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Thymus development in the zebrafish (*Danio rerio*) from an ecoimmunology perspective

Running title: Thymus development in zebrafish

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Abstract

The thymus is present in all gnathostome vertebrates and is an essential organ for the adaptive immune system via the generation of functional mature T-cells. Over the life span of mammals, the thymus undergoes morphological and functional alterations, including an age-related involution, which in humans starts in early life. Life history tradeoffs have been suggested as possible reasons for thymus involution. While in teleost fish, only a few studies have investigated alterations of thymus structure and function over different life stages, resulting in a fragmented database. Here, we investigated the

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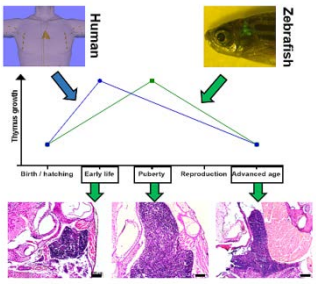
thymus growth of zebrafish (*Danio rerio*) from early life, throughout puberty and reproductive stage, up to 1-year-old. We assessed thymus growth by histological and morphometric analyses and thymocyte numbers. Thymus function was assessed by measuring the transcripts of the thymocyte marker genes, *ikaros*, *tcrα*, and *tcrδ*. Additionally, we analyzed gonad maturity and tail homogenate vitellogenin concentrations to align thymus status with the status of the reproductive system. Our results showed that the zebrafish thymus, in contrast to the human thymus, grew strongly during early life and puberty but started to undergo involution when the fish reached the reproductive age. The involution was characterized by reduced thymus area and thymocyte number, altered histoarchitecture, and decreasing thymocyte marker gene transcript levels. Our findings suggest that age-related changes of the zebrafish thymus do exist and could be partly explained in terms of resource tradeoffs, but also in terms of the ontogenetically late development of a functional adaptive immune system in teleosts.

Graphical Abstract

Comparison of thymus development between human and zebrafish at specific life stages.



Research Highlights

Zebrafish thymus growth peaks at puberty and age-related involution starts at reproduction, in contrast to mammals where thymus involution starts in early life, suggesting different ecological and evolutionary pressures on thymus development.

Keywords: Zebrafish; Thymus; Involution; Ecoimmunology; Tradeoffs; Life history

1. Introduction

The thymus is a primary lymphoid organ that provides the microenvironment for the development of a diverse, self-tolerant T-cell repertoire (Boehm, Hess, & Swann, 2012; Boehm & Swann, 2013). Evolutionary, it is present in all gnathostome vertebrates, while agnathans only possess a thymus-equivalent tissue (Bajoghli et al., 2011). In mammals, the thymus structure and function vary during the lifetime (Rezzani, Nardo, Favero, Peroni, & Rodella, 2014, Peel & Belov, 2017). In the case of humans, thymus development starts during embryogenesis and reaches its maximum size and output of naive T-cells during early life. Afterward, the thymus enters a period of gradual decline which is characterized by a remodeling of the histoarchitecture, a reduction in size, and production of naive T-cells (Contreiras et al., 2004; Haynes, Sempowski, Wells, & Hale, 2000; Manley, Richie, Blackburn, Condie, & Sage, 2011; Palmer, 2013; Quaglino et al., 1998; Rezzani et al., 2014; Taub & Longo, 2005). The exact time point of human thymus involution is a debated topic (Rezzani et al., 2014). The original consensus was that the onset of thymus involution occurs at puberty, in association with the increasing levels of sex steroid hormones and with the decreasing growth hormone production. It is now accepted that human thymus regression begins during childhood but may become more evident during puberty (Flores, Li, Sempowski, Haynes, & Hale, 1999; Haynes et al., 2000; Manley et al., 2011; Steinmann, Klaus, & Muller-Hermelink, 1985; Taub & Longo, 2005; Tosi et al., 1982).

The age-related regression of the thymus appears to span across all vertebrates, although there are considerable variations concerning the timing and extent of thymus involution (Contreiras et al., 2004; Quaglino, Accorsi, Boraldi, & Ottaviani, 2014; Shanley, Aw, Manley, & Palmer, 2009; Torroba & Zapata, 2003; Peel & Belov, 2017, Cockburn, 1992). This variation raises such questions as which factors select for the age-related involution of the thymus, and whether these factors are uniform across vertebrates, or whether

they vary with the life history of species. Even though thymus involution means a loss of immune capacity (Shanker, 2004; Shanley et al., 2009), there might be an adaptive function (Boehm & Swann, 2013; Quaglino et al., 2014). For instance, decreasing of thymus function with age could reduce the risk of autoimmune diseases (Aronson, 1993). Moreover, since peripheral T-cell populations are long-lived and can proliferate, the thymus may no longer be essential for the continued survival of the organism, according to the "disposable soma" theory (Kirkwood & Rose, 1991; George & Ritter, 1996). While, the hypothesis of "antagonistic pleiotropy" postulated that genes which benefit a highly efficient thymus function early in life may come along with costs later in life, and therefore evolutionary processes would select for an optimized peripheral T-cell repertoire during early life, together with thymus involution as a side effect later in life (Turke, 1994; Dowling & Hodgkin, 2009; Shanley et al., 2009). The question of age-related changes in thymus structure and function can also be addressed using an ecoimmunology perspective. Ecoimmunology seeks to understand the physiological, ecological, and evolutionary causes of variations in immune systems between individuals and between species, and how organisms allocate their limited resources between the competing demands of fitness functions like growth, reproduction, and maintenance - including immunity (Schmid-Hempel, 2003; Downs, Adelman, & Demas, 2014). An important source of variation among individuals is age. Resource-based arguments have been suggested to explain the age-related thymus involution in humans as a tradeoff between maximizing available resources for reproduction while reducing resources in an energy-intensive organ like the thymus (George & Ritter, 1996; Kirkwood & Rose, 1991; McDade, 2003; Shanley et al., 2009).

Thymus function implies high physiological costs. This is due to the stringent selection processes being active in the thymus as the output of T-cells to the periphery accounts for less than 10 % of all generated thymocytes (Quaglino et al., 2014). After the establishment of the peripheral T-cells populations, which occurs during early life, the loss of thymus function may be tolerable for aging organisms as the peripheral T-cells are long-lived and can undergo clonal expansion (George & Ritter, 1996). Furthermore, Shanley et al. (2009)

emphasized the life history tradeoffs potentially associated with a robust immune response. In that, natural selection may favor a strong immune response early in life and a finely tuned repression of immune functions later in life to accommodate successful reproduction.

