



Notch pathway is required for protection against heat stress in spermatogonial stem cells in medaka

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Abstract Gamete production is a fundamental process for reproduction; however, exposure to stress, such as increased environmental temperature, can decrease or even interrupt this process, affecting fertility. Thus, the survival of spermatogonial stem cells (SSCs) is crucial for the recovery of spermatogenesis upon stressful situations. Here, we show that the Notch pathway is implicated in such survival, by protecting the SSCs against thermal stress. First, we corroborated the impairment of spermatogenesis under heat stress in medaka, observing an arrest in metaphase I at 10 days of heat treatment, an increase in the number of spermatocytes, and downregulation of *ndrg1b* and *sycp3*. In addition, at 30 days of treatment, an interruption of spermatogenesis was observed with a strong loss of spermatocytes and

spermatids. Then, the exposure of adult males to thermal stress condition induced apoptosis mainly in spermatogenic and supporting somatic cells, with the exception of the germinal region, where SSCs are located. Concomitantly, the Notch pathway-related genes were upregulated, including the ligands (*dll4*, *jag1-2*) and receptors (*notch1a-3*). Moreover, during thermal stress presenilin enhancer-2 (*pen-2*), the catalytic subunit of γ -secretase complex of the Notch pathway was restricted to the germinal region of the medaka testis, observed in somatic cells surrounding type A spermatogonia (SGa). The importance of Notch pathway was further supported by an *ex vivo* approach, in which the inhibition of this pathway activity induced a loss of SSCs. Overall, this study supports the importance of Notch pathways for the protection of SSCs under chronic thermal stress.

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Introduction

Spermatogenesis is a cellular process necessary for the formation of male gametes from spermatogonial stem cells (SSCs), which proliferate through successive synchronous divisions and differentiate into millions of spermatozoa (Sz) (La and Hobbs 2019; Schulz et al. 2010). To maintain continuous spermatogenesis throughout the male reproductive life, a

specialized microenvironment of the testes, known as SSCs niche, regulates the properties of self-renewal, quiescence, size, or the ability of the SSCs to differentiate and proliferate (Chiarini-Garcia et al. 2001; de Rooij 2017; Kitadate and Kobayashi 2010; La and Hobbs 2019; Losick et al. 2011; Mäkelä and Hobbs 2019; McIntyre and Nance 2020; Nishimura et al. 2016; Nóbrega et al. 2010). Moreover, the number of germ cells is maintained by a fine-tuned balance between proliferation and apoptosis, whose regulation takes place by genes such as *amh* (anti-müllerian hormone) or *ndrg1b* (n-myc downstream regulated gene 1b), and p53, respectively (Arias Padilla et al. 2021; Morinaga et al. 2007; Ohta et al. 2003; Rodríguez-Marí et al. 2010). This ability of the testes to maintain the homeostasis in SSCs and the decision among self-renewal, differentiation, or apoptosis is what allows them to survive under adverse conditions (La and Hobbs 2019).

Temperature is one of the most relevant environmental factors that directly affects reproduction (Alix et al. 2020). While during early development, it can affect the course of gonadal differentiation, in adult stages increasing temperature can influence the quality and quantity of gametes in the testes, thus compromising spermatogenesis and ultimately reproductive success (Blackshaw and Massey 1978; Butzge et al. 2021; Hansen 2009; Hayashi et al. 2010; Hutson et al. 2013; Mieusset and Bujan 1995; Sarida et al. 2019; Setchell 1998). In mammals, the effects of how hyperthermia compromises the different types of germ cells have been studied (Carlsen 2003; Hansen 2009), mainly with focus on apoptosis (Lue et al. 1999). In case of teleost fish, which are poikilothermic animals, it has been observed that under chronic exposure to increasing temperatures, only SSCs remain as a remnant, which have the ability to regain the germ line (Ito et al. 2008a). This resilient characteristic that allows the fish germ line to overcome a stress factor, and the interaction of SSCs in the testes, led us to think that the protection of germ cells may rely on a protective mechanism that would be specifically activated by environmental cues.

It was observed that one of the main signaling pathways involved in the regulation of the proliferation and quiescence of different stem cells (Blanpain et al. 2006; Conboy and Rando 2002), including primordial germ cells (PGCs) (Garcia and Hofmann 2013; McIntyre and Nance 2020; Xavier et al. 2013),

is the Notch pathway. This is a highly conserved juxtacrine signaling pathway and is composed of four receptors (Notch 1–4) that interact with their ligands, delta 1, 3–4, jagged 1–2 (Bray 2006; Henrique and Schweisguth 2019). After binding to the ligand in the canonical Notch signal pathway, the receptor is activated by the γ -secretase complex, where proteolysis occurs within the transmembrane domain (Fortini 2002; Selkoe and Kopan 2003; Zhang et al. 2000). After cleavage, it is translocated to the nucleus where it associates with DNA-binding proteins, to activate transcription of cis target genes, such as *hes1* and *hes5* (Fischer and Gessler 2007; Kageyama et al. 2007). Despite the numerous studies on the Notch pathway in the regulation of cell proliferation, little is known about its participation in the regulation of the proliferation of germ cells under an environmental stressor, such as temperature.

On these regard, in a previous study, it was suggested that *Pen-2*, the catalytic subunit of γ -secretase complex, acts as an anti-apoptotic gene, protecting spermatogonia from temperature during testis differentiation in fish (Fernandino et al. 2011). This catalytic member of the γ -secretase complex has been shown to play an important function in the survival of cells, protecting them from apoptosis (dos Santos et al. 2008). Selective knock-down of *pen-2* in developing zebrafish embryos resulted in strong induction of the p53-dependent apoptosis cascade in whole animal (Campbell et al. 2006). Although the function of *Pen-2* has been highly studied in brain (Fortini 2002; Xia 2019) and cancer (Nowell and Radtke 2017), its participation on the gonad has not been fully elucidated. As in teleost fish, it has also been established that mammalian germ cells experience apoptosis via the p53 cascade during exposure to high temperatures (Ohta et al. 2003); however, as other stem cells (Wabik and Jones 2015), germ cells have a protective mechanism to avoid the damage induced by apoptosis by entering into a transient state of cell-cycle called quiescence. Therefore, germ cells must retain the appropriate information and totipotency, for later recovering the reproduction (Kadekar and Roy 2019).

