. Each aquarium was equipped with a porous ceramic shelter tube (150 mm L; 50 mm Ø) and standard filtration and aeration devices. Water conditions were kept within limits, i.e., temperature between 20-25 °C and conductivity between 130-180 μS/cm. Fish were fed tubifex worms and brine shrimp twice a week. Over the duration of the observation period, no apparent loss of weight was detected. Fish were transferred to these home aquaria 14 days prior to the beginning of the study.

There were three groups of fish: control males (CM, *n* = 8), control females (CF, *n* = 9), and 17MT-treated females (TF, *n* = 18). There were several losses: two designated TF died prior to the onset of treatment (i.e. on day 17, abbreviated as d17), and three fish (TF) were sacrificed following hormone

treatment (d59) for preparation of cleared and counter-stained specimens (Taylor and Van Dyke, 1985). On d127, gonads of four fish from each group were sacrificed for inspection. The study was conducted over a period of 457 days commencing in January 1996 and ending in May 1997.

Experiment 2

Subjects

Fifty-six female and eight male *B. niger* (SL 90.2 ± 7.2 mm; range: 76.4-108.6 mm; weight 10.6 ± 2.8 g; range: 5.8-17.3 g; body depth 24.2 ± 2.3 mm; range: 19-29.9 mm, body depth 8.6 ± 1.2 mm; range: 5.9-12 mm), imported from Nigeria at the end of the purported breeding season (November 1998) were obtained through a local tropical fish vendor (Quality Tropicals, Inc. Wallington, N.J.). Typically, specimens measuring from 90 mm upward had a fully developed gonad. All subjects were housed and maintained the same way as described in Experiment 1.

Following initial x-ray photography the fish were assigned to four groups. The main experimental group contained 35 treated females (TF), which underwent treatment with 17MT. The control groups contained four control males (CM) and 21 control females (CF). In addition four males were also treated (TM) with 17MT for a total of six weeks. Females were studied over the course of seven weeks divided in time periods of one week. Each time period is noted as week n where n indicates the number of weeks of treatment elapsed before fish were sacrificed. This experiment ran for a total of seven weeks, after weeks 1, 2,

3, 4, 5, 6, and 7 following EOD recordings, 8 fish (5 TF, 3CF) were removed from their tanks and sacrificed for histological analyses (bone, gonads). One of these treated fish (per week) and two control fish (during week 6) (total n = 9) were cleared and counterstained for further fin ray analysis (Taylor and Van Dyke, 1985).

General hormone treatment

Hormone treatment began on d17 (i.e. following a two week adaptation of the fish to their individual tanks) when dissolved 17MT was added to water (2 mg/l). Then, again every seven days, an equal dose was added for six treatments (d23, d30, d37, d44, and d51). Since the exact hormone levels necessary to elicit androgen effects in *B. niger* were not known (but were approximated based on data by Bass and Hopkins, 1985), the described procedure was adopted to effect a steady increase of 17MT over the 6-week treatment period. Water conductivity was maintained within limits (130-180 μS/cm) by weekly exchanging one third of the water volume in all tanks.

Experiment 1

On d59, hormone withdrawal began by changing the water four times every two days, followed by the regular weekly water change. Thus, fish were exposed to 17MT for a period of six weeks.

Experiment 2

In comparison to experiment 1 in which all fish were treated for a six-week period, in experiment 2 the length of treatment depended on the week the treated fish were assigned to be sacrificed. It is important to mention that androgen was administered to the treatment groups for a maximum of six weeks, which means that fish in week seven were treated with 17MT for six weeks and a rapid water change over three days removed most of the 17MT. Week seven fish therefore had a dramatically reduced hormone exposure for the last week.

Notes on use of synthetic 17MT.

Since this paper focused only on possible effects of male sexual steroids on selected targets (EO, anal-fin complex) rather than on specific physiological hormonal action of the fish's natural androgens, such as testosterone, dihydrotestosterone, and 11-ketotestosterone, the use of 17MT was selected.

Synthetic 17MT can be aromatized to a methyl-estradiol analogue, 17α-methylestradiol (Hornung, Jensen, Korte, Kahl, Durhan, Denny, Henry and Ankley, 2004). Evidence for this was provided by Ankley, Jensen, Kahl, Korte, Makynen, (2001); Parrott and Wood (2002); Zerulla, Lange, Steger-Hartmann, Panter, Hutchinson and Dietrich (2002) who found both androgenic and estrogenic effects in 17MT-treated juvenile and adult fathead minnows (*Pimephalis promelas*). A common phenomenon in aquaculture is so-called 'paradoxical feminization' in 17MT-treated tilapia (Nakamura, Kobayashi, Chang and Nagahama, 1998).

Cyclic reproduction is typical of riverine species such as *B. niger*. The onset of gametogenesis precedes the onset of the rainy season, and thus the fish's breeding season by about a month (Bénech and Quensière, 1985; Kirschbaum, 1995). By inference, one could assume that during this period, gonadal steroids also exert control over the expression of the fish's secondary sexual dimorphisms. Thus, the exposure of females in this study to six weeks of 17MT treatment could have mimicked a pre-breeding preparatory phase directing the expression of these dimorphisms.

Electric organ discharge

EODs were recorded every day for the first 100 days (Experiment 1). They were obtained with a pair of Ag/AgCl electrodes fitted to the far ends of the aquarium while fish were resting in their shelter. EODs were pre-amplified (custom-modified AD625, high frequency band pass filter) and analyzed with the help of a digital storage oscilloscope (Hitachi, Model VC-6023). The instrument allowed inspection of pre-trigger events so that the full-length EOD could be displayed and analyzed (see Figure 2.2 in Chapter 2).

The duration of the four EOD phases (P1, P2, P3, and P4) was taken from oscilloscope-stored, amplitude-normalized EOD waveform displays. The duration of the four phases was defined as follows: P1 (minor head-negative phase), from the point of just detectable origin (decline) on the 'noise-free' baseline to the intersection of the ascending P1 wave with the baseline; P2 (major head-positive phase), from the end point of P1 to the intersection of the descending P2 wave

with the baseline; P3 (major head-negative phase), from the end point of P2 to the intersection of the ascending P3 wave with the baseline; P4 (minor head-positive phase), from the end point of P3 to the first noticeable 'contact' with the baseline. The EOD-associated peak power spectrum frequency (PPSF) was measured with a spectrum analyzer (Hewlett-Packard, model 3582A, range 0-25 kHz, resolution 100 Hz).

In experiment 2, EODs were recorded in the same manner as in experiment 1, but only every other day for a period of 7 weeks, to correlate 17MT effects with changes in behavior (EOD) and structure (electrocyte geometry, anal-fin morphology).

Temperature correction (see also chapter 2)

Temperature affects EOD phase duration and associated PPSFs (Herfeld, 1998; Higgins, pers. communication, 1997). Therefore, EOD measures were corrected for 25 °C (using empirically established values *for* Q_{10}: PPSF, 1.59; P1, 0.52; P2, 0.54; P3, 0.42; and P4, 0.51). Effects due to small fluctuations in water conductivity as determined under constant temperature conditions were not significant.

Radiography

Experiment 1. Live fish from all three groups were radiographed several times throughout the study. Baseline data were collected on all groups three days prior to recording of EODs (d -3). The effects of hormone treatment and withdrawal on anal-fin expansion and anal-fin indentation were monitored nine

more times throughout the study (d37, d59, d101, d184, d216, d281, d334, d391, and d457). Both control groups were monitored three more times (d101, d184, and d216).

Experiment 2. Following initial x-ray photography the fish were assigned to the two main groups (control vs. treatment). Anal-fin ray bases were analyzed to confirm males. Before fish were sacrificed each week they were x-rayed for the second time.

Fish used in both experiments were anesthetized with buffered (sodium bicarbonate) tricaine methane sulphonate (MS-222, Sigma) and radiographed (Hewlett-Packard, model Faxitron 43807 N, Kodak ReadyPack II Industrex M film) under low-intensity radiation (30-40 KVP) for 30-40 s, depending on the size of the fish. Following radiography the fish were revived in highly aerated water and returned to their respective home aquaria. The mortality rate for this procedure was zero.

Posterior ventral body wall indentation and anal-fin expansion

Morphometrics and meristics (i.e. counts of the number of fin rays, number of fin rays with expansion, and the position-number of first and last ray with expansion) were obtained from radiographs and/or cleared and counter-stained specimens (Experiments 1 and 2).

Experiment 1.

To quantify the extent of the indentation (see Fig. 1.3 in chapter 1), (i) the maximum, dorsally-directed indentation was determined using the base of the associated ray as a point of reference (ray 12), (ii) from this point straight lines rostrad and caudad, respectively, using the bases of the adjoining next four rays as a reference were drawn, (iii) from the respective first and last fin-ray bases, the perpendicular projections on these lines were obtained, (iv) the endpoints of these projections were connected, and (v) the resulting triangle allowed the computation of 'b/a' (tan β) as indentation measure.

The structure of the anal-fin ray bases in treated females differed from that in non-treated females. This change in each individual anal-fin ray base was assigned to four expansion types approximating the differences in shape and extent of ossification from maximum expansion (types A and B) to lesser degrees of expansion (types C, D) (Fig. 3.1). Expansion was never observed to affect rays beyond ray 20.

Three-dimensional analysis of anal-fin bases

In experiment 1 radiographic images only allowed an assessment of one lateral surface area of the expanded or non-expanded ray base (see Figs 3.1, 3.6). In experiment 2, a three dimensional analysis of the anal fin bases was conducted in order to verify that treatment induced changes in the entire ray base.

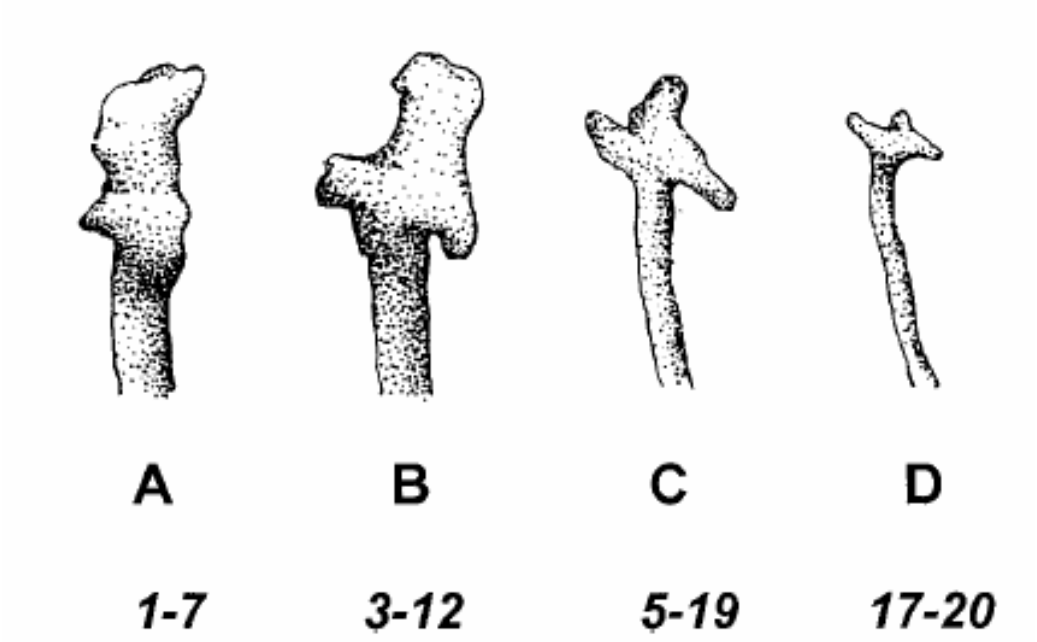

Figure 3.1. Anal-fin ray expansion types (A, B, C, and D) in 17MT-treated female *B. niger*. Position of individual bone types was associated with their characteristic range of fin-ray position numbers (italics).

Fish were cleared and counterstained with alcian blue for cartilage and alizarin red S for bone (Taylor and Van Dyke, 1985). From each fish, ray 12 was excised (i.e. from the site of maximal indentation) (Figure 3.8) and examined with a Nikon Eclipse E400 microscope (magnification 10X). Following examination of the lateral face (identical with that observed in the radiograph), the ray was rotated in steps of 90º to examine its posterior, anterior and the two lateral faces.

Data analysis

EOD measurements (P1, P2, P3, P4, and PPSFs) were pooled for consecutive four-day periods beginning with dd17-20 and ending with dd97-100. These data sets were subjected to two-way ANOVAs (with repetitions) with three

groups (TF, CF, CM) and twenty-one 4-day time blocks, followed by Newman-Keuls multiple post-hoc comparison tests (α = 0.05). EOD data taken during the time of hormone treatment were further analyzed for 'best-fit' functions using both linear and non-linear correlation analyses. Data on body wall indentation (tan β) were subjected to single factor ANOVAs (with repeated measures within groups across days of radiography, and independent measures across groups for days of radiography), followed by multiple post-hoc comparison tests (α = 0.05). Within-group comparisons between two days were made using paired t-tests.

All procedures were in compliance with local, state and federal regulations and protocols and were approved by the Hunter College and American Museum of Natural History Institutional Animal Care and Use Committees (IACUC) (Protocol # PM/SH/AV/7/00).

Results

Experiment 1

Androgen treatment of female *B. niger* resulted in male-like EODs, body wall indentation, and anal-fin ray expansion. Some of these changes were immediate and receded following hormone withdrawal (EOD), while others developed more slowly and were apparently permanent (indentation and bone formation). Androgen administration affected targets in treated females that are normally involved in the male's reproductive behavior, i.e. its courtship signal (EOD waveform), indentation, and expansion; 17MT did not affect rays of the dorsal or caudal fins.

Electric Organ Discharge

The PPSFs in females were lower than those in males and the duration of phases P1, P2, and P3 in control females was slightly, but consistently longer and that of P4 shorter than in control males (Figs. 3.2 and 3.3 A-D). An EOD-related sexual dimorphism is known to exist in sexually mature specimens of this species during their breeding season (Serrier and Moller, 1989; Jacob, in Moller, 1995, p. 181). Under the present conditions, however, with fish held in captivity and not in breeding conditions, these findings were consistent with data for *G. petersii* (Landsman, 1993 b, 1995). In *B. niger,* the apparent difference in all five EOD measures was not statistically significant (Table 3.1 e).

PPSF

17MT treatment affected the fish's EOD and resulted in a decrease in the associated PPSFs (Fig. 3.2). The steepest drop in PPSF, from an average of about 6,500 Hz (pre-treatment level) to about 4,700 Hz, occurred within the first two treatment cycles. Further hormone treatment (on d30, d37, d44, and d51) resulted in a slower frequency decrease to 4,100 Hz at the end of treatment.

	TF	*CF*	*CM*
TF	**a**		
PPSF	21-76, 81-84, 93-96		
P1	25-100		
P2	29-68		
P3	21-84, 89-92		
P4	29-96		
CF	**b**	**d**	
PPSF	25-68	61-64	
P1	29-68	97-100	
P2	37-64	ns	
P3	25-76	ns	
P4	25-100	57-80	
CM	**c**	**e**	**f**
PPSF	25-68	ns	ns
P1	25-100	ns	81-84, 89-100
P2	29-68	ns	ns
P3	25-80	ns	ns
P4	25-100	ns	ns

Table 3.1
Significant post-hoc comparisons (Newman-Keuls; α = 0.05) on five EOD indicator responses (PPSF – peak power spectrum frequency, and phase duration of P1, P2, P3, and P4). Note. The first 4-day period (days 17-21) in groups listed across in header (TF, treated females; CF, control females; CM, control males) were compared with consecutive 4-day periods in groups TF, CF, and CM (left side of table). Within-group comparisons: a, d, f; between-group comparisons: b, c, e. See text for details and explanations. ns, not significant.

Following hormone withdrawal beginning on d59, the PPSF reached pre-treatment levels (about 6,500 Hz) within 14 days with the steepest increase following the 3rd rapid water change on d64.

A two-way ANOVA (groups x time) indicated both significant ($p < .001$) main and interaction effects. The respective *F*-values were: F(2, 567) = 219.6 (between groups), F(20, 567) = 11.67 (within groups), and F(40, 567) = 5.49 (interaction). Post-hoc comparisons are listed in Table 3.1. Figures 3.2 and 3.3 illustrate the results from all fish alive at the time of sampling; the ANOVA was prepared only on those fish alive at the end of the study.

Within-group (TF) comparisons indicated a significant difference in PPSF between dd17-20 and the following 14 four-day groups (days 21-76: Table 3.1 a). By d45 PPSFs had attained their lowest level and remained at that level through d56. (Note: 17MT was only administered from d17 through d58). From d77 through the end of the sampling period (d100), PPSFs did not differ from dd17-20 (with the exception of dd81-84 and dd93-96; see 'Long-Term Effects'). There was no significant difference within both control groups (Table 1, parts d, f) with the exception of dd61-64 in CF (see Fig. 3.2) and dd81-84 and dd89-100 in CM (see Long-Term Effects). Between-group comparisons showed no difference between control groups (Table 1, part e), but significant differences between both controls (dd17-20) and treated females (from d25 through d68; Table 3.1, parts b, c).

The time course of hormone action on the PPSF was best expressed by two linear functions: y, mean PPSF in Hz; and x, time of treatment in days (Fig. 3.2, insert). The first, $y = -320x + 12{,}799$ ($r^2 = 0.962$) describes the steep drop in PPSF over the first 14 days of hormone treatment, and the second, $y = -24x + 5{,}570$ ($r^2 = 0.648$), illustrated the gradual leveling off during the remainder of treatment.

