Scientific Committee on Health, Environmental and Emerging Risks

SCHEER

Revision of Annexes III and IV of Directive 2010/63/EU on the protection of animals used for scientific purposes regarding accommodation parameters and methods of killing for zebrafish, and accommodation parameters for Passerine birds

The SCHEER adopted this document via written procedure on 25 September 2023

ABSTRACT

This scientific Opinion evaluates the current state of the art considering key accommodation parameters to maintain the welfare of zebrafish in captivity for scientific purposes. In addition, euthanasia methods (*e.g.* hypothermic shock) for zebrafish were evaluated. Furthermore, housing requirements were evaluated for maintaining the welfare of a number of Passerine bird species kept in captivity.

Sophisticated housing systems are available for zebrafish holding facilities such as flow-through and/or recirculating aquaculture systems. Water quality parameters were presented for zebrafish housing systems. The temperature range recommended for zebrafish housing systems is 24°C to 29°C, with an optimum temperature of 28°C, as is currently common practice. It is important to keep the noise level as low as possible and the light level constant, irrespective which light dark cycle (mostly 14/10 or 12/12 hours, light versus dark) is applied in the housing facility. Some form of enrichment (*e.g*. social, physical, visual, nutritional) in the system is recommended. In addition, health control measures should be in place to monitor for potential introduction of contaminants and pathogens causing disease. An optimal stocking density is 5 adult fish/L, whereas the maximum is considered 10 fish/L. The presence of less than 5 fish per tank is possible under certain conditions, however, this is not recommended for prolonged periods of time.

Besides an overdose of anaesthetics, hypothermic shock, also known as rapid chilling, can be considered a reliable and safe method of euthanasia in zebrafish equal or older than 16 days post fertilization (dpf). A proper hypothermic shock protocol should be followed ensuring that no direct contact of the fish to the crushed ice is possible.

Regarding Passerine birds, in this Opinion, 'captivity' is defined as holding birds within an enclosure (*e.g.* a cage or an aviary) that can be for short- or long-term periods. Both practically and physiologically, 'short term' can be justified as being up to one circadian cycle, *i.e.* up to 24 hours. Therefore 'short term' was defined as a period of 24 hours, for which the housing conditions may deviate from the conditions recommended in the Opinion. For Passerine birds in captivity beyond 24 hours, housing conditions were evaluated for starlings, sparrows and great and blue tits, as these are the most common Passerine birds used for scientific purposes. For starlings and house sparrows group housing is considered necessary. For great and blue tits in captivity, there is no preference for either being housed singly or in groups but in most situations single housing is preferable due to their territorial behaviour. In all cases, tits should have auditory contact with other conspecifics.

Keywords: zebrafish housing, zebrafish hypothermic shock, Passerine bird housing

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1. SUMMARY

Following the mandate from the European Commission, this scientific Opinion evaluates 1) the current state of the art considering key accommodation parameters to maintain the welfare of zebrafish in captivity for scientific purposes; 2) euthanasia methods for zebrafish with focus on the use of hypothermic shock; and 3) housing requirements for maintaining the welfare of a number of Passerine birds kept in captivity.

Besides the sometimes limited available literature, current practices at various European scientific institutes were also considered for answering the questions posed in the mandate.

Zebrafish

Sophisticated housing systems are available for zebrafish holding facilities such as flow-through and/or recirculating aquaculture systems. Water quality is of utmost importance in terms of temperature, conductivity, hardness and alkalinity, pH, presence of nitrogen compounds, and oxygen. These parameters should be checked on a regular basis and may need to be adapted when necessary. Stability of water parameters is often more important than the actual value. Although water temperature of the natural habitat of zebrafish spans a wide range (below 15°C to almost 35°C) the temperature range recommended for zebrafish housing systems is 24°C to 29°C, with an optimum temperature of 28°C, as is currently common practice. It is important to keep the light level constant, irrespective which light-dark cycle (mostly 14/10 or 12/12 hours, light versus dark) is applied in the housing facility. Gradual light changing, using dawn-dusk phases, might reduce startle reflexes as light intensity changes. Noise levels should be as low as possible and constant over time avoiding sudden loud noises and vibration. In addition, health control measures should be in place to monitor for potential introduction of contaminants and pathogens causing disease.

As zebrafish is a shoaling species, prolonged single housing is not recommended, but can be required during a limited period for specific reasons. Adult zebrafish should be kept in conditions that are neither overcrowded nor underpopulated. In order to allow shoaling, a minimum of 5 fish/tank is recommended. The general consensus is that the optimal stocking density is 5 adult fish/L while a maximum of 10 fish/L is considered reasonable. The presence of less than 5 fish per tank is possible under certain conditions, however, this is not recommended for prolonged periods of time. Considering the stocking density of 5 fish/L, the tank size and shape should allow the fish to perform their natural behaviour and swimming activity. In the tanks themselves some form of enrichment (*e.g*. social, physical, visual, nutritional) is recommended. When placing physical attributes inside a tank, specific considerations should be made for the composition of the materials used in view of possibility for cleaning/sterilization and/or possible release of potentially toxic components. In addition, the enrichment objects may reduce the available free swimming spaces, and this should be considered in view of number of fish housed.

The commonly used authorized method for euthanasia of zebrafish is an overdose of anaesthetics while hypothermic shock is regularly used on the basis of exemptions by the responsible authorities. Hypothermic shock, also known as rapid chilling, is recommended to be allowed as an additional authorized method. It can be considered a reliable and safe method of euthanasia in zebrafish depending on the age of the zebrafish. The temperature applied during hypothermic shock should at least be 20°C

below the husbandry temperature. A smaller difference of temperatures may not result in a hypothermic shock due to fish's capacity to adapt to the new decreased temperature. A proper hypothermic shock protocol should be followed ensuring that no direct contact of the fish to the crushed ice is possible, and a sufficient exposure time of 5 min in animals of 16 days post fertilization (dpf) and older before final confirmation of death. As for younger stages much longer times are needed, other methods than rapid chilling are recommended to be applied for zebrafish of 5 dpf to 15 dpf, *e.g*. an overdose of anaesthesia followed by decapitation and/or maceration. The following conditions should apply when rapid chilling is used as method for euthanasia; age ≥ 16 dpf, zebrafish (*Danio rerio*): body size ≤ 5 cm, husbandry temperature equal to or above 24°C, temperature of rapid chilling ≤ 4°C, allowing a temperature difference of at least 20°C with the maintenance temperature. The temperature of ≤ 4°C should be ensured during the whole procedure. Similar to the use of anaesthetics, confirmation of death of the fish shall be determined after the use of rapid chilling for zebrafish euthanasia. Hypothermic shock might also be considered appropriate for other small tropical fish in general as long as they are housed with temperatures consistently equal to or above 24°C.

Passerine birds

Directive 2010/63/EU Annex III on Requirements for Establishments and the Care and Accommodation of Animals currently includes accommodation parameters for domestic fowl, domestic turkeys, quails, ducks and geese, pigeons and zebra finches. This encompasses the majority of avian species used in research and testing in the European Union; however, a need has been identified to define standards for some additional species of Passerine birds. The order of Passeriformes birds includes over 6,500 species, with diverse behaviour, physiology and ecology, representing over half of all known species of birds. Only a limited number of species are, however, used for research and need to be held in captivity. This Opinion is therefore restricted to the species most commonly used; starlings (*Sturnus vulgaris*), house sparrows (*Passer domesticus*), and great and blue tits (*Parus major* and *Cyanistes caeruleus*). The recommendations are based on an approach of considering the natural history and behaviour of each species or group of animals, using the literature, current good practices and expert judgement to determine which features of the natural environment should be replicated, as far as practicable, within the laboratory.

In this Opinion, 'captivity' is defined as holding birds within an enclosure (*e.g.* a cage or an aviary) that can be for short- or long-term periods. Both practically and physiologically, 'short term' can be justified as being up to one circadian cycle, *i.e.* up to 24 hours. This Opinion therefore defines 'short term' as a period of 24 hours, and the species-specific standards set out in this Opinion apply whenever birds are held for period in excess of 24 hours. However, even when birds are held for shorter periods of time, animal welfare needs must be met. A maximum of 24 hours holding should be sufficient to perform minimally invasive procedures and/or measurements on the birds and allow holding overnight if necessary to avoid predation risks at certain times of day or release of the birds in unfavourable weather conditions.

Based on literature and expertise in various aviaries throughout Europe, recommendations for the housing conditions of starlings, sparrows and tits were formulated. Special emphasis was on animal density and housing conditions such as enclosure enrichment based on the social and actual behaviour of the three Passerine

species. The environmental enrichment could be provided by making available sufficient perches, water baths and foraging variation including live feed.

For starlings, enclosures need to be of adequate size to ensure that enough birds can be group housed, to promote social behaviour and synchronised flight. Group size should at least consist of four starlings.

House sparrows are group living birds and do not fare well in isolation. The enclosures for housing need special environmental enrichment to allow the sparrows their natural behaviour. They do not require a lot of space but rather structure where they can form groups, hide from each other's view, and forage in crevices and niches. For single sex, a group size of 2 animals is sufficient, while mixed sex groups should not be smaller than 6 animals, and have equal sex ratios or fewer males than females.

Great tits and blue tits are very territorial and do not tolerate other birds in their territory. They are not truly 'social species' and they have special requirements regarding both social and single housing. They are omnivorous birds, with a clear fluctuation in food preference throughout the season, that has partly to do with food availability. For tits in captivity, in most situations single housing is preferable. When group housing is needed, groups need to consist of one single sex. For mixed sex housing, the only exception is when one male and one female are housed in one enclosure during the breeding season. When groups are formed, they always need to enter the enclosure at the same time. In all cases, tits should have auditory contact with conspecifics.

It may be possible to adapt the discussed housing conditions for other small Passerines. However, some caution needs to be taken when translating the housing recommendations for the starlings, sparrows and tits to other small Passerines, based on their social, food and space requirements, as these may deviate significantly.

2. MANDATE FROM THE EU COMMISSION SERVICES

2.1. Background

Directive 2010/63/EU on the protection of animals used for scientific purposes (hereafter "the Directive") provides requirements for establishments and for the care and accommodation of animals used in research and testing. The Directive contains several Annexes containing inter alia more precise legally binding standards for specific provisions in the Directive. The present request for a scientific opinion relates to two of these Annexes:

2.1.1. Annex III on the standards of accommodation and care as required by Article 33 of the Directive

Annex III consists of two parts. Section A contains general requirements applicable to all animals within the scope of the Directive, including on physical facilities, the environment and its control as well as the care of the animals. Section B contains detailed specifications for the care and accommodation for the most commonly used species of mammals, birds, amphibia and reptiles, including specific tables of dimensions of enclosure systems and stocking densities.

Annex III was based on Appendix A of the Council of Europe Convention ETS 123 developed by Expert Working Groups each responsible for a species or group of species[1].

The recommendations were drafted on the basis of the available scientific evidence or, where unavailable, on the good practice at the time. These were then introduced into the EU legislative framework through Commission Recommendation 2007/526/EU.

In 2010, the Directive incorporated many of these recommendations into its Annex III. However, only those recommendations that all operators could comply with at all times under the Directive could be included to establish today's legally binding accommodation and care standards to safeguard the welfare of animals when kept in captivity for scientific purposes in the EU.

2.1.2. Annex IV on the methods of killing appropriate for animals bred, supplied or used in scientific procedures, as set out in Article 6 of the Directive

Methods in Annex IV were based on a 2005 Opinion of the Scientific Panel on Animal Health and Welfare (AHAW) of EFSA[2] with final adopted list as a result of the co-decision negotiations. Under Article 6 of the Directive, other methods of killing can be authorised, either when the method is to be considered at least as humane as those in Annex IV, or when necessary for scientific purposes. In the case of the former, Member States are required to provide an annual report on methods authorised which have been considered to be at least as humane as those set out in Annex IV.

Currently, the European Commission is undertaking a targeted review of Annexes III and IV focused primarily on

- the addition of accommodation and care standards for certain species and sub-groups of species not included or fully addressed in Annex III (Section B) to ensure harmonisation of appropriate welfare standards for these species, and
- methods of killing that have either been authorised at national level as being at least as humane as those in Annex IV, or where additional scientific evidence has been published on the suitability of existing methods, or supportive of new methods.

Following an analysis of the feedback from Member States and stakeholder organisations for the revision of these two Annexes, there are a small number of issues where considerations for inclusion or exclusion have generated insufficient evidence base or contradictory views. The current Opinion is limited to these points. Scientific evidence provided to DG ENV as part of the feedback is listed at the end of this document.

2.2. Background to the specific question for a scientific Opinion

2.2.1. Key accommodation parameters to maintain the welfare of zebrafish kept in captivity for scientific purposes

Currently, Annex III contains only general requirements for the accommodation of fish. EU statistics show, however, that around 2,700,000 fish are used annually in the EU, UK

[1] Revision of Appendix A (coe.int)
[2] "Aspects of the biology and welfare of animals used for experimental and other scientific purposes". Animals used for scientific purposes - Environment - European Commission (europa.eu)

and Norway. Due to their importance as research models, more detailed requirements to safeguard their welfare are needed in Annex III.

A review of the scientific evidence has shown, however, the lack of specific recommendations for any fish species except zebrafish (Danio rerio). Zebrafish nevertheless represent almost 17% of the total number of fish used in research and testing. In addition, the use of zebrafish is also required for regulatory toxicity studies, making it desirable to establish harmonised standards for their accommodation.

There is abundant scientific literature, including systematic reviews, on conditions described for housing and care of zebrafish (see references 1-10 at the end). However, there is a significant diversity of views (including by Member States and stakeholders) on suitable limits for the parameters defining quality of water and on appropriate standards for enclosure sizes and stocking densities in published papers and recommendations.

Parameters that were considered are: water supply and quality; oxygen, nitrogen compounds, pH, and salinity; water temperature; lighting; noise and maximum stocking density in relation to the stage of maturity of fish. However, to align with the level of detail in the current Annex III, only those parameters that are crucial and specific for the maintenance of zebrafish welfare should be considered.

For these reasons, it is necessary to request a scientific Opinion to identify and establish standards for the key parameters for the accommodation and care of zebrafish for consideration for Annex III.

2.2.2. Housing requirements to maintain the welfare of Passerine birds kept in captivity for scientific purposes

Statistical data from the EU show that great tit (Parus major) and blue tit (Cyanistes caeruleus) were the two most used species with no species-specific standards included in Annex III. Around 20,000 tits used for scientific purposes are reported annually.

In a recent consultation with Member States and stakeholder organisations it was confirmed that most studies with tits are conducted in the wild, but in some studies the maintenance of these animals in captivity is necessary. However, there seems to be no specific standards to define the conditions for keeping tits in captivity to ensure their welfare.

As a result, the conditions applied for tits kept in captivity are based on other similar species already defined in the current Annex III (e.g., Zebra finches). In some cases, studies conducted in tits provide enclosure details and which are subsequently used as reference (11,12).

In addition, tits are territorial birds and except for breeding purposes, tits in captivity are often housed individually as reported in several publications. The periods of time varied (between two days and two months) and details of the dimensions of the enclosures were given (13-17). However, only one publication was identified as recommending enclosure dimensions for tits, both in isolation and in group (18).

Consequently, a scientific opinion is requested on appropriate housing standards for tits required to be kept in captivity as part of a research study (using a similar template to those other bird groups detailed in Annex III), provided sufficient scientific evidence/information on best practice is available.

2.2.3. Hypothermic shock as a method of humane killing for zebrafish used for scientific purposes

Overdose of anaesthesia is a commonly used method listed in Annex IV for killing fish. However, there is evidence that some anaesthetics used in euthanasia of fish can be aversive (19). 11 Member States have reported authorisation of hypothermic shock (known as rapid cooling) for zebrafish, several annually since 2015. An exemption can only be authorised when the authorities have evaluated the method (rapid cooling in this case) to be at least as humane as methods already accepted in Annex IV on the basis of scientific evidence (20-24). There seems to be a general agreement that a competent use of rapid cooling is a humane method of killing of zebrafish when unwanted potential pharmacological effects from anaesthetics on experimental results must be avoided (25).

Even if regularly authorised, there seems to be lack of standardisation of this method in terms of temperature, time of exposure, etc. in relation to the age/size of the fish. Scientific literature provides evidence that immersion for a duration of five minutes for fry over 16 days post fertilisation and for adults is sufficient. However, for younger fry, times to guarantee death are much longer.

A number of Member States requested rapid cooling to be accepted as a humane method also for other species of fish, although the scientific evidence for this is scares (26). Most studies have been done in zebrafish. However, other small tropical fish, such as medaka (Oryzias latipes), are also used in research.

While the European Commission believes there is sufficient evidence for the inclusion of rapid cooling (hypothermic shock) as a humane method by immersing fish in water at less than 4ºC, advice is necessary in relation to the detailed conditions.

2.3. Terms of Reference

In view of the above, the European Commission asks SCHEER to issue a scientific Opinion on:

1. Key accommodation parameters to maintain the welfare of zebrafish kept in captivity for scientific purposes

- Which key parameters and their respective ranges are supported by sufficient scientific evidence in order to be considered for legally binding standards for the housing of zebrafish?
- On the basis of the current scientific evidence, which maximum stocking densities should be considered for zebrafish?

N.B. The scope of the Directive covers fish from the stage of independently feeding larval forms[3]. Zebrafish is considered to reach this level of maturity five days post fertilisation when maintained at approximately $+28^0C$[4]. Therefore, the parameters to be considered for zebrafish should not be extended to life stages before five days post fertilisation.

2. Housing requirements to maintain the welfare of Passerine birds kept in captivity for scientific purposes

[3] Directive 2010/63/EU, Article 1(3)(a)(i)
[4] Commission Implementing Decision 2020/569/EU, Annex III, Part B, Section B, point 1.2

- Is there sufficient scientific evidence to consider legally binding space allowances for keeping of Passerine birds in captivity, and if so, what should those be?
- Is there sufficient scientific evidence to require that, except for breeding purposes, Passerine birds should be individually housed to safeguard their welfare in captivity?

3. Hypothermic shock as a method of humane killing for zebrafish used for scientific purposes

- Under which conditions (minimum temperature fish need to be kept prior to the hypothermic shock, temperature for the hypothermic shock, time of exposure) should hypothermic shock be used as a killing method for zebrafish to ensure its humanness?
- Should the method of hypothermic shock be limited only to zebrafish 16 days post fertilisation or older?
- With the current scientific evidence, should the method of hypothermic shock be limited to zebrafish, or should its use also be allowed for other small tropical fish? If so, how should 'small' be defined?

It is important to bear in mind when formulating the Opinion that standards incorporated in Annexes III and IV will become legally binding and will require a case-by-case exemption by the authorities (which can only be granted on the basis of a scientific justification) should these not be possible to be complied with.

2.4. Deadline

SCHEER's Opinion would be appreciated by the end of August 2022 to contribute to the preparation of Commission Review of Annexes III and IV of the Directive and present it at the Member State National Contact Points meeting in November 2022.

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3. OPINION

Background

Directive 2010/63/EU on the protection of animals used for scientific purposes provides requirements for establishments and for the care and accommodation of animals used in research and testing. The Directive contains several Annexes containing *inter alia* more precise legally binding standards for specific provisions in the Directive. The present request for a scientific Opinion relates to two of these Annexes, Annex III on the standards of accommodation and care as required by Article 33 of the Directive, and Annex IV on the methods of killing appropriate for animals bred, supplied or used in scientific procedures, as set out in Article 6 of the Directive. However, especially in Annex III, not all animals kept and used for scientific purposes are specifically mentioned. The SCHEER was requested to produce an Opinion on key accommodation parameters to maintain the welfare of zebrafish and Passerine birds, and on the use of hypothermic shock as killing method of zebrafish.

Question 1.

Key accommodation parameters to maintain the welfare of zebrafish kept in captivity for scientific purposes

- Which key parameters and their respective ranges are supported by sufficient scientific evidence in order to be considered for legally binding standards for the housing of zebrafish?

- On the basis of the current scientific evidence, which maximum stocking densities should be considered for zebrafish?

Currently, sophisticated housing systems are available for zebrafish holding facilities such as flow-through and/or recirculating aquaculture systems. Water quality is of utmost importance, and major recommendations based on the data presented in Section 6.1.2 are shown in Table 3.1.

Table 3.1 Water parameters to be considered in zebrafish housing systems

Water parameter	**Recommendations**	**(Most) often used**	**Source**
Temperature	24-29°C	Juveniles-adults: 26-28°C Embryo-larval stages: 26-28.5°C	Villamizar *et al*., 2012, Aleström *et al.*, 2020,
Conductivity	150-1700 µS/cm	500-1000 µS/cm	Collymore *et al.*, 2015, Geisler *et al*., 2016, Lawrence *et al*., 2019, Aleström *et al*., 2020
Total/general hardness	40-250 mg/L $CaCO_3$	40-180 mg/L $CaCO_3$	OECD, 2019
pH	6.5-8		Aleström *et al*., 2020.
Nitrogen compounds	NH_3/NH_4^+<0.1[a] mg/L, NO_2^- < 0.3 mg/L, NO_3^- < 25 mg/L		Aleström *et al.*, 2020
Dissolved oxygen	> 5 mg/L		Collymore *et al.*, 2015; Cartner *et al.*, 2019

[a] or below detection limit. 0.1 mg/L indicates the total amount of ammonia, $NH_3+NH_4^+$. This corresponds to 0.002 mg/L of NH_3 at 28°C and pH 7.5.

The parameters indicated in Table 3.1 should be checked on a regular basis. Depending on the parameter and the housing conditions (static or recirculating), they may be measured and adjusted daily (T, pH), weekly (conductivity, nitrogen) or monthly (hardness, oxygen). The frequency of oxygen measurements may need to be increased for static housing conditions (*e.g.* weekly). In facilities where the system measures the parameters automatically, it is important to double check the measurements regularly with an external device. Furthermore, it should be clear what to do when water parameters deviate from the allowed ranges. This ensures that action can be taken rapidly to ensure fish welfare. Stability of water parameters is often more important than the actual value. In addition, health control measures should be in place to monitor for potential introduction of contaminants and pathogens causing disease.

