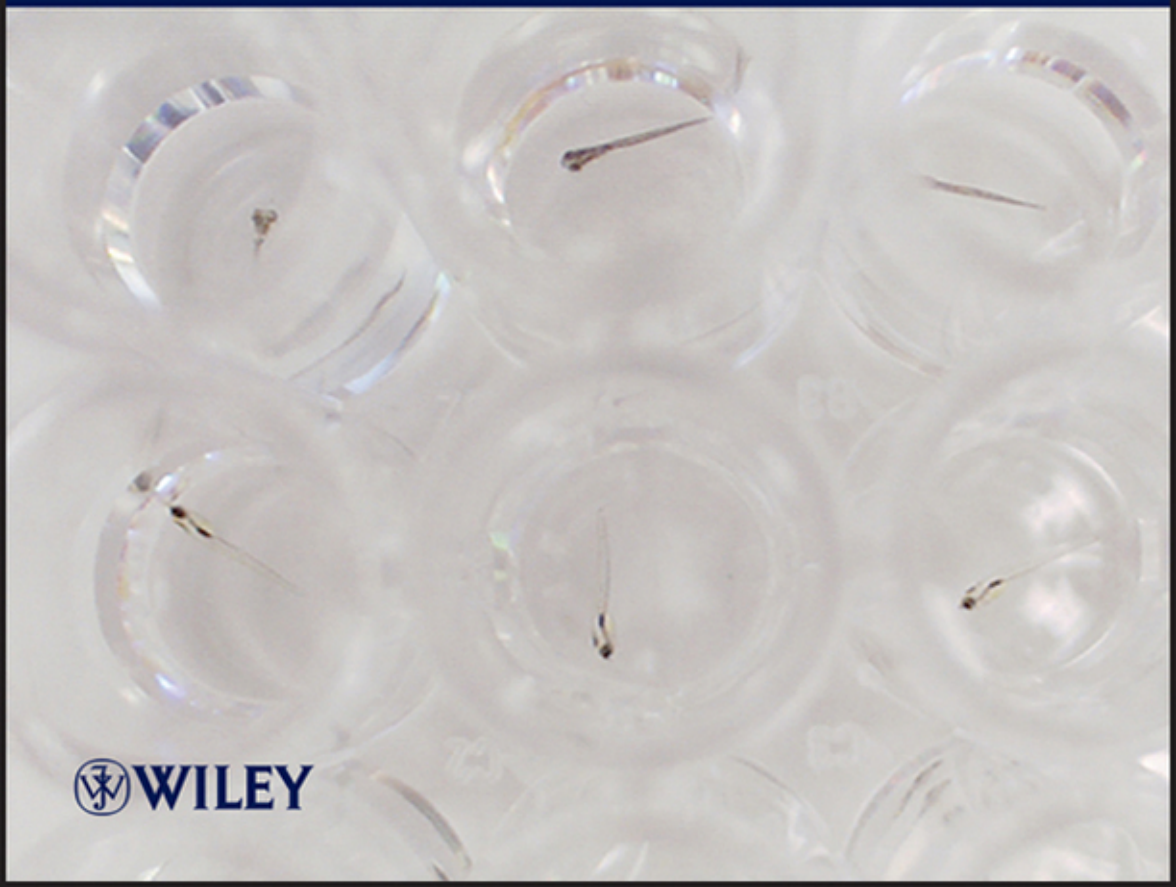
Zebrafish

Methods for Assessing Drug Safety and Toxicity

Edited by *Patricia McGrath*



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Preface

The zebrafish model organism is increasingly used for assessing compound toxicity, safety, and efficacy and numerous studies confirm that mammalian and zebrafish toxicity profiles are strikingly similar. This convenient, predictive animal model can be used at an intermediate stage between performing cell-based assays and conventional animal testing. Although *in vitro* assays using cultured cells are commonly used to evaluate potential drug effects, they are frequently not predictive of the complex metabolism that affects drug efficacy and causes toxicity in animals. Therefore, many compounds that appear effective *in vitro* fail during costly animal trials.