A comparative approach is essential to understand better which selective factors favor the thymus involution. Among the vertebrates, the available information suggests that teleost species have a highly diverse pattern of thymic involution, which varies in timing, extent (complete or partial involution), and whether it is permanent or seasonal (Cockburn, 1992). However, most of the research on the thymus in fish is fragmentary and focused on certain life stages or methods, resulting in an incomplete picture. Using zebrafish (*Danio rerio*) as model species, the present study aims to 1) describe the developmental changes in the thymus from the juvenile to the reproductive life stage and 2) examine if the developmental changes of zebrafish thymus relate to the developmental changes of the reproductive system. By exploring the association between age-related changes in the thymus and the reproductive system, we will add to the discussion of resource-based life-history tradeoffs as a selective force for thymus involution. The zebrafish was selected as a model organism because it has been extensively used to investigate the embryonic and larval ontogenesis of the thymus (Danilova et al., 2004; Dee et al., 2016; Lam et al., 2002; Langenau et al., 2004; Willett, Cortes, Zuasti, & Zapata, 1999; Willett, Zapata, Hopkins, & Steiner, 1997) and owing to the availability of transgenic lines. Transgenic zebrafish allow direct and detailed observation of marker expression in various tissues and organs throughout embryogenesis, even during later larval and adult development. In the present study, we used a transgenic lck:GFP line, which is fluorescent for the thymocyte marker (*lck*), and allowed the precise preparation of the thymus for dissection and evaluation. Moreover, we included a wild-type line because (1) the transgenic line may differ in some properties from wild-type fish, and (2) repeating the experiment in two different zebrafish lines enabled reproducibility of the findings. We assessed thymus development through size changes (thymus area and thymocyte number) and morphological changes (histological appearance, immunostaining of Proliferating Cell Nuclear Antigen

(PCNA)). Additionally, we analyzed transcript expression levels of specific thymocyte marker genes. We selected markers of different thymocyte stages to gain insight into their overall maturation pattern: the regulator of lymphocyte differentiation *ikaros,* which is expressed by early stages of thymocytes; and the T-cell receptor family *tcrα* and *tcrδ*, which are expressed in later thymocyte stages and also by mature T-cells (Carpenter & Bosselut, 2010; Seelye, Chen, Deiss, & Criscitiello, 2016; Willett, Kawasaki, Amemiya, Lin, & Steiner, 2001). In order to relate thymus development to the development of the sexual system, we assessed the reproductive status of the fish using gonad histology and the quantification of vitellogenin (vtg) protein in fish tail homogenates.

2. Material and methods

2.1 Fish culture

This study used two different lines of zebrafish, a wild-type (WT) provided by Prof. Thomas Braunbeck's laboratory (University of Heidelberg, Germany) and a lck:GFP transgenic line developed by Nikolaus Trede's laboratory (Harvard Medical School, USA) (Langenau et al., 2004) provided by Dr. Adam Hurlstone's laboratory (Manchester University, UK). Both lines were raised at the Centre for Fish and Wildlife Health (University of Bern, Switzerland).

The WT line was reared in glass aquaria in a flow-through system, while the lck:GFP was reared in Techniplast tanks in a recirculating water system (Flooren and Nooijen, Nederweert, Netherlands). Upon arrival, the eggs were transferred into Petri dishes and placed in a thermo-incubator (Binder, Tuttlingen, Germany) set to 27°C for five days. On day six, we transferred the newly hatched larvae to the growing out tanks at a starting density of ten larvae/L. The fish density was then adjusted to eight fish/L between 2-3 weeks post-fertilization (wpf) and to four fish/L between 16-19 wpf. Water parameters were as follows: temperature 26.3^{o}C (±0.49), conductivity 612 µS (±33), hardness 7-14 German degrees (°dH), both nitrite and nitrate levels were below 1 mg/L. The light cycle was 14:10 light-dark, respectively. We fed the fish three times per day, morning and evening with Gemma 75, 150, or 300

(fish size-dependent) and with Sander's artemia in the afternoon. Over the weekend, fish were fed only once a day with Gemma. All experiments were conducted under institutional and national guidelines for the care and use of laboratory animals, license number BE95/16.

2.2 Fish sampling

To investigate the age-related changes of the thymus, we sampled the fish at the following time points: 5, 8, 10, 28, and 52 wpf for the lck:GFP, and 5, 8, 10, 12, 18, 21, and 61 wpf for the WT. To understand if the spawning cycle influences the thymus, we sampled fish at 28 wpf "pre-spawning" and "post-spawning" lck:GFP females. "Pre-spawning" females were isolated in a female-only tank eight days before the start of the spawning experiment to allow for mature oocyte development (Connolly, Dutkosky, Heah, Sayler, & Henry, 2014). The "post-spawning" females were conditioned the following way: one male and one female were placed in a 2-liter Techniplast tank with an in-tank breeding device (Pentair Aquatic Eco-Systems, Apopka, USA) overnight. The next morning, the breeding pairs were allowed to spawn for one hour after the room lights turned on. We then sampled only the females that successfully spawned.

For sampling, the fish were euthanized with an overdose (500 mg/l) of MS-222 (Syndel, Washington, USA) buffered with sodium bicarbonate ($NaHCO_3$). For both WT and lck:GFP zebrafish, we applied the following methods. We determined the total length and body weight of the fish. For gonad histological analyses, we placed the trunk part of the body containing the visceral organs and gonads in neutral buffered 4 % formalin. For the thymus histological analysis, we placed the zebrafish heads in Histochoice (Lucerna-Chem AG, Luzern, Switzerland). Samples older than 12-weeks required an additional decalcification step, which was performed using either Decalcifier Rapid (Biosystems, Muttenz, Switzerland) for one hour or Decalcifying Solution-Lite (Sigma-Aldrich, Buchs, Switzerland) for 40 mins. Additionally, the tails of the fish were cut behind the anal fin, weighted, and then frozen in liquid nitrogen

and kept at -80°C until further processed for vtg quantification by enzyme-linked immunosorbent assay (ELISA).

Only the lck:GFP fish were used for thymocyte counting and RT-qPCR analyses. We extracted the lck:GFP thymus under a Leica fluorescent microscope equipped with a green filter (excitation/emission wavelength of 488/507 nanometers). From one group of fish, the left thymus was transferred in RPMI medium (Thermofisher Scientific, Reinach, Switzerland) for thymocyte isolation and counting (see 2.4), while the right thymus was fixed for histology. From the second group of fish, both the left and the right thymus were placed into lysis buffer (PicoPure RNA Isolation Kit, Thermofisher Scientific) for 30 mins at 42^{o}C, and then stored at -80^{o}C until RT-qPCR analysis.

2.3 Histological analyses

The fixed samples were subjected to routine paraffin embedding procedures (OECD Histopathology Guidance Document, 2010). Sections were cut at a thickness of 5 µm. Half of the thymus slides were stained with hematoxylin and eosin (H&E), while the other half were prepared for immunohistochemistry.

2.3.1 Gonad staging

The gonads were examined on H&E-stained coronal sections of the trunk part of the zebrafish body. The staging of gonad maturation status was done according to the OECD Histopathology Guidance Document (2010), while the ovaries were classified into pre-pubertal and pubertal groups following the criteria of Chen & Ge (2013). Females with ovaries containing only primary growth stage oocytes were classified as pre-pubertal. The pubertal stage starts with the appearance of the cortical alveolar oocyte stage. Females that had started spawning and displayed mature ovaries were classified as post-pubertal.

2.3.2 Thymus morphometric measurements

The morphometric measurements (area) of transversal sections from the whole thymus included the parenchyma, the perivascular space, and the capsule. They were performed on H&E-stained sections using the Olympus CellSense Software. During zebrafish development, the thymus undergoes various morphological three-dimensional changes (Lam et al., 2002). However, only the two-dimensional thymus area can be measured on histological sections, which may vary with section plane and section level. To account for this variation, we performed morphometric analyses of serial thymus sections for all fish to quantify the variation of the thymus area with the section plane. On that basis, we were able to identify the section plane that displays the maximum thymus area. We show an example of how the thymus area changes with the section plane in Fig. S1. The morphometric data shown in the main results refer to the maximum thymus area, which was selected as representative of thymus growth. The pre-trials also revealed that there was no significant difference in thymus area growth between the left and right thymus; therefore, only one thymus was used for morphometric measurements. Head measurements were taken on the same slide that was used for thymus area measurement. The thymus area was expressed as an absolute area, while the relative thymus area referred to the normalization of the thymus area by the head area. The normalization step was done to relate thymus growth to the overall body growth of the fish.

