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Chironomus larvae as a dietary vehicle for *Nothobranchius furzeri*

Effects of an enrichment with Punicic acid

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1 Summary

Several studies have demonstrated that specific fatty acids, such as punicic acid, can have a negative impact on cancer cell viability. The molecular mechanisms allowing this feature are still poorly understood but evidence suggests the involvement of a newly characterized cell death pathway: ferroptosis.

We want here to assess the influence of punicic acid on hepatic tumor development in a vertebrate model: *Nothobranchius furzeri*. *N. furzeri* is a rising new model to study the ageing processes and that has been shown to spontaneously develop a high incidence of hepatic tumors correlated with age. The aim of this thesis is to use larva of the *Chironomus riparius* species, a favored food in aquaculture that can exhibit rapid changes in its fatty acid content depending on its diet, to enrich the diet of the fish with punicic acid.

A complete rearing of *C. riparius* has been installed in our laboratories to reproduce a complete life cycle of this insect to produce a continuous production of enriched food for the fish. The development and survival of this insect is greatly influenced by environmental conditions. Indeed, the survival and growth of the larva are highly influenced by their diet and by the water parameters, such as temperature, pH, hardness, and conductivity. On another hand, adult reproduction is affected by the relative humidity in the air. The efficiency of this rearing therefore depends on all these factors influencing different stages of the life cycle of this insect.

Several methods have been used to enrich the diet of *C. riparius* larvae. One method that allowed its enrichment is a diet change method, allowing a division of the rearing into a larva growth part and an enrichment part using a mix of porcine gelatin and pomegranate seed oil, that contains a large proportion of punicic acid, as an enrichment food. In order to obtain more convincing results, this experiment will have to be replicated.

It is true that the use of these *C. riparius* larvae to enrich the diet of *N. furzeri* requires a large production, which implies great constraints; While the use of brine shrimp (*Salina nauplii*) for the same purpose requires fewer constraints and seems more efficient.

However, the use of this *C. riparius* enrichment method could be promising in other research themes, such as ecotoxicology for which this species is often used as a model, and which do not require such mass production as required here.

2 Résumé

Plusieurs études ont montré que certains acides gras, comme l'acide punicique, présentent un effet cytotoxique sur différentes lignées de cellules cancéreuses. Le mécanisme moléculaire permettant ces caractéristiques est encore partiellement incompris, mais des signes suggèrent qu'il implique un processus de mort cellulaire programmée récemment caractérisé : la ferroptose.

Nous voulons ici tester l'influence de l'acide punicique sur le développement tumoral hépatique d'un modèle vertébré : *Nothobranchius furzeri*. *N. furzeri* est un modèle de plus en plus utilisé pour étudier les processus de vieillissement et qui démontre une grande incidence de développement de tumeurs hépatiques corrélée à l'âge. Le but de ce mémoire est d'utiliser des larves de *Chironomus riparius*, aliment privilégié en aquaculture qui montre également la capacité de présenter des changements rapides dans sa composition en acide gras en fonction de son alimentation, dans le but de les utiliser comme moyen d'enrichir l'alimentation du poisson en acide punicique.

Un élevage de *C. riparius* a été installé dans nos laboratoires, permettant de reproduire le cycle de vie complet de cet insecte et ainsi permettre une production continue de larves enrichies pour nourrir *N. furzeri.* Le développement et la survie de cet insecte sont grandement influencés par les conditions environnementales. En effet, la survie et la croissance de sa forme larvaire sont grandement influencés par son alimentation, ainsi que par les paramètres de l'eau tels que la température, le pH, la dureté et la conductivité de l'eau. La reproduction des adultes est quant à elle beaucoup influencée par l'humidité relative de l'air. L'efficacité de cet élevage dépend donc de tous ces facteurs, influençant différentes étapes du cycle de vie de cet insecte.

Plusieurs méthodes ont été utilisées pour enrichir l'alimentation de la larve de *C. riparius*. Une méthode qui a permis son enrichissement est une méthode de changement de régime, permettant de diviser l'élevage en une partie de croissance larvaire et une partie d'enrichissement utilisant un mix de gélatine de porc et de l'huile de pépin de grenade, huile contenant une grande proportion d'acide punicique, comme nourriture d'enrichissement. Afin d'obtenir des résultats plus probants, cette expérience devra être répliquée.

Il est vrai que l'utilisation de ces larves de *C. riparius* pour enrichir l'alimentation de *N. furzeri* nécessite une grande production de celles-ci, ce qui implique de grandes contraintes. L'utilisation d'artémias (*Salina nauplii*) dans ce même but demande quant à elle moins de contraintes, et semble plus efficace.

Cependant, l'utilisation de cette méthode d'enrichissement de *C. riparius* peut se montrer prometteuse dans d'autres thèmes de recherches telles que l'écotoxicologie pour lesquelles cette espèce est souvent utilisée comme modèle et qui ne nécessitent pas une production de masse telle que requise ici.

3 Introduction

Previous studies demonstrate cytotoxic effects of specific combinations of fatty acids on cancer cell lines *in vitro*. We would like to build on this line of research by testing the anti-carcinogenic effects of dietary fatty acids on a fish vertebrate model (*Nothobranchius furzeri*), which have been reported to be prone to develop age-related liver neoplasia, by enriching their typical laboratory diets: *Artemia salina nauplii* (shrimp brine) and *Chironomus riparius* larvae (bloodworms). Within this report, I will describe the enrichment of bloodworms with pomegranate seed oil, which is rich in punicic acid, a conjugated linolenic acid that has been described to present beneficial effects on obesity, diabetes and to have anti-cancerous activity. The main aim of this punicic acid enrichment of bloodworms is to later use them as dietary vehicle for a punicic acid enrichment of *Nothobranchius furzeri*.

4 State of the art

4.1 Fatty acids

4.1.1 Structure, functions and nomenclature

Fatty acids are a class of organic molecules characterized by a single carbon chain with a terminal carboxyl group. They each contain a specific amount of carbon atoms, ranging between two to 40, and can also contain double bonds. The number of double bonds indicates the level of unsaturation of the fatty acid: a saturated fatty acid (SFA) does not present any double bonds in its carbon chain; on the contrary a monosaturated fatty acid (MUFA) contains a single double bond in its carbon chain; a polysaturated fatty acid (PUFA) contains more than one double bond in its carbon chain. The precise position of the double bond on the carbon chain and the conformation of the double bond are also important for the identity of the fatty acid. A double bond can take two different conformations: a double bond is in a *trans* conformation if the two adjacent carbons are positioned on each side of the double bond; conversely the double bond is in a *cis* conformation if the two adjacent carbons are positioned on the same side of the double bond (Figure 1).

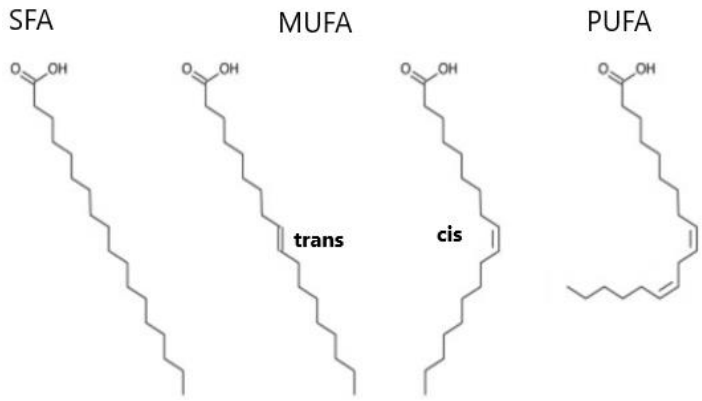


Figure 1: Global structure of a fatty acid: saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA). This figure also illustrates the two conformations for the double bond in the unsaturated forms: trans or cis conformation.

Fatty acids are essential in various biological mechanisms in living organisms, including serving as signaling messengers, as receptors, as antigens and as hormones[1]. They are also largely implicated in the storage of energy. Those functions are made possible by the specific conformation of fatty acids. For example, structural lipids like glycerophospholipids and sphingolipids are amphiphile molecules, containing an apolar subunit and a polar subunit, that form a major part of membranes. The polar subunit is often a phosphate group, but it can be another biomolecule, such as a disaccharide. The apolar subunit is composed of either two fatty acids esterified to a glycerol or a fatty acid amidified to a sphingosine which is a biomolecule formed by the reaction between a fatty acid and a serine (Figure 2). The storage form of fatty acid is triglycerides, molecule formed by the esterification of a glycerol to three carbon chains (Figure 2).

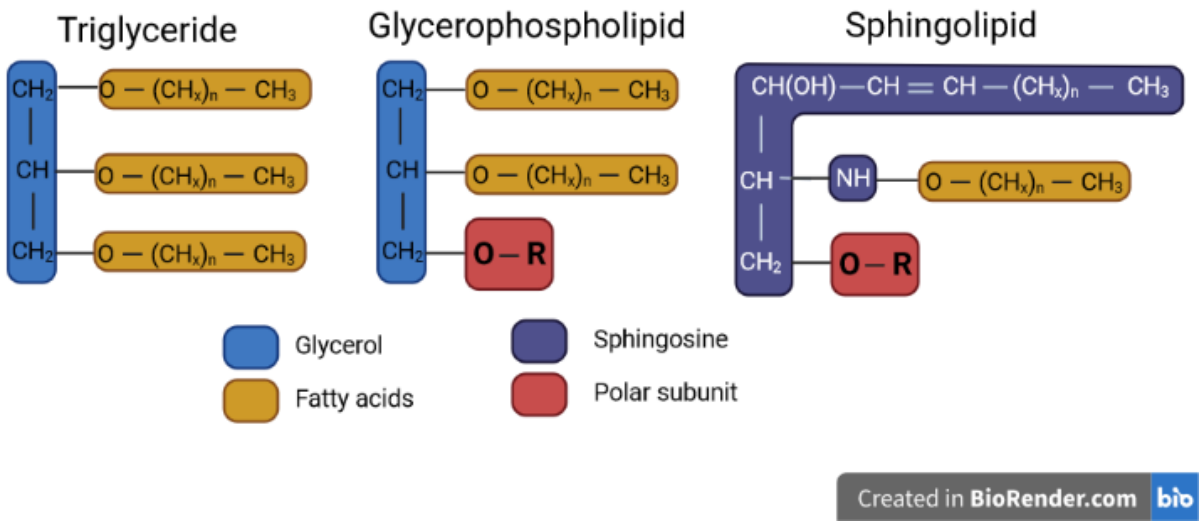
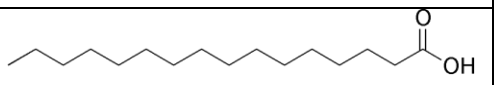
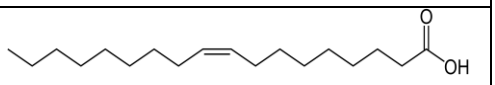
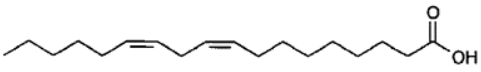
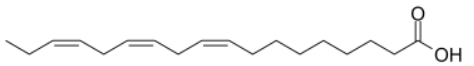
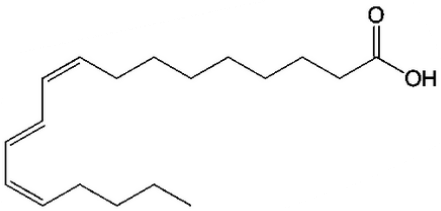
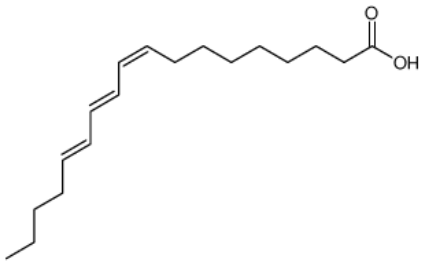
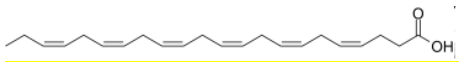


Figure 2: Different types of lipids. Triglycerids are formed by the esterification of a glycerol with three free fatty acids. Glycerophospholipids are amphiphile molecules formed by the esterification of a glycerol with two free fatty acids and a polar molecule. Sphingolipids are formed by the esterification of a free fatty acid and a sphingosine that is attached to a polar molecule. Created by Biorender.com

Fatty acids can be named based on their number of carbons. For example, a fatty acid is considered as a short chain fatty acid (SCFA) if it contains less than six carbons; a fatty acid is considered as a medium chain fatty acid (MCFA) if it contains between six and 12 carbons; a fatty acid is considered as a long chain fatty acid (LCFA) if it contains between 13 and 21 carbons; and finally, a fatty acid is considered as a very long chain fatty acid (VLCFA) if it contains more than 21 carbons[2,3]. The number of carbons greatly influences the chemical properties of the fatty acid (i.e. melting point and viscosity)[3]. Fatty acids can also be named based on their level of saturation (SFA, MUFA and PUFA) and the position of the double bonds in relation to the terminal carbon. The nomenclature for this type of classification is to use a Δ associated with the position and conformation of each double bond (for example, as shown in Table 1, oleic acid can be identified as C18:1(Δ^{9cis})). Another nomenclature can be used for nutritional purposes, which focuses on the number of double bonds in a fatty acid and on the position of the first carbon involved in the furthest double bonds from the carboxylic function (oleic acid is then identified as C18:1 ω-9) (Table 1). A final distinction is whether fatty acids are considered as non-essential fatty acid or essential depending on whether they can be synthetized by the organism or not. We also distinguish semi-essential fatty acid which are fatty acids that cannot be synthetized by the organism at certain developmental stages or if their synthesis is limited and not sufficient for their use.

Table 1: Nomenclature used to refer to a fatty acid, the saturation level and the structure of some common fatty acids

Name	Nomenclature Δ^x	Nomenclature ω	Saturation	Structure
Palmitic acid	C16:0	C16:0	SFA	
Oleic acid	C18:1(Δ^{9cis})	C18:1 ω-9	MUFA	
Linoleic acid	C18:2 ($\Delta^{9cis,11trans}$)	C18:2 ω-7	PUFA	
α-linolenic acid	C18:3 (cis $\Delta^{9,12,15}$)	C18:3 ω-3	PUFA	
Punicic acid	C18:3 ($\Delta^{9cis,11trans,13cis}$)	C18:3 ω-5	PUFA	
α-eleostearic acid	C18:3 ($\Delta^{9cis,11trans,13trans}$)	C18:3 ω-5	PUFA	
Docosahexaenoic acid	C22:6(cis $\Delta^{4,7,10,13,16,19}$)	C22:6 ω-3	PUFA	

4.1.2 Metabolism

In the context of the use of fatty acids for energy, we can distinguish two major metabolism pathways: fatty acid synthesis and fatty acid β-oxidation.

4.1.2.1 Fatty acid synthesis

Fatty acids can be synthesized in most animal cells. This synthesis is initiated by the influence of different factors, including a high ATP cellular content, a high concentration of glucose in the blood and a high concentration of citrate. The latter is an intermediate of the Kreb cycle that is exported out of the mitochondria towards the cytosol when in excess where it can be cleaved into oxaloacetate and acetyl-CoA by citrate lyase. The acetyl-CoA can react with CO_2 to form malonyl-CoA, a reaction catalyzed by acetyl-CoA carboxylase (ACC) (Figure 3). Malonyl-CoA is the primary component of fatty acid synthesis. The enzyme that allows the *do novo* synthesis of fatty acids is the fatty acid synthase (FAS)[4]. FAS have two binding sites

which allow for the attachment of the fatty acid in formation and malonyl-CoA that will be added to this fatty acid to form the carbon chain (two carbons at the time). The fatty acid is released from the FAS by a thioesterase, which will form the carboxyl group. This process leads to the formation of palmitic acid (C16:0). Further elongation of the carbon chain of the fatty acid is made by an elongase and desaturations are made by specific desaturases (Figure 3 and 4)[5].

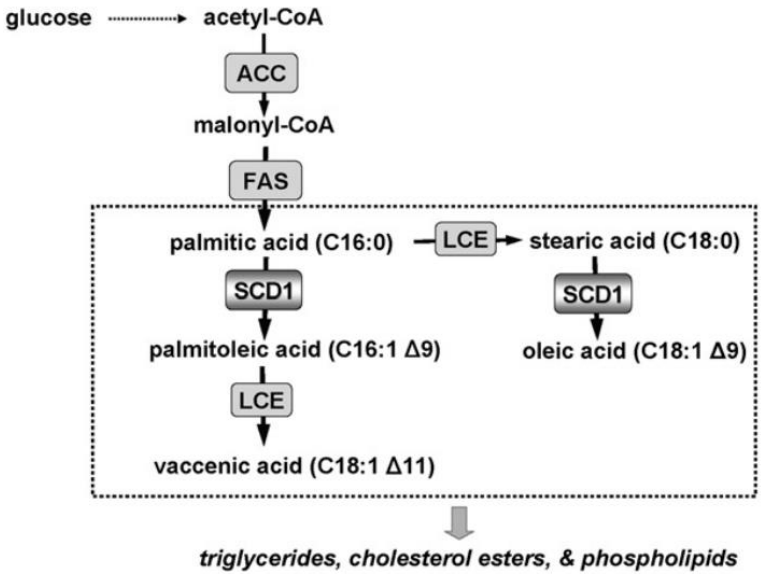


Figure 3: *de novo* lipogenesis[4]. This process begins with the formation of malonyl-CoA from acetyl-CoA by acetyl-CoA carboxylase (ACC). Malonyl-CoA is added to fatty acids in formation by fatty acid synthase (FAS) until the formation of palmitic acid (C16:0). At that point, fatty acids can be elongated by long chain fatty acid elongase (LCE) and desaturated by specific desaturases (SCD1).

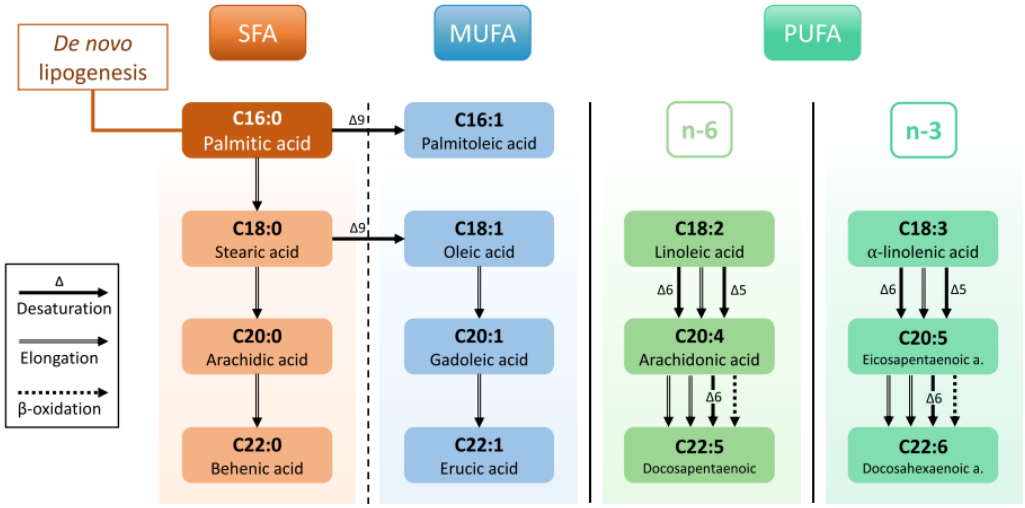


Figure 4: Synthesis of common fatty acids in animals[5]. Palmitic acid can be elongated and desaturated to form other fatty acids. Desaturation is done by specific desaturases (Δx). Some fatty acids cannot be synthesized from palmitic acid, those fatty acids (or their precursor) must be found in the diet.