Currently, there is no single reference source for toxicity testing using this emerging model organism. Investigators seeking general information on toxicity methods and results currently refer to toxicology textbooks that focus on mammalian models. The target readership of this timely book includes students (undergraduates and graduate level) and professionals in all biomedical sciences, including drug research and development, environmental testing, and product safety assessment.

This initial volume describes methods for assessing compound-induced toxicity in all major organs, including heart (Chapters 4, 5, 6, and 11), liver (Chapters 8, 9, and 11), kidney (Chapter 11), central nervous system (Chapters 10, 11, 12, 13, and 14), eye (Chapters 15 and 16), ear (Chapter 19), hematopoietic system (Chapter 7), and overall development (Chapters 2 and 3).

This vertebrate model offers several compelling experimental advantages including drug delivery directly in the fish water, small amount of drug required per experiment, statistically significant number of animals per test, and low cost. Animal transparency makes it possible to visually assess compound-induced effects on morphology and fluorescently labeled probes and antibodies can be used to localize and quantitate compound effects in physiologically intact animals. Compounds can be assessed using wild-type, mutant, transgenic, knockdown, and knock-in animals. In addition, several chemical-induced disease models, phenocopies, designed to identify potential drug candidates, are described (Chapters 14, 16, 17, 18, 19, and 21). Assays used to develop disease models can also be used to assess compound-induced toxicity on specific end points. Several widely used cell-based assay techniques have been adapted for use with this small model organism and quantitative morphometric image analysis (Chapters 10, 14, and 18) and microplate formats (9, 16, and 17) offer unprecedented throughput for assessing compound effects in whole animals. Additional analytical tools adapted for use with zebrafish, including ECG (Chapter 6) and motion detectors (Chapters 10, 12, 13, 15, and 18), are described.

Improvements in breeding and spawning, which address requirements of industrial scale screening, are discussed (Chapter 1). As a reference source to be used as a companion document for assessing data presented in individual chapters, we have

reprinted a description of zebrafish stages during organogenesis. An interesting recent development that successfully pairs this emerging model with an emerging market need is the use of zebrafish for assessing safety of nanoparticles (Chapter 20), which are now incorporated in virtually all product categories. In addition, the unique ability of this animal to regenerate tissue and organs offers potential for compound screening for cell-based therapies (Chapter 22).

An important recent development impacting wider use of zebrafish for toxicity testing is that the Organization for Economic Cooperation and Development (OECD), an international organization helping governments tackle the economic social and governance challenges of the globalized economy, is developing standards for using zebrafish to assess chemical toxicity.

Further supporting wider use of this emerging model organism, the European Union recently enacted Registration, Evaluation, Authorisation and Registration of Chemicals (REACH) legislation that requires toxicity assessment for any chemical imported or manufactured in the region and is expected to have far-reaching impact on new product introductions and animal testing, including zebrafish.

Confounding interpretation of drug-induced toxicity and limiting wider acceptance of this model organism, reported results show that inter- and intralaboratory standards vary widely, although cooperation among academic and industry laboratories to develop standard operating procedures for performing compound assessment in zebrafish is increasing. Understanding all aspects of current toxicology testing will facilitate more uniform approaches across industries and enhance acceptance from regulatory authorities around the world. Full validation of this model organism will require assessment of large numbers of compounds from diverse classes in a wide variety of assays and disease models. I hope that methods and data reported here will facilitate standardization and support increased use of zebrafish for compound screening.

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Chapter 1

The Reproductive Biology and Spawning of Zebrafish in Laboratory Settings*

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1.1 INTRODUCTION

There is growing demand for new, robust, and cost-effective ways to assess chemicals for their effect on human health, particularly during early development. Traditional mammalian models for toxicology are both expensive and difficult to work with during embryonic stages. The zebrafish (*Danio rerio*) has a number of features that make it an excellent alternative model for toxicology studies, including its small size, rapid external development, optical transparency during early development, permeability to small molecules, amenability to high-throughput screening, and genetic similarity to humans (Lieschke and Currie, 2007; Peterson et al., 2008).

