The Effect of Stress on Zebrafish Lactase Expression

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Abstract

Lactase is a digestive enzyme, and its principal function is to break down lactose, a disaccharide found in milk. The main site for lactase expression is the intestines, however, it is also expressed in other tissues, including the brain. Because the primary substrate, lactose, is not present in the central nervous system, it can be assumed that lactase serves another function besides lactose breakdown outside the digestive system. In C57BL/6NCrl mice, lactase expression is higher in the ventral hippocampus after chronic social defeat stress in comparison to controls. This suggests that lactase expression is to some extent affected by stress. Although lactose metabolism is only necessary for mammals, some other animals – including the zebrafish (*Danio rerio*) – possess a gene that codes for lactase. Research on the zebrafish lactase gene is scarce, and the expression pattern of its two transcripts, the primary *lct-201* and the secondary *lct-202*, is not known.

This study focused on measuring lactase expression in adult wild type zebrafish – both on the gene and on the protein level as enzymatic activity. The effect of stress on lactase expression was also examined by applying two different stress models: netting handling stress as a form of physiological stress, and chronic social defeat as a model for psychosocial stress.

Real-time polymerase chain reaction (q-RT-PCR) showed *lct-201* expression in all five tissues investigated in this study – the forebrain, the mid-hindbrain, higher intestines, lower intestines, and skeletal muscle, whereas *lct-202* was only expressed in the higher and lower intestines. The expression level of *lct-201* in the muscle was only fifth of that in the lower intestines. Lactase activity assay on the whole brain and whole intestines displayed enzymatic activity in both tissues, with the activity in the intestines being more than seven-fold compared to the brain. q-RT-PCR on both stressed and control fish whole brain and intestines revealed higher lactase expression in the stressed fish intestines, however, the effect was only seen with a primer pair targeting both transcripts simultaneously, and not for either of them separately. Lactase expression was on average approximately 40 % higher in physiologically and 55 % higher in psychosocially stressed fish in comparison to their respective controls. Neither physiological nor psychosocial stress affected lactase expression in the brain.

These findings suggest that the two zebrafish lactase transcripts have distinct expression patterns, which might imply different functional roles for *lct-201* and *lct-202.* Furthermore, these results indicate that lactase is expressed in the zebrafish brain, suggesting that it has a specific function in

the central nervous system. Based on the findings in this study, lactase gene expression might be connected to experienced stress – both physiological and psychosocial.

List of Abbreviations

aa	Amino acid
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
ctrl	Control
DMSO	Dimethyl sulfoxide
FCC	Food chemical codex
g	Gram
h	Hour
HPA	Hypothalamus-pituitary-adrenal
HPI	Hypothalamus-pituitary-interrenal
KO	Knockout
l	Liter
LA	Lactase activity
LCT or *lct*	Lactase gene
M	Molar
m	Meter
µ	Micro (10^{-6})
min	Minute

mRNA	Messenger RNA
n	Nano (10^{-9})
PBS	Phosphate-buffered saline
q-RT-PCR	Real-time polymerase chain reaction
RFU	Relative fluorescence unit
rpm	Revolutions per minute
s	Second
SEM	Standard error of mean
SNP	Single nucleotide polymorphism
SQ	Starting quantity
str	Stressed
WT	Wild type

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1 INTRODUCTION

Lactase gene is well known for its role in the development of lactose intolerance, a condition affecting approximately two thirds of the world's population (Storhaug et al. 2017). Commonly, the expression of this gene is high in infants, but declines in the course of development. This leads to inadequate digestion of milk sugar, lactose, during adulthood – which in turn is a causative to a variety of gastrointestinal symptoms (Forsgård 2019).

The main location of lactase expression is the small intestine, which is rather intuitive. In fact, the localization of lactase enzyme has been acknowledged for well over a hundred years, as was demonstrated by Plimmer in 1906. Through modern technologies, lactase expression has been observed, however, also outside the digestive system. Proteomic and transcriptomic studies detect expression in the brain of some mammals – including human (Wang et al. 2019). A previous finding in our group showed that chronic social defeat, a model for chronic psychosocial stress, induced lactase expression in the ventral hippocampus of an inbred mouse strain (Laine et al. 2018). This provokes the question of other, yet to be discovered, functionalities of the protein.

Despite having unique properties in digestion of milk, the lactase gene is also present in many non-mammals. According to Ensembl database, the human gene has over 400 orthologues (Yates et al. 2020). The high level of conservation seconds that lactase is not only a digestive enzyme, but rather a more complex molecule. Interestingly, the lactase gene also exists in zebrafish – and as proved by RNA sequencing, is also expressed (White et al. 2017, Bastian et al. 2021). Unpublished *in situ* hybridization studies by Rouhiainen and Chen show lactase expression in the zebrafish brain, which makes the species a compelling subject for studying the function of this gene in the central nervous system.

To this day, very little is known of the function of lactase outside the digestive system. Due to the lack of lactose in the brain, it can be presumed that the lactase gene serves another purpose besides lactose breakdown. Findings from the study by Laine et al. (2018) suggest that lactase expression might be connected to stress, but the pathway for this remains unknown.

In this study, I aim to shed light on the functionality of the lactase gene in the brain by measuring its expression levels in zebrafish before and after both psychosocial and physiological stress. I also

intend to identify the possible expression pattern differences between two splice variants of the zebrafish gene.

2 REVIEW OF THE LITERATURE

2.1 Lactase

2.1.1 Function

The lactase gene codes for lactase, which is best known for its role in digestion of lactose, a sugar molecule found in mammalian milk. Lactose is a disaccharide that is formed by two simpler sugars, glucose and galactose, attached via a glycosidic bond (Forsgård 2019) (Fig. 1). In order for lactose to be used as an energy source in the body, the glycosidic bond needs to be broken down. Resulting monosaccharides can then be taken up by enterocytes in the intestine. Lactase has enzymatic activity, through which it catalyzes the hydrolysis of the glycosidic bond (Swallow 2003). Lactase belongs to Glycoside Hydrolase Family 1 (Lombard et al. 2014).

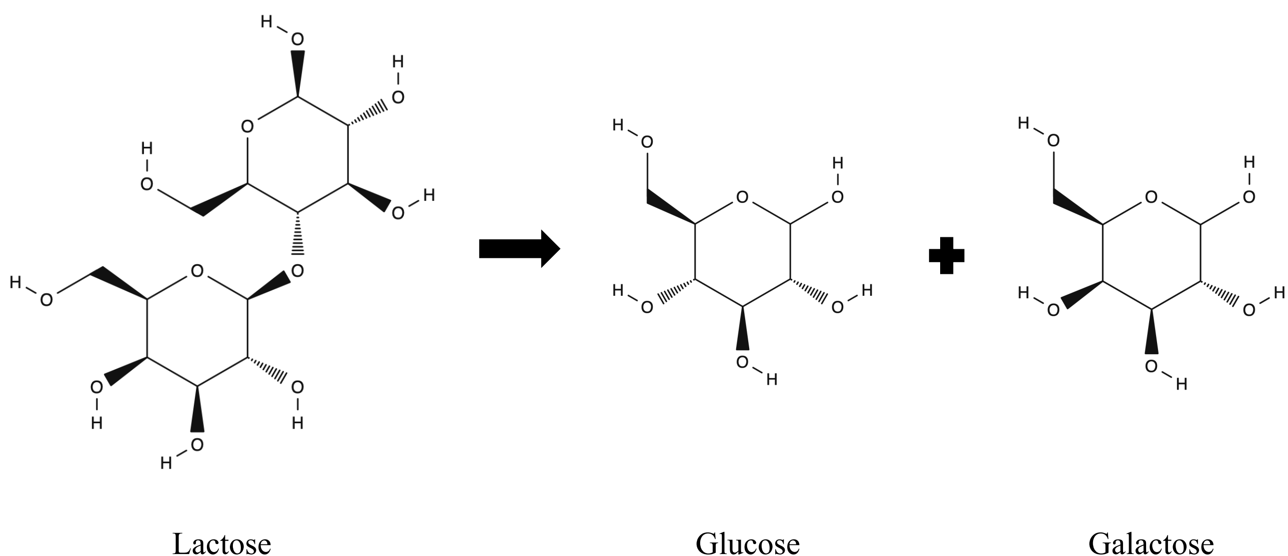

Fig. 1. The breakdown of lactose into glucose and galactose.

Lactase can be referred to as lactase-phlorizin hydrolase, because it also has an enzymatic site for phlorizin (Kraml 1972). Phlorizin is a flavonoid molecule found in fruit trees, especially apple (Ehrenkranz et al. 2005). It inhibits glucose transport through the renal tubule and therefore lowers blood glucose levels (Rossetti et al. 1987). Due to these properties, phlorizin has been studied as a treatment for type II diabetes. Enzymatically active sites for both lactose and phlorizin are located in the same protein subunit (Mantei et al. 1988).

In human, the lactase enzyme is synthesized as a large precursor molecule, which is then proteolytically cleaved and glycosylated in a complex manner (Naim et al. 1987). The initial translation product, which is synthesized in the endoplasmic reticulum, is pre-pro-lactase (Montgomery et al. 2007) (Fig. 2). It contains a short signal peptide sequence that guides the pre-pro-enzyme to the correct cellular compartment and is then cleaved by a peptidase (Mantei et al. 1988). Resulting pro-lactase is then cleaved and undergoes complex glycosylation in the Golgi apparatus (Naim et al. 1987). This processing results in the formation of a mature lactase molecule, which will locate in the intestinal lumen.

Fig. 2. The structure of human pre-pro-lactase.

The pre-pro-lactase consists of 1927 amino acids (aa), of which 19 form the signal peptide sequence in the N-terminus (Mantei et al. 1988). The pro-region cleaved in the maturation step makes up the second part of the translation product and is 849 aa. The third part of the product is the 1061 aa mature lactase (Naim et al. 1994). The C-terminus of lactase contains a short, 19 aa, hydrophobic anchor sequence that binds it to the cell membrane (Mantei et al. 1988). This is followed by a 26 aa tail, that remains in the cytosol (Naim et al. 1994).

In human, the lactase gene is located in the reverse strand of chromosome 2 (Yates et al. 2020). There are two isoforms, *LCT-201* and *LCT-202*, of which the latter is discarded through nonsense

mediated decay. The primary isoform, *LCT-201*, gene product is 6273 base pairs (bp), contains 17 exons and it is protein coding. The cytogenic location of the gene is 2q21.3 (OMIM).

