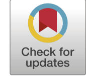
Journal of Sea Research 174 (2021) 102081

Contents lists available at ScienceDirect

Journal of Sea Research

journal homepage: www.elsevier.com/locate/seares



Adult female European perch (*Perca fluviatilis*) from the Baltic Sea show no evidence of thiamine deficiency

Johan Gustafsson[a,*], Karin Ström[a], Linus Arvstrand[a], Lars Förlin[b], Lillemor Asplund[a], Lennart Balk[a]

[a] Department of Environmental Science, Stockholm University, SE-106 91 Stockholm, Sweden
[b] Department of Biological and Environmental Sciences, University of Gothenburg, SE-405 30 Gothenburg, Sweden

ARTICLE INFO

Keywords:
Fish
Health
Apoenzyme
Seasonal variation
Transketolase
Vitamin B_1

ABSTRACT

Deficiency of thiamine (vitamin B_1) has been demonstrated in several species in the northern hemisphere and is suggested as a cause for declining populations. European perch from the Baltic Sea show negative temporal trends for several health biomarkers and poor recruitment of unknown cause. In this study, thiamine status of perch liver from the Baltic Sea was studied with emphasis on seasonal variation. During spring the thiamine concentration increased, reached a higher level during the summer and then decreased again during autumn. Despite this variation the thiamine concentration was always sufficient in the perch liver. These results indicate that direct thiamine deficiency is an unlikely explanation for the health effects observed in adult female perch from the Baltic Sea.

1. Introduction

The Baltic Sea is a brackish semi-enclosed sea located in the northern parts of Europe. The brackish water is home to several marine and fresh water fish species of which one emblematic species is the European perch (*Perca fluviatilis*). The perch in the Baltic Sea have for a long time shown negative trends for health biomarkers such as reduced relative gonad size and induced ethoxyresorufin-O-deethylase (EROD) activity indicating exposure to planar substances in areas located far from anthropogenic point sources (Balk et al., 1996; Hanson et al., 2009; Hansson et al., 2006a, 2006b; Linderoth et al., 2007). More recent studies have also shown increased plasma glucose concentration indicating effects on carbohydrate metabolism; and decreasing percentage of white blood cells indicating effects on immune function (Ericson et al., 2017; Förlin et al., 2017a, 2017b). The cause for these changes in health biomarkers is currently unknown. Temporal trends for populations of perch in the Baltic Sea are inconsistent, but populations are declining in some parts of the central Baltic Sea and poor recruitment has been reported from the coastal areas, where the fish in this investigation were sampled (Bryhn et al., 2020; Eklöf et al., 2020; Ljunggren et al., 2010; Olsson, 2019).

Thiamine (vitamin B_1) is a vitamin that is present in cells mainly as non-phosphorylated thiamine (T), thiamine monophosphate (TMP) and thiamine diphosphate (TDP) (Nelson and Cox, 2017). Among these, TDP is the biologically active form and functions as a cofactor for several enzymes. These enzymes are involved in several crucial steps for converting nutrients into energy and synthesising building blocks in the cell. Some examples of enzymes in the central metabolism where TDP is an essential cofactor is transketolase in the pentose phosphate pathway, pyruvate dehydrogenase as a final step in glycolysis and α-ketoglutarate dehydrogenase in the citric acid cycle (Nelson and Cox, 2017). Thiamine is mainly produced by plants and algae, but also by some bacteria and fungi in the aquatic environment (Goyer, 2010). The primary source of thiamine for fish is via their diet (Harder et al., 2018; Niimi et al., 1997).

Thiamine deficiency has been demonstrated for several aquatic species in the Baltic Sea such as European eel (*Anguilla anguilla*), Atlantic salmon (*Salmo salar*), sea trout (*Salmo trutta*) cod (*Gadus morhua*) and blue mussel (*Mytilus sp.*), but also in birds such as the common eider (*Somateria mollissima*) and herring gull (*Larus argentatus*) (Balk et al., 2016; Balk et al., 2009; Engelhardt et al., 2020; Mörner et al., 2017). Visually observable effects of thiamine deficiency include reproductive disturbances of Atlantic salmon leading to offspring mortality and swim-up syndrome, i.e. loss of equilibrium in the water (Åkerman and Balk, 1998; Fitzsimons, 1995; Mac et al., 1985). A sublethal effect, and indicator of thiamine deficiency, is latency of TDP dependent enzymes; i.e. the proportion of a TDP dependent enzyme not bound to TDP, also known as apoenzyme (Blair et al., 1999; Turck et al., 2016). In the

* Corresponding author.
E-mail address: Johan.Gustafsson@aces.su.se (J. Gustafsson).

https://doi.org/10.1016/j.seares.2021.102081
Received 2 December 2020; Received in revised form 25 May 2021; Accepted 15 June 2021
Available online 19 June 2021
1385-1101/© 2021 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

latency measurement, the enzymatic activity in a sample (endogenous activity) is compared to the enzymatic activity after the sample has been saturated with cofactor (the maximum activity) (Hansson, 2012). The endogenous activity is divided with the maximum activity to give the latency in percentage (Hansson, 2012). In an animal with sufficient amount of cofactor, the latency for the enzyme would be 0%, any higher latency indicates deficiency of the cofactor. For latency measurement some sample preparation is performed on the tissue directly after dissection. This requires complicated and time-consuming work in the field. Therefore, in addition to investigating potential thiamine deficiency in perch, we also investigated if perch liver tissue could be frozen directly and all sample preparation be performed in the laboratory at a later stage.

To assess the thiamine status of an animal the concentration of thiamine and its phosphorylated forms are also measured. This is usually expressed as the sum of the concentrations for T, TMP and TDP (SumT). The necessary concentration of SumT to avoid latency varies between species and tissues. Thus, the SumT concentration threshold where latency is observed needs to be established in new tissues and species investigated. Furthermore, data on thiamine status of healthy species is rare, except for humans, thus there is a need for establishing baseline data both to interpret the deficiency in other species but also provide baseline data to investigate potential future thiamine deficiency in the perch.

Thiamine status has previously been investigated in several fish species but not in perch. The changes in health biomarkers observed for perch in the Baltic Sea highlights the importance of investigating thiamine status and deficiency as a possible cause (Balk et al., 1996; Ericson et al., 2017; Förlin et al., 2017a, 2017b; Hanson et al., 2009; Hansson et al., 2006a, 2006b; Linderoth et al., 2007). Furthermore, in most studies where thiamine status has been evaluated, sampling has been performed at a single time point, thus there is limited data available on possible seasonal variation of thiamine status. Data on seasonal variation could provide valuable insights for identifying the cause(s) and mechanism(s) of thiamine deficiency, in perch and other species; and potentially revealing when populations are most susceptible to thiamine deficiency. In this work the thiamine status of perch from the Baltic Sea has been studied with emphasis on variation during spring, summer and autumn, the usually ice free period of the year.