2.3.3 Immunofluorescence

We stained sections of the thymus with a commercially available polyclonal antibody (pAb) targeting an immunogen containing the amino acid sequence 31 and 222 of the proliferating cell nuclear antigen (PCNA) from zebrafish (cat. Number SAB2701819, Sigma-Aldrich). This pAb has been previously used for PCNA staining in zebrafish (Ng Chi Kei, Currie, & Jusuf, 2017). Slides were deparaffinized, permeabilized with PBS-Tween 0.05 % for 5 mins, and incubated with 5 % donkey serum (Sigma-Aldrich) for 1 hour to block nonspecific binding sites. Afterward, the slides were incubated overnight at

4°C with the anti-PCNA pAB (dilution 1:1000), or in PBS-Tween 0.05 % without the pAB (negative control). The next day, the slides were washed in PBS-Tween 0.05 % and incubated for 2 hours with the secondary Ab donkey anti-rabbit (dilution 1:700, cat. Number A-21206, Thermofisher Scientific). Finally, the slides were washed with PBS-Tween 0.05 % before mounting. The slides were kept in the dark at 4°C until observation under the Nikon 80i fluorescent microscope.

2.4 Determination of thymocyte number

The isolation for thymocytes was done using a procedure modified from Paiola et al. (2017). The left thymus of lck:GFP zebrafish was dissected under a Leica fluorescent microscope, placed in RPMI medium (containing HEPES and sodium bicarbonate), and mechanically disaggregated. The resulting tissue slurry was passed through a 35 µm cell strainer to remove the non-thymocytes. The purity of the obtained suspension was confirmed by flow cytometry. The isolated thymocytes were counted in a Neubauer chamber. The counting was done in duplicate unless the deviation between the first two counts was greater than 30 %, then a third count was conducted. The cell count number was normalized by the fish wet weight to express the thymocyte number per mg of fish.

2.5 Transcript analysis of the thymus

RNA was isolated from both parts of the thymus using the PicoPure™ RNA Isolation Kit (Thermofisher Scientific), following the manufacturer's instructions, with some minor modifications, to include a DNase treatment step (Qiagen, Hombrechtikon, Switzerland). For each fish, we transformed 150 ng of thymus RNA into cDNA. The cDNA was synthesized using the GoScript Reverse Transcriptase Mix (Random Primers, Promega, Dübendorf, Switzerland) following the manufacturer's instructions. The cDNA was diluted 1:3 with molecular grade H_2O (Promega) and stored at -20°C until RT-qPCR.

Gene transcription levels were measured with an Applied Biosystems 7500 Fast RT-qPCR machine (Thermofisher Scientific), using SYBR Green PCR

Core Reagents (Promega) and specific primers detailed in Table 1. We targeted the T-cell receptor family members, which are markers of mature thymocytes (*tcrα* and *tcrδ*), and the regulator of lymphocyte differentiation (*ikaros*), which is a marker of early-stage thymocytes. A sample volume composed of 6.25 µl of GoTag Master Mix, 3.125 µl of molecular H_2O, 0.625 µl of the primers (10 µM), and 2.5 µl of cDNA was added for each well of the 96-well Fast Optical reactions plates (Thermofisher Scientific). Each sample was run in duplicate. The qPCR program was as follows: 95°C for 2 mins (activation) and then 50 cycles repeating 3 s at 95°C (denaturation) and 30 s at 60°C (annealing/elongation), finished with a dissociation step. The Ct results were converted into fold change with the 2^{-ddCt} method (Livak & Schmittgen, 2001). The Ct results were normalized with the zebrafish reference gene elongation factor alpha (*ef1a*) and were presented relative to the delta Ct average value of the 5 wpf fish.

2.6 Vitellogenin (vtg) quantification

Frozen tail samples (cut behind anal fin as described in OECD TG 234, 2011) were covered with 10 x amount of homogenization buffer (50 mM Tris-HCl with 1 % protease inhibitor, pH 7.4, 4□C) and a metal bead (5 mm) was put in each 2 ml reaction tube. Samples were homogenized in a Tissue-Lyser (Qiagen, Hilden, Germany) at a frequency of 20 Hz 2 x 3 mins, until fully homogenized. Samples were then centrifuged with a rotation frequency of 40.000xG/21.000 RPM for 30 mins at 4□C. Afterwards, the supernatant was immediately frozen at -80□C for later *vtg* measurement. *Vtg* concentrations in homogenate were measured using a direct non-competitive sandwich ELISA with biotin-conjugated antibodies and streptavidin-HRP as developed by Holbech et al. (2001) and modified by Morthorst, Holbech, & Bjerregaard (2010). The detection limit is 0.2 ng/ml homogenate, and the quantification limit is 40 ng/ml based on a 200 x dilution of the homogenate. This method is included in OECD TGs 229, 230, and 234 and has been used in multiple studies over the last decades (e.g., Andersen, Kinnberg, Holbech, Korsgaard, & Bjerregaard, 2004; Baumann et al., 2013; Kinnberg, Holbech, Petersen, & Bjerregaard, 2007).

2.7 Statistics

All statistical tests were performed with NCSS statistical software version 12, and the data were visually represented with GraphPad Prism version 8.4.0. The response variables were as follows: thymus morphometric area, thymocyte number, thymic immune gene expression, and vtg protein concentration. For each response variable, we determined the statistical differences between time points (age) or between reproductive stages (undifferentiated, pre-pubertal, pubertal, and post-pubertal/reproducing). The datasets of the response variables were tested for normal distribution and equal variances between groups, using the Shapiro-Wilk test and the Levene test, respectively. The analysis showed that the data were not normally distributed, and therefore, we used the Mann-Whitney Test (if two groups) or Kruskal-Wallis One-Way ANOVA on Ranks (if more than two groups) followed by a Dunn's-Bonferroni post hoc test. Differences between groups were considered statistically significant if $p < 0.05$.

3. Results

3.1 Zebrafish growth

The growth patterns of both zebrafish lines (WT and lck:GFP) were comparable (Fig. 1A-C). Total body length, weight, and head area increased rapidly for both lines until 20 wpf (corresponding to the adult reproductive stage). Afterward, growth continued, since zebrafish, like other teleosts, have indeterminate growth, but the growth rate slowed down (Fig. 1A-C).

3.2 Thymus growth

3.2.1 Age-related changes of thymus area (morphometry) and thymocyte number

In both lines, the absolute thymus area increased between 5-8 wpf, but was only significant for the lck:GFP line (Fig. 2, Kruskal-Wallis, $p < 0.05$). At 10 wpf, there was a significant increase in the absolute thymus area in the WT compared to the 8 wpf (Fig. 2, Kruskal-Wallis, $p < 0.05$). Comparable to the

absolute thymus growth data, the relative thymus area non-significantly increased from 5 wpf until 10 wpf for both lines (Fig. 2) and then started to decrease after 10 wpf. During the period of regular active reproduction (18-52 wpf), the decrease of the thymus area accelerated in both lines. Absolute and relative thymus areas were significantly decreased in 28 and 52 wpf zebrafish compared to the maximum thymus area at 10 wpf for both lines (Kruskal-Wallis, $p < 0.05$). Similar to the thymus area results, the thymocyte number significantly increased from 5 to 10 wpf fish, and then significantly decreased towards 52 wpf (Kruskal-Wallis, $p < 0.05$ Fig. 2A). The same pattern was shown when the thymocyte number was normalized by fish weight (mg of wet weight) (Fig. 2A).

