Use of Medaka in Toxicity Testing

Stephanie Padilla,[1] John Cowden,[1] David E. Hinton,[2] Bonny Yuen,[2] Sheran Law,[2] Seth W. Kullman,[3] Rodney Johnson,[4] Ronald C. Hardman,[2] Kevin Flynn,[4] and Doris W.T. Au[5]

[1]United States Environmental Protection Agency, Research Triangle Park, North Carolina
[2]Nicholas School of the Environment and Earth Sciences, Duke University, Durham, North Carolina
[3]North Carolina State University, Raleigh, North Carolina
[4]U.S. Environmental Protection Agency, Duluth, Minnesota
[5]City University of Hong Kong, Hong Kong, China

ABSTRACT

Small aquarium fishes are increasingly used as animal models, and one of these, the Japanese Medaka (*Oryzias latipes*), is frequently utilized for toxicity testing. While these vertebrates have many similarities with their terrestrial counterparts, there are differences that must be considered if these organisms are to be used to their highest potential. Commonly, testing may employ either the developing embryo or adults; both are easy to use and work with. To illustrate the utility and breadth of toxicity testing possible using medaka fish, we present protocols for assessing neurotoxicity in developing embryos, evaluating toxicant effects on sexual phenotype after treatment with endocrine-disrupting chemicals by sexual genotyping, and measuring hepatotoxicity in adult fish after treatment with a model hepatotoxicant. The methods run the gamut from immunohistology through PCR to basic histological techniques. *Curr. Protoc. Toxicol.* 39:1.10.1-1.10.36. © 2009 by John Wiley & Sons, Inc.

Keywords: medaka • neurotoxicity • hepatotoxicity • endocrine disruption

INTRODUCTION

Use of alternative species for toxicity testing provides many distinct advantages compared to the commonly used rodent laboratory species. First and foremost, an alternative species such as fish is usually smaller than normal laboratory rodents, allowing the researcher to process many more animals in a given period of time, using smaller amounts of toxicant per animal. These features allow for more animals per dosage group and/or the use of a larger number of dosage groups. In addition, the researcher may use laboratory fish (e.g., the medaka) as a surrogate species because it is closer phylogenetically to the investigator's target species than are the traditional laboratory rodent species, as is the case in ecotoxicity studies. The investigator may be interested in using fish to study the mechanisms of toxicity. Alternatively, an investigator could be interested in studying developmental toxicology in an easily accessible system, without the confounders of the maternal metabolism and health and the inability to observe embryonic development. The oviparous development of fish permits treatment of the embryos in vitro and their microscopic examination over time.

The Japanese Medaka (*Oryzias latipes*), a small (2 to 4 cm) freshwater fish (Fig. 1.10.1), have been used for over 50 years for toxicity testing. They are very hardy, harbor few diseases, tolerate wide latitudes in salinity and temperature, and are easily reared in a laboratory environment. In all types of toxicity tests, small fish are a convenient vertebrate model that can be used as an ecological sentinel (i.e., a rapid screen to predict mammalian toxicity) or as a detailed mechanistic investigational tool (Law, 2001; Shima and Mitani, 2004; Hinton et al., 2005). Testing may include multigenerational exposures, as well

Published online February 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471140856.tx0110s39

Figure 1.10.1 Adult female (upper) and male (lower) medaka. Note the egg clutch attached to the female's abdomen.

as acute or chronic exposure of either the adults or developing embryos to mixtures or individual compounds. Moreover, sophisticated experimental tools are available, e.g., stable transgenic lines (Fu et al., 2000; Wayne et al., 2005) and morpholino knock-down techniques (Carl et al., 2002; Paul-Prasanth et al., 2006).

In this unit, Basic Protocols 1 and 2 provide basic methods for maintaining, handling, and breeding medaka, and for collecting embryos. Basic Protocol 3 describes embryonic development of medaka, while Support Protocol 1 discusses chemical exposure of embryos for toxicology studies. Support Protocol 2 and Basic Protocol 4 address pronase dechorionation of embryos and whole-mount antibody staining of hatched medaka, respectively, techniques which are used in experimental neurotoxicology studies. Basic Protocol 5 and the Alternate Protocol provide two PCR techniques for determining sexual genotypes, applicable to studying toxicants that disrupt endocrine function. Basic Protocols 6, 7, 8, and 9 present methods that are used to detect toxic alterations in the intrahepatic biliary passageways of medaka.

BASIC PROTOCOL 1

MAINTAINING AND HANDLING ADULT MEDAKA

Medaka are very hardy, making them ideal for laboratory studies. They can tolerate wide ranges of salinity and temperature, as long as the transition is gradual. There are two very informative and comprehensive sources for answers to animal husbandry questions regarding medaka care and feeding: (1) the medaka Web site (*http://biol1.bio.nagoya-u.ac.jp:8000*) and (2) a pamphlet published by Carolina Biological Supply (Kirchen and West, 1976). Both of these resources provide step-by-step directions for establishing a medaka colony. Below are a few general suggestions.

Materials

Water for aquaria: prepare treated water from a public water supply by removing the chemicals added during water disinfection, using a reverse osmosis system (readily available in many different sizes and relatively inexpensive)

Japanese Medaka fish, male and female (e.g., Carolina Biological Supply)

Tropical fish food (e.g., Otohime B, Ashby Aquatics) *or* purified casein-based diet (DeKoven et al.,1992)

Test materials (toxicants)

5-gallon or 10-gallon freshwater aquaria with flow-through system for water exchange, temperature controlled

1. Set up 5-gallon aquaria with 24 to 30 fish per aquarium or 10-gallon aquaria with 50 to 100 pairs in each (Davis et al., 2002).

   Adult males and females are easily distinguished from one another (Fig. 1.10.1). The male's dorsal fin is usually notched and larger than the female's dorsal fin. Also, the male's anal fin is usually larger than the female's. A breeding female usually has a much larger abdomen than the male.

   Do not cohabitate medaka with other fish, including zebrafish.

2. Maintain water temperature at 25°C to 27°C and a pH between 5.5 and 7.0.

   Although medaka can withstand wide ranges (7° to 30°C) in temperature, the optimum is 25°C to 27°C.

   At these temperatures, the pH of the aquarium water should be between 5.5 and 7.0, but exact pH is not as critical as water cleanliness (see step 4).

3. Feed medaka sparingly, 2 to 3 times per day (consider the use of automatic feeders) with commercially available tropical fish food or other diets or a purified casein-based diet.

   If food accumulates on the bottom of the tank, that is an indication that the fish are being fed too much food.

4. Ideally, keep medaka in a flow-through system, where they are usually less stressed because there is less likelihood of ammonia build-up.

   If the animals are kept in a static system (i.e., with no flow-through), replace a proportion (e.g., half) of the water on a periodic basic (e.g., once a week).

   Normal life-span is 2 to 3 years. Normal breeding age is from 6 months to 1 year.

5. Maintain medaka (which prefer bright light) on a light/dark cycle of either 14:10 or 16:8.

6a. *To perform toxicity testing using a flow-through system:* Adjust the concentration of the test chemical as needed, usually in the supply tank that feeds the aquarium. Depending on the identity and toxicity of the test chemical, take care to dispose of the water exiting the aquaria in the appropriate manner.

6b. *To perform toxicity testing using static conditions:* Change the solutions every 24 hr to maintain the test material concentrations and limit stress on the fish due to ammonia build up.

   This 24-hour schedule for solution changes also allows frequent renewal of the chemical that is being tested and helps ensure consistent concentration of the test chemical.

BREEDING MEDAKA AND COLLECTING EMBRYOS

BASIC PROTOCOL 2

The medaka Web site (*http://biol1.bio.nagoya-u.ac.jp:8000*) and Carolina Biological Supply pamphlet (Kirchen and West, 1976) are informative sources for instructions and tips on breeding of medaka. Normally each female generates 30 to 50 eggs per day, with spawning occurring within 1 hr after the beginning of the light cycle each day. Eggs usually hatch in 8 to 11 days, depending on temperature, and the fry (i.e., newly hatched larvae) are adults at 6 to 8 weeks. Breeding fish require more food than nonbreeding fish, especially more protein. One of the most popular feeding supplements for breeder fish is artemia (brine shrimp); fish are fed with them at least once a day. Often, if medaka are not breeding well or are not producing an optimal number of eggs, the frequency of artemia feeding should be increased. General guidelines for collecting, preparing, and incubating medaka eggs are described below.

Materials

Medaka colony of mixed sexes (see Basic Protocol 1)
Embryo rearing medium (1× ERM; see recipe) *or* marine water (see recipe)
Test materials (toxicants)

Dip-net
Shallow dish containing aquarium water
Wide-mouth pipet or forceps
Paper towels
20-ml vials *or* 96-well microtiter plates

1. Observe the female medaka for fertilized eggs, which are attached in a clutch to her lower abdomen ∼1 hr after the beginning of the light cycle.
2. Using a dip-net, catch a female that has a cluster of eggs attached and transfer to a small, shallow dish with aquarium water.
3. Quickly remove the eggs using either a wide-mouthed pipet or a pair of forceps.
4. Gently return the female to the aquarium.
5. Continue this procedure with other presenting females until all the needed eggs are collected.
6. To separate the eggs, gently roll them on a moist paper towel.

   The eggs are attached to one another by filaments, which are broken by the rolling process.
7. Place the eggs in 2 ml aerated embryo rearing medium (1× ERM) in a 20-ml vial or 96-well microtiter plate with aerated 1× ERM. Change the medium each day.

   There should be at least 200 μl of ERM per egg, with no more than 10 eggs in the container. Therefore, if placing eggs in a vial, there should be 10 eggs per 2 ml of ERM. If placing them in a 96-well microtiter plate, there should be 1 egg in 200 μl of ERM in each well.

   Before placing the eggs in the vial or microtiter plate, they may be incubated for ∼10 min in marine water (Instant Ocean, Aquarium Systems; 1/2 cup/1 gallon water) or a methylene blue solution (1g methylene blue/100 ml water) to limit bacterial or fungal growth.
8. For toxicant exposures during development, incubate the eggs in 20-ml glass vials or 96-well microtiter plates (Oxendine et al., 2006). See Support Protocol 1.

BASIC PROTOCOL 3

EMBRYONIC DEVELOPMENT OF MEDAKA

Medaka progress from fertilized egg to swimming fish in 8 to 11 days. An excellent source for pictures of the developing medaka is *http://biog-101-104.bio.cornell.edu/BioG101_104/tutorials/Medaka/stage_36.html.* An abbreviated depiction of the medaka egg development is shown in Figure 1.10.2 (adapted from Iwamatsu, 2004). The developmental stages of medaka described below refer to Figure 1.10.2.

Stage 0. The unfertilized egg is characterized by small oil droplets (od) randomly distributed throughout the embryo.

Stage 1. The fertilized egg is characterized by fewer oil droplets and the ability to exclude methylene blue.

Stage 2b. At 1 hr after fertilization, the oil droplets are combining and moving toward the vegetal pole, and the blastodisc (bd) is apparent.

Stage 4. By 2 hr, the egg is usually in the 4-cell stage, and the oil droplets are larger and more concentrated at the vegetal pole.

Stage 8. By 4 hr, the embryo is in the early morula stage, with 64 to 128 blastomeres in three layers.

Stage 11. By 8 hr, the embryo is in the late blastula stage.

Stage 13. At 13 hr, the embryo is in the early gastrula stage, with the blastoderm beginning to expand over the yolk (i.e., epiboly), and the embryonic shield begins to appear.

Stage 15. By 18 hr, the embryo has achieved 50% epiboly (i.e., the blastoderm has covered ~1/2 of the yolk) at the mid-gastrula stage.

Stage 16. By 21 hr, the embryo has reached the late gastrula stage and 75% epiboly, and the embryonic body (em) is clearly visible.

Stage 17. By the end of the first day, the embryo has reached the early neurula stage, and the yolk is completely covered. The rudimentary head (head) and Kupffer's vesicle (kv) are visible.

Stage 20. By 1 day plus 8 hr, the embryo has reached the 4-somite stage. Brain and spinal cord are developing and the optic (eye) and otic (ev) buds appear.

Stage 22. About midway through the second day, the embryo has reached the 9-somite stage. The three main regions of the brain are apparent (fb, mb, hb), as well as the ear (ea) and the body cavity. The optic cups and lenses have formed, and the heart appears.

Stage 25. By 2 days, the embryo has reached the 18- to 19-somite stage. The heart has started beating and circulation is beginning. The otoliths are visible and the liver begins to appear.

Stage 29. By 3 days, the embryo has reached the 34-somite stage. Pigmentation is developing in both the retina of the eye as well as numerous melanophores. The pineal gland (pi) and air bladder (ab) are apparent.

Stage 31. By the fourth day, the embryo has reached the gill blood vessel–formation stage. The pectoral fin (pf), the rudimentary kidney (pn), inner ear, and gall bladder (gb) are all apparent.

Stage 34. By the fifth day, the embryo has reached the pectoral fin blood–circulation stage. The embryo is long enough that the tip of the caudal fin reaches the eye, and the pectoral fins have begun to move.

Stage 36. By the sixth day, the heart development in the embryo has reached the stage at which the atrium and ventricle now lie side by side.

Figure 1.10.2 *(on next two pages)* Embryonic development of the medaka. Stage 0 is the unfertilized egg, stage 1 is the activated, fertilized egg, and stage 2b shows formation of the blastodisc. See Basic Protocol 3 for descriptions of the stages. Abbreviations: ab, air (swim) bladder; at, attaching filament; bc, body cavity; bd, blastodisc; bl, beak-like mass of cells; bv, blood vessel; ca, cortical alveolus; cd, Cuvierian duct; ch, chorion; dl, dorsal lip of the blastopore; ea, ear (otic) vesicle; em, embryonic body; ev, ear (otic) vesicle rudiment; ey, eye; fb, forebrain; gb, gall bladder; gp, guanophores; gt, gut tube; h, heart rudiment; hb, hindbrain; kv, Kupffer's vesicle; l, lens; lj, lower jaw; lv, liver; mb, midbrain; mf,fin fold; ml, membrane labyrinth; mp, micropyle; no, notocord; od, oil droplet; op, olfactory pit; ot, otolith; pf, pectoral fin; pi, pituitary; pr, pronephros; sc, spinal cord; sp, spleen; uj, upper jaw. (Reprinted from *Mechanisms of Development*, vol 121, Takashi Iwamatsu, Stages of normal development in the medaka *Oryzias latipes*, pp. 605–618, 2004, with permission from Elsevier.)