A major underpinning of the use of zebrafish in this arena is their great fecundity, which supports high-throughput analysis and increases the statistical power of experiments. Adult female zebrafish can spawn on a daily basis, and individual clutch sizes can exceed 1000 embryos (Spence and Smith, 2005; Castranova et al., 2011). However, consistent production at these high levels is greatly dependent upon sound management of laboratory breeding stocks, which must be grounded in a thorough understanding of the reproductive biology and behavior of the animal. Management practices must also address key elements of husbandry, most notably water quality, nutrition, and behavioral and genetic management.

*Some information in this chapter was originally published in Harper and Lawrence, The Laboratory Zebrafish, CRC Press/Taylor and Francis Group, 2011. Used with permission.

Zebrafish: Methods for Assessing Drug Safety and Toxicity, Edited by Patricia McGrath.

1.2 OVERVIEW OF ZEBRAFISH REPRODUCTIVE BIOLOGY AND BEHAVIOR

1.2.1 Natural History

Zebrafish are native to South Asia, and are distributed primarily throughout the lower reaches of many of the major river drainages of India, Bangladesh, and Nepal (Spence et al., 2008). This geographic region is characterized by its monsoonal climate, with pronounced rainy and dry seasons. Such seasonality in rainfall profoundly affects both the physicochemical conditions in zebrafish habitats and resource availability. These factors also shape reproductive biology and behavior.

Data gathered from the relatively small number of field studies suggest that zebrafish are primarily a floodplain species, most commonly found in shallow, standing, or slow-moving bodies of water with submerged aquatic vegetation and a silt-covered substratum (Spence et al., 2008). Environmental conditions in these habitats are highly variable in both space and time. For example, pooled environmental data from zebrafish collection sites in India in the summer rainy season (Engeszer et al., 2007) and Bangladesh in the winter dry season (Spence et al., 2006) show that pH ranges from 5.9 to 8.5, conductivity from 10 to 2000 μS, and temperature from 16 to 38°C. These differences, which reflect changes in seasonality and geography, provide strong evidence that zebrafish are adapted to wide swings in environmental conditions. Results of laboratory experiments demonstrating their tolerance to both thermal (Cortemeglia and Beitinger, 2005) and ionic (Boisen et al., 2003) fluctuations support this hypothesis.

Zebrafish feed mainly on a wide variety of zooplankton and insects (both aquatic and terrestrial), and to a lesser extent, algae, detritus, and various other organic materials (McClure et al., 2006; Spence et al., 2007a). Gut content analyses of wild collected animals indicate that they feed primarily in the water column, but also take items off the surface and the benthos (Spence et al., 2007a).

Zebrafish are a shoaling species, most often occurring in small schools of 5–20 individuals (Pritchard et al., 2001), although shoals of much larger numbers have been observed (Engeszer et al., 2007). Reproduction takes place primarily during the monsoons, a period of resource abundance (Talwar and Jhingran, 1991). Fish spawn in small groups during the early morning, along the margins of flooded water bodies, often in shallow, still, and heavily vegetated areas (Laale, 1977). There has also been at least one report of fish spawning during periods of heavy rain later on in the day (Spence et al., 2008). Females scatter clutches of eggs over the substratum, and there is no parental care. The eggs, which are demersal and nonadhesive, develop and hatch within 48–72 h at 28.5°C. After hatching, larvae adhere to available submerged surfaces by means of specialized cells on the head (Eaton and Farley, 1974). Within 24–48 h post hatch, they inflate their gas bladders and begin to actively feed on small zooplankton. Larval fish remain in these nursery areas as they develop, and move into deeper, open water as they mature and floodwaters recede (Engeszer et al., 2007).

1.2.2 Reproductive Cycle and Controlling Factors

Zebrafish typically attain sexual maturity within 3–6 months post fertilization in laboratory settings, although this may vary considerably with environmental conditions, most importantly rearing densities, temperature, and food availability (Spence et al., 2008). Consequently, it may be more appropriate to relate reproductive maturity to size rather than age. Data from a number of studies indicate that a standard length of approximately 23 mm corresponds with attainment of reproductive maturity in this species (Eaton and Farley, 1974; Spence et al., 2008).