3.2.2 Age-related changes of thymus area in relation to the development of the reproductive system

The reproductive status (pre-pubertal, pubertal, and post-pubertal/reproducing) of female zebrafish was assessed using gonad histology according to the classification of Chen & Ge (2013). The gonad histological staging of the fish was complemented by the determination of tail vitellogenin (vtg) protein levels, and the onset of reproduction was confirmed by monitoring the spawning events.

Females of the pre-puberty stage contained only perinuclear, pre-vitellogenic oocytes in their ovaries. Accordingly, the vtg protein levels in these animals were close to the detection limit (Fig. 3). During the female puberty stage, i.e., the transition from the sexually immature to the sexually mature stage, the oocytes accumulate increasing levels of vtg, resulting in a non-significant increase of vtg levels in the fish body (Fig. 3). Finally, mature oocytes appeared, and the fish started to spawn. This reproductive stage was characterized by significantly elevated vtg protein contents (Kruskal-Wallis, $p < 0.05$, Fig. 3).

When plotting the thymus area versus the reproductive stage as revealed by gonad histology, it is evident that both the absolute and relative thymus area increased from the undifferentiated to the pre-pubertal and pubertal life stages

(Fig. 4). The thymus area begun to decrease when the fish reached the reproductive period (Fig. 4, Kruskal-Wallis, $p < 0.05$). This pattern appeared in both the wt and the transgenic lines of zebrafish. The decrease of the thymus area continued throughout the reproductive period.

In actively reproducing lck:GFP adult females, we examined whether the thymus area and thymocyte number changed between pre- and post-spawning individuals by sampling females that had been prevented from spawning and possessed ovaries with a high percentage of mature eggs, as well as females immediately after spawning. These two reproductive stages should differ in their endocrine status and resource status. However, no significant difference in the thymus area and thymocyte number between pre- and post-spawning females was observed (Fig. S2AB, Mann-Whitney). Similarly, the vtg protein levels showed no significant difference between pre- and post-spawning females (Fig. S2C, Mann-Whitney).

3.2.3 Age-related changes of thymus histology

The histological changes within the lck:GFP zebrafish thymus between 5-52 wpf were gradual and did not include degenerative or pathological features (Fig. 5). At 5 wpf (Fig. 5A), the shape of the thymus consisted of a broad basis towards the pharyngeal epithelium and a column- or horn-like extension towards the dorsal head region. At the upper end of the dorsal horn, the thymus was covered by a cartilage arch. The organ was enclosed in a thin fibrous capsule, with a layer of thymic epithelial cells (TECs) directly under the capsule. The thymus parenchyma was separated into the cortex, with densely packed basophilic thymocytes, and the medulla with loosely packed thymocytes. The medulla contained many eosinophilic roundish TECs, which were dispersed between the thymocytes. In the cortex, the TECs were less abundant and had a more irregular shape. Moreover, macrophages and large eosinophilic myoid cells with fibrillar inclusions around the nucleus were present, both in the cortex and medulla. Occasionally, rodlet cells could be found. Thymus vascularization was not yet well developed at 5 wpf. Hassall bodies were absent through all life stages investigated.

At 8 and 10 wpf (Fig. 5B-C), the dorsal horn had grown. Adipocytes occasionally occurred in the space between the thymic horn and the cartilage arch (Fig. 5F) in 10 wpf fish. The cortex-medulla separation was well developed. The medulla was composed of a framework of eosinophilic TECs with intermingled basophilic thymocytes. Blood vessels and occasionally connective tissue septae were more prominent than at 5 wpf.

In actively reproducing adult zebrafish (28 wpf, Fig. 5D), the extension of the dorsal horn was further reduced. In parallel, the space between the thymus and the cartilage arch was extended and regularly filled with adipocytes. Adipocytes could also be seen at the lateral and basal borders of the thymus but remained outside of the thymic capsule and did not infiltrate the thymus (Fig. 5G), in comparison to humans, where the adipocytes enter the perivascular space and become part of the aging thymic tissue. The cortex-medulla separation was less visible in 28 wpf zebrafish than in the previous stages. The thymus parenchyma was still densely packed with thymocytes and TECs, but the frequency of mitotic thymocytes appeared to be reduced. There was an increase in the number and partly also the size of myoid cells.

At 52 wpf (Fig. 5E), the extension of the dorsal horn was strongly reduced, and an overall size decrease of the thymus was noticeable. There was an increased number of adipocytes surrounding the thymus. The separation between cortex and medulla was still partly visible. Mitotic thymocytes were rare. The parenchyma contained many prominent myoid cells. Degenerative changes like cystic degeneration of TECs (as it occurs in the aging human thymus) were absent.

In addition to the thymus histological analysis with H&E staining, the thymus histological sections were stained with a PCNA antibody, a marker for proliferating cells, to investigate the thymus cell turnover. The PCNA staining was present in all zebrafish stages analyzed but at differing intensity. Maximum staining intensity was observed at 8-10 wpf; afterward, the staining intensity decreased gradually. (Fig. 5H-I). The PCNA staining was mainly visible in the cortical area.

3.2.4 Age-related changes of thymocyte marker gene transcripts

The mRNA levels of *ikaros* showed a significant increase from 5 to 8 wpf (Kruskal-Wallis, $p < 0.05$). Afterward, at 52 wpf the mRNA levels of *ikaros* significantly decreased (Fig. 6A, Kruskal-Wallis, $p < 0.05$). The expression of *tcrα* transcripts also showed a significant decrease from 8, 10, and 28 to 52 wpf (Fig. 6B, Kruskal-Wallis, $p < 0.05$). No significant differences were observed between age groups for the *tcrδ* mRNA expression (Fig. 6C, Kruskal-Wallis). Furthermore, regarding each age group, no significant differences in *ikaros, tcr*α, and *tcr*δ expression existed between genders (data not shown).

4. Discussion

The results of the present study provide the first description of the thymus development of zebrafish from the juvenile life stage to 1-year-old adult fish. Previous studies on thymus development in zebrafish focused on the embryonic and larval life stages. These studies showed that thymus differentiation was initiated and completed during the embryonic and larval life stages (Langenau et al., 2004; Willett et al., 1999; Willett et al., 2001; Willett et al., 1997). However, concerning the fate of the zebrafish thymus during later life, there was only the study of Lam et al. (2002), which provided a morphological description of zebrafish thymus from week 1 to week 15 post-fertilization. In our study, we assessed several hallmarks of thymus structure and function (Lynch et al., 2009), including thymus size (area measurements), cellularity, histology, and molecular markers of thymocyte differentiation.

The key finding of the present study showed that the zebrafish thymus continuously grows during the juvenile period, reaches the maximum size at puberty, and starts regressing when the fish reach the reproductive stage. At 52 wpf, the thymus size and cellularity reach values comparable, or even lower, than those of 5 wpf zebrafish. The reduction of thymus size is accompanied by a decrease of mitotic activity, a progressive loss of the cortex/medulla demarcation, and an increased presence of adipocytes around the thymus. Also, the mRNA levels of thymocyte marker genes (*ikaros* and

tcrα) tend to be reduced compared to the pre-pubertal and pubertal stages. All these changes point to a gradual loss of thymic function in reproducing zebrafish.

