

A lifespan atlas of the killifish testis defines a mid-age remodeling phase in vertebrate testicular aging

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Abstract

Background:

Reproductive aging in vertebrates is commonly interpreted as a gradual decline; however, whether aging of the male gonad proceeds linearly or involves discrete transitions in tissue state remains unclear. The turquoise killifish (*Nothobranchius furzeri*), an exceptionally short-lived vertebrate, enables lifespan-wide resolution of both rapid germline establishment and subsequent aging within months.

Results:

Our staged histological atlas shows that the testis progresses from a simple gonadal primordium to complete spermatogenesis within three to four weeks post-hatching revealing an exceptionally compressed developmental program largely independent of somatic growth variation. Proliferative activity peaks in early adulthood, marking maximal spermatogenic output, but declines abruptly at mid-age rather than gradually. This inflection point coincides with coordinated repression of germline, mitotic, and meiotic programs and activation of extracellular matrix remodeling, angiogenic, inflammatory, and stromal pathways. These molecular shifts are accompanied by structural remodeling, including expansion of the interstitial compartment and accumulation of collagen-rich matrix. Late-life testes exhibit comparatively modest additional changes, suggesting stabilization of a remodeled, low-proliferative niche.

Conclusions:

Testicular aging in the turquoise killifish unfolds as a process separated by a discrete mid-life remodeling that links testicular decline to somatic niche remodeling. This defined transition provides a mechanistic entry point for dissecting vertebrate reproductive aging and establishes the killifish as a uniquely powerful model for identifying interventions that preserve germline function.

Introduction

Aging of the male germline results in reduced fertility, increased genomic instability, and alterations in the somatic microenvironment of the testis. These changes not only impair an individual's reproductive success [1] but can also adversely affect the health of the offspring [2, 3]. Despite growing recognition of these processes, vertebrate models that allow comprehensive, lifespan-wide studies of testicular development and aging within experimentally tractable time scales and at low-cost maintenance remain limited, as most research still relies on rodent and non-human primate models [4].

Commonly used ray-finned fishes, such as zebrafish [5] and medaka [6, 7] offer low-cost maintenance; however, they undergo sex differentiation gradually over several weeks to months and reach sexual

maturation at around 3–4 months of age. In contrast, the turquoise killifish (Fig. 1A) (*Nothobranchius furzeri* Jubb, 1971) has evolved in ephemeral savannah pools that dry out seasonally, and its life cycle is adapted accordingly. Embryos undergo diapause during dry periods, and upon hatching at the onset of rainy season, fish grow explosively, can reach sexual maturity in less than 3 weeks (both in wild and laboratory conditions), and complete their entire lifespan within only a few months [8–10]. Such an accelerated life history implies a swift progression through gametogenesis during all developmental stages to expedite the rapid onset of germline development [11] and maturation [9, 10]. Despite this extreme life cycle, the timeline and lifelong dynamics of testicular development in this species have not yet been systematically described.

The remarkably short life cycle makes the turquoise killifish the shortest-lived model vertebrate and a powerful model for aging research [12, 13]. Importantly, it recapitulates many canonical hallmarks of vertebrate aging [14–16], including reproductive senescence in both sexes [17] and a recently identified decline in sperm quality and function [18]. However, it remains unclear how these organism-level reproductive changes in fertility relate to the timing and nature of cellular and molecular remodeling within the testis across the lifespan.

In ray-finned fishes, the seminiferous compartment in the testis is organized into functional units called spermatocysts, in which germ cells develop synchronously and are enclosed by Sertoli cells [19, 20]. The number and proliferative activity of Sertoli cells determine overall testicular size and spermatogenic capacity [21]. This feature contrasts with mammals, in which Sertoli cells are largely quiescent after puberty, and provides a convenient quantitative readout of testicular remodeling induced by physiological and/or environmental signals. [22]. This fish-specific feature might have important implications for aging, as lifelong Sertoli-cell proliferation and a necessity for continuous cyst turnover render fish spermatogenesis particularly vulnerable to age-related disruptions in niche function and coordination.

Across vertebrates, spermatogenic output is tightly coupled to the somatic microenvironment of the testis, in which Sertoli cells play a central role in supporting germ cell proliferation, meiotic entry, and differentiation, thereby defining a functional germline niche [23]. Age-related alterations in this somatic environment have been shown to impair spermatogenesis independently of intrinsic germ cell defects, highlighting the importance of niche integrity for lifelong germline maintenance [24]. However, how rapidly such somatic changes emerge and how tightly they are coordinated with germline decline across the lifespan remains poorly resolved in short-lived vertebrate models.

In this study, we compiled a detailed histological atlas of killifish spermatogenesis across defined age classes, capturing a rapid transition from embryonic gonad dominated by early germ cell stages to those containing fully differentiated spermatozoa within 3–4 weeks post hatching. We then analyzed cell cycling activity revealing a steep age-dependent decline in proliferative activity and dynamics. Transcriptomic profiling uncovered activation of fibrotic and immune pathways, accompanied by repression of mitotic, meiotic and spermatogenic gene networks. Finally, by integrating morphological,

cellular, and molecular data, we propose a model in which declining germline function is tightly coupled to somatic remodeling and inflammation during testicular aging. Together, these results establish the turquoise killifish as a rapid and tractable vertebrate model for dissecting the mechanisms that drive male reproductive aging.

Results

Here, we establish a comprehensive framework for studying testicular development and aging in the turquoise killifish. We (i) generate a staged histological atlas of spermatogenesis across defined age classes, (ii) quantify age-dependent changes in germ cell proliferation using PCNA immunolabeling and EdU incorporation, and (iii) integrate these data with transcriptomic profiling of young, mid-aged, and old testes. Together, these analyses provide a high-resolution view of how germline maturation and decline, somatic remodeling, and immune activation emerge and progress during aging in a short-lived vertebrate.

Rapid completion of all spermatogenesis stages in annual killifish

To characterize how larval testicular tissue develops into a mature organ within a few weeks, we generated a detailed histological atlas and evaluated serial sectioned gonads for the presence of the most advanced germ-cell type at each stage from 0 to 29 days post hatch (dph), by which time all males possessed testes containing spermatozoa.

Upon hatching, the testicular primordium consisted exclusively of spermatogonial and somatic gonadal cells (Additional file 1: Fig. S1). Based on cell morphology, the largest germ cells were identified as A-type spermatogonia (large oval cells, with high cytoplasmic volume and an euchromatic nucleus). Between 6 dph and 8 dph, entry into meiosis became evident, with the first primary spermatocytes (SPC I) recognizable by their reduced cell size, densely stained chromatin and the organization of multiple cells within a spermatocyst. From this point onwards, testis gained a typical triangular shape. By 10 dph, secondary spermatocytes (SPC II) appeared, indicating progression through the second meiotic division (Fig. 1B). Appearance of SPC II was followed by first detection of spermatids, having reduced cell diameter and round nuclei, while still having some cytoplasm. Spermatids were found from 14 dph onwards (Additional file 1: Fig. S1). Flagellated late spermatogenic cells with morphology consistent with spermatozoa were observed by 18 dph (Fig. 1B, Additional file 1: Fig. S1). The presence of spermatozoa in our dataset reflects completion of spermatogenesis rather than a direct assessment of reproductive performance. Later stages showed complete spermatogenesis with a predominance of spermatids and mature spermatozoa in the lumina of lobules, indicating a widely established spermatogenic process (Fig. 1B, Additional file 1: Fig. S1). Overall, testicular development in turquoise killifish spanned approximately 3–4 weeks, progressing from isolated germ and somatic cells at hatching to a fully differentiated organ. This remarkable expansion is likely facilitated by a high

proliferation rate, as evidenced by the frequent observation of germ cells in mitotic metaphase/anaphase in histological sections (Additional file 2: Fig. S2).

Next, we evaluated the most advanced germ-cell stage present in each male at the respective developmental time points (2–29 dph) (Fig. 1C). We found a stepwise progression observed in histological sections (Fig. 1B, Additional file 1: Fig. S1), with A-type spermatogonia representing the most advanced stage at early larval stages, followed by the emergence of primary spermatocytes, secondary spermatocytes, and subsequently post-meiotic germ cells. By 18 dph, spermatozoa were detected as the most advanced germ-cell type, demonstrating that complete spermatogenesis is achieved within less than three weeks post-hatch and all sampled males had developed spermatozoa by 29 dph, confirming that all individuals consistently reached complete spermatogenesis within the first month post-hatch (Fig. 1C).

Spermatogenesis progression is only partially linked to the body size

Killifish larval and juvenile period is characterized not only by rapid testicular development (Fig. 1B-C, Additional file 1: Fig. S1) but also by rapid growth [9]. However, during this period notable differences in body size emerge (Additional file 3: Fig. S3). Therefore, we asked whether faster growing males within the same age cohort show faster progression through spermatogenesis. To determine whether body size influenced the rate of spermatogenic progression, we compared all consecutive stages (e.g., SPG-A → SPG-B, SPG B → SPC I) with total body length and weight (Additional file 3: Fig. S3). However, the only significant effect of body size (both weight and length) was observed during the SPC I → SPC II transition (Fig. 1D). Males containing only primary spermatocytes were significantly smaller and lighter than those with secondary spermatocytes, indicating that progression through spermatogenesis at this stage is linked to body size. Next, we tested whether overall body condition is linked to the maturation by analyzing the condition factor (K) in males aged 18–29 dph across SPC II, SPT, and SPZ stages. Interestingly, all males from 18 dph onwards grouped by distinct spermatogenesis stages showed no significant differences in condition factor (Fig. 1E). Altogether, our results suggest that progression through spermatogenesis is primarily linked to age, and the condition factor does not determine final maturation stage (presence of spermatozoa).

Spatial organization and cytoarchitectural progression of spermatogenesis of the adult testis

Having characterized the timing of testicular maturation, we next examined the spatial organization of germ cells and the overall cytoarchitecture of the testis from adult males. To visualize the germline compartment, we performed DEAD-box helicase 4 (DDX4) immunohistochemistry and transmission electron microscopy (TEM), which together delineates germline, somatic, and extracellular structures within the testicular lobules.

Based on hematoxylin-eosin staining, the adult killifish testis displays a restricted lobular organization consistent with the restricted type of spermatogenesis. In this arrangement, early-stage spermatogonial germ cells are confined to the distal ends of the lobules, while progressively differentiated cysts occupy more medial and proximal regions, with mature spermatozoa accumulating near the efferent duct (Fig. 2A,a-c). DDX4 staining confirmed restricted lobular organization (Fig. 2B,a-a") and further demonstrated that DDX4 expression is high in early-stage germ cells (located in lobule margins), weaker in primary spermatocytes (SPC I), and not detected in later stages, thereby marking a clear transition from early germ cells towards spermatozoa (Fig. 2B,b-c").

TEM revealed the ultrastructure of the adult killifish testis with a cystic organization of germ cells. Spermatocysts containing germ cells are enclosed by cytoplasmic extensions of Sertoli cells (Fig. 2C,a-b). Early stage spermatogonia are large cells with predominantly euchromatic nuclei containing a prominent nucleolus, abundant cytoplasm, mitochondria, and Golgi apparatus. Primary spermatocytes (SPC I) exhibit reduced cell size compared to early-stage spermatogonia and show nuclei with more condensed and granular chromatin, consistent with entry into meiosis. Secondary spermatocytes (SPC II) display further chromatin condensation and reduced cell size; moreover, transitional stages between SPC I and SPC II were observed, suggesting a high rate of gametogenesis (Fig. 2C,c). Interstitial tissue is located adjacent to the spermatogenic cyst delineated by Sertoli cells and includes Leydig cells, as well as elements of the testicular vasculature, such as blood vessels containing erythrocytes. This region illustrates the close spatial relationship between spermatogenic cysts and the surrounding interstitial components (Fig. 2C,c,d). Post-meiotic stages are represented by spermatids, characterized by nuclear condensation and the subsequent formation of flagellar structures, including the axoneme and associated mitochondria (Fig. 2C,e). Mature spermatozoa (SPZ) are released into the lumina and have highly condensed nuclei, a lack of cytoplasm and elongated flagellum (Fig. 2C,f).

Testicular remodeling during aging happens at mid-age

Having established the spatial organization and cellular differentiation pattern of spermatogenesis in the adult killifish, we next asked how these histological and cellular features change with age at the molecular level. To reveal whether fish testicular tissue is prone to aging we performed bulk RNA-Seq on testes from young (1.5-month-old, mo), mid-aged (4 mo), and old males (6 mo) (Fig. 3A). The designation of mid-aged was based on lifespan of turquoise killifish, in which this interval corresponds to approximately 40–50% of the median lifespan under laboratory conditions [25, 26]. Principal component analysis revealed a clear age-dependent separation of transcriptomes. The largest transcriptional divergence occurred between young and mid-aged males (Fig. 3A right), suggesting that major molecular remodeling of the testis takes place early during aging. Based on the principal component (PC) analysis old testes clustered separately but closer to the young group along PC2, indicating that late-stage testes may converge toward a relatively stable expression profile. Analysis of differentially expressed genes (DEGs) found 1775 DEGs in mid-aged vs young (1344 upregulated, 431 downregulated) (Fig. 3B left, Additional file 4: Tab. S1), 1003 DEGs in old vs mid-aged (522 upregulated, 481

downregulated) (Fig. 3B middle, Additional file 5: Tab. S2) and 1988 DEGs in old vs young (1446 upregulated, 542 downregulated) (Fig. 3B right, Additional file 6: Tab. S3).

