

EZS European Zebrafish Society e.V.



Prof. S. Schulte-Merker
Mendelstraße 7
48149 Münster
Tel.: +49 251 8335424

Mail: office@ezsociety.org

Web: <http://www.ezsociety.org>

Karlsruhe Municipal Court, VR 103672

Tax ID Number: 34002-39263

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Dr. Susanna Louhimies
Policy Coordinator, Unit B2
DG Environment
European Commission
Brussels

Dear Dr. Louhimies,

My name is Stefan Schulte-Merker, I am heading a research institute at the University of Münster (Germany). I have used zebrafish as a model system in the field of biomedicine for 30 years. As you know, zebrafish (*Danio rerio*) are currently the second most widely used vertebrate model in academic research, behind mice. I also serve as the current president of the European Zebrafish Society (EZS), an aggregation of roughly 180 research team leaders across Europe working with zebrafish. I am writing this letter also on behalf of the board of EZS (19 members; <https://www.ezsociety.org/board>).

The revisions Annex III and IV of the EU-Directive 2010/63/EU contain wording regarding the age at which zebrafish individuals are classified as embryos versus larvae. The guideline for such distinction has been, as in many other species, the point of 'independent feeding'. This criterion is also used in the 'Thought Starter' of the revisions, but rather than adhering to the currently accepted 120 hours post fertilization (hpf), the text now suggests 72hpf as the time from which independent feeding is possible. This is factually incorrect. I have provided some examples from the literature that illustrate this below:

- (1) Zebrafish embryos rely for the first week on yolk supply. It is only at 76hpf that intestinal epithelium starts to differentiate, a process that is fully completed only at 126hpf. The hallmark paper describing these intestinal morphogenesis steps is Ng et al. (Dev Biol, 2005)¹.
- (2) The paper goes on: "With the opening of the anus, the intestinal tract at 102hpf is now a completely open-ended tube, lined by a monolayer of polarized epithelial cells." Hence, before 102hpf there is no fully open intestinal tube, and it is anatomically impossible for zebrafish embryos to digest food before 102hpf. Before 102hpf then, zebrafish cannot be classed as 'independently feeding'.
- (3) In order to swim in a coordinated manner, zebrafish embryos need to develop a gas filled swim bladder. This happens between 96 and 132hpf, and before this point in time, coordinated swimming behaviour is impossible. This, in turn, also means that hunting (and uptake of food) before this point in time is not possible.

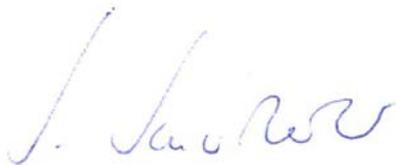
Considering the chronology of these anatomical and physiological developments, it is no coincidence that in zebrafish embryos the yolk is used up around 120hpf, as only at this time point full differentiation of the intestine and the ability to swim and hunt is established. Hence, the point of 'independent feeding' in zebrafish embryos cannot be possibly put at 72hpf, but should remain at 120hpf.

A 2020 paper² published by a working group commissioned by the Federation of European Laboratory Animal Science Associations (FELASA) suggests best practice for the housing and husbandry of zebrafish. In this publication, it is also clearly stated that 120hpf is the critical timepoint at which a zebrafish can be regarded as free-living, in line with the definitions in the present Directive.

There is additional literature on the topic, but I trust that the above information is sufficient to avoid making an erroneous statement in the Annex.

Please let me know whether I can add further to the discussion.

Very best wishes,



¹ Ng AN, de Jong-Curtain TA, Mawdsley DJ, White SJ, Shin J, Appel B, Dong PD, Stainier DY, Heath JK. Formation of the digestive system in zebrafish: III. Intestinal epithelium morphogenesis. Dev Biol. 2005 Oct 1;286(1):114-35. doi: 10.1016/j.ydbio.2005.07.013. PMID: 16125164.

² Aleström P, D'Angelo L, Midtlyng PJ, et al. Zebrafish: Housing and husbandry recommendations. Laboratory Animals. 2020;54(3):213-224. doi:10.1177/0023677219869037