Our study showed that thymus regression in adult zebrafish appears to take place at an accelerated rate relative to other aging processes. Outbred zebrafish kept under laboratory conditions have an average life span of about 40 months (Gerhard et al., 2002; Gilbert, Zerulla, & Tierney, 2014). Age-related phenotypic changes of zebrafish include altered spinal curvature, fading of colors, the appearance of β-galactosidase in the skin, reduced swimming endurance, and decreased frequency of successful breeding attempts of males. However, all these changes typically manifest in middle-aged (15-25 months) or old-aged (> 25 months) zebrafish (Gerhard et al., 2002; Gilbert et al., 2014; Kanuga et al., 2011; Kishi, 2004), i.e., in individuals that are older than the fish of our study (maximum age: 12 months). Precocious aging is a characteristic of thymus involution in other vertebrates (Quaglino et al., 2014; Shanley et al., 2009), and our data indicates that this is also the case for zebrafish.

How can the age-specific growth pattern of the thymus be understood from a life history perspective?

Immunity is costly, and as a result, immune defense investments require a compromise of investment towards other fitness-relevant life-history traits, e.g., growth or reproduction (Schmid-Hempel, 2003; Sheldon & Verhulst, 1996). Priorities in resource needs differ between the different life stages of an individual, which may drive the age-related variations of immunity. The thymus is believed to be an energy-expensive organ because of the costs required for continuous production of immunocompetent T-cells. The investments into the thymus must be balanced with its benefits for health and continued survival. This balance varies over an individual's life-span (McDade, 2003; Shanley et al., 2009; Urlacher & Kramer, 2018, Palmer et al., 2018). For comparison, this may be exemplified by a short discussion on the age-related changes of the human thymus.

The human thymus shows a rapid expansion of size and thymocyte proliferation activity during the late fetal period and reaches maximum size during infancy (Chinn, Blackburn, Manley, & Sempowski, 2012; Lynch et al., 2009; Palmer, 2013; Rezzani et al., 2014; Shanley et al., 2009). Infancy comprises the first 1-2 years of a human's life, followed by childhood, a life stage unique to humans covering the period between infancy and the juvenile stage (2 to 10 years of age) (Bogin & Smith, 1996). It is commonly reported that the human thymus continues to grow until puberty and then begins to involute; however, this is not the case. Both the size of the thymic parenchyma and T-cell production show a gradual decrease already in the infancy/early childhood years, around 2-3 years of age, and only thymic connective tissue continues to increase until puberty (Lynch et al., 2009; McDade, 2005; Rezzani et al., 2014; Steinmann et al., 1985).

The substantial expansion of the human thymus during infancy, a period of rapid growth, leads to a resource conflict (McDade, 2003; McDade, 2005). In human life, the risk of death due to infectious disease is the greatest during infancy. To develop an optimal immune defense, the organism has to take into account not only the available resources but also the risk and virulence of infection (Boots, Donnelly, & White, 2013; McDade, 2003). The existing resource conflict between growth and immunity during human infancy can be resolved by the use of adipose reserves of the baby, supply of immune components via breastfeeding, and by adaptive resource management (McDade, 2003). If the newborn becomes sick with an infection, then the immune function is prioritized, thus resulting in a slower growth rate of the infant (Urlacher & Kramer, 2018). The resource conflict between immune and growth investments continues throughout the childhood and juvenile period of humans. However, the somatic body growth slows during these times, except for the developing brain, which continues to require a high level of resources (Kuzawa et al., 2014). At puberty, growth levels off, and resource needs for growth may be exchanged with resource needs for reproduction (McDade, 2003, 2005). Notably, since the human organism establishes competent and long-lived peripheral T-cell populations early in life, resource investments into thymus maintenance may be gradually reduced in favor of competing life-

history traits like (brain) growth and reproduction (Dowling & Hodgkin, 2009; McDade, 2003; McDade, 2005).

Can the age-specific changes of thymus development in zebrafish also be understood from a life history perspective?

The pattern of age-related changes of the zebrafish thymus is in contrast to that of humans. The zebrafish life-cycle starts with the embryo period during which the organism depends on the yolk reserves for both nutrition and immunity, followed by the larval period once exogenous feeding begins (Belanger, Balon, & Rawlings, 2010). After 2-4 weeks, metamorphosis is completed, and the juvenile period starts (Singleman & Holtzman, 2014). The adult stage begins with the onset of spawning, which usually occurs around 3-4 months post-fertilization. The initial differentiation of the zebrafish thymus happens during the embryonic and larval periods (Lam et al., 2002, 2004; Langenau et al., 2004; Willett et al., 1999). However, as shown by the present study and by Lam et al. (2002), expansion and rapid growth of the thymus takes place only during the juvenile period of zebrafish. This thymus development pattern appears to be more generally realized in teleost fish, with the thymus being the first lymphoid organ to differentiate morphologically (Davidson & Zon, 2004; Rombout, Huttenhuis, Picchietti, & Scapigliati, 2005; Seemann, Knigge, Olivier, & Monsinjon, 2015; Zapata, Diez, Cejalvo, Gutiérrez-de Frías, & Cortés, 2006) and becoming only functional in the juvenile period (Falk-Petersen, 2005; Lam et al., 2004; Mulero, García-Ayala, Meseguer, & Mulero, 2007; O'Neill, 1989; Parichy, Elizondo, Mills, Gordon, & Engeszer, 2009; Tatner & Manning, 1983; Hunt & Rice, 2008). This late onset of thymus development and adaptive immune systems appears to be expressed in all teleost species that have been studied to date.

According to the ecoimmunological "pace of life" hypothesis, the rate of immunological development should differ between long-lived (slow pace of life) and short-lived (fast pace of life) species (Lee, 2006; Sandmeier & Tracy, 2014). For instance, mammals with a short gestation period like mice, rats, or hamsters, have a relatively immature adaptive immune system at birth

compared to long-lived mammals like primates or dogs (Martin, Weil, Kuhlman, & Nelson, 2006; Martin, Hasselquist, & Wikelski, 2006). In contrast, the late development of the thymus and adaptive immunity occurs in both long-lived fish species like cod, *Gadus morhua* (Falk-Petersen, 2005) or the Antarctic *Harpagifer* sp. (O'Neill, 1989) as well as in short-lived species like zebrafish or medaka, *Oryzias latipes* (Ghoneum & Egami, 1982). This delayed growth of the zebrafish thymus, in contrast to the human thymus, might partly be explained by the evolutionary trait of a late-developing adaptive immune system in teleost fish. Or it might imply that resource tradeoffs are not a driving factor for the age-related thymus changes seen in zebrafish. Concerning the competition for resources between growth, reproduction, and immunity in developing fish, the larval stage is characterized as the peak-intensity for growth, which is costly for the organism and requires major resource investments (Pedersen, 1997; Rombough, 1994). During the juvenile stage, body growth slows down (Conceição, Dersjant-Li, & Verreth, 1998; Wieser, 1994), which implies that resources become available for investment in immune system development. The juvenile period thus might represent a "window of opportunity" for investing resources into the development of immunity, including the pronounced growth of the thymus. Support for such an energetic flexibility of the juvenile period comes from our recent finding that pathogenic infection of juvenile rainbow trout was not associated with a reduction of body growth, indicating that there was no relevant resource conflict between the energy needs for growth and the activation of the immune system (Wernicke von Siebenthal et al., 2018). This finding is in contrast to humans, where infections during infancy or childhood result in decreased body growth (Urlacher & Kramer, 2018).