Heatmaps of variance-stabilized and normalized expression values highlight age-associated restructuring of the testicular transcriptome (Fig. 3C). Samples cluster primarily by age groups as also shown in the PCA plot (Fig. 3A), indicating global shifts in expression programs rather than isolated gene-level changes. While young and old testes form relatively coherent groups, mid-aged samples display increased within-group heterogeneity and partial sub-clustering, consistent with a transitional phase of testicular aging. As bulk RNA-Seq captures tissue-level transcriptomes, the observed changes likely reflect a combination of altered cell-type proportions and transcriptional regulation within individual cell populations. Together with the histological and proliferation data presented below, these results point to a pronounced tissue-level transition occurring at mid-age.

Proliferation decline is an early and prominent tissue-level feature of testicular aging

To understand the sequence of events underlying testicular aging, we integrated transcriptomic, immunofluorescence, and histological datasets. Analysis of cell-cycle–associated transcripts revealed that both mitotic and meiotic regulators were strongly upregulated in young testes while downregulated in mid-aged and old testes (Fig. 4A), indicating that decline of proliferative capacity represents an early and prominent tissue-level feature of testicular aging in turquoise killifish. Notably, genes involved in DNA replication and cell-cycle progression [27–29], including *proliferating cell nuclear antigen (pcna)*, *cyclin-dependent kinase 9 (cdk9)*, *coiled-coil domain containing 45 (ccdc45)*, *DNA topoisomerase II alpha (top2a)*, *GIN5 complex subunit 1 (gins1)*, *GIN5 complex subunit 4 (gins4)*, and members of the *minichromosome maintenance complex (mcm)*, showed high expression in young testes followed by a gradual age-dependent decrease. In addition, transcripts linked to chromatin organization and sister chromatid cohesion [30, 31], such as *stromal antigen 3 (stag3)*, *structural maintenance of chromosomes 1B (smc1b)*, and *establishment of sister chromatid cohesion N-acetyltransferase 2 (esco2)*, were downregulated, consistent with reduced proliferative and cell-cycle activity in aging testes.

In addition to canonical cell-cycle regulators, the cell-cycle–associated [32–34] expression cluster also included genes such as ras homolog enriched in brain (*rheb*), DNA polymerase nu (*poln*), pim kinase 2 (*pim2*), and mitogen-activated protein kinase kinase 1 (*map2k1*) which exhibited a non-linear age-dependent expression pattern. These genes showed a strong upregulation in young testes, reduced expression in mid-aged individuals and a partial re-upregulation in aged testes, although not reaching the levels observed in the young group. While not core components of the cell-cycle machinery, these genes are known to influence cell-cycle progression indirectly and have additional roles in cellular signaling, stress responses and mitochondrial organization. Their expression dynamics therefore parallel the overall age-related trends observed within this cell-cycle–associated cluster.

Given the limited temporal resolution of the bulk RNA-Seq dataset, which captures only three discrete age groups, we next employed PCNA antibody staining as an *in-situ* validation of the transcriptomic findings. This approach enabled direct spatial and quantitative assessment of proliferative activity in the testis across a substantially broader time window, spanning much of the killifish lifespan in captivity (1–10 months), and thus allowed finer resolution of age-associated changes in testicular proliferation dynamics. Quantification of the PCNA-positive area in testes from 1- to 8-10-month-old males revealed a rapid increase in proliferation during early adulthood, reaching a maximum around 2 months post-hatch (Fig. 4B). This peak was followed by a pronounced decline during mid-age, after which proliferative activity remained low and became nearly undetectable in the oldest individuals (8–10 months) (Fig. 4B).

EdU pulse–chase reveals age-dependent slowdown of cell-cycle progression

To further support proliferation data and uncover cell-cycle dynamics during aging, we employed EdU labeling capable of marking cells in S phase and then track the expansion of labelled lineages. Based on the data of proliferative activity using PCNA labelling, we compared young (1.5 mo) and mid-aged (4 mo) males (Fig. 5A) using EdU. Following intraperitoneal injection, EdU incorporation was detectable from 2 hours post injection (hpi) onward in both young and mid-aged males, indicating successful delivery and uptake in both age groups (Fig. 5B).

Quantification at 2 to 48 hpi revealed a significantly larger EdU-positive area in young males (Fig. 5C), consistent with a larger or more rapidly expanding cohort of cycling cells at early chase times. During the 10–48 hours post-injection chase, EdU-positive cells advanced toward the efferent duct, consistent with progression through meiotic differentiation. Aged males displayed substantially smaller EdU-positive area, indicating reduced cell-cycle progression evaluated by single EdU pulse.

At 24 and 48 hpi chase in young males we already observed plateauing of the percentage of EdU positive area likely reflecting extensive proliferation of testicular cells which in turn dilute the signal. Therefore, extended chase images (96–120 hpi) were used to qualitatively assess the most advanced germ cell stage retaining detectable EdU signal rather than to quantify progression kinetics. At 96 hpi, young males showed EdU-positive lumina containing spermatozoa (Fig. 5D). Importantly, pre-meiotic S-phase is the final stage at which EdU can be incorporated, once cells enter meiosis, no further EdU incorporation is expected. Thus, the presence of EdU-labeled spermatozoa is consistent with completion of spermatogenesis within 96 h among the labeled cohort. In contrast, mid-aged males failed to advance labeled cells to late spermiogenic stages within the given chase period of 120 hpi, consistent with slower transit or reduced effective cohort entry.

Together, these findings reinforce our RNA-Seq–based conclusion that testicular cell proliferation and germline differentiation decline sharply by mid-age. Although proliferation was quantified relative to total testicular area, and age-associated stromal expansion alters the relative proportion of germinal and non-germinal tissue (Fig. 6A-B), the spatial restriction of fibrotic remodeling to central regions indicates that

the decline in proliferation is not merely a consequence of compositional change. Instead, the data support a biologically meaningful reduction in proliferative capacity during mid-age transition.

Mid-age coincides with stromal expansion and extracellular matrix-associated transcriptional activation

Since we identified reduced cell cycling as a key feature of testicular aging, we next asked whether gross morphological changes are also linked to testicular aging and accompany the molecular shift.

Histological analysis revealed that there is a gradual expansion of interstitial regions, and accumulation of extracellular matrix. These changes were spatially heterogeneous: peripheral zones containing early germ cells remained largely preserved, whereas the central parenchyma showed fibrous-like extracellular matrix (ECM) deposition. Quantification of non-germinal tissue demonstrated a gradual increase during aging (Fig. 6A). Mallory trichrome staining confirmed increased collagen-rich ECM (Fig. 6B), indicating establishment of a remodeled stromal architecture in mid-aged males.

Transcriptomic profiling mirrored this trajectory. Young testes showed minimal activation of ECM-related genes (Fig. 6C). In contrast, ECM- and adhesion-associated genes including representative integrins (*itga6b*, *itgb8*) [35], metalloproteinases (*mmp11a*, *mmp14b*) [36], and matrix regulators TIMP metalloproteinase inhibitor 3 (*timp3*), lysyl oxidase like 4 (*loxl4*), and laminin subunit alpha 2 (*lama2*) [37–40] were upregulated in both mid-aged and old males. A subset of remodeling genes cathepsin L1 (*ctsl.1*) and serpin family F member 1 (*serpinf1*) [41, 42] was elevated only at mid-age, suggesting a transient remodeling wave. Interestingly, mid-aged males subclustered into two groups. The variability indicates differences in remodeling status among individuals, with some males displaying modest changes and others exhibiting strong upregulation of remodeling-associated genes. Gene Ontology enrichment analysis (GO) supported these observations, with strong overrepresentation of cell adhesion, cell–cell adhesion, ECM organization, and supramolecular fiber assembly categories (Fig. 6D), each showing a predominance of upregulated genes (> 75%) (Additional file 7: Tab. S4). In line with gene-level and GO analyses, KEGG pathway enrichment highlighted ECM–receptor interaction and signaling pathways related to somatic remodeling in mid-aged testes, supporting the ongoing stromal reorganization at this stage (Additional file 8: Tab. S5).

Vascular-related and signaling pathways are activated at mid-age, confirmed by angiogenesis-related GO terms

Along with ECM remodeling, genes linked to vascular and endothelial signaling such as kinase insert domain receptor (*kdr*), tyrosine kinase with immunoglobulin like and EGF like domains 1 (*tie1*), and Ras interacting protein 1 (*rasip1*) [43–45] showed increased expression beginning at mid-age. GO categories including vasculature development, tube morphogenesis, blood vessel development, and angiogenesis were among the most strongly enriched (Fig. 6D), with the angiogenesis module containing 38 upregulated vs. 3 downregulated genes (Additional file 6: Tab. S3; Additional file 7: Tab. S4). Consistently,

KEGG pathway enrichment analysis identified the Apelin signaling pathway (Additional file 8: Tab. S5), a known regulator of endothelial function and angiogenic remodeling [46], as significantly enriched at mid-age in turquoise killifish. These findings indicate that vascular remodeling accompanies the mid-age transition, consistent with the expansion of interstitial tissue.

Inflammatory and stress-responsive transcripts remain elevated with age

Transcriptomic analysis revealed an induction of inflammatory and stress-associated genes, including representative transcription factors CCAAT enhancer binding protein beta (*cebpb*) and Jun B proto-oncogene b (*junbb*), cytokine pathway mediators interferon regulatory factor 1 (*irf1*) and signal transducer and activator of transcription 1 (*stat1*), as well as macrophage-like markers macrophage expressed 1 (*mpeg1*) and lysozyme C (*lyz*). [47–49]. Signaling components involved in fibroblast recruitment, such as PDGF ligands/receptors and MAPK pathway members [50], were similarly upregulated (Additional files 4–6: Tab. S1-3; Additional file 9: Fig. S4). Consistently, KEGG pathway analysis of mid-aged versus young testes identified enrichment of the cytokine–cytokine receptor interaction pathway, supporting activation of immune-related signaling during the mid-age transition (Additional file 8: Tab. S5). GO biological process terms related to response to chemical stimulus, response to organic substance, response to endogenous stimulus and granulocyte leukocyte chemotaxis migration were significantly enriched (Fig. 6D, Additional file 7: Tab. S4), showing broad upregulation in mid-aged testes.

Germline-associated transcription is lost in parallel with stromal activation

Finally, to assess how the germline responds to stromal remodeling, we analyzed the expression dynamics of genes associated with stemness, early-stage germ cells, and germline maintenance. This module displayed the opposite pattern to the ECM and inflammatory–stromal programs described above. Key regulators of germ cells and niche interactions, including homeobox protein Nanog (*nanog*), male germ cell-associated kinase (*mak*), integrin subunit alpha 6a (*itga6a*), dynein axonemal heavy chain (*dnah*), and hyaluronan mediated motility receptor (*hmmr*) [51–54] were highly expressed in young testes but showed a marked reduction in mid-aged males and were almost completely lost in old fish (Additional file 9: Fig. S4). This indicates a functional decline of germline-associated transcriptional programs. Importantly, the loss of germline gene expression occurred in parallel with activation of stromal, inflammatory, and ECM-remodeling programs identified in the preceding heatmaps, together suggesting a shift from a germ cell–dominant tissue toward a more stromal, fibrosis-like, and low-proliferative testicular environment during aging. Collectively, these findings support the notion that the major remodeling phase occurs at mid-age, after which transcriptional changes appear more heterogeneous and of lower magnitude.

Discussion

The male gonad undergoes profound changes across the lifespan, encompassing rapid testicular morphogenesis, establishment of spermatogenesis, sustained germline output during adulthood, and progressive age-associated decline. While functional deterioration of male fertility has been documented in several fish species [18, 55], the extent to which this decline reflects coordinated cellular and transcriptional remodeling within the testis has remained unclear. By integrating staged histology with transcriptomic profiling across the lifespan, we demonstrate that testicular aging is characterized by a pronounced mid-life transition marked by loss of germline-associated programs and concurrent activation of stromal, inflammatory, and extracellular matrix–remodeling pathways. These findings indicate that reproductive aging in fish is not a gradual linear process but instead involves a temporally defined remodeling phase that reshapes the cellular and molecular architecture of the testis.

The developmental analysis shows that the turquoise killifish reared under laboratory conditions completes the full spermatogenic program within one month after hatching, consistent with the accelerated life history of turquoise killifish [9, 56]. Despite substantial variability in juvenile growth rates, our data indicate that testicular development and maturation are largely driven by chronological age rather than somatic growth. This suggests that the extremely rapid spermatogenic cycle requires a minimal, fixed time window. In contrast to other teleost models such as zebrafish [57] and medaka [58], which reach sexual maturity at a comparable body size but after several months of post-embryonic development, the turquoise killifish exhibits a markedly accelerated testicular differentiation program within 3–4 weeks.