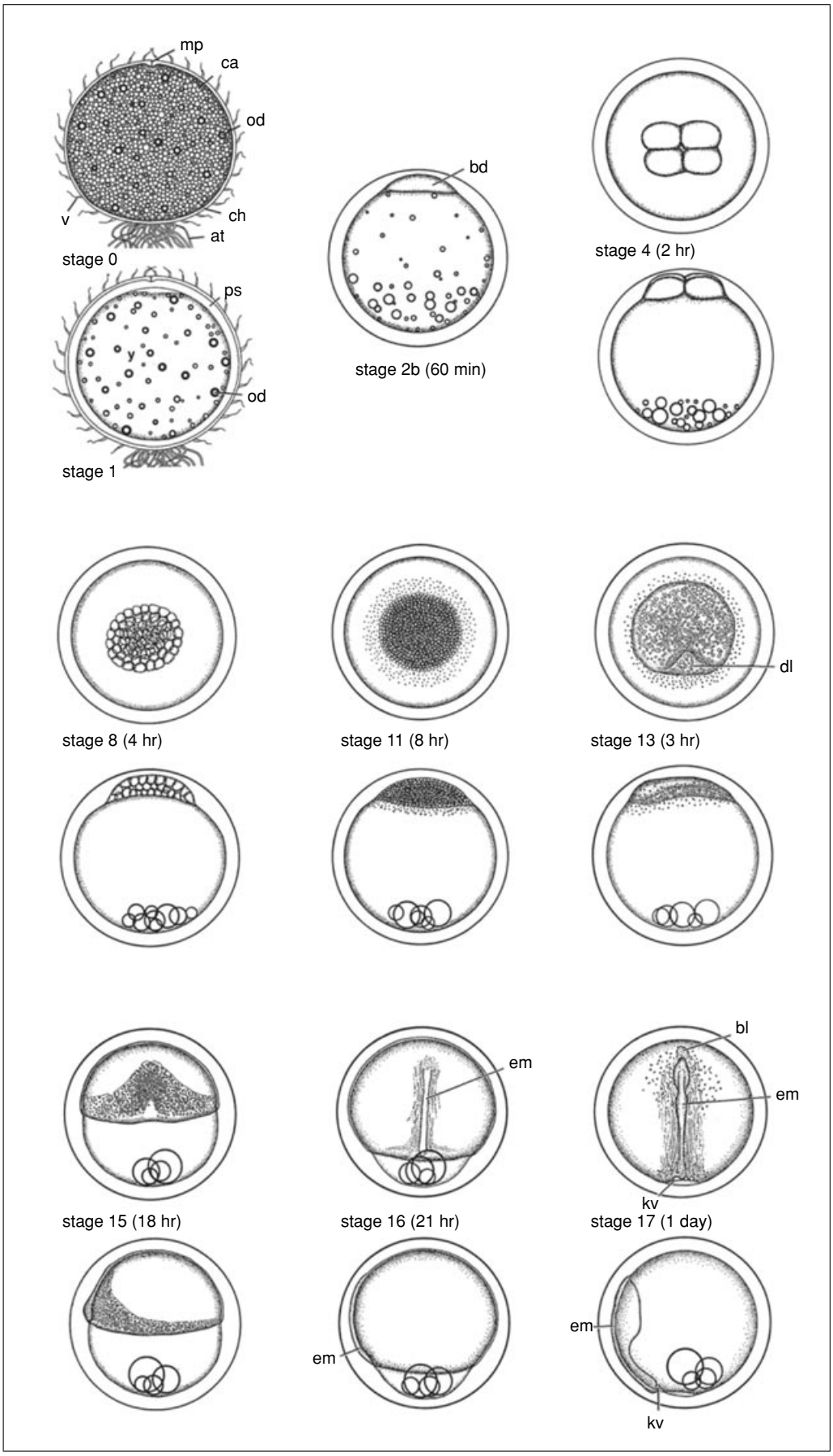

Figure 1.10.2 *(continues on next page)*

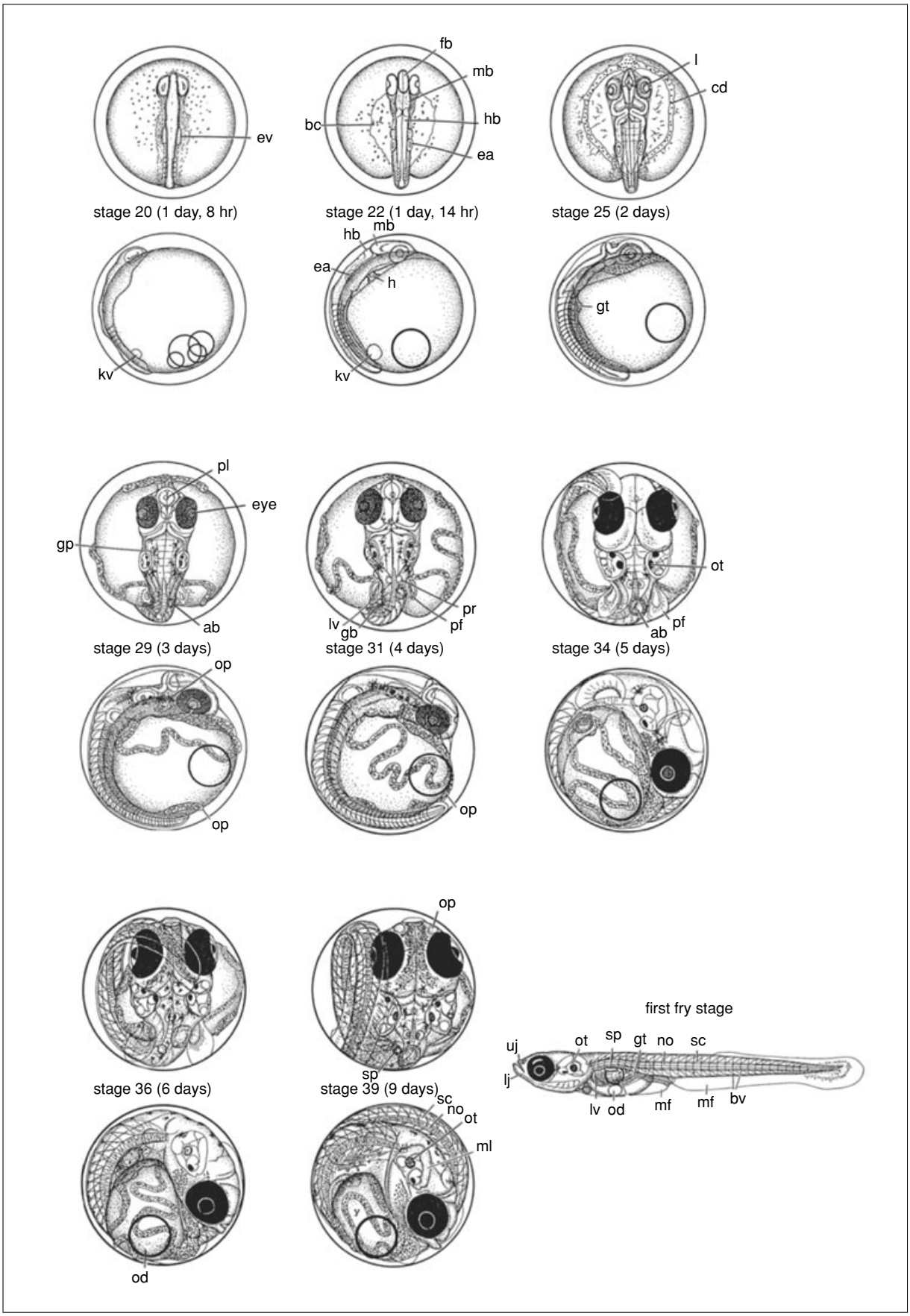

Figure 1.10.2 *(continued)*

Stage 39. By the ninth day, the embryo has reached the hatching stage. The embryo will secrete hatching enzyme, which softens and dissolves the chorion. The action of this hatching enzyme, accompanied by movement of the embryo, will cause the chorion to break open, releasing the embryo tail first.

The newly hatched fry is ~4.5 mm long and is considered to be at the first fry stage until fin rays appear on the caudal and pectoral fins. In the ensuing 3 months the fry will reach adulthood.

SUPPORT PROTOCOL 1

CHEMICAL EXPOSURE OF EMBRYO AND ADULT MEDAKA

Developmental exposure of the medaka embryo to a chemical allows the investigator the distinct advantage of observing the embryonic development through the transparent chorion (covering of the egg) to note changes. The chorion, however, may act as a barrier to some chemicals being tested, and this factor should be taken into consideration. In general, chemicals with positive logP values (octanol/water partition coefficient; a measure of the hydrophobicity of the chemical) are likely to cross the chorion (Helmstetter and Alden, 1995). An alternative is to dechorionate the embryo (see Support Protocol 2) and then proceed with chemical exposure, although dechorionation may cause higher than usual mortality in the embryos. Because medaka development is regular and predictable, it is especially suited to answering experimental questions regarding the effects of a toxicant during a particular window of development. For example, if an investigator were interested in the effects of a certain chemical on heart development, using a medaka model would allow exposure of the embryo solely during the period of heart development.

Toxicant exposure of adult fish can be accomplished either in a static system or in a flow-through system. Volatile chemicals may pose special problems, but suggestions on how to accomplish this type of exposure are in the literature (Walker et al., 1985). When exposing adult fish for more than 24 hr in a static system, a proportion of the aquaria water should be renewed each day to limit the stress on the fish. It is possible that solubility of the toxicant in fish water is an issue, and this may be solved by first solubilizing the toxicant in dimethyl sulfoxide, followed by addition of a measured small volume of that solution to the fish water. In our experience, a final concentration of no more than 1% (v/v) dimethyl sulfoxide should be used for developmental exposures (Oxendine et al., 2006).

EXPERIMENTAL NEUROTOXICOLOGY TECHNIQUES FOR MEDAKA

Several features of the medaka make it an excellent species for neurotoxicity studies. Histological references exist for both the adult medaka brain (Anken and Bourrat, 1998) and development of the embryonic nervous system (Kage et al., 2004). Unlike mammalian model systems, external development and optically clear embryos allow continual monitoring of the early development of the nervous system during chemical exposure. The protocols outlined below were selected because they are applicable for toxicity assays, including neurotoxicology studies. Support Protocol 2 describes the use of pronase for dechorionation of the medaka embryo. This protocol is designed to dechorionate large numbers of embryos rapidly. Basic Protocol 4 describes nervous system staining of medaka fry using whole-mount antibody staining.

SUPPORT PROTOCOL 2

Pronase Dechorionation of Medaka Embryos

The chorion of the medaka may act as a barrier to chemicals and reagents, making its removal desirable in some experimental situations. It is possible to dechorionate the medaka embryo using mechanical means, but the present protocol outlines pronase dechorionation because this method is more convenient for dechorionating large numbers of embryos. This protocol for pronase-mediated dechorionation is modified from

Villalobos et al. (2000). Typically, the earliest stage for successful dechorionation is stage 20 (day1 + 8 hr), although there have been reports of younger stages being successfully dechorionated (pers. comm., Dr. Ron Hardman, Duke University).

Materials

Stage 20 or older medaka embryos (see Basic Protocols 2 and 3)
Embryo rearing medium (1× ERM; see recipe)
1% (w/v) pronase solution (see recipe)
50% 1× ERM (see recipe)/50% phosphate-buffered saline (PBS; *APPENDIX 2A*)
PBS
4% (w/v) paraformaldehyde: prepared by diluting 16% (w/v) paraformaldehyde for Zamboni's fixative (see recipe) with PBS

6-well, microtiter plates
34°C water bath
Test tube rocker or rotator (e.g., MaxiRotator, LabLine)

1. Sort embryos by stage into 1× ERM.

   If using a 6-well plate, use 5 ml of 1× ERM per well. Up to 200 embryos can be dechorionated simultaneously in a single well.

2. Place the sorted embryos into 1% pronase solution.

3. Incubate at 34°C in a water bath with continuous gentle agitation.

4. After ∼1 hr, check the embryos for changes in chorion (holes or cracks, embryo protoplasm protruding, "crinkly" appearance of chorion; see Fig. 1.10.3B).

5. Place the embryos with alterations, in the chorion into fresh 1× ERM (without pronase).

   The residual pronase on the chorion will be sufficient to continue and complete the dechorionation.

6. Leave the embryos with unchanged chorions in the 1% pronase solution and return them to the 34°C water bath, with continual agitation. Check periodically (every 15 to 30 min) for changes in the chorion, placing embryos showing changes into a new well containing 1× ERM (without pronase).

7. Because embryos can dechorionate at any time in the pronase-free 1× ERM, monitor them while checking the embryos undergoing 1% pronase digestion.

8. Once the embryos have been dechorionated, either spontaneously or by gentle pushing out of the chorion using forceps (see Fig. 1.10.3E), rinse off residual pronase with at least two changes of fresh 1× ERM.

   At this stage, the dechorionated embryos may be used for chemical exposures (see Support Protocol 1) without the chorion acting as a barrier. Alternatively, the dechorionated embryos may be fixed for histochemical procedures.

9. Before fixing the embryos, rinse in 1× ERM, then 50% 1× ERM/50% PBS, followed lastly by a PBS rinse, by swirling ∼5 sec in one solution and transferring the embryos to the new solution.

10. Place embryos in 4% paraformaldehyde and incubate either 2 hr at room temperature or overnight at 4°C, with rocking to enable better fixative penetration.

    If opting for a different fixative in a different buffer, adjust the post-ERM rinses accordingly.

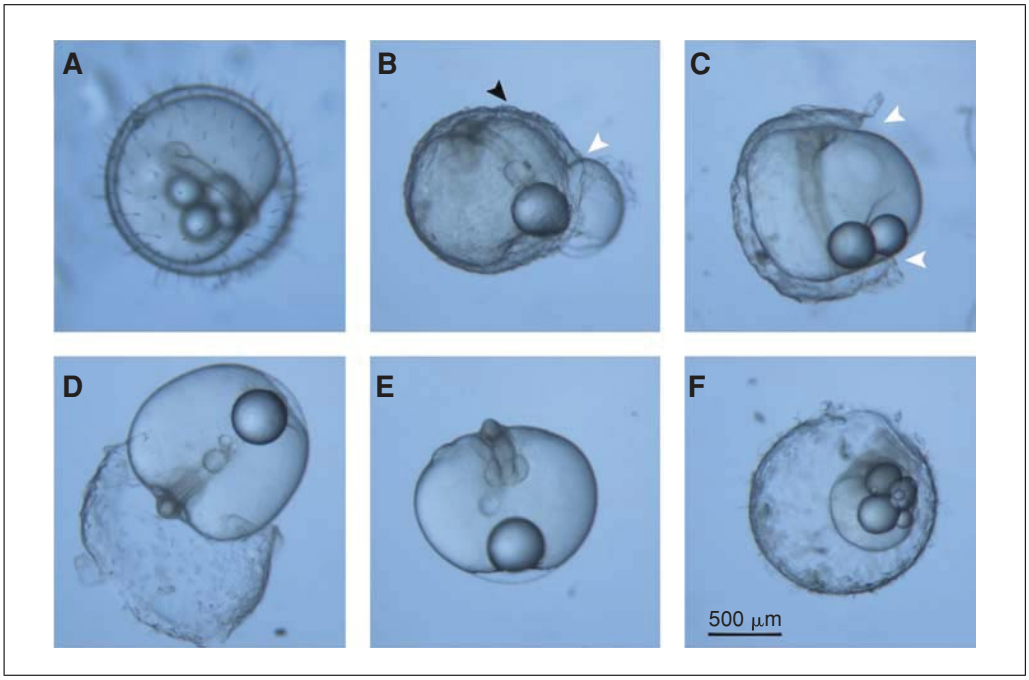

Figure 1.10.3 Pronase dechorionation of medaka embryos. (**A**) After separating from clutch, the embryo still has hair fibers, and the surface of the chorion has a smooth appearance. (**B**) Following incubation in 1% pronase at 34°C, the appearance of the chorion has changed; the hair fibers have been digested and the chorion has a wrinkled appearance (black arrowhead). Small holes have formed in the chorion through which the embryo is protruding (white arrowhead). (**C**) Continued incubation in residual pronase causes large holes in the chorion (white arrowheads). (**D**) Once the chorion has digested sufficiently, it can be easily removed from the embryo. (**E**) An embryo following pronase dechorionation, ready for subsequent experimentation. (**F**) An embryo that has been completely destroyed as a consequences of pronase overdigestion. The scale indicated by the bar in panel F is identical in panels A to E. Pictures were taken using an Olympus SZH10 dissection microscope (Olympus America) with a Leica DFC480 (Leica Microsystems) digital color camera.

