

Organisation/company	Country	Table of contents	Please indicate the line numbers of the text on which you comment, if appropriate	Please upload your file (max. 1MB per file)	Read the Privacy Statement: https://health.ec.europa.eu/document/61437b93-3572-4c98-905f-eba30377b0f7_en	If the case, please explain which is your legitimate interest which would be harmed by the publication of your input.
Champalmaud Foundation - Fish Platform	Portugal	6.1.6.2. Hypothermic shock	Please consider including in this Opinion/ Recommendations, the following conditions for euthanasia by rapid chilling: (i) chilled water should be clean water collected from the housing system/ tank of the fish. This is required to ensure that fish die due to the rapid temperature change without contribution of additional variables such as a sudden change in other water parameters. (ii) maximum density of fish in the euthanasia bath is not addressed in this Opinion and it is not referred to in publications as far as I am aware. Nevertheless, as fish are placed inside the chilled water, water temperature rises as a consequence of the higher temperature of the fish body. Temperature control should be ensured before, during and until at least 5 minutes after fish immersion. Maximum number of fish euthanized at the same time on a single euthanasia event should be adjusted to the container capacity (in Liters), type/composition of the container and its surface area in contact with the cold source, to ensure temperature stability before, during and until at least 5 minutes after fish immersion. (iii) For good thermal conduction and temperature stability of the chilled water, it should be used a container able to preserve temperature. Examples of good containers for chilled water for euthanasia: thermos boxes; a metal tray (or other good thermal conductor) for iced water, placed inside a larger container filled with ice for temperature preservation. Attached is an example of a common protocol for thermic shock used at the main Zebrafish Resource Center, in USA.		ZIRC_Hypothermal_Euthanasia.pdf	SCHEER has included several parameters for euthanasia of zebrafish by rapid chilling. Summary, Page 8. Opinion, Page 22. Text, Page 57. "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. For further convenience of the reader additional information is added on Page 57. 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.
Champalmaud Foundation - Fish Platform	Portugal	6.1.3.2. Stocking density and aquarium enrichment	Page 44, Line 28-29 : Regarding the sentence "paramecia or rotifers (for young larvae 28 [at a high density for the first ~5 days of feeding]) and artemia (for 10 dpf larvae and 29 adults)." Consider revision. Many zebrafish facilities worldwide provide rotifers until 30 dpf or later, and artemia thereafter (source: personal communication on Zebrafish Husbandry Association online meetings). Attached (and references below) are two publications that include successful feeding protocols where rotifers are used as single live feed until 30 dpf or later: (i) Monteiro JF, Martins S, Farias M, Costa T, Certal AC. The Impact of Two Different Cold-Extruded Feeds and Feeding Regimens on Zebrafish Survival, Growth and Reproductive Performance. J Dev Biol. 2018 Jun 21;6(3):15. (ii) Lawrence et al (2015) Successful Replacement of Artemia salina nauplii with Marine Rotifers (Brachionus plicatilis) in the Diet of Preadult Zebrafish (Danio rerio). Zebrafish (2015) pp.		Lawrence_et_al_2015.pdf; Monteiro_et_al_2018.pdf	I do not object to publication of my contribution, including my personal data, on internet SCHEER agrees. Text added on Page 45. Also rotifers may be used until 30 dpf and later (Lawrence et al. 2015; Monteiro et al. 2018).
Champalmaud Foundation - Fish Platform	Portugal	6.1.2.2. Water parameters	Page 30, Line 14-16: Please review the information regarding the effect of temperature on sex determination. From what I know, the publications available suggest the opposite of what is written here, that higher temperatures result in a higher proportion of males, not females as indicated in this Opinion. Attached are two examples of publications suggesting this. The references are also cited below: (i) (PDF) Geoffrey B, Wedekind C. Effects of global warming on sex ratios in fishes. J Fish Biol. 2020 Sep;97(3):596-606. doi: 10.1111/jfb.14429. Epub 2020 Jul 27. Erratum in: J Fish Biol. 2021 Jun;98(6):1495. PMID: 32524610 Link: https://pubmed.ncbi.nlm.nih.gov/32524610/ (ii) Valdivieso A, Ribas L, Montielón-Cetino A, Orbán L, Piferrer F. Exposure of zebrafish to elevated temperature induces sex ratio shifts and alterations in the testicular epigenome of unexposed offspring. Environ Res. 2020 Jul;186:109601. doi: 10.1016/j.envres.2020.109601. Epub 2020 Apr 25. PMID: 32371278. Link: https://pubmed.ncbi.nlm.nih.gov/32371278/		Geffroy_2020_Effects_of_global_warming_on_sex_ratio_in_fishes.pdf; Valdivieso_et_al_2020.pdf	I do not object to publication of my contribution, including my personal data, on internet SCHEER agrees with the comment. Thank you for observing this. Text modified accordingly. Page 31. 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 (35°C) result in a higher proportion of male off-spring (Geffroy and Wedekind 2020; Valdivieso et al. 2020).
