THE SWEDISH 3Rs CENTER

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Acclimatisation of zebrafish prior to experiments

A support material from the Swedish 3Rs Center

Photo: USER_59395, Mostphotos

Contents

Background

This document is part of a series of acclimatisation documents that the Swedish 3Rs Center has produced on behalf of the Swedish National Committee for the Protection of Animals Used for Scientific Purposes. The series consists of three parts with a focus on mice, rats and zebrafish respectively. This part is intended for everyone who works with zebrafish as laboratory animals and can be used as a support when writing acclimatisation routines of the animal facility, which is a requirement according to the Directive (2010/63/EU) of the European Parliament and of the Council on the protection of animals used for scientific purposes, as well as chapter 16, 7 § of the Swedish research animal legislation (Statens jordbruksverks föreskrifter och allmänna råd (2019:9) om försöksdjur).

The material is based on scientific literature and the Swedish animal welfare legislation. In addition, the Swedish 3Rs Center, in collaboration with veterinary students at the Swedish University of Agricultural Sciences, has collected experiences from people and organisations who work with mice, rats and zebrafish as laboratory animals in Sweden. The data collection was carried out through a questionnaire in order to get an overview of how acclimatisation is carried out in Swedish research animal organisations. At the end of the material, the references that have been used are listed. Please use the list of references to get deeper and more detailed information about the subjects we address. You are also welcome to contact the Swedish 3Rs Center if you have any further questions.

Introduction

Acclimatisation is a term that can be used in a variety of contexts. In this document, we use the term to describe the process that the body goes through to adapt to changes that are often linked to the environment. During an acclimatisation period, the animal is given an opportunity to recover from the stress that a change entails, and physiologically adapt to the new conditions. Acclimatisation is of importance both for the welfare of the animals and for more reliable research results. The length of the acclimatisation process depends on several different factors, such as the cause of stress, new environmental conditions and the animal's individual circumstances.

According to the Directive (2010/63/EU) of the European Parliament and of the Council on the protection of animals used for scientific purposes, Annex III point 3.1.a, introduction of new animals shall be a part of the health strategy that every facility must have. In point 3.7 there is also a requirement that facilities must have habituation programs for the animals. Furthermore, the Commission Recommendation of 18 June 2007 on guidelines for the accommodation and care of animals used for experimental and other scientific purposes (2007/526/EC), general section, 4.4 states that a period of acclimatisation is needed to allow animals to recover from transport stress, to become accustomed to a new environment and to get used to new husbandry and care practices. According to the Commission's recommendation, a period of acclimatisation is necessary even if the animals are perceived to be in good health. The amount of time required depends on what the animals have been subjected to. For example, a longer acclimatisation period may be required after long international transports that disrupt the animals' circadian rhythm, compared to after shorter transports within the country.

In chapter 16, 7 § in the Swedish Research Animal Regulations (Statens jordbruksverks föreskrifter och allmänna råd (2019:9) om försöksdjur) it is clear that there must be a written plan for assessing the physical and psychological well-being of the experimental animals. The plan must also include habituation and training programs adapted to the experimental animals and animal experiments in question. This plan must be produced by a laboratory animal veterinarian or, where applicable, by an expert. If an ethologist is associated with the facility, the plan must be developed in consultation with this ethologist. Thus, there are legal requirements for experimental animals to be acclimatised, but it is not clear how the acclimatisation is to be carried out. Therefore, the approaches vary between different organisations in Sweden.

What is stress?

It is known that stress in laboratory animals can affect research results negatively. In a stressed animal, several different physiological functions and systems are affected, which leads to an increased variation in the results. For example, the levels of adrenaline, norepinephrine, cortisol and corticosterone in the blood become elevated. These hormones are secreted to some extent in an unstressed state, but production increases in stressful situations in order to prepare the body to flee or fight. Stress over a longer period of time also affects the animal's ability to maintain the body's balance, which negatively affects the immune system and increases the risk that the animal will suffer an illness.