Although water temperature of the natural habitat of zebrafish spans a large range (below 15°C to almost 35°C), the temperature range recommended for zebrafish housing systems is 24°C to 29°C, with an optimum temperature of 28°C, as is currently common practice. In view of the recommended water temperatures indicated in the table above, the temperature range (21-25°C) as presented in some OECD guidelines (*e.g.* OECD TG 203 the Fish Acute Toxicity Test) is not considered to be in line with current scientific practices and may need to be adapted.

It is critical that the light level in a zebrafish facility is kept constant irrespective of whether a 14/10 Light/Dark cycle or a 12/12 Light/Dark cycle is applied in the housing facility. It is essential that the dark phase is completely dark. The use of dawn-dusk phases has been suggested as a form of visual enrichment for zebrafish in facilities, as it may reduce the startle reflex when the light goes on. Transition times ranging between 20 to 40 minutes have been used. In terms of light intensity, the general recommendation for adult fish is 54-334 lux at the water surface. Too much light accelerates the growth of algae, hindering observation of the fish and fish vision itself, both of which are important factors for animal welfare.

Zebrafish are thought to adapt to their environment regarding noise levels although sudden loud noises and vibration should be avoided. Where possible, equipment causing noise or vibration should be separated from fish-holding facilities. Although there are no clear recommendations for noise levels in zebrafish housing facilities, it can be recommended to keep noise levels as low as possible and constant over time. It should be noted that fish will adapt to the stimuli present in their environment and may become stressed when these change or when the fish are moved to unfamiliar surroundings.

Although no specific recommendation for tank sizes can be formulated, it is recommended that adult zebrafish should be kept in conditions that are neither overcrowded nor underpopulated. In order to allow shoaling, a minimum of 5 fish/tank is recommended. The general consensus is that the optimal stocking density is 5 adult fish/L, while a maximum of 10 fish/L is considered reasonable. Considering the stocking density of 5 fish/L, the tank size and shape should allow the fish to perform their natural behaviour and swimming activity. Therefore, water volumes smaller than 1L should not be used for adult fish. In the tanks themselves, some form of enrichment (*e.g*. social, physical, visual, nutritional) is recommended.

As zebrafish is a shoaling species, prolonged single housing is not recommended but can be required during a limited period for specific reasons. Visual/olfactory access to conspecifics should be a minimum requirement for individually housed fish. In addition, enrichment could be provided, similar to that provided in the other tanks of the facility, when there is a need to individually house fish.

Question 2.

Housing requirements to maintain the welfare of Passerine birds kept in captivity for scientific purposes

- Is there sufficient scientific evidence to consider legally binding space allowances for keeping of Passerine birds in captivity, and if so, what should those be?
- Is there sufficient scientific evidence to require that, except for breeding purposes, Passerine birds should be individually housed to safeguard their welfare in captivity?

Answer regarding space allowances:

As Passerine birds encompass a large number of different species, this Opinion is limited to four of the most commonly used Passerine species used in scientific research, starlings, house sparrows and the great and blue tit. The guidance for enclosures for the housing of birds, as described within this Opinion, applies to birds used in scientific procedures that are regulated by the Directive, and held in captivity for more than 24h.

A description of short-term holding of birds is proposed, as birds may be re-released to the wild. Both practically and physiologically 'short term' can be justified as being up to one circadian cycle, *i.e.* up to 24 hours. This Opinion therefore defines 'short term' as a period of 24 hours, and the species-specific standards set out in this Opinion apply whenever birds are held for periods in excess of 24 hours. However, even when birds are held for shorter periods of time, animal welfare needs must be met. A maximum of a 24 -hour time period of permitted holding should be sufficient to allow holding overnight, if necessary, for example to avoid predation risks at certain times of day, or to wait for unfavourable weather conditions to end before the release of the animals.

There is no or limited published scientific evidence for legally binding space allowances for passerine birds. Based on expert opinion and current practice as used in a number of European bird facilities, recommendations could be formulated for the long-term housing conditions of starlings, sparrows, and great and blue tits.

It may be possible to adapt the recommended housing conditions described below for other small Passerines. However, some caution needs to be taken when translating the housing recommendations for the starlings, sparrows and tits to other small passerines, based on their social, food and space requirements, as these may deviate significantly.

Starlings

In order to meet the species-specific needs of starlings as sociable, active birds, starlings should be housed in appropriate groups and given environmental stimulation that facilitates natural behaviour. Terrestrial foraging for invertebrates, flight, water bathing and perching are all essential behaviours for starlings. Enclosures also need to be of adequate size to ensure that enough birds can be group housed, to promote social behaviour and enable all birds to fly simultaneously. To permit these behaviours and minimise the risk of aggression, sufficient resources, and space, are necessary. A minimum group size of four starlings is strongly recommended. Table 3.2 shows recommended housing conditions for starlings. Relatively small enclosures should be long and narrow (for example 2 m by 1 m) to enable birds to perform short flights.

Table 3.2 Recommended enclosure conditions relative to number of starlings present

Group size	Minimum floor area (m^2)	Minimum height (cm)	Minimum length of food trough per bird (cm)	Minimum length of perch per bird (cm)
4 to 6	2	200	5	30
7 to 12	4	200	5	30
13 to 20	6	200	5	30
For each additional bird	0.25	200	5	30

between 21 to 50				
For each additional bird above 50	0.15	200	5	30

House sparrows

House sparrows require an environment where they can form groups, hide from each other's view, forage in crevices and niches. This can be provided by enrichment objects (e.g. trees and/or leafy branches) with hiding places, and/or ceiling length hessian cloth providing visual barriers in the enclosure. The stocking density can be increased if a visual barrier is provided. When mixed-sex groups are housed it is advised to provide house sparrows with nest boxes, because house sparrows will build nests and breed even if no nest boxes are available. Breeding can only be prevented by keeping the sexes separately. For single sex, the group size shall be at least 2 animals, while mixed sex groups should not be smaller than 6 animals, and be composed of equal numbers of males and females, or contain fewer males than females. Individual housing may be needed for animal care reasons (*e.g.* quarantine or recovery), in which case single birds fare well as long as they have sight and/or sound contact to other sparrows. Long-term individual housing is not recommended. Recommended housing conditions are presented in Table 3.3.

Table 3.3.a Recommended enclosure conditions relative to number of house sparrows present

Number of birds with no visual barriers		Enclosure sizes		
Group size	Approximate minimum volume per bird (m^3)	Minimum floor area (m^2)	Minimum height (cm)	Minimum volume (m^3)
2* to 10	0.4	2.4	180	4.4
11 to 20	0.4	4.8	180	8.7
21 to 30	0.4	7.3	180	13.1
For each additional bird above 30	0.4	Add m^2 according to increased volume (0.11 m^2 per bird)	180	-

* For mixed sex groups, the minimum number of birds should be 6. Mixed groups of 6 to 10 animals should be composed of equal numbers of males and females, or contain fewer males than females.

Table 3.3.b Recommended enclosure conditions including visual barriers relative to number of house sparrows present

Number of birds in presence of visual barriers		Enclosure sizes		
Group size	Approximate minimum volume per bird (m^3)	Minimum floor area (m^2)	Minimum height (cm)	Minimum volume (m^3)
2* to 15	0.3	2.4	180	4.4
16 to 35	0.25	4.8	180	8.7
36 to 60	0.2	7.3	180	13.1
For each additional bird above 60	0.2	Add m^2 according to increased volume (0.11 m^2 per bird)	180	-

* For mixed sex groups, the minimum number of birds should be 6. Mixed groups of 6 to 10 animals should be composed of equal numbers of males and females, or contain fewer males than females.

These stocking densities may temporarily be exceeded after hatching, until the young become independent from their parents, usually after 6 weeks. Also, these periods with the presence of increased numbers in family groups will typically not cause welfare deficits, such as increased levels of stress or aggression.

Great tit and blue tit

Tits show very territorial behaviour and do not tolerate conspecifics in their territory. They are not truly a 'social species' and they have special requirements regarding both social and single housing. For tits in captivity, there is no strong preference for either being housed singly or in groups, but in most situations single housing is preferable. Groups always need to consist of one single sex, although males will not easily tolerate other males. The only exception is when one male and one female are housed in one enclosure during the breeding season. When groups are formed, they always need to enter the enclosure at the same time. In all cases, tits should have auditory contact with other conspecifics. Recommended enclosure sizes are presented in Table 3.4.

Table 3.4 Recommended enclosure conditions relative to number of great tits or blue tits present.

Group size	Minimum floor area (m^2) per bird	Minimum height (cm)	Minimum number of feeders	Minimum length of perch per bird (cm)
1[a]	0.30	45	2	120
1[b]	3.00	180	1	100
2-10[c] (single sex)	1.00	180	2	40
1 female + 1 male	2.00	180	2	100

[a] There can be three situations in which small enclosures may be used for housing: (i) directly after catching, tits can be singly housed in small enclosures for a limited period of time (first 48h after catching the tits from

the wild); (ii) for juvenile birds, before their first moult; and (iii) in all other situations for a maximum of four weeks.
[b] For a prolonged period of time.
[c] Larger group sizes than 10 animals may incidentally be housed for short periods, although this is not recommended in view of increased risk of aggressive behaviour.

There is much similarity in the way great tits and blue tits are housed, and the proposed housing conditions can be generalised for the two tit species. The enclosure dimensions could also be valid for other smaller passerines such as pied flycatchers (*Ficedula alba*), blackcaps (*Sylvia atricapilla*), stonechats (*Saxicola torquata*) and other tit species. However, some caution needs to be taken when translating the housing recommendations for the tits to other small passerines, since their social, food and space requirements may deviate significantly.

Answer regarding individual housing:

- Is there sufficient scientific evidence to require that, except for breeding purposes, Passerine birds should be individually housed to safeguard their welfare in captivity?

Starlings and house sparrows should be housed in groups. For starlings a minimum group size of four is recommended, while for sparrows a minimum group size of two is sufficient. For tits in captivity single housing is preferable in most situations. When group housing is needed, groups need to consist of one single sex. For mixed sex housing, the only exception is when one male and one female are housed in one enclosure during the breeding season. In all cases, tits should have auditory contact with conspecifics.

Question 3.

- Hypothermic shock as a method of humane killing for zebrafish used for scientific purposes.

Hypothermic shock, also known as rapid chilling, can be considered a reliable and safe method of euthanasia in zebrafish. When compared to other methods authorised in Annex IV of EU Directive 2010/63, there are no indications that this method causes more stress or suffering. A proper hypothermic shock protocol should be followed ensuring that no direct contact of the fish to the crushed ice is possible, and a sufficient exposure time of 5 min for animals of 16 dpf and older before final confirmation of death. Because younger animals (< 16 dpf) would require much longer exposure times, rapid chilling is not recommended. For young zebrafish of 5 dpf to 15 dpf, an appropriate euthanasia method would be, for example, an overdose anesthesia followed by decapitation and/or maceration.

The following conditions should apply when rapid chilling is used as method for euthanasia; zebrafish (*Danio rerio*): age ≥ 16 dpf, body size ≤ 5 cm, husbandry temperature equal to or above 24°C, temperature of rapid chilling ≤ 4°C, allowing a temperature difference of at least 20°C. The temperature of ≤4°C should be ensured during the whole procedure. Similar to the use of anaesthetics, confirmation of death of the fish shall be determined after the use of rapid chilling for zebrafish euthanasia.

As the mode of action is a physical disruption of body functions that seems similarly effective in other fish species, it might also be considered appropriate for tropical fish in general, as long as they are of similar size and housed with temperatures consistently equal to or above 24°C. In addition, it should be verified that intended fish species do

not perceive cold as painful, and they do not express anti-freeze proteins (which might be assessed *in vitro*).

4. MINORITY OPINIONS

None

5. DATA AND METHODOLOGIES

5.1. Data/Evidence

The SCHEER, on request of Commission services, provides scientific Opinions on questions concerning health, environmental and emerging risks. The scientific assessments carried out should always be based on scientifically accepted approaches, and be transparent regarding the data, methods and assumptions that are used in the risk assessment process. They should identify uncertainties and use harmonised terminology, where possible, based on internationally accepted terms. In its scientific work, the SCHEER relies on the Memorandum on Weight of Evidence (WoE) and uncertainties (SCHEER, 2018), *i.e.* the search for relevant information and data for the SCHEER comprises the identification, collection and selection of possible sources of evidence in order to perform a risk assessment and/or to answer the specific questions being asked. For each line of evidence, the criteria of validity, reliability and relevance need to be applied and the overall quality has to be assessed. The SCHEER Memorandum (SCHEER, 2018) classifies results of analysis for human and environmental risks as follows:

- Strong weight of evidence: Coherent evidence from a primary line of evidence (human, animal, environment) and one or more other lines of evidence (in particular mode/mechanistic studies) in the absence of conflicting evidence from one of the other lines of evidence (no important data gaps)

- Moderate weight of evidence: good evidence from a primary line of evidence but evidence from several other lines is missing (important data gaps)

- Weak weight of evidence: weak evidence from the primary lines of evidence (severe data gaps)

- Uncertain weight of evidence: due to conflicting information from different lines of evidence that cannot be explained in scientific terms

- Weighing of evidence not possible: No suitable evidence available

The SCHEER noted that Passerine birds consist of a large number of different bird species of which only a limited number is used in scientific research. Even then, animals may be caught and handled for only a short period of time before they are released again immediately after the handling (*e.g.* for fitting external telemetry devices or blood sampling). The great tit (*Parus major*) and blue tit (*Cyanistes caeruleus*) are the two

most used species that are kept in captivity for research purposes, and/or bred in captivity. In addition, house sparrows (*Passer domesticus*) and starlings (*Sturnus vulgaris*) are used to a lesser degree. This Opinion considering requirements for the welfare of Passerine birds kept in captivity for scientific purpose will therefore be limited to tits, sparrows and starlings.

Especially for answering the questions posed in the mandate regarding housing conditions for zebrafish and Passerine birds, SCHEER included for the WoE also the expert judgement of scientists running housing facilities within Europe.

5.2. Methodologies

To address the terms of reference of this Opinion, scientific data on the housing conditions of zebrafish and Passerine birds were collected, as well as information on methods for euthanasia of zebrafish. For the evaluation of the housing conditions for Passerine birds an extensive inventory of current and up-to-date housing conditions was conducted by contacting a number of institutes holding birds in captivity.

5.3. Literature research

A literature search was conducted for aspects of zebrafish welfare and killing methods as indicated below because a considerable body of literature is available. A literature search for Passerine birds was not considered necessary as the available literature is limited and known to the members of the working group.

For the zebrafish literature search, the following key words were used: Fish, husbandry, euthanasia, hypothermia, housing conditions, water parameters, rapid chilling, water quality, holding density, stocking density.

The Commission library service performed a literature search for publications up to 2023 based on the key words indicated above. The search terms used and results are listed in Table 5.1. This search resulted in 130 published articles. A call for information was published between 25th January and 27th February 2023. In addition, the SCHEER made use of reports by other organisations on this topic (*e.g.* FELASA), as well as on information provided by the mandating DG. Additional literature provided by the working group members was considered and evaluated.

Each document and line of evidence were assessed for relevance, validity and reliability on a 0-3 scale and then the overall WoE was assessed by combining the scores.

Table 5.1: Results from literature search on aspects of zebrafish housing and euthanasia methods

Key words including MeSH terms	No of entries
Fish, husbandry, euthanasia, hypothermia, housing conditions, water parameters, rapid chilling, water quality, holding density, stocking density	
PubMed	107
Find-eR and Science Direct search	23
Total	130

References

SCHEER. (2018). Scientific Committee on Health, Environmental and Emerging Risks, Memorandum on weight of evidence and uncertainties. Revision 2018. Adopted by written procedure on 26 June 2018.

(https://health.ec.europa.eu/publications/memorandum-weight-evidence-and-uncertainties-revision-2018_en).

6. ASSESSMENT

6.1. Zebrafish

6.1.1. Introduction

The report on the statistics on the use of animals for scientific purposes in the Member States of the European Union and Norway in 2019, was released by the European Commission on 19 July 2022. EU statistics show that approximately 2,560,000 fish are used annually for scientific purposes and for the creation and maintenance of genetically altered animal lines for research purposes. The use of fish represents 24.6% of the total number of animals of any species used (-7.5% with respect to 2018); zebrafish represents almost 17% of the total number of fish used in research and testing. By looking at the numbers described in previous reports published by EU on the use of animals, *e.g.* the 2019 report on the statistics on the use of animals for scientific purposes in the Member States of the European Union in 2015-2017, it is evident that the use of zebrafish is actually rather high, and the percentage with respect to the total number of animals used is extremely relevant. Especially for the activity of creating new genetically altered animal lines, zebrafish are important, as in 2017 the main species used for this purpose were mice and zebrafish, 75% and 23% of the total respectively. In this context it is important to note that the significant increase in the use of other fish from 2018 onwards is a result of incorporation of the data from Norway into EU reports, where substantial amounts of salmon is being used for research purposes.

Table 6.1 Use of fish for research purposes in the European Union[a]

	2015	2016	2017	2018[b]	2019[b]
Zebrafish	338,815	513,011	499,763	461,521	517,193
Other fish	936,252	791,726	719,932	2,304,216	2,042,339

a) Data extracted from ALURES – ANIMAL USE REPORTING - EU SYSTEM as available up to 2019 (accessed March 3rd 2023) (https://webgate.ec.europa.eu/envdataportal/content/alures/section2_number-of-uses.html)

b) The increase in number of "other fish" in the reporting system can be explained by the inclusion of Norway in the reporting system.

The increasing interest in the use of the zebrafish model is not limited to the European area, as demonstrated by the trend over time for the number of publications retrieved in the PubMed search system by using zebrafish as 'key word' (*i.e.* present in the title and/or in the abstract). The trend is shown in Figure 6.1: it appears that till 1994 the number was stable well below 100 papers/year. In the year 2000 the number was >600 papers, rapidly increasing to around 1000 in 2003, doubled in 2010, exceeding 4000 in

2019 and around 4500-4700/year in the last three years. Over the years, the percentage of papers studying zebrafish embryos ranged from 30 to 50% of the total number of publications on zebrafish.

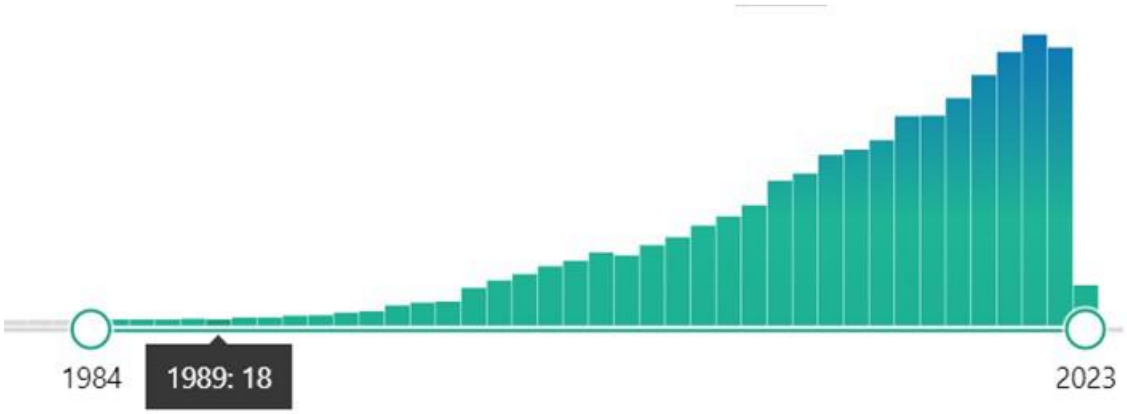

Figure 6.1. Increase in research papers using zebrafish between 1984 and 2023 as retrieved in PubMed (https://pubmed.ncbi.nlm.nih.gov/?term=zebrafish). Accessed 14 February 2023

In 2020, a report was published on various welfare and housing conditions of zebrafish (Aleström *et al.*, 2020). The report was prepared by a joint Working Group on zebrafish housing and husbandry recommendations, with members of the European Society for Fish Models in Biology and Medicine (EUFishBioMed) and of the Federation of European Laboratory Animal Science Associations (FELASA). The report contained, among others, background information on the natural habitat of zebrafish, including temperature and pH range (see Figure 6.2, Aleström *et al*., 2020).

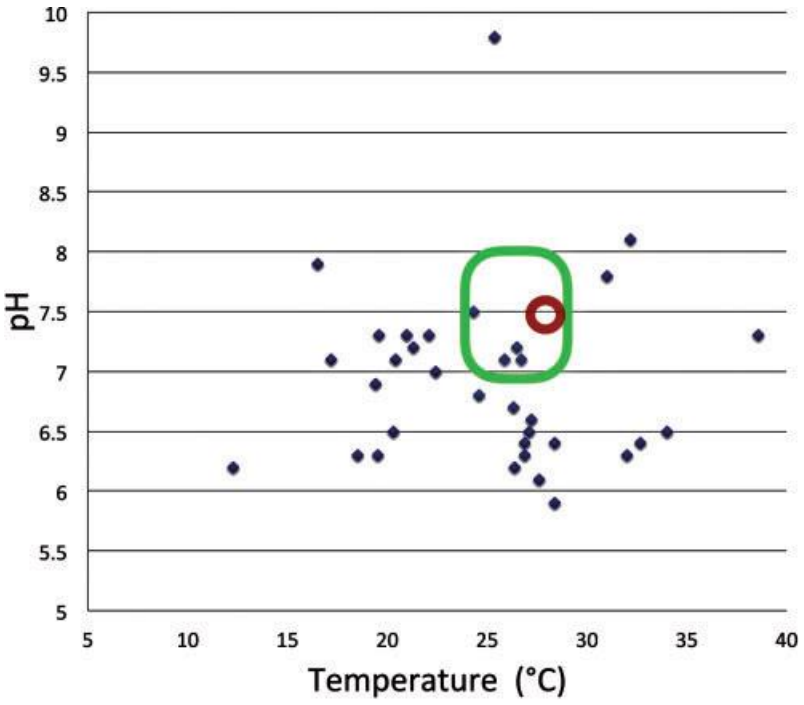

Figure 6.2 Temperature and pH ranges in natural habitats of zebrafish (Aleström *et al*., 2020)

In nature, zebrafish have a presence in a very wide habitat. Temperatures and pH levels were measured at 35 natural zebrafish habitats at altitudes between 14 m and 1576 m above sea level (Figure 6.2, blue dots). Ranges recommended for zebrafish housing systems (pH 6.5–8 and 24–29°C; green area) and values commonly referred to in literature being optimal for reproduction (pH 7.4–7.5 and 28°C; red circle) are indicated in Figure 6.2 (Aleström *et al*., 2020).

