

# NIH Animal Program Director Guidelines for Zebrafish Larvae Incubators

## BACKGROUND:

Zebrafish are poikilotherms and have a wide range of temperatures that are acceptable for growth and development. The rate of zebrafish embryo development is temperature dependent. For purposes of developmental research, maintaining a consistent incubator temperature is essential to standardize the stage of development at a given time post fertilization, but a consistent temperature is not critical for the health or welfare of the embryo.

Laboratory zebrafish embryos and larva are normally maintained in incubators during the first week (7 days) after egg fertilization. Zebrafish embryos dechorionate (hatch) at approximately 72 hours post fertilization [3 days post fertilization (dpf)]. Most zebrafish larva are placed into the animal facility aquatic system after 5 dpf. OLAW guidance addresses and recognizes zebrafish as 'animals' at dechoriation ("hatching") in accordance with the PHS Policy. The Guide and PHS Policy require vertebrate laboratory animals receive a level of care appropriate to the species through all its life stages.

Zebrafish larva are covered by the PHS Policy when they are maintained in an incubator. A common practice among zebrafish investigators is to maintain zebrafish embryos and larva in petri dishes or similar containers in an incubator in their laboratory which is often located outside a central animal facility. The Guide and PHS Policy require rooms be considered "satellite animal holding facilities" when they hold animals (including zebrafish larva) >24 hours outside a central animal facility. The IC ACUC is required to inspect satellite animal holding facilities semi-annually.

Zebrafish larva are different from mammalian neonates in ways that significantly affect the level of care and monitoring necessary to ensure appropriate zebrafish larva husbandry procedures are accomplished and animal welfare issues are addressed. Zebrafish larva are not able to feed upon hatching and are chiefly sustained by nutrients derived from yolk which is not depleted until 7 dpf (19). Since the embryos do not feed, there is little to no fouling of the water due to feed or wastes. Consequently, the embryos can go at least three days before needing water change or top-off in most cases. Zebrafish larva will only manifest signs of ill health in the absence of external feeding after 7 dpf (20). The criterion of nutritional independence for developmentally immature animals is subject to empirical verification and has found support in international regulations for the welfare of immature vertebrates (17).

Active feeding by zebrafish cannot commence at hatching because brain structures required for detecting and catching prey have not developed. At hatching, larva lack taste buds (21, 22), have poor visual acuity (14), and cannot swim effectively as they lack a swim bladder and have deficient motor control (23, 24). Therefore, the period between hatching (3 dpf) and nutritional independence (8 dpf) is an extension of the embryonic stage during which the larva develops sensory and motor functions required for living independent of its yolk.

During the first week of zebrafish development, larvae movements are simple reflexes that do not provide evidence for a capacity for suffering. Thus during the first week, zebrafish larvae can respond to simple stimuli but have not reached the point in brain development where stimuli can be experienced as

aversive. Pain perception and suffering requires that an animal have both the neural apparatus for detecting noxious stimuli as well as the mental ability to interpret such stimuli as aversive (1). While many studies have demonstrated that adult zebrafish show evidence of higher order cognition by being responsive to a variety of learning protocols (e.g. 2, 3, 4, 5), including learning to avoid aversive stimuli (6, 7, 8, 9); there is no evidence of higher order cognition in zebrafish during the first week of development. Developmental studies examining learning (12), reward (13), social (14, 15) and fright (16) behaviors have found that these functions associated with higher order cognition become operational only in older fish.

Room temperature is normally inconsequential to the internal temperature of an incubator. The incubators are designed to maintain a steady temperature to standardize the environment for zebrafish embryo and larva development. Frequent incubator opening disrupts the incubator's internal environment. The incubator will maintain a temperature range acceptable to the health and development of the embryo during short term room temperature fluctuations or a power outage lasting less than a day. Gradual cooling or warming of the incubator during a prolonged power outage would present little consequence to the larvae other than delayed development and reduced metabolism (28) during cooling, and accelerated larval development during gradual warming.

**ACCEPTABLE CARE AND MONITORING:** The following practices comprise an acceptable level of care for zebrafish larvae maintained in a laboratory incubator between 3 and 8 dpf:

- Health monitoring checks every three days or more frequently if required by the experiment.
- Daily feeding is not necessary because of the young hatchlings utilization of its yolk during this early life stage.
- Incubators do not need to be opened to monitor the health of the zebrafish larvae in the event of a power outage or room temperature fluctuation. Opening the incubator door disturbs the microenvironment and causes temperature fluctuations.

**INCUBATOR LABELING AND REPORTING:** The following practices should be followed to foster compliance with the PHS Policy regarding animal holding facilities:

- The zebrafish incubator should be labeled "Zebrafish Incubator" so it is easily identifiable during ACUC inspections.
- Incubators containing zebrafish embryos should be labeled as 'Contains Live Zebrafish Embryos'.
- The investigator should identify the location of the incubators on the Animal Study Proposal.
- The IC Animal Program should maintain a list of rooms which contain zebrafish incubators to aid in assessing whether zebrafish larva should be euthanized or relocated in response to a prolonged power outage or extreme room temperature.
- Room environmental monitoring is not required to assure zebrafish larva health and well-being.

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--Approved by the Animal Program Directors Committee, 26 August 2013.