Based on these antecedents, we considered to evaluate the participation of Notch pathway in the mechanism of protection of germ cells against thermal stress in medaka *Oryzias latipes*. In this fish, the niche has been well characterized, where SSCs are directly surrounded by Sertoli cells and that undergo asymmetric

divisions (Nakamura et al. 2012; Nishimura et al. 2016). Moreover, the induction of high temperature exposure on the decrease in the number of germ cells has been well reported (Selim et al. 2009), making this species an excellent model to study the effects of temperature on SSCs maintenance.

Materials and methods

Source of medaka

All experiments were performed with adults of medaka *Oryzias latipes* from the hi-medaka strain (ID: MT835) supplied by the National BioResource Project (NBRP). Fish were kept under controlled laboratory conditions for this species (Kinoshita et al. 2012). Briefly, 35 male fish were reared in 10 L fish tank at 25 °C ± 1 under a photoperiod of 14 h light /10 h of darkness for 2 weeks. Then, for the heat stress experiment, the temperature was raised to 33 °C ± 1 for 30 days. The control group was kept under normal conditions of the rearing room at 25 °C ± 1. For sampling, 5 fish from each group had their testes removed for subsequent analysis at 3, 10, and 30 days after the beginning of the experiment (Fig. 1A). Fish were handled in accordance with the Universities Federation for Animal Welfare Handbook on the Care and Management of Laboratory Animals (www.ufaw.org.uk) and internal institutional regulations (CICUAE-UNSAM 17/21).

Ex vivo experiments with DAPT

To carry out ex vivo experiments with the testicular sections, fish were anesthetized by cooling on ice and then euthanized. The testes were then dissected under the stereomicroscope, using sterile forceps and scissors. The gonads were removed and placed in 1 M phosphate buffer saline (PBS) with 1 × streptomycin penicillin (Gibco; Thermo Fisher Scientific™, Waltham). They were then rinsed, subsequently cut into pieces of about 1 mm, and left in L15 medium (Gibco) with antibiotic overnight at 25 °C (Fig. 4A). Then, 2 to 4 pieces of testis were placed in each well with 1 ml of medium with DAPT (Selleckchem, Houston), a γ -secretase inhibitor that indirectly inhibits Notch, at concentrations of 12.5 μ M and 25 μ M. The control was incubated with DMSO (Sigma-Aldrich;

Merck, Rahway), at a final concentration of 0.1%, which was used as a diluent of DAPT. Incubation was performed for 24 h in a stove (Thermo Fisher Scientific™) at 25 °C (NT) or 33 °C (HT) and 5% CO₂.

Total RNA extraction and real-time PCR

Half of each testis was removed from males for gene expression analysis. Total RNA extraction was carried out using 300 μ L of TRIzol® Reagent (Invitrogen; Thermo Fisher Scientific™), according to the manufacturer's instructions. RNA from each sample (500 ng) was used to cDNA synthesis using the SuperScript II enzyme (Invitrogen).

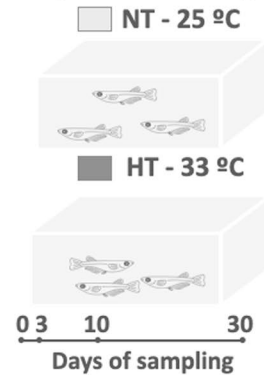
Real-time PCR primers are listed in Table Supplement 1. Gene-specific qPCR was performed using the SYBR green master mix (Applied Biosystem; Merck). The amplification protocol consisted of an initial cycle of 1 min at 95 °C, followed by 10 s at 95 °C and 30 s at 60 °C for a total 40 cycles. The subsequent quantification method was performed using the geometric mean of reference gene values for ribosomal protein L7 (*rpl7*) (Castañeda Cortés et al. 2019; Zhang and Hu 2007) and elongation factor alpha (*ef-1a*) (Arias Padilla et al. 2021). Additionally, the variation of reference gene (*rpl-7* and *ef-1*) was analyzed in the in vivo and ex-vivo treatments, showing no differences in temperature treatment and time of exposure (Supplementary Fig. S1A) and DAPT exposure and temperature treatment (Supplementary Fig. S1B).

Histology, cell quantification, and IF

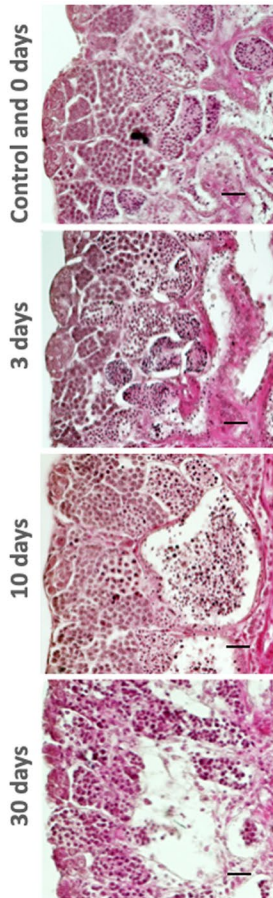
The remaining half of the testis was sampled for conventional histology and IF; tissues were first fixed in Bouin's solution and processed according to standard protocols for the preparation of hematoxylin and eosin-stained histological sections. Then, they were transversally sectioned using a DM 2125RT microtome (Leica, Wetzlar, Germany) at 4–5 μ m thickness.