2. Material and methods

2.1. Evaluation of sample preparation techniques

A detailed description of samples and methods used for evaluation of different sample preparation techniques is presented in the Supplementary material and summarized in Fig. S1. In short, two samples from each perch liver were taken. One sample was snap-frozen in liquid nitrogen and sample preparation was performed after 25 days. The extract was split into 2 parts and stored at −80 °C for 165 and 44 days, respectively, before instrumental analysis, these two methods are referred to as "Frozen Liver". The other liver sample was directly after sampling subjected to homogenisation and centrifugation. The acquired supernatant was split into 3 parts. One part was directly subjected to instrumental analysis, this method is referred to as the "Fresh" method and is considered the control method with no disturbance caused by the sample preparation or storage condition. The two other supernatant aliquots were stored at −140 °C for 165 and 44 days, respectively, before instrumental analysis. These two methods are referred to as "Frozen supernatant". The list of chemicals used can be found in the Supplementary material.

2.2. Samples

2.2.1. Samples for establishing the correlation between SumT and latency

The perch were caught in October 2015 at the environmental monitoring site Kvädöfjärden located in the Småland archipelago, Sweden (58° 2′N, 16° 46′E). All specimens included in the study were females 20 to 30 cm in total length. Upon sampling, the fish was killed by a blow to the head and the liver was dissected. One part of the liver was immediately frozen in liquid nitrogen and later used for analysis of T, TMP and TDP. Another part of the liver was used for latency measurement. Homogenization was performed using a Potter-Elvehjem homogenizer at 0 °C with 1.0 mL of 0.25 M sucrose solution added per gram of sample. The homogenate was prepared with 4 up-and-down strokes at 400 rpm, and then additional sucrose solution was added to adjust the sample concentration to 20% liver tissue in sucrose solution. The diluted homogenate was thereafter subjected to subcellular fractionation in a 2 mL Eppendorf tube by centrifugation at 10000 $g_{average}$ at 4 °C for 10 min in an Eppendorf 5415R centrifuge (Eppendorf, Hamburg, Germany). The supernatant was carefully collected with a pipette, without unsettling the pellet. The supernatant was mixed and aliquots were put in cryotubes, directly frozen in liquid nitrogen, and later stored at −140 °C until analysis of the transketolase activity. Ethical permission for the sampling was approved by Stockholm Northern Research Ethics Committee (Dnr. N209/14).

2.2.2. Samples for seasonal variation of thiamine concentration (SumT)

Perch were caught in the Stockholm archipelago (Sweden) around the island Nämdö (59° 11N, 18° 42′E) starting 14th of May and ending 8th of October 2018. In total, sampling was performed at 18 different occasions with five or six fish analysed per sampling occasion, giving in total 91 specimens. The perch were caught with gill nets and then stored in a corf (a cage in the water) close to the site of capture, this is a standard procedure used in the Swedish environmental monitoring program. After three days the fish were killed by decapitation. The liver was dissected and snap-frozen in liquid nitrogen and then stored in −140 °C until further analysis of T, TMP and TDP. Only livers from females between 15 and 30 cm in total length were included in the analysis. This is the size range where effects on carbohydrate metabolism and immune function are observed on the perch in the Baltic Sea. However, few short specimens were caught making the average total length over the whole sampling period 26 ± 2 cm (mean ± standard deviation). Ethical permission for the sampling of perch was approved by the Swedish Board of Agriculture (Dnr 4171–2018).

2.3. Enzyme activity measurement

Transketolase activity was measured on the 10000 g supernatant. The frozen sample for transketolase measurement was thawed quickly by vigorously shaking the cryo tube under a stream of cold tap water. When the sample was thawed it was placed on ice and the enzymatic measurement took place directly. This procedure was performed for each specimen without interruption and completed before the next specimen was thawed and enzymatically analysed. The measurements were performed in 25 mM Tris-Cl buffer, pH 7.0, at 30 °C and otherwise according to Tate and Nixon (1987) using a Shimadzu UV-2600 UV–Vis Spectrophotometer (Shimadzu Corporation, Kyoto, Japan). For measurement of maximum activity, TDP was added to the cuvette to a final concentration of 100 μM. Protein was quantified according to Lowry et al. (1951) with bovine serum albumin as the standard. The results were expressed as nmol NADH consumed per minute and mg supernatant protein.

2.4. Concentration measurement of T, TMP and TDP (SumT)

The determination of T, TMP and TDP was performed using high performance liquid chromatography (HPLC) with fluorescence detection. The method by Brown et al. (1998) with modifications from Kankaanpää et al. (2002) was used. Details of the method can be found in the Supplementary material. In short, trichloroacetic acid (TCA) was added to 0.2 g liver sample. The liver was homogenized in a Potter

Elvehjem homogenization tube with a pestle. Additional TCA was added and the sample was put in boiling water for 10 min. More TCA was added and the extract was centrifuged. The supernatant was isolated and washed with ethyl acetate:hexane 3:2. The sample was derivatised with potassium hexacyanoferrate to enable fluorescence detection. The derivatised sample was filtered and analysed the same day using HPLC with fluorescence detection.

2.5. Statistics

All statistical analysis was performed using GraphPad Prism version 8.4.3 (GraphPad Software, San Diego, California). The SumT seasonal variation data ($n = 91$) was tested for normality using the Shapiro–Wilk test. Due to the low number of samples available for the enzymatic activity measurements in this study ($n = 15$), normality test was not performed. However both SumT and enzymatic activity measurements have in previous studies, with larger data sets, been shown to follow a normal distribution (Balk et al., 2016; Balk et al., 2009). The correlation between SumT and transketolase latency was evaluated using a linear regression model. The seasonal variation of SumT was evaluated using a linear and a second order regression model of the average concentrations at each time point. Seasonal variation of relative proportions of T, TMP and TDP was evaluated using a linear and a second order regression model. Evaluation of differences in relative proportions of T, TMP and TDP between the 15 fish with the highest SumT and the 15 fish with the lowest SumT was done by student's *t*-test for T, TMP and TDP, respectively. Prior to the t-test, all groups were found to have equal variance according to the Brown-Forsythe test.

3. Results

3.1. Evaluation of sample preparation techniques

The method of freezing liver tissue gave rise to false positive latencies, Fig. S2 in Supplementary material. Thus the method of freezing supernatant was further used in the study. Detailed results and discussion of the sample preparation techniques are shown in the Supplementary material.

3.2. Correlation between SumT and transketolase latency

No correlation can be seen between SumT and transketolase latency due to no latency being present in the range 11–17 nmol SumT/g liver tissue, Fig. 1. The observed variation in latency is only due to the variation from the analytical method, thus the latencies vary around 0%.