In addition to growth, reproduction competes with immunity for resources. During the juvenile period of zebrafish, the initially undifferentiated gonads pass through a non-functional protogynic stage before they differentiate into ovaries or testes (Maack & Segner, 2004). Puberty, which involves the activation of the hypothalamic-pituitary-gonad axis and the maturation of germ cells in the gonads, occurred at the end of the juvenile period of our experiment; observed during the histological examination of the gonads. While

this stage was associated with a stop of thymus growth, the involution began when the gonads became mature and active reproduction started. From then on, the thymus rapidly involuted. This finding points to a resource tradeoff between reproductive activity and thymus maintenance. The adult zebrafish is a fractional spawner, which means that the female lays up to 200 eggs every 3 – 7 days (Connolly et al., 2014). Unfortunately, to the best of our knowledge, only a general energy budget exists for zebrafish (Augustine, Gagnaire, Floriani, Adam-Guillermin, & Kooijman, 2011; Chizinski, Sharma, Pope, & Patino, 2008) but no information on the specific costs of reproduction is available. However, the continuous production of high amounts of eggs by the females and the mating behavior of the males should require substantial energy investments. This assumption is also substantiated by the vtg data: while vtg protein levels showed an increase already during the puberty period, concomitant with the increasing number of vitellogenic oocytes in the ovaries, a significant increase took place only in the adult, actively reproducing fish (see Fig. 3). Thus, the reproductive investment appears to be significantly higher during the sexually mature period than during the pubertal period. The vtg data provide additional evidence that resource tradeoffs between immunity and reproduction contribute to the thymus involution in adult zebrafish. A tradeoff between immunity and growth should not be relevant for the adult fish, since growth is slowing down with the onset of reproduction (see Fig. 1). In this context, it should be emphasized that the life history of zebrafish is characterized by a short life cycle, with an early onset of reproduction. From the viewpoint of physiological investments, this life history model should prioritize investments into reproduction over investments into long-term maintenance and survival (Shanley et al., 2009).

If life-history influences age-related thymus changes, then species with contrasting reproductive strategies should display contrasting patterns of thymus development. For instance, in mammals, a distinct variation of the timing of thymus involution has been reported for marsupials, and this variation appears to be related to the reproductive mode (semelpar versus iteropar) of the species (Peel & Belov, 2017). Concerning fish, the problem in examining the relation between thymus involution and reproductive modes

comes from the fact that for most fish species, only insufficient data on age-related thymus changes concerning the reproductive status are available. Cockburn (1992) highlighted that fish species which reproduce only once in their lifetime (for instance, semelparous Pacific salmon species or annual cyprinodonts) experience a complete involution of the thymus before puberty. When maturation begins for this species, they have to allocate substantial resources into reproduction, and the intensive terminal investment in reproduction correlates with early thymus involution. However, the situation is more complex for other reproductive modes. For example, concerning iteroparous teleost species (e.g., the three-spined stickleback (*Gasterosteus aculeatus)* (Bigaj, Dulak, & Plytycz, 1987), anglerfish (*Lophius piscatorius)* (Fänge & Pulsford, 1985), sea bass (*Dicentrachus labrax*) (Good, Finstad, Pollara, & Gabrielsen, 1966) and carp (*Cyprinus carpio)* (O'Neill, 1989) who have a seasonal spawning mode, it has been reported that the thymus does not involute at all with age. For other seasonal spawning fish species, the thymus has been reported to involute before sexual maturity is reached (e.g., brown trout, *Salmo trutta*) (Deanesly, 1927) or when the reproductive age is reached (e.g., *Antarctic silverfish, Pleurogramma antarcticum*) (O'Neill, 1989), plaice *(Pleuronectes platessa*) (Lele, 1934), the Mexican tetra, also known as the blind cave fish *(Astyanax mexicanus)* (Hafter, 1952) and cichlids (Fishelson, 1995)). For fractional spawners, contrasting findings exist. For example, in zebrafish, the thymus regression is correlated with the start of reproduction, while Ghoneum and Egami (1982) reported that, for medaka (*Oryzias latipes*), the thymus continued to grow after the onset of reproduction and involuted only later in life.

From this short overview, it is evident that the currently available database is too fragmentary to support firm conclusions on whether teleost fish species compromise between resource needs for immunity and reproduction. More comprehensive studies on the relation between the immune system and reproductive investments of fish are needed to conclude that the timepoint of thymus involution systematically varies with the reproductive mode of species.

5. Conclusion

For the first time, this study provides a holistic insight into the developmental changes of thymus size, cellularity, histoarchitecture, and T-cell transcripts, over critical life stages (pre-pubertal, pubertal, sexually mature, and reproductive) of zebrafish - a teleost species. Our results indicate that thymus development and involution may be driven in part by resource limitations, but also the peculiarities of the adaptive immune system ontogeny in fish appear to play a role.

Future research should evaluate the potential role of sex steroids in thymus involution in teleost species. From mammals, we know that sex steroids influence both thymus differentiation and thymus involution (Staples et al., 1999; Zoller & Kersh, 2006). Recent research has shown that the thymus of fish expresses estrogen receptors and that their thymus development and functionality are sensitive to estrogens (Paiola et al., 2018; Paiola et al., 2017; Seemann et al., 2015). Thus, the relation between sex steroids and thymus involution is an intriguing avenue to explore.

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Author Contributions

H.S. and C.B. formulated the concept for the special edition. H.S, L.K. and J.R. designed the methodology. H.S. and L.K. collected and analyzed the data. A.D. and H.H. analyzed the vtg data. H.S. and C.B. wrote the manuscript

with contributions from L.K. and J.R. L.K. composed all figures and tables under supervision from H.S. All authors contributed critically to the drafts and gave final approval for publication.

Data availability statement

The data that support the findings of this study are available from the authors upon reasonable request.

Conflict of interest

The authors declare that there is no conflict of interest.

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Figure legends

Figure 1. Bar charts representing the age-related wet weight (mg) (A), total length (cm) (B), and head area (μm^2) (C) of the lck:GFP and wild-type (WT) zebrafish (mean ± SD). The fish n-numbers are given above the bar charts, while the arrows indicate fish n-numbers of data points with lower means.

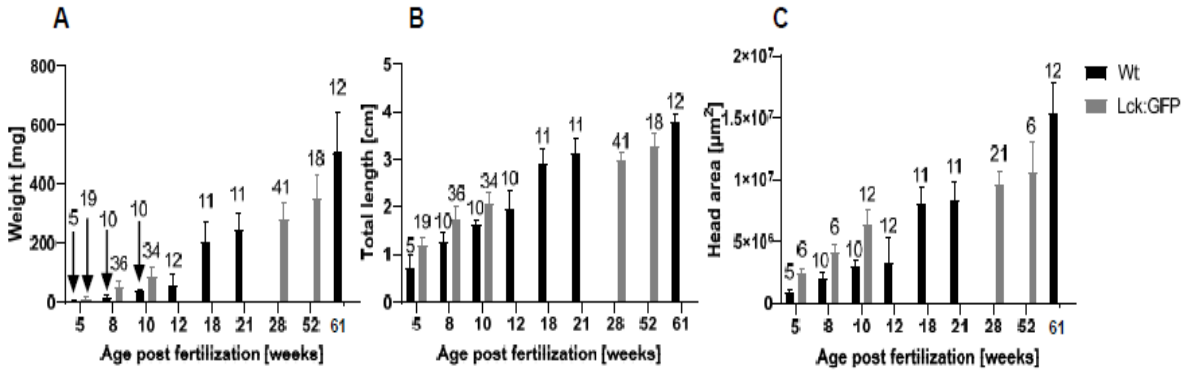


Figure 2. Bar charts representing the age-related absolute and relative thymus area of lck:GFP (A) and wild-type (WT) zebrafish (B) (mean ± SD). The relative thymus area was determined by the division of the absolute thymus area by the head area. A) shows the thymocyte number and the thymocyte number per milligram of fish only for the lck:GFP zebrafish line. The different letters indicate significant differences between the time points (Kruskal-Wallis One-Way ANOVA on Ranks, $p < 0.05$). The fish n-numbers are given above the bar charts, while the arrows indicate fish n-numbers of data points with lower means.

