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The husbandry of zebrafish (*Danio rerio*): A review

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Abstract

The zebrafish (*Danio rerio*) has recently emerged as a pre-eminent vertebrate biomedical research model. The same favorable characteristics that have contributed to its popularity as a model of human disease and development; *i.e.* high fecundity, small size, rapid generation time, optical transparency during early embryogenesis, have also long endeared it to investigators in numerous other disciplines, including animal behavior, fish physiology, and aquatic toxicology. Despite this, the scientific rigour of zebrafish husbandry techniques is poorly developed. While there is a considerable body of literature on zebrafish that has both direct and indirect relevance to their husbandry, this information is from disparate sources, and little of it is has been applied to developing standard protocols. This review is an attempt to integrate the available scientific information related to zebrafish biology and culture into an overview of the field that can be used to improve the efficiency with which this important model animal is used in research. The review also highlights those areas in which further studies are needed.

Keywords: Zebrafish; *Danio rerio*; Husbandry; Aquaculture; Management

Contents

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1. Introduction

Over the past twenty years, the zebrafish (*Danio rerio*) has emerged as a pre-eminent vertebrate model for studying genetics and development (Fishman, 2001), and more recently, for human disease and the screening of therapeutic drugs (Penberthy et al., 2002; Sumanasa and Lin, 2004). A number of favorable attributes, including its small size, rapid development and generation time, optical transparency during early development, tractability in forward genetic screens, and genetic similarity to humans (*e.g.* Lamason et al., 2005) have fueled its rise in popularity for biomedical studies, and will likely continue to spur growth in its use in other areas of research, particularly as the drafts of its genome sequence are further refined and the already impressive array of available tools and methods with which to study it expands (Dahm and Geisler, 2004; Jekosch, 2004).

Given the considerable importance of zebrafish as an experimental model, along with the significant economic costs associated with their large-scale use and the establishment and maintenance of culturing facilities, it is to some extent surprising that their husbandry is poorly developed. When compared with other commonly cultured fish species, such as tilapia (*e.g.* Lim and Webster, 2006); channel catfish (*e.g.* Tucker and Robinson, 1990); and carp (*e.g.* Billard, 1995), published husbandry standards are wholly inadequate. The exchange of information about husbandry techniques between the numerous research facilities housing zebrafish has been largely non-existent, and any advances that have been made are often employed in isolation and without the benefit of peer review. An unfortunate outcome of this situation is that many zebrafish facilities likely operate at sub-optimal levels, a situation that is only further exacerbated when investigators new to the model are unable to consult scientifically rigorous resources when devising standard operating procedures for newly established culturing facilities.

While published husbandry standards are lacking, the zebrafish is an otherwise well-studied animal. Long before its emergence as a developmental model, zebrafish were used to study a variety of aspects of fish biology (Laale, 1977). Over the years, researchers have capitalized upon the same practical advantages that now endear the zebrafish to the biomedical research community to study fish reproduction (Hisoaka and Firlit, 1962a,b; Eaton and Farley, 1974; Niimi and LaHam, 1974) shoaling (McCann et al., 1971; Engeszer and Ryan, 2004), aquatic toxicology (Hill et al., 2005), osmoregulation (Boisen et al., 2003), and olfaction (Bloom and Perlmutter, 1974; van den Hurk and Resink, 1992). More recently, groups have published studies in on zebrafish natural history (McClure et al., 2006), nutrition (Carvalho et al., 2006), and reproductive behavior (Spence and Smith, 2005). This research history potentially furnishes the zebrafish researcher

with a rich body of literature on their biology that can be directly or indirectly applied to husbandry. However, while this information is published, it is disparate and placed in journals that may not typically be consulted by most members of the zebrafish research community.

2. The natural history of zebrafish

A more holistic understanding of zebrafish in their native environment, including habitat preferences, reproductive behavior, and diet is necessary for both the refinement of husbandry standards and the optimization of their use in a wide array of biomedical and behavioral genetics studies. However, published information on the natural history of zebrafish is limited, and lags behind the significant amount of genetic and developmental data available for this species. In practice, little of the available behavioural and ecological information on zebrafish has been used to develop husbandry protocols, but this trend must change as the model continues to grow in popularity and is applied to different areas of research, such as behavioral genetics (*e.g.* Miklosi and Andrew, 2006). The importance of such information to husbandry is exemplified by the fact that data from the few field studies that have been conducted are referenced several times or more in various sections of this review. The following is a brief summary of the available data, along with suggestions for further work that could be used to improve the care of zebrafish in research facilities. A fuller treatment of the behaviour and ecology of the zebrafish is given by Spence et al. (in review).

2.1. Distribution and habitat preferences

Zebrafish are indigenous to South Asia, and are broadly distributed across parts of India, Bangladesh, Nepal, Myanmar, and Pakistan (Rahman, 1989; Barman, 1991; Talwar and Jhingran, 1991; Menon, 1999; Bhat, 2003). This geographic region has a monsoon climate, with pronounced rainy and dry seasons that have a profound effect on habitat parameters, including water chemistry and resource abundance. Zebrafish have been reported to occur in a wide variety of habitat types within this region, including irrigation ditches and rice fields, man-made fish ponds, upper reaches of rivers, and even fast flowing hill streams (Menon, 1999; Daniels, 2002; Bhat, 2003). However, the results of two field surveys conducted in India (McClure et al., 2006) and Bangladesh (Spence et al., 2006), when taken together, provide the most comprehensive description of the habitat preferences of this species to date. McClure et al. (2006) sampled 6 sites within the Ganges River drainage in the Indian states of West Bengal and Uttar Pradesh and found zebrafish in three of these sites, which included rice paddy and the quieter waters of two foothill streams (McClure et al., 2006). In Bangladesh, Spence et al. (2006) conducted surveys at 23 sites in the Ganges and Brahmaputra drainages and found zebrafish in 9 of these locations. As in the Indian study, zebrafish were found only in still or slow moving waters, including shallow ponds, a number of which were connected to rice cultivation. Interestingly, zebrafish were associated with aquatic vegetation in most or all of these sites. Notably, in field and laboratory-based behavioural studies wild and domestic zebrafish also showed a preference for spawning in sites associated with aquatic vegetation (Spence et al., accepted for publication).

Both of these surveys also described a number of additional physico-chemical properties of the water at sites in which zebrafish were found, including pH, temperature, salinity, transparency (Secchi disk), depth, and percent canopy (Table 1). In summary, these observations suggest that zebrafish seem to generally prefer still or slow moving, slightly alkaline (pH ~ 8.0), water of relatively high clarity (~>35 cm). These data are useful in that they may be directly applied to the design of optimum water chemistry parameters for zebrafish in the laboratory, as production in captivity may be highest when fish are housed in water that reflects that to which they are adapted in nature (Buttner et al., 1993). Future field studies should more fully investigate conditions during the summer months, over a broader range of habitat types, to determine the full range of habitat preferences of zebrafish in nature, making it possible to better match water chemistry parameters for laboratory stocks.

2.2. Reproduction and behavior

As zebrafish research is to a large degree predicated on consistent production of large numbers of embryos, information on the reproductive biology and behavior of the animal in the wild is of clear relevance for husbandry. Remarkably, little is known about the species in this regard. Much of the data that is available is largely derived from observations made by collectors during zoological surveys (*i.e.* Barman, 1991), although some more detailed descriptions have been reported, especially recently (McClure et al., 2006; Spence et al., 2006).