Champalmaud Foundation - Fish Platform	Portugal	1. SUMMARY	Page 7, Line 35-38: Besides what is recommended on these lines, I suggest including reference to the following: when using physical enrichment inside a tank there is a decrease in total tank capacity and area available for swimming. Therefore, the presence of physical enrichment inside a tank should also be a factor to consider when defining the total number of fish per tank. Page 7, Line 42-44: Removal of sentence "The temperature applied during hypothermic shock should at least be 20°C below the husbandry temperature." This sentence may induce the wrong application of rapid chilling if not complemented with additional information. The complete conditions are stated on lines 3-7 of page 8, in the same section of the document, so deletion of the proposed sentence should not produce lack of information. Page 8, Line 3-7. Please consider additional conditions for euthanasia with rapid chilling: (i) chilled water should be clean water collected from the housing system/ tank of the fish. This is required to ensure that fish die due to the rapid temperature change without contribution of additional variables such as a sudden change in other water parameters. (ii) maximum density of fish in the euthanasia bath is not addressed in this Opinion and it is not referred to in publications. Nevertheless, as fish are placed inside the chilled water, water temperature rises as a consequence of the higher temperature of the fish body. Temperature control should be ensured before, during and until at least 5 minutes after fish immersion. Maximum number of fish euthanized per euthanasia cycle should be adjusted to the capacity, material and surface area of the chilled water recipient, to ensure temperature stability before, during and until at least 5 minutes after fish immersion. Page 8, Line 6-7. Consider adding "at least" to the sentence "Temperature difference of "at least" 20°C"			Page 7: SCHEER agrees. Text added page 7. In addition, the enrichment objects may reduce the available free swimming spaces, and this should be considered in view of number of fish housed. Page 45 text added. In addition, the enrichment objects may reduce the available free swimming spaces, and this should be considered in view of number of fish housed. Page 7-: SCHEER disagrees. The statement is necessary to indicate that at least a temperature difference of 20°C is necessary to induce hypothermic shock, otherwise a more slow temperature decrease may result, that may induce a temperature adaption in the fish.. The information on page 8 gives proper conditions to apply the method of hypothermic shock. Text added for further explanation. PAGE 7/8 and page 56. A smaller difference of temperatures may not result in a hypothermic shock due to fish's capacity to adapt to the new decreased temperature. Page 8, Line 3-7. SCHEER disagrees. This is text of the summary. Not everything can be presented in the summary. For further convenience of the reader additional information is added on Page 57. 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 4°C should be ensured during the whole procedure. Similar to the use of anaesthetics, confirmation of death of the fish should be determined after the use of rapid chilling for euthanasia of zebrafish. Page 8, Line 6-7. SCHEER agrees. Text "at least" added on page 8, 22 and page 57.
Champalmaud Foundation - Fish Platform	Portugal	ABSTRACT	Page 2, line 5: Consider replacing the term "killing" by the word "euthanasia" or by "humane killing". This is because euthanasia per definition implies the application of humane killing methods that minimize the suffering of the animal. The term "killing" refers to any method humane or not			I do not object to publication of my contribution, including my personal data, on internet SCHEER agrees. Where appropriate text modified. Annex IV specifically indicates Methods of "killing" animals. In this respect the word "killing" complies with the language used in the legislation. However, where appropriate the word "killing" is replaced by "euthanasia".
