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Diplom-Biologist Florian Schmidt

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Oral examination: .....

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Characterization of effects along the hypothalamic-pituitary-thyroid axis of the zebrafish (*Danio rerio*) after exposure to thyroid-disrupting chemicals.

Referees: Prof. Dr. Thomas Braunbeck
Centre for Organismal Studies, Heidelberg

Prof. Dr. Werner Kloas
IGB, Dep. 5, Inland Fisheries, Berlin
Institute of Biology, Humboldt University Berlin

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbst verfasst und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe. Des Weiteren erkläre ich, dass ich an keiner Stelle ein Prüfungsverfahren beantragt oder die Dissertation in dieser oder einer anderen Form bereits anderweitig als Prüfungsarbeit verwendet oder einer anderen Fakultät als Dissertation vorgelegt habe.

Langnau am Albis, 07.12.2011

Florian Schmidt

"Some men see things as they are and say why –
I dream things that never were and say why not."

George Bernard Shaw

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Abbreviations

EDC	endocrine-disrupting chemical
ELISA	enzyme-linked immunosorbent assay
FSH	follicle-stimulating hormone
HE	hematoxylin-eosin (staining)
hpf	hours post-fertilization
LH	luteinizing hormone
OECD	Organisation for Economic Co-Operation and Development
PAS	periodic acid-Schiff (staining)
PI	*pars intermedia* (pituitary)
PN	*pars nervosa* (pituitary)
PBS	phosphate-buffered saline
PPD	proximal *pars distalis* (pituitary)
PTU	propylthiouracil
rER	rough endoplasmic reticulum
RPD	rostral *pars distalis* (pituitary)
rT3	reverse triiodothyronine
T2	diiodothyronine
T3	triiodothyronine
T4	tetraiodothyronine (thyroxin)
TH	thyroid hormone
TSH	thyroid-stimulating hormone
US EPA	United States Environmental Protection Agency

Contents

Summary

The present thesis characterizes the effects in the zebrafish caused by the disruption of key mechanisms of thyroid hormone production, i. e. the thyroid hormone peroxidases (*via* propylthiouracil, PTU) and the sodium-iodide symporter (*via* potassium perchlorate, PER), along the hypothalamic-pituitary-thyroid axis. Furthermore, stimulating effects of exogenous administration of T4 were noted.

Besides macroscopical endpoints, the thyroid and the liver were histologically and ultrastructurally evaluated. The pituitary as the main control organ of the endocrine system was histologically and immunohistochemically investigated. To quantify the effects caused by the thyroid-disrupting substances morphometrical evaluations in the pituitary were accomplished. Furthermore, whole body concentrations of T4 were measured *via* ELISA.

PTU and PER led to clear signs of thyroid activation, i. e. hyperplasia and hypertrophy, whereas T4 led to a clear inactivity of the thyroid. Nevertheless, depending on the mode of action of the goitrogen, different alterations in the thyroid could be detected both at the histopathological and the ultrastructural level. Interestingly, both the inhibiting substances and T4 as stimulating substance led to a decrease of whole body T4 concentrations.

For the first time, clear feedback-induced increases of TSH-producing cell populations in the fish pituitary after exposure to PTU and PER were described. The downstream located liver only showed glycogen depletion and minor ultrastructural effects. A brief comparison of amphibians with the zebrafish revealed similar sensitivities depending on the test substance.

The present thesis show that the zebrafish is a very rewarding model for the detection of goitrogens due to its high sensitivity and easy laboratory handling. Nevertheless, further research has to be conducted on the entire hypothalamic-pituitary-thyroid axis and its feedback mechanisms. Finally, molecular biological aspects, e. g. gene activations, mRNA detection etc. could lead to a deeper understanding of the principal regulatory processes and to further endpoints that could be used to detect thyroid-disrupting substances. A full comprehension and an appropriate interpretation of effects of goitrogens against the background of chemical regulatory purposes are only possible, if these aspects are fully clarified.

Zusammenfassung

Die vorliegende Arbeit beschreibt Effekte entlang der Hypothalamus-Hypophysen-Schilddrüsen Achse des Zebrabärblings, hervorgerufen durch die Hemmung der Peroxidasen sowie des Natrium-Iodid Symporters in der Schilddrüse durch die beiden Substanzen Propylthiouracil (PTU) und Kaliumperchlorat (PER). Des Weiteren wurden stimulierende Effekte durch die Belastung mit T4 beobachtet.

Neben makroskopischen Veränderungen wurden die Schilddrüse und die Leber histologisch und ultrastrukturell sowie die Hypophyse als Kontrollorgan des kompletten Hormonsystems histologisch und immunhistochemisch untersucht. Weiterhin wurden morphometrische Messungen in der Hypophyse sowie Konzentrationsbestimmungen von Thyroxin im kompletten Fisch durchgeführt.

PTU und PER führten zu einer deutlichen Aktivierung (Hyperplasie und Hypertrophie) der Schilddrüse, wohingegen T4 die Schilddrüsenaktivität klar inaktivierte. Nichtsdestotrotz unterscheiden sich die beobachteten Veränderungen aufgrund der unterschiedlichen Wirkmechanismen der verwendeten Substanzen. Interessanterweise führten alle Substanzen zu einem Rückgang der Thyroxinkonzentration im Fisch.

Zum ersten Mal konnten deutliche Effekte aufgrund der negativen Rückkopplung in der Hypophyse von Fischen gezeigt werden (Anstieg der TSH-produzierenden Zellen). Die Leber als essentielles Organ in der Umwandlung von T4 in das aktive Hormon T3 zeigte nur einen unspezifischen Abbau von Glykogen sowie weitere unspezifische ultrastrukturelle Veränderungen. Der Vergleich mit dem etablierten Testorganismus *Xenopus laevis* zeigte, dass der Zebrabärbling eine vergleichbare Empfindlichkeit gegenüber goitrogenen Substanzen aufweist.

Die vorliegende Arbeit zeigt, dass der Zebrabärbling ein lohnendes Model für den Nachweis von goitrogenen Substanzen ist. Allerdings müssen weitere Studien an der gesamten Schilddrüsenachse sowie den Rückkopplungsmechanismen durchgeführt sowie molekularbiologische Endpunkte charakterisiert werden. Ein vollständiges Verständnis und eine angemessene Interpretation von Effekten hervorgerufen durch goitrogene Substanzen kann nur erreicht werden, wenn diese Aspekte vollständig geklärt sind.

Chapter 1

Introduction

1.1 Historical background of the thyroid

The thyroid gland was first described by Galenos of Pergamon (130-201 AD) in De Voce (Gross et al. 1952, Rolleston 1936). In the 16th century the gland was described in detail by Vesalius. The name thyroid is derived from the greek word "thyreos" meaning shield due to the gland´s shield-like appearance. The term was first used by Thomas Warton (1614-1673) in his 1656 published work "Adenographica" (Singer et al. 1962). First signs of abnormal alterations of the thyroid were already known from Assyrian bas-reliefs, which described goiter formation. Similar alterations were mentioned in medical chronicles of ancient Egypt, China, India, and pre-Christian Rome. Plinius (23-79 AD) made the bad quality of drinking water responsible for several goiter formations in ancient Rome (Naturalis Historia quoted in Hertz (1943)). The abnormal goiter formations and the function of the shield-like organ itself remained mysterious for long. Galenos postulated that the thyroid gland secreted substances to moisture the larynx. Leonardo da Vinci, on the other hand, thought that the thyroid is supposed to support the beautiful shape of the neck (Bargmann 1939). In the 19th century Henle, Stannius, and Gerlach postulated the thyroid is a "blood vessel gland" similar to the lymph nodes, which were seen as "lymph vessel glands" (Bargmann 1939). The microscopic structure of the thyroid was first described by King (1836), although he still believed that the single follicles communicated with each other. It was not until 1841 when Heinrich Adolf von Bardeleben postulated that each follicle is an independent structure, not communicating with adjacent follicles (Singer et al. 1962).

A possible correlation between abnormal goiter formation and iodine-deficiency was soon hypothesized. Rogerius (1170; cited in Castiglioni (1958)), Prosser (Prosser 1769), Coindet (Coindet 1820), Prout (Prout 1834), and von Basedow (von Basedow 1840) recommended to eat seaweed, burnt sponges or dried deer thyroids to fight goiter formation. A few years later, Baumann (1895) succeeded in extracting an iodine-binding, amorphous fluid of the thyroid and named it "thyroiodine". In 1915, the iodine-containing substance was first isolated and crystallized by Edward Calvin Kendall, who postulated the first – but wrong – chemical structure (Kendall 1915). The correct chemical structure of thyroxine or tetraiodothyronine was than published in 1926 by Harington (Harington 1926a, b).

In 1952, triiodothyronine was identified by Gross and Pitt-Rivers (1952). This key discovery led to countless studies on thyroid hormones to date.

1.2 Thyroid phylogeny, morphology, and development

The vertebrate thyroid gland developed out of the acranian endostyle, which is essential for the filtration of food particles. This typical lower chordate organ is located ventrally to the Kiemendarm and responsible for the production of mucus. Morphologically, the endostyle consists of 8 different cell groups (Fig. 1.1), and already at this taxonomic level, cells from zone 7 of the endostyle have the ability to bind iodide, secrete glycoproteins, peroxidases, and thyroid hormones (Barrington et al. 1965, Barrington 1957, Dunn 1974, 1980, Fredriksson et al. 1993, Thorndyke 1978).

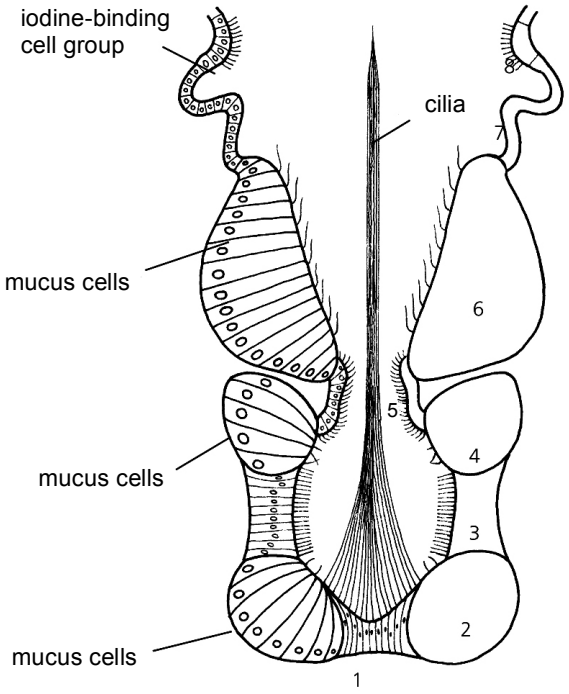

Fig. 1.1: Ascidian endostyle with the different functional cell groups (1 to 8). Cells from zone 7 have the capability to bind iodine, secrete glycoproteins, peroxidases, and thyroid hormones (schematic according to Barrington 1957).

Furthermore, in *Ciona intestinalis* and *Halocynthia roretzi*, it could be shown that cDNA clones of genes for thyroid peroxidase are restricted to zone 7 of the endostyle, i.e. the zone corresponding to the thyroid (Ogasawara et al. 1999). In cephalochordates and lamprey ammocoetes, thyroid hormones could be identified in the endostyle as well (Eales et al. 1997). Interestingly, even non-vertebrate species like *Ciona intestinalis* display clear signs of classical vertebrate effects when exposed to thyroid-disrupting chemicals (Patricolo et al. 2001). The most primitive vertebrates that definitely display a follicular thyroid gland are the jawless fish (agnathans; for further details on phylogeny see Fig. 1.2). In this context, lampreys are of highest interest, because these are the only vertebrates with a larval endostyle metamorphosing to an adult follicular thyroid gland (Kluge et al. 2005, Wright et al. 1976). During metamorphosis of the ammocoete into the adult lamprey, the endostyle detaches from the pharynx to form single scattered follicles. From a descriptive point of view, the processes in lamprey development exactly describe the phylogenetic transition from the acranian endostyle into the follicular thyroid gland in vertebrates. Thyroid follicle distribution in fish makes this transition easy to understand, since fish usually display single thyroid follicles distributed along the ventral aorta (Eales 1979, Raine and Leatherland 2000, Raine et al. 2001, Wabuke-Bunoti and Firling 1983, Wendl et al. 2002). Cartilaginous fish (chondrichthyes) display an encapsulated thyroid gland, and in higher vertebrates (amphibian, reptiles, birds, and mammals) the thyroid is a one- or two-lobed encapsulated gland.

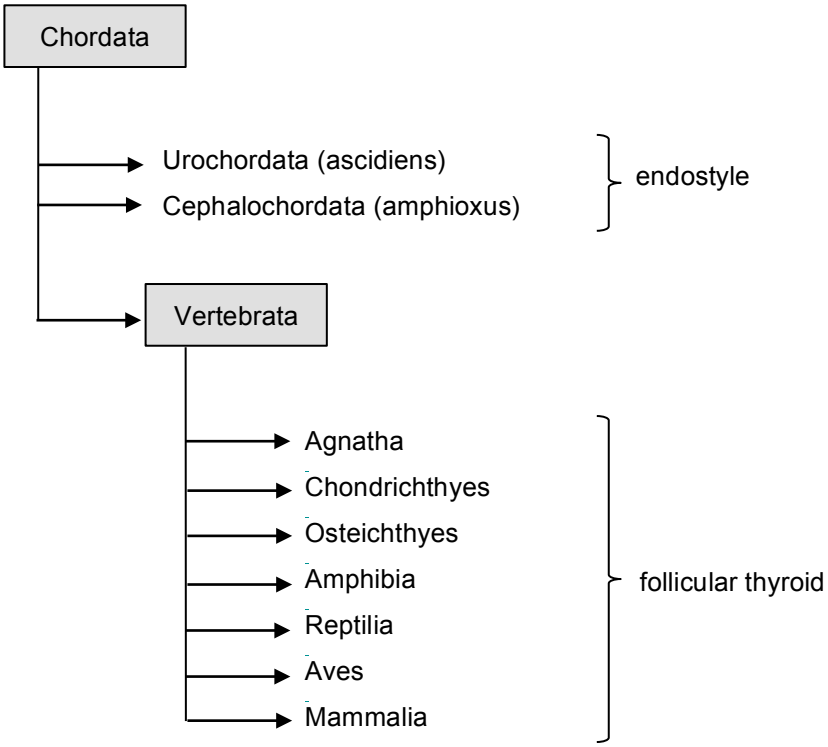

Fig. 1.2: Phylogenetic overview of the endostyle and follicular thyroid distribution throughout the chordates.

It has long been known that the thyroid gland is of endodermal origin. In fish, the primordium evaginates from the pharyngeal epithelium and adopts a position near the cardiac outflow tract under the influence of *pax2.1* and *pax8* (Alt et al. 2006a, Elsalini et al. 2003, Wendl et al. 2002). The first thyroid follicle differentiates around 55 hpf (Alt et al. 2006b), and thyroid hormone production starts at 72 hpf (Elsalini et al. 2003).

The key component of the thyroid is the follicle, which consists of a single-layered epithelium surrounding a PAS-positive lumen. The epithelium of the follicles itself usually consists of cuboidal thyrocytes with a basally located nucleus. The thyrocytes display microvilli at the apical border to the colloid which can be seen as a reminder of the origin of the gland from the endoderm showing a striking

relationship to the gastrointestinal tract. Furthermore, it was found that numerous vertebrate species display a central flagellum at the apical surface as well (Fujita 1963, Muramoto 1964, Tashiro and Sugiyama 1964), although this could not be observed in zebrafish examined in this study. The lumen of the single follicles is filled with colloid. In humans, the colloid is composed of 19S thyroglobulin, larger iodoproteins, and smaller proteins fractions, i.e. an albumin-like protein and a pre-albumin fraction (Anderberg et al. 1980, 1981). There are no cellular inclusions in the colloid visible. In general, the follicles have close contact to the blood vessel system to ease thyroid hormone secretion and distribution in the body.

1.3 Thyroid hormones

In humans, thyroid hormones are essential for the development of several tissues (brain, heart, kidney, skeletal muscle, and ear; Brouwer et al. 1998, Hauser et al. 1998) and for energy metabolism (Hennemann 1986, Kadenbach et al. 1995, Nelson et al. 1995). The synthesis of thyroid hormones depends on sufficient iodide supplies derived from the environment. In terrestrial vertebrates, almost all iodide is obtained from food by transport from the gut, where any elemental iodine is converted to iodide. Some geographical regions, called “goiter zones”, lack sufficient iodine and endemic goiter develop. For this reason, iodinated nutrients, e. g. table salt, are used. While iodide deficiency is not expected in fish living in sea water, which is relatively rich in iodide and iodate, it might be expected in fish living in fresh water, where iodide levels range from 1 µg/dl to less than 0.01 µg/dl (Eales 1979). However, plasma iodide levels in fish are generally higher than the normal human level. For a functioning thyroid hormone synthesis, sufficient iodide supply is essential. In contrast to tetrapods, which usually maintain their iodide supply via the intake of food, fish have the capability to take up huge amounts of iodide via their extensive gill surface (Hunn et al. 1966). The effectiveness of the branchial iodide pump was clearly shown in brook trout which showed elevated plasma iodide concentrations even after starvation for several weeks (Higgs and Eales 1971). After entering the plasma, iodide is transported to the thyroid follicles. Certain teleost species, especially clupeiformes, possess a plasma pre-albumin iodide binding protein (Leloup 1970) probably reducing filtration loss of

iodide at renal or gill exchange surfaces (Eales et al. 1993). Reaching the thyrocytes, the iodide is co-transported together with sodium into the cells by specialized transporters called the sodium-iodide symporter. This process is ATP-dependent. Inside the thyrocytes, it diffuses, following the electrical gradient, by a specialized channel, from the cell to the lumen at the apical membrane (Rodriguez et al. 2002), where thyroid hormone synthesis is located (Nilsson et al. 1992).

Thyroid hormone synthesis starts at the rough endoplasmic reticulum, which produces thyroglobulin – a large glycoprotein. This protein contains tyrosine residues which can be iodinated and than coupled to form T4 and to a minor extent T3. This process takes place in the colloid under the influence of a special enzyme called thyroperoxidase, using H_2O_2 as a substrate (Nunez et al. 1982). Once tyrosine residues are coupled to form T4 (and T3), secretion of thyroid hormones begins. For this case, it is essential for the thyrocyte to ingest the thyroglobulin, which happens *via* endocytosis and hydrolysis with the help of lysosomes. Peptidases break down thyroglobulin to its constituent amino acids. Abundant monoiodothyronine and diiodothyronine are deiodinated and much of the iodide recycled within the thyroid (Eales et al. 1993). To date, it is not clear how the free thyroid hormones are secreted, either by diffusion or by transport.

Once secreted, only a small fraction of the thyroid hormones are free in the plasma, the majority is bound to plasma proteins such as thyroxin-binding protein, transthyretin, and albumin. Among these plasma proteins, only thyroxin-binding protein seems to be fully selective for thyroid-hormone transport alone (Robbins et al. 1986). In contrast to humans, fish together with ungulates, carnivores, and amphibians do not express thyroxin-binding protein (Larsson et al. 1985). In amphibian larvae and teleost fish transthyretin is the most important plasma carrier (Power et al. 2000, Power et al. 2001, Yamauchi et al. 1993). It has been shown that the affinity of transthyretin to T3 is much higher in amphibians and teleost fish (Power et al. 2000, Power et al. 2001, Prapunpoj et al. 2000). Nevertheless, the role of thyroid hormone-binding proteins is not limited to plasma carriers but to maintain a stable pool of thyroid hormones to be released when needed (Robbins et al. 1986). The impact of the thyroid hormones on the target tissues is determined by (1) the peripheral concentration of thyroid hormones, (2) the presence and activities of deiodinases (type I, II, and III) and (3) the presence of thyroid

hormone transporters in the cell membranes (Friesema et al. 2005). It is long known, that T3 is the biologically active hormone and deiodinases type I and II catalyze outer-ring deiodination to generate T3 whereas deiodinase type III inactivates both T3 and T4 by inner-ring deiodination to the inactive forms T2 and reverse T3, respectively (Hennemann 1986). On the other hand, the presence of thyroid hormone transporters is crucial, because thyroid hormone action is only mediated in the target cells. Around three decades ago, it was common ground that thyroid hormones enter the target cells by simple diffusion. To date, numerous reports on ATP-dependent transporters were published (Hennemann et al. 2001). An overview of thyroid hormones and their deiodination processes are shown in Fig. 1.3.

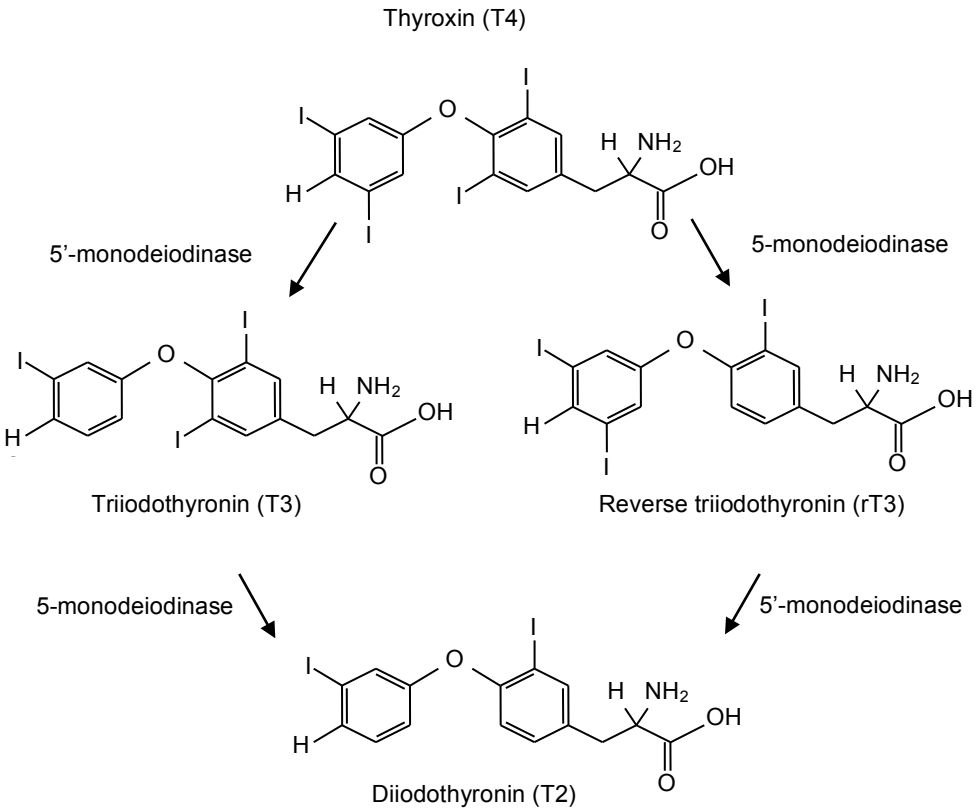

Fig. 1.3: Thyroid hormones and the different deiodination processes (redrawn from Eales et al. 1993).

1.4 The hypothalamic-pituitary-thyroid axis

In general, the main regulator of the endocrine system is the pituitary closely connected to the hypothalamus. Regulatory pathways of the thyroid system start with the reception of external and internal sensory information reaching the brain and the hypothalamus. From here, the information is redirected to the pituitary either *via* TRH like in higher vertebrates or in case of teleost fish *via* a direct neuronal connection to endocrine cells in the pituitary through the hypophyseal stalk (Peter et al. 1990). TSH activates thyroid hormone production and in consequence T4 and a reduced amount of T3 are secreted by the follicles (Eales et al. 1999). As a pro-hormone, T4 secreted by the thyroid gland has to be converted to the biologically active hormone triiodothyronine (T3) by an outer ring deiodination (Fig. 3; Eales et al. 1999, Leatherland et al. 1990). This process takes place in peripheral tissues, mainly the liver (Darras et al. 1998, Eales et al. 1999). Control of the thyroid hormone secretion appears to be primarily regulated by a feedback loop, with both T4 and T3 having a negative effect on the release of TSH in the pituitary (Larsen et al. 1997, Pradet-Balade et al. 1997, 1999, Yoshiura et al. 1999). For an overview of the hypothalamic-pituitary-thyroid axis see Fig. 1.4.

Thyroid hormone levels significantly fluctuate during development and life span. From amphibians, it is known that during metamorphic climax T3 and T4 reach their highest peaks. Having finished metamorphosis concentration levels of both T3 and T4 are reduced to much lower levels (Shi 2000). In salmonids, thyroid hormones follow seasonal rhythms (Leatherland et al. 1980a). Thyroid hormone levels are high during gonad development and gametogenesis. During their potamodromous migration, serum levels rapidly decrease (Leatherland 1982). Nowadays, it is widely accepted that thyroid hormone secretion is subject to different daily or seasonal changes (Boujard et al. 1992, Eales 1979, Leatherland 1982, 1993, Spieler 1993). Furthermore, during development, it is generally assumed that thyroid hormone levels are usually higher during the embryonic and larval phases than in the adult phase.

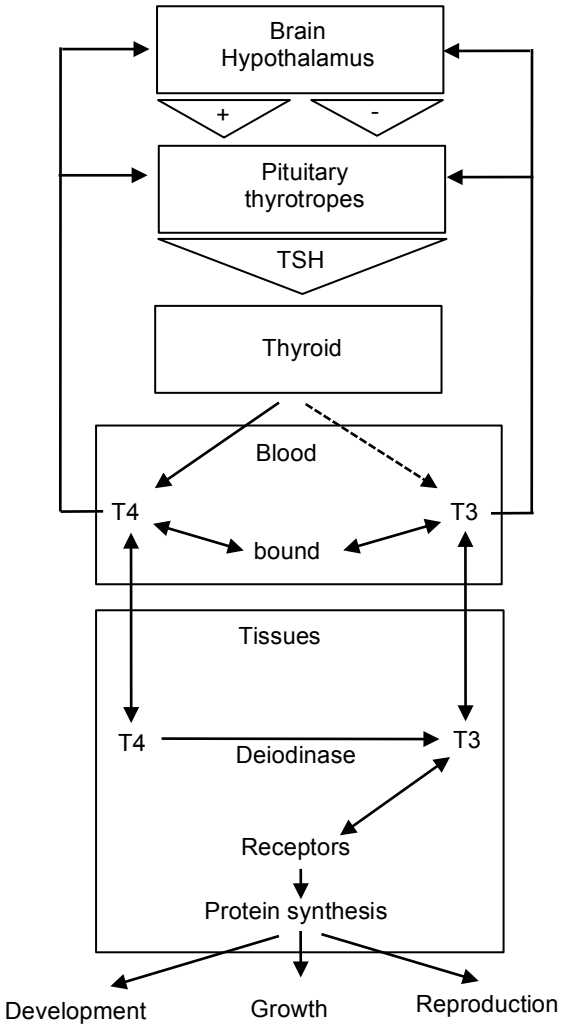

Fig. 1.4: Overview of the hypothalamic-pituitary-thyroid axis in teleost fish (redrawn from Eales et al. 1993).

1.5 Thyroid hormones in teleost fish

In vertebrates, one of the best-known examples of thyroid hormone action is found in amphibian tadpoles metamorphosing into the juvenile form (definitive phenotype), which is dependent on the presence of thyroid hormones. Around 100 years ago, Friedrich Gudernatsch published his works on the influence of macerated equine thyroid glands on tadpole development (Gudernatsch 1912, 1917). These works initiated the interest in similar "metamorphic" processes in other vertebrate groups. Among teleosts, one of the best-known examples for metamorphic processes is the transformation of pelagic bilaterally-symmetrical flounder larvae into the asymmetrical benthic juveniles and the smoltification of salmonid fish. Both of these developmental processes are dependent on thyroid hormone action. Besides these popular examples, numerous other developmental processes are dependent on thyroid hormones. Table 1.1 gives a brief overview of reported actions of thyroid hormones in fish.

Table 1.1: Reported actions of thyroid hormones in fish (Leatherland 1994).

Thyroid hormone action	Reference
Seasonal changes in thyroid hormone activity (including responses to changes in ambient temperature)	2, 5
Migration	2, 5
Ontogeny and development	2, 5
Reproduction	2, 5, 6
Growth	1, 2, 4, 5
Intermediary metabolism	2 3, 4, 5
Osmotic and ionic regulation	2, 4, 5
Nervous system development and function	2, 4, 5
Pigmentation	2, 4, 5

References:
1 = Donaldson et al. (1978)
2 = Eales (1979)
3 = Eales (1988)
4 = Gorbman (1969)
5 = Leatherland (1982)
6 = Leatherland (1987)

1.6 Thyroid hormone disruption by environmental contaminants

Apart from these well-known effects of thyroid hormones on the development and metabolism of fish, the deficiency of these hormones is not well understood. In humans, it has long been known that adequate production of thyroid hormones is essential in mothers during early pregnancy to allow correct neurological development of the fetus (Berbel et al. 2010). The deficiency of thyroid hormones during fetal and neonatal periods in humans results in impaired brain development and neurological malformations (Nicholson et al. 1972, Rabie et al. 1980, Xiao et al. 1998, Zoeller et al. 2007). In fish, similar effects have hardly been examined, but the similarity of fish thyroid development and molecular and functional studies of thyroid follicles demonstrated a high degree of conservation with higher vertebrates.

Studies on alterations of the thyroid system in teleost fish have increased in the last couple of years (1) because of the importance of thyroid hormones in the development, and (2) to develop a suitable analogue for the human thyroid system to screen for goitrogens. Since the late 1980s, research on endocrine-disrupting chemicals has mainly focused on effects on reproductive biology (Gray et al. 2002, Jobling 1998, Tyler et al. 1998), but the awareness of potential risks by thyroid system-disrupting chemicals is increasing (Brar et al. 2010, Brucker-Davis 1998, Raldua et al. 2009, Rolland 2000, Shi et al. 2009, Zoeller 2003). Thus, in 2009 an OECD guideline has been established to detect adverse effects on the amphibian thyroid system with *Xenopus laevis* as test model (OECD 2009). Besides specific substances designed to inhibit thyroid hormone synthesis like PTU, numerous other substances are known to exert negative effects on the hypothalamic-pituitary-thyroid axis (Table 1.2).

Table 1.2: Overview of several chemicals/chemical classes known to affect the thyroid system (adapted from Crofton 2007).

Chemicals	Mechanisms	Effects on THs	References
Perchlorate, chlorate, bromate, thiocyanate, nitrate	Competition/block of NIS	Decreased thyroidal synthesis of T3 and T4	Van Sande et al. 2003, Wolff 1998
Methimazole, propylthiourea, amitrole, mancozeb, soy isoflavones, benzophenone 2, 1-methly-3-propyl-imidazole-2-thione	Inhibition of TPO	Decreased thyroidal synthesis of T3 and T4	Biegel et al. 1995, Capen 1997, Doerge and Sheehan 2002, Hurley 1998, Schmutzler et al. 2007
Hydroxyl-PCBs, EMD 49209, pentachlorophenol	Altered binding to serum transport proteins	Unknown	Lans et al. 1993, Schroder-van der Elst et al. 1998, Van den Berg et al. 1998
Acetochlor, phenobarbital, 3-methylcolanthrene, PCBs, 1-methyl-3-propyl-imidazole-2-thione	Upregulation of glucuronylsyltransferases or sulfotransferases (*via* CAR/PRX or AhR	Increased biliary elimination of T3 and T4	Biegel et al. 1995, Brucker-Davis 1998, Hood and Klaassen 2000, Hurley 1998, Liu and Klaassen 1996
TCPOBOP, pregnenolone-16α-carbonitrile, TCDD, rifampicin, phenobarbital, oltipraz	Upregulation of OATPs or MCT transporters *via* CAR/PRX or AhR	Increased biliary elimination of T3 and T4	Guo et al. 2002, Jigorel et al. 2006, Petrick and Klaassen 2007, Staudinger et al. 2001
Hydroxylated PCBs, triclosan, pentachlorophenol	Inhibition of sulfotransferases (SULTs)	Decreased sulfation of THs	Schuur et al. 1998, Wang et al. 2004, Wang and James 2006
FD&C Red dye #3, propylthiouracil, PCBs, octyl-methoxycinnamate	Inhibition or upregulation of deiodinases	Decreased peripheral synthesis of T3	Capen 1998, Klammer et al. 2007, Morse et al. 1993, Visser et al. 1979
Tetrabromobisphenol A, bisphenol A, hydroxyPCB	Direct or indirect alterations in TR-TRE binding	Altered activation of TH dependent gene transcription	Gauger et al. 2004, Kitamura et al. 2005, Moriyama et al. 2002

In general, there are five ways for endocrine-disrupting chemicals to exert toxic effects along the hypothalamic-pituitary-thyroid axis (Fig. 1.5): (1) Competition for receptor binding. Special chemicals, e. g. polychlorinated/polybrominated diphenyl chemicals, polybrominated flame retardants, bisphenol-A, and triclosan are known to compete for thyroid hormone receptors in different tissues (Marsh et al. 1998, McKinney et al. 1994, Moriyama et al. 2002, Veldhoen et al. 2006). Nevertheless, compared to the estrogen receptor, very little is known about the ability to interact with the thyroid hormone receptor. It has been shown by crystallography that the pocket where the ligand binds to the receptor is internal and provides a tight fit for T3, and much less for T4 and other thyroid hormone analogues (McGrath et al. 1994). Despite extensive work, amiodarone, GC-1 and NH-3 are the only three pharmaceutical compounds found so far with capacity to bind to the thyroid receptor (Furlow et al. 2004, Lim et al. 2002, van Beeren et al. 1996, Yoshihara et al. 2003).

A recent review by Zoeller (2005) indicates that other interactions of environmental contaminants with the thyroid receptor-mediated cascade may be of greater importance: (2) Inhibition of biosynthesis of thyroid hormones. The medical treatment of hyperthyroidism in humans uses specially designed anti-thyroid drugs to specifically inhibit thyroid hormone production. Among these substances, methimazole, carbimazole, and propylthiouracil are the best-known examples. Another important group of substances are isoflavones, in particular genistein, and daidzein (Divi et al. 1997), whose main dietary source are soy products, peas, bean nuts, grain products, coffee and tea (Boker et al. 2002). These substances share a common mode of action: the inhibition of the thyroid peroxidase, which is essential for the biosynthesis of thyroid hormones. Other substances, e.g. perchlorates, thiocyanates, and nitrates competitively inhibit iodide uptake, which results in distorted hormone synthesis as well.