Evidence gathered to date indicates that zebrafish occur in small shoals (5–20 individuals) in slow moving

Table 1
Physico-chemical characteristics of wild zebrafish habitats

Locality	Habitat description	pH	Salinity (ppt)	Depth (cm)	Transparency (cm)	Water temperature (C°)	Current	Substratum	Ref
India, Uttar Pradesh	Tributary of Song River	8.2	Not recorded	Not recorded	>35	27	0.1 (m s^{-1})	Clay, silt cobble, boulders	McClure et al. (2006)
India, Uttar Pradesh	Side channel of Pasuni River	8	Not recorded	Not recorded	>35	32	0.05 (m s^{-1})	Clay, silt cobble, boulders	McClure et al. (2006)
India, West Bengal	Rice paddies connected to Bhairab River	7.9	Not recorded	Not recorded	>35	34	0 (m s^{-1})	Clay, silt cobble, boulders	McClure et al. (2006)
Bangladesh (Khulna District)	Ditch surrounding series of ponds	8	0	80	51	20	Still	Mud	Spence et al. (2006)
Bangladesh (Khulna District)	Isolated channel of Golomari River	8	0	50	19	20	Still	Mud	Spence et al. (2006)
Bangladesh (Mymensingh District)	Cultivated pond connected to paddy fields	8	0.6	15	15	20.5	Still	Mud	Spence et al. (2006)
Bangladesh (Mymensingh District)	Isolated pond	8	0.6	40	15	19.5	Still	Mud	Spence et al. (2006)
Bangladesh (Mymensingh District)	Isolated pond	8	0.6	103	30	16.5	Still	Mud	Spence et al. (2006)
Bangladesh (Mymensingh District)	Semi-natural pond	8	0.4	96	31	21	Still	Mud	Spence et al. (2006)
Bangladesh (Mymensingh District)	Ditch connected to paddy fields	8	0.4	50	50	23	Still	Mud	Spence et al. (2006)
Bangladesh (Mymensingh District)	Semi-natural pond	8	0	65	15	33	Still	Mud	Spence et al. (2006)
Bangladesh (Mymensingh District)	Channel feeding into field station	8	0	75	15	33	Still	Mud	Spence et al. (2006)

or standing water in floodplain water bodies (Pritchard et al., 2001). Zebrafish are asynchronous, batch spawners that breed in small groups, with females scattering clutches of eggs over the substratum with no parental care (Breder and Rosen, 1966). As with other fishes adapted to monsoonal climate regimes, reproduction is likely cued by the arrival of the rains (Munro, 1990; Talwar and Jhingran, 1991), although observations of females with mature ova during the dry season prompted Spence et al. (2006) to speculate that reproduction is more likely dependent upon food availability, the level of which is positively correlated with increased rainfall (Spence et al., 2006). Eggs are demersal, and depending on conditions (temperature, water chemistry, *etc.*), hatch within 4–7 days into free-swimming larvae. Male zebrafish are territorial around potential sites of oviposition (Spence and Smith, 2005), and also adopt an alternative mating tactic of chasing females (Goolish et al., 1998). Females exert mate choice (Spence and Smith, 2006a) that may be linked to olfactory cues (Gerlach, 2006).

Laboratory experiments have demonstrated that larval zebrafish prefer to associate with kin, using olfactory (Mann et al., 2003; Gerlach and Lysiak, 2006) and visual (Engeszer and Ryan, 2004) cues. This observation suggests that shoals of larvae in the wild are likely to be close relatives, although this has not been confirmed. The degree of genetic relatedness of individuals in shoals of adults is also unknown, although experimental evidence indicates that an individual's preference to associate with siblings switches to avoidance after sexual maturity, suggesting that at some point larval or juvenile fish disperse away from natal shoals (Gerlach and Lysiak, 2006). This premise is supported by population genetics studies that have shown high levels of genetic variability and a weak genetic structure among fish collected from four different sites in India, at least based on microsatellite markers (Gratton et al., 2004).

2.3. Lifespan

To date, there have been no reported studies detailing the lifespan of zebrafish in the wild. Spence et al. (2006) speculated that zebrafish are primarily an annual species, based upon size distribution of specimens collected in monthly samples over the course of a year, and assessment of their reproductive strategy (spawning continuously upon maturity). However, these data are limited, and refer to only a single population. Documented life spans for laboratory zebrafish can exceed five years (Gerhard et al., 2002). Further collection of age-class data for wild populations will facilitate comparisons of the physiological ages between wild-caught and laboratory housed animals, which will be useful for the design of biomedical studies, particularly those that involve aging.

2.4. Diet

Information on the dietary preferences of wild zebrafish is of relevance to husbandry because it may aid in the design of diets and feeding protocols that better reflect species-specific digestive physiology and feeding behavior than are currently used in captivity. There are some data available regarding the dietary habits of zebrafish in nature. Zebrafish are generalists, consuming a wide variety of benthic and planktonic crustaceans, in additional to worms and insect larvae (Dutta, 1993; Spence et al., in press). Their reported tendency to consume dipteran larvae has led to proposals for their use in mosquito control (Shrestha, 1990). McClure et al. (2006) analyzed the gut contents of zebrafish sampled from three different sites in India, and found that insects, mostly of terrestrial origin, were the predominant prey item. These authors reasoned that this pattern was the result of microhabitat preferences of the species, as pools and stream margins where zebrafish were most often found were also areas where terrestrial insects were more likely to fall into the water. While these data are indeed suggestive, additional studies conducted during periods of high rainfall are necessary to gain a more complete understanding of the dietary habits of this species in nature.

3. Zebrafish in culture

3.1. Water chemistry

A primary factor underlying the rise of the zebrafish as an experimental model animal is their tolerance of a wide range of environmental conditions in captivity. Their adaptability is a reflection of their distribution in the wild, as they are found across a range of habitat types that vary considerably in their physico-chemical properties as a result of local geology and pronounced seasonal fluctuations in rainfall patterns (Talwar and Jhingran, 1991). However, it is important to recognize that there is an energetic cost to fish in operating outside their optimum range of environmental parameters. Animals maintained under sub-optimal conditions must devote an increasing proportion of energy towards maintaining homeostasis, rather than on growth, gamete production, and immune function (Wooton, 1998). A

consequence of sub-optimal conditions is a decrease in growth rates, the number and quality of offspring, and ultimately, survival (Haywood, 1983). Thus, it is vital to determine optimal ranges of water quality parameters for zebrafish in captivity, so that mortalities can be minimized, and fish grow rapidly and consistently produce large numbers of high-quality embryos upon attainment of sexual maturity.

3.1.1. Temperature

Temperature is one of the most important physical parameters to consider in fish culture operations because of the profound effects it exerts on biological and chemical processes in living systems (Boyd, 1979). Poikilothermic animals, such as fish, display varying degrees of tolerance to changes in temperature, as well as a more narrow optimum range in which they perform best (Kelsch and Neill, 1990). Zebrafish can be classified as eurythermal, as they exhibit a tolerance for wide temperature ranges. Data from controlled laboratory experiments (Cortemeglia and Beitinger, 2005; Schaefer and Ryan, 2006) indicate that zebrafish have a maximal thermal tolerance range of 6.7–41.7 °C, which puts them in a similar class with one of the most eurythermal fish species known, the sheepshead minnow (*Cyprinodon variegatus*) (Bennett and Beitenger, 1997). It is important to note that the range of tolerance in both studies was strongly influenced by acclimation temperature; *i.e.* fish that are acclimated for a period of time at lower temperatures can extend their lower temperature tolerance further than fish acclimated to higher temperatures (Cortemeglia and Beitinger, 2005; Schaefer and Ryan, 2006). This pattern of temperature flexibility has also been documented in natural populations; recorded observations of water temperature from nine different sites at which zebrafish were collected in Bangladesh ranged from 16.5 to 33 °C (Spence et al., 2006). These data provide strong evidence that the broad tolerance shown by zebrafish in laboratory experiments is not an artificial response, but rather is representative of conditions they experience in nature.