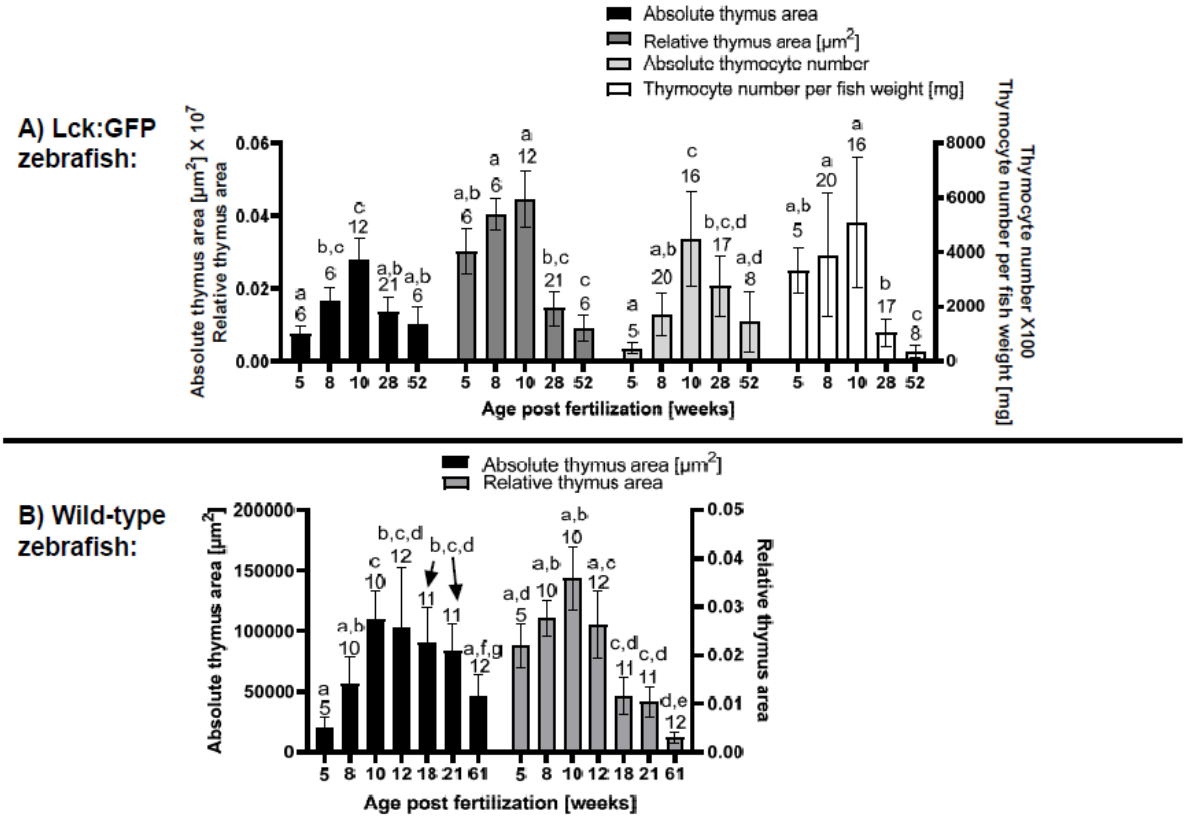


Figure 3. Bar charts representing vitellogenin (*vtg*) protein concentration (ng/ml) of tail homogenate of female lck:GFP zebrafish (mean ± SD) in relation to the stages of sexual development: pre-puberty, puberty, and sexually mature. The staging was done based on gonad histology, according to Chen & Ge (2013). The different letters indicate the significant differences between the stages of sexual development (Kruskal-Wallis One-Way ANOVA Ranks, $p < 0.05$). The fish n-numbers are given above the bar charts.

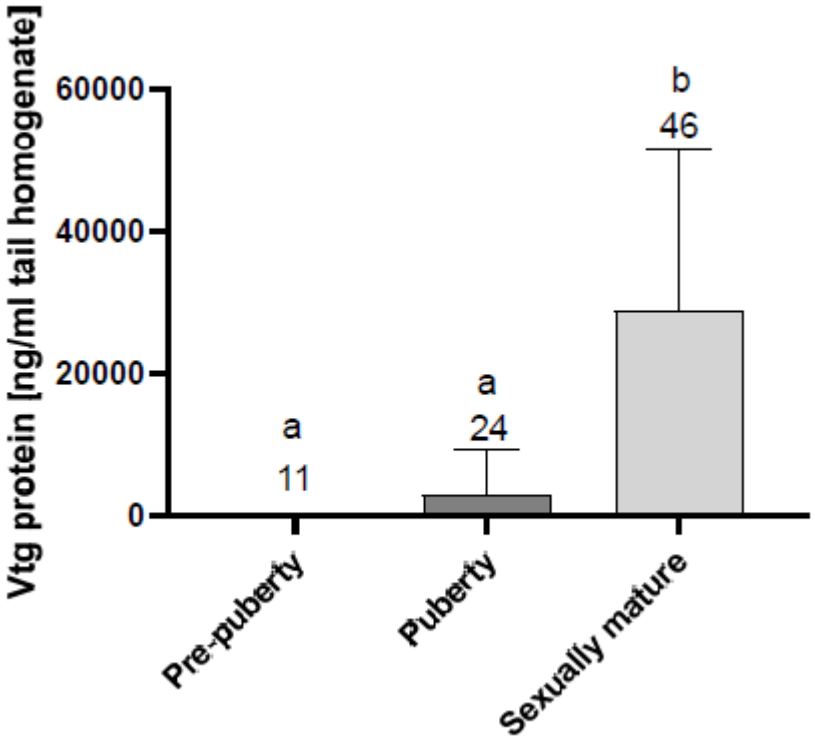


Figure 4. Bar charts representing absolute (A) and relative (B) thymus area of the female of both lck:GFP and wild-type (WT) zebrafish (mean ± SD) in relation to the stages of sexual development: undifferentiated (5 wpf), pre-puberty (8-10 wpf), puberty (8-10 wpf), onset of spawning (12 wpf) and regular spawning (18 wpf). The relative thymus area was determined by the division of the absolute thymus area by the head area. The different letters indicate significant differences between the stages of sexual development (Kruskal-Wallis One-Way ANOVA Ranks, $p < 0.05$). The fish n-numbers are given above the bar charts.