This acceleration likely reflects an evolutionary compression of gonadal development, including rapid establishment of the spermatogonial stem cell niche and early differentiation of supportive somatic cells, potentially followed by earlier engagement of the hypothalamic–pituitary–gonadal axis as maturation proceeds [59, 60]. Such coordinated timing may enable spermatogenesis to initiate at or near hatching in annual turquoise killifish. In contrast, zebrafish [57] and medaka [58] initiate spermatogenesis substantially later. Importantly, the accelerated development of the testis in the turquoise killifish does not represent an isolated organ-specific phenomenon. Instead, it parallels accelerated post-hatching somatic growth program found in killifish brain tissue expanding several-fold faster than in zebrafish [61]. The rapid progression of testicular differentiation thus likely reflects a global compression of post-embryonic development associated with the annual life history strategy, rather than a gonad-specific acceleration.

Interestingly, spermatogenesis in adult turquoise killifish males remains accelerated relative to zebrafish [20]. By directly tracking S-phase-labeled germ cells using EdU pulse-chase experiments, we reveal that this early-life acceleration is not maintained throughout adulthood but diminishes with age. Unlike previous studies that inferred aging effects primarily from static histological or transcriptomic snapshots, our pulse–chase approach directly quantifies age-dependent changes in spermatogenic progression kinetics *in vivo*. This age-associated alteration in spermatogenic progression broadens our understanding of spermatogenic dynamics across teleost fish, complementing findings from zebrafish and [62] medaka [63]. By integrating dynamic cell-cycle tracking with histological and transcriptomic

profiling in a short-lived vertebrate, our study bridges cellular kinetics with molecular remodeling of the niche across the lifespan. Together, these data suggest that the age-related slowdown and restriction of spermatogenesis reflect conserved limitations in germ cell dynamics and their supporting microenvironment [64] rather than a killifish-specific phenomenon. Impaired spermatogenesis is further evident by downregulation of mitotic and meiotic regulators detected by bulk RNA-Seq, demonstrating that testicular cell proliferation declines sharply between young and mid-aged males. A similar early decline in proliferative activity has been described in aging mammalian testes [65–67].

Importantly, the age-associated reduction in proliferative activity is not supported by EdU pulse–chase data alone but is independently observed by PCNA immunolabeling across a broad age range, indicating a robust decline in the fraction of cycling testicular cells. While single-pulse EdU labeling supports age-associated differences in advancement through spermatogenesis, age-dependent variation in EdU uptake, label dilution, or cohort attrition cannot be excluded. Accordingly, the absence of EdU-labeled late spermatogenic stages in mid-aged testes should be interpreted as consistent with slower transit or reduced effective cohort entry rather than definitive kinetic slowing.

Together with the proliferative decline, the somatic environment undergoes extensive remodeling. Histology revealed reduction of germinal compartment and accumulation of collagen-rich extracellular matrix, consistent with fibrosis-like stromal remodeling described in aging mammalian testes [68, 69]. Correspondingly, genes associated with adhesion, ECM organization, and matrix remodeling were strongly upregulated, and GO analyses confirmed enrichment of these pathways. Vascular and endothelial signaling programs become activated at the same stage, consistent with altered metabolic or structural demands in the remodeled tissue. Mechanistically, ECM stiffening has been shown to impair testicular endocrine and structural function, supporting the idea that the stromal remodeling observed here is likely to play a causal role in reproductive aging [70]. However, direct quantitative assessment of extracellular matrix deposition and tissue mechanics was not performed. Mallory trichrome provides qualitative evidence of collagen-rich matrix, while transcriptomic changes reflect ECM-associated transcriptional activation rather than confirmed matrix accumulation. Orthogonal assays will be required to directly quantify ECM composition and its mechanical properties.

While our data reveal a tight temporal and transcriptional association between stromal remodeling and germline decline, they do not establish direct causality. Functional perturbation of ECM and inflammatory pathways will be required to determine whether these processes actively drive germline aging. Furthermore, although multiple independent readouts converge on ECM-associated changes at mid-age, direct quantification of matrix deposition and tissue mechanics will be required to establish the extent and functional impact of extracellular matrix remodeling during testicular aging.

In mammals, age-associated disruption of immune homeostasis, whether through barrier dysfunction or altered interstitial immune activity, has been linked to impaired spermatogenesis and niche dysfunction [71]. In our dataset, mid-age further marks the onset of sustained inflammatory and stress-responsive gene expression. However, concepts of immune cells regulation and testicular environment are distinct

between mammals and fishes. The mammalian testis is classically considered an immune-privileged organ primarily due to the presence of the blood–testis barrier [72]. However, accumulating evidence indicates that immune cells are physiologically present within the testis and represent an integral component of the testicular microenvironment. In particular, macrophage populations have been shown to participate in testicular tissue remodeling in mammals, establish close spatial associations with somatic and germ cells, and contribute to the regulation of testicular function during development and homeostasis [73].

In teleost fishes, this concept of immune privilege is implemented differently. Although Sertoli cells surround all germ cells, their intercellular junctions do not form an early, continuous blood–testis barrier comparable to that of mammals, resulting in a stage-dependent and only partial immune privilege. Instead, functional barrier properties emerge late during spermatogenesis, typically at the end of or after meiosis, such that postmeiotic haploid germ cells are preferentially shielded from the vascular compartment and immune system. In several species, these junctions restrict the entry of large molecules into mature cysts, consistent with a functionally selective rather than absolute barrier [59].

In our dataset, cytokine-associated transcription factors, interferon pathway mediators, and macrophage-like markers become robustly upregulated from mid-age onward and remain elevated into old age. Given the late and partial nature of immune privilege in teleost testes, such immune activation may disproportionately affect premeiotic and meiotic germ cell stages and the surrounding somatic niche, without requiring overt disruption of barrier integrity. While our data do not allow us to directly distinguish whether these immune signatures originate from the interstitial compartment or reflect increased immune access to the germinal compartment, their temporal coincidence with progressive disorganization is consistent with altered immune–somatic crosstalk contributing to testicular aging. Similar inflammatory signatures have been associated with reproductive aging and niche disruption in mammals [74, 75], supporting the idea that immune-related remodeling represents a conserved component of testicular aging across vertebrates. Consistent with this interpretation, immune-related signatures identified here reflect persistent transcriptional changes at the tissue level; their cellular origin and functional activation will require future cell-type–resolved and protein-level validation.

As stromal and inflammatory programs intensify, germline identity markers show progressive downregulation. The transcriptional decline during aging includes genes with established roles in germline maintenance and spermatogenic differentiation, particularly those involved in meiotic progression and sperm flagellar assembly (e.g. *nanog*, *hsf2bp*, *dnah*, *rsph*, *ift*). These genes decline markedly at mid-age, consistent with reduced proliferative capacity. Reduced expression of germline regulators has been similarly reported in other models of reproductive senescence [67, 76].

The pattern observed in turquoise killifish, characterized by a rapid onset of reproductive competence followed by an age-associated slowdown of spermatogenic progression, aligns with broader life-history trade-offs described across vertebrates. Early maturation is often achieved at the expense of late-life reproductive performance, reflecting selection for increased early-life fertility at the cost of reproductive

maintenance later in life [77]. A similar diversity of life-history strategies is evident in reptiles, including lizards, where substantial interspecific variation in age at first reproduction and reproductive lifespan highlights trade-offs between rapid post-hatching development, early reproduction, and reproductive longevity [78]. Also, our data suggests that the major remodeling wave occurs at mid-age and that late-life testes represent a relatively stable remodeled state rather than undergoing continuous deterioration, consistent with non-linear aging patterns [79, 80].

Based on our integrated analyses, we propose a unifying conceptual model describing testicular development and aging in turquoise killifish (Fig. 7). This model is characterized by an exceptionally rapid expansion of the male germline immediately after hatching, reflecting the accelerated life history of this species and adaptation to ephemeral habitats [8, 10, 81, 82]. Following completion of spermatogenesis, testicular cells proliferation and differentiation intensify, with cell-cycling activity progressively increasing and reaching a maximum around 2 months of age. This peak represents a phase of highly efficient spermatogenesis, marked by minimal stromal activation and limited extracellular matrix deposition. Subsequently, the testis undergoes an abrupt transition into a remodeling phase, during which proliferative activity in both mitotic and meiotic germ cells sharply decline. This shift coincides with the activation of somatic programs associated with extracellular matrix remodeling, inflammation, and angiogenesis, ultimately leading to progressive fibrosis-like remodeling and erosion of the germline function. In late stages, the testicular tissue stabilizes in a remodeled state characterized by persistently low cell-cycling activity, reduced germline output, and a mixed stromal transcriptional signature. Together, this model captures a fundamental remodeling from a germ cell-dominated, highly prolific tissue to a somatically remodeled, low-regenerative environment, providing a framework for understanding how accelerated aging impacts reproductive capacity in short-lived vertebrates.

Conclusion

Our study establishes an integrated developmental and aging framework for the testis of the annual turquoise killifish. By combining detailed histology, proliferation assays, and transcriptomic profiling across the lifespan, we identified a distinct mid-age transition as the principal turning point in testicular aging. This transition is characterized by the well-defined decline in testicular cell proliferation, activation of ECM-remodeling and vascular programs, the emergence of persistent inflammatory signaling, and subsequent downregulation of germline identity markers. Rather than a gradual continuum, aging in the killifish testis proceeds through a rapid reorganization of the somatic niche that ultimately stabilizes into a chronically remodeled late life state. Although we do not resolve aging at a continuous temporal resolution, the concordant histological, proliferative, and transcriptomic shifts observed between young and mid-aged males clearly define this interval as the dominant inflection point in testicular aging.

Together, these findings establish the turquoise killifish as a powerful vertebrate model for resolving both the construction and remodeling of male reproductive function with high temporal precision. The identification of a discrete mid-age remodeling phase defines a tractable window for mechanistic

dissection and paves the way for testing genetic, environmental, and pharmacological interventions aimed at preserving germline function during aging.

Material and methods

We aimed to define the temporal and mechanistic organization of testicular development and aging across the lifespan of the turquoise killifish. We generated a staged histological atlas from hatching through adulthood, quantified proliferative activity, performed bulk RNA-Seq of young, mid-aged, and old testes to resolve age-associated transcriptional changes. Morphological remodeling was assessed using histology and trichrome staining.

Housing

Original parental broodstock of killifish MZM-222 outbred strain was obtained from Leibniz Institute on Aging and cultured under standard conditions [56]. Fish were fed twice a day with a dry diet (Gemma micro 500, Skretting) and once a day with frozen bloodworms (AQUARIMEX 5100016). Adults were provided with plastic cups filled with fine black sand with weekly collection intervals. Collected embryos were incubated at 28°C until reaching golden-eye stage (pre-hatching stage where the iris is gold-pigmented), transferred to Petri dishes filled with wetted coconut fiber and incubated for 3 weeks. Embryos were then hand-picked, hatching was triggered by their immersion in 2% cold humic acid extract (Hu-Ben Torben, H-10126). Larvae were cultured at 28°C provided with *Artemia* sp. (Ocean Nutrition, Sep-Art Artemia Cysts) ad libitum for 8 days. After that dry diet and frozen blood worms were provided twice a day each.

Sampling and histological analyses

Turquoise killifish were sampled at defined developmental and adult ages for histological, analyses. Fish were randomly selected from multiple tanks and clutches. Each individual represented one biological replicate.

From 0 days post-hatch (dph) to 29 dph fish were sampled at 2–3 days intervals ($n \geq 8$ for each time point). Body length and weight were measured immediately after euthanasia. Condition factor (K) was calculated as $K = 100 \times (\text{body weight} / \text{body length}^3)$. Collected tissues were fixed either in Bouin's solution or 4% formaldehyde at 4°C, washed thoroughly in PBS, dehydrated through graded ethanol series, and embedded in JB-4 resin (Sigma-Aldrich, EM0100) or paraffin (Sigma-Aldrich, P3683). Sections were cut at 4 µm thickness stained using a standard protocol for hematoxylin and eosin staining [83, 84].

Similar procedure for JB4 sectioning was adopted for males sampled from 1 month post-hatch till the termination of experiment. To analyze the proportion of non-germinal tissue the whole section was imaged using a 40x and automatic sample stitching. Next, non-germinal tissue was manually measured in Fiji and related to total testicular area per section.

Tissue samples for Trichrome Stain were embedded in wax using standard procedures [85] and stained with Mallory Trichrome Stain according to manufacturer's instructions (Sigma Aldrich, HT10516) and imaged (Olympus BX63).

Immunohistochemistry

For antibody labelling to visualize testicular organization, tissues from 3 representative males (2-month-old) were fixed in 4% formaldehyde and processed as for paraffin sectioning as described above. Rehydrated sections were subjected to antigen retrieval and blocked [86]. The primary antibody against DDX4 (GTX128306, GeneTex) was diluted 1:300 in antibody dilutant (DAKO) and then applied to the slides overnight at 4°C in a humidified chamber. Slides were then washed and incubated for 2 h at room temperature with a goat anti-rabbit secondary antibody conjugated to Alexa Fluor™ 594 (A-11012, Thermo Fisher) at a dilution of 1:500. Finally, the slides were mounted with Fluoroshield containing DAPI (F6057, Sigma-Aldrich) and imaged using a confocal microscope (Olympus FV3000).