The thermal preference or optimum for zebrafish has not been formally defined. The maintenance temperature of 28.5 °C recommended by Westerfield (1995) is almost universally cited for zebrafish in culture. This optimum is supported experimentally by at least one published study, in which zebrafish showed a marked increase in growth when held at 28±0 °C as opposed to lower and/or stochastically and predictably fluctuating temperatures (Schaefer and Ryan, 2006). However, based upon their wide range of tolerance in nature, it is highly probable that their actual preference range extends considerably above and below this temperature, and the wider range of 24–30 °C recommended by Matthews et al. (2002) is probably more appropriate.

While the general adherence to Westerfield's (1995) recommendation has resulted in temperature being perhaps the most universally consistent environmental parameter in zebrafish husbandry and research, it should be understood that deviation of holding and rearing temperatures outside the range of thermal preference may impact and potentially compromise research in a number of ways. Elevated rearing temperatures have been shown to disrupt the production of steriodogenic enzymes that regulate sexual differentiation, thereby skewing sex ratios of laboratory populations (Uchida et al., 2004). Maintenance of cellular function by molecular chaperone proteins may be less efficient at variable temperatures, thereby impacting the performance of some fish species at temperatures outside their thermal optimum (Place and Hofmann, 2001). Given these and other potential problems, it is imperative that the thermal preferences for zebrafish are more clearly defined by large-scale experiments that monitor the effects of various holding temperatures on a number of parameters, including growth, reproduction, and stress. This information can then be used to optimize the design of experiments, especially in situations that require rearing or maintenance of animals at temperatures outside the optimum range.

3.1.2. pH

Like temperature, the pH of water in aquatic systems also exerts profound effects on biological processes in fish, as well as the function of the microbial community that supports them. In closed recirculating aquaculture systems, the optimal pH range for the bacterial flora in biofilms that metabolize nitrogenous wastes excreted by fish is between 7 and 8 (Masser et al., 1999). While most freshwater fish can tolerate a wider pH range of ~6.0–9.5, it is generally practical to maintain most freshwater fish at a pH in the 7–8 range in order to promote good health of biofilters and stable water quality (Timmons et al., 2002). However, all fishes display a specific range of preference where growth, feed conversion, and reproduction is optimal, and consequently, the goal of pH management in culture is to balance these needs with the requirement of bacteria in biofilms such that production of both is maximized.

Limited data from field studies suggest that zebrafish are encountered in slightly alkaline waters. Spence et al. (2006) reported an average pH of 8.0 across nine zebrafish habitats in Bangladesh, and McClure et al. (2006) found a similar mean pH of 8.0 at 3 sites in India.

Waters in the Ganges River drainage that support zebrafish have also been reported to be typically alkaline, with an average pH in excess of 8.0 (Payne et al., 2003). Further habitat studies, during both the rainy and dry seasons, are needed to determine whether this pattern is reflective of a true ecological preference or it is simply an artifact of limited sample size.

The optimal pH for zebrafish in captivity has never been determined. The maintenance pH that most zebrafish facilities strive for is between 7.0–8.0, which is within the general range recommended for freshwater fish (Alabaster and Lloyd, 1980) and this clearly is sufficient to successfully rear and breed zebrafish in laboratory settings (Brand et al., 2002). However, systematic studies detailing growth and reproductive performance of zebrafish at different levels of pH have not been conducted. Experiments that address breeding in particular would be instructive, as spawning in a number of other cyprinid fish species (*i.e. Catla catla*, *Labeo rohita*) adapted to monsoonal climate regimes is thought to be controlled by changes in water chemistry, including pH, brought about by the onset of monsoon rains (Sinha, 1985).

3.1.3. Hardness

Water hardness is a measure of the quantity of divalent ions, primarily calcium and magnesium, and to a lesser extent, iron and selenium, in water (Wurts, 2002). Fish require these ions for biological function, and they must either be provided to fish in captivity in their water and/or diet. The most important of these ions, calcium, is required by fish for ossification, blood clotting, and a number of other biological and physiological processes (Wurts, 1993). The degree of hardness may also affect osmoregulation and is often (although not always) related to the buffering capacity of the water. Finally, hardness may also influence the pathology of certain diseases (Canadian Council on Animal Care, 1984).

It is likely that water hardness values are not consistent across zebrafish facilities, principally because the methods used to buffer pH in recirculating systems are not consistent. Regulation of alkalinity *via* the direct addition of sodium bicarbonate, without concomitant addition of calcium and/or magnesium salts, can result in low hardness values (Buttner et al., 1993; Wurts, 1993). If crystalline forms of calcium carbonate, such as crushed coral or aragonite, are employed, then hardness values will be higher. Source water also influences hardness values. Municipal supplies will have varying degrees of hardness, depending on the local geology. If distilled or reverse osmosis water is utilized, then addition of calcium and magnesium salts (usually as part of a formulated mix) is required to bring hardness values to within 75–200 mg/L $CaCO_3$, the generally recommended range for freshwater aquatic animals (Wurts, 2002).

Zebrafish have been classified as a “hard water” species, preferring hardness values in excess of 100 mg/L $CaCO_3$ (Brand et al., 2002). However, experimental evidence documenting this is limited. Zebrafish showed decreased resistance to soft environments in a comparative study with goldfish (*Carrasius auratus*) and ayu (*Plecoglossus altivelis*), a pattern the authors attributed to the presumption that zebrafish were not often subjected to low calcium conditions in nature (Chen et al., 2003). A review of the literature does not reveal any recorded hardness values for habitats within the natural range for zebrafish, although the alkaline pH reported in three references (Payne et al., 2003; McClure et al., 2006; Spence et al., 2006) is likely to be indicative of the calcareous nature of underlying geology in those habitats and, therefore, may be supportive of the suppositions of Chen et al. (2003). Systematic experiments investigating the effect of varying ranges of hardness on reproduction, growth, and disease resistance could further strengthen this classification, and would be helpful in determining guidelines for zebrafish culture.

3.1.4. Salinity

Salinity is the measure of the total concentration of all dissolved ions in water (Buttner et al., 1993). Freshwater fishes are hyperosmotic to the media in which they live, and thus tend to gain water and lose salts by diffusion across the gills and skin. Consequently, they must maintain their internal water and salt balance by excreting copious amounts of dilute urine while actively transporting ions back into the blood *via* chloride cells on the gill epithelium. The energetic cost of this process varies with the salinity of the external medium, and all freshwater fish exhibit a preferred level of salinity where this cost is minimized. Maintaining fish above or below this optimum is possible (the degree to which depends on the tolerance of the species), but because fish must expend more energy in doing so, it can compromise growth, survival, and reproduction.

Zebrafish are a freshwater fish, but are tolerant of a wide range of salinities that technically extend to brackish conditions. Sawant et al. (2001) found that embryos reared in salinities of up to 2 parts per thousand (ppt) displayed similar rates of survival and hatching to controls reared at ~0.3 ppt. These authors also showed that post-gastrulation embryos were more tolerant of changes in salinity than were younger embryos,

surviving 1–2 h pulses of up to 14 ppt. Zebrafish also appear to be tolerant of low salinities. Boisen et al. (2003) found that zebrafish were able to maintain plasma and whole body concentrations of ions in salinities as low as 35 μM sodium chloride, although this had a negative impact on egg production and survivability. Again, these observations reflect their adaptation to the variability their native habitats, which vary widely due to underlying geology and seasonal fluctuation in rainfall; recorded salinities range from ~0.1–0.6 ppt (Payne et al., 2003; Spence et al., 2006).