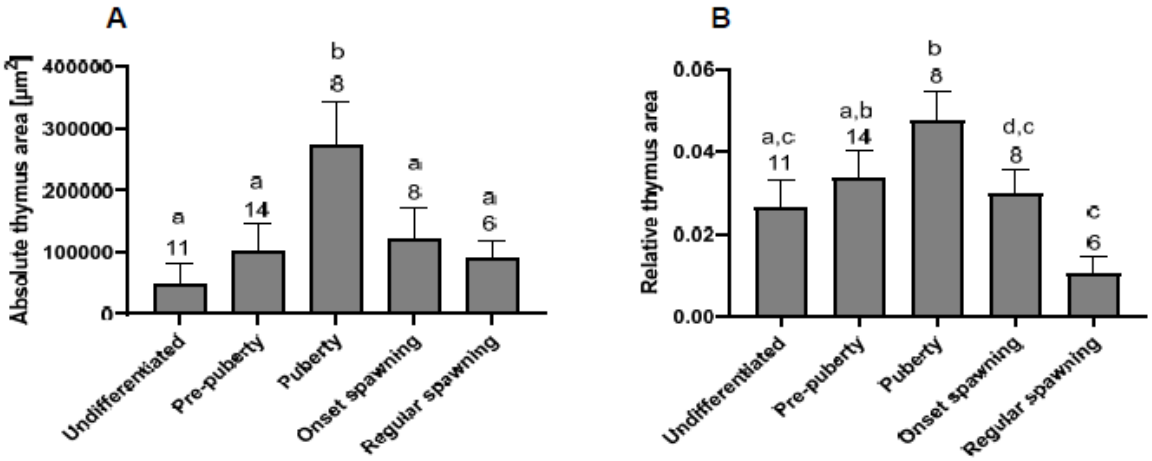


Figure 5. Transversal histological thymus sections from lck:GFP zebrafish stained with Hematoxylin and Eosin (H&E) or PCNA antibody showing cellular proliferation (green immunofluorescence). Figures A - E: 5, 8, 10, 28 and 52 weeks post-fertilization (wpf) (H&E); Figures F and G: adipocytes in the space between the dorsal end of the thymus and the cartilage arch (H&E); Figures H and I: PCNA immunostaining at 10 and 52 wpf. Bar scale of all pictures: 100 µm. A - Adipocytes, C - cortex, M - medulla.

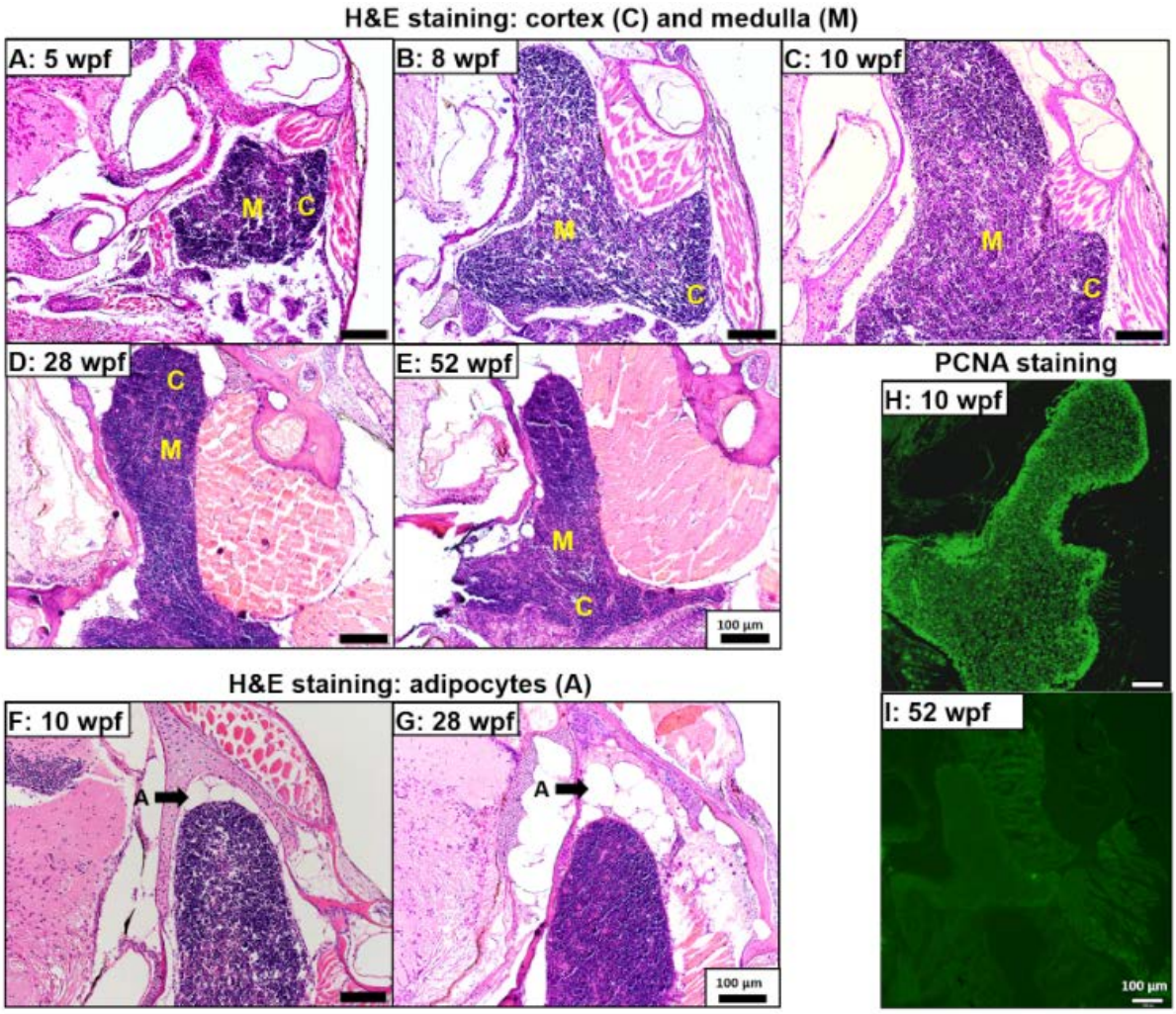
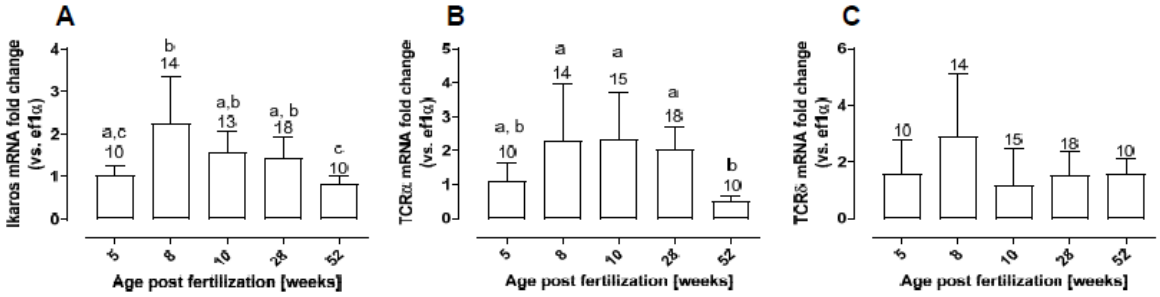


Figure 6. Bar charts representing relative fold change of *ikaros* (A), *tcrα* (B) and *tcrδ* (C) in the thymus of lck:GFP zebrafish (mean ± SD). As a reference gene, *ef1α* was used. The different case letters indicate significant differences between the time points (Kruskal-Wallis One-Way ANOVA on Ranks, $p < 0.05$). The fish n-numbers are given above the bar charts.



Table

Table 1. Sequences of zebrafish primers used in the present study with corresponding accession number and references.

Accession number	Gene name	Primer Forward 5' to 3'	Primer Reverse 5' to 3'	References
NM_131263.1	*ef1α*	CTGGAGGCCAGCTCAAACAT	TCAAGAAGAGTAGTACCGCTAGCATTAC	(Tang, Dodd, Lai, McNabb, & Love, 2007)
AF416372.1	*ikaros*	AGAAGGGTAACCTGCTCCGACAC	GGGCTTTCCAACCGAATGAGT	(Ma et al., 2012)
AF425590.1	*tcrα*	TTACTGCGAGGAGACAGGC	TCCTCAGCCAGAAGATGCC	(Yoon et al., 2015)
KX009743	*tcrδ*	GTGGCCGCCGGATTCTTTCCTCA	TTTGTGGATGGTGGGGTGGTAGT	(Wan et al., 2016)