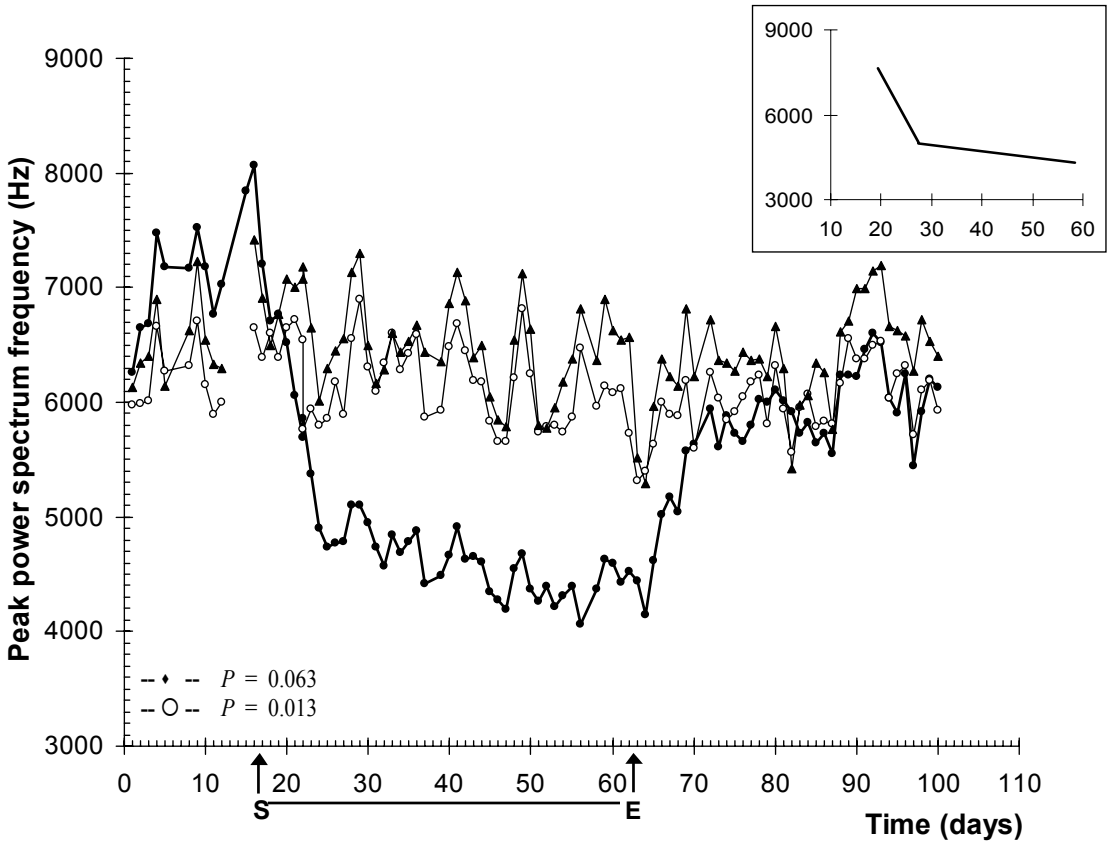

Figure. 3.2. Time course illustrating the effects of 17MT administration on EOD-associated peak power spectrum frequency (PPSF) in female *B. niger* (●) and comparisons with non-treated females (○ and males (▲). S, E – Start and end of hormone treatment. Insert shows two linear best-fit functions to describe hormone action (y, mean PPSF in Hz; x, time of treatment in days): y = -320x + 12,799 (steep drop over the first 14 days) and y = -24x +5,570 (gradual leveling off during remainder of treatment). *P* -values in left lower corner indicate outcome of regression analysis on both control groups over the period from d17-100 (long term effects: see text for details).

EOD phase duration

The effects of 17MT on the individual phase duration (P1-4) and the corresponding data for the two control groups (CM, CF) are illustrated in Figure 3.3 A-D. In treated females, beginning with dd17-20, over the course of the 100-day sampling period, the duration of P1, P2, and P3 first increased and then

decreased, while P4 first decreased and then increased. Congruent with the androgen effects on the PPSF, these changes in phase duration in TF also became apparent within the first two treatment cycles and reversed during the two weeks following hormone withdrawal.

Two-way ANOVAs showed both significant ($p < .001$) main and interaction effects. The *F*-values for P1, P2, P3, and P4 were respectively F(2,567) = 165.31, 145.79, 710.18, and 562.21 (between groups); F(20, 567) = 12.09, 3.15, 13.02, and 20.88 (within groups); and F(40, 567) = 6.18, 2.18, 13.64, and 10.56 (interaction). Post-hoc comparisons are listed in Table 3.1. Within TF, significant differences in phase duration appeared between dd17-20 and dd25-100 (P1); dd29-68 (P2); dd21-84, dd89-92 (P3); and dd29-96 (P4), respectively.

While there were no statistically significant differences for P2 and P3 over time within both control groups, such differences were found in CF between the first 4-day block (dd17-20) and dd97-100 (P1) and dd57-80 (P4); and also in CM between dd17-20 and dd81-84, dd89-100 (P1).

Both control groups (using dd17-20 as comparison) differed significantly in phase duration (P1-4) from treated females (Table 3.1 b, c). The presence of 17MT resulted in maximum, time-dependent changes as follows (interaction effects): for P1 from d22 to d68, for P3 from d25 to d64, and for P4 from d29 to d79 (17MT was only administered from d17 through d58). Although there was a plateau-like increase in P2 from d37 to d64, these values were not significantly different from the remaining sampling days (see Long-Term-Effects).

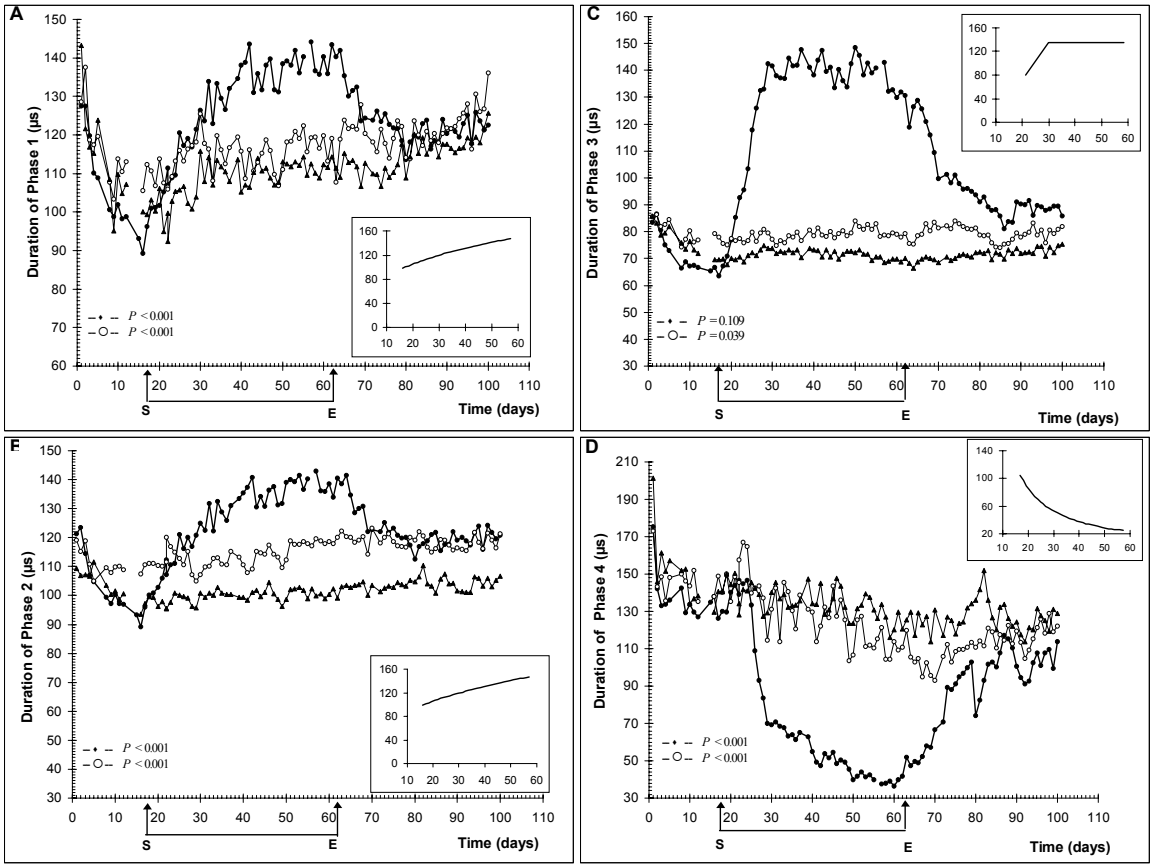

Figure. 3.3. Time course illustrating the effects of 17MT administration on EOD phase duration (A: P1, B: P2, C: P3, and D: P4) in female *B. niger* (●) and comparisons with non-treated females (○ and males (▲). S, E – Start and end of hormone treatment. Inserts show best-fit functions to describe hormone action: power functions for P1: $y = 40.6x^{0.319}$, P2: $y = 41.4x^{0.312}$, and P4: $y = 3087x^{1.197}$, and two linear functions for P3 (dd15-30): y = 6.4x – 46.5, P3 (dd30-58): y = 0.03x + 139.8. *P* -values in left lower corner indicate outcome of regression analysis on both control groups over the period from d17-100 (long term effects: see text for details).

The time course describing changes in phase duration during hormone treatment is shown in Fig. 3.3 A-D (inserts). The best-fit functions were power functions for P1: $y = 40.6x^{0.319}$ ($r^2 = 0.894$); P2: $y = 41.4x^{0.312}$ ($r^2 = 0.908$), and two linear functions for P3 (dd15-30): y = 6.4x – 46.5 ($r^2 = 0.972$); P3 (dd30-58): y = 0.03x + 139.8 ($r^2 = 0.003$); and P4: $y = 3087x^{1.197}$ ($r^2 = 0.702$).

Long-term effects

Fish were initially housed in a 1,200-l communal tank prior to transfer to 20-l aquaria. During a 2-week adaptation period, the duration of all four EOD phases exhibited a decrease in duration ranging from 20-60 μs. This drop in phase duration and the associated increase in PPSF were attributed to the acclimation of the fish to their smaller holding tanks and the weekly water changes. Over the entire sampling period, i.e. from dd17-100, a steady but slight incline in the duration of P1 and P2, and a decrease in P4 and PPSF in both control groups were noticed. These long-term changes were significant with the exception of PPSF and P3 in CM (see *p*-values in Figs. 3.2, 3.3). These small changes could reflect a slow adaptation process of the fish to their new environment. Treated fish also, at the end of the sampling period, adjusted their new baseline range at respectively shorter phase durations and higher PPSFs. This is reflected in the difference between dd17-20 and dd81-84, dd93-96 (PPSF), dd89-92 (P3), and dd89-100 (P4).

Anal fin

The total anal-fin ray count in *B. niger* was 27-30 (branched and unbranched rays). The maximum, dorsally directed indentation in males coincided with ray numbers 10-12 (see Fig. 1.3 in chapter 1) with basal fin-ray expansion observed in control males on ray numbers 6-17 and treated females on ray numbers 3-20.

Posterior ventral body wall indentation

During the period of hormone treatment, extensive reddening of the dorsal margin of the anal fin was observed (starting week 2), signaling massive vascularization in support of possible tissue (muscle) growth. Fig. 3.4 illustrates the time course of the effects of 17MT on the body wall indentation in female *B. niger* and the comparisons with both control groups (CM, CF). On d-3 (pre-treatment), there was a significant difference among groups ($F(2, 32) = 18.07$, $p < .001$) with multiple-comparison tests indicating a significant difference between CM ($\tan \beta = 0.215 \pm 0.042$, $n = 8$) and both CF ($\tan \beta = 0.112 \pm 0.035$, $n = 9$) and designated TF ($\tan \beta = 0.109 \pm 0.047$, $n = 18$). Thus, although their EOD waveforms were indistinguishable, males and females could be clearly distinguished based on their external, sexually dimorphic body wall indentation.

17MT treatment significantly affected the shape of the posterior ventral body wall, which developed a male-like indentation [$F(6, 56) = 10.36$, $p < .001$]. By the end of the hormone treatment period (d59: $\tan \beta = 0.244 \pm 0.045$, $n = 16$), the indentation in treated females was no longer different from that seen in males [d-3: $\tan \beta = 0.215 \pm 0.042$, $n = 8$; $t(22) = 1.51$, $p > .05$]. Although, following hormone removal, there was a steady decline in the indentation measure over the ensuing twelve months, the indentation remained significantly different (greater) from its pre-treatment condition. None of the post-treatment values d184 trough d457) differed significantly from one another. This finding contrasted sharply with the relatively fast return (10-14 days) of the masculinized EOD waveform to the pre-treatment, i.e. female condition.

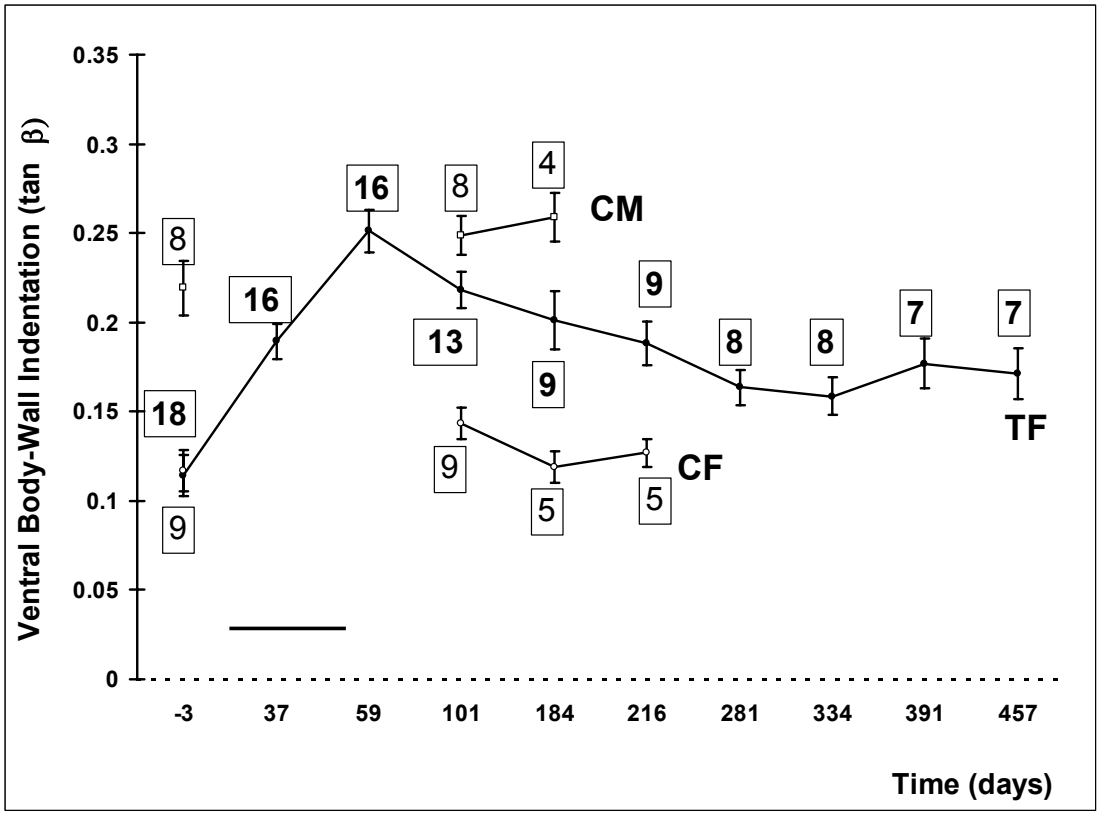

Figure. 3.4. The figure shows the time course illustrating the effects of 17MT (horizontal bar) on the ventral body wall indentation in female *B. niger* ± SEM. The numbers in the individual boxes indicates the number of fish. Comparisons with control groups are indicated. Treatment resulted in male-like indentation; following hormone removal, indentation remained significantly different from pre-treatment condition. TF, treated females; CF, control females; CM, control males.

A comparison of the indentation angles in TF on two post-treatment days (d101, 184) with those in CM and CF found them to be significantly different from both controls assuming a middle position between the two (d101: CM, tan β = 0.244 ± 0.032, n = 8, TF, tan β = 0.209 ± 0.037, n = 9; CF, tan β = 0.143 ± 0.023, n = 6; $F(2,24) = 16.09$, $p < .001$; d184: CM, tan β = 0.255 ± 0.025, n = 4, TF, tan β = 0.196 ± 0.049, n = 9; CF, tan β = 0.116 ± 0.021, n = 5; $F(2, 15) = 14.56$, $p <$

.001). On d216, TF (tan β = 0.182 ± 0.038, *n* = 9) still exhibited a significantly stronger indentation than CF [tan β = 0.122 ± 0.019, *n* = 5; t(12) = 3.30, p = .006].

Control fish were radiographed during the course of the study (CF: d101, 184, 216; CM: d101, 184). The indentation angle was compared within each group on two days (d-3 vs. d101; paired t-test). There was no significant change over time in either (Note that while Fig. 3.4 illustrates means ± SEM of all fish alive at the time of recording, all ANOVAs with repeated measures were performed only on data of those seven fish, TF, that were still available at the end of the study.)

Basal anal-fin ray expansion

In females, the bases of the first two rays are elongated and differ from all others. From the third ray on, the fin-ray bases are rounded dorsally and point rostrad. This configuration was observed in all other rays, but the bases diminish in size caudally (Fig. 3.5 A). The effect of 17MT, after six weeks of treatment (up to d58), on basal fin-ray bone structure in female *B. niger* was substantial (Fig. 3.5 B). Starting with the third ray, the bases began to expand, attained a maximum between rays 10 and 12, and gradually decreasing from ray 13 on. The first three fin-ray bases were not affected, resembling those of control fish. From ray 20 on, there were no more expansions noticeable (Fig. 3.5 B). The expansions observed between rays 3 and 20 differed in shape and size and were defined earlier (Fig. 3.1). In males, as in females, the bases of the first two rays are elongated. Starting with the third ray, the bases are expanded rostrad; they

appear bigger beginning with ray 9, and on ray 12, are maximally expanded, followed by a decrease in expansion (Fig. 3.5 C). Fin ray expansion and body wall indentation in TF were more pronounced than in CM, which was attributed to the fact that these males were not in breeding condition.

The temporal progression of expansion is illustrated in Fig. 3.6. Prior to treatment, none of the defined expansion types (A-D) was present. All types were apparent though by d59 (when treatment had stopped) and maintained their expression at different rates throughout the sampling period (except type A). Midway through treatment (d37), small transparent appendages (not categorized as a bone expansion type) on rays 5-19 began to appear.