3.3. Seasonal variation of thiamine concentration (SumT)

The average SumT concentration in liver varied between 15 and 23 nmol/g between May and October, Fig. 2. Both the linear and second order regression models were significant but since the second order function explained much more of the data compared to the linear function (higher R^2-value) the second order model was chosen. In the second order function all the terms were significant at a 99% confidence interval and the R^2 was 0.71. When comparing the time point with the lowest SumT (14th of May) to the time point with the highest SumT (27th of August) the increase in average is 34%. The lowest and highest individual SumT concentration measured during the sampling period was 12 nmol/g and 31 nmol/g, respectively.

The dominating form of thiamine is TDP (83%) followed by TMP (16%) and T (1%), Fig. 3. These proportions remained constant during the whole sampling period, Fig. 3.

The relative proportions in SumT for the 15 fish with the highest SumT (Group A, average SumT 25 nmol/g) is compared to the relative proportions for the 15 fish with the lowest SumT (Group B, average SumT 14 nmol/g), Fig. 4. There is no significant difference between the groups for any of the three proportions calculated ($p > 0.05$).

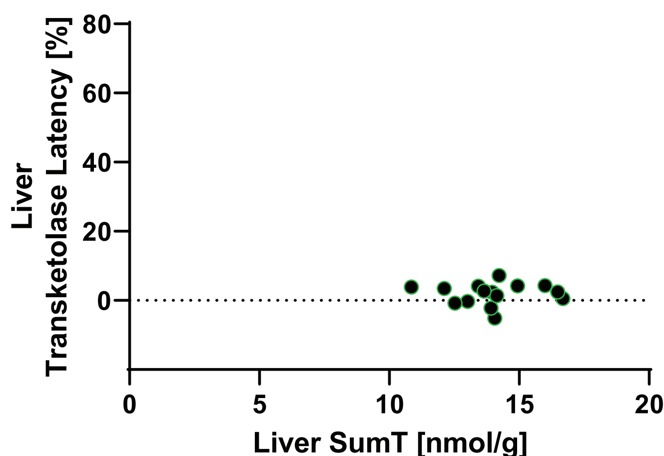


Fig. 1. Correlation between liver SumT and latency of transketolase in perch liver from Kvädöfjärden in the Baltic Sea ($n = 15$). The correlation is not significant ($p > 0.05$).

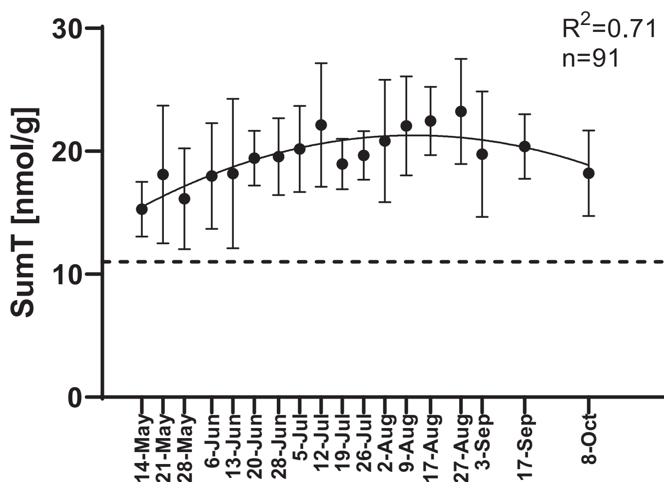


Fig. 2. Average concentration of SumT with 95% confidence interval from May until October in perch liver from Nämdö in the Baltic Sea ($n = 91$). The dotted line shows the lowest concentration where no latency was observed in the transketolase measurement.

4. Discussion

Since no latency was observed in this study it is not possible to determine a threshold concentration of SumT where latency occurs for perch liver, Fig. 1. Although it is clear that no latency is observed above 11 nmol SumT/g liver. The lowest average SumT concentration measured in this study is on the 14th of May where the average SumT was 15 nmol/g, Fig. 2. This average SumT concentration is above the 11 nmol/g where no latency was observed. The lowest individual SumT concentration measured in the seasonal variation portion of this study was 12 nmol/g which also is above the 11 nmol/g where no latency was observed. Thus, even the lowest individual concentration of SumT measured from May until October corresponds to no latency. In species suffering from thiamine deficiency the proportions of T, TMP and TDP can be shifted between the specimens with the highest SumT and lowest SumT. For instance, this effect is observed for cod from the Baltic Sea where the proportion of TDP increased and the proportion of TMP decreased in thiamine deficient specimens (Engelhardt et al., 2020). The unchanged relative proportions of T, TMP and TDP measured in this

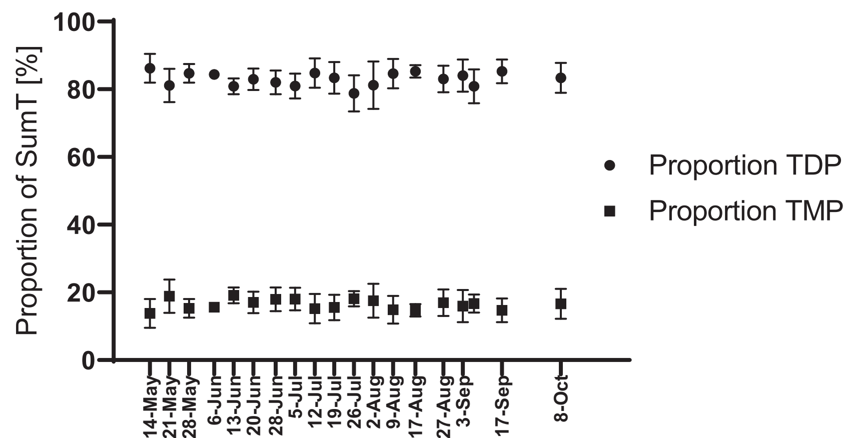


Fig. 3. Average proportions of TMP and TDP in SumT with 95% confidence interval (n = 91). No significant differences are present between time points for each phosphorylated form ($p > 0.05$).

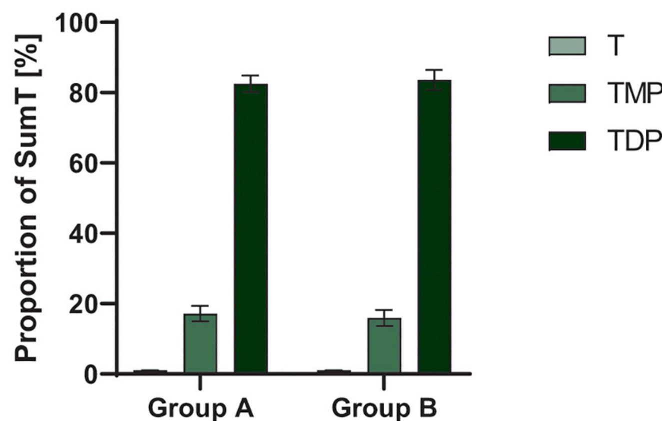


Fig. 4. Average proportion with 95% confidence interval of T, TMP and TDP in the 15 fish with the highest SumT (Group A) and the 15 fish with the lowest SumT (Group B). The average SumT for Group A is 25 nmol/g and for Group B 14 nmol/g.

study further indicates that sufficient amount of thiamine is present in adult female perch from the Baltic Sea, Figs. 3 and 4. However, the observed seasonal variation of SumT indicates that a minimum concentration of SumT may occur outside of the sampling period. Future studies are required to investigate if thiamine deficiency could be present outside of the period investigated in this study.