2.1.2 Gene expression

Lactase gene expression is developmentally regulated (Montgomery et al. 1999) (Fig. 3). The expression begins prior to birth and continues throughout the nursing period (Auricchio 1994). In humans, lactase expression begins at around 24 weeks of gestation and increases considerably during the third trimester – reaching its peak at full-term birth (Montgomery et al. 2007). In most mammals, the expression is decreased around the time of weaning. This then leads to low levels of lactase activity through adult phase. The temporal decline in expression is caused by a *cis*-acting regulatory element (Wang et al. 1995). Low lactase expression – or complete lack thereof – is the cause of lactose intolerance in humans.

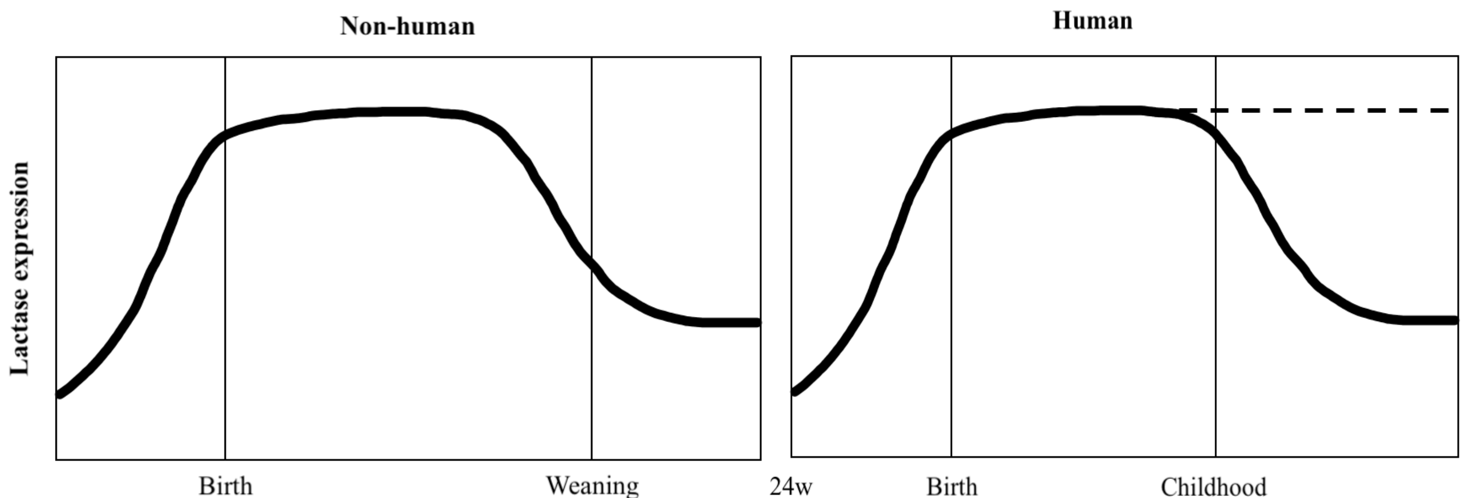

Fig. 3. The developmental pattern of lactase expression in human and non-human mammal intestine. Adapted from reference (Montgomery et al. 2007).

The main site of lactase expression is the small intestine, more specifically the jejunum. The amount of lactase decreases gradually when moving towards the ileum (Ugidos-Rodríguez et al. 2018). Before weaning, however, lactase is present in all parts of the small intestine (Auricchio

1994). In humans, lactase messenger RNA (mRNA) is localized on the apical side of enterocytes in the microvillus membrane (Montgomery et al. 2007).

Several transcription factors are known to affect the promoter activity of the lactase gene. A GATA family transcription factor, GATA-6, has been shown to stimulate the lactase gene promoter *in vitro* (Fitzgerald et al. 1998). In another study by Krasinski et al. (2001), they found that GATA-5 together with another transcription factor, HNF-1α, coactivates the promoter and increases lactase gene expression. OCT-1, which is closely linked to the lactase persistence phenotype, also enhances lactase promoter activity (Lewinsky et al. 2005).

RNA sequencing studies have shown that despite having a specific function in the intestine, lactase is also expressed in other tissues. Interestingly, lactase expression has been detected in the brain of both mice (Soumillon et al. 2013) and humans (Wang et al. 2019). Yu-Chia Chen and Ari Rouhiainen from our group have shown that lactase is also expressed in the zebrafish brain in two separate *in situ* hybridization experiments (unpublished data). A proteomic and transcriptomic study by Wang et al. (2019) detected lactase expression in 30 different tissues, both within and outside the digestive system in humans. According to mouse transcriptomic studies, lactase is expressed in the following brain regions: dorsal raphe nucleus (Bonthuis et al. 2015), hippocampal formation (Keane et al. 2011) – including dentate gyrus (Ramos et al. 2013), and medial prefrontal cortex (Gregg et al. 2010).

The amount of lactase gene expression or the degree of lactase enzymatic activity are not induced by increasing the amount of dietary lactose in humans (Gilat et al. 1972). The regulation of lactase expression is therefore not dependent on diet, but rather on more complicated factors. In C57BL/6NCrl mice, lactase was found to be differentially expressed in the ventral hippocampus after chronic social defeat stress (Laine et al. 2018). This finding points that stress might be one of the components affecting lactase gene expression. In fact, an early *in vitro* study on rat intestine found an increase in lactase activity after administrating dexamethasone, a glucocorticoid (Simon-Assman et al. 1982). A more recent study by Nanthakumar et al. (2003) demonstrated that administration of cortisone acetate increased lactase activity in jejunal xenografts. The results for the effect of glucocorticoids on lactase expression have not, however, been conclusive. The lactase gene in humans does not contain a binding site for a glucocorticoid receptor (Boll et al. 1991), which suggests that the expression is not directly affected by glucocorticoids, but rather by secondary activators.

2.1.3 Lactase in zebrafish

Lct in zebrafish is located in the reverse strand of chromosome 9. The full size of the gene area is 29679 bp. There are two known transcripts: *lct-201* and *lct-202* (Fig. 4), of which both code for protein (Yates et al. 2020). *Lct-201* is considered the principal isoform, whereas *lct-202* is a splice variant (Rodriguez et al. 2018). In terms of size, *lct-201* is a bit smaller, 5703 base pairs, compared to 6496 base pairs of *lct-202.* Both consist of 17 exons, of which 14 are shared between these transcripts. Exons 1 to 3 are different for *lct-201* and *lct-202* (Yates et al. 2020).

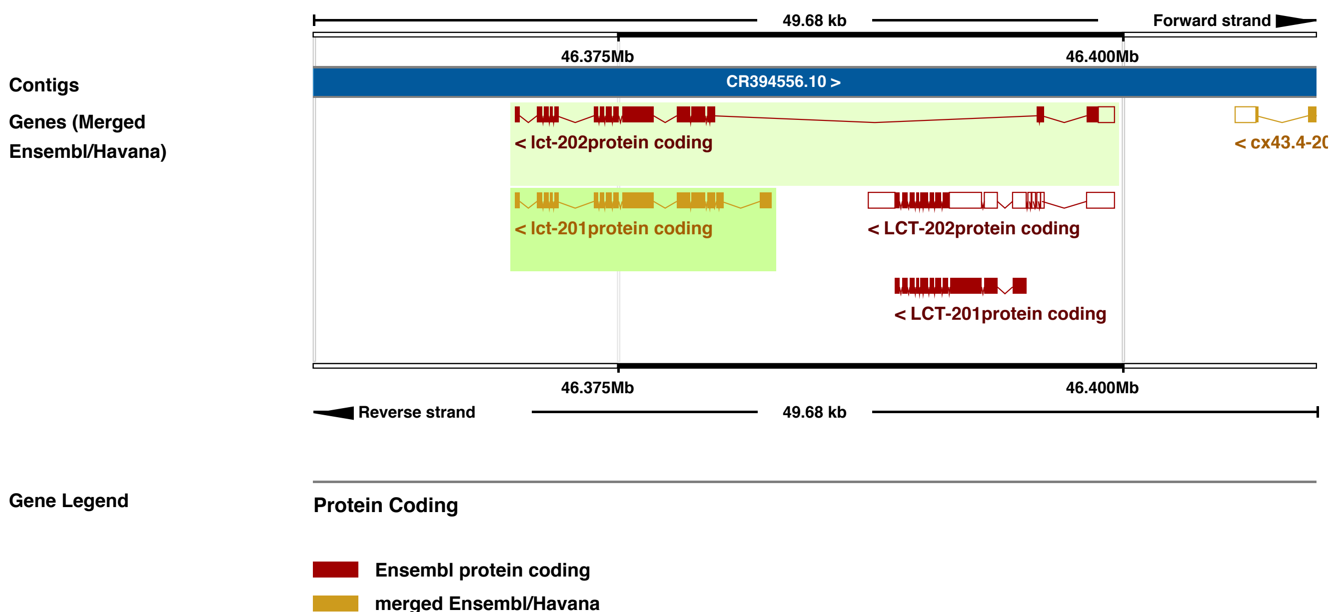

Fig. 4. The zebrafish lactase gene. Image from Ensembl release 103 (Yates et al. 2020).

Based on transcriptomics data, *lct* is expressed at low to medium levels at the larval stage (White et al. 2017). At fully formed stage, *lct* is expressed in at least intestine, liver, head, tail and head kidney tissues (Bastian et al. 2021). These locations of expression have been detected by RNA sequencing. *In situ* hybridization showed that *lct* is also expressed in the brain of zebrafish (Rouhiainen, unpublished; Chen, unpublished), as it is in mice and humans.

Based on gene ontology, the subcellular location of the lactase primary product in zebrafish is the endoplasmic reticulum (Gaudet et al. 2011). This is not entirely in agreement with the fact, that the zebrafish lactase also contains a transmembrane helical structure, just like its human counterpart (UniProt Consortium 2021). Automatic computational assertion also places lactase in the integral component of the membrane.