BASIC PROTOCOL 4

Whole-Mount Antibody Staining of Cranial Nerves in Hatched Medaka Fry

This protocol describes whole-mount antibody staining of cranial nerves of stage 39 hatched medaka fry using anti-acetylated α-tubulin. Tubulin is a microtubule component, and anti-acetylated α-tubulin is expressed in the peripheral and central nervous system. Although this protocol uses anti-acetylated α-tubulin antibody, the same technique could be used with any antibody for target protein expressed in the hatched fry, including neural and non-neural tissues. It has been modified from a published protocol (Ishikawa and Hyodo-Taguchi,1994) to accommodate Alexa Fluor fluorescent secondary antibodies. The steps are carried out in 2-ml plastic tubes with caps and volumes of all reagents are 1 ml, unless otherwise indicated.

Materials

Medaka fry, hatched (stage 39; see Basic Protocol 3)
Zamboni's fixative (see recipe)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
1% (w/v) trypsin in PBS, freshly prepared
4% (w/v) paraformaldehyde: prepared by diluting 16% (w/v) paraformaldehyde for Zamboni's fixative (see recipe) with PBS
High-Triton PBS (see recipe)
Low-Triton PBS (see recipe)
Primary antibody: mouse anti-acetylated α-tubulin (Sigma)

Secondary antibody: fluorophore-conjugated anti-mouse antibody (e.g., Alexa Fluor 555 donkey anti-mouse IgG, Invitrogen)
Fluorescence mounting medium, e.g., glycerol or Vectashield (Vector Labs)

2-ml clear plastic tubes with caps
Test tube rotator or rocker (e.g., 4631 MaxiRotator, LabLine)
Glass depression slides
Coverslips
Fluorescence microscope

Fix and wash fry

1. Fix live hatched fry in 2-ml tubes containing 1 ml Zamboni's fixative for 2 to 3 hr at 4°C.

   Up to 10 fry have been successfully fixed in a single 2-ml tube.

2. Wash in 1 ml of PBS for 3 to 10 days at 4°C with continuous gentle rocking.

Digest fixed fry with trypsin

3. Incubate in 1 ml freshly prepared 1% trypsin in PBS for 3 hr at 4°C with continuous gentle rocking.

4. Rinse thoroughly in 1 ml PBS to remove residual trypsin, by transferring the fixed fry into a tube containing the fresh solution briefly, then transferring to the new tube containing fresh solution.

5. Wash in 1 ml high-Triton PBS for 2 to 3 days at 4°C, with continuous gentle rocking.

6. Wash in 1 ml low-Triton PBS for 30 min at 4°C, with continuous gentle rocking.

Incubate with primary antibody

7. Incubate in 1 ml mouse anti-acetylated α-tubulin diluted 1:1000 (v/v) in low-Triton PBS for 2 to 3 days at 4°C, with continuous gentle rocking.

8. Remove the primary antibody solution and store at 4°C for reuse.

   Primary antibody solutions can be used multiple times. The 1:1000 mouse anti-acetylated α-tubulin has been successfully used in our laboratory up to three times.

9. Wash in 1 ml low-Triton PBS for 6 hr at 4°C, with continuous gentle rocking.

Incubate with secondary antibody

10. Select an appropriate fluorescent secondary antibody for the desired emission spectrum. Incubate in 1 ml of the selected fluorophore-conjugated anti-mouse secondary antibody diluted to a final concentration of 5 μg/ml in low-Triton PBS for 2 to 3 days at 34°C, in the dark, with continuous gentle rocking.

11. Recover the secondary antibody dilution and store at 4°C, in the dark.

    Secondary antibody solutions can be used multiple times. Both Alexa Fluor 555 donkey anti-mouse IgG (Invitrogen) and Alexa Fluor 488 chicken anti-mouse (Invitrogen) secondary antibody dilutions have been used successfully in our laboratory up to three times.

12. Rinse thoroughly in 1 ml low-Triton PBS to remove excess secondary antibody dilution.

Visualize nervous system

13. Place the embryos on a glass depression slide in 1 ml low-Triton PBS. Remove as much low-Triton PBS surrounding the embryos as possible. Add glycerol (or other suitable fluorescence mounting medium, such as Vectashield) to the embryos.

14. Add a coverslip and examine the embryos on a fluorescence microscope.

If staining is successful, the central and peripheral nervous system of the hatched fry should be fluorescent. The Alexa Fluor 555 donkey anti-mouse IgG secondary antibody (Invitrogen) has an excitation wavelength of 555 nm, with an emission wavelength of 565 nm. The Alexa Fluor 488 chicken anti-mouse secondary antibody (Invitrogen) has an excitation wavelength of 495 nm and an emission wavelength of 519 nm.

DETERMINING SEXUAL GENOTYPE IN MEDAKA AFTER ENDOCRINE DISRUPTION

In the United States, the Food Quality Protection Act as well as other legislation requires that chemicals be evaluated for their potential to disrupt the endocrine systems of humans and other animals. In addition, the member countries of the Organization for Economic Cooperation and Development (OECD) are interested in developing test methods for similar evaluations of chemicals for possible effects on both humans and wildlife.

In wildlife, the concern is primarily for population-level effects due to adverse impacts on adult reproduction and/or on reproductive development. In general, two types of short-term testing protocols have been developed for assessing chemicals for these types of effects in fish: (1) those that assess reproductive potential directly by exposing adult animals and measuring fecundity and fertility (Ankley et al., 2001; Seki et al., 2006) and (2) those that determine early life-stage effects by exposing developing embryos and measuring effects on the development of the normal reproductive phenotype (Orn et al., 2003, 2006; Seki et al., 2003). The tests that expose embryos seem to be more sensitive than adult reproduction tests. Most of the endpoints used in such tests involve measuring various gender-specific phenotypic effects, e.g., induction or suppression of egg protein vitellogenin (VTG) production by the liver, development of secondary sex characteristics (i.e., ovipositor in male mosquitofish, anal fin papillae in medaka, fat pad and nuptial tubercles in fathead minnows), and/or occurrence of sex reversal or intersex conditions in the gonads (i.e., gonads with both male and female structures).

Such endpoints can be used to analyze for chemical effects by comparing the sexual phenotype ratio in treated fish to the ratio observed in controls. For example, based on gonadal histology, the sex ratio in 17α-ethynylestradiol (EE2)–treated zebrafish embryos is skewed toward females, compared to controls (Orn et al., 2003). Detection of these types of effects, when weakly expressed, requires relatively large sample sizes. Alternatively, the sexual genotype can be inferred using sex-linked characteristics, such as pigment variations in each individual, and compared to the sexual phenotype of the same individual (Papoulias et al., 1999; Balch et al., 2004).

Assessing the effects of chemicals on reproductive development based on concordance of the sexual genotype and phenotype in individual fish requires far fewer animals than methods based on changes in population-level phenotypic sex ratios; however, there are several limitations in these methods. Errors can be caused by crossover between the actual sex-determining gene and the sex-linked trait, e.g., in medaka the error is $\sim$2% when using the presence of leucophore pigment cells in the skin to identify genetic females (Wada et al., 1998). In addition, strains of fish that carry sex-linked gene variants are sometimes less robust than the wild-type strains. This not only makes them more difficult to use in routine chemical assessments, but the results obtained may also be less representative across the species. Thus, it is preferable when possible, to directly identify the sex-determining gene in such studies.

Matsuda and coworkers (Matsuda et al., 2002) identified the sex-determining gene (*DMY*) in medaka. Since the *DMY* gene is present on the Y chromosome of every somatic cell in males, DNA isolated from any tissue is appropriate for determining genotypic

sex, including often unutilized tissues, e.g., tail fin, pectoral fin, gills. In this section, we outline two quick and efficient PCR protocols for identifying the *DMY* gene in individual medaka using a small tissue sample, noninvasively obtained from the medaka caudal fin. The choice between the two protocols depends largely on the availability of equipment. The first protocol relies on fairly commonplace laboratory equipment: a standard thermocycler for PCR, gel electrophoresis equipment to separate PCR products, and an ultraviolet (UV) transilluminator to visualize the PCR products. The second protocol uses quantitative real-time PCR (qRT-PCR), which requires a specialized thermocycler. For both protocols, any method can be used to isolate the DNA, as long as the resulting DNA is of sufficient quality for PCR. Examples of appropriate commercial DNA isolation kits include Tri Reagent (Sigma), TRIzol (Invitrogen), and DNeasy Kits (Qiagen).

PCR/Gel Electrophoresis Protocol for Determining Sexual Genotype

BASIC PROTOCOL 5

Once DNA has been extracted, the presence of *DMY* (i.e., male) or its absence (i.e., female) can be determined using routine PCR amplification methods, followed by gel electrophoresis (Shinomiya et al., 2004). Various primer sets for different amplicons (the amplified product of PCR) have been used (Matsuda et al., 2002; Ohmuro-Matsuyama et al., 2003; Suzuki et al., 2005; Otake et al., 2006). Often, two primers are chosen to produce both a sex-linked gene amplicon (*DMY*; NCBI accession no. AB071534), which will be present only in males, and an additional amplicon from a somatic gene with a sequence that is similar to DMY(e.g., *DMRT1*; NCBI accession no. AY442916), which will be present in both males and females and is a positive control.

Materials

10× PCR buffer, without $MgCl_2$ (Sigma, cat. no. P2317)
25 mM $MgCl_2$
10 mM (each nucleotide) dNTP mix
PG17.19: 5′-GAA CCA CAG CTT GAA GAC CCC GCT GA-3′ (Inui et al., 2003)
PG17.20: 5′-GCA TCT GCT GGT ACT GCT GGT AGT TG-3′ (Inui et al., 2003)
JumpStart *Taq* DNA polymerase (Sigma, cat. no. D4184)
DNA sample from fish: prepared using a DNA preparation kit (e.g., DNeasy Kits, Qiagen)
DEPC-treated water (e.g., see *UNIT 2.9*)
NuSieve 3:1 agarose (Cambrex, cat. no. 50091)
1× TBE buffer (*UNIT 2.2*)
10 mg/ml ethidium bromide stock solution

PCR tubes, appropriate to thermocycler
Thermocycler (e.g., PTC-200, MJ Research)
UV transilluminator

Additional reagents and equipment for performing agarose gel electrophoresis (*UNIT 2.2*)

1. Prepare the master mix for PCR amplification (for a 48 μl reaction mix) as follows:

   5 μl of 10× PCR buffer (1× final)
   3 μl of 25 mM $MgCl_2$ (1.5 mM final)
   1 μl of 10 mM dNTP mix (0.2 mM final, each dNTP)
   4 μl of PG17.19 (80 nM final)
   4 μl of PG17.20 (80 nM final)
   1 μl of 2.5 U/μl JumpStart *Taq* DNA polymerase (2.5 U final)
   30 μl of DEPC-treated water (0.5 U/μl final).

   Keep on ice.

2. Add 48 µl of the master mix, followed by 2 µl of sample to each PCR tube.

3. Carry out PCR using the following amplification cycles:

Initial step:	3 min	94°C	(initial denaturation)
35 cycles:	30 sec	94°C	(denaturation)
	30 sec	60°C	(annealing)
	2.5 min	72°C	(extension)
Final:	5 min	72°C	(final extension).

4. Prepare a 1% (w/v) NuSieve 3:1 agarose gel in TBE. Add 0.4 µg ethidium bromide/ml of the agarose solution, using a 10 mg/ml ethidium bromide stock solution.

5. Electrophorese samples at up to 10 V/cm and visualize the amplicons on a UV transilluminator.

   It is possible to use a higher voltage to shorten electrophoresis time because high resolution and band quality are not required.

   With the primers used in this protocol, male DNA will produce a single band at ~1900 bp, while female DNA will produce no amplicon at all.

ALTERNATE PROTOCOL

High-Throughput Quantitative Real-Time PCR Protocol for Determining Genotypic Sex

Alternatively, a method with higher throughput than the method described in Basic Protocol 5 has been developed and used successfully in our laboratory. It is based on qRT-PCR and takes advantage of a 96-well-plate format to increase analysis efficiency. For this type of *DMY* analysis, the DNA isolation/purification is identical to that of the PCR/agarose gel–based analysis. Once the DNA is extracted from the tissue sample, the presence of *DMY* (identifying the individual as a male) is determined on a real-time thermocycler, eliminating the need to run an agarose gel for amplicon identification. This method greatly increases the throughput of the analysis by taking advantage of 96-well-plate formats and automated data analysis, allowing the simultaneous analysis of at least 90 unique DNA samples.

The protocol utilizes a Taqman assay, a variant of qRT-PCR, to detect amplification of the *DMY* gene in each well of the 96-well plate. Starting at the designed primers, DNA polymerase moves downstream, synthesizing a new strand from the template strand, and, via its 5′ exonuclease activity, the polymerase removes any bases that would impede its progress down the template strand. A Taqman probe, present in the PCR mix, anneals to the template between the two primers in the path of the DNA polymerase. The Taqman probe is a short sequence of DNA (~20 bp) with a reporter fluorophore on one end and a quencher on the other end. The proximity of these two moieties allows interaction, prohibiting the fluorescence of the reporter. When the exonuclease activity of the DNA polymerase cleaves the probe, the reporter and its quencher are separated, allowing the reporter to fluoresce upon excitation. The quantity of fluorescence from the reporter during the exponential phase of PCR is directly proportional to the amount of target sequence in the sample.

During PCR, amplicon production goes through three phases: (1) the exponential phase, where doubling of amplicon is occurring every cycle and the reaction is very specific and precise; (2) the linear phase, where reagents are becoming rate limiting, the reaction slows, and amplicons may start to degrade; and (3) the plateau phase, where the reaction has stopped, with no additional product formed (see Fig. 1.10.4). During real-time PCR, fluorescent measurements are taken during the exponential phase, while with traditional PCR, the reaction is often terminated in the plateau phase and amplicons are detected with a gel.

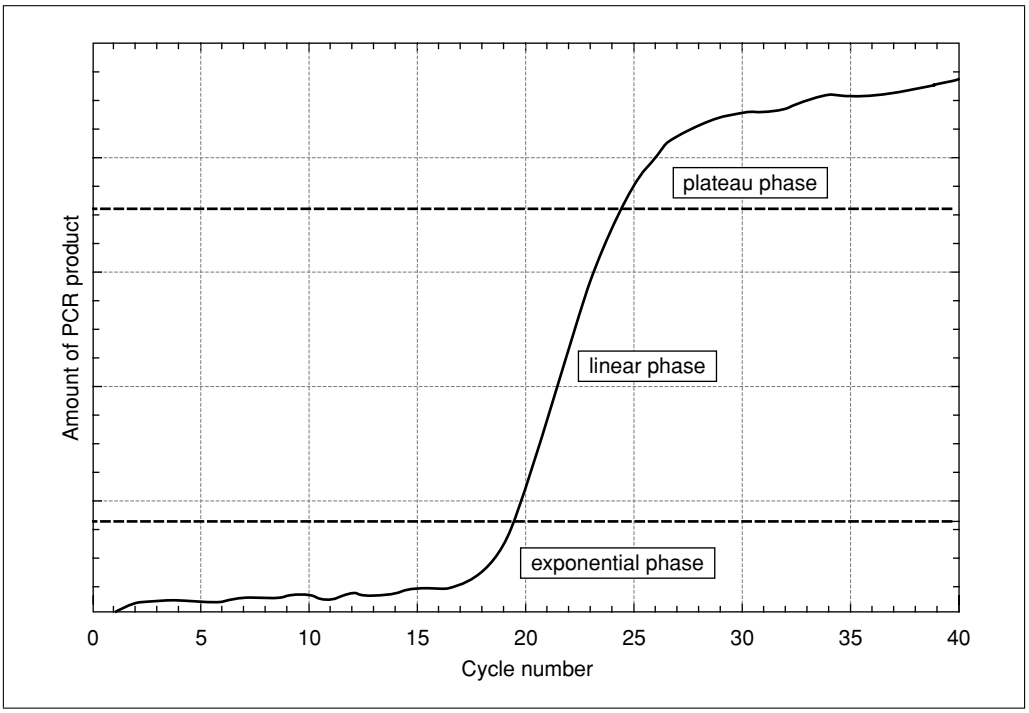

Figure 1.10.4 The three phases of PCR. In the exponential phase, doubling of product occurs in each cycle, and accurate and precise quantification is possible. In the linear phase, reagents become rate limiting, the reaction slows, and products may start to degrade. In the plateau phase, the reaction stops, and no additional product is formed.