Salinities probably vary to a considerable degree among zebrafish facilities. Recommendations for husbandry are typically in the region of 0.25 ppt (Brand et al., 2002), but in practice some facilities run as high as 1.0 ppt (personal observation). Given the considerable impact that operation at sub-optimal salinities can have on survival, growth, and reproduction, formal studies delineating this optimum salinity would be valuable. Until such parameters are determined, maintaining zebrafish stocks at stable salinities within the general range of 0.25–0.75 ppt is the most appropriate course of action.

3.1.5. Dissolved oxygen

Dissolved oxygen is a highly important parameter in fish cultivation (Boyd, 1979). Low levels of dissolved oxygen are responsible for more fish mortalities in culture than any other parameter (Timmons et al., 2002). Fish require oxygen for respiration, and demand depends upon a number of factors, including body size, feeding rate, activity levels, and temperature. The availability of dissolved oxygen in the water is determined by water temperature, salinity, and water quality (Boyd, 1979).

The dissolved oxygen requirements of zebrafish have not been determined. In general, small-bodied, tropical fish such as zebrafish typically have high metabolic rates and, therefore, consume more oxygen per unit weight than larger fish (Helfman et al., 1997). This fact, coupled with their relatively high maintenance temperatures, stocking density, and levels of feed input that are typical of intensive zebrafish facilities necessitate that dissolved oxygen levels be maintained at or just under saturation (~7.8 mg/L at 28.0 °C) to ensure health of the fish. A number of warmwater species, such as tilapia (Popma and Masser, 1999), are tolerant of lower levels of dissolved oxygen, and it may be possible that zebrafish fall into this category, given that they are likely to encounter oxygen-poor environments in nature. However, until this is demonstrated, the most conservative approach is to maintain dissolved oxygen levels close to saturation for laboratory stocks. There is a clear requirement for detailed studies of zebrafish performance at varying levels of dissolved oxygen for developing recommended standards for culture.

3.1.6. Nitrogenous wastes

In freshwater fishes, ammonia is excreted across the branchial epithelium *via* passive diffusion, and to a lesser extent, in feces (Wilkie, 2002). It is also produced during the decomposition of decaying organic matter (*i.e.* dead fish, uneaten food). Two forms of ammonia exist at equilibrium in artificial aquatic systems, and the ratio of the highly toxic ammonia to the non-toxic ammonium increases with pH and temperature. Levels of NH3 in excess of 0.02 ppm are typically toxic to aquatic animals and, therefore, must be eliminated in closed recirculating systems; this is accomplished by nitrifying bacteria that oxidize ammonia/ammonium into nitrate (Buttner et al., 1993). The intermediate product of this conversion, nitrite is toxic to fish, and can be problematic in freshwater systems at concentrations in excess of 1 ppm (Buttner et al., 1993), although small-bodied fish seem to be generally less sensitive to nitrite toxicity than larger fish (Lewis and Moriss, 1986). Anecdotal evidence suggests that larval zebrafish are tolerant of nitrite levels of up to 2.0 ppm, (personal observation), though this has not been formally investigated. Nitrates are generally not toxic to fish (Bromage et al., 1988), although prolonged exposure of fish to levels in excess of 200 ppm may be problematic (Camargo et al., 2005). Zebrafish do not seem to be overtly impaired by chronic nitrate levels of up to 100 ppm (personal observation), though whether or not long-term exposure to elevated levels is a source of chronic stress is unknown. Studies delineating tolerance levels of zebrafish of both developing and mature zebrafish to ammonia, nitrite, and nitrates are needed to define the range of standards for these parameters in captivity.

4. Nutrition, diet, and feeding practices

4.1. Nutritional requirements

The specific nutrient (protein/amino acid, lipid, carbohydrate, mineral and vitamin) requirements of cultivated species must be known in order to be able to properly design adequate diets and feeding protocols. These requirements must be determined for each life stage; larval, juvenile, and adult, through controlled experiments that test the effects of varying dietary components on survival, growth, disease/stress resistance, and reproduction. Remarkably, this information is almost entirely lacking for zebrafish. This is undoubtedly

due in part to the fact that zebrafish can be maintained and successfully spawned (to some degree at least) under a wide variety of diets and feeding regimes, including those that are likely to be sub-optimal (personal observation).

The limited data that is available on the nutritional requirements of zebrafish centers around their demand for essential fatty acids. Zebrafish display a pattern of fatty acid metabolism typical of freshwater fishes, and have the ability to elongate and convert the essential polyunsaturated fatty acids (PUFAs) linoleate (18:2n-6) and linolenate (18:3n-3) into the physiologically more important highly unsaturated fatty acids (HUFAs), most notably eicosapentaenoic acid (20:5n-3; EPA), docsahexaenoic acid (22:6n-3; DHA), and arachidonic acid (20:4n-6; AA) (Tocher et al., 2001). Fishes vary widely in their specific requirements for essential fatty acids, but can generally be classified by their relative demand for n-3 and n-6 fatty acids in the diet. Some species require a higher ratio of n-3 FA, some require equal amounts of both, and others require higher proportions of n-6 FA (Watanabe, 1982). Zebrafish, like many other warm-water fishes, belong to the latter class. In a series of studies, Meinelt et al. reared zebrafish on formulated feeds containing varying ratios of n-3 and n-6 PUFAs, and found that growth and fertilization rates were positively correlated with the level of n-6 PUFA in the diet (Meinelt et al., 1999, 2000). Given the somewhat limited nature of these studies, further experiments in different life stages, strains, and with larger sample sizes, are required to justify feeding zebrafish diets with higher n-6:n-3 PUFA ratios. Such data would also clearly have important implications for the formulation of processed feeds for zebrafish.

Generally however, even the most basic nutritional studies have yet to be performed with zebrafish. Requirements for proteins and amino acids, lipids, and to a lesser extent, carbohydrates, vitamins, and minerals should all need to be delineated in feeding studies with different strains and life stages. Until such data become available, the best approximation of zebrafish requirements can probably be drawn from studies of cyprinid species that occupy reasonably similar feeding niches in nature, including the golden shiner (*Notemigonus crysoleucas*), and fathead minnow (*Pimephales promelas*) (Lochman and Phillips, 1996). Some work of a general nature has been also been completed with tropical freshwater fish, and may be applied to zebrafish (Sales and Janssens, 2003). However, since at least some of the requirements of zebrafish may be specific, relying on data derived for other species may not be the most scientifically sound approach.

4.2. Diet

Whatever the nutritional requirements of zebrafish turn out to be, they must be met in the diet. Choice of food is of central importance for fish health and productivity. Fish in captivity may be fed live prey items, artificial diets, or some mixture of the two. It is feasible to feed zebrafish a diet based on live food items throughout their lifespan. This is also preferable because live diets like *Artemia* or rotifers (*Brachionus* sp.) typically possess balanced nutritional profiles (Watanabe et al., 1983) and therefore are most likely to meet the requirements of zebrafish. Bloodworms (Chironomid larvae), which are an important component of the diet of wild zebrafish (Spence et al., in press) are readily available from local suppliers and may be used to feed adults. In general, live feeds are also visually and chemically attractive to fish, and are highly digestible (Cahu and Zambonino Infante, 2001). Since the specific nutritional requirements of zebrafish have yet to be determined, and may be fundamentally different from even closely related species, it may be unwise to feed an exclusively artificial diet, especially since systematic studies of adult zebrafish performance on artificial or processed diets are not available.

There are two ways in which artificial diets may be utilized in a scientifically justifiable manner for zebrafish. The first is as a supplement to live diets. Artificial feeds can be used to deliver specific nutrients that may not be present in sufficient levels in live prey items. For example, *Artemia* may be deficient in certain fatty acids or in stabilized vitamin C (Lavens and Sorgeloos, 1996). In these instances, a prepared feed containing known levels of these nutrients can be added to the diet to help ensure that these dietary requirements are adequately met.