In this study, the thiamine level in perch liver seems to be higher during the late summer than in the spring and autumn, Fig. 2. A potential explanation for the slightly higher thiamine concentration during the summer is that the perch has a higher metabolic rate and a higher food intake during the summer since it is a poikilothermic animal (Volkoff and Rønnestad, 2020). This increased metabolic activity during the summer could possibly lead to higher concentrations and activities of thiamine dependent enzymes, which would require more thiamine as cofactor. Another possible explanation is that the seasonal variation of SumT is connected to the reproduction cycle of the perch. The perch spawn during the spring, around the start of the sampling period in this study. Adult female perch allocate thiamine to their eggs beginning prior to spawning season, and it is possible that this process decrease tissue thiamine concentrations in the adult female. When the adult female perch have spawned the thiamine levels increase again. At the start of the autumn the adult female perch start to produce eggs and thiamine is transferred to the eggs which decreases the concentration of thiamine in the adult female perch. Previous studies have found an association between SumT concentration in adult female Salmonines and SumT concentration in the eggs (Balk et al., 2016). While no signs of thiamine deficiency in perch could be detected in this study, thiamine deficiency has been detected in several species of the same food web as the perch (Balk et al., 2016). It is possible that the cause of thiamine deficiency in these other species also influences the seasonal variation observed in this study.

In contrast to the results for the perch in this study, blue mussels from the Baltic Sea show a strong decrease in SumT during July (Balk et al., 2016). Also in this study a small but not statistically significant decrease was seen in SumT in the middle of July, Fig. 2. In Lake Michigan, USA, the SumT in alewife (*Alosa pseudoharengus*) tended to be lowest during spring, slightly elevated in the summer, and slightly reduced in the fall (Tillitt et al., 2005). This is the same trend as seen in this study. However, for bloater (*Coregonus hoyi*) there was no variation depending on season (Tillitt et al., 2005). In the same study large inter-year variations were also observed with up to three times differences in SumT concentration. These results highlight that thiamine status is dynamic and the temporal as well as spatial variation should be considered when evaluating thiamine status.

As perch grow, their food preferences change. Perch between 12 and 20 cm in total length feed mainly on crustaceans while perch larger than 20 cm feed mainly on fish (Lappalainen et al., 2001). Since the food differs depending on the size of the perch the thiamine status could also differ. The perch used in this study were females of 15 to 30 cm in total length. The thiamine status of shorter and thus younger perch is not known, it would therefore be of interest to study thiamine status in younger and smaller perch to find out if it differs from the larger perch investigated in this study. The thiamine status of smaller and younger perch could be interesting considering the poor recruitment of perch in the Baltic Sea (Ljunggren et al., 2010).

Thiamine deficiency is detected in many species with highly varying spatial and temporal distribution. In this study a species without obvious signs of thiamine deficiency has been identified. This is important information when mapping the spread of thiamine deficiency and trying to identify the mechanisms and causes for the deficiency. The negative trends for health biomarkers of perch from the Baltic Sea is another concern. The changes in EROD activity, relative gonad weight and glucose concentration have somewhat levelled off in recent years but remain at levels indicating reduced health compared to the 1990s (Mustamäki et al., 2020). In this study the affected health could not be explained by thiamine deficiency. Further research into the poor health of perch from the Baltic Sea should thus consider other factors than direct thiamine deficiency as the primary cause for the poor health. In the study of Eklöf et al. (2020) the population decline of perch was attributed to the increase of the three-spined stickleback (*Gasterosteus aculeatus*) which has caused a regime shift. This regime shift leads to a

so-called trophic cascade, where high densities of stickleback favours the growth of filamentous algae (Eklöf et al., 2020). These algae produce toxic substances such as hydroxylated brominated diphenyl ethers (OH-PBDEs), which, among other things, disrupt the cell's energy production, the oxidative phosphorylation (OXPHOS) (van Boxtel et al., 2008; Legradi et al., 2014; Malmvärn et al., 2008). High concentrations of OH-PBDEs have been found in perch from the Baltic Sea (Dahlgren et al., 2016). The regime shift combined with disrupted energy production may be important factors for the health and poor recruitment of perch in the Baltic Sea. Further studies are underway to investigate the effect of OH-PBDE on the perch from the Baltic Sea.

5. Conclusions

The thiamine content in perch from the Baltic Sea varies from spring until autumn. However, data from several time points and two locations in the Baltic Sea show no signs of thiamine deficiency in adult female perch. Using transketolase latency as a marker, it appears unlikely that thiamine deficiency is the direct cause of the poor health in adult perch from the Baltic Sea.

Declaration of Competing Interest

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

Acknowledgments

Henrik Dahlgren and Jari Parkkonen are greatly acknowledged for the sampling, Hanna Gustavsson for her assistance with the chemical analysis, and Tracy Collier and Josefin Engelhardt for valuable comments on the manuscript. The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (Formas) (Contact Dnr: 942-2015-944), Göte Borgströms stiftelse för fiske- och vattenvård, Stiftelsen Hållbara Hav and the National Marine Monitoring Programme (Swedish Environmental Protection Agency) are acknowledged for financially supporting the project.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seares.2021.102081.