For cell quantification, we followed our previous work (Arias Padilla et al. 2021). Briefly, three gonadal sections for each testis ($n=3-4$ per sample points) were counted, setting the distance between each section to avoid counting the same cell twice. To reduce technical errors, each section was counted twice in all experiments. All the section photographs

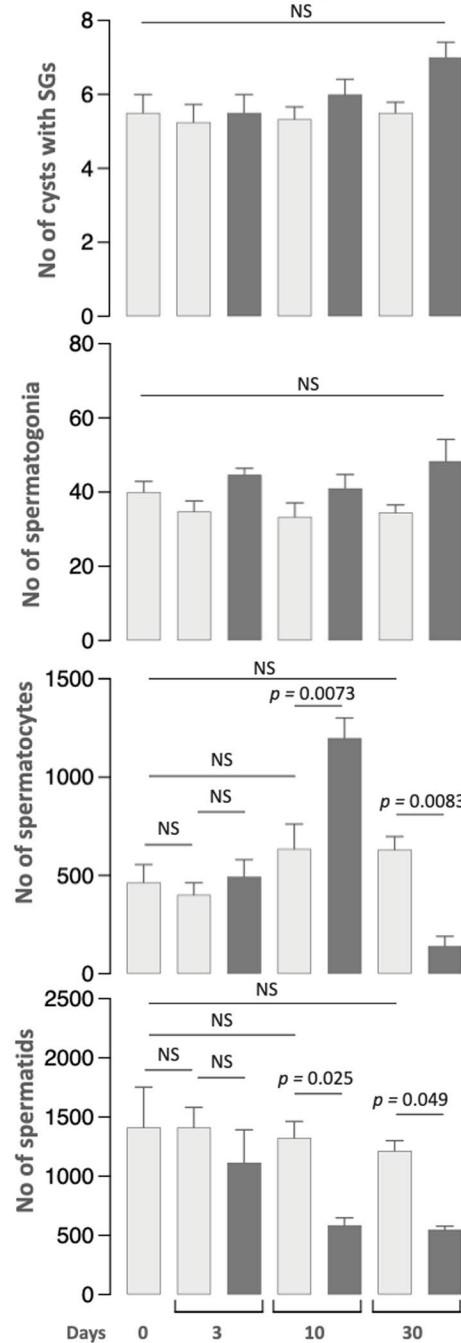
A Experimental design



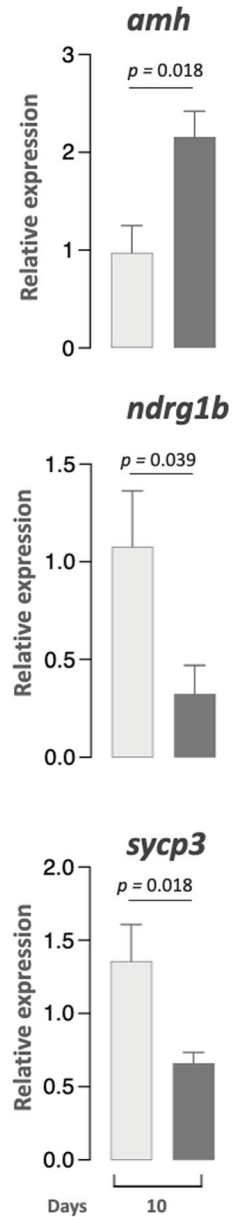
B Histology



C Spermatogenesis cells quantification



D qPCR



were taken using an Eclipse E600 microscope (Nikon, Tokyo). Then, images were analyzed using the FIJI software (<https://imagej.nih.gov/ij/>). Individual cells were counted manually with the Cell Counter plugin for FIJI. Spermatogonia were counted based on the

description of Iwasaki et al. (2009) and spermatogenic cells following Schulz et al. (2010).

For IF, sections were washed with 0.1 M PBS (pH 7.4) and blocked in 0.1 M PBS containing 0.5% bovine serum albumin (Sigma-Aldrich) and 0.5% Triton X100 for 60 min before overnight incubation

Fig. 1 Inhibition of spermatogenesis in males reared at high temperature. Experimental design: The spermatogenesis was analyzed in adults reared at normal (NT — 25 °C, light grey) and high (HT — 33 °C, dark grey) temperature for 30 days (A). Histology: Transversal sections of testis stained with hematoxylin and eosin (H&E) of adult male reared at HT for 0 (and control), 3, 10, and 30 days (B). Spermatogenesis cells quantification: Number of spermatogonia (SGs: type A undifferentiated spermatogonia and type A differentiated spermatogonia as SGa; type B spermatogonia as SGb), number of cysts with SGs, number of spermatocytes (leptotenic/zygotenic primary spermatocytes; pachytenic primary spermatocytes; diplotenic spermatocytes/metaphase I) and number of spermatids (early, intermediate and final spermatids) (C). qPCR: Quantification of *amh*, *ndrg1b*, and *sycp3* transcript abundance at 10 days in testis of males reared at NT and HT (D). Spermatogenic cell quantifications were compared by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test, for comparing the mean of each column with the mean of every other column. For transcript abundance quantification *amh*, *ndrg1b*, and *sycp3* values were normalized against geometric mean of reference genes *rpl7* and *ef-1a*. Continuous variables were compared between two groups by the unpaired two-tailed Student's *t*-test. All differences were considered as statistically significant for $P < 0.05$. Scale bar represents 20 μm

at 4 °C with primary antibody, anti-pen-2 antibody (1:200, rabbit, LS-C135520, LSBio, Seattle). A negative control was also run to check the specificity without the primary antibody. After incubation, the sections were washed twice in PBS and incubated at room temperature (RT) for 90 min with Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibodies (Thermo Fisher Scientific™, A-11008) at a dilution of 1:2000 in PBS. After incubation, sections were rinsed twice with PBS and mounted with Fluoromount mounting medium (Sigma-Aldrich) containing 4',6-diamidino-2-phenylindole (DAPI, 5 $\mu\text{g}/\text{ml}$, Life Technologies; Thermo Fisher Scientific™).

TUNEL assay

The presence of apoptosis in gonad was detected through the In situ Cell Death Detection Kit, Fluorescein (Roche, Basel). Sections were treated according to the manufacturer's manual, with a step of permeabilization with 0.1% Triton X-100, 0.1% sodium citrate in PBS 1X solution. Fluorescein was observed under a Nikon Eclipse E600 microscope. For the quantification of positive TUNEL-positive cells, 2 slides per testis were quantified for each individual ($n = 3$ per sample points).