Stress is a difficult concept to define. It usually refers to the body's response to an external stimulus, called a stressor. The response can be both physiological and behavioural. Stressors can consist of a variety of things and what constitutes a stressor can differ between individuals. Examples of stressors are social hierarchy, an injury, exhaustion, or difficulty managing the temperature of the environment. Stress can often be linked to a change in the animal's environment that the animal is trying to cope with. Stress does not have to be negative, but prolonged or severe stress can affect the individual negatively, especially if the individual lacks control over the situation.

There are a number of different parameters that can be used to measure stress in fish. Those parameters include levels of glucose and the hormones cortisol and corticosterone in the blood, the animal's behaviour, reproductive performance and altered feed consumption. In zebrafish, cortisol can be measured by terminating the fish and extracting cortisol from tissues, a method commonly used for fry and small individuals. Another method is to measure cortisol in the water, as fish release cortisol via the gills. Several behavioural patterns of zebrafish are associated with increased stress levels, such as erratic swimming pattern, little or no interest in food, and remaining at the bottom of the aquarium. In order to get a proper estimation of the fish’s stress level, it is necessary to assess several different parameters. It has, for example, been proven difficult to determine the stress level of fish solely by measuring cortisol. When cortisol is only measured once, it is also unclear what is actually being measured. This is due to cortisol normally being present in the body and has been shown to be an indication of the zebrafish's experience and general well-being, rather than only its stress response. In other fish species, acclimatisation of cortisol levels has also been shown to depend on the temperature of the water.

To consider when acclimatising zebrafish

To ensure proper animal welfare and secure reliable scientific results, several factors need to be considered when planning the acclimatisation of zebrafish after stressful events and when getting them accustomed to new routines. Such factors include:

- Previous situations the fish have been exposed to and how they were prepared for those situations.
- What the aim of the research is and which parameters are required.
- How quickly the fish are moved to the aquarium after transport.
- Water parameters, including temperature, conductivity (salinity), pH, hardness (KH), ammonia ($NH3$) and ammonium ($NH4$).
- Handling.

We will take a closer look on these factors below.

Other factors that likely influence the length of acclimatisation needed include:

- Changes in routines, feed and the design of the environment.
- Circadian rhythm and brightness of the light.
- The sex and age of the animals.
- Regrouping.

What does research and empirical evidence say about zebrafish acclimatisation?

Scientific literature addressing the subject of acclimatisation of zebrafish prior to experiments is very limited. In addition to scientific literature on zebrafish, this section is therefore largely based on proven experience with zebrafish and research on other fish species. Hence, this part may give an indication of how to acclimatise zebrafish prior to experiments, but probably does not give a completely accurate picture, which readers should keep in mind.

Acclimatisation when changing water parameters

Aquatic animals can be sensitive to differences in water parameters, such as salinity level, mineral composition, temperature and level of acidity. Because of this, acclimatisation of fish has been centred primarily around their physiological adaptation to a new water quality in order to optimise survival immediately after relocation. Zebrafish, for example, have been shown to have a considerable ability to adapt to changes in water hardness (the amount of minerals in the water), but this was accompanied by changes in the gills. The study describing these changes showed, amongst other things, that the expression profile of various genes in the gills were altered by the change in the chemical composition of the water. It also showed that around 7 days of acclimatisation were required for those changes to return to baseline after the hardness of the water had been slowly altered over 7 days prior to the acclimatisation period. For some of the measured parameters, even 14 days of acclimatisation, after getting used to the new water quality, were found to be insufficient. The authors therefore stated that the acclimatisation must be carefully planned before attempting to change the hardness of the water.

Moving zebrafish into a new aquarium

During transport, the quality of the transport water deteriorates due to the fish consuming oxygen and the proportion of carbon dioxide increasing. This leads to an acidification of the water. The acidification is beneficial to some extent, as it shifts the equilibrium from the highly toxic unionised ammonia (NH3) to the much less toxic ionised form ammonium (NH4+). When the transport bag is opened, the carbon dioxide is released and the pH of the water increases. The balance then shifts back towards the toxic unionised ammonia, which can seriously hurt, or even kill, the fish. For those working with fishes, it is therefore important to have knowledge of basic water chemistry.