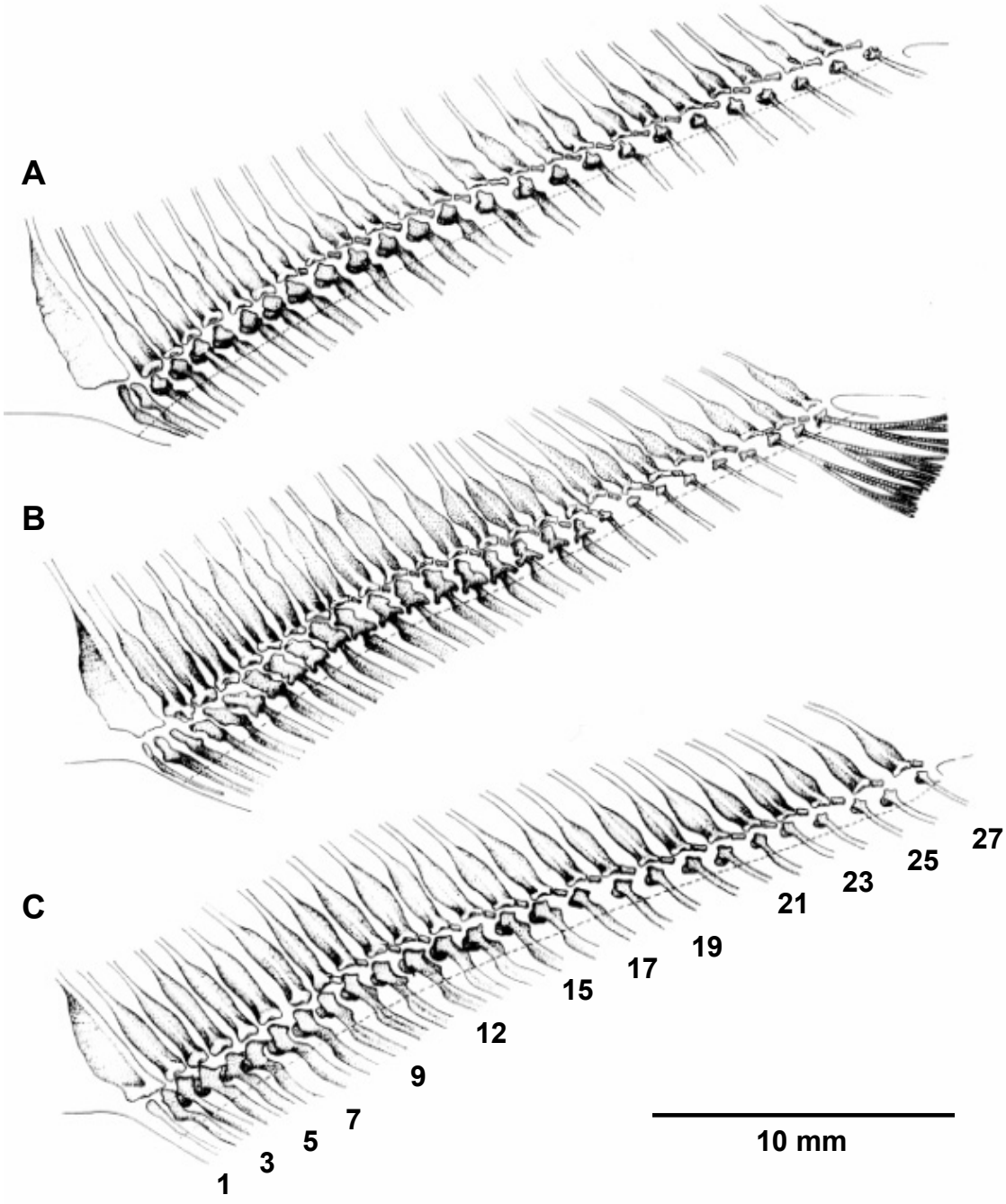

Figure 3.5. *Camera lucida* drawings (taken from cleared and counter-stained specimens that were sacrificed following hormone treatment on day 59) showing the condition of the bases of anal-fin rays in untreated female *B. niger* (A), 17MT-treated females (B), and untreated males (C). Note appearance of massive fin ray expansion and expanded distal pterygiophores in treated females. Expansion and body wall indentation were more pronounced than in control males (C) possibly due to the fact that untreated males were not in full breeding condition. Fin-ray numbers are indicated in C.

On average by d59, based on a survey of all specimens, these appendages, depending on the position of the fin ray, had developed into types B (range: rays 3-12), C (range: rays 5-19), and D (range: rays 17-20), respectively. Type A (range: rays 1-7) reflected a general widening and lengthening of the pre-treatment condition (e.g. rays 5 and 6 in Fig. 3.5 B). Thus, the maintenance of bone growth in the absence of externally administered 17MT continued when EOD characteristics had long reverted to pre-treatment levels (Figs. 3.2, 3.3).

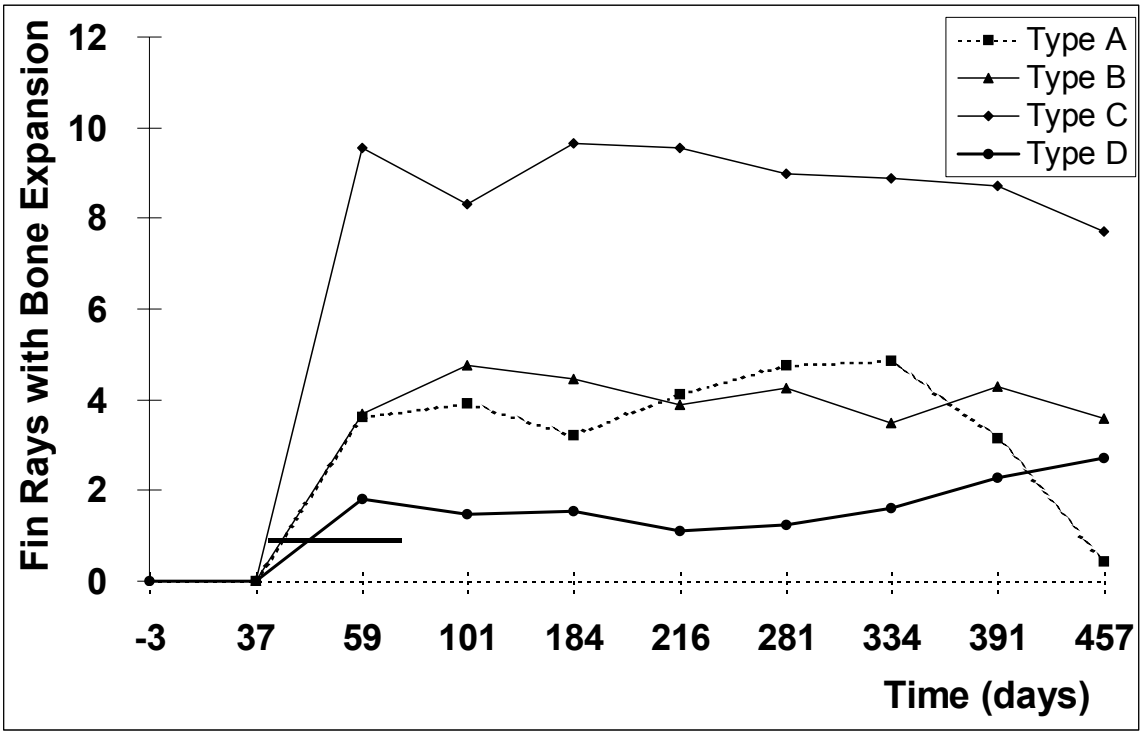

Figure 3.6. Time course illustrating the progression of fin ray expansion. Prior to hormone treatment, none of the defined expansion types (A-D) was present (see Fig. 3.5). All expansions became apparent by day 59 (when treatment had stopped) and maintained their expression at different rates throughout the sampling period in the absence of externally administered 17MT. Horizontal bar indicates treatment.

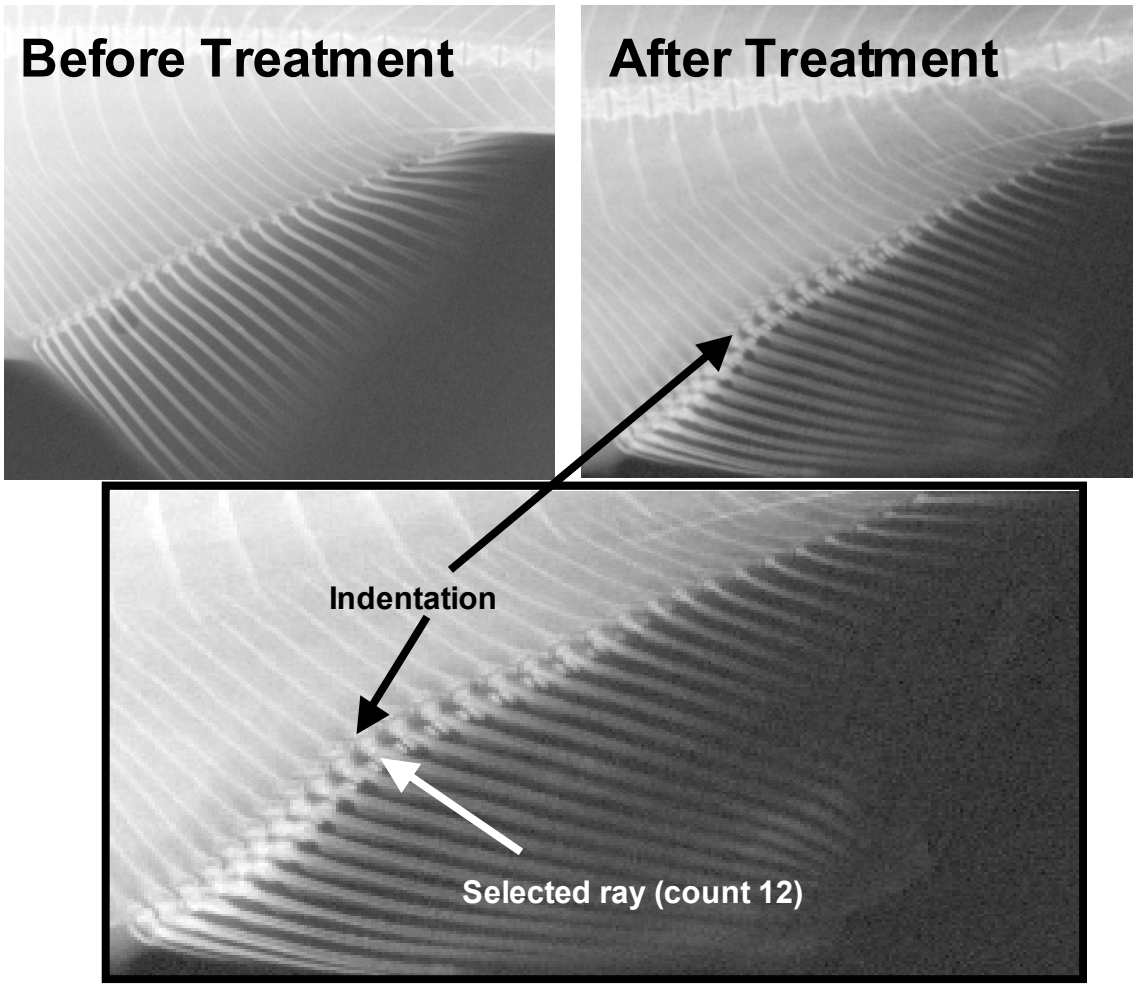

Figure 3.7. Radiographs of the anal fin of a female *B. niger* before and after treatment with 17MT. Ray 12 (white arrow) was selected based on maximal indentation (black arrows).

Results

Experiment 2

Three-dimensional analysis of anal-fin bases

The radiographs in Fig. 3.7 illustrate the morphology of the female anal fin before and after 17MT treatment. Clearly, androgen caused a major structural modification of individual anal-fin ray bases. The expansion ('spurs', Voustianiouk, 2003) is accompanied by the male-typical indentation of the fin's dorsal margin. The image, by default, allows only a surface view of one lateral face of the fin-ray base. To examine the extent to which anabolic action had affected the entire ray, individual rays were excised from nine cleared and stained specimens (selected at different stages of treatment) (Fig. 3.8).

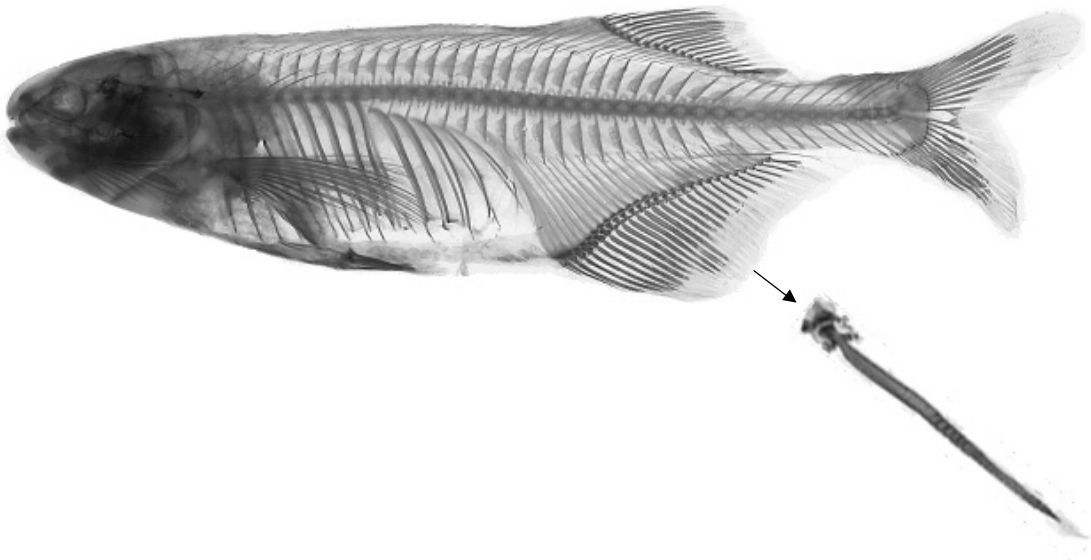

Figure 3.8. Cleared and counterstained specimen of *B. niger*. Ray 12 was excised and shown enlarged.

Following examination of the lateral face (identical with that observed in the radiograph), the ray was rotated in steps of 90º to examine its posterior, anterior and the opposite lateral faces (Fig. 3.9).

Fin rays in *B. niger* are composed of a shaft and a base. The anterior and posterior views (faces) show the position of the distal pterygiophore, and also that the rays are split at their base. These features were not detectable on the lateral views (which are represented in all radiographs).

In control fish, expansions (spurs) do not exist on the base of the anal fin-rays in all four views. However, small protuberances can be identified on the anterior and posterior faces, where the spurs typically exist in males. After five weeks of treatment with 17MT, the two protuberances present on each side of the base of the fin ray, in the lateral views, expanded significantly into spurs as identified by the arrows in Figure 3.8. After six weeks of treatment these spurs almost doubled in size (first and third lateral view). It is interesting to note that despite hormonal withdrawal, they appeared to grow still during week 7. The time course suggested that growth starts with a lengthening of the spur to maximum extension followed by thickening at the base.

Adding to the androgen-sensitive structures of the mormyrid anal fin support system was the finding that individual ray shafts increased in thickness by almost 70% over a 6-week treatment period as compared to these structures in control individuals. Thus, hormone treatment affected still another support structure of the anal-fin complex.

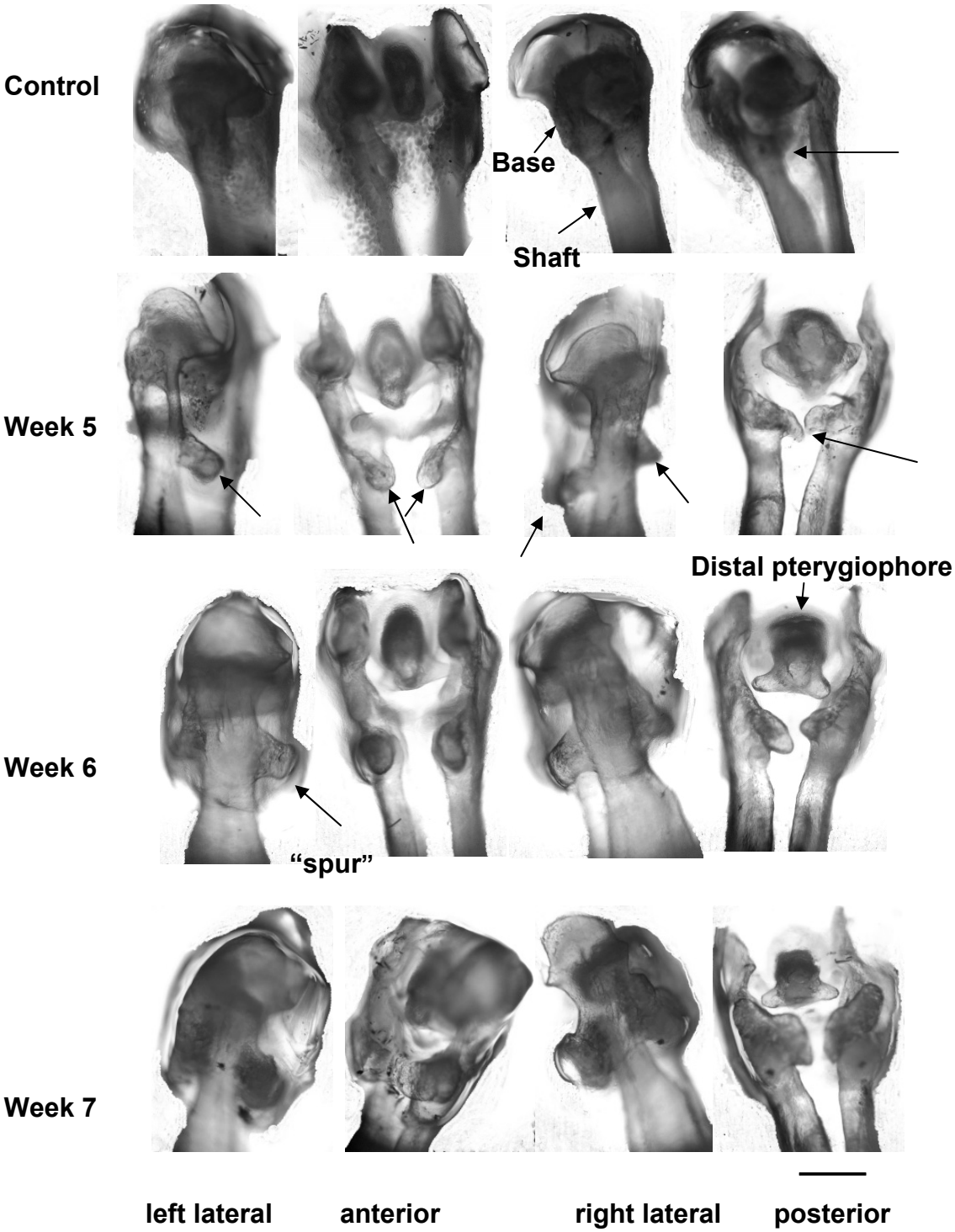

Figure 3.9. Microphotographs, cleared and counterstained, (10x) of four representative anal-fin ray bases (ray 12) from control and three 17MT-treated fish (weeks 5, 6, 7). Shown are four views; → spurs. Horizontal bar: 1 mm.