Both *lct-201* and *lct-202* protein products contain a 23 aa signal peptide. The former consists of 1900 aa in total, whereas the latter has 1898 aa (UniProt Consortium 2021). The two contain a 25 aa transmembrane sequence in the C-terminal end. The only amino acid differences between the translation products are within the first 250 aa (Fig. 5), and the rest of them is identical. The shared active sites are both nucleophilic. The molecular function for both translation products is the same: glycosidase and hydrolase activity.

```
MMKILGVFLSLGVLAIFWSYSNADEQEFMLLAGPLTTSKTLHLEEQYVFNCGGPLPQQSK 60
MMKILGGFLSLGVLAIFWSYINADEQEFMLLAGPLTTSQNLPSEEQYVFNCRGPLPQQSK 60
****** ************* *****************:.*  ******** ********

DYFLYLQRRGVTNFKVPLSWSHILPTGDANQPHEETVMCFKTLVQQLTESGIKPLLVLHR 120
DYFLYLQRRGVTNFKVLLSWSHILPTGDANQPHEETVMCFKTLVQQLTESGIKPLLVLHR 120
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SAVPELFRAKYGGWENPLLVQMFEQYAGFVFSTFRDHVDTFVTFSHLHELQDRQLKNALQ 180
SAVPELFRAKYGGWGNPLLVQIFEQYAGFVFSTFRDHVDTFVTFSHLHELQHEELQNALQ 180
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SHENAYKVCHQRFTGLRLSLGIKASDVTSIHQMGSKIKTHVDFLSVHMQFNCKMQTALTE 240
SHENAYKVCHQQFKGLRLSLGIRASHATSIYQMGSKIMTHVDFLSVHMQFNCKMQTALTE 240
***********:*.********:**..***:****** **********************

ELRK--IVSGQKSLLIYQLTVNDCDGYEHDFQPLTVVLGVLQNKDYKIIGCDITHVFEKL 298
ELRKVQIVSGQKSLLIYQLTVNDCDGYEHDFQPLTVVLGVLQNKDYKIIGCDITHVFEKL 300
****  ******************************************************

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DYEETPRSLQKEDAMSVFNQKSASAFSYQKVWEKFKTQTEAERDQFLSGSFPVDFEWSVS 360
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SESFKVEGGSAEHGKGETIWDRFNHEAGVNESILGCDSYHKVDYDVYLLRGMMAPNYQFS 420
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ISWARIFPTGRKESFVEKGAAYYDKMINTLLQSGIEPTVTLHHWDLPQALQESGGWINDS 480
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QVPSKSKVVWEKFSPQTKFERQLYHYGTFSEGFQWGVSSSAYQVEGGWNADGKGPSVWDT 898
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SLSIIAAVTVILLVYGLVKASKKQKHPAGEYIDLEKKDKF 1900
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Fig. 5. Alignment of *lct-201* and *lct-202* translation products. Top row is the protein product of *lct-202* and bottom row that of *lct-201*. Highlighted sequences represent following annotations: magenta= signal peptide, green= coiled coil, red= active site, yellow= transmembrane. Produced with Clustal Omega (1.2.4) multiple sequence alignment tool.

2.1.4 Phenotypes associated with lactase

Lactose intolerance is a very common condition, affecting around two thirds of the world's population. It can be divided into three subtypes: congenital lactase deficiency, secondary lactase deficiency and adult-type lactase deficiency (Berni Canani et al. 2016) (Table 1).

Table 1. The three subtypes of lactose intolerance.

	Congenital lactase deficiency	**Secondary lactase defieciency**	**Primary lactase defienciency**
Cause	Lack of lactase enzymatic function	Primary condition causing damage to intestinal cells	Normal age-dependent decline of lactase expression
Pattern of inheritance	Autosomal recessive	Not heritable	Autosomal recessive
Mutated gene	*LCT*	None	*MCM6*
Mutation type	Several known mutations in the coding region, resulting in non-functional enzyme	None	Several known SNPs, C/T-13910 key mutation in Europe
Prevalence	Rare	Not known	Very common, in over two thirds of adults
Symptoms	Diarrhea, malnutrition, slowed development	Diarrhea, discomfort, flatulence	Diarrhea, discomfort, flatulence
Age of onset	Newborn	Any age	Childhood or adolescense
Management	Completely lactose free diet	Treatment of the primary condition, in which case lactase deficiency can be reversed; low-lactose diet	Low-lactose diet

Congenital lactase deficiency manifests almost immediately after birth as lack of lactose absorption, resulting in chronic diarrhea and slowed development due to malnutrition (Lifshitz 1966). The symptoms can be reversed by switching to a lactose-free diet (Di Constanzo & Berni Canani 2018). Congenital lactase deficiency is inherited in an autosomal recessive manner and it is one of the Finnish heritage diseases (Savilahti et al. 1983). Several mutations in the lactase gene coding region have been identified as the cause, including a nonsense mutation, $\text{Fin}_{\text{major}}$ – the most common mutation in the Finnish population (Kuokkanen et al. 2006). Other mutation types causing truncated protein structures have also been found.

Secondary lactase deficiency is caused by intestinal damage generated as a result of a primary condition (Di Constanzo & Berni Canani 2018). Such damage can be caused by an intestinal disorder – such as Crohn's disease or celiac disease, infections, food allergies, bacterial imbalance or cancer treatments. Secondary lactase deficiency can be reversed as the intestinal damage is cleared. The symptoms are similar to those of primary lactose intolerance.

Adult-type lactase deficiency, or primary lactose intolerance, affects most of the world's population. There is a vast difference in the frequency of the condition between populations, with the Middle East having the highest prevalence (Storhaug et al. 2017). On the contrary, Northern and Western Europe are among the regions with the lowest prevalence. The ability to digest lactose is considered to be the result of a selective pressure, and historic dairying practices are associated with a lower prevalence of lactose intolerance among certain populations (McCracken 1971).

Primary lactose intolerance is caused by the decline of lactase gene expression after weaning (Forsgård 2019). This leads to a decreased amount of lactase enzymatic activity in the intestines, causing lactose to pass on in the digestive system without being degraded. Symptoms, such as bloating, diarrhea and discomfort, occur when the undigested lactose reaches the colon (Ingram et al. 2009). The symptoms are alleviated on a low-lactose diet (Di Constanzo & Berni Canani 2018).

Whether the decline of lactase expression occurs after infancy, is determined by genetic factors (Di Constanzo & Berni Canani 2018). Several variants are known to affect the ability to digest lactose in adulthood, and a single nucleotide polymorphism (SNP) C/T_{-13910}, located upstream from the lactase gene is the most prevalent, primarily in the European population. The homozygous C/C_{-13910} genotype is associated with lactase intolerance, whereas heterozygous or homozygous T/T_{-13910} genotypes are linked to lactase persistence (Enattah et al. 2002).

