The social status of the male Nile tilapia (*Oreochromis niloticus*) influences testis structure and gene expression

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Abstract

Dominant and territorial behaviour are known social phenomena in cichlids and social stress influences reproduction and growth. The gonadotropic hormones trigger spermatogenesis and subordinate males have typically lower levels of gonadotropins than dominant males. In this study, we compared testis morphology and gene expression of dominant and subordinate Nile tilapia males (d- and s-males) in socially stable communities. The d-males had the highest gonadosomatic index but they were not the largest animals in the majority of studied cases. Long-term d-males showed large groups of Leydig cells and hyperplasia of the tunica albuginea due to numerous cytochrome-P450-11β-hydroxylase (Cyp11b) expressing myoid cells. Increased Cyp11b expression in d-males was reflected by elevated 11-ketotestosterone plasma values. However, immunofluorescence microscopy and expression analysis of selected genes revealed that most s-males conserved their capability for spermatogenesis and are, therefore, ready for reproduction when the social environment changes. Moreover, in s-males gene expression analysis by quantitative RT-PCR showed increased transcript levels for germ line-specific genes (*vasa*, *sox2* and *dmc1*) and Sertoli-specific genes (*amh*, *amhrII* and *dmrt1*) whereas gene expression of key factors for steroid production (*sf1* and *cyp11b*) were reduced. The Nile tilapia is a promising model to study social cues and gonadotropic signals on testis development in vertebrates.

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Introduction

The Nile tilapia, *Oreochromis niloticus* L., is a robust food fish of which several million tons are produced annually worldwide (FAO 2008). This African cichlid fish came into the focus of research because knowledge about reproduction and physiology is necessary for effective growth in aquaculture (Li & Webster 2006). Meanwhile, *O. niloticus* has become a valuable model for gonad development and gene expression studies (Matta et al. 2002, Davis et al. 2008, de Alvearenga & de Franca 2009, Kobayashi & Nagahama 2009, Lacerda et al. 2010, Soler et al. 2010). Genetic factors together with environmental cues determine the phenotypic gender of this gonochoristic species (Baroiller et al. 2009a,b). Reproduction in nature requires the coordination of gonad development and social behaviour. Seasonal changes in gonad structure, onset of puberty or social effects on fertility, are controlled by the endocrine system along the brain–pituitary–gonad axis. FSH and LH mediate neuronal signals to the gonads and stimulate spermatogenesis (Weltzien et al. 2004, Levavi-Sivan et al. 2010). Spermatogenesis in fish occurs in seminiferous tubules that consist of numerous cysts filled with germ line cells. Within a single cyst all germ line cells are derived from a single spermatogonium by synchronous mitotic divisions. After several rounds of mitotic divisions the germ line cells also enter meiosis simultaneously. The germ line cells in a developing cyst are in close contact with somatic Sertoli cells that also form the cyst wall. The spermatogenic tubule is surrounded by a thin layer composed of basal lamina and peritubular myoid cells. The space between the tubules is referred to as intertubular compartment or interstitium and is filled with steroidogenic Leydig cells (LC), blood vessels, macrophages, mast cells, neuronal cells and connective tissue cells (reviewed in Schulz et al. (2010)). The testis is enclosed by an epithelium with a thin subjacent layer of collagenous connective tissue termed tunica albuginea (TA) by most authors (discussed in Koulish et al. (2002)). Little is known about the ultrastructure of the TA in teleost testis. A study on protogynous fish (*Thalassoma bifasciatum*) reported continuity between the subjacent layers close to the outer epithelium and the interstitium (Koulish et al. 2002). In *O. niloticus* most but not all of the undifferentiated spermatogonia are located close to the...
TA (Vilela et al. 2003). This organisation may be interpreted as an intermediate form between the restricted and unrestricted spermatogonial distribution. In the first type undifferentiated spermatogonia are restricted to the vicinity of the TA, whereas in the unrestricted type they are located along the entire tubule (Grier et al. 1980, Grier 1981, Parenti & Grier 2004, Schulz et al. 2010).

Only few data exist about the regulative network of endocrine and paracrine factors and about the interaction between the different testis cell types. The steroid hormone 11-ketotestosterone (11-KT) is the major androgen in fish and promotes spermatogenesis. Cytochrome-P450-11β-hydroxylase (Cyp11b) catalyses the conversion from testosterone to 11β-hydroxysterone, the immediate precursor of 11-KT. In the male gonad this enzyme was found to be present mainly in LC (Devlin & Nagahama 2002, Fernandes et al. 2007, Zhang et al. 2010). 11-KT synthesis is stimulated by gonadotropins (Billard et al. 1991, Nagahama 1994, Ikeuchi et al. 2001). In the Japanese eel, activin B is stimulated by 11-KT while the Amh level is reduced and this is thought to trigger spermatogenesis (Miura et al. 1991, Nagahama 1994, Ikeuchi et al. 2001). These data are supported by the analysis of an Amh receptor-deficient Medaka mutant (Morinaga et al. 2007) whose phenotype led the authors to conclude that Amh inhibits gonial proliferation in adult testis and initiates premature meiosis in males.