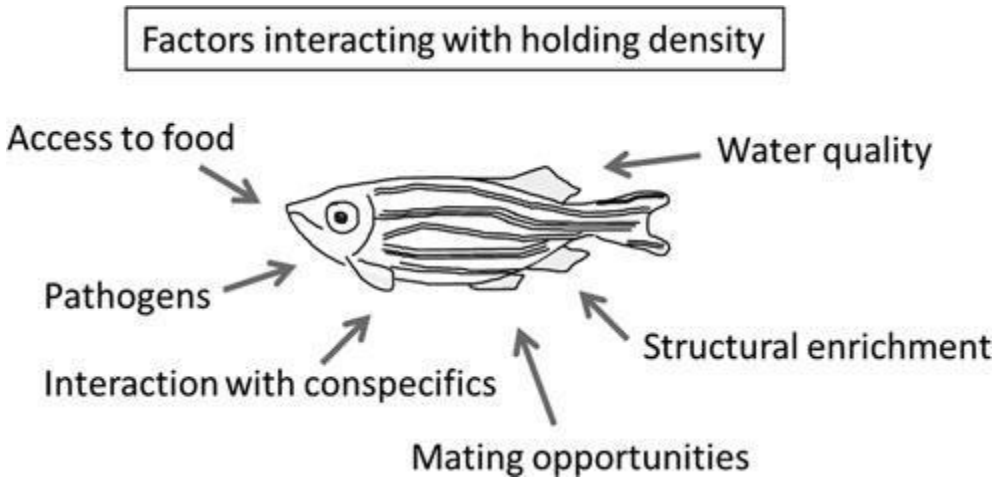

Figure 6.3 Environmental factors affected by the holding density of zebrafish (Andersson and Kettunen 2021)

Holding density is crucial for the welfare of zebrafish. Zebrafish are a shoaling species, and in their natural environment, live in large groups of conspecifics. The holding density does not only correspond to available space per fish but will also affect other factors relevant for fish welfare, such as the access to food and the resulting water quality, including oxygen levels and waste products (Figure 6.3, Andersson and Kettunen 2021).

Reviewing the literature has clearly demonstrated how crucial density is for the welfare of zebrafish. It affects a wide array of parameters, including growth, reproduction, stress response, behaviour, water quality, and pathogenic outbreaks. Lee *et al.,* (2022) reviewed current housing conditions regarding both physical and social aspects, and reported a fundamental lack of knowledge of how zebrafish interact with many biotic and abiotic features in their natural environment to support ways to optimise zebrafish health and well-being in the laboratory. Optimising the welfare of zebrafish may be achieved by a careful evaluation of a number of parameters (*e.g.* survivorship, growth, health, reproduction, cortisol levels, and behaviour) as suggested by Lee *et al.,* (2022). Especially, animal density should be included when creating universal holding guidelines for laboratory fish and must be kept constant between experiments when varying other parameters (Andersson and Kettunen 2021).

It should be realised that for the keeping and housing of fish, a number of general requirements are existing worldwide. Annex III of Directive 2010/63/EU already contains general requirements on care and accommodation of fish. This Opinion specifically addresses recommendations regarding care and accommodation of zebrafish.

The proposals for methods of euthanasia as described in this Opinion refer to the zebrafish used for scientific purposes. The SCHEER is aware that for other (farmed) fish species, more specific rules for euthanasia are still lacking. Council Regulation No 1099/2009 provides general aspects of killing of fish as described in Article 3 (1) "Animals shall be spared any avoidable pain, distress or suffering during their killing and related operations". In one European Commission report (COM(2018) 87 final), it was concluded that it was not appropriate to propose specific requirements on the protection of fish at the time of killing, as the evaluated evidence suggested that the objectives of the Regulation may equally be achieved by voluntary measures. Still, a more recent evaluation by the European Commission indicated that current practices are not in agreement with current scientific and technological development (SWD(2022) 328 final). Killing farmed fish is described in the "Aquatic Code" as regularly updated and published by the World Organisation for Animal Health (WOAH, Paris, France). It should be noted that for farmed fish several methods of killing including rapid cooling in ice water were considered to result in poor fish welfare (WOAH 2022). Furthermore, it is important to note in this context that the conditions for the killing of farmed fish cannot be compared to the killing of zebrafish in a laboratory context (*e.g.* species of fish; housing temperature of farm fish often not allowing 20°C drop in temperature). Therefore, further research on the methods and procedures used for the killing of (farmed) fish is recommended.

Conclusions

The zebrafish is one of the fish species most used for research purposes in the European Union and worldwide. Considering its natural habitats, the zebrafish has a very broad habitat range with temperatures from 12°C up to over 35°C. For the keeping and housing of fish, general requirements exist worldwide. For the European Union, these are described in Directive 2010/63/EU Annex III on *Requirements for Establishments and the Care and Accommodation of Animals.* In the mandate, recommendations are asked for more specific parameters on zebrafish housing conditions.

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6.1.2. Welfare aspects

6.1.2.1. Zebrafish housing systems

Zebrafish have been kept in laboratories for decades (Westerfield 2007, Avdesh *et al*., 2012, Lee *et al*., 2022). However, the housing conditions may not be optimal when compared to the natural habitat including physical and social parameters as suggested by Lee *et al*. (2022).

Literature on optimal tank sizes for zebrafish housing is limited. Both tank size and zebrafish density were noted to affect several behaviour parameters of zebrafish although the outcomes were ambiguous (Shishis *et al*., 2022). Maierdiyali *et al*. (2020) reported that fish that lived in small tanks behaved less boldly, had poor stamina, and spent much time on movement. One male and one female were housed in varying tank sizes between mini (water volume 40 cm^3), small (water volume 80 cm^3), medium (water volume 400 cm^3), and large (water volume 1500 cm^3). In both studies low numbers of animals were used in the investigational groups, so it is difficult to extrapolate the outcomes to larger fish groups, and the relevance for determination of optimal housing conditions regarding tank size and fish density is limited. However, these studies do show that housing conditions affect animal behaviour, and it seems likely also animal welfare.

While initial housing was simple, self-designed and small scale, nowadays sophisticated housing systems are commercially available. The type of housing system will depend on the local situation and the specific research question. Ultimately the selected aquaculture system should provide a stable and favourable environment that produces and maintains healthy and (re)productive fish and supports specific research goals. The waste secreted by the fish and food residues in the water results in the presence of toxic compounds (*e.g*. ammonia and nitrite) that need to be removed. There are mainly two type of aquaculture systems used for zebrafish housing that deal with the removal of waste differently: flow-through and recirculating. Static and semi-static systems may also be used provided appropriate control of water quality is available.

Flow-through aquaculture systems

In flow-through systems, clean water is pumped into the fish tank causing an overflow of the water including the waste products. The water flow should be calibrated in function of the fish load in the tank. To improve the efficiency of waste removal, the output should take water from the bottom of the tank. The benefit of the flow-through system over a recirculating system is better disease control. This system requires fresh water to be available at all times. Because of the continuous flow of fresh water, it requires larger amounts of water and energy to heat up the water compared to recirculating aquaculture systems. The advantage of flow-through systems is that a (bio)filter for water intake is not needed as the water is continuously refreshed.

Recirculating aquaculture systems

In a re-circulating system, suspended solids and the fish waste products are removed from the water after which the 'cleaned' water is reused. The advantage of this system is that it uses much less water and energy compared to the flow-through systems. A recent survey that was held on 98 zebrafish facilities in 20 different countries indicated that

most facilities (>80%) use a re-circulating aquaculture system (Lidster *et al*., 2017). There are several commercial recirculating housing systems for zebrafish on the market that have very similar basic operating principles. Most systems are designed to remove solids, soluble toxic waste products and pathogens from the water (Lawrence and Mason, 2012). Solid waste, such as fish faeces and uneaten food, needs to be removed from the water as it can clog the system and produces toxic ammonia. Removal of solid waste is achieved by settling the water into a sedimentation tank in combination with pumping system water through a filter pad or rotating drum. The next step is the removal of soluble waste such as ammonia, which is produced by the fish and the catabolism of uneaten food and solid waste. Ammonia is highly toxic to the fish and is typically removed by biological filtration. The biological filter contains a high-surface substrate on which nitrifying bacteria attach and grow and that is in contact with the aquarium water. Nitrifying bacteria convert ammonia into nitrite and then nitrate. Nitrate is tolerated by fish at much higher concentrations (Learmont and Cavalho 2015). The nitrate is removed from the system by daily water changes, typically at least 10% of the total water volume. However, water changes are dependent on the housing conditions (*e.g*. fish density; body weight; feeding rates; tank volume), and therefore the water quality should always determine the water exchange rate (see below). To remove microbes that are potentially pathogenic to the fish, the recirculating water flows through a disinfection unit which often consists of an ultraviolet steriliser. The water quality in recirculating systems can be very dynamic. To control for water quality, housing systems need to be checked regularly for pH, temperature, ammonia, nitrite and nitrate and adjusted when any of these parameters are out of range (see below).

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6.1.2.2. Water parameters

Temperature

The temperature directly affects a broad range of biologically important parameters of zebrafish, such as developmental rate, food intake, growth and behaviour (Tsang *et al*., 2017). Although zebrafish naturally occur in a wide range of temperatures from 6.7°C to 41.7°C (Cortemeglia and Beitinger, 2005; Schaefer and Ryan, 2006; Aleström *et al*., 2020), they are not expected to thrive in the outside borders of this range in a laboratory situation.

In a laboratory context and for experimental purposes, if changes are gradual, zebrafish can often adapt to decreasing or increasing temperatures, although this depends on the specific experimental conditions. Their temperature tolerance, described as the critical thermal minimum and maximum (CTmin and CTmax) can then shift (Cortemeglia and Beitinger, 2005; Schaefer and Ryan, 2006). As sudden temperature changes can cause stress, it is important to ensure that the temperature of the inflowing water is the same as that of the aquarium (Reed and Jennings, 2011). The temperature of the room is also important, especially when fish are removed from the system when they are, for example, isolated for egg production or stay in experimental set-ups outside of the system. A study has shown that small fish (1 g) may cool at a rate of 1.8°C per minute when the temperature of the water is lower than their body temperature, rapidly affecting their metabolism (Cartner *et al*., 2020). When the temperature gradually decreases (for example, in a system where the temperature regulation breaks down but is set up in a climate chamber), zebrafish can tolerate temperatures as low as 22-23°C without their metabolism being severely affected (Matthews *et al*., 2002).

Regarding animal welfare, the temperature at which early life stages are raised has an effect on development and mortality of all following life stages of the zebrafish. At a constant temperature of 28.5°C, standardised stages of development were described for zebrafish during the first 120 hours (Kimmel *et al*., 1995). In a study examining the effect of temperature and temperature cycles on growth, larvae grew fastest at a constant temperature of 28°C (Villamizar *et al*., 2012). Zebrafish raised at >30°C have an accelerated pace of life, which means they mature faster on average and have a shorter lifespan (Sfakianakis *et al*., 2011). Larvae reared at 32°C showed more abnormalities than larvae reared at 28.5°C and 30.5°C. When exposed to temperatures above 34.5°C, there was >25% mortality after 96 h (Pype *et al*., 2015).

The sex ratio of the population is also affected by temperature. Usual housing temperatures (28°C) result in a higher proportion of females, while higher temperatures (up to 35°C) result in a higher proportion of male off-spring (Geffroy and Wedekind, 2020; Valdivieso *et al.,* 2020).

It was previously recommended that the temperature in zebrafish housing systems is typically 24-29°C (Aleström *et al*., 2020). In practice, facilities often choose a constant water temperature of 28.5°C for early life stages (embryos and larvae), although many facilities also use 26°C or 27°C. For adults, many facilities follow reference works and use temperatures between 26 and 29°C, most often 28°C (Westerfield 1993; Cartner *et al.,* 2020).

OECD Test Guidelines (TGs) for testing of chemicals also include parameters for temperatures for different life stages of zebrafish: 26±1°C for embryo development in the Fish Embryo Acute Toxicity (FET) Test (OECD TG 236, 2013), 26±1.5°C in the fish Early Life Stage Toxicity Test (OECD TG 2010, 2013), and 27±2°C as optimal temperature for sexual development in the Fish Sexual Development Test (OECD TG 234, 2011). However, it was noted that OECD TG 203 describes significantly lower temperatures for adult zebrafish in the Fish Acute Toxicity test (21-25°C), which is not in line with current scientific practices (OECD TG 203, 2019). In specific circumstances, e.g. embryo development, even temperatures above 30°C have been used (Urushibata *et al*., 2021), although it has also been reported that 31°C is the maximum temperature for acceptable housing conditions (Westerfield, 2007). Also, when moving to the higher temperatures, it should be kept in mind that oxygen solubility is much lower at higher temperatures. This can especially be a problem in static systems with separate aquaria.

Conductivity, hardness and alkalinity

Fish homeostasis is directly affected by water salinity, as the entire body and the large surface area of the gills are in direct contact with the water (Hoshijima and Hirose, 2007). In terms of conductivity, zebrafish can also adapt to a wide range. Furthermore, later developmental stages can tolerate higher conductivities. However, the optimum for fish health and the tolerable rate of fluctuations have not yet been determined (Tsang *et al.*, 2017).

Multiple interdependent water parameters are relevant for describing the salt content of the water including conductivity, total hardness and alkalinity or carbonate hardness. Conductivity is affected by both alkalinity and hardness, which is why it is recommended to determine these parameters separately to get a more accurate picture of water quality in a given system (Hammer, 2020).

Electric conductivity is mainly determined by sodium and chloride levels for reconstituted water based on sea salts on the one hand and by calcium and carbonate when tap water is mixed with reverse osmosis (RO) water on the other hand. Aleström *et al*. (2020) recommended a conductivity range for zebrafish between 150 and 1700 µS/cm (Aleström *et al*., 2020). This is a broad range and conductivity also varies considerably between facilities. Some sources report ranges between 180 and 350 µS/cm (Brand *et al*., 2002; Geisler *et al*., 2016; Tsang *et al*., 2017), while many facilities use 500-600 µS/cm (Collymore *et al*., 2015; Varga, 2016). After surveying 19 facilities, a mean conductivity of 800 µS/cm (600-1000 µS/cm) was found (Lawrence *et al*., 2016). Sometimes, it may be useful to increase the conductivity, such as during transport or when there is a disease outbreak in the facility. Because of the higher conductivity, the fish have to spend less energy on osmoregulation. As a result, there is more energy left for the immune system and stress response. Also, pathogens are less resistant to high conductivity (Harper and Lawrence, 2016).

(Total) water hardness or general hardness (GH) indicates the concentration of divalent metal ions (Ca^{2+} /Mg^{2+}). It is usually measured as mg/L of $CaCO_3$ equivalents (*i.e*., the hardness corresponding to those determined by a given concentration of $CaCO_3$). Other units may less frequently be used, such as: German degrees or Degrees of General Hardness (dGH; 1 dGh=17.85 mg $CaCO_3$/L), French degrees (°fH; 1°fH=10 mg $CaCO_3$/L). In function of hardness, water may be classified as soft (<60 mg $CaCO_3$/L), moderately hard (60 - 120 mg $CaCO_3$/L), hard (120-180 mg $CaCO_3$/L), very hard (>180 mg $CaCO_3$/L). Hardness strongly affects the toxicity of chemicals, particularly of metals,

by affecting their bioavailability. At high hardness levels, metal toxicity substantially decreases. Therefore, all official procedures for aquatic ecotoxicology testing recommend the control of hardness and a preferred range for performing the test. The recommended range for freshwater fishes (including *Danio rerio*) is 40- 250, preferably <180 mg $CaCO_3$/L (OECD TG 203, 2019). As a consequence, it is also one of the parameters that must be checked in holding water. Hardness is one of the key parameters (together with pH and dissolved organic carbon) required for the development of models linking metal bioavailability to toxicity in freshwaters (Biotic Ligand Models, BLM) (Di Toro *et al*., 2001).

The alkalinity of water (carbonate hardness - KH) is a measure of CO_3^- concentration (CO_3^{2-} and HCO_3^-). It is strictly linked to hardness and is also often expressed in mg/L $CaCO_3$. It is an important indicator of the buffering capacity of the water. When alkalinity drops, pH can also drop very quickly, endangering fish health and welfare. A survey of 19 facilities worldwide found an average alkalinity of 90 mg/L (47-133 mg/L) $CaCO_3$ (Lawrence *et al.*, 2016). Hammer (2020) proposed a range of 50 to 75 ppm which equals 50-75 mg/L $CaCO_3$.

When hardness and/or alkalinity values become too high, part of the water can be renewed with purified Reverse Osmosis (RO) water to stabilise it. When it becomes too low, $NaHCO_3$ or $CaCO_3$ can be added (Hammer, 2020). However, it is important to keep monitoring the resulting values for total conductivity and pH at all times.

pH

In nature, zebrafish are exposed to a wide range of pH values (Tsang *et al*., 2017). In natural aquatic ecosystems pH is influenced by many factors, the most important of them being primary productivity that may produce very high pH variability (up to 2-3 pH units or more in eutrophic water bodies) during the daily cycle. Photosynthesis increases the pH during the day while it decreases with respiration during the night. Other factors affecting pH are oxidation of ammonia, respiration and decay of organic materials (Newell and Brocca, 2022). Although the optimal pH range for zebrafish in experimental animal facilities is not known, sudden changes in the pH should be avoided. Similar to other water parameters (*e.g.* temperature), the stability of pH values is often more important for the health and welfare of fish than the absolute pH value (Tsang *et al*., 2017). Adding a buffer (such as $NaHCO_3$) stabilises pH, but it is equally important to locate and address the source of acidification (*e.g*. lots of organic waste, too low refreshment rate). Finally, pH affects the behaviour of dissociating substances (*e.g*. ammonia, see next section) and the solubility and bioavailability of metals, of which toxicity increases at low pH values. Aleström *et al*. (2020) recommended a pH range from 6.5 to 8. In the biological filter, bacteria are also exposed to the pH values and fluctuations occurring in the system. For the optimal functioning of these organisms, a pH above 7 should be maintained (Tsang *et al*., 2017; Aleström *et al*., 2020). Therefore, a pH range from 7 to 8 ensures optimal health of both the fish and the biological filter. Official procedures for aquatic ecotoxicology testing recommend the control of pH and a preferred range for performing the test. The recommended range for freshwater fishes (including *Danio rerio*) is 6.0-8.5 (OECD TG 203, 2019).

Carbon dioxide is produced by aquatic organisms (animals and plants) during respiration and dissolves in water to form carbonic acid (a weak acid), that dissociates to form bicarbonate ion and hydrogen, as in the reaction below:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$

The equilibrium carbonic acid-bicarbonate is the most common buffering system in natural waters.

The amount of free CO_2 in water depends on pH, temperature, and hardness. In surface water, in equilibrium with the atmosphere, the amount of free CO_2 can never reach levels that may be dangerous for fish. However, free CO_2 in groundwater is frequently supersaturated relative to its equilibrium with atmospheric partial pressure, up to levels that may be dangerous for fish (Vesper and Edenborn, 2012). The response of fish to high concentrations of free CO_2 is variable in the different species and in different environmental conditions. A safe level could be estimated around 20 mg/L (Martens *et al*., 2006). Therefore, if groundwater is used as water source, the concentration of free CO_2 should be checked and, if necessary, degassing systems must be used.

Nitrogen compounds

In aquatic systems with a biofilter, ammonia is converted into nitrite and nitrite is converted into nitrate through oxidation steps mediated by bacteria (*Nitrosomonas*, *Nitrosococcus*, *Nitrobacter)* colonising the filter. Ammonia and nitrite levels should be kept as close to 0 as possible. A properly functioning biofilter should ensure that total ammonia (NH_3/NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) concentrations remain below 0.1 mg/L (or below detection limit if detection limit is higher), 0.3 mg/L and 25 mg/L, respectively (Aleström *et al*., 2020). Both ammonia and nitrite levels are preferably as close to 0 mg/L as possible. The toxicity of ammonia on aquatic animals is strictly determined by pH and temperature, (and to a limited extent by conductivity), that regulate the balance between highly toxic NH_3 and far less toxic NH_4^+ (Table 6.2, Figure 6.4). The higher the pH and temperature and the lower the conductivity, the more toxic NH_3 is present (Harper and Lawrence, 2016). Acute effects on some fish (no data on zebrafish included) have been demonstrated in laboratories at concentrations as low as 0.1 mg NH_3/L and chronic effects at concentrations as low as 0.02 mg NH_3/L (WHO, 1986). Hammer (2020) recommended NH_3 levels below 0.05 mg/L and total ammonia nitrogen is recommended below 1 mg/L for long-term exposure (Timmons and Ebeling, 2013).

Nitrite is less toxic than ammonia but more toxic than nitrate, and Hammer (2020) recommended taking action if nitrite levels approach 0.5 mg/L, while Aleström *et al*. (2020) recommended keeping nitrite below 0.3 mg/L. Nitrate itself is much less toxic but must be disposed of as it is not further degraded by the bacteria in the biofilter. In the absence of plants, only water renewal can lower nitrate levels (Harper and Lawrence, 2016). Nitrate levels of 50 mg/L are often considered safe for long-term exposure of fish (Hammer, 2020), while Aleström *et al*. (2020) recommend an upper limit of 25 mg/L.