Discussion

This study has described the effects of exogenous 17MT on subadult female mormyrids, *B. niger*. Hormone treatment caused masculinization of their EOD waveform (elongation), anal-fin rays (expansion), and changes in the posterior ventral body wall (indentation). All these traits typically take part in the male's courtship behavior. The results infer an immediate and reversible effect of androgens on the EOD-generating structures (electrocytes) and a gradual, delayed long-term effect on bone and body wall indentation. Within two weeks, following hormone withdrawal, the male-typical EOD seen in 17MT-treated females returned to the female waveform, whereas both bone (anal-fin ray expansion) and body wall indentation had not reverted to pre-treatment levels by the end of the 15-month study.

Plasticity of androgen-induced behavioral transformations (EOD)

As reported for several other mormyrid species (Bass and Hopkins, 1983, 1985; Landsman, 1995; Landsman and Moller, 1988; Voustianiouk, 2003), female *B. niger* responded to androgen administration with male-like changes in their EOD waveform. Within only a few days, the presence of 17MT began to cause a decrease in PPSF associated with a lengthening of phases P1, P2 and P3, and a shortening of P4. By the end of the second week, androgen treatment had achieved its maximum effects notwithstanding further hormone treatment. Comparably, Bass (1986 b) illustrated qualitative 17MT-, and estradiol-induced changes in the fish's EOD waveform. Both treatments resulted in a noticeable

increase in the amplitude of P1 and a decrease in the amplitude of P3. (Note: age and sex of the fish in Bass' study were not specified.)

Electrocytes in mormyrids are morphologically dimorphic with those in males being significantly larger, thicker, and possessing more membrane infoldings than those in females (Bass, 1986 a; Freedman et al., 1989). In this study, androgen treatment correlated with an elongation of EOD phases P2, and P3 (see Fig. 3.3) which are generated, respectively, by a depolarization of the posterior and anterior faces of electrocytes (Bass and Volman, 1987 ; Bass, Denizot, and Marchaterre, 1986a). However, the morphological change in electrocyte volume (along the anterior face) accounts for only one phase to increase in duration (Phase 3; Freedman et al., 1989). This is due to the increase in time for current to travel through the electrocyte to depolarize its anterior face. There is no equivalent increase in electrocyte tissue at its posterior face. Thus, as Phase 2 also increases in duration, ionic properties of membrane channels may also be affected by gonadal steroids resulting in higher membrane capacitance of the posterior face.

In androgen-treated females the thickening of the electrocytes mimicked the male condition which leads to an increase in EOD duration (Bass *et al*., 1986a; Freedman *et al*., 1989). In support of the capacitance hypothesis were findings on steroid effects on the duration of individual action potentials generated by each electrocyte membrane that showed a 2-3 fold increase in duration as compared to potentials recorded from untreated mormyrid fish (Bass and Volman, 1987). Similar effects were observed in pulse-type South American

gymnotiform fish (Hagedorn and Carr, 1985). In gymnotiform fish, both androgens and estrogens seem to alter specific ionic conductances in excitable cells that are involved in reproductive behavior (Ferrari and Zakon, 1993; Ferrari, McAnelly and Zakon, 1995; McAnelly and Zakon, 1996; Dunlap et al., 1997).

The typical long-term or seasonal changes of male EOD durations are genomic and adjusted by androgens (Bass and Hopkins, 1983, 1985; Landsman, 1995). Franchina and Stoddard (1998) proposed that short-term modulations in EOD duration and amplitude of a South American gymnotiform *Brachyhypopomus pinnicaudatus*, with a waveform similar to *B. niger* are due to an alteration of ion channels on the electrocytes. These changes are thought to be under the control of melatonin secretion from the pineal gland, which in turn is affected by ambient light levels (Stoddard, Markham, and Salazar, 2003).

In 17MT-treated female *B. niger*, the most pronounced effect on phase duration was on P3 which, on average, almost doubled from 80 to 140 μs (Fig. 3.3 C). The time course of hormone effects on P2 and P3, respectively, points to the differential response of the electrocytes to androgenic hormones. While the slow increase in P2 duration (due to the increase in membrane capacitance) was best described by a power function, the rapid increase in P3 duration (due to the thickening of anterior face of the electrocyte) occurred in a linear fashion during the first 2 weeks (see Fig. 3.3 B, C; inserts).

The current study illustrated, in a systematic fashion for *B. niger*, the reversibility of androgen-induced EOD transformations following hormone withdrawal. Both EOD characteristics (PPSF and phase duration) reverted to

their pre-treatment and/or control group (i.e. untreated female) conditions. This phenomenon, however, is not universal among mormyrid species. The reversibility of androgen-induced EOD changes in females seems to reflect the natural, permanently or seasonally expressed EOD-related sexual dimorphism in males of several given species. Landsman (1993 b) found that adult *G. petersii* imported during their breeding season showed an EOD-related sexual dimorphism that totally vanished after 14 days in captivity.

Landsman (1993 b) noticed that the shortening of phases P2 and P3 (resembling the female condition) was accompanied by a significant drop in both plasma testosterone and 11-ketotestosterone levels. In a separate study, Voustianiouk and Perrotti (cit. in Landsman, 1995) established that, following hormone withdrawal, the androgen-induced male EOD reverted in treated female *G. petersii* within three weeks. Both adult *Brienomyrus brachyistius* long-biphasic (listed as *B*. sp. 2 by Alves-Gomes and Hopkins, 1997) and *Brienomyrus brachyistius* tri-phasic (listed as *B*. sp. 3 by Alves-Gomes and Hopkins, 1997), show an EOD-related sexual dimorphism (Hopkins, 1981). However, while the androgen-induced male-like EOD waveform in female 'long bi-phasics' reverted within 24 days (Bass and Hopkins, 1983), it remained nearly unchanged in female 'tri-phasics' after 25 days post-treatment (Bass and Hopkins, 1985).

Further, male 'long biphasics' kept in captivity for 3-6 months lost their male-typical EOD character reverting to the female waveform; this was not the case in captive male 'tri-phasics'. In freshly imported male *B. brachyistius* (from

Nigeria), the EOD reverted to the shorter female-like condition (Landsman and Moller, 1993).

Sexual dimorphisms of the anal- and/or dorsal-fin complex

Mormyridae are not unique in expressing external, structural sexual dimorphisms. Such dimorphisms affecting osteological characters of both anal and dorsal fins, and contributing to reproductive behavior are well known in both 'non-teleost' and other teleost fishes and, depending on the species, can be seasonal or permanent as follows:

Adult male *Polypterus* (all species) can be unambiguously distinguished from adult females on the basis of a much larger anal fin, more strongly-developed anal-fin musculature and often a higher number of anal-fin rays (Bartsch and Britz, 1996). Interestingly, the indentation of the body wall in *Polypterus* males resembles that observed in male mormyrids, as described by Budgett (1907) for *Polypterus* and Boulenger (1909) for mormyrids. A sexual dimorphism affecting spinulation, length, and diameter of the dorsal-fin spine characterizes male *Ageneiosus* (bottlenose catfish) during the breeding season (Ferraris, 1988). Males of the livebearing Poeciliidae, among them guppies (*Poecilia*), mosquitofish (*Gambusia*), and swordtails (*Xiphophorus*) transform select anal-fin rays into a functional gonopodium (e.g. Rodriguez-Sierra and Rosa-Molinar, 1990; Rosa-Molinar et al., 1996).

Androgen-induced structural transformation

The anabolic effects of 17MT on anal-fin ray expansion in female *B. niger* supported our hypothesis that, taking part in the fish's reproductive display (anal-fin reflex), an increase in bone surface could provide for added muscle tissue to facilitate this behavior. As stated earlier, within days following hormone treatment, the entire length of the dorsal margin of the anal fin showed intensive vascularization, possibly pointing to muscle tissue formation. Inasmuch as the electrocyte geometry, responsible for the shape and duration of the fish's courtship signals, is androgen-sensitive, so are the underlying morphological structures involved in exhibiting the anal-fin reflex.

Adding to the androgen-sensitive structures of the mormyrid anal-fin support system was the finding that individual ray shafts increased in thickness as compared to these structures in control individuals. Thus, hormone treatment affected still another support structure of the anal-fin complex. These findings corroborated those by Greisman and Moller (2005) who discovered this sexual dimorphism in natural populations of adult male *Gnathonemus petersii*. Additional studies on this species have separated the contributions of testosterone, dihydrotestosterone, and 11-ketotesosterone to the expression of structure and behavior (Voustianiouk, 2003) which has further strengthened the hypothesis that androgens selectively affect those characters that participate in the male fish's reproductive behavior:

Plasticity of androgen-induced structural transformations

In contrast to the EOD waveform that reverted within two weeks to its pretreatment level following hormone withdrawal, androgen-induced bone and possibly muscle growth (judged by the extent of body wall indentation) seemed to be permanent. Although there was a gradual decline in bone volume (based on the presence of expansion types and the degree of indentation), neither measure reverted to pre-treatment levels. The observed decline, possibly due to a loss of calcium and muscle tissue, might have been due to captivity and dietary factors, which is not an uncommon phenomenon. This explanation, however, is somewhat weakened by the fact that male controls (CM) showed no decline in both measures throughout d184 by which time treated females (TF) did already show a slight reversal (see indentation measure, Fig. 3.4).

An alternative explanation might be that under natural conditions in males, mostly testosterone, but not estradiol, directs the expression and maintenance of expanded fin rays and muscle. A number of arguments make this hypothesis plausible: control males in this study, just attaining sexual maturity and still indistinguishable in their EODs from females, might have produced sufficient levels of their own androgens to maintain expansion and indentation. In a separate study, non-gonadectomized, 17MT-treated male *B. niger* (caught out of breeding season) discharged a male-typical, elongated EOD and further increased both already existing fin ray expansion and body wall indentation. But in contrast to treated females in the present study, following hormone withdrawal, all three characters developed further, i.e. “more” male-like, indicating a possible

role of the male's own androgens (Moller et al., 2004). Treatment with non-aromatizable androgen (dihydrotestosterone) and its subsequent withdrawal in treated juvenile *G. petersii* resulted in immediate changes in EOD and gradual changes in body wall indentation (Landsman and Moller, 1988; Voustianiouk, 2003). Estradiol treatment of female *B. brachyistius* and *G. petersii* did not result in any detectable anal-fin ray expansion (Gannon, unpubl. data). Fin ray data on both species were obtained from specimens deposited at the AMNH (*B. brachyistius:* 98316, 98317, 98321; *G. petersii*: 226657). (*G.petersii* specimens were used in Landsman et al., 1990; *B. brachyistius* specimens were treated by R. E. Landsman.)

Several mormyrid species regress in their expression of sexually dimorphic displays (EODs) and morphology (Okedi, 1969) comparable to the seasonal differentiation of courtship displays in many avian and mammalian species (Wingfield and Farner, 1980). Adult *G. petersii*, for example, caught outside their breeding season (November/December) are not only indistinguishable in their EOD waveform (Landsman, 1993 a, 1995), but can hardly be sexed on the basis of the shape of their external ventral body wall. As pointed out earlier, the fish's response to hormone withdrawal and/or captivity seems to reflect the apparent species-typical seasonality or permanence of the EOD-related sexual dimorphism.

Chapter 4

Effects of 17α-methyltestosterone on electric organ tissue in *Brienomyrus niger:* a light microscopic survey

Summary

The results of this experiment have confirmed, for *B. niger*, that the electric organ is sensitive to androgens as had been demonstrated for several other species. Administration of 17MT increased the thickness of the individual electrocytes, a surface proliferation, especially along the anterior face of the electrocytes. Total electrocyte thickness was about two times greater in 17MT-treated males and females than in controls. For females, a two factor ANOVA (time x treatment) showed a significant effect for treatment, but none for time of treatment. There were no differences between control males and females, and between treated males and treated females. There was, however, a significant difference between treated females and treated males, and also between control and treated males.

Introduction

The sexually dimorphic EOD reported for many mormyrid species is either permanent or, depending on environmental conditions, seasonal with low water conductivity levels (rainy season) triggering gonadal recrudescence and higher levels gonadal regression (dry season). The increase in plasma androgens during the fish's breeding season affects anabolic action on both the muscle-derived electric organ resulting in an elongation of the EOD as well as on the anal-fin complex resulting in the expression of an externally visible indentation of the ventral posterior body wall (see Chapters 1, Fig. 1.1; Chapter 3, Fig. 3.3) (Landsman, 1995; Landsman et al., 1990; Moller et al., 2004; Greisman and Moller, 2005). Out of breeding season, the EOD waveforms of male and female *Gnathonemus petersii* (Landsman, 1993 b; Moller, personal communication) and *Brienomyrus niger* (Serrier and Moller, 1989) are indistinguishable.

Previous studies have found that in androgen-treated females, the male condition is mimicked in that electrocytes thicken, leading to an increase in EOD duration (Bass *et al*., 1986 a; Freedman *et al*., 1989). Bass, Segil, and Kelley (1986 b) and that the steroid sensitivity of the electric organ correlates with high levels of androgen-binding activity in electrocytes. Such androgen-binding activity in the electric organ is several folds greater than in other myogenic structures such as trunk musculature (Bass et al., 1986 b).

The previous chapter demonstrated that (1) the electric organ discharge (four phases, see Chapter 2, Fig. 2.2) in *B. niger* is characterized by a sexual dimorphism which is expressed in the duration of the discharge (2) how exogenous 17MT affects the fish's secondary sexually dimorphic traits, i.e. behavior (electric organ discharge, anal-fin reflex) and structure (basal anal-fin ray expansion and anal-fin musculature, Herfeld and Moller, 1998; Voustianiouk, 2003). It has long been known that androgens can have masculinizing effects on the electric organ discharge in female mormyrid fish such that the duration of individual phases increases (Bass and Hopkins, 1983, 1985; Bass and Volman, 1987; Landsman, 1993 b, 1995; Landsman et al., 1990). For this to happen the underlying morphological structure, the electric organ itself must undergo corresponding modifications (Bass, 1986 a; Freedman et al., 1989). In many species a sexually dimorphic EOD is a result due to steroid action on the electroplates (electrocytes) (Bass and Hopkins, 1983, 1984, 1985; Landsman, 1995).

Gonadal steroids act directly on the EOD-generating structural elements, the electrocytes of the electric organ, resulting in sex-specific EOD waveforms (African Mormyridae: Hopkins, 1981, 1986; Bass and Hopkins, 1983, 1984, 1985; Bass 1986 a, b; Freedman et al., 1989; South American Gymnotiformes: Dunlap and Zakon, 1998), and possibly also on the central pacemaker system to set the sex-specific EOD rate in gymnotiforms (Dunlap and Zakon, 1998) or discharge patterns in mormyrids (Bass et al., 1986 b).

The electric organ in *B.niger* is located in the tail (Figure 4.1a). The electrocytes that make up the electric organ are flattened disk-shaped multi-nucleated cells with a diameter of up to several millimeters and a length of only 10-50 μm (Fig. 4.1b). One hundred or more disks lie packed together in four parallel columns in the elongated and cylindrical caudal peduncle of an electric fish. In males, the basic structural elements of the electric organ, the electrocytes (Bennett, 1971 a) are significantly larger and thicker, and possess more membrane infoldings than those in females (Bass, 1986 c).

Each electrocyte has a complex silt-root-like process emerging from one flat face or the other (usually posterior). These rootlets are smallest in diameter at the point where they fuse with the electrocyte face. They repeatedly fuse with others, increasing in diameter until they reach a large trunk where they receive synaptic input from electromotor nerve axons. This entire root system is called a “stalk” (Bennett and Grundfest, 1961, Bennett, 1971 a) or a “pedicule” (Szabo, 1960, 1961) (Figure 4.1c). The electrocytes have penetrating stalks, and are innervated on the posterior side and the innervated stalk immediately makes its first penetration through to the anterior side. The stalk travels across the anterior surface for a distance before making a second penetration back through to the posterior side near the margin of the cell (Figure 4.1c).

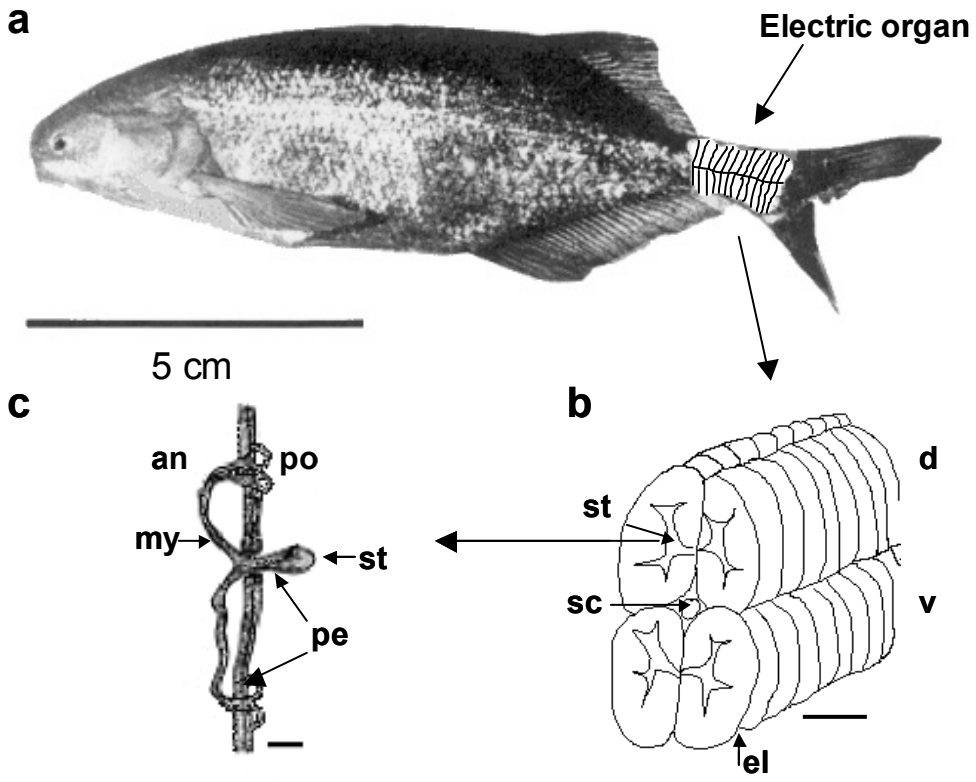

Figure 4.1. (a) The electric organ in *B. niger* is located in the caudal peduncle (arrow). (b) The organ consists of four columns of serially arranged electrocytes; horizontal bar 1 mm (c) single electrocyte illustrating penetrating stalk system; horizontal bar - 30 μm; (an – anterior, po – posterior, d – dorsal, v – ventral; my – myofilaments, st – stalk, pe – location where stalk penetrates cell; el – electrocyte, sc – spinal cord). Adapted from (a) Serrier and Moller, 1989, (b) Bass and Volman, 1987, (c) redrawn and modified for a double penetrating stalk system typical of *B. niger*, after Bass and Volman, 1987.