(3) Chemical-induced cytotoxicity. Certain chemicals are known to affect direct cellular injury (cytotoxicity). Onderwater et al. (1998) report of cytotoxicity of a series of mono- and di-substituted thiourea in rat hepatocytes. Recent studies by Golli-Bennour et al. (2010) revealed that amiodarone has clear toxic effects on three cell lines including hepatocytes, epithelial cells and renal cells. In these tissues, the toxic effect was highest in the renal and epithelial cells maybe limiting

its use as an anti-arrhythmic drug. (4) Accelerated clearance of hormone. In mammals, one of the best-known mechanisms for inducing thyroid neoplasia is by treating animals with hepatic enzyme inducers. As glucuronidation of thyroxin is a rate-limiting step in biliary excretion of T4 substances, which induce hepatic thyroxin glucuronyltransferase have been reported to increase thyroid neoplasia by a chronic compensatory stimulation of the thyroid gland by pituitary TSH resulting in follicular cell hypertrophy and hyperplasia (McClain 1989).

Another example is deiodination in peripheral tissues, which is responsible for the production of the biologically active hormone T3, but also for the clearance of thyroid hormones by producing rT3 or T2. An example of a substance affecting de-iodination of T4 is found in studies in which rats fed FD&C Red No. 3, (erythrosine), showed lower circulating levels of 3,5,3' triiodothyronine (T3), but increased levels of 3,3',5'-triiodothronine (reverse/inactive T3). An inhibition of 5'-deiodinase, the enzyme involved in deiodination of T4 to 3,5,3'-triiodothyronine, (T3), is proposed as a mode of action (Capen et al. 1989).

(5) Effects on plasma protein binding. Besides certain chemicals affecting thyroid plasma proteins by increasing or decreasing plasma transport protein production, there are substances that can bind to these proteins, thus limiting transport capabilities (Ishihara et al. 2003). Studies with polychlorinated biphenyl ethers have found little evidence for receptor binding, although they interacted with transthyretin and thyroid binding globulin (Cheek et al. 1999).

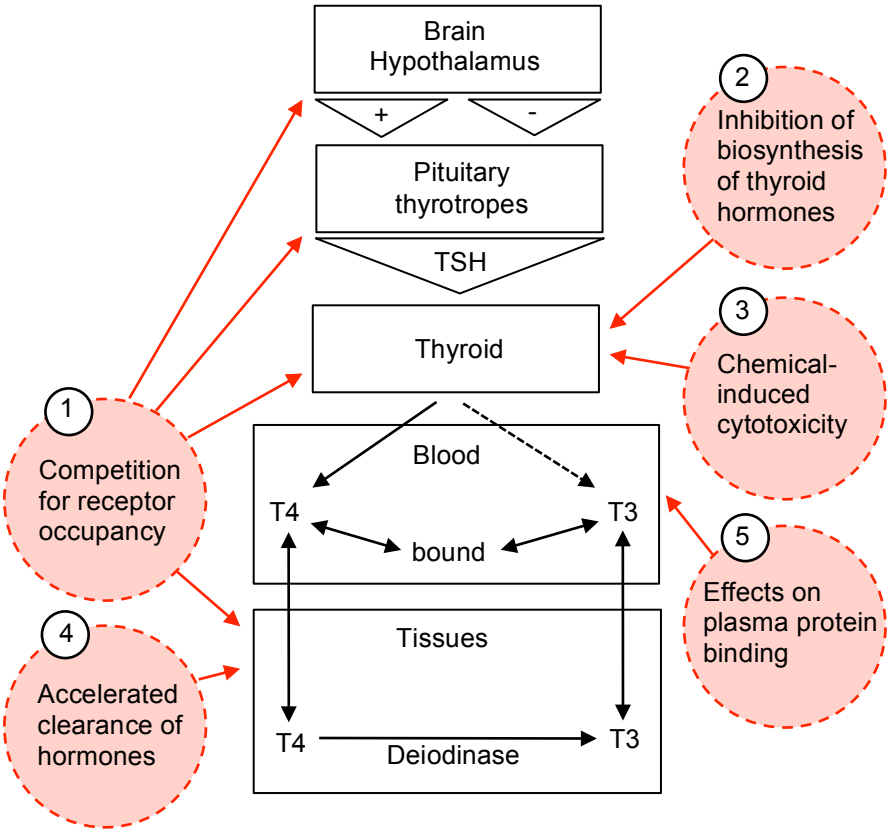

Fig. 1.5: Possible toxic effects of thyroid-disrupting chemicals along the hypothalamic-pituitary-thyroid axis.

1.7 The zebrafish (*Danio rerio*) as a test organism

The test species used for the *in vivo* experiments in this thesis is one of the most popular laboratory animals worldwide and belongs to the family of the Cyprinidae within the order Cypriniformes. Numerous reasons contributed to its worldwide use as laboratory species, e. g. easy handling, fully sequenced genome, well understood developmental patterns, and frequent spawning. Reproduction can easily be induced by offering adequate stimuli in terms of spawning weed. The collected eggs are fully transparent and, thus, well observable and easy to handle. Moreover, numerous official test guidelines (e. g. OECD 203, 210, 215, 229, 230, 234) exist with *Danio rerio* as one of the preferred test organism. For present chemical regulatory purposes, the zebrafish is one of the most important aquatic test organisms, and, thus, its possible usability in screening tests for goitrogens is highly appreciated.

1.8 Outline of the thesis

The substances used in the present thesis are rather characterized by their well-known mode of action than by their environmental relevance. Since very little is known about effects of endocrine-disrupting chemicals in teleost fish, the focus was clearly on elucidating possible effects along the hypothalamic-pituitary-thyroid axis with knocking out specific key steps in thyroid hormone synthesis. For inhibiting thyroid hormone synthesis (1) propylthiouracil was used to block thyroid peroxidases in the follicles, (2) potassium perchlorate was used to competitively inhibit iodide uptake into the thyrocytes, and (3) thyroxin was used as a stimulating substance (positive control). The effects were analyzed along the hypothalamic-pituitary-thyroid axis, and the sensitivities of the different endpoints were compared. In detail, the thyroid was histologically screened for alterations caused by the different substances. Additional ultrastructural studies in the thyroid were conducted to further substantiate the effects.

Since the activity of the thyroid is controlled and regulated by the pituitary, histological and immunohistochemical analyzes were carried out to highlight specific feedback-induced effects in the pituitary and the TSH-producing cells. In

teleost fish, the main site for the conversion of T4 to the biologically active T3 is the liver. This organ was also histologically and ultrastructurally investigated to elucidate effects either feedback-induced or directly caused by the different substances. Furthermore, T4 contents were measured to get precise information on the ability of the fish to compensate the exposure to different goitrogens. The key strategy was to get a complete overview of the fishes' reaction to substances inhibiting or stimulating different steps in thyroid hormone metabolism. Furthermore, overall suitability and sensitivity of the test species *Danio rerio* compared to the well-established model *Xenopus laevis* was conducted to clarify its potential use as screening model for thyroid inhibiting substances. As mentioned above, the zebrafish is one of the favored aquatic test organisms in today´s chemical regulation. Therefore its possible usability for the detection of goitrogens would be very beneficial.

Chapter 2

Alterations along the hypothalamic-pituitary-thyroid axis of the zebrafish (*Danio rerio*) after exposure to propylthiouracil.

2.1 Abstract

In the past, various approaches have been developed to detect adverse effects of pollutants on the thyroid of vertebrates, most of these with special emphasis on the South African clawed frog, *Xenopus laevis*. Although fish are primarily affected by thyroid-disrupting chemicals, studies into alterations of the thyroid of fish are scarce. Therefore, effects of the reference compound propylthiouracil on histopathology of the thyroid axis were analyzed in a modified early life-stage test with zebrafish (*Danio rerio*) exposed to proplythiouracil. The test substance induced dose-dependent alterations of thyroidal tissue concomitant with increases in the number of surrounding blood vessels. Despite this massive proliferation of the thyroid, zebrafish were not able to maintain thyroxin concentrations. The pituitary was affected displaying significant alterations in thyroid-stimulating hormone cell counts. Quantitative evaluation of pituitary surface areas revealed a dose-dependent increase of adenohypophyseal tissue. Distinct histopathological effects may contribute to a more easy identification and interpretation of alterations induced by thyroid-disrupting chemicals.

2.2 Introduction

Over the past 20 years, the potency of endocrine-disrupting chemicals (EDCs) has attracted attention in numerous toxicological and ecotoxicological studies (Bernanke et al. 2009, Blanton et al. 2007, Crisp et al. 1998, Hotchkiss et al. 2008, Kloas et al. 2009, Matthiessen 2003, Scholz et al. 2008, Scholz and Mayer 2008, Vos et al. 2000). So far, the main focus of EDCs was clearly on effects on reproductive biology (Gray et al. 2002, Jobling 1998, Tyler et al. 1998), but the awareness of potential risks by thyroid system-disrupting chemicals is increasing (Brar et al. 2010, Brucker-Davis 1998, Raldua et al. 2009, Rolland 2000, Shi et al. 2009, Zoeller 2003). Therefore, several approaches to detect adverse effects of pollutants on the thyroid system of vertebrates have been developed with special emphasis on the South African clawed frog, *Xenopus laevis* (Degitz et al. 2005, Kloas 2002, Opitz et al. 2005, Opitz et al. 2002) and an OECD guideline has recently been established to detect thyroid system-disrupting chemicals with

amphibians as test model (OECD 2009). On the other hand, although fish form the most versatile and heterogeneous vertebrate group with regard to anatomy, physiology, reproduction, behavior, and ecology (Damstra et al. 2002, Janz 2000, Lagler et al. 1977) – approx. 48 % of all vertebrates are fish, which occupy a great variety of ecological niches (Lagler et al. 1977) – studies on alterations of the thyroid system under the influence of pseudothyroid-acting substances are still scarce. However, in recent years, there is increasing evidence of effects both in the field (Baker et al. 2009, Brar et al. 2010, Iwanowicz et al. 2009, Moccia et al. 1977, 1981, Morgado et al. 2009, Schnitzler et al. 2008) and in the laboratory (Coimbra et al. 2007, Crane et al. 2006, Elsalini and Rohr 2003, Liu et al. 2008, Mukhi et al. 2007, Park et al. 2007, Patino et al. 2003, Picard-Aitken et al. 2007, Shi et al. 2009, van der Ven et al. 2006). So far, only a small selection of these chemicals has been tested under laboratory conditions, mostly with a focus on polychlorinated biphenyls, polyhalogenated aromatic hydrocarbons (Kirubagaran et al. 1989, Ram 1988, Ram et al. 1987), and, more recently, brominated flame retardants (de Wit 2002, Legler et al. 2003). Perchlorates as a group of water-soluble goitrogens have attracted some interest because of their potency to contaminate drinking-water (Buffler et al. 2006).

In many aspects, the thyroid system of fish is similar to the mammalian or the amphibian thyroid system, but there are some differences that have to be taken into account when studying effects of thyroid EDCs in fish. The regulation of thyroidal homeostasis is principally maintained by feedback mechanisms, in which both thyroxin (T4) and triiodothyronine (T3) have negative feedback effects on the release of thyroid-stimulating hormone (TSH) in the pituitary, which is the central control organ of the thyroid system (Yoshiura et al. 1999). Nevertheless, in contrast to mammals, in fish the central control of thyroid hormone is limited to the production and secretion of T4, which is transformed into the biologically active T3 in peripheral tissues, mainly the liver (Eales et al. 1999, Leatherland et al. 1990). Morphologically, the thyroid of fish also differs from the thyroid of higher vertebrates: Instead of a compact organ encapsulated by connective tissue, most fish thyroid follicles are loosely scattered in the gill region along the ventral aorta (Eales 1979, Raine et al. 2001, Wabuke-Bunoti et al. 1983). It has been shown that the thyroid is of endodermal origin. Under the influence of *pax2.1* and *pax8*, its primordium evaginates from the pharyngeal epithelium and adopts a

position near the cardiac outflow tract (Alt et al. 2006a, Elsalini et al. 2003, Wendl et al. 2002). The final localization then depends on the development of the ventral aorta demonstrating the connection between thyroidal tissue and adjacent arteries (Alt et al. 2006a). In mammals, the thyroid primordia fuse with the ultimobranchial body, which differentiates into C-cells during further development (De Felice et al. 2004). In lower vertebrates such as fish and amphibians, but also in birds, thyroid follicle cells do not merge with the ultimobranchial bodies, which are instead located elsewhere in the body (De Felice et al. 2004, Le Douarin et al. 1974, Le Lievre et al. 1975). In zebrafish, the first follicle differentiates around 55 hours post-fertilization (hpf), and T4-production can be revealed at around 72 hpf (Alt et al. 2006b, Elsalini and Rohr 2003). This thyroid follicle formed first corresponds to the most anterior follicle in the adult fish, and new follicles are added more caudally (Alt et al. 2006b). This aspect is important for evaluating histopathological changes in the zebrafish thyroid, since follicle maturity and, thus, size represents major endpoints.

Although the thyroid gland acts as the downstream-located hormone-producing gland, the key organ for regulation of, e.g., growth, development, reproduction, or adaption to environmental challenges along hormonal axes is the pituitary (Kasper et al. 2006). Regulatory pathways of the thyroid system start with the reception of external and internal sensory information reaching the brain and the hypothalamus. In contrast to higher vertebrates, the role of the thyrotropin-releasing hormone in the regulation of TSH release in fish is less well established (Janz 2000). Unlike mammals, teleost fish lack a portal system between the hypothalamus and the pituitary. Instead, there is a direct neuronal connection to endocrine cells through the hypophyseal stalk (Peter et al. 1990). The hypothalamus thus directly innervates the pituitary exerting control through secretion of several hormones – in this case, *via* TSH (Kime 1998). The functional significance of TSH is limited to the regulation of T4 release and iodide uptake by the thyroid follicles (Eales et al. 1999).

Morphologically, the pituitary in teleost fish is divided into two major parts: (1) the neurohypophysis (*pars nervosa*; PN), which folds down from the diencephalon and (2) the adenohypophysis, which pouches up from the roof of the oral cavity (Weltzien et al. 2004). During development, the neurohypophysis interdigitates

with the adenohypophysis, which on its part can be subdivided into (1) the *pars distalis* (PD), which can further be divided into the rostral *pars distalis* (RPD) and the proximal *pars distalis* (PPD) and (2) the *pars intermedia* (PI; for further details, see Fig. 2.1). Multiple studies have documented the principal distribution of adenohypophyseal cells in fish (Garcia Ayala et al. 2003, Kasper et al. 2006, Leunissen et al. 1982, Quesada et al. 1988, Ueda et al. 1983) and amphibians (Garcia-Navarro et al. 1988, Miranda et al. 1996a, Ogawa et al. 1995); the impact of thyroid-disrupting chemicals, however, has not been investigated so far.

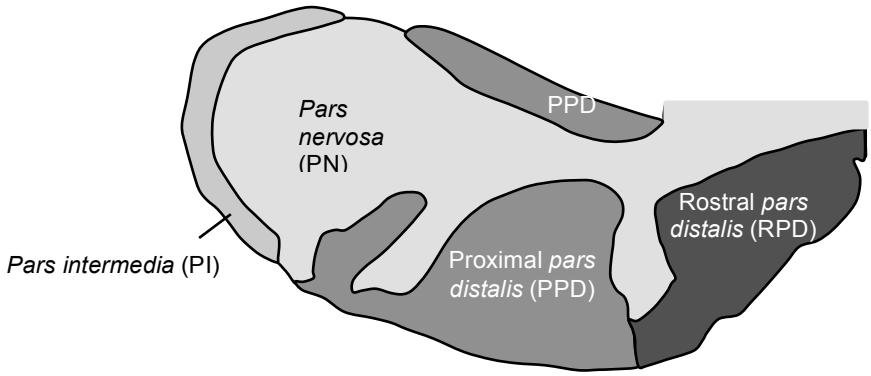

Fig. 2.1: Sagittal view of zebrafish (*Danio rerio*) pituitary.

The anti-thyroid drug, propylthiouracil (PTU) – a representative of the thionamide group – which contains a sulfhydryl group and a thiourea moiety within a heterocyclic structure, has been selected for its well-known mode-of-action on thyroid peroxidases. It inhibits the production of thyroid hormones by interfering with thyroid-peroxidase mediated iodination of tyrosine residues in thyroglobulin (Cooper 2005). In contrast to mammals, PTU has no effect on fish deiodinases (D1, D2 and D3) (Orozco et al. 2002, Orozco et al. 2003, Sanders et al. 1997, 1999, Valverde et al. 1997). In medical treatment, it is used for half a century to manage hyperthyroidism, especially Grave´s disease (Cooper 2005, Cooper et al. 2009, Momotani et al. 2000). The thyroid system-disrupting effects of PTU observed in mammals have also been shown in other vertebrate groups, especially amphibians (Grim et al. 2009, OECD 2009, Opitz et al. 2005, Opitz et al. 2006,

Opitz et al. 2009). Effects of PTU on fish, however, have hardly been documented (van der Ven et al. 2006).

The present study was designed to identify histological and ultrastructural changes in selected zebrafish organs along the hypothalamic-pituitary-thyroid axis. Thus, histological endpoints in the thyroid, i. e. distribution of the single follicles, hyperplasia, homogeneity of the follicles, colloid composition, hypertrophy, histological, and immunohistochemical identification of TSH-producing cells in the pituitary along with quantitative morphometrical evaluations, as well as histological and ultrastructural changes in the liver, as one of the main sites of deiodination, were examined.

2.3 Material and Methods

2.3.1 Animals and husbandry

Fertilized eggs from zebrafish (*Danio rerio*) were obtained from in-house breeding facilities of the Aquatic Ecology and Toxicology Group at the Center for Organismal Studies, University of Heidelberg. All experiments were conducted in compliance with the institutional guidelines for the care and use of animals as well as with permission by the regional animal welfare (AZ 35-9185.81/G-144/07). The exposure experiment involved aqueous exposure of 60 *Danio rerio* larvae for 5 weeks in two replicates. Fertilized eggs were initially raised in 20 cm Petri dishes in a KB 115 incubator (Binder, Tuttlingen, Germany) at a constant temperature of 27.0 ± 1.0 °C, which had been pre-exposed to 0, 2.5, 10, 25, and 50 mg/L 6-proyl-2-thiouracil (Sigma, Deisenhofen, Germany) for saturation. Three days after fertilization, the eggs were transferred into the 10 L flow-through exposure facilities (triplicate water change/d, 27.0 ± 1.0 °C, 12:12h light:dark cycle; oxygen saturation > 80 %) containing the same PTU-concentrations. Flow-through conditions guaranteed that ammonia, nitrite, and nitrate were kept below detection limits (0 - 5, 0.025 - 1 and 0 - 140 mg/L, respectively). After hatching, embryos were fed daily with Sera Micron (Sera, Heinsberg, Germany) and after one week with freshly raised *Artemia* nauplii (Sanders, USA) *ad libitum*. Excessive food and feces was removed from the aquaria at least twice daily.

2.3.2 Histology

After 5 weeks, each of the 60 fish per concentration group was anesthetized with a saturated solution of 4-ethylaminobenzoate (benzocaine, Sigma). Whole body length and weight were measured immediately after anesthetization. For histology, thirty fish were fixed in Davidson‘s fixative (Romeis 1989) for a minimum of 24 hrs at 4 °C. Whole fish were processed in a Leica TP 1020 Tissue Processor (Leica Microsystems, Wetzlar, Germany), embedded in Histoplast S (Serva, Heidelberg, Germany), and sectioned in horizontal and median planes at 2 µm thickness, respectively. For details on embedding see Table 2.1. Serial sections of the thyroid, the pituitary, and liver region were mounted on glass slides covered with an albumen-glycerin solution (Serva), stained with PAS (Romeis 1989); nuclei were counterstained with hematoxylin, and coverslipped with X-TRA Kitt (Medite, Burgdorf, Germany).

Table 2.1: Details of dehydration and embedding of zebrafish tissue.

Dehydration	Duration
80 % Ethanol	1 h
90 % Ethanol	1 h
90 % Ethanol	1 h
96 % Ethanol	1 h
96 % Ethanol	1 h
100 % Isopropanol	1 h
100 % Isopropanol	1 h
Xylene	1 h
Xylene	12 h
Xylene	4 h
Histoplast S	12 h
Histoplast S	12 h

2.3.3 Pituitary immunohistochemistry

For immunohistochemistry, the Vectastain ABC Kit for the detection of TSH (Vector Laboratories, Burlingame, USA) was used in the control and the highest concentration group, respectively. During this procedure, antigens were unmasked by heating the slides to 96 °C in 0.01 M citrate buffer (pH 6.0) followed by incubation in 1% H_2O_2 in phosphate-buffered saline (PBS, pH 7.4). Unspecific binding was reduced by double blocking sections with (1) 2% bovine serum albumin in PBS for 30 min at room temperature and (2) the blocking serum delivered by Vector Laboratories. Afterwards, sections were incubated overnight at 4 °C with a rabbit anti-human TSH antibody (AbD Serotec, Oxford, UK; cat. no. 8926-0004) diluted 1:250 in PBS. After three rinses in PBS, the antiserum was tagged with the biotinylated Vectastain secondary antibody and then incubated in a preformed avidin and biotinylated horseradish peroxidase complex. To visualize the antiserum, sections were incubated in 3,3‘-diaminobenzidine (Vector Laboratories) until desired staining intensities had developed. According to the manufacturer, the antiserum reacts with fish. The specificity had been shown previously by Grandi and Chicca (2004) in *Acipenser naccarii* and by Kasper et al. (2006) in *Oreochromis niloticus*. For instance, no immunostaining was observed after pre-incubation of the anti-human β-TSH antisera with an excess of the appropriate antigens (Grandi and Chicca 2004). Sections were slightly counterstained with Mayer´s hematoxylin (Romeis 1989), rehydrated and mounted for observation.

2.3.4 Ultrastructure

For ultrastructural studies, liver samples were fixed in 2.5 % glutardialdehyde in sodium cacodylate buffer (pH 7.4) at 4 °C for a minimum of 24 hrs and post-fixed with 1 % osmium ferrocyanide for two hours (Karnovsky 1971). After triplicate rinsing in sodium cacodylate buffer (pH 7.4), tissues were stained *en bloc* with 1 % uranyl acetate in maleic buffer (pH 5.2) overnight at 4 °C. The liver was dehydrated in a graded series of ethanol and embedded in Spurr´s medium (Spurr 1969). For localization of correct sectioning areas, semi-thin sections were cut on a Reichert-Jung Ultracut microtome (Leica Microsystems) and stained with

methylene blue / Azur II (Richardson et al. 1960). Afterwards, ultrathin sections of 60 - 80 nm were cut and counterstained with alkaline lead citrate (Reynolds 1963).

2.3.5 Imaging

For both histology and immunohistochemistry, light microscopy was performed with a Leitz Aristoplan microscope (Leitz, Wetzlar, Germany) equipped with a ColorView Soft Imaging Systems digital camera (Soft Imaging Systems, Münster, Germany). The surface areas of adeno-, neurohypophysis, and total pituitary were measured and the number of TSH-producing cells counted using the free software tool ImageJ 1.44 (National Institutes of Health, USA) to quantify the observed alterations. Ultrathin sections of the liver were examined in a Zeiss EM 10C (Carl Zeiss, Oberkochen Germany) transmission electron microscope.

2.3.6 Thyroid hormone extraction and ELISA

The methods for methanol-extraction of whole body THs were adopted from Shi et al. (2009) in zebrafish. Three zebrafish samples from each concentration group were homogenized in 0.5 ml ice-cold methanol with 1 mM PTU. The homogenates were dispersed by intermittent sonic oscillation for 5 min on ice and vortexed for 10 min. After centrifugation at 3,500 g at 4 °C for 20 min, the supernatants were collected, and the pellets were re-extracted with 0.5 ml ice-cold methanol/PTU and centrifuged again. The freshly collected supernatant was combined with the original supernatant and vacuum-dried overnight at room temperature. The samples were re-dissolved in 0.05 ml methanol, 0.2 ml chloroform, and 0.05 ml 0.11 M barbital buffer (pH 8.6; Sigma). The mixture was vortexed for 3 min and centrifuged at 3,500 g at 4 °C for 15 min. The upper layer was carefully collected and immediately used for the T4-measurements. The ELISA was performed with commercial kits (Diagnostic Automation/Cortez Diagnostics Inc., Calabasas, USA) according to the manufacturer‘s instructions.

2.3.7 Data Analysis

The non-parametric Kruskal-Wallis test was used to determine differences in whole body weight and length, T4-content, and the surface areas of the pituitary. 60 fish of each concentration group were used to determine whole body length, weight, and the surface areas of the pituitary. T4 content was measured in three animals per concentration group. Dunn‘s multiple comparison test was used for pairwise comparisons with the control group. For analyzing TSH-producing cell number the Mann-Whitney test was used to compare 5 individuals from the highest concentration group to 5 individuals from the control. All statistical analyses were performed using the software package GraphPad Prism 4.0a for Macintosh (GraphPad Software, Inc., La Jolla, USA). Differences were considered significant at $p < 0.05$ (*), highly significant at $p < 0.01$ (**), and highest significant at $p < 0.001$ (***).

2.4 Results

2.4.1 Whole body weight and whole body length

Both whole body weight and length showed a biphasic response pattern to PTU exposure (Fig. 2.2). If compared to the control group, a statistically significant decrease in whole body length was observed in fish exposed to 50 mg/L PTU. In contrast, mean values for whole body length from zebrafish treated with 2.5 and 10 mg/L were slightly higher than in control fish, with 25 mg/L reaching the control level again. Nevertheless, none of the observed increases in the lower concentrations showed any statistical significance. Whole body weight revealed a similar pattern with an increase at the low concentrations and a slight decrease at the highest concentration. However, in contrast to whole body length, a statistically significant increase in fish exposed to 2.5 mg/L PTU was observed, whereas the highest concentration only showed a slight, but non-significant decrease.

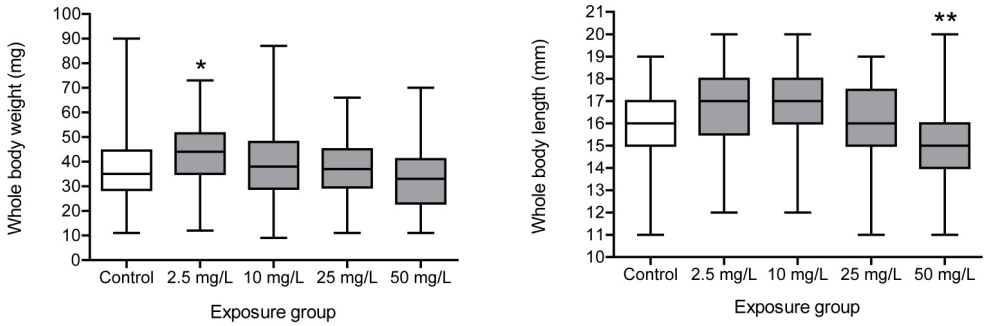

Fig. 2.2: Whole body weight and length of zebrafish (*Danio rerio*) after exposure to 0, 2.5, 10, 25, and 50 mg/L PTU. Measurements were performed with 60 animals after 35 days of exposure. Asterisks indicate significant differences between exposure and control groups (* $p < 0.05$, ** $p < 0.01$; Dunn's test).

2.4.2 T4 contents

ELISA measurements of T4 contents revealed a dose-dependent decrease of T4 with a significant reduction in the 50 mg/L concentration group (Fig. 2.3). Probably due to the small sample size of only three fish per treatment group, the 50 mg/L group was the only significant group compared to the control despite the dose-response curve.

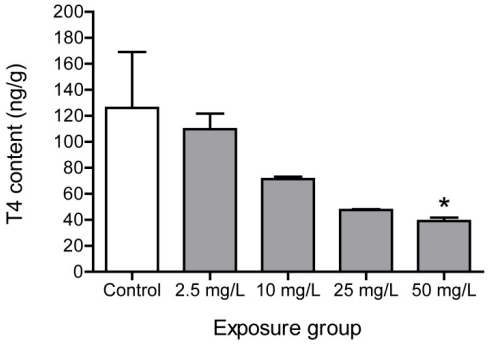

Fig. 2.3: Whole body contents of T4 in zebrafish (*Danio rerio*) exposed to 0, 2.5, 10, 25, and 50 mg/L PTU. Results are given as means ± SEM from three samples per replicate. Asterisks indicate significant differences between exposure and control groups (* $p < 0.05$; Dunn's test).

2.4.3 Macroscopical effects

From 10 mg/L PTU, clear macroscopical effects could be observed. The opercular region showed increasing red coloration due to massive blood aggregation. Furthermore, fish exposed to concentrations ≥ 25 mg/L displayed obvious goiter formations in the midline of the lower jaw region. Concomitant to the blood aggregation in the operculum region, the goiters displayed a clear red coloration resulting from massive blood aggregations (Fig. 2.4).

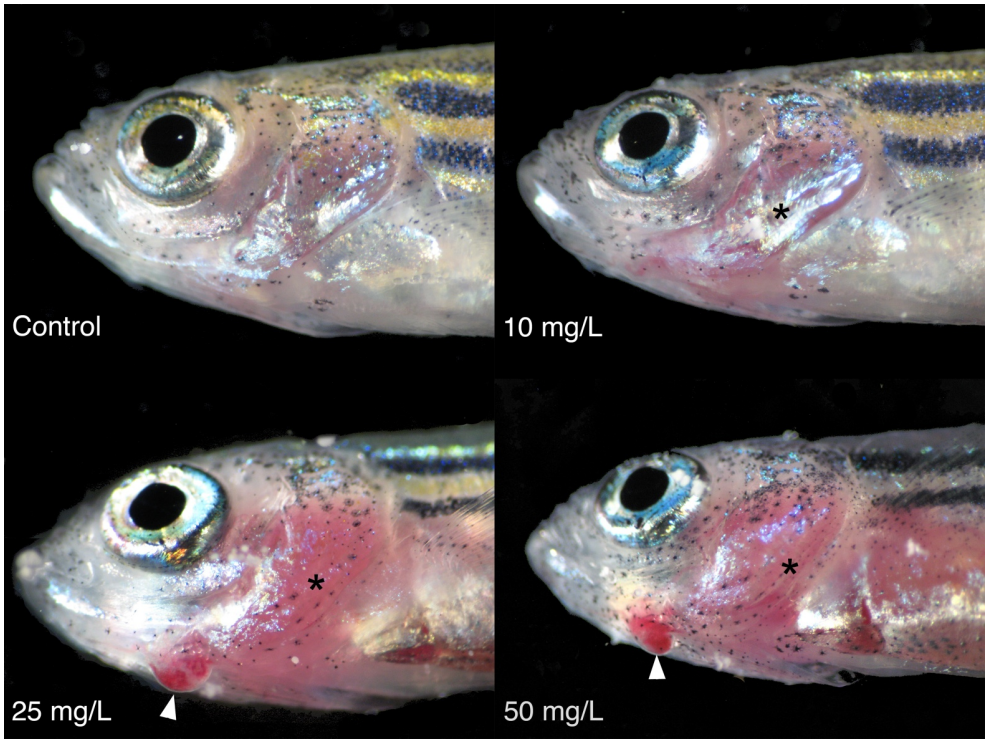

Fig. 2.4: Macroscopical pictures of the head region of zebrafish (*Danio rerio*) following exposure to 0, 10, 25, and 50 mg/L PTU. Increased red coloration due to blood aggregation in the opercular region (*) at concentrations ≥ 10 mg/L and goiter formation (►) at concentrations ≥ 25 mg/L are clearly detectable.

2.4.4 Histological alterations in the thyroid

PTU-related effects in the thyroid are summarized in Table 2.2. Light microscopical examination of thyroidal tissue exposed to PTU revealed complex dose-dependent effects. Whereas in the control group the spherically to ovally shaped thyroid follicles, consisting of cuboid to flat epithelia surrounding a homogeneously stained colloid, were loosely distributed in the connective tissue adjacent to the ventral aorta and its final rostral branching in the gill region (Fig. 2.5a, b), the PTU-exposed groups showed conspicuous deteriorations (Fig. 2.6).

Table 2.2: Semiquantitative evaluation of alterations in the thyroid of zebrafish (*Danio rerio*) induced by PTU.

PTU [mg/L]	Control	2.5	10	25	50
Follicles					
Total number		+	++	+++	+++
Size		+	+	+++	+++
Shape			++	+++	++++
Blood vessels					
Hyperemia			++	+++	++++
Colloid					
Reduced homogeneity			+	+++	++
Reduced density			+	+++	++
Colloid depletion	+	+	+	+	+
Foamy texture				+	+
Cellular inclusions				++	++
Epithelial cells					
Cell height			++	+++	+++
Stratification				++	+++
Cell crowding			+	++	+++

Data are given as means of observation in 20 individuals per exposure group: + = little developed; ++ = moderately developed; +++ = strongly developed; ++++ = very strongly developed

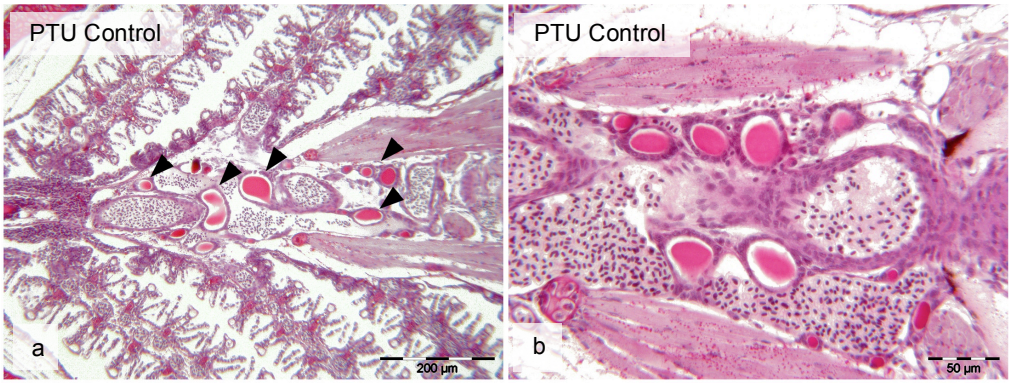

Fig. 2.5: Histological pictures of thyroidal tissue in the control zebrafish (*Danio rerio*). The isolated thyroid follicles are distributed adjacent to the ventral aorta in the gill region. Follicular shape is spherical to oval with a cuboidal epithelium and a homogeneously stained colloid. Sections of 2 µm thickness stained with periodic-acid Schiff (PAS) and Mayer‘s hematoxylin.

