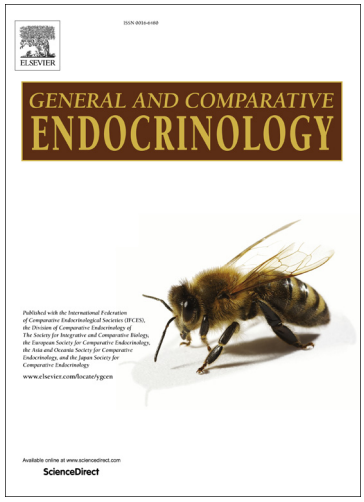

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Temperature-induced testicular germ cell loss and recovery in Nile tilapia *Oreochromis niloticus*

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Abstract

Water temperature is a critical external factor influencing gonadal development in fish. This research aimed to study the impact of elevated temperature on testicular germ cell survival and reproductive capacity of Nile tilapia. Male Nile tilapia were exposed to high temperatures of either 36 (HT1) or 37°C (HT2) for 3000 degree-days (DD) and thereafter returned to the control temperature of 27°C (CT) for 2200 DD. The deleterious effects on testicular germ and somatic cells were observed histologically, characterised by vacuolisation, atrophy and the loss of spermatogenic cells in testes with a more severe impact of HT2 compared to HT1. Interestingly, serum 11-ketotestosterone (11-KT) and testosterone (T) levels tended to be higher during the heat treatments than CT. Expression levels of germline-specific genes *piwil1*, *piwil2* and *nanos2* and Bcl-2 family genes, *bcl-xLb* and *baxa* were significantly reduced during the heat treatment compared to CT, more so in the HT2, while the levels of *nanos3* and *gfra1* transcripts were only significantly reduced in HT2, implying a significant loss of spermatogonial stem cell (SSC) and spermatogonia in HT2. The effect of HT2 is further evidenced by the significantly reduced sperm density and fertilisation rate compared to CT and HT1 at the end of the recovery period but complete sterility was not induced by HT2. Overall, the present study showed significant effects of HT2 on germ cell survival with histological changes in testes, reduced milt quality, increased 11-KT, and decreased expression of germline-specific genes, SSC marker genes and Bcl-2 family genes in testes which could therefore be potential target genes for sterilisation by genome editing.

Keywords: Heat treatment, testicular germ cells, germline-specific genes, Bcl-2 family genes, 11-KT, sterility

Abbreviations: 11-KT, 11-ketotestosterone; *11β-HSD, 11β-Hydroxysteroid dehydrogenase*; ANOVA, analysis of variance; *bax, B-cell lymphoma 2 associated X*; *bcl-2, B-cell lymphoma 2*; *bcl-xL, B-cell lymphoma-extra large*; *bim, Bcl-2 homology 3-only protein*; *box, Bcl-2-related ovarian killer*; dah, days after hatching; DD, degree-days; *dnd*, *dead end*; E_2, estradiol-17β; EIA, enzyme immunoassay;

ELISA, enzyme-linked immunosorbent assay; FADD, Fas-associated death domain; Fas, death receptor; *figla, factor in the germ line, alpha*; FSH, follicle stimulating hormone; *gfra1, Glial cell line-derived neurotrophic factor (GDNF) family receptor alpha-1*; GnRH, gonadotropin releasing hormone; GP, germ plasm; LH, luteinizing hormone; *mcl-1, myeloid cell leukemia 1*; *nanos*, RNA binding zinc finger proteins; *ngn3, neurogenin 3*; PGC, primordial germ cells; piRNA, piwi-interacting RNA; *piwil, P-element induced wimpy testis-like*; *pum*, *pumilio*; qRT-PCR, quantitative reverse transcription PCR; SSC, spermatogonial stem cell; T, testosterone; *vasa*, ATP-dependent RNA helicase of the DEAD-box family.

1. Introduction

Reproductive containment in farmed species is desired for sustainability to prevent introgression by escapees and improve productivity by avoiding gonadal maturation in aquaculture. Sterility can be achieved by suppressing germline-specific gene function or manipulating expression of apoptotic genes to induce apoptosis in germ cells (Wong and Zohar, 2015). This study aimed to rationalise the choice of candidate genes by using high temperature as a means to induce germ cell loss.

Water temperature is a critical environmental factor for fish gonadal development and spawning. High temperature treatment can induce sex reversal or suppress reproduction in fish. In genetically female zebrafish exposed to 37°C between 15 – 25 days after hatching (dah), oocytes at early diplotene stage regressed and masculinised (Uchida *et al*., 2004). In medaka, high temperature (33°C) inhibited proliferation of germ cells and induced female-to-male sex reversal associated with an increase in plasma cortisol (Hayashi *et al*., 2010). Baroiller *et al*. (2009a) suggested apoptosis and/or proliferation of primordial germ cells (PGCs) caused by high temperature during the labile period of sex differentiation could be a critical factor for sex reversal in fish. In addition, water temperature above the natural range for the species can hinder gonadal development and cause germ cell loss in teleosts (de Alvarenga and de França, 2009; Ito *et al*., 2003; Soria *et al*., 2008; Strüssmann *et al*., 1998; Uchida *et al*., 2004). Germ cell death caused by heat treatment was reported in male pejerrey (*Odontesthes bonariensis*), showing nuclear pyknosis or eosinophilia (Ito *et al*., 2003; Soria *et al*., 2008) and in pufferfish (*Takifugu rubripes*) (Lee *et al*., 2009). Sterility was reported in female Patagonian freshwater pejerry (*Patagonina hatcheri*) and Argentinian silverside (*Odontesthes bonariensis*) following exposure to 27 – 28.5 and 29°C, respectively for 112 – 135 days from 1 – 5 weeks after hatching (Strüssmann *et al*., 1998). Complete sterility was also reported in female Nile tilapia (Chitralada strain) and male Mozambique tilapia (*Oreochromis mossambicus*) by high temperature treatment at 37°C for 40 – 50 days from 3 dah (Nakamura *et al*., 2015; Pandit *et al*. 2015). On the other hand, heat treatment between 32 to 36.5°C for 10 days during the thermosensitive period in *O. niloticus* (10 – 30 dpf) induced masculinisation with 36°C being effective (Baroiller *et al*. 1995; Baroiller *et al.* 2009b). While several studies have shown temperature-specific effects of heat

treatments on gonadal germ cells and overall suppression of gametogenesis in fish, the underlying functional mechanisms leading to germ cell death remains unclear. In the present study, two temperature treatments (i.e. 36 and 37°C) have been selected and tested based on the reported impacts on germ cell survival in Nile tilapia albeit 1°C difference.

Piwil1 and *piwil2* are germline-specific argonautes known to maintain germline fate and suppress transposon activity together with piRNA (Houwing *et al*., 2007, 2008). In Nile tilapia, *piwil1* and *piwil2* transcripts are maternally deposited in eggs and exclusively expressed in both ovary and testis (Jin *et al*., 2019). In this species, both Piwil1 and Piwil2 proteins were present in various phases of testicular germ cells from spermatogonia to spermatid but not in spermatozoa (Xiao *et al*., 2013). *nanos2* and *nanos3* are RNA-binding zinc finger proteins and *nanos2* is known to be a key regulator for the maintenance and modulation of spermatogonial stem cells (SSCs) self-renewal (Hofmann, 2008; Sada *et al*., 2009; Suzuki *et al*., 2009). In Nile tilapia, *nanos2* is expressed exclusively in testis and transcripts appeared not to be maternally provided in eggs (Jin *et al*., 2019). *nanos2* has been reported as a marker for putative SSC in rainbow trout (*Oncorhynchus mykiss*) and Nile tilapia (Bellaiche *et al*., 2014; Lacerda *et al*., 2013). On the other hand, *nanos3* was reported to play an important role in PGC survival and migration (Doitsidou *et al*., 2002; Draper *et al*., 2007). The expression of *nanos3* was reported in undifferentiated spermatogonia and the early phases of differentiating spermatogenic germ cells in both mammals (Suzuki *et al*., 2009) and rainbow trout (Bellaiche *et al*., 2014). In Nile tilapia, *nanos3* is maternally transferred to the zygote and expressed in testis as well as in ovary (Jin *et al*., 2019). Glial cell line-derived neurotrophic factor (GDNF) family receptor alpha-1 (*gfra1*) was also suggested as a potential marker of SSCs, as it is expressed exclusively in single type A undifferentiated spermatogonia in Nile tilapia (Lacerda *et al*., 2013). Therefore, *piwil1*, *piwil2, nanos2*, *nanos3* and *gfra1* are considered as suitable markers for testicular germ cells at different stages.