RNA in situ hybridization

ISH was performed as previously described (Arias Padilla et al. 2021). Briefly, digoxigenin-labeled riboprobes were synthesized from linearized plasmid containing the full-length medaka cDNA of *oct-4* (a marker of undifferentiated spermatogonia, also known as *pou5f1*) (Froschauer et al. 2013; Wang et al. 2011), which was previously cloned into pGEM@-T Vector (Promega, Madison). Testicular explants from ex vivo treatments were fixed overnight in 4% RNase-free paraformaldehyde (PFA) at 4 °C, permeabilized using 20 $\mu\text{g}/\mu\text{l}$ proteinase K at RT, and hybridized at 68 °C overnight with *oct-4* digoxigenin (DIG)-labeled RNA probes. Hybridized probes were detected using an alkaline phosphatase-conjugated anti-digoxigenin antibody (1:2000; Roche) in the presence of nitro blue tetrazolium/5-bromo-4-chloro-3'-indolylphosphate substrates (Roche). Stained testicular explants were embedded in gelatin, cryostat sectioned at 14–16 μm thickness and photographed.

Statistical analysis

Values are presented as mean \pm standard error of the mean (SEM) for continuous variables and as percentages for categorical variables. Fold change and statistical analysis of RT-qPCR quantifications were performed using geometric mean in the FgStatistics software, based on the comparative gene expressions method (Pfaffl 2001). Statistical analyses were performed using Prism 9 (GraphPad Software, San Diego). Continuous variables were compared by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test, for comparing the mean of each group. Continuous variables were compared between two groups by the unpaired two-tailed Student's *t*-test. All differences were considered statistically significant for $p < 0.05$.

Results

Inhibition of spermatogenesis in males reared at high temperature

In this first step, the spermatogenesis process was analyzed in vivo in adults, by keeping adult males at normal (NT — 25 °C) or high temperature (HT

— 33 °C) for 30 days (Fig. 1A). Initially, the response of medaka to heat treatment was analyzed by histology of the testes. It was observed that the number of spermatogonia (SGs: type A undifferentiated spermatogonia and type A differentiated spermatogonia as SGa; type B spermatogonia as SGb) as well as the number of cysts with SGs did not change during treatment (Fig. 1B, C). Moreover, the inhibition of proliferation of SGa and SGb was supported by the upregulation of *amh*, a well-known regulator of germ cell proliferation (Morinaga et al. 2007; Ohta et al. 2003; Rodríguez-Marí et al. 2010), at 10 days of heat treatment (threefold compared to NT) (Fig. 1D).

On the other hand, we observed an increase in the number of spermatocytes (leptotenic/zygotenic primary spermatocytes; pachytenic primary spermatocytes; diplotenic spermatocytes/metaphase I) at 10 days, with a reduction in number at 30 days of heat treatment (Fig. 1A, C). Moreover, *ndrg1b*, characterized as a negative regulator of cystic germ cell proliferation (Arias Padilla et al. 2021), was downregulated at 10 days of heat treatment (Fig. 1D), correlating with the increase of spermatocyte number (Fig. 1C). In this regard, the spermatogenesis marker *sycp3* (synaptonemal complex protein 3), which has been associated with metaphase I of spermatogenesis in medaka (Iwai et al. 2006; Yuan et al. 2000), was downregulated, in accordance with the reduction in the number of spermatids (early, intermediate, and final spermatids) observed at 10 days of heat treatment (Fig. 1B, C, D).

Apoptosis of testis germ line at high temperature

To be able to determine the fate of germ cells loss in response to heat, the occurrence of apoptosis in the heat-treatment was evaluated. The apoptotic pathway, quantified by *p53* and *bcl2* transcript abundances, showed an upregulation in HT testis at 10 days (0.5-fold of *p53* and twofold of *bcl2*) in relation to NT (Fig. 2A), suggesting that the heat-treatment induced apoptosis. Finally, the analysis of apoptosis in the germ line by TUNEL assay showed an increased number of TUNEL-positive cells at 10 days of treatment (40-fold; Fig. 2B, C). Moreover, at 30 days, the number of TUNEL-positive cells was higher compared to the control group (80-fold; Fig. 2B–C). This greater number of apoptotic cells were found in the medullar region of the testis, whereas no apoptotic

cells were observed in the distal portion of the tubule (Fig. 2C), where SGa are localized (see below).

Identification of Pen-2 in adult testis

To better understand the regulators of germ cell (GC) number maintenance during heat treatment, the Notch pathway, one of the main signaling pathways involved in the regulation of the proliferation and quiescence of different stem cells (Blanpain et al. 2006; Conboy and Rando 2002), was evaluated. As a first step, the presence of Pen-2, the catalytic subunit of γ -secretase complex, was characterized in adult testes maintained at NT, observing Pen-2 positive cells in the distal portion of the testis lobule, which comprises the germinal region where the SSCs are located (Fig. 3 A, B). To verify the co-localization of Pen-2 with SGa, an ISH with *oct-4* riboprobe was performed. Immune reactive-Pen2 (Ir-Pen-2) cells were observed in somatic cells surrounding SGa (*oct-4* positive cells) (Fig. 3 C, D), indicating that Pen-2 is expressed most likely in Sertoli cells.

Upregulation of gonadal Pen-2 at high temperature

After characterizing the localization of Pen-2 in the testis, the next step was to analyze its expression during thermal treatment. The expression of Pen-2 (transcript and protein) was then analyzed in an in vivo adult treatment, by keeping adult males at NT and HT for 30 days (Fig. 3 F). Firstly, we quantified the transcript abundance of Notch pathway-related genes, such as *pen-2* and *hes-1* (a well-known Notch effector) (Henrique and Schweisguth 2019). In the heat treatment, both genes were upregulated at 3 (*pen2* 1.5-fold and *hes1* 2.5-fold) and 10 days (*pen2* and *hes1* twofold at HT respect to NT) of treatment, showing no differences at the end of the experiment (30 days) compared to NT (Fig. 3 E, F). Moreover, no differences in *pen-2* and *hes1* transcript abundance were observed at NT between sampling days (Fig. 3 E, F).