From 30 dph to the termination of the experiment, gonads were fixed in 4% formaldehyde in PBS. At least $n = 5$ individuals were analyzed per stage. Formaldehyde fixed samples were processed for paraffin embedding, sectioning and immunohistochemistry including antigen retrieval following a standard procedure [86]. Testicular sections were stained with anti-PCNA antibody conjugated with Alexa Fluor® 488 (ab201672, Abcam) at 1:300 dilution and counterstained with Fluoroshield with DAPI (F6057, Sigma-Aldrich). Images were acquired using Olympus BX63 fluorescence microscope using consistent settings across experimental groups. Image analysis was performed in Fiji[87] by measuring total testicular area and PCNA+ area expressed as percentage from the total area. Obtained data were normalized to measurements on 3 non-adjacent sections per male.

EdU proliferation analysis

Since PCNA detection provides rather a snapshot of the testicular state, we also employed EdU labelling to gain more insights into the dynamics of spermatogenesis. Anaesthetized males (6–9 males per sampling point and per age cohort) were intraperitoneally injected with a single dose of EdU 50mg/kg (BCK-EdU594IM100, baseclick GmbH) using a 10 µl nanofil syringe with 33 GA beveled needle (WPI) and sacrificed at 2-120 h intervals. Excised testes were fixed in 4% formaldehyde and processed for paraffin embedding and sectioning. EdU detection was performed according to manufacturer instructions using 5/6-Sulforhodamine 101-PEG3-Azide. Sections were counterstained with Fluoroshield with DAPI (F6057, Sigma-Aldrich) and imaged using a fluorescence microscope (Olympus BX63) for quantification of the EdU+ area. Image analysis was performed as described above for PCNA immunohistochemistry. For precise lineage tracing of EdU distribution confocal microscope imaging was later used (Olympus FV3000) followed by manual analysis of the individual spermatocysts showing EdU signal and their staging based on published literature [19].

Statistical analysis

Statistical analyses were performed using appropriate non-parametric methods. Group differences were assessed using the Kruskal–Wallis test followed by Dunn’s post hoc test, where applicable. When multiple pairwise comparisons were performed, *p* values were adjusted to control the false discovery rate using the Benjamini, Krieger and Yekutieli method.

Transmission electron microscopy

Testicular samples from 2-month-old killifish were cut into 2-3mm fragments and fixed into 2.5% glutaraldehyde (G5882, Sigma-Aldrich) in PBS (P4417, Sigma-Aldrich) and processed for TEM as described previously [88, 89]. Briefly, samples were dehydrated in acetone, embedded in Poly/Bed 812 (08791 – 500, Polysciences). Ultrathin sections were cut on a Leica ultramicrotome, double-stained with uranyl acetate and lead citrate mounted and imaged using TEM (JEOL 1400, Jeol).

Bulk RNA-Seq

For transcriptome sequencing we selected three age groups of turquoise killifish consisting young 1.5-month-old (*n* = 6), mid-aged 4-month-old (*n* = 7) and old 6-month-old (*n* = 7). Males were euthanized in MS222, dissected and testicular tissue was snap-frozen in a liquid nitrogen. Total RNA was extracted using TriReagent extraction and LiCl precipitation (Sigma) according to the manufacturer’s instructions. The concentration of total RNA was determined using a spectrophotometer (Nanodrop 2000; Thermo Fisher Scientific), and the quality of RNA was assessed using a Fragment Analyzer (Agilent, Standard Sensitivity RNA analysis kit, DNF-471). Two hundred fifty nanograms of total RNA were used for library preparation (NEBNext UltraExpress RNA Library Prep Kit ,E3330L, NEB) with poly-A enrichment (NEBNext Poly(A) mRNA Magnetic Isolation Module, E7490S, NEB). Libraries were pooled and sequenced using Illumina NextSeq 500 2x75 bp.

Bulk RNA-Seq analysis

RNA-Seq generated an average of 32.5 million read pairs per sample, ranging from 27.9 to 41.1 million. Adapter trimming and quality filtering were performed using bbduk.sh script v39.06 from BBMap software suite [90] with the following parameters: ktrim = r, k = 23, mink = 11, hdist = 1, tpe, tbo, qtrim = rl, trimq = 10, and minlen = 40, and ref=adapters.fa (provided by BBMap software). Ribosomal RNA reads were removed using again BBDuk with the parameters: ref=smr_v4.3_default_db.fasta.gz and k = 27. The rRNA reference database was obtained from the SortMeRNA project [91].

Reads were aligned to the *N. furzeri* reference genome using STAR v2.7.11b [92]. The genome index was generated using the Ensembl reference genome Nfu_20140520, release 115, with the corresponding gene annotation and sjdbOverhang set to 60. On average, 96.57% of reads mapped to the reference genome, including 93.57% uniquely mapped reads. Gene-level read quantification was performed using featureCounts v2.0.8 [93] in paired-end, strand-specific mode.

Differential expression analysis was performed in R v4.4.3 using DESeq2 v1.44.0 [94], and tested as the pairwise contrasts old vs young, mid vs young, and old vs mid, and *p* values were adjusted using the

Benjamini–Hochberg method. Genes were considered differentially expressed if they showed an adjusted p value ≤ 0.05 and $\log_2$ fold change >1 .

Volcano plots were generated using EnhancedVolcano v1.22.0 (Blighe et al., 2024) based on DESeq2 differential expression results. Heatmaps were generated (i) from all DEGs, (ii) for predefined gene sets related to cell cycle, apoptosis, fibrosis, inflammation, and spermatogenesis, based on curated gene lists containing DEGs associated with each process. After DESeq2 median of ratios normalization, variance-stabilized expression values were obtained using the DESeq2 vst function and mean centered per gene for visualization. Heatmaps were produced using Pearson's correlation distance and Ward's clustering method.

GO over-representation analysis was performed using clusterProfiler v2.60.1 [96] with the enrichGO function and the org.Dr.eleg.db annotation database v3.19.1 [97] on differentially expressed genes mapped to one-to-one zebrafish orthologs retrieved from Ensembl via biomaRt [98]. Only biological process ontology was used, with Benjamini–Hochberg adjustment and p valueCutoff = 0.1. *N. furzeri* Ensembl gene identifiers were converted to *N. furzeri* Entrez Gene IDs via biomaRt, and the enrichKEGG function was used to identify enriched KEGG T05163 pathways [99] based on DEGs from each pairwise comparison.

Declarations

Ethics

All procedures were performed in accordance with the Animal Research Committee of the Faculty of Fisheries and Protection of Waters (Vodňany, Czech Republic) and were approved by the Ministry of Agriculture of the Czech Republic (reference number: MZE-3972339723/2025-13143). Fish were maintained according to the principles based on the EU-harmonized Animal Welfare Act of the Czech Republic and Principles of Laboratory Animal Care in compliance with the national law (Act No. 246/1992 on the Protection of Animals Against Cruelty).

Consent for publication

All authors approved the final version of manuscript.

Data availability statement:

The raw data generated from bulk RNA-Seq are available in the Gene Expression Omnibus (GEO) under accession GSE320313. All data generated or analyzed during this study are included in the manuscript and its supplementary files.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this manuscript.

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Authors' contributions

Conceptualization: RF. Data curation: RF; TT. Formal analysis: RF, TT. Funding acquisition: RF, RS. Investigation: all authors contributed. Methodology: RF, TT, RS, HS. Project administration: RF, RS. Resources: RF, TT, RS, HS. Supervision: RF. Validation: RF, TT, RS, ATM. Visualization: RF, TT, ATM, DHSS. Writing – original draft: RF. Writing – review & editing: all authors contributed.

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Declaration of generative AI in scientific writing

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References

1. Dong S, Chen C, Zhang J, Gao Y, Zeng X, Zhang X. Testicular aging, male fertility and beyond. *Front Endocrinol.* 2022;13:1012119. <https://doi.org/10.3389/FENDO.2022.1012119/XML>.
2. Jimbo M, Kunisaki J, Ghaed M, Yu V, Flores HA, Hotaling JM. Fertility in the aging male: a systematic review. *Fertil Steril.* 2022;118:1022–34. <https://doi.org/10.1016/J.FERTNSTERT.2022.10.035>.
3. Yatsenko AN, Turek PJ. Reproductive genetics and the aging male. *J Assist Reprod Genet.* 2018;35:933. <https://doi.org/10.1007/S10815-018-1148-Y>.
4. Carrageta DF, Guerra-Carvalho B, Spadella MA, Yeste M, Oliveira PF, Alves MG. Animal models of male reproductive ageing to study testosterone production and spermatogenesis. *Rev. Endocr Metab Disord.* 2022;23:1341–60. <https://doi.org/10.1007/S11154-022-09726-9>.
5. Liew WC, Bartfai R, Lim Z, Sreenivasan R, Siegfried KR, Orban L. Polygenic sex determination system in zebrafish. *PLoS One.* 2012;7:e34397. <https://doi.org/10.1371/journal.pone.0034397>.

6. Tanaka M, Saito D, Morinaga C, Kurokawa H. Cross talk between germ cells and gonadal somatic cells is critical for sex differentiation of the gonads in the teleost fish, medaka (*Oryzias latipes*). *Dev Growth and Differ.* 2008;50:273–8. <https://doi.org/10.1111/j.1440-169X.2008.01015.x>.
7. Nishimura T, Yamada K, Fujimori C, Kikuchi M, Kawasaki T, Siegfried KR, et al. Germ cells in the teleost fish medaka have an inherent feminizing effect. *PLoS Genet.* 2018;14:e1007259. <https://doi.org/10.1371/journal.pgen.1007259>.
8. Cellerino A, Valenzano DR, Reichard M. From the bush to the bench: the annual *Nothobranchius* fishes as a new model system in biology. *Biol Rev.* 2016;91:511–33. <https://doi.org/10.1111/BRV.12183>.
9. Blažek R, Polačik M, Reichard M. Rapid growth, early maturation and short generation time in African annual fishes. *Evodevo.* 2013;4:24. <https://doi.org/10.1186/2041-9139-4-24>.
10. Vrtílek M, Žák J, Pšenička M, Reichard M. Extremely rapid maturation of a wild African annual fish. *Curr Biol.* 2018;28:R822–4. <https://doi.org/10.1016/j.cub.2018.06.031>.
11. Richter A, Mörl H, Thielemann M, Kleemann M, Geißen R, Schwarz R, et al. The master male sex determinant Gdf6Y of the turquoise killifish arose through allelic neofunctionalization. *Nat Comm.* 2025 16:1. 2025;16:1–18. <https://doi.org/10.1038/s41467-025-55899-7>.
12. Hu CK, Brunet A. The African turquoise killifish: A research organism to study vertebrate aging and diapause. *Aging Cell.* 2018;17. <https://doi.org/10.1111/accel.12757>.
13. Terzibasi E, Valenzano DR, Cellerino A. The short-lived fish *Nothobranchius furzeri* as a new model system for aging studies. *Exp Gerontol.* 2007;42:81–9. <https://doi.org/10.1016/J.EXGER.2006.06.039>.
14. Kim Y, Nam HG, Valenzano DR. The short-lived African turquoise killifish: an emerging experimental model for ageing. *Dis Model Mech.* 2016;9:115. <https://doi.org/10.1242/DMM.023226>.
15. Ruparelia AA, Salavaty A, Barlow CK, Lu Y, Sonntag C, Hersey L, et al. The African killifish: A short-lived vertebrate model to study the biology of sarcopenia and longevity. *Aging Cell.* 2024;23:e13862. <https://doi.org/10.1111/ACEL.13862>.
16. Di Fraia D, Marino A, Lee JH, Sacramento EK, Baumgart M, Bagnoli S, et al. Altered translation elongation contributes to key hallmarks of aging in the killifish brain. *Science.* 2025;389:eadk3070. <https://doi.org/10.1126/science.adk3079>
17. Žák J, Reichard M. Reproductive senescence in a short-lived fish. *J Anim Ecol.* 2021;90:492–502. <https://doi.org/10.1111/1365-2656.13382>.
18. Cattelan S, Valenzano DR. Age-dependent decline in sperm quality and function in a naturally short-lived vertebrate. *BMC Ecol Evol.* 2025;25:2-. <https://doi.org/10.1186/S12862-024-02343-X/TABLES/2>.
19. Nóbrega RH, Greebe CD, van de Kant H, Bogerd J, de França LR, Schulz RW. Spermatogonial stem cell niche and spermatogonial stem cell transplantation in zebrafish. *PLoS One.* 2010;5:1–16. <https://doi.org/10.1371/journal.pone.0012808>.