Androgen target

The mormyrid electric organ is an androgen-target tissue and behaves, in the presence of androgens, very much like other vertebrate target tissues such as the mammalian levator ani muscles (Venable, 1966 a, b; Galavazzi and Szirmai, 1971; Dube, Lesage, and Tremblay, 1976), the avian syrinx (Lieberburg and Nottebom, 1979), and the anuran larynx (Sassoon, Segil, and Kelley, 1983; Segil, Silverman, Kelley, and Rainbow, 1983) that all undergo an increase in the diameter of muscle fibers. Thus, the increase in overall thickness of myogenic electrocytes of 17MT-treated mormyrids was consistent with the above data showing steroid-induced hypertrophy of vertebrate muscle fibers. In support of androgen action on membrane properties are data by Erulkar and Wetzel (1985) who demonstrated that androgens can influence the conductance of acetylcholine-activated membrane channels in cultured myotubes of the anuran larynx musculature.

This chapter will investigate the effects of 17MT treatment on the electric organ of *B. niger.* The goal of this study was twofold, (a) to confirm, for *B. niger*, previous findings in related species, namely that androgens can masculinize electric organ tissue in subadult and adult females, and (b) to provide the time course of these changes that will be discussed as they relate to the corresponding 17MT-induced modifications affecting the electric organ discharge, gonads, and anal fin.

This study is a light microscopic survey that describes the organizational features underlying the anatomy of the mormyrid electric organ in *B. niger* and

was prompted by the discovery that gonadal steroids can, in juveniles and females, induce EODs typical of mature males in species with a known EOD-related sex difference (Bass and Hopkins, 1983, 1985, Bass, 1986 c).

The morphology of *B. niger* electrocytes will be quantitatively assessed and compared among untreated males and females (controls), and 17MT-treated males and females. It is hypothesized that the average thickness of electrocytes is greater in 17MT-treated females and comparable to treated males, whereas this measures for both control males and females (kept under captive conditions, i.e. out of breeding conditions) is significantly smaller and similar for both sexes.

Materials and Methods

Subjects

Twenty-one female *Brienomyrus niger* (SL 90.2 ± 7.2 mm; range: 76.4 - 108.6 mm; weight 10.6 ± 2.8 g; range: 5.8 - 17.3 g; body height 24.2 ± 2.3 mm; range: 19.1 - 29.9 mm), imported from Nigeria at the end of the supposed breeding season (November 1998) were obtained through a local tropical vendor (Quality Tropicals, Inc. Wallington, N.J.). Fish were housed separately throughout the study in 20 l aquaria (water volume: 13 l) and maintained on an L:D = 12:12 photoperiod with lights on at 8:30 h. Water conditions were kept within limits, i.e. temperature between 20-25 ºC and conductivity between 130-180 µS/cm. Fish were fed tubifex worms and brine shrimp twice a week. There were four groups of fish: control females (CF, n = 7), control males (CM, n = 3), 17MT treated females (TF, n = 14) and 17MT treated males (six weeks only) (TM, n = 4).

Hormone treatment and tissue procedures

A total of 14 females and 4 males were treated for 6 weeks with dissolved 17MT, which was added to the water (2 mg/l) every seven days for a total of six weeks. Water conductivity was controlled via weekly water changes (see Chapter 3). Each week, one control (CF) and one treated fish (TF) were sacrificed. In addition, two additional treated females were sacrificed at the end of week 3, week 6, and week 7. All males were sacrificed after six weeks of treatment. Thus the following number of subjects were available for histological analysis: week one, 1 CF, 1 TF; week two, 1 CF, 1 TF; week three, 1 CF, 4 TF; week four, 1 CF, 1 TF; week five, 1 CF, 1 TF; week six, 1 CF, 3 TF; week seven, 1 CF, 3 TF. At the end of week six, hormone treatment was ended with six rapid water changes over a period of three days. Thus, fish maintained during week seven were exposed to dramatically reduced hormone concentrations.

Fish were euthanized with an overdose of MS-222 (tricaine methanesulfonate). Fish were preserved in 4% buffered formaldehyde. The caudal peduncle (containing the electric organ) was sectioned off and stored in 0.1 M phosphate buffer (pH 7.2) followed by immersion in 30% sucrose in 0.1 M phosphate buffer solution prior to embedding in Tissue Tek, Oct 4583 compound. Samples were frozen at –80 °F and sectioned in the sagittal plane (5 µm) using a cryostat (Leica CM 3050 S). Every third section was transferred onto polylysin-covered slides. Sections were stained with a combination of Alcian blue and hematoxylin eosin. All sections were examined with a Nikon Eclipse E400 microscope.

Results

Electrocyte morphology

The electric organ of *B. niger* is located in the caudal peduncle (Fig. 4.1a). It consists of two columns of serially arranged, disk shaped cells (electrocytes or electroplaques; Bennett, 1971b) located on either side of the midline (Fig. 4.1b). The electric organ takes up about 2/3 of the caudal peduncle. At a standard length (SL) of about 12 cm the electric organ measures about 1.5 cm. The relative length of the electric organ is about 12% (10-14%, n = 21) of the SL.

Hormone-sensitive electrocytes

The morphology of electrocytes in control and 17MT-treated females, and control and 17MT-treated males was compared (Fig. 4.2, 4.3). Androgen treatment had a dramatic effect upon electrocyte morphology. Total electrocyte thickness was about two times greater in 17MT-treated males and females than in controls (Figure 4.3). For females, a two factor ANOVA (time X treatment) showed a significant effect for treatment, $F(1, 8) = 288.69$, $p < .0001$, but none for time of treatment, $F(6, 8) = 0.89$, $p = .54$. Since data for males were only taken at the beginning and at the end of the study, data were compared using t-tests. There were no differences between control males (CM 0.15 ± 0.02 mm) and control females (CF: 0.15 ± 0.01 mm), as well as between treated males (TM: 0.28 ± 0.02 mm) and treated females (TF: 0.32 ± 0.03 mm). But control males differed significantly from treated males $t(5) = 7.91$, $p = .005$.

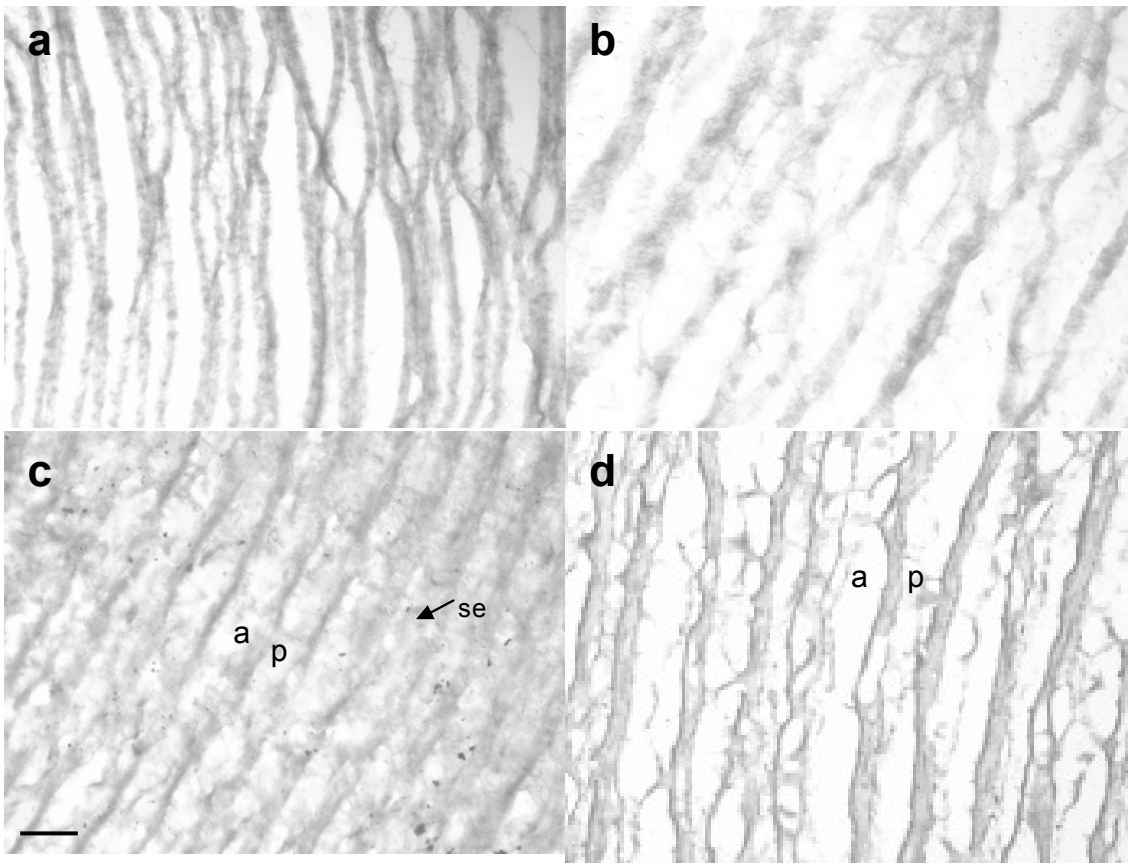

Figure 4.2. Histology of the electric organ in *B. niger*. Representative samples (cross-sections) of electrocytes obtained from (a) control female, (b) control male, (c) 17MT-treated female, and (d) treated male. a – anterior, p – posterior, se – septa, horizontal bar is 300 μm.

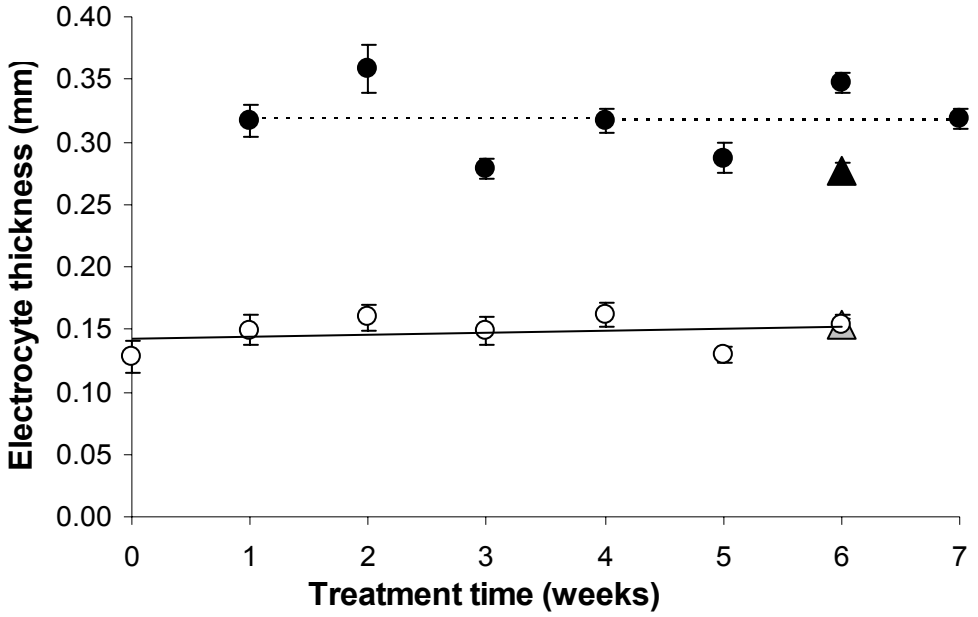

Figure 4.3. This figure shows the mean electrocyte thickness (± SEM) in Δ control males, ▲17MT treated males, ○ control females (solid line), and ● 17MT treated females (dotted line). Hormone treatment doubled the electrocyte thickness in males and females compared to electrocytes in untreated fish.

Discussion

Changes in electrocyte morphology

The results of this experiment have confirmed, for *B. niger*, that the electric organ is sensitive to androgens as has been demonstrated in several other species (Bass and Hopkins, 1983, 1985; Bass and Volman, 1987; Freedman et al., 1989). Exogenous 17MT administration increased the thickness of the individual electrocytes. Following Bennett (1971 a) who suggested that a thicker electrocyte is associated with a longer duration of the action potential or spike generated by the depolarizing electrocyte face, one would expect an

increase in EOD duration. This was in fact the case as was reported in the previous chapter (Chapter 3; Herfeld and Moller, 1998).

Testosterone induces an increase in electrocyte surface area (Bass et al., 1986 a), and overall changes in membrane capacitance (Hobbie, 1978). This can account for the observed changes in EOD duration as the increase was associated with the observed increasing surface proliferation, especially along the anterior face of the electrocytes already noted by Bass et al. (1986) in a different species.

It was suggested that the action potential generating properties of the electrocyte's membrane are controlled by both passive (area-related) and active (conductance-related) factors. Bass et al., (1986 a) found that testosterone induces not only an increase in electrocyte surface area, but also membrane capacitance. Androgen treatment correlates with an elongation of those two EOD phases that are generated by the depolarization of both anterior and posterior faces of the electrocyte (Bass and Volman, 1987). However, the morphological change in electrocyte volume (along the anterior face) accounts only for an increase in duration of phase 3. This is due to the increase in time for current to travel through the electrocyte to depolarize its anterior face. There is no equivalent increase in electrocyte tissue at its posterior face. Thus, as phase 2 also increases in duration (Chapter 3, Fig. 3.3), ionic properties of membrane channels of the posterior face may also be affected by gonadal steroids (see also Chapter 3).

The maximum change in electrocyte morphology was essentially in place following one week of hormone treatment, which was in good agreement with findings for a congeneric species (Freedman et al., 1989). These authors followed the initial daily time course and found that the electric organ had already substantially thickened by day 3, i.e. at a time when the generated electric organ discharge had also increased in duration. This too was born out by the current data on 17MT-induced changes in EOD duration (Chapter 3; Fig. 3.3).

Electrocyte as androgen target

The mormyrid electric organ is an androgen target tissue. Androgen binding activity in the electric organ of *Brienomyrus sp*. is several fold greater than in other myogenic structures such as trunk musculature (Bass, 1986 c). Steroid hormones also induce increases in the diameter of electrocytes in gymnotiform electric fishes (Hagedorn and Carr, 1985). Androgen receptors in the electrocytes of *Eigenmannia virescens*, a South American knifefish, have been identified through immunolabeling, which strongly supports the assumption that androgens act directly on peripheral tissue, i.e. the electric organ. Structurally and hormonally modified electrocytes determine changes in the EOD waveform, and under natural conditions, in the presence of endogenous androgens, a sexually dimorphic EOD (Dunlap and Zakon, 1998).

The effect of steroid hormones on sexually dimorphic structures, muscle and bone, has been studied in numerous vertebrate systems (sonic muscle in fish: Modesto and Canario, 2003; Connaughton and Taylor, 1995; larynx in

Xenopus leavis: Kelley, 1988; Potter, Bose and Yamaguchi, 2005). For example, steroid hormones control the expression of the sexually dimorphic avian syrinx (Lieberburg and Nottebom, 1979) and anuran larynx (Segil et al., 1983; Erulkar and Wetzel, 1985). Thus, the gonadal steroid induced anabolic effects on electric organ tissue, which with only a few exceptions, is myogenic, is consistent with data showing steroid induced hypertrophy of vertebrate muscle fibers.

Chapter 5

Effects of 17α-methyltestosterone on ovarian growth in adult *Brienomyrus niger*

Summary

The results of this study show that androgen-treated subadult and adult female mormyrid fish *B. niger* developed a dramatic ovarian hypertrophy that appeared to mirror the natural maturational process during seasonal gonadal recrudescence. There was no evidence of intersex gonads; thus 17MT and/or its metabolites were solely affecting ovarian growth. It seems plausible that an excessive presence of 17MT was aromatized (17α-methylestradiol) to an excessive presence of a methyl-estrogen analogue that affected ovarian growth.

Introduction

Among teleost fishes there exists a wide range of reproductive plasticity (Taborsky, 1994; Foran and Bass, 1999) as exemplified by the following scenarios: Juveniles develop into (1) either females or two permanent, distinct male phenotypes exhibiting alternative mating tactics (midshipman: Bass, 1996), (2) either females or two types of males exhibiting reversible changes in status and breeding conditions as territorial or non-territorial males (cichlids: Frayley and Fernald, 1982), (3) initial-phase males and females that both can further develop into dominant terminal-phase males (sex / role change; bluehead wrasse: Shapiro and Rasotto, 1993), (4) a monogamous male (protandrous male) changing sex to become the larger female in the group (anemone fish: Shapiro, 1992), and (5) into males and females that can repeatedly switch between the sexes (marine goby: Cole, 1990).

In this thesis, four sexual phenotypes among teleost fishes are distinguished: males, females, sequential hermaphrodites, and synchronous (simultaneous) hermaphrodites. Sexual differentiation follows the patterns of gonadal development. In gonochoristic species, gonads differentiate into either testes or ovaries, and remain such throughout the life of the adult fish. In sequential hermaphrodites, fish develop from male to female, or vice versa, from female to male through the resorption of the gonadal tissue of one sex, and the growth and maturation of the gonadal tissue of the opposite sex. Synchronous or simultaneous hermaphrodites have developed both ovaries and testes and alternately take the role of male and female during spawning (Yamamoto, 1969).

The teleost gonads develop as paired structures in the dorsal lining of the peritoneal cavity (Jobling, 1995). However, in some species like *B. niger*, fish possess only one gonad as a result of fusion or failure to develop fully (see also Dodd, 1977; Grier, 1981; Wallace and Selman, 1981; Billard, Fostier, Weil, and Breton, 1982; de Vlaming, Grossman, and Chapman, 1982; Nagahama, 1983; Billard, 1995; Tyler and Sumpter, 1996; and Brooks, Ryler, and Sumpter, 1997).