Heat stress is a major environmental stressor and it can cause germ cell loss through the stimulation of apoptotic pathways in fish (AnvariFar *et al*., 2017; Ito *et al*., 2008). Signalling pathways mediating apoptosis involve various molecules including Bcl-2 family proteins, caspases,

cytochrome c, p53, death receptor (Fas) and Fas-associated death domain (FADD). The apoptotic pathways are conserved in fish and mammals although some differences can be found such as the lack of a C-terminal region in the FADD in teleosts (AnvariFar *et al*., 2017). Bcl-2 family genes can be divided into two groups based on their role, proapoptotic: *B-cell lymphoma 2 associated X* (*bax*), *Bcl-2 homology 3-only protein* (*bim*), *Bcl-2-related ovarian killer* (*box*) and prosurvival: *B-cell lymphoma-extra large* (*bcl-xL*), *B-cell lymphoma 2* (*bcl-2*), *myeloid cell leukemia 1* (*mcl-1*) (Cory and Adams, 2002). Oltval *et al*. (1993) suggested that the balance between proapoptotic and prosurvival Bcl-2 family proteins can determine survival or death of cells, as demonstrated in mouse PGCs where the ratio of Bcl-x and Bax regulate the survival of PGCs and apoptosis (Rucker *et al*., 2000). In teleosts, however, it is unknown which Bcl-2 family members are involved in temperature-mediated germ cell apoptosis.

The high temperature-induced germ cell loss is likely to be associated with a dysregulation of sex steroid hormones as these play essential roles in the survival of germ cells (Billig *et al*., 1996; Lue *et al*., 1999). High temperature disrupts the gonadotropin releasing hormone (GnRH)-gonadotropins (follicle stimulating hormone, FSH and luteinizing hormone, LH) system in fish (Soria *et al*., 2008), resulting in a decrease in the production of sex hormones from steroidogenic cells in the gonads. Sterile male Mozambique tilapia, induced by heat treatment, displayed normally developed Leydig cells and comparable levels of sex steroid hormones (11-KT, T and E_2) to control fish (Nakamura *et al*., 2015). However, those results were obtained after a recovery period and the immediate impact of heat treatment on the production of sex hormones and germ cell loss in male Nile tilapia has not been reported.

This study aimed to build on the work of Nakamura *et al*. (2015) and Pandit *et al*. (2015) and investigate, at the molecular level, the impact of high temperature on germ cell loss in Nile tilapia. To do so, tilapia at 120 dah were chosen to be large enough to sample testes and blood for subsequent analyses. Exposure windows to heat treatment (36°C for 83 days or 37°C for 81 days) and recovery (27 °C for 82 days) were adapted from Pandit *et al.* (2015) (37°C for 60 days and 27°C for 90 days). In this work, key genes associated with both survival and apoptosis of germ cells were profiled to

investigate their potential role in association with germ cell loss. The study aimed to rationale potential candidate genes and help prioritise for future studies looking at new means of sterilisation in fish. To this end, the impact of high temperature (36 – 37°C) on the survival of germ cells and reproductive development was investigated in male Nile tilapia. The impact of the high temperature exposure on the germ cell survival and apoptosis was analysed through expression levels of SSC and/or spermatogonia markers (*nanos2, nanos3* and *gfra1*), testicular germ cell markers (*piwil1* and *piwil2*) and apoptosis-related genes (Bcl-2 family genes, *bcl-xLa, bcl-xLb, baxa, baxl, bcl-2* and *mcl-1a*). In addition, the treatment effects on male reproductive development was analysed through serum sex steroid levels, testis histology and sperm quality including sperm density, motility and fertilisation rate.

2 Materials and Methods

2.1 Ethics statement

All working procedures were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and were approved by the Animal Welfare and Ethical Review Body (AWERB) ethics committee of the University of Stirling.

2.2 High temperature treatment and sampling

All male (XY) progeny were produced by crossing YY supermale (Scott *et al*., 1989) with XX female at the tropical aquarium of the Institute of Aquaculture (University of Stirling). A total of 158 progeny were sexed throughout the experiment and all were males. Fish were reared at 27 ± 1°C and a photoperiod of 12L:12D. All male Nile tilapia at 120 dah (initial average weight of 3.1 ± 0.4 g, n = 180) were divided into three temperature groups: two high temperature treatments (HT), 35.9 ± 0.1°C (HT1), 36.9 ± 0.1°C (HT2) and a control temperature treatment of 26.7 ± 0.4°C (CT). An aerator was used for each 300 L tank and both water temperature and oxygen were monitored throughout the day with underwater sensors and a data logger (HOBO Pendant® Onset Computer Corporation). Water was exchanged daily and unionized ammonia (NH_3) levels remained < 0.1 µg/L and pH between 6.4

and 7.4 during the HT window. Timing of samplings was standardised between temperature treatments at 1200, 2100, 3000, 3800 and 5200 degree-days (DD, average daily water temperature multiplied by number of days) (Fig. 1). Each fish (n = 6 fish/treatment/time point except for 5200 DD which was n = 12) was sacrificed by an approved Schedule 1 method (overdose of Benzocaine and severing of spinal column) at 1200, 2100 and 3000 DD during the temperature treatments and at 3800 and 5200 DD during the recovery period at 27°C. Body weight, visceral somatic index (VSI), hepatosomatic index (HSI) and gonadal somatic index (GSI) were recorded. Each right and left lobe of testis was either fixed in Bouin's solution for histology (H&E stain) or stored in RNAlater at 4°C prior to RNA extraction for qPCR analysis, respectively.

2.3 Real-time qRT-PCR (quantitative reverse transcription PCR)

Total RNA from testis samples (n = 6 fish/treatment/time point) were extracted using TRI reagent (Sigma-Aldrich), with the tissue being homogenised prior to extraction using a bead-beater (BioSpec Products). Total RNA quality was provisionally checked by spectrophotometry (A260/A280 ratio was >1.8) and the RNA integrities were confirmed by checking for the presence of clearly defined ribosomal RNA bands on 1% agarose gel. The RNAs were treated with DNase I to remove gDNAs using DNA-free DNA Removal kit (Thermo Fisher). Then, 400 ng of DNase I treated RNAs were used for cDNA synthesis using High capacity cDNA reverse transcription kits (Applied Biosystems) with a blend of random hexamer & anchored oligo dT primer as 3:1 ratio in a 20 µL total reaction volume.

The Nile tilapia sequences of spermatogonia markers (*nanos2, nanos3* and *gfra1*), testicular germ cell markers (*piwil1* and *piwil2*), apoptosis-related genes (Bcl-2 family genes, *bcl-xLa, bcl-xLb, baxa, baxl, bcl-2* and *mcl-1a*) and reference genes (*ß-actin* and *elf1α)* were identified in NCBI and primers (Table 1) were designed using Primer-BLAST in NCBI. PCR products were cloned using pGEM T-easy vector systems (Promega) and sequenced (GATC Biotech) to generate standard curves of each target gene to allow absolute quantification. Efficiency and R^2 of the standard curve were higher than 90% and 0.995, respectively, for all genes, and the melting curve of each gene was

checked in each assay to assure the production of a single product (Table 1). Each reaction consisted of a total volume of 5 µL containing 1.3 µL of cDNA (1/5 diluted, approx. 5.2 ng RNA), 2.5 µL of Luminaris Color HiGreen qPCR master mix (Thermo Fisher), 0.3 µM of each forward and reverse primer and MilliQ water up to 5 µL. The qRT-PCR reaction followed the protocol: 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60 – 62°C for 60 sec, and melting curve analysis using LightCycler 480 (Roche) and 384-well plate. The absolute copy numbers of every target gene were calculated based on its standard curve, and the relative mRNA levels of all the samples were calculated by normalising to the levels of a geometric mean of *ß-actin* and *elf1α* for each time point.

2.4 Testis histology

Testis samples were fixed in Bouin's solution, then dehydrated, cleared and impregnated with paraffin wax using an automated Tissue Processor. The samples were embedded in paraffin wax using a histoembedder (Leica UK Ltd.). The wax blocks were trimmed and sectioned using a Rotary microtome (Leica UK Ltd.) at 5 µm thickness. The sections were transferred onto slides, dried and left overnight in oven. On the following day, these slides were stained with haematoxylin and eosin. Sagittal sections of testes were observed for 0 DD (n = 3 fish) and 1200 to 3800 DD samples (n = 6 fish/treatment/time point). Both sagittal and transverse sections were observed for 5200 DD testes samples (n = 12 fish/treatment).

2.5 Steroid hormone analysis

Blood samples (n = 6 fish/treatment/time point) were collected from the fish caudal veins and stored at 4°C overnight. Serum was collected after centrifugation (1,400 xG for 10 min) and stored at −20°C as aliquots until use. 50 µl of each serum sample was mixed with 1 mL of ethyl acetate and centrifuged at 1500 rpm for 10 min at 4°C, and the supernatants were evaporated using a vacuum centrifuge at 30°C for 40 min, then the extract was dissolved in an assay buffer provided by the enzyme immunoassay kits (EIA) for 11-KT and T (Cayman) (Chen *et al*., 2017), while serum samples were directly used to measure cortisol level by enzyme-linked immunosorbent assay (ELISA) (IBL

Int.) (Chabbi and Ganesh, 2017), according to the manufacturer's instructions. Intra- and inter-assay coefficients of variation (CVs) in 11-KT and T assays were less than 12.5% and 12.6%, respectively. The inter-CV in cortisol assay was 2.8%. Each assay was run with the serial dilution of unknown tilapia serum sample to confirm linearity of measurement by the assay (R^2 = 0.998 – 1) along with the standard curve samples to allow direct interpretation of hormone level in unknown samples (R^2 = 0.992 – 0.999).