Several published reports also indicate that artificial feeds may be utilized to rear zebrafish larvae — either as the sole food source or as a supplement to (and for) live prey items (Table 2). Goolish et al. (1999) found that zebrafish could be reared on processed diets with some success. However, rates of survival and growth of fish fed processed diets were markedly reduced when compared to those fed a control diet of *Paramecium* and *Artemia*. In a separate study, Önal and Langdon (2000) tested the efficacy of a yeast-based microparticulate diet encapsulated in either protein or gelatin capsules for first feeding zebrafish larvae, and found it possible to substitute up to 40% of *Artemia* in the diet with protein-walled capsules with no significant decreases of growth and survival. However, increases beyond this percentage resulted in greatly decreased growth and/or survival.

Table 2
Performance of larval zebrafish on various artificial diets

Diet	Approximate particle size (um)	Nutrient profile (%)					Treatment period	Larval density	Delivery method	Daily ration (dry mg)	Frequency	Water exchange	Mean length (mm)	Growth rate (mm/day)	Survival (%)	Reference
		P	L	F	C	A										
Microfeast L-10	5–10	44	12	2			4–21dpf	94/L	Manual, suspension	100 mg	3× daily	Recirc	6.0	0.15	72.8	Goolish et al. (1999)
Kyowa Fry Feed	250, 400	54	10	3			4–21 dpf	94/L	Manual, suspension	100 mg	3× daily	Recirc	6.4	0.17	60.4	Goolish et al. (1999)
OSI Microfood	100	37	30	12			4–21dpf	94/L	Manual, suspension	100 mg	3× daily	Recirc	5.8	0.13	50.1	Goolish et al. (1999)
Argent Hatchfry Encapsulon Grade 1	50–150	50	12	3	22		4–21 dpf	94/L	Manual, suspension	100 mg	3× daily	Recirc	6.4	0.17	64.6	Goolish et al. (1999)
Particle assisted rotational agglomeration	300–500	48	18		26		4–21 dpf	94/L	Manual, suspension	100 mg	3× daily	Recirc	6.3	0.16	80.0	Goolish et al. (1999)
Liquifry for egg layers	<10	5	18	0.2			4–21 dpf	94/L	Manual, suspension	100 mg	3× daily	Recirc	4.2	0.04	10.0	Goolish et al. (1999)
Dried egg yolk	250	17	31	2			4–21dpf	94/L	Manual, suspension	100 mg	3× daily	Recirc	5.3	0.10	84.3	Goolish et al. (1999)
Tetramin Baby Fish Food "E"	50	45	6	2			4–21 dpf	94/L	Manual, suspension	100 mg	3× daily	Recirc	6.2	0.16	51.1	Goolish et al. (1999)
Microfeast	5–10	44	12	2			14–22 dpf	80/L	Manual, suspension	50 mg	10× daily	Recirc	5.3	0.02	72.6	Önal and Langdon (2000)
Microfeast encapsulated in cross-linked protein-walled capsules	20–1000	44	12	2			14–22 dpf	80/L	Manual, suspension	50 mg	10× daily	Recirc	5.3	0.02	43.0	Önal and Langdon (2000)
Microfeast encapsulated in gelatin–alginated beads	20–1000	44	12	2			14–22 dpf	80/L	Manual, suspension	50 mg	10× daily	Recirc	5.9	0.08	92.3	Önal and Langdon (2000)
Commercial	200–400	60.4	22			10.8	6–27 dpf	18/L	Automatic feeder	100–120 mg	continuous	Recirc	10.6	0.33	56.0	Carvalho et al. (2006)
Experimental purified	100–400	60.2	4.5			7	6–27 dpf	18/L	Automatic feeder	100–120 mg	Continuous	Recirc	6.9	0.16	55.0	Carvalho et al. (2006)
Experimental practical	100–400	51.1	6.1			7.2	6–27 dpf	18/L	Automatic feeder	100–120 mg	Continuous	Recirc	10.3	0.32	73.0	Carvalho et al. (2006)
Experimental practical	100–400	51.1	6.1			7.2	6–27 dpf	18/L	Manual, dry	100–120 mg	3× daily	Recirc	7.9	0.20	84.0	Carvalho et al. (2006)

Diet manufacturer: Microfeast L-10, Microfeast Feeds, Bartlesville OK; Kyowa Fry Feed, Biokyowa, Inc., Cape Girardeau, MO.; OCI Microfood, Ocean Star Marine Lab, Inc., Hayward, CA.; Hatchfry Encapsulon, Argent Chemical Laboratories, Redmond, WA.; particle assisted rotational agglomeration, Rick Barrows, US Fish and Wildlife Service, Bozeman, MT; Liquifry, Interpret Ltd. UK; egg yolk, prepared in lab; Tetramin Baby Food, Tetra Werke, Germany; Commercial = AglosNorse, Ewos, Norway.
Nutrient profile: P = crude protein, L = crude lipid, F = crude fiber, C = carbohydrate, A = ash.

Finally, in the most promising of these trials, Carvalho et al. (2006) demonstrated that zebrafish reared exclusively on a practical artificial diet exhibited similar, although slightly reduced, growth and survival rates to fish fed *Artemia* alone from swim-up. However, these results were achieved only when the processed diet was delivered *continuously* during daylight hours. An important and consistent theme in all three of these studies is that fish reared on live diets always showed better performance, in terms of growth and survival, on live *versus* processed diets.

4.3. Feeding

Feeding practices are also of crucial importance in fish husbandry. The amount of feed presented at each feeding and the frequency of application are both important components of feeding protocols, and are often specific to both the species and application of its culture (*i.e.* breeding *versus* meat production) (National Research Council, 1993). These parameters may also have profound impacts on feed efficiency, growth rates, and ultimately, gamete production in cultured fish (Lee et al., 2000). None of these parameters has been defined for zebrafish.

In terms of ration size, there are two general approaches utilized in fish culture: feeding to satiation and body weight feeding. A derivative of the former method, the so-called "five-minute rule", is commonly employed in zebrafish facilities (personal observation). This technique requires that no more (or less) food should be presented to fish at each feeding than they can fully consume within 5 min. However, this particular rule-of-thumb is suspect, given that there are seldom uniform numbers of fish in tanks and different feed types have different residence times and nutrient leaching rates in the water column. The result is that fish may be chronically over- or under fed using this scheme, a situation that can lead to decreases in water quality, and/or depression of growth, reproductive function, and immune response.

Feeding by body weight involves providing a ration as a fixed percentage of fish body weight each day. In intensive culture systems, larval fish are typically fed more per day than adult fish, up to 50–300% of their body weight each day, compared to 1–10% for adults (Bryant and Matty, 1980, 1981). This method, which necessitates that managers have accurate estimates of total fish weight in the system, is commonly employed in commercial aquaculture, but rarely, if ever, utilized for zebrafish in research settings. However, it probably represents the most efficient and scientifically sound manner in which to determine feeding allowances for zebrafish. It would require a set of "average" weights to be established for larval, juvenile, and adult fish, correlated with age and total length, as well as a standard number of fish per tank. Feeding trials in which feeds are presented to fish at varying percentages of body weight can then be conducted to determine the most appropriate parameters for zebrafish.

Standards for frequency of feedings are also lacking for zebrafish. Data from other fishes generally indicate that the number of feedings required per day will generally decrease as fish get older (*e.g.* Pullin and Lowe-McConnell, 1982), but the number (as well as the amount of food in each) most appropriate for each life stage is often very species and environment (*e.g.* temperature) specific. It may be reasonable to speculate that because zebrafish are small-bodied and lack a true stomach, it would be best to present them with frequent, small meals throughout the day to promote maximal assimilation, but this remains to be demonstrated. At any rate, an ideal frequency can be readily determined *via* a series of simple experiments, and should be combined with the results of ration size studies to determine the most suitable feeding regimen for zebrafish.