The following should be done in order to transfer zebrafish to a new aquarium as safely as possible:

- The temperature of the water in the transport bag should slowly be adjusted to the water temperature of the system the fish will be introduced in. This can be done by placing the closed transport bag into a water bath or similar.
- Once the temperature is adjusted, the transport bag should be opened and the fish quickly netted over to an aquarium containing the water of the new facility. Care should be taken to transfer as little water from the transport bag into the new aquarium as possible.
- The water in which the fish has been transported should be disinfected and disposed of according to current local rules and regulations.

Acclimatisation after transport

Research has shown that both fry and adult zebrafish are highly stressed by transportation, as indicated by them displaying several signs of stress during transport and after arrival at the destination. Due to these signs of stress, the fish should be acclimatised prior to being handled, used in breeding or included in an experiment. The sparse collection of scientific studies and other publications about acclimatisation of zebrafish state that an acclimatisation period of 2–5 weeks is needed. The required length of acclimatisation likely correlates to the time the fish has spent in the transport bag. For fish that have been transported short distances, such as within a facility, it is likely enough to acclimatise the fish for a few days before they can be used in research. On the other hand, fishes that have been transported nationally or internationally probably require several weeks of acclimatisation.

A study on African sharptooth catfish (*Clarias gariepinus*) that was transported for 3 hours showed that the transportation increased the levels of cortisol in the plasma. After the transport it took 48 hours for the cortisol levels to return to normal. Studies on tambaqui (*Colossoma macropomum*) that were transported for 8 hours showed that both levels of cortisol and glucose in the plasma is elevated during transport. When it comes to glucose, the levels were drastically reduced after 24 hours and returned back to base levels after 72 hours. On the other hand, the levels of cortisol had still not returned to base levels after 96 hours, when the experiment was ended. In a different experiment, European perch (*Perca fluviatilis*) were transported for 4 hours. After the transport it took between 7 and 14 days before the levels of cortisol and glucose returned to base levels. In the same experiment the highest levels of cortisol and glucose were measured 2 days after the transportation.

Different ways to reduce the need for acclimatisation

It is possible that the need for acclimatisation can be reduced with the help of different measures taken prior to transportation. For example, zebrafish need a shorter period of acclimatisation after being transported for 72 hours if nitrifying bacteria are added to the transport water. Research on various fish species has also shown that skin problems and mortality is reduced if salt is added to the transport water. In addition, a study on Chinook salmon (*Oncorhynchus tshawytscha*) has shown that the stress response, in the form of glucose, cortisol and concentration of lactase, and thereby the time needed for acclimatisation, can be reduced by training fishes using positive reinforcement prior to transport. Fish that had been trained to associate unpleasant circumstances before transport with feeding also displayed a better chance of survival in connection to the transport.

How fast habituation to the new water is made can also affect the length of acclimatisation needed for the fish. In a research project on the fish species tambaqui, it was shown that if the fish got 30 minutes to habituate to the new water, the measures studied were returned to normal faster than if they were given 0 or 60 minutes for habituation. Moving the fish to the new water should therefore be done quickly, but not completely without habituation. For more information, see the section on moving zebrafish into a new aquarium above.

Acclimatisation after handling

Handling, in particular if it involves the fish being subjected to air, is very stressful and should be followed by a period of acclimatisation, if the stress response in itself is not of interest for the particular experiment. Zebrafish have shown to react quickly to being chased and captured by a net. Fifteen minutes after the chase, considerably elevated levels of cortisol were measured, which culminated after 30 minutes and were only back to base levels after 2 hours. During this time, variations in the expression of different types of mRNA were also measured. Studies that have investigated how levels of cortisol varies after handling in European perch and African sharptooth catfish measured that it required 4 and 6 hours respectively for the cortisol levels to return to normal.

In a study on Eastern mosquitofish (*Gambusia holbrooki*) the researchers drew the conclusion that it takes 3–4 hours for the behaviours of the fish to stabilise enough for a behavioural experiment that was initialised by the fish being moved to smaller, separate, containers. During acclimatisation, the fish were swimming faster and made fewer slow turns than normal. The literature study made in connection to the experiment showed that most researchers that performed similar experiments allowed the fishes less than an hour to acclimatise, which may have led to inaccurate results.