2.6 Fertilisation rate and sperm quality analyses

At the end of the recovery period, between 5000 – 5200 DD, fertilisation and sperm quality tests were conducted twice for each fish using a total of six independent batches of eggs (n = 2 egg batches/treatment). Semen from HT1, HT2 or CT males (n = 12 fish/treatment) were collected and stored on ice. Eggs of each batch collected from XX females were distributed into 12 Petri dishes, corresponding to one Petri dish for each semen sample. Semen collected from randomly picked CT (n = 2) were used to confirm egg viability (> 30% fertilisation rate in CT) in the fertilisation test of HT1 and HT2 groups. A small batch of eggs (n > 50) was fertilised *in vitro* by adding 1 µL of semen from each male for each egg batch used. 1 µL of semen was chosen due to the minimum semen volume of 2 µL which could be collected from fish in the HT2 group After 3 min of incubation, fertilised eggs were gently washed with the aquarium water and incubated in a tumbling egg system in isolation at 27 ± 1°C (total of 72 batches over the period). The fertilisation rate was determined at blastula stage (4 hours post fertilisation) under a stereomicroscope.

Following the setup of the fertilisation test and within 2 hours of semen collection, sperm motility, duration of motility and spermatozoa density were analysed from each collected semen sample (1 µL) using a hemocytometer. The motility was scored based on the percentage of motile sperm in a semen sample after dilution with a scale between zero and five: zero, no motility; one, 1 – 20%; two, 21 – 40%; three, 41 – 60%; four, 61 – 80% five, 81 – 100% motile (Fauvel *et al*., 1999). Distilled water was used as the dilution/activation medium. The time from the sperm activation to the end of sperm motility was recorded. Finally, spermatozoa density was estimated using immotile

spermatozoa and a hemocytometer. The number of spermatozoa was counted in five of twenty five squares within the central counting area of two chambers, and it was carried out in triplicate for each sample. The sperm density was calculated by this following formula: Spermatozoa density (cell/mL) = average cells count in 5 squares from two chambers in triplicates × 5 × dilution factor × 10,000.

2.7 Statistics

Data are presented as mean ± SEM. Statistical analysis was performed using Minitab 17 (Minitab Inc., State College, USA). Data was transformed when necessary to meet the homogeneity of variance and normal distribution. Significant differences in data between different developmental stages and temperatures (morphometric indices, expression level of germline-specific genes and Bcl-2 family genes and serum levels of 11-KT and T) were tested by two-way analysis of variance (ANOVA), followed by Tukey's HSD test ($p < 0.05$). Two-Sample t-test was used to evaluate the significant difference in sperm quality (motility, duration of motility, density and fertilisation rate) ($p < 0.05$).

3. Results

3.1 Mortality and morphometric indices

During the first window of the heat treatment (up to 1200 DD), mortalities in CT, HT1 and HT2 were 2.5, 3.3 and 4.2%, respectively. There was no mortality in CT and HT1 between 1200 and 2100 DD, but 12.3% in HT2. During the last phase of the heat treatment (2100 to 3000 DD), mortalities of 7.4 and 16.2% were observed in CT and HT2, respectively, and none in HT1. While mortalities in CT and HT1 were most likely caused by cannibalism as evidenced by eye and body injuries, dead fish in HT2 did not show any external injuries but empty stomach and poor condition.

Significant time, temperature and interaction effects were observed in body weight ($F = 147.83$; $p < 0.001$, $F = 103.62$; $p < 0.001$ and $F = 16.22$; $p < 0.001$, respectively) at 3000 and 5200 DD. At the end of the heat treatment (3000 DD), HT1 and HT2 mean body weight was 4.5 and 11.1 times lower than for CT, respectively (Table 2). HT1 and HT2 fish significantly gained weight during the recovery

period; however, the weights remained significantly lower than that of control at 5200 DD (1.9 and 2.9 times lower, respectively).

For the GSI, significant time and temperature effects were observed ($F = 132.89$, $p < 0.001$ and $F = 10.89$, $p < 0.001$, respectively) without an interaction effect ($F = 1.96$, $p = 0.159$) at 3000 and 5200 DD. GSI in HT1 and HT2 were significantly lower than CT at 3000 DD, while GSI at 5200 DD were comparable between the temperature treatment groups (Table 2).

For the HSI, significant time, temperature and interaction effects were observed ($F = 8.00$; $p = 0.008$, $F = 3.66$; $p = 0.038$ and $F = 32.68$; $p < 0.001$, respectively) at 3000 and 5200 DD. HT1 and HT2 fish has significantly lower HSI than that of CT at 3000 DD (1.9 and 2.3 times lower, respectively), but HSI of HT fish became significantly higher than CT at 5200 DD (1.7 and 1.5 times higher, respectively for HT1 and HT2).

For the VSI, significant time, temperature and interaction effects were observed ($F = 5.55$; $p = 0.025$, $F = 35.45$; $p < 0.001$ and $F = 57.18$; $p < 0.001$, respectively) at 3000 and 5200 DD. VSI of HT1 and HT2 fish were 1.7 and 2.5 times lower than that of CT at 3000 DD, respectively, but became comparable to CT at 5200 DD.

3.2 Testis histology

Prior to the heat treatment at 0 DD (120 dah), the average weight, total body length and height of 0 DD fish were 3.1 ± 0.4 g, 5.8 ± 0.2 cm and 1.8 ± 0.1 cm ($n = 6$). All testes at 0 DD weighed less than 0.01 g, but the three investigated groups observed histologically showed advanced stage of testicular germ cells (see Supplemental Fig. S1).

Throughout the experiment, CT fish developed mature testes showing active spermatogenesis with well-structured seminiferous lobules (Fig. 2A-E). At 1200 DD, a reduction in the number of spermatids was observed in both HT1 and HT2 groups with increased vacuolation; however, spermatogonia and spermatocytes were still observed (Fig. 2F&K). Leydig cell hyperplasia was also observed with an apparent dense eosinophilic cytoplasm in HT treated fish from 1200 DD to 3800 DD (Fig. 2F-I&K-N). At 2100 DD, the level of atrophy and vacuolation increased in testes from both HT1

and HT2 fish, but the number of testicular germ cells was greatly reduced in HT2 compared to HT1 (Fig. 2G&L). By the end of the temperature treatments (3000 DD), testes of HT2 fish displayed increased severity of atrophy and vacuolation with testes from 7 out of 12 fish (58.3%) showing almost no testicular germ cells at any stages (Fig. 2M). In contrast, testis from all HT1 fish at 3000 DD presented all phases of testicular germ cells despite an overall depletion of testicular germ cells and somatic cells (Fig. 2H). Testis from HT1 treated fish appeared to rapidly resume spermatogenesis with spermatozoa in the lumen of the seminiferous lobules by 5200 DD (Fig. 2I&J and Supplemental Fig. S2B). However, two different groups in HT2 were observed at 3800 DD: one was lacking almost all phases of testicular germ cells (33%, 2/6) (Fig. 2N1), the other contained spermatogonia but no other spermatogenic stages (67%, 4/6) (Fig. 2N2). At the end of the recovery period (5200 DD), the majority of HT2 fish appeared to recover as evidenced by the presence of spermatozoa in the lumen (75%, 9/12) (Fig. 2O2), while a few (25%, 3/12) still appeared to lack spermatozoa at this time (Fig. 2O1 and Supplemental Fig. S2C #3, 5 & 12). Testes from HT2 males with less spermatozoa (Supplemental Fig. S2C #1 & 11) or lack of spermatozoa (Supplemental Fig. S2C #3, 5 & 12) were semi-transparent (Supplemental Fig. S3C #1, 3, 5, 11 & 12) while the rest of the testes were whitish to pinkish in colour similar to CT and HT1 testes (see Supplemental Fig. S3). These HT2 males with less or lack of spermatozoa (Supplemental Fig. S2C #1, 3, 5, 11 & 12) also had a significantly lower GSI than the rest of HT2 males at 5200 DD ($p < 0.05$) but their body weight was not significantly different.

3.3 Sperm quality

No significant differences in sperm motility and duration of sperm motility were observed between treatments (Table 3). On the other hand, sperm densities of HT1 ($p = 0.037$) and HT2 ($p = 0.001$) were significantly lower than CT, showing 2- and 27-fold decreases compared to CT, respectively. The fertilisation rate was significantly reduced in HT2, compared to CT ($p < 0.001$) and HT1 ($p < 0.01$) treatments, being 72.5% reduced compared to the CT treatment (Table 3).