Because the *DMY* gene is only present in genotypic males, true quantification is not necessary, only verification of this sequence's presence within the DNA sample. Therefore, the samples normally used to generate a standard curve for quantification are omitted from the 96-well plate. In addition, to verify that no inhibitors of PCR are present in the sample and that the DNA is of sufficient quantity and quality, an endogenous positive control (EPC) is included. The EPC is another Taqman assay duplexed with the *DMY* assay that amplifies a segment of 18S DNA (or cDNA) with reaction-limiting primers and a spectrally distinct reporter fluorophore. Samples positive for the target (*DMY*, males) have increased fluorescence from the Taqman probes for both *DMY* and EPC. Samples negative for *DMY* (females) have increased fluorescence only for the EPC probe, and samples with PCR-inhibiting contamination or no DNA have only baseline fluorescence for both probes.

Materials

Taqman Universal PCR Master Mix (Applied Biosystems, cat. no. 4304437)
18S rRNA Taqman Assay Reagent (Applied Biosystems, cat. no. 4319413E)
DMY HTP forward primer (5′-TTC TGC TGG AAA GAC-3′)
DMY HTP reverse primer (5′-TCT CTG GCG GAC CAT GAT-3′)
DMY HTP probe (5′-FAM-CCA GTG CTT CAA ATG CGA GCA-BHQ-3′)
RNase-free water (e.g., see *UNIT 2.9*)
DNA sample from fish: prepared using a DNA preparation kit (e.g., DNeasy Kits, Qiagen)

Primer design software (e.g., Primer Express, Applied Biosystems)
96-well optical plates, prechilled
Refrigerated centrifuge with rotor adapted for 96-well plates, 4°C
Real-time thermocycler (e.g., model 7500, Applied Biosystems)

1. Design primers and a Taqman probe using the default parameters to detect an appropriate sized amplicon (~150 bp) unique to the *DMY* gene.
2. Prepare the master mix for PCR amplification (for a 22.5 μl reaction mix):

   12.5 μl TaqMan Universal PCR master mix (1× final)
   0.25 μl 18S rRNA reagent (0.2× final)
   1.75 μl DMY HTP forward primer (0.7 nM final)
   1.75 μl DMY HTP reverse primer (0.7 nM final)
   4.0 μl DMY HTP probe (200 nM final)
   2.24 μl RNase-free water.

   Keep on ice.
3. Using a prechilled 96-well optical plate, load each well with 22.5 μl of the master mix and add 2.5 μl of the DNA sample. Include three types of controls on each plate: no-template controls (NTC), *DMY* positive controls (DNA sample from a known male), and *DMY* negative controls (DNA sample from a known female).
4. Seal, vortex, and centrifuge the plate 5 min at 500 × *g*, 4°C, to mix the samples and remove any bubbles.

   Air bubbles in the well will alter the fluorescence measurement.
5. Carry out PCR using the following amplification cycles:

Initial steps:	2 min	50°C	(activation)
	5 min	95°C	(denaturation)
30 cycles:	15 sec	95°C	(annealing)
	1 min	60°C	(extension).

   The 50°C incubation step activates the AmpErase UNG in the Taqman PCR master mix.

   These amplification cycles produce increasing fluorescence in the DMY channel (emission maximum ~520 nm) in males and not in females. Simultaneously, the fluorescent signal in the EPC channel (emission maximum ~550 nm) should increase in every well.
6. Determine the genotypic sex of each individual based upon the fluorescence in the *DMY* channel.

   As mentioned previously, quantification of DMY is not necessary, the specific number of DMY copies in the sample being irrelevant. The important information is the presence of DMY based on sufficient fluorescence to exceed a critical threshold (C_t). Individuals that exceed the C_t are genotypic males (DMY positive), and those that do not exceed the C_t are genotypic females (DMY negative).

   The C_t is determined empirically by comparing the amplification plot (the fluorescence) of known males and females and setting the C_t so the known males exceed it and the known females do not. Often the fluorescence from females will not exceed the baseline from the no-template control. If the fluorescence does increase over no-template controls, it occurs in the last cycles of amplification.

METHODS FOR DETECTING TOXIC ALTERATIONS IN THE INTRAHEPATIC BILIARY PASSAGEWAYS OF MEDAKA

Small aquarium fish such as medaka and zebrafish are becoming widely used laboratory fish models (Shima and Mitani, 2004; Alestrom et al., 2006) for studies of acute and chronic toxicity. The liver and its biliary system is a target for various toxicants, and a related group of methods for determining toxic responses of the intrahepatic biliary system is presented below. Basic Protocol 6 presents a general method for histological preparation of medaka fry that is different from mammalian liver preparations. The next three protocols can be used together to microscopically evaluate toxicant effects on cell

proliferation (Basic Protocol 7) and cell death (Basic Protocol 8). The identification of the affected cells as biliary epithelial cells and/or bile preductular epithelial cells is accomplished by staining of their cytokeratin filaments (Basic Protocol 9). This method allows assignment of toxic effects specifically to the liver epithelial cells by colocalization of cell proliferation or apoptosis markers with the cytokeratin marker in the same tissue section, using a combination of primary antibodies (anti-pan-cytokeratins and anti-PCNA, or anti-pan-cytokeratins and ApopTag).

Previous studies from our laboratory have shown that the intrahepatic biliary system of channel catfish (*Ictalurus punctatus*), trout (*Oncorhynchus mykiss*), or medaka (*Oryzias latipes*) have numerous transitional passageways (bile preductules) between canaliculi of hepatocytes and the biliary ductules (Hampton et al., 1988; Okihiro and Hinton, 2000; Hardman et al., 2007). We have shown that these transitional passageways (1) occur commonly in the parenchymal compartment, (2) are lined by epithelial cells that share junctional complexes with hepatocytes, and (3) form numerous passageways (Hampton et al., 1989). Together, these structures comprise the intrahepatic biliary passageway, only recently well defined in three-dimensional reconstructions of living, "see-through" medaka (STII strain; Hardman et al., 2007). Due to their location, swelling of these cells may distort the lumens of the intrahepatic biliary passageway, causing transient or longer alteration in bile flow, resulting in toxic states.

The intrahepatic biliary system of the medaka, like that of rodent models, responds to toxic injury as exhibited by chronic toxicity associated with hepatic tumorigenesis (Okihiro and Hinton, 1999, 2000) or independent of carcinogenesis by way of degeneration (Wolf and Wolfe, 2005). In addition, gall bladder lesions and cystic alteration in the bile duct have been reported in medaka treated chronically with bromodichloromethane, a water disinfection residual (Toussaint et al., 2001).

The following group of protocols provides the means to distinguish a spectrum of alterations that accompany intrahepatic biliary toxicity. Toxicity of α-naphthyl isothiocyanate (ANIT; a reference biliary toxicant) is well described in rodent models where responses include cytotoxicity (e.g., impaired mitochondrial function, small biliary epithelial cell necrosis) in biliary epithelium of bile ductules and ducts, cholestasis (Woolley et al., 1979; Hill and Roth, 1998; Orsler et al., 1999; Waters et al., 2002), and biliary tree arborization, i.e., biliary epithelial cell hyperplasia resulting from decreased bile secretion/transport and bile duct(ule) obstruction (Connolly et al., 1988; Alpini et al., 1992; Masyuk et al., 2003). Using the protocols provided below, our studies with ANIT in the see-through (STII) medaka were able to differentiate biliary epithelial cells and passageways from other liver components, determine whether exposure resulted in apoptosis of these cells, and determine whether surviving cells underwent proliferation to repopulate the system.

Anesthesia, Sacrifice, Fixation, and Paraffin Embedment of Medaka Fry

BASIC PROTOCOL 6

This protocol describes methods for histological preparation of whole medaka. Because of the medaka's small body size, whole fish are processed and embedded in paraffin for morphological analysis of the liver. Fixation and staining of whole fish, rather than liver alone, provides some advantages. Toxicant(s) may affect more than one organ (e.g., apoptosis and/or proliferation of cells may occur both in liver and other sites such as kidney or gut), and whole mounts allow simultaneous morphological examination and analysis of multiple organs. In addition, simultaneous localization of protein(s) of interest (e.g., pan-cytokeratins) or other specific cellular reactions in various organs can provide in situ biological controls.

Decalcification of the fixed organism (commonly performed) will allow sectioning of the entire carcass for survey histopathology and avoidance of sectioning artifacts caused by

presence of bones. However, the decalcification fluid may affect the antigenic properties of the tissue in subsequent immunohistochemical procedures. Thus, we avoid its use and instead use the mechanical approach described in the following protocol.

Materials

Medaka (Basic Protocol 1), treated with toxicant (Basic Protocol 3) and nontreated or vehicle-treated controls
Ethyl 3-aminobenzoate methanesulfonate (MS-222; Sigma-Aldrich)
GPHS fixative (see recipe)
70%, 85%, 95%, and 100% (v/v) ethanol
Xylene
Paraffin (Paraplast Plus, EMS)

Plastic Pasteur pipets
Dissecting tools, including scalpel and curved forceps
Glass vials of appropriate size, with caps
Test tube rotator or rocker (e.g., 4631 MaxiRotator, LabLine)
Tissue molds and embedding rings (EMS)
Rotary microtome (e.g., MICROM, International GmbH)
Poly-L-lysine-coated glass slides (EMS)

Fix whole fish

1. Sacrifice the fish through anesthetic overdose by immersion in a dedicated exposure tank of chilled water containing 1% (w/v) MS-222.
2. Remove the fish from the tank, and with a plastic Pasteur pipet, immediately flush with 10 vol GPHS fixative over the gills and into the mouth and pharynx.
3. Either decapitate the fish or remove the following bones from the head region to prevent sectioning artifacts (Fig. 1.10.5):

   Skull roof
   Otoliths
   Operculum.

4. To release gas from swim bladder to facilitate immersion of fish in the fixative, use a sharp scalpel to cut the body wall along dorso-ventral path from midway between the anal vent and anal fin at the ventral midline to the area of the head kidney (rostral trunk). Press the lateral body wall gently with curved forceps, releasing gas bubbles (see Fig. 1.10.5E).
5. Open the abdominal cavity by making a cut through the ventral body wall along the midline from the anal vent to the pectoral girdle, facilitating entry of fixative to internal organs. Flush inside with additional fixative, using a plastic Pasteur pipet.
6. Cut away and discard the pectoral, pelvic, dorsal, anal, and caudal fins.
7. Immerse a single fish in 20 vol GPHS fixative in a glass vial of appropriate size. Cap and incubate overnight at 4°C, with rotation.

Process tissues

8. Aspirate and discard the fixative. Dehydrate the tissue by incubating at room temperature, with, rotation in two changes (40 min and 10 min) of each of the following solutions:

   70% ethanol
   85% ethanol
   95% ethanol.

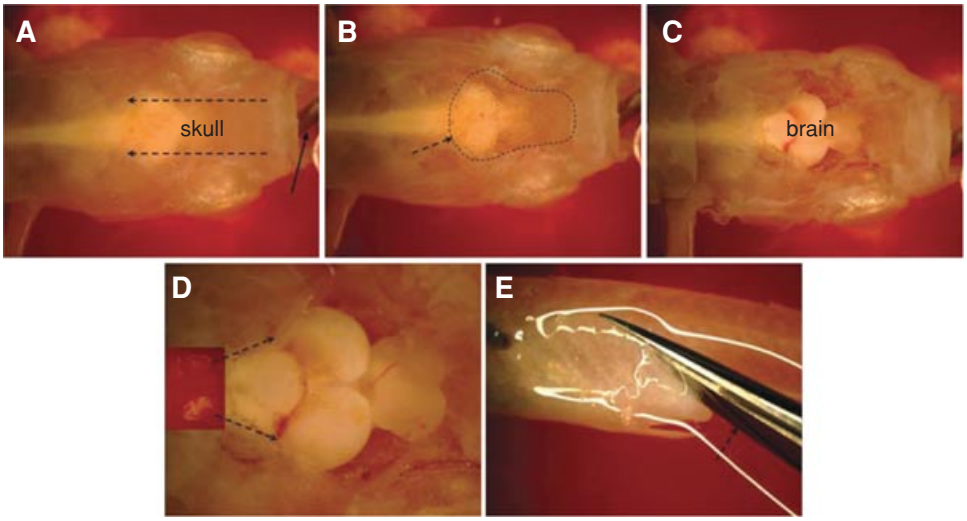

Figure 1.10.5 Specific steps in the removal of skull roof and otoliths of STII medaka. (**A**) Mount fish on a wax plate and secure in position by pinning the mouth, and gently scrape away scale and skin of the skull roof with curved forceps by moving forceps backward from the mouth. (**B**) Remove the skull roof from the parietal with fine forceps, as indicated by the arrow and dotted lines to (**C**) expose the brain. (**D**) Locate the otoliths in the cranial grooves between the midbrain and hindbrain (indicated by arrows), and pull the otoliths out by inserting fine forceps along the side of cranium. Note: Extra care needs to be taken when removing otoliths, as brain is very soft tissue. The insert depicts a pair of otoliths. (**E**) Release of gas from swim bladder by cutting the body wall along dorso-ventral path from midway between the anal vent and anal fin at ventral midline to the area of the head kidney and pressing the lateral body wall gently with curved forceps.

Incubate in three changes (40 min, 40 min, and 10 min) of 100% ethanol at room temperature with rotation.

9. Clear the tissue by incubating in three changes of xylene for 30 min each at room temperature, with rotation.

10. Infiltrate the tissue with paraffin by incubating in four changes of Paraplast Plus for 60 min each at 58°C to 60°C.

Orient and embed samples

11. Cover the bottom of the tissue mold with melted paraffin.

12. Place the tissue mold on the benchtop at room temperature, and as the paraffin cools, quickly orient the specimen with the left lateral surface down. Pour melted paraffin over the specimen. Place the embedding ring onto the mold and complete filling with paraffin.

13. After the block has solidified, remove it with the embedding ring intact and store up to several months at 4°C, until ready to section.

14. Using a rotary microtome, generate 5-μm sections and mount on poly-L-lysine coated glass slide.

At this point, the glass slides are ready for immunohistochemical labeling, as described in Basic Protocols 7, 8, and 9.

BASIC PROTOCOL 7

Immunohistochemistry for Cell Proliferation (PCNA)

Cell proliferation is measured by detecting the proliferating cell nuclear antigen (PCNA), a protein synthesized in early G_1 and S phases of the cell cycle. Hepatocytes are the largest cells, each containing a single nucleus and one prominent nucleolus. The nuclei of proliferating cells will be stained darker than nonproliferating cells. See Figure 1.10.6 for results using this method.

Materials

Paraffin sections of toxicant-treated and control medaka on a poly-L-lysine glass slide (Basic Protocol 6)
Xylene
70%, 95%, and 100% (v/v) ethanol
10 mM citrate buffer, pH 6
3% (v/v) hydrogen peroxide in phosphate-buffered saline (PBS; *APPENDIX 2A*)
1× PBS
10% (v/v) normal goat serum, diluted in PBS containing 0.1% (v/v) Tween 20
Primary antibody: mouse anti-PCNA antibody (Dako, cat. no. M0879), diluted 500-fold in antibody diluent (Dako, cat. no. S0809)
Secondary antibody: goat anti-mouse-HRP conjugated immunoglobulins (DakoCytomation Envision+ System-HRP; Dako, cat. no. K4006)
Peroxidase substrate: 3,3′-diaminobenzidine solution (DAB, Dako)
Harris' hematoxylin
Permount (Fisher Scientific)

Microwave oven
Humidified chamber of appropriate size (home made or commercially available)
Coverslips
Light microscope

1. Deparaffinize the tissue sections with the following solutions:

   Xylene (three times for 5 min each)
   100% ethanol (three times for 3 min each)
   95% ethanol (5 min)
   70% ethanol (5 min)
   Water (two times for 5 min each).