Under favorable conditions, zebrafish spawn continuously upon attainment of sexual maturation (Breder and Rosen, 1966). Females are capable of spawning on a daily basis. Eaton and Farley (1974) found that females would spawn once every 1.9 days if continuously housed with a male, and Spence and Smith (2006) reported that females were capable of producing 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 chemistry, nutrition, behavioral setting, etc.) is suboptimal or if the fish are used for production frequently (Lawrence, 2007).

Olfactory cues play a determining role in zebrafish reproduction and spawning behavior (Fig. 1.1). The release of steroid glucuronides into the water by males induces ovulation in females (Chen and Nartinich, 1975; Hurk and Lambesrt, 1983). Gerlach (2006) reported that females exposed to male pheromones showed significant increases in spawning frequencies, clutch size, and egg viability when compared with females held in isolation. Upon ovulation, females release pheromones that in turn

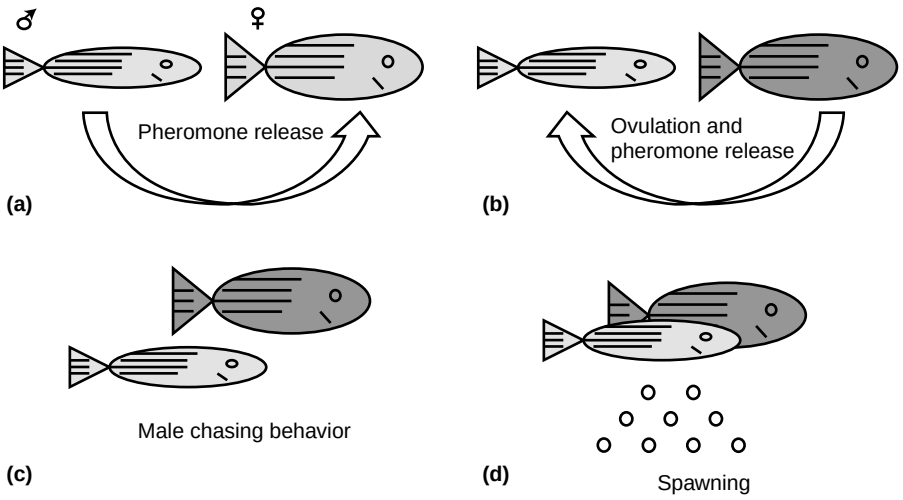

Figure 1.1 Simplified model of olfactory control of zebrafish reproduction. (a) Male (yellow) releases pheromone into water in vicinity of female (blue). (b) Female smells pheromone, which triggers ovulation (indicated by female color change to green), which is then followed by female release of postovulatory pheromones. (c) Male senses pheromones, which trigger mating and chasing behavior. (d) Spawning. (See the color version of this figure in Color Plates section.)

prompt male mating behavior that immediately precedes and elicits oviposition and spawning (Hurk and Lambesrt, 1983). Pheromonal release in some cases also appears to suppress reproduction, as holding water from "dominant" female zebrafish has been shown to inhibit spawning of subordinate females (Gerlach, 2006).

Reproduction in zebrafish is also influenced by photoperiod. Ovulation most typically occurs just prior to dawn (Selman et al., 2005) and spawning commences within the first few hours of daylight (Spence et al., 2006; Engeszer et al., 2007). However, spawning is not strictly limited to this time period. Zebrafish will breed in the laboratory throughout the day, particularly during the evenings, although spawning is most reliable and intense in the early morning (personal observation). In the wild, zebrafish have also been observed spawning during the afternoon following the onset of heavy rain (Spence et al., 2008).

1.2.3 Reproductive Behavior

Zebrafish display ritualized courtship behaviors prior to and during spawning. During courtship, males swim in tight circles or hover, with fins raised, above a spawning site in clear view of nearby females. If females do not approach, males will chase them to the site, snout to flank. When spawning, a male swims parallel to a female and wraps his body around hers, triggering oviposition and releasing sperm simultaneously (Spence et al., 2008). This ritualized mating behavior and the fact that males are known to establish and defend territories indicate that females are selective (Darrow and Harris, 2004; Spence and Smith, 2005). This is supported by the fact that females will produce larger clutches and spawn more frequently when paired with certain males (Spence and Smith, 2006).