Table 6.2 shows the percentage of highly toxic ammonia (NH_3) in the total ammonia content depending on temperature and pH in the conditions relevant for zebrafish. Table modified according to Emerson *et al*. (1975). Figure 6.4 shows the relation between the presence of NH_4^+ and NH_3 depending on the pH.

Table 6.2 Changes in fraction of NH_3 depending on temperature and pH

Changes in fraction of NH_3 depending on temperature and pH

Temperature (° C)	pH								
	6,0	6,5	7,0	7,5	8,0	8,5	9,0	9,5	10,0
20	0,0397	0,125	0,396	1,24	3,82	11,2	28,4	55,7	79,9
21	0,0427	0,135	0,425	1,33	4,10	11,9	29,9	57,5	81,0
22	0,0459	0,145	0,457	1,43	4,39	12,7	31,5	59,2	82,1
23	0,0493	0,156	0,491	1,54	4,70	13,5	33,0	60,9	83,2
24	0,0530	0,167	0,527	1,65	5,03	14,4	34,6	62,6	84,1
25	0,0569	0,18	0,566	1,77	5,38	15,3	36,3	64,3	85,1
26	0,0610	0,193	0,607	1,89	5,75	16,2	37,9	65,9	85,9
27	0,0654	0,207	0,651	2,03	6,15	17,2	39,6	67,4	86,8
28	0,0701	0,221	0,697	2,17	6,56	18,2	41,2	68,9	87,5
29	0,0752	0,237	0,747	2,32	7,00	19,2	42,9	70,4	88,3
30	0,0805	0,254	0,799	2,48	7,46	20,3	44,6	71,8	89

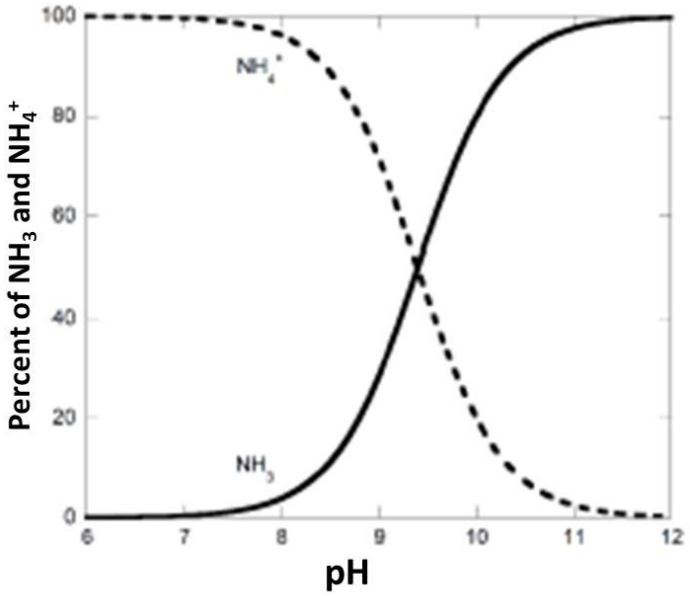

Figure 6.4. Percent of un-ionised (NH_3, solid line) and ionised (NH_4^+, dashed line) ammonia at 20°C as a function of pH (modified after Emerson *et al.,* 1975)

Oxygen

Typically, a dissolved oxygen concentration of 6-8 mg/L is recommended in recirculating systems (Collymore *et al*., 2015; Hammer, 2020). Maximum oxygen saturation in freshwater at 28°C is 7.8 mg/L, thus this corresponds to an almost complete saturation of the water at 28°C. OECD Test Guidelines recommend a minimum threshold of 60% saturation (5 mg/L at 28°C) (OECD TG 210, 2013; OECD TG 203, 2019).

Dissolved oxygen in tanks is affected by temperature, fish density and microbial load. The oxygen concentration can drop rapidly when there is no (more) water inflow. This is important to realise when temporarily removing small aquaria with high densities of fish from a recirculating system for experiments, cleaning, or other manipulations. Microorganisms growing on the walls and organic waste in the tank also consume oxygen. It is therefore important to clean the tanks at regular intervals (Hammer, 2020). Supersaturation (>100% DO) can also occur, for example due to leaks in the pumping system or rapid changes in temperature. Supersaturation can lead to "Gas Bubble Disease" (Murray *et al*., 2020).

Conclusions

The major recommendations based on the data presented above are presented in Table 6.3. Overall, the WoE for the selection of relevant parameters indicated in Table 6.3 is strong.

Table 6.3 Water parameters to be considered in zebrafish housing systems

Water parameter	**Recommendations**	**(Most) often used**	**Source**
Temperature	24-29°C[a]	Juveniles-adults: 26-28°C Embryolarval stages: 26-28.5°C	Villamizar *et al*.,2012; Aleström *et al.*, 2020
Conductivity	150-1700 µS/cm	500-1000 µS/cm	Collymore *et al.*, 2015; Geisler *et al*., 2016, Lawrence *et al*., 2016, Aleström *et al.*, 2020
Total/general hardness	40-250 mg/L $CaCO_3$	40-180 mg/L $CaCO_3$	OECD, 2019
pH	6.5-8		Aleström *et al*., 2020.
Nitrogen compounds	NH_3/NH_4^+<0.1[b] mg/L, NO_2^- < 0.3 mg/L, NO_3^- < 25 mg/L		Aleström *et al.*, 2020
Dissolved oxygen	> 5 mg/L		Collymore *et al*., 2015; Hammer, 2020

[a] 28°C is considered the optimal housing temperature. However, temperatures of 30-31°C are also acceptable, as there is insufficient data to conclude that housing zebrafish at these temperatures reduces animal welfare.
[b] or below detection limit, if detection limit > 0.1 mg/L. 0.1 mg/L indicates the total amount of ammonia, $NH_3+NH_4^+$. This corresponds to 0.002 mg/L of NH_3 at 28°C and pH 7.5.

Table 6.3 shows a preferred housing temperature of 24-29°C, with an optimal temperature of 28°C (WoE strong). However, there is insufficient data to conclude that housing zebrafish at 30-31°C reduces animal welfare (WoE weak). The parameters indicated in the table 6.3 (WoE strong) should be checked on a regular basis. Depending

on the parameter and the housing conditions (static or recirculating), they may be measured and adjusted daily (T, pH), weekly (conductivity, nitrogen) or monthly (hardness, oxygen). The frequency of oxygen measurements may need to be increased for static housing conditions (*e.g.* weekly). In facilities where the system measures the parameters automatically, it is important to double check the measurements regularly with an external device. Furthermore, it should be clear what to do when water parameters deviate from the allowed ranges. This ensures that action can be taken rapidly to ensure fish welfare. Stability of water parameters is often more important than the actual value.

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6.1.3. Zebrafish housing conditions

6.1.3.1. General aspects

General conditions for lighting and noise are presented in DIRECTIVE 2010/63/EU OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 22 September 2010 on the protection of animals used for scientific purposes. (EU, 2010), Annex III Section A 2.2 (a) addresses the lighting, "Where natural light does not provide an appropriate light/dark cycle, controlled lighting shall be provided to satisfy the biological requirements of the animals and to provide a satisfactory working environment." Annex III Section A 2.3 (a) addresses noise in an animal facility: "Noise levels including ultrasound, shall not adversely affect animal welfare."

Regarding light it is critical that the photoperiod in a zebrafish facility is kept constant (Villamizar *et al.,* 2014). The most commonly used photoperiods are 14/10 Light/Dark cycle (Brand *et al.,* 2002, Matthews *et al*., 2002) and 12/12 Light/Dark cycle (Lawrence 2007). Furthermore, it is essential that the dark phase is completely dark (no disturbing light from nearby devices) because this can affect egg production (Adatto *et al.,* 2016). For embryos and juvenile fish, values of 500-1100 lux are indicated for specific experimental procedures (OECD TG 236, 2013; OECD TG 203, 2019). The general recommendation for adult fish is 54-334 lux at the water surface (Matthews *et al.,* 2002). Prolonged light exposure above 300 lux was suggested to be detrimental to adult

zebrafish (Zynda, 2020). In one facility, light is used up to 700 lux without obvious signs of discomfort (pers comm. De Vrieze, Radboudumc, Dept. of Otorhinolaryngology & Radboud University Zebrafish Facility, Nijmegen, The Netherlands). However, concerning light intensity, insufficient data are available in the primary literature. Too much light accelerates the growth of algae, hindering fish vision, which is an important factor for animal welfare. An intensity of 300 lux centrally between housing systems, at 1 m height, is generally considered to be common practice. Although it is recommended to distribute light as uniformly as possible, most systems with zebrafish are illuminated from above, which creates a gradient in intensity (Lieggi *et al.,* 2020; Zynda, 2020). Targeted lighting on the tanks or LED strips mounted on the rack can provide optimum standardised light intensity. Alternatively, wall-mounted LED lighting panels can be used to distribute light evenly between rows. The use of dawn-dusk phases has been suggested as a form of visual enrichment for zebrafish in facilities, but very little is known about its consequences (Stevens *et al.,* 2021). Introducing dusk and dawn would reduce the startle reflex (Lidster *et al.,* 2017). Transition times ranging between 20 minutes (Wilkes *et al.,* 2012) and 40 minutes (Woodward *et al.,* 2019) have been used.

Zebrafish has been recognised as a well-established model organism in hearing and balance research especially in the area of genetic impacts on hearing (Whitfield, 2002, Sheets *et al*., 2021, Popper and Sisneros, 2022). The zebrafish model can also be used to evaluate potential ototoxicity of chemotherapeutic agents like cisplatin and potentially otoprotective compounds in real time (Niihori *et al*., 2015, Barallo-Gimeno and Llorens, 2022). Popper and Sisneros (2022) stated in their review on hearing assessment that human-generated (anthropogenic) sound added to the environment has the potential to disrupt the detection of biologically relevant sounds, alter behaviour, impact fitness, and produce stress and other effects that can alter the well-being of animals. A considerable difference may occur between laboratory housing conditions and natural habitat of zebrafish. When natural soundscapes were evaluated for five different natural habitats, it was observed that sound pressure levels in the natural habitat (range 98-126 dB) showed a clear difference from sound pressure levels under large scale housing conditions (range 122-143 dB) habitats (Lara and Vasconcelos, 2019). In addition, high noise levels (150 ± 10 dB) can lead to hearing loss and changes in behaviour (Wong *et al.*, 2022), and at 150 dB even an increase in mortality in zebrafish <5 dpf (Lara and Vasconcelos 2021). As sound is a form of vibration also vibration may affect zebrafish behavioural and brain functions (Wang *et al.,* 2021). On the other hand, classical music at 65-75 dB twice daily for 2 hours resulted in less anxiousness in tests and decreasing stress levels as indicated by reduced inflammatory cytokines (Barcellos *et al*., 2018). So, an increase in noise above the continuous 50-55 dB background was found to have beneficial effects on the zebrafish.

Fish can be acutely sensitive to sounds, even at very low levels. Noise levels within experimental facilities should be kept to a minimum, and the examples above show that high noise levels in zebrafish housing conditions need to be avoided. Where possible equipment causing noise or vibration, such as power generators or filtration systems, should be separated from fish-holding facilities. Fish reared in a particular environment will adapt to the stimuli presented there and may become stressed if moved to unfamiliar surroundings. In general, zebrafish are thought to adapt to their environment regarding noise levels although sudden loud noises and vibration should be avoided. (Matthews *et al*., 2002, CCAC 2020). Currently, there are no clear recommendations for noise levels in zebrafish housing facilities.

Conclusions

Regarding light, it is critical that the photoperiod in a zebrafish facility is kept constant irrespective whether a 14/10 Light/Dark cycle or a 12/12 Light/Dark cycle is applied in the housing facility (WoE strong). It is essential that the dark phase is completely dark. The use of dawn-dusk phases has been suggested as a form of visual enrichment for zebrafish in facilities, as it may reduce the startle reflex when the light goes on. Transition times ranging between 20 minutes and 40 minutes have been used. For light intensity, the general recommendation for adult fish is 54-334 lux at the water surface (WoE moderate). Too much light accelerates the growth of algae, hindering fish vision, which is an important factor for animal welfare.

Zebrafish are thought to adapt to their environment regarding noise levels although sudden loud noises and vibration should be avoided. Where possible, equipment causing noise or vibration should be separated from fish-holding facilities. Although there are no clear recommendations for noise levels in zebrafish housing facilities, it can be recommended to keep noise levels as low as possible and constant over time (WoE weak). It should be noted that fish will adapt to the stimuli present in their environment and may become stressed when these change or when the fish are moved to unfamiliar surroundings.

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6.1.3.2. Stocking density and aquarium enrichment

Stocking density

Commercially available laboratory aquaria typically offer a broad range of housing tanks -- ~1 L to ~10 L -- and the numbers of fish that are kept in each can vary depending on laboratories' requirements. However, there is evidence that suggests optimal stocking densities, and this is broadly based on a range of welfare considerations. A systematic review of the literature on stocking density of zebrafish (Andersson and Kettunen, 2021) considered the welfare outcomes of different stocking densities according to a series of endpoints: growth; reproduction; stress response; water quality and pathogens; rearing of larvae). The framework used in this systematic review is used here as a guidance, but the evidence is extended based on more recent studies. The evidence is somewhat contradictory in places: some studies tend to suggest that higher stocking densities are always a bad thing, others that smaller densities are worse.

In one multicentre study with eight zebrafish facilities, reproduction and rearing was evaluated to estimate effects of stocking density (Castranova *et al.,* 2011). A large variety in clutch size and spawning success was observed, however, the stocking densities used (3, 6, or 12 fish/L) did not result in significant differences on the breeding results. So, the authors concluded that a stocking density of 12 fish/L had no negative effect on breeding performance.

Growth: Whether growth parameters are a welfare indicator is a matter of debate and somewhat contentious, and there is conflicting evidence on stocking density and growth[5]. Some studies have indicated that there is a negative correlation between growth and group size with the consensus being that optimal density should be no higher than 7.5 fish/L. In one study high stocking density (37 and 74 fish/L) resulted in reduced body mass and growth in both males and females, and reduced survival in early life stages (Ribas *et al.,* 2017). However, one of the main factors may be availability of food in larger groups; in support of this, varying the feeding regime in accordance with group size removed the differences in growth between 2 fish/L and 20 fish/L (Andersson and Kettunen, 2021).

Stress response: Several studies have examined physiological stress responses to different stocking densities (through the release of cortisol). There have been mixed findings, with some studies showing no difference in cortisol between fish between 4 and 40 fish/L, and some showing significantly higher cortisol in fish kept at densities > 5 fish/L (Ramsey *et a*l, 2006; Pavlidis *et al*., 2013; Ribas *et al.,* 2017) One key difference appears to be the size of the tank: experiments that have varied the tank size have found higher cortisol in groups in larger tanks compared to smaller, irrespective of stocking density (Pavlidis *et al.*, 2013).

Behaviour: Behavioural outcomes that could be considered welfare indicators in fish include anxiety responses and social behaviour. Group-housed fish show *higher* stress reactivity and anxiety than long-term individually housed fish (Parker *et al*., 2012;

[5] Stocking density is defined here in terms of number of fish *per* litre of water.

Shams *et al.*, 2015), but this may reflect the degree of change associated with removal from the home environment to the test environment (*i.e*. anxiety is measured on individual fish, and individually-housed fish are therefore experiencing less of a social change during testing than group-housed fish). An additional consideration is aggression which, although part of the typical social behaviour of the species during formation of social hierarchies, can be a welfare concern if it is frequent or inescapable (Graham *et al*., 2018). Aggression is higher in fish kept at low densities (1 fish/L or lower), and this correlated with higher anxiety behaviours and stress levels (Andersson *et al.,* 2022).

Water quality: This subject is covered in detail in Section 6.1.2.2. With respect to stocking density, higher stocking densities are associated with a larger build-up of waste and therefore the potential for increased pathogens.

There is currently very little in the literature considering the *welfare* of younger developing (larval/juvenile) zebrafish in terms of stocking density; for this reason, much of the evidence is based on physical parameters.

Conclusions

Studies have tended to focus on welfare issues associated with higher, rather than lower, stocking densities; the evidence suggests that lower stocking densities could be a challenge from the perspective of social enrichment (*i.e.* the presence of conspecifics and welfare implications of small social groups). The evidence suggests that adult zebrafish should be kept in conditions that are neither overcrowded nor underpopulated, and the consensus that the optimal stocking density is 5 adult fish/L (WoE strong). In order to allow shoaling, a minimum of 5 fish/tank is recommended, whereas the maximum is considered 10 fish/L (WoE weak to moderate). The presence of less than 5 fish per tank is possible under certain conditions; however, this is not recommended for prolonged periods of time. Considering the stocking density of 5 fish/L, the tank size and shape should allow the fish to perform its natural behaviour and swimming activity.

Enrichment

According to Annex III, there is a legal requirement to provide enrichment in the husbandry for all animals used in scientific research. The provision in Annex III Section B, Species-specific Section, 11.4 specifically mentions that "Fish shall be provided with an appropriate environmental enrichment, such as hiding places or bottom substrate, unless behavioural traits suggest none is required". Environmental enrichment was described by Newberry (1995) as "*an improvement in the biological functioning of captive animals resulting from modifications to their environment*". The provision of adequate (species-specific) enrichment is widely accepted in terrestrial species as being essential for welfare (Young, 2013). There are several ways in which an environment can be enriched, including:

- 'social' enrichment (*i.e.* the presence of a stable group of conspecifics; this is covered in the discussions of stocking density);
- 'behavioural' enrichment (this may overlap with physical enrichment, but specifically includes the use of toys/puzzles/etc. to encourage animals to actively interact with their environment);
- 'physical' enrichment (the provision of physical stimuli in the environment, such as 'hides' or 'natural' substrates for manipulation);

- 'nutritional' enrichment (such as live feed if appropriate for a species);
- 'sensory' enrichment (including the use of sensory [auditory, visual or olfactory] stimuli).

The functional significance of providing enrichment in zebrafish is less well established than in some other species, but the weight of evidence supports its use (WoE moderate). Recent papers (Stevens *et al*., 2021, Gallas-Lopes *et al*., 2023) have summarised the research evidence for enrichment in zebrafish and considered enrichment to have an overall positive impact on animal welfare. As with stocking density, there is currently a gap in our knowledge about the use of enrichment for welfare purposes in juveniles/larvae (≥5 dpf). In addition, it should be noted that an inherent difficulty with judging the quality of enrichment studies is that it is hard to evaluate the benefit of the enrichment objectively. For example, preference tests (*i.e.* do the animals spend time with the enrichment device, or prefer to consume the nutritional enrichment) are inherently circular in their interpretation. For this reason, it is difficult to ascertain their benefit. Plastic grass or plastic aquarium plants can be used as enrichment for the tanks that house zebrafish. However, grass type of autoclavable plastic green can get fish trapped and injured, and aquarium type plastic plants cannot be autoclaved and are very difficult to disinfect. Parts of the home aquarium type of plastic plants were observed in the faeces of zebrafish indicating oral uptake (pers. comm. Dr B. Schmid, Deutsches Zentrum für Neurodegenerative Erkrankungen e. V. (DZNE), Munich, Germany).

Physical enrichment: A comprehensive study on preference for different putative forms of enrichment showed that the presence of substrate on the bottom of a tank (*e.g*. gravel) or even a picture of the substrate placed under the tank is preferred to barren environments (Schroeder *et al*., 2014). Of note, this only needs to be a picture affixed to the base of the tank, as opposed to actual substrate (Schroeder *et al*., 2014). This, and other, studies have found that zebrafish spend more time in close proximity to 'structures' in their environment, suggesting they have preference for this, over barren, environments. Several studies (reviewed in Stevens *et al*., 2021) have shown that the presence of physical complexity in the environment reduces anxiety (both in terms of physiological and behavioural measures), increases exploratory behaviour, increases brain size and learning performance, and increases 'positive' social interactions (although, some studies have notably found increases in aggression associated with environmental complexity (Bhat *et a*l., 2015). Also, some studies did not report an effect of tank enrichment on fish behaviour and cortisol levels (Wilkes *et al*., 2012; Collymore *et al*., 2015). In addition, the enrichment objects may reduce the available free swimming spaces, and this should be considered in view of number of fish housed.

It should be noted that the material used for objects as physical enrichments may have an impact on the fish as well. Most physical objects are currently made from plastic that may be associated with the presence and release of softeners/plasticisers (*e.g.* phthalates), possibility for plastic uptake by the fish, and biofilm formation on the objects. With the exception of avoiding possible exposure to released chemicals from the objects, it is currently not possible to formulate specific recommendations for physical enrichments (Aleström *et al*., 2020). In addition, it has to be considered that water conditions can be affected by blockage of waterflow. Therefore, expected benefits of structural enrichment have to be carefully balanced against potential detrimental effects.

Sensory enrichment: As well as the visual enrichment mentioned above (*i.e.* the pictures of gravel substrate affixed to tank bases), one study found that auditory enrichment, in the form of the playing of classical music to zebrafish, reduced physiological stress markers (including cortisol decrease, and decreases in pro-inflammatory markers), and reduced stress responses to a novel environment (Barcellos *et al*., 2018).

Food as enrichment: There are several manufactured diets that are commercially available and may be considered as being nutritionally complete (Siccardi III *et al*., 2009; Karga and Mandal, 2017). Evidence suggests very little difference in performance (growth, development, breeding) on these various diets (Siccardi III et al., 2009; Karga and Mandal 2017). However, the consensus at a recent zebrafish husbandry meeting (see Osborne *et al.,* 2016 for overview) was that offering additional live feeds, at all free-feeding (*i.e.* >4 dpf) life stages, should be considered important for welfare, by offering fish the opportunity to perform natural prey capture behaviour and for avoiding build-up of uneaten food at the base of the tank (which may encourage unnatural feeding behaviour). Types of live food available include paramecia or rotifers (for young larvae [at a high density for the first ~5 days of feeding]) and artemia (for 10 dpf larvae and adults). Also, rotifers may be used until 30 dpf and later (Lawrence *et al.,* 2015; Monteiro *et al.,* 2018).