20. Leal MC, Cardoso ER, Nóbrega RH, Batlouni SR, Bogerd J, França LR, et al. Histological and stereological evaluation of zebrafish (*Danio rerio*) spermatogenesis with an emphasis on spermatogonial generations. *Biol Reprod.* 2009; 81(1):177–187. <https://doi.org/10.1095/biolreprod.109.076299>.
21. Schulz RW, Menting S, Bogerd J, França LR, Vilela DAR, Godinho HP. Sertoli Cell Proliferation in the Adult Testis—Evidence from Two Fish Species Belonging to Different Orders. *Biol Reprod.* 2005;73(5):891–898. <https://doi.org/10.1095/biolreprod.105.039891>.
22. Petersen C, Söder O. The Sertoli Cell – A Hormonal Target and ‘Super’ Nurse for Germ Cells That Determines Testicular Size. *Horm Res Paediatr.* 2006;66:153–61. <https://doi.org/10.1159/000094142>.
23. Griswold MD. Spermatogenesis: The Commitment to Meiosis. *Physiol Rev.* 2016;96:1–17. <https://doi.org/10.1152/PHYSREV.00013.2015>.
24. Smith LB, Walker WH. The regulation of spermatogenesis by androgens. *Semin Cell Dev Biol.* 2014;30:2–13. <https://doi.org/10.1016/J.SEMCDB.2014.02.012>.
25. Žák J, Reichard M. Fluctuating temperatures extend median lifespan, improve reproduction and reduce growth in turquoise killifish. *Exp Gerontol.* 2020;140:111073. <https://doi.org/10.1016/J.EXGER.2020.111073>.
26. Baumgart M, Di Cicco E, Rossi G, Cellerino A, Tozzini ET. Comparison of captive lifespan, age-associated liver neoplasias and age-dependent gene expression between two annual fish species: *Nothobranchius furzeri* and *Nothobranchius korthause*. *Biogerontology.* 2015;16:63–9. <https://doi.org/10.1007/S10522-014-9535-Y>.
27. Labib K, Gambus A. A key role for the GINS complex at DNA replication forks. *Trends Cell Biol.* 2007;17:271–8. <https://doi.org/10.1016/J.TCB.2007.04.002>.
28. Vermeulen K, Van Bockstaele DR, Berneman ZN. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif.* 2003;36:131. <https://doi.org/10.1046/J.1365-2184.2003.00266.X>.
29. Arellano M, Moreno S. Regulation of CDK/cyclin complexes during the cell cycle. *International Journal of Biochemistry and Cell Biology.* 1997;29:559–73. [https://doi.org/10.1016/S1357-2725\(96\)00178-1](https://doi.org/10.1016/S1357-2725(96)00178-1).
30. Hopkins J, Hwang G, Jacob J, Sapp N, Bedigian R, Oka K, et al. Meiosis-Specific Cohesin Component, Stag3 Is Essential for Maintaining Centromere Chromatid Cohesion, and Required for DNA Repair and Synapsis between Homologous Chromosomes. *PLoS Genet.* 2014;10:e1004413. <https://doi.org/10.1371/JOURNAL.PGEN.1004413>.
31. Peters JM, Tedeschi A, Schmitz J. The cohesin complex and its roles in chromosome biology. *Genes Dev.* 2008;22:3089–114. <https://doi.org/10.1101/GAD.1724308>.
32. Liu Z, Tian M, Ding K, Liu H, Wang Y, Fu R. High expression of PIM2 induces HSC proliferation in myelodysplastic syndromes via the IDH1/HIF1- α signaling pathway. *Oncol Lett.* 2019;17:5395. <https://doi.org/10.3892/OL.2019.10256>.

33. Sato T, Umetsu A, Tamanoi F. Characterization of the Rheb-mTOR Signaling Pathway in Mammalian Cells: Constitutive Active Mutants of Rheb and mTOR. *Methods Enzymol.* 2008;438:307. [https://doi.org/10.1016/S0076-6879\(07\)38021-X](https://doi.org/10.1016/S0076-6879(07)38021-X).
34. Drosten M, Barbacid M. Targeting the MAPK Pathway in KRAS-Driven Tumors. *Cancer Cell.* 2020;37:543–50. <https://doi.org/10.1016/J.CCELL.2020.03.013>.
35. Barczyk M, Carracedo S, Gullberg D. Integrins. *Cell Tissue Res.* 2009;339:269. <https://doi.org/10.1007/S00441-009-0834-6>.
36. Cabral-Pacheco GA, Garza-Veloz I, Rosa CCD La, Ramirez-Acuña JM, Perez-Romero BA, Guerrero-Rodriguez JF, et al. The Roles of Matrix Metalloproteinases and Their Inhibitors in Human Diseases. *Int J Mol Sci.* 2020;21:9739. <https://doi.org/10.3390/IJMS21249739>.
37. Martins SG, Ribeiro V, Melo C, Paulino-Cavaco C, Antonini D, Naidu SD, et al. Laminin- α 2 chain deficiency in skeletal muscle causes dysregulation of multiple cellular mechanisms. *Life Sci Alliance.* 2024;7:e202402829. <https://doi.org/10.26508/LSA.202402829>.
38. Liu R, Li B, Zi J, Zhang R, Yu M, Zhou J, et al. The dual role of LOXL4 in the pathogenesis and development of human malignant tumors: a narrative review. *Transl Cancer Res.* 2024;13:2026–42. <https://doi.org/10.21037/TCR-23-2003/COIF>.
39. Fan D, Kassiri Z. Biology of Tissue Inhibitor of Metalloproteinase 3 (TIMP3), and Its Therapeutic Implications in Cardiovascular Pathology. *Front Physiol.* 2020;11:546314. <https://doi.org/10.3389/FPHYS.2020.00661/XML>.
40. Karamanos NK, Theocharis AD, Piperigkou Z, Manou D, Passi A, Skandalis SS, et al. A guide to the composition and functions of the extracellular matrix. *FEBS J.* 2021;288:6850–912. <https://doi.org/10.1111/FEBS.15776>.
41. Park SY, Son K, Kim J, Kim K, Joo S, Kim B, et al. Cathepsin L as a dual-target to mitigate muscle wasting while enhancing anti-tumor efficacy of anti-PD-L1. *Nat Com.* 2025;16:1. 2025;16:10706-. <https://doi.org/10.1038/s41467-025-64500-0>.
42. Becerra SP, Notario V. The effects of PEDF on cancer biology: mechanisms of action and therapeutic potential. *Nat Rev Cancer.* 2013;13:258–71. <https://doi.org/10.1038/NRC3484>.
43. Zhang Y, Kontos CD, Annex BH, Popel AS. Angiopoietin-Tie Signaling Pathway in Endothelial Cells: A Computational Model. *iScience.* 2019;20:497. <https://doi.org/10.1016/J.ISCI.2019.10.006>.
44. Xu K, Chong DC, Rankin SA, Zorn AM, Cleaver O. Rasip1 is required for endothelial cell motility, angiogenesis and vessel formation. *Dev Biol.* 2009;329:269–79. <https://doi.org/10.1016/J.YDBIO.2009.02.033>.
45. Wettschureck N, Strilic B, Offermanns S. Passing the vascular barrier: Endothelial signaling processes controlling extravasation. *Physiol Rev.* 2019;99:1467–525. <https://doi.org/10.1152/PHYSREV.00037.2018/ASSET/IMAGES/LARGE/Z9J0031929080022.JPEG>.
46. Helker CSM, Eberlein J, Wilhelm K, Sugino T, Malchow J, Schuermann A, et al. Apelin signaling drives vascular endothelial cells towards a pro-angiogenic state. *Elife.* 2020;9:1–44. <https://doi.org/10.7554/ELIFE.55589>.

47. Medzhitov R. Origin and physiological roles of inflammation. *Nature* 2008 454:7203. 2008;454:428–35. <https://doi.org/10.1038/nature07201>.
48. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell*. 2006;124:783–801. <https://doi.org/10.1016/J.CELL.2006.02.015/ASSET/8793559C-2439-44D5-A8B9-E680D9CBB773/MAIN.ASSETS/GR3.JPG>.
49. Lurie RH, Platanias LC. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol*. 2005;5:375–86. <https://doi.org/10.1038/nri1604>.
50. Heldin CH, Westermark B. Mechanism of action and in vivo role of platelet-derived growth factor. *Physiol Rev*. 1999;79:1283–316. <https://doi.org/10.1152/physrev.1999.79.4.1283>
51. Jinno A, Tanaka K, Matsushime H, Haneji T, Shibuya M. Testis-specific mak protein kinase is expressed specifically in the meiotic phase in spermatogenesis and is associated with a 210-kilodalton cellular phosphoprotein. *Mol Cell Biol*. 1993;13:4146. <https://doi.org/10.1128/MCB.13.7.4146>.
52. Wang X, Shen G, Yang Y, Jiang C, Ruan T, Yang X, et al. Biallelic pathogenic variants in DNAH3 cause male infertility in humans and mice. *Elife*. 2024;13. <https://doi.org/10.7554/ELIFE.96755.1>.
53. Struijk RB, Mulder CL, van Daalen SKM, de Winter-Korver CM, Jongejan A, Repping S, et al. ITGA6 + Human Testicular Cell Populations Acquire a Mesenchymal Rather than Germ Cell Transcriptional Signature during Long-Term Culture. *Int J Mol Sci*. 2020;21:1–20. <https://doi.org/10.3390/IJMS21218269>.
54. Chambers I, Silva J, Colby D, Nichols J, Nijmeijer B, Robertson M, et al. Nanog safeguards pluripotency and mediates germline development. *Nature* 2007 450:7173. 2007;450:1230–4. <https://doi.org/10.1038/nature06403>.
55. Liyanage SSN, Bosworth BG, Martin KA, Wood KR, Nowicki AE, Abernathy JW, et al. Age-related reproductive performance and transcriptome profiling of testis in male blue catfish, *Ictalurus furcatus*. *BMC Genomics*. 2025;26:817. <https://doi.org/10.1186/S12864-025-12014-W>.
56. Polačik M, Blažek R, Reichard M. Laboratory breeding of the short-lived annual killifish *Nothobranchius furzeri*. *Nat Prot*. 2016;11:1396–413. <https://doi.org/10.1038/nprot.2016.080>.
57. Maack G, Segner H. Morphological development of the gonads in zebrafish. *J Fish Biol*. 2003;62:895–906. <https://doi.org/10.1046/J.1095-8649.2003.00074.X>.
58. Zhang J, Huang JQ, Li YY, Wang N, Sun J, Wang TZ, et al. Histological observations of Medaka(*Oryzias latipes*) gonad sexual differentiation and development. *Zool Res*. 2013;34:464–70. <https://doi.org/10.11813/J.ISSN.0254-5853.2013.5.0464>.
59. Schulz RWRW, Renato L, França D, Lareyre JJ, Legac F, Chiarini-garcia H, et al. Spermatogenesis in fish. *Gen Comp Endocrinol*. 2010;165:390–411. <https://doi.org/10.1016/j.ygcen.2009.02.013>.
60. Schulz RW, Crespo D, Chauvigné F, Cerdà J. Spermatogenesis: The development from stem cells to sperm and its regulation by hormones and growth factors. *Encyclopedia of Fish Physiology*. 2024; 652–70. <https://doi.org/10.1016/B978-0-323-90801-6.00042-2>.

61. Coolen M, Labusch M, Mannioui A, Bally-Cuif L. Mosaic Heterochrony in Neural Progenitors Sustains Accelerated Brain Growth and Neurogenesis in the Juvenile Killifish *N. furzeri*. *Curr Biol*. 2020;30:736–745.e4. <https://doi.org/10.1016/j.cub.2019.12.046>.
62. Sposato AL, Hollins HL, Llewellyn DR, Weber JM, Schrock MN, Farrell JA, et al. Germ cell progression through zebrafish spermatogenesis declines with age. *Development*. 2024;151. <https://doi.org/10.1242/dev.204319>.
63. Duangkaew R, Kezuka F, Ichida K, Boonanuntanasarn S, Yoshizaki G. Aging- and temperature-related activity of spermatogonial stem cells for germ cell transplantation in medaka. *Theriogenology*. 2020;155:213–21. <https://doi.org/10.1016/j.theriogenology.2020.05.049>.
64. Li T, Xu W, Yin L, Zhang J, Dhikhirullahi O, Zhang Z, et al. Effects of Aging on Mammalian Spermatogenesis From a Cellular Perspective. *Mol Reprod Dev*. 2025;92:e70061. <https://doi.org/10.1002/MRD.70061>.
65. Paul C, Robaire B. Ageing of the male germ line. *Nat Rev Urol*. 2013;10:227–34. <https://doi.org/10.1038/nrurol.2013.18>.
66. Morales E, Pastor LM, Horn R, Zuasti A, Ferrer C, Calvo A, et al. Effect of ageing on the proliferation and apoptosis of testicular germ cells in the Syrian hamster *Mesocricetus auratus*. *Reprod Fertil Dev*. 2003;15:89–98. <https://doi.org/10.1071/RD02071>.
67. Ozawa M, Mori H, Endo T, Ishikawa-Yamauchi Y, Motooka D, Emori C, et al. Age-related decline in spermatogenic activity accompanied with endothelial cell senescence in male mice. *iScience*. 2023;26:108456. <https://doi.org/10.1016/J.ISCI.2023.108456>.
68. Xu Y, Hu P, Chen W, Chen J, Liu C, Zhang H. Testicular fibrosis pathology, diagnosis, pathogenesis, and treatment: A perspective on related diseases. *Andrology*. 2025;13:1322–32. <https://doi.org/10.1111/ANDR.13769>.
69. Stöckl JB, Schmid N, Flenkenthaler F, Drummer C, Behr R, Mayerhofer A, et al. Age-related alterations in the testicular proteome of a non-human primate. *Cells*. 2021;10:1306. <https://doi.org/10.3390/CELLS10061306/S1>.
70. Huang J, Sun L, Yin Y, Yang H, Zeng K, Huang P, et al. High matrix stiffness triggers testosterone decline in aging males by disrupting stem Leydig cell pool homeostasis. *Cell Rep*. 2025;44:116207. <https://doi.org/10.1016/J.CELREP.2025.116207>.
71. Fijak M, Meinhardt A. The testis in immune privilege. *Immunol Rev*. 2006;213:66–81. <https://doi.org/10.1111/J.1600-065X.2006.00438.X>.
72. Jiang XH, Bukhari I, Zheng W, Yin S, Wang Z, Cooke H, et al. Blood-testis barrier and spermatogenesis: lessons from genetically-modified mice. *Asian J Androl*. 2014;16:572. <https://doi.org/10.4103/1008-682X.125401>.
73. Hosseini S, Moody SC, Fietz D, Indumathy S, Schuppe HC, Hedger MP, et al. The changing landscape of immune cells in the fetal mouse testis. *Histochem Cell Biol*. 2022;158:345–68. <https://doi.org/10.1007/S00418-022-02129-6>.