References

Åkerman, G., Balk, L., 1998. Descriptive studies of mortality and morphological disorders in early life stages of cod and salmon originating from the Baltic Sea. Am. Fish. Soc. Symp. 41–61.
Balk, L., Larsson, Å., Förlin, L., 1996. Baseline studies of biomarkers in the feral female perch (*Perca fluviatilis*) as tools in biological monitoring of anthropogenic substances. Mar. Environ. Res. 42, 203–208.
Balk, L., Hägerroth, P.-Å., Åkerman, G., Hanson, M., Tjärnlund, U., Hansson, T., Hallgrimsson, G.T., Zebühr, Y., Broman, D., Mörner, T., Sundberg, H., 2009. Wild birds of declining European species are dying from a thiamine deficiency syndrome. Proc. Natl. Acad. Sci. 106, 12001–12006.
Balk, L., Hägerroth, P.-Å., Gustavsson, H., Sigg, L., Åkerman, G., Muñoz, Y.R., Honeyfield, D.C., Tjärnlund, U., Oliveira, K., Ström, K., Stephen, M.D., Karlsson, S., Ström, M., van Manen, M., Berg, A.-L., Halldórsson, H.P., Strömquist, J., Collier, T. K., Börjeson, H., Mörner, T., Hansson, T., 2016. Widespread episodic thiamine deficiency in Northern Hemisphere wildlife. Sci. Rep. 6, 38821.
Blair, P.V., Kobayashi, R., Edwards, H.M., Shay, N.F., Baker, D.H., Harris, R.A., 1999. Dietary thiamin level influences levels of its diphosphate form and thiamin-dependent enzymic activities of rat liver. J. Nutr. 129, 641–648.
Brown, S.B., Honeyfield, D.C., Vandenbyllaardt, L., 1998. Thiamine analysis in fish tissues. In: Early Life Stage Mortality Syndrome in Fishes of the Great Lakes and Baltic Sea. American Fisheries Society, Symposium, pp. 73–81.
Bryhn, A., Sundelöf, A., Lingman, A., Florin, A.-B., Petersson, E., Vitale, F., Sundblad, G., Strömberg, H., Wennhage, H., Wickström, H., Ahlbeck Bergendahl, I., Olsson, J., Lövgren, J., Persson, J., Sundin, J., Lundström, K., Edsman, L., Ogonowski, M., Ulmestrand, M., Lindmark, M., Bergenius, M., Sjöberg, N., Holmgren, N., Renman, O., Naddafi, R., Fredriksson, R., Larsson, S., Axenrot, T., Dekker, W., Hekim Pekcan, Z., 2020. Fisk-och skaldjursbestånd i hav och sötvatten 2019: Resursöversikt [Fish and shellfish stocks in marine and freshwater areas 2019: Synthesis of the fisheries resources] (In Swedish).
Dahlgren, E., Lindqvist, D., Dahlgren, H., Asplund, L., Lehtilä, K., 2016. Trophic transfer of naturally produced brominated aromatic compounds in a Baltic Sea food chain. Chemosphere 144, 1597–1604. https://doi.org/10.1016/j.chemosphere.2015.10.024.
Eklöf, J.S., Sundblad, G., Erlandsson, M., Donadi, S., Hansen, J.P., Eriksson, B.K., Bergström, U., 2020. A spatial regime shift from predator to prey dominance in a large coastal ecosystem. Commun. Biol. 3, 1–9.
Engelhardt, J., Frisell, O., Gustavsson, H., Hansson, T., Sjöberg, R., Collier, T.K., Balk, L., 2020. Severe thiamine deficiency in eastern Baltic cod (*Gadus morhua*). PLoS One 15.
Ericson, Y., Olsson, J., Franzén, F., Förlin, L., Larsson, Å., Parkkonen, J., Faxneld, S., Danielsson, S., Nyberg, E., Ek, C., 2017. Faktablad från Integrerad kustfiskövervakning 2017: 3: Kvädöfjärden (Egentliga Östersjön) 1988–2016 (In Swedish).
Fitzsimons, J.D., 1995. The effect of B-vitamins on a swim-up syndrome in Lake Ontario lake trout. J. Great Lakes Res. 21, 286–289.
Förlin, L., Larsson, Å., Parkkonen, J., Ericson, Y., Ek, C., Faxneld, S., Danielsson, S., Nyberg, E., Olsson, J., Franzén, F., 2017a. Faktablad från integrerad kustfiskövervakning 2017:2. Holmöarna (Bottniska viken) 1989–2016 (In Swedish).
Förlin, L., Larsson, Å., Parkkonen, J., Ericson, Y., Ek, C., Faxneld, S., Danielsson, S., Nyberg, E., Olsson, J., Franzén, F., 2017b. Faktablad från Integrerad kustfiskövervakning 2017:4. Torhamn (södra Egentliga Östersjön) 2002–2016 (In Swedish).
Goyer, A., 2010. Thiamine in plants: aspects of its metabolism and functions. Phytochemistry 71, 1615–1624.
Hanson, N., Förlin, L., Larsson, Å., 2009. Evaluation of long-term biomarker data from perch (*Perca fluviatilis*) in the Baltic sea suggests increasing exposure to environmental pollutants. Environ. Toxicol. Chem. An Int. J. 28, 364–373.
Hansson, T., 2012. Comparison of two measures of missing cofactor in cofactor-dependent enzymes: proportion versus relative increase. Open Biomark. J. 5.
Hansson, T., Lindesjöö, E., Förlin, L., Balk, L., Bignert, A., Larsson, Å., 2006a. Long-term monitoring of the health status of female perch (*Perca fluviatilis*) in the Baltic Sea shows decreased gonad weight and increased hepatic EROD activity. Aquat. Toxicol. 79, 341–355.
Hansson, T., Schiedek, D., Lehtonen, K.K., Vuorinen, P.J., Liewenborg, B., Noaksson, E., Tjärnlund, U., Hanson, M., Balk, L., 2006b. Biochemical biomarkers in adult female perch (*Perca fluviatilis*) in a chronically polluted gradient in the Stockholm recipient (Sweden). Mar. Pollut. Bull. 53, 451–468. https://doi.org/10.1016/j.marpolbul.2006.04.014.
Harder, A.M., Ardren, W.R., Evans, A.N., Futia, M.H., Kraft, C.E., Marsden, J.E., Richter, C.A., Rinchard, J., Tillitt, D.E., Christie, M.R., 2018. Thiamine deficiency in fishes: causes, consequences, and potential solutions. Rev. Fish Biol. Fish. 28, 865–886.
Kankaanpää, H., Vuorinen, P.J., Sipiä, V., Keinänen, M., 2002. Acute effects and bioaccumulation of nodularin in sea trout (*Salmo trutta* m. *trutta* L.) exposed orally to Nodularia spumigena under laboratory conditions. Aquat. Toxicol. 61, 155–168.
Lappalainen, A., Rask, M., Koponen, H., Vesala, S., 2001. Relative abundance, diet and growth of perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) at Tvaerminne, northern Baltic Sea, in 1975 and 1997: responses to eutrophication? Boreal Environ. Res. 6, 107–118.
Legradi, J., Dahlberg, A.K., Cenijn, P., Marsh, G., Asplund, L., Bergman, Å., Legler, J., 2014. Disruption of oxidative phosphorylation (OXPHOS) by hydroxylated polybrominated diphenyl ethers (OH-PBDES) present in the marine environment. Environ. Sci. Technol. 48, 14703–14711. https://doi.org/10.1021/es5039744.
Linderoth, M., Ledesma, M., Noaksson, E., Widell, B., Zebühr, Y., Balk, L., 2007. Seasonal testosterone UDP-glucuronosyltransferase activity and biliary steroids in Eurasian perch: Response to leachate exposure. Ecotoxicol. Environ. Saf. 68, 49–56. https://doi.org/10.1016/j.ecoenv.2006.08.001.
Ljunggren, L., Sandström, A., Bergström, U., Mattila, J., Lappalainen, A., Johansson, G., Sundblad, G., Casini, M., Kaljuste, O., Eriksson, B.K., 2010. Recruitment failure of coastal predatory fish in the Baltic Sea coincident with an offshore ecosystem regime shift. ICES J. Mar. Sci. 67, 1587–1595.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
Mac, M.J., Edsall, C.C., Seelye, J.G., 1985. Survival of lake trout eggs and fry reared in water from the upper Great Lakes. J. Great Lakes Res. 11, 520–529.
Malmvärn, A., Zebühr, Y., Kautsky, L., Bergman, Å., Asplund, L., 2008. Hydroxylated and methoxylated polybrominated diphenyl ethers and polybrominated dibenzo-p-dioxins in red alga and cyanobacteria living in the Baltic Sea. Chemosphere 72, 910–916. https://doi.org/10.1016/j.chemosphere.2008.03.036.
Mörner, T., Hansson, T., Carlsson, L., Berg, A.-L., Muñoz, Y.R., Gustavsson, H., Mattsson, R., Balk, L., 2017. Thiamine deficiency impairs common eider (*Somateria mollissima*) reproduction in the field. Sci. Rep. 7, 1–7.
Mustamäki, N., Franzén, F., Persson, S., Tollerz Bratteby, U., Tärnlund, S., Pettersson, M., Olsson, J., Förlin, L., Larsson, Å., Parkkonen, J., others, 2020. Faktablad från Integrerad kustfiskövervakning 2020, p. 1 (In Swedish).
Nelson, D.L., Cox, M.M., 2017. Principles of Biochemistry, 7th ed.
Niimi, A.J., Jackson, C.J.C., Fitzsimons, J.D., 1997. Thiamine dynamics in aquatic ecosystems and its biological implications. Int. Rev. der gesamten Hydrobiol. und Hydrogr. 82, 47–56.
Olsson, J., 2019. Past and current trends of coastal predatory fish in the Baltic Sea with a focus on perch, pike, and pikeperch. Fishes 4, 7.
Tate, J.R., Nixon, P.F., 1987. Measurement of Michaelis constant for human erythrocyte transketolase and thiamin diphosphate. Anal. Biochem. 160, 78–87.