Conclusions

When keeping zebrafish in a laboratory environment, enrichment needs to be made available, that could be based on physical, visual, nutritional and social aspects (WoE moderate). For example, this could include a visual image of substrate affixed to the base of the tank or some form of physical stimulus within the tank. However, when placing physical attributes inside a tank, specific considerations should be made for the composition of the materials used in view of possibility for cleaning/sterilization and/or possible release of potentially toxic components. Potential long-term consequences of physical enrichments, both in terms of benefits and harm, are yet unknown, and more research on this subject is recommended (WoE weak). Although there is little objective evidence that offering live feeds improves welfare, the consensus among users is that offering live feeds is likely to be beneficial as it encourages natural behaviour (WoE weak).

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6.1.3.3. Solitary housing

Zebrafish are a shoaling species, and, in their natural environment, live in large groups of conspecifics. Solitary (individual) housing can be required in the laboratory for husbandry reasons or as part of a protocol. For example, fish may require solitary housing for quarantine (in the case of a disease outbreak) or for genotyping purposes (to identify carriers of a transgene, for example). There is mixed evidence about the effects of isolation on welfare (Parker *et al.,* 2012; Pagnussat *et al.,* 2013; Collymore *et al.,* 2015; Onarheim *et al*., 2022;). Pagnussat *et al.* (2013), for example, demonstrated that short term isolation resulted in increased cortisol and more variability in behavioural responses to a novel environment, suggesting increased stress in these individuals. However, several others have found that longer term isolation actually induces lower cortisol and less variability in behavioural responses (Onarheim *et al*., 2022; Parker *et al.,* 2012). Of note, one study (Parker *et al.,* 2012) found that there were no differences either in behaviour or cortisol between individually housed fish and fish housed in pairs/small groups with no physical access to one another. This offers social enrichment (*i.e.* visual/olfactory access to conspecifics).

Conclusions

As zebrafish is a shoaling species (WoE strong), prolonged single housing is not recommended, but can be required during a limited period for specific reasons. Visual/olfactory access to conspecifics should be a minimum requirement for individually housed fish. In addition, enrichment could be provided similar to the situation in the other tanks of the facility when fish are individually housed.

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6.1.4. Mating

Since zebrafish are photoperiodic breeders with onset of mating during dawn and the early light period, a firm control of the light cycle is required. Therefore, fish should not be removed from the habituated light regime for mating to avoid disturbing the circadian rhythm. Additionally, the mating should take place in the same water conditions (temperature, pH, nitrite, nitrate, hardness etc.) as the normal husbandry to avoid stress by adaptation processes. Especially, when using isolated mating tanks outside actively temperature-controlled husbandry systems, the dropping to room temperature may inhibit mating or reduce the amount of eggs produced. Mating tanks can be reduced in size compared to normal husbandry (for a maximum time of 1 day only) but should not be smaller than 300 mL in volume for 6 fish (Goolish *et al.,* 1998), albeit ensuring water quality equal to normal husbandry parameters.

Since zebrafish tend to eat their own eggs, the system should be constructed in a way to collect the eggs safely. This might be addition of marbles to the mating tank (producing chinks not accessible for the adults) or the use of grid floors above a solid floor to separate the eggs from the adults. Fish regularly used for breeding should be fed with energy-rich food. Polyunsaturated acids can improve fecundity and larvae quality (Nowosad *et al.,* 2017). The mating pairs might be 1:1, but a relation of 1 male for 2 females is usually more effective (Westerfield, 2007).

Design of tilted mating cages resulting in water levels varying between deep and shallow areas (height of water column about 1x the height of the fish that the body is at least covered completely) may mimic natural mating situations and can result in improved embryo yield (Sessa *et al.,* 2008). Commercial constructions for designing this kind of setup are available.

In vitro fertilisation is a possible alternative for rare or important genetically modified lines, but due to the involved procedures (anaesthesia and egg / sperm collection) is not considered the standard breeding procedure in facilities.

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6.1.5. Health control (contaminants/pathogens)

Good housing conditions are not only defined by maintaining the key water parameters within acceptable ranges but also by the absence of contaminants and pathogens (Sanders and Farmer, 2019). For this, an effective water purification and/or monitoring system must be in place. With regular monitoring, use of municipal or other local tap water can be acceptable but it has to be considered that water quality may change over the year, *e.g*. due to seasonal effects (Aleström *et al.,* 2020). To rule out significant variations of water quality, appropriate filtration and purification systems must be used before the water is added to the housing system (Kent *et al.,* 2009). Filtration and purification systems can include mechanical, chemical (*e.g*. active carbon) and biological (nitrification) filter systems (Aleström *et al*., 2020).

While filtration and purification systems like reverse osmosis or deionization reduce most contaminants like chlorine, they tend to be less effective for heavy metal ions like copper. As copper is toxic to larvae and adults at concentrations as low as 1 µM (Johnson *et al.,* 2007; Vicario-Parés *et al.,* 2018; Zhang *et al.,* 2015) and hard to detect at these low concentrations with commercially available analytics, it is best to avoid in the facility use of copper altogether in all surfaces and plumbing that come in contact with husbandry water.

In addition to contaminants, the zebrafish facility should also be free of known pathogens. Water purification systems and use of UV light can at least reduce the presence of pathogens in the water, but many pathogens are commonly found in the environment. These can easily spread in the facility if improper hygiene measures are in place (Collymore *et al.,* 2016; Kent *et al.,* 2020; Mocho *et al.,* 2022a, 2022b). Therefore, routinely health monitoring should be performed on euthanized fish and environmental samples from the facility in accordance with EU Directive 2010/63 Annex III, Section A 3.1a. To mitigate pathogen outbreaks, proper hygiene and quarantine measures should be in place.

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Overall conclusions

Sophisticated housing systems are available for zebrafish holding facilities such as flow-through and/or recirculating aquaculture systems. Water quality is of utmost importance, and major recommendations based on the data are presented in Table 6.3 (WoE strong). The parameters indicated in Table 6.3 should be checked on a regular basis. Depending on the parameter, they may be measured and adjusted daily to monthly. In facilities where the system measures the parameters automatically, it is important to double check the measurements regularly with an external device. Furthermore, it should be clear what to do when water parameters deviate from the allowed ranges. This ensures that action can be taken rapidly to ensure fish welfare. Stability of water parameters is often more important than the actual value. In addition, health control measures should be in place to monitor for potential introduction of pathogens causing disease.

Although water temperature of the natural habitat of zebrafish spans a large range (below 15^0C to over 35^0C) the temperature range recommended for zebrafish housing systems is 24°C to 29°C, with an optimum temperature of 28°C, as is currently common practice (WoE strong). In view of the recommended water temperatures indicated in Table 6.3, the temperature range (21-25°C) as presented in some OECD test guidelines (*e.g.*, OECD TG 203 the Fish Acute Toxicity Test) is considered not to be in line with current scientific practices and may need to be adapted.

Regarding light it is critical that the photoperiod in a zebrafish facility is kept constant, irrespective whether a 14/10 Light/Dark cycle or a 12/12 Light/Dark cycle is applied in the housing facility (WoE strong). It is essential that the dark phase is completely dark. The use of dawn-dusk phases has been suggested as a form of visual enrichment for zebrafish in facilities, as it may reduce the startle reflex when the light goes on. Transition times ranging between 20 to 40 minutes have been used. The general recommendation of light intensity for adult fish is 54-334 lux at the water surface (WoE

moderate). Too much light also accelerates the growth of algae, hindering fish vision, which is an important factor for animal welfare.

Zebrafish are thought to adapt to their environment regarding noise levels although sudden loud noises and vibration should be avoided. Where possible equipment causing noise or vibration should be separated from fish-holding facilities. Fish reared in a particular environment will adapt to the stimuli presented there and may become stressed if moved to unfamiliar surroundings. Although there are no clear recommendations for noise levels in zebrafish housing facilities, it can be recommended to keep noise levels as low as possible and constant over time (WoE weak).

Although no specific recommendation for tank sizes can be formulated, it is recommended that adult zebrafish should be kept in conditions that are neither overcrowded nor underpopulated. In order to allow shoaling, a minimum of 5 fish/tank is recommended (WoE moderate). The presence of less than 5 fish per tank is possible under certain conditions, however, this is not recommended for prolonged periods of time. The maximum is 10 fish/L (WoE weak to moderate). There is a general consensus that the optimal stocking density is 5 adult fish/L. The tank size and shape should allow the fish to perform their natural behaviour and swimming activity. Therefore, smaller water volumes than 1 L should not be used for adult fish. There is limited scientific literature that studied the relationship between tank size and natural behaviour and swimming activity. But tank size does have an effect on zebrafish behaviour. In the tanks themselves some form of enrichment (*e.g*. social, physical, visual, nutritional) is recommended (WoE moderate). In addition, health control measures should be in place to monitor for potential introduction of contaminants and pathogens causing disease.

As zebrafish is a shoaling species (WoE strong), prolonged single housing is not recommended, but can be required during a limited period for specific reasons. Visual/olfactory access to conspecifics should be a minimum requirement for individually housed fish. In addition, enrichment could be provided similar to the situation in the other tanks of the facility when there is a need to individually house fish.

6.1.6. Methods of euthanasia

In EU directive 2010/63, Annex IV the following euthanasia methods are listed as acceptable for fish in general:

- Anaesthetic overdose
- Concussion/percussive blow to the head
- Electrical stunning (special equipment required)

In an international survey regarding euthanasia methods employed for zebrafish, where multiple answers were possible, 70% used anaesthesia overdose, 40% of its respondents used hypothermic shock, while none of the respondents reported using electrical stunning (Lidster *et al.,* 2017).

Physical means of euthanasia have not been reported as the small size of zebrafish makes application of concussion unfeasible (Köhler *et al.,* 2017). Physical methods are more often employed as a second step on unconscious animals as confirmation of death, as described in Annex IV section 2. In this case, the destruction of the brain has to be ensured as neural activity could persist in decapitated heads (Van De Vis *et al.,* 2003,

Verheijen and Flight, 2008). For small fish whole body maceration has been considered as an option (Close *et al.,* 1996).

Killing fish by means of electricity is known as electrocution while electronarcosis is caused by electrical stunning. Electrocution can be a method of euthanasia while electronarcosis would be a two-step procedure with a follow up method to confirm death of the animal to avoid recovery. The electric shock disrupts brain activity resulting in unconsciousness within seconds and if prolonged is followed by failure of respiratory and cardiac function. In flawed applications this can cause considerable pain and damage to the fish with strong muscle contractions or seizures that can result in muscle ruptures, bleeding or broken spines (Sharber *et al.,* 1994; Snyder, 2003). Varying effects even within the same species have been observed due to dependency to field strength, time of exposure, conductivity, pH and water temperature (Snyder, 2003). In addition, effects of alternating current differ from direct current or pulsed direct current (Snyder, 2003). By now, electrical stunning in general is accepted as a humane slaughter method for farmed fish like trout or salmon when correctly applied (EFSA, 2004, 2009; Jung-Schroers *et al.,* 2020; Schroeder *et al.,* 2021). It has to be noted though, that European regulations for animal slaughter have a different purpose compared to euthanasia as applied for animals housed and used for scientific purposes.

For zebrafish, so far electrocution has not been applied on a broad basis for euthanasia out of safety concerns and lack of commercially available equipment (Lidster *et al.,* 2017). Only very recently the first report appeared, demonstrating the proof of principle (Mocho *et al.,* 2022; Teulier *et al.,* 2018). This report proposes electrocution as an acceptable alternative especially for early larval stages (Mocho *et al.,* 2022). Before electrocution can be safely used as euthanasia method, effective parameters have to be established *e.g.* for electrical current, voltage and exposure time in regard to different sizes of zebrafish or influence of water conductivity to ensure unconsciousness in fish and avoid unnecessary stress and pain (EFSA, 2004; Kenney *et al.,* 2017; Kuroda *et al.,* 2019; Lines and Kestin, 2004).

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6.1.6.1. Anaesthetics

An anaesthetic overdose is considered a safe and effective method for killing zebrafish that is well established (Neiffer and Stamper, 2009; Matthews and Varga, 2012; Collymore *et al.,* 2016; Martins *et al.,* 2016; CCAC, 2020; Schroeder *et al.,* 2021). Various anaesthetics have been shown to be suitable for euthanasia (Table 6.4). Even though tricaine (MS-222) is traditionally by far most often used, it was recently shown that lidocaine and/or propofol are promising alternatives (Collymore, 2020; Davis et al., 2022; Ferreira et al., 2022a, 2022b; Von Krogh *et al.,* 2021).

To perform euthanasia by anaesthetic overdose, an immersion bath is prepared in which the transferred fish loses consciousness quickly. Death occurs due to suffocation within minutes, but fish must remain in the solution for at least 10 minutes after operculum movements have ceased (Leary *et al.,* 2020). The anaesthetics differ in time needed for the onset of unconsciousness, depending on how aversive they are perceived until that timepoint and the recovery rate for how many fish would regain consciousness if transferred back to fresh water after a given time. For refinement purposes, some of these properties have been compared of the most commonly used anaesthetics, but as these are among other things strongly dependent on dose, water parameters like pH or temperature, water solubility and the age of the euthanized fish, so far, no single standard procedure has emerged as superior in all relevant categories.

Even though some chemical agents have been reported to be perceived as aversive by zebrafish (Readman *et al.,* 2013; Wong *et al.,* 2014), their continued use is justified as the benefits (easy application, quick loss of consciousness) are likely to outweigh potential distress. Still, continuous refinement is to be expected (Von Krogh *et al*., 2021; Schroeder *et al.,* 2021). Larval fish younger than 16 days post fertilization are resistant to death by suffocation due to passive oxygen uptake and might need much longer treatments or additional measures to confirm death (Collymore, 2020).

Table 6.4, **List of commonly used anaesthetics for euthanasia by overdose of adult zebrafish**

Substance	Dose	Reference
Tricaine (MS-222)	>200 mg/L	Collymore *et al.* 2016, Collymore 2020, Ferreira *et al.* 2022a,b, Von Krogh *et al.* 2021
Benzocaine[a]	>250 mg/L	CCAC 2020, Von Krogh *et al.* 2021
Isoeugenol[b]	>50 mg/L	CCAC 2020, Von Krogh *et al.* 2021
Etomidate	>6 mg/L	Ferreira *et al.* 2022a, Von Krogh *et al.* 2021
2-Phenoxyethanol[a]	>2 mL/L	CCAC 2020, Von Krogh *et al.* 2021
Lidocaine	>400 mg/L	Collymore *et al.* 2016, Collymore 2020, Von Krogh *et al.* 2021
Propofol	>100 mg/L	Davis *et al.* 2022
Propofol + Lidocaine	20 mg/L + 100 mg/L	Ferreira *et al.* 2022a,b

[a] Concentrations for benzocaine and 2-Phenoxyethanol are less well investigated for use in euthanasia for zebrafish, but it is generally accepted that they are safe to use as 5-10x of the anaesthetic dose.

[b] Isoeugenol was recently demonstrated to be acting more as a local anaesthetic than systemic anaesthetic (Machnik *et al.,* 2023). Therefore, it should not be used for euthanasia.

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6.1.6.2. Hypothermic shock

The euthanasia method of hypothermic shock, also referred to as "rapid chilling" or "rapid cooling", describes the induction of death by rapid transfer of the fish from the long-term adapted husbandry temperature (usually 26-28° C for zebrafish) to ice-cold water. It should be clearly differentiated from the term "hypothermia" referring to a gradual and slow decrease in temperature to immobilize poikilothermic animals or having a subnormal body temperature. The method is considered as less stressful, faster and more reliable as an overdose of anaesthetics (Matthews and Varga, 2012) and is widely accepted for zebrafish as well as other small, warm-water laboratory fish, and several countries, including the USA (Leary *et al*., 2020; NIH, 2020) and Canada (CCAC, 2020) even regard it as the preferred method of euthanasia.

Fish as poikilothermic animals are somewhat adapted to changes in the body temperature as this can occur in the natural environment (Donaldson *et al*., 2008). As a physiologic reaction to cold environment, most fish reduce body activity including neuronal activity by a reduced blood flow to the Central Nervous System (CNS; Van Den Burg *et al.,* 2005). While cold-water fish species do express antifreeze proteins when exposed to cold water temperatures as a constant situation to inhibit ice crystal formation in the tissue, no temperature functional antifreeze proteins have been described in zebrafish. To date no cold sensitive nociceptors have been described in different fish species like trout (Ashley *et al*., 2007).

Concerns about the method are usually expressed based on conclusions drawn from data from fish species adapted to and favouring much lower temperatures than fish traditionally used in laboratory settings for biomedical research like zebrafish or medaka. Main concerns were: it is too slow, fish secret stress hormones, ice crystals are formed in the tissue and fish might be only unconscious or immobilised by the cold and are not effectively killed. Current literature available for fish housed in sub-tropical to tropical water temperatures dispels these concerns, especially when comparing hypothermic shock to other accepted methods of euthanasia for fish like the overdose of anaesthetics, which is the only applicable method for small-sized fish in the laboratory environment.

Studies available on zebrafish (Wilson *et al*. 2009) and bony breams (Blessing et al., 2010) confirm that rapid chilling induces loss of consciousness (defined by loss of swimming ability as well as cessation of opercular beat rate) which is reached very quickly within up to 10 seconds, usually even much quicker (Wilson *et al*., 2009; Ferreira *et al*., 2022a,b). Compared to overdose of anaesthetics (up to 1 min), this reduces the time of conscious perception drastically. Already with a decrease in temperature to 11°C a reduction in neuronal activity was noted when cooling was investigated for anaesthesia (Leyden *et al.*, 2022). To ensure death, exposure times between 30 s and 5 min were reported for zebrafish of 16 dpf to 90 dpf (Wallace *et al*., 2018), suggesting that the exposure period for zebrafish starting from 16 dpf should be 5 min minimum. Although Leary *et al.* (2020) recommends to always keep the fish in the euthanizing solution for 10 min after cessation of opercular beat, literature clearly shows that no recovery is possible after 5 min (Wallace *et al*., 2008; Wilson *et al.,* 2009, Ferreira *et al*., 2022a). The method has been applied effectively in fish of a body size up to 13.5 cm of body length (Blessing *et al*., 2010). Death should be confirmed after applying rapid chilling methods, *e.g.* rigor mortis and/or decapitation. Typical signs of stress like gasping or erratic swimming are reduced or absent when compared to an overdose of anaesthetics (Blessing *et al*., 2010). An increase in cortisol is detectable, but this increase is similar to

the levels measured in established methods of anaesthetic overdose like Tricaine (MS 222) or clove oil (Ferreira *et al*., 2022b). There was no formation of ice crystals due to the relatively short contact to cold water (few minutes), as the temperature of the water is still above freezing point (Wilson *et al*., 2009). Histological integrity of the tissue is less affected compared to chemical methods of euthanasia (Ferreira *et al*., 2022a). There must be no risk of direct contact of the fish to the crushed ice to avoid skin damage. Incubation of fish directly on crushed ice instead of water is lethal but will prolong the procedure because the contact area for the cold convection is reduced and the animals will suffocate additionally. Compared to an anaesthetic overdose, the rapid chilling method is at least similarly or even more reliable as there is no recovery, as demonstrated by placing fish classified as dead into husbandry water and observing whether they will regain any signs of vitality (Wilson *et al*., 2009; Blessing *et al*. 2010; Ferreira *et al*., 2022a,b). To ensure death, most studies include also time series of exposure after stop of the opercular beating, before re-placing the fish in housing water. Time ranges reported do last from 30 s (Wallace *et al*., 2018) to 2 min (Ferreira *et al*., 2022a; Wilson *et al*., 2009).
While it was shown also in larger poikilothermic animals (toad) that the body core temperature follows rapidly the ambient temperature (Shine *et al*., 2015) thereby, never reaching a difference between these two values of more than 1°C at any time point and thus indicating that the method is not slow in effect, data from bony bream do show a dependency between size of the fish and the onset of effect (Blessing *et al*., 2010). Therefore, the maximum size of fish where the method can be applied should be limited to a size where data confirm a safe and quick effect. The method has been applied effectively in fish other than zebrafish of a body size up to 13.5 cm of body length (Blessing *et al.,* 2010). The method is effective for zebrafish and seems similarly effective in other small (approximately 5 cm) tropical fish species.

The rapid transition to very cold water disrupts vital physiological and metabolic functions causing death. For this process, the temperature gradient between the adapted husbandry temperature and the cold water of rapid chilling is essential. The critical thermal minimum temperature is at least 20°C below the adapted temperature. A smaller difference of temperatures may not result in a hypothermic shock due to fish's capacity to adapt to the new decreased temperature. This seems to be a consistent pattern as it is quite similar for different fish species (Currie *et al.,* 1998), indicating that the method can be considered suitable for a variety of fish with characteristics similar to zebrafish (Danio rerio): body size ≤ 5 cm, husbandry temperature > 24°C, temperature of rapid chilling ≤ 4°C.

It should be realised that the method of performing euthanasia of (zebra)fish is highly dependent on the life stage of the zebrafish. Limitations of the method have to be considered when applying it to embryos before hatching, eleuthero-embryos (post-hatch until start of self-feeding) and early larval stages. Embryos and early larvae do not have developed gills and breathe via diffusion through the epidermis. This makes them more resistant to temperature changes (Köhler *et al*., 2017) as well as to the effect of chemical anaesthetics. For zebrafish larvae of at least 14 days (26°C -28°C husbandry temperature) rapid chilling was reliable when the animals were incubated for at least 20-40 min in the cold water (Strykowski and Schech, 2015; Köhler *et al*., 2017). For younger stages below 14 dpf, even longer periods are needed up to 60 min and even 12 hours (Wallace *et al*., 2018). Therefore, for stages before day 16, other methods should be applied as neither overdose of anaesthetics nor rapid chilling are reliable enough to

be regarded as safe (Wallace *et al*., 2018). For very early life stages (≤ 4 dpf) recently lidocaine hydrochloride (1 g/L) buffered with sodium bicarbonate (2 g/L), and mixed with ethanol (50 mL/L) was suggested to be the most suited anaesthetic treatment for euthanasia (Mocho *et al.,* 2022).