5. Reproduction and breeding techniques

5.1. Reproduction

Relatively little is known about zebrafish breeding and reproductive behavior, particularly in natural settings. However, results of a number of laboratory-based experiments, along with anecdotal observations stemming from years of use as a research model organism provide a reasonable picture of reproduction in this animal. Zebrafish are asynchronous, batch spawners that, under favorable conditions, spawn continuously upon attainment of sexual maturation (Breder and Rosen, 1966). Females are capable of spawning on a nearly daily basis. Eaton and Farley found that females would spawn once every 1.9 days if continuously housed with a male (Eaton and Farley, 1974), and Spence and Smith (2005) showed that females in their experiments were capable of producing viable clutches every day over a period of at least 12 days, though variance in egg production was substantial. This interval is likely to be greater when the environment (water quality, diet, social situation, *etc.*) is sub-optimal or if the fish are used for production frequently.

Olfactory cues play a vital role in zebrafish reproduction. The release of steroid glucuronides into the water by males induces ovulation in females (Chen

and Nartinich, 1975; van den Hurk and Lambert, 1983). After ovulation, females release hormones that in turn prompt male mating behavior that immediately precedes and elicits oviposition and spawning (van den Hurk and Lambert, 1983). Pheromones also appear to have the ability to suppress reproduction, as holding water from "dominant" female zebrafish has been shown to inhibit spawning of subordinate females (Gerlach, 2006). This information should be considered in the design of broodstock management regimes; for example, a combination of increased chemical filtration and rate of water replacement on recirculating systems may help to reduce potential decreases in the reproductive output of broodstock brought about by pheromonally mediated dominance interactions between females.

Reproduction in zebrafish is strongly regulated by photoperiod. Zebrafish most commonly spawn at dawn, within the first few hours of daylight, in both the laboratory (Selman et al., 1993) and the wild (Spence et al., 2006). However, spawning does not seem to be strictly limited to this time period. In captivity, zebrafish will breed throughout the day, particularly during the evenings prior to an imposed dark period, and it is also possible to strip viable eggs from females throughout the day (personal observation). In the wild, zebrafish have also been observed spawning during the afternoon following the onset of heavy rain (R. Spence, pers. comm.). So while clearly there is a strong relationship between photoperiod, ovulation, and spawning, control is not absolute.

There also appears to be some element of mate choice in zebrafish. Ritualized mating behavior and the establishment and defense of territories on the part of males suggest that females may be selective (Darrow and Harris, 2004; Spence and Smith, 2005). This supposition is supported by the fact that females have been shown to produce larger clutches and spawn more frequently when paired with certain males (Spence and Smith, 2006a). However, the selective basis of female choice is unclear. Spence and Smith (2006a,b) found that territorial males had a marginally higher reproductive success than non-territorial males at low densities, though there was no difference at higher fish densities, and that male dominance rank did not correlate with female egg production. Male defense of territories may be one cue that females use to select males. Both male and female zebrafish show a strong preference for oviposition site, selecting and preferentially spawning over gravel *versus* silt in both laboratory and field-based experiments (Spence et al., accepted for publication). Fish also showed a preference for vegetated over non-vegetated sites. Therefore, male defense of desirable spawning locations, over which females are choosy, may be the basis to the zebrafish mating system.

Females may also select males based on their genotype. Many fish, including zebrafish, use olfactory cues to differentiate between kin and non-kin, and this mechanism may be utilized during breeding to avoid mating with close relatives. For example, female rainbow fish *Melanotaenia eachamensis* and guppies *Poecillia reticulata* prefer unrelated over related males based on visual and olfactory cues (Hughes et al., 1999; Arnold, 2000). Zebrafish also appear to use olfactory cues in social and mating contexts. Using odor plume tests, Gerlach and Lysiak showed that adult female zebrafish chose the odors of non-related, unfamiliar (reared and maintained separately) males over those of unfamiliar brothers for mating (Gerlach and Lysiak, 2006). The underlying genetic basis of this preference is unknown, but may be the major histocompatibility complex (MHC) genes that are important in kin recognition in other fish species (Apanius et al., 1997).

Clearly, years of breeding zebrafish (in many cases, *via* sib-mating) in captivity demonstrates that female choice based on any one or all of these factors may be readily overridden. However, a more comprehensive understanding of reproductive behaviors may facilitate the design of improved spawning techniques and breeding programs that can ultimately help increase spawning efficiency in laboratory breeding facilities.

5.2. Breeding techniques

The most basic, and the first formally described breeding technique for laboratory zebrafish involves placing marbles at the bottom of holding or special breeding tanks. When fish spawn over the marbles, the eggs drop into the spaces in between, preventing egg cannibalism and facilitating their subsequent collection (Westerfield, 1995; Brand et al., 2002). While this method may be effective to some extent, it is generally over-simplified and impractical for use in large culturing facilities with hundreds or thousands of tanks. Despite its shortcomings, it is still frequently cited in the methods sections of zebrafish papers, and is often used by investigators breeding zebrafish for the first time.

The majority of zebrafish breeding facilities currently utilize a dedicated breeding tank technique that adheres to the following general principles: a small (typically <1 L) plastic mating cage or box with a mesh or grill bottom is placed inside a slightly larger container that is filled with water. Fish (pairs or small groups) are then added to the box in the evening. When the fish spawn (usually the following morning; see above discussion), the fertilized eggs fall

through the "floor" of the inner box and are thereby protected from cannibalism by adults (Mullins et al., 1994).

This technique has proven to be generally effective and, consequently, derivations of the trap design are manufactured by a number of aquaculture and laboratory product supply companies. Available products vary slightly in size, shape, depth, and total volume, as well as adjustability. Surprisingly, the effects of these parameters on reproductive success have not been formally investigated. The only published study explored the effects of varying the size of the breeding cage itself on spawning success and egg production; fish were kept in the same recirculating water throughout the experiment (Goolish et al., 1998). The results, which showed no difference in spawning success between control cage of 3.5 L and test cages of 500, 400, 300, 200, and 100 ml, and reduced production in 200 and 100 ml sizes, are of only minimal applicability to current practices. The total volume of water in static breeding cages is one parameter that should be tested, since the excretion of nitrogenous wastes by fish may decrease water quality and breeding conditions in cages, particularly in smaller volumes. This is of particular interest because many products available on the market are very small to maximize space efficiency. Whether or not this is an acceptable trade-off remains to be determined.

Another parameter that requires investigation is whether or not the origin of water used to fill mating cages influences spawning success. Many zebrafish facilities are designed in such a way that water used for breeding is taken directly from the sumps (after filtration) of the recirculating systems themselves, rather than using an off-system source of water for this purpose. A key difference between these two approaches has to do with metabolites; specifically sex pheromones. While effective biological filtration eliminates ammonia and nitrites, it does not remove steroid or steroid conjugates. Pheromones, most notably steroid glucuronides, have been shown to enhance, and also suppress reproduction in zebrafish (van den Hurk et al., 1987; Gerlach, 2006). Therefore, these compounds, in addition to nitrogenous waste products and phosphates, might have impacts on spawning. Using water from off-system reserves may help avoid these problems, if they exist. Experiments comparing these conditions would be simple in both concept and design, and could be readily conducted with minimal resources.