Dominant and territorial behaviour are well known in cichlids (Turner & Robinson 2000). Such behaviour gives an advantage for the dominant/territorial (d)-male for mating with a female (Kelly 2008). In addition, stable social hierarchies save energy and reduce the risk of injury (Kaufmann 1983). Dominant behaviour is accompanied by an elevation of GnRH1 levels in the hypothalamus (White et al. 2002, Burmeister et al. 2005). Experiments with the cichlid Astatotilapia burtoni showed increased levels of GnRH1, FSH, LH and plasma 11-KT, as well as an increased size of the gonad in socially ascending males while subordinated (s)-males show the opposite effect (Francis et al. 1993, White et al. 2002, Renn et al. 2008, Maruska & Fernald 2011, Maruska et al. 2011). The social ascent to become a d-male proceeds within only few hours and is promoted by the immediate-early gene (egr-1) in the brain of A. burtoni (Burmeister et al. 2005). Once a stable social status is reached, the expression of egr-1 remains unchanged, but the expression of that gene is apparently only required to initiate the social status change. The stability of the social structure in cichlids (Dijkstra et al. 2007, Maruska et al. 2011) is under endocrine control. 11-KT feeds back from testis to GnRH-containing hypothalamus (White et al. 2007, Maruska et al. 2011). The social ascent to become a d-male proceeds within only few hours and is promoted by the immediate-early gene (egr-1) in the brain of A. burtoni (Burmeister et al. 2005). Once a stable social status is reached, the expression of egr-1 remains unchanged, but the expression of that gene is apparently only required to initiate the social status change. The stability of the social structure in cichlids (Dijkstra et al. 2007, Maruska et al. 2011) is under endocrine control.

Figure 1 Morphological and cytological differences between dominant (d) and subordinate (s) Nile tilapia males. (A) Photograph of a stable community (18 months old) with dark coloured s-males and females near the water surface and a pale d-male that controls the area around the bottom of the tank. (B and C) Body-colouration and eye-darkening pattern for a d-male (B) and an s-male (C). Iris and sclera from the d-male are completely pale. Insets in B and C show the whole animal (yellow bar = 30 cm). (D–F) Light microscopic comparison of paraffin-embedded testis cross-sections (4 μm) from a d- and two s-males (sample ID58, 59 and 64, respectively, see Supplementary Table 1, see section on supplementary data given at the end of this article). Immunolocalisation of Vasa protein (brown stain) counterstained with methyl-green. s-male1 in D shows an example for a fully degenerated testis. Note that the degenerated testes completely lacks Vasa protein. Asterisks indicate large groups of Leydig cells (LC) – a higher magnification of this area is shown in Supplementary Figure 3, see section on supplementary data given at the end of this article. Cross-sections are orientated with the efferent ducts located towards the top of the picture. (E and F) Details of light micrographs from D. E shows the extended tunica albuginea (TA, arrow) and LC between spermatogenic tubules in a d-male. (F) TA (arrow) and spermatogenic tubules of an s-male in comparison to E. E and F same magnification.

Comparative studies between dominant and subordinate Nile tilapia males with detailed cytological description of gonads are not available to date. To fill this gap and to link social status to morphological and functional features of the gonads we compared testes from long-term stable d- and s-males by histological criteria and by comparing the expression of key genes.

Results

Dominant and subordinate males in communities with stable social hierarchy

We have evaluated 20 Tilapia communities and observed the behaviour and measured body and testis weight from a total of 20 dominant and 72 subordinate males. The observation of a community started with about 15 pubescent, 4–5 months old fish in one tank. The percentage of females in the communities varied between 30 and 70%. Within the next 4 weeks the social hierarchy was established and aggressive behaviour of individuals was frequently observed. During this time, males with paler body colour appeared which is a typical attribute of dominance. At the end of the first week only one white d-male was present and defended a territory around the bottom of the tank. All other members of a group stayed close to the water surface. In every tank we always observed the formation of stable social structures (Fig. 1A). The d-male developed the typical eye colour pattern with pale iris and sclera (Fig. 1B and C) and randomly attacked all other individuals. Only d-males established a spawning site. A tube at the bottom of the tank was preferentially used for mating (Fig. 1A). Spawning and successful reproduction occurred throughout the year. We observed that the dominance of a particular male can persist for more than 2 years. However, changes in dominance also occurred occasionally followed by the establishment of a new dominant–subordinate relationship. The replacement was accomplished by a slow change in the eye colour pattern over several days. The animals chosen for sampling were between 0.5 and 3 years old and remained at least for 5 months in the status of a d-male. The d-male was compared with one or several subordinates from the same tank and all animals were killed at the same time.

The calculated gonadosomatic index (GSI) showed higher values for the d-male in comparison with the s-males in all of the 20 communities (Fig. 2, Supplementary Table 1 and Supplementary Figure 1, see section on supplementary data given at the end of this article). The plot of the body weight against the quotient between GSI of any male from one community and the corresponding d-male’s GSI revealed that the d-male was not necessarily the largest animal of a community (Fig. 2). Interestingly, the GSI of the subordinates varied within a broad range on values near the GSI of dominants to very small values (Supplementary Figure 1, see section on supplementary data given at the end of this article). The increased testis weight from d-males was the result of a larger width but not length of the organ (Fig. 1D, Supplementary Table 1, see section on supplementary data given at the end of this article).

Both, d- and s-males, exhibited numerous Vasa-positive germ line cells in cysts. Also, the large single germ line cells were specifically stained. Most of them were located close to the TA (Fig. 1E and F). The testis in d-males showed large groups of interstitial cells and the lumen of the spermatogenic tubules appeared wider.