Additionally, the localization of the Pen-2 in testis of fish reared at high temperature was characterized (Fig. 3 G–J). At the beginning of treatment (NT — 0 days), Pen-2 showed expression in somatic cells surrounding SGa of the distal portion of the lobule and in the medullar region of the testis (Fig. 3 G, G'). Under high temperature treatment, the expression of

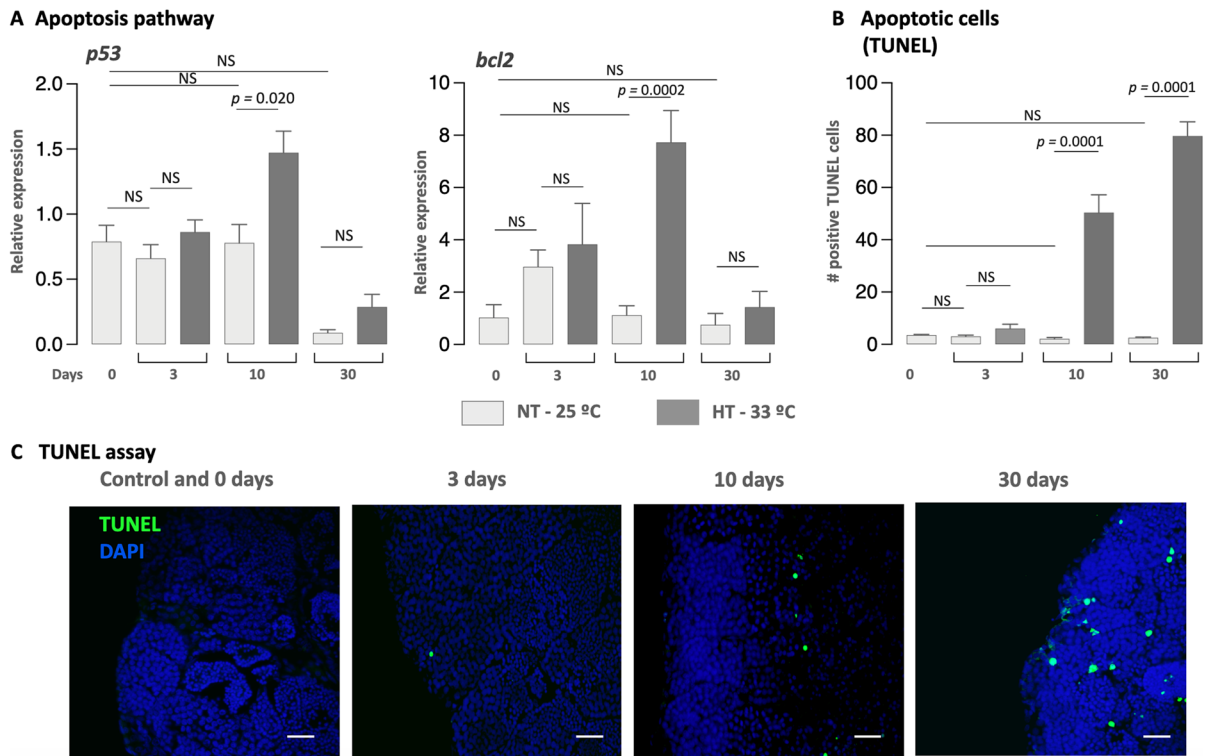


Fig. 2 High temperature induces apoptosis in testis germ line. Apoptotic pathway: Quantification of *p53* and *bcl2* transcript abundance at 0, 3, 10, and 30 days in testis of males reared at normal (NT — 25 °C, light grey) and high (HT — 33 °C, dark grey) temperature (A). Apoptotic cells: quantification of apoptotic cells by TUNEL assay at different sampling time and thermal treatment (B). TUNEL assay: Transversal section of the testis with TUNEL assay showing apoptotic cells (green) and nuclei stained with DAPI (blue) at 0 (and control), 3, 10,

and 30 days (C). For transcript abundance quantification *p53* and *bcl2* values were normalized against geometric mean of reference genes *rpl7* and *ef-1a*. Apoptotic cell quantifications were compared by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test, for comparing the mean of each column with the mean of every other column. *P*-values are indicated when differences between treatment at the same sampling day differ significantly ($P < 0.05$). NS, not statistically significant. Scale bar represents 20 μm

Pen-2 was restricted to the distal portion of the lobule, where the SGa are localized (Fig. 3 H–J, 3H'–J').

Activation of Notch pathway in testis under high temperature treatment

Taking into account the upregulation of Pen-2/*pen-2* and *hes1*, two key players in the Notch pathway, in testes kept at high temperature, the transcript levels of ligands and receptors of this pathway were measured at 10 days of treatment. The transcript abundance of the receptors *notch-1a* and *notch3* (Fig. 4A, B), as well as the ligands *dll4*, *jag1a*, *jag1b*, and *jag2* (Fig. 4C–F), was upregulated at high temperature compared to the control. In contrast, *jag2b* did not show any difference between NT and HT at 10 days

of treatment (Fig. 4G). This upregulation of receptors and ligands upon temperature treatment supports the activation of the Notch pathway in testis upon heat stress.