3.4 Cortisol and sex steroid hormones

Serum cortisol levels were not significantly different between treatments during the whole experimental period with levels ranging from 124 and 275 ng/mL (data not shown). Significant time, temperature and interaction effects were observed in serum 11-KT (F = 5.48; p =0.001, F = 5.37; p = 0.007 and F = 8.87; $p < 0.001$, respectively) while T levels showed significant interaction effects (F = 4.90; $p < 0.001$) without significant time and temperature effect. Serum 11-KT and T levels in fish from HT1 and HT2 were higher than that of CT at 1200 and 2100 DD with significant differences between HT1 and CT (Fig. 3). Levels then decreased to comparable levels than in CT fish at 3000 DD. During the first phase of the recovery window at 3800 DD, both serum 11-KT and T levels in HT1 and HT2 fish were significantly lower than that of CT fish, but levels returned to CT levels by 5200 DD (Fig. 3).

3.5 Gene expression pattern

3.5.1 Germline-specific genes (*piwil, nanos* and *gfra1*)

Significant time, temperature and interaction effects were observed in relative gene expression levels of *piwil1* (F = 47.91, $p < 0.001$; F = 145.07, $p < 0.001$ and F = 8.29, $p < 0.001$, respectively) and *piwil2* (F = 30.49, $p < 0.001$; F = 86.43, $p < 0.001$ and F = 5.27, $p < 0.001$, respectively). Expression levels of *piwil1* and *piwil2* in testis of HT1 and HT2 fish remained significantly lower than CT during the high temperature exposure window (1200 to 3000 DD) and the first phase of recovery (3800 DD), with HT2 being significantly lower than HT1 from 2100 to 3800 DD (Fig. 4A&B). During the last phase of the recovery period (3800 to 5200 DD), HT1 fish showed comparable expression levels to CT of both *piwil1* and *piwil2*; however, the levels remained significantly lower in HT2 compared to CT and HT1 at 5200 DD.

Significant time, temperature and interaction effects were observed in relative gene expression levels of *nanos2* (F = 14.60, $p < 0.001$; F = 69.63, $p < 0.001$ and F = 8.22, $p < 0.001$, respectively) and *nanos3* (F = 3.37, p =0.008; F = 35.65, $p < 0.001$ and F = 3.41, p = 0.001, respectively). Expression levels of *nanos2* in both HT1 and HT2 fish were significantly lower than CT during the high temperature exposure window (1200 to 3000 DD) with HT2 being significantly lower than HT1

(1200 to 3000 DD) (Fig. 4C). On the other hand, only HT2 fish showed significantly lower expression levels of *nanos3* compared to CT during the heat treatment period (1200 to 3000 DD), while HT1 showed comparable expression levels to CT (Fig. 4D). During the recovery period (3800-5200 DD), expression levels of *nanos2* in HT2 fish remained significantly lower than CT and HT1, while the levels of *nanos3* increased in both HT1 and HT2 and reached the similar level to CT.

Significant time, temperature and interaction effects were observed ($F = 3.88$, $p = 0.003$; $F = 18.60$, $p < 0.001$ and $F = 2.76$, $p = 0.005$, respectively) in relative expression level of *gfra1*. Only HT2 fish showed significantly lower *gfra1* levels than CT fish between 1200 and 2100 DD (Fig. 4E). Following the return of all fish to control temperature, *gfra1* levels in HT1 fish were significantly higher than CT at 3800 DD, and no significant differences were observed between treatments at 5200 DD.

Testes from HT2 males sampled at 5200 DD appeared to contain less spermatozoa and even showed an apparent complete lack of spermatozoa in some cases (Supplemental Fig. S2C #1, 3, 5, 11 & 12). Expression levels of *piwil1*, *piwil2*, *nanos2* and *nanos3* was significantly lower in these testes than for the rest of the HT2 males (Supplemental Fig. S2C #2, 4, 6 – 10) ($p < 0.05$) however there was no significant difference in *gfra1* (data not shown).

3.5.2 Bcl-2 family genes

Significant time, temperature and interaction effects were observed in relative gene expression levels of *bcl-xLa* ($F = 7.96$, $p < 0.001$; $F = 22.59$, $p < 0.001$ and $F = 3.42$, $p = 0.001$, respectively), *bcl-xLb* ($F = 37.82$, $p < 0.001$; $F = 172.97$, $p < 0.001$ and $F = 11.10$, $p < 0.001$, respectively), *baxa* ($F = 45.84$, $p < 0.001$; $F = 78.97$, $p < 0.001$ and $F = 6.73$, $p < 0.001$, respectively), *baxl* ($F = 14.31$, $p < 0.001$; $F = 8.45$, $p < 0.001$ and $F = 2.46$, $p = 0.012$, respectively), *bcl-2* ($F = 9.50$, $p < 0.001$; $F = 7.64$, $p = 0.001$ and $F = 3.28$, $p = 0.001$, respectively) and *mcl-1a* ($F = 5.40$; $p < 0.001$, $F = 4.47$; $p = 0.014$ and $F = 3.17$; $p = 0.002$, respectively).

The expression level of *bcl-xLa* in HT2 fish were significantly lower than CT and HT1 at 2100 and 3800 DD (Fig. 5A). The pattern of *bcl-xLb* and *baxa* mRNA levels were similar to those of *piwil1*

and *piwil2* (Fig. 5B & C, Fig. 4A & B). The expression levels of *bcl-xLb* and *baxa* were significantly lower in both HT1 and HT2 fish than CT fish from 1200 to 3800 DD with HT2 being significantly lower than HT1 from 2100 to 3800 DD, and levels remained significantly lower in HT2 compared to CT and HT1 at 5200 DD (Fig. 5B & C). No significant differences in expression levels of *baxl* between temperature treatment groups were observed except for *baxl* levels in HT1 at 1200 and 3800 DD which were significantly higher than CT (Fig. 5D). No changes in the expression levels of *bcl-2* between temperature treatment groups were observed during the high temperature treatment (Fig. 5E). During the recovery period, HT1 fish showed significantly higher levels of *bcl-2* than CT at 3800 DD and HT2 fish at 5200 DD. The expression levels of *mcl-1a* between temperature treatment groups were not different except for *mcl-1a* levels in HT1 at 1200 DD which were significantly higher than CT and *mcl-1a* levels in HT2 at 3800 DD which were significantly lower than CT and HT1 (Fig. 5F).

Within HT2 males at 5200 DD, it was also noted that testes with less or lack of spermatozoa (Supplemental Fig. S2C #1, 3, 5, 11 & 12) showed significantly lower expression levels of *bcl-xLb* and *baxa* and significantly higher expression of *bcl-2* than the rest of HT2 male testes (Supplemental Fig. S2C #2, 4, 6 – 10) ($p < 0.05$) (data not shown).

4. Discussion

The aim of this study was to explore potential targets which play an important role in the survival or apoptosis of germ cells by utilizing HT as a means to induce germ cell death. Male tilapia exposed from 120 dah to HT treatments (36 and 37°C) for a duration of 3000 DD showed a significant loss of testicular germ cells compared to the control fish kept at 27°C. However, the HT treatments did not induce complete sterility in Nile tilapia. Importantly, the impact of HT on spermatogenesis studied at morphometric, histological, endocrine, gene expression and sperm quality levels, differed between 36 and 37°C with more acute and lasting effects being observed in fish exposed to 37°C.

In contrast to previous work performed in tilapia (Nakamura *et al*., 2015; Pandit *et al*. 2015), this study examined the impact of temperature-induced germ cell loss at the molecular level. Both *piwil1* and *piwil2* have previously been reported to be expressed in various phases of testicular germ

cells except for mature sperm in Nile tilapia (Xiao *et al*., 2013). In CT fish, both *piwil1* and *piwil2* expression levels gradually increased during the study in line with the progression of spermatogenesis. The significantly reduced levels of *piwil1* and *piwil2* in HT fish were associated with the suppression of spermatogenesis and reduction in testicular germ cells, which were more acute in fish exposed to 37°C. The expression levels of both *piwil1* and *piwil2* in HT2 fish increased significantly during the recovery period compared to levels during the heat treatment. However, the significantly lower expression in HT2 compared to CT and HT1 during the recovery period was in accordance with the observed reduced functionality of the testis/sperm at the end of the study.