IBS	Portugal	3. OPINION	Taking into account the current practices of zebrafish maintenance, there are still some aspects and ideas that could be improved or, in other words, should be agreed upon by the scientific community using zebrafish as an animal model. In our view, there is still a lack of consensus regarding specific environmental enrichment, particularly when fish need to be kept isolated. It remains uncertain which type of enrichment would be most suitable in such situations. Despite that, the feeding of the animals should be more homogeneous within facilities (the frequency, the type of food: live food and /or dry food). Furthermore, different methods of euthanasia raise questions. In Portugal, for instance, special authorization is required for the use of rapid cooling as a euthanasia method, necessitating a justification for choosing this approach over alternatives such as MS222. According to the literature, rapid cooling is considered less stressful than MS222. In my opinion, this method should be accepted and authorized alongside the use of MS222. Another area that lacks clear guidelines is the use of analgesics for certain procedures, such as fin clipping. I believe analgesia should be administered for fin clipping and implemented consistently across all fish facilities. Regarding water parameters and the acceptable range of values, I believe more stringent regulations should be applied specifically to conductivity values, to avoid such differences between facilities. In addition, this stringent regulation should be applied to the light intensity values. Overall, the initiatives are very welcome and should be translated into guidelines for zebrafish welfare and maintenance.			I do not object to publication of my contribution, including my personal data, on internet Thank you for your comments. On the subject of your comments the SCHEER Opinion provides a number of recommendations within the limits of the mandate submitted to the SCHEER. The proposed housing conditions in the opinion are considered crucial for the welfare of the animals, and based on the current knowledge as presented in the literature. As is foreseen most (if not all) recommendations by the SCHEER might be translated into legislation during the current revision of Annexes III and IV of Directive 2010/63/EU on the protection of animals used for scientific purposes. Further development of dedicated guidelines and harmonisation is up to the scientific community of scientists involved in zebrafish research.
Animal Welfare Academy; German Animal Welfare Federation (Akademie für Tierschutz; Deutscher Tierschutzbund e.V.	Germany	6.2. Passerine birds	Chapter 6.2. -We support the recommendations in this chapter which will improve the welfare of passerine birds used for scientific purposes.			I do not object to publication of my contribution, including my personal data, on internet Thank you for your support
Animal Welfare Academy; German Animal Welfare Federation (Akademie für Tierschutz; Deutscher Tierschutzbund e.V.	Germany	6.1.7. Recommendations	*Page 58, Lines 44-45 Please change to: Hypothermic shock, also known as rapid chilling, is considered a reliable and safe method of euthanasia in zebrafish when used in combination with non-aversive anaesthetics. Please delete this sentence: "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." Explanation: -Applying solely a hypothermic shock cannot be considered a less stressful, faster and more reliable method of humane killing. Rapid cooling of fish in ice water does not meet the standards of the World Organisation for Animal Health and has "been shown to result in poor fish welfare" (WOAH, 2020). Rather, it is likely to be only perceived as a sufficient method by mistake as the shock induced by rapid cooling renders the fishes incapable of moving, but loss of swimming activity and lack of eye rotation reflex cannot be considered an assured sign of loss of consciousness. Even so called typical "signs of stress like gasping or erratic swimming are reduced or absent when compared to an overdose of anaesthetics" could be misinterpreted due to the immobilisation, thence escape attempts and signs of stress are not shown by the animal. Ferreira et al. (2022a) also state "the evidence that is available to date is not sufficient to understand what zebrafish feel during rapid cooling euthanasia". *Page 59, Lines 20-22 Please change to: In addition, it should be verified that intended fish species do not perceive cold as painful, and they do not express anti-freeze proteins. These assessments shall only be done with non-animal methods (NAMs) e.g. fish neural cell culture systems and proteomic techniques to determine if the fishes express anti-freeze proteins. Explanation: -We recommend to adapt the wording in order to avoid the SCHEER report indirectly resulting in a call for the performance of animal tests, especially as they will be of high severity just to verify that intended fish species do not perceive cold as painful. If this has to be investigated, this shall only be done with non-animal methods (NAMs) e.g. fish neural cell culture systems and proteomic techniques to determine if the fishes express anti-freeze proteins. In conclusion, rapid cooling should only be used in combination with non-aversive anaesthetics to ensure a more reliable and safer method of euthanasia in zebrafish. References: -Ferreira JM, Lufs F, Jorge S, Monteiro SM, Olsson IAS, Valentim AM. (2022a). Anesthesia Overdose Versus Rapid Cooling for Euthanasia of Adult Zebrafish. Zebrafish 19 (4): 148-159. https://doi.org/10.1089/zeb.2022.0001 -Ferreira JM, Jorge S, Félix L, Morello GM, Olsson IAS, Valentim AM. (2022b). Behavioural Aversion and Cortisol Level Assessment When Adult Zebrafish Are Exposed to Different Anaesthetics. Biology 11(10): 1433. https://doi.org/10.3390/biology11101433 -World Organisation of Animal Health – WOAH (2020). Aquatic Code. Article 7.3.6., Point 4. https://www.woah.org/en/what-we-do/standards/codes-and-manuals/aquatic-code-online-access/?id=169&L=1&htmlfile=chapter_welfare_stunning_killing.htm (last access 06.06. 2023)			Page 58, Lines 44-45 5 SCHEER disagrees with the comment to add text "when used in combination with non-aversive anaesthetics". It is clearly presented in the Opinion that rapid chilling should be followed by confirmation of the death of the animals (e.g. page 22, twice on page 57). Text modified. Similar to the use of anaesthetics, confirmation of death of the fish should be determined after the use of rapid chilling for euthanasia of zebrafish. Page 60 line 21. SCHEER agrees regarding the limited evidence on stress induction and suffering during rapid chilling. However, evidence for the contrary are also not present. To accommodate the comment the text has been modified to include the words "with the current scientific knowledge" indicating the existing uncertainty. Page 59 lines 20-22 SCHEER agrees. SCHEER indeed does not want to promote animal testing. Text indicating possible use of in vitro methods has been added on Page 22 and Page 61. Text added. "(which might be assessed in vitro).