Females may exert choice on the basis of several combined factors. The quality of a spawning site is clearly important, as 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., 2007b). If given the choice, fish will also spawn preferentially in vegetated versus nonvegetated sites (Spence et al., 2007b) and in shallow versus deep water (Sessa et al., 2008; Adatto et al., 2011).

Male defense of territories may be one cue that females use to select males. Spence and Smith (2005, 2006) found that territorial males had a marginally higher reproductive success than nonterritorial 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. This fact, coupled with female preferences for substrate, depth, and structure for spawning, suggests that male defense of desirable spawning locations over which females are choosy may be the basis to the zebrafish mating system.

Females appear to select males based on their genotype. Many fish, including zebrafish, use olfactory cues to differentiate between kin and nonkin, and this mechanism may be utilized during breeding to avoid inbreeding. Zebrafish also appear to use olfactory cues to make social and reproductive decisions. Using odor plume tests, Gerlach and Lysiak (2006) showed that adult female zebrafish chose the

odors of nonrelated, unfamiliar (reared and maintained separately) males over those of unfamiliar brothers for mating. 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).

1.3 SPAWNING TECHNIQUES AND TECHNOLOGY

1.3.1 In-Tank Strategies

One general approach to breeding zebrafish in the laboratory is to simply provide a spawning site or substrate directly in holding tanks, while fish remain "on system" or in flow. This type of technique relies on the "natural" production of fish kept in mixed sex groups with minimal manipulation of individuals. Another important feature of this basic approach is that because fish remain on flow, water quality is regulated and maintained throughout breeding events. Finally, it also largely minimizes the handling of fish, which can be a stressful event (Davis et al., 2002).

The first formally described technique for breeding laboratory zebrafish is the most basic example of an in-tank breeding method. In this approach, glass marbles are placed at the bottom of holding tanks to provide a spawning substrate for the animals. Fish spawn over the marbles, and the eggs drop into the spaces in between, preventing egg cannibalism and facilitating their subsequent collection by siphoning (Westfield, 1995; Brand et al., 2002). While this method may be effective to some extent, it is generally 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.

A slightly more advanced in-tank approach involves placing a breeding box or container in holding tanks that fish will spawn over during breeding events (Fig. 1.2a). A common feature of this method is that the box/container will have a mesh-type top through which spawned eggs drop and are subsequently protected from cannibalism. The box will also typically have some plastic plants affixed to it to make it more attractive as a spawning site. This type of method is more facile than the marbles technique, as boxes can be moved freely in and out of holding tanks as desired. It also better facilitates the collection of staged embryos from groups of fish, and can also be used for breeding pairs. This method is utilized relatively infrequently, and thus no commercially fabricated equipment of this type is available. When this method is chosen, the box/container must be custom-made to fit with the needs of the particular facility in which it is being utilized.

Another form of in-tank breeding involves the use of a specially manufactured crossing cage that is designed to fit inside holding tanks. The fish to be crossed are netted out of holding tanks and transferred to the crossing cage. Eggs are collected after breeding takes place by siphoning or after removal of the fish from the tank. This method allows for production of time-staged embryos because it can include a divider to separate males and females until eggs are needed for experiments. This technology