Unlike *piwil1* and *piwil2* which are expressed in various phases of testicular germ cells (Xiao *et al*., 2013), *nanos2*, *nanos3* and *gfra1* are known as SSC-specific markers in mammals and fish (Bellaiche *et al*., 2014; Suzuki *et al*., 2009). The expression patterns of *nanos2*, *nanos3* and *gfra1* in CT fish were not significantly altered during the study period, implying that there was no significant change in the number of SSC and/or spermatogonia during this period. Interestingly, the expression levels of *nanos3* and *gfra1* were reduced in HT2 fish only, while *nanos2* was downregulated in both HT1 and HT2 fish. Suzuki *et al*. (2009) reported differential expression of *gfra1*, *nanos2*, *nanos3* and *ngn3* (*neurogenin 3*) within undifferentiated spermatogonial stages and proposed that SSCs are *gfra1* & *nanos2* positive and *nanos3* & *ngn3* negative, while *nanos3* & *ngn3* are widely expressed in undifferentiated spermatogonia in mice. Likewise, Lacerda *et al*. (2013) reported both *gfra1* and *nanos2* as SSC markers in Nile tilapia with *gfra1* being expressed exclusively in single type A undifferentiated spermatogonia (presumptive SSCs), while *nanos2* is also expressed in type A differentiated spermatogonia. Therefore, the decrease of *gfra1* expression levels in fish exposed to HT2 reflects a significant reduction of single type A undifferentiated spermatogonia at 37°C but not at 36°C (HT1). The role of *nanos3* in spermatogenesis in this species has not yet been reported but *nanos3*-expressing spermatogonia were only significantly decreased in HT2 fish, suggesting *nanos3* as a marker of undifferentiated spermatogonia similar to *gfra1* in this species. Further investigation is required to reveal the function of *nanos3* during spermatogenesis. On the other hand, the expression levels of *nanos2* suggest that type A differentiated spermatogonia might be significantly reduced by

both temperature treatments, more so in fish exposed to 37°C. These differences in the relative expression patterns of *nanos2*, *nanos3* and *gfra1* between fish exposed to heat treatment in the present study support a more acute effect of 37°C than 36°C on the survival of different phases of spermatogonia in Nile tilapia. It was further supported by histological changes in testes and the significantly lower sperm density in fish exposed to 37°C due to the loss of spermatogonia.

The present study profiled apoptosis-related genes to investigate the apoptosis mechanisms in germ cells under HT. Apoptosis is a process of programmed cell death which is essential for normal development and maintenance of normal cellular homeostasis in metazoan (Danial and Korsmeyer, 2004). The initiation of apoptosis is firmly regulated as once apoptosis has begun cell death is inevitable (Böhm and Schild, 2003). Apoptosis is initiated by two pathways, extrinsic and intrinsic pathways (Chauhan *et al*., 1997); the extrinsic pathway is mediated by the binding between extracellular death ligands and cell-surface death receptor (Wajant, 2002) while the intrinsic pathway is initiated by interaction between Bcl-2 family proteins and mitochondria (Danial and Korsmeyer, 2004). It is known that in the intrinsic pathway, a change in ratio between prosurvival and proapoptotic Bcl-2 family proteins is the decisive step for the initiation of apoptosis (Jia *et al*., 2007; Oltval *et al*., 1993; Rucker *et al*., 2000). Among Bcl-2 family genes, *bcl-xL* genes are known to be pro-survival genes and their downregulation has been reported to cause the release of cytochrome c and activation of caspase cascades (Brockhaus and Brüne, 1999). In the current study, long-term exposure to HT induced the downregulation of *bcl-xLb* but not *bcl-xLa*, which suggests differential functions between these paralogs under heat stress. The expression pattern of *bcl-xLb* was similar than for *piwil1* and *piwil2,* suggesting that a decrease of pro-survival gene expression may be correlated to the loss of germ cells. On the other hand, pro-apoptotic genes, *bax* genes can be separated into three clades, *baxa*, *baxb* and *baxl*; *baxa* is clustered with mammalian homologs while *baxb* and *baxl* are absent in amphibians and mammals (Li *et al*., 2017). In this study, *baxa* and *baxl* genes were investigated and HT suppressed the expression of *baxa* but appeared to have no effects on the expression of *baxl*, suggesting also potential functional differences between these two genes under heat stress. Similar to the expression patterns of *bcl-xLb*, *piwil1* and *piwil2*, levels of *baxa* transcripts

were significantly lower in HT fish with more severe impact at 37°C. The downregulation of pro-apoptotic gene expression might serve as a protection mechanism in testes under HT or possibly as a result of testicular germ cell loss. Among six Bcl-2 family genes investigated in this study, *bcl-xLb* and *baxa* expression levels were significantly altered by the long-term HT treatments in Nile tilapia, while *bcl-xLa, baxl, mcl-1a* and *bcl-2* were not significantly affected. Given that the imbalance between pro-survival and pro-apoptotic Bcl-2 family proteins is the trigger of apoptosis (Jia *et al*., 2007; Oltval *et al*., 1993; Rucker *et al*., 2000), it can be speculated that the significant decrease of *bcl-xLb* and *baxa* at 36°C and 37°C compared to 27°C might disrupt the rheostat of pro-survival/pro-apoptotic Bcl-2, which then in turn might cause testicular germ cell loss through apoptosis. Further functional analysis of Bcl-2 family genes is desired to elucidate the apoptotic pathway of testicular germ cells under HT in this species.

The significant growth retardation caused by HT exposure was accompanied by a significant reduction in HSI and VSI at the end of the HT window, indicating the low energy status and metabolism in HT fish (Lambert and Dutil, 1997; Nunes *et al*., 2011; Wootton *et al*., 1978). In addition, the significantly lower GSI values at the end of HT reflect the suppressive effects of HT on spermatogenesis. Interestingly, these effects were transient as GSI but also HSI and VSI showed signs of recovery at the end of the recovery window demonstrating the high resilience of this species to HT. These effects on growth, lower HSI, VSI and GSI at the end of the HT window could be associated with reduced appetite and food intake as shown in fish under heat stress (Azaza *et al*., 2008; Brett, 1979; Handeland *et al*., 2008; Pandit and Nakamura, 2010). At the end of the recovery window, HT male tilapia showed significantly reduced sperm density compared to control fish exposed to 27°C with a more severe reduction in HT2 fish, further supported by a significantly reduced fertilisation rate in HT2 compared to CT and HT1 fish. This link between sperm density and fertilisation rate has previously been reported in a number of teleost species (Aas *et al*., 1991; Tvedt *et al*., 2001). However, it was noted that both motility of sperm and duration of motility were not significantly different between HT and control fish, implying the fitness and viability of the sperm were similar to CT (Stoss and Holtz, 1983). In the current study, heat treatment at 37°C did not result in sterility

indicating that some SSCs were able to survive and proliferate to become functional spermatozoids in fish returned to a favourable temperature. The contrasting results obtained in the present study compared to a previous study in Mozambique tilapia showing a complete lack of testicular germ cells in fish exposed to 37 ± 0.5°C for 50 days from 3 dah (Nakamura *et al*., 2015), could be explained by the different developmental stages and species. As larval germ cells are in a more vulnerable state due to the insufficient support and protection provided by testicular somatic cells such as Sertoli cells (Boekelheide *et al*., 2000), a complete loss of germ cell could be induced. Even though HT treatment failed to induce full sterility in male tilapia in this study, it gave us an insight into the molecular mechanisms underlying germ cell loss induced by heat stress which could not be done at a larval stage.

Germinal epithelium is the source of germline stem cells containing Sertoli cells and spermatogenic cells (Schulz *et al*., 2010). In Nile tilapia, temperature modulates Sertoli cell proliferation as shown in seasonal fish (de Alvarenga and de França, 2009). The number of Sertoli cells dictates the capacity of the testis to support testicular germ cells as each Sertoli cell is able to support a fixed number of germ cells (Matta *et al*., 2002). Therefore, residual vacuolated Sertoli cells observed in some atrophic germinal epithelium in HT fish is likely to contribute to the loss of testicular germ cells. There was also notable Leydig cell hyperplasia observed in fish exposed to HT while their body weight and GSI were significantly lower than fish exposed to 27°C, indicating a higher proportion of Leydig cells in HT fish compared to control fish. The Leydig cell hyperplasia is commonly related to atrophic tubules in mammals (Greaves, 2012). In addition, elevated androgen levels were reported in diffuse Leydig cell hyperplasia in mammals (Akingbemi *et al*., 2004; Wilson and Netzloff, 1983). Thus, the significant increase in circulating plasma androgens (T and 11-KT) levels in HT fish is thought to be caused by the Leydig cell hyperplasia without an apparent effect on plasma cortisol levels. Stressors modulate fish reproduction through endocrine and paracrine pathways including androgens and cortisol by affecting the hypothalamus–pituitary–interrenal axis (Schreck, 2009). The lack of HT effects on cortisol might be due to the chronic rather than acute nature of the stressor, although significant effects on growth may suggest otherwise. In addition, the prolonged high levels of androgens in HT fish might accelerate the testicular germ cell death. It was

reported that high plasma androgen levels can cause irreversible damage to SSCs and especially when germ cells are damaged by external factors (*e.g.* cytotoxic treatment, irradiation) they are more vulnerable to excessive androgen (Dohle *et al*., 2003; Meistrich *et al*., 2003). Excessive 11-KT related to impaired spermatogenesis was also reported in *figla* (*factor in the germ line, alpha*)-over-expressed XY tilapia (Qiu *et al*., 2015). Taken together, it can be postulated that the overabundance of 11-KT and T induced by HT might accelerate the loss of testicular germ cells but it is unclear whether the increase of androgens is also the consequence of the germ cell loss. Further investigation is required to understand the temperature-mediated control of androgen production and its effect on germ cell survival.