<p>Animal Welfare Academy, German Animal Welfare Federation (Akademie für Tierschutz, Deutscher Tierschutzbund e.V.)</p>	<p>Germany</p>	<p>6.1.6.2. Hypothermic shock</p> <p>*Page 53, Lines 19-23 Please change to: The method is considered as less stressful, faster and more reliable as an overdose of anaesthetics (Matthews and Varga, 2012) while several countries, including the USA (Leary et al., 2020; NIH, 2020) Canada (CCAC, 2020) even regard it as the preferred method of euthanasia. Nevertheless, rapid cooling of fish in ice water does not meet the standards of the World Organisation for Animal Health which has "been shown to result in poor fish welfare" (WOAH, 2020). *Page 54, Lines 13-18 Please change to: Studies available on zebrafish (Wilson et al., 2009) and bony breams (Blessing et al., 2010) confirm that rapid chilling induces a 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 to take effect drastically. However, this should not be defined as "loss of consciousness". Rather, it is likely to be misleading, as the shock induced by rapid cooling renders the fishes incapable of moving without the desired effect of them losing consciousness. Therefore, loss of swimming activity and lack of eye rotation reflex cannot be considered an assured sign of loss of consciousness. *Page 54, Line 28 Please add: However, the absence of these signs could be misinterpreted due to the immobilisation induced by the rapid cooling, hence escape attempts and signs of stress are not shown by the animal. Ferreira et al. (2022a) also state "the evidence that is available to date is not sufficient to understand what zebrafish feel during rapid cooling euthanasia". *Page 55, Line 4 Please add: Furthermore, to ensure that the water temperature is at least 20°C below the fishes' adapted temperature, the number of individuals added to the ice water must also be taken into account. With high body density, the temperature of the water can rise again and prolong the hypothermic process. This also applies to animals that swim at the top and may not be completely covered by water. *Page 55, Lines 31-33 Please change to: Rapid chilling is considered a reliable and safe method of euthanasia in zebrafish when used in combination with non-aversive anaesthetics (Ferreira et al., 2022b), although it is highly dependent on the life stage of the zebrafish (W&E strong). *Page 55, Lines 40-43 Please change to: A proper protocol should be followed ensuring that no direct contact of the fish to the crushed ice is possible, and it should also be taken care that the body temperature of the fishes doesn't increase the water temperature. *Page 55, Line 8 Please add: Nevertheless, this shall not lead to the performance of additional animal tests, especially as these will be of high severity. The assessment shall only be done with non-animal methods (NAMs) e.g. fish neural cell culture systems and proteomic techniques to determine if the fish express anti-freeze proteins. References: *Ferreira JM et al. (2022a). Zebrafish 19 (4): 148-159. https://doi.org/10.1089/zeb.2022.0001 *Ferreira JM, et al. (2022b). Biology 11(10):1433. https://doi.org/10.3390/biology11101433 *Machnik, P. et al. (2023). Communications Biology, 6(1):309. https://doi.org/10.1038/s42003-023-04695-4</p>	<p>I do not object to publication of my contribution, including my personal data, on internet</p>	<p>Page 53 lines 19-23 SCHEER disagrees. The WOAHA describes specifically the killing of farmed fish, generally of much larger size than the small laboratory fish as discussed on page 51. However, also the issue of farmed fish is shortly discussed on page 26, for which SCHEER has added on page 27 the following text. 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). Page 54 lines 13-18 SCHEER disagrees. Sufficient data indicate that rapid cooling reduces neuron activity. Also the lack of survival after rapid cooling indicates the induction of early unconsciousness. There is no indication that cooling is less efficient than the use of an overdose of anaesthetics for euthanasia of zebrafish based on the observable indicators of unconsciousness like behaviour or opercular movement. Added is a reference on reduction of neuronal activity in a study on the use of cooling as anaesthetic. (Leyden et al., 2020). Text added page 55. 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., 2020). Page 54 line 28. SCHEER disagrees. Sufficient data indicate that rapid cooling reduces neuron activity. Also the lack of survival after rapid cooling indicates the induction of early unconsciousness. There is no indication that cooling is less efficient than the use of an overdose of anaesthetics for euthanasia of zebrafish based on the observable indicators of unconsciousness like behaviour or opercular movement. Text added page 55. Already with a decrease in temperature to 11°C a reduction in neuronal activity was noted when cooling was investigated for anaesthesia (Leiden et al., 2020). Page 55 line 4. SCHEER agrees. Text added page 57. 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 of 0°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 at 4°C should be ensured during the whole procedure. Similar to the use of anaesthetics, confirmation of death of the fish should be determined after the use of rapid chilling for euthanasia of zebrafish. Page 55 lines 32-33. SCHEER disagrees. At several locations it is indicated that when using rapid chilling the procedure should be followed by confirmation of death of the animals. There is no need to repeat this here as well. See also comment line 12 above. Page 55 lines 40-43 SCHEER disagrees. The issue of possible temperature rise is already addressed in the text above on page 57. Page 55 line 8 SCHEER agrees. SCHEER indeed does not want to promote animal testing. Text indicating possible use of in vitro methods has been added on Page 22 and Page 61. Text added. "(which might be assessed in vitro)".</p>