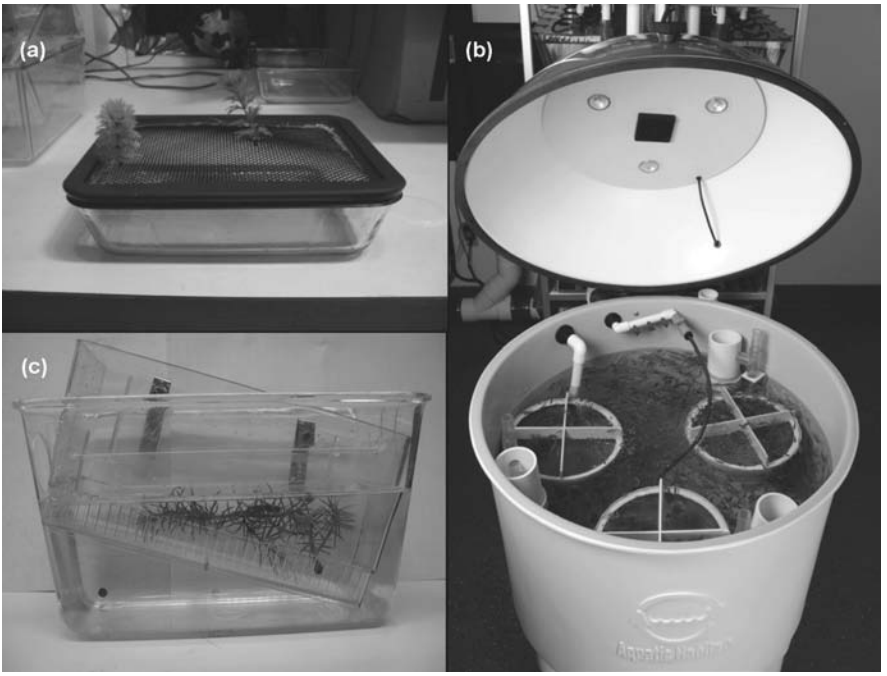

Figure 1.2 Representative examples of zebrafish spawning technology. (a) In-tank breeding container. (b) MEPS™. (c) Typical static tank mating tank with insert.

has a number of drawbacks, including the fact that all fish in the housing tanks where breeding is taking place must be either in the crossing cage or transferred to other tanks so that eggs are not cannibalized. This requires extensive handling of animals, offsetting one inherent advantage of the in-tank breeding methodology. Second, in most cases, flow of clean water into tanks must be either shut off or reduced to prevent spawned eggs from being flushed out of the tanks. There may be means by which to collect these eggs when flow remains on, but if not, another strength of the in-tank system is taken away when using this method. Finally, although various cages of this type are commercially available, they are often system vendor specific, which limits their applicability to the users of the associated system.

The most recent development in in-tank breeding technology is the Mass Embryo Production System (MEPS™), designed by Aquatic Habitats, an aquatic animal housing system manufacturer. The MEPS™ is a large spawning vessel, with a holding capacity of 80 or 250 L, which can be plumbed directly into any existing recirculating or flow-through system. The MEPS™, which can house large populations (up to 1000 or more) of breeding fish, contains one or more spawning platforms, which are specially fabricated funnels capped with plastic mesh screens that can be located at various depths inside the vessel (Fig. 1.2b). When the spawning platforms are placed inside the vessel, fish breed over and on the platforms, and spawned eggs fall through the mesh into the associated funnels. The eggs are then pumped through an attached

tube into separate collection screens by means of pressurized air directed into the funnels, allowing eggs to be collected without disturbing the fish. The units also have the capability to be run on altered photoperiods via the use of an attached light cycle dome with a programmable light cycle dimmer.

The MEPS™ system capitalizes upon several attributes of the general in-tank breeding approach, including consistent water quality and minimal handling of animals, with the added benefits of reduced labor input and increased space efficiency. When used properly, this technology is capable of supporting high-level egg production on the order of tens of thousands of embryos per event, and is therefore well suited for experimental applications requiring large numbers of time-staged eggs. However, this approach is not without its limitations and specific challenges. For example, its use is limited to experiments where the individual identity of parents is not necessary, which excludes it from being used for certain types of genetic screens, which are an important component of the zebrafish model system. The performance of fish in this type of breeding unit is also very dependent upon management. Detailed understanding of reproductive behavior and biology of the fish is imperative to maximize efficiency, and therefore the MEPS™ may be less suitable for newly established zebrafish laboratories where such expertise is not available.