Overall this work has provided a new perspective, at the molecular level, of mechanisms underlying testicular germ cell loss under heat stress. Importantly, this research suggested the functional importance of *piwil* genes, in particular, in relation to germ cell loss and proliferation which prioritises them over the other candidates as a potential target for gene editing to induce sterilisation of Nile tilapia. At the same time this work looked at the molecular regulation of apoptosis in tilapia and it highlighted potential subfunctionalisation of gene roles within the Bcl-2 gene family that should be further investigated. HT treatment clearly impacted on spermatogenesis in Nile tilapia with the acuteness of the response being apparently temperature-dependent; however, given the observed physiological impacts on growth, energy status and survival, it can be concluded that thermal induction of sterility is not a commercially viable approach to be taken forward.

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Table 1. Primers used for qRT-PCR and values of the standard curve

Oligo name		Oligo sequence (5'-3')	Size	Product length	Tm (°C)	Accession No.	Standard curve		
							Efficiency	R^2	Range
piwil1	F	ATGATCGTGGGCATCGACTGCTA	23	97	62	XM_003445546.2	0.968	0.998	$10\text{-}10^7$
	R	ACCACCTGCTCATGCTTTGGTTG	23						
piwil2	F	TGCCATCAAGAAGCTGTGCTGTG	23	73	62	XM_003445662.2	0.909	1.000	$10\text{-}10^6$
	R	CTGGGAAATTGTGCGGACGTTGA	23						
nanos2	F	CGGGAAAGTTTTCTGCCCCATCC	23	140	62	XM_005448855.1	0.944	0.999	$10\text{-}10^7$
	R	AGAACTTGGCCCCTGTCTCCATC	23						
nanos3	F	GGAGTGTGACATGAGCCGAGCTA	23	116	62	XM_005460553.1	0.903	0.996	$10\text{-}10^7$
	R	AACTCGTTAGTGCACATTCGCGG	23						
gfra1	F	AAGCGACCAAACAGCACGGTAAG	23	92	60	XM_003441935.3	0.924	0.999	$10\text{-}10^7$
	R	GTCTGTTTTCCACACAGCAGCCA	23						
bcl-xLa	F	GGCTTTATGACACCGGCACAACA	23	110	62	XM_003456961.4	0.911	0.995	$10\text{-}10^6$
	R	TGCACCTGGAGTATGAACAGGCA	23						
bcl-xLb	F	GGAGGATGGGACCGCTTTACAGA	23	109	62	XM_003442737.4	0.920	0.998	$10^4\text{-}10^7$
	R	GGAGGATGGGACCGCTTTACAGA	23						
baxa	F	CGGTCGGGGTTTTCTTGGCA	20	128	60	XM_019357746.2	0.981	0.999	$10\text{-}10^7$
	R	TCCTCCTGTGCCCATTTCCC	20						
baxl	F	GCTCGAGTCAGTTCAGACTGGGT	23	83	62	XM_003456558.5	0.950	0.999	$10\text{-}10^7$
	R	AAACTCCCTCGGGTCTCTGTGTG	23						
mcl-1a	F	TATGTCATTTGTAGCGAAGAGCCT	24	124	60	XM_013264682.1	0.906	0.998	$10\text{-}10^7$
	R	GCAGTTGTCCCTGCCCTTTT	20						
bcl-2	F	ACCCCTGCTCGTTAGGGTGA	20	140	60	XM_003437902.4	0.905	0.996	$10\text{-}10^6$
	R	CACTGCGCTGGTTGTAGCGA	20						
β-actin	F	ATCTACGAGGGTTATGCCCTGCC	23	118	62	KJ126772.1	0.934	0.996	$10\text{-}10^7$
	R	CTGTGGTGGTGAAGGAGTAGCCA	23						
elf1α	F	CTGGACAAACTGAAGGCTGAGCG	23	116	62	NM_001279647.1	0.967	0.999	$10^2\text{-}10^7$
	R	AAGTCTCTGTGTCCAGGGGCATC	23						

Table 2. Morphometric indices (body weight, GSI, HSI, and VSI) at 3000 and 5200 DD. Data are presented as mean ± SEM (n = 6 fish/treatment/time point). Superscripts denote statistically significant differences between temperature and time points ($p < 0.05$) except for GSI where it denotes significant differences between temperature at each time point ($p < 0.05$)

	3000 DD			5200 DD		
	CT	**HT1**	**HT2**	**CT**	**HT1**	**HT2**
Body weight (g)	226.7 ± 26.2^{ab}	50.1 ± 9.4^{d}	20.4 ± 2.5^{e}	350.7 ± 30.9^{a}	189.4 ± 21.6^{bc}	120.6 ± 8.9^{c}
GSI (%)	0.17 ± 0.09^{a}	0.05 ± 0.01^{b}	0.03 ± 0.00^{b}	0.56 ± 0.09^{a}	0.40 ± 0.07^{a}	0.33 ± 0.07^{a}
HSI (%)	2.96 ± 0.11^{a}	1.58 ± 0.16^{b}	1.26 ± 0.15^{b}	1.71 ± 0.15^{b}	2.83 ± 0.26^{a}	2.48 ± 0.19^{a}
VSI (%)	13.04 ± 0.65^{a}	7.54 ± 0.18^{b}	5.21 ± 0.15^{c}	7.01 ± 0.63^{bc}	8.68 ± 0.41^{b}	7.65 ± 0.24^{b}

Table 3. Sperm quality of milt collected from CT, HT1 and HT2 fish at the end of the recovery period (5200 DD). Data are presented as mean ± SEM (n = 12). Superscripts denote statistically significant differences between temperature treatments ($p < 0.05$)

Treatments	**CT**	**HT1**	**HT2**
Motility score (0-5)	4.8 ± 0.1^{a}	4.7 ± 0.1^{a}	4.3 ± 0.3^{a}
Motility duration (min)	2.6 ± 0.1^{a}	2.4 ± 0.0^{a}	2.5 ± 0.2^{a}
Spermatozoa density ($\times 10^{9}$) (cell/mL)	2.81 ± 0.55^{a}	1.47 ± 0.13^{b}	0.10 ± 0.02^{c}
Fertilisation rate (%)	41.5 ± 4.6^{a}	35.0 ± 3.4^{a}	11.4 ± 1.8^{b}

Figure legends

Figure 1. Diagram representing the experimental design with three temperature treatments, showing sampling points based on degree-day (DD) for different temperature groups. 0 DD began at 120 dah. The black bar indicates heat treatment period and white bar shows rearing at control temperature of 27°C.

Figure 2. Histology of representative testes at 1200, 2100, 3000, 3800 and 5200 DD. (A – E) CT, fish exposed to 27°C; (F – J) HT1, fish exposed to 36°C; (K – O) HT2, fish exposed to 37°C. N1 & O1 are representative histology of lack of testicular germ cells, while N2 & O2 represents re-proliferated teste in the recovery period. SG, spermatogonia; SC, spermatocytes; ST, spermatids; SZ, spermatozoa; V, vacuolation. SG (arrowhead), atrophy (arrow) and Leydig cell hyperplasia (*) are indicated.

Figure 3. Serum sex steroid levels in Nile tilapia exposed to temperature treatments (CT, 27°C; HT1, 36°C; HT2, 37°C). (A) 11-ketotestosterone, 11-KT, and (B) testosterone, T, levels at 1200, 2100 and 3000 DD during the treatment exposure window and 3800 and 5200 DD during the recovery period (n = 6). Data are presented as mean ± SEM. Superscripts denote significant differences between temperature treatment groups at each time point ($p < 0.05$).

Figure 4. Normalised relative expression level of selected germline-specific genes in testis. (A) *piwil1,* (B) *piwil2,* (C) *nanos2,* (D) *nanos3* and (E) *gfra1*. Data are presented as mean ± SEM (n = 6). Superscripts denoted significant differences between treatments at a given time ($p < 0.05$). CT, 27°C; HT1, 36°C; HT2, 37°C.

Figure 5. Normalised relative expression level of Bcl-2 family genes in testis. (A) *bcl-xLa*, (B) *bcl-xLb*, (C) *baxa*, (D) *baxl*, (E) *bcl-2* and (F) *mcl-1a*. Data are presented as mean ± SEM (n = 6). Superscripts denoted significant differences between treatments at a given time ($p < 0.05$). CT, 27°C; HT1, 36°C; HT2, 37°C.