4.1.2.2 Fatty acid β-oxidation

Fatty acid β-oxidation takes place when glucose concentration in the blood and in the tissues is low. It occurs in every cell and more specifically in the liver and the striated skeletal and cardiac muscle cells. This is a slower process than glycolysis, but it has a much higher potential of energy creation. β-oxidation takes place within the mitochondrial matrix and begins with the creation of a double bond between the α and β carbon (the two closest carbons from the coenzyme A) and the formation of $FADH_2$ by an acyl CoA dehydrogenase (ACDH). Then, H_2O is added to form a hydroxyl group on the β carbon by an enoyl CoA hydratase (ECH). The third step in the β-oxidation process is the transformation of the hydroxyl group into a ketone group and the formation of a NADH molecule by a β-ketoacyl dehydrogenase (βKADH). The three last steps prepare the acyl-CoA for its cleavage by a thiolase into an acetyl-CoA and an acyl-CoA with two less carbons. The resulting acyl-CoA can restart this cycle again, until it is completely divided into acetyl-CoA[6] (Figure 5).

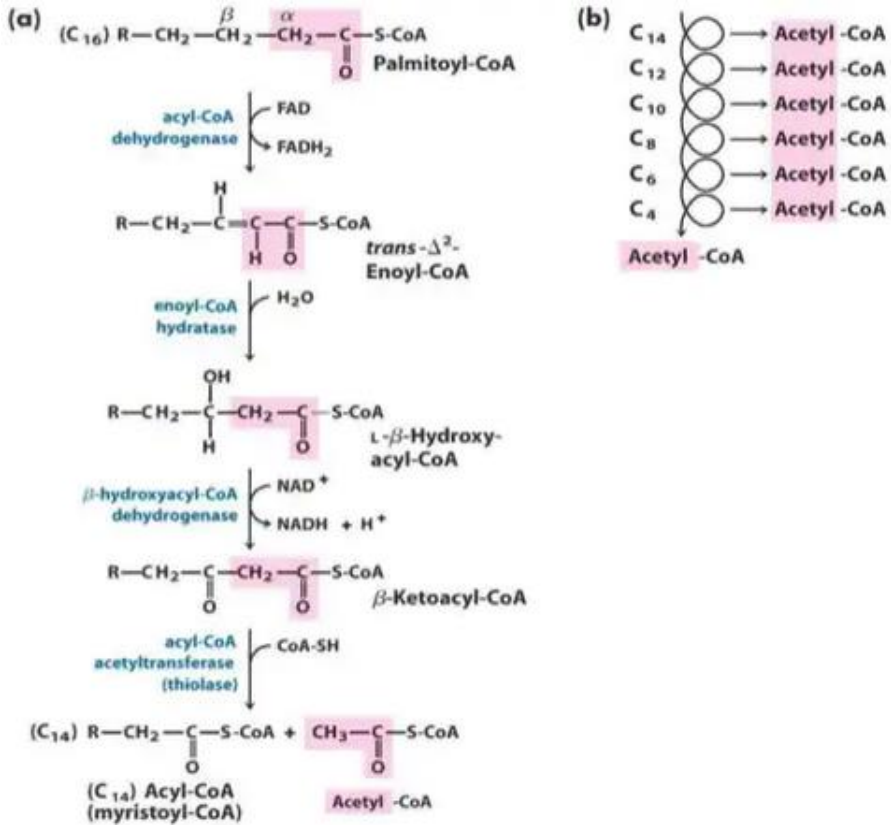


Figure 5: β-oxidation is completed by a succession of 4 reactions catalyzed by different enzymes (in blue) (a) until the entire fatty acid is broken down into acetyl-CoA molecules(b)[6]

4.2 Cancer

Cancer is the second leading cause of death worldwide and was responsible for 20 million cases and 10 million deaths in 2020 worldwide[7] (Figure 6). Cancer is described as a rapid proliferation and growth of cells with abnormal phenotypes which can invade other tissues and spread throughout the entire organism. This disease can touch numerous cell types and tissues. Cancers can be classified by the tissue affected or by the organ where the disease emerged. Depending on the tissue affected, we distinguish carcinoma, sarcoma, myeloma, leukemia, and lymphoma. Carcinoma emerges from epithelial tissues and account for80 to 90% of all cancer cases. Sarcoma emerges from supportive and connective tissues, representing less than 1% of all cancer cases. Myeloma develops from plasma cells, lymphoma from lymphocytes and leukemia from blood-forming cells. Cancers developing from blood cells represent less than 10% of all cancer cases together. Cancer can also be characterized by the type of organs affected (Figure 6).

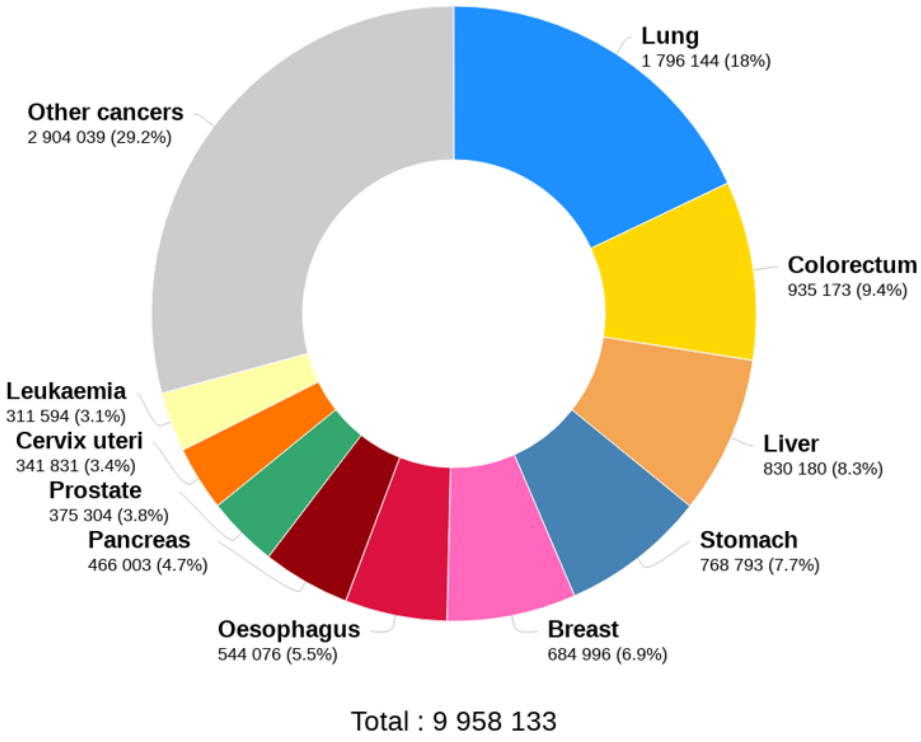


Figure 6: Risk of mortality from all types of cancer worldwide in 2020[7].

Cancer arises from a long process of exposition to precancerous factors, including but not limited to the environment, viral infections, heredity, hormone levels, and lifestyle. The development from a normal cell a cancerous cell can take several years[9]. During this time, the cells will undergo phenotypic changes. Specific genes, normally regulating the growth and the division of the cell and repairing DNA, will be mutated. The expression of proto-oncogenes is upregulated in cancer cells whereas tumor suppressor genes are downregulated. Together, these changes lead to uncontrolled cell growth and division.

Tumors will form by different stages[10]:

- Hyperplasia: cells that have undergone mutations multiple uncontrollably, leading to an excessive number of cells in the tissue. Hyperplasia results in benign tumors.
- Dysplasia: the cells begin to look abnormal and continue to grow, the tissue looks disorganized.
- *In situ* cancer: cells lose their characteristics and de-differentiate. They are no longer performing their usual functions.
- Invasive cancer: cancer cells begin to invade tissues through the circulation system. The cancer can then spread throughout the body to form metastases.

4.2.1 Hallmarks of cancer

Cancer cells are characterized by their propension to maintain proliferative signals, evade growth suppressors, and are associated with a replicative immortality. These three hallmarks allow a periodic proliferation of cancer cells. They can induce the formation of new blood vessel (angiogenesis) and activate the invasion of other tissues to produce metastasis. They are resistant to typical cell death programs and can evade immune destruction. They can create micro-environment beneficial for the tumor development. They present a genome instability and specific mutations. Finally, they need a complete overhaul of their energetic metabolism to sustain their modified phenotypes[11] (Figure 7).

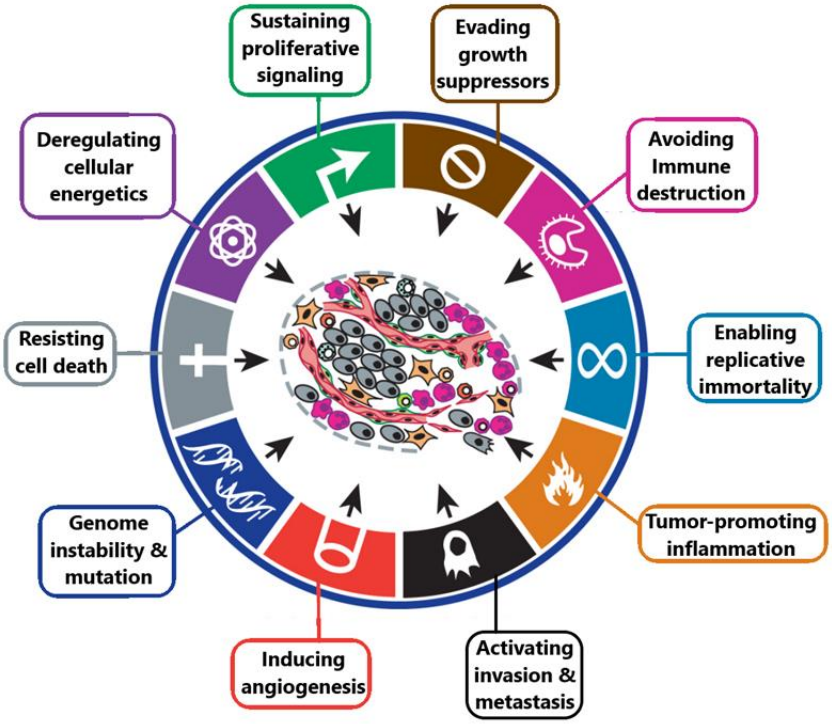


Figure 7: Hallmarks common to all cancer cells that are reported to cancer cells (in grey) and cells in the tumor microenvironment[11].

4.2.2 Cancer energetic metabolism

All cells mainly rely on glucose through glycolysis for their income in energy. This process leads to the formation of Acetyl-CoA which can be fermented into lactate under anaerobic conditions (which produces ATP but also create an acidification of the medium). Under aerobic conditions Acetyl-CoA enters the Krebs cycle to produce NADH and $FADH_2$ which can be used in the oxidative phosphorylation process to finally produce ATP (Figure 8). Another way of getting energy is the β-oxidation of fatty acids, developed in the section 4.1.2.2. Indeed, this slower process leads to the formation of $FADH_2$ and NADH in addition to replenishing the pool of Acetyl-CoA for the Krebs cycle (Figure 5).

4.2.2.1 Glucose metabolism: Warburg effect

In cancer cells, glycolysis is upregulated to produce a higher quantity of pyruvate. This allows a higher production of energy but also allows the use of glucose to synthesize other biomolecules[12]. This is key for cancer cell growth and proliferation. In healthy non-cancerous cells, glycolysis is regulated by ATP feedback inhibition due to the activity of phosphofructokinase 1 (PFK1). Cancer cells can counter this feedback by upregulating the expression of phosphofructokinase 2 (PFK2). PFK2 catalyzes the reaction of fructose-1-phosphate (F1P) into fructose-2,6-biphosphate (F26BP) which acts as an allosteric activator of PFK1 (Figure 8).

From pyruvate, cancer cells primarily use the lactate fermentation process to produce energy (even if the oxidative phosphorylation process is also used). This is called the Warburg effect and it allows for a rapid production of energy while also producing an acidic cellular microenvironment (by exportation of the protons produced).

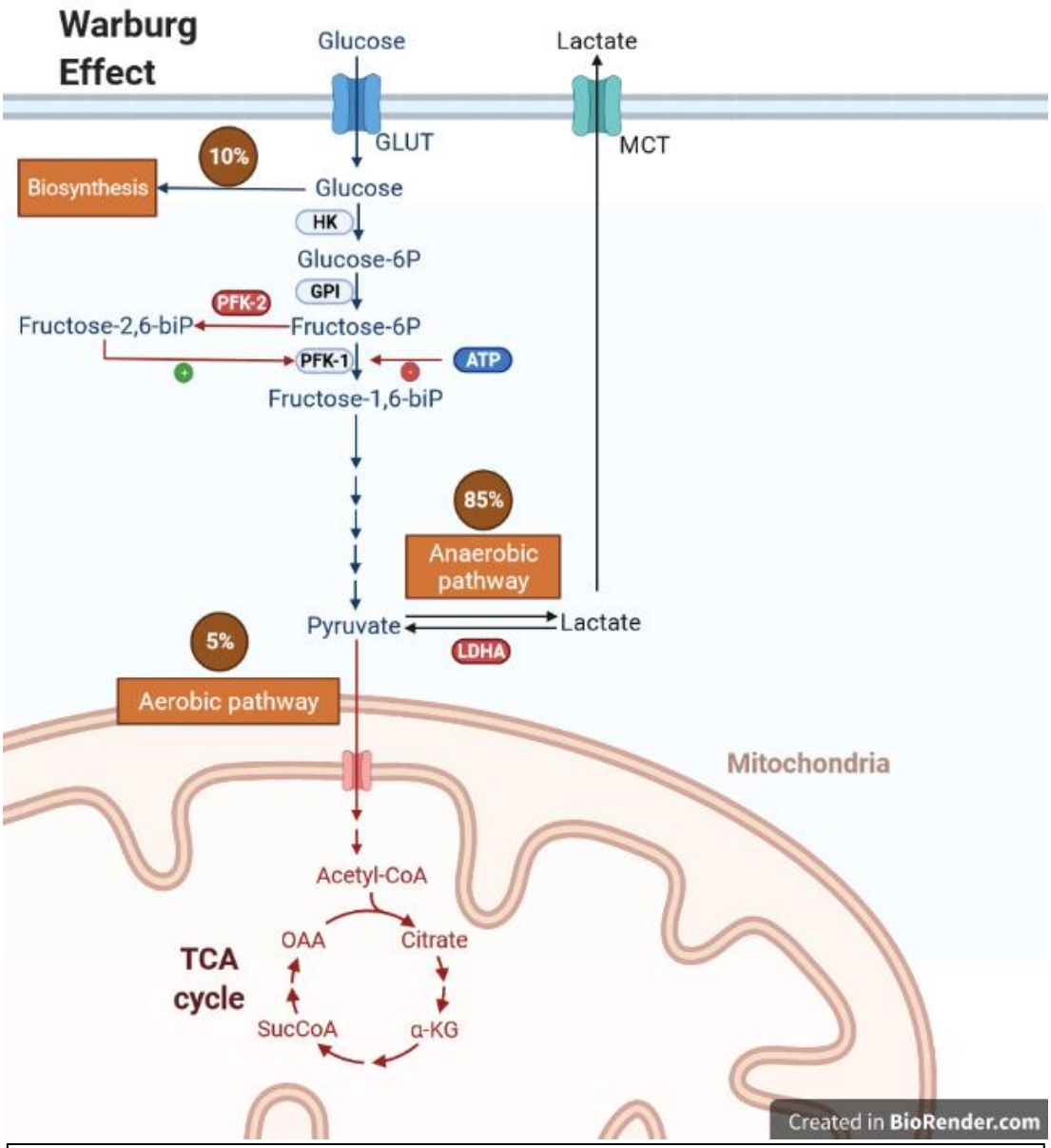


Figure 8: Warburg effect. The upregulation of phosphofructokinase-2 (PFK-2) allows the formation of fructose-2,6-biphosphate that has an activating effect on phosphofructokinase-1 (PFK-1), which diminish the inhibiting effect of high concentration of ATP on this enzyme. Most of the pyruvate from glycolysis is derived towards the anaerobic pathway due to an upregulation of lactate deshydrogenase (LDHA). 10% of the intake of glucose is diverted from the glycolysis pathway to be used for biosynthesis. Created by biorender.com

The Warburg effect is, at first, an advantage for the cancer cell because it will improve its ability to evade immune destruction and invasiveness by creating a specific microenvironment. However, this acidosis, at one point, will become unfavorable for the cancer cells. Therefore, to adapt, cancer cells will reduce their reliance on glucose and shift towards the use of fatty acids as their main energy source.

4.2.2.2 *Fatty acid metabolism*

This shift in the main source of energy in the cell is mandatory for the survival of cancer cells. But as cancer cells are in an environment with poor access to blood vessels, they cannot readily rely on exogenous fatty acid intake. To overcome this, cancer cells produce *de novo* fatty acids in parallel with β-oxidation. *De novo* fatty acid synthesis in cancer cells is primarily derived from glutamine[13]. The overexpression of the enzymes FAS, ACC, and citrate lyase allows for an upregulation of *de novo* synthesis of fatty acids in the cytosol and a down regulation of ACC2, which under normal conditions inhibits the mitochondrial production of acetyl-CoA from fatty acid[14] (Figure 9).

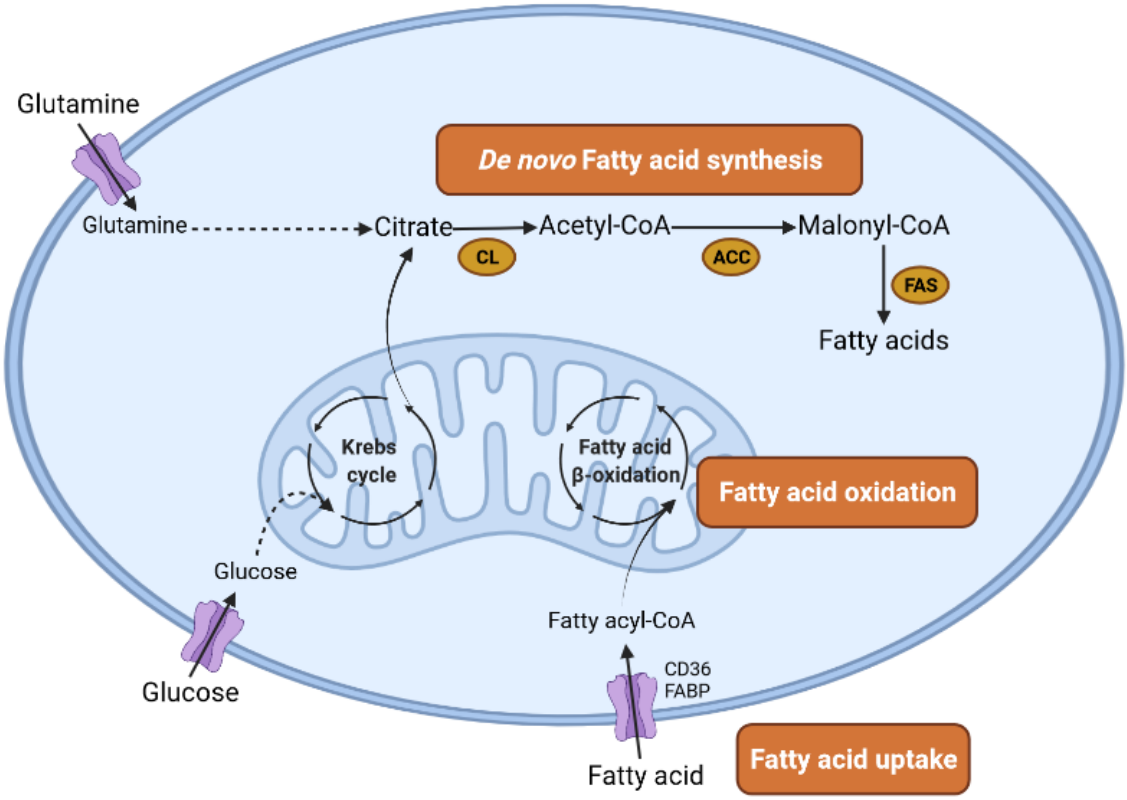


Figure 9: Altered fatty acid metabolism in breast cancer cells. Exogenous fatty acids that enter the cancer cell via fatty acid transporters (CD36 and FABP) are sent to the mitochondria to produce energy. In the meantime, *de novo* fatty acids are synthetized by citrate lyase (CL), acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) from citrate coming from the Krebs cycle and from glutamine decarboxylation [modified from 14]. Created by Biorender.com

4.2.3 Fatty acid cytotoxicity

Several studies have demonstrated that specific fatty acids, such as PUFAs can have a negative impact on cancer cell viability. The molecular mechanisms allowing this feature are still poorly understood but evidence suggests the involvement of a newly characterized cell death pathway: ferroptosis[15]. Ferroptosis differs from other cell death pathways due to the specific peroxidation of membrane lipids. The shift towards fatty acid utilization in cancer cells associated with this specific cytotoxicity opens a novel approach to cancer treatment and/or prevention.