Various protocols are available in the literature. In general, rapid cooling is achieved by submerging the fish in ice-water (*e.g.* one part water five parts crushed ice) resulting in a temperature ≤ 4°C when ice remains present. Contact of the fish with the ice should be avoided. The number of fish euthanised should be monitored carefully in order to avoid a temperature rise of the water. When feasible clean water used for housing the fish may be preferable over other sources of water to keep water conditions constant. Good quality of containers should be used in order to preserve the low temperature. The temperature of ≤ 4°C should be ensured during the whole procedure. Similar to the use of anaesthetics, confirmation of death of the fish shall be determined after the use of rapid chilling for euthanasia of zebrafish.

Conclusions

Commonly used methods for euthanasia of zebrafish are an overdose of anaesthetics and hypothermic shock, also known as rapid chilling (WoE strong). Rapid chilling is considered a reliable and safe method of euthanasia in zebrafish, although it is highly dependent on the life stage of the zebrafish (WoE strong). When compared to other methods authorised in Annex IV of EU Directive 2010/63 there are indications that this method does not cause more stress or suffering. The mode of action for rapid chilling is a physical disruption of body functions that seems similarly effective in other small (maximum size approximately 5 cm) tropical fish species. It might also be considered appropriate for fish in general as long as they are housed with temperatures equal to or above 24°C consistently. The critical thermal minimum temperature of the water should at least be 20°C below the husbandry temperature. A proper protocol should be followed ensuring that no direct contact of the fish to the crushed ice is possible, and a sufficient exposure time of 5 min for animals of 16 dpf and older before final confirmation of death (WoE strong). Because for younger stages much longer times are needed, other methods than rapid chilling are recommended to be applied for zebrafish of 5 dpf to 15 dpf, *e.g.* an overdose of anaesthesia followed by decapitation and/or maceration (WoE strong). The following conditions should apply when rapid chilling is used as method for euthanasia of zebrafish (*Danio rerio*): age ≥ 16 dpf, body size ≤ 5 cm, husbandry temperature equal to or above 24°C, temperature of rapid chilling ≤ 4°C, allowing a temperature difference of at least 20°C. The temperature of ≤ 4°C should be ensured during the whole procedure. Similar to the use of anaesthetics, confirmation of death of the fish shall be determined after the use of rapid chilling for euthanasia of zebrafish.

As the mode of action is a physical disruption of body functions that seems similarly effective in other fish species, it might also be considered appropriate for tropical fish in general, as long as they are of similar size and housed with temperatures consistently equal to or above 24°C (WoE weak). In addition, it should be verified that intended fish species do not perceive cold as painful, and they do not express anti-freeze proteins. When the use of hypothermic shock is not feasible, the euthanasia should be performed by other methods as listed in Annex IV (2).

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6.1.7. Recommendations

- It is recommended to regularly monitor the water quality for a variety of parameters including temperature, salinity, alkalinity and water hardness, pH, presence of nitrogen compounds, and oxygen. Depending on the parameter, they may be measured and adjusted daily (temperature; pH) weekly (conductivity; nitrogen), or monthly (general hardness; oxygen). A temperature range of 24°C - 29°C is recommended, with an optimum temperature of 28°C, as is currently common practice. The various parameters for water quality are presented in Table 6.3 in more detail.
- In view of the recommended water temperatures, the temperature range (21-25°C) as presented in some OECD test guidelines (*e.g.* OECD TG 203 the Fish Acute Toxicity Test) is considered not in line with current scientific practices for housing conditions for zebrafish. In cases where lower temperatures are not specifically required for the performance of the test methods, they may need to be adapted regarding zebrafish housing conditions.
- The health status of the fish should be regularly monitored.
- Some form of enrichment is recommended such as physical enrichment like structural hiding places, visual enrichment like a picture affixed to the base of the tank, or placed outside the tank, and/or nutritional enrichment including live food. The so-called social enrichment (*i.e.* visual/olfactory access to conspecifics) of the presence of a stable group of conspecifics is also important because zebrafish are a shoaling species. When placing physical attributes inside a tank, the composition of the materials should be considered in regard to and how it might affect cleaning/sterilization, and/or possible release of potential toxic components.
- Studies have tended to focus on welfare issues associated with higher, rather than lower, stocking densities; the evidence suggests that lower stocking densities could

be a challenge from the perspective of social enrichment (*i.e*. the presence of conspecifics and welfare implications of small social groups). The evidence suggests that adult zebrafish should be kept in conditions that are neither overcrowded nor underpopulated, and the consensus is that the optimal stocking density is 5 adult fish/L. In order to allow shoaling, a minimum of 5 fish/tank is recommended, whereas the maximum is considered 10 fish/L. Considering the stocking density of 5 fish/L, the tank size and shape should allow the fish to perform their natural behaviour and swimming activity.

- A specific tank size cannot be recommended, as volume and fish density are critical parameters. There is a general consensus that the optimal stocking density is 5 fish/L while a maximum of 10 fish/L is reasonable.
- As zebrafish is a shoaling species, prolonged single housing is not recommended, but can be required during a limited period for specific reasons. Visual/olfactory access to conspecifics should be a minimum requirement for individually housed fish. In addition, enrichment could be provided similar to the situation in the other tanks of the facility when fish are individually housed.
- Hypothermic shock, also known as rapid chilling, is considered a reliable and safe method of euthanasia in zebrafish. When compared to other methods authorised in Annex IV of EU Directive 2010/63, with the current scientific knowledge there are no indications that this method causes more stress or suffering. As the mode of action is a physical disruption of body functions that seems similarly effective in other tropical fish species, it might also be considered appropriate for fish in general as long as they are housed with temperatures above 25°C consistently.
- A proper hypothermic shock protocol should be followed ensuring that no direct contact of the fish to the crushed ice is possible, and a sufficient exposure time of 5 min for animals of 16 dpf and older before final confirmation of death. Because for younger stages much longer times are needed, other methods than rapid chilling are recommended to be applied for zebrafish of 5 dpf to 15 dpf, *e.g*. an overdose of anaesthesia followed by decapitation and/or maceration. The following conditions should apply when rapid chilling is used as method for euthanasia of zebrafish (*Danio rerio*): age ≥ 16 dpf, body size ≤ 5 cm, husbandry temperature equal to or above 24°C, temperature of rapid chilling ≤ 4°C. Otherwise, the killing should be completed by other methods as listed in Annex IV (2).
- As the mode of action is a physical disruption of body functions that seems similarly effective in other fish species, it might also be considered appropriate for tropical fish in general, as long as they are of similar size and housed with temperatures consistently equal to or above 24°C. In addition, it should be verified that intended fish species do not perceive cold as painful, and they do not express anti-freeze proteins (which might be assessed *in vitro).*

6.2. Passerine birds

6.2.1. Introduction

Directive 2010/63/EU Annex III on *Requirements for Establishments and the Care and Accommodation of Animals* currently includes accommodation parameters for domestic fowl, domestic turkeys, quails, ducks and geese, pigeons and zebra finches. This encompasses the majority of avian species used in research and testing in the European Union; however, a need has been identified to define standards for some additional species of passerine bird. In addition, the abovementioned annex of the Directive contains a number of general requirements for the housing and care of animal species, including birds.

Statistics on experimental animal use produced by Member States categorise birds as either domestic fowl or 'other' species (ALURES database, European Commission, 2022). Most birds used in research and testing are domestic fowl; official UK statistics also listed domestic turkeys and quail separately until 2013[6]. According to ALURES, there were almost 125,000 uses of 'other' birds in the EU and Norway in 2019; 60% were for basic research, of which the majority of uses (80%) were for ethology, animal behaviour or animal biology research. The great tit (*Parus major*) and blue tit (*Cyanistes caeruleus*) were the two most used 'other' birds, after the turkey, according to information provided by the Member States to the European Commission.

A 2010 review of passerine bird use in research estimated that over 300,000 individuals were used in experiments worldwide annually. The review identified publications on 40 different passerine species, with the three most commonly used being the zebra finch (*Taeniopygia guttata*), the European starling (*Sturnus vulgaris*), and the house sparrow (*Passer domesticus*). Parids, corvids, various finches and American sparrows accounted for many of the others (Bateson and Feenders, 2010). Passerines are used in fundamental research, for example to study neural, sensory and cognitive aspects of their song, including vocal learning (Benichov *et al.,* 2016; Polzin *et al.,* 2021). Passerine species are also used to study the physiology of flight and navigation, cognition, foraging and behaviour (Thorogood *et al.,* 2018; Halfwerk and Van Oers, 2020; Aronsson and Gamberale-Stille, 2021; Sam *et al.,* 2021; Tomotani *et a.l,* 2021). For a review of house sparrow use in basic and applied biology, including metabolic, immunological and genetic studies, see Hanson *et al*. (2020). Other, less common uses include ecotoxicity testing (Werner *et al*., 2021).

The avian order Passeriformes is characterised by a specially structured palate, special syringeal anatomy, a distinct insertion of the forearm muscle, sperm with coiled heads and a foot with three toes pointing forward and one backwards which also is capable of independent action.

The order includes over 6,500 species, with diverse behaviour, physiology and ecology, representing over half of all known species of birds. Only a limited number of species are, however, used for research and need to be held in captivity. This Opinion will be restricted to the species most commonly held; house sparrows (*Passer domesticus*),

[6] gov.uk/government/collections/animals-in-science-statistics

starlings (*Sturnus vulgaris*) and great and blue tits (*Parus major* and *Cyanistes caeruleus*).

The recommendations presented in the Opinion are for the housing and care of birds used in scientific procedures regulated by Directive 2010/63/EU. They are based on an approach of considering the natural history and behaviour of each species or group of animals, using the literature, current good practices and expert judgement to determine which features of the natural environment should be replicated, as far as practicable, within the laboratory. The recommendations provided in this Opinion are to help ensure compliance with Directive Article 33 (1b), which requires Member States to *ensure that any restrictions on the extent to which an animal can satisfy its physiological and ethological needs are kept to a minimum*.

In this Opinion, 'captivity' is defined as holding birds within an enclosure (*e.g.* a cage or an aviary). Bird species already included in Annex III and commonly used in research will mainly have been bred in captivity and are likely to be humanely killed, using a technique listed in Annex IV, following procedures. In contrast, passerines such as sparrows, starlings and tits are more likely to be wild-caught or bred from parents captured in the wild. They may also be re-released to the wild, following short-term captivity either as part of a protocol or following procedures (Bateson and Feenders, 2010).

The fact that Passerines may be re-released to the wild makes it necessary to define short-term captivity within this Opinion. This is primarily for animal welfare reasons, because wild-caught birds can exhibit high levels of stress for a period of time if they are immediately placed into large enclosures, where this stress can easily lead to panic flights. As birds do not yet know the boundaries of the new enclosure, there is a high risk for injuries. When kept short term, it is typically less stressful for birds to be kept in a smaller space, with the addition of a lack of opportunity for flight and thus less injury. It may also be necessary to hold birds until it is safe to release them, for example to avoid predation risks at certain times of day or at unfavourable weather conditions.

There is no empirical evidence with respect to bird health or welfare which indicates when a given captivity period can be defined as 'short-term' (*e.g.* 24 hours). For example, the British Trust for Ornithology implements a 24-hour limit for holding birds within its bird ringing scheme. This is in place for practical reasons, to ensure consistency and to avoid any impact of captivity on behaviour or survival rates (N. Bugg, pers. comm.). A period of 24 hours was also chosen as constituting 'captivity' in Bateson and Feenders (2010). Moreover, a recent review of guidance on defining 'short term' accommodation for animals, in a range of sectors, has reported both practical and physiological justification for 'short term' being up to one circadian cycle, *i.e.* up to 24 hours (Warwick *et al.,* 2023). This Opinion therefore defines 'short term' as a period of 24 hours, and the species-specific standards set out below apply whenever birds are held for periods in excess of 24 hours. However, even when birds are held for shorter periods of time, animal welfare needs must be met.

There may be reasons to temporarily hold birds in smaller enclosures (*e.g.* in a test arena, Skinner box or metabolism cage for scientific purposes). The Directive permits Member States to allow exemptions from the requirements of Annex III for scientific, animal-welfare or animal-health reasons. If a project includes holding individuals in smaller enclosures exceeding 24 hours, this may be regarded as a procedure (*i.e.*

reaching the minimum threshold of pain, suffering and distress as defined in Article 3(1)) which should be included in a project authorisation application to the Competent Authority.

Similar to other birds, consideration should be given to avoiding capturing and using passerine birds at times when they would be breeding or migrating (the latter depending on the species), unless this is necessary for scientific reasons.

This document should be read and used in conjunction with the background information to the sections of the current Annex III of Directive 2010/63/EU that address birds. In addition, the Council of Europe published in 2003 a report on principles for housing and care of laboratory birds, particularly around the needs for a good quality and quantity of space, the desirability of outdoor access wherever practicable, and the need for social housing and environmental enrichment (Council of Europe, 2003). Although this report was published in 2003, the principles within it still hold true.

Conclusion

A description of short-term holding of birds is proposed, as birds may be re-released to the wild. Both practically and physiologically 'short term' can be justified as being up to one circadian cycle, *i.e.* up to 24 hours. This Opinion therefore defines 'short term' as a period of 24 hours, and the species-specific standards set out in this Opinion apply whenever birds are held for periods in excess of 24 hours (WoE moderate to strong). However, even when birds are held for shorter periods of time, animal welfare needs must be met. A maximum of 24 hours holding should be sufficient, to allow holding overnight, if necessary, for example to avoid predation risks at certain times of day, or to wait for unfavourable weather conditions to end.

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6.2.2. Starlings (*Sturnus vulgaris*)

Background and rationale

This section of the document largely follows, and is based upon, a chapter on the European starling written by Melissa Bateson of Newcastle University, in the forthcoming 9th edition of the UK Universities Federation for Animal Welfare (UFAW) *Handbook on the Care and Management of Laboratory and Other Research Animals* (Bateson, 2023). We strongly recommend that all those responsible for housing, caring for or using starlings in research consult this chapter, which includes further detail on all the topics below and also includes guidance on refining common laboratory procedures.

Natural history and behaviour

The European starling (*Sturnus vulgaris*), currently occurs worldwide apart from Antarctica. The species is adapted to foraging on short grass and nesting in cavities, so it is common in farms and built-up areas. Most populations are migratory, *e.g*. some birds from north-eastern European populations over-winter in Iberia and Africa. Immature birds show a fairly complex migration behaviour, with considerable migration activity after fledging and before the autumn moult. The relatively long and pointed wings of the starling are an adaptation for fast flight (Bateson, 2023).

Starlings are primarily adapted for terrestrial foraging by walking on the ground and probing the bill into the soil to find invertebrates. They will perform this important natural behaviour in the wild and in captivity. Wild individuals also eat fruit such as apples, cherries and grapes, and animal feed such as pig pellets, which can conflict with human interests.

Starlings are highly sociable throughout the year. In winter, they form large feeding flocks and communal roosts that may number thousands of birds. Starlings are known for their spectacular murmurations, in which flocks of birds fly tightly together and change direction in a closely-coordinated manner. The species is highly vocal, and both sexes sing apart from during the breeding season, when only the males sing. Their song is complex and they are capable of learning new songs, and mimicry, throughout their lives (Bateson, 2023).

The starling does not have a strong social structure, but dominance hierarchies form in captivity, in which males are dominant to females and adults to juveniles (Bedford *et al*., 2017). Individuals may defend preferred perching positions or feeding sites, and birds may fight by gripping with the feet and stabbing with their bills, usually without serious injury (Bateson, 2023).

In order to minimise restrictions on the extent to which starlings can satisfy their physiological and ethological needs, their housing standards need to allow: adequate space and height for flight and group housing appropriate numbers of birds; perching; natural foraging behaviours; and sufficient resources to minimise competition. All of this was taken into account when defining the minimum standards recommended in Table 6.6, and is further explained below.

Enclosures

Wild starlings are estimated to travel up to 20 km a day between feeding and roosting sites (Feare, 1984). To facilitate flying and walking exercise and desirable natural behaviours, group housing in large, outdoor aviaries with environmental enrichment is the ideal. Outdoor housing also permits natural light and reduces feather damage, whilst minimising the need for disturbance from human caretakers. Effective protocols will need to be in place for observing and catching the birds, and allowances made for the fact that environmental conditions will be difficult to control (Bateson, 2023).

If outdoor aviaries are not feasible, starlings may be housed indoors with a good quality and quantity of space, and with special attention paid to lighting regimes as set out below.

Enclosure dimensions and layout

Enclosures shall be long and narrow (for example 2 m by 1 m) to enable birds to perform short flights. It is clear that small enclosures are unsuitable for starlings. For example, very small cages (e.g. ~0.15 m^3) are associated with abnormal behaviour (*e.g*. somersaulting stereotypies) and 'pessimistic' cognitive biases that could indicate anxious or depressed states (Matheson *et al.,* 2008; Brilot *et al*., 2010; Feenders and Bateson, 2011). Starlings housed in groups of up to six, in small cages of ~0.05 m^3 displayed decreased preening and increased agonistic behaviour and heart rate, indicating acute stress (Nephew and Romero, 2003). Furthermore, a larger enclosure will reduce the risk of collisions due to migratory restlessness.

It was not possible to find any published, empirical evaluations of enclosure size for starlings. As a starting point, we referred to the minimum enclosure floor areas for pigeons in the current Directive 2010/63/EU Annex III (2 m^2, with a height of 200 cm) and consulted expert practitioners who keep starlings at universities and institutes in Belgium, Germany and the UK. Table 6.5 below summarises the practices used regarding enclosure dimensions and stocking densities.

Table 6.5 Enclosure dimensions and number of birds as currently used in some aviaries at research institutes.

Establishment	Floor area (m^2)	Height (cm)	Volume (m^3)	Number of birds (n)	Volume per bird (m^3)
1	8.4	250	21	20	1
2 (indoor)	3.5	220	7.8	15	0.5
2 (outdoor)	26	220	57.2	Up to 110	0.5
3	6	200	12	25 20 15	0.48 0.6 0.8
4	11.5	260	29.9	20	1.5
5 (indoor)	6.25	280	17.5	Thrush (*Turdus* spp.), similar size to starling	
5 (outdoor)	12	250	30	10 birds (thrushes)	

On this basis, it is suggested that 0.7 m^3 per starling is appropriate and feasible, and this is therefore recommended for starlings in Directive 2010/63/EU Annex III. This agrees with the average recommendation of 0.7 m^3 per starling made in the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement report on laboratory bird husbandry (Hawkins *et al*., 2001). According to practitioners, it is unusual to keep over 30 starlings, so the Table should reflect common practice; however, a space allocation of 0.15 m^2 per additional bird over 50 individuals is included, should the need arise (this is the same as for additional pigeons in the current Annex III). As an example, this would provide 100 birds with 0.39 m^3 each.

Starlings can produce large quantities of faeces. Although essential for animal health and hygiene, cleaning can be stressful for birds, with implications for both animal welfare and science, so reductions in cleaning frequency and disruption are desirable. Lower stocking densities and larger floor surfaces, mean that the large quantities of faeces produced by starlings are less concentrated, and thus require less frequent cleaning.

For pigeons and zebra finches, the current Annex III states that '*enclosures shall be long and narrow (for example 2 m by 1 m) to enable birds to perform short flights*'. It is recommended to use the same requirement for starlings housed in relatively small enclosures, given their flocking behaviour and need for flying exercise.

Group size

The minimum group size recommended by Bateson (2023) is four birds. There is evidence that starlings place a high value on social contact; isolated birds will forgo foraging to be close to a group of conspecifics (Vasquez and Kacelnik, 2000).

Feeding and watering

Starlings are omnivores. They eat invertebrates including insects and their larvae, soft fruits in autumn, and seeds and cereals in autumn and winter. Captive birds can be fed *ad libitum* on commercial poultry (chick or turkey) or game bird starter crumbs, or dry cat or dog food, provided the animal protein content is around 30% and the fat content

around 10% (Bateson, 2023). However, these diets are monotonous and should be supplemented with dietary enrichment.

Suitable supplements for starlings include live or dried invertebrates (*e.g.* mealworms or commercial insect-based mixes insectivorous birds) and low-sucrose fruit such as apple pieces, and berries, as the Sturnidae cannot digest sucrose, so high sucrose fruit should be avoided (Martínez del Rio, 1990). Foraging enrichment can be provided by creating a 'probing substrate' (see below) and placing invertebrate prey in this. Starlings do not appear to require grit (Bateson, 2023).

Although starlings are social and gregarious, they need to be provided with sufficient feeders and water sources for all birds to eat or drink simultaneously, to reduce the risk of aggression. It was not possible to find empirical evidence for food trough length per bird, but practitioners felt that the 5 cm allocated to pigeons in the Annex would also be suitable for starlings. It should be permissible for birds to be fed from circular feeders designed for poultry, using the circumference as trough length, as this is common practice and works well.

Identifying individuals

Starlings can be individually identified with plastic, rubber or aluminium leg rings (bands) after ~7 days post-hatch. Rings with an inner diameter of 4.2 to 4.3 mm are usually appropriate for starlings. Rings may be printed with numbers and/or come in different colours to aid identification without the need for catching. More than one ring can be accommodated on each leg to enable a larger number of birds to be identified from a distance (Bateson, 2023), which will be essential in large enclosures.

A microchip can be mounted on a leg ring to allow non-invasive automated identification of a bird when it is close to a microchip reader. This can also facilitate automated remote weighing of birds or automated recording of feeder visits (Bateson, 2023). This is also of value in large enclosures, as birds do not have to be caught.