74. Frungieri MB, Calandra RS, Bartke A, Matzkin ME. Ageing and inflammation in the male reproductive tract. *Andrologia*. 2018;50. <https://doi.org/10.1111/AND.13034>.
75. Zhang W, Xia S, Xiao W, Song Y, Tang L, Cao M, et al. A single-cell transcriptomic landscape of mouse testicular aging. *J Adv Res*. 2022;53:219. <https://doi.org/10.1016/J.JARE.2022.12.007>.
76. Zhang W, Xia S, Xiao W, Song Y, Tang L, Cao M, et al. A single-cell transcriptomic landscape of mouse testicular aging. *J Adv Res*. 2023;53:219–34. <https://doi.org/10.1016/J.JARE.2022.12.007>.
77. Ellison PT, Ottinger MA. A Comparative Perspective on Reproductive Aging, Reproductive Cessation, Post-Reproductive Life, and Social Behavior. 2014.
78. Shine R. Life-history evolution in reptiles. *Annu Rev Ecol Evol Syst*. 2005;36:23–46. <https://doi.org/10.1146/annurev.ecolsys.36.102003.152631>
79. Somel M, Guo S, Fu N, Yan Z, Hu HY, Xu Y, et al. MicroRNA, mRNA, and protein expression link development and aging in human and macaque brain. *Genome Res*. 2010;20:1207–18. <https://doi.org/10.1101/GR.106849.110>.
80. Shen X, Wang C, Zhou X, Zhou W, Hornburg D, Wu S, et al. Nonlinear dynamics of multi-omics profiles during human aging. *Nat Aging* 2024 4:11. 2024;4:1619–34. <https://doi.org/10.1038/s43587-024-00692-2>.
81. Astre G, Moses E, Harel I. The African turquoise killifish (*Nothobranchius furzeri*): biology and research applications. *Laboratory Fish in Biomedical Research: Biology, Husbandry and Research Applications for Zebrafish, Medaka, Killifish, Cavefish, Stickleback, Goldfish and Danionella Translucida*. 2022;:245–87. <https://doi.org/10.1016/B978-0-12-821099-4.00011-0>.
82. Valenzano DR, Benayoun BA, Singh PP, Zhang E, Etter PD, Hu CK, et al. The African Turquoise Killifish Genome Provides Insights into Evolution and Genetic Architecture of Lifespan. *Cell*. 2015. <https://doi.org/10.1016/j.cell.2015.11.008>.
83. Nayak R, Franěk R, Šindelka R, Pšenička M. Enhancement of zebrafish sperm production via a large body-sized surrogate with germ cell transplantation. *Commun Biol*. 2023;6:412. <https://doi.org/10.1038/s42003-023-04800-7>.
84. Torres-Martínez A, Tichopád T, Pšenička M, Franěk R. Triploidy alters hormonal and paracrine signaling to promote male development in zebrafish. *Mol Cell Endocrinol*. 2026;615:112740. <https://doi.org/10.1016/J.MCE.2026.112740>.
85. Franěk R, Tichopád T, Fučíková M, Steinbach C, Pšenička M. Production and use of triploid zebrafish for surrogate reproduction. *Theriogenology*. 2019;140:33–43. <https://doi.org/10.1016/J.THERIOGENOLOGY.2019.08.016>.
86. Havlíková Z, Gao L, Hubálek M, Vazačová M, Stundl J, Stundlova J, et al. Crispr-Cas13d Mediated Germ Cell Ablation in Sterlet Sturgeon: Advancing Surrogate Reproduction Technologies for Endangered Species. 2025. <https://doi.org/10.2139/SSRN.5364348>.
87. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods* 2012 9:7. 2012;9:676–82. <https://doi.org/10.1038/nmeth.2019>.

88. Tichopád T, Vetešník L, Šimková A, Rodina M, Franěk R, Pšenička M. Spermatozoa morphology and reproductive potential in F1 hybrids of common carp (*Cyprinus carpio*) and gibel carp (*Carassius gibelio*). *Aquaculture*. 2020. <https://doi.org/10.1016/j.aquaculture.2020.735092>.
89. Gao L, Franěk R, Tichopád T, Rodina M, Gela D, Šindelka R, et al. Mitochondria as indispensable yet replaceable components of germ plasm: insights into PGCs specification in sturgeons. *Reproduction*. 2025;169. <https://doi.org/10.1530/REP-24-0441>.
90. Bushnell B. BBMap: A Fast, Accurate, Splice-Aware Aligner. 2014.
91. Kopylova E, Noé L, Touzet H. SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. *Bioinformatics*. 2012;28:3211–7. <https://doi.org/10.1093/BIOINFORMATICS/BTS611>.
92. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 2013;29:15–21. <https://doi.org/10.1093/bioinformatics/bts635>.
93. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*. 2014;30:923–30. <https://doi.org/10.1093/BIOINFORMATICS/BTT656>.
94. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15:550-. <https://doi.org/10.1186/S13059-014-0550-8/FIGURES/9>.
95. Blighe Kevin, Rana Sam, Lewis Mam. EnhancedVolcano: Publication-ready volcano plots with enhanced colouring and labeling. R package. 2024. <https://doi.org/doi:10.18129/B9.bioc.EnhancedVolcano>.
96. Xu S, Hu E, Cai Y, Xie Z, Luo X, Zhan L, et al. Using clusterProfiler to characterize multiomics data. *Nature Protocols* 2024 19:11. 2024;19:3292–320. <https://doi.org/10.1038/s41596-024-01020-z>.
97. Carlson M. Genome wide annotation for Zebrafish. R package version 3.19.1. 2024.
98. Durinck S, Spellman PT, Birney E, Huber W. Mapping identifiers for the integration of genomic datasets with the R/Bioconductor package biomaRt. *Nat Protoc*. 2009;4:1184–91. <https://doi.org/10.1038/NPROT.2009.97>.
99. Reichwald K, Petzold A, Koch P, Downie BR, Hartmann N, Pietsch S, et al. Insights into Sex Chromosome Evolution and Aging from the Genome of a Short-Lived Fish. *Cell*. 2015;163:1527–38. <https://doi.org/10.1016/J.CELL.2015.10.071>.

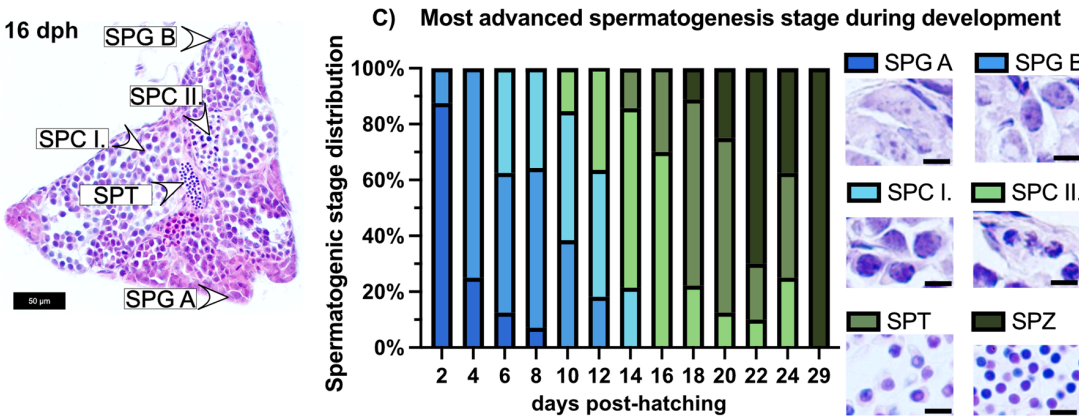
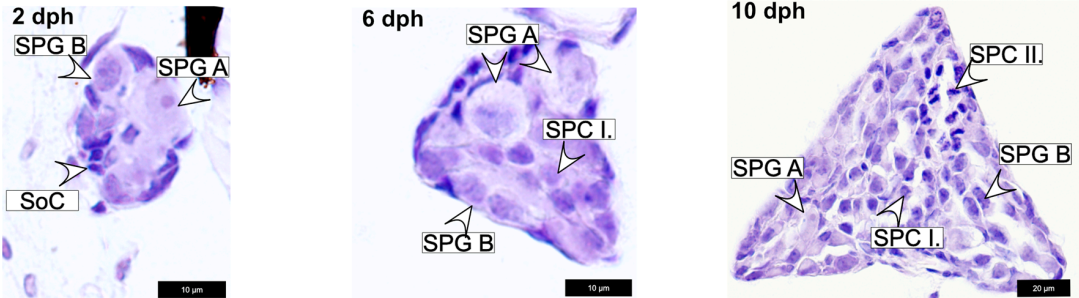
Figures

Fig. 1

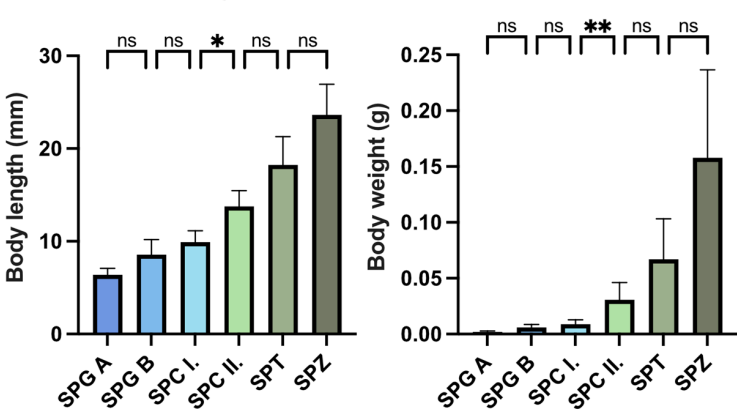
A) Adult killifish male (red tail morph) and female



B) Spermatogenesis progression post-hatching



D) Transition from SPC I. to SPC II. is the only spermatogenic phase associated with body size



E) Condition factor is not associated with final maturation

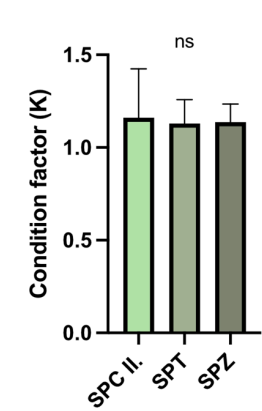


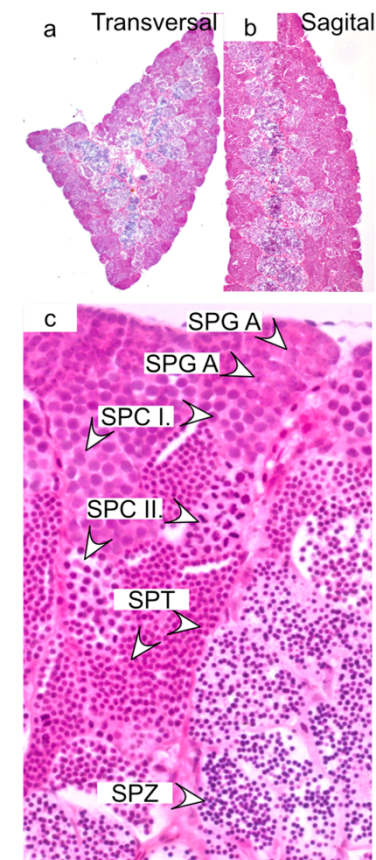
Figure 1

Rapid testicular development and spermatogenesis in turquoise killifish. A) An artistic artwork showing adult turquoise killifish male and appearance of adult female B) Representative histological sections illustrating the progression of spermatogenesis during early post-hatching development. At 2 dph and 6 dph, the most advanced germ cell stages are spermatogonia (arrowheads) and primary spermatocytes, respectively. At 10 dph, secondary spermatocytes become evident. By 16 dph advanced spermatogenic