<p>Animal Welfare Academy, German Animal Welfare Federation (Akademie für Tierschutz, Deutscher Tierschutzbund e.V.)</p>	<p>Germany</p>	<p>6.1.6.1. Anaesthetics</p> <p>*Page 52, Lines 29-32 Please change to: Even though tricaine (MS-222) is traditionally by far most often used, it was recently shown that a combination of lidocaine and propofol as well as clove oil are promising and less aversive alternatives (Collymore, 2020; Davis et al., 2022; Ferreira et al., 2022a; Von Krogh et al., 2021; Ferreira et al., 2022b). Furthermore, for non-invasive procedures, also Isoeugenol can be used (Machnik et al., 2023). Explanation: *Ferreira et al. (2022b) demonstrated that a combination of lidocaine and propofol as well as clove oil are promising and less aversive anaesthetics that can be used with zebrafish. Additionally, Machnik et al. (2023) recommends the use of Isoeugenol as an anaesthetic in non-invasive procedures, e.g. relocating the zebrafish to a new water container. References: *Ferreira JM, Jorge S, Félix L, Morello GM, Olsson IAS, Valentim AM. (2022b). Behavioural Aversion and Cortisol Level Assessment When Adult Zebrafish Are Exposed to Different Anaesthetics. Biology 11(10): 1433. https://doi.org/10.3390/biology11101433 *Machnik, P. et al. (2023). Recordings in an Integrating central neuron reveal the mode of action of Isoeugenol. Communications Biology, 6(1), 309. https://doi.org/10.1038/s42003-023-04695-4</p>	<p>I do not object to publication of my contribution, including my personal data, on internet</p>	<p>Page 52 lines 29-32. SCHEER disagrees. Isoeugenol was found to be acting as local anaesthetic rather than to have systemic anaesthetic activity in a recent study by Machnik et al., 2023. SCHEER considered isoeugenol without systemic activity not suited for euthanasia. Table 6.4 shows commonly used anaesthetics for euthanasia. Based on the study by Machnik et al., 2023 text was added below Table 6.4. 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.</p>
<p>Animal Welfare Academy, German Animal Welfare Federation (Akademie für Tierschutz, Deutscher Tierschutzbund e.V.)</p>	<p>Germany</p>	<p>2.2.3. Hypothermic shock as a method of humane killing for zebrafish used for scientific purposes</p> <p>*Page 12, Lines 9-10 Please change to: Scientific literature provides indications that immersion for a duration of five minutes for fry over 16 days post fertilisation and for adults could be sufficient when used in combination with an overdose of anaesthetics. Explanation: *We recommend combining several methods to ensure more rapid loss of consciousness and limit pain perception, e.g. using overdoses of anaesthetics before inducing a hypothermic shock. This is also suggested by Ferreira et al. (2022a), stating "after the loss of equilibrium induced by anaesthesia, this method can be used to accelerate euthanasia". This will not only combine the advantages out of two methods (anaesthetics with rapid cooling) but also lead to "minimizing the duration of exposure to chemicals that may affect post-mortem analyses" (Ferreira et al., 2022a). Valid candidates for an anaesthetic protocol could be the use of a combination of propofol with lidocaine and clove oil (Ferreira et al., 2022b) before applying the hypothermic shock. *Page 12, Lines 12-13 Please change to: 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 scarce (26) and rapid cooling of fish in ice water does not meet the standards of the World Organisation for Animal Health which has "been shown to result in poor fish welfare" (WOAH, 2020). Explanation: *Applying solely a hypothermic shock cannot be considered a less stressful, faster and more reliable method of humane killing. Rapid cooling of fish in ice water does not meet the standards of the World Organisation for Animal Health and has "been shown to result in poor fish welfare" (WOAH, 2020). Rather, it is likely to be only perceived as a sufficient method by mistake as the shock induced by rapid cooling renders the fishes incapable of moving, but loss of swimming activity and lack of eye rotation reflex cannot be considered an assured sign of loss of consciousness. Even so called typical "signs of stress like gasping or erratic swimming" could be misinterpreted due to the immobilisation, hence escape attempts and signs of stress are not shown by the animal. Ferreira et al. (2022a) also state that "the evidence that is available to date is not sufficient to understand what zebrafish feel during rapid cooling euthanasia". *In conclusion, rapid cooling should only be used in combination with non-aversive anaesthetics to ensure a more reliable and safer method of euthanasia in zebrafish. References: *Ferreira JM, et al. (2022a). Zebrafish 19 (4): 148-159. https://doi.org/10.1089/zeb.2022.0001 *Ferreira JM et al. (2022b). Biology 11(10): 1433. https://doi.org/10.3390/biology11101433 *World Organisation of Animal Health – WOAHA (2020). Aquatic Code. Article 7.3.6., Point 4. https://www.woah.org/en/what-we-do/standards/codes-and-manuals/aquatic-code-online-access/?id=169&L=1&href=chaptre_welfare_stunning_killing.htm (last access 06.06. 