5.3. Spawning efficiency

Clearly, the growing popularity of the zebrafish as an experimental model indicates that the ability of facility managers to induce reliable egg production in their zebrafish stocks does not appear to be a major problem in the field. However, it is unclear as to how efficient this process is, and should be, across rearing facilities. Although zebrafish appear to spawn under a wide range of conditions in the laboratory, reductions in the overall egg production may reflect sub-optimal husbandry parameters, and could constitute an unacceptable situation as far as fish welfare is concerned. Therefore, data on the average broodstock spawning parameters (*i.e.* spawning success, fertility, clutch size, inter-spawn interval, reproductive longevity for standard zebrafish wild-type strains, *etc.*) should be collected from a wide number of culturing facilities. Such information would facilitate the design of experiments that could be used to help establish standards in broodstock production and quality for the zebrafish research community.

6. Larval rearing

6.1. Larval biology

In typical laboratory conditions (28.5 °C), zebrafish larvae typically hatch within 2.5–3 days post-fertilization (dpf) (Westerfield, 1995). After hatching, they adhere to hard surfaces by means of specialised cells on the head (Laale, 1977), displaying low levels of spontaneous activity until ~5 dpf, when they inflate their gas bladders by swallowing air at the water surface (Goolish and Okutake, 1999). Larvae subsist largely on yolk-sac reserves until the onset of exogenous feeding, which is coincident with completion of a number of physiological steps, including the development of a functional digestive system (Pack et al., 1996; Holmberg et al., 2004) and the ability to regulate position in the water column (gas bladder inflation (Goolish and Okutake, 1999). Depending on conditions, this transition takes place between 5–6 dpf (Kimmel et al., 1995). The yolk is quickly exhausted after this point, with total absorption occurring ~7 dpf (Jardine and Litvak, 2003). From this point on (and preferably before), feed must be provided on a nearly constant basis to meet nutritional demands, which are highest during this life stage (Dabrowski, 1986; National Research Council, 1993).

6.2. Diet and nutrition

Selection of the appropriate diet is essential to larval survival. Live prey items such as *Paramecium*, rotifers, and *Artemia* are preferable, due to the fact that they are generally more nutritionally balanced, visually and chemically attractive, digestible, and distributed evenly

in the water column than artificial diets (Cahu and Zambonino Infante, 2001). Artificial diets may be utilized (see above), but in general survival and growth rates are depressed when compared with performance on live diets (Goolish et al., 1999; Önal and Langdon, 2000; Carvalho et al., 2006).

Zebrafish are gape-limited predators, particularly during the larval stage. First feeding zebrafish have a maximal gape size of ~100 μm (L. Patricia Hernandez, personal communication), and prey items must accordingly be within a similar range. *Paramecium* (150–200 μm) and rotifers (~250 μm) are ideally sized. *Artemia* nauplii are much larger, typically on the order of 500×100–150 μm ($L \times W$), but also can be taken by first feeding larvae, as it is possible to successfully rear zebrafish on them exclusively (Carvalho et al., 2006). Presumably, larvae may consume nauplii by breaking off smaller pieces during the first few days of presentation, until gape size increases enough for them to swallow the whole organism, utilizing the typical teleost ram-suction feeding behavior (personal observation). Current data support the general approach of utilizing *Paramecium* or rotifers in first feeding zebrafish until they are able to efficiently feed on *Artemia*.

Larval fish utilize both chemical and visual cues to locate and ingest prey (Cahu and Zambonino Infante, 2001). Visual feeding behavior is dependent upon both motion and color of the prey item (D'Abramo, 2002), while olfactory detection of specific molecules, including free amino acids, also promotes successful capture of food particles (Kolkovski et al., 1997; McElligott and O'Malley, 2005). Feeding and prey tracking behaviors in zebrafish larvae appear to be strongly visual, as individuals kept in dark conditions feed poorly or not at all (McElligott and O'Malley, 2005). The role of olfaction in zebrafish feeding behaviors has not been fully explored, but zebrafish larvae exhibit a behavioral response to free amino acids as early as 4 dpf, just prior to the onset of exogenous feeding (Lindsay and Vogt, 2004). Observations of improved performance of zebrafish larvae reared on live diets *versus* formulated artificial feeds (Goolish et al., 1999; Önal and Langdon, 2000; Carvalho et al., 2006) probably reflect the superiority of natural feed sources over prepared inert feeds in both of these areas (Cahu and Zambonino Infante, 2001).

The nutrient profile of the diet must meet the demands of the fish. Since the nutrient requirements of zebrafish are largely unknown (see above), current practices are based on guesswork. For instance, it is not possible to rear zebrafish larvae using *Paramecium* alone, presumably because the nutrient profile of these protozoans is not adequate to support dietary demands during the larval to juvenile transition (metamorphosis) that occurs approximately three weeks post-fertilization (Brown, 1997). While it is not altogether clear which nutrients *Paramecium* lack, there are several plausible explanations for its poor performance as a sole food source for zebrafish. Ciliate cultures fed only bacteria have decreased levels of polyunsaturated fatty acids and certain amino acids (Boëchat and Adrian, 2006). It is also possible that bacteria-fed *Paramecium* lack essential minerals such as iodine, a precursor of thyroid hormone, which is important for initiating metamorphosis in fishes (Brown, 1997; Moren et al., 2006). Because *Artemia* nauplii and some formulated diets can be used as sole food sources for larval zebrafish with varying degrees of success (*i.e.* Carvalho et al., 2006), they presumably possess nutrient profiles that better reflect the requirements of developing zebrafish. However, in order to be able to eliminate a great deal of the uncertainty involving the most appropriate diet(s) for zebrafish larvae, the nutritional requirements of this life stage should be determined.

6.3. Water quality

The requirement of larval zebrafish to feed almost continuously must be balanced with the need to maintain adequate water quality in their environment. High food inputs have a tendency to result in poor environmental conditions (low dissolved oxygen, elevated ammonia, *etc.*) that will negatively impact growth and survival of larvae. Therefore, the excretory products of the fish, as well as decomposing uneaten feed, must be removed by water exchange. Historically, larvae were reared in static containers up until 21 dpf (Westerfield, 1995). This technique, which requires manual water changes and siphoning of solid wastes on a daily basis, is not practical for larger zebrafish facilities.

A more time efficient and biologically sound approach involves placing tanks of larvae directly on recirculating systems, with initially low rates of flow. Depending on the rate of water exchange, a proportion of nitrogenous wastes and solids are automatically flushed from tanks, eliminating the need for daily, laborious water exchange. The degree of flow is important; if flow rates are too high, especially within the first 5 days after gas bladder inflation, there will be a negative impact on survival, presumably because larvae are forced to expend energy on maintaining their position in the water column (Bagatto et al., 2001). Elevated flow rates may also make prey items more difficult to capture, particularly for early larval fish with restricted swimming capabilities.

Alternatively, rates of water turnover must be sufficiently high to maintain a satisfactory water quality. Information on precise water quality parameter thresholds during various developmental stages for zebrafish is lacking, but would be useful in determining idealized rates of flow in nurseries.

6.4. Growth rates and survival

The efficacy of a rearing protocol can typically be measured in growth rate and survival of larvae. The results of published studies detailing larval growth vary widely. For example, in a recent study, Biga and Goetz (2006) reported an average total length (TL) of ~4.7 mm at 28 days post-fertilization. These results contrast markedly with those of Carvalho et al. (2006) who documented average total lengths of up to 14.3 mm TL for the same time period. This nearly four-fold difference is remarkable, but is probably indicative of discrepancies in rearing environment, specifically diet. Although a number of factors, including rearing densities and genetic differences between strains, are likely to be involved, the salient difference between the two studies is in their feeding protocols: Biga and Goetz fed their fish sterile *Paramecium* only, whereas fish in the Carvalho et al. study were reared exclusively on *Artemia* nauplii. This example highlights the need for establishing standardized, biologically based rearing protocols.