2.4.4.1 Follicles

From the lowest PTU concentration, increasing numbers of thyroid follicles and, to lower extent, proliferations of follicle size were detectable (Fig. 2.6a). Both effects were strongest in concentration groups ≥ 25 mg/L, but could already be observed at 2.5 mg/L with a dose-dependent increase. Following exposure to concentrations ≥ 2.5 mg/L PTU, follicle shape changed, with an increase in the numbers of papillary in- and outfoldings. In terms of histology, the above-mentioned goiter observed at concentrations ≥ 25 mg/L PTU consisted of numerous small follicles and connective tissues embedded in an enlarged capillary network responsible for the red coloration already observed macroscopically. Although there were hardly any goiters visible at 10 mg/L, histologically detectable goitrous tissue was detectable from 10 mg/L PTU. The most conspicuous structural modification was the appearance of follicles in the gills, which was detectable at concentrations ≥ 10 mg/L (Fig. 2.6f). In contrast, at 2.5 mg/L, follicles were randomly distributed, but restricted to the pharyngeal region.

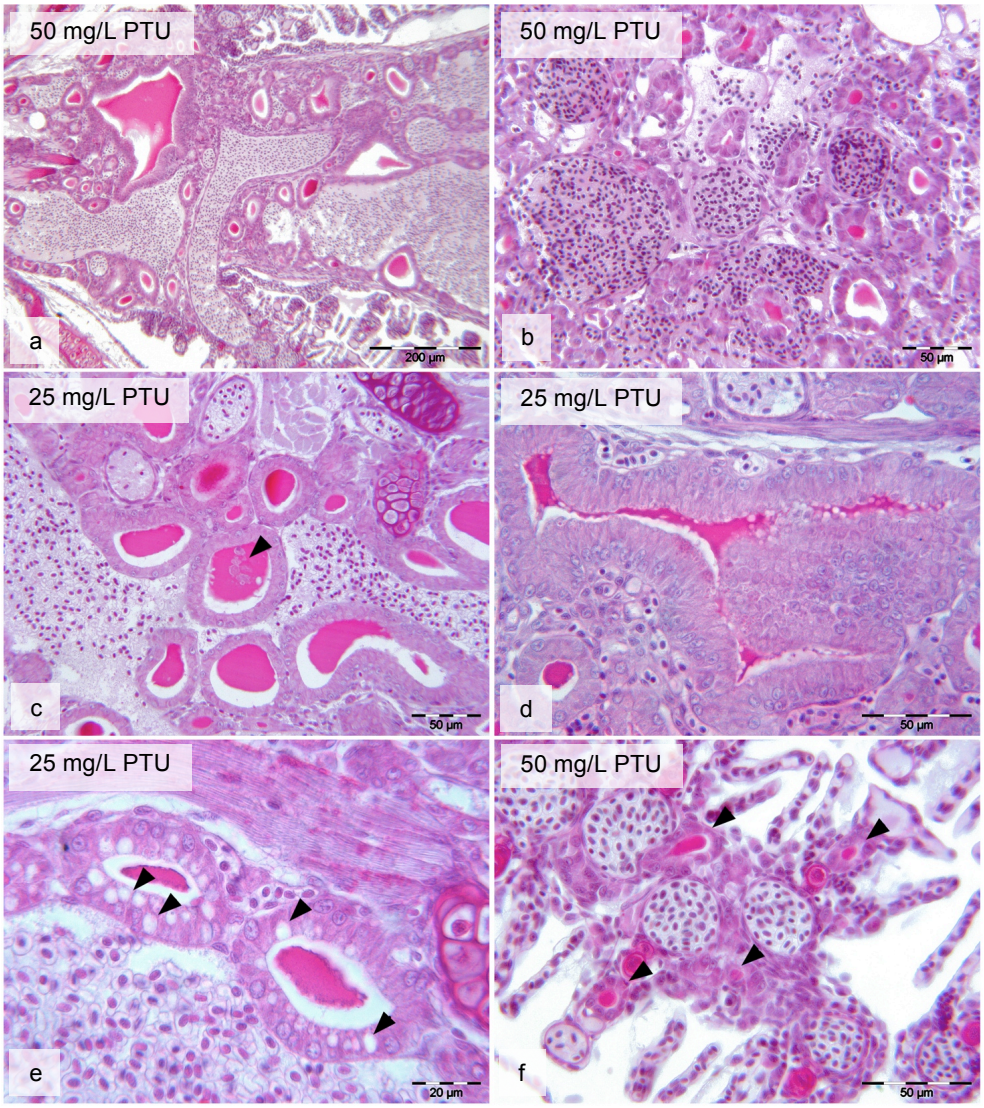

Fig. 2.6: Histopathological effects in thyroidal tissue of zebrafish (*Danio rerio*) exposed to 25 and 50 mg/L PTU. Exposure to PTU resulted in massive hyperplasia and hyperemia with an increased number of small follicles embedded in adjacent blood capillaries (Figs. a, b). In the 25 and 50 mg/L concentration groups, inclusion bodies in the colloid were visible (Fig. c: ►). At concentrations ≥ 10 mg/L, severe hypertrophy of thyroid follicles could be detected with stratification and cell crowding (Fig. d). One individual in each of the 25 and 50 mg/L concentration groups showed mostly apically located vesicles in the epithelial cells (Fig. e: ►). Some individuals displayed small follicles scattered across the gills (f: ►). Sections of 2 µm thickness stained with periodic-acid Schiff (PAS) and Mayer‘s hematoxylin.

2.4.4.2 Architecture of the blood vessel supply

One of the most striking effects was the architecture of the blood vessels surrounding the thyroid follicles. From concentrations ≥ 2.5 mg/L, a concentration-dependent proliferation of the adjacent blood vessels was evident with massive hyperemia at ≥ 25 mg/L (Fig. 2.6a, b). These observations are in line with the macroscopically detected coloration of the entire opercular region.

2.4.4.3 Alterations in colloid appearance

Whereas colloid homogeneity and colloid density/contents were only slightly modified in single individuals at the lowest concentration, there were clear-cut alterations in exposure groups ≥ 10 mg/L: In contrast to the controls and the lowest concentration, PAS-staining revealed a blotchy and foamy texture with heterogeneous tinctorial properties. Throughout all experimental groups, colloid depletion could be detected; however, not in a dose-dependent manner. From 10 mg/L, an increased number of cellular inclusions was visible in the colloid of several individuals (Fig. 2.6c). Shrinking phenomena were evident in all experimental groups and should not be misinterpreted as effects by PTU.

2.4.4.4 Alterations of epithelial cells

As in controls, epithelial cells in individuals exposed to 2.5 mg/L PTU displayed a cuboid to flat appearance with only few exceptions showing a slight increase in cell height resulting in a moderately columnar appearance. At concentrations ≥ 10 mg/L, epithelial cell height consistently increased to a columnar cell shape with stratification and cell-crowding (Fig. 2.6d). The nucleus, centrally located in cuboidal cells, migrated towards the basal part of the columnar-shaped thyrocytes. At 25 mg/L and 50 mg/L PTU, an increasing number of individuals showed large vesicles in the apical part of the cell (Fig. 2.6e).

2.4.4.5 Histological and immunohistochemical alterations in the pituitary

On consecutive PAS-stained control sections, a clear separation of the neurohypophysis from the adenohypophysis was visible with the neurohypophysis slightly digitating into the adenohypophysis (Fig. 2.7a). With respect to overall dimensions of the pituitary on longitudinal sections across the pituitary, only the 25 mg/L PTU concentration group revealed a statistically significant increase of pituitary surface area in consequence of PTU exposure (Fig. 2.8). As a member of

the glycoprotein family, TSH, together with FSH and LH, reacted PAS-positive and could be detected in the proximal *pars distalis* along the border to the *pars nervosa*, which digitated into the adenohypophysis. Although differentiation of TSH-producing cells from FSH- and LH-producing cells was not possible with PAS-staining, profound morphological changes in consequence of exposure to PTU could already be detected in PAS-stained sections. Especially at 25 and 50 mg/L PTU exposure, a clear proliferation of adenohypophyseal tissue was visible (Fig. 2.7b). Morphometrical analysis revealed a significant increase in adenohypophyseal tissue in the two highest concentration groups (Fig. 2.8). This proliferation mostly occurred in the proximal *pars distalis* of the adenohypophysis (Fig 2.7b). Thus, given the similar pituitary volumes of control and exposed fish, the ratio between adeno- and neurohypophysis had changed (Fig. 2.9). PTU led to a clear dose-dependent increase of this ratio with significant changes from 10 mg/L onwards. Surface area measurements of the neurohypophysis did not reveal any significant proliferations (Fig. 2.8).

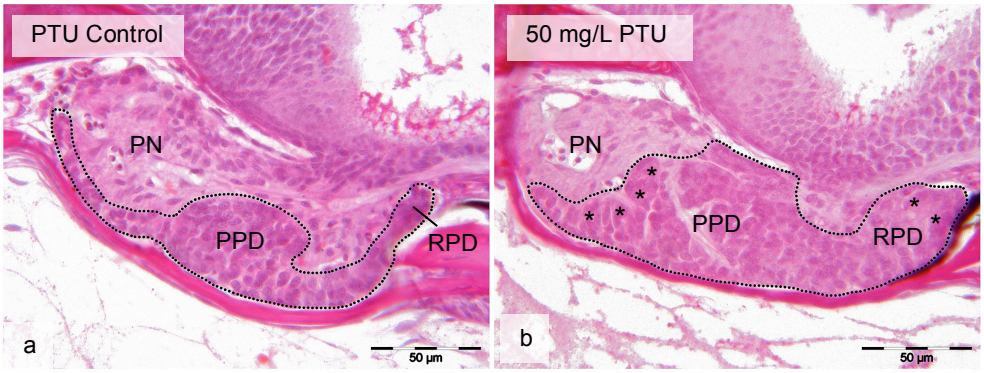

Fig. 2.7: PAS-stained sagittal sections of zebrafish (*Danio rerio*) pituitaries exposed to PTU. The adenohypophysis is encircled by a dotted line to highlight the changes in the ratio between adeno- and neurohypophysis. A massive proliferation of PAS-positive cells (*) mostly located in the proximal *pars distalis* is evident. PI – *pars intermedia* (adenohypophysis); PN – *pars nervosa* (neurohypophysis); PPD – proximal *pars distalis* (adenohypophysis); RPD – rostral *pars distalis* (adenohypophysis). Sections of 2 µm thickness stained with periodic-acid Schiff (PAS) and Mayer's hematoxylin.

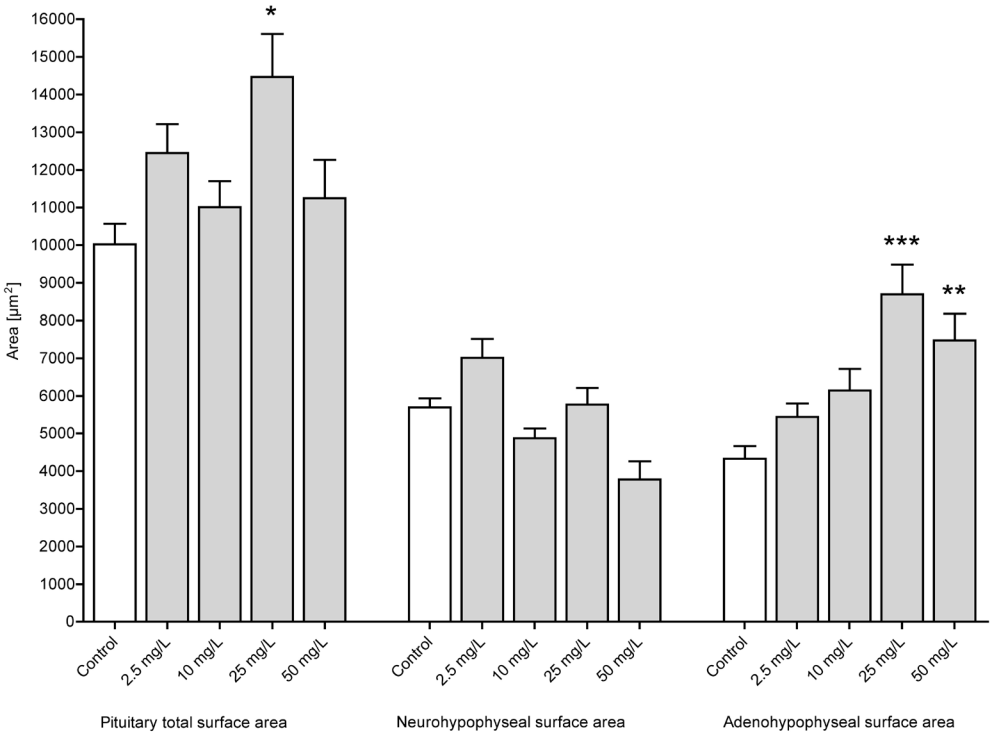

Fig. 2.8: Pituitary surface areas of zebrafish (*Danio rerio*) after exposure to 0, 2.5, 10, 25, and 50 mg/L PTU. Measurements were performed with 60 animals after 35 days of exposure. Asterisks indicate significant differences between exposure and control groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Dunn's test).

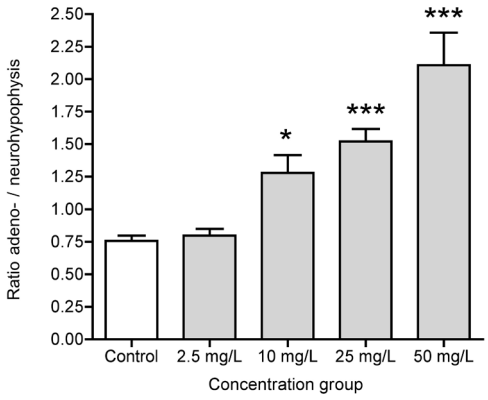

Fig. 2.9: Ratio of adeno- to neurohypophyseal tissue of zebrafish (*Danio rerio*) after exposure to 0, 2.5, 10, 25, and 50 mg/L PTU. Measurements were performed with 60 animals after 35 days of exposure. Asterisks indicate significant differences between exposure and control groups (* $p < 0.05$, *** $p < 0.001$; Dunn's test).

Immunohistochemical staining with anti-TSH antibodies revealed a rather homogeneous distribution of TSH-producing cells (Fig. 2.10). In the control group, TSH-positive cells were mainly limited to the rostral *pars distalis* and the *pars intermedia* with a few cells located in the proximal *pars distalis* (Figs. 2.10a). This situation changed after exposure to 50 mg/L PTU (Fig. 2.10b): The most striking proliferation occurred in the proximal *pars distalis*, where cells located at the border of the proximal *pars distalis* to the *pars nervosa* proliferated. This observation is in line with the aforementioned proliferation of adenohypophyseal tissue. Quantification of TSH-producing cell counts in the control and the highest concentration group led to a statistically significant increase after exposure to PTU (Fig. 2.11).

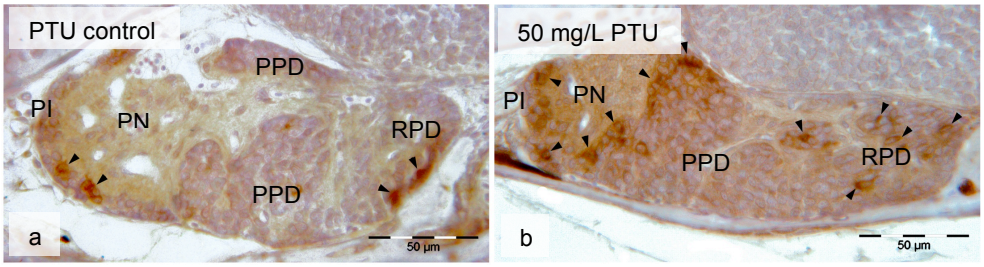

Fig. 2.10: Sagittal sections of immunohistochemically stained zebrafish (*Danio rerio*) pituitaries exposed to 0 and 50 mg/L PTU. The samples show clear proliferations of TSH-producing cells (►) especially in the proximal *pars distalis*. Sections of 2 µm thickness immunostained with an anti-TSH antibody (nuclei were counterstained with Mayer's hematoxylin). PI – *pars intermedia* (adenohypophysis); PN – *pars nervosa* (neurohypophysis); PPD – proximal *pars distalis* (adenohypophysis); RPD – rostral *pars distalis* (adenohypophysis).

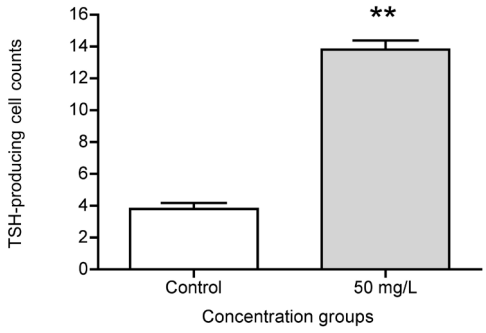

Fig. 2.11: TSH-producing cell counts of zebrafish (*Danio rerio*) pituitaries after exposure to 0 and 50 mg/L PTU. Statistical analysis was conducted on five immunostained sections respectively (** $p < 0.01$; Mann-Whitney test).

2.4.4.6 Further histological and ultrastructural alterations in the liver

PTU-related effects in the liver are summarized in Table 2.3. Histologically, these alterations by PTU were restricted to a moderate depletion of glycogen deposits, which was concentration-dependent up to the highest concentration group of 50 mg/L (Fig. 2.12). Interestingly, at concentrations ≥ 25 mg/L the numbers of mitochondria decreased. Following exposure to PTU, the rough endoplasmic reticulum of zebrafish underwent a moderate reduction and displayed some fenestration (Fig. 2.13).

Table 2.3: Semiquantitative evaluation of alterations in the liver of zebrafish (*Danio rerio*) induced by PTU.

PTU [mg/L]	Control	2.5	10	25	50
Glycogen					
Glycogen depletion	+	+	++	++	++
Mitochondria					
Decrease in number			+	++	++
Rough endoplasmic reticulum					
Fenestration			+	++	++
Reduction in amount			+	++	++

Data are given as means of observation in 10 individuals per exposure group: + = little developed; ++ = moderately developed; +++ = strongly developed; ++++ = very strongly developed

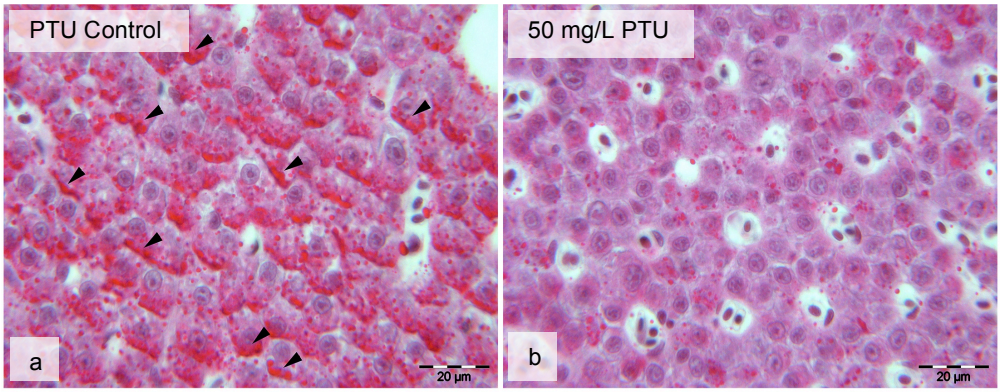

Fig. 2.12: Histopathological alterations in hepatocytes of control (a) and PTU-exposed zebrafish (b). The well-visible glycogen deposits in the control (a: ►) are depleted in the higher concentration groups. Sections of 2 µm thickness stained with periodic-acid Schiff (PAS) and Mayer's hematoxylin.

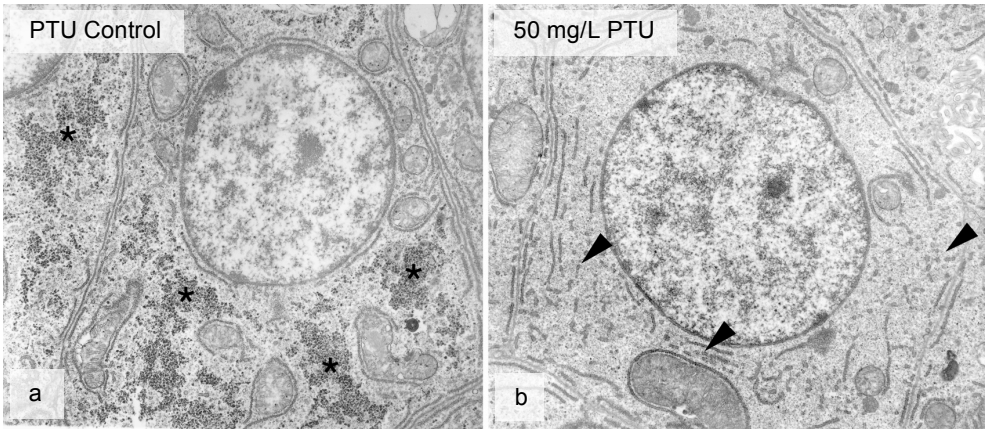

Fig. 2.13: Ultrastructural alterations in hepatocytes of control (a) and PTU-exposed zebrafish (b). In the control fish, glycogen storage deposits are clearly visible (a: *), whereas the exposed fish hardly display any glycogen in the cytoplasm. Moderate reductions and fenestrations of the rough endoplasmic reticulum are observable in PTU-exposed zebrafish (b: ►). Furthermore, the number of mitochondria is decreased. Magnification: 10,000x.

2.5 Discussion

In the present study, histological, immunohistochemical, and ultrastructural alterations of the thyroid, the pituitary, and the liver in the zebrafish by the potent anti-thyroid drug PTU were evaluated in order (1) to reveal reactions of the thyroid system to potential endocrine-disrupting chemicals, (2) to determine the sensitivity of the zebrafish thyroid system to endocrine-disrupting chemicals, and (3) to investigate changes in the pituitary as the main control organ of the endocrine system.

The evaluation of suitable endpoints is highly linked with the understanding of the underlying regulating endocrinological mechanisms and the mode of action of the test substance. For the interpretation of effects by thyroid system-disrupting chemicals, it is indispensible to understand the different endpoints, since these endpoints might also provide indications as to the underlying mode of action. Out of the numerous possibilities for disruption along the hypothalamic-pituitary-thyroid axis, PTU inhibits thyroid hormone synthesis by interfering with thyroid peroxidase-mediated iodination of tyrosine residues in thyroglobulin (Cooper 2005). In teleost fish, the hypothalamic-pituitary-thyroid axis is regulated by feedback mechanisms, but the critical step in the synthesis of the biologically active hormone T3 is located in peripheral tissues, mainly the liver. This aspect has to be taken into account, when judging thyroid system-disrupting chemicals.

Gross morphology of zebrafish exposed to PTU revealed striking effects in the thyroid and associated tissues with massive effects in the supporting blood vessels. The proliferation observed for adjacent blood vessels led to severe hyperemia at concentrations $\geq$ 25 mg/L. This effect was most prominent in goitrous tissue, where the connection between follicles and supporting blood vessels was highly evident. Connors et al. (1988, 1991) reported correlations between TSH-concentration and thyroid gland blood flow in rats. Although TSH-concentrations were not measured in the present study, immunohistological staining of TSH-producing cells in pituitaries of the highest concentration group revealed a significant increase of these cells indicating an elevation of TSH concentrations. As a consequence, the effects observed in the present study would be caused by

elevated TSH concentrations caused by the negative feedback in the hypothalamic-pituitary-thyroid axis due to the exposure to PTU.

Hyperplasia and hypertrophy are two alternatives to accomplish thyroid activation in various species (Goleman et al. 2002b, Grim et al. 2009, Miranda et al. 1996a, Yamasaki et al. 2002). In this study, hyperplasia of thyroidal tissue was most sensitive and most prominent from the lowest PTU concentration. In contrast, thyrocyte hypertrophy was only detectable at concentrations ≥ 10 mg/L. Thus, at least in juvenile fish, the first step to up-regulate TH-production *via* TSH seems to be an increase in cell number (hyperplasia) rather than an increase in cell size (hypertrophy). TSH-production is under the negative feedback influence of both T4 and T3 (Pradet-Balade et al. 1999, Yoshiura et al. 1999). The activating effect of TSH is mediated *via* a G-protein-coupled TSH-receptor (Farid et al. 2004), which is mainly expressed in thyroidal tissue and the gonads (MacKenzie et al. 2009). Nevertheless, the factors that trigger hyperplasia remain unclear and need further exploration. The proliferation of the follicles was most prominent at concentrations ≥ 25 mg/L, where several small follicles could be detected in the gills. Limited space in the central pharyngeal region and increasing demand for THs could lead to an invasion of thyroid tissues into the gills. Together with this invasion, a proximal swelling of the supporting blood vessels was detected. Although gill tissues seemed not to be effected *per se*, a displacement was obvious, leading to the question whether reduced functionality of gill tissues could lead to any respiratory consequences for the fish. In the present study, no signs of respiratory problems could be detected, but oxygen concentration in the tanks was kept near saturation throughout the experiment. There are studies demonstrating that embryonic fish are capable of surviving oxygen rates of 5 % when acclimated to none-lethal oxygen concentrations (Rees et al. 2001, Strecker et al. 2011) showing numerous adaptions, e. g. metabolic rate reduction or increased ventilation rates, hematocrit and hemoglobin oxygen affinity, which was detected in common sole (*Solea solea*; Dalla Via et al. 1994, Rankin et al. 1993).

A very interesting endpoint was the observation of changes in the quality of the colloid. As the main site of TH synthesis and storage, it was likely to detect alterations especially with PTU inhibiting thyroid peroxidases in the colloid itself. In the present study, PAS-staining was used because of the special staining

properties of the colloid, although standard HE-staining is adequate to detect alterations in the colloid as documented in other publications (Crane et al. 2005, Degitz et al. 2005, Patino et al. 2003). In contrast to studies with perchlorate (Crane et al. 2005, Mukhi and Patino 2007, Patino et al. 2003), colloid depletion could not be identified as a significant PTU-induced effect. Partially or totally depleted follicles were present in each exposure group including the control, although complete depletion could never be observed in this study. Apparently, the different inhibiting mechanisms of perchlorate and PTU lead to distinct histological effects. Nevertheless, concentrations ≥ 10 mg/L led to clear-cut alterations in colloid homogeneity and density, and from 25 mg/L a foamy, granular texture was well visible in contrast to the homogenous, smooth texture in controls. Studies from Anderberg et al. (1980, 1981) on human thyroids revealed that the colloid is composed of 19S thyroglobulin, larger iodoproteins and smaller protein fractions (an albumin-like protein and a pre-albumin fraction). In these studies, exposure to carbimazole, which belongs to the same group of chemicals as PTU, leads to a decrease of the larger thyroglobulin aggregates compared with the relative amount observed in the colloid from normal human thyroid tissue. This decrease was explained with an insufficient capacity to iodinate thyroglobulin. Such assumptions could provide an explanation for the different staining properties and the granular texture observed in the exposure groups. Opitz et al. (2006) described peripheral vacuolation in ethylenethiourea-exposed *Xenopus laevis* tadpoles as a sign for activated follicles. This effect was hardly visible in the present study; however, at concentrations ≥ 25 mg/L cellular inclusions probably due to cellular blebbing were detectable, mostly inside the colloid, but some at the periphery as well. A study from Pitsiavas et al. (1997) on amiodarone-induced ultrastructural changes in rat thyroids reports on inclusion bodies found in the thyreocytes as well as in the colloid. Allen (Allen 1992) described similar effects on rats after exposure to excess iodine. Although explanations are still scarce, the observed effects could reflect ongoing cytotoxic processes induced by the test substance. In the present study, no inclusion bodies were found in the thyrocytes but only in the colloid. For several mono- and di-substituted thiourea compounds, cytotoxic effects could be observed (Onderwater et al. 1998). Eventually, these cytotoxic effects might be an explanation for the inclusion bodies detected in the colloid. Adams et al. (1986) suggested that the inclusion bodies encapsulated the

toxic agent to avoid further toxicity to the cell. In this case, it would make sense to dispose such structures outside the cells, for example in the colloid as observed.

Epithelial cell height represents a classical parameter to detect thyroid activation (Eales et al. 1993, Goleman et al. 2002b, Miranda et al. 1996b). Goleman et al. (2002a) suggested cell height to be the most sensitive parameter for evaluating perchlorate-exposed *Xenopus laevis* tadpoles. In the present study, an increase in epithelial cell height was conspicuous at concentrations ≥ 10 mg/L; however, it did not reach the same sensitivity as effects observed in the follicles. Increases in cell height usually coincided with cell crowding and stratification best observable in the highest concentration. These effects are clear signs of a massive hypertrophy triggered by a stimulation of TSH. Ultrastructural studies on amiodarone-exposed rats and PTU- and methimazole-exposed white leghorn chicks revealed massive distortions of the cytological architecture, namely increased dilation of the rough endoplasmic reticulum and Golgi fields and an increase of secondary lysosomes which hints at a massive activation of protein synthesis (cf. chapter 5; Handa et al. 1980, Pitsiavas et al. 1997).

The effects observed in the thyroid are clear signs of an activation triggered *via* the hypothalamic-pituitary-thyroid axis. The critical hormone involved in this process is TSH, which belongs to the glycoprotein family having an α-subunit identical to FSH and LH. The β-subunit is structurally distinct and confers hormone-specific functions (Pierce et al. 1981). Until now, it has been unclear if the exposure to thyroid-inhibiting substances leads to any morphological changes in the pituitary of zebrafish or only induces elevated metabolic activity of an unchanged number of pituitary cells. Studies in Sprague-Dawley rats report on measurably increased TSH-concentrations after exposure to certain EDCs (O'Connor et al. 1999), but it was not clear how this was achieved. The present study documents that the decreasing negative feedback induced by exposure to PTU is responsible for proliferations of basophilic cells within the proximal *pars distalis*. Morphometrical analysis clearly showed that the increase of adenohypophyseal tissue followed a concentration-dependent pattern up to 25 mg/L. Interestingly, the highest exposure group showed a slight decrease, but still statistically significant from the control. Concomitant with adenohypophyseal proliferation, the neurohypophysis slightly decreased, although showing no statistical significance. If these morphological

alterations lead to any physiological problems is unknown. We could not detect any signs of abnormal behavior of the fish throughout the experiment. Additional tests are necessary to clarify potential consequences.

To describe the morphological alterations in the pituitary, the ratio of adeno- to neurohypophysis turned out to be a very precise indicator. The effects are in agreement with studies on Wistar rats showing basophilic cell proliferations after exposure to PTU (Mellert et al. 2003). It is known that TSH-producing cells together with LH- and FSH-producing cells are basophilic and react PAS-positive in histological staining. The control and the highest concentration group revealed a significant increase of TSH-producing cells of the highest exposure group after immunostaining. The increased number of cells is thus capable of producing the TSH concentrations required to stimulate the thyroid. Since only the control and the highest concentration group were immunostained, it is unknown whether this effect is already observable in the lower concentrations, although the measurement of adenohypophyseal surface area implies such an increase. These treatment-related changes of TSH-cell counts contribute to compensate for reduced TH production due to PTU exposure. Nevertheless, ELISA-measurements of T4 contents revealed an obvious decrease of T4 implying the inability of the thyroid system to compensate for the inhibition by PTU. The inability to produce sufficient amounts of T4 could be due at least in part to the enormous concentrations of PTU used in the study, which are far from being environmentally relevant.

An important aspect of pituitary regulation is the responsiveness to the negative feedback loop. In our experiments, fish were exposed for 35 days, starting directly after fertilization. At early stages of zebrafish larval development, the absence of thyrotropic hormones does not affect thyroid hormone production or growth of follicles and both processes (Alt et al. 2006b). On the other hand, nothing is known about the response of the pituitary to environmental exposure at early larval stages. The first thyroid follicles appear around 60 hours post fertilization (Alt et al. 2006b), and an increasing number of follicles appear after the onset of thyroid hormone (T4) production at around 72 hpf (Elsalini et al. 2003). At least in the first few days of development, maternally derived thyroid hormone is likely to compensate for the lack of zygotic thyroid hormone following exposure to endocrine disruptors, although the role of thyroid hormones in early larval

development is not clear (Power et al. 2001). The fact that the thyroid gland does not depend on TSH at early larval stages leaves the question when the feedback loop of the inhibited thyroid is established.

Macroscopically observable alterations could be found in whole body length and weight. Considering the importance of thyroid hormones for somatic growth and development, a continuous decline in these two endpoints could be expected. However, a biphasic response pattern was observable in both whole body weight and length with an increase in the lowest concentrations – in case of whole body weight statistically significant – and a decrease in the highest concentration, which was significant in the case of whole body length. One possible explanation for this observation could be reactive over-compensation *via* TSH. The inhibitory effect of PTU is based on competing with thyroglobulin-linked tyrosine residues and diverting oxidized iodide away from hormone synthesis (Cooper 2005). The concentration-dependent competitive inhibition probably leads to partially blocked thyroid synthesis still capable of producing certain amounts of T4, which were detected in the ELISA. As PTU does not affect fish deiodinases, the conversion of T4 to the biologically active hormone T3 should not be affected. In the present experiment, T3 concentrations were not measured, but studies from van der Ven et al. (2006) revealed that T3 concentrations remained stable in zebrafish at concentrations of 10 mg/L PTU. In their study, a significant decrease of T3 could be detected at PTU-concentrations of 100 mg/L, which is far beyond the concentrations tested in this study. The highest measurements of whole body weight and whole body length in the present study were determined at 2.5 mg/L PTU. Higher exposure groups showed continuous decreases. Since the van der Ven study (2006) did not show any decrease of T3 at 1 mg/L and 10 mg/L it can be assumed that PTU-concentrations of 2.5 mg/L will not induce T3 decreases either. However, it is likely that PTU-concentrations above 10 mg/L will cause a decline in T3 levels which may mediate, at least in part, the observed effects on whole body weight and length. These results could explain the biphasic response pattern of whole body length and weight as well as the severe proliferations in the thyroid.

Although the liver is the main site of deiodination (Morin et al. 1993), histologically detectable effects were scarce. PAS-staining revealed clear-cut glycogen depletion at concentrations ≥ 10 mg/L PTU. Since glycogen is not

directly related to the regulation of the hypothalamic-pituitary-thyroid axis, this effect is most probably a general stress symptom caused by the altered thyroid status. Ultrastructurally, decreased numbers of mitochondria and fenestration and reduction of the rough endoplasmic reticulum were common features of concentrations ≥ 10 mg/L. Apparently, fenestration and reduction of the rough endoplasmic reticulum are common reactions of fish hepatocytes to toxic insults, since similar reactions were observed after *in vivo* exposure to various substances of rainbow trout and zebrafish, respectively (Braunbeck et al. 1990b). However, specific reactions of the liver with respect to the regulation of thyroidal status could not be observed, rather general toxic effects directly caused by the substance. It is known that PTU has side effects in humans in the treatment of hyperthyroidism (Benyounes et al. 2006, Cooper 1999). There are reports of anti-mitochondrial antibody production in humans (Parker 1982) which could explain the reduced number of mitochondria. Numerous studies report on necrosis of different severity (Benyounes et al. 2006, Deidiker et al. 1996); however, no signs of necrosis were visible in the present study, which can possibly be related to the much lower PTU concentrations used in this study together with species-specific differences.