Because of this extreme plasticity it is not surprising that exogenous steroid hormones or their mimics (endocrine disruptors) can alter the endocrine milieu, and thus affect the natural (normal) expression of sexual differentiation and/or maturation (Folmar, Denslow, Rao, Chow, Crain, Enblom, Marcino, and Guilette, 1996; Guillette, Woodward, Crain, Pickford, Rooney, and Percival, 1999). The genetic sex-determining mechanisms in many teleost species can be altered by administration of exogenous gonadal steroids to early lifetime stages (larvae or fry) during which organizational effects can still be implemented. As a rule, androgens cause genetic females to develop into functional males, and estrogens cause genetic males to develop into functional females provided these hormones are administered early enough during a critical period, i.e. when gonads have not begun synthesizing sex hormones. In contrast, exogenous synthetic androgen administration during later stages affects mainly secondary sex characters. This has long been known as demonstrated in the classical studies on adult female poeciliid fishes (*Xiphophorus helleri*: Regnier, 1938; *Gambusia affinis affinis*: Turner, 1941; *Poecilia maculatus*, *P. variatus*: Grobstein, 1940, 1948) that resulted in masculinization of the fish's anal fin. Pickford and Atz

(1957) reviewed a host of studies dealing with the masculinizing effects of androgenic hormones on morphological, secondary sexual characters.

In commercial aquaculture, the synthetic steroid 17α-methyltestosterone (17MT) is used to induce sex reversal in teleost fish (Hunter and Donaldson, 1983). The magnitude of sex reversal, however, depends on various factors including dose, diet, temperature, timing and duration of treatment, and mode of administration (Higgs, Fagerlund, Eales and McBride, 1982; Mirza and Shelton, 1988). Successful masculinization of genetic and gynogenetic females into phenotypic males was reported by Olito and Brock (1991), Schmelzing and Gall (1991), Chevassus and Krieg (1992), Nakamura (1994), Feist, Yeoh, Fitzpatrick and Schreck (1995) and Malison and Garcia-Abiado (1996). Yamazaki (1983) and Donaldson and Hunter (1982) established all-female populations of salmon through estradiol treatment. Masculinization in common carp (*Cyprinu carpio*) (Komen, Lodder, Huskens, Richter and Huisman, 1989 and Nagy, Bercsenyi and Csany, 1981) and *Tilapia nilotica* (Nakamura and Iwahashi, 1982) was induced using doses of 17MT at 50-100 µg/g diet; higher doses, however induced sterile gonads (Donaldson and Hunter, 1982 and Komen et al., 1989 and Goetz, Donaldson, Hunter and Dye, 1979) and paradoxical feminization (Nakamura et al., 1998).

Reproduction in teleosts occurs in cycles controlled by gamete development and maturation. Ovarian development and the final release of mature oocytes is correlated with endogenous and exogenous stimuli (for review

see Lam, 1983; Peter, 1983; Idler and Ng, 1983; Nagahama, Yoshikuni, Yamahita, Tokumoto, and Katsu, 1995).

Exogenous factors such as temperature, water quality and water level (de Vlaming, 1974), photoperiod, availability of food, and social factors (e.g. visual, chemical or tactile contact with conspecifics) are important to stimulate and activate the endocrine pathways of the hypothalamo-pituitary-gonadal (HPG). axis. In teleost fish, the most important endogenous hormones are the hypothalamic gonadotropin releasing hormone (GnRH), the pituitary gonadotropin GTH I and GTH II (= FSH and LH in mammals, Prat, Sumpter, and Tyler, 1996), sex steroids and prostaglandins.

Gonadal steroids play a central role in sexual differentiation, i.e. growth and development of reproductive organs, and control of the reproductive cycle in vertebrates, including fish. Estrogens (estradiol-17ß, E2) control ovarian growth whereas androgens (11-ketotestosterone, 11KT, and testosterone, T) stimulate testicular growth and development (Yamamoto, 1969; Nagahama, 1994; Nakamura et al., 1998). The notion, however, that androgens are exclusively male and estrogens exclusively female hormones has been equally dispelled for teleost fishes as it has been for other vertebrate classes. In fact, T and androstenedione are major hormones secreted by the teleost ovary, and are precursors of 17β-estradiol and estrone, respectively.

Tropical freshwater fishes often show reproductive cycles that are related to dry and rainy seasons: reproduction takes place during the rainy reason and regressed gonads are found during the dry season (Lowe-McConnell, 1975;

Schwassmann, 1980). In both the Neotropical Gymnotiformes (knifefishes) and the African Mormyriformes breeding cycles are correlated with the alternation of rainy and dry seasons (Nawar, 1960 a, b, Okedi, 1969; Provenzano, 1984; Kirschbaum, 1979, 1987; Zakon, Mills, and Ferrari, 1991; Schugardt, 1997). Most mormyrid fishes of tropical Africa breed during the rainy season and cease reproductive activity during the dry season. Kirschbaum (1979) has shown that gonadal recrudescence can be induced under laboratory conditions by decreasing water conductivity, increasing the water level, and imitating rainfall, whereas gonad regression was brought about by continuous increase of conductivity alone.

Proliferation of oogonia and formation of new oocytes occur throughout the fish's reproductive life. Sexual differentiation and gonadal development has been extensively investigated in several teleosts with emphasis on farm-raised species that are of commercial importance (Nakamura et al., 1998; Coward, Bromage, Hibbitt, and Parrington, 2002). The detailed histological description of gonadal differentiation and development for zebrafish (*Danio rerio*) (Maack and Segner, 2003), Japanese rice fish, the medaka (Hamaguchi, 1992), and fathead minnow (*Pimephales promelas)* (van Aerle, Runnalls, and Tyler, 2004) have, in part, served as a reference for the current assessment of ovarian growth in *B. niger*. There are only a few studies that cover gonadal histology and reproduction in weakly electric fish (*Gymnotus carapo*: Barbieri and Barbieri, 1984, 1985; *Sternopygus marcrurus*: Zakon et al., 1991; *Mormyrus rume proboscirostris, Campylomormyrus cassaicus*: Schugardt, 1997).

The endocrine control of oocytic maturation in teleosts resembles that of higher vertebrates: hypothalamic signals cause the pituitary to release gonadotropins (GTH), which in turn stimulate production of C21 steroids by ovarian follicular cells. Gonadotropin-I (GTH-I) induces T production in the thecal layer of the ovarian follicles, with T subsequently being converted to E2 in the granulosa layer (Nagahama, 1994). The conversion of androgens to estrogens is controlled by ovarian cytochrome P450 aromatase, an enzyme that is most active during vitellogenesis (Chiang, Yan, Guiguen, Postlethwait, and Chung, 2001; Kishida and Callard, 2001; Trant, Gavasso, Ackers, Chun, and Place, 2001). Plasma ovarian androstenedione and testosterone is converted to estrogens in the brain and pituitary, both of which contain large amounts of aromatase.

Vitellogenesis is a critical period during oocytic growth during which vitellogenin (VTG), a liver-derived glyco-lipophospho-protein, is taken up from the bloodstream through membrane receptor-mediated endocytosis (Brooks et al. 1997; Prat et al., 1996; Perazzolo, Coward, Davail, Normand, Tyler, Pakdel, Schneider, and Lemenn, 1999; Kwon, Prat, Randall, and Tyler, 2001). VTG is the major nutritional source for the developing embryo (Specker and Sullivan, 1994).

The premise underlying the studies reported in chapters 3 and 4 was that secondary sexually dimorphic traits, i.e. behavior (the fish's electric organ discharge and its courtship-associated anal-fin reflex) and structure (anal-fin ray expansion, supposed anal-fin musculature) would be affected by exogenous androgens. The results of these studies supported this hypothesis. In support of

the extreme plasticity in teleost sexual differentiation, contrasting to that in mammals, behavioral and structural traits were masculinized (Herfeld and Moller, 1998; Voustianiouk, 2003). Thus, the expectation that in mormyrids, with no apparent natural sex change strategy, sexual differentiation should occur during some defined period during early development was not met.

Androgen treatment induced sex reversal in subadult and adult females from a short electric organ discharge to the longer, male-typical courtship signal, and induced both the structural basis for and the ability to display the male-typical anal-fin reflex. Hormone withdrawal caused a complete reversal of behavior (loss of the anal-fin reflex in androgen-treated females), partial muscle hypotrophy, but apparently no bone resorption (Chapter 3; Herfeld and Moller, 1998; Voustianiouk, 2003).

This chapter will investigate the effects of exogenous 17MT treatment on the fish's gonadal development and test the hypothesis that androgen treatment can masculinize, i.e. transform ovarian into testicular tissue in subadult and adult female *B. niger*. Alternatively, it can be hypothesized that such treatment at this developmental stage is ineffective, *or* due to excessive aromatization of 17MT to estradiol results in some form of overexpression of ovarian tissue (see notes on the use of 17MT in Chapter 3, Material and Methods).

Material and Methods

Subjects

Twenty-three female *Brienomyrus niger* imported from Nigeria at the end of the supposed breeding season (November 1995) were obtained through a local tropical vendor (Quality Tropicals, Inc. Wallington, N.J.). Fish measures: SL 90.2± 7.2 mm; range: 76.4-108.6 mm; AFL 25.3 ± 2.3 mm, range 20.3-29.7 mm; body weight 10.6 ± 2.8 g, range: 5.8-17.3 g; body height 24.2 ± 2.3 mm, range: 19.1-29.9 mm; body depth 8.6 ± 1.2 mm (5.9-12 mm). Fish were housed separately throughout the study in 20 l aquaria (water volume: 13 l) and maintained on an L:D = 12:12 photoperiod with lights on at 8:30 h. Water conditions were kept within limits, i.e. temperature between 20-25 ºC and conductivity between 130-180 µS/cm. Fish were fed tubifex worms and brine shrimp twice a week.

Procedures

A total of 23 fish were divided into two groups: controls (CF, n = 7) and 17MT treated females (TF, n = 16). Water-dissolved 17MT (2 mg/l) was administered for 6 weeks, added to the tanks every seven days for a total of six weeks. Water conductivity was controlled via weekly water changes (see Chapter 3). Each week, one control and one treated fish were sacrificed. At the end of week three, three additional treated females were sacrificed; at the end of week five, one additional treated female; at the end of week six, three additional treated females; and at the end of week seven, two additional treated females

were sacrificed. Following hormone treatment, at the end of week six, rapid water changes over three days removed most of 17MT. During week seven, fish were therefore exposed to reduced hormone concentrations.

To assess gonadal changes induced by 17MT treatment in B. niger, after each treatment the designated number of fish were euthanized following IACUC guidelines with an overdose (500 mg l^{-1}) of MS-222 (tricaine methanesulfonate, Sigma). The ovary was immediately removed, weighed (Sartorius, BP61S scale to the nearest 0.1 mg), and morphometrics taken with a pair of calipers (length, width, and depth). These procedures were approved by the Hunter College Institutional Animal Care and Use Committees (IACUC) (Protocol # PM/SH/AV/7/00).

Gonadosomatic index (GSI)

The GSI is a good measure of gonad maturation and spawning readiness, based on the assumption that proportionally larger gonads indicate greater development (West 1990). This measure is a good indicator for assessing the seasonal gonadal development and spawning regimens (De Vlaming et al., 1982). The index is defined by GSI = gonadal weight / (fresh body weight – gonadal weight) x 100, and was computed to quantify changes in gonadal size affected by MT treatment.

Following fresh weight, length, and width measurements, gonads were fixated in 10% formaldehyde and transferred to 70% ethanol. Gonads were stored in 0.1 M phosphate buffer (pH 7.2) followed by immersion in a 30%

sucrose solution in 0.1 M phosphate buffer prior to embedding with Tissue Tec, Oct 4583 compound. Samples were frozen at – 80 °F. Gonads were sectioned in the sagittal plane (5 - 7 µm), using a Leica (CM 3050 S) cryostat. Every third section was transferred onto polylysin-covered slides. Sections were stained following Domagk (in Romeis 1989: no. 1535) with hematoxylin-eosin.

To evaluate if the chosen sections were representative of all cell types typical of a given treatment, i.e. were they present throughout the entire gonad, all ovaries were sectioned entirely from rostral to caudal. Every third section was selected and inspected. In all cases, there was no evidence of a polarized distribution of oocytes.

Gonad histology – assessment of maturational stages

Sections were analyzed and photographed under a Nikon, Eclipse E400 light microscope, using computer-assisted software (SPOT RT Software v. 3.5); digitized images were stored on disk for future assessment. Following Childress, Taylor, Cailliet, and Price (1980) and Davis (1982), the diameters of the 10 largest oocytes of each ovary were measured to determine the average diameter. This measure was corrected for shrinkage due to formaldehyde fixation (West, 1990). Although no individual oocyte was ever exactly circular, the average diameter provides useful descriptive information of ovarian maturation.

Classification of ovarian development

Most teleosts exhibit the same pattern of oocyte growth (Tyler and Sumpter, 1996). Based on a set of physiological, biochemical, morphological, and histochemical criteria, ovarian development has been assigned to discrete developmental stages (Nagahama, 1983, 1994; Selman, Wallace, and Barr, 1986; Bromage and Cumaranatunga, 1988; Tyler and Sumpter, 1996; Coward and Bromage, 1988).

1. Chromatin nucleolar stage (oogonial proliferation)
2. Early perinucleolar stage (oogenesis)
3. Late perinucleolar stage (primary growth and folliculogenesis)
4. Cortical alveolar stage
5. Early vitellogenesis
6. Maturing/late vitellogenesis
7. Mature stage (germinal vesicle migration and breakdown)

These normal ovarian developmental stages were observed to emerge during the 6-week, 17MT-induced growth patterns in *B. niger.* The description of these stages was adapted from several authors: Bromage and Cumaranatunga, 1988; Tyler and Sumpter, 1996; Carrasson and Bau, 2003; Quintana et al., 2004 (gymnotiform fishes); Leino, Jensen, and Ankley, 2005 (fathead minnow); Coward et al., 2002 (teleost fishes reviewed).

> *1. Chromatin nucleolar stage (primary growth phase)*
> "At the beginning of the primary growth phase oocytes are very similar to oogonia in that they contain scant cytoplasm and large, centrally located nuclei, but each oocyte becomes invested with a layer of follicle cells (Khoo, 1979). The small oocyte has a large nucleus surrounded by a thin layer cytoplasm. The cytoplasm contains no cortical alveoli or yolk bodies. In the course of oocytic

growth, the nuclei increase in size and multiple nucleoli appear. The cytoplasm also increases in volume and becomes strongly chromophilic due to the presence of aggregates of ribonucleoprotein particles".

2. Early perinucleolar stage (oogenesis)
"The oocyte is slightly enlarged and the nucleolus has split into several small nucleoli, which spread towards the periphery of the nucleus. The cytoplasm shows uniform dark staining" (Cararasson and Bau, 2003).

3. Late perinucleolar stage (primary growth and folliculogenesis)
"The oocyte is on average almost double the size of the former stage. Also, there are some vacuoles in the cytoplasm, whose presence usually characterizes the next (cortical alveoli) stage. Towards the end of this stage the chorion begins to form" (Cararasson and Bau, 2003).

4. Cortical alveoli stage (secondary growth phase)
"The secondary growth phase involves the accumulation of yolk within the oocytes. Numerous vesicles/ alveoli appear in the margin of the oocyte. These vesicles generally increase in size and number until they fill the cytoplasm. The temporal distribution and size of the cortical alveoli varies quite widely amongst teleosts" (Gilkey, Jaffe, Ridgway, and Reynolds, 1978).

5. Early vitellogenic stage
"The most critical period of oocyte growth is vitellogenesis (a process common to all oviparous animals). In non-mammalian vertebrates, the principal events responsible for the enormous growth of oocytes involve the sequestration and packaging of a liver-derived plasma precursor, vitellogenin, into yolk protein. Initially only few and small yolk bodies are visible and are located centrally dispersed around the cortical alveoli. As the oocyte grows, the yolk bodies become larger and more numerous and displace the cortical alveoli, pushing them to the periphery of the oocyte."

6. Late vitellogenic stage
"The yolk accumulates in fluid-filled spheres, which migrate to the center of the oocyte and fuse to form a continuous mass. As this occurs, the yolk vesicles are displaced toward the periphery (Prat et al., 1996; Perazzolo et al., 1999). Vitellogenesis ceases once oocytes are fully developed; oocytes then undergo maturation and ovulation follows triggered by appropriate hormonal stimulation" (Masui and Clarke, 1979).

> *7. Mature stage (germinal vesicle breakdown).*
> "The start of the mature stage is indicated by the peripheral migration of the nucleus and the dissolution of its membrane. The final stage of oocyte maturation is difficult to follow because of the shrinkage and distortion of these cells during normal processing."

Statistics

Scoring of stages of maturity

Based on this classification scheme, each fish was assigned a numerical score to denote its ovarian stage of maturity, judged by the presence of the most dominant type of oocytes: ***1*** – stage 1 (presence of oogonia), ***2*** – combining stages 2 and 3 (perinucleolar growth), ***3*** – stage 4 (cortical alveolus oocytes), ***4*** – combining stages 5 and 6 (early to late-vitellogenic oocytes). Mature stage 7 oocytes were never observed. From hereon, a reference to stage of development is always made to these newly defined stages 1-4.

Morphometrics, meristics

1) Within a pre-selected sampling area (6.5 μm^2) on 10 selected sections per fish, the number of follicles and oocytes at various stages of development were counted (Miles-Richardson, Kramer, Fitzgerald, Render, Yamini, Barbee, and Giesyet, 1999 a; Miles-Richardson, Pierens, Nichols, Kramer, Snyder, Snyder, Render, Fitzgerald, and Giesy, 1999 b; Smith, 1978). 2) Oocytes were classified according to size and main morphological characteristics (see: scoring stages of maturity). 3) The total surface area of all follicles and oocytes per

developmental stage was measured and expressed as a percentage of the sampling area.

Statistical procedures

Morphometrics were analyzed with one-way ANOVA (main factor: treatment), followed by the Tukey "Honest Significant Difference" (HSD) test (α = 0.05).

Results

General evaluation of gonads

When examining the fish's gonads under a dissecting microscope, two main facts became immediately clear. First, the hypothesis that 17MT treatment can masculinize, i.e. transform ovarian into testicular tissue in subadult and adult female *B. niger* had to be rejected. There was never any evidence for intersex gonads (ovotestes), i.e. some form of hermaphroditic gonad. Second, the alternative hypothesis that androgen treatment at this developmental stage causes an overexpression of ovarian tissue, due perhaps to excessive aromatization of 17MT to an estrogen was supported. Subadult females developed huge ovaries typical of mature, ripe fish, almost ready to spawn (Figure 6.1). The time course of the actual increase in androgen-induced volume and the quantitative growth pattern of the ovaries are illustrated in Figure 6.2 and Figure 6.3. At the end of week 3, vitellogenic oocytes become clearly visible at the surface. Gonadal weight differed significantly from control values by the end

of week 3 when it had increased 5-fold $F(7, 67) = 2.7399$, $p = 0.015$; difference between control (0.05 g) and week-3 gonads (0.36 g): $p = .019$; Tukey's HSD, $p = .019$. Neither total body weight nor size of the fish was affected by hormone treatment (Figure 6.4, 6.5).