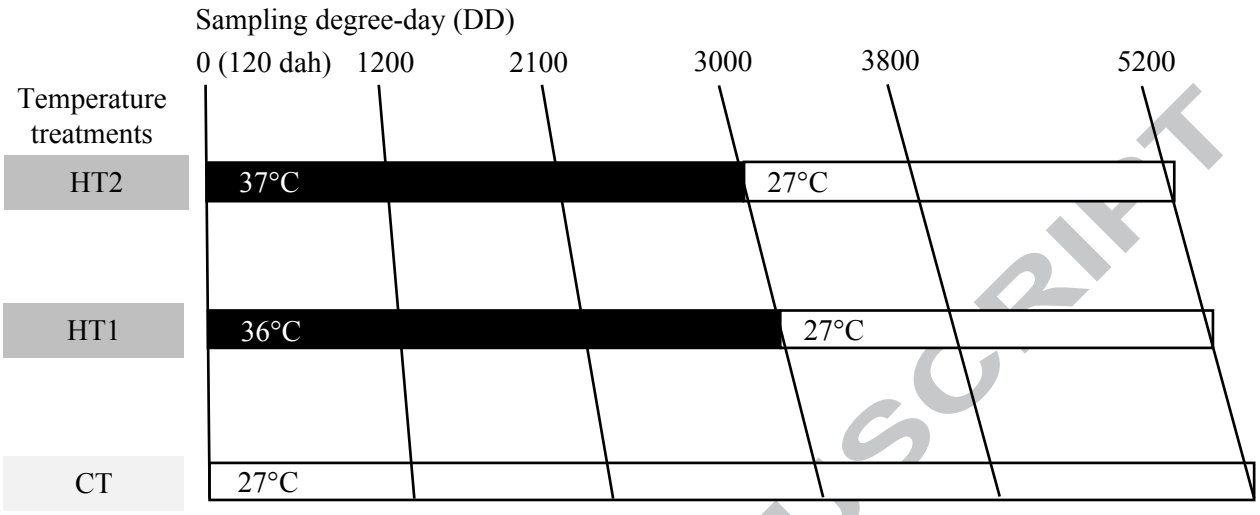

Figure 1.

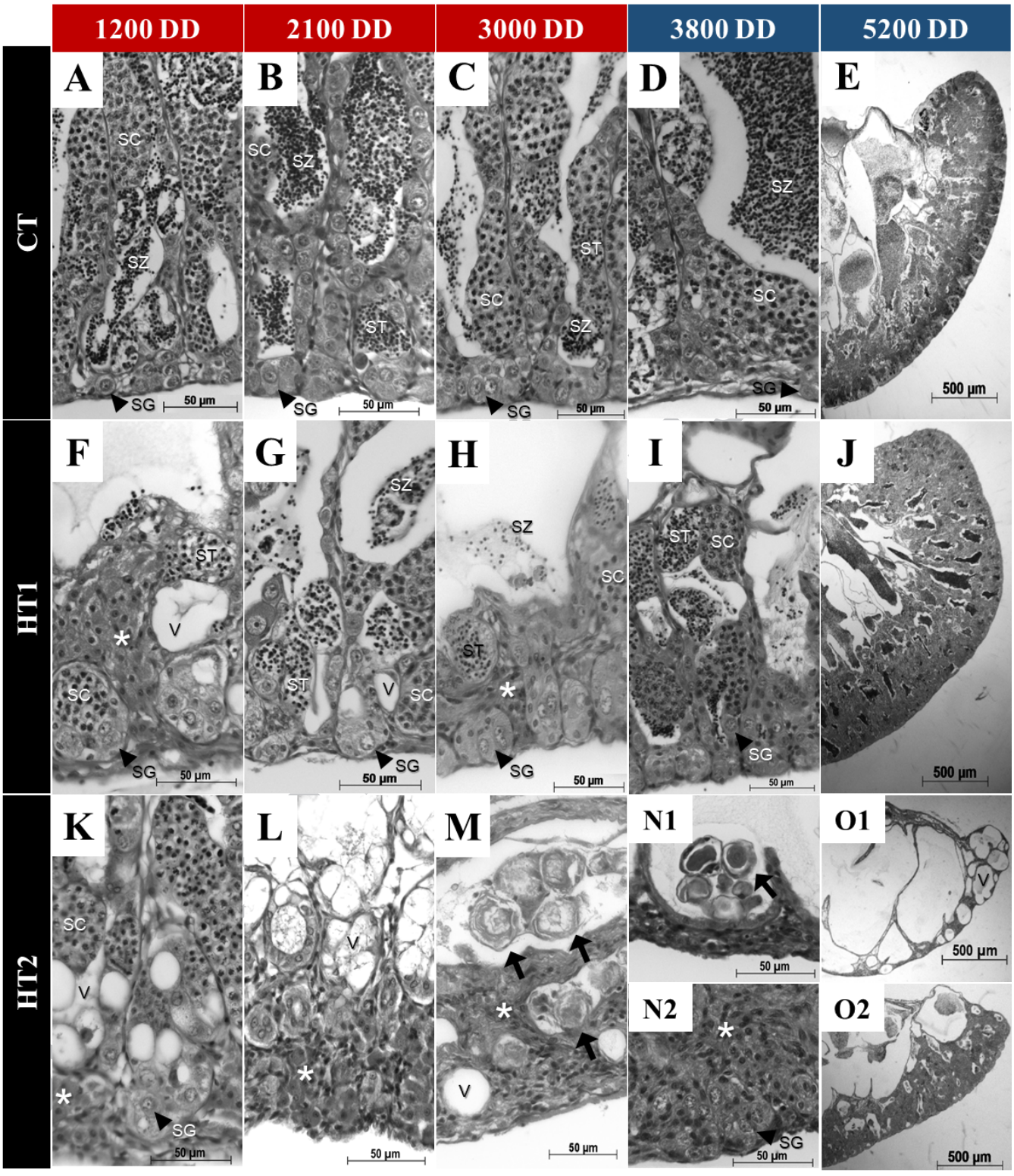
1200 DD
2100 DD
3000 DD
3800 DD
5200 DD
CT
HT1
HT2
A
B
C
D
E
F
G
H
I
J
K
L
M
N1
N2
O1
O2
SC
SZ
ST
V
SG
50 µm
500 µm

Figure 2.

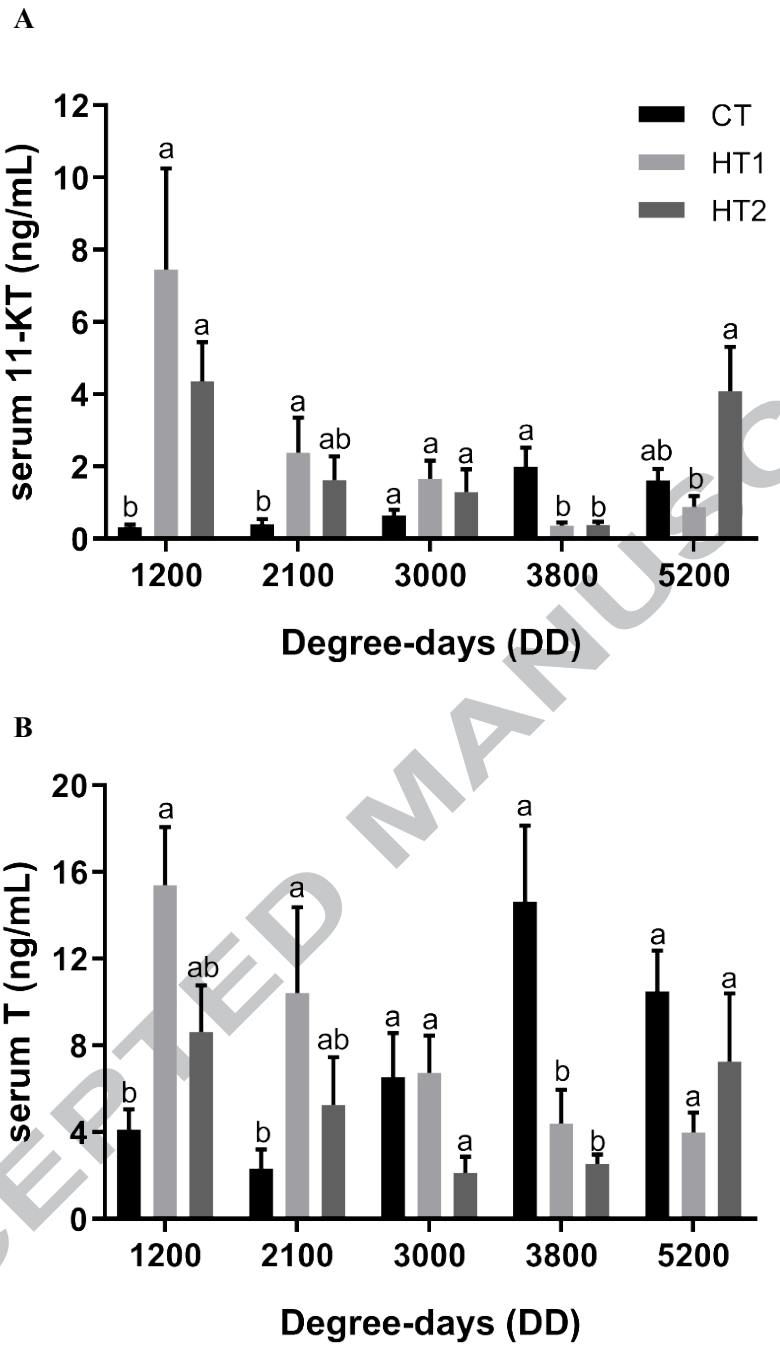

Figure 3.