2.6 Conclusions

The present study clearly shows that the zebrafish is sensitive to the effects of PTU and thus may become a useful tool for studying potential thyroid disruptors. The hypothalamic-pituitary-thyroid axis showed distinct effects, especially in the thyroid itself and also in the pituitary. Compared to studies in *Xenopus laevis*, the sensitivity of histologically detectable parameters in the thyroid was as high as or even higher than in *Xenopus* (Degitz et al. 2005). The liver revealed rather generalized (unspecific) toxic effects of PTU. Future studies should include downstream markers of thyroid function (e. g. cholesterol levels) to further elucidate these effects. The fact that there are severe morphological changes in the pituitary leaves open questions whether these changes have negative effects on the regulation of the endocrine system. Further studies are necessary to clarify the responsiveness of the pituitary to inhibiting test substances, since in fish reactions

to goitrogens at very early larval stages are unknown and since the establishment of the functioning negative feedback loop remains unclear. Moreover, prolonged test covering the completion of sexual development should be considered to reveal possible effects on sex determinations and gonad development.

Chapter 3

Effects of the anti-thyroidal compound potassium-perchlorate on the thyroid system of the zebrafish

3.1 Abstract

The increasing pollution of aquatic habitats with anthropogenic compounds has led to various test strategies to detect hazardous chemicals. However, information on effects of pollutants in the thyroid system in fish, which is essential for growth, development and parts of reproduction, is still scarce. Other vertebrate groups such as amphibians or mammals are well-studied; so the need for further knowledge especially in fish as a favored vertebrate model test organism is evident. Modified early life-stage tests were carried out with zebrafish exposed to the known thyroid inhibitor potassium perchlorate (0, 62.5, 125, 250, 500 and 5000 µg/L) to identify adverse effects on the hypothalamic-pituitary-thyroid axis. Especially higher perchlorate concentrations led to conspicuous alterations in the thyroidal tissue architecture and to effects in the pituitary. In the thyroid, severe hyperplasia at concentrations ≥ 500 µg/L together with an increase in follicle number could be detected. The most sensitive endpoint was the colloid, which showed alterations at ≥ 250 µg/L. The tinctorial properties and the texture of the colloid changed dramatically. Interestingly, effects on epithelial cell height were minor. The pituitary revealed significant proliferations of TSH-producing cells and adenohypophyseal tissue. The liver as the main site of T4 deiodination showed severe glycogen depletion at concentrations ≥ 250 µg/L. In summary, the thyroid system in zebrafish showed effects by perchlorate from concentrations ≥ 250 µg/L, thus documenting a high sensitivity of the zebrafish thyroid gland for goitrogens. In the future, such distinct alterations could lead to a better understanding and identification of potential thyroid-disrupting chemicals.

3.2 Introduction

The thyroid system in vertebrates is essential for growth, development, and aspects of reproduction (Brown et al. 2004, Cyr and Eales 1988, Leatherland 1994, Power et al. 2001). Whereas it is well-studied in mammals (Momotani et al. 2000, Schreiber 2002, York et al. 2003) and amphibians (Grim et al. 2009, Huang et al. 2001, Regard and Mauchamp 1971, Tata 2006), information on the thyroid system in fish is scarce and effects of industrial chemical compounds have hardly been

studied (Bradford et al. 2005, Grau 1988, Schmidt and Braunbeck 2011, van der Ven et al. 2006).

The present study was designed to elucidate the thyroid-disrupting effects of potassium-perchlorate, a known thyroid function inhibitor (Wolff 1998). Perchloric acid and its salts are strong oxidizers and used in pyrotechnics, explosives, and jet or rocket fuels (von Burg 1995). Furthermore, perchlorate salts have been used in the medical treatment of specific hyperthyroid conditions and as a provocative test for the release of thyroid hormones (Martino et al. 1986, Wenzel and Lente 1984). Naturally occurring perchlorate is formed in the atmosphere leading to trace levels in precipitation, which can concentrate geologically in some locations such as northern Chile (Urbansky et al. 2001) or West Texas (Dasgupta et al. 2005), but the majority of environmental perchlorate is of anthropogenic origin.

Environmental perchlorate pollution of ground and surface waters has mainly been documented in the United States, where 4 % of public water contains perchlorate. Several states have reported perchlorate concentrations of 8 µg/L up to 3.7 g/L (Urbansky 1998). These data are well in the range of the US EPA (2002) Tier II acute and chronic effects screening levels for ecotoxicological effects (5 and 0.6 mg/L, respectively) and necessitate additional data on adverse effects in freshwater fish.

Perchlorates together with thiocyanates and nitrates are known to affect thyroid function (Wolff 1998, Wyngaarden et al. 1953) by competitive inhibition of the sodium iodide symporter, which is responsible for the uptake of iodide in the thyrocytes (Tonacchera et al. 2004), with perchlorate being most potent inhibitor of the sodium iodide symporter followed by thiocyanate and nitrate. Wyngaarden et al. (1953) found that perchlorate is 10-times more potent than thiocyanate and 300-times more potent than nitrate in inhibiting radioiodine uptake in the rat thyroid. In Chinese hamster ovary cells transfected with human sodium-iodide symporter, Tonacchera et al. (2004) reported that concentrations of ClO_4^-, SCN^-, I^-, and NO_3^- required for 50 % inhibition of radioiodine uptake were in the ratios 1:15:30:240.

Thyroid hormone activity is exceptionally important during early development in amphibians and fish being responsible for the completion of metamorphosis

(Einarsdottir et al. 2006, Miwa and Inui 1987, Shi 2000). The importance of thyroid hormones in this phase of development of fish is exceptionally evident in flatfish, which are dependent on thyroid hormones to metamorphosize to the asymmetrical juvenile (Einarsdottir et al. 2006, Miwa and Inui 1987). In zebrafish, the first thyroid follicle differentiates around 55 hours post-fertilization and thyroxin (T4) production starts around 72 hours post-fertilization (Alt et al. 2006a, Elsalini and Rohr 2003). The first follicle corresponds to the anterior-most follicle in the adult (Alt et al. 2006b), which is important for evaluating histopathological samples, since follicle size is a major endpoint.

Although the thyroid gland acts as the downstream hormone-producing gland, the key organ for regulation of, e.g., growth, development, reproduction or adaption to environmental challenges along hormonal axes is the pituitary (Kasper et al. 2006). Regulatory pathways of the thyroid system start with the reception of external and internal sensory information reaching the brain and the hypothalamus. In contrast to higher vertebrates, the role of the thyrotropin-releasing hormone in the regulation of thyroid-stimulating hormone (TSH) release in fish is less well established (Janz 2000). Unlike mammals, teleost fish lack a portal system between the hypothalamus and the pituitary gland. Instead, there is a direct neuronal connection to endocrine cells through the hypophyseal stalk (Peter et al. 1990). The hypothalamus thus directly innervates the pituitary exerting control through secretion of several hormones – in this case, *via* TSH (Kime 1998). The functional significance of TSH is limited to the regulation of T4 release and iodide uptake by the thyroid follicles (Eales et al. 1999).

Morphologically, the pituitary in teleost fish is divided into two major parts: (1) the neurohypophysis (pars nervosa; PN), which folds down from the diencephalon and (2) the adenohypophysis, which pouches up from the roof of the oral cavity (Weltzien et al. 2004). During development, the neurohypophysis interdigitates with the adenohypophysis, which on its part can be subdivided into (1) the pars distalis (PD), which can further be divided into the rostral pars distalis (RPD) and the proximal pars distalis (PPD) and (2) the pars intermedia (PI; for further details, see Fig. 2.1). Multiple studies have documented the principal distribution of adenohypophyseal cells in fish (Garcia Ayala et al. 2003, Kasper et al. 2006, Leunissen et al. 1982, Quesada et al. 1988, Ueda et al. 1983) and amphibians

(Garcia-Navarro et al. 1988, Miranda et al. 1996a, Ogawa et al. 1995); the impact of thyroid-disrupting chemicals, however, has hardly been examined (Schmidt and Braunbeck 2011).

The importance of the thyroid system in fish and the potency and distribution of perchlorate make further studies indispensable to properly evaluate effects of thyroid-disrupting chemicals and to identify possible environmental risks. Therefore, this study was designed to describe histological effects in the thyroid, to quantify histological and immunohistological alterations in the pituitary, and to record histological and ultrastructural effects in the liver in a modified early life-stage test with the zebrafish (*Danio rerio*).

3.3 Material and Methods

3.3.1 Animals and husbandry

Fertilized eggs from zebrafish (*Danio rerio*) were obtained from in-house breeding at the Department of Aquatic Ecology and Toxicology, Centre for Organismal Studies, University of Heidelberg. All experiments were conducted in compliance with the institutional guidelines for the care and use of animals as well as with permission by the regional animal welfare (AZ 35-9185.81/G-144/07). Fertilized eggs were initially raised in 20 cm petri dishes in a KB 115 incubator (Binder, Tuttlingen, Germany) at a constant temperature of 27.0 ± 1.0 °C and exposed to 0, 62.5, 125, 250, 500, and 5000 µg/L potassium perchlorate (Sigma, Deisenhofen, Germany). Three days after fertilization, the eggs were transferred into 10 L flow-through exposure facilities (triplicate water change per day, 27.0 ± 1.0 °C, 12:12h light:dark cycle). After hatching, embryos were fed daily with Sera Micron (Sera GmbH, Heinsberg, Germany) and from 7 days post-fertilization with freshly raised *Artemia* nauplii (Sanders, USA) *ad libitum*. Excessive food and feces were regularly removed from the aquaria.

3.3.2 Exposure studies

The exposure experiment involved aqueous exposure of *D. rerio* larvae for 5 weeks. Sixty embryos were placed in each of the two replicate tanks per concentration. Throughout the exposure, all tanks were inspected daily for dead embryos, which were immediately removed from the tanks. After 5 weeks, fish were anesthetized with a saturated solution of 4-ethylaminobenzoate (benzocaine; Sigma). Whole body length and weight were measured immediately after anesthetization, being the basis for the calculation of the condition factor according to the formula body weight [mg] × 100 / body length^3 [mm^3]. For histology, fish were fixed in Davidson‘s fixative (Romeis 1989), for ultrastructural studies fish were fixed in glutardialdehyde and stored at 4 °C until further use.

3.3.3 Histology

For histology, thirty fish were dehydrated *in toto* in a graded series of ethanol using a Leica TP 1020 Tissue Processor (Leica Microsystems, Wetzlar, Germany). After embedding in Histoplast S (Serva, Heidelberg, Germany), fish were sectioned at 2 µm thickness in horizontal and median planes, respectively. Serial sections of the thyroid, the pituitary, and the liver region were mounted on glass slides covered with an albumen-glycerin solution (Serva), stained with PAS (Romeis 1989) (nuclei were counterstained with hematoxylin), and embedded with glass coverslips using X-TRA Kitt (Medite, Burgdorf, Germany).

3.3.4 Pituitary immunohistochemistry

For immunohistochemistry, the Vectastain ABC Kit for the detection of TSH (Vector Laboratories, Burlingame, USA) was used in the control and the highest concentration group, respectively. During this procedure, antigens were unmasked by heating the slides to 96 °C in 0.01 M citrate buffer (pH 6.0) followed by incubation in 1% H_2O_2 in phosphate-buffered saline (PBS, pH 7.4). Unspecific binding was reduced by double-blocking sections with (1) 2% bovine serum albumin in PBS for 30 min at room temperature and (2) the blocking serum delivered by Vector Laboratories. Afterwards, sections were incubated overnight at 4 °C with a rabbit anti-human TSH antibody (AbD Serotec, Oxford, UK; cat. no.

8926-0004) diluted 1:250 in PBS. After three rinses in PBS, the antiserum was tagged with the biotinylated Vectastain secondary antibody and then incubated in a preformed avidin- and biotin-labelled horseradish peroxidase complex. To visualize the antiserum, sections were incubated in 3,3‘-diaminobenzidine (Vector Laboratories) until desired staining intensities had developed. According to the manufacturer, the antiserum reacts with fish. The specificity has been shown previously by Grandi and Chicca (2004) in *Acipenser naccarii*, and by Kasper et al. (2006) in *Oreochromis nilotics*, as well as Schmidt and Braunbeck (2011) in zebrafish. For instance, no immunostaining was observed after pre-incubation of the anti-human β-TSH antisera with an excess of the appropriate antigens (Grandi and Chicca 2004). Sections were slightly counterstained with Mayer´s hematoxylin (Romeis 1989), rehydrated and mounted for observation.

3.3.5 Ultrastructure

For ultrastructural studies, liver samples were fixed in 2.5 % glutardialdehyd in 0.1 M sodium cacodylate buffer (pH 7.4) at 4 °C for a minimum of 24 hrs and postfixed with 1 % osmium ferrocyanide for two hours (Karnovsky 1971). After triplicate rinsing in sodium cacodylate buffer, tissues were stained *en bloc* with 1 % uranyl acetate in 0.05 M maleic buffer (pH 5.2) overnight at 4 °C. Liver was dehydrated in a graded series of ethanol and embedded in Spurr´s medium (Spurr 1969). For localization of the required area, semi-thin sections were prepared on a Reichert-Jung Ultracut microtome (Leica Microsystems) and stained with methylene blue / azur II (Richardson et al. 1960). Afterwards, ultrathin sections (60 - 80 nm) were cut and counterstained with alkaline lead citrate (Reynolds 1963).

3.3.6 Imaging

For both histology and immunohistochemistry, light microscopy was performed with a Leitz Aristoplan microscope (Leitz, Wetzlar, Germany) equipped with a ColorView Soft Imaging Systems digital camera (Soft Imaging Systems, Münster, Germany). The surface areas of adeno-, neurohypophysis, and total pituitary were measured and the number of TSH-producing cells counted using the free software tool ImageJ 1.44 (National Institutes of Health, Bethesda, USA) to quantify the

alterations observed. Liver samples from the control and the highest concentration group were quantitatively examined using AnalySIS (Soft Imaging Systems) to measure the distance between two cell nuclei centers. Ultrathin sections of the liver were examined in a Zeiss EM 10C (Carl Zeiss, Oberkochen Germany) transmission electron microscope.

3.3.7 Thyroid hormone extraction and ELISA

The methods for methanol-extraction of whole body thyroid hormones (THs) were adopted from Shi et al. (2009) in zebrafish. Zebrafish samples were homogenized in 0.5 ml ice-cold methanol containing 1 mM 6-propyl-2-thiouracil. The homogenates were disrupted by intermittent sonic oscillation for 5 min on ice and vortexed for 10 min. After centrifugation at 3,500 g for 20 min at 4 °C, the supernatants were collected and the pellets were re-extracted with 0.5 ml ice-cold methanol/PTU and re-centrifuged. The freshly collected supernatant was combined with the original supernatant and vacuum-dried overnight at room temperature. The samples were re-dissolved in 0.05 ml methanol, 0.2 ml chloroform, and 0.05 ml 0.11 M barbital buffer (pH 8.6; Sigma). The mixture was vortexed for 3 min and centrifuged at 3,500 g for 15 min at 4 °C. The upper layer was carefully collected and immediately used for the T4-measurements. The ELISA was performed with commercial kits (Diagnostic Automation/Cortez Diagnostics Inc., Calabasas, USA) according to the manufacturer‘s instructions.

3.3.8 Quantitative morphometric analysis and data analysis

The non-parametric Kruskal-Wallis test was used to determine differences in whole body weight and length, the condition factor, T4 contents, hepatocyte distance (i.e. cell size), and the surface areas of the pituitary. 60 fish of each concentration group were used to determine whole body length, weight and the surface areas of the pituitary. T4 contents were measured in three animals per concentration group. Dunn’s multiple comparison test was used for pairwise comparisons with the control group. For analyzing TSH-producing cell number, the Mann-Whitney test was used to compare 5 individuals from the highest concentration group to 5 individuals from the control. All statistical analyses were performed using the software package GraphPad Prism 4.0a for Macintosh

(GraphPad Software, Inc., La Jolla, USA). Differences were considered significant at $p < 0.05$ (*), highly significant at $p < 0.01$ (**), and highest significant at $p < 0.001$ (***).

3.4 Results

3.4.1 Whole body weight, whole body length, and condition factor

Overall body proportions revealed clear alterations in the higher concentration groups (Tab. 3.1). Whole body weight did not show any statistical aberrance. Concentrations ≥ 500 µg/L showed a slight, however not statistically relevant decrease. Instead, whole body length revealed a statistically significant increase at 125 µg/L, whereas the other exposure groups were not affected. However, the condition factor decreased throughout the concentration groups with significant alterations at concentrations ≥ 125 µg/L (Fig. 3.1).

Table 3.1: Whole body length, whole body weight, and condition factor of zebrafish (*Danio rerio*) after 35 days of exposure to potassium perchlorate.

Potassium-perchlorate [µg/L]	Control	62.5	125	250	500	5000
Effects on body proportions						
Whole body length (mean)	18.3 ± 1.3	18.3 ± 2.0	19.0 ± 2.0	18.5 ± 2.3	18.6 ± 1.7	18.4 ± 1.0
Whole body weight (mean)	61.0 ± 13.4	61.5 ± 16.6	64.4 ± 15.9	60.3 ± 18.5	57.8 ± 13.6	56.5 ± 8.6
Condition factor (mean)	0.99 ± 0.10	0.99 ± 0.12	0.93 ± 0.13	0.94 ± 0.16	0.89 ± 0.10	0.91 ± 0.09

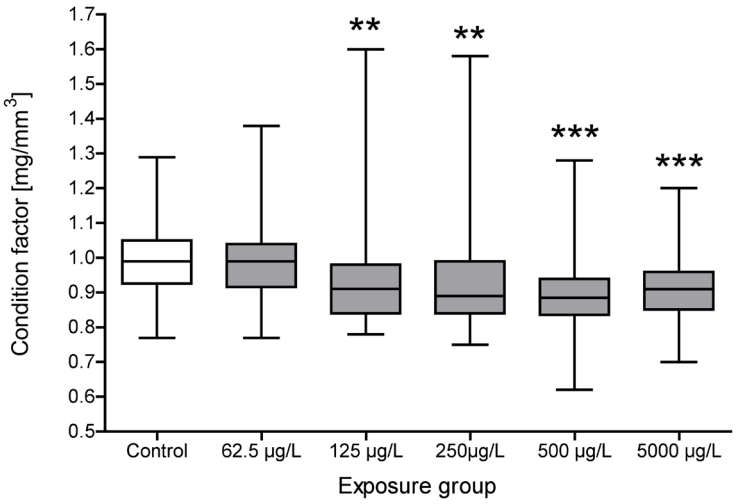

Fig 3.1: Perchlorate-induced changes in the condition factor of zebrafish after 35 days of exposure (n = 60; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Dunn's test).

3.4.2 T4 contents

T4 concentrations showed a clear-cut dose-dependent decrease after exposure to perchlorate with a highly significant reduction at 5 mg/L (Fig. 3.2). Probably due to the small sample size of only three fish per treatment group, the 5 mg/L group was the only significant group compared to the control despite the clear dose-response curve; yet, the trend is obvious from much lower concentrations.

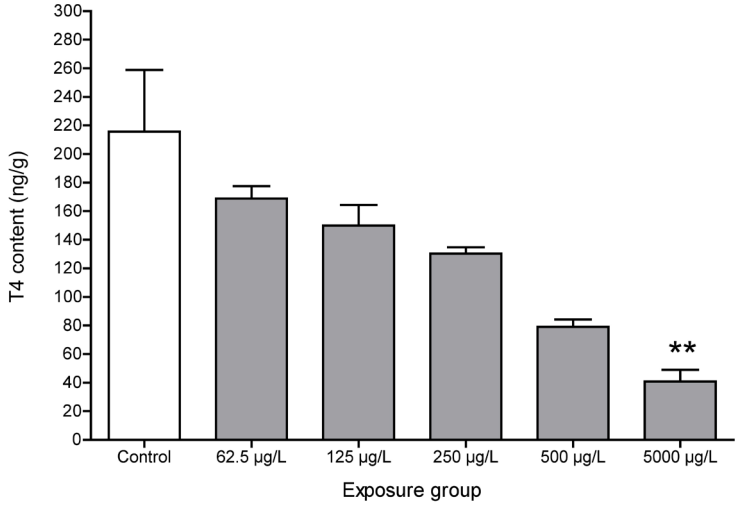

Fig. 3.2: Whole body contents of thyroxin (T4) in zebrafish exposed to 0, 62.5, 125, 250, 500, and 5000 µg/L perchlorate (n = 3; ** $p < 0.01$, Dunn's test).

3.4.3 Histopathological alterations in the thyroid

Histopathological examination revealed conspicuous alterations, especially at concentrations ≥ 500 µg/L (Tab. 3.2). The thyroids of the control group featured round to ovally-shaped follicles with a homogeneously stained colloid surrounded by cuboid to flat epithelia (Fig. 3.3a, b). The thyroids of the exposure groups ≥ 500 µg/L, however, displayed severe alterations in the follicle size and shape as well as an increased total number (Fig. 3.4a). Follicle size was diminished at 5 mg/L (Fig. 3.4a, b). In one individual in the highest concentration group small follicles in the gills could be detected (Fig. 3.4c). Moreover, the shape of the follicles was affected at the two highest concentrations, showing slight papillary in- and outfoldings (Fig. 3.4b).

The thyrocytes themselves were not as sensitive as the follicles, showing slight alterations at concentrations ≥ 500 µg/L. The most sensitive endpoint, however, was the colloid which showed first signs of heterogeneities at 250 µg/L. Compared to the control group, the texture of the colloid at concentrations ≥ 250 µg/L started to show a foamy appearance, and the colloid density decreased (Fig. 3.4d). Distinct colloid depletions could be seen at concentrations ≥ 500 µg/L together with a moderate reduction in colloid homogeneity (Fig. 3.4e). Some samples displayed a blotchy colloid. Occasionally, cellular inclusions could be seen throughout all exposure groups including the control (Fig. 3.4f). Additionally, all sample groups showed shrinking artifacts from the dehydration process, which should not be mistaken for actual effects caused by perchlorate.

Table 3.2: Semiquantitative evaluation of histological alterations in the thyroid and the liver of zebrafish (*Danio rerio*) induced by potassium perchlorate.

Potassium-perchlorate [µg/L]	Control	62.5	125	250	500	5000
Effects in the thyroid						
Follicles						
Total Number					++	++++
Size						+
Shape					+	+
Colloid						
Reduced homogeneity					++	+++
Reduced density				+	++	+++
Colloid depletion					++	+++
Foamy texture				+	++	+++
Epithelial cells						
Cell height					+	+
Effects in the liver						
Glycogen depletion				++	+++	++

Data are given as means of observation in 20 individuals per exposure group: + = little developed; ++ = moderately developed; +++ = strongly developed; ++++ = very strongly developed

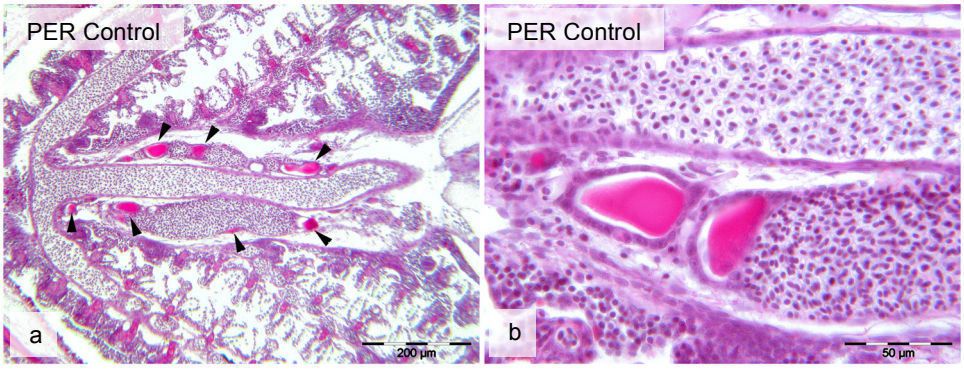

Fig. 3.3: Histology of thyroidal tissue in the control group of zebrafish. The single thyroid follicles (a: ►) are distributed adjacent to the ventral aorta in the gill region. Follicle shape is round to oval with a cuboidal epithelium and a homogeneously stained colloid

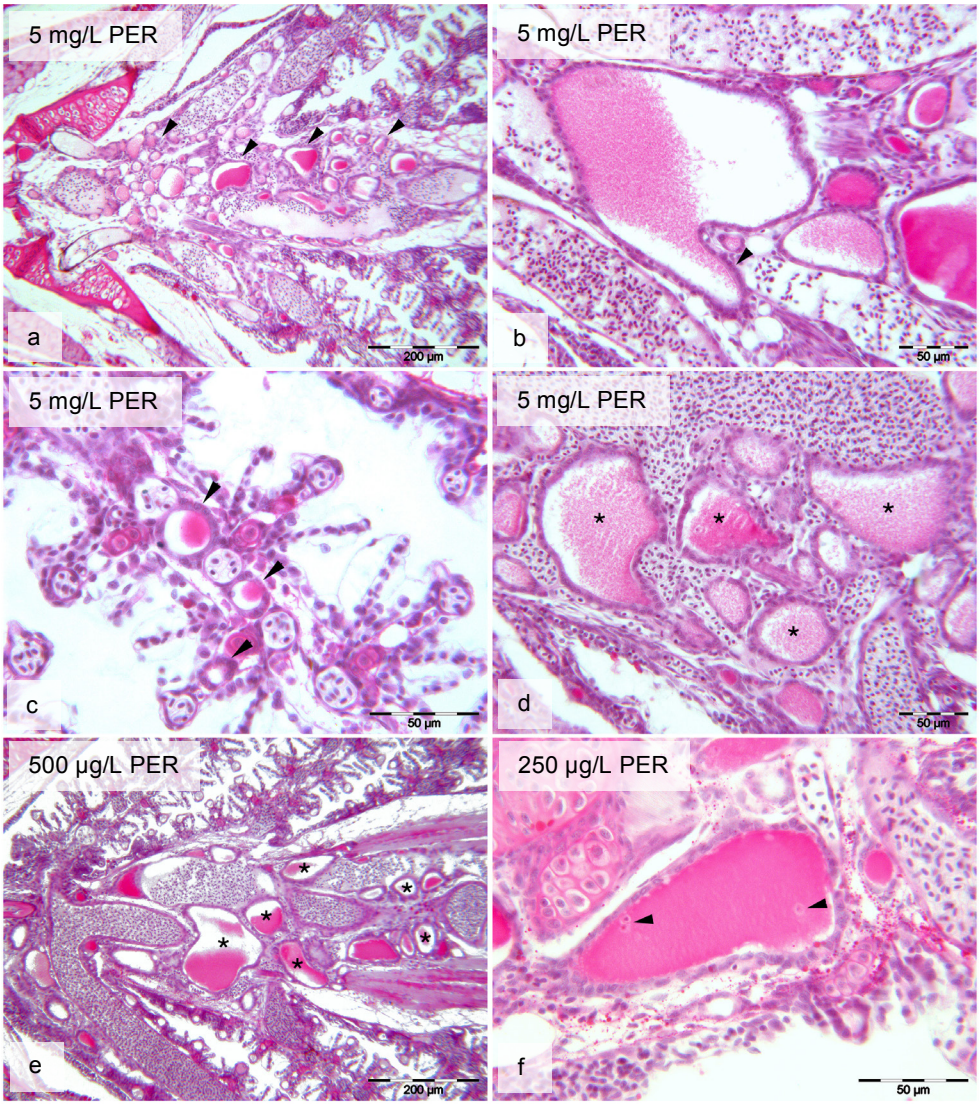

Fig. 3.4: Effects of thyroidal tissue exposed to 250, 500, and 5000 µg/L perchlorate, respectively. Exposure to perchlorate resulted in massive hyperplasia caused by an increased number of small follicles (a: ►). Especially in concentrations ≥ 500 µg/L, numerous follicles featured papillary in- and outfoldings (b: ►). One sample of the highest concentration group showed follicles in the gill archs (c: ►). The most sensitive endpoints were alterations in the colloid, which showed a dose-dependent increase from ≥ 250 µg/L. Primarily, a foamy texture and a reduced density was observable (d and e: *). Occasional appearances of cells (or cell fractions) within the colloid could be seen throughout all exposure groups including the control (f: ►).

3.4.4 Histological and immunohistochemical alterations in the pituitary

On consecutive PAS-stained control sections, a clear separation of the neurohypophysis from the adenohypophysis was evident with the neurohypophysis slightly digitating into the adenohypophysis (Fig. 3.5a). With respect to overall dimensions of the pituitary on longitudinal sections, none of the exposure groups revealed any statistically significant increase of pituitary surface area in consequence of PER exposure (Fig. 3.6).

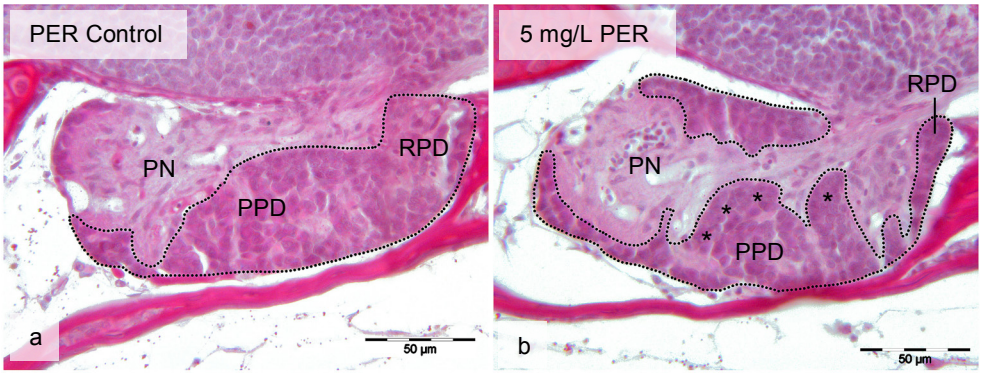

Fig 3.5: PAS-stained sagittal sections of zebrafish (*Danio rerio*) pituitaries exposed to perchlorate. The adenohypophysis is encircled by a dotted line to highlight the changes in the ratio between adeno- and neurohypophysis. Moderate proliferation of PAS-positive cells (*) mostly located in the proximal *pars distalis* is evident. PI – *pars intermedia* (adenohypophysis); PN – *pars nervosa* (neurohypophysis); PPD – proximal *pars distalis* (adenohypophysis); RPD – rostral *pars distalis* (adenohypophysis). Sections of 2 µm thickness stained with periodic-acid Schiff (PAS) and Mayer's hematoxylin.

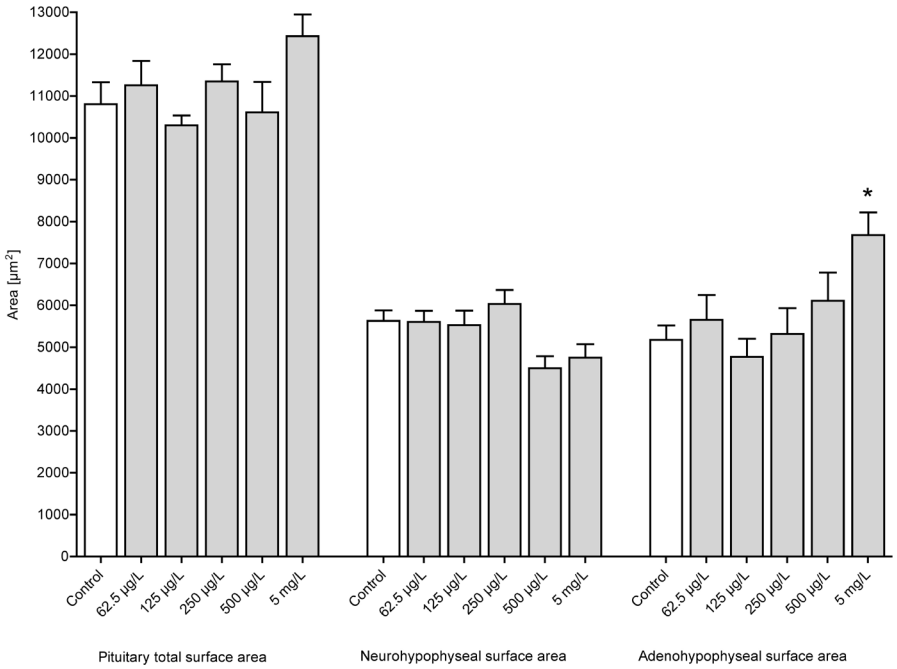

Fig 3.6: Pituitary surface areas in zebrafish (*Danio rerio*) after 35 days exposure to 0, 62.5, 125, 250, 500 and 5000 µg/L PER (n = 60; * $p < 0.05$; Dunn's test).

As a member of the glycoprotein family, TSH, together with FSH and LH, reacted PAS-positive and could be detected in the proximal *pars distalis* along the border to the *pars nervosa*, which digitated into the adenohypophysis. Although differentiation of TSH-producing cells from FSH- and LH-producing cells was not possible with PAS-staining, profound morphological changes in consequence of exposure to PER could already be detected in PAS-stained sections: Especially at concentrations ≥ 500 µg/L, there was a moderate proliferation of adenohypophyseal tissue (Fig. 3.5b). Morphometric analysis revealed a significant increase in adenohypophyseal tissue at the highest PER concentration (Fig. 3.6). This proliferation mostly occurred in the proximal *pars distalis* of the adenohypophysis (Fig. 3.5b). Thus, given the similar pituitary volumes of control and exposed fish, the ratio between adeno- and neurohypophysis had changed (Fig. 3.7). PER led to a clear dose-dependent increase of this ratio with a significant change at the highest exposure group. Surface area measurements of the neurohypophysis did not reveal any significant proliferations (Fig. 3.6).