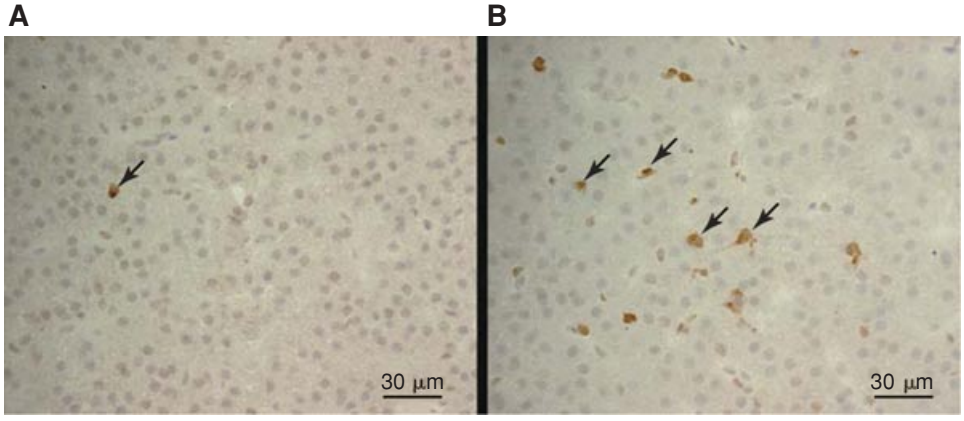

Figure 1.10.6 Immunohistochemical labeling of STII medaka livers with anti-PCNA antibodies. Proliferating cells (indicated with arrows) are labeled brown. (**A**) Control liver. (**B**) ANIT-treated liver. See Table 1.10.1 for an interpretation of the results in conjunction with other staining methods.

2. Pretreat the tissue by immersing the sections in 10 mM citrate buffer, pH 6.0, and boiling for three cycles of 3 min each in a microwave oven, with 3-min pauses between each boiling step.
3. Let the solution sit on bench for 30 min.
4. Rinse twice in water for 2 min each.
5. Quench endogenous peroxidase by incubating 5 min in 3% hydrogen peroxide in PBS.
6. Rinse twice in PBS for 2 min each.
7. Block the sample by incubating in 10% normal goat serum for 20 min.
8. Incubate in primary antibody 1 hr in a humidified chamber, at room temperature.

   The humidified chamber can be as simple as a closed container with the antibody-treated slides and an additional water slide, with the antibody solution and the water contained on the slides in "wells" formed by a marker pen. The humidity is important in preventing evaporation of reagents.
9. Rinse three times in PBS for 5 min each.
10. Incubate in secondary antibody 30 min in a humidified chamber at room temperature.
11. Rinse three times in PBS for 5 min each.
12. Apply DAB to cover, and incubate 3 to 6 min.
13. Rinse 1 min in running tap water.
14. Counterstain the specimen 1 min in Harris' hematoxylin to cover.
15. Rinse 1 min in running tap water.
16. Dehydrate the sample for 1 min in each of the following:

    70% ethanol
    95% ethanol
    100% ethanol (three times)
    Xylene (three times).
17. Mount the specimen in Permount and add a coverslip.
18. Visualize the cells under a light microscope and count both total cells and proliferating cells (those with brown stained nuclei; see Fig. 1.10.6).
19. Calculate the cell proliferation index as follows:

    PCNA positive cells/total cells × 100%

Immunohistochemistry for Apoptosis (ApopTag)

BASIC PROTOCOL 8

DNA fragmentation associated with apoptosis in the liver is detected quantitatively using a commercial detection kit according to the terminal deoxyribonucleotidyl transferase-mediated nick end labeling (TUNEL) method. See the detection kit supplier's Web site for more information about the detection method, and see Figure 1.10.7 for results using this method.

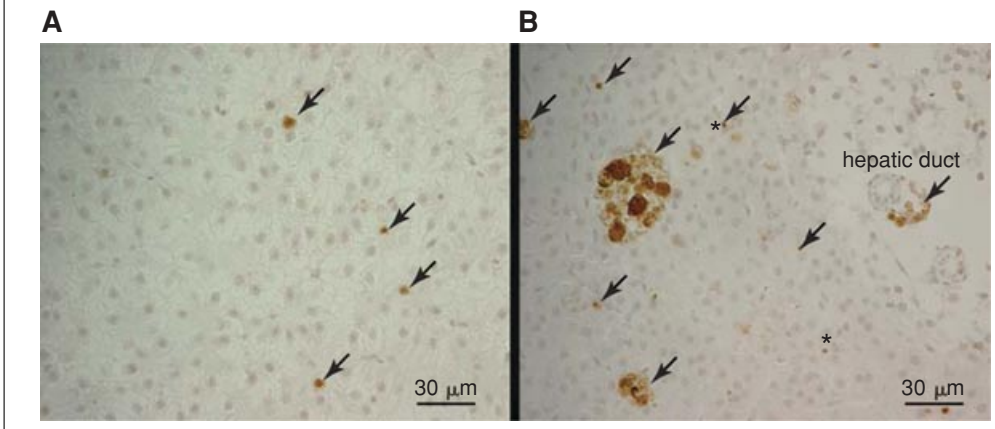

Figure 1.10.7 TUNEL assay of STII medaka livers. Apoptotic cells indicated by arrows are labeled brown. Asterisks (*) indicate biliary preductular epithelial cell. (**A**) Control liver. (**B**) ANIT-treated liver. See Table 1.10.1 for an interpretation of the results in conjunction with other staining methods.

Materials

Paraffin sections of toxicant-treated and control medaka on poly-L-lysine coated slide (Basic Protocol 6)
Xylene
70%, 95%, and 100% ethanol
10 mM citrate buffer, pH 6.0
3% (v/v) hydrogen peroxide/phosphate-buffered saline (PBS; *APPENDIX 2A*)
PBS
10% (v/v) normal goat serum, diluted in PBS containing 0.1% (v/v) Tween 20
Apop Tag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore) containing:
  Equilibration buffer
  TdT (terminal deoxyribonucleotide transferase)
  Stop/wash buffer
  Anti-digoxigenin conjugate
  DAB substrate solution
Harris' hematoxylin
Permount (Fisher Scientific)
Boiling water bath
Room temperature and 37°C humidified chamber of appropriate size (home made or commercially available)
Coverslips
Light microscope

1. Deparaffinize the tissue section by incubating in the following series:

   Xylene (three times for 5 min each)
   100% ethanol (three times for 3 min each)
   95% ethanol (5 min)
   70% ethanol (5 min)
   Water (two times for 5 min each).

2. Pretreat tissues in 10 mM citrate buffer, pH 6.0, boiling for three cycles of 3 min, with 3-min pauses between each boiling step.

3. Let the solution sit on the bench for 30 min.

4. Rinse twice in water for 2 min each.

5. Quench endogenous peroxidase by incubating 5 min in 3% hydrogen peroxide PBS.
6. Rinse twice in PBS for 2 min each.
7. Apply 75 μl/5 cm^2 of equilibration buffer for at least 10 sec.
8. Tap off excess equilibration buffer. Apply 55 μl/5 cm^2 working strength TdT (terminal deoxyribonucleotidyl transferase) and incubate 1 hr in a humidified chamber at 37°C.
9. Agitate sections in stop/wash buffer for 15 sec. Then incubate 10 min.
10. Rinse three times in PBS for 2 min each.
11. Apply 65 μl/5 cm^2 anti-digoxigenin conjugate and incubate 30 min in a humidified chamber at room temperature.
12. Rinse four times in PBS for 2 min each.
13. Apply 75 μl/5 cm^2 DAB for 3 to 6 min.
14. Rinse 1 min in running tap water
15. Counterstain the specimen 1 min in Harris' hematoxylin.
16. Rinse 1 min in running tap water.
17. Dehydrate the specimen for 1 min in each of the following:

    70% ethanol
    95% ethanol
    100% ethanol (three times)
    Xylene (three times).

18. Mount the specimen in Permount and add a coverslip.
19. Visualize the cells under a light microscope and count both total cells and dead cells (TUNEL-positive cells; see Fig. 1.10.7).
20. Calculate the degree of apoptosis in the liver as the apoptotic index as follows.

    TUNEL-positive cells/total cells × 100%

Immunocytochemistry for Pan-Cytokeratins (AE1/AE3)

BASIC PROTOCOL 9

Biliary epithelial cells and/or bile preductular epithelial cells are localized and detected by the application of Zymed's monoclonal mouse anti-cytokeratin (Pan; clone: AE1/AE3) antibody, which is a specific stain for the cytokeratin filaments of the biliary epithelial cells (Fig. 1.10.8).

Materials

Paraffin section of toxicant-treated and control medaka mounted on poly-L-lysine coated slide (Basic Protocol 6)
Xylene
70%, 95%, and 100% (v/v) ethanol
10 mM citrate buffer, pH 6.0
3% (v/v) hydrogen peroxide in phosphate-buffered saline (PBS; *APPENDIX 2A*)
PBS
10% (v/v) normal goat serum, diluted in PBS containing 0.1% (v/v) Tween 20
Primary antibody: mouse anti-AE1/AE3 antibody (Zymed, cat. no. 08-0132), diluted 200-fold in antibody diluent (Dako, cat. no. S0809)

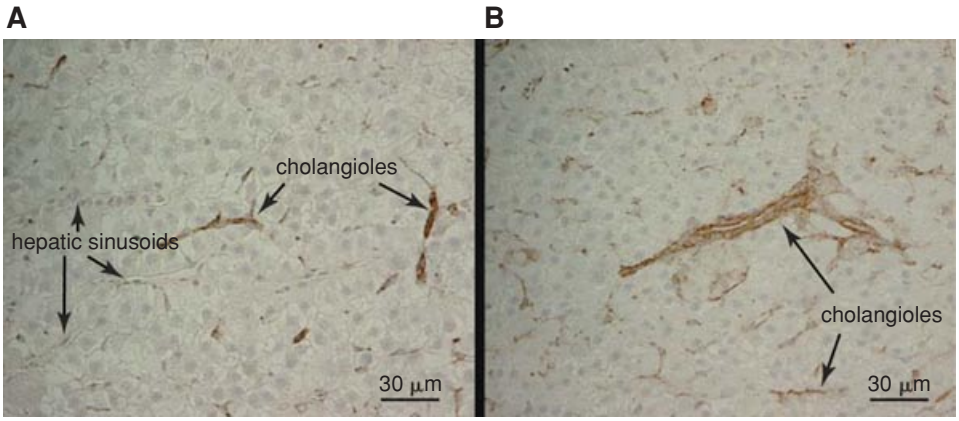

Figure 1.10.8 Immunohistochemical labeling of STII medaka liver with anti-pan-cytokeratin antibodies. Positive cells are labeled brown. (**A**) Control liver showing hepatic parenchyma with hepatic sinusoids and bile cholangioles indicated by arrows. (**B**) ANIT-treated liver showing biliary epithelial cells of hepatocellular tubules and cholangioles. Significant swelling of biliary epithelial cells is seen following ANIT exposure. See Table 1.10.1 for an interpretation of the results in conjunction with other staining methods.

Secondary antibody: goat anti-mouse-HRP conjugated immunoglobulins (DakoCytomation Envision+ System-HRP; Dako, cat. no. K4006)
Peroxidase substrate: 3,3′-diaminobenzidine (DAB, Dako)
Harris' hematoxylin
Permount (Fisher Scientific)

Boiling water bath
Room temperature and 37°C humidified chambers (home made or commercially available)
Coverslips
Light microscope

1. Deparaffinize the tissue section in the following solutions:

   Xylene (three times for 5 min each)
   100% ethanol (three times for 3 min each)
   95% ethanol (5 min)
   70% ethanol (5 min)
   Water (two times for 5 min).

2. Pretreat the specimen by immersing sections in 10 mM citrate buffer, pH 6.0, and boiling for three cycles of 3 min each in a microwave oven, with 3-min pauses in between.
3. Let the solution sit on bench for 30 min.
4. Rinse twice in water, 2 min each.
5. Incubating 5 min in 3% hydrogen peroxide in PBS to quench endogenous peroxidase.
6. Rinse twice in PBS, 2 min each time.
7. Incubate 20 min in 10% normal goat serum to block.
8. Incubate 1 hr in primary antibody in a humidified chamber at 37°C.
9. Rinse three times in PBS for 5 min each.

10. Add secondary antibody and incubate 30 min in a humidified chamber at room temperature.
11. Rinse three times in PBS for 5 min each time.
12. Apply DAB solution for 3 to 6 min.
13. Rinse 1 min in running tap water.
14. Counterstain the specimen 1 min in Harris' hematoxylin.
15. Rinse 1 min in running tap water.
16. Dehydrate the specimen in the following solutions:

    70% ethanol (1 min)
    95% ethanol (1 min)
    100% ethanol (three times, 1 min each)
    Xylene (three times, 1 min each).

17. Mount in Permount and add a coverslip.
18. Visualize the cells under a light microscope and identify the biliary epithelial cells by the stained cytokeratin filaments in the cytoplasm. Count the number of positively stained cells.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Embryo rearing medium (ERM), 1×

10 g NaCl
0.3 g KCl
0.4 g $CaCl_2 \cdot 2\ H_2O$
1.63 g $MgSO_4 \cdot 7\ H_2O$
Deionized water to 1000 ml

Stir ~15 min at room temperature until completely dissolved. Adjust the pH using sodium bicarbonate solution (0.25 g sodium bicarbonate in 20 ml of deionized water) to pH ~7. Store up to 6 months at room temperature (in absence of microbial contamination). Dilute this 10× ERM to 1× with water before use.

GPHS fixative

Pour 80 ml HistoChoice MB fixative (Amresco) into a measuring cylinder, slowly, to avoid foaming. Add 0.2 ml 25% glutaraldehyde (EMS; 0.05% final) and 12.5 ml 16% (v/v) paraformaldehyde (see recipe; 2% final). Bring to 100 ml with water. Add 1 g sucrose (Sigma-Aldrich; 1% final) and mix well. Prepare fresh or store overnight at 4°C. Alternatively, place stocks, aliquots, into small vials and store at −20°C. Thaw and return to room temperature before use.

High-Triton PBS

500 µl Triton X-100 (Sigma Aldrich)
0.25 g bovine serum albumin (Fraction V; Fisher Scientific, cat. no. BP1605)
2.5 ml of 2% (w/v) NaN_3
Add phosphate-buffered saline (PBS; *APPENDIX 2A*) to 50 ml
Store up to 6 months at room temperature (in absence of microbial contamination)

2% NaN_3 solution is prepared by dissolving 2 g of NaN_3 in water to a final volume of 100 ml.

Low-Triton PBS

150 µl Triton X-100 (Sigma-Aldrich)
0.25 g bovine serum albumin (Fraction V; Fisher Scientific)
2.5 ml 2% (w/v) NaN_3
Add phosphate-buffered saline (PBS; *APPENDIX 2A*) to 50 ml
Store up to 6 months at room temperature (in absence of microbial contamination)

Marine water

8 g Instant Ocean (Aquarium Systems)
Deionized water to 500 ml

Stir overnight until well mixed. Check that the final pH is ~8 and adjust, if necessary.