Figure 2 Gonadosomatic indices (GSIs) and body weight data of all examined animals shown as the quotient of (GSI/ GSI d-male of the same community) plotted against the body weight. Therefore, the value for the d-male in each community is 1. Filled symbols represent d-males, open symbols represent s-males. Green squares indicate the community that was used for plasma 11-KT measurement (Table 2). Triangles in blue and red indicate the two communities that were used for qRT-PCR and histology in parallel. Symbols highlighted with ID numbers are the samples analysed by qRT-PCR in this study (Fig. 5). The numbered blue triangles correspond to specimens investigated in parallel by IF and TEM. Symbols highlighted with asterisks are ascending males with GSI values near to the d-male. Males with completely degenerated testes (#) show low quotients. A further diagram (GSIs plotted against age) is shown as Supplementary Figure 1, see section on supplementary data given at the end of this article. All GSI values and ID numbers are presented in Supplementary Table 1, see section on supplementary data given at the end of this article.
than in s-males (Figs 1D, 3, 4 and Supplementary Figure 3, see section on supplementary data given at the end of this article). Furthermore, the TA was expanded to a multi-layered structure in all d-males (Fig. 1E) that were dominant for at least 5 months. We counted the absolute number of tubules and cysts in representative cross-sections shown in Fig. 1D. In d-males about 30% more tubules were found. The quotient between tubules and cysts was identical for d- and s-male. Interestingly, the percentage of the area with interstitial cells, most likely LC, comprised 44% in d-male and only 4% in the s-male. In our samples from subordinates we also noticed different degrees of testis degeneration. This ranged from some liquid-filled cysts on the testicular surface to a complete germ cell-depleted testis in five cases (Fig. 1D and Supplementary Figure 2, see section on supplementary data given at the end of this article).

**Gene expression analysis of testes from dominant and subordinate males by qRTP-PCR**

We compared the relative transcript levels between d- and s-males of two different communities by qRTP-PCR (Fig. 5). Selected marker genes (Table 1) of the germ line (vasa, sox2 and dmc1), of somatic cells (amh, amhrI

![Figure 3](image-url) Immunohistological analysis of Vasa- and PCNA protein expression in testis cross-sections (4 μm) from Nile tilapia d- (ID58; A–C) and s-males (ID59; D–F). The orientation of the cross-sections is as shown in Fig. 1D with the efferent ducts located on the dorsal side (top). (A and D) Vasa protein (red) is present in cysts and large, single germ line cells. The fluorescence intensity is decreased from spermatogonia (A and B) to spermatocytes (Sc) and is lost in spermatids (St). The subcellular localisation of Vasa is cytoplasmic. (B and E) Brightfield picture overlaid with DNA staining (DAPI, converted to black) to illustrate the testis structure consisting of tubules (dashed line in A and C) and interstitium (* in B–F) including Leydig cells (LC). Lumen (Lu) of a spermatogenic tubule. (C and F) Identification of proliferating cells by PCNA staining (green); Vasa is shown in red and DNA in blue (DAPI). PCNA is located in the nuclei and appears from green to light blue due to the overlay with DNA staining in blue with different fluorescence intensities. Most spermatogonia are positive for PCNA. PCNA-labelled Sertoli cells are marked with a white arrow. Nuclei of early spermatogonia (A*) stained very faintly (DAPI). Erythrocytes (ery) appear in a bright green due to endogenous fluorescence (C and F). Scale bar in F: 20 μm (all pictures).

![Figure 4](image-url) Immunolocalisation of Cyp11b protein (green fluorescence) in testes cross-sections (4 μm) from d- and s-males showing the region close to the tunica albuginea (TA). DAPI staining of DNA (blue) is merged with the green channel. (A and B) Testis from a d-male (ID58) at different magnifications. Myoid cells in different orientations (myo) and interstitial cells show strong Cyp11b signals. (C and D) Testis from an s-male (ID59) in comparison. Only few myoid cells were stained in s-males. Bright green dots in C are autofluorescing erythrocytes (ery). Erythrocytes in B and D appear in orange because of the additional overlay with the red channel to show autofluorescence. In part D, a single Cyp11b-positive cell (myoid boundary cell) from s-male can be seen (white arrow). Leydig cells (LC), spermatogenic cysts (cy), interstitium (*), border of tubules (dashed line) and myoid boundary cells (white arrow). Lumen (Lu) of a spermatogenic tubule. The dimension of the TA in B and D is highlighted (dotted line). Bars: 50 μm (A, also valid for C) and 10 μm (D, also valid for B).
and dmr1) and typical for steroidogenic cells (sf1, cyp11b and cyp19a1) were generally expressed in all samples.

The genes vasa and sox2 are expressed in spermatogonia and spermatocytes of Nile tilapia (Kobayashi et al. 2002, Supplementary Figure 4, see section on supplementary data given at the end of this article) while dmc1 is expressed only in meiotic cells (Kajiura-Kobayashi et al. 2005). In s-males the relative abundance of the analysed germ line-specific genes was higher than in d-males (Fig. 5). We also investigated one of the s-males with a completely degenerated testis (Fig. 1D and Supplementary Figure 2D, see section on supplementary data given at the end of this article). There were no vasa or dmc1 transcripts detectable and sox2 expression was extremely weak (data not shown) indicating the absence of germ line cells.