4.2.3.1 Ferroptosis

Ferroptosis is a relatively newly described cell death pathway[16]. It is involved in numerous diseases where this program is dysregulated[17]. Ferroptosis is an iron-mediated form of death cell which results in the production of ROS from the phospholipids of the plasma membrane. The regulation of the pathway of ferroptosis appears to be driven by three main players. The first one, the antioxidant glutathione (GSH) plays a major role in neutralizing ROS primarily derived from oxidative phosphorylation. During ferroptosis, GSH can be recruited by glutathione peroxidase (GPX4) to produce lipid-OH (L-OH) from lipid-peroxide (L-OOH), which acts as the second player in this regulation. L-OOH are formed from unsaturated fatty acids by the oxidation of their double bonds. Some fatty acids are more prone to this phenomenon, especially conjugated fatty acids[18]. This reaction occurs due to the formation of fatty acid phosphatidylethanolamines (PUFA-PEs) from phospholipids by the enzymes ACSL4 and LPCAT3. PUFA-PE can then be transferred to a lipoxygenase (LOX) to be transformed into L-OOH. The third contributor to this pathway is iron, which will influence GSH and L-OOH in addition to its direct effect by the Fenton reaction; $Fe^{++} + H_2O_2 \rightarrow Fe^{+++} + OH^- + OH^*$. If the cellular input of Fe^{++} is increased, higher levels of ROS will be formed from the phospholipids. These ROS can be neutralized by GSH, but they still have a negative impact on the neutralization of L-OOH by saturating the GSH-GSSG system. Iron is also a cofactor for LOX, promoting the formation of L-OOH (Figure 10).

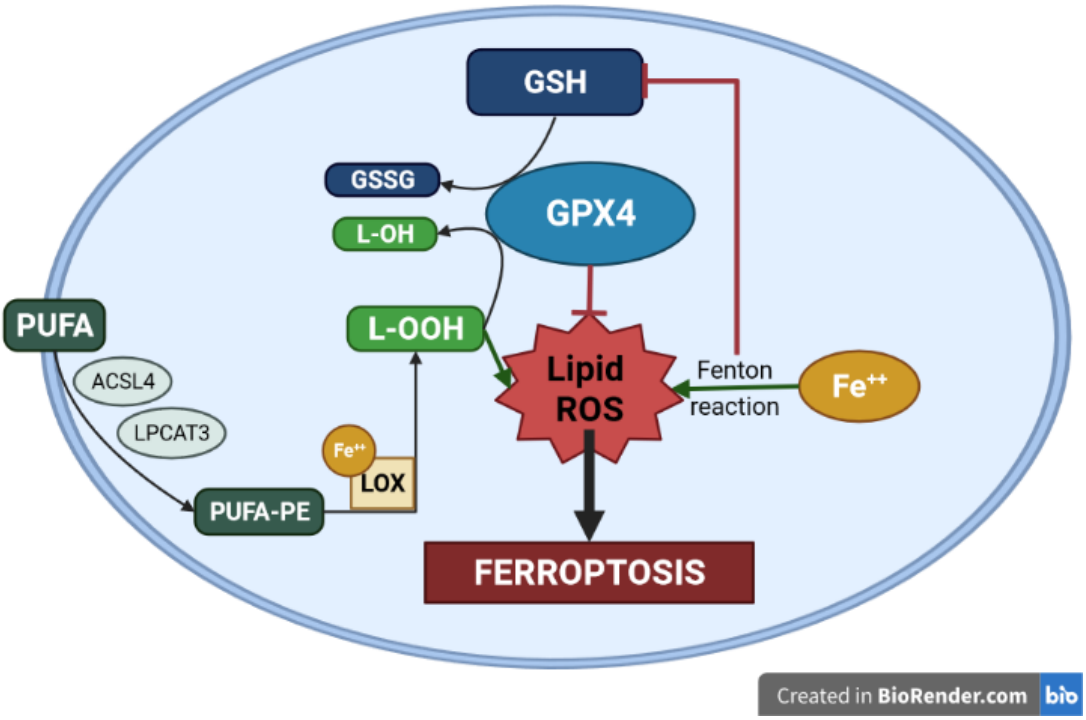


Figure 10: Regulatory pathways of ferroptosis: Gluthatione (GSH) is recruited by glutathione peroxidase (GPX4) to reduce lipid-peroxide (L-OOH) in lipid-alcohol (L-OH) by its oxidation (GSSG). L-OOH are formed from phospholipids by the enzymes ACSL4 and LPCAT3 that catalyze the formation of phosphatidylethanolamine (PUFA-PE). PUFA-PE are transferred to a lipoxygenase (LOX) that catalyze their transformation into L-OOH. Iron (Fe^{++}) is central in ferroptosis due to the Fenton reaction that create reactive oxygen species (ROS) that can form lipid ROS and saturate the recovery of GSH from GSSG and do to the fact that it is a cofactor for LOX[modified from 15]. Created by Biorender.com

4.2.3.2 Conjugated linolenic acid and punicic acid

This project is focusing on the effects of a specific PUFA: punicic acid (PunA). This fatty acid is found in large amounts in pomegranate seed oil (reaching levels of approximately 80% of the entire fatty acid fraction) and is an isomer of the essential fatty acid α-linolenic acid (ALA)[8,19]. Plants possess enzymes that can isomerize ALA and is then responsible for the formation of all isomers of ALA. Conjugated linolenic acids (CLnA) are a set of fatty acids regrouping all the isomers from ALA. They contain three double bonds with at least two of them conjugated with each other (two double bonds which are not separated by a methylene group). They are well studied due to their numerous beneficial effects on human health. They appear to present positive effects on obesity, inflammatory disorders, diabetes, as well as presenting antioxidant effects and a protective effect against the development of cancer[20]. PunA is an ω-5 PUFA with three conjugated double bonds, making it prone to peroxidation, a characteristic that is reported to present and reduce obesity and improve insulin resistance[20]. Furthermore, direct PunA treatment appears to have an anti-cancerous activity in several lines of human cancer cells (SiHa, LNCaP, MDA-ER-7, PC-3) by inhibiting their proliferation[20,21]. It is preponderant to determine if PunA can have a similar impact against cancer cells *in vivo*[20,22].

4.3 *Nothobranchius furzeri* as fish model

Nothobranchius furzeri (African Turquoise Killifish) is a promising new model to study the evolution of cancer. Indeed, it had been shown that this small and prolific vertebrate model presents rapid ageing processes, including a high incidence of age-related histopathological changes like hepatic neoplasms[23]. This processes has emerged due to the constraining characteristics of their natural habitat[24].

4.3.1 Ecology of the natural habitat of *N. furzeri*

This annual killifish lives in ephemeral pools in semi-arid areas of Africa that are created during the rainy season. Due to the constraint of the short duration of the rainy season, killifish must complete their life cycle before each dry season. Therefore, during the rainy season, the fishes will hatch and reach maturity within three weeks (Figure 11). Eggs resist the dry season due to their desiccant-resistant property and their capacity to enter multiple facultative embryonic diapause stages (making the range of their embryonic development from two weeks to three years)[25]. Their lifespan is mainly influenced by the duration of their habitat, and depending on the region and the strain, killifish possess different lifespans, ranging from three to seven months[26]. The shortest lifespan measured from *N. furzeri* is the GRZ lab strain originally collected from Zimbabwe.

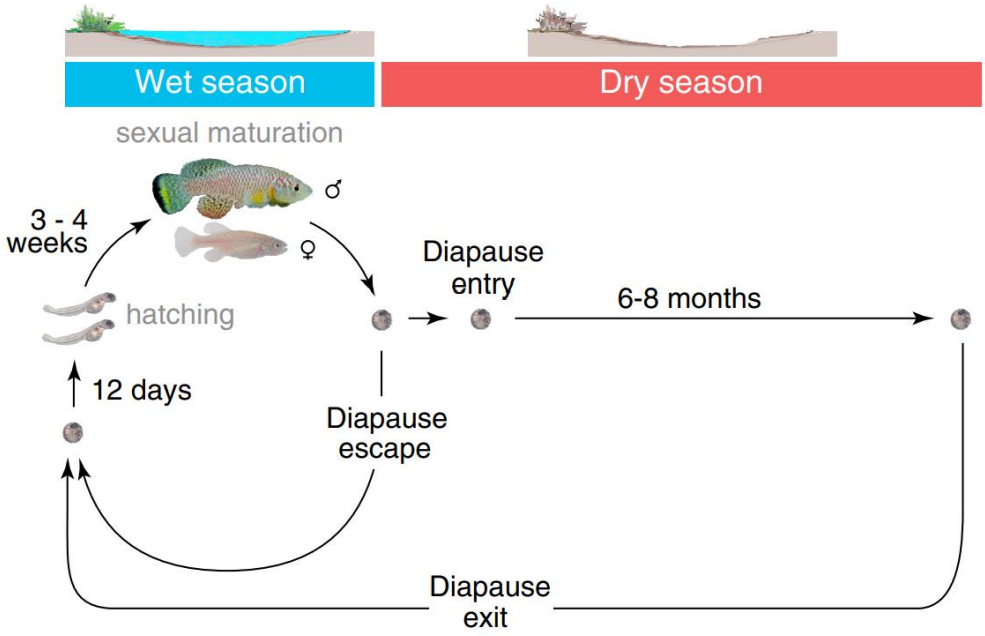


Figure 11: Turquoise killifish life cycle: during the wet season, the apparition of ponds allows the resumption of the metabolic activities of the eggs which exits from their diapause state. The hatched fish achieve sexual maturation and reproduce during the wet season. Some of the eggs will enter a diapause state that allows the survival during the dry season when the pond is desiccated[24].

Sexual maturation is accompanied by a pronounced sexual dimorphism (Figure 12). Male fish present bright colors, with the most distinct color on their caudal fin, which also differs between strains[27]. The GRZ strain have yellow fins whereas the MZM strain have red fins. Females of both strains are pale in color and are therefore more difficult to be distinguished[28] (Figure 12).

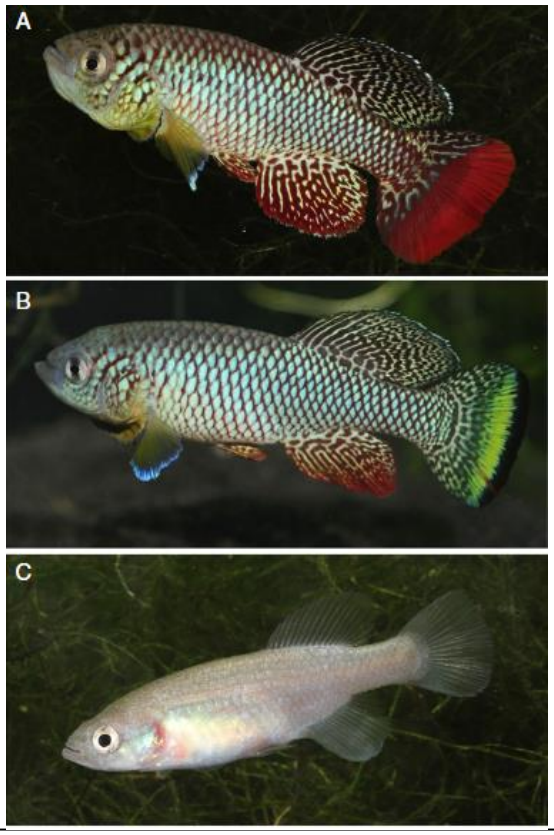


Figure 12: Sexual dimorphism between male (MZM red form in (A) and GRZ yellow form in (B)) and female (C) in *Nothobranchius furzeri* strains[25].

4.3.2 Advantages for laboratory use

N. furzeri are easily maintained in the laboratory. Indeed, they are small fishes (maximum length of 7 cm) that can tolerate a large range of temperature (15-35°C) and salinity (300-500 µS/cm²). They are also prolific fishes that reproduce daily with the females depositing up to 50 eggs/day under optimal conditions[25]. *N. furzeri* have the shortest vertebrate lifespan recorded in captivity (six months on average). Moreover, *N. furzeri* also display typical vertebrate aging phenotypes including spinal curvature, cognitive impairment, decrease in fecundity, and muscle degeneration[29]. In addition, age-related histopathological lesions have been measured in aged *N. furzeri*, with liver as the most impacted organ. Those lesions contain a high incidence of hepatic neoplasia (35%). *N. furzeri* is then a promising new fish model for studies on aging and on tumorigenesis[23,25].

4.3.3 Rearing of N. furzeri

We can divide the general rearing of *Nothobranchius furzeri* in 3 main stages:

4.3.3.1 Egg stage

Eggs can develop in peat, on top of peat or in water, the choice often depends on the level of developmental control required by the experimenters. Egg development in peat most closely mimics natural conditions but does not allow for easy developmental monitoring. In contrast, egg development on top of peat or in water can easily be observed. The development of the eggs is also influenced by temperature; lower temperature induces diapause while higher temperatures leads to a faster embryonic development[25].

We can observe different levels of egg development. Diapause eggs can present different stages: in the first one, eggs are yellow and do not show any visible structure of the future fish (Figure 13A). We can easily differentiate this stage from dead eggs which are white (Figure 13D). The first visible sign of post-diapause and of resumption of active development is the apparition of black eyes through the egg envelope (Figure 13B). When the eggs are ready to hatch, the eyes will appear gold (Figure 13C).

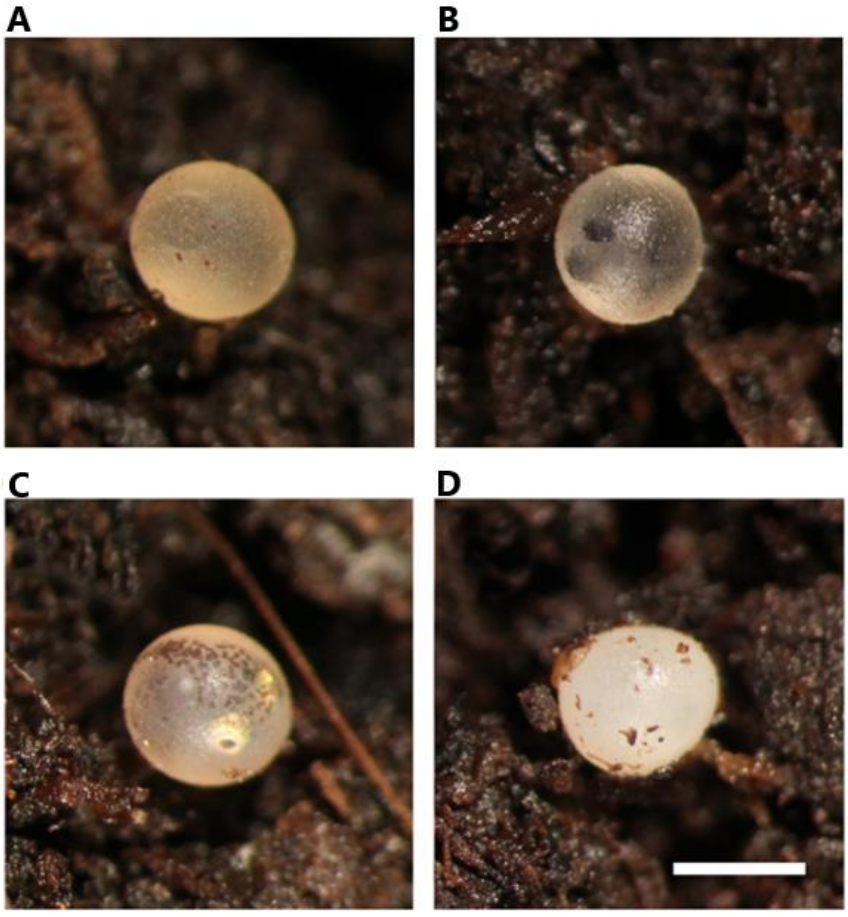


Figure 13: Different stages of *Nothobranchius furzeri* eggs development. Diapaused eggs (A), black-eye stage (B), golden-eye stage (C) and dead egg (D)[25].

4.3.3.2 Juvenile stage

Ready-to-hatch eggs are placed in a small aquarium filled until a maximum depth of 3 cm with cold water (15-16°C) to stimulate hatching. The aquarium is still put in a warm environment (25-27°C) to allow rapid development. Once hatched, larvae are fed twice a day with living *artemia*.

4.3.3.3 Adult stage

At the age of 7 days, juvenile fishes are transferred to a bigger aquarium maintained at room temperature (21-22°C) and filled with water adjusted for salt and pH by a reverse osmosis system. The passage to adulthood results in dramatic phenotypic changes. At this point, it is important to limit the density of the fish to a maximum of 1L/fish. Male killifish can present aggressive behavior when in contact with other males and therefore are often reared in separate tanks.

Breeding is performed in aquariums containing a spawning place. This spawning place consists of a jar filled with sand. Breeding fish are placed in this aquarium with a sex ratio of 3:1 (female:male)[25].

4.3.4 Diet of N. furzeri

There is no standardized artificial diet used for *N. furzeri* husbandry. They are typically fed with live and/or frozen food[25]. Larvae and juveniles (from hatching until 1-2 weeks) are fed with live baby brine shrimp (*Artemia salina*) while adults receive both *Artemia* and *Chironomus* larvae. *Chironomus* larva are often used in aquaculture as a diet as they present a high content of amino acids (56% of protein on dry weight)[30] associated with a high quantity of essential fatty acid[31].

4.4 Chironomus larvae

Chironomus riparius are a non-biting midge that is largely used as food in aquaculture, especially for ornamental fish species. *C. riparius* have a well-known and short life cycle (ranging from 15 days to several months, depending on the food source and temperature)[32]. Females are capable of laying large amounts of eggs simultaneously and can allow a rapid growth of the colony. *C. riparius* is a ubiquitous insect belonging to the benthic macroinvertebrates[32–34]. It has an essential role in the trophic chain as it is part of the diet of most fishes (in its larvae form) and some birds (in its adult form)[32]. Therefore, *C. riparius* is responsible for a major transfer of nutrients from the water to the air.

4.4.1.1 Life Cycle of Chironomus riparius

C. riparius is an holometabolous insect, and therefore they undergo a complete metamorphosis during their life cycle, which can be separated into four main stages presenting different features: eggs, larvae, pupa, and adults (Figure 14).