Tillitt, D.E., Zajicek, J.L., Brown, S.B., Brown, L.R., Fitzsimons, J.D., Honeyfield, D.C., Holey, M.E., Wright, G.M., 2005. Thiamine and thiaminase status in forage fish of salmonines from Lake Michigan. J. Aquat. Anim. Health 17, 13–25.

Turck, D., Bresson, J.-L., Burlingame, B., Dean, T., Fairweather-Tait, S., Heinonen, M., Hirsch-Ernst, K.I., Mangelsdorf, I., McArdle, H.J., Naska, A., Nowicka, G., Pentieva, K., Sanz, Y., Siani, A., Sjodin, A., Stern, M., Tomé, D., Van Loveren, H., Vinceti, M., Willatts, P., Lamberg-Allardt, C., Przyrembel, H., Tetens, I., Rakic, J.G., Ioannidou, S., de Sesmaisons-Lecarré, A., Forss, A.C., Neuhauser-Berthold, M., 2016. Dietary reference values for thiamin. Eur. Food Saf. Auth. J. 14.

van Boxtel, A.L., Kamstra, J.H., Cenijn, P.H., Pieterse, B., Wagner, M.J., Antink, M., Krab, K., van der Burg, B., Marsh, G., Brouwer, A., 2008. Microarray analysis reveals a mechanism of phenolic polybrominated diphenylether toxicity in zebrafish. Environ. Sci. Technol. 42, 1773–1779.

Volkoff, H., Rønnestad, I., 2020. Effects of temperature on feeding and digestive processes in fish. Temperature 7, 307–320.

Supplementary material

Adult female European perch (*Perca fluviatilis*) from the Baltic Sea show no evidence of thiamine deficiency

Johan Gustafsson*[1], Karin Ström[1], Linus Arvstrand[1], Lars Förlin[2], Lillemor Asplund[1], Lennart Balk[1]

[1]Department of Environmental Science, Stockholm University, SE-106 91, Stockholm, Sweden

[2]Department of Biological and Environmental Sciences, University of Gothenburg, SE-405 30, Gothenburg, Sweden

*Corresponding Author: Email: Johan.Gustafsson@aces.su.se Telephone: +4686747227

2. Material and Methods

Chemicals

Bovine serum albumin (A4378), magnesium chloride (Ultra M2670), sodium chloride (S7653), NAD^+ (N1511), NADH (N8129), D-ribose 5-phosphate (R7750), sucrose (Ultra S7903), thiamine (T4625), TDP (C8754), TMP (T8637), triosephosphate isomerase (T2391), Tris-Cl (T3253) and D-xylulose 5-phosphate (15807) were purchased from Sigma Aldrich (St. Louis, MO, USA). DL-dithiothreitol (43819) was purchased from Fluka (Riedel-deHaën, Germany). Acetonitrile LiChrosolv (1.14291.2500), hydrochloric acid 30% Suprapur (1.00318.1000), potassium dihydrogen phosphate p.a. (1.04873.1000), potassium hexacyanoferrate (III) p.a. (1.04973.0250) and trichloroacetic acid p.a. (1.00807.1000) were purchased from Merck (Darmstadt, Germany). Ethyl acetate (8037), n-hexane (8044) and dipotassium hydrogen phosphate anhydrous (0241) were purchased from J. T. Baker (Deventer, the Netherlands). Sodium hydroxide (1.3303-1) was purchased from AkzoNobel (Bohus, Sweden). Water was obtained from a MilliQ Integral 3 system (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.1 Evaluation of sample preparation techniques

The perch were caught in October 2015 a few kilometres North-East of the island Nämdö (59°11´N, 18° 42´E) in the Stockholm archipelago, Sweden. Six female perch between 25 and 35 cm in total length were caught overnight with gill nets (mesh size 30–33 mm bar length) and carefully released from the net directly when taken up. The fish were placed in a water tank on board the boat and transported to the shore. Further transportation to the laboratory was performed in an aerated water tank with water from the fishing site. At the laboratory, the fish were stored in an aerated water basin with water also from the fishing site at a temperature of 11 °C and light/dark cycle for 3 days until sampling.

Upon sampling, the fish was killed by a blow to the head and the liver was dissected. The different preparation techniques and storage conditions evaluated are illustrated in Figure S1. Before each storage step the sample was snap-frozen in liquid nitrogen. From each liver two pieces from the central part was removed. One piece was used for the "Frozen Liver" methods and one for the "Fresh" and "Frozen supernatant" methods. The liver piece for the methods "Frozen liver" was directly frozen in liquid nitrogen and 20 to 30 days later thawed, homogenized and centrifuged in two steps (Förlin et al., 1994). The supernatant after the second centrifugation was mixed and split into two aliquots which were stored at -80 °C for different amounts of time before enzymatic analysis. For the three methods "Fresh" and "Frozen supernatant" (1 and 2), homogenization and one step centrifugation of the liver piece was performed immediately after dissection. The supernatant acquired from the centrifugation was split into three aliquots. Two of the aliquots were stored at -140 °C for different amounts of time, these two methods are referred to as "Frozen supernatant". The third aliquot was analyzed directly without any prior storage, this method is referred to as "Fresh". This method is considered as the control method with no disturbance caused by the

sample preparation or storage condition, a fact that was later verified due to no observed latency.