Amh, its type II receptor AmhrII and Dmrt1 are typically expressed in Sertoli cells of teleosts (Klüver et al. 2007, Supplementary Figure 6, see section on supplementary data given at the end of this article, Kobayashi et al. 2008). Transcript levels of all three genes were reduced in testes from d-males. Steroid biosynthesis is a typical LC function. We estimated aromatase expression (cyp19a1) since the enzyme also plays a role in testis development (reviewed in Schulz et al. (2010)). Transcript levels of cyp19a1 were increased in s-males. In summary, the gene expression profile indicated normal spermatogenesis in d- and s-males, respectively, while cyp11b necessary for 11-KT production was elevated in d-males.

**Spermatogenesis in dominant and subordinate males**

To evaluate the spermatogenic activity in d- and s-males and to identify a possible arrest of spermatogenesis in s-males we analysed cross-sections of 8 d-males and 18 s-males histologically and by immunofluorescence microscopy (IF). In Fig. 3, we show sections of representative d- and s-males that were also analysed by qRT-PCR and EM in this study (IDS8 and IDS9). We used antibodies specific for pre-meiotic germ line cells (Vasa) and for mitotic cells (PCNA, p-Histone H3). The estimation of the nuclear diameter after DNA staining allows the identification of different germ line cell types. But secondary spermatogonia and spermatocytes from leptotene to diplolene show similar nuclear diameters.

**Table 1** Primer pairs (5′–3′) used for quantitative real-time RT-PCR analysis.

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<th>Reverse primer</th>
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<tr>
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<tr>
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<td>ACTACCCAGCAGCACTCCT</td>
<td>AAGCTGAGACAGCAGGAGA</td>
<td>395</td>
</tr>
<tr>
<td>sf1</td>
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<td>TACGGATCAAGGGCTTTCA</td>
<td>AACTGTGTGGTGAGGAGA</td>
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<tr>
<td>cyp11b</td>
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</tr>
<tr>
<td>cyp19a1</td>
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</tr>
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<td>TGAAGCCCTGGCCGCCGCA</td>
<td>CCAAGACATGCTCACCACAC</td>
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</tr>
</tbody>
</table>

*This work. †Further information was given in Supplementary Figure 6, see section on supplementary data given at the end of this article. ‡RBEST http://reprobio.nibb.ac.jp. ‡jiri et al. (2008) cyp11b, long form. §Kobayashi et al. (2002).旅游业 xml 会话.

www.reproduction-online.org
and for this reason we used the higher Vasa concentration in spermatogonia as an additional criterion for identification. Double IF staining with anti-Vasa/anti-PCNA and anti-PCNA/anti-p-Histone H3 antibodies revealed mitotically active germ line cells and Sertoli cells in both d- and s-males. PCNA was detected generally in cyst-forming spermatogonia, but also in some Sertoli cells mostly located around primary spermatogonia or early secondary spermatogonia (Fig. 3). Spermatids were most frequently positive for p-Histone H3 (Supplementary Figure 5, see section on supplementary data given at the end of this article).

In summary, we have identified mitotic spermatogonia, meiotic spermatocytes and haploid spermatids and spermatozoa in the testes of d- and also in s-males with GSI > 0.07. We have not seen obvious differences in the relative abundance of specific germ cell stages to each other. Only s-males with germ cell-depleted testis and the lowest GSIs had apparently abolished spermatogenesis. Our histological analysis revealed that most s-males showed all stages of spermatogenesis and proliferating cells, but the dynamics of spermatogenesis cannot be assessed by the available morphological data. Considering the lower GSI in s-males caused by the reduced number of LC and spermatogenic tubules we expect a reduced potential for spermatogenesis in s-males compared with d-males.

**Dominant males exhibit large groups of LC and a thickened TA**

LC and myoid cell were identified based on their characteristic ultrastructural features. In d-males LC form large clusters outside the seminiferous tubules (Fig. 6A and B) and show characteristic smooth endoplasmic reticulum and mitochondria with tubular cristae (Fig. 6C and D) indicating active steroid production (Nakamura & Nagahama 1989). LC are located in close proximity only to interstitial myoid cells or to other LC (Fig. 6E) as has been observed in Medaka testes (Gresik et al. 1973).

The thickened TA of the testis from d-males contained several cell layers of elongated myoid cells (Fig. 6F). These cells are typical smooth muscle cells as shown by the presence of electron-dense myofilaments, dense bodies, dense plaques and caveolae at their cell periphery (Fig. 6G and H). The myoid network from the TA appears to be connected with myoid cells from

![Figure 6](https://www.reproduction-online.org)
the interstitium (Fig. 6F). LC are not part of the TA. In contrast, s-males exhibit always a thin TA consisting of an epithelium and a thin subjacent layer that also includes some myoid cells.

The testis of s-males is further characterised by a reduced number of LC (Fig. 7A and B). In s-males large parts of the interstitium were loosely organised (Fig. 7D). Between myoid cells covered by fibrous material numerous gaps appeared, possibly as the result of LC loss (Fig. 7C and D). Macrophages and mast cell-like cells were present in the interstitium that was not seen in d-males (Fig. 7E–H). That observation indicates inflammatory reactions and/or tissue remodelling in the testis of s-males.