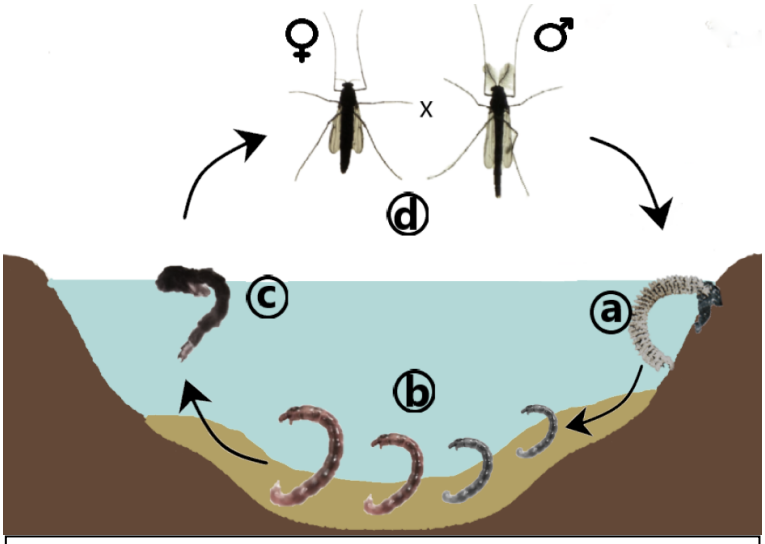


Figure 14: Life cycle of *Chironomus riparius* presenting the egg ropes (a), the larva in their 4 instar forms (b), the pupa (c) and the male and female adults (d)

4.4.1.1.1 Eggs

The eggs are laid by the flying female on the surface of the water: between 265 and 722 eggs are laid together, protected by a gelatinous matrix, and attached to the edge of the water by a gelatinous stalk forming an egg rope[35–37] (Figure 15). The shape of the egg rope and the organization of the eggs inside the egg ropes is characteristic of the species. The eggs hatch after several days, depending mostly on the species and the temperature[36].

Figure 15: Egg mass of *Chironomus riparius*[35]. a. Global view of the egg rope. b. Detailed view of the arrangement of the eggs inside the egg mass.

4.4.1.1.2 Larvae

Larvae hatch by breaking the chorion of the egg. They are primarily planktonic, swimming freely in the water column. This early planktonic stage will rapidly reach the benthic zone to bury itself into the substrate[36] (Figure 14). They are non-selective filter feeders, and feed on the benthic substrate by creating a tube around them with the secretion of silk-like threads by their salivatory glands[36]. The purpose of this tube is to create a current of water allowing the intake of their diet. Moreover, the tube also serves as a protection against predators. Their growth is divided by four successive molts[32], the last one resulting in the formation of the pupa. The developmental stages after each molt are called instars. The main differences between instars is the weight and length of the individuals[38,39], the size of their head capsule[36,39] and the color of the larvae. Indeed, from the 3rd instar, the larvae of *C. riparius* will produce hemoglobin which provides them with their characteristic red color (from which the name bloodworm originates)[32,36]. However, this hemoglobin differs from human hemoglobin as it is only composed of one subunit and therefore is morphologically closer to myoglobin. Although its functions are still to transport O_2 and CO_2 to the tissue via the blood (hemolymph). The purpose of this part of the life cycle is to store sufficient amounts of energy for the remaining life stages (i.e., metamorphosis, flying, mating and creation of reproductive cells)[36]. The larval stage is a major part of the life cycle of *C. riparius* and can last between 10 days to a month. Although this duration is primarily influenced by water temperature and food consumption[40], other environmental factors such as oxygen concentration, water pH, ionic concentration and water hardness are also important[41]. The weight and the maturation of the larvae are also dependent on temperature. Higher temperatures lead to increased levels of maturation but reduced levels of weight gain, resolving in smaller 4th instar larva. The speed of development is also influenced by sex; females develop slower than males but are heavier and bigger than them, especially during the last instar[36,42].

4.4.1.1.3 Pupa

The pupa is an immobile stage allowing for the complete metamorphosis to take place. Since the adult is aerial, the metabolism of the larvae will change drastically to prepare itself for this metamorphosis[36]. Pupation take place inside the tube of the larvae which can be rebuilt to better match its new purpose. The metamorphosis can take between a few hours to a couple of days and is highly dependent on temperature. When the metamorphosis takes place, the pupa matures to progress towards the next phase. It will swim towards the surface of the water (helped by air that accumulates under the pupal skin around the thorax of the adult)[36]. Once in contact with the air, the pupa will hatch, taking less than a few minutes. The hatched adult is capable of flight almost immediately[36].

4.4.1.1.4 Adult

The adult is the only aerial phase of the life cycle of *C. riparius*. It is at this stage that the species will reproduce. This stage typically lasts less than two weeks, in which reproduction takes place by the formation of a mating swarm mainly formed by the males and is believed to attract females[32]. Fecundation will take place in flight and females will lay their eggs at the surface of the water in a gelatinous substance[36].

From this point, the larval stage of *Chironomus riparius* will be mentioned as bloodworms (BW) and the adult stage will be mentioned as *C. riparius*.

4.4.2 Composition

The precise BW composition of amino acids, carbohydrates and lipids depends on their diet, the season and on the species[34]. Depending on those factors, the range of caloric content in *Chironomidae* larvae ranges between 4.6 and 6.1 kcal/g of larval mass[30]. Therefore, it is important to use the same diet, at the same time of the year or in stable environmental factors for laboratory use and to take the taxonomic affiliation into account when using BW[34].

Since BW live in sediment, feeding by filtration of the water, it is difficult to observe if they select their food as well as analyzing their gut content[43]. This poses an issue for enrichment experiments because it is difficult to determine their natural diet, and since their composition is largely influenced by their diet, differences can arise.

It is also important to consider that the pool of nutrients can be modified by the BW after ingestion. This metabolism primarily depends on the life history traits of the BW and on the nature of the nutrients[43]. A last point to keep in mind is that their composition will also change depending on their instar stage. Especially during the last instar of their larval stage, BW will present an increase in their lipid content to prepare for the pupal stage[44].

Carbohydrates are the primary component that is used for the replenishment of energy. It is stored as glycogen, which is lysed in trehalose (Figure 16), a glucose disaccharide, for its use and its mobilization across the different tissues of the organism[45,46]. BW shows an average of 56% of dry weight in proteins[30]. Lipids are stored in small lipid droplets, composed of a triglyceride core surrounded by a coat of phospholipids and proteins. This organelle is

called a lipid droplet. They are found in almost every tissue of the body. In adipocytes, the lipid droplets can occupy most of the intracellular space[45].

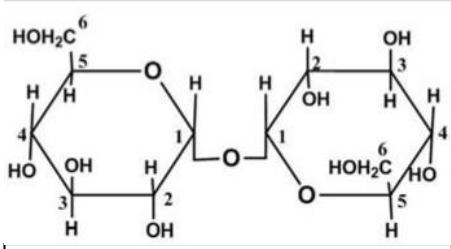


Figure 16: Molecular structure of trehalose[43]

The fatty acid content in BW differs greatly depending on their diet, but there is always a major part of this content represented by PUFAs, ranging from 27 to 49% of the total mass of fatty acids in BW, with a majority of C18 PUFAs[43]. Another major part of the total fatty acid content is represented by MUFAs, ranging from 23 to 42% of the total mass of fatty acids in BW. The rest of the fatty acid content is SFAs, ranging from 22 to 28% of the total mass of fatty acids in BW[43].

4.4.3 Metabolism

Metabolism in insects is greatly mediated by the fat body. They will primarily use carbohydrates for their residual energy needs and will store fatty acids as triglycerides.

4.4.3.1 Fat body

The fat body is an organ specific to insects. It is a highly dynamic tissue dispersed across the internal cavity of the insect. One of its primary functions is to regulate the use of energy by the body. To fulfill this function, adipocytes from the fat body are specialized for storing lipids (as triglycerides) and carbohydrates (as glycogen). Moreover, the fat body is an endocrine gland which will regulate the use and the storage of those energy molecules. This feature of the fat body is a key element in the coordination between the growth and the life history traits of the insect[45]. Finally, the fat body is involved in other metabolism processes. It is responsible for the synthesis of most of the hemolymph's proteins, including antimicrobial peptides, and for the detoxification of xenobiotics[45,47].

4.4.3.2 Carbohydrates

Carbohydrates are stored in the form of glycogen. This molecule can be formed from dietary glucose and circulating trehalose under *ad libitum* conditions. Glucose can enter the cell using specific transporters (GLUT1) and be derived into uridine-biphosphate-glucose (UDP-G). The UDP is removed to form a glycosidic bond with the glycogen molecule[46] (Figure 17).

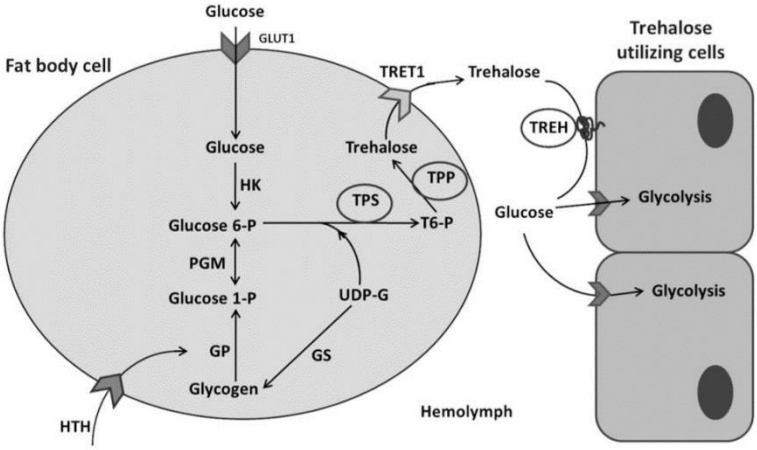


Figure 17: Metabolism of carbohydrates in the fat body cell.
The storage form is glycogen, and the mobile form is trehalose[43].

Glycogenolysis in insects is similar to the process characterized in mammals; glucose-1-phosphate (G1P) is formed by the catalysis of glycogen by glycogen phosphatase (GP). G1P is then isomerized into G6P by phosphoglycerate mutase (PGM). G6P can enter glycolysis to produce energy or be conjugated to UDP-G to form trehalose. In insects, the glycogenolysis is promoted in the fat body by a specific neuropeptide, hypertrehalosemic hormone (HTH). The formation of glucose can also be done from circulating trehalose. This process is catalyzed by a membrane trehalase (TREH).

In insects, the circulating form of carbohydrates is trehalose. The formation of this molecule is possible due to two specific enzymes. Trehalose-6-phosphate synthase (TPS) catalyze the reaction between G6P and UDP-G to form trehalose-6-phosphate (T6P). Trehalose-phosphate phosphatase (TPP) catalyzes the formation of trehalose from T6P. Trehalose can be exported to the hemolymph by trehalose transporter 1 (TRET1).

4.4.3.3 Lipids

Triglycerides in the fat body are synthetized from dietary fatty acids, carbohydrates, and peptides[45]. The content of FAs in BW is reflected by the content of the diet but can also be influenced by FA metabolism. Strandberg observed arachidonic acid (ARA, C20:4n6) and eicosapentaenoic acid (EPA, 20:5n3) in BW fed only a C18 PUFA diet, suggesting that they are capable of FA elongation and desaturation. Conversely, they did not find docosahexaenoic acid (DHA, 22:6n-3) in BW fed a DHA-rich diet, implying that DHA was retro-converted into EPA or oxidized for energy[43]. The FA content in BW quickly changes in response to the diet. This is especially true for PUFAs, and more intensively towards the synthesis of EPA by precursors. Since EPA is one of the precursors for eicosanoids and other bioactive lipids that play an active role in essential metabolic function of the insect (like reproduction, immunity control and ion transport)[43].

4.4.4 Rearing

Table 2: Rearing characteristics advised by the literature for *Chironomus riparius*

Parameters		Advised levels
Water	NH_4	< 18mg/L[43]
	NO_3	< 2 mg/L[33]
	Conductivity	> 540 µS/cm[48]
	Temperature	15 → 27°C[40], 21°C[33,42]
		23°C +/-0.6°C[49]
	pH	7[43,50], 8.2[38,42,44,48,51]
	Hardness	< 400 mg CaCO3/L[32], 210 mg CaCO3/L[44]
	O2 saturation	> 60%[52]
Room	Temperature[43,50]	20+/-1-2°C[43,52,53], 15+/-1°C[32]
	Relative humidity	> 60%[48]
	Luminosity	Twilight 30' at 5-10µmol/m².s[51,54], 16:8 (1000 lux)[32,33,40,42–44,48–53]
BW	Box	44mL/BW[33,40,42], Surface 4cm²/BW and Volume 5 mL/BW[38], Surface 5cm²/BW and Volume 10 mL/BW[44], Surface 2 cm²/BW[32,42], Surface 10 cm²/BW and Volume 40 mL/BW[53]
	Sand	Depth 5-10 mm[52]
		Grain diameter <500µm[38,44], Grain diameter 200-500µm (avoid swallowing)[51], Grain diameter 50-200 µm[42]
		Sand/water = ¼ in volume[43,54]

	Food	1,4 mg TM/BW fed daily[33,42], 0,6-1,2 mg TM/BW (depending on the temperature) fed daily[40], 1,5 µg TM/BW twice a week[38], 1 mg C/BW fed every other day[43], 0,25 → 1 mg Trouvit/BW fed daily[50], 2 mg TM/BW fed daily[32], 0,2 mg TM/BW fed daily[48], 0,25-0,5 mg TM/BW (depending on the age of BW) fed daily[52]
Adults		30x30x30cm[52], 1mx1mx50cm[48]
		Fed on sucrose[48,52]

5 Materials and methods

5.1 *Chironomus riparius* Rearing

The rearing is kept in the insectarium, in the same room as Drosophila. This room is monitored to stay at a constant temperature of 23±2°C and at a photoperiod of 16 hours. The *Chironomus* species is an holometabolous insect presenting two distinct habitats during its life cycle: the eggs, larvae and pupae are aquatic while adults are aerial and therefore the rearing of this species is held in two different cages.

5.1.1 Culture box

The culture box (CB) consists of a plastic box of 24 dm^2 (60x40cm) filled with 8 to 10L of tap water and 1L of autoclaved sand with a grain diameter between 180 and 350 µm. This aquatic environment in aerated with an air pump, allowing the release of a few bubbles/seconds (Figure 18A). Two egg ropes are added to this culture box to allow a surface of approximatively 5cm^2/larvae.

BW are fed goldfish flakes (GFF) three times a week (Monday, Wednesday, and Friday). To allow a faster sinking of GFF in the water, we grind the food using a mortar and a pestle and mixing it with 10 times their volume of tap water. Six drops of this mix are deposited on top of the water of each culture box. Prior to the feeding, half of the water is replaced by fresh tap water to reduce algal growth.

This rearing is kept until the apparition of the first pupae. At this time, the culture box is moved into the reproduction box.

5.1.2 Reproduction cage

The reproduction cage (RB) consists of a wooden cube of 1m^3 (1x1x1m) whose edges are connected by a net and with a door that can be locked without crushing the tube bringing the air flow to the culture box placed in this cage. Behind this door, another net is placed to prevent the escape of adults while opening the cage to feed them and to harvest the egg ropes (Figure 18B). The culture box containing pupae is placed into this cage that can allow the formation of a swarm.

Egg ropes are laid at the surface of the water, attached to the edges of the box by their gelatinous matrix. Egg ropes are harvested before the cleaning and feeding of the box by dismantling the part of this matrix connected to the box and sucking them out into a pipette.

The adults are fed by the addition of a petri dish at the surface of the water, containing some cotton that is humidified and on which a pinch of crystallized sugar is added (Figure 18B). The CB placed in the RB follow the same protocol of rearing as the one outside the cages.

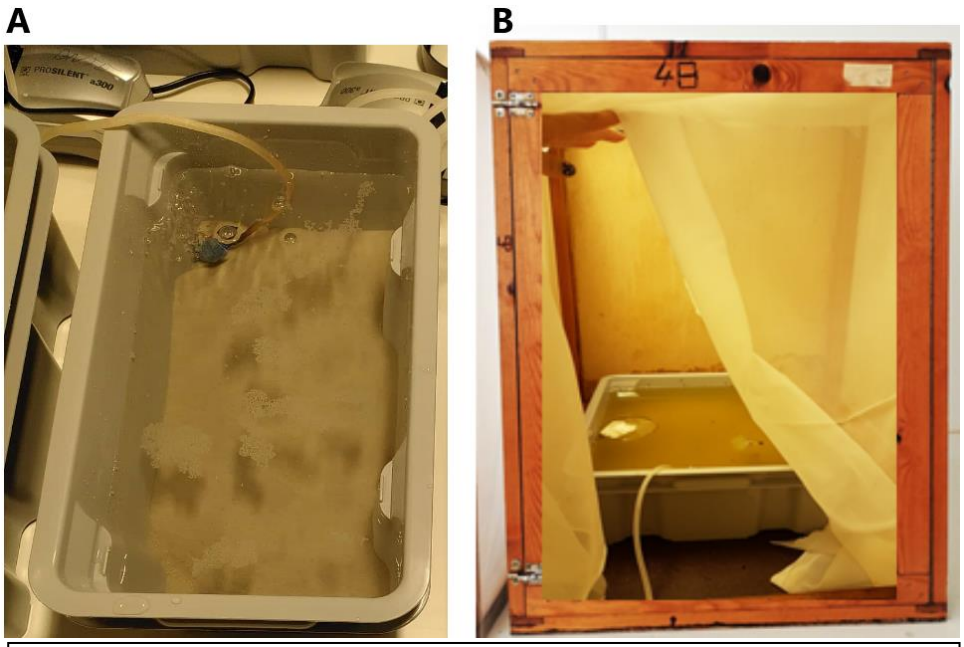


Figure 18: Pictures of the *Chironomus riparius* rearing. A: Culture box (CB) half full of tap water and sand (1/8 in volume), connected to an air pump. B: Reproduction box (RB) containing a CB supplemented by a petri dish containing humidified cotton with sugar.

5.1.3 Enrichment rearing

This experimental type of rearing is limited to the culture box since this project is focusing on the larval form of *C. riparius* (BW). We tried two different types of experimental rearing, in the first we fed BW only with the enrichment food and in the second we fed BW GFF for a three-week period followed by a switch to enrichment food for 7 days.

5.1.3.1 Full enrichment experiment

Two egg ropes were put into a culture box of 24 dm² filled with 1L of autoclaved sand and 8L of tap water aerated with an air pump. Hatched BWs were fed three times weekly Basal Feed Matrix (BFM) for four weeks. At the end of the experiment, bloodworms are harvested by emptying the water from their culture box through a sieve with a mesh of 0,3 mm. To retrieve every bloodworm, multiple washing of the culture box is performed. This washing is done by filling the culture box half-full of tap water, by mixing the sand with this water to recover BW and by emptying the water from the culture box through a sieve. Between each wash, the bloodworms harvested are put in a petri dish with enough water to contain them. The harvesting is completed when no further BW can be recovered from the sand.

5.1.3.2 Diet-switch experiment

This type of rearing begins with conditions described in the section 5.1.1: two egg ropes are put into a culture box of 24 dm² filled with 1L of fine sand and 8L of tap water aerated with an air pump. They are fed GFF three times a week with for a 3-week period.

The last week of the experiment, the feeding was switch to the specific food tested. The number of culture boxes used for this rearing correspond the number of specific foods tested plus a box which continues to receive Goldfish Flakes and a box that is left fasted. At the end of the experiment, bloodworms are harvested as described just above in the section 5.1.3.1.

5.2 Gas Chromatography coupled with a Flame Ionization Detector (GC-FID)

Gas Chromatography (GC) is a technique used to separate and analyze different components in a mixture. For this specific type of chromatography, we are using gases, or volatilized liquids, that are vaporized into a capillary column using a carrier gas, called the mobile phase, that is continuously injected into the column and that will carry the sample along the column. The inner wall of the capillary column is coated with a stationary phase that has an affinity which differs depending on the components of the samples. The difference of affinities between the different gases and the stationary phase will cause a difference of separation speed in the column, partitioning the different components of the sample depending on this affinity. The separation of the different components is influenced by the column temperature, the carrier gas flow rate, the column length, and the amount of sample injected. At the end of the column, a flame ionization detector (FID) detects the ions created by their combustion.