The few published reports of survival rates in the literature are also inconsistent. The most detailed information comes from dietary studies. In their evaluation of the performance of larval zebrafish on ten different artificial diets, Goolish et al. (1999) reported survival rates in the range of ~15–80% through 28 days post-fertilization, depending on diet and method of food delivery. Mean survival among treatment groups in the study by Önal and Langdon (2000) ranged from ~40–98% for a similar time period, with the best performance resulting from a diet of 40% GAB (commercial larval feed encapsulated in a gelatin–alginate bead) and 60% *Artemia* nauplii. Carvalho et al. (2006) reported mean survival ranging from 55–86% at 27 dpf, again depending upon diet and frequency of application. The variance in the data from these studies stems from the fact that all three were trials of artificial diets and feeding methods. In practice, survival should generally be much higher, with zero mortality not uncommon (Diekmann and Nagel, 2005; Lawrence et al., in press).

In general, current data suggest that best practices for the rearing of larval zebrafish are those that involve the delivery of large amounts of live zooplankton (initially ciliates and most importantly *Artemia*) under low to moderate rates of water exchange that promote stable and favorable water quality. Formulated diets may be used, but are much less effective than live feeds; even when delivered continuously, growth and survival of clutches fed on these feeds are reduced when compared to clutches reared on live diets. Further work is needed to develop artificial diets capable of producing results comparable to those achieved using more labor intensive live feeds. Additionally, more specific information on the precise water quality and nutrient requirements of zebrafish larvae would also be helpful in further refining both diets and rearing protocols.

7. Adult maintenance

7.1. Holding densities

The density at which fish are held in culture exerts profound impacts on their health, productivity, and welfare (Ellis et al., 2002). In general, exposing fish to crowded conditions results in decreases in growth rate (Procarione et al., 1999) and immune function (Wedemeyer, 1996; Suomalainen et al., 2005). The inverse relationship between these factors and rearing density has been linked to stress, indicated by the fact that the stress hormone cortisol is often elevated in the plasma of fish held in crowded conditions (Vijayan and Leatherland, 1990; Barton and Iwama, 1991). What ultimately evokes this stress response at elevated densities is unclear; the two most commonly proposed factors are an increase in aggressive behaviors or crowding itself (Ellis et al., 2002).

Overt, antagonistic behavioral interactions between individuals during the establishment of territoriality and the formation of dominance hierarchies contributes to stress of fish in captivity, with plasma cortisol levels being higher in subordinate than in dominant individuals (Pottinger and Pickering, 1992; Fox et al., 1997). It has been proposed that such direct behavioral interactions are highest at intermediate densities (Pickering, 1992) as territories are easiest to defend at low densities and impossible to defend at higher densities (McCarthy et al., 1992). Zebrafish have been shown to exhibit antagonistic behavior and to establish dominant–subordinate relationships (Larson et al., 2006), and so the possibility that these relationships between density and aggression may contribute to stress in captivity deserves further investigation.

Crowding itself, in the absence of overt aggressive behaviors, may also cause stress to fish. Crowded conditions lead to elevated cortisol levels in many species, including sea bream, (*Sparus aurata*), common carp,

(*Cyprinus carpio*), and rainbow trout (*Oncorhynchus mykiss*) (Pickering and Pottinger, 1987; Tort et al., 1996; Ruane et al., 2002). Zebrafish also appear sensitive to crowding. Adult zebrafish do show increases in whole-body cortisol levels when subjected to high-density conditions (40 fish/L) (Ramsay et al., 2006) and breeding efficiency is negatively impacted in crowded conditions (60 fish/L) (Goolish et al., 1998). In both cases, water quality was held constant, suggesting that some aspect of crowding itself elicited the negative effects.

Reduced performance and impaired health of fish at elevated densities may also be caused by increased competition for resources and a decrease in water quality (Ellis et al., 2002). Because zebrafish in research settings are typically fed *ad libitum*, competition for food is less likely to be a factor than is water quality. In the absence of formal data for zebrafish, one may at least initially distinguish between density problems related to water quality and those caused by social environment and behavior by examining growth rates and size variation in clutches (Jobling, 1995). In this model, initially proposed by Jobling for rainbow trout, clutches with high mean growth rates and high variance in size indicate good water quality and social environment. Clutches with low mean growth rates and low variance are indicative of poor water quality, whereas clutches with low mean growth rates and high size variance are more suggestive of a poor social environment.

A further issue involving rearing densities is the effect that they may have on sex ratio. Environmental conditions, including temperature, pH, behavioral interactions, and rearing densities have been shown to influence primary sexual differentiation in some fishes (Godwin et al., 2003). The effect of densities on sex ratios has been described in several species, including the American eel, (*Anguilla rostrata*) (Krueger and Oliveira, 1999), and the paradise fish (*Macropodus opercularis*) (Francis, 1984). It has been suggested that zebrafish are also sensitive to rearing densities, based upon anecdotal observations that clutches reared at low densities often show a female bias (Brand et al., 2002). Although recent work suggests that it is actually growth rates caused by variations in food consumption that influence the expression of the sexual phenotype in zebrafish, (Lawrence et al., in press) densities do have an effect on the amount of food that each fish consumes, and thereby may influence growth rate and, in turn, sexual development.

It is important to note that classifications of densities in zebrafish research tend to vary considerably depending on the experimental setting. For example, the actual densities (fish/liter) in the "high" density treatments (0.25 fish/liter) utilized in one recent behavioral study by Spence and Smith (2006b) are nearly 200 times lower than the "high" density treatment (40 fish/liter) in Ramsay et al.'s (2006) paper on crowding. Therefore, the raw data must be taken into account when interpreting the results of density-related studies, especially if such data are to be applied to developing standards for husbandry. Given the potential effects of density on the health and productivity of zebrafish at different developmental stages, standard rearing and holding densities should be established to help minimize stress in laboratory populations.

7.2. Genetic breeding programs

Closed populations of laboratory strains of animals such as zebrafish are subject to a continuous loss of genetic diversity stemming from founder effects, genetic drift, and population bottlenecking (Stohler et al., 2004). These evolutionary forces, which are further intensified by the subsequent isolation of the founding population from its source as well as inconsistent propagation methodologies, may lead to decreases in production and long-term viability of strains. These same factors also contribute to the genetic divergence of different populations of laboratory strains, which if unaccounted for, could compromise the reproducibility of experiments.

Members of the zebrafish research community have long recognized that the ability to maintain high-quality, genetically uniform wild-type laboratory strains of zebrafish over the long-term is of vital importance for the use of the animal as an experimental model (Hawkins et al., 2001). The Zebrafish International Resource Center (ZIRC), which was formed in 1998 in part to help meet this need, does indeed propagate and disseminate wild-type strains to research groups around the world, though they have not made the details of their genetic breeding program public. Further, it is also unclear whether the other global stock centers in Tuebingen (Germany), and London utilize the same breeding program, or if there is routine interbreeding of stocks between the three facilities. As a further complication, other large facilities separately maintain and distribute their own versions of wild-type strains throughout the zebrafish community.

The poor coordination of breeding and stock distribution highlights the need for the zebrafish research community to make standardization of wild-type, mutant, and transgenic strains of zebrafish a priority. Discrepancies between the main stock centers, if and where they exist, should be eliminated or minimized, and a standard protocol for maintaining the genetic integrity of lab strains

should be developed and implemented throughout the zebrafish research community.

8. Conclusions

Given the growing importance of the zebrafish as a research model system, it is imperative that standards for its husbandry be developed. Several decades of scientific work on a range of aspects of zebrafish biology, when considered holistically, provide an excellent springboard for research directed at questions relating to husbandry, including nutrition, breeding, behavior, and external environmental requirements. The application of a more comprehensive understanding of the animal as a whole organism, rather than on the basis on its constituent parts, can only serve to improve the reproducibility of experiments, efficiency of use, and its welfare in a research environment.

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