The C/T_{-13910} SNP is located in intron 13 of the *MCM6* (minichromosome maintenance complex component 6) gene (Enattah et al. 2002) (Fig. 6). This gene area functions as an enhancer to the lactase gene, and binding of transcription factors to the enhancer provokes transcription of the gene it targets. OCT-1, a transcription factor, has been found to bind strongly to the T_{-13910} variant and consequently increase lactase promoter activity (Lewinsky et al. 2005). Additionally, the SNP variant affects the DNA methylation patterns in both lactase gene enhancer and promoter areas: C/C_{-13910} genotype is linked to a higher and T/T_{-13910} genotype to a lower level of methylation (Leseva et al. 2018).

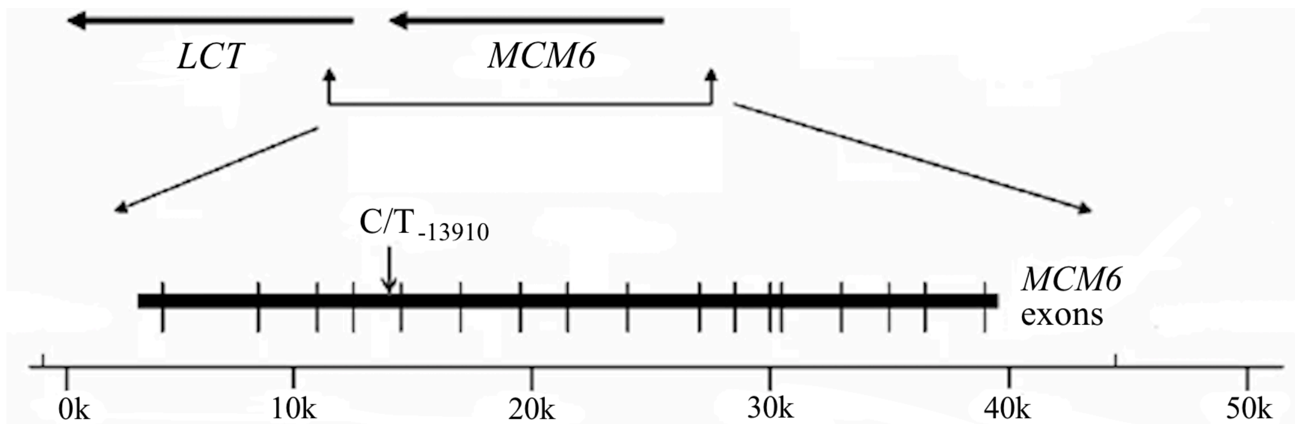

Fig. 6. The locus for C/T$_{-13910}$ SNP underlying primary lactose deficiency. Adapted from reference (Enattah et al. 2002).

In the human lactase gene, a SNP rs2164210 is associated with neuroblastoma (Maris et al. 2008). Neuroblastoma is a malignancy affecting adrenal medulla and paravertebral sympathetic ganglia in early developmental stages (Tsubota & Kadomatsu 2018).

2.2 Zebrafish as a model organism

2.2.1 Zebrafish in stress research

The zebrafish is widely used in medical research and it is considered as one of the classical model organisms for vertebrates. Besides their original application in developmental biology, zebrafish are now used to study pathological mechanisms in several fields of science. There are many advantages to using a zebrafish model – for example, they are cost-efficient, they develop quickly, and the zebrafish genome is fully sequenced and easily accessible (Kari et al. 2007).

Studies on pharmacological agents affecting the nervous system show similar effects in zebrafish and humans, which points that these species might share analogous neural networks (Lieschke & Currie 2007). The adult zebrafish brain contains all the major subdivisions found in human brain: the forebrain, the midbrain and the hindbrain (Vaz et al. 2019) (Fig. 7). There are also many homologous smaller structures, for example the hippocampus, the cerebellum, and the diencephalon, which compose of the same cell types and are formed through similar differentiation pathways as in humans. Familiar neurotransmitters, such as serotonin, histamine, and dopamine, are found in both mammal and zebrafish brain (Panula et al. 2006). These similarities support the use of zebrafish as a model organism in neuroscientific research.

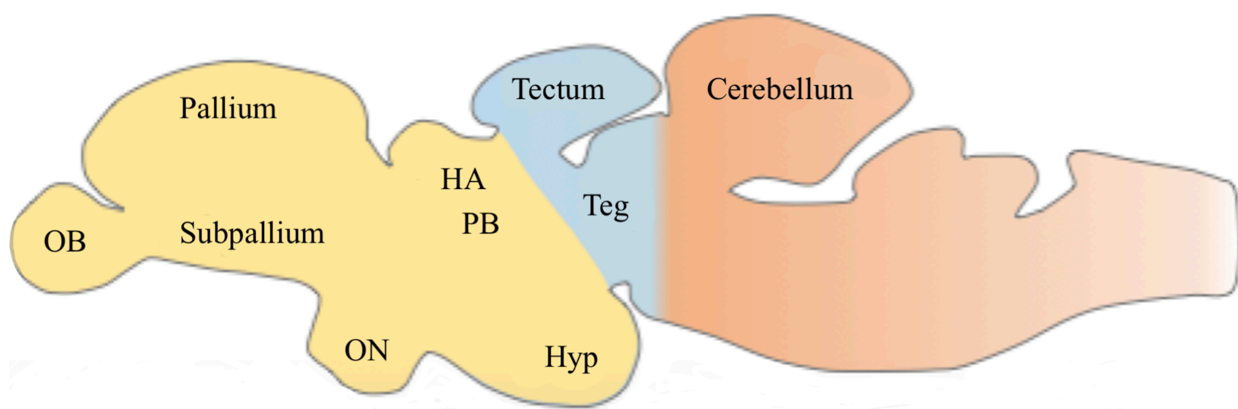

Fig. 7. The organization of the adult zebrafish brain. Yellow= forebrain, blue= midbrain, orange= hindbrain. HA= habenula, Hyp= hypothalamus, OB= olfactory bulb, ON= optic nerve, PB= pineal body, Teg= tegmentum. Adapted from reference (Vaz et al. 2019).

Stress response, the physiological response of the body to a threat, is similar in fish and other vertebrates (Schreck et al. 2016). It is mediated by hormonal systems, which produce corticosteroids and catecholamines that regulate the secondary stress response. The hormonal system in zebrafish responsible for catecholamine secretion is the hypothalamic-sympathetic-chromaffin cell axis (Wendelaar Bonga 1997). The chromaffin cells are located in the head kidney and are homologous to mammalian adrenal medulla. Corticosteroids – or cortisol in fish, on the other hand, are produced by the hypothalamus-pituitary-interrenal (HPI) axis, which is similar to mammalian hypothalamus-pituitary-adrenal (HPA) axis (Fig. 8). The interrenal cortisol-producing cells do not form a gland but are rather spread within the head kidney.

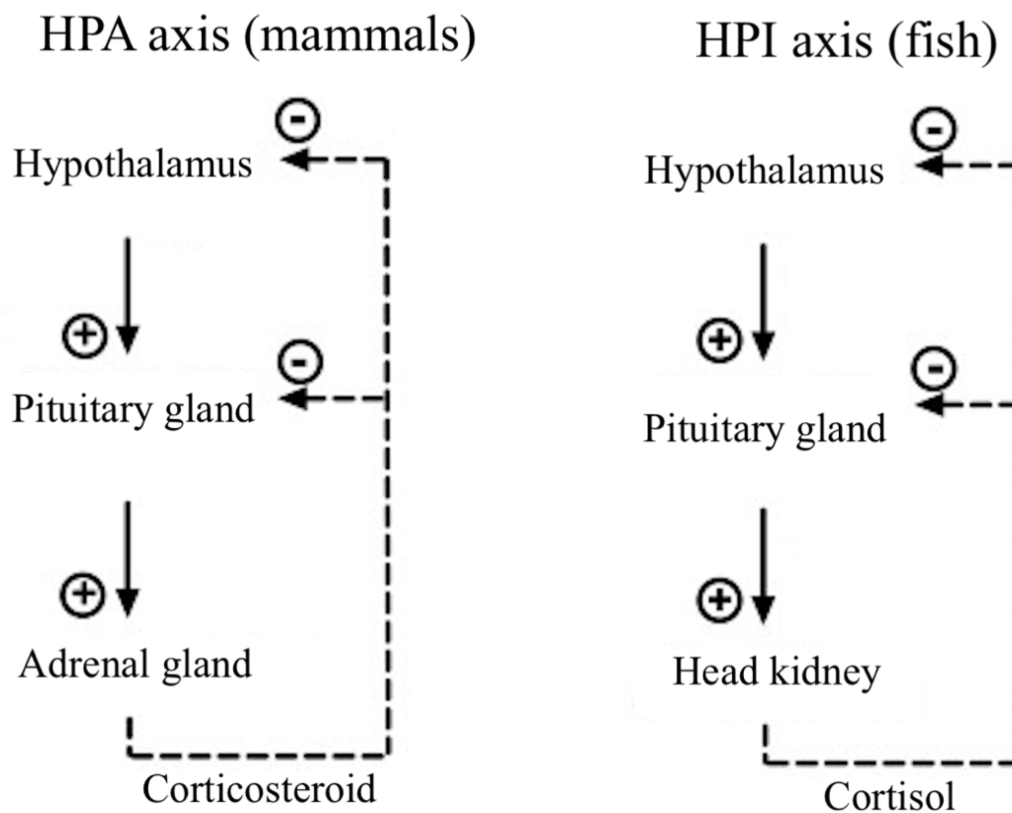

Fig. 8. The similar organization of the mammal HPA and fish HPI axis. Adapted from reference (Steenbergen et al. 2011).

2.2.2 Assessing stress in zebrafish

Besides sharing neuroanatomical structures and physiological stress mechanisms with mammals, zebrafish also display anxiety-like or depressive-like behaviors. These behaviors can be interpreted as stress-related.

A widely used behavioral paradigm, the novel tank diving test, is applied to assess how the fish responds to an unfamiliar environment (Buccafusco 2009) (Fig. 9). Typically, at first the fish dives to the bottom of the tank and remains there for a while, until gradually moving towards the upper part. The tendency to dwell on the bottom of the tank is considered anxiety-like behavior, which is induced by the stress generated by the novel environment (Levin et al. 2007). Different factors can be measured: the latency to enter the top part of the tank, the amount of these entries, the time spent in the top part as well as the number of erratic movements, which is considered an anxiety-like trait (Egan et al. 2009).

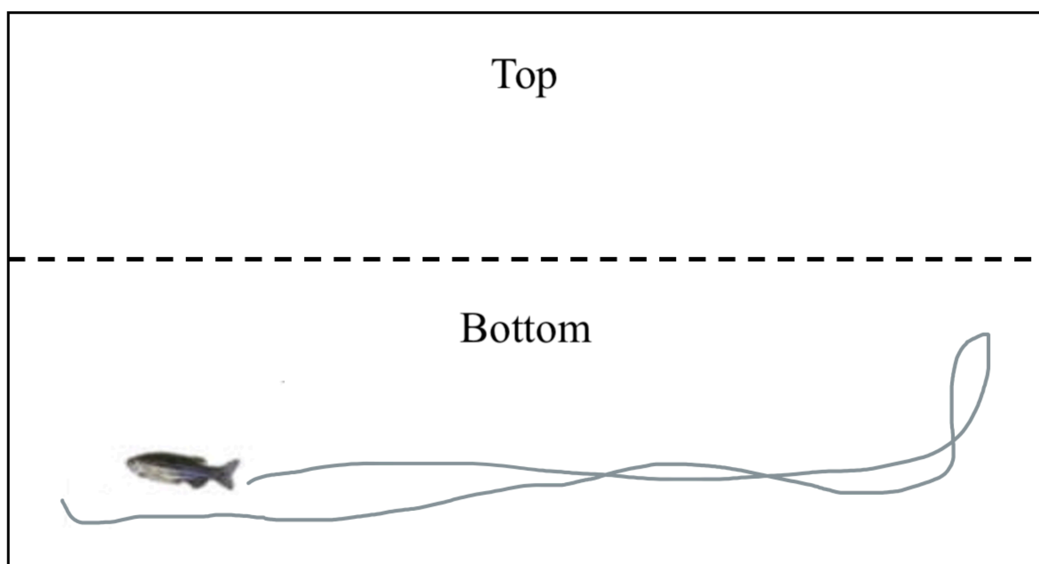

Fig. 9. The novel tank diving test. Adapted from reference (Egan et al. 2009).

Another behavioral effect found in zebrafish after stress is related to their interactions with their shoal mates. In a 2008 study by Speedie and Gerlai, they found a significant decrease in the

distance between any two fish in a shoal when exposed to a zebrafish-derived alarm substance. In the same study, the alarm substance exposure also caused freezing, which is a fear response.

The stress experience in zebrafish can also be assessed through cortisol level measurements. Being exposed to a stressor increases the levels of whole-body cortisol (Ramsay et al. 2009). Combining behavioral testing with cortisol level measurements can provide a more accurate assessment of the stress response in zebrafish.

3 AIMS

In this study, my aim was to investigate the expression levels of lactase in selected zebrafish tissues. The more specific aims were the following:

1. To show that both lactase mRNA and protein are expressed in zebrafish tissues.

2. To determine whether the two zebrafish lactase transcripts, *lct-201* and *lct-202*, have different expression patterns.

3. To investigate the effect of both physiological and psychosocial stress on lactase expression in zebrafish.

4 MATERIALS AND METHODS

4.1 Animal experiments

4.1.1 Animals

Adult male zebrafish from two different lines maintained by Pertti Panula's group were used in these experiments. The wild type (WT) line (Turku) was used in real-time polymerase chain reaction (q-RT-PCR) experiments to determine baseline and post-stress lactase expression levels, and in the lactase activity assay to measure lactase enzymatic activity. Samples obtained from homozygous lactase-knockout (*lct*-KO) zebrafish were used in q-RT-PCR primer validation and in a substrate-specificity test for the lactase activity assay. The *lct*-KO line – with an 11 bp frameshift mutation causing a premature stop codon in exon 8 – was generated with CRISPR-Cas9 technology, and fish from F3 generation were used in experiments.

4.1.2 Stress models