Paraformaldehyde, 16% (w/v), for GPHS fixative

Dissolve 16 g paraformaldehyde powder (Sigma) in 100 ml water by heating to ~60°C under a fume hood. Add 1 N KOH, drop by drop, stirring until the solution clears. Prepare fresh or store overnight at 4°C.

Paraformaldehyde, 16% (w/v), for Zamboni's fixative

Dissolve 8 g paraformaldehyde (Sigma-Aldrich) in water, bringing the volume to 50 ml. Add 10 N NaOH to help with dissolving (~1 drop of 10 N NaOH/10 ml of water). Stir and heat (not higher than 60°C) in a fume hood until dissolved. Prepare fresh, store overnight at 4°C, or store at −20°C.

Pronase solution, 1% (w/v)

For 10 ml add 0.1 g pronase (protease from *Streptomyces griseus*; Sigma-Aldrich, cat. no. P8811) to a final volume of 10 ml 1× ERM (see recipe) and vortex. Store at −20°C.

The brownish-tan color is normal.

Sodium phosphate (dibasic), 0.2 M

Dissolve 1.42 g Na_2HPO_4 in water, bringing the volume to 50 ml. Store up to 3 months at room temperature.

Sodium phosphate (monobasic), 0.2 M

Dissolve 1.38 g NaH_2PO_4 in water, binging the volume to 50 ml. Store up to 3 months at room temperature.

Trypsin, 1 % (w/v)

Dissolve 0.1 g trypsin (Sigma Aldrich, cat. no. T9201) in 10 ml PBS (*APPENDIX 2A*). Store at −20°C.

Zamboni's fixative

390 ml 0.2 M Na_2HPO_4 (see recipe)
110 ml 0.2 M NaH_2PO_4 (see recipe)
25 ml 16% (w/v) paraformaldehyde (see recipe)
15 ml saturated picric acid (EK Industries, cat. no. 8539)
10 ml distilled water
Adjust pH to 7.3 to 7.4, if necessary
Store up to 6 months at room temperature

COMMENTARY

Background Information

Advantages of using medaka for neurotoxicity studies

High fecundity, external fertilization, and development in an optically clear chorion make medaka ideal for studying nervous system ontology and pathology. Medaka have long been used in toxicology, and an array of well developed molecular and genetic tools make medaka suitable for mechanism/mode-of-action studies, as well. Several mutant medaka strains have been identified with disruptions in nervous system development (Ishikawa, 2000; Kitagawa et al., 2004; Yoda et al., 2004). Molecular methods of potential use for neurotoxicology studies include transgenesis (Winn, 2001), in situ hybridization (Kage et al., 2004), cell death assays (Iijima and Yokoyama, 2007), proliferation assays (Candal et al., 2005), and behavioral endpoints (Eisenberg and Dudai, 2004; Park et al., 2005). The completion of a draft medaka genome (Kasahara et al., 2007) will provide additional tools for identifying components of neurotoxicant pathways.

Dechorionating medaka embryos

We prefer the method of dechorionating embryos described in Support Protocol 2 because it allows large numbers of embryos to be dechorionated simultaneously. In this method, medaka embryos are bathed in pronase for dechorionation. Young embryos have an internal pressure that forces the embryos out of small holes in the chorion, and bathing the entire chorion in pronase reduces the likelihood of the embryo oozing out through a single small hole, which is usually the case in mechanical dechorionation.

Another enzymatic method of dechorionation uses medaka hatching gland enzyme. Although it is an endogenous protein, the hatching gland enzyme is not specific to the chorion and will digest embryonic tissue. Additionally, different preparations of hatching gland enzyme yield different activity levels, requiring empirical determination of appropriate concentrations and incubation times. In contrast, pronase dechorionation allows for strict concentration control across experiments.

There have also been several reports of mechanical dechorionation of medaka embryos. The primary advantage of chemical dechorionation is the ability to dechorionate a large number of embryos rapidly and simultaneously. Moreover, the chorion of younger medaka embryos (<48 hr) is extremely resilient, and the force required for mechanical dechorionation often leads to accidental embryo damage.

Staining the nervous system of medaka embryos

Basic Protocol 4 describes whole-mount antibody staining of hatched medaka fry to examine the nervous system structure. Measuring changes in protein expression following chemical exposure is one method used to characterize neurotoxicant effects and determine mechanism of action. In addition to expression levels, whole-mount antibody staining allows the visualization of proteins within the context of the entire animal, providing valuable structural information. While Basic Protocol 4 is designed for examining protein expression patterns in hatched medaka fry, it could be adapted for younger embryos.

Analysis of genotypic sex

Identifying the genotypic sex of individuals is very useful for evaluating chemical treatment effects on the development of gonads and secondary sex characteristics. Several test methods incorporating genetic sex determination of medaka as part of the data analysis are being developed to evaluate chemicals for endocrine-disrupting activity. In most of these protocols, the fish are exposed to the test agent or chemical, at a nontoxic level, by dissolving the chemical in the water (usually without carrier solvents), which is constantly delivered to the fish in the exposure aquaria. Usually several dose (concentration) levels are tested simultaneously to allow evaluation of the lowest-observed-effect-levels (LOEL) and no-observed-effect-levels (NOEL) for each test chemical. The goal of studies using genotypic sex information is the development of new methods for evaluating chemicals that are more sensitive and cost effective, and use fewer animals.

For example, genotypic sex information can be used for assessing the long-term reproductive and developmental effects of chemicals on fish. In fish exposed to endocrine disrupting chemicals (EDC), secondary sex characteristics are often ambiguous, e.g., the papillae on the anal fin of male medaka, normally an easily evaluated male phenotype. This ambiguity can be easily overcome by segregating the fish into pairs, sampling the dorsal or ventral tip of each caudal fin, and identifying the genotypic sex of each fish with qRT-PCR (Alternate Protocol). From this

information unambiguous breeding groups can be assembled.

Genotypic sex data is also helpful when analyzing data from treatments that change primary gender-specific phenotypes. For example, the gonadal tissue of fish exposed to EDCs during development can express histological structure of both sexes (i.e., intersex gonads; Fig. 1.10.9C). Under some conditions, the expressed gonadal phenotype is completely opposite of the genetic sex of the individual (i.e., sex reversed; Fig. 1.10.9D). Without genotypic sex data, complete sex-reversal can only be determined by changes in the population sex-ratio, which requires much larger sample sizes for detection.

Immunohistochemistry of the hepatobiliary system

Bile synthesis and transport, performed by the hepatobiliary system, are essential life functions and fundamental to the elimination and removal of metabolic byproducts. Impairment of bile synthesis and transport (cholestasis) often results in morbidity and mortality due to the accumulation of endogenous and exogenous substances and their metabolites within the liver (Alpini et al., 2002; Wolkoff and Cohen, 2003).

Previous studies have shown that the hepatobiliary systems of channel catfish (*Ictaulurus punctatus*), rainbow trout (*Oncrhynchus mykiss*) and medaka (*Oryzias laptipes*) contain numerous biliary passageways (bile preductules) between hepatocelluar canaliculi and biliary epithelial cell (BEC) delimited bile ductules (Hampton et al., 1988; Okihiro and Hinton, 2000; Hardman et al., 2007). These transitional biliary passageways are anatomically analogous to peri-portal canals of Hering and oval cells (i.e., putative progenitor cells) in the mammalian liver (Fausto and Campbell, 2003).

Recent in vivo studies in medaka livers has shown that they are replete with bile preductular epithelial cells closely associated with the transitional biliary passageways (bile preductules; Hardman et al., 2007). From previous investigations, injury to preductular epithelial cells may distort bile preductular lumina and alter intrahepatic bile flow, leading to cholestasis. It is, therefore, essential to recognize these cells in normalcy and disease, and to understand the spectrum of responses of the

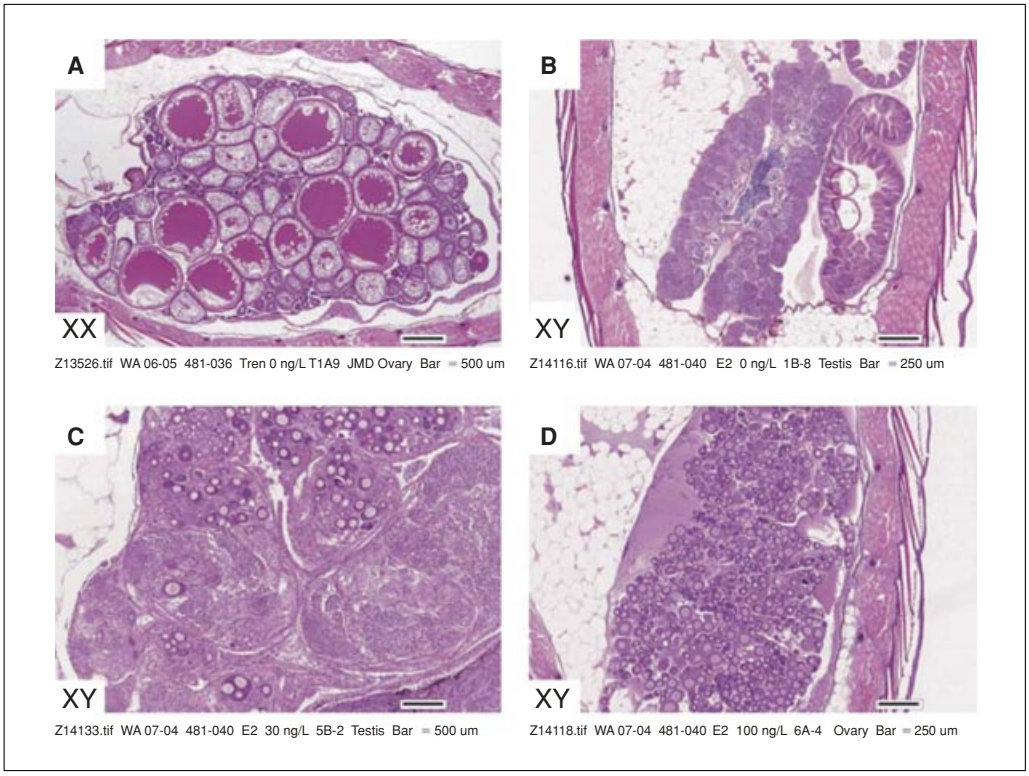

Figure 1.10.9 Representative histological sections from medaka, stained with hematoxylin and eosin. (**A**) A normal ovary from an unexposed XX fish; (**B**) a normal testis from an unexposed XY fish; (**C**) a gonad from a XY fish exposed to 17 β-estradiol, showing substantial amounts of both testicular and ovarian tissues (intersex); and (**D**) a gonad from a XY fish exposed to 17 β-estradiol, with almost exclusively ovarian tissues.

hepatobiliary system to xenobiotics that target preductular epithelial cells.

Previous studies have used light and transmission electron microscopy to examine and distinguish preductular epithelial cells in rainbow trout liver (Hampton et al., 1985). However, due to the small size (<2 μm in diameter), these cells are difficult to identify using conventional light microscopic analysis; therefore, biliary toxicity is not uniformly recognized. Examination of preductular epithelial cells by transmission electron microscopy is often time consuming and requires expertise. Thus, data from analysis of these initial biliary epithelial cells is limited.

Histochemical localization of magnesium-dependent ATPase has been employed by researchers to identify the biliary system in rainbow trout (Hampton et al., 1985, 1988), in which deposits of lead sulfide mark sites of enzyme activity along the plasma membrane of biliary passageways (i.e., canaliculi, bile preductules, ductules and bile ducts). This method is, however, not routinely used due to lack of resolution and safety hazards in using lead.

Immunohistochemical localization of preductular epithelial cells using anti-pan-cytokeratin antibodies (Zymed, 18-0132) provides a much safer and convenient alternative. Anti-pan-cytokeratin antibodies recognize cytokeratin filaments of biliary epithelial cells, including both bile preductular epithelial cells and cuboidal epithelial cells in medaka liver (demonstrated in this unit), as well as in rainbow trout (Okihiro and Hinton, 2000) and striped bass (Bunton, 1993). This method allows colocalization of proteins of interest in the same tissue section using a combination of primary antibodies (anti-pan-cytokeratins and anti-PCNA, or anti-pan-cytokeratins and ApopTag), which can provide valuable information about the toxic mechanism of some drugs (e.g., target cell types, cytotoxic and/or stimulative).

For the purposes of demonstration of the procedures described in this unit and their relationship to induction of intrahepatic biliary toxicity in medaka, we have followed procedures recently published by Hardman et al. (2007). Larval medaka were exposed to a range of aqueous concentrations of ANIT for a 24- to 72-hr duration, and a concentration of 5 μM was selected for use. We also employed, in adult medaka, a single intraperitoneal injection of 40 mg/kg of ANIT in DMSO carrier, and sampled anesthetized fish at 48 to 72 hr post exposure.

Critical Parameters and Troubleshooting

Maintenance of medaka

Medaka are normally very disease free. If they become sick, it is likely that they are stressed. The common stressors are rapid changes in pH or temperature, inconsistent light/dark cycle, increased levels of ammonia, overcrowding, and under- or over-feeding.

Breeding medaka and collecting embryos

The same variables that are important in maintaining a healthy medaka colony (see above) are also very important in successful egg production: light, food, temperature, and pH. Moreover, those same parameters tend to be important in that order. Light intensity and light duration are two factors that should be investigated immediately if egg production is not optimal or sporadic.

Dechorionating embryos

Pronase will digest both the chorion and the embryo. Therefore, the critical element of this protocol is to remove the embryo from the 1% pronase solution before the pronase can digest the embryo (see Fig. 1.10.3F). Monitoring the embryo periodically is essential for preventing destruction of the embryo, as even small holes in the chorion will quickly result in embryo digestion. Lowering the incubation temperature will slow the reaction.

Whole-mount staining for hatched fry

Support Protocol 2 is designed for hatched fry. It must be adapted for younger embryos by dechorionating the embryos, shortening the trypsin digestion to accommodate smaller amounts of embryonic tissue, and reducing the number of washes.

When working with hatched fry, trypsin digestion is the most important step in the method and should not be shortened (pers. commun., Dr. Yuji Ishikawa, National Institute of Radiological Sciences, Japan). While the trypsin digestion is essential for proper probe penetration, it may have detrimental effects on morphology (see Fig. 1.10.10C). Longer fixation times help maintain morphological integrity, but can prevent probe penetration in thicker tissue regions (compare Fig. 1.10.10E to 1.10.10A,B). The length of fixation and trypsin digest will vary according to the target tissue; longer trypsin digests will be required for target tissues, possibly at the expense of more superficially located structures.