Inhibition of Notch pathway in ex vivo testis explants at high temperature

Based on the upregulation of Notch pathway and the restricted expression of Pen-2 in the germinal region of the distal lobule, our next step was to inhibit this pathway under high temperature treatment. Given the complexity of the Notch pathway and the way it affects the fate of many cell types in different tissues (Campbell et al. 2006), an ex vivo testis explants approach was used to analyze the protective role of

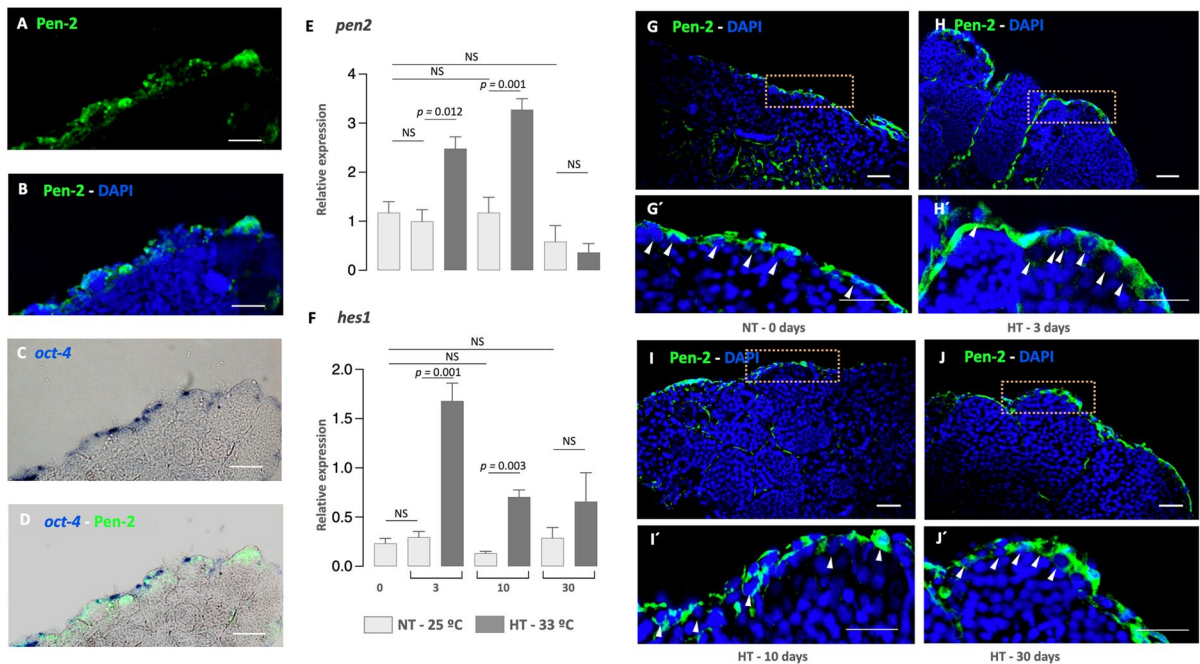


Fig. 3 Pen-2 is upregulated during thermal treatment in male. Transversal sections of the distal portion of the testis lobule in adult males reared at NT, with Pen-2 expression (green, immunofluorescence, IF) and nuclei stained with DAPI (blue) (A, B). Co-localization of Pen-2 (green, IF) with *oct-4* (blue, in situ hybridization) (C, D). Notch pathway: transcript abundance levels of *pen-2* and *hes1* at 0, 3, 10, and 30 days of treatment. (E–F). IF of Pen-2 (green) in testis of male reared at HT at 0 (G), 3 (H), 10 (I), and 30 (J) days of thermal treatment, and nuclei stained with DAPI (blue). Magnification of each

testis (dotted orange line) at different sampling time at 0 (G'), 3 (H'), 10 (I'), and 30 (J') days of thermal treatment. SGa is indicated with arrowhead (G'–J'). Transcript abundance quantification was performed using the geometric mean method and *pen-2* and *hes1* values were normalized against geometric mean of reference genes *rpl7* and *ef-1a*. *P*-values are indicated when transcript abundance between treatment at the same sampling day differs significantly ($P < 0.05$). NS, not statistically significant. Scale bars represent 20 μ m

this cell-to-cell signaling (Fig. 5A). Initially, apoptosis was induced from 3 h of exposure, with an upregulation in *p53* (Supplementary Fig. S2A). Moreover, no differences were observed in *p53* transcription between 3, 12, and 24 h of high temperature exposure, taking the last one to the following ex vivo experiment. Additionally, a dose-dependent response showed the highest conditions for blocking the Notch action ranged between 12.5 (moderate downregulation of *hes1*) and 25 μ M (high inhibition of *hes1*) of DAPT (Supplementary Fig. S2B).

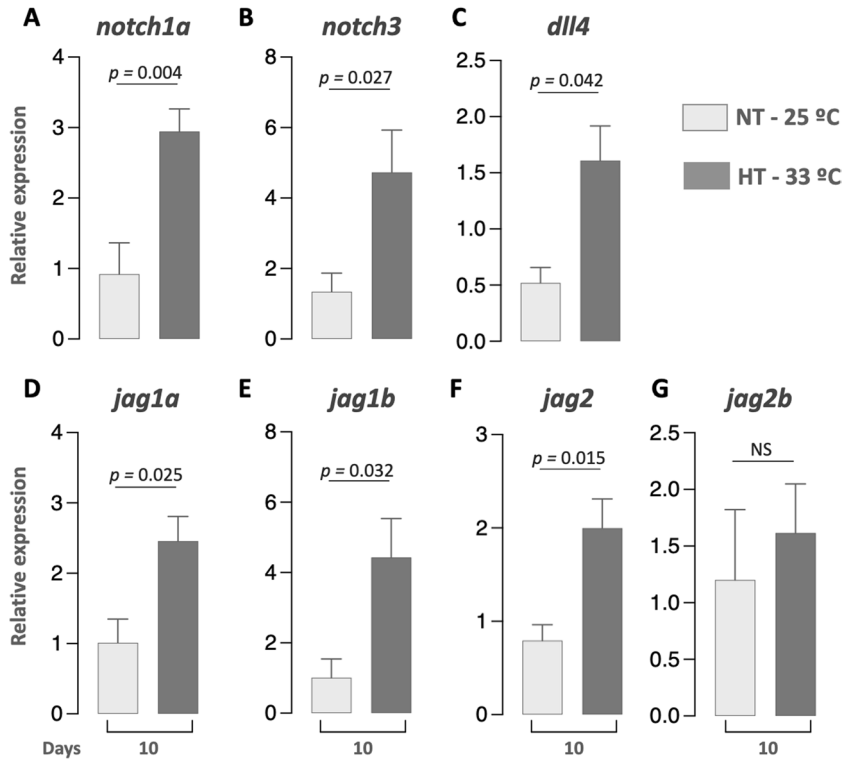
Finally, as we had previously observed *hes1* expression in testicular explants is upregulated at high temperature; however, when testicular explants subjected to high temperature are treated with the Notch pathway inhibitor DAPT, a loss of *hes1* upregulation was observed (Fig. 5B), showing that Notch pathway response was inhibited. Moreover, the

expression of the stem cell marker *oct-4* was only affected by temperature treatment, showing an upregulation (Fig. 5C). Interestingly, in the temperature with DAPT treatment, a reduction of these levels was observed in testicular explants, presenting levels similar to the control treatment. This suggests a reduction of SGa and a protective effect of Notch on germ cell fate under heat stress.