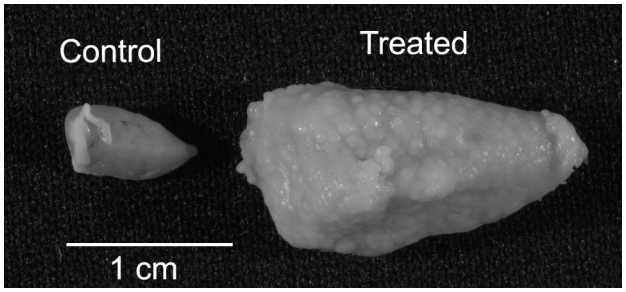

Figure 5.1. Effects of 17MT on ovarian growth in *B. niger*. By week three, gonadal weight had increased 5-fold.

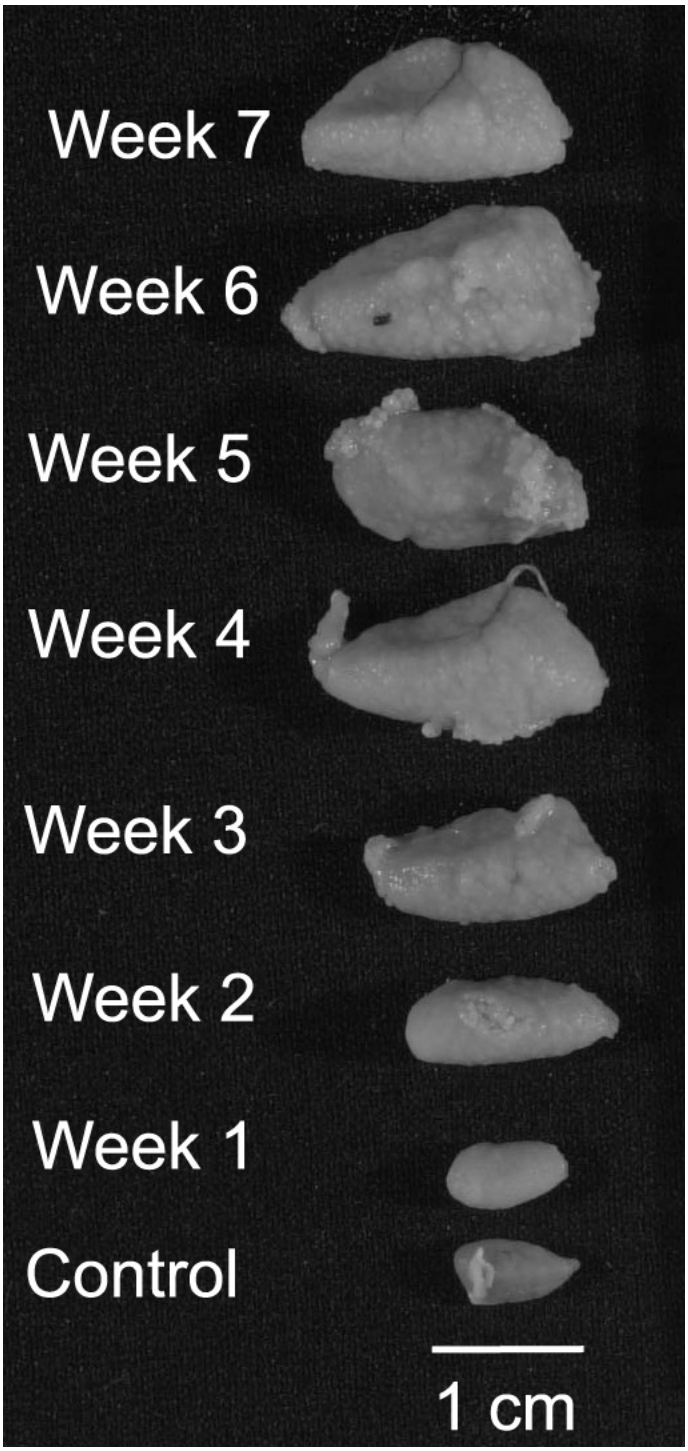

Figure 5.2. Time course of 17MT-induced ovarian growth over a period of six weeks in *B. niger*. During week 7, hormone had been removed. Note the clear appearance of vitellogenic oocytes from week 3 on.

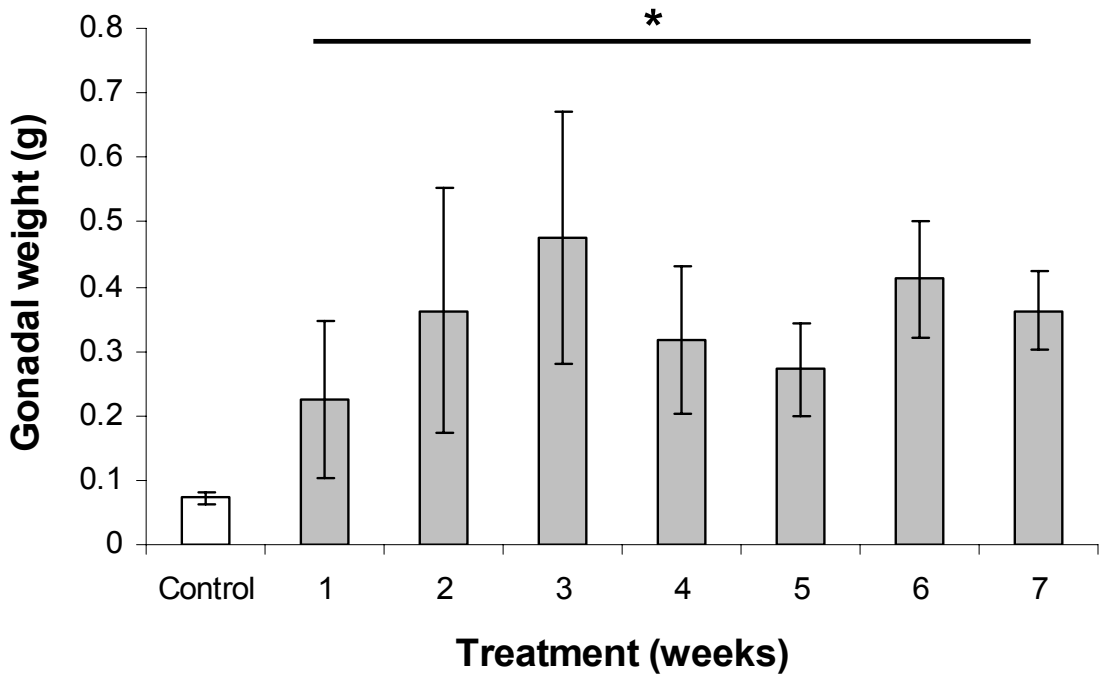

Figure 5.3. The mean gonadal weight (± SEM) is shown as a function of androgen treatment over time. By the end of week three, gonadal weight had increased 5-fold. Asterisk (*) denotes significance between the control fish and all treated fish ($p < .05$).

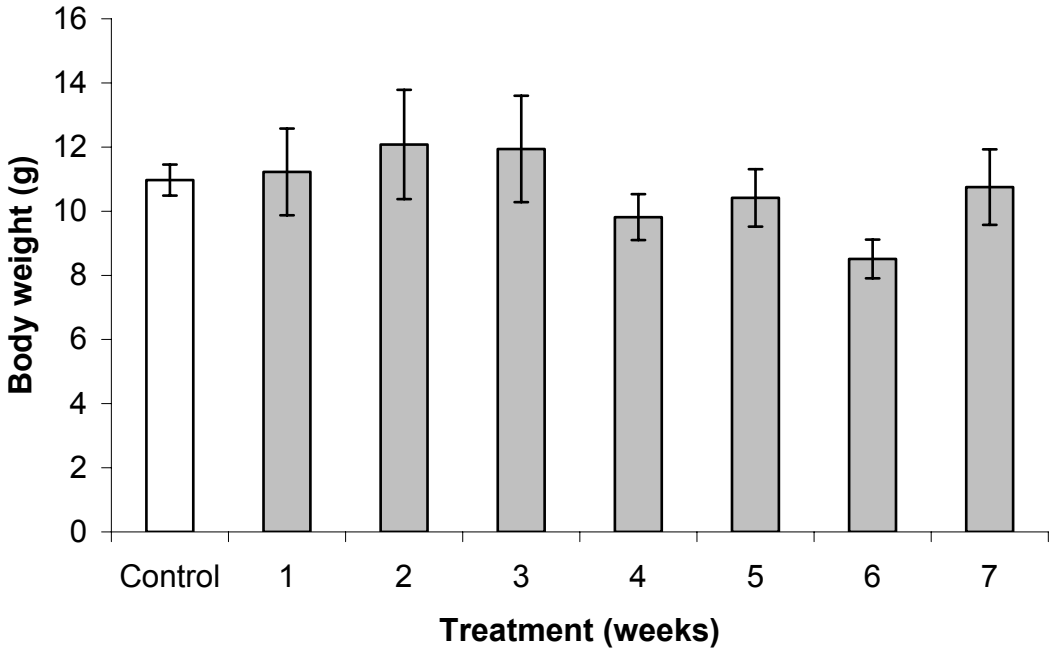

Figure 5.4. The mean body weight (± SEM) is shown as a function of androgen treatment. Treatment had no significant effect on body weight ($p<.05$).

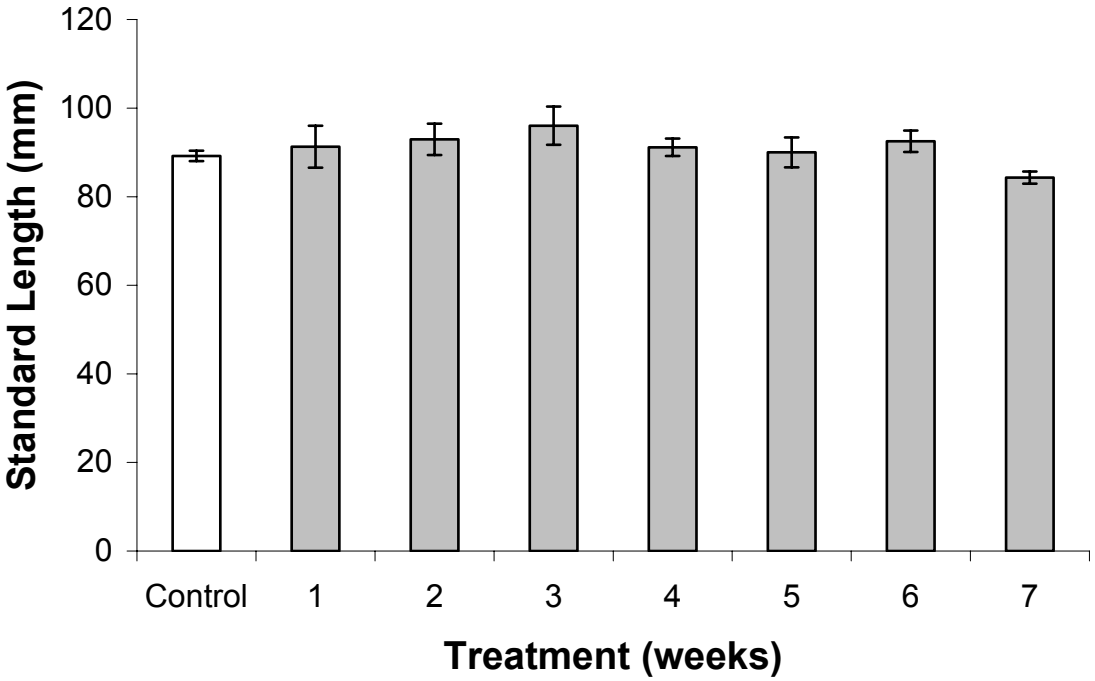

Figure 5.5. The mean standard length (± SEM) is shown as a function of androgen treatment. Treatment had no significant effect on growth in length over the 6 week treatment period ($p<.05$).

Gonadosomatic Index (GSI)

GSI values in untreated fish averaged 0.6 and increased significantly over the time of treatment $F(7, 67) = 2.68$, $p = .016$, seven-fold by week 3 and five-fold by the end of the sampling period ($p = .06$ and .034 respectively, Tukey's HSD) (Figure 5.6). These values were higher than previously reported values for other mormyrids in particular (Schugardt, 1997) and other teleosts in general (Jensen, Korte, Kahl, Pasha, and Ankley, 2001; Ankley et al. 2001).

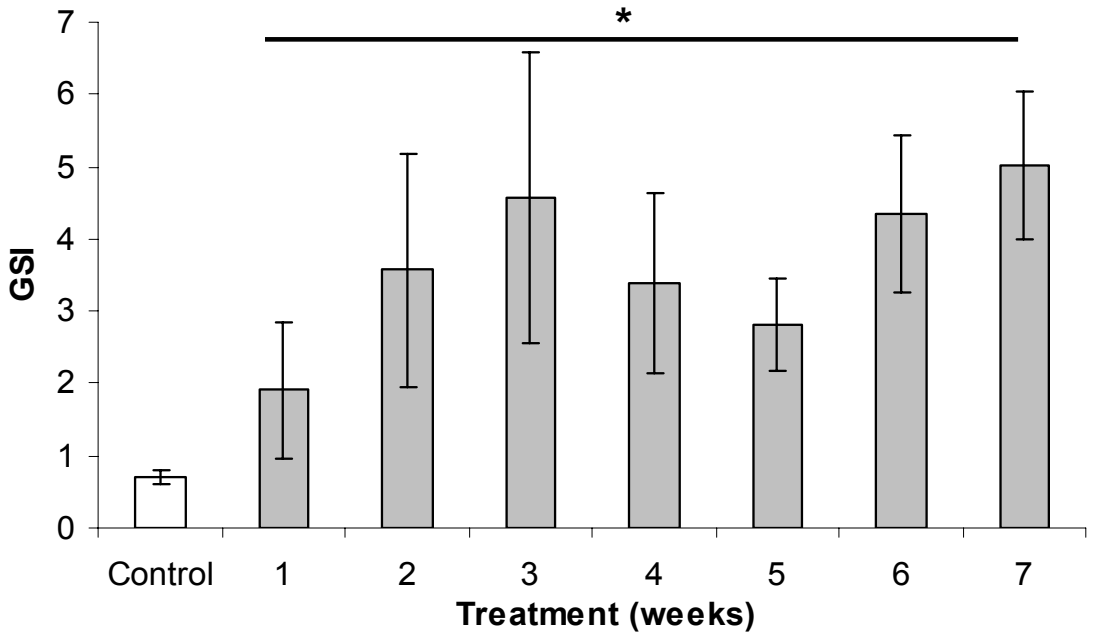

Figure 5.6. This figures shows the mean gonadosomatic index (± SEM) of the control and seven weeks of treatment with 17MT. Maximum level was attained following 3 weeks of treatment, and all weeks of treatment differed significantly from the control group ($p < .05$). Asterisk (*) denotes significance between the control fish and all treated fish.

Histology

Figure 5.7 a, b illustrate the presence of oocytes in various stages of development induced by 17MT treatment over a period of 6 weeks. To follow the time course of ovarian growth under the influence of 17MT, the data are presented in two ways: (1) Relative occurrence of stages 1-4, i.e. number of oocytes at a given developmental stage as percentage of all oocytes counted in a fixed sampling area (Figure 5.8), and (2) the cumulative surface area of oocytes at a given developmental stage as percentage of the sampling area (Figure 5.9). In untreated fish (controls), only stages 1 and 2 were present. In treated fish by the end of week 3, all four stages were present. The density of early oogonia across treatment remained fairly constant (about 60%) and provided a ‘reserve’ of sort for future eggs. The data show that only a small number of oocytes matured as 17MT treatment progressed. Oocytic growth remained stable even after the hormone had been withdrawn. None of the control fish showed any gonadal maturation with the exception of fish at the end of the study with stage 2 oocytes (weeks 6 and 7). In terms of volume taken up by developing oocytes, their relative distribution is obviously biased towards stages 3 and 4 ovaries (Figure 5.9).

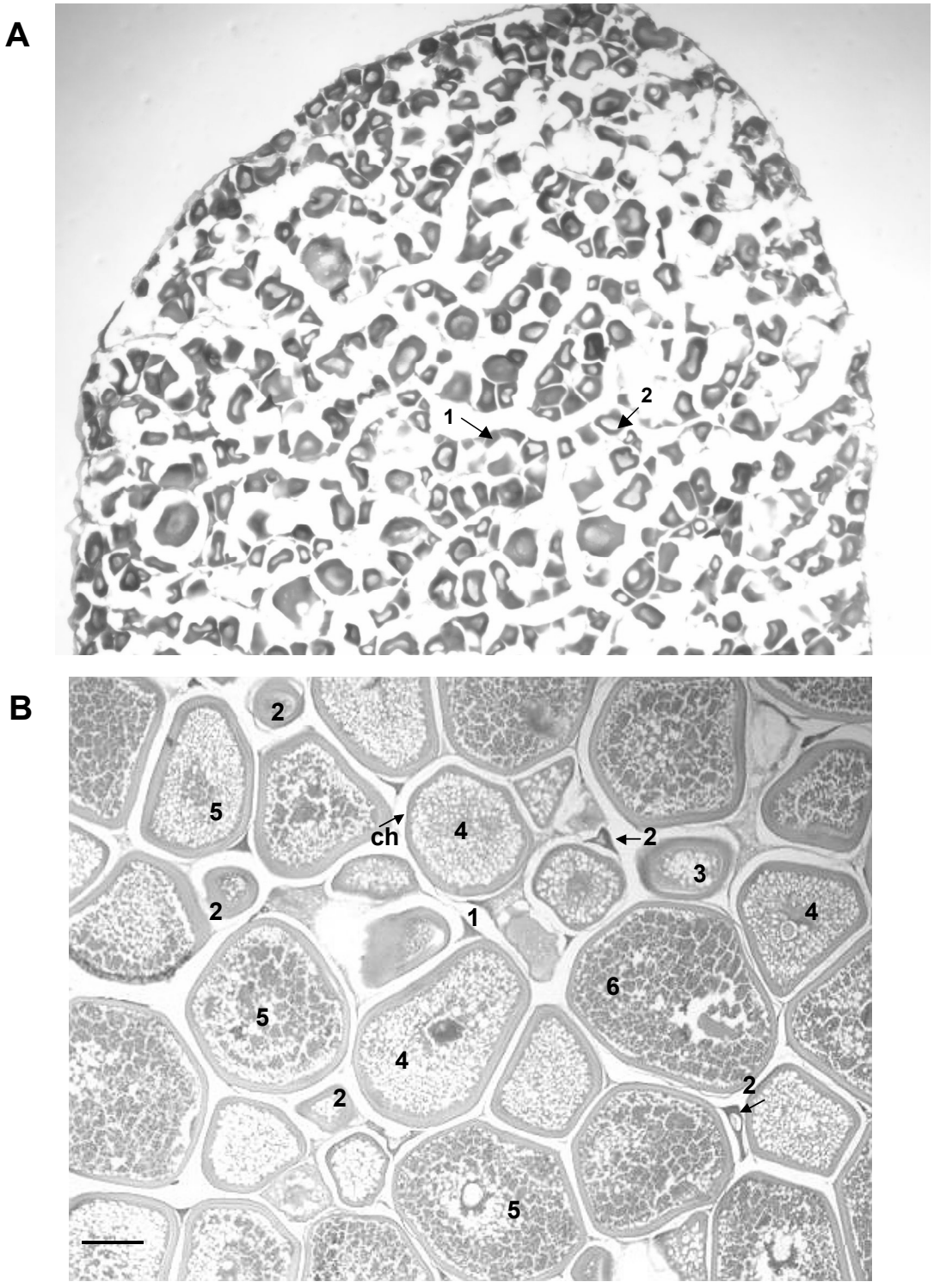

Figure 5.7. Histology of ovary (cross-section) (4 x magnification). (a) control (b) 17MT treated fish. 1. chromatin nucleolas stage, 2. early perinucleolar stage, 3. late perinucleolar stage, 4. cortical alveoli stage, 5. early vitellogenic stage 6. late vitelogenic stage, ch –chorion, horizontal bar gives the scale of 200 µm.