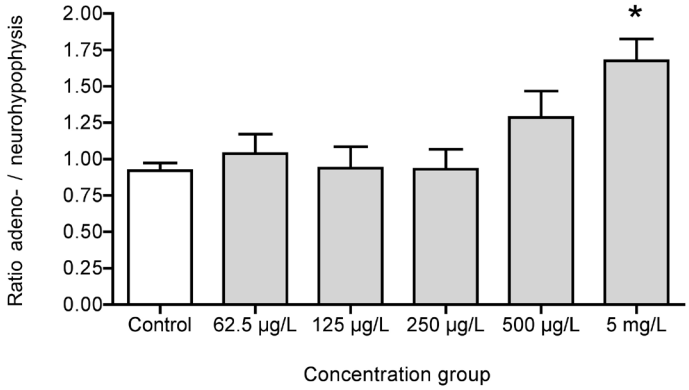

Fig 3.7: Ratio of adeno- to neurohypophyseal tissue of zebrafish (*Danio rerio*) after 35 days exposure to 0, 62.5, 125, 250, 500 and 5000 µg/L potassium perchlorate (n = 60; * $p < 0.05$; Dunn's test).

Immunohistochemical staining with anti-TSH antibodies revealed a rather homogeneous distribution of TSH-producing cells (Fig. 3.8). In the control group, TSH-positive cells were mainly limited to the rostral *pars distalis* and the *pars intermedia* with a few cells located in the proximal *pars distalis* (Fig 3.8a). This situation changed after exposure to 5 mg/L PER (Fig. 3.8b): The most conspicuous proliferation occurred in the proximal *pars distalis*, where cells located at the border of the proximal *pars distalis* to the *pars nervosa* had proliferated. This observation is in line with the aforementioned proliferation of adenohypophyseal tissue. Quantification of TSH-producing cell counts in the control and the highest concentration group led to a statistically significant increase after exposure to PER (Fig. 3.9).

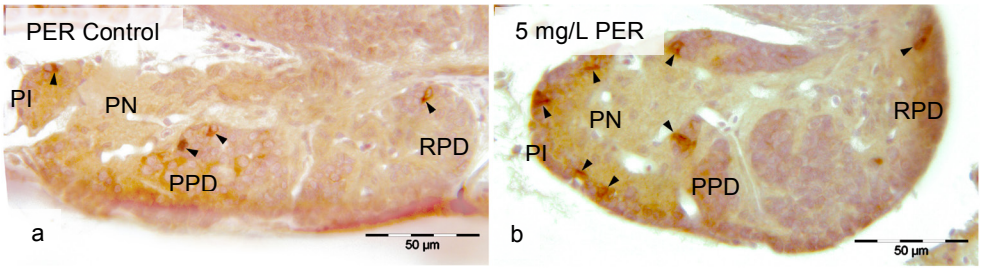

Fig 3.8: Sagittal sections of immunohistochemically stained zebrafish (*Danio rerio*) pituitaries exposed to 0 and 5 mg/L potassium perchlorate for 35 days. The samples show a moderate proliferation of TSH-producing cells (►) especially in the proximal *pars distalis*. Sections of 2 µm thickness immunostained with an anti-TSH antibody (nuclei were counterstained with Mayer's hematoxylin). PI – *pars intermedia* (adenohypophysis); PN – *pars nervosa* (neurohypophysis); PPD – proximal *pars distalis* (adenohypophysis); RPD – rostral *pars distalis* (adenohypophysis).

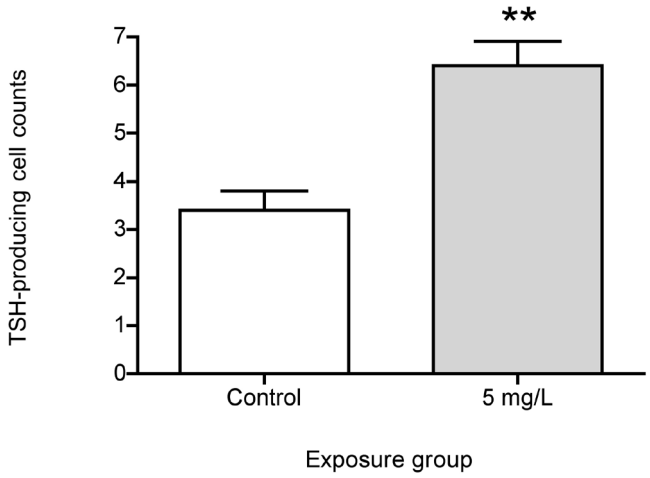

Fig 3.9: TSH-producing cell counts of zebrafish (*Danio rerio*) pituitaries after 35 days exposure to 0 and 5 mg/L potassium perchlorate (n = 5;** $p < 0.01$; Mann-Whitney test).

3.4.5 Histopathological and ultrastructural alterations in the liver

Liver samples revealed histologically and ultrastructurally detectable moderately to strong depletions of glycogen storage deposits from 125 µg/L (Fig. 3.10, 3.11). Morphometric analyses of hepatocyte dimensions did not reveal any quantifiable alterations. Ultrastructurally, lysosome-like bodies, increasing lipid concentrations, myelinated bodies, and fenestrated rER were detectable at concentrations ≥ 250 µg/L. Moreover, moderate condensations of nucleolar RNA could be detected in concentrations ≥ 500 µg/L (Fig. 3.11).

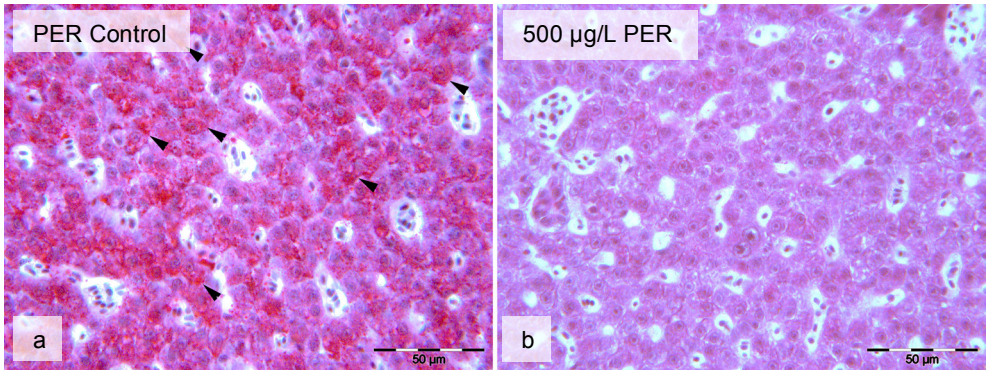

Fig 3.10: Histopathological alterations in hepatocytes of control (a) and potassium perchlorate-exposed zebrafish (b). The well-visible glycogen deposits in the control (a: ►) are depleted at the higher concentration groups. Sections of 2 µm thickness stained with periodic-acid Schiff (PAS) and Mayer's hematoxylin.

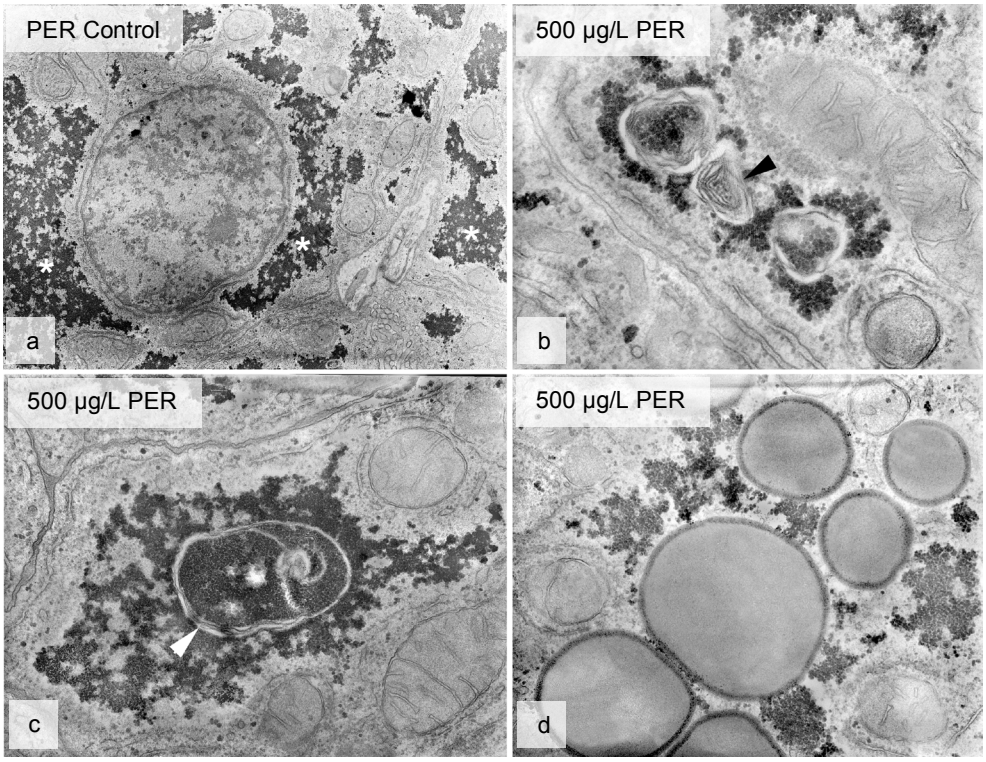

Fig. 3.11: Ultrastructural alterations in hepatocytes of control (a) and potassium perchlorate-exposed zebrafish (b-d). In the control fish, extended glycogen storage deposits are clearly visible (a: *). The exposed samples show myelinated bodies (b: ►), lysosome-like bodies (c: ►), and lipid droplets (d), Magnification: a 5,800x; b 34,000x; c, d 19,000x.

3.5 Discussion

In the present study, alterations of the thyroid system of the zebrafish by potassium perchlorate were used to determine distinct effects caused by inhibited iodide uptake. To assess effects of endocrine-disrupting chemicals, it is essential to examine appropriate endpoints since these endpoints (1) can provide indications as to the underlying mode of action of the chemical and (2) show different sensitivities in the single endpoints, which are essential to properly grade the effect, and (3) to characterize morphological and morphometric changes in the pituitary as the main control organ of the endocrine system.

Anti-thyroid agents are characterized by their potential to inhibit thyroid hormone production, thus, leading to reduced circulating levels of thyroid hormones (Brucker-Davis 1998). *Via* a negative feedback loop, declining thyroid hormone concentrations activate thyroid hormone synthesis to up-regulate and maintain hormone levels in the body. Nevertheless, the mode of action of the inhibitor plays an important role in the characteristic effects evident from alterations of thyroidal tissue. In contrast to thiourea-based anti-thyroid agents, which inhibit thyroperoxidase (Cooper 2005), perchlorate competitively inhibits the sodium-iodide symporter and thus the uptake of iodide (Wolff 1998).

In the present study, histopathological observations indicated a clear-cut thyroid activation evidenced by conspicuous alterations in the follicles. Contrary to Goleman et al. (2002a), who suggested epithelial cell height as the most sensitive endpoint in *Xenopus laevis* tadpoles, only mild increases in epithelial cell height could be detected, which, nonetheless, indicates an activation of thyroidal tissue. The most sensitive endpoint turned out to be follicle number, which showed a clear-cut increase at the two highest perchlorate concentration groups. One sample of the 5 mg/L group even displayed small follicles migrating up gill arches. The only other observable effects in the thyroid were little developed increases in follicle size and alterations in follicle shape. The changes in the epithelial cell height and follicle appearance are likely the result of negative feedback-induced secretion of thyroid-stimulating hormone (TSH) by the pituitary, which is known to react to exposures of thyroid-disrupting chemicals by intensified production of TSH. The activating effect of TSH is mediated *via* a G-protein-coupled TSH-

receptor (Farid and Szkudlinski 2004), which is mainly expressed in thyroidal tissue and the gonads (MacKenzie et al. 2009). Despite this knowledge, the factors that trigger a proliferation of thyroid follicles rather than a proliferation of follicle size are unclear and need further investigation.

In the pituitary, proliferation of TSH-producing cells could be detected as a clear sign of increased TSH-production. The alterations observed are in line with corresponding effects in mammals (Siglin et al. 2000). Nevertheless, the activation of thyroidal tissue to maintain thyroid function cannot be considered successful regarding declining T4 concentrations. ELISA measurements clearly show a dose-dependent decrease of T4 being significant at the highest concentration group (cf. Fig. 3.2). The attempt to up-regulate the production of thyroid hormones must be regarded as relatively ineffective, since even at 62.5 µg/L perchlorate a clear decrease of T4 contents is observable. It remains unknown whether this decline has any consequences on the development of the fish. Neither macroscopical changes nor behavioral abnormalities were evident in our experiments. However, contrary to results by Mukhi et al. (2005), the condition factor significantly decreased at concentrations ≥ 125 µg/L (cf. Fig. 3.1), which can be expected taking into consideration the inhibiting effect of perchlorate on TH synthesis together with dose-dependent decreases of T4 concentration.

Besides alterations in the follicles, the colloid turned out to be the most sensitive endpoint in this study. In line with various other studies (Crane et al. 2005, Mukhi and Patino 2007, Patino et al. 2003), colloid depletion increased with higher perchlorate concentrations (cf. Fig. 3.4). The inhibiting effect of perchlorate is based on its ability to compete for the sodium-iodide symporter. In this context, the question of whether or not perchlorate is actively transported by the symporter is still controversial. Recent studies in mammals showed that perchlorate is actively taken up (Dohan et al. 2007, Tran et al. 2008), but not metabolized (Anbar et al. 1959). In the present study, the most intriguing observations were the dose-dependent changes of the texture and the reduced density of the colloid, which both occurred at concentrations ≥ 250 µg/L (cf. Fig. 3.4). It is known that perchlorate leads to iodide efflux from the thyroid gland in rats (Scranton and Halm 1965, Surks 1967). Furthermore, in mice, perchlorate rapidly increases the secretory response of the thyroid to TSH, including both iodide and iodothyronines

(Rousset et al. 1977). An increased iodide secretion would automatically lead to decreasing levels of iodide in the colloid itself due to the inhibiting effect of perchlorate on the sodium-iodide symporter. In humans, the colloid consists of 19S thyroglobulin, larger iodoproteins and smaller protein fractions (Anderberg et al. 1980, 1981). In these studies, exposure to carbimazole resulted in depleted thyroglobulin aggregates. Assuming a similar colloid composition in zebrafish, the efflux of iodide and thyroglobulin due to the exposure to perchlorate would result in an altered composition of the colloid and thus to different PAS-staining properties.

Apart from the conspicuous alterations in the colloid, it is not clear if the thyroid is the only target for the inhibiting effect of perchlorate. In terrestrial vertebrates, almost all iodide is obtained from the food by transport from the gut (Eales and Brown 1993). Fish, on the other hand, have the capacity to take up iodide from the ambient water across their extensive gill surface (Hunn and Fromm 1966). Additionally, it is known from salmonids that they can obtain substantial amounts from the diet (Gregory and Eales 1975). Nevertheless, the main contributor to iodide intake is suggested to be the branchial iodide pump (Higgs and Eales 1971). However, the transporting mechanism for iodide across the gill surface is still not fully understood, but there is evidence, that it is the same sodium-iodide symporter that is responsible for the uptake in the thyroid follicles as well (Hunn and Fromm 1966). In this case, perchlorate would not only inhibit the uptake of iodide in the thyroid, but also in the gills.

ELISA measurements clearly showed that exposure to perchlorate leads to decreasing T4 contents. Usually, thyroid hormone production is regulated *via* negative feedback-induced stimulation by TSH, which is produced in the pituitary. TSH belongs to the glycoprotein family with an α-subunit identical to FSH and LH. The β-subunit is structurally distinct and confers hormone-specific functions (Pierce and Parsons 1981). Morphological alterations in the pituitary caused by influencing the negative feedback loop are hardly investigated. Studies in Sprague-Dawley rats report on measurably increased TSH concentrations, but not on the underlying mode of action (O'Connor et al. 1999). Principally, there are two different ways of increasing TSH concentrations: (1) Elevation of metabolic activity or (2) proliferation of TSH-producing cells. It is likely that both of these

processes contribute depending on the severity of the inhibition. In the present study, it could be shown that at the highest concentration group a moderate proliferation of TSH-producing cells take place. This proliferation leads to clear alterations of pituitary morphology, which could be shown by morphometric measurements of pituitary surface areas. The total surface area slightly increased at the highest exposure group, although not being statistically significant. This increase reflects adenohypophyseal proliferation especially at 5 mg/L. Simultaneously, neurohypophyseal tissue decreased at concentrations ≥ 500 µg/L. Whether these morphological alterations in the pituitary lead to any physiological problems is unknown. The present experiment did not reveal any signs of abnormal behavior throughout the experiment. In consequence, further studies are necessary to clarify potential problems.

The observed effects are in agreement with studies on zebrafish and Wistar rats showing basophilic cell proliferations after exposure to PTU (Mellert et al. 2003, Schmidt and Braunbeck 2011). To describe the effects in the pituitary, the ratio of adeno- to neurohypophysis turned out to be a very precise indicator (cf. Fig. 3.7). The proliferation of TSH-producing cells at the highest concentration group probably reflects the ongoing inhibition by perchlorate. The fish tries to compensate by increasing TSH-producing cell counts (cf. Fig. 3.9) in order to produce the required amounts of TSH to stimulate the thyroid. Since only the control and the highest concentration group were immunostained, it is unknown whether this effect already exists at lower concentrations, although the measurement of adenohypophyseal surface area suggests such an increase. Nevertheless, since T4 contents decrease, the up-regulation of TSH-production cannot be regarded successful, at least at high concentrations of PER. In this context, it would be interesting to investigate the threshold at which the fish is still able to compensate the inhibitory effect. It is likely, that this threshold depends not only on the concentration of the goitrogen, but also on the duration of exposure. In our experiment, fish were exposed for 35 days, starting directly after fertilization. At early stages of zebrafish larval development, the absence of thyrotropic hormones does not affect thyroid hormone production or growth of follicles and both processes (Alt et al. 2006b). Likewise, thyroid-inhibiting substances do not have any effect over the first few days of development, since maternally derived thyroid hormone is likely to compensate for the lack of zygotic thyroid hormone

following exposure to endocrine disruptors, although the role of thyroid hormones in early larval development is not fully clear (Power et al. 2001).

The impact on the negative feedback axis raises the question if any downstream-located organ or tissue shows effects as well. In this regard, the liver is a promising organ because in fish it is the main site for the conversion of T4 to the biologically active hormone T3 and the regulation of major fractions of circulating active T3 (Cheng et al. 2007). Histologically, however, the only observable effect in the liver was glycogen depletion at concentrations ≥ 250 µg/L perchlorate. In general, glycogen regression is described as either hormone-induced (Gluth and Hanke 1985) or as an unspecific stress reaction (Braunbeck 1994, Braunbeck et al. 1992). Thyroid hormone levels are known to be able to affect glycogen contents *in vivo* in the liver of red sea bream (*Chrysophrys major*; Woo et al. 1991) and *in vitro* in hepatocytes isolated from the silver sea bream (*Sparus sarba*; Leung and Woo 2010), thus providing evidence for the observed effects. In addition, a reduction of cellular energy reserves generally indicates higher energy requirements of individuals (Braunbeck 1992), and long-term exposure to high concentrations of toxicants also lead to a depletion of glycogen (Segner and Braunbeck 1990).

The ultrastructural analysis further revealed lysosome-like bodies (glycogenosomes) enclosing glycogen within membranes, indicating pathological glycogen degradation. Similar effects were reported in rat hepatocytes under conditions of massive glycogenolysis (Thorne-Tjomsland and Jamieson 1996, Weis 1972). Besides glycogen depletion and lysosome-like bodies, increasing lipid concentrations, the occurrence of myelinated bodies, and fenestrated rER could be detected. Some individuals also displayed moderate condensations of nucleolar RNA. All these alterations can be seen as general stress symptoms of the fish as adaptions to the exposure to environmental contaminants. It was shown that stress can lead to an increase of free fatty acids, responsible for rising lipid concentrations in fish (Braunbeck et al. 1992, Mazeaud et al. 1977). These lipid reservoirs possess protective functions like inactivation of contaminants from active metabolism (Segner and Braunbeck 1990), which could explain their appearance in samples exposed to perchlorate. Myelinated bodies can be regarded as a general stress symptom after exposure to contaminants. On the other hand, they can be seen as an adaptive mechanism under contaminant-induced stress for

compensation of an increased conversion of cellular components (Braunbeck et al. 1990a). In addition, mitochondria with myelin-like and glycogen enclosures could be detected. These abnormal mitochondrial alterations are potentially linked to oxidative stress (Saraswathy and Rao 2009) and cell death (O'Gorman et al. 1997). Apparently, fenestration of the rER is a common reaction of fish hepatocytes to toxic insults, since similar reactions were observed after *in vivo* exposure of rainbow trout and zebrafish to various substances, respectively (Braunbeck et al. 1990b). Nevertheless, all of these ultrastructural effects cannot be seen as specific reactions of the liver to the regulation of thyroidal status, but rather general toxic effects directly caused by the substance itself. In this context, an interesting study by Chen et al. (2009) on quail chicks revealed that liver tissue is not protected against hypothyroid conditions. Decreased plasma TH concentrations lead to an over-expression of 5'-deiodinase in the liver. This suggests that the liver responded to hypothyroidism with an increased T3 production. Moreover, the study discovered that young quail chicks were influenced by hypothyroidism in the first two weeks of perchlorate exposure, but not after 7.5 weeks. The present study discovered a dose-dependent decrease of T4 concentration; however, T3 was not measured. Further studies should concentrate on possible correlations in fish. The absence of any other effect in the liver probably corresponds to the biokinetics and the rather short half-life time of perchlorate in the body. The half-life time in rats have been determined to vary from approx. 8 h (Eichler and Hackenthal 1962), < 8 h (Kutzim et al. 1980), to approx. 20 h (Goldman and Stanbury 1973). Perchlorate is not metabolized in the body and it appears to be virtually excreted unchanged both in the rat (Eichler and Hackenthal 1962) and in man (Anbar et al. 1959).

3.5 Conclusions

In conclusion, the present study shows that the most sensitive endpoints for perchlorate exposure of zebrafish are alterations in the colloid which reflect the mode of action of perchlorate as an inhibitor of the sodium iodide symporter. Epithelial cell height did not show comparable sensitivities or severities. As a side effect, the liver showed strongly developed depletions of glycogen storage deposits and additional unspecific ultrastructural effects. Future studies should include

downstream markers of thyroid function such as cholesterol levels to further characterize the reaction to T4 inhibitors. One of the most interesting findings were the morphological alterations in the pituitary. These changes leave open questions on the regulation of the endocrine system and responsiveness of the pituitary to inhibiting test substances. Based on the results of this study, further research in the uptake of iodide, especially across the gill surface is essential to fully understand the inhibiting mechanisms of sodium iodide symporter-inhibiting substances. Finally, prolonged tests covering the completion of sexual development should be considered to reveal potential effects on sex determination and gonad development.

Chapter 4

Effects of thyroxin exposure on larval zebrafish (*Danio rerio*) development

4.1 Abstract

The effects of 0.25, 0.5, 1 and 2 µg/L L-thyroxin (T4) on the hypothalamic-pituitary-thyroid axis as well as on growth, body performance and body composition were investigated by 35 d exposure of 60 zebrafish (*Danio rerio*) larvae. Thyroidal status was analyzed by light and electron microscopic analyses of the thyroid and the liver as the main site of teleost T4 to T3 conversion as well as histological and immunohistochemical analyses of the pituitary as the main control organ of the endocrine system. The thyroid hormone status was investigated by whole-body T4 measurements *via* ELISA. Exposure to T4 resulted in a biphasic response pattern. Weight and length were significantly increased with the lowest T4 concentration (0.25 µg/L), but continuously declined with an increase of T4 concentrations. Both thyroid and pituitary did not show any histologically detectable alterations after exposure to T4. Ultrastructurally, however, the thyroid showed a clear decline of organelle contents. In the liver, a concentration-dependent decrease of glycogen contents could be detected; ultrastructurally, only a minor decrease of the rough endoplasmic reticulum could be detected at the highest concentration. Morphometric analysis of cell nuclei distance revealed a significant decrease at 2 µg/L T4 exposure. In conclusion, the fish showed an anabolic response to T4 in the lower concentrations, whereas higher concentrations resulted in a catabolic response.

4.2 Introduction

Various aspects of fish development as well as osmoregulation, metamorphosis, temperature tolerance, migration, oxygen consumption and metabolism are at least partly dependent on a well-balanced thyroid hormone metabolism (Donaldson et al. 1979, Higgs et al. 1982, Weatherley and Gill 1987). Numerous pseudothyroid-acting substances are known to have the capability of disrupting thyroid homeostasis, and there is increasing evidence of effects both in laboratory fish (Coimbra and Reis-Henriques 2007, Crane et al. 2006, Elsalini and Rohr 2003, Liu et al. 2008, Mukhi and Patino 2007, Park et al. 2007, Patino et al. 2003, Picard-Aitken et al. 2007, Schmidt and Braunbeck 2011, Shi et al. 2009, van der Ven et

al. 2006) and in field fish (Baker et al. 2009, Brar et al. 2010, Iwanowicz et al. 2009, Moccia et al. 1977, 1981, Morgado et al. 2009, Schnitzler et al. 2008). The effects of thyroid-inhibiting substances are rather well-known, but thyroid hormone-mimicking substances have hardly been studied in fish, especially effects on the entire hypothalamic-pituitary-thyroid axis. In fact, the impact of exogenous administration of thyroid hormones (T4 or T3) have only been looked at in few studies (Ansal and Kaur 1998, Fagerlund et al. 1984, Higgs et al. 1992, Kumar et al. 1991, Lam and Sharma 1985, Reddy and Lam 1992). Eales (1979) stated that exogenous administration of thyroid hormones usually results in modified body proportions and accelerated development of skin, bones and scales. However, the mechanism of growth stimulation is not clear; a likely assumption appears to be through synergism with growth hormone (Farbridge and Leatherland 1988, Weatherley and Gill 1987). Nevertheless, a direct action of thyroid hormones on somatic growth, cartilage and bone development cannot be ruled out (Higgs et al. 1982).

Following previous communications on the effects of propylthiouracil and perchlorate, which act as thyroid inhibiting substances, a modified early life-stage test with zebrafish was carried out to investigate effects of T4 on developing zebrafish larvae. In detail, the study was conducted to elucidate (1) effects in the thyroid, (2) feedback-induced effects in the pituitary, (3) alterations in the liver as the main site for T4 conversion, (4) changes in whole-body T4-concentration, and (5) effects on body proportions.

4.3 Material and Methods

4.3.1 Animals and husbandry

Fertilized eggs from zebrafish (*Danio rerio*) were obtained from the in-house breeding facilities of the Aquatic Ecology and Toxicology Group at the Centre for Organismal Studies, University of Heidelberg. All experiments were conducted in compliance with the institutional guidelines for the care and use of animals as well as with permission by the regional animal welfare (AZ 35-9185.81/G-144/07). The exposure experiment involved aqueous exposure of 60 *Danio rerio* larvae for 5 weeks in two replicates. Fertilized eggs were initially exposed to 0, 0.25, 0.5, 1, and 2 µg/L L-thyroxin (Sigma, Deisenhofen, Germany) in 20 cm Petri dishes in a KB 115 incubator (Binder, Tuttlingen, Germany) at a constant temperature of 27.0 ± 1.0 °C; Petri dishes had been pre-exposed to L-thyroxin for saturation. Three days after fertilization, the eggs were transferred into the 10 L flow-through exposure facilities (triplicate water change/d, 27.0 ± 1.0 °C, 12:12h light:dark cycle; oxygen saturation > 80 %) containing the same T4-concentrations. Flow-through conditions guaranteed that ammonia, nitrite, and nitrate were kept below detection limits (0 - 5, 0.025 - 1 and 0 - 140 mg/L, respectively). After hatching, embryos were fed with Sera Micron (Sera, Heinsberg, Germany) on a daily basis and after one week with freshly raised *Artemia* nauplii (Sanders, USA) *ad libitum*. Excessive food and feces was removed from the aquaria at least twice daily.

4.3.2 Light microscopy

After 5 weeks, each of the 60 fish per concentration group was anesthetized with a saturated solution of 4-ethylaminobenzoate (benzocaine, Sigma). Whole body length and weight were measured immediately after anesthetization. For histology, thirty fish were fixed in Davidson‘s fixative (Romeis 1989) for a minimum of 24 hrs at 4 °C. Whole fish were processed in a Leica TP 1020 Tissue Processor (Leica Microsystems, Wetzlar, Germany), embedded in Histoplast S (Serva, Heidelberg, Germany), and sectioned in horizontal and median planes at 2 µm thickness, respectively. For details on embedding, see Table 4.1. Serial sections of the thyroid, the pituitary, and the liver region were mounted on glass slides

covered with an albumen-glycerin solution (Serva), stained with PAS (Romeis 1989). Nuclei were counterstained with hematoxylin, slides were coverslipped with X-TRA Kitt (Medite, Burgdorf, Germany). Additionally, liver samples from the control and the highest exposure group were quantitatively evaluated. The distance between two cell nuclei centers of neighboring cells (i. e. cell size) was measured *via* AnalySIS (Soft Imaging Systems, Münster, Germany) for quantitative morphometric analysis.

Table 4.1: Details on dehydration and embedding of zebrafish tissue.

Dehydration	Duration
80 % Ethanol	1 h
90 % Ethanol	1 h
90 % Ethanol	1 h
96 % Ethanol	1 h
96 % Ethanol	1 h
100 % Isopropanol	1 h
100 % Isopropanol	1 h
Xylene	1 h
Xylene	12 h
Xylene	4 h
Histoplast S	12 h
Histoplast S	12 h

4.3.3 Pituitary immunohistochemistry

For immunohistochemistry, the Vectastain ABC Kit for the detection of human TSH (Vector Laboratories, Burlingame, USA) was applied to the control and the highest concentration group. Antigens were unmasked by heating the slides to 96 °C in 0.01 M citrate buffer (pH 6.0) followed by incubation in 1% H_2O_2 in phosphate-buffered saline (PBS, pH 7.4). Unspecific binding was reduced by double-blocking sections with (1) 2% bovine serum albumin in PBS for 30 min at room temperature and (2) the blocking serum delivered by Vector Laboratories. Afterwards, sections were incubated overnight at 4 °C with a rabbit anti-human TSH antibody (AbD Serotec, Oxford, UK; cat. no. 8926-0004) diluted 1:250 in PBS. After three rinses in PBS, the antiserum was tagged with the biotinylated Vectastain secondary antibody and then incubated in a preformed avidin and biotinylated horseradish peroxidase complex. To visualize the antiserum, sections were incubated in 3,3‘-diaminobenzidine (Vector Laboratories) until desired staining intensities had developed. According to the manufacturer, the antiserum reacts with fish. The specificity had been shown previously by Grandi and Chicca (2004) in *Acipenser naccarii* and by Kasper et al. (2006) in *Oreochromis nilotics* as well as Schmidt and Braunbeck (2011) in zebrafish. For instance, no immunostaining was observed after pre-incubation of the anti-human β-TSH antisera with an excess of the appropriate antigens (Grandi and Chicca 2004). Sections were slightly counterstained with Mayer´s hematoxylin (Romeis 1989), rehydrated and mounted for observation.

4.3.4 Electron microscopy

For ultrastructural studies, liver and lower jaw samples were fixed in 2.5 % glutardialdehyde in 0.1 M sodium cacodylate buffer (pH 7.6; Sigma) at 4 °C for a minimum of 24 hrs and post-fixed with 1 % osmium ferrocyanide for two hours (Karnovsky 1971). After triplicate rinsing in sodium cacodylate and 0.05 M maleate (pH 5.2; Sigma) buffers , tissues were stained *en bloc* with 1 % uranyl acetate in maleate buffer overnight at 4 °C. The samples were dehydrated in a graded series of ethanol and embedded in Spurr´s medium (Spurr 1969). For localization of correct sectioning areas, semi-thin sections were cut on a Reichert-Jung Ultracut microtome (Leica Microsystems, Nussloch, Germany) and stained

with methylene blue / Azur II (Richardson et al. 1960). Afterwards, ultrathin sections of 60 - 80 nm were cut and counterstained with alkaline lead citrate (Reynolds 1963).

4.3.5 Imaging

For both histology and immunohistochemistry, light microscopy was performed with a Leitz Aristoplan microscope (Leitz, Wetzlar, Germany) equipped with a ColorView Soft Imaging Systems digital camera (Soft Imaging Systems). Ultrathin sections of the liver and the thyroid were examined in a Zeiss EM 10C transmission electron microscope (Carl Zeiss, Oberkochen, Germany).

4.3.6 Thyroid hormone extraction and ELISA

The methods for methanol-extraction of whole body THs from zebrafish larvae were adopted from Shi et al. (2009): Zebrafish samples were homogenized in 0.5 ml ice-cold methanol with 1 mM PTU. The homogenates were dispersed by intermittent sonic oscillation for 5 min on ice and vortexed for 10 min. After centrifugation at 3,500 g at 4 °C for 20 min, the supernatants were collected, and the pellets were re-extracted with 0.5 ml ice-cold methanol/PTU and re-centrifuged. The freshly collected supernatant was combined with the original supernatant and vacuum-dried overnight at room temperature. The samples were re-dissolved in 0.05 ml methanol, 0.2 ml chloroform, and 0.05 ml 0.11 M barbital buffer (pH 8.6; Sigma). The mixture was vortexed for 3 min and centrifuged at 3,500 g at 4 °C for 15 min. The upper layer was carefully collected and immediately used for the T4 measurements. The ELISA was performed with commercial kits (Diagnostic Automation/Cortez Diagnostics Inc., Calabasas, USA) according to the manufacturer‘s instructions.

4.3.7 Data analysis

The non-parametric Kruskal-Wallis test was used to determine differences to controls for whole body weight, length, (n = 60) and T4 contents. T4 contents were measured in three animals per concentration group. Dunn‘s multiple comparison test was used for pairwise comparisons with controls. All statistical analyses were

performed using the software package GraphPad Prism 4.0a for Macintosh (GraphPad Software, Inc., La Jolla, USA). Differences were considered significant at $p < 0.05$ (*), highly significant at $p < 0.01$ (**), and highest significant at $p < 0.001$ (***).

4.4 Results

4.4.1 Whole body weight and length

Exposure to T4 resulted in a biphasic response pattern of both whole body weight and length (Fig. 4.1). The lowest concentration group (0.25 µg/L) led to a transient, but statistically significant increase of whole body weight followed by a gradual decline with increasing concentrations. The highest exposure group still showed values above controls, however not statistically significant.

Likewise, compared to controls, whole body length significantly increased at 0.25 and 0.5 µg/L T4 exposure. Again, this transient increase was followed by a slight decline at ≥ 1 mg/L T4; in contrast to body weight, however, body length data fell below controls at higher T4 concentrations (Fig. 4.1). Besides these alterations, no further macroscopically detectable effects were seen.