Discussion

The sustainability of spermatogenesis is mainly brought about by a decision between two types of division, self-renewal of themselves for the maintenance of the stem cell pool or the differentiating division for continuous production of spermatozoa. In the present study, we demonstrated that

Fig. 4 Notch pathway is upregulated in testis at 10 days of heat treatment. Transcript quantification of different Notch pathway receptors (*notch1a*, 3; **A–B**) and ligands (*dll4*, *jag1a-b*, *jag2-2b*; **C–G**) at 10 days in testis of males reared at normal (NT — 25 °C, light grey) and high (HT — 33 °C, dark grey) temperature. Transcript abundance quantification was performed using the geometric mean method and *notch1a*, 3, *dll4*, *jag1a-b*, and *jag2-2b* values were normalized against geometric mean of reference genes *rpl7* and *ef-1a*. *P*-values are indicated when transcript abundance between treatment at the same sampling day differs significantly ($P < 0.05$)



exposure to high temperature in adult medaka males induces the loss of spermatogenesis, observing an initial arrest in metaphase I with a final loss

of spermatogenic cells, with the exception of GCs. Moreover, in the germinal region of the testis, where SSCs are located, the activation of the Notch

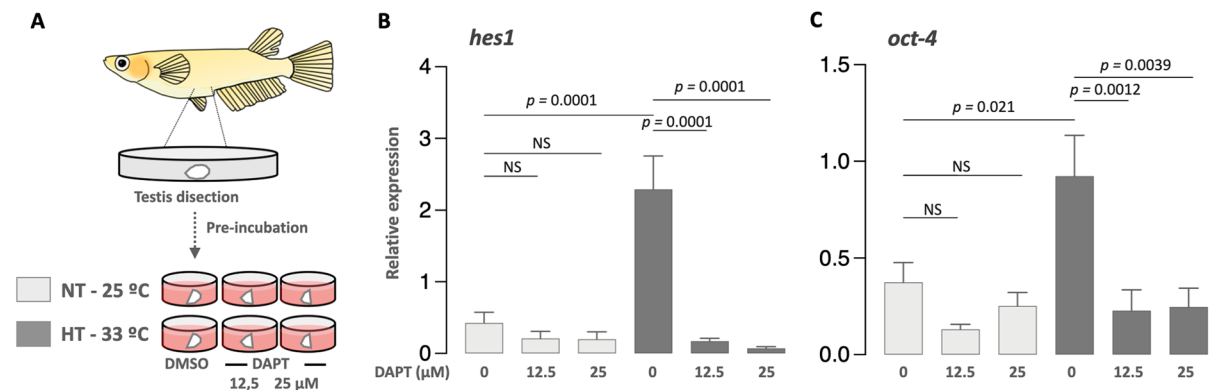


Fig. 5 Notch pathway protects type A spermatogonia from heat-induced loss. Experimental design of ex vivo approach, in which adult testis explants were incubated during 24 h at control (NT — 25 °C, light grey) and high (HT — 33 °C, dark grey) temperature with DAPT (γ -secretase inhibitor; 12.5 and 25 μ M) in triplicates (**A**). Transcript abundance levels of *hes1* (**B**, Notch pathway cis target genes) and *oct-4* (**C**, stem

state marker gene) at different treatments. Transcript abundance quantification was performed using the geometric mean method, and *hes1* and *oct-4* values were normalized against geometric mean of reference genes *rpl7* and *ef-1a*. *P*-values are indicated when transcript abundance between treatment at the same sampling day differs significantly ($P < 0.05$)

pathway was observed. Additionally, the inhibition of this pathway under thermal stress triggered a fast impairment of spermatogenic germ stem cells, supporting that the activation of the Notch pathway is essential for the maintenance of the germ line (Supplementary Fig. S3), what could ensure the re-establishment of the spermatogenic cycle.

Although increasing temperatures promote spermatogenesis by differentiation of spermatogonia (Alvarenga and França 2009), prolonged persistence at high temperatures causes reduction in the spermatogenic cell line due to an increase in apoptosis in most cells (Ito et al. 2008b; Rockett et al. 2001), with the exception of type A spermatogonia (Ito et al. 2008a, b; Majhi et al. 2009). These stem cells are kept in a quiescent state, a strategy that protects them from stress by inhibiting differentiation-activating signals, either intrinsic or through cell-to-cell interactions (Chen et al. 2017; Liao et al. 2014; Zhou et al. 2017). For this reason, the interpretation of the mechanism that allows these cells to persist during stress would have important implications for understanding the reduction of male fecundity, sperm quality, or even infertility. In our experimental model medaka, thermal stress showed to induce the loss of spermatogenic line, from spermatocytes to spermatozoa. The induction of apoptosis was earlier observed by the upregulation of apoptotic-related genes, such as *p53* and *bcl2*, indicating that both the apoptotic and anti-apoptotic pathways are active under temperature stress. Interestingly, Notch pathway has been observed to protect different cells from apoptosis (Chadwick et al. 2010; Zetterberg et al. 2006). Moreover, the protective action of apoptosis from Notch was corroborated in zebrafish embryos using morpholinos against *pen-2*, and as in our work, using DAPT as an inhibitor of this γ -secretase complex (Zetterberg et al. 2006). On this regard, we observed apoptotic cells mainly in the medullary region of the testis, but not in the distal portion of the lobule, where SSCs are localized. In addition, an impairment of SGa upon treatment with the Notch pathway inhibitor was observed under heat treatment, due to a decrease in *oct-4*, possibly due to differentiation of SGa into SGb. These results suggest that SGa are less susceptible to apoptosis than other germ cells, further suggesting the existence of a protective mechanism of SGa differentiation under heat stress.