The method “Frozen supernatant 2” is the methodology that has been used since 2004 to evaluate thiamine status in wild animals (Balk et al., 2016, 2009; Engelhardt et al., 2020; Mörner et al., 2017). The two methods “Frozen liver” are the methodology used for preparation and storage of hepatic subcellular fractions in the Swedish environmental monitoring program for fish health which has been running since 1988 (Sandström et al., 2005).

Homogenization of the fresh perch liver in the methods “Frozen supernatant 1 and 2“ and “Fresh” was performed using a Potter-Elvehjem homogenizer at 0 °C with 1.0 mL of 0.25 M sucrose solution added per gram of sample. The homogenate was prepared with 4 up-and-down strokes at 400 rpm, and then additional sucrose solution was added to adjust the sample concentration to 20% liver tissue in sucrose solution. The diluted homogenate was thereafter subjected to subcellular fractionation in a 2 mL Eppendorf tube by centrifugation at 10 000 $g_{average}$ at 4 ˚C for 10 min in an Eppendorf 5415R centrifuge (Eppendorf, Hamburg, Germany). The supernatant was carefully collected with a pipette, without unsettling the pellet. The supernatant was mixed and aliquots were put in cryotubes, directly frozen in liquid nitrogen, and later stored at -140 ˚C until analysis of the transketolase activity.

Preparation of the thawed perch liver in the two methods “Frozen liver” (1 and 2) were performed in a slightly different manner in that different buffers were used and a second centrifugation was added. Homogenization of the liver was performed in a 0.1 M Na/K-phosphate buffer containing 0.15 M KCl (pH 7.5). After a first centrifugation at 10 000 g (20

min at 4 °C) the 10 000 g supernatant was ultracentrifuged at 105 000 g (60 min at 4 °C) (Förlin et al., 1994). Aliquots of the 105 000 g supernatant (cytosolic fraction) were immediately frozen and stored at -80°C until transketolase activity measurements. Ethical permission for the sampling was approved by Stockholm Northern Research Ethics Committee (Dnr. N209/14). Differences in latencies between the sample preparation techniques were evaluated with Analysis of Variance (ANOVA).

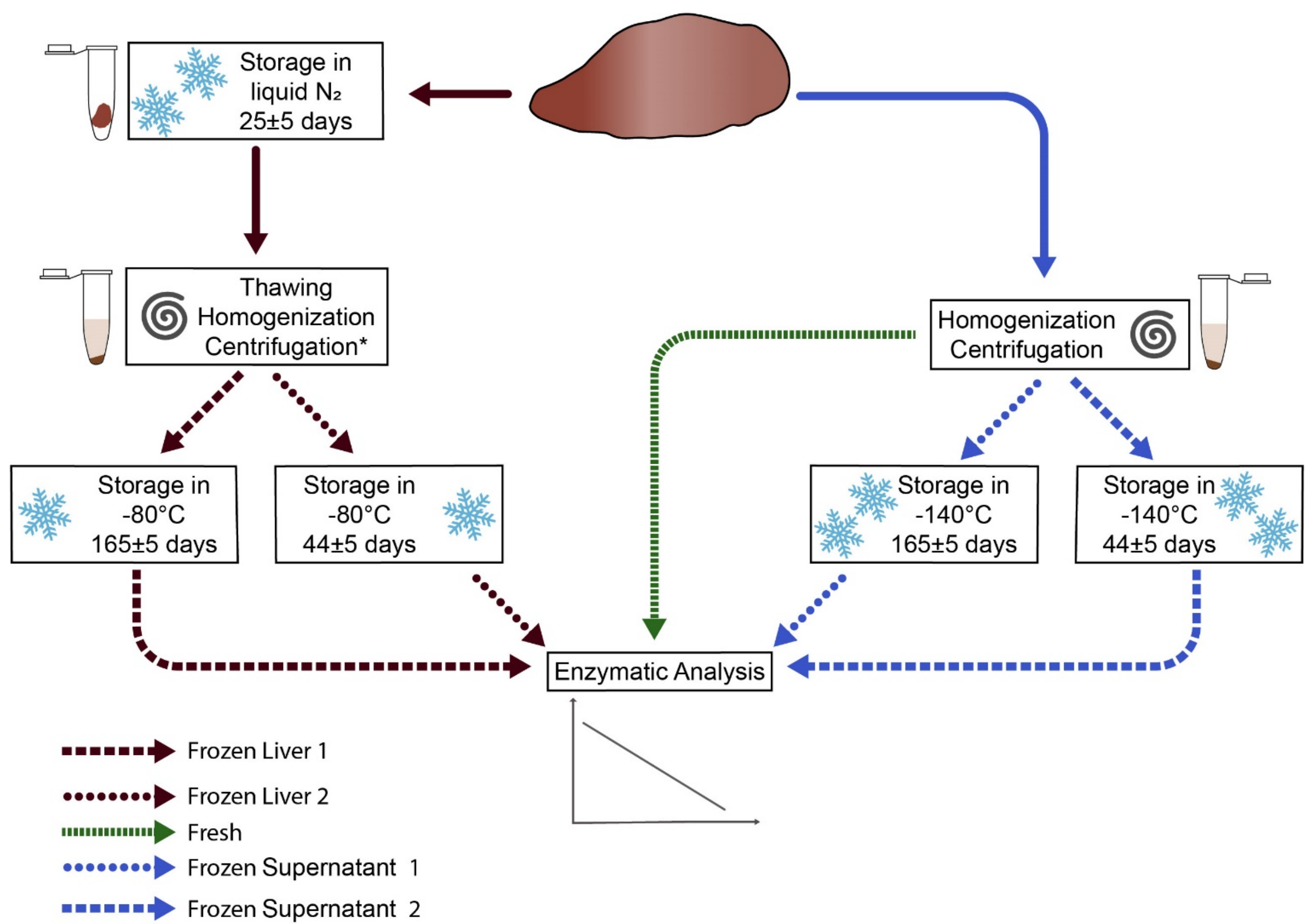


Figure S1. Illustration of the different sample preparations and storage conditions evaluated for measurement of latency on perch liver. *Centrifugation was performed in two steps.

2.5 Measurement of T, TMP and TDP (SumT)

The samples were rapidly thawed at the start of the analysis and then kept on ice between the different preparation steps. A volume of 2.1 mL of 2% trichloro acetic acid (TCA) was added to 0.2 g liver sample. The liver was homogenized in a Potter Elvehjem homogenization tube with a pestle (10 strokes at 420 rpm). The pestle was rinsed with 1.5 mL 2% TCA before putting the sample in boiling water for ten minutes. The sample was cooled on ice and 1.5 mL of 10% TCA was added. The sample was further homogenised with the pestle and the pestle was rinsed with 1.5 mL 10% TCA. The extract was centrifuged at 14.000 g for 15 minutes at 4 °C. The supernatant was isolated and washed three times through addition of 10 mL 3:2 ethyl acetate:hexane. An aliquot of the sample extract (850 µL) was derivatised by addition of 150 µL 0.2% potassium hexacyanoferrate in 0.72M sodium hydroxide. The derivatised sample was filtered through a 0.22 µm PTFE filter before HPLC analysis the same day.