**d-males show Cyp11b expressing myoid cells and elevated amounts of Cyp11b protein**

To analyse the 11-KT producing potential of testes from d- (three individuals) and s-males (six individuals) we used a specific antibody to determine the localisation of Cyp11b expressing cells by IF. Furthermore, we used one d- and one s-male, respectively, to quantify the number of Cyp11b-positive cells by flow cytometry and the amount of Cyp11b protein by immunoblotting.

In paraffin sections a strong cytoplasmic Cyp11b signal in the vicinity of the nucleus was apparent in the cells of the thickened TA and around the tubules in the testis of d-males (Fig. 4A). Our ultrastructural analysis revealed myoid cells as the most prominent cell type of the thickened TA (Fig. 6F). The Cyp11b-positive cells of the TA show the typical elongated myoid cell shape (Fig. 4B) while in transversal sections they have a regular spherical shape (Figs 4B and 6F). Therefore, we conclude that the Cyp11b-positive cells of the TA represent myoid cells. In testes from s-males the thickened periphery was not observed and Cyp11b-positive myoid cells were rare in the entire testis (Fig. 4C and D). Controls devoid of the primary antibody were without fluorescence (not shown). Cyp11b was also detected in cells of the...
Table 2 Plasma 11-ketotestosterone (11-KT) levels in a socially stable Nile tilapia community.

<table>
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<th>Plasma 11-KT (ng/ml)</th>
<th>GSI</th>
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<td>d-male</td>
<td>15.4</td>
<td>0.41</td>
</tr>
<tr>
<td>s1</td>
<td>1.5</td>
<td>0.132</td>
</tr>
<tr>
<td>s2</td>
<td>2.0</td>
<td>0.149</td>
</tr>
<tr>
<td>s3</td>
<td>3.6</td>
<td>0.168</td>
</tr>
<tr>
<td>s4a (3 h)</td>
<td>23.3</td>
<td>0.312</td>
</tr>
<tr>
<td>Female</td>
<td>1.7</td>
<td>ND</td>
</tr>
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</table>

s1–s3: s-males; s4a: ascending male (3 h after removal of the d-male).

connective tissue layer around epithelial cells of efferent ducts but there was no apparent difference between d- and s-males (not shown).

To quantify Cyp11b-positive cells we used primary testis cells for flow cytometry. Specific binding of anti-Cyp11b antibody was detected with an EGFP labelled secondary antibody. Amongst the testis cells of a d-male 58% of the cells showed a positive Cyp11b reaction while the number for an s-male was 21% (Fig. 8A). In compliance with these data immunoblotting of testis extracts revealed higher levels of Cyp11b protein in d-males compared with s-males (Fig. 8B).

Measurement of plasma 11-KT levels in d- and s-males

To prove an elevated 11-KT biosynthesis in d-males we measured the plasma 11-KT concentration in several s-males and the corresponding single d-male (Table 2). In this d-male the 11-KT value was 15.4 ng/ml while in s-males the 11-KT values were much lower (1.5, 2.0 and 3.6 ng/ml) and close to the level of a female control (1.7 ng/ml). These data corroborate the observed higher Cyp11b expression and the increased number of Cyp11b-positive cells in d-males.

We observed in all communities that apart from the white coloured d-male one or more males existed that were lighter coloured than others. Such s-males were also characterised by comparatively high GSI (Fig. 2 and Supplementary Figure 1, see section on supplementary data given at the end of this article) and might become an ascending male after removal of the d-male. We were able to identify the new d-male in the population after about 30 min because this male assumed a greyish-white colour in an otherwise black–coloured community and showed the typical aggressive behaviour of d-males. In a single case we determined the 11-KT concentration 3 h after ascent in a new d-male and obtained the high value of 23.3 ng/ml.

Discussion

Social hierarchy and spermatogenesis in d- and s-males

In our study, we observed a stable social hierarchy among Nile tilapia reared in 800-l aquaria, and such a social structure has also been observed for Oreochromis sp. in nature. Typically, d-males occupy a territory and defend it for several weeks (Turner & Robinson 2000).

We compared the GSIs from O. niloticus d- and s-males and in general, the d-males showed twice the values as in s-males and only ascending candidates reached values close to the d-male of a community. Interestingly, the d-male of a community with the highest GSI was not necessarily the male with the largest body weight. Lower social status is associated with suppressed growth, probably caused by decreased hepatic IGF1 values and reduced food intake (Vera Cruz & Brown 2007). On the other hand, a d-male needs much more energy to establish or to defend a territory than the others. Obviously, several phenomena seem to interfere with each other (Vera Cruz & Brown 2007). We can exclude food limitations for s-males in our long-term communities because the animals were fed ad libitum. For A. burtoni it was reported that non-territorial s-males in schools together with females and in absence of d-males show faster growth than territorial d-males (Hofmann et al. 1999). The body weight is apparently not a prerequisite to become a d-male, but early experiences as dominant individual with territoriality and with courtship are more essential (Fraley & Fernald 1982, Huntingford et al. 1990).

In our long-term communities we have observed that social stress was sufficient to cause fin disorders, increased skin mucus and occasionally gall bladder hypertrophy in s-males with low GSI (F Pfennig 2010, unpublished observations). Pathological reactions such as bile retention and immunosuppression were also reported for cichlid s-males (Faisal et al. 1989, Earley et al. 2004). Permanent stress affects testis development negatively (Wendelaar Bonga 1997, Celino et al. 2009, Schreck 2010, Trubiroha et al. 2011) and this conclusion is also supported by the observation that temperature decrease leads to spermatogenic arrest at late pachytene spermatocytes in sexually mature Nile tilapia males (Vilela et al. 2003).