Breeding animals

Starlings become sexually mature at one year of age. They will attempt to breed if housed in mixed-sex aviaries with nest boxes, exhibiting natural reproductive behaviours including singing, copulation, solicitations, nest construction, laying and incubation (see Calisi *et al.,* 2011). Male birds will also defend a territory immediately around the nest site during the breeding season. Although starling eggs will hatch in captivity, suitable food for starling chicks is not commercially available and they will die unless the adult birds are able to forage in natural grass. For this reason, researchers who require starling eggs or chicks usually obtain them from nest boxes in the wild (M. Bateson, G. Feenders, pers. comm.). Nest boxes should therefore not be routinely provided in mixed-sex housing in aviaries. However, it has been reported that wild-caught starlings may be more apathetic, and fearful, than hand-reared birds under some circumstances (Jayne *et al.,* 2013). If it is necessary and feasible to breed starlings, the adults should be able to access adequate areas of natural grass to enable them to forage for soil invertebrates that they can feed to the chicks.

Environmental conditions

Starlings evolved in temperate regions and their annual cycle of reproduction coincides with seasonal fluctuations in climate and food supply. Their physiological states, and behaviours, are sensitive to environmental cues including temperature and photoperiod

(Bateson and Feenders, 2010). They will do well in outdoor enclosures, in temperate climates, provided that some shelter is available. In the laboratory, temperatures of 14 to 20°C are common practice (Bateson, 2023) but it was not possible to find any empirical evidence regarding appropriate temperature ranges for starlings.

There is no information on the humidity requirements of starlings or the effects of changes in humidity (Bateson, 2023). If water baths are provided to encourage natural bathing behaviour (see below), these will also enable birds to increase the humidity within their micro-climate.

Seasonal onsets of breeding and moult are regulated by day length, so the photoperiod is very important for starlings (Nicholls *et al.,* 1988; Dawson, 2007). The natural seasonal cycle for indoor starlings can be maintained by altering the light schedule weekly, to correspond with outside day length.

It may be necessary to manipulate day length, for example to stimulate moulting. The welfare consequences of altering the natural seasonal cycle are unknown. For more on this topic, see Bateson (2023).

Light quality is also very important for good health and welfare in starlings. If natural light is not available, rooms should be lit with high-frequency fluorescent lights (>150 Hz) (Bateson, 2023). Conventional low-frequency fluorescent lights (100 Hz in Europe and 120 Hz in the USA) and cathode ray tube monitors are not suitable for rooms holding starlings, as it is believed that they may be able to perceive the flicker from these monitors (Bateson, 2023). There are several sources of evidence for this; in preference tests, starlings prefer high-frequency (>30 kHz) over low-frequency (100 Hz) lighting (Greenwood *et al.,* 2004); myoclonus (involuntary muscle twitching) is induced in starlings exposed to fluorescent lighting and cathode ray tube monitors flickering below 150 Hz (Smith and Evans, 2005); and birds are less active and have higher basal corticosterone levels under low-frequency lighting, suggesting that they may find it more stressful (Goldsmith *et al.,* 2005; Smith *et al*., 2005). There are also inconsistencies in mate choice in low- as opposed to high-frequency lighting (Evans *et al*., 2006).

As for all day-active birds, full-spectrum lighting should also be provided for starlings, *e.g.* by using specialist UV lamps. This is because starlings have an additional retinal cone type tuned to UV wavelengths, so housing them without UV light will deprive them of visual information, potentially preventing normal behaviours. Bateson (2023) cites evidence suggesting that starlings may prefer a light environment containing UV (Greenwood *et al*., 2002), and that being housed in a UV-deficient light environment causes higher basal corticosterone levels (indicating stress) and behaviour changes (Maddocks *et al*., 2002).

Environmental stimulation

Starlings need perches, water baths and foraging enrichment. Although the species is social and lives in groups, provision of all these items needs to be sufficient for all birds to use them simultaneously, to prevent competition and potential aggression (*e.g.* Boogert *et al.,* 2006). The enclosure should be of an adequate size to accommodate appropriate enrichment, whilst permitting free flight and increased activity associated with migration periods.

Enclosures should be provided with plenty of perches at a variety of heights; birds will usually spend most of their time on the highest perch available and this will be especially

valuable during husbandry, which is likely to be stressful. Males are dominant over females in captivity and occupy higher perches (Bedford *et al*., 2017). Perches that move (*e.g.* ropes) will help to conserve muscle strength and agility. Perches of varying thicknesses and textures (*e.g.* natural branches) will help maintain healthy claws and feet and enable bill-wiping (Witter and Cuthill, 1992). Perches should not be located directly over food and water dishes to avoid fouling.

It is important to consider the need to protect starlings from the elements, and to enable them to feel secure, in outside enclosures. These should include an area for roosting that is protected from the weather. Protective cover, *e.g.* in the form of evergreen trees or branches, is likely to reduce perceived predation risk in starlings, which may reduce anxiety and encouraging birds to use other available enrichment (Bateson, 2023).

Water bathing appears to be a strong behavioural requirement and is probably important for feather and skin maintenance (Brilot *et al.,* 2009). Trays of bathing water at least 20 cm in diameter and not more than 3 cm deep should be provided, and will need to be replaced daily due to fouling (Bateson, 2023). Starlings will attempt to bathe in their drinking water unless suitable baths are provided, and birds deprived of bathing water show increased signs of predation-related anxiety (Brilot and Bateson, 2012).

Starlings will choose to work for food by searching for it in a substrate such as sand even if the same food is freely available (Inglis and Ferguson, 1986; Bean *et al*., 1999). Starlings will 'pay' the cost of having to open a heavily weighted door to access a cage housing with a turf probing tray, which shows that this foraging enrichment is highly valued (Asher *et al.,* 2009). It is therefore essential to provide a substrate for starlings to probe, in order to facilitate this vital natural behaviour. Ideally, the entire floor of the enclosure should be covered with a substrate such as bark chippings, but if this is not possible, trays of sand, bark chips or turf should be provided that are large enough. For example, a tray can be filled with cocoa shell garden mulch and white blowfly (*Calliphora vomitoria*) maggots (Gill, 1994). The starlings housed at Establishment 1 (Table 6.5) are provided with a probing box for foraging (Bateson, 2023).

Catching and handling

It is possible to catch birds effectively, and without causing significant stress, in large enclosures, provided that there is a good protocol in place for catching birds and staff are well trained, competent and empathetic. Starlings will not fly in the dark, so it is possible to turn off the room lights and use a small torch to locate birds before capturing them using a net with padded edges. If there is a requirement for birds to fly from one enclosure to another, this can be achieved by turning off the lights in the original enclosure and allowing the birds to fly into an adjacent, lit holding facility. Starlings can also be trained to enter a small transport cage by reinforcing this behaviour with a preferred treat such as mealworms (Bateson, 2023).

Health and welfare checks

Effective health monitoring and surveillance can easily be achieved when birds are housed in large enclosures – it can be argued that an individual with poor health or welfare can be identified more quickly when animals are better able to display a wide range of behaviours. For example, using the water bath, and singing, can be used as indicators that welfare is good (E. Jonckers, pers. comm.). An example assessment protocol shared with the SCHEER includes knocking on the animal room door before entering, then standing completely still and watching the birds fly and interact with one

another. The observer pays attention to posture, perching position, feather condition, any 'grounded' birds, and the presence of any blood or diarrhoea on the walls, perches or substrate. Every second week, each bird is caught, weighed and examined, including noting the condition of the feathers, beak and tongue, legs, feet and claws (as practised in Establishment 1 of Table 6.5). This works well in an enclosure with a volume of 21 m^3.

Disease surveillance is also essential in outdoor housing, because starlings can carry zoonotic pathogens. It has been reported that most bacteria in the droppings of wild starlings did not belong to the specific types most often found in humans, suggesting that starlings are unlikely to present an infection risk for staff (Gautsch *et al*., 2000). However, avian influenza (AI) can occur in wild starlings, but with mild symptoms that could go undetected (Perkins and Swayne, 2003; Ellis *et al*., 2021). Based on a visual health check that might show indications for disease, a more extensive clinical investigation may be performed. In some aviaries, incoming starlings are routinely screened for common pathogens including Salmonella, Yersinia and coccidia; also, newly caught birds are isolated for further screening and parasite treatment. It is advisable that incoming birds are quarantined, with enhanced biosecurity, for four weeks (Bateson, 2023).

Conclusions

In order to meet the species-specific needs of starlings as sociable, active birds, starlings should be housed in appropriate groups and given environmental stimulation that facilitates desirable, natural behaviours (WoE strong). Therefore, a minimum group size of four starlings is strongly recommended (WoE strong). Terrestrial foraging for invertebrates, flight, water bathing and perching are all essential behaviours for starlings. It is therefore important to ensure that enclosures are large enough to contain sufficient resources, and space, to permit these behaviours and minimise the risk of aggression. Enclosures also need to be of adequate size to ensure that enough birds can be group housed, to promote social behaviour and synchronised flight, yet with a low enough stocking density to avoid the rapid build-up of faeces, which would increase cleaning frequency and cause the birds avoidable stress. The proposed engineering standards are considered feasible and achieve a reasonable compromise between the needs of starlings and humans (WoE moderate to strong).

Table 6.6 shows recommended housing conditions for starlings as based on the information presented above (WoE moderate to strong). Relatively small enclosures should be long and narrow (for example 2m by 1m) to enable birds to perform short flights.

Table 6.6 Recommended enclosure conditions relative to number of starlings present.

Group size	Minimum enclosure size (m^2)	Minimum height (cm)	Minimum length of food trough per bird (cm)	Minimum length of perch per bird (cm)
4 to 6	2	200	5	30
7 to 12	4	200	5	30
13 to 20	6	200	5	30
For each additional bird	0.25	200	5	30

between 21 to 50				
For each additional bird above 50	0.15	200	5	30

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6.2.3. House sparrows (*Passer domesticus*)

Natural history

House sparrows *Passer domesticus* are small songbirds native to Eurasia and northern Africa, which have been introduced and established on every continent bar Antarctica (Saetre *et al.,* 2012; Nakagawa and Pick, 2016; Hanson *et al*. 2020). House sparrows may be one of the most widespread birds of the world, in large part due to them living in close association with humans, typically in rural areas like farms, but more also in urban habitats (Saetre *et al.,* 2012). House sparrows are often found on farms all around the world, foraging in stables, barns, and other human shelters, and are even well known to even enter cafés and houses in search for food (Hanson *et al.,* 2020). Oftentimes the nests are located indoors, too, if access allows. The adults feed on grains, seed, and left-over human food and animal feed, while the young are fed insects by their parents until after fledging and leaving the nest (Anderson, 2006).

Male and female house sparrows are of equal size, but their plumage differs by sex. Males have a distinctive black bib, and black eye mask which females lack (Anderson, 2006). This plumage trait was hailed as a text-book example for signalling social dominance, but recent meta-analyses across several populations and datasets failed to support this notion (Sánchez-Tójar *et al*., 2018). However, the male ornament is positively associated with age (Nakagawa and Burke, 2008).

House sparrow females live on average for 3.4 years, with males living on average 0.4 years longer (Schroeder *et al.,* 2012a). The maximum observed lifespan in captivity is 13 years (Schroeder, unpublished data), while wild birds have been observed to reach 9-13 years (Klimkiewicz and Futcher, 1987; Schroeder *et al*., 2012a).

These group-living birds typically form socially monogamous pair bonds, and are hole-nesting breeders. Males reduce the size of their testes over winter, and when the testes grow again in spring, the males are become more interested in copulation and other reproductive behaviours. In the presence of females, males will then start building nests and display to females. They typically choose openings under the eaves, in walls, or other sheltered cavities for their nest, but also willingly accept nest boxes (Anderson, 2006). The male builds a nest in the cavity, which the female will inspect before she chooses one. Cavities may be re-used for multiple broods per breeding season by the same pair, with up to 6 attempts per season (Westneat *et al.,* 2014). The female will lay between 3 and 6 eggs, approximately one per day (Westneat *et al*., 2014). Males and females both care for the brood, taking turns incubating the brood for approximately 14 days, after which the chicks hatch, all typically within 24 hours. Then, both parents provide the young with food and warmth, visiting the nest on average between 7 and 8 times per hour with food (Schroeder *et al*., 2012b; Schroeder *et al.,* 2016), depending on food availability, age and number of the chicks, and daylength. Loud noise can be

detrimental to successful provisioning (Schroeder *et al.,* 2012c). Chicks will fledge at approximately 14 days old, after which they will often remain in a sibling/family group (Anderson, 2006).

House sparrows are not migratory and may use their nests also in the winter for shelter at night, where they mostly sleep singly, often in the nest that they have bred in during the summer, or one in close vicinity. Socially monogamous pairs may stay together across years and can be found sleeping in adjacent nest boxes in winter (Sánchez-Tójar *et al*., 2017). Young birds may prospect multiple nest boxes for appropriate sleeping locations, with older birds are more territorial to their, often better sheltered, nest boxes for longer times (Sánchez-Tójar *et al*., 2017).

Enclosure for adult birds

There are no recommended guidelines available for the husbandry of house sparrows.

Layout and size

The following text has been informed by the combined experience of animal caretakers and researchers working with captive house sparrows (more than two decades of keeping house sparrows at the Max Planck Institute for Ornithology in Seewiesen, and in Radolfzell, and at Imperial College London), and research papers with house sparrows that mention housing conditions (Girndt *et al.,* 2017; Girndt *et al*., 2018; Matsushima *et al*., 2019; Simons *et al.,* 2019; Vargas-Pellicer *et al*., 2019; Plaza *et al*., 2020). House sparrows thrive in aviaries that resemble structured old-fashioned farm buildings such as stables. They do not require a lot of space but rather structure where they can form groups, hide from each other's view, and forage in crevices and niches. At the Max Planck Institute for Ornithology and at Imperial College London the layout and size of the aviaries was modelled after the former. Sparrows were/are kept in aviaries ranging from 90-120 cm wide and 270-400 cm long, with a height of 180-220 cm (see references above). One of these compartments can hold comfortably 10 birds, more in the presence of visual barrier (*e.g.* ceiling-length hessian cloth separating the ends of the aviary from each other).

For larger groups, these compartments are combined with each other, and larger areas can house more birds per area.

Table 6.7. Inventory of current housing conditions for house sparrows

Establishment	Floor size (area m^2)	Height (cm)	Volume (m^3)	Maximum number of birds	
				In presence of visual barrier	No visual barrier
1	0.9x2.7 (2.43)	190	4.37	15	10
1	1.8x2.7 (4.86)	190	8.75	35	20
1	2.7x2.7 (7.3)	190	13.12	60	30

2	4.5x5.0 (22.5)	180	40.5	200	n.a.
3	1.0x3.0 (3.0)	200	6.0	15	10
3	2.0x3.0 (6.0)	200	12.0	35	20
3	3.0x3.0 (9.0)	200	18.0	60	30
3	4.0x3.0 (12.0)	200	24.0	120	60

These stocking densities may temporarily be exceeded after hatching, until they become independent from their parents, usually after 6 weeks. Also, these periods with the presence of increased numbers will not typically cause welfare deficits, such as increased levels of stress or aggression.

Captivity by group size and individual housing

House sparrows are living in loosely arranged groups and do not fare well in isolation. Typically, for mixed sex groups, the initial group size should not be smaller than 6 birds. Mixed-sex groups with fewer than 6 birds are not recommended unless monitored closely because aggressive interactions can lead to injuries. There is some experience that housing mixed sex groups with lower numbers of animals (*i.e.* 2 or 4), is possible provided that an equal number of male and females is present, or contain fewer males than females. If injuries occur the aggressive individuals need to be identified and removed from the flock. Single sex groups should comprise at least 2 birds.

Individual housing may be needed for animal care reasons (*e.g.* quarantine or recovery), in which case birds fare well as long as they have sight and/or sound contact to other sparrows. Long-term individual housing is not recommended.

Individual identification, including sex

Typical recommendations for birds apply. Split rings with individual number engraved for individual identification are appropriate. For house sparrows, if used, RFID tags are better implanted under the skin than attached to the ring. This is due to the house sparrows' nature to explore small crevices where they may run risk entangling their feet in the environment.

Sex can only be identified visually after the moult in the first autumn after fledging, when the sexually-dimorphic plumage has developed fully.

Breeding/non-breeding

During the breeding season in the environmental conditions given, it is advised to provide house sparrows with nest boxes when in mixed-sex groups, because house sparrows will build nests and breed even if no nest boxes are available. To prevent breeding, sexes must be kept separately.

Breeding will generally only be successful in larger groups, in smaller groups than 6 they may become aggressive and this can lead to injuries. However, also smaller groups may be possible provided that the group has an equal sex ratio, or fewer males than females. During the breeding season, nesting material (*e.g.* coconut fibres, horse hair, etc) must be provided. It is advised to provide more nest boxes than males present, to reduce aggression. Furthermore, it is advised to leave the fledglings with their parents for extended parental care.

Environmental conditions

As sparrows are ubiquitous nearly all over the world, typical the outside environmental conditions where the sparrows have been caught are suitable for captivity. Sparrows fare well even in extreme cold temperatures – in aviaries exposed to ambient temperatures they do well even in -15ºC, if provided with non-frozen water. They appear to be more vulnerable to extreme heat, so it is advised to provide sufficient shade and water in temperatures above 30ºC.

Enrichments

Perches must be provided, as should regular sand- and water baths.

House sparrows require structure in their aviaries, *e.g.* hessian cloth (also called burlap) curtains that break up the line of sight. Further enrichments that help reduce aggression consist of providing hiding places and crevices, leafed branches, cardboard rolls to hide in, wooden pallets, hessian curtains alongside the wall where sparrows enjoy crawling behind. Care needs to be applied when choosing fabric for enrichment – fabric with long and robust fibres (*e.g.* nylon) should be avoided because sparrows will play with these and get entangled if they cannot bite through or rip the fibres.

Nest boxes can be provided year-round, but note the comment above that more boxes must be provided than males present to prevent aggression.

Capturing and handling of captive birds

Besides the general requirements as indicated in the Directive 2010/63/EU, no species specific handling of the animals is necessary.

Conclusions

House sparrows require an environment where they can form groups, hide from each other's view, forage in crevices and niches (WoE strong). This can be provided by enrichment objects with hiding places, and/or ceiling length hessian cloth providing visual barriers in the enclosure. The stocking density can be increased if a visual barrier is provided. When mixed-sex groups are housed, it is advised to provide house sparrows with nest boxes, because house sparrows will build nests and breed even if no nest boxes are available. Breeding can only be prevented by keeping the sexes separately. For single sex a group size of 2 animals is sufficient, while mixed sex groups should not be smaller than 6 animals, and have an equal sex ratio, or fewer males than females. Individual housing may be needed for animal care reasons (*e.g*. quarantine or recovery), in which case birds fare well as long as they have sight and/or sound contact to other sparrows. Long-term individual housing is not recommended. Recommended housing conditions are presented in Table 6.8 (WoE moderate to strong).

Table 6.8. Recommended enclosure conditions relative to number of house sparrows present.

Enclosure sizes			Number of birds in presence of visual barriers		Number of birds with no visual barriers	
Minimum floor area (m^2)	Minimum height (cm)	Minimum volume (m^3)	Maximum number of birds	Approximate minimum volume per bird (m^3)	Maximum number of birds	Approximate minimum volume per bird (m^3)
2.4	180	4.4	15	0.3	10	0.4
4.8	180	8.7	35	0.25	20	0.4
7.3	180	13.1	60	0.2	30	0.4
Add m^2 according to increased volume (0.11 m^2 per bird)	180	-	Above 60	0.2	Above 30	0.4

These stocking densities may temporarily be exceeded after hatching, until they become independent from their parents, usually after 6 weeks. Also, these periods with the presence of increased numbers will not typically cause welfare deficits, such as increased levels of stress or aggression.

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6.2.4. Great tit and blue tit (*Parus major* and *Cyanistes caeruleus*)

Introduction

In view of the limited availability of reviewed literature, this section of the Opinion is largely written based on discussions with a network of researchers that have kept or keep tits in captivity for scientific purposes. This community of researchers has shared their unpublished experiences on tit housing.

Natural history

Tits are little, agile birds with strong bills and short legs. The family of *Paridae* comprises 67 species typically inhabiting wooded terrestrial habitats in the Nearctic, Palaearctic, Oriental and Afro tropical regions. In Europe 9 species occur, with great tit (*Parus major*) and blue tit (*Cyanistes caeruleus*) being the most common and widespread ones. In this Opinion we concentrate on the European species *i.e.* great tit and blue tit.

Natural food for these birds predominantly consists of insects including larvae, spiders and other invertebrates. Outside the breeding season, seeds, fruits and berries are also taken, and buds in spring. More than all other tit species, the blue tit also feeds on nectar from willows. Due to their strong bill and socially learned skills, tits are able to open rather hard shelled seeds like those of sunflowers and many conifer species. Great tits and blue tits usually are among the most frequent visitors at bird feeders. Thanks to their natural curiosity and inquisitive behaviour, tits are also able to find new food sources, even man-made ones, such as opening milk bottles in the UK to reach the cream (Fisher and Hinde, 1949).

Tits build their nests in natural or artificial hollows, which they usually do not build by themselves. Great tits and blue tits show clear seasonal patterns. Tits regress their testes and gonads during the non-breeding period (Lambrechts and Perret, 2000; Silverin *et al*., 2008), enabling them to adapt to the winter conditions including changes in foraging conditions and temperature changes. Due to photoperiodic changes, especially long days, birds start to invest in reproductive function and gear up their reproductive system again from March onwards (Lambrechts and Perret, 2000; Silverin *et al*., 2008).