1.3.2 Static Tank Strategies

The alternative to in-tank breeding strategies is to remove fish from holding tanks and to spawn them in off-system or "static water" breeding chambers. This general approach, which is utilized in the great majority of zebrafish breeding facilities, adheres to the following general principles: a small (typically <1 L) plastic mating cage or insert 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 insert in the evening. When the fish spawn, the fertilized eggs fall through the "floor" of the insert 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 static tank 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 of inserts in the static spawning chamber (Fig. 1.2c). A very small number of studies have explored the effects of variations of these parameters on reproductive success and spawning efficiency. Sessa et al. (2008) showed that fish set up in crossing cages in which spawning inserts were titled to provide a deep to shallow water gradient showed statistically significant increases in egg production when compared with fish set up in cages in which the inserts were not titled (no gradient). Fish that were set up in chambers with titled inserts displayed both a preference to spawn in shallow water and specific breeding behaviors that were limited to the tilted physical configuration. Indeed, this behavior is the basis of a newly developed approach for collecting large numbers of developmentally synchronized embryos from groups of fish in a static breeding vessel (Adatto et al., 2011).

Little else has been published in this area, although a study of the effects of varying the size of the breeding insert itself on spawning success and egg production 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 (Goolish et al., 1998). However, since this particular study was conducted in recirculating water (test chambers were placed inside large on-system tanks), it does not present a clear picture of the effect of chamber size on breeding efficiency in static tanks.

There are a number of strengths to the static tank approach. Virtually any type of experiment can be supported using this technique, as fish of any desired genotype can be set up in pairs or smaller groups in a varying number of crosses. Because fish are removed from holding tanks, the effects of behavioral hierarchies established in holding tanks that can be counterproductive to breeding are negated. Static tank technologies also allow for direct manipulation of water quality parameters; changes in water chemistry, such as decreases in salinity, pH, and temperature, are thought to promote spawning in fish adapted to monsoonal climate regimes (Murno, 1990). These factors may also affect reproduction in zebrafish (Breder and Rosen, 1966).

There are drawbacks to static tank breeding strategies. Because the chambers are off-flow, water quality conditions in the spawning setups deteriorate over time. Although this has not been formally investigated, metabolites such as total ammonia nitrogen and carbon dioxide accumulate in the water and are likely to have a negative effect on spawning. Tanks may be flushed with fresh water to offset these potential problems, but this represents added labor. Using static setups also necessitates that fish are handled constantly, which may be a source of long-term stress for breeding populations.

1.4 DETERMINING FACTORS FOR REPRODUCTION IN LABORATORY STOCKS OF ZEBRAFISH

That zebrafish will readily spawn under a wide range of conditions in captivity has undoubtedly played an important role in their rapid rise to prominence as a model organism. This flexibility also suggests that there is considerable spread in reproductive performance of laboratory stocks. Indeed, there are a number of key husbandry factors that impact breeding efficiency, including water quality, nutrition, behavior, and genetic management. A detailed understanding of how each of these factors contributes to reproductive success is vital to maximizing production of zebrafish in controlled settings. These concepts are touched upon briefly below. For a more in-depth treatment of these subjects, see reviews by Lawrence (2007) and Spence et al. (2008).

1.4.1 Water Quality

Zebrafish tolerate a wide range of environmental conditions in captivity. This flexibility is a reflection of their distribution in the wild, as they are found across

a range of habitat types that vary considerably in their physicochemical properties as a result of local geology and pronounced seasonal fluctuations in rainfall patterns (Talwar and Jhingran, 1991). However, it should be recognized that there is an energetic cost to fish in operating outside their optimum range of environmental parameters. Animals maintained under suboptimal conditions must devote an increasing proportion of energy toward maintaining homeostasis, rather than on growth, reproduction, and immune function (Wooton, 1998). Consequently, one major consequence of fish being held under suboptimal conditions is a decrease in the number and quality of offspring (Haywood, 1993). Thus, it is vital to manage water chemistry as close to optimal as possible to ensure that fish allocate resources to reproductive function.

Stability within a given range of each parameter is also crucial, and may be more important than maintaining at optimum, especially for a generalist species like zebrafish. Adapting to constantly fluctuating environmental conditions is energy intensive, and can be a source of chronic stress that manifests itself in decreases in number and quality of offspring (Wooton, 1998; Conte, 2004).