Our experiments revealed ongoing spermatogenesis and proliferating testis cells also in s-males and no cell cycle arrest was apparent. The maintenance of spermatogenesis in s-males may enable them to reproduce by a ‘sneaking’ strategy. The fact that the s-males in a community are characterised by testes in a wide range of physiological states allows to quickly replace d-males if the necessity arises (Goncalves-de-Freitas & Nishida 1998). Complete testis degeneration in s-males and germ cell depletion was seen in 5 out of 72 cases. We speculate that those s-males were highly suppressed over a long time and such individuals should have low FSH plasma levels (Weltzien et al. 2004, Levavi-Sivan et al. 2010, Schreck 2010, Maruska et al. 2011). FSH seems to be necessary for gonial proliferation and has anti-apoptotic activity on germ line cells in fish (Ohta et al. 2007, Iwasaki et al. 2009, Garcia-Lopez et al. 2010).
Histological and gene expression studies give evidence for different steroidogenic potential of d- and s-males

The number of steroid-producing LC is positively correlated with spermatogenesis in teleost fish (Kanamori et al. 1985, Loir et al. 1989, Nobrega & Quagio-Grassiotto 2007). We demonstrated that stable d-males had testes with large groups of LC. Our estimation of the areas occupied by different testis cell types revealed a very high proportion (44%) of LC in d-males. A similarly high value was reported only for Myleus ternetzi at mature stage (Loir et al. 1989). In s-males, this value dropped to 4%, probably due to a loss of LC. Inflammatory reactions and tissue remodelling and subsequent regression of the fish testis are known from seasonal changes in other teleosts (Loir et al. 1989, Koulish et al. 2002, Lo Nostro et al. 2004, Chaves-Pozo et al. 2005). Macrophages, which typically accompany these processes, were particularly abundant in s-males.

The second striking difference in testis structure between d- and s-males was the thickened TA in d-males. Most likely the myoid cells of the TA formed a contractile network with peritubular myoid cells, a structural arrangement referred to as ‘Y’ zone by Koulish et al. (2002). This structure was also observed in our histological analysis. A contractile testis wall presumably squeezes out the mature sperm during mating. Contractile properties of the TA are also known from mammals (Banks et al. 2006). We can only speculate about the factors that trigger the strong development of myoid cells in d-males, but androgens are likely candidates because of their known stimulating effect on muscle development and growth in vertebrates (Schulz et al. 2010).

Remarkably, the myoid cells of the TA in d-males expressed Cyp11b, necessary for 11-KT biosynthesis. Therefore, the myoid cells could be an additional source for 11-KT apart from the numerous LC. This cytological observation is corroborated by the elevated cyp11b transcript levels, a twofold increase in the number of Cyp11b expressing cells estimated by flow cytometry, and the detection of higher amounts of Cyp11b protein estimated by immunoblotting. Finally, a tenfold increased plasma 11-KT level in a representative d-male confirmed the higher androgen production in d-males.

We showed that in s-males transcript levels for Sertoli cell genes such as amh, amhtr1 and dmrt1 and for germ line-specific genes were higher than in testes of d-males although the histological analysis revealed a reduced number of spermatogenetic tubules in s-males. The reason for this discrepancy presumably is the strongly different representation of specific cell types in s- and d-males and the quantification of the transcript level with reference to housekeeping genes. Due to the large number of LC and myoid cells in d-males the number of Sertoli cells represents a smaller fraction of the total number of testis cells in d-males compared with s-males. Hence, the high transcript concentrations for Sertoli- and germ cells in s-males do not reflect an up-regulation in the respective cell types. Since the number of germ line cells depends on the number of Sertoli cells (Schulz et al. 2005) the marker genes for both cell types show the same trend in the RT-PCR analysis. The reduced expression of cyp11b in s-males is in accordance with the histological analysis and confirms the lower steroidogenic potential of s-males. Further studies by other techniques such as in situ hybridisation, laser dissection or histomorphometry are necessary to quantify the cell type-specific expression of genes of interest.

Role of 11-KT in stable d-males

We determined the plasma 11-KT values and measured tenfold higher level in d-males than in s-males. This observation is in compliance with data from the cichlids A. burtoni and Pundamilia nyererei (Parikh et al. 2006, Dijkstra et al. 2007). We presume that d-males in stable Nile tilapia communities have elevated plasma 11-KT due to the social status and the permanent presence of potential challengers. That is in agreement with the ‘challenge hypothesis’ that predicts higher androgen levels during the course of territory establishment and defence amongst others (discussed by Oliveira et al. (2002) and by Maruska & Fernald (2010)).

In our study, we investigated the long-term effect of social dominance in Nile tilapia males. This system allows the research of the naturally originated hierarchy. This situation is in contrast to the artificial pairing studies with A. burtoni males (Maruska & Fernald 2010). We were also able to identify ascending males that will become the new d-male after removal of an actual d-male. In undisturbed communities the replacement of a d-male by another male is rarely seen. In these rare cases the change in dominance takes several days whereas the experimentally induced change proceeded within 30 min. We did not determine the 11-KT concentration before removing the d-male, but 3 h after removal a high 11-KT level was found in the ascending male. We assume that two effects contributed to this high 11-KT level: first, the ascending male is recruited from males with relatively high GSI close to the d-male and hence the number of LC and steroidogenic capacity is already elevated. Secondly, a quick ascent in social status is accompanied by a rapid elevation of plasma 11-KT values in A. burtoni (Maruska & Fernald 2010) and this might also hold for Nile tilapia.

inhibition might also hold for the fish testis but it seems likely that Amh acts locally since at the distant spermatocytic ducts we observed numerous LC as a result of active proliferation (Schulz et al. 2005).