Related to apoptosis, in our experiments, we observed a large difference in the time course of apoptotic response to temperature between in vivo and ex vivo experiments. These differences in response could rely on the fact that exposure in a whole organism presents different regulations that does tissue culture, both at the cellular and paracrine levels, as well as at the systemic and endocrine levels, which implies a limitation in ex vivo exposure experiment. The whole organism presents different strategies to cope with a rise in temperature at the systemic level, such as the elevation of cortisol through a cerebral translation by corticotropin-releasing hormone (Crh), for the synthesis of this glucocorticoid in the adrenal/interrenal, and its modulatory action in the organs target, such as the gonad (Wang et al. 2011); e.g., it has been observed in fish like Japanese eel and pejerrey that a rise in temperature is accompanied by an increase in cortisol, the major stress hormone, through the activation of Crh in the brain (Fernandino et al. 2012; Mommsen et al. 1999; Ozaki et al. 2006). This increase in cortisol produces a concomitant elevation in androgen levels, as a secondary effect, which promotes an initial increase in spermatogenesis (Mommsen et al. 1999; Ozaki et al. 2006). Then, under chronic thermal stress, this effect decreases and an increase in apoptosis can be detected, as in our study, as well as in experiments carried out in other species (Ito et al. 2008a). In contrast, the ex vivo culture does not present these homeostatic modulations to external factors, so its responses can be accelerated by direct contact with the stressor, in this case temperature. Always bearing in mind the limitations of ex vivo approach, the combination of in vivo and ex vivo experiments has allowed us to elucidate the regulation of the Notch pathway in the protection of SGa against an increase in temperature, a mechanism that takes on greater relevance in the present scenario of global warming/climate change.

In the present work, we analyzed the expression of the Notch pathway member Pen-2, a protein of the γ -secretase complex that is essential for the correct cleavage and signal transduction inside the cell (Zhang et al. 2000). During thermal stress, *pen-2* was highly expressed and, interestingly, restricted to the distal portion of the lobule surrounding SSCs, presumptively in Sertoli cells (Alix et al. 2020; Nishimura et al. 2016; Schulz et al. 2010). On this regard, our observation would

establish a cell-to-cell communication that protects the SSCs from high temperature-induced apoptosis, with the Notch pathway holding an important role in such protective mechanism. Besides the germ cell protection, the Notch signaling has been related to the regulation of self-renewing state and/or prevention of differentiation of SSCs through the regulation of *Nanos2*, *Stra8*, *Bcl6b*, *Cxcr4*, and other genes in A^{undiff} spermatogonia (Chen et al. 2005; Garcia et al. 2014, 2017; Oatley et al. 2006; Sada et al. 2012). Notch negative regulation occurs through the activation of Jag1 (jagged 1) expression in germ cells expressing Jag1 (Garcia et al. 2014, 2017; Mäkelä and Hobbs 2019), a ligand that was highly expressed in our study on thermal treatment in medaka. In case of the receptor NOTCH1, it is expressed in undifferentiated germ cells and Sertoli cells of mouse testis, whereas the ligand DLL4 is ubiquitously expressed in germ cells and in some Sertoli cells (Murta et al. 2013). During embryogenesis, the constitutive activation of NOTCH1 signaling in Sertoli cells caused the exit of gonocyte from the quiescence status (Garcia et al. 2013). In flies, Notch signaling directly controls germline stem cell development and maintenance (Kimble and Crittenden 2007; McIntyre and Nance 2020), reinforcing the importance of cell-to-cell Notch signaling in the regulation of germ cell differentiation. In this regard, our results support a strong link between the activation of the Notch pathway and the inhibition of spermatogenesis by increasing temperature. Moreover, the inhibition of SGa proliferation was observed at a cellular level by the loss of the sperm lineage, and also molecularly by the increase of genes related to the inhibition of germ cell proliferation in fish, such as *amh* (Morinaga et al. 2007). Moreover, an initial increase in spermatocyte proliferation is observed with increasing temperature, which is corroborated by a decrease in the cystic proliferation inhibitor *ndrg1b* (Arias Padilla et al. 2021). However, these spermatocytes are arrested in metaphase I, as also corroborated by a decrease of sperm markers, such as *sycp3* (Iwai et al. 2006; Yuan et al. 2000). However, if exposure to high temperatures persists, a drastic decrease in the number of spermatocysts and spermatids is observed, resulting in a testis with a limited number of remnants spermatogonia. Interestingly, although our results in medaka support the activation of the Notch pathway, including receptors,

ligands, γ -secretase, and the respective effectors of this pathway, the activation of Notch signals seems to work in the opposite direction to that described above, wherein the input signal (high temperature) seems to be sensed by SGa while the output seems to be in the somatic cells with a high expression of Pen-2, presumably Sertoli cells (Nishimura et al. 2016). This differential activation by temperature in medaka would promote the protection through a state of quiescence and the concomitant inhibition of SGa differentiation (Supplementary Fig. S3) by the suppression of spermatogenesis.

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Author contribution ODMA: conceptualization, methodology, analysis; AFB: methodology and visualization; RSH: funding acquisition, conceptualization, writing — reviewing and editing, JIF: funding acquisition, conceptualization, investigation, supervision; writing — original draft preparation, writing — reviewing and editing.

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Data availability All data generated or analyzed during this study are included in this article and its supplementary information files. Raw data are available from the authors upon reasonable request.

Declarations

Ethics approval This animal experiment was reviewed and approved by the Institutional Committee for the Care and Use of Animals for Experimental Purposes—Universidad Nacional de San Martín, Argentina (approval number: CICUAE-UNSAM 17/21).

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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