Tits are very territorial and do not tolerate other birds in their territory. Breeding pairs settle as early as October and may occupy territories until the brood has fledged, when they might start roaming in larger areas with family bonds staying together for up to 3 weeks. Sometimes a second brood may follow in the same season. Outside the breeding season tits usually engage in larger fission-fusion flocks (often mixed-species flocks with other tits, nuthatch (*Sitta europaea*), treecreeper (*Certhia sp.*) and goldcrests (*Regulus sp*.) roaming around through larger areas, sometimes performing short migrations. Especially in the Northern and Eastern range of the European distribution, great tits and blue tits leave their summer areas during some winters and migrate to milder areas within Europe. In central, southern and western Europe, adult birds (particularly males) stay in or close to their breeding territories all year round and remain locally dominant throughout winter. For those birds, tree cavities, nest boxes and other hollow-like shelters within their winter territories are crucial to survive cold winter nights. Generally, individual night roosts in hollows are used frequently all through the year, although the extent to which this happens varies between populations and species. Tits do not tolerate other birds roosting in the hollows in their territory in winter.

Tits are omnivorous birds, with a clear fluctuation in food preference throughout the season. This has partly to do with food availability, with arthropods being less available from the autumn onwards throughout the winter. Also, the lower temperatures during winter in seasonal habitats cause tits to change their food from protein rich to more fat rich diets, likely in order to adhere to the changing demands in fat storage (Krams *et al*., 2010).

Although tits are active during the day, they have a foraging peak early during the day to compensate for fat loss during the night and show another increase in foraging activity during the afternoon, in order to fatten up for the night. Most of their locomotion consists of climbing and hopping, interrupted by short flights. Even on migration, they do not fly larger distances but typically move over a few hundred metres from one shelter to the next.

Juvenile tits become independent from their parents after an extended period of parental feeding, both before and after fledging. Nestlings fledge when they have reached an age of about 18-21 days after hatching, after which the parents remain feeding their offspring for about seven to 14 days more.

Enclosures

There is much similarity in the way great tits and blue tits (both referred to as "tits" hereafter) are housed. Therefore, the proposed housing conditions can be generalised for the two tit species. The enclosure dimensions could also be valid for other smaller Passerines such as pied flycatchers (*Ficedula alba*), blackcaps (*Sylvia atricapilla*), stonechats (*Saxicola torquata*) and other tit species. However, some caution needs to be taken when translating the housing recommendations for the tits to other small Passerines, since their social, food and space requirements may deviate significantly.

Although wild tits can be seen interacting with other Passerines, they are not social species as is meant in the Directive. Therefore, they have special requirements regarding social and single housing. For tits, group housing in aviaries may be generally preferred throughout the year. However, depending on the season, wild tits show large variation in the extent and form of sociality. Males are especially known to not tolerate other individuals within a certain range during the pre-breeding and breeding period. Hence, although tits are found to group with other birds outside the breeding season in the wild, they do not form social bonds with these birds.

It was not possible to find published, empirical evaluations of enclosure size for tits. We therefore surveyed the scientific community with experience in tit housing. We asked them what enclosure sizes they were using, how many animals were housed in these enclosures and what their positive and negative experiences were with other enclosure sizes or bird numbers. In general, tits are housed in two types of enclosures. Birds are kept in smaller enclosures (Table 6.8) for a limited period (up to about 4 weeks and 2 months in one case). When institutes house tits for a prolonged period of time (*e.g.* for weeks or months), they usually house the tits in larger enclosures (Table 6.9).

The sizes of the small enclosures vary from 0.2 m^2 to 0.6 m^2 floor surface. Birds are always kept singly in these small enclosures for periods ranging from a few days to several months. At two institutes, the floor space of small enclosures was relatively small (<0.25 m^2). In one case birds were kept for a few weeks, in the other only for few days and both for behavioural testing. The general experiences with housing tits in enclosures smaller than 0.3 m^2 floor size were negative, with higher stress levels and more

stereotypic behaviours associated with stress. All experiences with housing tits in small enclosures suggests a minimal floor space of 0.30 m^2, with their width being about twice the length. A maximum of 4 weeks is suggested for this type of housing.

In one case at establishment 2, birds were kept together in smaller enclosures (1.8 m^2), though there were two separate compartments in these cages. At establishment 9, experience was gathered housing breeding pairs in double or triple smaller enclosures (1.35 m^2). Breeding success was much lower compared to housing in larger enclosures (Table 6.9), indicating suboptimal housing conditions. Other experiences are variable with these small enclosures with stereotypic behaviours observed. When birds are housed in cages for longer than about four weeks, stereotypic behaviours are sometimes observed. Table 6.8 shows the current practice for housing tits in cages at various research institutes in Europe.

Table 6.8 Small enclosures used in research facilities for solitary housing of great tits and blue tits

Establishment	Floor size (area m^2)	Height (cm)	Volume (m^3)	Number of birds (duration) /material – contact
1	0.80 x 0.41 (0.33)	50	0.16	1 (weeks) / solid with wire mesh – sound no visual
2	1.80 x 0.45 (0.81)	80	0.65	1 (2 months)
3	1.15 x 0.60 (0.69)	90	0.62	1 (4 weeks) / plywood, sound no visual
4	0.56 x 0.36 (0.20)	55	0.11	1 (4 weeks great tit: 3 days blue tit) / wire mesh – sound visual
5	0.80 x 0.45 (0.36)	35	0.13	1 (weeks) / solid with wire mesh – sound no visual
6	1.0 x 0.60 (0.60)	50	0.30	1 (weeks) / solid with wire mesh – sound no visual
	2.0 x 0.9 (1.8)	80	1.44	2 (weeks) / solid with wire mesh – sound no visual
7	0.81 x 0.50 (0.41)	40	0.16	not allowed anymore in Germany (Bavaria)
	1.22 x 0.5 (0.61	50	0.31	1 (weeks)/ solid with wire mesh – sound no visual
8	0.60 x 0.35 (0.21)	55	0.12	1 (few days) / plywood, sound no visual
9	0.90 x 0.50 (0.45)	50	0.23	1 (weeks; great tits and blue tits)/ solid with wire mesh front – sound and visual

Even in larger enclosures, (often referred to as aviaries or holding rooms by members of the scientific community) (Table 6.9), birds are often kept singly or in pairs for breeding purposes. This is done mostly to avoid aggression between individuals or because of practical reasons such as the ease of capturing individuals without having to stress the whole group, ease of welfare checks, and because data needs to be collected on single

individuals. Floor surfaces vary, but the heights of the aviaries are often between 1.8 and 2.5 meters.

Table 6.9 Enclosure sizes used in research institutes for single and group housing of captive great tits and blue tits

Establishment	Floor size (area m^2)	Height (cm)	Volume (m^3)	Number of birds (duration) – contact (m^3/bird)
10	1.2 x 3.4 (4.1) 1.6 x 2.5 (4.0)	250	10.3 10.0	1 or 9 (1 week) / wire mesh – visual sound (9:1.1 m^3 – 1:10.3 m^3)
4	2.0 x 1.5 (3.0)	200	6.0	1 (weeks) / solid with wire mesh front – visual and sound (6.0 m^3)
11	2.9 x 2.9 (8.4)	250 180	21.0 15.1	6-8 (months) / visual and sound (2.6/1.89 m^3)
6	3.9 x 2.45 (9.6)	217	20.7	8 (weeks)/indoor flights (2.6 m^3)
7	4.0 x 1.0 (4.0)	220	8.8	1 (months) / visual (wild birds) and sound (8.8 m^3)
8	3.0 x 4.0 (12.0)	200	24.0	12 (9 months) / inside room (2.0 m^3)
9	4.0 x 1.9 (7.6) 2.0 x 2.0 (4.0)	190 200	14.4 8.0	2-7 (months) / solid with wire mesh front – sound (7.2-2.1 m^3) 2 (months) / indoor flights (4.0 m^3)

On the basis of these experiences, it is suggested that if birds are kept in groups in aviaries or indoor holding rooms, the minimal space per bird that is needed is about 2 m^3 at 2 m height (Tables 6.8 and 6.9). This is assuming that there is enough opportunity to hide and perch space to sit, in order to avoid aggressive encounters. If birds are kept in breeding pairs (one single male with one female), the suggested minimal space per bird increases to about 4 m^3 (at 2 m height) per individual. Experiences are that floor space, together with the holding room height, determine the number of birds that can be housed, although a minimal height of about 1.8 meters is preferable.

Single housing

For tits in captivity, there is no preference for either being housed singly or in groups. As mentioned before, tits are territorial birds that do not tolerate other individuals when they are confined, except for in certain situations. Experiences with group housing have been mixed. Therefore, in most situations, single housing is preferable. Tits thrive well during single housing and birds show decreased levels of stress when housed individually when compared to the same birds during social group housing (Van Der Meer and Van Oers, 2015).

It is recommended to keep tits singly in smaller enclosures for the first 48 hours after capture, before putting them in larger groups. This is to enable effective monitoring of

food consumption and welfare during these first days. Easy access to water and food is necessary during this first period after catching the tits from the wild. Tits habituate typically in about 48 hours to captive housing conditions and these first 48 hours are crucial.

Great and blue tits can be hand reared in captivity (Van Oers *et al*., 2004), and the newly fledged birds should be kept in small groups in small wire mesh enclosures (3 - 4 birds, 0.1 m^2) after fledging, and singly housed after being able to feed independently (no later than 30-35 days after hatching). It is advised to house these birds singly, until juvenile moult (about 60 days after hatching). Mortality is generally much higher when they are kept in groups in larger enclosures immediately after gaining independence. The mortality in the wild is around 60% in the first week right after fledging (Naef-Daenzer *et al.,* 2001).

When individually housed, tits should always have auditory contact to at least one other conspecific.

Group housing

Groups always need to consist of one single sex, although males will not easily tolerate other males. The only exception is when one male and one female are housed in one enclosure during the breeding season. When groups are formed, they always need to enter the aviary at the same time. If extra birds need to be added to an existing group, it is advised strongly to remove the group first and put the whole new group in a new aviary. Groups will form stable hierarchies within a week.

Based on the information presented above, Table 6.10 present recommended enclosures for the housing of tits.

Table 6.10 Recommended enclosure conditions (cages and aviaries/holding rooms) relative to number of great tits or blue tits present

Group size	Minimum enclosure size (m^2) per bird	Minimum height (cm)	Minimum number of feeders	Minimum length of perch per bird (cm)
1[a]	0.30	45	2	120
1[b]	3.00	180	1	100
2-10[c] (single sex)	1.00	180	2	40
1 female + 1 male	2.00	180	2	100

[a] There can be three situations in which small enclosures may be used for housing. 1) Directly after catching, tits can be singly housed in small enclosures for a limited period of time (first 48h after catching the tits from the wild); 2) for juvenile birds, before their first moult; and 3) in all other situations for a maximum of four weeks.

[b] For a prolonged period of time.

[c] Larger group sizes than 10 animals may incidentally be housed for short periods, although this is not recommended in view of increased risk of aggressive behaviour.

Individual identification, including sex

Individual marking is possible with conventional bird rings made of metal or plastic on the bird's tarsus from the fifth day after hatching onwards. National institutions

organising scientific bird ringing provide lists with the most appropriate ring sizes for the various species.

In juvenile plumage, the sex of the tits cannot be inferred from coloration or morphology with large accuracy, unless somebody is very experienced (up to 90% accuracy). This is also true for most species in adult plumage except for the great tit, where adult males can be identified by the broad black colouration on breast and especially between the legs on the belly and – with experience – for the blue tit, where males have a deeper ultramarine blue crown and a wider eye-stripe (but see Scott, 1993).

Breeding vs non-breeding

Outside of the breeding season, adult birds should be housed in single-sex groups. During the breeding season, single pairs (one male and one female) can be housed in a large cage or aviary. No other birds can be allowed in these aviaries, since tits are highly territorial during this period. At least two nest boxes need to be provided, in order to allow both female and male to roost in a box during the night. More nest boxes are preferred, since females prefer to choose a nest box for building a nest. Females lay clutches ranging from 5-12 eggs and will restart laying after removal of full clutches. Eggs can be left for incubation by the female, but chicks should not be left to be reared by the parents since success is very low. This because chicks rely on green caterpillars to grow and to produce the coloration of their beaks. This is a signal for the parents to feed them. Without the green caterpillars they will not develop this coloration, which is a signal for the parents to stop feeding. Moreover, males can become aggressive to the female and the chicks, and rearing success if very low (based on experience in institute 9).

During the breeding season, birds can also be kept in single-sex groups in aviaries. No nest boxes should be provided in the case of female groups, since they may start building nests and laying eggs, also in the absence of a male. Birds can also be housed in individual cages during the breeding season as long as they have auditory contact to at least one other conspecific. This means that at least one conspecific (same or different sex) should be in the same room.

Environmental conditions

Tits tolerate temperatures well below zero and are also known to live in areas with extreme heat spells. Still, mild temperatures are optimal for the birds and heat seems especially stressful to them. Catching them from aviaries/cages at high temperatures is very stressful to them and they can even die. Therefore, enough cool places should be available when temperatures rise above 30^0C. Large temperature changes are also not tolerated very well.

As with other birds, tits can be very sensitive to lighting conditions. Preferably they should be kept under natural day and night cycles that follow the local day and night. Light intensities should be high enough to avoid shading in cages. Rooms should be lit with high-frequency fluorescent lights (> 150 Hz). See also the text that was written for starlings.

Humidity should preferably be above 20%, especially during moulting periods.

Tits will be less stressed when they are experiencing natural sounds. Strong noise should be avoided, such as slamming of doors, human activity or air conditioning sounds. For

example, white-noise was shown to affect tit foraging capability (Halfwerk and Van Oers, 2020).

Small birds in aviaries attract other animals that might be predatory to them. Rats are known to predate on tits during the night. This can be avoided by electric wiring or double mesh with space between the two mesh parts. Sparrow hawks are regularly seen to be around aviaries in several institutes. They hunt during the day and attack through the mesh. Double mesh will avoid casualties.

Enrichments

Cages for singly housing should typically allow birds to make small hops and flights between perches. They can consist of a wooden cage with at least three perches. They can have a wire mesh front and bedding that allows to take up moisture, in order to avoid fungal growth. A watering bath and at least one extra water supply should be available. A variety of food types should be provided at various places, to help prevent picky birds from avoiding certain food types or spaces in the cage. Dry food (for example egg food), life insect food (*e.g.* mealworms or wax moth larvae) and sunflower seeds or (crushed) peanuts, fruit (apple slices or berries) can also be provided. Foraging enrichment in the form of new food types works well for tits.

For wild caught birds, enough hiding places should be available both in cages as well as in aviaries. Enrichment in cages, such as hiding places, is necessary, although these hiding places should preferably be small and elongated. Experiences with small cardboard bird boxes or plastic tubes show that birds want to hide in these small places. Experiences with larger hiding places, where birds experience darkness (such as nest boxes connected to the cage) can lead to casualties. In those cases, birds prioritise fleeing and hiding over foraging, which should be avoided.

In aviaries, enough perching space should be available. Great tits will explore all parts of the aviary, but tend to be higher up than 1 meter in general. Evergreen trees such as conifers provide permanent hiding and roosting places. Nest boxes are roosting and hiding places as well, and as many should be provided as there are birds in the group. Fresh branches in spring and summer provide birds with insects and leaf buds to eat. Other possibilities for enrichment include opportunities for extractive foraging, places to hide seeds, paper to shred, things to crawl into or natural materials to manipulate. These materials should be chosen carefully so that the birds cannot become entangled in them.

Capturing and handling of captive birds

Tits can be caught by hand or using small capturing nets from small cages. Larger nets can be used in aviaries. If possible, the manipulation of lights can also be used to assist capture. When lights are switched off, tits will freeze and can be caught with more ease.

Healthcare

Disease surveillance is extremely important for tits since wild birds are known to carry a wide diversity of diseases (Holzinger-Umlauf *et al.*, 1997; Lawson *et al.*, 2012; Williams *et al.*, 2021). Two main health threats for tits in captivity are avian pox or avipoxvirus (Lawson *et al.*, 2012) and *Psittacosis* (Williams *et al.*, 2021), where the second is also a health threat for personnel. Avian pox is a virus causing external pustules or internal diphteria-like symptoms. Wild individuals are known to be able to recover from the symptoms, but in captivity the avipoxvirus is known to spread at much higher rates, without the chance of recovery. *Psittacosis*, ornithosis or parrot fever, is a bacterial

infection caused by the *Chlamydia psittaci* bacterium that is also known to cause severe pneumonia in humans.

Conclusions

Tits show very territorial behaviour and do not tolerate conspecifics in their territory. They are not truly a 'social species' and they have special requirements regarding both social and single housing. In order to avoid unnecessary stress for tits which migrate according to changing seasons, considerations might be made to avoid keeping those in captivity during the migration time. For tits in captivity, there is no strong preference for either being housed singly or in groups, but in most situations single housing is preferable. Groups always need to consist of one single sex, although males will not easily tolerate other males. The only exception is when one male and one female are housed in one enclosure during the breeding season. When groups are formed, they always need to enter the enclosure at the same time. In all cases, tits should have auditory contact with other conspecifics. Recommended enclosure sizes are presented in Table 6.11 below (WoE moderate to strong).

Table 6.11 Recommended minimal enclosure sizes (cages and aviaries/holding rooms).

Group size	Minimum enclosure size (m^2) per bird	Minimum height (cm)	Minimum number of feeders	Minimum length of perch per bird (cm)
1[a]	0.30	45	2	120
1[b]	3.00	180	1	100
2-10 (single sex)	1.00	180	2	40
1 female + 1 male	2.00	180	2	100

[a] There can be three situations in which small enclosures may be used for housing: (i) directly after catching, tits can be singly housed in small enclosures for a limited period of time (first 48h after catching the tits from the wild); (ii) for juvenile birds, before their first moult; and (iii) in all other situations for a maximum of four weeks.
[b] For a prolonged period of time.

There is much similarity in the way great tits and blue tits are housed, and the proposed housing conditions can be generalised for the two tit species. The enclosure dimensions could also be valid for other smaller passerines such as pied flycatchers (*Ficedula alba*), blackcaps (*Sylvia atricapilla*), stonechats (*Saxicola torquata*) and other tit species (WoE weak). However, some caution needs to be taken when translating the housing recommendations for the tits to other small passerines, since their social, food and space requirements may deviate significantly.

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Discussion

This Opinion sets out accommodation parameters and guidance for starlings, house sparrows, and great and blue tits. This complements Directive 2010/63/EU Annex III, which includes domestic fowl, domestic turkeys, quails, ducks and geese, pigeons and zebra finches. Other avian species are also used in research, testing and education, but in view of low numbers used, it is not currently deemed necessary, or practicable, to add them to the Annex. However, it is still essential to minimise any restrictions on the extent to which these species can satisfy their physiological and ethological needs when they are housed for use in procedures regulated by the Directive.

Housing, husbandry and care protocols for avian species not mentioned in Annex III or this Opinion should therefore be carefully researched and defined in consultation with a range of experts. Researchers in the field, user groups, attending veterinarians, animal technologists and care staff can all provide useful insights. In some cases, staff at zoos, animal collections and wildlife rehabilitation centres may also have useful experience and expertise that can help to optimise laboratory housing to better meet the animals' welfare needs. Useful general principles around good practice for housing passerines in the laboratory are set out in Bateson and Feenders (2010, see above). It should be noted that for other bird species, husbandry conditions are included in the UFAW Handbook on the Care and Management of Laboratory and Other Research Animals, 9th Edition (in press, 2023).

7. RECOMMENDATIONS FOR FUTURE WORK

Passerine birds

The information in the literature on housing conditions is limited. Authors should include relevant details of bird housing, husbandry and care in materials and methods sections of publications, or as supplementary material if necessary. For example, this could include enclosure length, width and height, diet, perch dimensions and materials, information about dust- and water baths, light quality and light/dark phases, methods for catching and welfare assessment protocols. This information is currently lacking in many publications, although the conditions it describes can profoundly affect animal welfare, and therefore the quality of the science. Providing adequate detail will enable more effective interpretation of results and conclusions, sharing of good practice, better replication of conditions by others, and allow systematic reviews of housing conditions and their impact on animal welfare and science.

Welfare assessment protocols for birds should be further developed, shared and used to provide objective information about welfare levels in different housing and husbandry conditions (*e.g.* in relation to group sizes and composition, single housing, enclosure sizes, enclosure sanitation regimes, methods for capturing individuals housed in the laboratory, etc.). It may be possible to do this in conjunction with ongoing housing and research, avoiding the need for separate studies.

8. REFERENCES

As this Opinion discusses a number of different subjects, the references are included after each (sub)chapter.

9. PUBLIC CONSULTATION

After the adoption (2 May 2023) and publication of the preliminary Opinion on the "Revision of Annexes III and IV of Directive 2010/63/EU on the protection of animals used for scientific purposes regarding accommodation parameters and methods of killing for zebrafish, and accommodation parameters for Passerine birds", a public consultation period was started from 12 May 2023 to 12 June 2023.

A total of 19 comments was received of which 15 regarding the text on zebrafish and four on the text of Passerine birds. Six organisations and one individual commented on the preliminary Opinion. Based on the comments the Opinion was adapted in several locations where appropriate. Especially the text on rapid cooling for the euthanasia of

zebrafish was extended with further considerations when using the rapid cooling protocol for euthanasia.

10. LIST OF ABBREVIATIONS

ALURES	AnimaL Use Reporting – EU System (EC)
BVAAWF	British Veterinary Association Animal Welfare Foundation (UK)
CCAC	Canadian Council on Animal Care (Canada)
CTmax	Critical temperature maximum
CTmin	Critical temperature minimum
dpf	days post fertilization
EC	European Commission
EFSA	European Food Safety Authority
EU	European Union
FELASA	Federation of European Laboratory Animal Science Associations (Belgium)
FRAME	Fund for the Replacement of Animals in Medical Experiments (UK)
LED	Light-emitting Diode
NIH	National institutes of Health (USA)
OECD	Organization for Economic Co-operation and Development (France)
RO	Reversed Osmosis
RSPCA	Royal Society for the Prevention of Cruelty to Animals (UK)
SCHEER	Scientific Committee on Health, Environmental and Emerging Risks (EC)
SWD	Staff Wording Document (EC)
UFAW	Universities Federation for Animal Welfare (UK)
WoE	Weight of Evidence