This study provides information about the structural differences in testis development between d- and s-males of an economically important fish species. Differently developed gonads as a function of social cues make Nile tilapia males a promising model for studying the function of gonadal hormones and their gonadotropic control.

Materials and Methods

Fish maintenance and sampling

Nile tilapia (O. niloticus L.) were kept in circulating 800-l freshwater glass tanks (AQUA SCHWARZ GmbH, Göttingen, Germany) at 26°C and under constant photoperiod cycle (14 h light:10 h darkness). Five per cent of the water was exchanged daily and the conductivity of the water was adjusted to 450 μS by addition of Coral Pro Salt (Red Sea Europe, Verneuil s’Avre, France) to deionised water. Fish were fed ad libitum (around 1% of biomass) once daily with commercial trout pellets (Trouvix F3P Select, Skretting; Trouw Nutrition Deutschland, Burgheim, Germany). One community consisted always of siblings from one set of parents. There was no selection for size or sex before the experiment. The killed d-males were at least 5 months continuously dominant. The behaviour and social status were controlled daily. We identified each animal by its individual fin shape. Animal culture was performed according to the German national regulations and animal welfare.

Fish were stunned mechanically before they were killed by decapitation. All animals of a community used for experimental analysis were killed at the same time. The weight of body and testis was estimated and gonadosomatic indices were calculated (GSI = gonad weight/body weight × 100; plots were created with Origin 8.5; OriginLab, Northampton, MA, USA). Testes were removed immediately and sliced into fragments of 5 mm thickness from the central part of the organ. Specimens were frozen in liquid nitrogen and stored at −80°C until RNA or protein extraction. Adjacent pieces of the same size were frozen in liquid nitrogen and stored at −80°C thickness from the central part of the organ. Specimens were embedded in paraffin, sectioned at 4 μm and transferred to ω-lysine coated slides. Paraffin cross-sections were deparaffinised and successively rehydrated through xylo/ethanol series. Antigen unmasking was performed in 10 mM citrate buffer (18 ml of 100 mM citric acid, 82 ml of 100 mM sodium citrate and 900 ml deionised water, pH 6) in a pressure cooker for 7 min, followed by three washing steps with PBS. Unspecific binding sites were blocked with 3% w/v BSA in PBST (PBS with 0.1% v/v Tween 20). The samples were incubated overnight at 4°C with primary antibodies. The following primary antibodies and dilutions were used for IF (and IHC): anti-zfVasa purified antiserum (rabbit, Knaut et al. 2000) 1:100 (1:1000 for IHC); anti-PCNA MAB (mouse, M0879; DAKO, Glostrup, Denmark) 1:100; anti-phospho-Histone H3 – protein A purified IgG-antiserum (rabbit, #06-570; Abcam, Cambridge, UK) 1:100; anti-Sox2 affinity purified antiserum (rabbit, NB110-79875; Acris, Herford, Germany) 1:500 and anti-(Medaka)-Cyp11b1 antiserum (rabbit, ab71561, Abcam) 1:50. Slides were washed with PBST and the primary antibodies were detected for IF with appropriate secondary antibodies conjugated with Alexa 488, DyLight 488 or Rhodamine Red-X (Invitrogen or Jackson ImmunoResearch, West Grove, PA, USA). After washing with PBST, DNA was stained with DAPI (1:20 000; stock solution 1 mg/ml in DMSO) and mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). Anti-Vasa and anti-Sox2 antibodies were also detected immunohistochemically using a biotinylated anti-rabbit antibody (#BA-1000, Vector) 1:1000, followed by binding of streptavidin-conjugated HRP (1 mg/ml, Jackson ImmunoResearch) 1:5000 and terminal staining with the substrate NovaRed (Vector Laboratories) according to the manufacturer’s instructions. Methylgreen (1:100; stock solution 1% v/v in 20% v/v EtOH) was used for counterstaining. Sections were analysed in a fluorescence microscope (AxioLab, Zeiss) with Neofluar objectives (Zeiss). Documentation was done with Spot cam model 1.40 and the software SpotAdvanced 4.0.9 (Diagnostic Instruments, Heights Sterling, MI, USA). Overview pictures of sections were taken on a BZ-8100E microscope (Keyence) with Plan Apo objectives (Nikon) and assembled with the BZ analyser software (Keyence). Software Image J 1.41 with plug in MBF ‘Image J for microscopy’ (free software) was used for quantification of areas with defined cell types on testis sections (Vasa IHC).