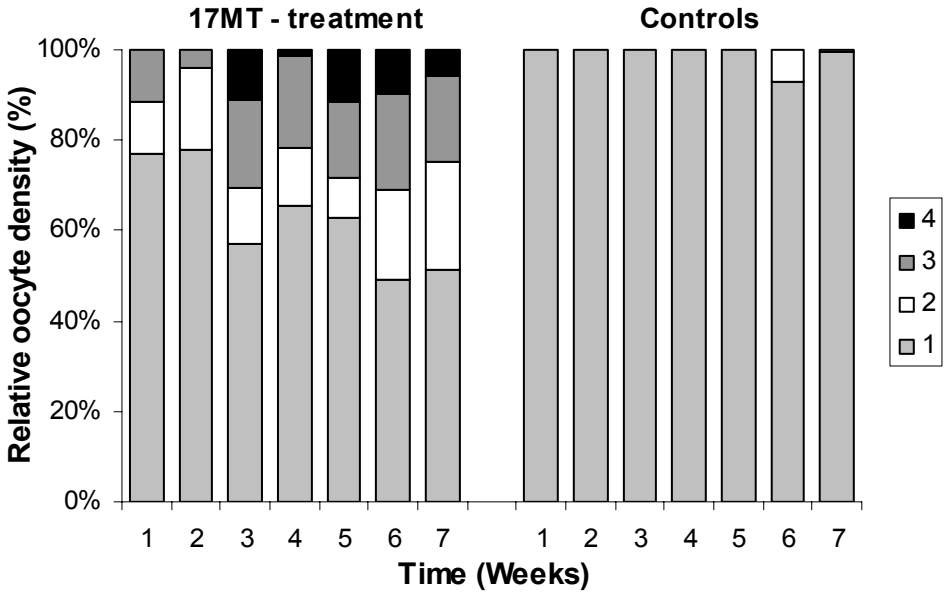

Figure 5.8. Density distributions for 17MT treated and control fish over the 7 weeks. Relative occurrence of stages 1-4. Number of oocytes at a given developmental stage as percentage of all oocytes counted in a fixed sampling area.

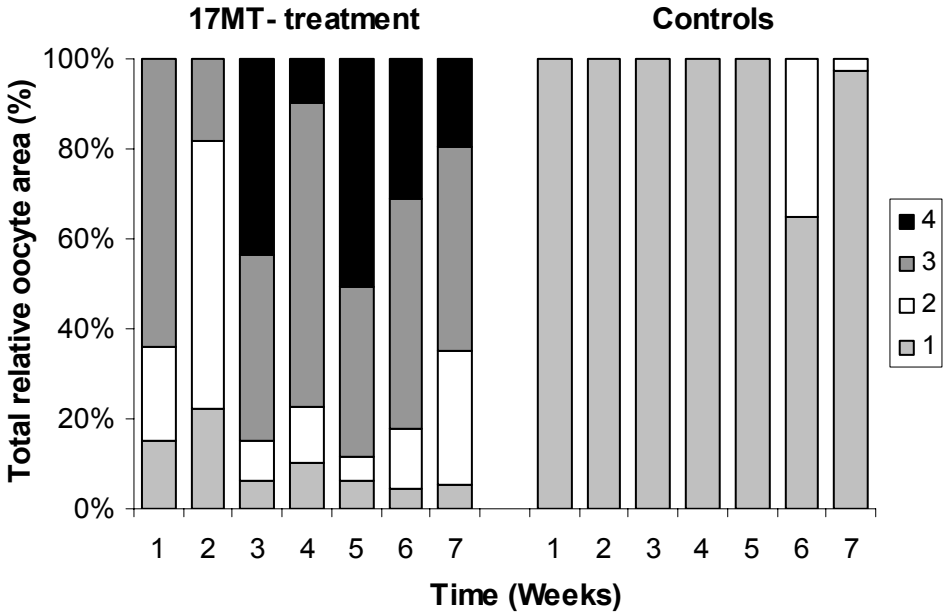

Figure 5.9. Cumulative surface area of all oocytes at a given developmental stage (1-4) as percentage of the available fixed sampling area for 17MT treated and control fish in the 7 weeks of treatment.

Discussion

Hermaphroditic phenotypes

The results of this study have shown that androgen-treated subadult and adult female mormyrid fish *B. niger* developed a dramatic ovarian hypertrophy that appeared to mirror the natural maturational process during seasonal gonadal recrudescence. There was no evidence of intersex gonads; thus 17MT and/or its metabolites were solely affecting ovarian growth. A comparison of the observed gonadal measures (GSI) with published data (Schugardt, 1997) indicated that the observed late treatment effects (weeks 5-6) even surpassed the normal GSI values of treated females, suggesting an anomalous overexpression of the fish ovaries. It seems plausible that an excessive presence of 17MT was aromatized to an excessive presence of a methyl-estrogen affecting ovarian growth (17α-methylestradiol: Hornung at al., 2004). It is known that 17MT produces both androgenic and estrogenic effects in juvenile and adult fathead minnows (Ankley et al, 2001; Parrott and Wood, 2002; Zerulla et al., 2002).

The androgenic effects on gonadal tissue differed most profoundly from those on behavior (EOD) and structural tissue (chapters 3, 4) that resulted in complete masculinization of the corresponding female traits. Thus, late lifetime androgen administration does not affect primary sexual characters, but does affect secondary ones. Structural and behavioral characters are not affected in estradiol-treated adult mormyrids (Landsman et al., 1990; Voustianiouk, 2003). Voustianiouk found that T mostly affected changes in the fish's EOD, whereas the non-aromatizable dihydrotestosterone (DHT) affected structural components

most (anal-fin rays). In the current study, metabolism of 17MT to a DHT analogue could explain the masculinization effects on structure in *B. niger*. Landsman et al. (1990) and Voustianiouk (2003), however, did not investigate the effects of their E2 implants on ovarian growth. (It would be interesting to obtain their voucher specimens for such an analysis.)

The present findings together with those by Landsman and Voustianiouk suggested early lifetime organization for some, but not all reproductive traits. Work in progress is now showing that early lifetime DHT exposure results in hermaphroditic adult phenotypes, only this time also affecting gonadal tissue (ovotestis) (Moller, Kirschbaum, Schugardt, and Dowling, in prep.).

Ecology

Most species, including *B. niger*, with asynchronous oocyte development have protracted spawning seasons with multiple spawnings (de Vlaming, 1983). Thus, the ability to produce multiple batches of eggs that reach maturity and can be spawned at different times may present an advantage in habitats that provide the appropriate stimulation for the HPG axis. The rapid changes of water conductivity due to the onset of the rainy season provide such stimulation for many weakly electric fishes (Kirschbaum, 1987, 1995). In habitats that experience multiple rainy seasons, mormyrids and gymnotiforms spawn triggered by a change in the ionic content of the water. The histological examination showed that all stages of sexual maturity were present in ovaries of females treated for 3-6 weeks with MT. This indicates that *B. niger* is an asynchronous

spawning species. Spawning occurs over an extended period during the rainy season, and therefore, as a multiple spawner, it is likely that only a small proportion of oocytes are released at any one time.

General Discussion

Undertaking this project required knowledge about the maturational time line for *B. niger* and an identification of this species' discrete developmental stages, which were recognized by prominent osteological sexual dimorphisms that affect the support structures of the anal-fin. These dimorphisms were fully developed when the sex ratio had attained 1:1.

Once these stages were known, the study proceeded with an investigation of androgen-induced transformations in juvenile and adult fish. 17MT exposure induced male-typical transformations of reproductive structural and behavioral traits in juveniles of both sexes, as well as in subadult and adult females. These transformations were partial, and masculinized only secondary sexual traits, namely (1) large expansions of the anal fin ray base and thickening of fin ray shafts, (2) inferred increases in attached muscle leading to an indentation of the dorsal margin of the anal fin, (3) structural modifications of the electric organ, i.e. a thickening of the anterior face of the individual electrocytes, and (4) temporal changes in the electric discharge it generates. Following hormone withdrawal, osteological modifications remained permanent, whereas the male-typical elongation of the EOD and body wall indentation returned to pre-treatment conditions. 17MT treatment did *not* masculinize female gonads, but, on the

contrary, resulted in a dramatic hypertrophy of gonadal tissue and induction of normal oocytic development. There was no evidence of intersex gonads.

Normal development

Males reach maturity (> 90mm) when their testis produce androgens and activate osteogenesis at the base of anal fin rays. At the onset of the rainy season, changes in water conductivity elicit gonadal recrudescence (Kirschbaum, 1995) and increased androgen levels exert anabolic effects on the electric organ resulting in a seasonally dimorphic EODs and seasonally prominent body wall indentations. At the end of the breeding season, androgen levels drop and the anabolic effects are reversed (loss of EOD dimorphism and indentation).

Solving a paradox

An fascinating finding of this study was the dual function of exogenous 17MT affecting secondary sexual traits in subadult and adult females, i.e. in exerting opposite activational effects on target tissues: masculinization of structure and muscle, and hyper-feminization of the ovaries. Although the courtship related anal-fin reflex could not be elicited in female *B. niger*, it could be elicited in T- and 11KT-treated female *G. petersii,* and ceased after hormone withdrawal (Voustianiouk, 2003). The adult androgen-treated female could be called a pseudo-hermaphrodite. The *G. petersii* data suggest that in addition to the EOD-generating structures, locomotor control via spinal motor neurons are masculinized as well. This leaves an intriguing question as to whether these

pseudo-hermaphroditic phenotypes are able to reproduce, i.e. can properly respond to normal breeding-condition females, in which case masculinization must have had also affected sensory control.

Although this study did not include hormone assays, the 17MT treatment paradox could be resolved by reviewing the possible metabolic fate of 17MT. Two pathways seem plausible, aromatization to an E_2 analogue and metabolic reduction to a DHT analogue. An excessive presence of E_2 could affect ovarian growth and maturation, and DHT could stimulate osteogenesis as shown by Voustianiouk (2003).

As reported in Chapter 3, synthetic 17MT can be aromatized to a 17α-methylestradiol (Hornung et al., 2004; Ankley et al., 2001; Parrott and Wood, 2002; Zerulla et al., 2002). The enzyme cytochrome P450 aromatase catalyzes the synthesis of estrogens from androgens (Simpson, Michael, Agarwal, Hinshelwood, Bulun and Zhao, 1997). Aromatase is found in the brain, gonads, and other peripheral tissues including the placenta and the adipose tissue in mammals (Simpson, Mahendroo, Means, Kilgore, Corbin and Mendelson, 1993).

Callard, Petro, and Ryan (1981) reported that the levels of this enzyme (cytochrome P450) in the brain of teleost is 100- 1,000-fold higher than in the brain of mammals. Aromatase was attributed to the regulation of reproductive behavior (Pasmanik and Callard, 1988; Pasmanik, Schlinger, and Callard, 1988).

In goldfish, *Carassius auratus*, aromatase levels were highest during the time of spawning and lowest during periods of reproductive inactivity (Pasmanik and Callard, 1988).

17MT is also metabolized to a DHT analogue, a fact well known to body builders relying on the anabolic effects on muscle. The conversion is realized by an enzyme, 5 α-reductase that is found in levels comparable to that in mammals in the teleost brain, spinal cord, and pituitary gland (Pasmanik and Callard, 1985). In goldfish (*Carassius auratus*) and toadfish (*Opsanus tau*) of both sexes, the cyclic changes in 5 α-reductase were opposite than those recorded for aromatase; thus enzyme levels in both brain and pituitary were maximal when fish were reproductively inactive, and lowest during spawning. Circulating gonadal steroids were high when aromatase was high and low when 5 α-reductase was maximal (Pasmanik and Callard, 1985). This seems to reflect the natural endocrine demand when during gonadal recrudescence (both ovarian growth and spermiation) E_2 is needed.

Developmental plasticity, sexual bipotentiality

The extreme plasticity and heterogeneity in sexual differentiation and dimorphisms in many teleost species requires an open genetic program so that gonadal steroids can to various degrees affect both primary and secondary sexual traits at any time of development.

In several species of teleosts, gonadal sex can be determined relatively late in development. Salmonids treated with testosterone during embryonic (egg) and/or fry stages completely change to males (or to sterile males, depending on hormone concentration) (Feist et al., 1995). The question is to what extent and at what developmental period masculinization or feminization is achieved naturally, or can be achieved through hormone manipulation. Clearly, adult-treated female

B. niger expressed male traits that they normally would not, thus demonstrating developmental bipotentiality into adulthood. On the other hand, ongoing research on mormyrid fish in our laboratory has shown that early lifetime exposure of a related species (*Mormyrus rume proboscirostris*) to 17MT resulted in extreme gonadal anomalies including intersex (ovotestis) and duplication of gonads (mormyrids normally possess only one gonad). These results clearly point to early organizational effects affecting the expression of primary sexual characters (Moller et al. in prep.). In salmon exogenous estradiol causes 100% female phenotypes (Hunter and Donaldson, 1983). Piferrer, Zanuy, Carrillo, Solar, Devlin, and Donaldson (1994) tested the importance of E_2 in the normal development of genetic female chinook salmon by blocking aromatization for a short period (2 hrs); genetic females were transformed into functional males. Partial or complete gonadal sex reversal has been reported in over 50 species through administration of gonadal steroids, their antagonists, or aromatase inhibitors (Devlin and Nagahama, 2002).

Endocrine disruptors

The selective sensitivity of *B. niger* and *M. rume* to gonadal steroids, and androgens in particular, elevates these species to model status. They could serve as a potent indicator to monitor and detect exogenous, toxic steroids and/or their mimics.

About 80,000 chemicals have been introduced into the environment within the last 50 years. Some of these chemicals may pose extensive threat to wildlife

and human populations (Tyler, Jobling, and Sumpter, 1998). Among these chemicals are those that can interfere with the endocrine system and thus adversely affect reproduction and development (endocrine disruptors, EDs). Their presence in the environment affects behavior, secondary sexual characteristics, and gonads.

Probably the most convincing field data documenting the effects of exposure to EDs and reproductive anomalies have come from studies on fish as most of these chemicals are discharged or end up in the aquatic environment (Jobling, Nolan, Tyler, Brighty, and Sumpter, 1998). Androgenic chemicals cause morphological masculinization of reproductively mature female fathead minnows (Ankley et al., 2001, Ankley, Jensen, Makynen, Kahl, Korte, Hornung, Henry, Denny, Leino, Wilson, Cardon, Hartig, and Gray, 2003) and estrogenic compounds induce plasma vitellogenin in males (Ankley et al., 2001; Harries, Shean, Jobling, Matthiessen, Neall, Routledge, Rycroft, Sumpter, and Tylor, 1996). Major studies on ecotoxicology have involved three freshwater species, the medaka, *Oryzias latipes*, zebrafish, *Danio rerio* Hamilton and fathead minnow, *Pimephales promelas* Rafinesque. Structural anomalies induced by exposure to environmental estrogens are manifest in ovotestis, duct disruption, or the increased frequency of atretic oocytes (Jobling, Sheahan, Osborne, Matthiessen, and Sumpter, 1996; Gimeno, Komen, Gerritsen, and Bowmer, 1998; Flammarion, Brion, Babut, Garric, Migeon, Noury, Thybaud, Tyler, and Palazzi, 2000; Kinnberg, Korsgard, and Bjerregaard, 2000; Metcalfe, Metcalfe,

Kiparissis, Niimi, Foran, and Benson, 2000; Nolan, Jobling, Brighty, Sumpter, and Tyler, 2001).

A vast literature reports about the EDs most prominent effects on the expression of primary sexual characters. Mormyrid fish, however, such as *B. niger* could also indicate the effects of EDs in the form of anomalies affecting the endocrine-controlled reproductive structures.

Osteoporosis

A surprising result in the experiment reported in chapter 3 was that once the bases of anal fin rays had expanded in adult females they remained expanded for many months following hormone withdrawal while in the absence of 17MT anabolic action on muscle and myogenic tissue, i.e. the electric organ ceased. What maintains androgen-induced bone tissue in female mormyrid fish?

Along with calcium-regulating hormones, sex hormones participate in the expression of skeletal sexual dimorphisms and maintain mass and strength of bone in adults (Falahati-Nini, Riggs, Atkinson, O'Fallon, Eastell, and Khosla, 2000). Testosterone is important for skeletal growth and a source of estrogen, which in turn maintains bone in both sexes. Insufficient levels of estrogen lead to bone loss and fractures. In women, estrogen deficiency is considered the main cause of post-menopausal osteoporosis, and hormone replacement therapy is recommended for its prevention.

It is safe to assume that androgens and not estrogens caused fin ray expansion in female *B. niger* as has been described for another mormyrid *G. petersii* (Voustianiouk, 2003). DHT, a non aromatizable androgen but not

estradiol caused the anal-fin ray expansion in these fish. It is further plausible to assume that, following hormone removal, the fish's own estrogen maintained the expanded base of anal-fin rays. Future studies depriving females of endogenous estrogen (ovarioectomy) could provide more definitive answers.

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