T, TMP and TDP was determined using an L-7100 high performance liquid chromatograph (HPLC) from Merck-Hitachi coupled to an L-7200 autosampler (Merck-Hitachi). The separation was performed with a Luna NH_2, 5 µm 100 Å, 250 x 4.6 mm analytical column with a phenomenex NH_2 (4 x 3.0 mm) guard column. The columns were kept at 30 °C using a column oven. An isocratic elution was performed with 45% acetonitrile and 55% 0.085M potassium phosphate buffer at pH 7.5 at a flow of 1.0 mL/min with a total run time of 25 minutes. Injection volume was 10 µL and detection was performed with an L-7485 fluorescence detector (Merck-Hitachi) with excitation at 375 nm and emission at 433 nm.

Quantification was performed using an external standard calibration curve. The calibration standards were subjected to the full sample preparation before instrumental analysis. The standards were treated like the samples with the exception that derivatisation was performed with a 0.05% potassium hexacyanoferrate solution. In every batch of samples one blank and one reference sample was included. The reference sample was an eider liver previously analysed 133 times. The SumT quantification of the reference sample was always within plus/minus two standard deviations of the previously established mean concentration.

Standards of T, TMP and TDP were prepared in 0.1 M HCl and kept at 4 °C for no longer than 3 months. The phosphate buffer used as mobile phase in the HPLC analysis was prepared fresh every day or every second day. The TCA solutions were kept at 4 °C. The ethyl acetate:hexane 3:2 solution was kept at 4 °C. The 0.2% potassium hexacyanoferrate in 0.72 M NaOH was prepared fresh on a daily basis.

3. Results

3.1 Evaluation of sample preparation techniques for enzyme activity measurement

In Figure S2 the average measured transketolase latencies prepared with the different methods are shown with 95% confidence intervals. For the control method "Fresh" the latency varied around 0%. The latencies for the two methods "Frozen supernatant" also varied around 0% and none of them is significantly different from the control method "Fresh". For the two methods "Frozen liver" the average latencies were 82% and 32%. There is a strong significant difference ($p<0.001$) between the control method "Fresh" and both of the methods Frozen liver 1 and Frozen liver 2. There is also a significant difference between "Frozen liver 1" and "Frozen liver 2" ($p<0.001$), showing that the effect is more pronounced for longer storage time.

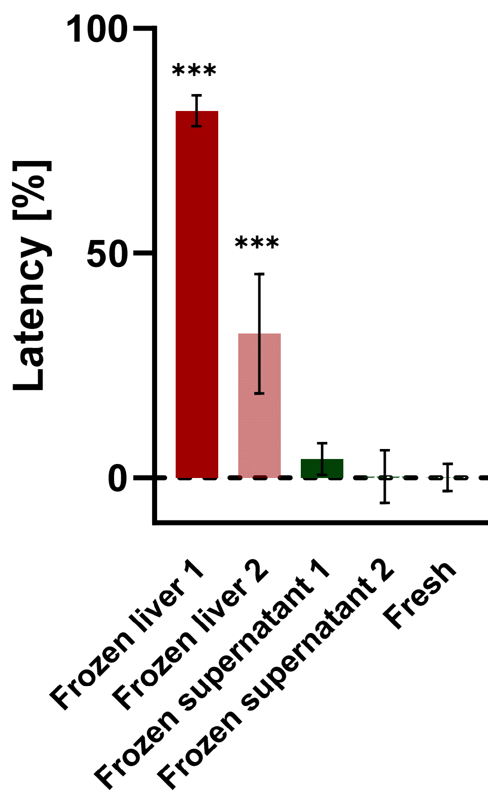


Figure S2. Average latency of transketolase with 95% confidence interval in perch liver prepared with different preparation and storage methods. Stars indicate significant differences ($p<0.001$) towards the control method “Fresh” (n=6 per method).

4. Discussion

Different sample preparation and storage methodologies have been evaluated for measuring latency of the TDP dependent enzyme transketolase in perch liver. Large differences were detected between the investigated methods. Due to differences in buffer type and centrifugation steps between the methods “Frozen liver” and “Frozen supernatant” it is not possible to with certainty pinpoint the reason of the difference. However, the lack of sample preparation on fresh tissue is suspected to be the major contributor of the false positive latencies. This leads to that samples collected in the Swedish environmental monitoring program for fish health cannot be used for measurements of the transketolase latency. One option to avoid advance in-field sample preparation but still achieve correct sampling is to transport live fish in water tanks to the laboratory where the sampling and preparation of the 10 000g supernatant can be performed (Engelhardt et al., 2020).

5. References

Balk, L., Hägerroth, P.-Å., Åkerman, G., Hanson, M., Tjärnlund, U., Hansson, T., Hallgrimsson, G.T., Zebühr, Y., Broman, D., Mörner, T., Sundberg, H., 2009. Wild birds of declining European species are dying from a thiamine deficiency syndrome. Proc. Natl. Acad. Sci. 106, 12001–12006.

Balk, L., Hägerroth, P.-Å., Gustavsson, H., Sigg, L., Åkerman, G., Muñoz, Y.R., Honeyfield, D.C., Tjärnlund, U., Oliveira, K., Ström, K., Stephen, M.D., Karlsson, S., Ström, M., van Manen, M., Berg, A.-L., Halldórsson, H.P., Strömquist, J., Collier, T.K., Börjeson, H., Mörner, T., Hansson, T., 2016. Widespread episodic thiamine deficiency in Northern Hemisphere wildlife. Sci. Rep. 6, 38821.

Engelhardt, J., Frisell, O., Gustavsson, H., Hansson, T., Sjöberg, R., Collier, T.K., Balk, L., 2020. Severe thiamine deficiency in eastern Baltic cod (*Gadus morhua*). PLoS One 15.

Förlin, L., Goksøyr, A., Husøy, A.M., 1994. Cytochrome P450 monooxygenase as indicator of PCB/dioxin like compounds in fish. Biomonitoring Coast. waters estuaries 135–150.

Mörner, T., Hansson, T., Carlsson, L., Berg, A.-L., Muñoz, Y.R., Gustavsson, H., Mattsson, R., Balk, L., 2017. Thiamine deficiency impairs common eider (*Somateria mollissima*) reproduction in the field. Sci. Rep. 7, 1–7.

Sandström, O., Larsson, Å., Andersson, J., Appelberg, M., Bignert, A., Ek, H., Förlin, L., Olsson, M., 2005. Three decades of Swedish experience demonstrates the need for integrated long-term monitoring of fish in marine coastal areas. Water Qual. Res. J. 40, 233–250.