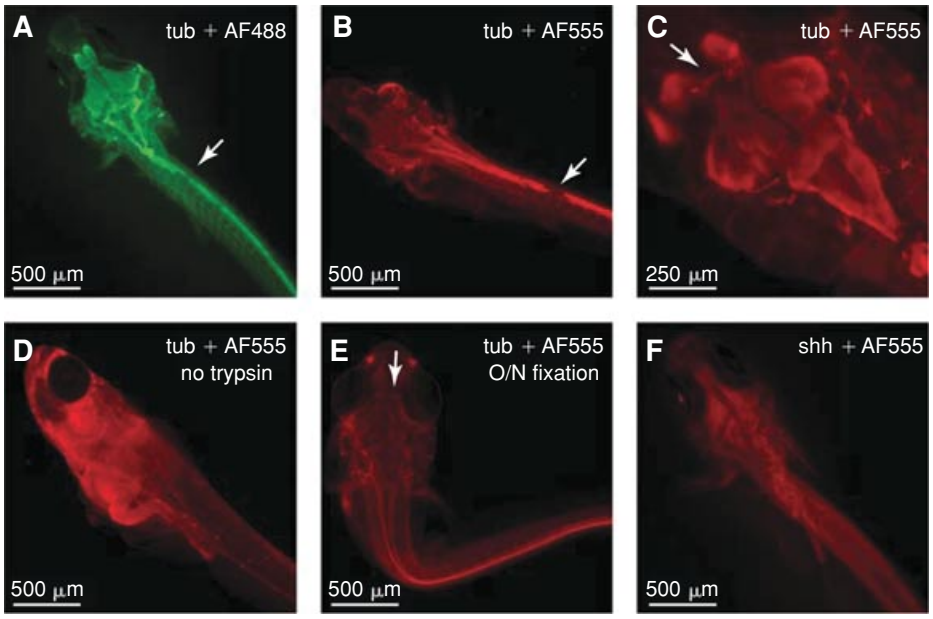

Figure 1.10.10 Anti-acetylated α-tubulin fluorescent staining of hatched medaka fry. (**A-C**) Successful antibody staining of the medaka fry nervous system. (**D-F**) Technical difficulties encountered while performing anti-acetylated α-tubulin fluorescent antibody staining protocol. (**A**) Dorsal view of a hatched medaka fry developed with Alexa Fluor 488 chicken anti-mouse secondary antibody, resulting in a fluorescent green nervous system. (**B**) Dorsal view of a hatched medaka fry developed with Alexa Fluor 555 donkey anti-mouse secondary antibody, resulting in a fluorescent red nervous system. (**C**) A higher magnification ventral view of the head of a hatched medaka fry. The optic chiasm (white arrow) is clearly visible as are the regions of the brain. (**D**) Lateral view of an artifactually stained hatched fry immunostained for anti-acetylated α-tubulin without trypsin digestion (**E**) Ventral view of a hatched fry fixed overnight at 4°C, followed by immunostaining. As shown in panels A and B (white arrows), short fixation time combined with long washes can produce poor morphology. Longer fixation times result in better morphology, though probe penetration is compromised (compare panel E with panel C—optic chiasms indicated by arrowhead). (**F**) Dorsal view of an embryo demonstrating nonspecific background fluorescence. The background fluorescence seen in this embryo is likely due to nonspecific binding of the primary antibody. Sonic hedgehog is a morphogenic protein that patterns the ventral neural tube and should be expressed in the ventral neural tube and brain. Scale is indicated for each panel. Photographs were taken using a Nikon Eclipse TE200 (Nikon) upright compound fluorescence scope with a Spot RT Slider Model 2.3.1 digital camera (Diagnostic Instruments). Abbreviations: tub, mouse anti-acetylated α-tubulin; AF488, Alexa Fluor 488 chicken anti-mouse secondary antibody; AF555, Alexa Fluor 555 donkey anti-mouse secondary antibody; O/N, overnight; shh, mouse anti-sonic hedgehog. For the color version of this figure go to *http://www.currentprotocols.com*.

When using Basic Protocol 4, an important parameter to consider in fluorescent antibody staining is nonspecific background fluorescence (not related to the binding of the antibody to target protein). This sometimes results from binding of the primary and/or secondary antibody to nonspecific protein targets (see Fig. 1.10.10F). Blocking in phosphate buffer plus Triton helps eliminate nonspecific binding of the primary antibody.

Primary antibodies generated against proteins from other species may not recognize the homologous medaka protein. Select antibodies directed against the most conserved protein regions of medaka and the antibody-producing species, or use antibodies which have shown cross-reactivity in immunohistochemical assays across many species.

Antibody binding affinity and protein levels will also affect the sensitivity of this assay and should be considered during the antibody incubations. In general, a weakly expressed protein or a low affinity–binding antibody will require longer incubation and/or more concentrated antibody dilutions.

Too much primary or secondary antibody also leads to background fluorescence, so optimal dilutions of both primary and

secondary antibodies should be determined empirically.

Nonspecific fluorescence may also come from the embryo itself (autofluorescence) or as a result of fixation (fixative-induced fluorescence). Medaka fry have autofluorescent pigmented cells along the surface of their body. Fixation induces extensive background fluorescence, even with fixation times as short as 1 hr. This protocol eliminates both autofluorescence and fixative-induced fluorescence, most likely as a result of extensive washing.

Controls will help distinguish fixative-induced fluorescence and background fluorescence from successful antibody staining. The following controls should be included: no antibody control, primary antibody–only control, and secondary antibody only control. For the no-antibody control, follow Basic Protocol 4, but do not add primary antibody or secondary antibody. Fluorescence in the no-antibody control is attributable to fixative-induced fluorescence or autofluorescence. For the primary antibody–only control, follow Basic Protocol 4, but do not add secondary antibody. Similarly, for the secondary antibody–only control, follow Basic Protocol 4, but do not add primary antibody. When compared with the no-antibody control embryos, fluorescence seen in either the primary antibody–only control or secondary antibody–only control embryos indicates nonspecific background fluorescence. These three control conditions are important for determining optimal antibody concentrations.

In Basic Protocol 4, the secondary antibody determines the fluorescence emission color and will only emit fluorescence when exposed to the appropriate excitation wavelengths. Examining embryos under several excitation wavelengths is a simple way to distinguish background fluorescence from specific antibody staining. Fluorescence from successful antibody staining should be specific to a particular excitation condition, while background fluorescence will typically appear in several excitation conditions. This simple method does not replace the controls outlined above, but will give a rapid assessment of staining success.

Analysis of genotypic sex

If using the traditional PCR method followed by gel electrophoresis (Basic Protocol 5), the most important parameter to monitor is the amplicon size as verified on the gel. The size of the bands on the gel for each fish need to be used to verify both that PCR was not inhibited and if applicable, that the correct product was amplified. If using the high-throughput method (Alternate Protocol), the 18S rRNA reagents verify that PCR was not inhibited. Without this control, it would be impossible to determine whether an individual is a female or if t inhibitors of PCR were present.

Immunohistochemistry of the hepatobiliary system

Excessive background staining. Sometimes, because of the variability of specimens and preparation techniques, certain tissues may give excessive backgrounds or false-positive staining. Where this is encountered, consider the following:

1. The suggested color-substrate development time range is approximate because the rate of color development is influenced by differences in specimen composition and room temperature. Shortening the color-substrate development time should reduce background staining.

2. Another probable cause of excessive background staining could be due to high endogenous peroxidase activity of the specimen. Increased incubation time of peroxidase block should reduce this background staining.

3. Incomplete removal of paraffin and/or incomplete rinsing of slides could attribute to excessive background staining. Use fresh xylene or toluene baths and fresh solutions in buffer baths, to reduce background staining.

4. Commonly, sections that become desiccated during staining procedures also show increased background staining. Use a humidified chamber and wipe only three to four slides at a time before applying reagent to diminish likelihood of desiccation.

5. Nonspecific binding of reagents to tissue sections could also contribute to high background staining. This may be addressed by application of a blocking solution containing an irrelevant protein, thereby reducing the background.

6. Last, but not least, is concentration of primary antibody (i.e., anti-PCNA or anti-pan-cytokeratin). To prevent overstaining, reduce primary antibody concentration. We recommend final dilutions ranging from 1:200 to 1:2000 for the pan-cytokeratins, and 1:500 to 1:2000 for anti-PCNA.

When using the ApopTag Peroxidase Kit, start with control liver sections and deploy a range of working strength TdT. This commonly results in a single labeled nucleus in 100 total nuclei. Then, employ the identical conditions and procedures to establish the

rate of apoptosis in exposed individuals. In practice, we find that dilutions in the range of 1:5 to 1:16 (instead of the manufacturer-recommended 1:3) result in a decrease in the nonspecific staining.

Weak staining. This occurs when sections retain excess solution after washing with PBS and/or when slides are not incubated long enough. This problem can be solved by gently tapping wet slides to remove excess solution or by carefully wiping around a section to remove excess solution. In addition, increasing incubation time and/or increasing concentration of primary antibodies (i.e., anti-PCNA and anti-pan-cytokeratins) may yield satisfactory results.

In the case of the detection of apoptosis using the ApopTag Peroxidase Kit, we occasionally encounter apoptotic nuclei with a pale tan color. In our experience these nuclei, although positive, will not be discernible after counterstaining to reveal tissue architecture. In this case, we recommend a lighter background of the DAB substrate and achieve this using a 1:20 rather than a 1:50 manufacturer-recommended dilution. Such an adjustment enables detection of apoptotic nuclei in stained sections of tissue.

Anticipated Results

Dechorionating embryos

Chorions of some embryos digest more quickly than others, even in staged embryos. Hence, it is not unusual for the embryos to dechorionate asynchronously. Careful monitoring should yield a high percentage of successfully dechorionated embryos, although some embryo loss is typical.

Whole-mount staining neurotoxicity studies

At the end of Basic Protocol 4, the three control conditions (no antibody, primary antibody only, and secondary antibody only) should have little to no fluorescence. In the experimental embryos, the nervous system should fluoresce under appropriate excitation conditions. Antibody staining patterns should accurately reflect endogenous protein expression. Several factors may affect antibody staining, including poor probe penetration, poor morphology, and background fluorescence. Poor probe penetration may result exclusively in surface staining (see Fig. 1.10.10D). Sensitivity of the assay is determined by the affinity of the primary antibody and the abundance of endogenous target protein.

Analysis of genotypic sex

In both Basic Protocol 5 and the Alternate Protocol, the data output is the same: identification of an individual medaka as a genotypic male (XY) or a genotypic female (XX). The biological error rate (i.e., the spontaneous prevalence in wild populations of disconcordance between phenotypic and genotypic sex) appears to be 1% or less (Shinomiya et al., 2004). However, in our facility to date, we have not seen a genotype-to-phenotype mismatch in the several hundred unexposed (control) fish that have been analyzed. The protocols detailed in this unit provide efficient and accurate determination of the genotypic sex of medaka.

Immunohistochemistry of the hepatobiliary system

See Table 1.10.1 for analysis of immunohistochemisty results.

Time Considerations

Growing medaka

Medaka eggs usually hatch in <2 weeks, and the fish are sexually mature by 3 to 4 months. Adult medaka have a life-span of 2 to 3 years under normal laboratory conditions.

Collecting embryos

It usually takes only a couple of hours to collect, clean, and distribute the embryos into vials or a microtiter plate

Performing toxicology experiments

The length of time for performing the toxicology experiment depends, of course, on the exposure paradigm. Acute experiments in embryos may only take hours, while chronic experiments in adults may take months.

Dechorionating embryos

The length of time for dechorionation (Support Protocol 2) will vary based on embryo age, incubation temperature, and pronase concentration. In general, most chorions will show effects of 1% pronase digestion at 34°C in 2 hr or less. The chorions of later stage medaka embryos are easier to digest than earlier stage medaka embryos, and could take less time to dechorionate.

Whole-mount staining

The amount of washing and length of antibody incubation determine the time required (see Basic Protocol 4). In general, extended washing reduces background staining, but can cause morphology problems. Times

Table 1.10.1 Possible Outcomes and Interpretations in Liver Sections from Toxicant-Treated Versus Control Medaka[a]

Cell proliferation index[b]	Apoptotic index[c]	Pan-cytokeratin[d]	Interpretations[e]
−	−	−	No adverse effect of toxicant on liver
+	+	−	Hepatocytes independent of biliary passageways targeted by toxicant
−	+	+	Biliary epithelial cells targeted by toxicant, as in acute toxicity
+	+	+	
+	−	+	Remodeling of intrahepatic biliary passageways suggested, depending on duration (e.g., chronic cell loss or proliferation over 1 to 3 weeks)
−	−	+	Chronic toxicity repaired, but biliary passageway architecture remains altered

[a]The minus sign (−) indicates no difference detected between treatment and control. The plus sign (+) indicates significant difference detected between treatment and control fish. For both the treatment and control groups n = liver sections from 5 to 7 fish.
[b]Determined using Basic Protocol 7.
[c]Determined using Basic Protocol 8.
[d]Staining performed using Basic Protocol 9 distinguishes hepatocytes from other types of cells and may be used in combination with other stains.
[e]Our intent here was to show how the method can be used to identify definitively biliary epithelia and to determine quantitative changes within the intrahepatic biliary cellular compartment with respect to cell proliferation and/or programmed cell death (apoptosis). Additional possible outcomes not mentioned in the table include alteration of the structure of the biliary cells (swelling or shrinkage) after treatment, hypertrophy or hyperplasia of the tissue, and/or alteration of the space lined by the biliary cells.

for antibody incubation are determined empirically, and depending upon the particular antibodies, the entire procedure can take from 9 to 19 days.

Analysis of genotypic sex

The time it takes to extract DNA from samples depends on the technique used. In our laboratory, we routinely use DNeasy kits (Qiagen), which extract PCR-ready DNA in ∼2 hr. A digestion step in these kits can be completed in as little as 30 min or can be extended to an overnight incubation, allowing flexibility within the protocol. Once DNA is extracted, a 96-well plate can be manually loaded with PCR master mix and appropriate samples in ∼30 min, followed by ∼1.5 hr of qRT-PCR (Alternate Protocol). Alternatively, samples and master mix can be loaded into PCR-appropriate tubes and run on a traditional thermocycler in about the same time (Basic Protocol 5). However, the presence of the amplicons then need to be verified by gel electrophoresis, which takes a couple more hours with far less throughput than real-time PCR.

Immunohistochemistry of the hepatobiliary system

After exposure to the toxicant, hepatic immunohistochemistry procedures may be carried out in 1 to 3 weeks. The fixation, embedding, and sectioning of the paraffin blocks is the most time-consuming portion of the protocols (4 days; Basic Protocol 6). However, the paraffin blocks may be archived and used for more efficient assessment using the various staining techniques. The performance of the other tests—proliferating cell nuclear antigen (PCNA; Basic Protocol 7), immunohistochemistry of anti-pan-cytokeratin (Basic Protocol 8), and apoptosis (Basic Protocol 9)—require a day each, but the tests can be carried out simultaneously on sectioned, paraffin-embedded material. Finally, the semiquantitative determination of proliferating cells and of apoptosis requires ∼1 hr per liver from one animal.

Acknowledgements

This research was supported by National Center for Research Resources (NCRR) grants 1 RO1 RRo18583-04 to DEH, R21CA106084-01A1 to SWK, and 5 P42 ES10356 to R.T. Di Giulio.

The authors thank Drs. Ram Ramabhadran and Kevin Crofton for their careful review of the manuscript.

This manuscript has been reviewed by the National Health and Environmental Effects Research Laboratory of the U.S. Environmental Protection Agency, and approved for

publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

John Cowden contributed the protocols related to experimental neurotoxicology techniques (Support Protocol 2 and Basic Protocol 4). Rodney Johnson and Kevin Flynn provided methods for determining sexual genotypes in medaka after endocrine disruption (Basic Protocol 5 and Alternate Protocol). Bonny B. H. Yuen, Ronald C. Hardman, David E. Hinton, Sheran Law, Seth W. Kullman, and Doris W. T. Au contributed the protocols used for detecting toxic alterations in the intrahepatic biliary passageways (Basic Protocols 6, 7, 8, and 9).

Literature Cited

Alestrom, P., Holter, J.L., and Nourizadeh-Lillabadi, R. 2006. Zebrafish in functional genomics and aquatic biomedicine. *Trends Biotechnol.* 24:15-21.

Alpini, G., Aragona, E., Dabeva, M., Salvi, R., Shafritz, D.A., and Tavoloni, N. 1992. Distribution of albumin and alpha-fetoprotein mRNAs in normal, hyperplastic, and preneoplastic rat liver. *Am. J. Pathol.* 141:623-632.

Alpini, G., Baiocchi, L., Glaser, S., Ueno, Y., Marzioni, M., Francis, H., Phinizy, J.L., Angelico, M., and Lesage, G. 2002. Ursodeoxycholate and tauroursodeoxycholate inhibit cholangiocyte growth and secretion of BDL rats through activation of PKC alpha. *Hepatology* 35:1041-1052.