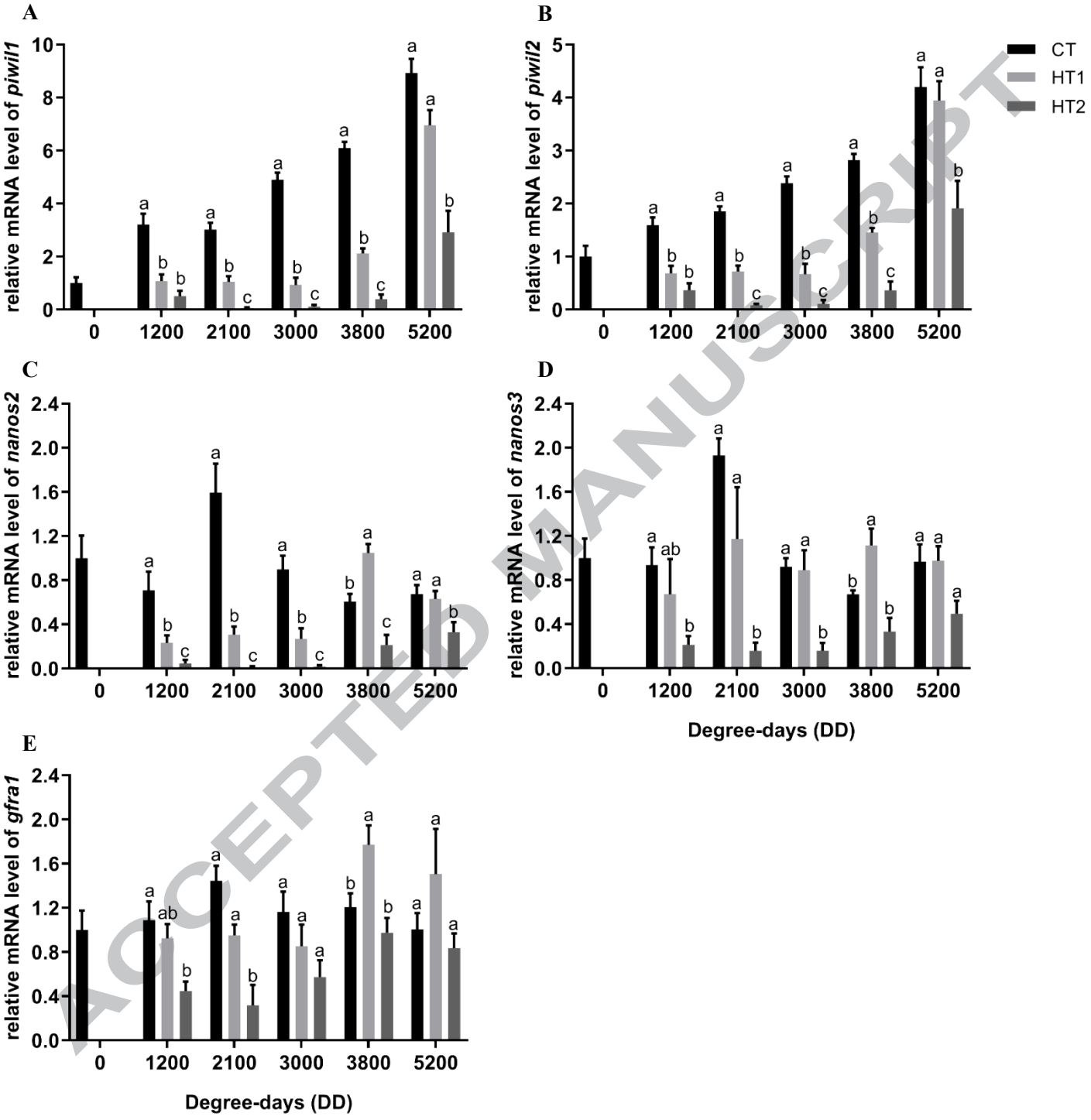

Figure 4.

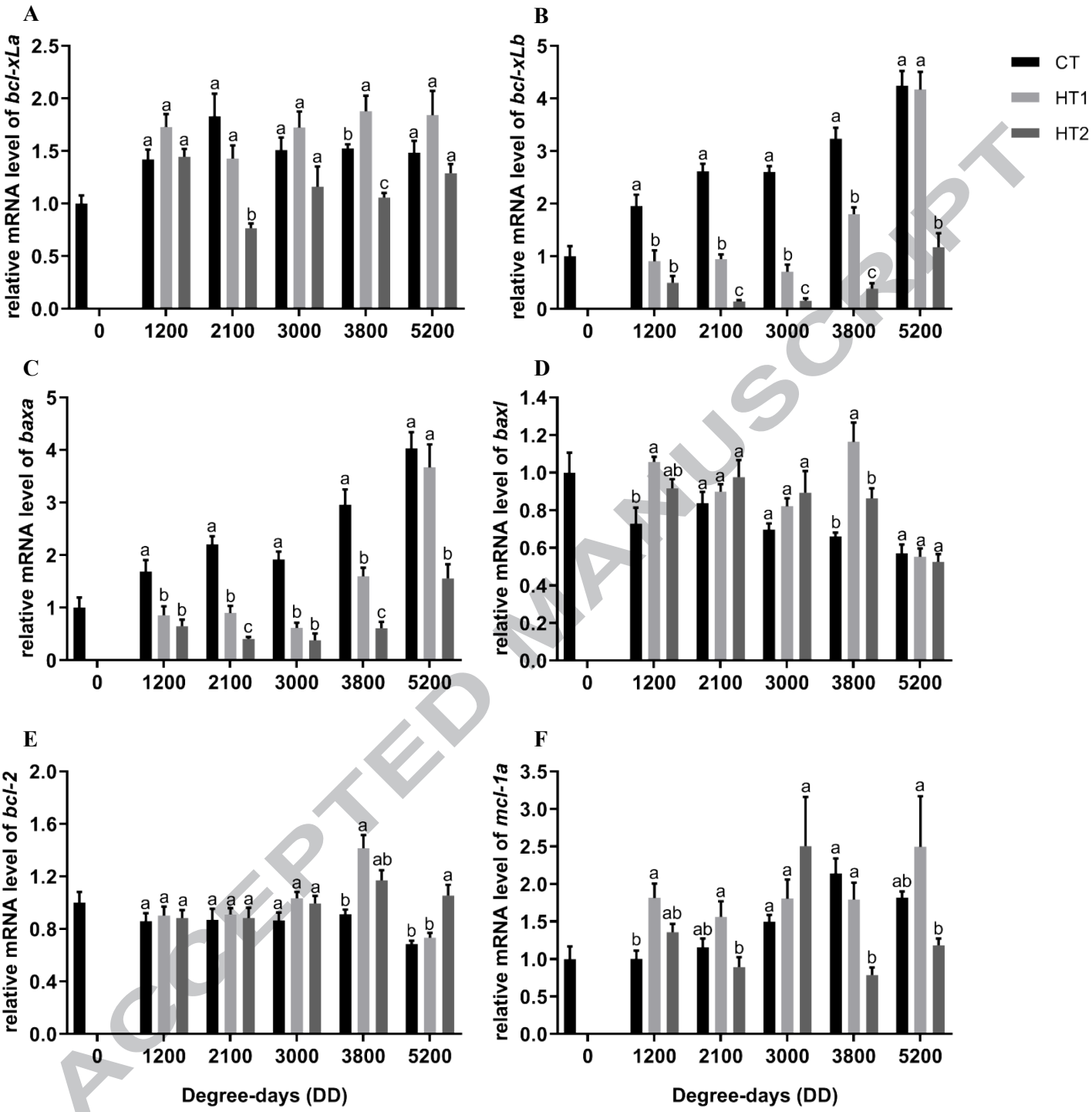

Figure 5.

Paper highlights:

- High temperature changed expression of testicular germ cell markers and bcl-2 family genes.
- Heat-induced germ cell loss was apparent with hormonal and histological changes in the testis.
- When returned to favourable temperature, germ cells appeared to proliferate producing functional spermatozoa.