Netting handling was chosen as a model for physiological stress. The fish were kept in a net in shallow water for three minutes, followed by a three minute rest; this was then repeated three times, after which the fish were dissected for brain and whole intestines.

For psychosocial stress, a chronic social defeat model was adopted (Fig. 10). In this protocol, the experimental fish is repeatedly placed in the same tank with an unfamiliar larger-sized aggressor fish – this repeated social subordination is a psychosocial stressor. An experimental fish was placed in a tank inhabited by an aggressor fish, and physical interaction was allowed for 15 min. The conflicting fish were then separated by a transparent partition, providing visual but no physical contact for 24 h. On the next day, the experimental fish was placed in a tank with a new aggressor, and the subordination and co-habitation were repeated. This procedure was carried on for ten consecutive days, after which the fish were dissected for brain and intestines.

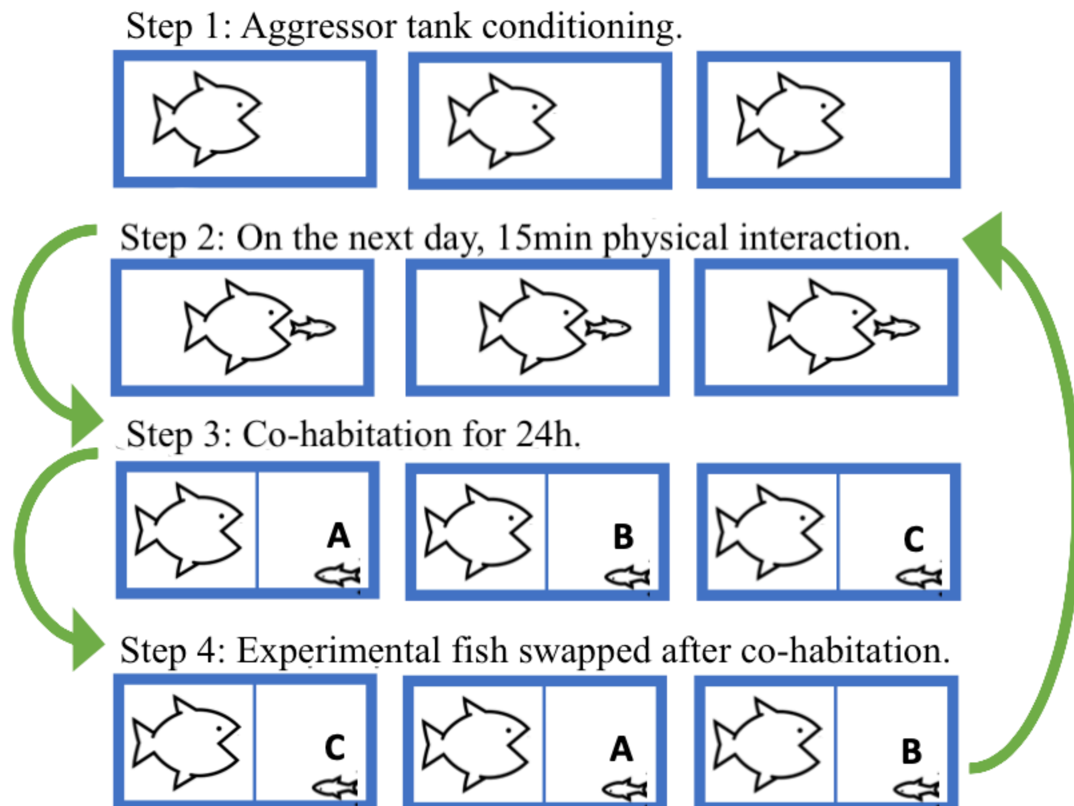

Fig. 10. The zebrafish chronic social defeat stress protocol. Adapted from source (Chen, unpublished).

All the fish that underwent either of the stress procedures were 5-month-old Turku WTs. Each experimental fish was paired with a sibling, that did not undergo a stress procedure and were therefore used as controls. The aggressor fish in the chronic social defeat were 15-month-old Turku WTs.

All of the animal experiments and dissections were performed by members of the Panula group. I received zebrafish tissue samples for the lactase activity assay and complementary DNA (cDNA) samples for the q-RT-PCR experiments.

4.2 Quantification of lactase expression

4.2.1 Primer design

q-RT-PCR was carried out to measure lactase expression levels in five different zebrafish tissues. I designed three primer pairs for this purpose using NCBI primer design tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Two of the pairs were designed to amplify one of the isoforms exclusively (*lct-201* and *lct-202*), and the third to amplify both of them simultaneously (*lct-20X*) (Table 2). *Rpl13a* was selected as a housekeeper gene to be used for the normalization of the expression levels in the first experiment, where lactase expression levels in different tissues were measured. For this, I used previously published primers (Xu et al. 2016). Additional housekeeper gene, *b-actin1* (F: CGAGCAGGAGATGGGAACC, R: CAACGGAAACGCTCATTG), was used for the quantification of lactase expression after stress.

Table 2. The zebrafish lactase -targeting q-RT-PCR primer sequences.

Transcript	Forward sequence (5'→3')	Reverse sequence (5'→3')
lct-201	ACTGTAGAGGTCCACTGCCA	TGGGACTGGAGAGCATTTTGAA
lct-202	AAAAATGCTCTCCAGTCCCA	GAGGGACTTTTGACCGCTGA
lct-20X	TGACACTCTGCGTGTTGACT	AAGGCACTTGAGATGGAGGC

4.2.2 cDNA samples

I received cDNA samples from the Panula group from the Zebrafish Unit in the University of Helsinki. Approximately 1 µg of RNA was used for the synthesis of each cDNA reaction.

4.2.3 q-RT-PCR

cDNA samples were amplified with 2.5 µM primers in CFX384 Real-Time PCR cycler (Bio-Rad Laboratories, Hercules, CA, USA) using iQ SYBR Green Supermix (Bio-Rad Laboratories,

Hercules, CA, USA). Each reaction was 10 µl in total, with 2 µl of cDNA template diluted in Milli-Q® water (Table 3). Each sample was run in triplicate and with a standard curve (Table 4) present on each assay plate. The q-RT-PCR program had 40 amplification cycles, and a melt phase to determine the melting temperature of each amplification product (Table 5).

Table 3. The cDNA dilutions used for q-RT-PCR.

	lct-201	*lct-202*	*lct-20X*	*rpl13a*	*b-actin1*
Forebrain	1:40	1:10	1:40	1:100	
Mid-hindbrain	1:40	1:10	1:40	1:100	
Whole brain	1:125		1:125	1:250	1:250
Lower intestine	1:200	1:100	1:100	1:200	
Upper intestine	1:100	1:100	1:100	1:300	
Whole intestine	1:100	1:100	1:100	1:150	1:150
Muscle	1:20	1:10	1:20	1:200	

Table 4. The cDNA concentration for each standard dilution in ng/µl.

	lct-201	*lct-202*	*lct-20X*	*rpl13a*
Standard 1	0.625	0.625	0.625	0.625
Standard 2	0.342	0.342	0.342	0.342
Standard 3	0.187	0.187	0.187	0.187
Standard 4	0.102	0.102	0.102	0.102
Standard 5	0.056	0.056	0.056	0.056
Standard 6	0.030	0.030	0.030	0.030
Standard 7	0.017	0.017	0.017	0.017
Standard 8		0.009		
Standard 9		0.005		

Table 5. The q-RT-PCR program. Steps 2–4 were repeated 40 times. Steps 6 and 7 represent the melt curve, where the temperature was increased 0.5 °C every five seconds.

Step	Temperature (°C)	Duration (s)
1	95	180
2	95	10
3	55	30
4	72	30
5	95	10
6	65	5
7	95	5

4.2.4 Statistical analysis

Starting quantity (SQ) values for each sample were obtained from CFX Maestro v3.1.1517.0823 software (Bio-Rad Laboratories, Hercules, CA, USA) and normalized to corresponding housekeeper SQ values. For the quantification of lactase expression after stress, mean of the SQ values of both housekeepers were calculated and used for normalization of the lactase SQ values. All the values were also corrected for dilution factors. Statistical analysis was conducted with GraphPad Prism v8.0.0 for Windows (GraphPad Software, San Diego, CA, USA). Kruskal-Wallis test and Dunn's *post hoc* test were performed to detect expression level differences in different tissues. For comparing lactase expression levels in stressed (str) and control (ctrl) fish tissues, Mann-Whitney U test was conducted. Results were plotted with GraphPad Prism v8.0.0 for Windows as bar graphs of SQ mean ± standard error of mean (SEM) with individual values as dots. The difference between group means was considered significant when p value was less than 0.05 (* < 0.05, ** < 0.01, *** < 0.001).

4.3 Lactase activity assessment

4.3.1 Tissue samples

Frozen brain and intestines from sixteen 5-month-old Turku WT fish were used for lactase activity assessment. Four whole brains and two whole intestines were pooled together to maximize the protein concentration of the homogenates, totaling four replicates per tissue type.

4.3.2 Sample preparation

The samples were transferred from dry ice to Precellys® tubes containing cold 0.03 % Triton™ X-100 (Sigma Aldrich, Saint Louis, MO, USA) in phosphate-buffered saline (PBS) (Biotop Oy, Turku, Finland) with a cOmplete™ Protease Inhibitor Cocktail tablet (Hoffmann-La Roche, Basel, Switzerland). The amount of 0.03 % Triton™ X-100 in PBS was adjusted according to the amount of tissue per sample, with 400 µl used for brain and 600 µl for intestine samples. The samples were then homogenized in Precellys® 24 Tissue Homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) at 2000 rpm for 2x30 s with a 10 s break in between. The tubes were then centrifuged at 10000 rpm for 10 min at +4 °C to reduce the amount of foam formed in the homogenization process. Homogenates were then transferred to microcentrifuge tubes and placed on ice.