The analyses of fatty acids were performed on a GC Trace 1310 containing a capillary column Restek RT 2560 (100m x 0.25 mm x 0.2 µm) covered with a piscyanopropyl polysiloxane as a stationary phase and hydrogen as a mobile phase. The results from the analyses performed on this GC were processed using Chromeleon 7 software. This GC was used to analyze the fatty acid composition in enriched BW. After their harvest, BW are lyophilized in order to be used in a Bligh and Dyer protocol which will allow the extraction of the hydrophobic part of the samples. As long chain fatty acids are not volatile enough to be used in GC, they are firstly converted into a methyl ester that can pass through the column of the GC. The results from the analyses

5.2.1 Standards

To perform a correct analysis of the results given by the GC, different standards are made. These standards contain a known concentration of identified fatty acids.

5.2.1.1 Calibration standard

This standard is used to identify the peaks in the GC results of our samples. This standard contains 41 identified fatty acids in their methyl ester form at a known concentration dissolved in a hexane solvent.

5.2.1.2 Injection standard

This standard is used to compensate the variation due to the GC analysis and, to compensate any differences between sample injections in the GC. This standard contains C11:0 in its methyl ester form dissolved in a hexane solvent.

5.2.1.3 Extraction standard

This standard is used to assess the validity of the results obtained for each sample by the GC. This standard contains a known volume of a specific fatty acid (C19:0) that is not present in the sample. This specific fatty acid is dissolved in a chloroform solvent and is added at the beginning of the extraction process of the Bligh and Dyer, described in the following section. At the end of the analysis, the recovery of this specific fatty acid (quantity retrieved after the GC compared to the quantity added in the beginning) corresponds to the quality of the analysis of every sample.

5.2.2 Freeze-dryer

This technique is used to remove the majority of water from a substance by sublimation. This can be done by heating a frozen sample at low pressure. This technique allows to retrieve a substance with only 1-5% of their initial water composition, while maintaining the volume and the properties of the sample. We can divide the lyophilization in three steps. The freeze-drying was performed on a CHRIST Gamma 1-16 LSCplus freeze-dryer.

5.2.2.1 Freezing

During this stage, the sample is cooled to temperatures in the range of -20 to -80 °C. It is best to apply a rapid freezing to prevent crystallization, and then denaturation of the sample.

5.2.2.2 Primary drying

The frozen sample is directly transferred from the freezer to the freeze dryer that apply a relative void of 100 µbar around the sample with a rising temperature. This allows the passage of the water from the solid state to the gaseous state without boiling (no liquid state). The water vapor lost from the sample is trapped in a condenser to decrease the concentration of water in the air, allowing a continuous sublimation flow. This process can remove about 80-90% of the water from the sample.

5.2.2.3 Secondary drying

This last stage is meant to remove the residual water that is left trapped at the surface of the sample. To do so, the void is increased towards 5 µbar, with a constant temperature. At the end of this stage, the sample contains less than 5% of its initial water.

5.2.3 Bligh and Dyer

The Bligh and Dyer technique allows for the extraction of fatty acids from biological samples based on a differential affinity for the solvents (liquid-liquid extraction). This method will also prepare the sample for their analysis in the GC. The Bligh and Dyer protocol is divided in three steps: extraction, methylation and vial preparation.

5.2.3.1 Extraction

Samples retrieved from the lyophilization stage are weighed, then crushed to form a homogenized powder that is mixed with a solution of chloroform/methanol/water 2:2:1.8 (v/v/v). Centrifugation of this mix results in two phases: the upper water-rich phase containing the proteins and carbohydrates and the lower organic-rich phase containing the non-polar components. This step ends with the sampling of the organic-rich phase, containing all types of lipids.

<u>Detailed protocol:</u>

- Mix of 10 to 50 mg of the dry sample with 800 µL of Milli-Q water and silica beads.
- Homogenization of the sample with two cycles of 30 seconds in a MagNA lyser.
- Transfer of the homogenate in a GC tube with the addition of 2x1mL of methanol.
- Add 1mL of Internal Standard (C19), the making of which is described in the GC section that follows.
- Add 1mL of chloroform and 1mL of Milli-Q water.
- Perform a 10-minute centrifugation at 3000 rpm on this mix to form the two phases.
- Take 1,7 mL of the lower phase.

5.2.3.2 Methylation

This step is used to prepare the fatty acids for the GC. Indeed, the long-chain fatty acids are not volatile enough for their use in a GC. Their methyl-ester form is therefore used for analysis. To do so, the solvent of the sample is evaporated on a nitrogen flow. The fatty acids remaining are mixed with methanol in acidic/basic conditions at high temperatures to add the methyl group to fatty acids.

Detailed protocol:

- Sample were evaporated on a nitrogen flow at 30°C.
- 0.5 mL KOH/MeOH (0.1 M) were added to methylate the neutral lipids and phospholipids fractions of the sample.
- Incubated 1 hour in water at 70°C
- 0.2 mL HCl/MeOH added to methylate the free fatty acids fraction of the sample.
- Incubated 15 minutes in water at 70°C.

5.2.3.3 Vial preparation

This last step allows the formation of the mixture that will be analyzed by the GC. The injection standard described in the section 5.2.1.2 is added to every sample during this step.

Two different vials are prepared: a storage vial, in which the mix is made, and an injection vial, that will be injected in the GC. This allows to retain the samples for potential further analysis.

Detailed protocol:

- 1 mL Hexane and 500 µL Milli-Q water was added to the methylated samples.
- Samples were centrifuged (5 minutes at 2500 rpm) to form two phases.
- X µL of the upper phase were added to an empty storage vial.

X corresponds to the volume of sample added in each vial. Its value depends on the initial concentration of fat in the samples. The amount of fatty acids to be detected by the GC but not too much to saturate and damage the GC.

For BW, X corresponds to 400 µL. For the analysis of the food given to the bloodworms, X corresponded to a volume ranging from 50 to 400 µL.

- Add (720-X) µL of n-Hexane to each storage vial.
- Add 80 µL of injection standard to each storage vial.
- Take 250 µL of the 800 µL contained in the storage vial to put it in an injection vial.

5.3 Enrichment food used

5.3.1 Basal Feed Matrix (BFM)

BFM is a mix of every essential nutrient for zebrafish except for fatty acids. This mix was made to allow a FA enrichment in zebrafish. Indeed, we can add up to 14% of a FA of interest (in dry weight) to this mix.

To prepare BFM, we begin by creating a specific premix: minerals, vitamins soluble in water, vitamins soluble in fat and a Grindomix (containing casein, arginine, threonine, dextrin, gelatin, cellulose, bacterial agar, carboxymethylcellulose) (Table 3-5). All the premixes are then combined with specific fatty acid to complete the BFM without the experimental oil (Table 6).

Table 3: Proportion of ingredients contained in the mineral premix

Ingredient	Proportion (%)
$CaHPO_4$	29.5500
$Ca(H_2PO_4)_2.H_2O$	21.7000
$Na_2SeO_3.5H_2O$	0.0011
$NaHCO_3$	8.9500
KCl	10.0000
NaCl	17.2400
KI	0.0200
$MgCl_2$	6.3700
$MgSO_4.7H_2O$	3.4300
$MnSO_4.H_2O$	0.2000
$FeSO_4.7H_2O$	1.5000
$CuSO_4.5H_2O$	0.0400
$ZnSO_4.7H_2O$	1.0000

Table 4: Proportion of ingredients contained in the water-soluble vitamin premix

Ingredient	Proportion (%)
Vitamin C	5.600
Vitamin B1	0.627
Vitamin B2	1.344
Vitamin B6	0.504
Vitamin B5	1.579
Vitamin H1	4.479
Vitamin B12	0.003
Vitamin B3	3.359
Vitamin H	0.011
Choline chloride	39.194
Inositol	5.599
E161g	1.120
α-cellulose	36.401

Table 5: Proportion of ingredients contained in the liposoluble vitamin premix

Ingredient	Proportion (%)
Vit A acetate	0.253
Vitamin D3	0.253
Vit E acetate	86.364
Vitamin K3	5.612
Butylated hydroxy-anisole (BHA)	3.788
Butylated hydroxy-toluene (BHT)	3.788

Table 6: Proportion of ingredients contained in the basal feed matrix (BFM)		
Ingredient		**Proportion (g/50g of BFM)**
Grindomix	Casein	20.75
	Gelatin	4.00
	L-arginine	0.50
	L-threonine	0.50
	Dextrin	5.00
	Cellulose	3.50
	Carboxymethylcellulose	1.50
	Bacterial agar	0.50
Water-soluble vitamin premix		0.48
Liposoluble vitamin premix		2.80
Mineral premix		3.25
Experimental oil		**7.20 (added after the formation of BFM)**

5.3.2 Emulsion

This enrichment food is made based on the adapted oil emulsion method from Tamaru et al. used to enrich artemia in our lab [Ariane2021][55].

- 5 mL of warm water in a 50 mL falcon.
- 5 mL of pomegranate seed oil (PSO) added to the falcon.
- 250 µL of Tween80 added to the falcon.
- Mixed in a vortex mixer until obtaining a homogenized yellow/egg white mixture.
- Stored at -20°C until use.

5.3.3 Gelatin mixture

This enrichment food consists of a minimized version of BFM that allows the oil of interest to be water-soluble. Two different mixtures have been used that differs in their proportion of oil in it.

- 10 mL of water in a 50 mL falcon.
- 2g of PSO added to the falcon.
- Mixed 30 seconds in a vortex mixer.
- 1g for Gel1 and 3g for Gel2 of porcine gelatin added to the falcon.
- Mixed in a vortex mixer until obtaining a homogenized mixture that looks like a gel.

Immediately mix the mixture after adding the porcine gelatin to the falcon to prevent the formation of lumps and an uneven incorporation of the oil into the gelatin.

- Stored at -80°C overnight.
- Freeze-dried to obtain a solid block.
- Grinded to form a powder.

5.3.4 Rearing food without fatty acids

To reduce the parameters outside of our control in this enrichment experiments, we tried to use the same food as the controls but supplemented with PunA. To do so we had to firstly extract the FA from this rearing food. To perform that, we used two protocols:

5.3.4.1 Bligh and Dyer

Based on the protocol described in the 5.2.3 section. At the end of the extraction part of this procedure, we obtain two phases: an organic phase, in which the fat is retrieved, and a hydrophilic phase, in which the amino acids and sugars are recovered. The idea here is to recover the hydrophilic phase and to freeze dry it to retrieve the food without the fat.

- Extraction part of the Bligh and Dyer protocol (Section 5.2.3.1) performed on GFF.
- 3 mL taken from the upper phase
- Stored at -80°C overnight.
- Freeze-dried.

5.3.4.2 Soxhlet

Based on Soxhlet extraction protocol that allows to quantify the complete proportion of fatty acids contained in the sample by a solid-liquid extraction process[56]. The extraction is performed by multiple washes of the sample by an extractant solvent (ether) that can solubilize the fraction to extract from the sample.

The Soxhlet extractor contains three main parts (Figure 19). The extraction part contains the solid sample and allow the contact with the liquid extraction solvent. The extraction solvent containing the fatty acids extracted from the sample falls into the distillation flask by the siphon. The distillation flask is heated to evaporate the extraction solvent but not the fatty acids. The gaseous extraction solvent goes up into the condenser to liquefy the solvent the fall back into the extraction part of the Soxhlet extractor. This design allows a continuous wash of the sample by the extraction solvent

- Sample and distillation flask weighed.
- Sample putted in a filter in the extraction part of the Soxhlet extractor.
- Extraction solvent (ether) added to the extraction part of the Soxhlet extractor until it goes into the siphon.
- Heat source and condenser turned on to start a 6-hour extraction period.

Ether heats fast; the heat of the heat source and the water debit from the condenser must be adapted to prevent a violent boiling and a correct time of sample wash.

- Ether removed from the distillation flask (siphoning plus two-hour steam room and 30-minute desiccator period).
- Distillation flask weighed to calculate the mass of fatty acid extracted from the sample.

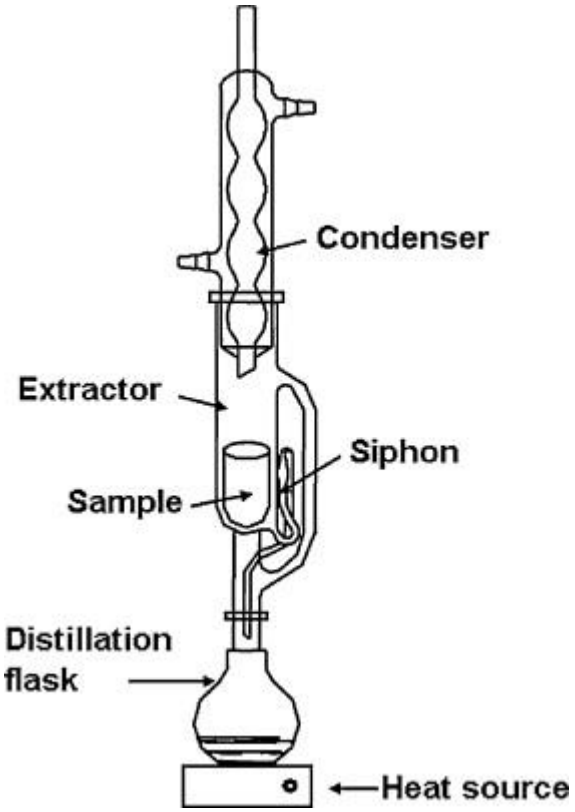


Figure 19: Conventional Soxhlet extractor: the heat source allows the ebullition of the solvent (ether) contained in the distillation flask. The condenser allows the cool down of gaseous ether which falls on the sample into the extractor. Ether accumulates in the extractor until it is allowed to fall back into the distillation flask, bringing the lipids with it. The majority of the fatty acid fraction of the sample is extracted after several washes[56].

This standard procedure of Soxhlet extraction can be initialized by an HCl hydrophilization of the sample to enhance the fraction of fatty acid extracted by the Soxhlet extraction method. This is performed by the boiling of HCl in presence of the sample in the distillation flask that is directly connected to the condenser. An-hour period of slight boiling is sufficient to perform this HCl hydrophilization. The sample are then rinsed with demineralized water to remove the HCl and prepare it for the Soxhlet extraction.

5.4 Development of bloodworm analysis

5.4.1 Survival of bloodworm

BWs from a same CB were weighed after their freeze-dry to retrieve the total mass of BW produced by every condition. This allowed to assess the relative survival rate of each CB compared to the control condition. But this kind of approximation cannot be used to compare the survival rate of different experiments.

5.4.2 Length of bloodworm

To assess the well-being of BW after the enrichment, the length of each BW was assessed using an approximation with ImageJ, an opensource software using Java to process images. To do so, a picture is taken of the petri dish of each sample with a ruler just after the BW harvest. Indeed, ImageJ can translate pixels in a picture into a specified number of a specified unit.

5.5 Environmental analysis

5.5.1 Temperature and relative humidity of the *C. riparius* rearing room

To assess the evolution of temperature and the relative humidity in the BW rearing room, we used a HOBO UX100-003 Temp/RH Logger. This device can record temperature and relative humidity in an indoor environment with a 3,5% accuracy. The records can be analyzed by HOBOWARE, a software specifically designed for this device. The device was left in the rearing room to record the temperature and relative humidity, which was collected at the same time as the rearing care (three times a week). Between the 3rd and the 4th of November 2021, the logger was also set to record temperature and relative humidity of the BW rearing room every 15 minutes.

To assess the evolution of temperature in the water of the culture box, we used an iButton® Device. The device was set to record the temperature every 15 minutes in a falcon tube filled at a quarter by sand. This falcon tube was immerged into the water of a culture box for a week, between the 3rd and the 10th of November. The records of the iButton can be analyzed by OneWireViewer, a software specifically designed for this device. The sensitivity of this indirect measurement induce a loss of sensitivity of maximum 1°C[57].

5.5.2 Water parameters analysis

Three main components of the water were assessed: the NH_4, NO_3 and NO_2 concentrations. Those analysis were performed using JBL tests, commonly used in aquacultures to test water parameters in aquarium to assess if the bacterial degradation process of those components is disturbed. Different dyes are used to color the water depending on the NH_4, NO_3 and NO_2 concentration.

Table 7: Chemical composition of the JBL tests

Parameters	Reagent	Composition
NH_4	1	trisodium citrate, purified water
	2	sodium hydroxide, solution of sodium hypochlorite, purified water
	3	thymol, sodium nitroprusside, purified water, isopropanol
NO_3	1	sulfanilamide, acid tartaric, tartrazine, zinc
	2	1,3-diaminobenzene dihydrochloride, acid hydrochloric acid, purified water
NO_2	1	sulfanilamide, acid acetic, tartrazine, purified water
	2	naphthyl-ethylenediamine dihydrochloride, ethanol, purified water

- 5 mL of water is prepared for every test (NH_4, NO_3 and NO_2).
- 4 drops of the first reagent, 4 drops of the second reagent and 5 drops of the third reagent for NH_4 test is added to the water sample for NH_4 test.
- Left to rest for 5 minutes.
- a spoon of the first reagent and 3 drops of the second reagent for NO_3 test is added to the water sample for NO_3 test.
- Left to rest for 7 minutes
- 5 drops of the first reagent and 5 drops of the second reagent for NO_2 test is added to the water sample for NO_2 test.
- Left to rest for 3 minutes.
- Compare the color of every sample with the color scale of the water (Figure 20).

5.6 Statistics

All statistical analyses were performed using the RStudio software (version 1.4.1717)[58]. For each analysis, the statistical distribution of the values was assessed using the Shapiro-Wilk normality test and an analysis of the homogenous distribution of the residuals of the data. The differences between conditions are compared using an ANOVA I analysis. The differences between treatments are considered significative under a threshold of p value = 0.05.

6 Results

6.1 Rearing optimization

In order to improve the efficiency of our rearing, we assessed different characteristics that can influence the well-being, reproduction and survival of bloodworms (BW).

6.1.1 Water parameters

The water concentrations of NH_4, NO_3, and NO_2 were measured directly from tap water, from water of the reproduction box (RB) and from the reverse osmosis water used for the *N. furzeri* rearing using the JBL test protocol described in the section 5.5.2. There is no significant difference for the NH_4 concentration, being at less than 0.05 mg of NH4/L of water, which is the smallest concentration that can be detected by this test. The RO water contained less NO_3 than the two other water: less than 0.5 mg of NO_3/L of water compared to 1mg/L for the tap and RB water. For the NO_2 content, the tap and RO water contained 0.05 mg of NO2/l of water, which was less than the RB water that presented 0.1mg/L (Figure 20).

Figure 20: JBL test for NH_4, NO_3 and NO_2 in tap water (TAP), reverse osmosis water (RO) and from a reproduction box (RB)

Other water characteristics, such as pH, conductivity and hardness come from the information provided by the Walloon Brabant water management body, INBW[59], as shown in the Table 8.

Table 8: Levels of pH, conductivity and hardness of the tap water from Louvain-la-Neuve[59] compared to the levels recommended for a *C. riparius* rearing[32,38,42–44,48,50,51]. The hardness is expressed in French degrees, each one corresponding to 3.4mg of OH^-/L or 6 mg of CO_3^{--}/L of water.

Characteristic	INBW	Recommended for BW
pH	7,7	6-9
Conductivity (µS/cm)	610	> 540
Hardness (°f)	33	< 40

6.1.2 Water temperature

The temperature in the water of the culture box (CB) ranged daily from 19.686°C to 21.688°C. The minimum of the day was measured between 11PM and 3AM while the maximum was reached between 11AM and 6PM. Half of the volume water of the CB tested was exchanged for fresh tap water on the 5th day, according to the protocol detailed in the section 5.1.1. This exchange caused a drop of 1°C of the water temperature recorded by the iButton®, from 21.187°C to 20.186°C, however this drop was recovered in a 2-hour period (Figure 21).