2023)</p>	<p>I do not object to publication of my contribution, including my personal data, on internet</p>	<p>These comments refer to the text of the mandate as provided by the European Commission. This mandate has been published by the European Commission and the text can not be changed by the SCHEER.</p>
<p>Animal Welfare Academy, German Animal Welfare Federation (Akademie für Tierschutz, Deutscher Tierschutzbund e.V.)</p>	<p>Germany</p>	<p>ABSTRACT</p> <p>*Page 2, Lines 21-24. Please change to: 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) if combined with the use of non-aversive anaesthetics. A proper hypothermic shock protocol should be followed ensuring that no direct contact of the fish to the crushed ice is possible, the resulting decrease of the water temperature is ~ 20 °C and that the number of individuals added to the ice water does not increase the water's temperature level. Explanation: *We recommend combining several methods to ensure more rapid loss of consciousness and limit pain perception, e.g. using overdoses of anaesthetics before inducing a hypothermic shock. This is also suggested by Ferreira et al. (2022a), stating "after the loss of equilibrium induced by anaesthesia, this method can be used to accelerate euthanasia". This will not only combine the advantages of two methods (anaesthetics with rapid cooling) but also lead to "minimizing the duration of exposure to chemicals that may affect post-mortem analyses" (Ferreira et al., 2022a). Valid candidates for an anaesthetic protocol could be the use of a combination of propofol with lidocaine and clove oil (Ferreira et al., 2022b) before applying the hypothermic shock. Additionally, to ensure that the water temperature is at least 20°C below the fishes' adapted temperature, the number of individuals added to the ice water must also be taken into account. With high body density, the temperature of the water can rise again and prolong the hypothermic process. References: *Ferreira JM, Lufs F, Jorge S, Monteiro SM, Olsson IAS, Valentim AM. (2022a). Anesthesia Overdose Versus Rapid Cooling for Euthanasia of Adult Zebrafish. Zebrafish 19 (4): 148-159. https://doi.org/10.1089/zeb.2022.0001 *Ferreira JM, Jorge S, Félix L, Morello GM, Olsson IAS, Valentim AM. (2022b). Behavioural Aversion and Cortisol Level Assessment When Adult Zebrafish Are Exposed to Different Anaesthetics. Biology 11(10): 1433. https://doi.org/10.3390/biology11101433</p>	<p>I do not object to publication of my contribution, including my personal data, on internet</p>	<p>SCHEER disagrees with the comment to add text "when used in combination with non-aversive anaesthetics". It is clearly presented in the Opinion that rapid chilling should be followed by confirmation of the death of the animals (e.g. page 22 and twice on page 56). Similar to the use of anaesthetics, confirmation of death of the fish should be determined after the use of rapid chilling for euthanasia of zebrafish. The SCHEER answers the question in the mandate whether "rapid chilling" is by itself an appropriate method for euthanasia of zebrafish. SCHEER presents information that indeed rapid chilling can be used as independent method for euthanasia of zebrafish of 16 dpf and older. Details of the rapid chilling method are presented in the Summary and the Opinion as answers to the questions of the mandate. In addition, an overdose of anaesthetics by itself is already an accepted method for euthanasia of zebrafish.</p>
<p>Netherlands Institute of Ecology</p>	<p>Netherlands</p>	<p>6.2.3. House sparrows (Passer domesticus)</p> <p>Page 19line 9-10: Here, the text suggests that mixed-sex groups of house sparrows should not be less than 6 animals. We have very good experience housing mixed-sex groups of 2 birds (1male/1female) and 4 birds (2males/2females). The caveat being that this has been done with equal sex-ratios (i.e. equal number of males and females). Under the conditions, we have not observed increased amounts of aggression (relative to aviaries of 6 or 8 birds). Our birds are monitored daily (twice daily during the week/daily on weekends), and these groups of 2 and 4 birds have all remained healthy and breed successfully. Thus I think it is entirely possible to have mixed sex groups of less than 6 birds. Page 73 line 16-17: same comment as above regarding the size of mixed sex groups of house sparrows. Page 74 line 17-18: We have had mixed sex groups of 1 male and 1 female house sparrow (i.e. a pair) breed successfully in our aviaries. This includes the birds building nests, laying eggs, feeding nestlings, and successfully fledging young. Thus I feel it is entirely possible for groups of less than 6 birds to breed successfully and thus suggest that smaller group sizes should be allowed provided the group has an equal sex ratio. These statements are based on our experience housing and breeding house sparrows since 2020. Further, our protocols were established via discussions with others housing house sparrows for research. Each year we have very successful breed house sparrows under captive conditions. Additionally, I have > 10 years experience working with house sparrows in captivity and in the wild.