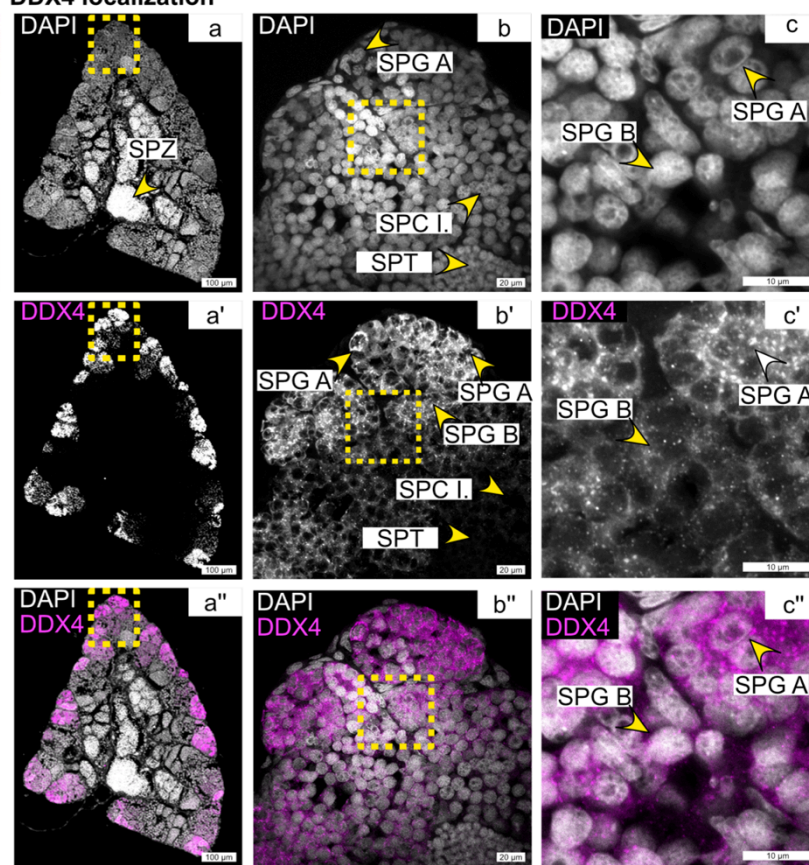
cyst with spermatozoa is present. Arrowheads show distinct germ cell stages. **C)** Quantification of the most advanced spermatogenic stage present in individual testes at successive developmental time points (2–29 dph). Stacked bars represent the proportion of individuals reaching given spermatogenic stage. Abbreviations: type A spermatogonia (SPG A), type B spermatogonia (SPG B), primary spermatocytes (SPC I), secondary spermatocytes (SPC II), spermatids (SPT), and spermatozoa (SPZ). Scale bars: 5 mm. **D)** Body weight (left) and body length (right) of males grouped according to the most advanced spermatogenic stage present. Differences among stages were analyzed using the Kruskal–Wallis test followed by Dunn’s multiple comparisons test with adjustment for family-wise error rate (FWER). Significance is indicated by asterisks ($*p < 0.05$; $**p < 0.01$). The transition from SPC I to SPC II represents the only stage transition significantly associated with somatic size. Bars represent mean $\pm$ SEM. **E)** Condition factor of males at SPC II, SPT, and SPZ stages. No significant differences were detected (Kruskal–Wallis test, $P \geq 0.05$). Bars represent mean $\pm$ SEM.

Fig. 2

A) H&E staining



B) Killifish shows restricted type of spermatogenesis revealed by DDX4 localization



C) Ultrastructure of killifish testicular tissue

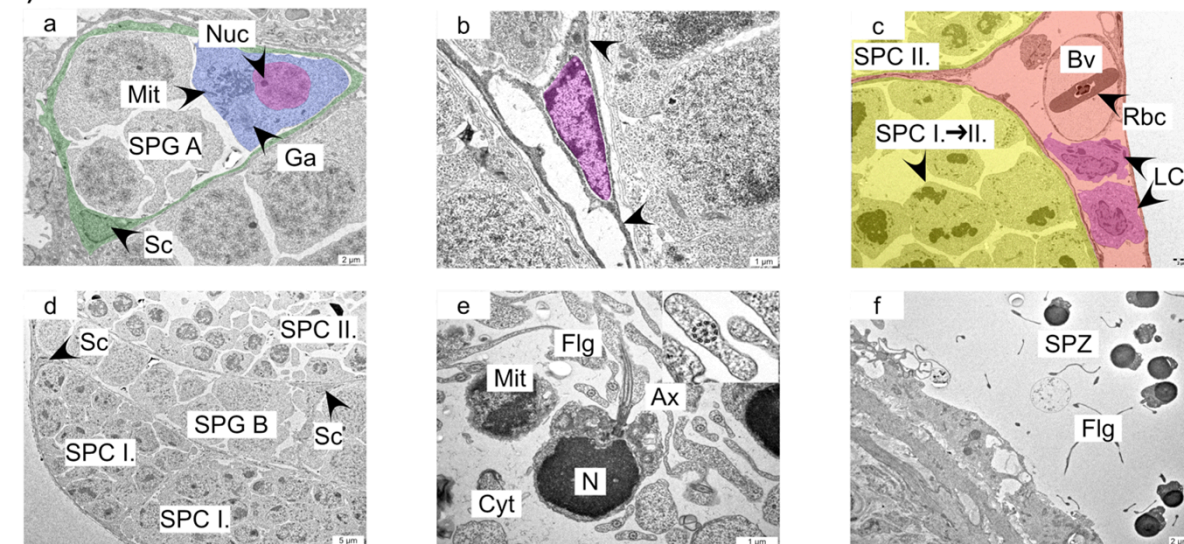


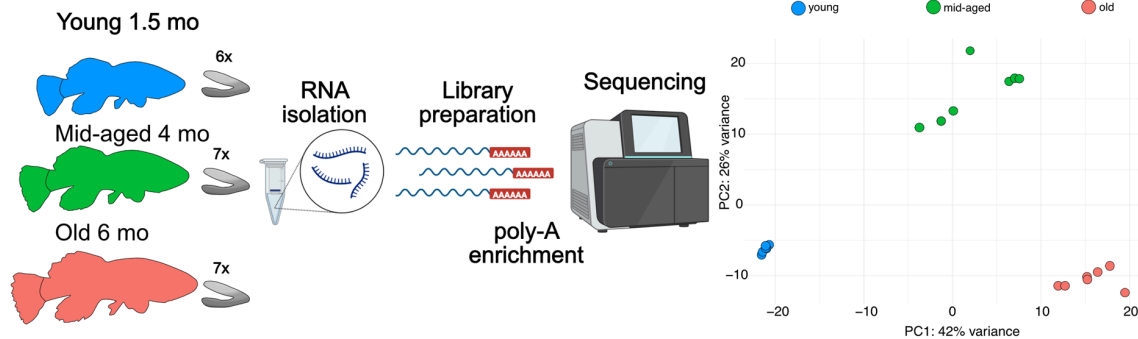
Figure 2

Characterization of adult testicular tissue in turquoise killifish. A) Histological organization of the adult turquoise killifish testis. Transverse (a) and sagittal (b) sections show the lobular architecture of the testis. Higher magnification (c) illustrates the spatial organization of spermatogenic cysts containing type A spermatogonia (SPG A), type B spermatogonia (SPG B), primary spermatocytes (SPC I), secondary spermatocytes (SPC II), spermatids (SPT), and spermatozoa (SPZ). Arrowheads indicate

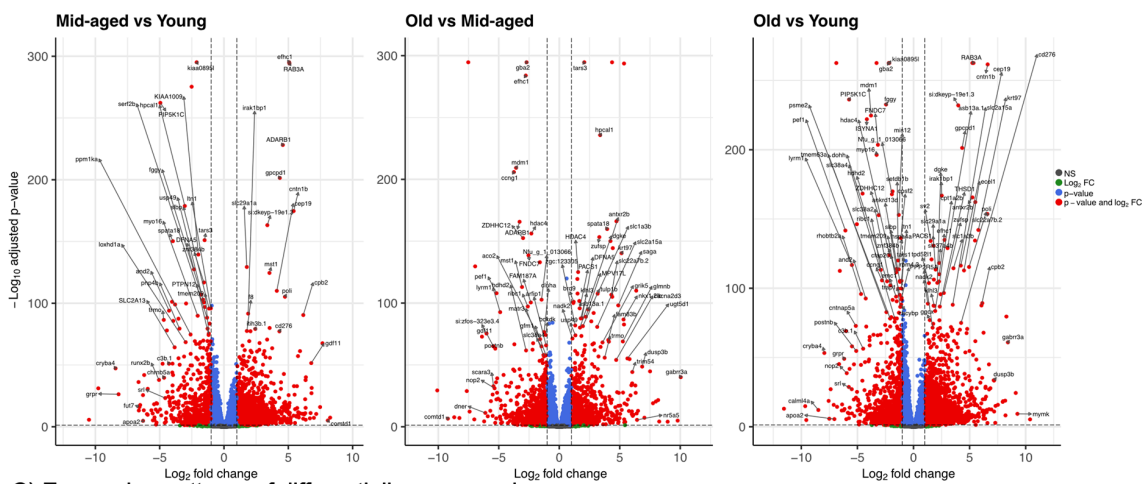
representative germ cell types. **B)**Restricted type of spermatogenesis in adult turquoise killifish revealed by DEAD-box helicase 4 (DDX4) immunolocalization. Overview images show nuclear staining (DAPI, grayscale) and DDX4-positive germ cells (magenta) across the testicular lobule (a-a"). Higher-magnification images (b-c") demonstrate stage-specific localization of DDX4 in containing spermatogonia and early meiotic cells, with reduced or absent signal in late spermatids and spermatozoa. Distinct spermatogenic stages are indicated. The white dashed rectangle indicates the region shown at higher magnification in the panel to the right. Abbreviations: type A spermatogonia (SPG A), type B spermatogonia (SPG B), primary spermatocytes (SPC I), secondary spermatocytes (SPC II), spermatids (SPT), and spermatozoa (SPZ). **C)**Ultrastructural analysis of adult killifish testicular tissue by transmission electron microscopy. Representative micrographs show morphology of a spermatocyst delineated by Sertoli cell (Sc) and its cytoplasmic extensions (green). The cyst contains type A spermatogonia (SPG A) (cytoplasm in blue, nuclei in magenta) with prominent nucleolus (Nuc), mitochondria (Mit), and Golgi apparatus (Ga) (a). Detail on Sertoli cell (nuclei in magenta) and its cytoplasmic extensions (arrowheads) enclosing each spermatocyst (b). Individual spermatocysts (yellow) are in proximity of interstitial compartment (red) containing a blood vessel (Bv) with red blood cells (Rbc) and Leydig cells (Lc, magenta) (c). Bottom panels depict cysts containing SPG B and SPC I/II (d), differentiating spermatids with developing flagellum (Flg), axoneme (Ax), nucleus (N), cytoplasm (Cyt), and mitochondria (Mit), and mature spermatozoa released into the lumen with fully formed flagellum (Flg).

Fig. 3

A) Sampling and analysis



B) Visualization of differentially expressed genes by volcano plots



C) Expression patterns of differentially expressed genes across age groups

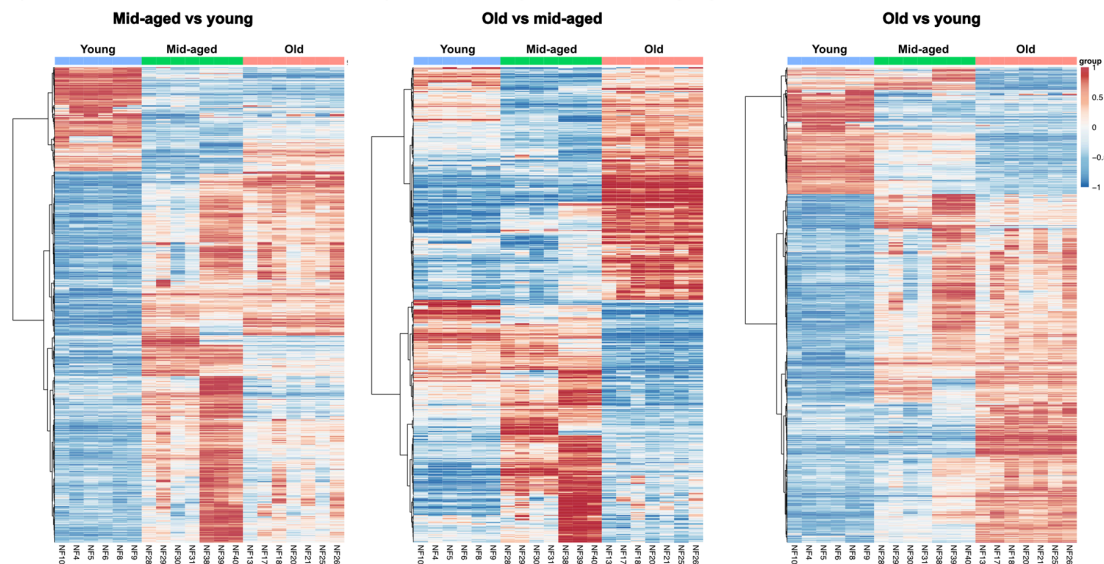


Figure 3

Schematic overview of sampling and RNA-Seq workflow. **A)** Sampling design: testes were collected from young (1.5-month-old, $n = 6$), mid-aged (4-month-old, $n = 7$), and old (6-month-old, $n = 7$) males, followed by RNA isolation, poly(A)⁺ enrichment, library preparation, and sequencing. Principal component analysis (PCA) shows clear age-dependent separation of transcriptomic profiles. **B)** Volcano plots show differentially expressed genes in pairwise comparisons between age groups (mid vs young, old vs mid-

age, and old vs young). Genes are plotted according to log2 fold change and $-\log_{10}$ adjusted p value; significantly regulated genes are highlighted. **C)** Heatmaps display variance-stabilized expression values of genes identified as differentially expressed in the indicated pairwise comparisons. Although gene selection is based on pairwise contrasts, expression levels are shown across all three age groups to provide a comprehensive view of age-related expression dynamics.