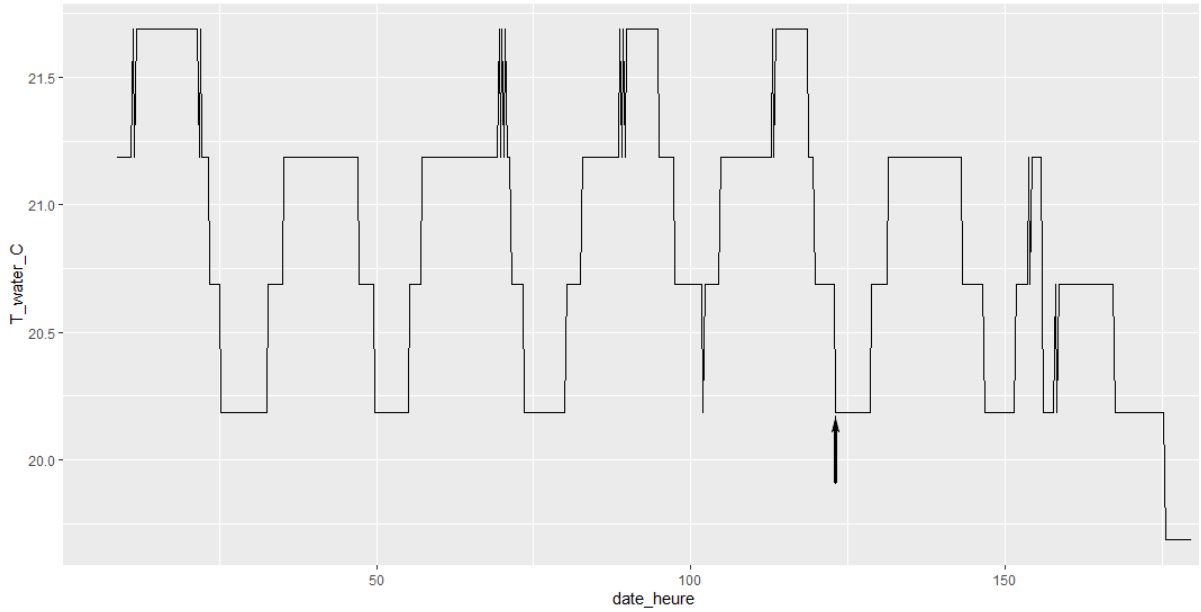


Figure 21: Graph of the evolution of water temperature of a culture box (CB) from *C. riparius* rearing during a week of recording. The y-axis represents the temperature of the water in the CB in °C. The x-axis represents the hour after the start of this experiment. The black arrow represents the recording of the drop of temperature due to the change of water in the CB

6.1.3 Room temperature and relative humidity

The temperature in the rearing room remained relatively stable during the light-on period, showing a global difference of 1.77°C between 9AM and 11PM. The room temperature presents a drop of more than 2°C at 11 PM which is maintained throughout the light-off period (Figure 22).

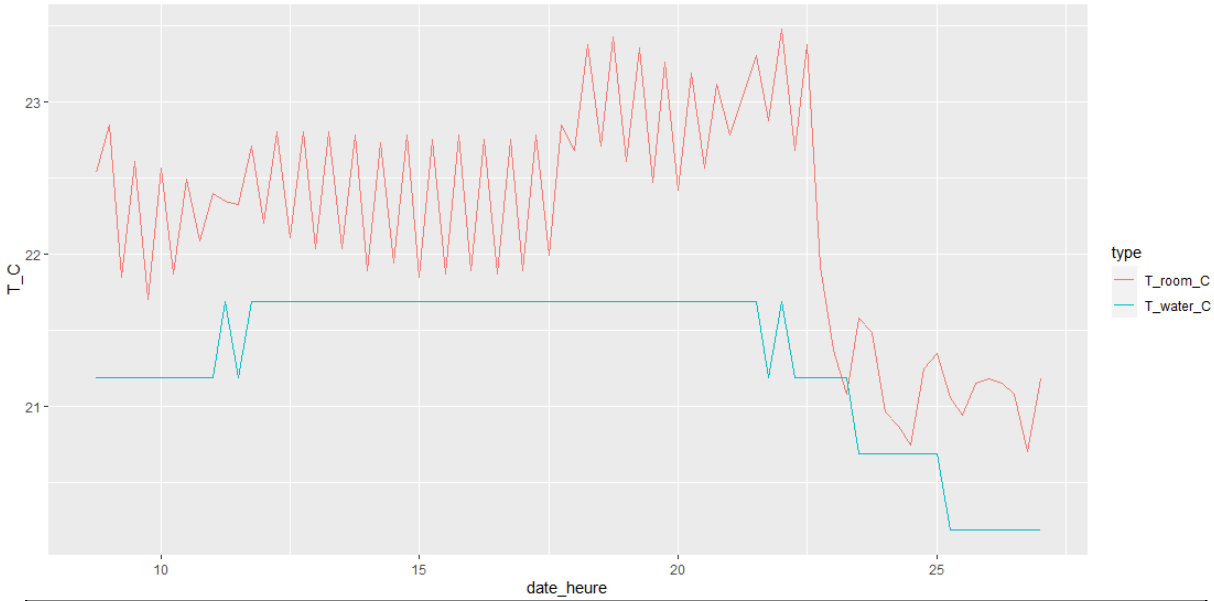


Figure 22: Graph of the evolution of water temperature of a culture box (CB) from *C. riparius* rearing (in blue) and the room temperature (in red) recorded every 15 minutes over a 27-hour recording period

The relative humidity (RH) recorded by the HOBO Logger varied between 38.53% and 51.93%. This variation seems to be dependent of the time of the day, with an increase around 11AM towards maximum values in the beginning of the afternoon and a decrease around 8PM towards the minimum values around 9PM. The variation of RH seems to be less pronounced during the night (Figure 23).

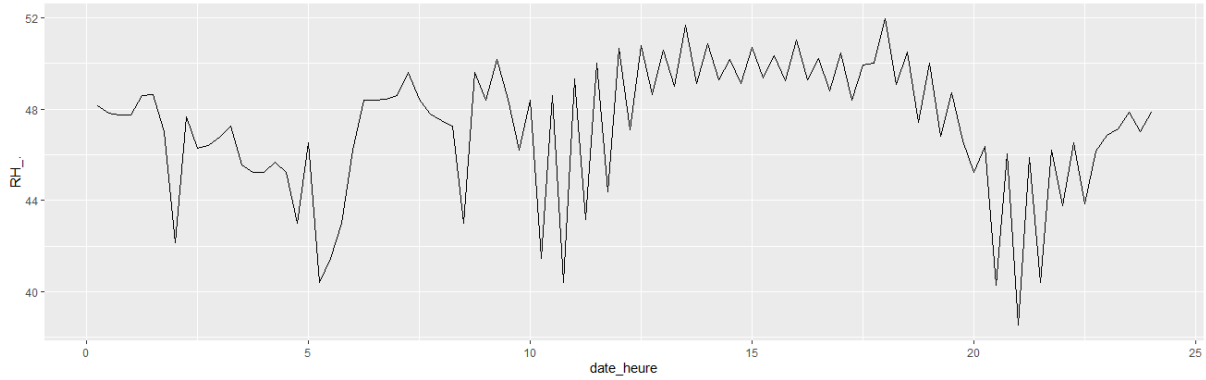


Figure 23: Graph of the evolution of relative humidity (RH) in the *C. riparius* rearing room over a 24 hour-period. The y-axis represents the percentage of RH in the room. The x-axis represents the hour of the day.

The data collected with the HOBO Logger over a multiple month-period in the rearing room reveals the variation of relative humidity. The relative humidity correlates with the number of egg ropes hatched (r=0,407) (Figure 24).

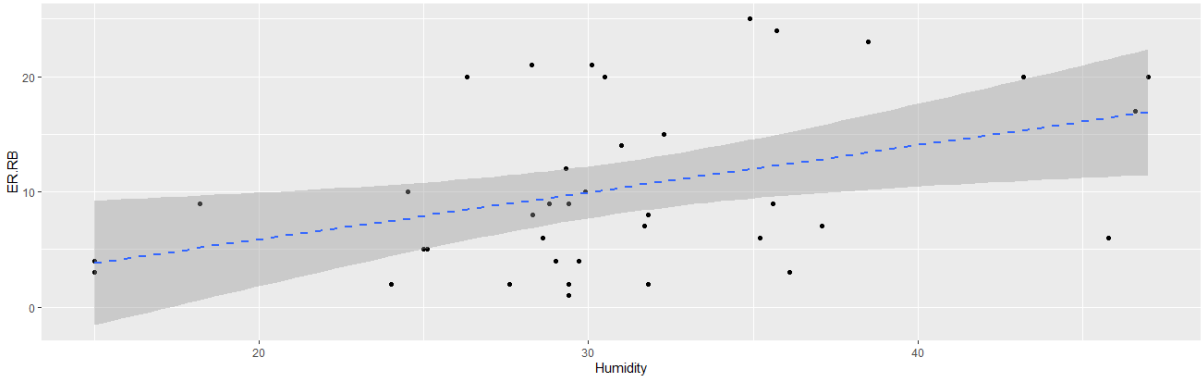


Figure 24: Graph of the number of egg ropes laid/reproduction box (ER_RB) as a function of the relative humidity of the rearing room (Humidity).

6.1.4 Rearing food

At the beginning of this master thesis, the rearing food used for our colony, Goldy from Sera©, stopped to be produced by the fabricant. To assess which food use to replace Goldy, we did a diet-switch experiment, like described earlier in the eponym section, with three different brands of fish food usually used in *Chironomus sp.* rearing: Goldfish Flakes (GFF) and Tetramin Flakes (TMF) from Tetra© and Novobel (NB) from JBL©.

The survivability of the BW after this experiment was assessed by the total weight of the BW harvested from each CB. GFF and NB show a good rate of survivability compared to the fasted trial. TMF, on the other hand showed a poor rate of survival (Figure 25).

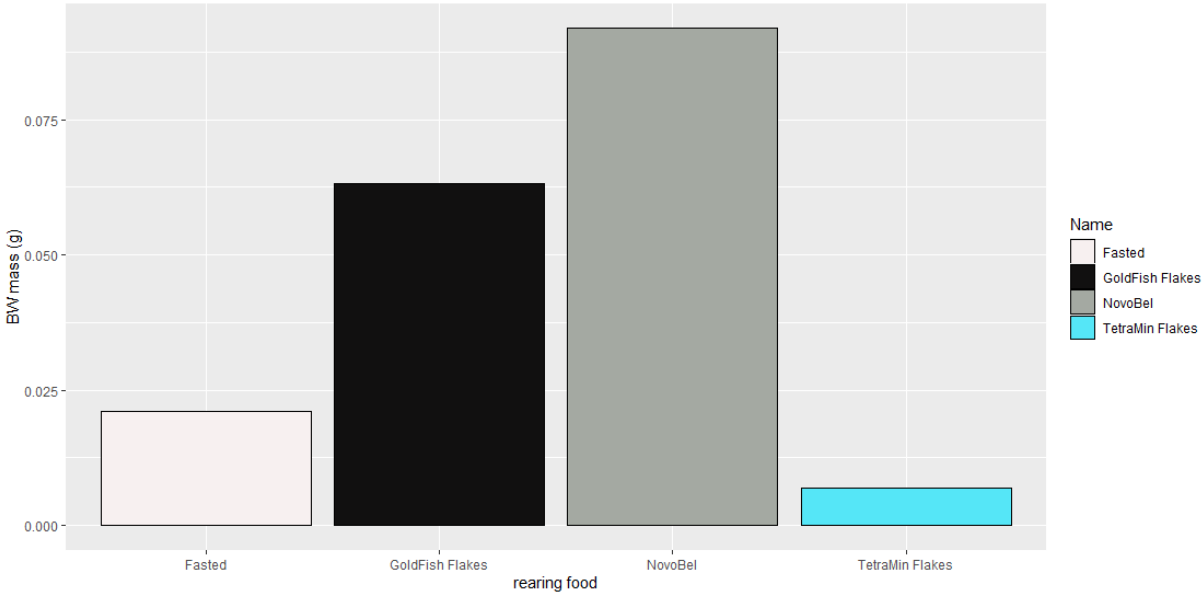


Figure 25: Graph of the mass of BW harvested from each diet condition: fasted in white, goldfish flakes in black, novobel in grey and tetramin flakes in blue

6.2 Enrichment experiment

6.2.1 Enrichment foods

Fatty acid profiles were recorded on the enrichment foods itself to assess their relative levels of enrichment. GFF was used as a control food and BFM was enriched in olive oil. Figure 26 reveals that these two foods do not contain PunA, in contrast PunA was detected in the three other foods.

The largest concentration of PunA was recovered in the emulsion with 355.72±50.10 mg/g of food. The concentration of PunA in Gel1 and Gel2 was respectively 232.80±22.92 mg/g and 159.78±30.14 mg/g of food. Considering the way those foods were made, we expect to have 100%, 60% and 40% of PSO in the emulsion, Gel1 and Gel2 respectively (See section 5.3.2 and 5.3.3). If we have 350 mg of PunA/g of the PSO used, like the emulsion suggests, we see that we recover 65.44% and 44.92% of PSO in Gel1 and Gel2 used respectively. This could be due to variation in sample compositions and errors in the results from the emulsion; indeed, this enrichment food was highly concentrated in oil and only 50 µL of a diluted portion of the sample of emulsion was injected in the GC. Another explanation is that the emulsion does not allow a complete recovery of the oil in the sample.

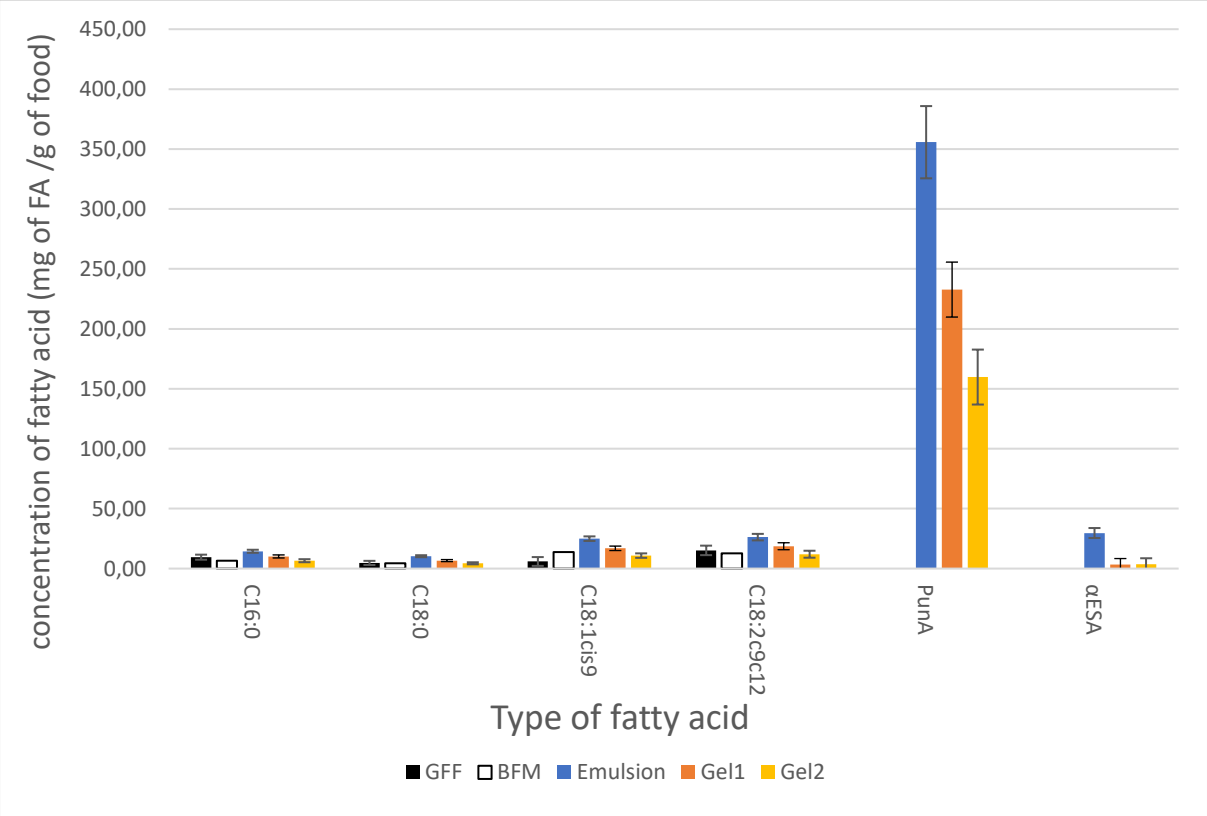


Figure 26: Graph of the concentration of the most concentrated fatty acids recovered from the fatty acid profile of different diets: GFF in black, BFM in white, emulsion in blue, Gel1 in orange and Gel2 in yellow

PunA was not the only CLnA recovered from the enriched diets; α-eleostearic acid (αESA) was also recovered from the diets. The most probable origin of this αESA emergence is the isomerization of the PunA. A proportion of PunA compared to PunA+αESA concentration was performed to differentiate the three enrichment foods enriched with PunA. The proportion of PunA recovered in each enriched diet was 91.62±0.64%, 97.99±3.08% and 97.08±3.64% for the emulsion, Gel1 and Gel2 respectively (Figure 27). The proportion of PunA

recovered in the emulsion is significatively lower compared to the two mixes of gelatin (p value under 0.0001). The proportion of PunA recovered in Gel1 is also significatively higher compared to Gel2 (p value equals to 0.0438).

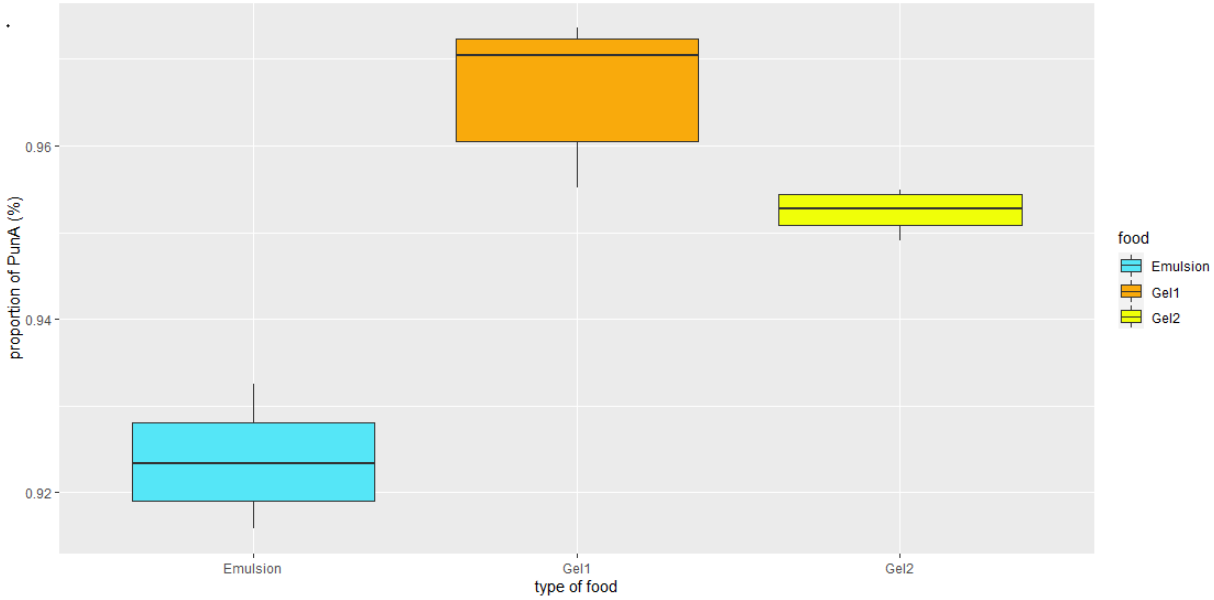


Figure 27: Graph of the proportion of PunA in the three enrichment diets compared to the total of CLnA recovered in it. Emulsion in blue, Gel1 in orange and Gel2 in yellow.