</p>	<p>I do not object to publication of my contribution, including my personal data, on internet</p>	<p>Page 19line 3.4: enrichment objects and visual barriers can also be achieved by placing trees and/or leafy branches in the aviary. Agreed text modified. Added. (e.g. trees and/or leafy branches) Page 19line 9-10: SCHEER disagrees to include this lower number in the answer to the mandate. The housing of lower numbers than 6 animals is presented for one facility only. It is included in the main text as possibility on page 76. Page 19 line 18. For clarification the text has been modified. For single sex, the group size shall be at least 2 animals, while for mixed sex groups such groups should not be smaller than 6 animals, and composed of equal numbers of males and females. Page 73 line 16-17: information on smaller group size is included on page 76. Agreed. Text modified and added. Page 76 text added. 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. Page 74 line 17-18 Agreed. Text modified and added as page 76 text added. 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.</p>
<p>Radboudumc, dept. of Otorhinolaryngology & Radboud University Zebrafish Facility</p>	<p>Netherlands</p>	<p>6.1.3.1. General aspects</p> <p>lines 19-20 (page 38, section 6.1.3.1) Based on my survey, I do not see any data supporting the claim that "Prolonged light exposure above 300 lux was suggested to be detrimental to adult zebrafish". Perhaps this is the result of a growing overinterpretation by several papers over the course of 20 years. Therefore, I urge the authors to either provide the appropriate references to support the suggested SCHEER guidelines (citing research papers using broad-spectrum white light), or relax the criteria on light levels.</p>	<p>I do not object to publication of my contribution, including my personal data, on internet</p>	<p>section_6.1.3.1_publication_comment_of_my_contribution, Radboudumc.doc SCHEER agrees and has modified the text on page 39. 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 1m height, is generally considered to be common practice.</p>
<p>Toxicon GmbH</p>	<p>Germany</p>	<p>6.1.3. Zebrafish housing conditions</p> <p>p. 38, I2: I it is very welcome to adapt the OECD guidelines to the current scientific knowledge of housing conditions (e.g. water temperature 28°C versus 21-25°C, avoiding noise etc.) working daily with them, and further to consider if these changes might have an impact on study outcomes, compared to the current housing standards? However, animal welfare should be placed first in my opinion, thank you so much for your improvements of animal welfare wherever possible. I only had ideas which I have forwarded, maybe they might support a bit. Thanks!</p>	<p>I do not object to publication of my contribution, including my personal data, on internet</p>	<p>Thank you for your support</p>

Toxicolicon GmbH	Germany	6.1.6.1. Anaesthetics p. 52, l. 32: In the case that several anaesthetics in overdose induce more aversive behaviors during euthanasia induction and prolonged times of suffering in fish, it might be considered to replace the anaesthetics with the "worst profile" (longest duration/suffering until death) for fish killing with the hypothermic shock method. p. 53 l. 7: The restriction to the age of zebra fish should be mandatory as mentioned due to otherwise prolonged killing phases. In my opinion due to animal welfare the time of suffering has always to be kept as short as possible p. 52 l. 43: There might be place for improvement to define a maximum time span until the fish loses consciousness by the killing method?	I do not object to publication of my contribution, including my personal data, on internet	Page 52 line 32 SCHEER disagrees, as the fields of comparing various methods of euthanasia with each other is still developing. At this moment, a preference for one or the other method cannot be concluded. The development of continuous refinements is already expressed in the text. Page 53 line 7 SCHEER agrees and has indicated in the answer to Question 3 of the mandate on page 21 and other locations in the Opinion, that the use of hypothermic shock should only be used for zebrafish 16dpf . Page 52 line 43 SCHEER agrees, however, evaluating all procedures for the indicated anaesthetics was outside the scope of the SCHEER mandate. In general it is stated that immersion in a bath with anaesthetics in which the fish are transferred, the fish lose consciousness quickly.