In Chinook salmon it has also been seen that the temperature the fish live in may affect the body’s reaction to stress. The study describes that glucose levels in salmons that lived in warmer waters became higher than for the fish living in colder waters. Even though the cortisol levels increased in a similar matter, they lowered the slowest for salmons living in colder water. When the researchers confined the fishes for a longer period of time, the cortisol levels increased the fastest and the glucose levels became the highest for the fish that lived in warmer waters, compared to those living in colder waters.

When and how are zebrafish acclimatised in Sweden?

In a survey on acclimatisation, initiated by the Swedish 3Rs Center, 10 participants answered the questions about how they acclimatise zebrafish. The relatively low number of respondents, combined with the fact that it is not clear which organisation the respondents belong to, should be considered when conclusions are drawn. The purpose of the survey was to investigate when and how acclimatisation of zebrafish was carried out in Sweden.

Zebrafishes are usually acclimatised upon arrival at a new facility

Participants that answered yes to the question **Are zebrafish acclimatised when first arriving at your facility?** were given the opportunity to describe in free text how long their zebrafish were acclimatised for. There were six respondents (60 %) who answered yes to the question and described the length of the acclimatisation period. The respondents all acclimatised their zebrafish for at least a week upon arrival but often even longer, up to several months. Other respondents did not acclimatise zebrafish upon arrival at a new facility, did not know if it was performed at their facility, or chose not to answer the question.

When the survey respondents were asked **Are zebrafish acclimatised when they are regrouped at your facility?** there were three respondents (30 %) who answered yes and described how it was performed. The respondents acclimatised their zebrafish for one to two weeks following regrouping. The remaining respondents did not acclimatise zebrafish when regrouping, did not know if it was performed at their facility, or chose not to answer the question.

To the question **Are zebrafish acclimatised when they are moved to a new location within your facility?** there were two respondents (20 %) who answered yes and described the length of the acclimatisation period. Both respondents acclimatised their zebrafish for at least a week upon moving them within the facility. The other respondents did not acclimatise zebrafish when moving them within the facility, did not know if it was done at their facility, or chose not to answer the question.

There were two respondents (20 %) who answered yes to the question **Are zebrafish acclimatised when the circadian rhythm is changed at your research facility?** One of the respondents acclimatised for two weeks. The other described that a change in the circadian rhythm was performed gradually, with two to three hours of change per day, to give the fish opportunity to acclimatise. Other respondents did not acclimatise zebrafish to an altered circadian rhythm, did not know if it was carried out at their facility or chose not to answer the question.

Receiving and rearing are important factors regarding how zebrafish are acclimatised

The survey participants were given the opportunity to briefly describe in free text how they carried out acclimatisation of zebrafish. We received six descriptions of approaches, these are listed below.

- They are put in tanks and get to grow up before they are used for breeding or in experiments.
- It depends on the water parameters, I change the parameters slowly over a period of about a week. If it is about embryos, they are moved directly to the new environment.
- We have them in their home aquariums for a week before we start experiments. Almost always, however, it involves the fish being raised within the facility and then used in experiments as adults. The period from fertilisation to the start of the experiment is the period I think of as the acclimatisation period.
- When purchasing fishes to the facility, they arrive in bags. These are placed in designated aquariums for temperature acclimatisation. The bags are opened and water is changed successively in the bags over a few hours to adapt to the water quality in the new facility. During experiments, the same water as in the facility is used, so adaptation to a new water quality is not necessary. When moving fish to test aquariums, they are acclimatised for about a day before the start of the test.
- Adult fish arrive in bags. The bags are placed in aquarium water until the temperature in the bag corresponds to that of the aquarium. After that, the fish are moved to the aquarium using a net. The water in which the fish were transported is disinfected and then poured out, it is not emptied into the aquarium due to biosecurity and the stress hormones that remain after transport.
- The fish are left alone with normal feeding etcetera in the new environment.