6.2.2 Extraction of fatty acid from the food

6.2.2.1 Bligh and Dyer method

For the fabrication of this free-FA commercial food, we used 471 mg of tetramin flakes (TMF) divided in 10 samples to test the level of recovery allowed by this method. During the extraction process of the Bligh and Dyer, 3 mL of the water-rich phase of each sample was removed to a petri dish which was put in the freezer for freeze-drying. After the freeze-drying, only 30 mg out of the 471 mg were retrieved.

6.2.2.2 Soxhlet method

This method of fatty acid extraction from the commercial food can be done with or without HCl hydrolyzation of the sample prior to the extraction itself, as explained in the section 5.3.4.2. The respective mass of TMF samples used for the Soxhlet extraction was 3.025g and 0.776g with and without HCl hydrolyzation. After this extraction, 0.392g and 0.087g of fatty acid were extracted in these two samples. This corresponds to 12.95% and 11.24% of the total mass of the samples (Table 9).

Table 9: Mass of samples from the extraction of fatty acid by Soxhlet

Type of extraction	With HCl hydrolyzation	Without HCl hydrolyzation
Initial mass of the sample (g)	3,025	0,776
Mass after extraction (g)	2,633	0,689
Mass of fatty acids extracted (g)	0,392	0,087
Proportion of fatty acid extracted (%)	12,95	11,24

A fatty acid profile was performed on the food after the fatty acid extraction to assess the remaining fatty acids in those modified commercial GFF food. The concentration of fatty acid retrieved in those samples were compared to the same commercial food which has not undergo a fatty acid extraction process. The same spectrum is observed for the three foods, but with some differences in their concentration. There is still 5.785mg/g and 17.221mg/g of food in the food extracted with Soxhlet with and without HCl hydrolyzation respectively (Figure 28). This corresponds to 11% and 32% of the amount retrieved in the initial GFF.

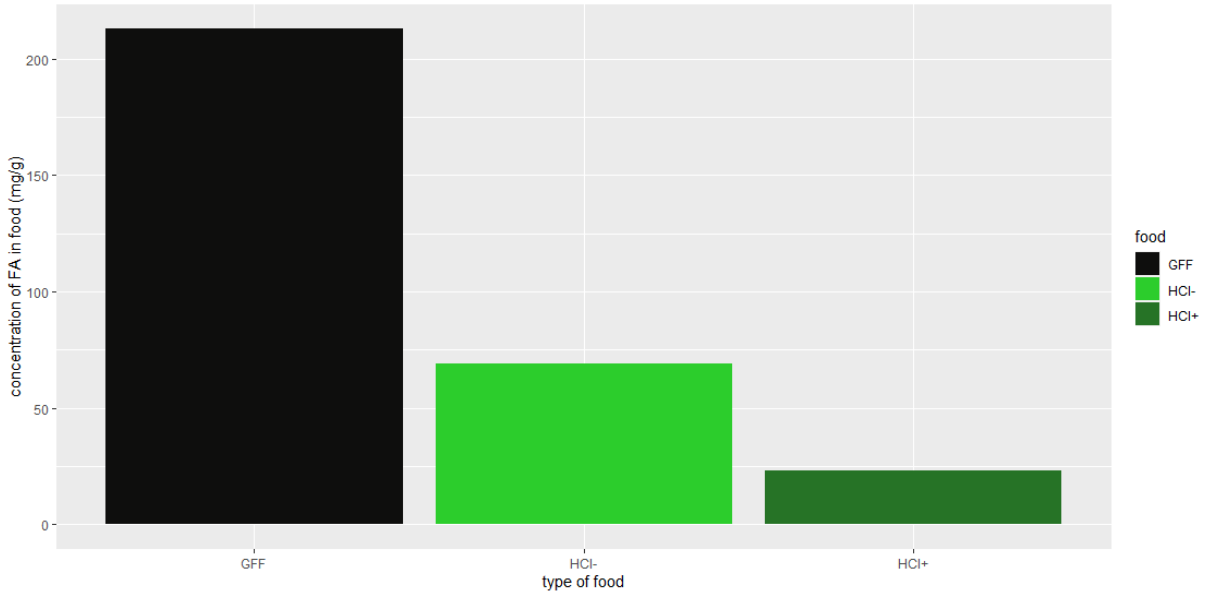


Figure 28: Graph of the total concentration of the fatty acid fraction in three different diets: GFF in black, GFF after a Soxhlet extraction without HCl hydrolyzation in light green and with HCl hydrolyzation in dark green

6.2.3 Complete rearing enrichment experiment

To assess the possibility to enrich BW with this method we used BFM enriched with olive oil. Two culture boxes were prepared as described in the section 5.1.3.1. One culture box was reared with GFF and the other with BFM. After a month of rearing, the BW were harvested, and the mass of each sample was used to assess the survival rate linked to each diet. Based on mass, the BW reared entirely with BFM had a low survival rate, with a retrieval of 12,5% of the amount from the GFF condition (Figure 29).

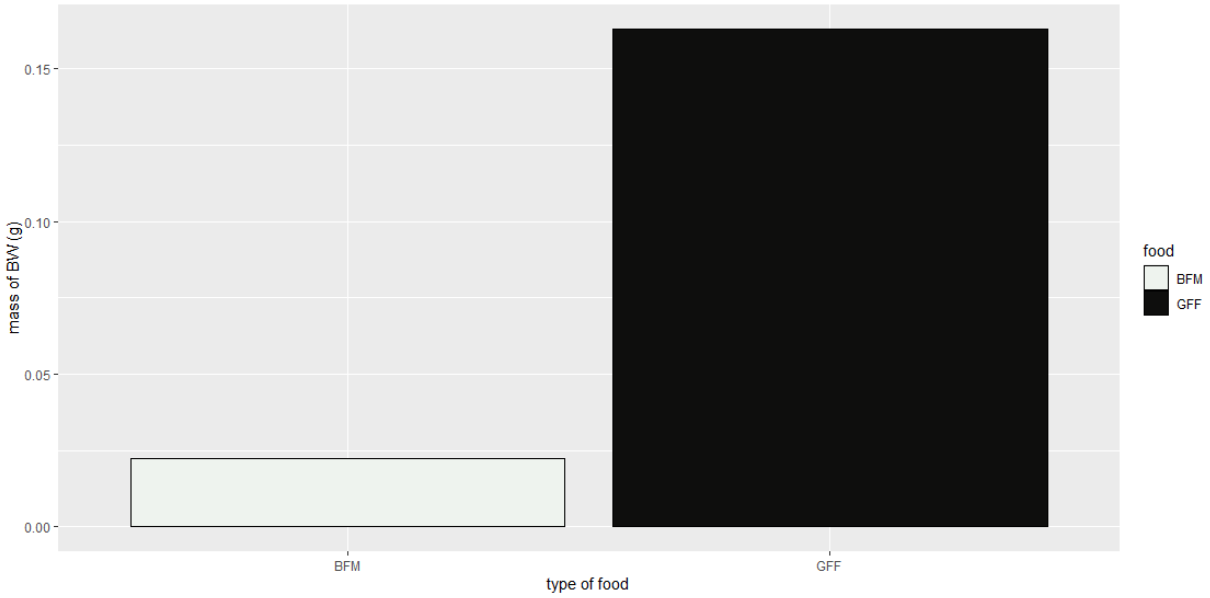


Figure 29: Graph of the mass of BW recovered after a complete rearing enrichment experiment depending on the diet: GFF in black and BFM in white.

6.2.4 Diet-switch enrichment experiment

For this experiment, we prepared five CB as described in the section 5.1.3.2: two controls, one fed with GFF and the other left fasted, and three enrichment foods; Emulsion, Gel1 and Gel2. After the week of enrichment, the BW were harvested, and the total weight of each condition were used to assess the survival rate associated with the condition. The control condition continuously fed with GFF resulted in a low level of survival, with only 10.2 mg of BW retrieved. The three enrichment foods tested presented similar rate of survivals, with 75.3mg, 73mg and 68.9 mg retrieved for the emulsion, Gel1 and Gel2 respectively, all higher than the 57.3mg of the fasted condition (Figure 30).

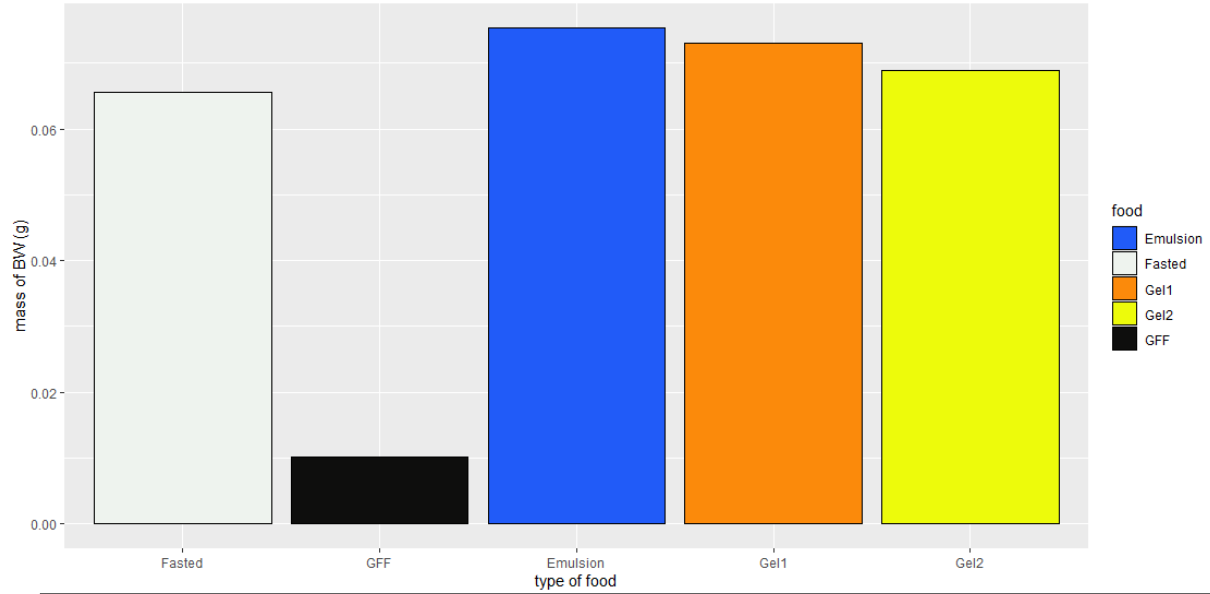


Figure 30: Graph of the mass of BW recovered after a diet-switch rearing enrichment experiment depending on the diet: BW left fasted in white, fed GFF in black, Emulsion in blue, Gel1 in orange and Gel2 diet in yellow

PunA was not detected in the fasted BW or those fed with GFF, whereas PunA were detected from the collected BW fed the three enriched diets. The level of PunA measured from collected BW varied depending on the enrichment food. For example, 0.487mg of PunA was recovered per gram of BW fed the emulsion diet. In contrast, 1.269mg and 2.739 mg of PunA was measured per gram of BW fed the mix of Gel2 and Gel1 respectively (Figure 31). However, the PunA levels did not significatively differ between the two gel mixtures due to a large variation of sample concentration (p value equals 0.2222). Indeed, one sample presented a concentration of more than twice the concentration of the two others. Since the other concentrations of fatty acids in this sample was not significatively different from the two others, this difference of PunA concentration must come from a wrong partition of the samples at the beginning of the Bligh and Dyer, the richer BW integrated in the samples first. A small portion of α-eleostearic acid (αESA) was also retrieved in the BW. Depending on the enrichment food, the proportion of PunA compared to the complete CLnA fraction in the BW was different, 69%, 80% and 78% for the emulsion, Gel1 and Gel2, respectively.

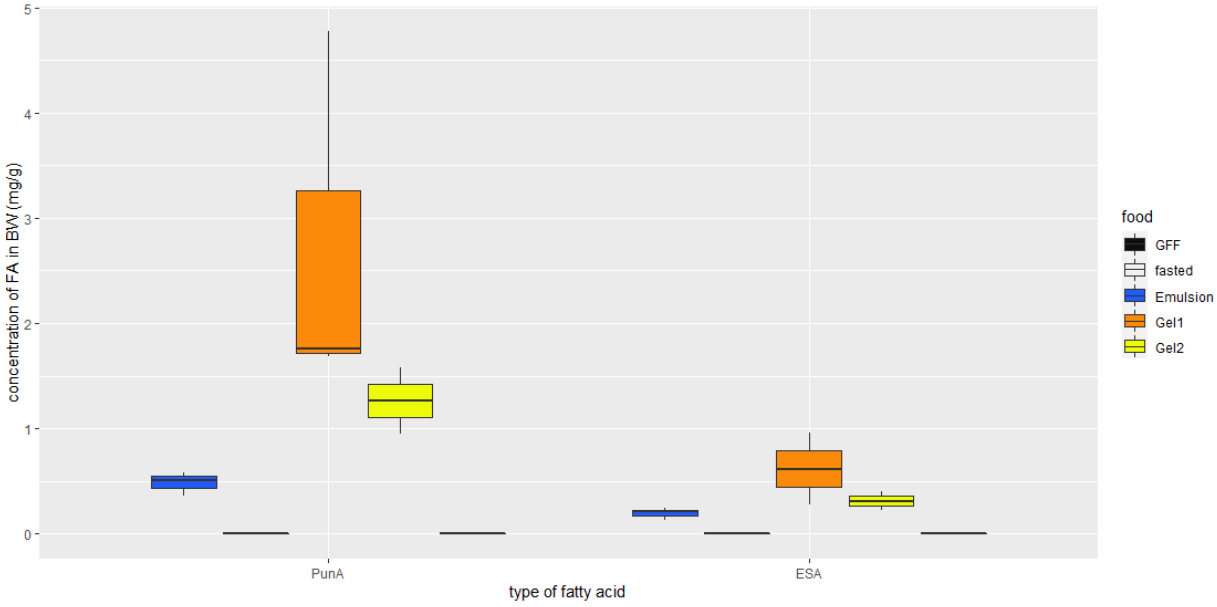


Figure 31: Graph of the level of enrichment with PunA in BW depending on the diet: GFF in black, fasted in white, emulsion in blue, Gel1 in orange and Gel2 in yellow.

PunA only represents 1.35±0.33% in BW fed with the emulsion, 7.66±3.11% and 3.45±1.03% in Gel1 and Gel2, corresponding to a total mass of PunA of 0.037mg, 0.194mg and 0.087mg/CB respectively (Appendix).

7 Discussion

The main goal of this master thesis was to assess the possibility to enrich *Chironomus riparius* larva (BW) with punicic acid (PunA) to offer this enriched diet to *Nothobranchius furzeri*, in order to assess the effects of PunA. During this master thesis, I created three different enrichment diets that successfully enriched BW in PunA. However, this process was impacted due to rearing issues, which had to be addressed throughout the project. The first part of this discussion deals with the rearing issues encountered, and the second part of the discussion focuses on the PunA food enrichment results.

7.1 Rearing control

The usual rearing food Goldy was removed from the market at the beginning of the project and had to be replaced by an adequate substitute. This food normally allows a rapid and proliferent production of individuals. Multiple diets usually used to rear BW were tested: goldfish flakes (GFF), tetramin flakes (TMF) and novobel (NB). GFF was the best diet for our rearing; However, the diet did not result in the same reproduction level of *C. riparius*. In addition, our *C. riparius* colony suffered a mass extinction, which could be due to a contaminated food box. Indeed, the only factor that changed during this period was the switch of GFF to TMF as the rearing food of the colony. Moreover, the experiment on the multiple diets described just before happened in the same period, and the only condition that showed a poor survival rate was the one fed with TMF. Those results supported us in our decision to use GFF as the new rearing food. New egg ropes were sent to us from the Swedish University of Agricultural Sciences. Although this allowed for us to rebuild our colony, the initial development rate could not be recovered. As a result, numerous steps were made to improve our rearing conditions to allow for a sustainable production of BW, which would allow us to test the enrichment process. This step was complex as there are numerous factors that can influence each life cycle stage of *C. riparius*.

The temperature of the water was first assessed. Indeed, a too low water temperature could negatively impact the growth and development of aquatic forms of *C. riparius*. Similarly, a too high water temperature can also hinder the development of the BW[40]. However, Figure 21 indicates that the water temperature stays within the 21 and 23°C recommended (Table 2), even after the replacement of the water, as described in section 5.1.1. In our rearing facility, the water temperature was at the lowest part of this temperature fork at the time we recorded it, but not low enough to induce the slowdown of our rearing development, and not too high to have an impact on reproduction or survivability. Nevertheless, the water temperature is largely influenced by the room temperature which is set to be 23°C. Tap water added to culture box (CB) induces a drop in the water temperature but not more than 1°C, and which was compensated within 2 hours.

Concerning the relative humidity in the room, values from 15 to 52% were recorded between February and November 2021 (Appendix A). This factor has no influence on BW but can impact the reproduction of the adults. Indeed, values below 60% correlates with a significant decrease in the number of egg ropes[52], which was corroborated by our values (Figure 24). This factor is important to change if we want to optimize our rearing. However, we shared the room with another team working on *Drosophila melanogaster* and could not control this parameter. The water parameters such as composition, pH, conductivity and hardness, are another condition that can affect BW growth and survival. The level of ammonium, nitrite and nitrate were assessed in the CB after two weeks without water

exchange. However these levels were lower than those reported to be harmful for BW growth (Table 2)[60]. This was also the case for nitrite levels that were higher than compared to the tap water. This difference in nitrite levels is probably due to a lack of bacteria that can metabolize nitrite into nitrate, which take more time to develop than the bacteria degrading the ammonium into nitrite. The other parameters assessed were all in the range of the recommendations for BW rearing. The other rearing characteristics were adapted to fit in the recommended range of the literature (Table 2). But, as described in the table 2, those characteristics vary depending on the article consulted and there is no control over this type of rearing.

There are multiple potential reasons for the slowdown of our rearing. First of all, the fact that the relative humidity is lower than the minimum value for the reproduction of *C. riparius* may pose a major problem for a mass production of BW. Secondly, the choice of the rearing food is predominant for the life cycle of Chironomus *riparius*. Indeed, the first reduction of the rearing occured when we switched their diet from Goldy© to GFF and the second reduction when we switched from GFF to TMF. Finally, the *C. riparius* strain can also influence the characteristics fitted for them.

7.2 Enrichment in PunA

Moreover, concerning the enrichment in PunA, a full rearing with BFM enriched with olive oil was first tested. This highlighted two main problems with the enrichment. Firstly, the enriched food was not sufficient for the well-being of the BW. The rearing food is essential for the growth and survival of the BW, and this is especially true with the enriched food since it is completely artificially made and that it contains only the oil of interest. The second important observation was that an entire rearing with an enrichment food is not the best solution. Indeed, enriched food are made with a large proportion of oil to allow the best enrichment possible while keeping the potential to dissolve in water. This delicate balance will eventually break, and the oil will dissociate with its matrix resulting in the formation of a lipid layer on top of the water. When the oil comes out of the matrix, the fatty acid enrichment of BW is no longer possible. To counter that issue a food that is more stable in water could be created, or the period of enrichment can be shortened. Since the main goal of this thesis is to enrich BW to further use them as a dietary vehicle to enrich *N. furzeri* with PunA, a protocol that allows a mass production of BW needed to be created, which does not seem possible with this method. We opted for a shorter period of enrichment as Strandberg described that it was possible to switch the main composition of FA in BW in a 6-day period by changing their feeding[43].