Toxicolicon GmbH	Germany	6.2. Passerine birds First of all, I very much appreciate that for wild life passerine birds standardized accommodation parameters are set up p. 61 l. 4: To maintain as much space as possible for flying around and to define this for wild life animals in capture should always be considered with respect to feasibility of holding these birds. Therefore, a definition of length and of cages for all passerine birds should be developed and in my eyes mandatory. p. 61, l. 17: the captivity should be stopped as soon as stereotypic behavior occurs due to animal welfare p. 78, l. 14: For tits which migrate according to changing seasons, considerations might be made to avoid keeping those in captivity during the migration time	I do not object to publication of my contribution, including my personal data, on internet	6.2 Thank you for your support. Page 61 line 4 SCHEER agrees with the comment and has provided cage sizes in its answers to the mandate for three Passerine bird species (starlings, house sparrows, and great and blue tits) most used in research. These recommendations are presented in Tables 3.2-3.3-3.4 on pages 19-21 of the Opinion. The situation on page 61 described in lines 1-4, however, describes a situation when birds need to be held for a short period of time. In these situations, housing in small enclosures will make it easier to catch the birds, avoiding additional stress. Page 61 line 17. SCHEER agrees. However, in view of the short period of captivity (<24 hours) the SCHEER believes that such stereotypic behavior is not likely to develop. This issue is also addressed in the Directive, Article 33 (1)(d). p. 78, l. 14: For tits which migrate according to changing seasons, considerations might be made to avoid keeping those in captivity during the migration time. SCHEER agrees. Text has been added to the general introduction on birds page 63, and conclusions section of tits. Page 86. Page 63. 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. Conclusions section of tits. Page 86. In order to avoid unnecessary stress for tits which migrate according to changing seasons, it needs to be considered to avoid keeping those in captivity during the migration time.
Toxicolicon GmbH	Germany	7. Allow me question and ideas for further research: RECOMMENDATIONS p. 86, l. 22: Is there any existing guideline or guidance for capturing passerine birds? FOR FUTURE WORK p. 86 l. 26: Regarding food, if there is no standard food available, scientific recommendations for grit, sepias and minerals should be given where advised if available.	I do not object to publication of my contribution, including my personal data, on internet	Page 88 line 22 guidelines Yes there are. Bird catching and ringing for following migration of birds is generally regulated in the member states. Page 86 line 26 SCHEER agrees. However, the evaluation of food regimens for birds was outside the scope of the SCHEER mandate that was focused on housing conditions, and food conditions were not extensively evaluated. Also, food requirements are already indicated in Directive Annex III, Section A, 3.4. It is recommended in Chapter 7 under Recommendations that in reported studies as much as possible information is provided on the housing conditions of the animals used in the research including their diet. Page 17: Therefore, smaller water volumes than 1L should not be used for adult fish. In addition, indications for effect of tank size on behaviour are present on page 28, and text on page 51. Page 28. 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 (Shihis et al., 2022). Mairdiyali 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.
Eurogroup for Animals	Belgium	3. OPINION Page 17, lines 19 to 26 Page 21, lines 20 to 23	Eurogroup_for_Animals_comments_on_SCHEER_s_Preliminary_Opinion.pdf I do not object to publication of my contribution, including my personal data, on internet	Text on page 51. 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. SCHEER agrees and has added text referring to Mocho et al., 2022 on page 56. As indeed the killing of larvae is not in the scope of the Opinion this information was not included in the Opinion with the answers to the mandate. Page 56. For very early life stages (4dpf) 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).