While managing water quality for stability within optimum ranges is straightforward conceptually, it is a bit more challenging to achieve in practice, primarily because optimum environmental conditions for zebrafish for the most part have yet to be demonstrated experimentally. Until such data are available, the soundest practice is to base management on the best available scientific information. Observational data from years of experimental use along with concepts gleaned from biological studies of zebrafish allow for a reasonable place to start, however. A detailed treatment of each of these factors relative to the management of zebrafish is given in the review by Lawrence (2007).

1.4.2 Nutrition and Feeding

Nutrition and feeding are among the most important determinants of reproductive success—or failure—in zebrafish facilities. Therefore, to ensure efficient and scientifically sound management of breeding stocks, it is essential that managers and technicians possess a thorough understanding of fish nutrition and the different types of feeds available, as well as the techniques to deliver them.

While the specific nutritional requirements of zebrafish are yet to be determined, it possible to apply scientific principles of finfish nutrition, along with what zebrafish specific data does exist in the design of diets and feeding regimens that will support high levels of production. At the most general level, stocks should be fed balanced diets with adequate levels of essential nutrients: proteins, lipids, carbohydrates, vitamins, and minerals. Deficiencies in essential nutrients will result in reduced production, low growth, and decreased immune function, among other problems.

At minimum, it is also crucial to ensure that diets used for breeding populations of zebrafish contain adequate levels of specific nutrients known to support reproductive function in fish. Most notably, these include the highly unsaturated fatty acids (HUFAs) eicosapentaenoic acid (20:5*n*-3; EPA), docosahexaenoic acid (22:6*n*-3;

DHA), and arachidonic acid (20:4*n*-6; AA), all of which are of pivotal importance for the production of high-quality gametes and offspring (Watanabe, 1982), and have been specifically shown to enhance reproduction in zebrafish (Jaya-Ram et al., 2008). Certain vitamins, including retinoids and ascorbic acid in particular, are also known to be extremely important for long-term reproductive quality and health, and should be considered in diet selection (Dabrowski and Ciereszgo, 2001; Alsop et al., 2008).

The type of feed is also of critical importance. Zebrafish may be fed live prey items, processed diets, or some mixture of the two. 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 processed diet, especially since systematic studies of adult zebrafish performance on these diets are not available. Live prey items such as *Artemia* typically possess relatively balanced nutritional profiles (Watanabe, 1982) and therefore are most likely to meet much of the requirements of zebrafish. Processed diets may be included to the diet as a supplement to *Artemia*, as they can be used to deliver specific nutrients that may not be present in sufficient levels in *Artemia* or other live prey items. For example, *Artemia* are deficient in DHA and in stabilized vitamin C (Lavens and Sorgeloos, 1996). One way to address these inadequacies is to incorporate a prepared feed containing known levels of these nutrients into the diet to help ensure that these dietary requirements are adequately met and reproductive function is supported.

Finally, it is essential that feeds be stored and administered properly. This is particularly critical for processed feeds. The typical maximal shelf life of a processed feed does not exceed 3 months, when maintained in cool, dry conditions (Craig and Helfrich, 2002). Oxidation of feed components, particularly fatty acids, increases with temperature. Thus, feeds should be kept in airtight containers, refrigerated, and discarded after 3 months to ensure that fish stocks derive maximal nutritional benefit from their application. In terms of delivery, processed feeds should be fed dry to minimize leaching of water-soluble amino acids and vitamins upon administration (Pannevis and Earle, 1994; Kvåle et al., 2007).

1.4.3 Genetic Management

Small, 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). This loss of genetic diversity can cause a number of problems relative to reproductive potential of zebrafish breeding stocks. Continued breeding between close relatives will lead to accumulation of deleterious alleles in breeding populations. These alleles may directly affect a number of factors related to reproduction, including reduced quantity and quality of embryos. Reduced genetic diversity may also manifest itself in reduced spawning rates, as zebrafish show preference to associate with nonrelatives over siblings or closely related individuals (Gerlach and Lysiak, 2006). This mode of kin recognition, which is thought to help avoid inbreeding in natural populations, may result in decreased spawning rates when fish in a breeding population are closely related.