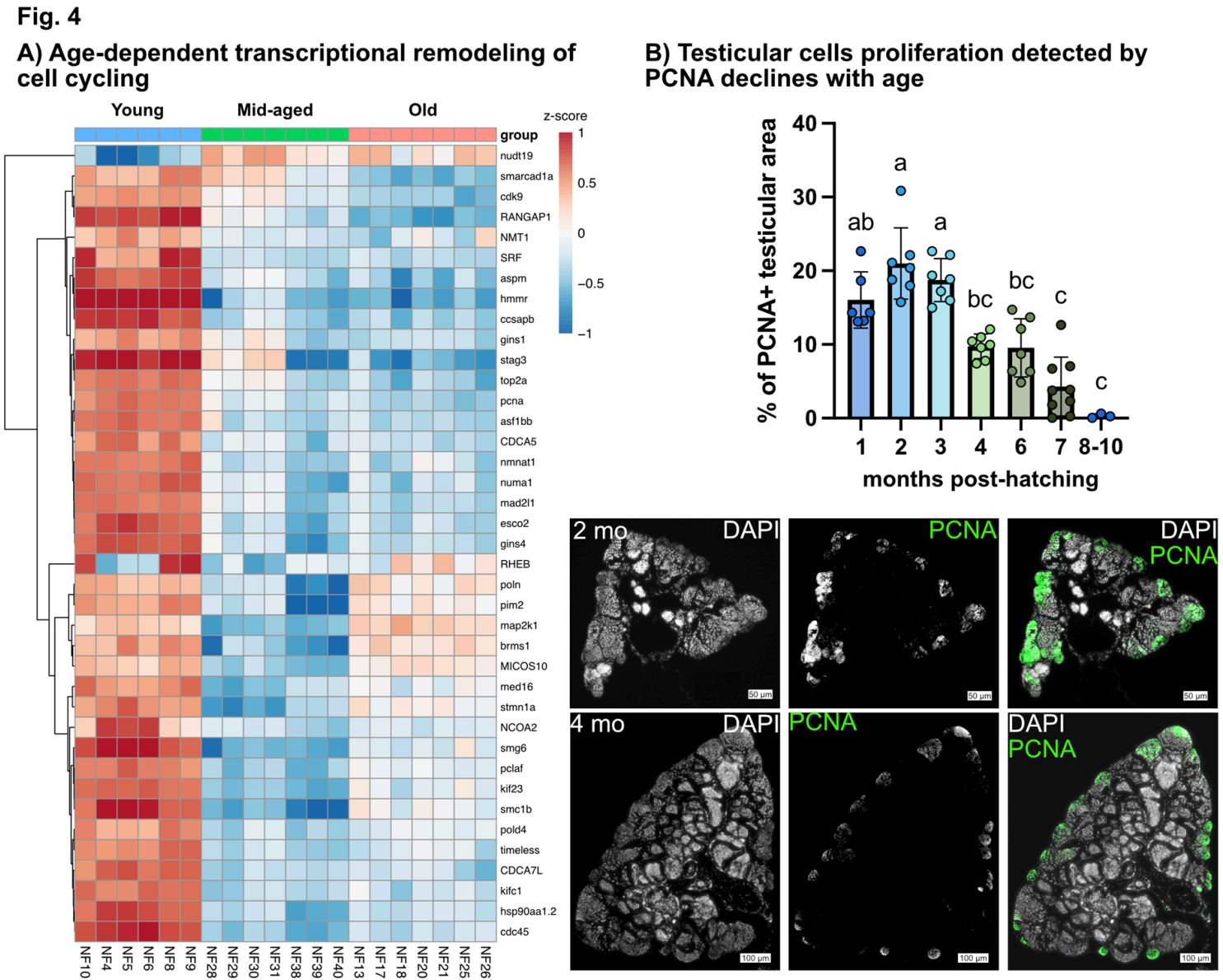


Figure 4

Age-dependent decline in testicular proliferation in turquoise killifish. **A)** Heatmap showing age-associated transcriptional changes in genes related to cell cycle in testes of young, mid-aged, and old males. Rows represent selected mitosis/meiosis-associated genes and columns represent individual samples; colors indicate relative expression levels. **B)** Quantification of testicular cell proliferation by Proliferating Cell Nuclear Antigen (PCNA) immunolabeling. Bar graph shows the proportion of PCNA-positive area relative to total testicular area across age groups. Bars represent mean $\pm$ SEM; individual data points are shown. Differences were analyzed using the Kruskal–Wallis test followed by Dunn’s test

for multiple comparisons with false discovery rate control (two-stage step-up method of Benjamini, Krieger and Yekutieli). Different letters indicate statistically significant differences between groups (adjusted $p < 0.05$). Representative testis sections from 2- and 4-month-old males stained for 4',6-diamidino-2-phenylindole (DAPI) (nuclei) and PCNA are shown below.

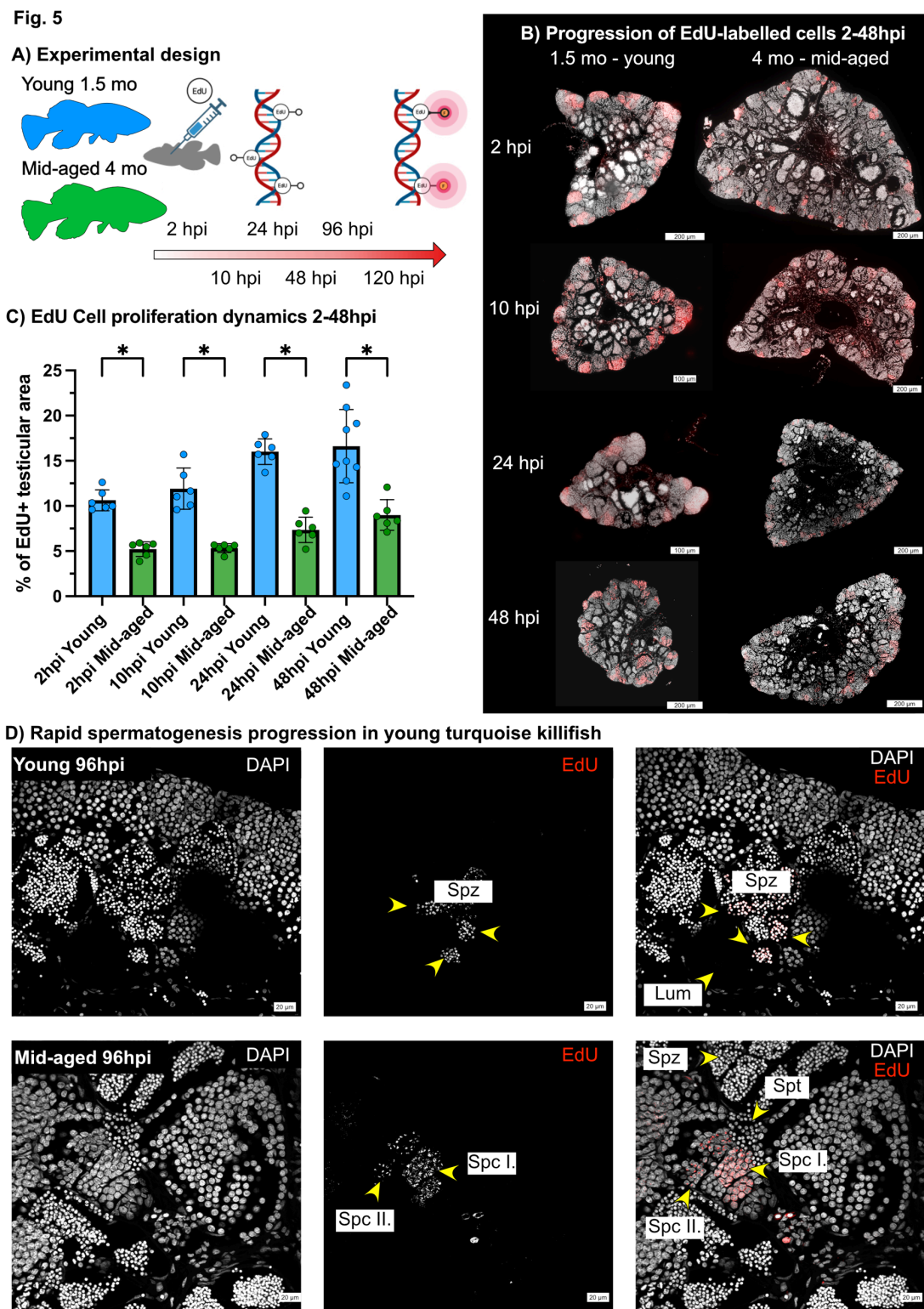
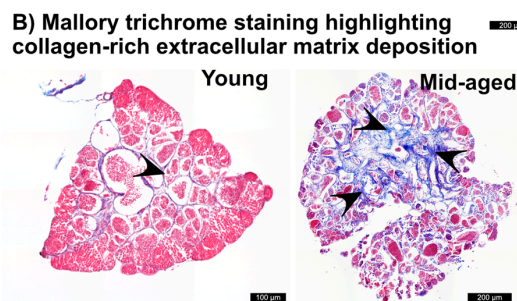


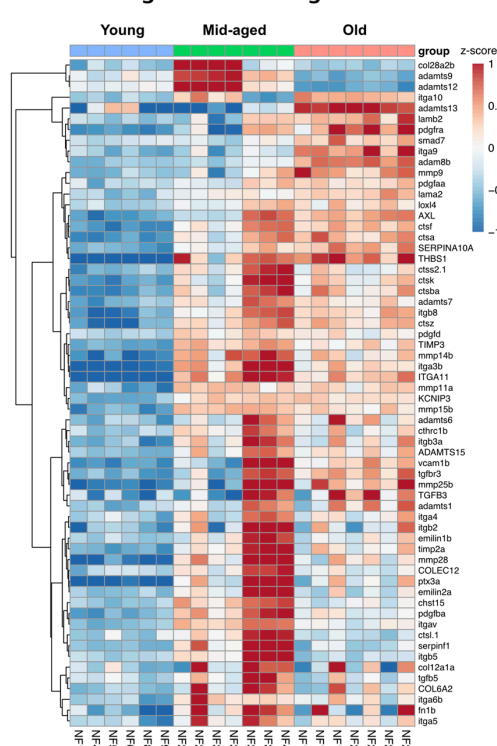
Figure 5

EdU pulse-chase assay reveals impaired spermatogenesis dynamics during aging in turquoise killifish. A) Schematic overview of the EdU pulse–chase experimental design and sampling time points. **B)** Representative testis sections showing EdU incorporation and its distribution at 2–48 hours post-injection (hpi) in young (1.5-month-old) and mid-aged (4-month-old) males. Images are shown as merged, DAPI – grey, EdU – red. **C)** Quantification of EdU-positive area relative to total testicular area at indicated time points. Differences at given sampling point between young and mid-aged fish were analyzed using the Kruskal–Wallis test followed by Dunn’s multiple comparisons test. Significance is indicated by asterisks ($*p < 0.05$). Bars represent mean $\pm$ SEM, individual data points are shown. **D)** High-magnification images illustrating rapid progression of EdU-labeled germ cells through spermatogenesis in young testes at early (2 hpi) and late (96 hpi) chase times. Germ cell types are indicated. EdU, 5-ethynyl-2'-deoxyuridine; hpi, hours post-injection; SPC I., primary spermatocyte; SPC II., secondary spermatocyte; Spt, spermatids; Spz, spermatozoa.

A) Quantification of germ-cell lacking areas



C) Age-dependent upregulation of ECM remodeling and stromal genes



cell population proliferation

immune response

supramolecular fiber organization

organic anion transport

cell-cell adhesion

cell adhesion

superoxide metabolic process

superoxide anion generation

cellular response to organic substance

response to organic substance

cellular response to chemical stimulus

response to oxygen-containing compound

response to endogenous stimulus

response to hormone

enzyme-linked receptor protein signaling pathway

cell-matrix adhesion

cell-substrate adhesion

cell adhesion mediated by integrin

integrin-mediated signaling pathway

blood vessel morphogenesis

blood vessel development

angiogenesis

vasculature development

granulocyte migration

neutrophil migration

leukocyte chemotaxis

granulocyte chemotaxis

neutrophil chemotaxis

cell chemotaxis

leukocyte migration

superoxide cell-cell anion fiber

cellular to stimulus substance

cell-matrix cell-substrate by integrin

blood angiogenesis vessel development

granulocyte leukocyte chemotaxis migration

number of genes

p.adjust

Progressive remodeling of the testicular niche supported by ECM transcriptional programs in turquoise killifish. A) Quantification of germ cell-lacking area in testes across aging. Bars represent mean $\pm$ SEM; individual data points are shown. Differences were analyzed using the Kruskal–Wallis test followed by Dunn’s multiple comparisons test with adjustment for family-wise error rate (FWER). Asterisks denote p values (* p < 0.05, ** p < 0.01). Representative histological sections from young and mid-aged males are

shown; arrowheads indicate expanded non-germinal regions. **B)** Mallory trichrome staining highlighting age-associated accumulation of collagen-rich extracellular matrix (arrowheads) in the testis in mid-aged males. **C)** Heatmap of differentially expressed genes associated with ECM remodeling and stroma-related pathways across age groups, showing transcriptional activation with aging. **D)** Gene Ontology (GO) enrichment shows terms related to ECM organization, cell–matrix adhesion, angiogenesis, and immune-related processes.

Supplementary Files

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