Anken, R. and Bourrat, F. 1998. Brain Atlas of the Medakafish *Oryzias latipes*. INRA Editions, Paris.

Ankley, G.T., Jensen, K.M., Kahl, M.D., Korte, J.J., and Makynen, E.A. 2001. Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*). *Environ. Toxicol. Chem.* 20:1276-1290.

Balch, G.C., Shami, K., Wilson, P.J., Wakamatsu, Y., and Metcalfe, C.D. 2004. Feminization of female leukophore-free strain of Japanese medaka (*Oryzias latipes*) exposed to 17β-estradiol. *Environ. Toxicol. Chem.* 23:2763-2768.

Bunton, T.E. 1993. The immunocytochemistry of cytokeratin in fish tissues. *Vet. Pathol.* 30:418-425.

Candal, E., Anadon, R., Bourrat, F., and Rodriguez-Moldes, I. 2005. Cell proliferation in the developing and adult hindbrain and midbrain of trout and medaka (teleosts): A segmental approach. *Brain Res. Dev. Brain Res.* 160:157-175.

Carl, M., Loosli, F., and Wittbrodt, J. 2002. *Six3* inactivation reveals its essential role for the formation and patterning of the vertebrate eye. *Development* 129:4057-4063.

Connolly, A.K., Price, S.C., Connelly, J.C., and Hinton, R.H. 1988. Early changes in bile duct lining cells and hepatocytes in rats treated with alpha-naphthylisothiocyanate. *Toxicol. Appl. Pharmacol.* 93:208-219.

Davis, C.R., Okihiro, M.S., and Hinton, D.E. 2002. Effects of husbandry practices, gender, and normal physiological variation on growth and reproduction of Japanese medaka, *Oryzias latipes*. *Aquat. Toxicol.* 60:185-201.

DeKoven, D.L., Nunez, J.M., Lester, S.M., Conklin, D.E., Marty, G.D., Parker, L.M., and Hinton, D.E. 1992. A purified diet for medaka (*Oryzias latipes*): Refining a fish model for toxicological research. *Lab. Animal Sci.* 42:180-189.

Eisenberg, M. and Dudai, Y. 2004. Reconcolidation of fresh, remote and extinguished fear memory in Medaka: Old fears don't die. *Eur. J. Neurosci.* 20:3397-3403.

Fausto, N. and Campbell, J.S. 2003. The role of hepatocytes and oval cells in liver regeneration and repopulation. *Mech. Dev.* 120:117-130.

Fu, L., Mambrini, M., Perrot, E., and Chourrout, D. 2000. Stable and full rescue of the pigmentation in a medaka albino mutant by transfer of a 17 kb genomic clone containing the medaka tyrosinase gene. *Gene* 241:205-211.

Hampton, J.A., McCuskey, P.A., McCuskey, R.S., and Hinton, D.E. 1985. Functional units in rainbow trout (*Salmo gairdneri*) liver: I. Arrangement and histochemical properties of hepatocytes. *Anat. Rec.* 213:166-175.

Hampton, J.A., Lantz, R.C., Goldblatt, P.J., Lauren, D.J., and Hinton, D.E. 1988. Functional units in rainbow trout (*Salmo gairdneri*, Richardson) liver: II. The biliary system. *Anat. Rec.* 221:619-634.

Hampton, J.A., Lantz, R.C., and Hinton, D.E. 1989. Functional units in rainbow trout (*Salmo gairdneri*, Richardson) liver: III. Morphometric analysis of parenchyma, stroma, and component cell types. *Am. J. Anat.* 185:58-73.

Hardman, R.C., Volz, D.C., Kullman, S.W., and Hinton, D.E. 2007. An in vivo look at vertebrate liver architecture: Three-dimensional reconstructions from medaka (*Oryzias latipes*). *Anat. Rec. (Hoboken)* 290:770-782.

Helmstetter, M.F. and Alden, R.W. III. 1995. Passive trans-chorionic transport of toxicants in topically treated Japanese medaka (*Oryzias latipes*) eggs. *Aquat. Toxicol.* 32:1-13.

Hill, D.A. and Roth, R.A. 1998. Alpha-naphthylisothiocyanate causes neutrophils to release factors that are cytotoxic to hepatocytes. *Toxicol. Appl. Pharmacol.* 148:169-175.

Hinton, D.E., Kullman, S.W., Hardman, R.C., Volz, D.C., Chen, P.J., Carney, M., and Bencic, D.C. 2005. Resolving mechanisms of toxicity while pursuing ecotoxicological relevance? *Mar. Pollut. Bull.* 51:635-648.

Iijima, N. and Yokoyama, T. 2007. Apoptosis in the medaka embryo in the early developmental stage. *Acta Histochem. Cytochem.* 40:1-7.

Inui, M., Adachi, T., Takenaka, S., Inui, H., Nakazawa, M., Ueda, M., Watanabe, H., Mori, C., Iguchi, T., and Miyatake, K. 2003. Effect of UV screens and preservatives on vitellogenin and choriogenin production in male medaka (*Oryzias latipes*). *Toxicology* 194:43-50.

Ishikawa, Y. 2000. Medakafish as a model system for vertebrate developmental genetics. *Bioessays* 22:487-495.

Ishikawa, Y. and Hyodo-Taguchi, Y. 1994. Cranial nerves and brain fiber systems of the medaka fry as observed by a whole-mount staining method. *Neurosci. Res.* 19:379-386.

Ishikawa, Y., Kage, T., Yamamoto, N., Yoshimoto, M., Yasuda, T., Matsumoto, A., Maruyama, K., and Ito, H. 2004. Axonogenesis in the medaka embryonic brain. *J. Compar. Neurol.* 476:240-253.

Iwamatsu, T. 2004. Stages of normal development in the medaka *Oryzias latipes*. *Mechan. Develop.* 121:605-618.

Kage, T., Takeda, H., Yasuda, T., Maruyama, K., Yamamoto, N., Yoshimoto, M., Araki, K., Inohaya, K., Okamoto, H., Yasumasu, S., Watanabe, K., Ito, H., and Ishikawa, Y. 2004. Morphogenesis and regionalization of the medaka embryonic brain. *J. Comp. Neurol.* 476:219-239.

Kasahara, M., Naruse, K., Sasaki, S., Nakatani, Y., Qu, W., Ahsan, B., Yamada, T., Nagayasu, Y., Doi, K., Kasai, Y., Jindo, T., Kobayashi, D., Shimada, A., Toyoda, A., Kuroki, Y., Fujiyama, A., Sasaki, T., Shimizu, A., Asakawa, S., Shimizu, N., Hashimoto, S., Yang, J., Lee, Y., Matsushima, K., Sugano, S., Sakaizumi, M., Narita, T., Ohishi, K., Haga, S., Ohta, F., Nomoto, H., Nogata, K., Morishita, T., Endo, T., Shin, I.T., Takeda, H., Morishita, S., and Kohara, Y. 2007. The medaka draft genome and insights into vertebrate genome evolution. *Nature* 447:714-719.

Kirchen, R.V. and West, W.R. 1976. The Japanese Medaka: Care and Development. Carolina Biological Supply, Burlington, NC.

Kitagawa, D., Watanabe, T., Saito, K., Asaka, S., Sasado, T., Morinaga, C., Suwa, H., Niwa, K., Yasuoka, A., Deguchi, T., Yoda, H., Hirose, Y., Henrich, T., Iwanami, N., Kunimatsu, S., Osakada, M., Winkler, C., Elmasri, H., Wittbrodt, J., Loosli, F., Quiring, R., Carl, M., Grabher, C., Winkler, S., Del Bene, F., Momoi, A., Katada, T., Nishina, H., Kondoh, H., and Furutani-Seiki, M. 2004. Genetic dissection of the formation of the forebrain in Medaka, *Oryzias latipes*. *Mech. Dev.* 121:673-685.

Law, J.M. 2001. Mechanistic considerations in small fish carcinogenicity testing. *Ilar. J.* 42:274-284.

Masyuk, T.V., Ritman, E.L., and LaRusso, N.F. 2003. Hepatic artery and portal vein remodeling in rat liver: Vascular response to selective cholangiocyte proliferation. *Am. J. Pathol.* 162:1175-1182.

Matsuda, M., Nagahama, Y., Shinomiya, A., Sato, T., Matsuda, C., Kobayashi, T., Morrey, C., Shibata, N., Asakawa, S., Shimizu, N., Hori, H., Hamaguchi, S., and Sakaizumi, M. 2002. *DMY* is a Y-specific dm-domain gene required for male development in the medaka fish. *Nature* 417:559-563.

Ohmuro-Matsuyama, Y., Matsuda, M., Kobayashi, T., Ikeuchi, T., and Nagahama, Y. 2003. Expression of *DMY* and *DMRT1* in the various tissues of the medaka, (*Oryzias latipes*). *Zoolog. Sci.* 20:1395-1398.

Okihiro, M.S. and Hinton, D.E. 1999. Progression of hepatic neoplasia in medaka (*Oryzias latipes*) exposed to diethylnitrosamine. *Carcinogenesis* 20:933-940.

Okihiro, M.S. and Hinton, D.E. 2000. Partial hepatectomy and bile duct ligation in rainbow trout (*Oncorhynchus mykiss*): Histologic, immunohistochemical and enzyme histochemical characterization of hepatic regeneration and biliary hyperplasia. *Toxicol. Pathol.* 28:342-356.

Orn, S., Holbech, H., Madsen, T.H., Norrgren, L., and Petersen, G.I. 2003. Gonad development and vitellogenin production in zebrafish (*Danio rerio*) exposed to ethinylestradiol and methyltestosterone. *Aquatic Toxicol.* 65:397-411.

Orn, S., Yamani, S., and Norrgren, L. 2006. Comparison of vitellogenin induction, sex ratio, and gonad morphology between zebrafish and japanese medaka after exposure to 17 alpha ethinylestradiol and 17 beta trenbolone. *Arch. Environ. Contam. Toxicol.* 51:237-243.

Orsler, D.J., Ahmed-Choudhury, J., Chipman, J.K., Hammond, T., and Coleman, R. 1999. ANIT-induced disruption of biliary function in rat hepatocyte couplets. *Toxicol. Sci.* 47:203-210.

Otake, H., Shinomiya, A., Matsuda, M., Hamaguchi, S., and Sakaizumi, M. 2006. Wild-derived XY sex-reversal mutants in the Medaka, *Oryzias latipes*. *Genetics* 173:2083-2090.

Oxendine, S.L., Cowden, J., Hinton, D.E., and Padilla, S. 2006. Adapting the medaka embryo assay to a high-throughput approach for developmental toxicity testing. *Neurotoxicology* 27:840-845.

Papoulias, D.M., Noltie, D.B., and Tillitt, D.E. 1999. An in vivo model fish system to test chemical effects on sexual differentiation and development: Exposure to ethinyl estradiol. *Aquat. Toxicol.* 48:37-50.

Park, Y.S., Chung, N.I., Choi, K.H., Cha, E.Y., Lee, S.K., and Chon, T.S. 2005. Computational characterization of behavioral response of medaka (*Oryzias latipes*) treated with diazinon. *Aquat. Toxicol.* 71:215-228.

Paul-Prasanth, B., Matsuda, M., Lau, E.L., Suzuki, A., Sakai, F., Kobayashi, T., and Nagahama, Y. 2006. Knock-down of *DMY* initiates female pathway in the genetic male medaka, *Oryzias latipes*. *Biochem. Biophys. Res. Commun.* 351:815-819.

Seki, M., Yokota, H., Matsubara, H., Maeda, M., Tadokoro, H., and Kobayashi, K. 2003. Fish full life-cycle testing for the weak estrogen 4-tert-pentylphenol on medaka (*Oryzias latipes*). *Environ. Toxicol. Chem.* 22:1487-1496.

Seki, M., Fujishima, S., Nozaka, T., Maeda, M., and Kobayashi, K. 2006. Comparison of response to 17 β-estradiol and 17 β-trenbolone among three small fish species. *Environ. Toxicol. Chem.* 25:2742-2752.

Shima, A. and Mitani, H. 2004. Medaka as a research organism: Past, present and future. *Mech. Dev.* 121:599-604.

Shinomiya, A., Otake, H., Togashi, K., Hamaguchi, S., and Sakaizumi, M. 2004. Field survey of sex-reversals in the medaka, *Oryzias latipes*: Genotypic sexing of wild populations. *Zool. Sci.* 21:613-619.

Suzuki, A., Nakamoto, M., Kato, Y., and Shibata, N. 2005. Effects of estradiol-17β on germ cell proliferation and *DMY* expression during early sexual differentiation of the medaka *Oryzias latipes*. *Zool. Sci.* 22:791-796.

Toussaint, M.W., Rosencrance, A.B., Brennan, L.M., Dennis, W.E., Beaman, J.R., Wolfe, M.J., Hoffmann, F.J., and Gardner, H.S. Jr. 2001. Chronic toxicity of bromodichloromethane to the Japanese medaka (*Oryzias latipes*). *Toxicol. Pathol.* 29:662-669.

Villalobos, S.A., Hamm, J.T., Teh, S.J., and Hinton, D.E. 2000. Thiobencarb-induced embryotoxicity in medaka (*Oryzias latipes*): Stage-specific toxicity and the protective role of the chorion. *Aquat. Toxicol.* 48:309-326.

Wada, H., Shimada, A., Fukamachi, S., Naruse, K., and Shima, A. 1998. Sex-linked inheritance of the 1f locus in the medaka fish (*Oryzias latipes*). *Zool. Sci.* 15:123-136.

Walker, W.W., Manning, C.S., Overstreet, R.M., and Hawkins, W.E. 1985. Development of aquarium fish models for environmental carcinogenesis: An intermittent-flow exposure system for volatile, hydrophobic chemicals. *J. Appl. Toxicol.* 5:255-260.

Waters, N.J., Holmes, E., Waterfield, C.J., Farrant, R.D., and Nicholson, J.K. 2002. NMR and pattern recognition studies on liver extracts and intact livers from rats treated with alpha-naphthylisothiocyanate. *Biochem. Pharmacol.* 64:67-77.

Wayne, N.L., Kuwahara, K., Aida, K., Nagahama, Y., and Okubo, K. 2005. Whole-cell electrophysiology of gonadotropin-releasing hormone neurons that express green fluorescent protein in the terminal nerve of transgenic medaka (*Oryzias latipes*). *Biol. Reprod.* 73:1228-1234.

Winn, R.N. 2001. Transgenic fish as models in environmental toxicology. *ILAR J.* 42:322-329.

Wolf, J.C. and Wolfe, M.J. 2005. A brief overview of nonneoplastic hepatic toxicity in fish. *Toxicol. Pathol.* 33:75-85.

Wolkoff, A.W. and Cohen, D.E. 2003. Bile acid regulation of hepatic physiology: I. Hepatocyte transport of bile acids. *Am. J. Physiol. Gastrointest. Liver Physiol.* 284:G175-G179.

Woolley, J., Mullock, B.M., and Hinton, R.H. 1979. Reflux of billiary components into blood in experimental intrahepatic cholestasis induced in rats by treatment with alpha-naphthylisothiocyanate. *Clin. Chim. Acta* 92:381-386.

Yoda, H., Hirose, Y., Yasuoka, A., Sasado, T., Morinaga, C., Deguchi, T., Henrich, T., Iwanami, N., Watanabe, T., Osakada, M., Kunimatsu, S., Wittbrodt, J., Suwa, H., Niwa, K., Okamoto, Y., Yamanaka, T., Kondoh, H., and Furutani-Seiki, M. 2004. Mutations affecting retinotectal axonal pathfinding in Medaka, *Oryzias latipes*. *Mech. Dev.* 121:715-728.