The enrichment method was also modified, since BFM is quite expensive and time-consuming to make. The first idea to create a new enrichment mix was to extract the fatty acids from the usual rearing food to allow its enrichment with PunA. The Bligh and Dyer method can be used to extract lipids from a biological mix to isolate them from the other components. This method was tested to separate a matrix containing every compound of the rearing food without the lipids. Unfortunately, the recovery was insufficient and the small portion recovered could not be used further for enrichment. Additionally, this method relies on the extraction of the compounds in an aqueous form in a methanol solvent that we freeze dry to form a dried matrix. Although this generally allows for an increased ease in enrichment, it also creates a substance that is strongly attached to the surface of the petri dish in which they were freeze dried. Another way to extract fatty acids from a mixture is the Soxhlet method. The extraction of lipids from GFF with this method allowed the formation of a matrix

similar in appearance to the initial food and in a sufficient amount to use it efficiently as an enrichment food. A fatty acid profile was made on this matrix to assess the efficacity of the fatty acid extraction. Unfortunately, 32% of the initial amount of fatty acid remained in this matrix. We tried to add an HCl hydrolyzation step prior to the fatty acid extraction with the Soxhlet method to enhance its efficacity. This successfully enhanced the fatty acid extraction, with only 11% of the initial fatty acid amount retrieved afterwards, but it caused a major denaturation of the remaining product. Indeed, the matrix looked like ash that was unsuitable for the use as food.

The second idea to create this enrichment mix was inspired by the method used by Le Clercq to enrich artemia with PunA[61]. The idea is to simplify the method by focusing on the enrichment itself, by making pomegranate seed oil (PSO) water soluble. In that aim, we created two different foods: an oil emulsion and a mix of PSO with gelatin. The oil emulsion consists of a mix of the PSO with water and an emulsifier, following the method adapted from Tamaru by Le Clercq[55,61]. For the mix with gelatin, we used a mix of PSO and water in which we added different amounts of pig gelatin to assess which proportion was the most adequate to create a homogenized mix that was also easy to use. Two mixtures (Gel1 and Gel2) seemed equally suitable, so we decided to use both. Those three enrichment foods were then used to enrich BW.

This new attempt to enrich BW with PunA was performed using a diet-switch experiment where the first three-week period focuses on the optimal growth and development of BW until their last instar and the last week focuses on their enrichment in PunA. Unfortunately, the control condition that was fed on GFF during the entire experiment resulted in the lowest survival rate, probably due to the fact that one of the two egg ropes of this CB did not hatch. Without the GFF survival rate, we cannot state the range of survival rate of the three experimental conditions, fed with the emulsion, Gel1 and Gel2 even though they presented a survival rate higher than the fasted condition.

Nevertheless, the level of enrichment for the three experimental conditions was still assessed. It highlighted the fact that the emulsion is not appropriate to enrich BW diet. Indeed, the BW fed with this diet presented the lowest concentration of PunA compared with the two others, whereas the emulsion itself contained the highest concentration of PunA. This can be explained by the fact that BW lives primarily burrowed in the sand at the bottom of the CB and that the emulsion stayed mostly at the surface of the water due to a lack of agitation to correctly mix it with water. In addition, the emulsion mix presents a higher concentration of other stereoisomers of PunA than the two other mixtures, which difference is retrieved in BW. The mixture that allowed the better concentration of PunA in BW was Gel1. Unfortunately, this concentration is not significantly different from the concentration in Gel2 due to the presence of an outliner in Gel1 that presented the double of PunA concentration compared to the two other samples of Gel1.

This experiment needs to be replicated in order to correctly assess the efficiency of the reduced enrichment period on the survival rate of the BW fed with the enriched diet and the level of enrichment possible with this method.

8 Conclusion

During this master thesis we showed that feeding BW with an enriched diet have a consequent impact on their survival rate. We succeeded to enrich their diet with PunA by using a diet-switch method that allowed to reduce the length of the enrichment period to its minimum. This method shows promising results, but we need more replicates to assess its efficiency.

In either case, the major inconvenience in the use of BW to enrich the *N. furzeri* diet is the rearing itself. Indeed, it requires a rearing of one month to obtain BW fitted for being eaten by the fish. Associated with the fact that the number of BW retrieved from each CB after this month of rearing could potentially feed 10 fishes for a day, we would need a rearing of at least 30 CB to feed those 10 fishes daily, plus the CB dedicated to maintains a complete life cycle to produce enough egg ropes to form those 30 CB a month.

There are two methods used to find a way to enrich the *N. furzeri* diet with PunA: BW and artemia. The main advantages of artemia compared to BW is that we only need two days to prepare them (egg until enriched nauplius ready to be used as food), their rearing requires less space and they are naturally fatter than BW, equals to 5-15%[62,63] and 5-10%[30,31,43] of the complete dry matter in artemia and BW respectively. The level of enrichment of artemia is also bigger than for BW: Le Clerq managed to obtain a concentration of PunA compared to the total fatty acid fraction ten times higher than the one obtained here[61].

In the project to enrich *N. furzeri* diet with PunA, artemia seems to be more efficient than BW. Even though BW could potentially better enrich *N. furzeri*, since they are consistently bigger, so they transport more fatty acid, but their rearing implies too many difficulties to obtain a correct amount of BW allowing a proper enrichment. However, this method could be of great use for other projects that do not require a high production. This is the case for the ecotoxicological research where *C. riparius* is a widely used model since it is ubiquitous and filter feeder so it will swallow the pollutants in their environment. A potential use of the enrichment method in that context would be to test the protective effect of specific fatty acids against the raising pollutants retrieved in our environment.

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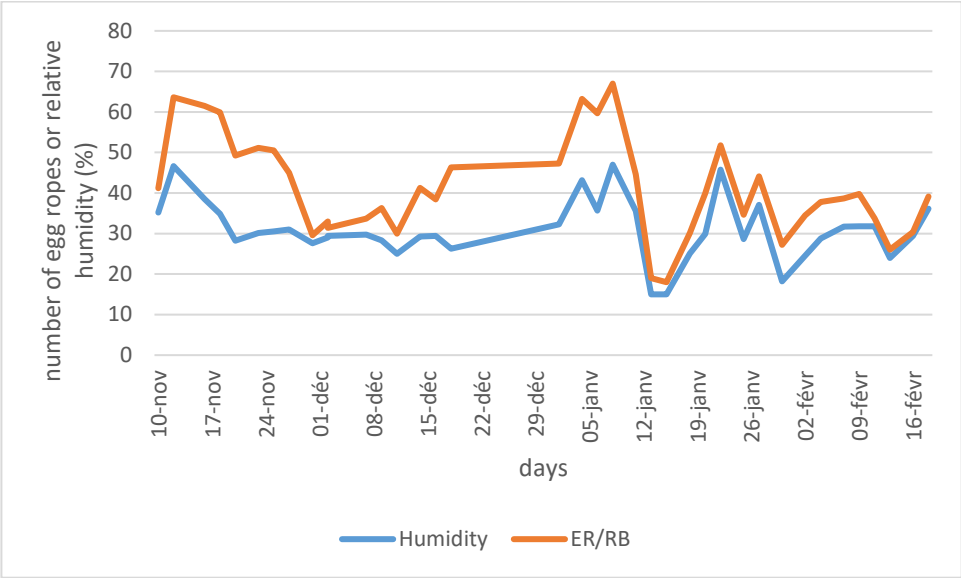
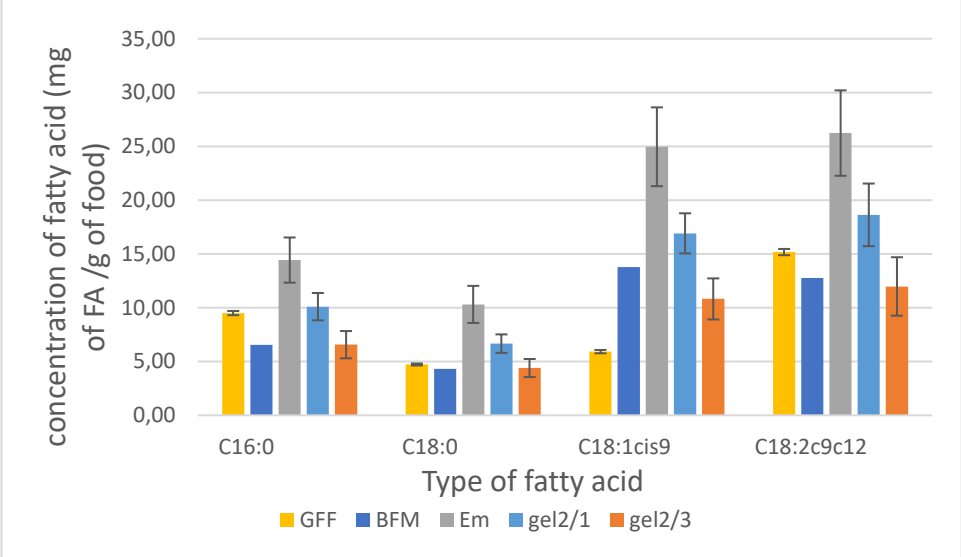
10 Appendix

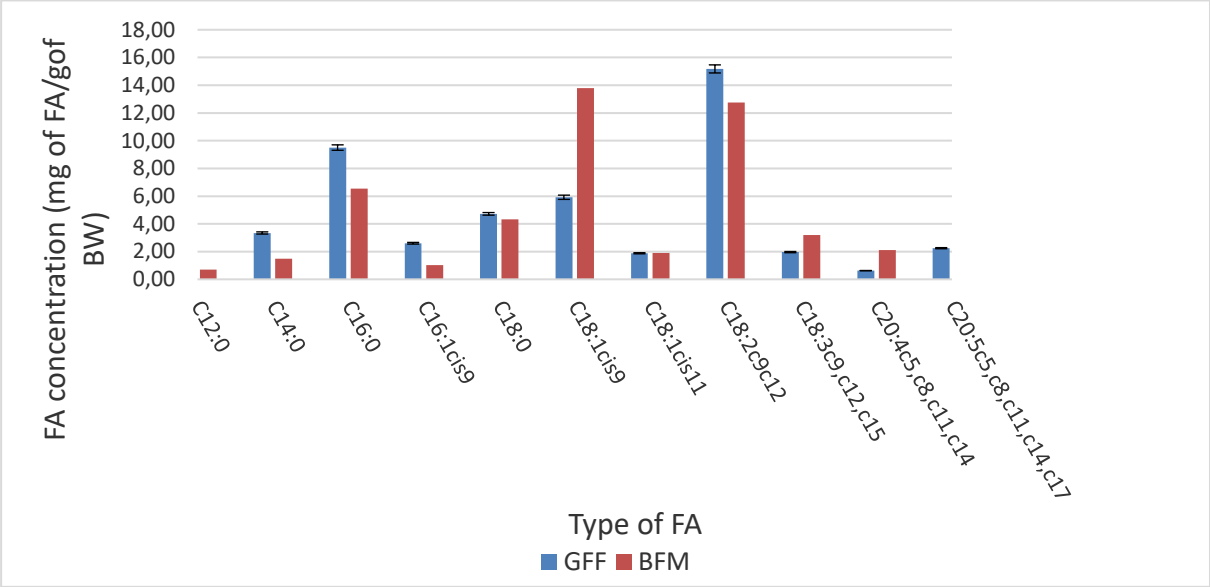
10.1 Appendix A – evolution of ER and HR



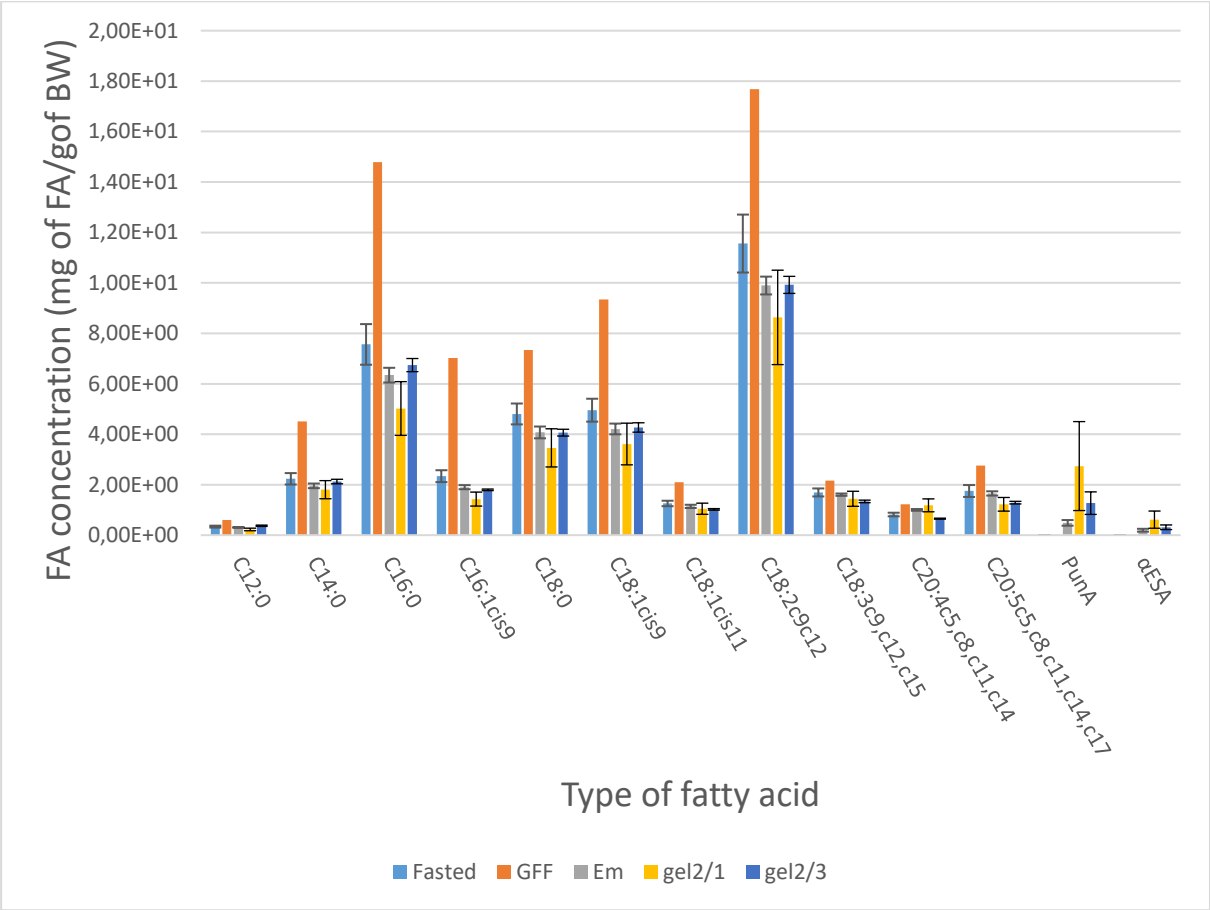
10.2 Appendix B – concentration of FA in enriched diet (CLnA hidden)



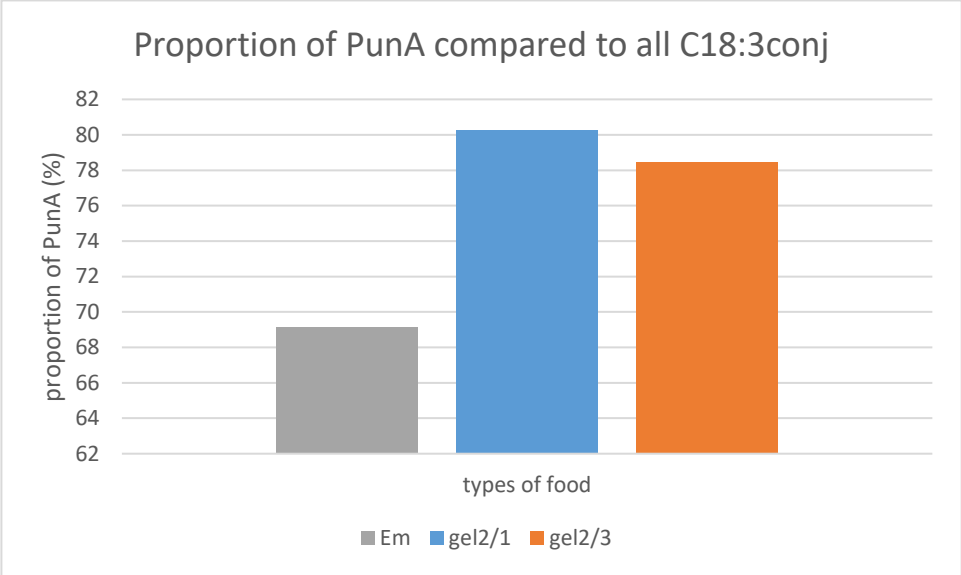
10.3 Appendix C – FA profile of the complete rearing enrichment experiment



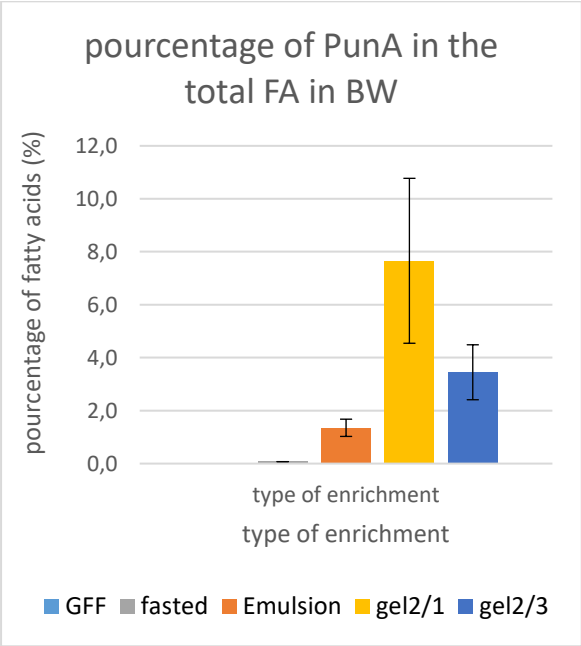
10.4 Appendix D – FA profile of the diet-switch enrichment experiment

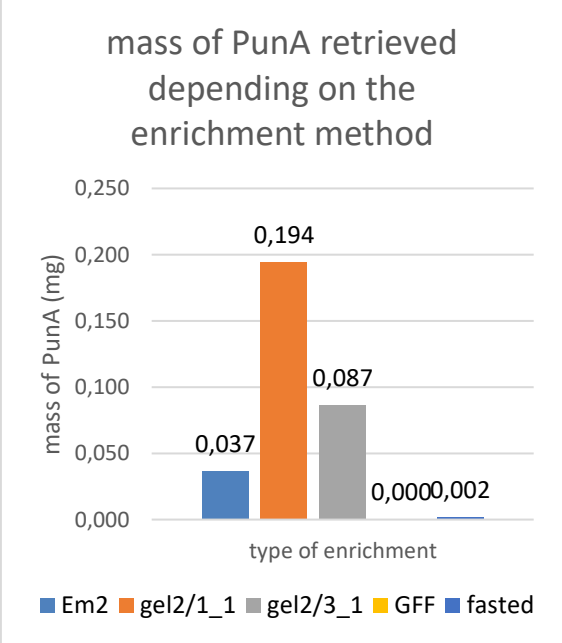


10.5 Appendix E – Proportion of PunA compared to the total CLnA fraction in BW



10.6 Appendix F – level of enrichment with PunA





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