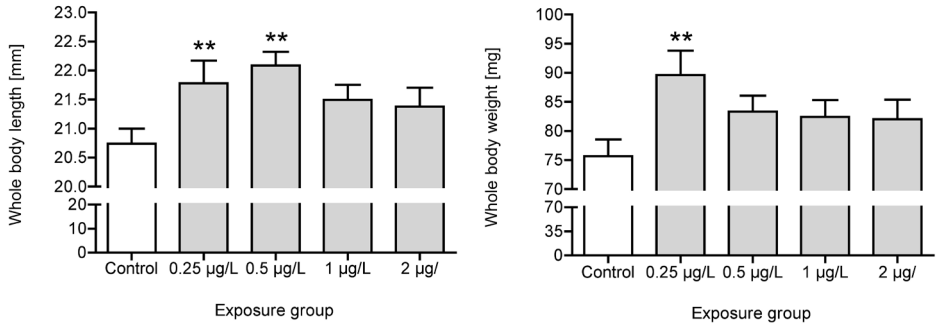

Fig. 4.1: Whole body weight and length of zebrafish (*Danio rerio*) after exposure to 0, 0.25, 0.5, 1, and 2 µg/L T4. Measurements were performed in 60 animals after 35 days of exposure. Asterisks indicate significant differences between exposure and control groups (** $p < 0.01$; Dunn's test).

4.4.2 Thyroxin (T4) contents

ELISA measurements of whole-body T4 contents revealed a dose-dependent decrease of T4 contents with a statistically significant reduction in the 2 µg/L exposure group (Fig. 4.2).

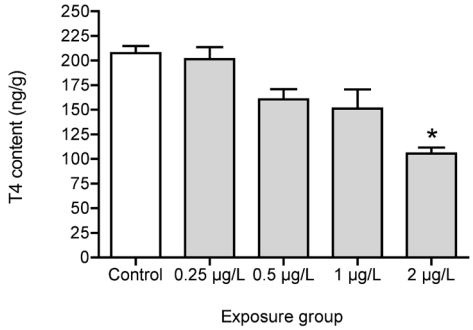

Fig 4.2: Whole body contents of thyroxin (T4) in zebrafish (*Danio rerio*) exposed to 0, 0.25, 0.5, 1, and 2 µg/L T4. Results are given as means ± SEM from three samples per replicate. Asterisks indicate significant differences between exposure and control groups (* $p < 0.05$; Dunn's test).

4.4.3 Histological and ultrastructural alterations in the thyroid

Following exposure to thyroxin, no changes could be detected in the thyroid at the light microscopical level (Fig. 4.3). In general, the thyroid gland consisted of 10 to 20 follicles aligned along the ventral aorta in the gill region. The follicles were spherical to ovally shaped and showed no conspicuous in- or outfoldings in any exposure group. The size of the follicles was not affected by T4 exposure and remained within the species-specific range of variability. The colloid, which turned out to be a very sensitive parameter in PER-exposed zebrafish, neither showed any alterations in tinctorial properties nor any infiltrations. Epithelial cell height was within the control range across all exposure groups. Blood vessel architecture in the gill region did not reveal any abnormalities.

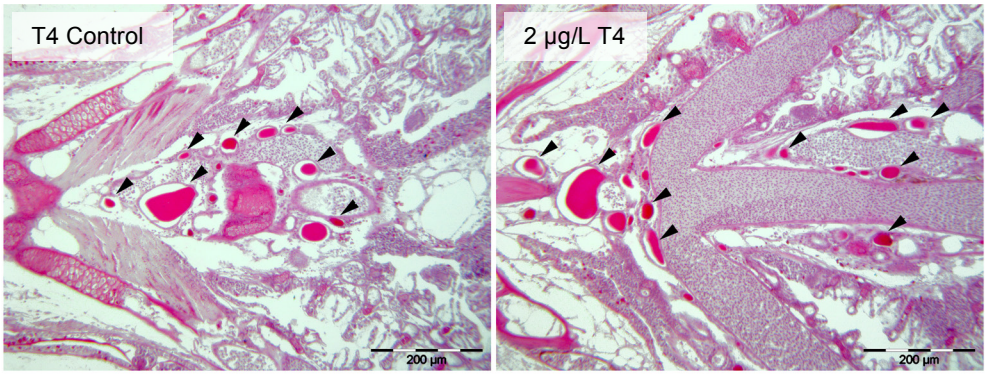

Fig. 4.3: Histology of thyroidal tissue in control zebrafish (*Danio rerio*) as well as zebrafish exposed to 2 µg/L thyroxin (T4). The isolated thyroid follicles (►) are distributed adjacent to the ventral aorta in the gill region. Follicles are spherical to oval and lined with a cuboidal epithelium. The colloid is homogeneously stained. No significant changes can be seen in consequence of T4 exposure. Sections of 2 µm thickness stained with periodic-acid Schiff (PAS) and Mayer's hematoxylin.

In contrast, at the ultrastructural level, a clear reduction in the overall amount of organelles could be detected (Fig. 4.4a – control, 4.4b – 2 µg/L T4). From ≥ 1 µg/L T4, the entire protein-synthesizing apparatus as well as the number of mitochondria are significantly less developed than in controls.

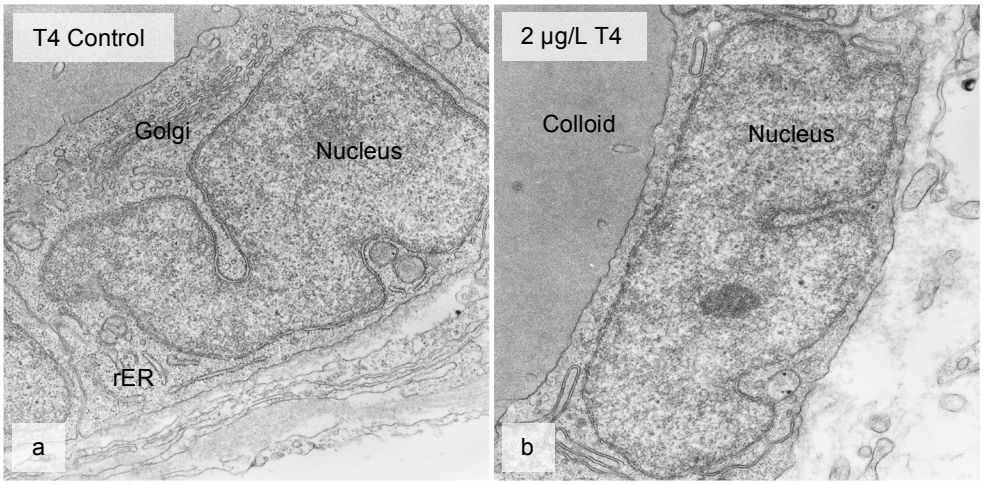

Fig. 4.4: Ultrastructure of thyroidal tissue in control zebrafish (*Danio rerio*) as well as zebrafish exposed to 2 µg/L thyroxin (T4). Thyroids of zebrafish exposed to thyroxin sample are characterized by a conspicuous lack of cell organelles, especially rough endoplasmic reticulum (rER), Golgi fields and mitochondria. Magnification: 16,000x.

4.4.4 Histological and immunohistochemical alterations in the pituitary

With conventional light microscopy, no conspicuous alterations were seen in the pituitaries of T4-exposed zebrafish (Fig. 4.5). Serial PAS-stained sections consistently revealed a clear-cut separation of the neurohypophysis from the adenohypophysis, with the neurohypophysis slightly interdigitating into the adenohypophysis. To further clarify feedback-induced effects on TSH-producing cell populations in the adenohypophysis, immunohistochemical staining was performed in controls and pituitaries of zebrafish from the highest exposure group, respectively (Fig. 4.6). TSH-producing cell counts were not altered at 2 µg/L exposure to T4. Both sample groups revealed a rather homogeneous distribution of TSH-producing cells.

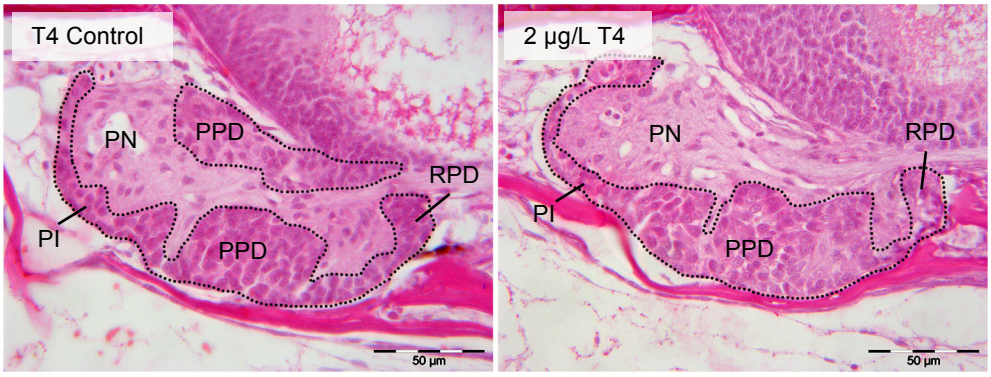

Fig. 4.5: PAS-stained sagittal sections of zebrafish (*Danio rerio*) pituitaries exposed to 0 and 2 µg/L thyroxin (T4). The adenohypophysis is encircled by a dotted line. PI – *pars intermedia* (adenohypophysis); PN – *pars nervosa* (neurohypophysis); PPD – proximal *pars distalis* (adenohypophysis); RPD – rostral *pars distalis* (adenohypophysis). Sections of 2 µm thickness stained with periodic-acid Schiff (PAS) and Mayer‘s hematoxylin.

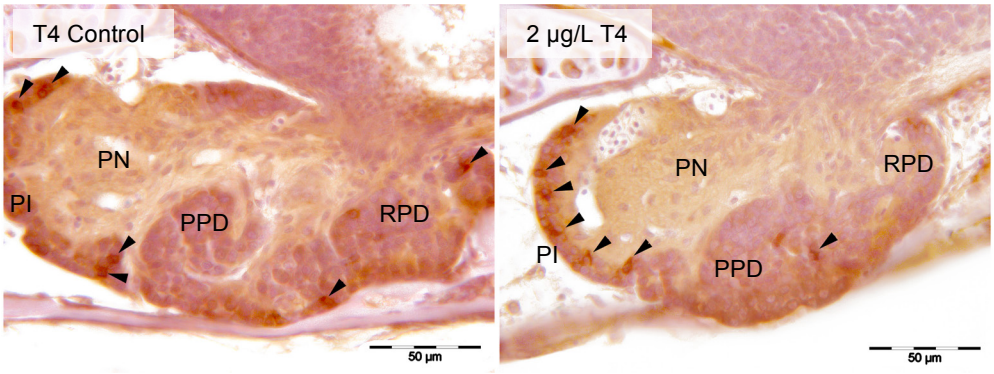

Fig. 4.6: Sagittal sections of immunohistochemically stained zebrafish (*Danio rerio*) pituitaries exposed to 0 and 2 µg/L T4. Immunostained TSH-producing cells are mostly located in the *pars intermdia* (►). Sections of 2 µm thickness immunostained with an anti-TSH antibody (nuclei were counterstained with Mayer's hematoxylin). PI – *pars intermedia* (adenohypophysis); PN – *pars nervosa* (neurohypophysis); PPD – proximal *pars distalis* (adenohypophysis); RPD – rostral *pars distalis* (adenohypophysis).

4.4.5 Histological and ultrastructural alterations in the liver

Histologically, PAS-staining revealed a moderate depletion of glycogen storage deposits in the hepatocytes (Fig. 4.7). This decrease was concentration-dependent with the lowest glycogen values in zebrafish at the highest T4 concentration. Furthermore, glycogen distribution seemed to be more heterogeneous in the higher exposure groups than in controls. Quantitative evaluation of cell distance (i. e. cell size) in the control and the highest T4 exposure revealed a statistically significant decrease of hepatocyte size (Fig. 4.8). Ultrastructurally, a slight decrease of the rough endoplasmic reticulum (rER; Fig. 4.9) could be observed.

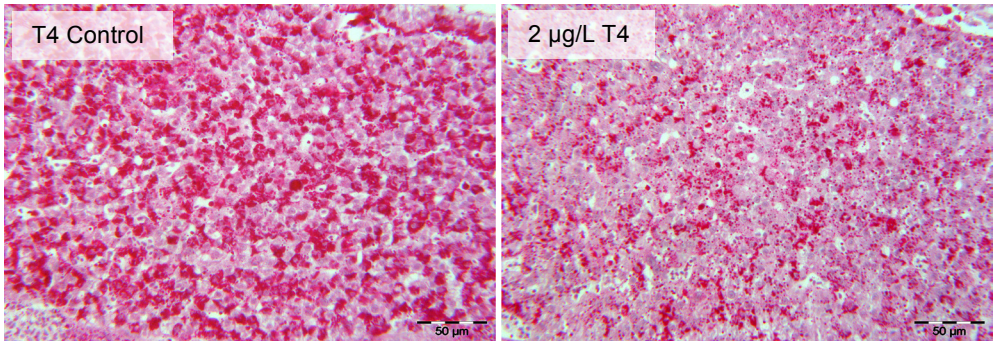

Fig 4.7: Histopathological alterations in hepatocytes. The well-visible glycogen deposits in the control (A) are depleted in the higher concentration groups. Sections of 2 µm thickness stained with periodic-acid Schiff (PAS) and Mayer's hematoxylin.

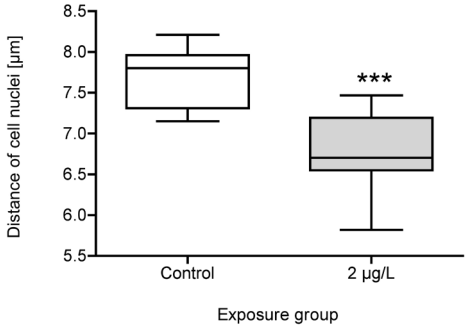

Fig. 4.8: Distance of hepatocyte nuclei in control and thyroxin (T4)-exposed zebrafish (*Danio rerio*). After exposure to T4, hepatocytes show a significant reduction in nuclei distance resulting from reduced cell size (*** $p < 0.001$; Mann-Whitney test).

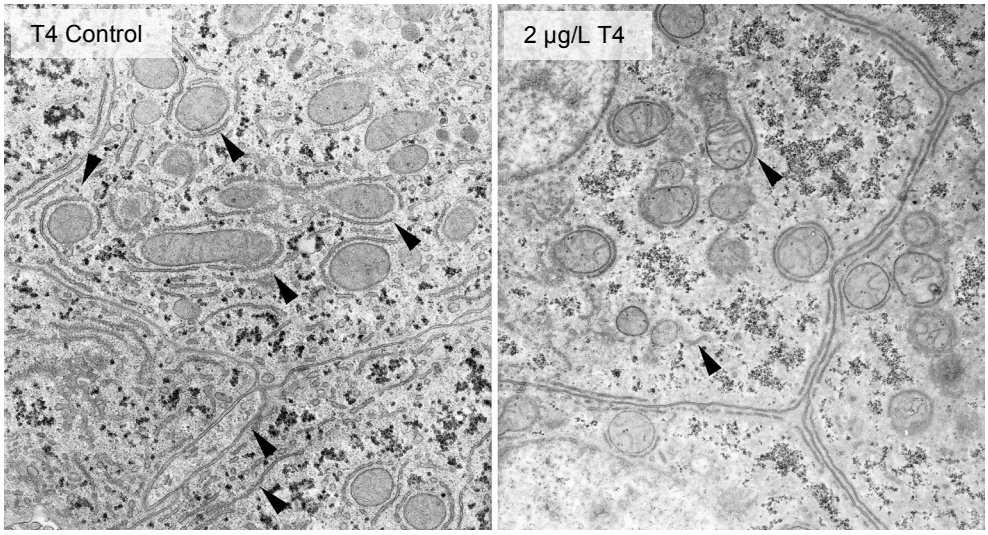

Fig. 4.9: Ultrastructural alterations in hepatocytes of control and T4-exposed zebrafish (*Danio rerio*): If compared to controls, hepatocytes show a moderate reduction of the rough endoplasmic reticulum after T4 exposure (►). Magnification: 12,500x.

4.5 Discussion

The results obtained in this study are in general agreement with previous studies showing that exogenous administration of thyroid hormones enhances growth in teleost fish (Ansal and Kaur 1998, Fagerlund et al. 1984, Higgs et al. 1992, Kumar et al. 1991, Lam and Sharma 1985, Reddy and Lam 1992). In zebrafish, the strongest gain in weight and length was induced by exposure to 0.25 and 0.5 µg/L T4, respectively. Higher concentrations resulted in a dose-dependent decrease. As early as 1973, Lam documented that excessive use of thyroid hormones may result in growth inhibition or even elevated mortality. The maximum concentration of 2 µg/L T4, as used in the present study, did not cause any increase in immobility or mortality. In fact, several studies on zebrafish found toxic effects of T4 exposure: Mukhi et al. (2007) reported on toxic effects at 10 nM (7.8 µg/L) T4, and Liu and Chan (2002) found toxicity at 30 nM (23.3 µg/L) even after short-term exposure. Both of these values are far above the highest concentration used in this study, which could explain the absence of any symptoms of macroscopically observable toxic effects. Since fish growth is generally attributed to enhanced protein synthesis, elevated weight and size at the lower exposure groups can be attributed to the anabolic effect of exogenous administration of T4.

Lin et al. (1994) found enhanced protein synthesis and liver hypertrophy in hybride tilapia (*Oreochromis nitloticus* x *O. aureus*) after exposure to T3. The only histologically observable alteration in the liver found in the present study was glycogen depletion. This effect is in line with studies from Fontaine et al. (1953) and Leray et al. (1970), who reported on decreasing glycogen levels in eel (*Anguilla anguilla*), rainbow trout (*Oncorhynchs mykiss*) and mullet (*Liza aurata*). On the one hand, this decline can easily be explained with higher energy demands of the fish during elevated growth. On the other hand, however, it is not clear why glycogen contents are lowest in the highest T4 exposure group, as this is not the concentration group showing the highest growth stimulation. Since glycogen depletion rather followed increasing T4 concentrations, unspecific stress-induced acceleration of metabolism might also play a role. Since Mukhi et al. (2007) reported acutely toxic effects at 7.8 µg/L T4, the highest concentration used in the present study might account for such sublethal stress phenomena. Likewise, other ultrastructurally observable effects such as the decrease of rER might also be

interpreted as first signs of toxic effects of T4 (Braunbeck 1998, Segner and Braunbeck 1998). Most likely, glycogen depletion together with reductions of rER and mitochondria are responsible for the morphometric reduction in hepatocellular size.

Interestingly, the thyroid did not show any histologically detectable effect, although it had been assumed that T4 exposure would lead to thyroid atrophy. Nevertheless, at the ultrastructural level, a clear inactivation of the thyrocytes was evident in terms of reduced organelle contents, which eventually accounts for the hormone synthesis. Coinciding with these findings, ELISA measurements of whole body T4 content revealed a dose-dependent decrease of T4 with a significant reduction in the highest exposure group. This indicates the dose-dependent inactivation of the thyrocytes, i.e. the progressive inability to produce thyroid hormones themselves. The inactivity of the thyroid did not have any feedback-induced effect in the pituitary. Neither morphological alterations nor discrepancies in TSH-producing cell counts could be detected. The fact that adequate (exogenous) amounts of T4 were present obviously prevented any feedback-induced effects on the pituitary.

4.6 Conclusions

In conclusion, this study revealed a biphasic response pattern to varying thyroxin levels concerning aspects of growth and metabolism. Lower doses predominantly had anabolic effects, whereas higher concentrations acted as catabolic agent. In contrast to several other studies using higher doses of T4, symptoms of acute intoxication could hardly be detected. In this context, it would be interesting to clarify whether prolonged exposure would cause toxic effects or whether T4 concentration would have to be increased. Thyroidal architecture was only affected at the ultrastructural level, but ELISA measurements revealed a dose-dependent decrease in T4-secretion, which coincides with reduced organelle contents of thyrocytes. Probably due to the exogenous availability of T4, no feedback-induced alterations in the pituitary and the amount of TSH-producing cells were seen. Besides effects on growth and development, the liver showed clear signs of glycogen depletion which partly reflects stimulated growth and partly unspecific

stress. Future investigations will attempt to further clarify the responsiveness of the hypothalamic-pituitary-thyroid axis to thyroid-stimulating substances.

Chapter 5

Ultrastructural alterations in zebrafish thyrocytes after exposure to the thyroid-disrupting chemicals propylthiouracil and perchlorate

5.1 Abstract

Histopathology is a common powerful and sensitive approach to evaluate effects of endocrine-disrupting chemicals in the thyroid gland. Since, however, in fish structural effects have hardly been examined at the electron microscopical level, zebrafish were exposed for five weeks to graded sublethal concentrations of the known goitrogens propylthiouracil (2.5, 10, 25, and 50 mg/L) and perchlorate (62.5, 125, 250, 500, and 5000 µg/L) in a modified early life-stage test. Whereas, propylthiouracil induced dose-dependent effects from the lowest test-concentrations in the rough endoplasmic reticulum, only higher concentrations of perchlorate were effective. With both substances, the number of lysosomes was increased and mitochondria showed irregular swelling of the intercristae space. Perchlorate-exposed thyrocytes displayed a massive increase in apical microvilli. At 50 mg/L, propylthiouracil induced first signs of necrosis. Ultrastructural changes in zebrafish thyroid cells could thus be documented to be specific for different endocrine-disrupting chemicals, most likely depending on the inhibiting mechanism of the test chemicals. The knowledge of subcellular changes in thyrocytes will help to better understand and interpret existing histological data.

5.2 Introduction

The thyroid system in vertebrates is essential for growth, development and aspects of reproduction (Brown et al. 2004, Cyr and Eales 1988, Leatherland 1994, Power et al. 2001). On account of these multiple functions, an OECD guideline has been established in 2009 with *Xenopus laevis* as test organism to detect adverse effects of potential thyroid-disrupting substances (OECD 2009). One important observational endpoint in this guideline is thyroid histology, which provides a powerful and most sensitive tool for evaluating effects caused by endocrine-disrupting chemicals (Grim et al. 2009). In contrast to light microscopical effects, however, subcellular effects, which are usually responsible for the manifestation of histologically observable alterations, have not been recorded.

The follicular thyroid gland is visible throughout the vertebrates, except for the larval lamprey (ammocoetes) which possesses an endostyle comparable to the ascidian endostyle. Parts of this organ metamorphosize to the follicular-arranged thyroid gland typically present in vertebrates (Fujita 1975). Among the different cell groups present in the ammocoetes' endostyle the type-2 and -3 cells are considered to be homologous to thyroid cells. The fine structure of these cells is characterized by a well-developed rough endoplasmic reticulum, although no dilated cisternae could be observed. In the infranuclear to the apical part of the cytoplasm numerous vesicles are detectable, i. e. lysosomlike dense bodies, and thyroglobulin-carrying vesicles. At the apical surface, cilia and microvilli can be noted projecting into the endostylar lumen (Hoheisel 1969, 1970). In higher vertebrates, the follicles of the thyroid are typically arranged as a compact organ encapsulated by connective tissue, however, the thyroid gland of teleost fish differs: Instead of a compact organ encapsulated by connective tissue, fish thyroid follicles are usually loosely distributed in the gill region along the ventral aorta (Eales 1979, Raine et al. 2001, Wabuke-Bunoti and Firling 1983); single follicles, however, may also occur in other organs (e.g. Agrawala and Dixit 1979, Sharma and Kumar 1982). Interestingly, the thyroid gland of the existing sarcopterygii display features which are retained in the thyroid glands of the tetrapods, i. e. the gland is compact and encapsulated (Chavin 1976). The follicles themselves consist of a single layer of epithelial cells closely connected to blood capillaries. The fine structure of the thyrocytes is rather well-documented in mammals, but has hardly been studied in fish, eventually due to their difficult accessibility. In mammals, the thyrocytes are characterized by the occurrence of microvilli at the apical surface (Braunsteiner et al. 1953, Dempsey and Peterson 1955, Monroe 1953). The cytoplasm is characterized by well-developed rough endoplasmic reticulum with somewhat dilated cisternae. Mitochondria, club-shaped or oval, with lamellar cristae, are distributed throughout the cytoplasm (Fujita 1975, Pitsiavas et al. 1997, Tsujio et al. 2007). The Golgi apparatus, consisting of smooth-surfaced vacuoles, lamellae, and vesicles, is generally located in the supranuclear region of the thyrocytes. In the subapical region near the Golgi apparatus at least three kinds of granules or vesicles could be identified: (1) small round, less dense vesicles, (2) large colloid droplets, and (3) small highly electron-dense granules (Fujita 1975). It was found that the large colloid droplets are containing reabsorbed colloid from

the follicle lumen (Bauer and Meyer 1965, Ekholm and Smeds 1966, Fujita 1969, Sheldon et al. 1964, Stein and Gross 1964). Nadler et al. (1964) showed, that the small, less dense vesicles contain thyroglobulin synthesized in the rER and transported into the follicle lumen. The electron-dense vesicles, which are mostly located in the apical or subapical regions of the thyrocytes are believed to be primary lysosomes due to their positive acid phosphatase reaction (Fujita 1975, Kosanovic et al. 1968, Seljelid 1965, 1967a, c, Wetzel et al. 1965, Wollman et al. 1964). It could be shown, that the primary lysosomes fuse with reabsorbed colloid droplets at the apical part of the cells making the colloid droplets positive to the acid phosphatase reaction. The droplets belong to the secondary lysosomes (Seljelid 1967d, Seljelid et al. 1971, Wetzel et al. 1965). As mentioned above, studies on teleost fish are scarce compared to mammalian studies, nevertheless, reports on the fine structure have been presented for the Japanese amberjack (*Seriola quinqueradiata;* Fujita and Machino 1965), the Japanese eel *(Anguilla japonica;* Fujita et al. 1966), the Asian sheepshead wrasse *(Semicossyphus reticulatus),* and the marbled rockfish (*Sebasticus marmoratus*; Suemasa et al. 1968). In general, the fine structure addressed in these studies reflects the mammalian thyroid fine structure with slight differences like aggregates of fine filaments in some lysosomelike dense bodies noted in *Seriola, Semicossyphus,* and *Sebasticus*. Furthermore, in the Atlantic stingray (*Dasyatis sabina),* and the Pacific hagfish (*Epaptretus stouti*) occasional apical cilia were found which are not present in mammals (Henderson and Gorbman 1971, Volkoff et al. 1999).

There are numerous goitrogens with different inhibitory modes of action. In the present study, perchlorate, an effective competitive inhibitor of the sodium-iodide symporter, and propylthiouracil, which blocks thyroid peroxidase, were used to provoke reactions of the thyrocytes of the zebrafish (*Danio rerio*). At the light microscopical level, the two substances lead to differing thyroid morphologies (Schmidt and Braunbeck 2011, Schmidt et al. 2011). Ultrastructurally, studies in mammals showed that exposure to methimazole, and propylthiouracil, leads to cuboidal to columnar epithelial cells with numerous microvilli in the apical region of the cells. Moreover, alterations in the mitochondria, proliferations in the rough endoplasmic reticulum as well as an accumulation of moderately dense vesicles could be detected (Fujita et al. 1963, Tsujio et al. 2007). Perchlorate exposure of *Bufo arenarum* tadpoles lead to conspicuous developments of the endoplasmic

reticulum and Golgi complex, as well as to an increase of mitochondria and colloid droplets (Miranda et al. 1996b). To our knowledge, similar information is not available for any teleost species.

The primary objective of the present study was to determine the ultrastructural effects of propylthiouracil and perchlorate on the thyroid of the zebrafish. Both substances were tested at concentrations used in histological studies both in the zebrafish and in *Xenopus laevis* to allow further comparisons between ultrastructure and histology. To the best of our knowledge, this is the first study dealing with ultrastructural alterations in teleost fish thyroid follicles after exposure to endocrine-disrupting chemicals. These results are expected to further our understanding and interpretation of existing histological data on alterations of the thyroid in fish.

5.3 Material and Methods

5.3.1 Animals and husbandry

Fertilized zebrafish (*Danio rerio*) eggs were obtained from the in-house breeding facilities of the Aquatic Ecology and Toxicology Group at the Centre for Organismal Studies, University of Heidelberg. All experiments were conducted in compliance with the institutional guidelines for the care and use of animals as well as with permission by the regional animal welfare commission (AZ 35-9185.81/G-144/07). Fertilized eggs were initially raised in 20 cm Petri dishes in a KB 115 incubator (Binder, Tuttlingen, Germany) at a constant temperature of 27.0 ± 1.0 °C under exposure to the different test solutions. Three days after fertilization, the eggs were transferred into 10 L-flow-through exposure tanks (triplicate water change per day, 27.0 ± 1.0 °C, 12:12 h light:dark cycle; oxygen saturation > 80%). Flow-through conditions guaranteed that ammonia, nitrite, and nitrate were kept below detection limits (0-5, 0.025-1, and 0-140 mg/L, resp.). After hatching, larvae were fed twice daily with Sera Micron (Sera, Heinsberg, Germany) for one week; subsequently, larvae were fed freshly raised *Artemia* nauplii (Sanders, USA) *ad libitum*. Excessive food and feces were regularly removed from the aquaria.

5.3.2 Exposure to endocrine disruptors

Exposures to endocrine disruptors were carried out under flow-through conditions with triplicate water exchange over periods of 5 weeks. Sixty embryos were placed in each of two replicate tanks containing nominal concentrations of 2.5, 10, 25, and 50 mg/L propylthiouracil (Sigma, Deisenhofen, Germany) or 62.5, 125, 250, 500, and 5000 µg/L perchlorate (Sigma). Throughout the exposure, all tanks were inspected daily for dead embryos, which were removed immediately. After 5 weeks, the fish were anesthetized with a saturated solution of 4-ethylaminobenzoate (benzocaine, Sigma).

5.3.3 Ultrastructure

For the ultrastructural studies, samples of the pharyngeal region were fixed in 2.5 % glutardialdehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4 °C for a minimum of 24 hrs and postfixed with 1 % osmium ferrocyanide for two hours (Karnovsky 1971). After triplicate rinsing in sodium cacodylate buffer (pH 7.4), tissues were stained *en bloc* with 1 % uranyl acetate in 0.05 M maleic buffer (pH 5.2) overnight at 4 °C, dehydrated in a graded series of ethanol and embedded in Spurr´s medium (Spurr 1969). For localization of the thyroid area, semi-thin sections were prepared on a Reichert-Jung Ultracut microtome (Leica Microsystems, Nussloch, Germany) and stained with methylene blue / azur II (Richardson et al. 1960). Afterwards, ultrathin sections of 60 nm were cut and counterstained with alkaline lead citrate (Reynolds 1963).

5.3.4 Imaging

Light microscopy of the semi-thin sections was performed with a Leitz Aristoplan microscope (Leitz, Wetzlar, Germany) equipped with a ColorView Soft Imaging Systems digital camera (Soft Imaging Systems, Münster, Germany). Ultrathin sections were examined in a Zeiss EM 10 transmission electron microscope (Carl Zeiss, Oberkochen, Germany).

5.4 Results

5.4.1 Controls

The thyrocytes of the control fish of both experimental groups were morphologically comparable to each other (Tables 5.1, and 5.2; Fig. 5.1). The epithelium enclosed a homogeneously stained colloid without any inclusions (Fig. 5.1a). The flat to cuboidal thyrocytes displayed a basally located nucleus with evenly dense chromatin and regularly shaped nucleoli (Fig. 5.1b). Overall, the number of organelles was small; most organelles were located in the apical part of the cells (Fig. 5.1e). At the border to the follicle lumen, few short microvilli were present (Fig 5.1c). Under higher magnification, the mitochondria appeared with spherical, ovoid, and elongated shapes (Fig. 5.1d, e). The endoplasmic reticulum comprised of slightly branched cisternae and Golgi fields were regularly present (Figs. 5.1d, e). In addition to the Golgi vesicles, electron dense vesicles were visible in the apical part of the cells (Figs. 5.1b, d, e).

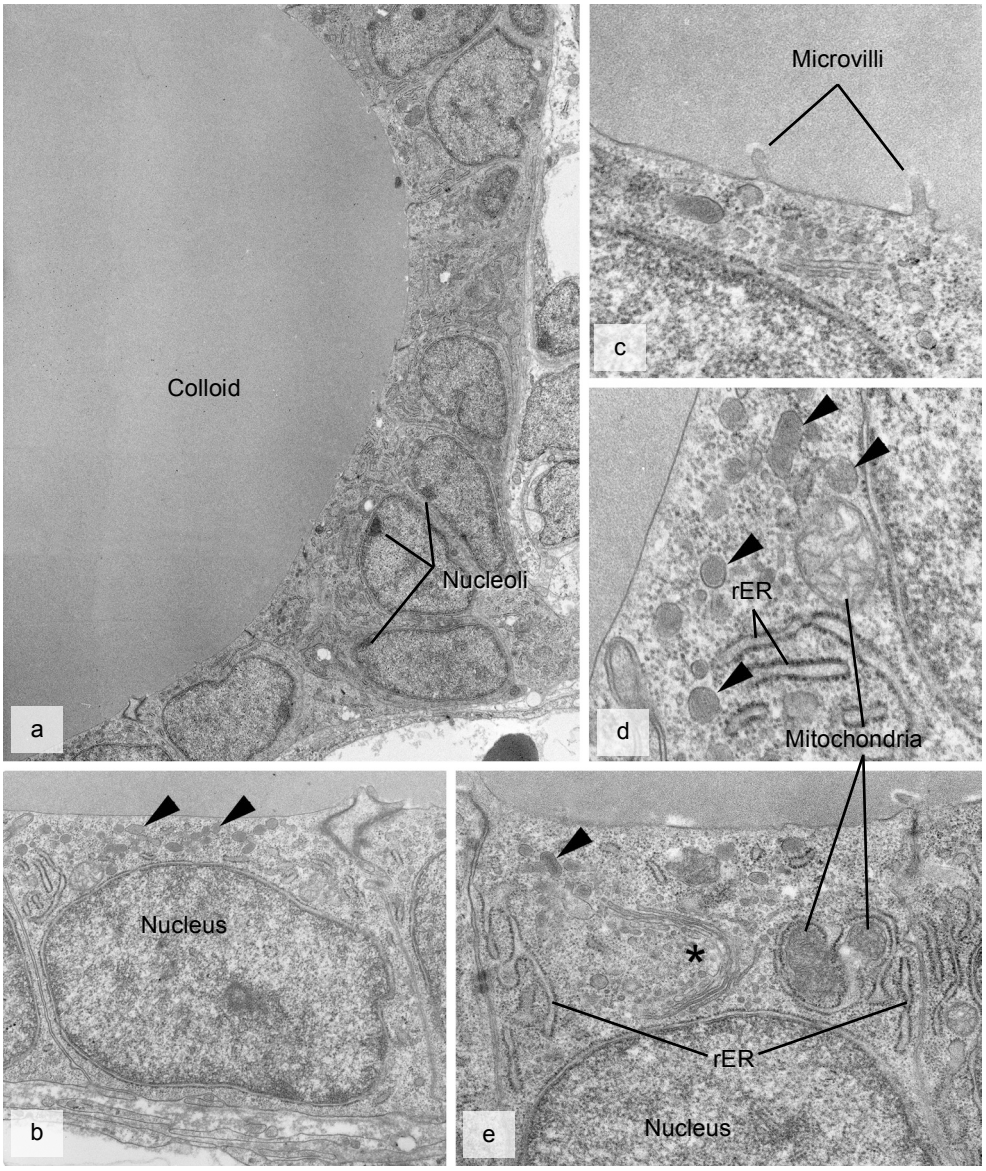

Fig. 5.1: Ultrastructure of thyroidal tissue in control zebrafish (*Danio rerio*): The epithelium encloses an evenly stained colloid devoid of any inclusions (a). The nucleus is basally located and most of the organelles can be found in an apical position (b, e). Mitochondria appear spherical to ovally shaped, the rough endoplasmic reticulum and Golgi fields (*) are of cistern-like appearance (d, e). At the apical pole of the thyrocytes, few electron-dense lysosomes (►), and, at the border to the colloid, some microvilli are detectable (b, c, d, e). Magnification: a – 2,000x, b – 10,000x, c – 12,500x, d – 40,000x, e – 31,500x.