Survey on acclimatisation in Sweden

During the autumn of 2022, a survey was sent out to the networks of animal welfare bodies, laboratory animal veterinarians and animal technicians with whom the Swedish 3Rs Center was in contact. The survey was also sent to the principal investigators who, during the years 2018–2022, had been granted ethical permission for research on any of the animal species in question. Recipients were able to spread the survey further, since the links were reusable. The survey was available in both Swedish and English. There were three versions of the survey and the recipient was asked to answer the ones corresponding to the animal species they worked with, mice, rats or zebrafish. The questions were the same in all three versions. All responses were anonymous and it is therefore not possible to determine whether several responses came in from the same organisation. A total of 153 people completed the survey, of which 105 people answered regarding mice, 38 people regarding rats and 10 people regarding zebrafish. A large majority of the respondents were researchers, see table 1. The next two largest groups consisted of laboratory animal technicians or other care staff and laboratory animal veterinarians.

Table 1. Distribution of the respondents' professions.

Profession	Number of respondents
Researcher	108
Animal technician or other care staff	15
Laboratory animal veterinarian	11
Laboratory assistant	8
Named Animal Care and Welfare Officer (NACWO)	7
Animal facility manager	2
License holder	1
Other	1
Total	153

Written plan for acclimatisation

According to the National Animal Welfare Report 2022, one of the most common insufficiencies found during physical controls of laboratory animal facilities is linked to the following control point "There are written plans for preventive animal health care, animal welfare-related measures and assessment of the physical and psychological well-being of the laboratory animals". This control point includes the written plan for acclimatisation. Also, the responses from our survey indicate that there may be insufficiencies in the written plan for acclimatisation. According to the survey, the shortcomings can be found in the design and accessibility of the plan, as well as in the application of the plan. A number of respondents were unsure of what the written plan contained and where it could be found. When asked what types of sources the written plan for acclimatisation was based on, the survey indicated that the experiences of the respondents were the most common source. For those who responded to the survey, legislation and scientific studies were two other important resources. One respondent specifically described that other scientific studies in the same field were consulted to apply a similar approach to acclimatisation in order to increase comparability between studies.

Conclusion

For those working with fish, it seems to be common knowledge that the animals need to be given the opportunity to get used to new water parameters immediately after transport. Since most zebrafish are then reared at the facility, further acclimatisation of the animals after other stressful events, such as handling and moving within the facility, has rarely been done. However, based on the studies used for the compilation of this support material, it seems as if acclimatisation should be considered on more occasions and studied further.

Scientific knowledge regarding acclimatisation of zebrafish before experiments is, until today, poor. Even so, each researcher should ensure that the zebrafish they use in research have been acclimatised for a sufficient period of time. This is highly important, as stressed animals can negatively affect research results. Based on the knowledge we drew attention to in this support material, it is not obvious how long zebrafish should be acclimatised for, but studies suggest 2–5 weeks after transport.

There are no recommendations for acclimatisation of zebrafish after handling, but based on other small fish species and depending on the type of experiment the fish is going to be used for, such acclimatisation should be done for 3–6 hours. It is also possible that fish may need a shorter period of acclimatisation if they are trained and used to what is going to happen, or to stressful events in general. For example, training could lead to the handler not having to chase the zebrafish as much with the net, which research has shown to increase cortisol levels in connection to handling. What we can determine is that the time needed for fish to acclimatise after being exposed to stress depends on a variety of factors and a balanced decision must therefore be made in each case.

Finally, we would like to emphasise that scientific literature should be consulted to provide guidance on how stress affects the type of research that is going to be conducted. In the case of the zebrafish, however, it is difficult to do. Knowledge of other fish species or your own studies can therefore be helpful. When the period of acclimatisation is being chosen, it is good to also consider whether it can be sufficient that the parameters relevant to the experiment return to stable levels, while others are still stabilising. For behavioural experiments or other studies of the whole fish, a longer acclimatisation period may be necessary for fish to acclimatise completely, unless the stress response is of interest to the experiment.

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