Epon histology and electron microscopy

After dissection the testes were fixed in modified Karnovsky’s fixative (2% v/v glutaraldehyde +2% v/v paraformaldehyde in 50 mM HEPES; Karnovsky 1965, Kurth et al. 2010) at 4°C overnight, and washed 2× in 100 mM HEPES and 2× in PBS. The specimens were postfixed with 1% w/v OsO4/PBS for 2 h on ice, washed with PBS and water and en bloc contrasted with 1% w/v uranyl acetate in water. The samples were then washed several times in water, dehydrated in a graded series of ethanol, infiltrated in epon 812 (epon/ethanol mixtures: 1:3, 1:1, 3:1 1.5 h each, pure epon overnight, pure epon 3 h) and embedded in flat embedding moulds. For light microscopy, semithin sections were mounted on glass slides and stained with 1% w/v toluidine blue and 0.5% w/v borax. For electron microscopy, ultrathin sections were collected on formvar-coated slot grids, stained with lead citrate and uranyl acetate according to Venable & Coggeshall (1965) and analysed on a FEI Morgagni 268 at 80 kV.

Immunoblotting

RIPA buffer (Boehringer Mannheim) including protease inhibitor mix (Roche, cOmplete ULTRA Tablets, Mini, EDTA-free, EASYpack) was added to the samples (35 mg tissue per 1 ml buffer). After homogenisation with a pestle the samples

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were sonicated twice in an ice bath. After centrifugation for 10 min at 14 000 \( g \) and 4 °C, the samples were partitioned in aliquots, frozen in liquid nitrogen and stored at −80 °C until use. Sample aliquots were separated by SDS-PAGE under reducing conditions and blotted onto nitrocellulose membrane according to standard protocols (Rosenberg 2005). Unspecific binding sites were blocked with 5% instant non-fat dry milk solution in TBST (TBS with 0.1% w/v Tween 20). Blots were incubated with rabbit polyclonal Cyp11b1 antiserum (see above, dilution 1:500) or for controls with mouse monoclonal anti-\( \gamma \)-tubulin-antibody (T5326, Sigma–Aldrich, 1:5000) overnight at 4 °C. After washing specific binding was detected using a HRP-conjugated anti-rabbit or anti-mouse IgG, respectively, and visualised by enhanced chemiluminescent detection (ECL Detection Kit, Amersham).

**Flow cytometry**

Whole testes were minced mechanically with a scalpel in prewarmed Hank's Balanced Salt Solution (Biochrom, Berlin, Germany) and resulting tissue fragments were treated with 0.34 mg/ml collagenase XI (Sigma–Aldrich) in DMEM (Biochrom) for 2 h at 27.5 °C. After incubation, cells were centrifuged and washed with prewarmed Hank's Balanced Salt Solution (Biochrom). Aggregates and debris were removed by centrifugation for 10 min at 125 \( g \) and by filtration steps with different pore sizes (100, 50 and 20 \( \mu \)m). After washing, the cells were harvested by centrifugation. For immunostaining testis cells were resuspended in PBS and fixed with 2% w/v paraformaldehyde for 45 min and permeabilised with 0.2% v/v Tween 20 added for the last 15 min of fixation. Cells were centrifuged (10 min at 220 \( g \)) and washed two times with PBST. Cells were resuspended in PBST including 3% w/v BSA and incubated for 45 min, then harvested by centrifugation and washed once. After resuspending in PBST including anti-Cyp11b1 antiserum (rabbit, ab71561, Abcam, diluted 1:500) and incubating overnight at 4 °C, cells were harvested by centrifugation and washed twice with PBST. Alexa488-conjugated anti-rabbit antibody (Invitrogen, diluted 1:500) was added and incubated for 5 min at 27.5 °C. After washing specific binding was detected using a HRP-conjugated anti-rabbit or anti-mouse IgG, respectively, and visualised by enhanced chemiluminescent detection (ECL Detection Kit, Amersham).

**RNA preparation and quantitative real-time PCR (qRT-PCR)**

Total RNA was prepared from testis fragments using TriFast (Peqlab) according to the manufacturer’s protocol. RNA concentrations and purity were measured by spectrophotometry (Nanodrop 1000, Peqlab). Residual genomic DNA was enzymatically eliminated with Desoxyribonuclease 1 (Ambion) and completeness of removal was examined by PCR. MMLV reverse transcriptase (Life Technologies) and random hexamers were used for the first-strand cDNA synthesis. qRT-PCR analysis was performed in three independent runs as described elsewhere using 18S rRNA as an endogenous reference and SybrGreen1 to detect PCR products (Kretzschmar et al. 2005). The amount of cDNA per PCR reaction corresponded to 65 ng of total RNA. The primers that have been used are listed in Table 1. Properties of designed primers were examined with AnnHyb version 4.936 (free software). Results were expressed as relative amounts of mRNA compared with the d-male using the \( 2^{-\Delta\Delta C_T} \) formula (Winer et al. 1999).

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-11-0292.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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**References**


de Alvarenga ER & de Franca LR 2009 Effects of different temperatures on testis structure and function, with emphasis on somatic cells, in sexually mature Nile tilapias (*Oreochromis niloticus*). *Biological Reproduction* **80** 537–544. (doi:10.1095/biolreprod.108.072827)

Banks FC, Knight GE, Calvert RC, Turmaine M, Thompson CS, Mikhailidis DP, Morgan RJ & Burnstock G 2006 Smooth muscle and...


Reproduction (2012) **143** 71–84

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Trubiroha A, Kroupova H, Frank SN, Sures B & Kloas W 2011 Inhibition of gametogenesis by the cestode Ligula intestinalis in roach (Rutilus rutilus) is attenuated under laboratory conditions. Parasitology 138 648–659. (doi:10.1017/S0031182010001514)


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