Table 5.1: Results of thyroid ultrastructural changes in zebrafish (*Danio rerio*) treated with propylthiouracil.

	Propylthiouracil				
	Control	2.5 mg/L	10 mg/L	25 mg/L	50 mg/L
Nucleus					
Irregular outline					+
Amount of heterochromatin					++
Mitochondria					
Proliferation			+	++	++
Swelling			+	++	++
Ruptured cristae		++	++	+++	+++
Lysosomes					
Proliferation		+	+	+	++
Lipofuscinogenesis					
Colloid					
Inclusions			++	++	++
Electron density	evenly stained	evenly stained	evenly stained	evenly stained	blotchy
Rough endoplasmic reticulum					
Proliferation		++	++	+++	+++
Fenestration		+	+	++	++
Dilation		+	+	++	++
Apical vesicles		+	+	+	++
Large electron-dense vesicles			++	++	++
Endo-/exocytosis activity		+	+	+	+
Microvilli	+	+	++	++	++
Necrosis	-	-	-	-	+
Cell height	+	+	++	++	+++

- = not observed; + = little developed; ++ = moderately developed; +++ = markedly developed

5.4.2 Propylthiouracil (PTU) exposure

Following exposure to propylthiouracil (PTU), thyrocytes displayed numerous dose-dependent effects (Table 5.1). The cell height dose-dependently increased to low columnar or even highly columnar thyrocytes in the highest exposure group. At 50 mg/L, condensed thyrocytes with electron-dense cytoplasm and shrunken nuclei (Fig. 5.2a) were visible, which can be regarded as a first sign of cell necrosis. The nucleus appeared less regular (Figs. 5.2a, b), and the amount of heterochromatin was increased at 50 mg/L PTU. First alterations in the rough endoplasmic reticulum (rER) were evident from the lowest concentration group (2.5 mg/L PTU) and were markedly increased at 25 and 50 mg/L (Figs. 5.2a, b).

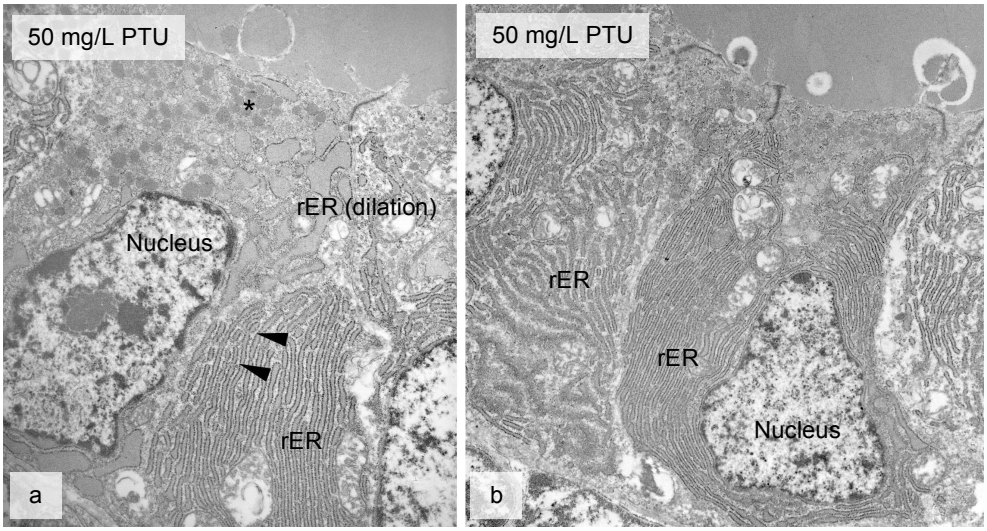

Fig. 5.2: Ultrastructure of PTU-exposed zebrafish thyroids. At 50 mg/L, an electron-dense cytoplasm and shrunken nuclei represent first symptoms of necrosis (a). Increased amounts of heterochromatin are visible (a, b). Marked proliferation, dilation and fenestration in the rough endoplasmic reticulum (►) are further alterations ((a, b). The apical regions display proliferations of lysosomes (*; a). Magnification: a – 10,000x, b – 4,000x.

In addition to massive proliferation, fenestration and dilation of the rER occurred (Figs. 5.2a, 5.3a). Mitochondria showed a moderate proliferation and swelling was visible (Fig. 5.3a, b). Additionally, they were characterized by extremely distorted cristae associated with a strong inflation of the inter-cristae space at concentrations ≥ 2.5 mg/L PTU with a marked increase at concentrations ≥ 25 mg/L, where the majority of the mitochondria was affected (Figs. 5.3a, b, c).

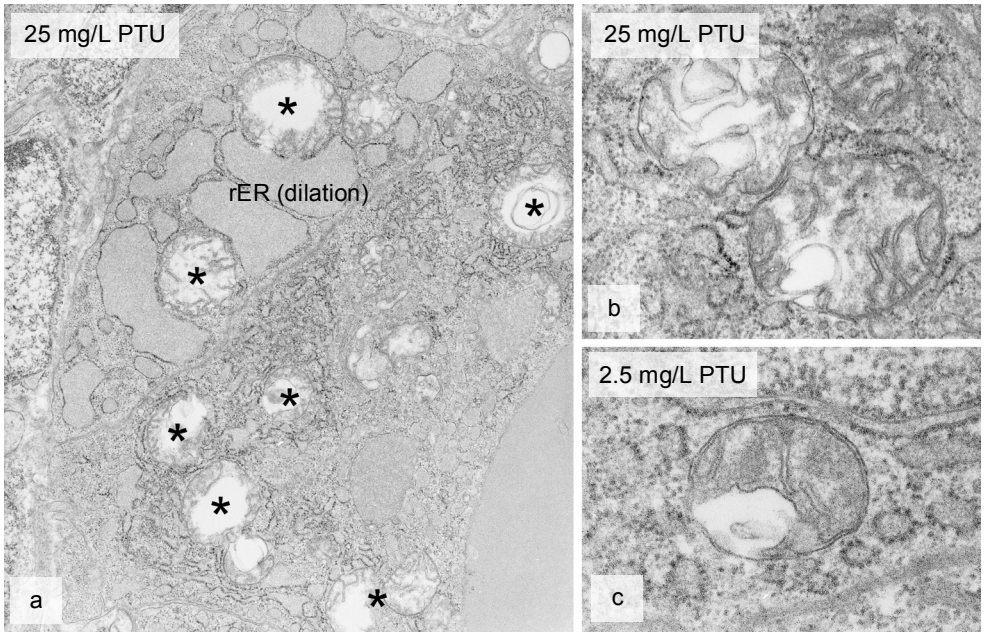

Fig. 5.3: Mitochondrial alterations in zebrafish exposed to PTU. Already at 2.5 mg/L, mitochondria showed irregular swelling of the intercristae space (c). The higher exposure groups displayed proliferations, and extensive swellings (*, a; b). Furthermore, massive dilation of the rER is visible (a). Magnification: a – 10,000x, b, c – 40,000x.

In the apical region of the cells, a strongly concentration-dependent proliferation of electron-dense bodies could be detected (Fig. 5.2a). At concentrations ≥ 10 mg/L PTU the colloid was interspersed with cytoplasmic inclusions (Fig. 5.4a). The electron density of the colloid remained intermediate, but exposure to 50 mg/L PTU led to cloudy tinctorial properties. From 2.5 mg/L PTU, there was a protrusion of apical vesicles into the follicular lumen, which was most prominent at 50 mg/L PTU (Fig. 5.4b). Together with the occurrence of apical vesicles, endo- or exocytosis was detectable at concentrations ≥ 10 mg/L PTU (Figs. 5.4b, c). Furthermore, the apical part of the thyrocytes displayed short microvilli, which moderately increased with PTU concentrations (Fig. 5.4b): Only at 10 mg/L PTU microvilli appeared slightly elongated.

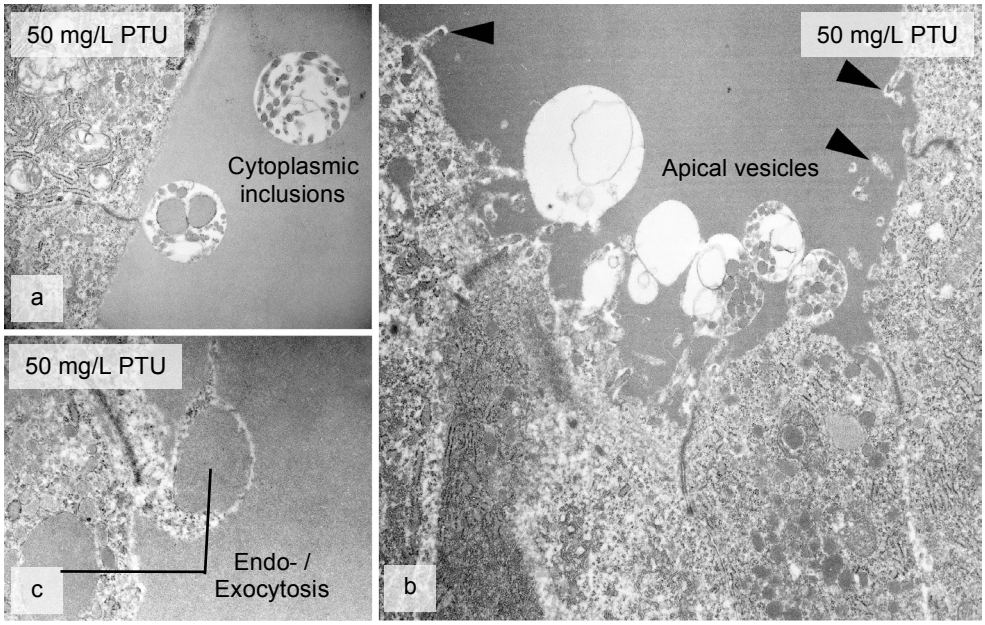

Fig. 5.4: Apical alterations in zebrafish thyrocytes caused by PTU exposure. At concentrations ≥ 10 mg/L PTU, cytoplasmic extrusions were evident (a): At concentrations ≥ 2.5 mg/L PTU, numerous apical vesicles were seen protruding into the follicular lumen (b). Bleb-like structures indicate endo- or exocytotic processes at concentrations ≥ 10 mg/L PTU (b, c). Moderate proliferation of microvilli can be observed at concentrations ≥ 10 mg/L (►; b). Magnification: a – 12,500x, b – 8,000x, c – 20,000x.

5.4.3 Perchlorate (PER) exposure

Exposure to perchlorate (PER) led to numerous effects, which, however, were only partially dose-dependent (Table 5.2). Epithelial cell height dose-dependently increased from flat to cuboidal in the controls to columnar in the highest exposure group.

Table 5.2: Results of thyroid ultrastructural changes in zebrafish (*Danio rerio*) treated with perchlorate.

	Perchlorate					
	Control	62.5 µg/L	125 µg/L	250 µg/L	500 µg/L	5 mg/L
Nucleus						
Irregular outline			+	++	++	+
Amount of heterochromatin			+		++	+
Mitochondria						
Proliferation				+	+	+
Swelling		+	+	+	+	+
Ruptured cristae		+	++	++	++	++
Lysosomes						
Proliferation		+	++	++	++	+++
Lipofuscinogenesis		+	++	+	++	+
Colloid						
Inclusions					+	+
Electron density	evenly stained	slightly slighter	slightly lighter	slightly lighter, cloudy	lighter	very light
Rough endoplasmic reticulum						
Proliferation					+	++
Fenestration					+	+
Dilation				+		+
Large electron-dense vesicles			+	+	+	++
Endo-/exocytosis activity				+	+	+
Microvilli	+	++	++	++	++	+++
Cell height	+	+	++	++	++	+++

+ = little developed; ++ = moderately developed; +++ = markedly developed

The nucleus started to reveal first effects at concentrations of 125 µg/L displaying an irregular outline and little increases of heterochromatin. These effects increased at concentrations of 250 and 500 µg/L (Fig. 5.6b) but were less evident at the highest concentration of 5000 µg/L. The rER was only affected in concentrations ≥ 500 µg/L showing moderate proliferations, and fenestrations (Fig. 5.5d). Additionally, slight dilations were observable at 250 µg/L and 5000 µg/L only. From the lowest PER concentration (62.5 µg/L), mitochondrial morphology was affected in terms of swellings. Part of them displayed dilation of the inter-cristae space and distortion of cristae (Fig. 5.5a). At concentrations ≥ 62.5 µg/L the number of electron-dense bodies with increasing lipofuscin agglomerations were elevated (Fig. 5.6a). The highest concentration group of 5000 µg/L revealed a further marked increase of electron-dense bodies located in the apical part of the thyrocytes (Figs. 5.5b, c, d). In contrast, the accumulation of lipofuscin did not seem to be dose-dependent; however, it was restricted to the thyrocytes of PER-exposed zebrafish. Only moderate increases of lipofuscin agglomerations could be found at 125 and 500 µg/L PER (Fig. 5.6a).

At ≥ 125 µg/L PER, large vesicles appeared usually in an apical location; at 125 µg/L PER, these vesicles showed rod-like shapes, and at 250 µg/L some fibre-like inclusions were present. Concentrations ≥ 62.5 µg/L revealed dose-dependent increases of the apically located microvilli (Fig. 5.5b, c). Both the number and the length of the microvilli were increased.

Concentrations ≥ 500 µg/L induced small amounts of colloid inclusions. The electron density of the colloid dose-dependently decreased at concentrations ≥ 62.5 µg/L (Fig. 5.5c). Apical vesicles were not present.

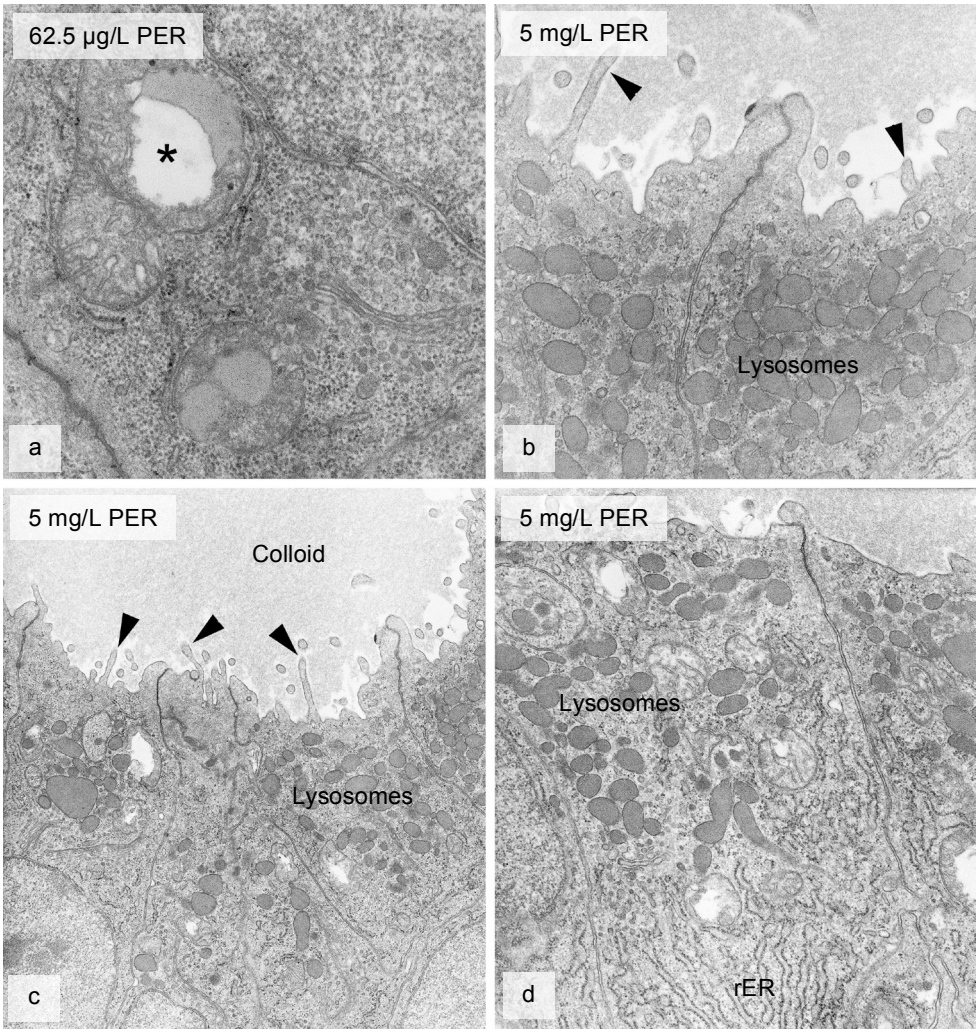

Fig. 5.5: PER-induced ultrastructural alterations in zebrafish thyrocytes: At concentrations ≥ 62.5 µg/L, mitochondria are swollen and display irregular swellings of the intercristae space (a). At concentrations of 5000 µg/L a marked increase of lysosomes mostly located in the apical part of the thyrocytes was visible (b, c, d). The rough endoplasmic reticulum showed moderate proliferation and some fenestration (d). The electron density of the colloid markedly decreased in the higher concentration groups (c). Proliferations of microvilli are observable in concentrations ≥ 62.5 µg/L (►). Magnification: a – 31,500x, b – 20,000x, c – 10,000x, d – 16,000x.

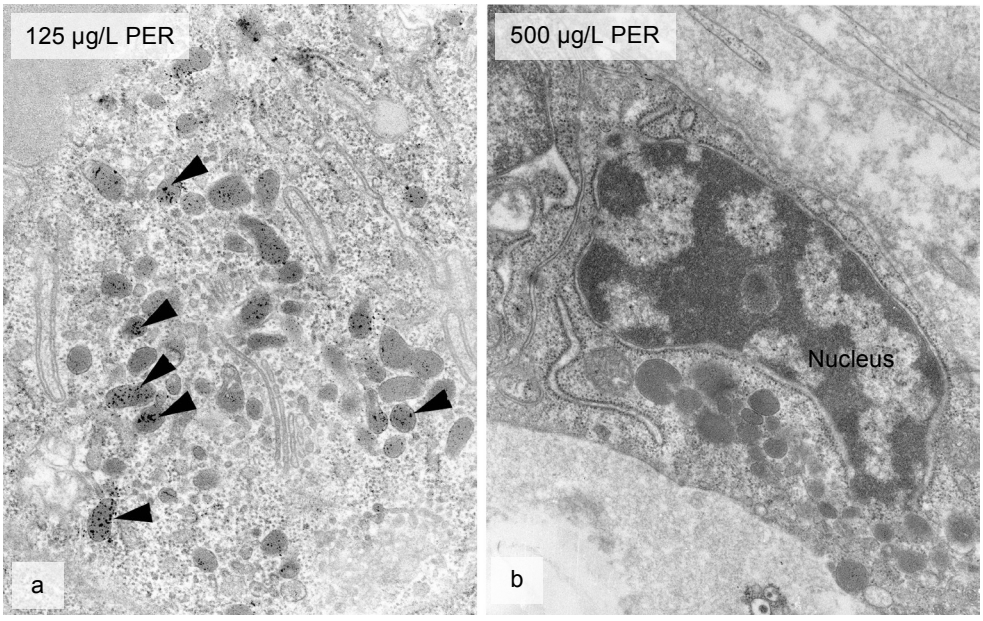

Fig. 5.6: Further alterations of PER-exposed zebrafish thyrocytes comprise a moderate increase of lipofuscin within lysosomes (►; a). Moreover, the nucleus showed increased amounts of heterochromatin, and an irregular outline, especially at 125 and 500 µg/L PER exposure (b). Magnification: a – 20,000x, b – 12,500x,.

5.5 Discussion

To date, most of the ultrastructural studies in the thyroid have been performed in higher vertebrates, which is probably at least partly due to the much easier accessibility of the gland. Principally, follicular ultrastructure in zebrafish thyroids is similar to mammalian (Fujita 1975, Pitsiavas et al. 1997, Tsujio et al. 2007) or avian ultrastructure (French and Hodges 1977, Handa and Chiasson 1980) except for the presence of apically located cilia or flagella, which to date could only be detected in mammals (Tashiro 1964, Tashiro and Sugiyama 1964), birds (Fujita 1963, Muramoto 1964), cyclostomes, stingrays, and hagfish (Fujita and Honma 1966, Henderson and Gorbman 1971, Volkoff et al. 1999). Most of the histologically detectable alterations in amphibians and fish caused by the exposure to endocrine-disrupting chemicals can be confirmed and specified more precisely at the ultrastructural level at very low exposure levels and, thus, help to understand the underlying modes of action. Hence, the present study was designed to elucidate the ultrastructural changes in zebrafish thyrocytes after exposure to propylthiouracil and perchlorate.

Results document that both propylthiouracil and perchlorate lead to severe ultrastructural alterations with clear-cut discrepancies due to the differing underlying modes of action. The occurrence of increased numbers of electron-dense bodies, microvilli, apically located luminal vesicles, and significant exo- and endocytotic activity are well-defined indicators of colloid reabsorption and thyroidal activation (French and Hodges 1977, Fujita 1975, Henderson and Gorbman 1971, Olen 1969) probably due to thyroid-stimulating hormones. The electron-dense bodies observed were addressed in numerous other studies (Fujita and Machino 1965, Henderson and Gorbman 1971, Leatherland et al. 1978, Leatherland and Sonstegard 1980) but to date it is still not of concluding certainty what these really are. Usually, the smaller, electron-dense droplets typically located in the apical or subapical regions are regarded as lysosomes, especially with the appearance of lipofuscin, whereas the larger droplets are regarded as colloid reabsorption from the follicle lumen. Wright et al. (1978) demonstrated the appearance of thyroglobulin in large (colloid) droplets. Nevertheless, this aspect definitely needs further clarification. The occurrence of fibre-like inclusions in some of the droplets of perchlorate-exposed fish has also been described in the

Japanese amberjack (*Seriola quinqueradiata*), which showed aggregates of numerous wavy fine filaments (Fujita and Machino 1965). These filaments were discussed as having an intimate relationship with old or altered droplets (Fujita and Machino 1965).

Interestingly, lysosomal architecture and microvilli appearance clearly differed between the two substances in that perchlorate caused much stronger lysosomal alterations than propylthiouracil: In addition to the mere increase, perchlorate led to pronounced lipofuscin agglomerations within the lysosomes. Even at the lowest perchlorate concentration, low amounts of lipofuscin could be detected. Lipofuscin is known as an age pigment that progressively accumulates within lysosomes in long-lived post-mitotic cells (Brizzee et al. 1969, Donato and Sohal 1981, Strehler 1964a, b). Studies in rats revealed a thyrocyte turnover time of about 0.5 years (Dumont et al. 1980), and in mice a turnover time of about 70 days could be detected (Galand 1967). In zebrafish, the thyroid cell turnover times are unknown and it is difficult to correlate from homeotherm mammals to poikilotherm zebrafish but eventually the turnover times might be high enough to enable accumulations of lipofuscin inside the lysosomes of zebrafish. On the other hand, lipofuscin is formed within secondary lysosomes (primary lysosomes fused with colloid droplets) due to an interplay of two processes, (1) the production of partially reduced oxygen species by mitochondria and (2) the autophagocytotic degradation within lysosomes (Brunk et al. 1992). The chemical-induced creation of reduced oxygen species strikingly coincides with mitochondrial architecture. After exposure to both propylthiouracil and perchlorate, mitochondrial swelling and proliferation in conjunction with extremely distorted cristae were detectable from the lowest concentrations, although lysosomal lipofuscin could only be detected in perchlorate-exposed fish. Interestingly, similar effects were observed in white leghorn chicken after exposure to propylthiouracil and methimazole (Handa and Chiasson 1980) and in iodide-treated BB/W rats (Li and Boyages 1994). Based on reviews by Hotchkiss et al. (2009), Skulachev (2006), Tsujimoto and Shimizu (2007), and Ulivieri (2010), the effects observed are regarded as clear signs of necrosis, which was most evident in the highest propylthiouracil group.

Mitochondrial damage is generally seen as a strong indicator of oxidative stress which can be reproduced in rats by using hyperoxia as a model of increased

generation of endogenous free radicals (Gille et al. 1989). Oxidative stress impairs free radical fluxes which could overcharge the capacity of the antioxidant defense system, thus leading to damaged cellular functions (Gille et al. 1989). In fact, perchlorate is known to be actively taken up by the thyrocytes (Dohan et al. 2007, Tran et al. 2008), but not to be metabolized (Anbar et al. 1959). At least inside rat thyrocytes, perchlorate leads to an iodide efflux (Scranton and Halm 1965, Surks 1967). It might be hypothesized that the perchlorate accumulation could easily interfere with the intracellular antioxidant defense system similar to iodide, which would then in turn lead to mitochondrial swelling.

Since, in contrast to perchlorate, propylthiouracil did not cause any lipofuscin agglomerations in the lysosomes, the aforementioned processes can only be used to explain perchlorate-induced mitochondrial alterations. The commonly known mode of action of propylthiouracil is the blockade of thyroid peroxidases which prevents thyroid hormone synthesis. The subsequent activation of thyroidal tissue *via* thyroid-stimulating hormone could lead to an increased influx of iodide and Na^+ into the thyrocytes *via* the sodium-iodide symporter. As shown by Li and Boyages (1994), both iodide excess and propylthiouracil exposure affected the mitochondria. It is known that mitochondria may show a sudden increase in the permeability of the inner mitochondrial membrane for solutes smaller than 1,500 Da, which may than result in mitochondrial swelling (Halestrap et al. 2002, Zoratti and Szabo 1995). Either way, most likely the affected mitochondria were not capable of producing sufficient amounts of ATP, depletion of ATP stores might than result in, e. g., the accumulation of lysosomes and the dysfunction of ATP-dependent processes, e. g. the sodium-iodide symporter, which in turn could affect iodide supplies. Finally, the decrease of ATP-levels will inevitably lead the cell towards necrotic death (Crompton 1999, Halestrap et al. 2002).

Conspicuous alterations by *in vivo* exposure to propylthiouracil were found within the rough endoplasmic reticulum. Apparently, rER proliferation, fenestration, and dilation are common reactions of thyrocytes exposed to substances inhibiting thyroid peroxidases, since similar reactions were observed *in vivo* after contamination of Wistar rats with methimazole (Tsujio et al. 2007) and exposure of white leghorn chicken to propylthiouracil (Handa and Chiasson 1980). The marked alterations of the rough endoplasmic reticulum suggests that both

propylthiouracil and − to a lower extent − perchlorate induce disruptions of the protein-sorting pathways leading to a drug-induced form of protein storage disease. The possible problems in protein production could further lead to the loss of essential proteins involved in cellular homeostasis, leading to cellular death.

The thyroids of especially perchlorate-exposed zebrafish displayed marked proliferation and elongation of the apical microvilli, which strikingly correlated with the staining properties of the colloid. Perchlorate is known to lead to iodide efflux from the thyroid gland in rats (Scranton and Halm 1965, Surks 1967). In mice, perchlorate rapidly increases the secretory response of the thyroid to thyroid-stimulating hormone, including both iodide and iodothyronines (Rousset et al. 1977). Furthermore, it is known that exposure of PER results in depleted colloid stores in zebrafish (Patino et al. 2003, Schmidt et al. 2011). In humans, the colloid consists of 19S thyroglobulin, larger iodoproteins and smaller protein fractions (Anderberg et al. 1980, 1981). In these studies, exposure to carbimazole resulted in depleted thyroglobuline aggregates. Assuming a similar colloid composition in zebrafish, the efflux of iodide and thyroglobuline due to the exposure to perchlorate would result in an altered composition of the colloid and, thus, to different electron density. In this context, the marked proliferation of apical microvilli could support colloid depletion due to the enormous surface multiplication. Furthermore, perchlorate concentrations ≥ 250 µg/L additionally revealed enhanced endo- or exocytotic activity which could also be found in propylthiouracil concentrations ≥ 2.5 mg/L. Interestingly, the time-dependent activating responses to thyroid-stimulating hormone seems to be dependent on the mode of action of the test substance, since propylthiouracil induces endo- and exocytotic activity prior to microvilli elongation whereas in perchlorate-exposed samples microvilli elongation occurs first.

In contrast, alterations in nuclear morphology cannot be considered as suitable endpoint due to its low sensitivity in propylthiouracil-exposed fish. Perchlorate-treated samples showed an increasingly irregular outline of the nucleus at concentrations ≥ 125 µg/L. In parallel, the amount of heterochromatin moderately increased at higher concentrations of both substances. Despite the relatively moderate extent, the nuclear changes could be interpreted as first reactions of the

thyrocytes leading to cellular death seen at the highest propylthiouracil concentration.

When dealing with effects of thyroid-disrupting chemicals the feedback mechanism which is responsible for thyroid homeostasis has to be taken into account. The thyroid is regulated by a negative feedback loop with the pituitary acting as the main control organ excreting thyroid-stimulating hormone (TSH). If the concentration of thyroid hormones decrease due to inhibiting substances, elevated concentrations of TSH are responsible for activating thyroid hormone synthesis. Against that background, studies describing the alterations of the thyrocytes after application of TSH alone are very interesting. Numerous studies address this aspect, e. g. in tadpoles (Neuenschwander 1972), chicken (Fujita 1963), and especially in rats (Fujita and Suemasa 1968, Lupulescu et al. 1968, Roos 1960, Seljelid 1965, 1967a, b, c, d, e, Wetzel et al. 1965, Wissig 1963). In these studies remarkable similarities to the present effects could be found, e. g. increase in cell height, dilation of rER cisternae, and alterations of the microvilli. Thus, the influence of thyroid-stimulating hormone on the ultrastructural appearance of the thyrocytes is remarkable. Nevertheless, this influence alone cannot explain the differences of the effects between the two test substances. Both perchlorate and propylthiouracil lead to increased stimulation by TSH, thus, the alterations of the thyrocytes should not deviate too much. The fact, that the thyrocytes exposed to perchlorate differ from thyrocytes exposed to propylthiouracil can only be explained with the intrinsic modes of action of the test substances. This aspect needs further clarification in the future.

In the context of endocrine disruption thyroid histopathology provides an excellent powerful and sensitive tool for the detection of thyroid-disrupting substances (Grim et al. 2009); histopathological endpoints in the thyroid, however, are usually limited to rather general endpoints such as hyperplasia and hypertrophy. In contrast, ultrastructural investigations not only confirm histopathological observations, but also provide much more detailed information to answer the question as to the underlying mechanisms of thyroidal activities. Especially at lower exposure levels, these thyroidal processes will be designed to compensate for disrupting (mostly inhibiting) effects of the goitrogens in zebrafish. The closer inspection of morphological processes revealed that fundamental cellular

parameters and functions such as the structural organization of the rough endoplasmic reticulum, mitochondrial architecture, the number of lysosomes together with lipofuscinogenesis and signs of colloid reabsorption (i. e. microvilli architecture), large electron-dense droplets, apically located luminal vesicles, and significant exo- and endocytotic activities, displayed clear-cut differences between the two substances due to the unique underlying modes of action.

5.6 Conclusions

In conclusion, the present study highlights the value of zebrafish as sensitive test organism in a non-mammalian model of thyroid goitrogenesis to improve our understanding of (fish) thyroid function and histopathological alterations. For the first time, goitrogen-induced alterations in zebrafish thyrocyte ultrastructure were evaluated in an attempt to further our understanding of histopathological data. Obviously, the proliferation of microvilli, large electron-dense droplets, apically located luminal vesicles and significant exo- and endocytotic activity are common features of thyroidal activation *via* thyroid-stimulating hormones. Nevertheless, the differential modes of action of goitrogens lead to different changes in thyrocyte architecture. Beside the massive proliferation of the rough endoplasmic reticulum in propylthiouracil-exposed zebrafish thyroids, the most surprising effect was severe alterations in mitochondrial morphology in both propylthiouracil- and perchlorate-exposed zebrafish. In fact, the occurrence of distinct indications of oxidative stress followed by first signs of necrosis induced by both test substances seems to be a key property of thyroid-inhibiting substances. The cytopathological observations appear even more important, since they document effects on thyroid development in zebrafish larvae; in larval and juvenile development, functional thyroid hormone synthesis is essential. The list of ultrastructural effects under different inhibiting situations provides a valuable resource for further comparative studies addressing mechanistic aspects of thyroid pathology as well as the evaluation and in-depth-interpretation of the existing histopathological endpoints for detection of thyroid-disrupting xenobiotics in zebrafish.

Chapter 6

Final discussion and conclusion

The zebrafish (*Danio rerio*) provides a valuable tool for evaluating different aspects of aquatic ecotoxicology and general toxicology. There are numerous official test guidelines with *Danio rerio* as the preferred test organism. Nevertheless, its usability for the detection of goitrogens has hardly been examined. Rather, for the evaluation of thyroid-disrupting chemicals, the OECD launched the amphibian metamorphosis assay in 2009 (OECD 2009). Nevertheless, the main test organisms in ecotoxicology are fish, and effects on their thyroid system have hardly been studied. Therefore, the purpose of this thesis was (1) to elucidate the usability of the zebrafish for the detection of thyroid-disrupting chemicals, (2) to describe effects of well-known reference compounds along the hypothalamic-pituitary-thyroid-axis (hpt-axis) with special emphasis on feedback-induced effects, and (3) to briefly compare the sensitivity of the zebrafish to the well-established test organism *Xenopus laevis*.