4.3.3 Measuring protein concentration

Bradford assay was performed to measure the amount of protein present in each homogenate. Two hundred and fifty microliters of Bradford Reagent (Sigma Aldrich, Saint Louis, MO, USA) brought to room temperature was pipetted to each well of a transparent 96-well plate. Five microliters of each standard dilution made from bovine serum albumin (BSA) (Sigma Aldrich, Saint Louis, MO, USA) in PBS (Table 6), along with 5 µl of the protein homogenate samples diluted 1:10 in PBS were pipetted in triplicate to the plate. The plate was then incubated at room temperature on a

shaker at 300 rpm for 5 min, followed by absorbance measurement with Hidex Sense microplate reader (Hidex, Turku, Finland) at 595 nm.

Table 6. The standard dilutions in Bradford assay as µg/µl.

	Protein concentration
Standard 1	0
Standard 2	0.25
Standard 3	0.5
Standard 4	1
Standard 5	1.4

The standard curve for the absorbance measurements was formed using Microsoft Excel® from Microsoft Office Professional Plus 2016 for Windows (Microsoft, Albuquerque, New Mexico, USA). Mean absorbance value for each triplicate was calculated and normalized by reducing the value of Standard 1 (blank). Standard curve was plotted, and the protein concentration of each homogenate was calculated using the standard curve equation.

4.3.4 Lactase activity assay

For the lactase activity assay, 1 µg/µl dilutions of each protein homogenate in PBS were prepared according to the protein concentration calculations. Total reaction volume of protein dilutions and substrate per well was 150 µl, with substrate concentration of 10 µM. The diluted homogenate was pipetted in triplicate on a black-wall 96-well plate with a transparent bottom. Triplicates of each standard dilution (Table 7) and a negative control (only PBS) were also pipetted to the plate. The standard dilutions were prepared using Pirkka lactase enzyme tablets (Vitabalans Oy, Hämeenlinna, Finland) produced in *Aspergillus oryzae* – with one tablet containing 4500 Food chemical codex (FCC) units. The substrate, fluorescein di(β-D-galactopyranoside) (Santa Cruz Biotechnology, Dallas, TX, USA) dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich, Saint Louis, MO, USA), was added to each well to induce a fluorescent reaction, which was then measured with

Hidex Sense microplate reader at 485 nm excitation and 520 nm emission as a continuous measurement every 5 min for 30 min in total.

Table 7. Standard dilutions for the lactase activity assay as FCC unit/µl.

	FCC unit concentration
Standard 0	0
Standard 1	$7.81x10^{-6}$
Standard 2	$1.56x10^{-5}$
Standard 3	$3.13x10^{-5}$
Standard 4	$6.25x10^{-5}$
Standard 5	$1.25x10^{-4}$
Standard 6	$2.50x10^{-4}$

4.3.5 Statistical analysis

The standard curve for the fluorescence measurements was formed using Microsoft Excel® for Windows. Mean relative fluorescence unit (RFU) value for each triplicate was calculated and normalized by reducing the value of Standard 0 (blank). Standard curve was plotted, and lactase activity value for each sample was calculated using the standard curve equation.

Graphic demonstration of the standard curve containing samples as dots was created using GraphPad Prism v8.0.0 for Windows.

Statistical analysis was performed with GraphPad Prism v8.0.0 for Windows. Mann-Whitney U test was applied to compare the means of lactase activity per mg of protein in the brain and intestines. Results were plotted as bar graphs of lactase activity (LA) as FCC units/mg of protein ± SEM with individual values as dots. The difference between group means was considered significant when p value was less than 0.05 (* < 0.05, ** < 0.01, *** < 0.001).

5 RESULTS

5.1 Quantification of lactase expression in different tissues

5.1.1 Expression levels in adult zebrafish tissues

The expression levels of the two lactase transcripts were studied in five different zebrafish tissues: the forebrain, the mid-hindbrain, the upper and lower intestines and the muscle.

The primary transcript, *lct-201*, was expressed in all of the aforementioned tissues (Fig. 11a). The expression level was highest in both of the intestine fractions, and lowest in muscle. The level of expression of *lct-201* in the muscle was only 21 % of that in the lower intestine (p= 0.002).

The secondary transcript, *lct-202*, was only expressed in the intestine (Fig. 11b). No expression in either of the brain areas or muscle was detected, hence all comparisons between either of the intestine fractions and other tissues reached statistical significance. No significant difference between the expression levels in the lower and higher intestines was observed.

The expression pattern for the *lct-20X* primer pair resembled that of *lct-201*, however, the relative expression in both intestine fractions was higher compared to other tissues (Fig. 11c). The *lct-20X* expression level in the muscle was 11 % of that in the lower intestine (p= 0.004) and 12 % of that in the upper intestine (p= 0.003).

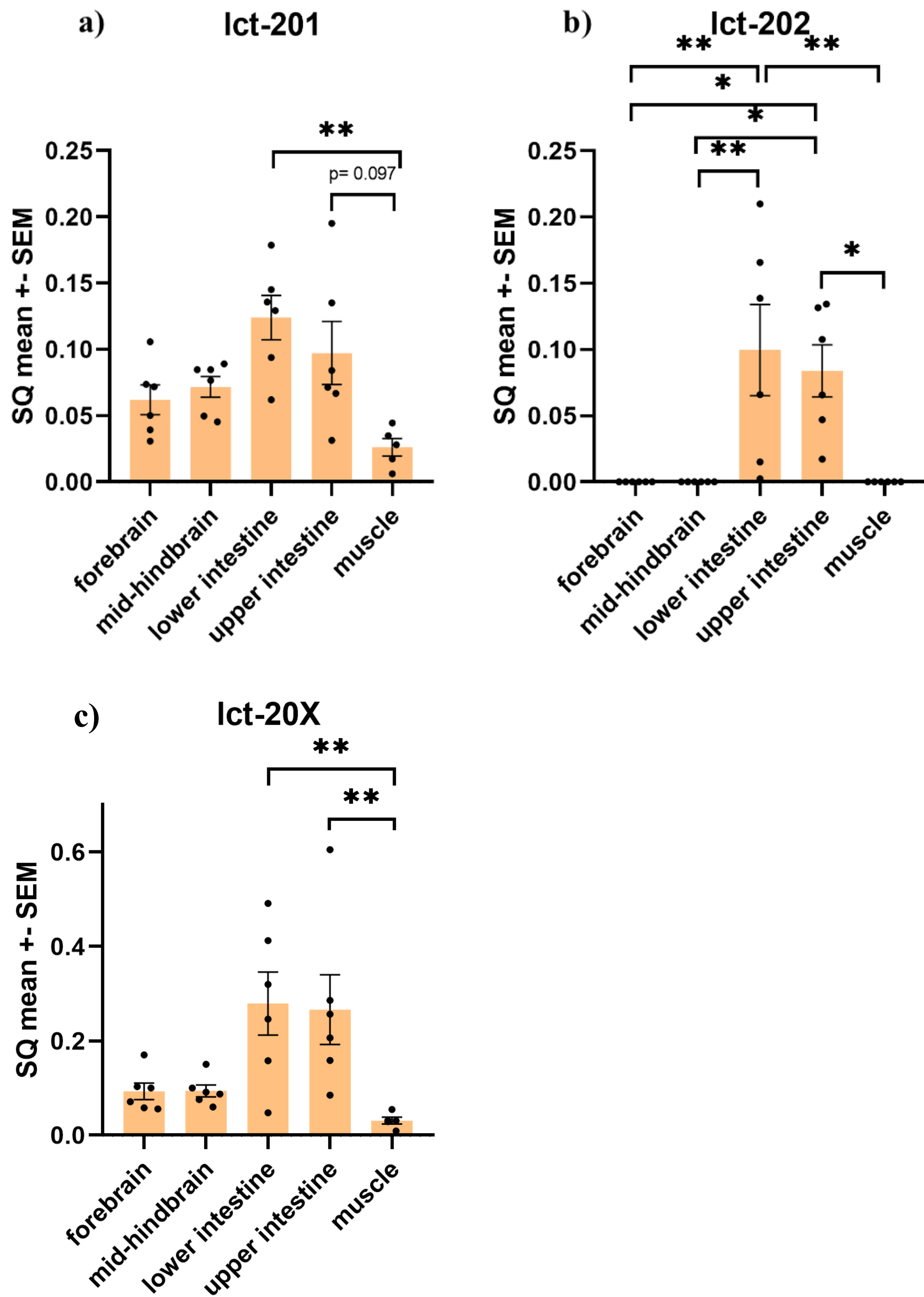

Fig. 11. *Lct* transcript expression levels in adult zebrafish tissues. Bar graphs showing normalized expression levels of the *lct* transcripts in five tissues. Kruskal-Wallis test (**a)** 14.85, p= 0.005; **b)** 26.7, p< 0.0001; **c)** 17.49, p= 0.0015) and Dunn's *post hoc* were calculated for each transcript (n=6). p values: * < 0.05, ** < 0.01. SQ mean ± SEM is shown.

5.2 Assessment of lactase activity in the brain

To confirm the presence of lactase in the zebrafish whole brain and intestines at a protein level, lactase activity assay was performed.

The amount of protein present in each sample was measured using the Bradford assay. Protein concentration in each homogenate sample was calculated using the standard curve equation. Intestine protein concentrations were on average 47 % of those in the brain. 1 µg/µl protein dilutions were used in the lactase activity assay, to ensure even amounts of protein in each reaction.

Fluorescence signal was detected in all tissue samples. The lactase activity values for each sample were calculated using the standard curve equation. Lactase activity was low in the brain, but higher in the intestine – with the average lactase activity in the brain being 14 % of that in the intestines (Fig. 12).

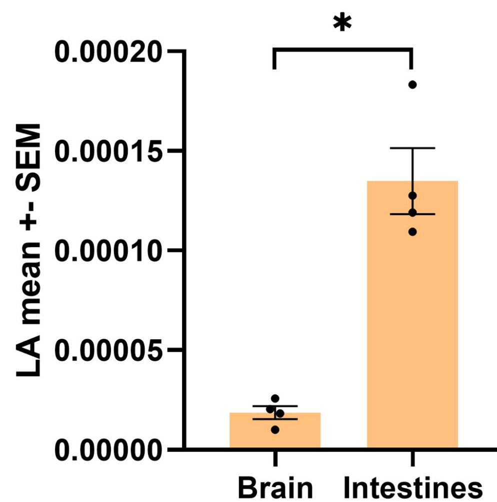

Fig. 12. Lactase activity in the zebrafish brain and intestines. Lactase activity calculated as FCC units per mg of protein. Difference in mean activity between the brain and intestines was compared using Mann-Whitney U test (p= 0.029). Lactase activity (LA) mean ± SEM is shown.

5.3 Quantification of lactase expression in the brain after stress

5.3.1 *Lct* expression after physiological stress

To study whether stress has an effect on lactase expression in the zebrafish, the expression levels in the brain and whole intestines of both stressed and control fish were measured. In this first round of experiments, the focus was on physiological stress – in this case, netting handling.

In *lct-201*, the primary gene product, there were no significant differences in expression between the stressed and control groups in either the brain (Fig. 13a) or the intestines (Fig. 13b).

For *lct-202*, the secondary transcript, the mean lactase expression was not significantly different between the stressed and control individuals in the intestines (Fig. 13c).

In the case of *lct-20X*, there was no stress effect on the lactase expression in the brain (Fig. 13d), whereas in the intestines, the expression level was higher in the stressed fish (Fig. 13e). *Lct-20X* was expressed at approximately 39 % higher level in the stressed group in comparison to the controls in the intestines (p= 0.018).

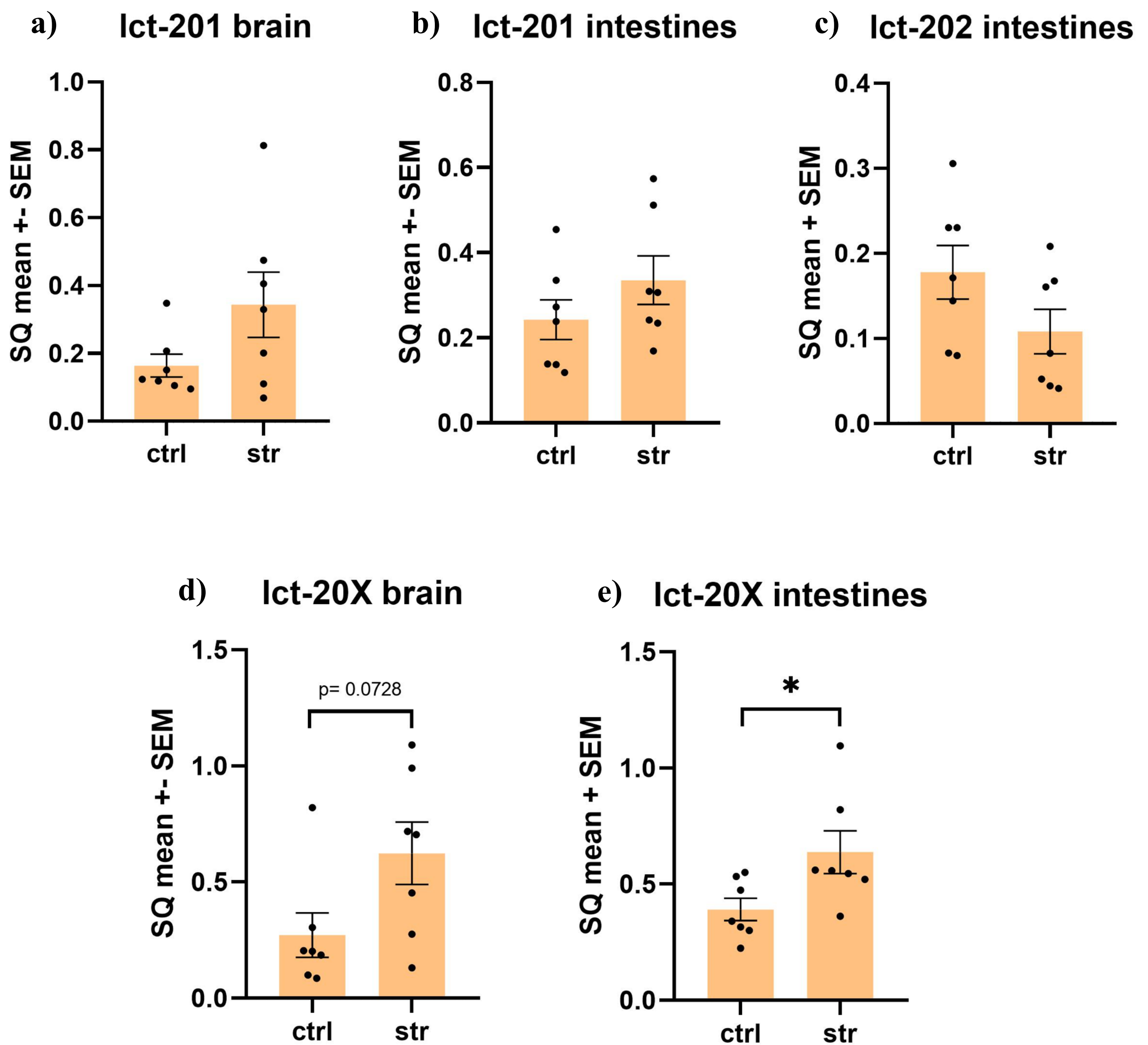

Fig. 13. *Lct* transcript expression levels in adult zebrafish brain and intestines after netting handling stress. Bar graphs showing normalized expression levels of the *lct* transcripts in the brain and intestines of stressed and control fish. Stress effect was evaluated using Mann-Whitney U test (**a)** p= 0.26, **b)** p= 0.26, **c)** p= 0.10, **d)** p= 0.073, **e)** p= 0.018) (n=7). p values: * < 0.05. SQ mean ± SEM is shown.

5.3.2 *Lct* expression after psychosocial stress

To further investigate the effect of stress on lactase expression in adult zebrafish, another stress model was applied. The expression levels of lactase in the brain and intestines of fish that underwent a chronic social defeat stress -treatment and untreated control fish were measured and compared.

In the brain, *lct-201* was evenly expressed between the stressed and control fish (Fig. 14a). In the intestines, no difference in expression was detected either (Fig. 14b).

Lct-202, the intestine-specific transcript, showed no stress effect in its expression levels (Fig. 14c).

Lct-20X displayed no effect of psychosocial stress in its expression in the brain (Fig. 14d). On the other hand, in the intestine fraction, *lct-20X* was expressed at a higher level in the stressed group in comparison to the controls (Fig. 14e). The expression level was approximately 55 % higher in the stressed fish compared to the controls in this tissue (p= 0.050).

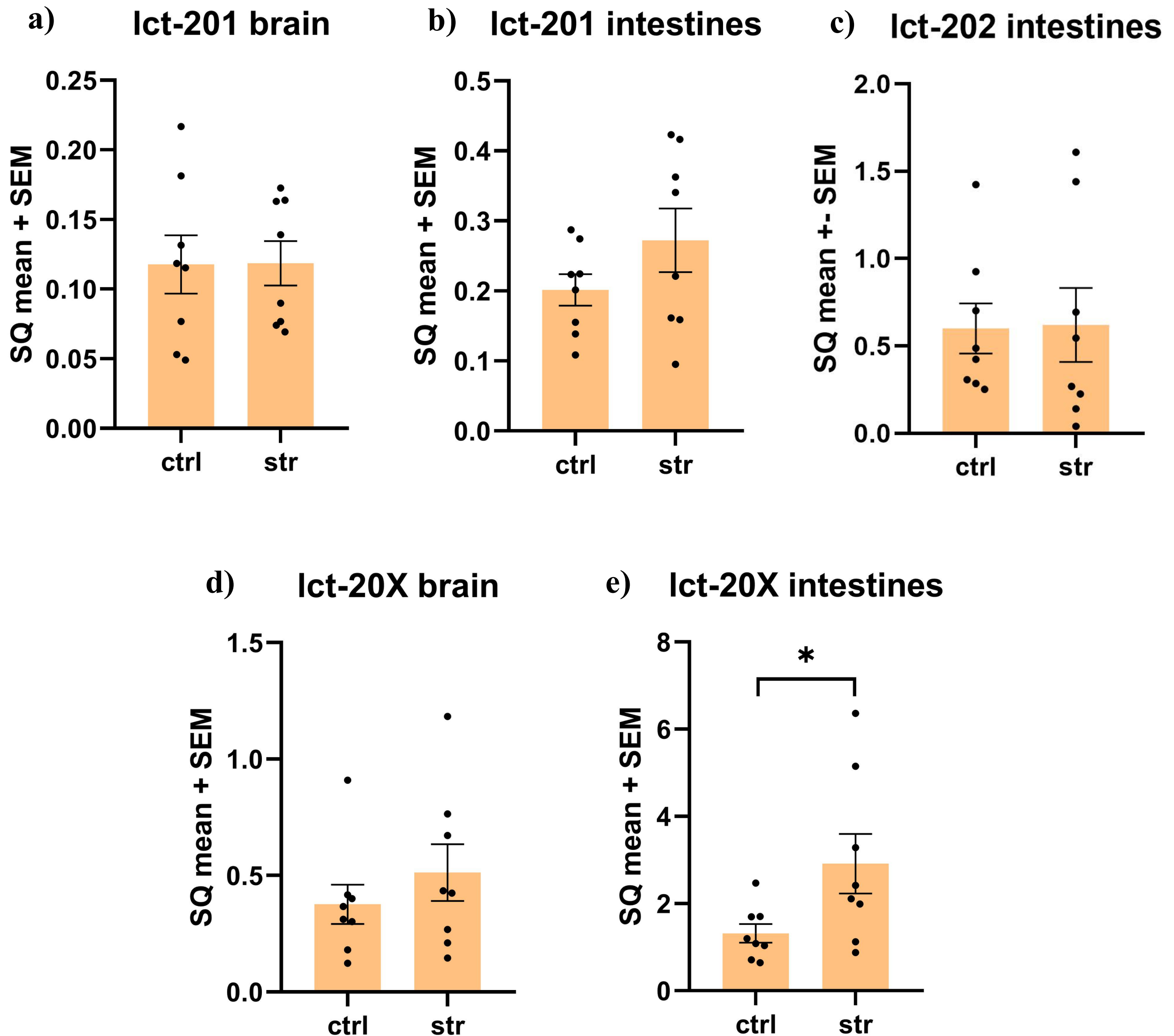

Fig. 14. ***Lct*** **transcript expression levels in adult zebrafish brain and intestines after chronic social defeat stress.** Bar graphs showing normalized expression levels of the *lct* transcripts in the brain and intestines of stressed and control fish. Stress effect was evaluated using Mann-Whitney U test (**a)** p= 0.96, **b)** p= 0.33, **c)** p= 0.65, **d)** p= 0.38, **e)** p= 0.050) (n=8). p values: * < 0.05. SQ mean ± SEM is shown.

6 DISCUSSION

This study was able to prove, that the two lactase transcripts, *lct-201* and *lct-202*, are both in fact expressed in WT zebrafish tissues. They show a distinctive expression profile, with *lct-201* being the only transcript expressed in the brain, whereas *lct-202* expression was limited to intestines in the tissues examined in this study. *Lct-202* did show some amplification in other tissues, however, based on the melt peak (Fig. S1), this product was unspecific. Therefore, the conclusion was made that this transcript is not expressed in the brain or in the muscle. The different patterns of expression suggest a separate biological function for the transcripts. Interestingly, the human lactase gene also has two transcripts, however, one of them is discarded through nonsense-mediated decay and only the other is coding for protein (Yates et al. 2020). The protein product of the primary human transcript contains four enzymatically active sites, of which three are coded by exons that are shared between the two transcripts (UniProt Consortium 2021). Whether the functions performed by the two different transcripts in zebrafish are executed by the one protein-coding human homologue remains unclear.

Of the five zebrafish tissues examined in this study – the forebrain, the mid-hindbrain, the upper and lower intestines and the muscle – the highest level of lactase expression was found in both of the intestine fractions. A similar pattern of intestine-focused expression is present in humans, since lactase is an important digestive enzyme needed for the proper digestion of milk and dairy products. Zebrafish diet, however, does not contain the milk sugar lactose, which is the primary substrate for lactase. In fact, the suggested diet for adult zebrafish is mostly protein – not carbohydrate-based (Westerfield 2007). Therefore, the substrate for zebrafish lactase is not necessarily a sugar compound that would be structurally similar to lactose. This raises the question of other – yet to be discovered – targets of enzymatic activity for lactase in zebrafish. In the zebrafish brain, there was no difference in lactase expression between the forebrain and the mid-hindbrain. One explanation for this could be the rather rough sectioning of the brain due to its small size. According to *in situ* hybridization studies, lactase expression in the mouse brain is concentrated in the hippocampal formation (Lein et al. 2007). More specifically, lactase seems to be expressed only by the neuronal cells of the dentate gyrus, a brain structure associated with adult-type neurogenesis (Purves et al. 2012), Whether the function, or the substrate, of lactase is the same in the brain and intestines calls for further investigation.

In addition to the findings regarding gene expression, I was able to show that lactase is also translated to protein in the zebrafish tissues. The higher lactase activity levels detected in the intestines compared to the brain are in line with the gene expression level difference discovered in these tissues. To ensure that the breakdown of the substrate was indeed specific to lactase, I performed a test with samples from *lct*-KO fish line. In this experiment, I detected substrate breakdown in the KO brain and intestines as well. This enzymatic activity, however, was only about third in the KO intestines compared to WT (t-test, $p< 0.002$). The likely explanation for this is, that there are other hydrolases present in the tissue capable of breaking down the substrate. The statistically significant difference in enzymatic activity between KO and WT tissue does nonetheless suggest, that this method is proficient in detecting functional lactase protein. For future experiments, a competitive inhibitor – namely lactose – could be applied to the lactase activity assay reactions to determine the level of unspecific enzymatic activity: the fluorescence signal produced by the reaction between lactase and the substrate should diminish when lactose is introduced – the remaining signal would represent the amount of unspecific activity.