For this end, the well-known test substances propylthiouracil (PTU) and perchlorate (PER) as inhibiting substances and thyroxin (T4) as stimulating substance were selected because of their well-known modes of action. For a complete evaluation, effects were traced along the hpt-axis, i.e. in the thyroid, the pituitary, and the liver as the main teleost site for T4 conversion to the biologically active T3. Since thyroid, pituitary and liver represent most important and easily accessible organs along the hpt-axis, the present study focused on all of these three organs to obtain a more comprehensive picture for the evaluation of thyroid-disrupting substances.

Exposure experiments of this study clearly showed that the zebrafish is a sensitive test organism for substances blocking thyroid peroxidases and substances interfering with the sodium-iodide symporter. These differing modes of action are responsible for the different histopathologically and ultrastructurally observable effects in the thyroid. Probably due to the analogous decrease of thyroid hormones in both exposure experiments and the resulting influence on the negative feedback axis, the alterations in the pituitary were identical in terms of increasing TSH cell counts. The downstream-located liver did not show specific reactions related to the modes of action of the test substance. Rather general effects such as glycogen depletion could be observed. Compared to the well-defined effects following PTU and PER exposure, T4 did not result in any histopathologically detectable effect in

the thyroid compared to the corresponding control group. Nevertheless, its thyroidal architecture was affected at the ultrastructural level, which revealed clear signs of inactivation, i.e. decreased organelle contents. Table 6.1 summarizes the sensitivity of the dose-dependent effects caused by PTU, PER and T4.

Table 6.1: Overview of the sensitivity of the dose-dependent histopathological and ultrastructural effects in the thyroid and the liver of zebrafish (*Danio rerio*) after exposure to PTU, PER and T4. Endpoints with effects in the exposed samples that were not dose-dependent are not listed.

Effects	PTU	PER	T4
Effects in the thyroid			
Follicles			
Total number	++++	+	
Size	++++	+	
Shape	+++	+	
Blood vessels			
Hyperemia	+++		
Colloid			
Reduced homogeneity	+++	+	
Reduced density	+++	++	
Colloid depletion		+	
Foamy texture		++	
Cellular inclusions			
Epithelial cells			
Cell height	+++	+	
Stratification	++		
Cell crowding	+++		
Mitochondria			
Proliferation	+++	++	
Swelling	+++	++++	
Ruptured Cristae	++++	++++	
Decreased number			+
Lysosomes			
Proliferation	++++	++++	
Decreased amount			+
Colloid			
Inclusions	+++	+	
Electron Density	+	++++	
Apical vesicles	++++		
Rough endoplasmic reticulum			
Proliferation	++++	+	
Fenestration	++++	+	
Dilation	++++	++	
Decreased amount			+
Microvilli	+++	++++	+
Cell shape	+++	++++	
Effects in the liver			
Glycogen depletion	+++	++	++

+ = less sensitive; ++ = moderately sensitive; +++ = sensitive; ++++ = very sensitive

Hyperemia and TSH-related effects

The gross morphology of zebrafish exposed to PTU revealed striking effects in the thyroid and associated tissues with massive effects in the supporting blood vessels. The proliferation observed for adjacent blood vessels lead to severe hyperemia at concentrations ≥ 25 mg/L PTU. This effect was most prominent in goitrous tissue, where the connection between follicles and supporting blood vessels was highly evident. Connors et al. (1988, 1991) reported correlations between TSH-concentration and thyroid gland blood flow in rats. Although TSH concentrations were not measured in the present study, immunohistological staining of TSH-producing cells in pituitaries of the highest concentration group revealed a significant increase of these cells indicating an elevation of TSH concentrations. As a consequence, the effects observed in the present study would be caused by elevated TSH concentrations as a consequence of the negative feedback in the hypothalamic-pituitary-thyroid axis due to the exposure to PTU.

Although PER-exposed fish revealed moderate increases in TSH-producing cells as well, no signs of hyperemia could be detected. One possible explanation could be the lower concentrations of PER used in the study leading to a lesser influence on the negative feedback regulation. Compared to PTU, PER-exposed samples only revealed moderate increases in TSH-producing cell counts in the pituitary. Another aspect could be the effectiveness of the substances themselves. As mentioned above, PTU blocks thyroid peroxidases which are directly responsible for the synthesis of TH. PER on the other hand “only” competitively interferes with iodide uptake across the basal membrane of the thyrocytes. It may be assumed that despite the presence of PER some amounts of iodide still “leak” through the membrane which can then be used for TH synthesis. Another point is the storage capacity of the follicles, where substantial amounts of iodide are stored in the colloid, which could act as a depot or buffer for the decreasing iodide transport across the basal membrane. This mechanism could easily compensate part of the inhibiting effect of PER, which then would lead to a lower effect on feedback-induced regulation *via* TSH compared to PTU.

The colloid as a sensitive parameter

In PER-exposed fish, the structure of the colloid turned out to be the most sensitive endpoint. In line with various other studies (Crane et al. 2005, Mukhi and Patino 2007, Patino et al. 2003), colloid depletion increased with higher perchlorate concentrations. The most intriguing observations were the dose-dependent changes of the texture and the reduced density of the colloid, which was detectable at concentrations ≥ 250 µg/L (histopathology) and ≥ 62.5 µg/L (ultrastructure), respectively. It is known that PER leads to iodide efflux from the thyroid gland in rats (Scranton and Halm 1965, Surks 1967). Furthermore, in mice, PER rapidly increases the secretory response of the thyroid to TSH, including both iodide and iodothyronines (Rousset et al. 1977). An increased iodide secretion would automatically lead to decreasing levels of iodide in the colloid itself due to the inhibiting effect of PER on the sodium-iodide symporter. In humans, the colloid consists of 19S thyroglobulin, larger iodoproteins and smaller protein fractions (Anderberg et al. 1980, 1981). In these studies, exposure to carbimazole resulted in depleted thyroglobulin aggregates. Assuming a similar colloid composition in zebrafish, the efflux of iodide and thyroglobulin due to the exposure to perchlorate would result in an altered composition of the colloid and, thus, to different PAS-staining properties. Strikingly correlating with the histological staining properties of the colloid (above all with colloid depletion) are ultrastructural effects: PER caused marked proliferation and elongation of the apically located microvilli. These changes could support colloid depletion due to the enormous surface multiplication. Furthermore, PER concentrations ≥ 250 µg/L additionally revealed enhanced endo- or exocytotic activity further contributing to the dose-dependent decrease of the colloid.

In contrast to the studies with perchlorate, colloid depletion could not be identified as a significant PTU-induced effect although it directly inhibits the thyroid peroxidases located at the border of the follicular lumen to the colloid. Partially or totally depleted follicles were present in every PTU exposure group including the control, although complete depletion could never be observed in the PTU study. Nevertheless, concentrations ≥ 10 mg/L PTU led to clear-cut alterations in colloid homogeneity and density, and from 25 mg/L PTU a foamy, granular texture was well visible in contrast to the homogenous, smooth texture in controls. Exposure to

carbimazole, which belongs to the same group of chemicals as PTU, leads to a decrease of the larger thyroglobulin aggregates compared with the relative amount observed in the colloid from normal human thyroid tissue (Anderberg et al. 1980, 1981). This decrease was explained with an insufficient capacity to iodinate thyroglobulin. Such assumptions could provide an explanation for the different staining properties and the granular texture observed in the PTU exposure groups. Moreover, ultrastructural analysis revealed PTU to induce endo- and exocytotic activity and microvilli elongation, which are both clear signs of colloid reabsorption.

Hyperplasia and hypertrophy – two mechanisms to activate the thyroid gland

Hyperplasia and hypertrophy are two alternatives to accomplish thyroid activation in various species (Goleman et al. 2002b, Grim et al. 2009, Miranda et al. 1996a, Yamasaki et al. 2002). These changes are likely the result of negative feedback-induced secretion of TSH by the pituitary, which is known to react to exposures to thyroid-disrupting chemicals by intensified production of TSH. TSH-production is under the negative feedback influence of both T4 and T3 (Pradet-Balade et al. 1999, Yoshiura et al. 1999). The activating effect of TSH is mediated *via* a G-protein-coupled TSH-receptor (Farid and Szkudlinski 2004), which is mainly expressed in thyroidal tissue and the gonads (MacKenzie et al. 2009). Both PTU and PER showed a distinct increase in follicle number, but rather moderate hypertrophy effects. Thus, at least in juvenile fish, the first step to up-regulate TH-production *via* TSH seems to be an increase in cell number (hyperplasia) rather than an increase in cell size (hypertrophy). Likewise, exposure to both substances resulted in follicle migration into adjacent gill arches. Limited space in the central pharyngeal region and increasing demand for THs could lead to such an invasion of thyroid tissue into the gills. Although gill tissues seemed not to be effected *per se*, a displacement was obvious, leading to the question whether reduced functionality of gill tissues could lead to any respiratory consequences for the fish. In the present studies, no signs of respiratory problems could be detected, but oxygen concentration in the tanks was kept near saturation throughout the experiments. There are studies showing that at least embryonic stages of fish may be capable of surviving oxygen rates of 5 % when acclimated to non-lethal oxygen

concentrations (Rees et al. 2001, Strecker et al. 2011) showing numerous adaptions, e.g. metabolic rate reduction or increased ventilation rates, hematocrit and hemoglobin oxygen affinity, which was detected in common sole (*Solea solea*) (Dalla Via et al. 1994, Rankin and Jensen 1993). Nevertheless, the factors that trigger a proliferation of thyroid follicle number rather than an increase of follicle size are unclear and need further investigation.

Effects on epithelial cell height

Epithelial cell height represents a classical parameter to detect thyroid activation (Eales and Brown 1993, Goleman et al. 2002b, Miranda et al. 1996b). Contrary to Goleman et al. (2002a), who suggested epithelial cell height as the most sensitive endpoint in PER-exposed *Xenopus laevis* tadpoles, only a minor increase in epithelial cell height could be detected in PER-exposed zebrafish, which, nonetheless, indicates an activation of thyroidal tissue. In PTU-exposed fish, an increase in epithelial cell height was conspicuous at concentrations ≥ 10 mg/L PTU; however, it did not reach the sensitivity of effects seen in the follicles, i.e. hyperplasia and hypertrophy. An increase in cell height usually coincided with cell crowding and (pseudo-)stratification of the epithelium best observable at the highest PTU concentration. These effects are clear signs of a massive hypertrophy triggered by a negative feedback-induced stimulation of TSH.

Is the thyroid the only target for perchlorate and propylthiouracil

One of the purposes of the present study was to provide a detailed description of the effects of PTU, PER and T4 along the hypothalamic-pituitary-thyroid axis together with feedback-induced effects. However, in case of PER, it is not clear if the thyroid is the only target for the inhibiting effect of the substance. In terrestrial vertebrates, almost all iodide is taken up *via* food in the gut (Eales and Brown 1993). Fish, on the other hand, have the capacity to take up iodide from the ambient water across their extensive gill surface (Hunn and Fromm 1966). Additionally, it is known from salmonids that they can obtain substantial amounts *via* the diet (Gregory and Eales 1975). Nevertheless, the main contributor to iodide intake is suggested to be the branchial iodide pump (Higgs and Eales 1971).

However, the transporting mechanism for iodide across the gill surface is still not fully understood, but there is evidence that it is the same sodium-iodide symporter that is also responsible for the uptake in the thyroid follicles (Hunn and Fromm 1966). In this case, PER would not only inhibit the uptake of iodide in the thyroid, but also in the gills; this is an aspect that has not yet been taken into account, but needs further clarification.

Ultrastructural effects – lipofuscin and mitochondrial damages

Besides the clear differences in histopathology, ultrastructural observations of the thyroid gland emphasize the influence of the mode of action of the test substance on the observable effect. Both PTU and PER stimulated distinct ultrastructural appearances. The most conspicuous effect in PTU-exposed samples were the massive alterations in the rER. Interestingly, lysosomal architecture and microvilli appearance clearly differed between the two substances, and PER caused much stronger lysosomal alterations than PTU. Besides the mere increase, it led to conspicuous lipofuscin agglomerations in the lysosomes of PER-exposed fish. Even at the lowest PER concentration group, low amounts of lipofuscin could be detected. Lipofuscin is known as an age pigment that progressively accumulates within lysosomes in long-lived post-mitotic cells (Brizzee et al. 1969, Donato and Sohal 1981, Strehler 1964a, b). Eventually, this contributes to the lipofuscinogenesis observed in perchlorate-exposed zebrafish thyrocytes.

On the other hand, lipofuscin is formed within secondary lysosomes due to an interplay of two processes, (1) the production of partially reduced oxygen species by mitochondria and (2) the autophagocytotic degradation within lysosomes (Brunk et al. 1992). The existence of reduced oxygen species is strikingly coinciding with mitochondrial architecture. In both exposure scenarios, mitochondrial proliferation and irregular swelling of the inter-cristae space were detectable from the lowest concentrations onwards, whereas lysosomal lipofuscin could only be detected in PER-exposed fish. Interestingly, similar effects were observed in white leghorn chicken after exposure to PTU and methimazole (Handa and Chiasson 1980) and in iodide-treated BB/W rats (Li and Boyages 1994). Based on reviews by Hotchkiss et al. (2009), Skulachev (2006), Tsujimoto and Shimizu

(2007), and Ulivieri (2010), the observed effects are regarded as clear signs of necrosis, which was most evident in the highest PTU exposure group.

In general, mitochondrial damage is seen as a strong indicator of oxidative stress, which can be reproduced in rats by studies using hyperoxia as a model of increased generation of endogenous free radicals (Gille et al. 1989). Oxidative stress impairs free radical fluxes, which could overcharge the capacity of the antioxidant defense system leading to damaged cellular functions (Gille et al. 1989). Furthermore, PER is known to be actively taken up by the thyrocytes (Dohan et al. 2007, Tran et al. 2008), but not to be metabolized (Anbar et al. 1959). At least inside rat thyrocytes, perchlorate leads to an iodide efflux (Scranton and Halm 1965, Surks 1967). It might be hypothesized, that the PER accumulation could easily interfere with the intracellular antioxidant defense system similar to iodide, which would then in turn lead to mitochondrial swelling. However, in contrast to PER, PTU did not cause any lipofuscin agglomerations in the lysosomes; thus, the aforementioned processes can only be used to explain PER-induced mitochondrial alterations.

The commonly known mode of action of PTU is the blockade of thyroid peroxidases, which prevents thyroid hormone synthesis. The subsequent activation of thyroidal tissue *via* thyroid-stimulating hormone could lead to an increased influx of iodide and Na^+ into the thyrocytes *via* the sodium-iodide symporter. As shown by Li and Boyages (1994), both iodide excess and PTU exposure affected the mitochondria. It is known, that mitochondria may show a sudden increase in the permeability of the inner mitochondrial membrane for solutes smaller than 1,500 Da, which may then result in mitochondrial swelling (Halestrap et al. 2002, Zoratti and Szabo 1995). Either way, most likely, the affected mitochondria were not capable of producing sufficient amounts of ATP; depletion of ATP stores might then result in, e.g., the accumulation of lysosomes and the dysfunction of ATP-dependent processes, e.g. the sodium-iodide symporter, which in turn could affect iodide supplies. Finally, the decrease of ATP-levels will inevitably lead the cell towards necrotic death (Crompton 1999, Halestrap et al. 2002).

Compared to histopathology, which, although being limited to rather general endpoints (usually hyperplasia and hypertrophy), already provides a powerful and sensitive tool for the detection of thyroid-disrupting substances (Grim et al. 2009), the ultrastructural investigations in the present study deliver first hints to the

underlying mechanisms of thyroidal attempts to compensate the inhibiting effects of the goitrogens tested in zebrafish. The closer analysis of the effects revealed that fundamental cellular functions displayed clear-cut differences between the two substances due to the underlying modes of action.

Feedback-induced effects in the pituitary

The effects observed in the thyroid are clear signs of an activation triggered *via* the hypothalamic-pituitary-thyroid axis. The critical hormone involved in this process is TSH, which belongs to the glycoprotein family having an alpha-subunit identical to FSH and LH. The beta-subunit is structurally distinct and confers hormone-specific functions (Pierce and Parsons 1981). The present exposure studies with PTU and PER document that the decreasing negative feedback is responsible for proliferation of basophilic cells within the proximal *pars distalis*. Morphometrical analysis clearly showed that the increase of adenohypophyseal tissue followed a concentration-dependent pattern in both exposure scenarios. However, it is unknown whether these morphological alterations lead to any physiological consequences. Although no signs of abnormal behavior of the fish throughout the experiments were observed, additional tests seem necessary to clarify potential consequences. Interestingly, the effects observed in the pituitary do not differ between PTU and PER, probably due to the same feedback-induced mechanism that caused TSH increases.

In order to describe the morphological alterations in the pituitary, the ratio of adeno- to neurohypophysis turned out to be a very precise indicator. It is known that TSH-producing cells together with LH- and FSH-producing cells are basophilic and PAS-positive in histological staining. The control and the highest concentration groups of both PTU and PER revealed a significant increase of TSH-producing cells after immunostaining. The increased number of cells is thus capable of producing the TSH concentrations required to stimulate the thyroid. These treatment-related changes of TSH-producing cell counts contribute to compensate for reduced TH production due to PTU and PER exposure. Nevertheless, ELISA-measurements of T4 contents revealed an obvious decrease of T4 in both exposure scenarios implying the failure of the thyroid system to compensate for the inhibition by PTU and PER. The inability to produce sufficient

amounts of T4 could be due at least in part to the enormous concentrations of PTU and to a lower extent of PER used in the studies.

An important aspect of pituitary regulation is the responsiveness to the negative feedback loop. In our experiments, fish were exposed for 35 days, starting directly after fertilization. At early stages of zebrafish larval development, the absence of thyrotropic hormones does not affect thyroid hormone production or growth of follicles and both processes (Alt et al. 2006). On the other hand, nothing is known about the response of the pituitary to environmental exposure at early larval stages. In zebrafish, the first thyroid follicles appear around 60 hours post fertilization (Alt et al. 2006b), and an increasing number of follicles appear after the onset of thyroid hormone (T4) production at around 72 hpf (Elsalini et al. 2003). At least in the first few days of development, maternally derived thyroid hormone is likely to compensate for the lack of zygotic thyroid hormone following exposure to endocrine disruptors, although the role of thyroid hormones in early larval development is not clear (Power et al. 2001).

Feedback-induced impacts on downstream-located organs

The impact on the negative feedback axis in both PTU- and PER-exposed fish raises the question if any downstream-located organ or tissue shows effects as well. In this regard, the liver is a promising organ because in teleost fish it is the main site for the conversion of T4 to the biologically active hormone T3 and the regulation of major fractions of circulating active T3 (Cheng et al. 2007). Histologically, however, the only observable effect in the liver in both exposure scenarios was glycogen depletion. In general, glycogen regression is described as either hormone-induced (Gluth and Hanke 1985) or as an unspecific stress reaction (Braunbeck et al. 1992, Braunbeck 1994). Thyroid hormone levels are known to be able to affect glycogen contents *in vivo* in the liver of the red sea bream (*Chrysophrys major*; Woo et al. 1991) and *in vitro* in hepatocytes isolated from the silver sea bream (*Sparus sarba*; Leung and Woo 2010), thus providing evidence for the observed depletion of glycogen contents. In addition, a reduction of cellular energy reserves generally indicates higher energy requirements of individuals (Braunbeck 1992), and long-term exposure to high concentrations of toxicants can also lead to a depletion of glycogen (Segner and Braunbeck 1990).

Similarly to the histologically detectable unspecific effects, only general toxic effects directly caused by the substances themselves were visible at the ultrastructural level. In this context, an interesting study by Chen et al. (2009) on quail chicks revealed that liver tissue is not protected against hypothyroid conditions. Decreased plasma TH concentrations lead to an over-expression of 5'-deiodinase in the liver. This suggests that the liver responded to hypothyroidism with an increased T3 production. Moreover, this study discovered that young quail chicks were influenced by hypothyroidism in the first two weeks of perchlorate exposure, but not after 7.5 weeks. The present studies on zebrafish discovered a dose-dependent decrease of T4 concentration; however, T3 was not measured. Future studies should concentrate on possible correlations in fish.

Macroscopical effects – whole body weight, length and goitrous tissue

Beside histopathological and ultrastructural effects along the hypothalamic-pituitary-thyroid axis, macroscopical effects were detectable in PTU, PER and T4 exposed fish. One of the most interesting findings was the appearance of goitrous tissue in PTU-exposed fish located at the lower jaw region. These goiters consisted of densely packed blood capillaries in close contact to small follicles. Neither PER nor T4 samples produced similar alterations. Effects of PTU and PER on whole body length and weight were moderate, but T4 revealed interesting effects: Both endpoints revealed a biphasic response pattern. The lowest concentration group (0.25 µg/L) led to a statistically significant increase of whole body weight followed by a moderate decline. The highest exposure group still showed increased values but not statistically significant. Compared to the control, whole body length significantly increased at 0.25 and 0.5 µg/L T4 exposure followed by a slight decline as well. Here, the values of the two highest concentration groups were slightly lower compared to the control group.

The results gained in this study are in agreement with several other studies showing that exogenous administration of thyroid hormones stimulates growth in teleost fish (Ansal and Kaur 1998, Fagerlund et al. 1984, Higgs et al. 1992, Kumar et al. 1991, Lam and Sharma 1985, Reddy and Lam 1992). The maximum weight and length gain was caused by 0.25 and 0.5 µg/L T4, respectively. Higher concentrations led to a dose-dependent decrease. Lam (1973) stated that the

excessive use of thyroid hormones either result in growth inhibition or mortality. The maximum T4 concentration used in the present study did not cause any increases in immobility or mortality, however, growth was inhibited in the higher concentration groups when compared to the lower exposure groups. Several studies on zebrafish found toxic effects of T4 exposure. Mukhi et al. (2007) reported toxic effects at 10 nM (7.8 µg/L) T4 and Liu and Chan (2002) found toxicity at 30 nM T4 (23.3 µg/L) even after short-term exposure. Both of these values are far above the highest concentration used in the present study, which could explain the absence of any macroscopically observable toxic effects of T4. Fish growth is generally attributed to enhanced protein synthesis; thus, higher weight and length levels at the lower exposure groups can be attributed to the anabolic effect of exogenous administration of T4. Lin et al. (1994) found enhanced protein synthesis and liver hypertrophy in tilapia after exposure to T3. In conclusion, exposure to T4 revealed a biphasic response pattern with respect to growth and metabolism. Lower T4 doses had anabolic effects, while higher doses acted as catabolic agent. In contrast to several other studies using higher doses of T4, intoxications could hardly be detected.

Xenopus laevis vs. Danio rerio – a brief comparison of two model test organisms

In comparison with the well-established amphibian metamorphosis assay and its model test organism *Xenopus laevis,* the sensitivity of the zebrafish is similar, but depends on the test substance. As mentioned above, the substances used in this study and their concentrations were analogically chosen to earlier studies in *Xenopus laevis* to allow a comparison of the relative sensitivities of both model test organisms. A detailed overview of the sensitivities of *Xenopus* compared to the zebrafish is given in Table 6.2. In contrast to *Xenopus laevis* or comparable anuran test models, which offer different additional validated endpoints related to metamorphosis such as hind limb length, snout to vent length, developmental stage, wet weight, thyroid histology and mortality, the zebrafish from this study were primarily investigated histologically and ultrastructurally. If present, additional macroscopical alterations were noted.

Table 6.2: Comparison of LOEC and NOEC data for amphibians and zebrafish exposed to PER and PTU

Chemical	Species	Endpoint	LOEC [mg/L]	NOEC [mg/L]	Reference
$KClO_4$	*B. arenarum*	Histopathology	340		Miranda et al. 1996b
NH_4ClO_4	*X. laevis*	Metamorphosis	0.06		Goleman et al. 2002a
		Histopathology	0.06		
		Whole-body T4	14		
NH_4ClO_4	*X. laevis*	Metamorphosis	0.005		Goleman et al. 2002a
		Tail resorption	0.020		
NH_4ClO_4	*X. laevis*	Metamorphosis	0.038		Goleman et al. 2002b
		Histopathology	0.038		
$NaClO_4$	*X. laevis*	Metamorphosis	14		Goleman et al. 2002b
		Histopathology	0.038		
NH_4ClO_4	*X. laevis*	Metamorphosis	0.07		Fort et al. 2000
$NaClO_4$	*X. laevis*	Histopathology	0.016		Tietge et al. 2005
		Metamorphosis	0.25		
$NaClO_4$	*X. laevis*	Histopathology	0.062	< 0.06	Tietge et al. 2005
$NaClO_4$	*X. laevis*	Histopathology	0.125	0.062	Schmidt 2006
$KClO_4$	*D. rerio*	Histopathology	0.250	0.125	Present study
		Ultrastructure	0.0625		
PTU	*X. laevis*	Metamorphosis	75		Opitz et al. 2005
PTU	*X. laevis*	Metamorphosis	1.5		Fort et al. 2000
PTU	*X. laevis*	Metamorphosis	10	5	Degitz et al. 2005
		Histopathology	10	5	
PTU	*X. tropicalis*	Metamorphosis	75		Mitsui et al. 2006
PTU	*X. tropicalis*	Metamorphosis	75	20	Carlsson and Norrgren 2007
PTU	*X. laevis*	Histopathology	10		Blank 2004
PTU	*D. rerio*	Histopathology	2.5		Present study
		Ultrastructure	2.5		

Exposure to perchlorate

In contrast, the amphibian thyroid gland seems to be slightly more susceptive to exposure to perchlorate compared to the thyroid of the zebrafish. The LOECs of the studies mentioned in Table 6.2 range from 5 µg/L to 340 mg/L, but the species and the endpoint have to be taken into account. 340 mg/L was measured in *Bufo arenarum*, the other values are all derived from experiments with *Xenopus laevis*. If only considering the histopathological results, the LOECs in experiments with *Xenopus laevis* range from 16 to 125 µg/L. In the studies by Goleman et al. (2002a, b), the tadpoles were exposed for 70 d, whereas Tietge et al. (2005) and Fort et al. (2000) exposed for only 14 d. Morphometrical evaluation of 21 d perchlorate-exposed *Xenopus laevis* tadpoles in the framework of the amphibian metamorphosis assay revealed first statistically significant alterations at concentrations ≥ 125 µg/L (LOEC); the NOEC was determined to be 62.5 µg/L (Schmidt 2006). The quantitative evaluation makes these values highly reliable.

In the zebrafish, first histologically detectable alterations were visible at concentrations ≥ 250 µg/L (LOEC). Ultrastructurally, first alterations were already detectable at the lowest concentration group of 62.5 µg/L (LOEC); thus, a NOEC could not be determined. The fact that histopathologically visible effects manifest themselves at the ultrastructural level makes the latter technique highly sensitive and should not mistakenly be compared to the histopathologically derived results in the amphibian studies.

Although amphibians seem to be slightly more sensitive to exposure to perchlorate, the histologically detectable effects differed from the effects observed in the zebrafish. Goleman et al. (2002a) suggested epithelial cell height to be the most sensitive parameter in *Xenopus laevis* tadpoles, which could be confirmed in own studies (Schmidt 2006). Nevertheless, in the present study on perchlorate-exposed zebrafish, the colloid turned out to be the most sensitive parameter. A common effect in both model test organisms was an increase in the number of follicles.

Exposure to propylthiouracil

In case of PTU, the LOECs of the amphibian studies mentioned in Table 6.2 are in the range of 1.5 to 75 mg/L. Morphometrical analyses of *Xenopus* tadpole thyroids showed that the LOEC after 21 day exposure was at 10 mg/L (Blank 2004). This concentration revealed first effects in epithelial cell height, follicle diameter, total diameter of the lobe, and maximal area of the gland.

Apparently, the zebrafish reacts slightly more sensitive to disruptions of the thyroid peroxidases compared to amphibians: The LOEC was determined to be 2.5 mg/L, the NOEC could not be derived. The follicles showed clear effects at concentrations of 2.5 mg/L as total number increased leading to proliferations of follicles along the ventral aorta. Furthermore, follicle size was already affected at 2.5 mg/L. At 10 mg/L PTU, almost every endpoint was affected including epithelial cell height. Interestingly, in contrast to *Xenopus laevis* tadpoles, the zebrafish revealed massive hyperemia visible as clear-cut increases of blood vessels and conspicuous red-colored goiters in the lower jaw region and colorations of the opercular region. In the tadpoles, markedly deformed marginal blood vessels were detected, but this effect is probably caused solely by the massive proliferation of the thyroid lobes (Blank 2004). Ultrastructurally, the zebrafish revealed first effects in the lowest concentration group (2.5 mg/L) – mainly in the rER. The fact that a NOEC could neither be determined for histopathology nor for the ultrastructure should be taken into account in future studies which should address this issue to precisely define the NOEC.

Exposure to thyroxin

The presence of T4 as a stimulating substance did not cause any histologically detectable effect in *Danio rerio*, although severe reductions in organelle contents could be detected at the ultrastructural level for concentrations ≥ 1 µg/L. In comparison to zebrafish, *Xenopus laevis* showed statistically significant increases in epithelial cell height at concentrations ≥ 1 µg/L (Schmidt 2006). At first sight, this result seems confusing, but it could be shown that overall tadpole development was markedly increased. Nieuwkoop and Faber (1956) divided tadpole development in 66 morphologically distinct stages. In the abovementioned study in

Xenopus laevis it could be shown that upon test termination the majority of the animals were in stage 57 and 58, whereas – with the exception of one animal – all tadpoles from the highest T4 concentration group (2 µg/L) were in stage 60 to 62. In this context, epithelial cell height was shown to generally increase during metamorphic development (Regard 1978). Thus, the measured increase of epithelial cell height only reflects the higher metamorphic developmental stage and cannot be regarded as direct effect of exogenous application of T4. It can be concluded that neither the zebrafish nor *Xenopus* tadpoles reveal histologically detectable effects at least in the used concentration range ≤ 2 µg/L.

Overall, it is evident that – depending on the test compound – zebrafish is roughly as sensitive or even slightly more sensitive as *Xenopus laevis*. Therefore, it will be a matter of discussion whether thyroid disruption could not also be measured in fish tests, which have to be carried out anyway in the context of various regulations. From an animal welfare point of view, it might also be argued that implementation of thyroid endpoints in existing fish testing protocols might not only allow a reduction of financial budgets, but also a reduction of the numbers of animals (in this case amphibians) spent during testing procedures.

The present thesis clearly documents that the zebrafish is a suitable test organism for the detection of goitrogens due to its similar sensitivity compared to *Xenopus laevis*. At the histological level, it could be shown that the hypothalamic-pituitary-thyroid axis reacts at different levels to the exposure to thyroid-disrupting substances. The main target and concurrently the most sensitive tissue was the thyroid gland itself, showing distinct histopathological alterations after exposure to both PTU and PER. At the ultrastructural level, the thyroid revealed an even higher sensitivity compared to classical histopathology, and the differences between the single substances were even more pronounced. Thus, dependent on the mode of action of the specific test substance, the reaction of the thyroid gland was unique and could therefore help to identify possible modes of action of unknown substances. In this context, further chemicals with different known modes of action should be tested and compared to evaluate the distinct histological and ultrastructural differences.

Furthermore, feedback-induced effects on the pituitary could be described. The detection of these effects is highly valuable because of the leading role of the pituitary controlling the entire endocrine system. It is unknown if morphological alterations in the pituitary as observed in this study can have possible consequences for other downstream-located glands of the endocrine system. The knowledge of these effects is very scarce and should definitely be extended in future research. In this context, the importance of endocrine feedback axes should be highlighted as the main contributor to a functioning hormonal homeostasis.

Another aspect concerning the pituitary that was not dealt with in the present study is the direct influence of substances on the pituitary itself instead of feedback-induced effects caused by the inhibition of downstream-located processes. This aspect should be considered in future studies to identify possible disruptors of the key organ of the endocrine system. On the downstream-located part, the liver as the main site for T4 conversion did not show specific reactions that could clearly be assigned to disruptions of hormone synthesis. Rather general aspects of stress like glycogen depletion or little developed alterations on the ultrastructural level could be detected. However, the presence of deiodinases is not restricted to the liver, which could be a possible explanation for the absence of clear effects. The wide-spread distribution of deiodinases possibly contributes to the fact that the

conversion of T4 to T3 in the peripheral metabolism is not that easily susceptible to a decline of T4. This decline was a very good indicator for the capability of the compensation of the inhibiting potency of the test substances and the ongoing inactivation of the thyrocytes, respectively. Nevertheless, measurements of T3 should be included in future experiments to precisely describe the ongoing production and conversion rates of thyroidal hormones together with their ratio.

In conclusion, the present thesis characterizes the effects caused by the disruption of different key mechanisms of thyroid hormone production, i.e. the thyroid hormone peroxidases and the sodium-iodide symporter, along the hypothalamic-pituitary-thyroid axis. Furthermore, stimulating effects of exogenous administration of T4 were noted. Depending on the mode of action of the goitrogen, different alterations in the thyroid could be detected both at the histopathological and the ultrastructural level. For the first time, clear feedback-induced increases of TSH-producing cell populations in the fish pituitary after exposure to PTU and PER were described which raises the question if other parts of the endocrine system are affected. The downstream located liver only showed glycogen depletion and minor ultrastructural effects, which can only be correlated with general stress symptoms. A brief comparison of amphibians with the zebrafish revealed similar sensitivities depending on the test substance. For general chemical regulatory purposes, one of the standard test organisms in ecotoxicology is the zebrafish. Its wide-spread use and acceptance makes it a very rewarding model for the detection of goitrogens due to its high sensitivity and easy laboratory handling, and it will definitely be included in routine chemical hazard and risk assessment. Moreover, due to the fact that the zebrafish is already used as standard test organism, it would save test animals and costs to also use it for the detection of thyroid-disrupting chemicals. Nevertheless, further research has to be conducted on the entire hypothalamic-pituitary-thyroid axis and its feedback mechanisms. Finally, molecular biological aspects, e.g. gene activations, mRNA detection etc. could lead to a deeper understanding of the principal regulatory processes and to further endpoints that could be used to detect thyroid-disrupting substances. A full comprehension and an appropriate interpretation of effects of goitrogens against the background of chemical regulatory purposes are only possible, if these aspects are fully clarified.

Chapter 7

Literature

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