The effect of stress on lactase expression remains inconclusive in this study. Lactase expression was indeed increased as a result of both physiological and psychosocial stress in the zebrafish intestine with the *lct-20X* primer pair targeting both of the transcripts. However, no stress effect was detected in the brain with *lct-20X*. *Lct-201* and *lct-202* when examined separately, did not display lactase expression differences between stressed and control animals in either the brain or the intestines, regardless of the stress model. These findings are inadequate to prove that lactase is upregulated in stressed zebrafish in comparison to controls. However, the results gained from these experiments do not oppose the possible connection between stress and lactase expression either.

One of the challenging aspects of this study is the genetic heterogeneity in zebrafish. Unlike with mice, for which it is relatively easy to obtain inbred individuals, zebrafish bred for experimental purposes are not genetically homogenous. Indeed, it has been shown that inbreeding in zebrafish leads to a general decline in fitness (Monson & Sadler 2010), and therefore should be avoided. The large variation observed in lactase expression levels in the control fish from the stress experiments might be due to this heterogeneity. The small sample size in this study is sensitive to the effect of individual outliers on the group mean. Increasing the number of samples per group could reduce the variance in expression levels.

Another obstacle I encountered during the experimental part of this thesis was the uneven expression of housekeeper genes in the q-RT-PCRs concerning the effect of stress on lactase levels.

Rpl13a, which was chosen as a housekeeper due to its relatively stable expression between different tissues in the first round of experiments, was not evenly expressed within the brain and intestines in the experiments on stressed and control fish (SQ mean= 1.15, stdev= 0.75). Because this problem was present in both stressed and control group, stress is not the explanatory factor. To overcome this issue, I also carried out the q-RT-PCRs with another housekeeper gene, *b-actin1*. Unfortunately, this did not yield better results: *b-actin1* was also uneven in its expression (SQ mean= 1.31, stdev= 1.19). I compared the expression levels of both housekeeper genes for each sample and there was a statistically significant positive correlation (Pearson's r= 0.70, *p*= 2.87×10^{-8}). In order to determine whether the expression level variance was caused by differences in cDNA synthesis efficacy, I measured the cDNA concentrations and compared those to the mean SQ values of both housekeepers. I found no correlation between the cDNA concentration and the expression level of either *rpl13a* (Pearson's r= -0.19, *p*= 0.35) or *b-actin1* (Pearson's r= 0.26, *p*= 0.19). From these analyses, I could deduce that differences in cDNA concentration between samples were not the cause of unstable housekeeper expression levels. To minimize the risk of having distorted lactase expression values due to a non-optimal housekeeper gene, the mean expression value of *rpl13a* and *b-actin1* was used for normalization. This approach is in fact supported as it reduces the normalization error caused by using only one housekeeper gene (Vandesompele et al. 2002).

One of the strenghts of this study is its novelty. To this day, there are no publications regarding the functionality of the zebrafish lactase gene – let alone its connection to stress. The pathway through which stress could affect lactase expression remains a question, although some suggestive connections have been found. Several studies have linked cortisol to increased lactase expression in intestinal cells both *in vivo* (Sangild et al. 1995, Chaudhry et al. 2008) and *in vitro* (Hauri et al. 1994), however, this effect is not consistent throughout the field (Andrés et al. 1984, Elnif et al. 2006). Lactose breakdown product, galactose, is a component of galactocerebrosides, which are glycosphingolipids enriched in myelin (Coelho et al. 2015). Considering that stress affects myelin thickness – as demonstrated by Laine et al. from our group in 2018 – altered lactase expression as a result of stress might be linked to myelin synthesis through galactose metabolism. Glucose, on the other hand, is associated to stress in a very straightforward manner: blood glucose levels are increased as a part of the fight-or-flight response. Thus, changes in lactase expression might be due to the increased need for lactose breakdown products. Unfortunately, this does not explain the phenomenon in fish.

The methods applied in this study are well established and generally reliable, which makes it a good reference for future studies. One approach to further investigate the relationship between lactase and stress is to compare the lactase activity levels in stressed and control fish. Another interesting future prospect is conducting behavioral experiments on *lct*-KO fish. This would demonstrate whether lactase has a complex behavioral function in zebrafish. Especially experiments measuring anxiety-like behavior, such as novel tank diving test, would hopefully shed light on the role of lactase in the brain.

All in all, this study suggests that lactase is expressed in the zebrafish brain, where it might have a functionality that is unique to the central nervous system. A clear connection between stress and lactase expression was not established, although the results offer a glimpse of hope for future studies regarding this topic. A great deal is yet to be discovered on the lactase gene and its function in zebrafish. The most important aspect, however, is how these results will be translated to humans. Whether research on lactase experiences a renaissance as the next hot topic in neuroscience after decades of only being associated with unpleasant intestinal problems, remains to be seen.

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9 APPENDIX

9.1 Supplemental figures

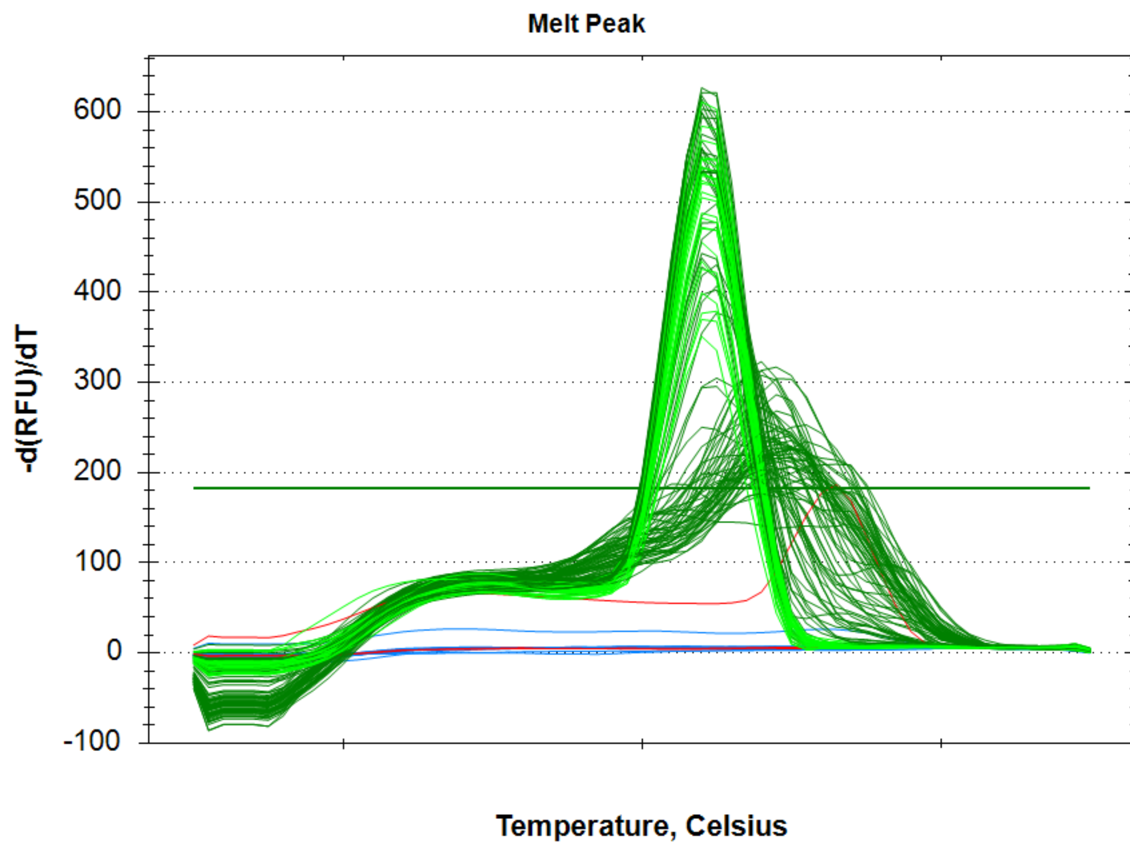

Fig. S1. The melt peak of *lct-202*. The actual melt peak formed by standard (light green) and intestine samples (dark green), with the incoherent curve consisting of the brain and muscle samples next to it (also dark green). Blue and red indicate no reverse transcriptase and no template control, respectively.